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Editorials

8th World Congress of Veterinary Dermatology

The Eighth World Congress of Veterinary Dermatology was held in Bordeaux, France, from 31st May to 4th June 2016. Over 2150 individuals participated in the congress including 1750 delegates from 72 countries. The scientific programme of the congress included state-of-the-art speakers, supporting reviews and original research in the areas of genetics/genomics, allergy, skin biology, therapeutics, infectious diseases and new diagnostic approaches. Twenty manuscripts with information from these presentations are found in this electronic publication – most of these manuscripts were published previously in the February 2017 issue of *Veterinary Dermatology*. In addition, this publication includes summaries and information from 11 workshops conducted during the congress. We thank the congress speakers, co-authors and workshop moderators, secretaries and participants for their important contributions to the discipline of veterinary dermatology.

Over 230 research papers and posters were also presented at the congress. Abstracts of this information were published previously in a special supplement of *Veterinary Dermatology* (Volume 27, Supplement 1, May 2016, available at http://onlinelibrary.wiley.com/doi/10.1111/vde.2016.27.issue-s1/issuetoc). Proceedings of the continuing education programme from the congress are available electronically on the World Association for Veterinary Dermatology (WAVD) website (available at http://www.wavd.org/images/pdf/WCVD8CEProceedings.pdf).

The success of the congress was made possible by the commitment, dedication and energy of the officers and members of all the organizing committees. Further, we would like to recognize the general assistance and financial support of our sponsors. We hope you find this issue of *Advances in Veterinary Dermatology* valuable in your efforts to bring the specialty of veterinary dermatology to a broader global audience.

Philip Roudebush
Biltmore Lake, North Carolina, USA

Sheila M.F. Torres
St Paul, Minnesota, USA

9th World Congress of Veterinary Dermatology

Sydney warmly invites you to attend the Ninth World Congress of Veterinary Dermatology (WCVD9) to be held from 22 to 24 October, 2020. Come and be part of this superb international scientific event in the dream destination of Australia. Our exciting programme will deliver the most recent scientific advances in veterinary dermatology in the new, purpose built International Convention Centre overlooking the spectacular Darling Harbour - the world's largest natural harbour and recognised as one of the world's most beautiful waterways. The Centre is located just 8 km from the airport and within the vibrant and modern Sydney metropolis; access to hotels, restaurants, iconic heritage and tourist sites are within walking distance and easily accessed by public transport.

The World Congress of Veterinary Dermatology has become a global event of unsurpassed status in the international sphere of veterinary science and education. The great success of the past Congress in Bordeaux, with record attendance from around the world, emphasizes the global nature of veterinary dermatology. WCVD9 delegates will have the option of attending lectures from world-class experts delivering cutting edge, state-of-the-art research to an extensive programme of both advanced and comprehensive continuing education in dermatology. Free communications, poster sessions, company symposia and workshops will allow for informal exchanges of ideas between experts and participants in a wide range of subjects as well as show-casing original scientific research. Our excellent scientific programme will be matched by a social programme that captures our warmth and relaxed hospitality.

While you are here, experience Sydney's natural beauty and culture around Sydney Harbour and the historic Rocks district; dine under the sails of the Sydney Opera House; bushwalk through Sydney Harbour National Park; explore famous coastal beaches or visit the Blue Mountains. Australia is an exciting destination boasting spectacular wilderness, rare wildlife, rich indigenous heritage, friendly hospitality, and world class wine and food. Australia is a perfect destination for WCVD9 and a meeting not to be missed.

Mandy Burrows
Murdoch, Western Australia
WCVD9 President

The 2016 Hugo Schindelka Medal and Lecture awarded to Professor David H. Lloyd

Professor David H. Lloyd was the recipient of the Hugo Schindelka Medal at the 8th World Congress of Veterinary Dermatology in Bordeaux, France. Hugo Schindelka is considered the father of veterinary dermatology, having taught at the University of Vienna, initiated the scientific approach to the specialty and published *Hautkrankheiten bei Haustieren Handbuch der Tierärztlichen Chirurgie und Geburtshilfe* (*Skin Diseases of Domestic Animals*) in 1903. The medal is awarded in his honour every 4 years by the World Association for Veterinary Dermatology (WAVD) for excellence in scholarship and publication in veterinary dermatology. David embodies the spirit of this award: a senior academician, a renowned scientist, a long-time mentor and a tireless contributor to specialty organizations. The Schindelka Medal was first awarded in 2008 to Dr George Muller and in 2012 to Professor Richard Halliwell.

Nominations for the award are invited globally from all Member, Affiliate and Provisional Organizations of WAVD. The 2016 Hugo Schindelka Selection Committee members were Doctors Richard Halliwell (Chair), Koji Nishifuji and Julie Yager. Criteria include contributions to organization and development of the discipline; excellence in teaching at both undergraduate and postgraduate levels in universities and other educational systems; development of diagnostic and therapeutic methods, courses and other teaching materials; and publication of a substantial volume of high-quality material with significant impact on the discipline.

Professor Lloyd received his veterinary degree from the Royal Veterinary College (RVC) of the University of London and his PhD from the University of Glasgow. David set up the Dermatology Unit at the RVC in 1979, held the Chair of Veterinary Dermatology there for many years and is now Emeritus Professor. Professor Lloyd has mentored 12 PhD and four Master's degree students and 15 clinical residents. Many of his former students now hold prominent positions in both academia and private practice. He has also played a leading role in undergraduate microbiology and dermatology teaching at the RVC and his enthusiasm, energy, ideas and vision continue to drive research and guide the dermatology group.

Professor Lloyd's research and publications have significantly shaped our understanding of bacterial skin disease. David conducted pioneering work on dermatophilosis, starting in the 1970s in Africa and in Europe. He and his research team have also addressed the challenge of antimicrobial resistance in public health, studied factors affecting skin colonization by staphylococci and are currently researching skin carriage of antibiotic-resistant staphylococci. He has published a substantial volume of high-quality material relating to veterinary dermatology. David has written 180 peer-reviewed publications ranging from the 'Incidence of cutaneous streptothricosis in Nigeria' in 1971 to a recent publication on whole-genome sequencing of *Staphylococcus pseudintermedius* isolates investigating the evolution of multidrug resistance. As part of this award, Professor Lloyd was invited to deliver the first Hugo Schindelka Memorial Lecture on a topic of his choice. Based upon his vast experience conducting research on bacterial skin diseases, he delivered 'Microbial interaction and disease control' on the opening day of the congress.

In addition to his academic achievements, Professor Lloyd has made major contributions to the growth of the specialty of veterinary dermatology in Europe and globally. In his nomination it was said by one of his colleagues, 'There cannot be a single significant development in veterinary dermatology in Europe over the past 30 years that he was not involved in and, in most cases, a driving force behind'. He is a founding member of the British Veterinary Dermatology Study Group, the European Society of Veterinary Dermatology, the European College of Veterinary Dermatology, the World Congress of Veterinary Dermatology Association (the forerunner of WAVD) and the Veterinary Wound Healing Association. David has held leadership positions in many of these organizations and is currently Treasurer and Administrative Committee Member of WAVD. In 2004, he served as President of the 4th World Congress of Veterinary Dermatology in Vienna, Austria. Perhaps his most important and lasting contribution is as founding editor of *Veterinary Dermatology*, the official journal of nine national and multinational dermatology organizations in its 27th year of publication.

As WAVD President, I had the honour of presenting the Hugo Schindelka Medal to Professor Lloyd during the Opening Ceremony of the 8th World Congress in June. He was recognized during the presentation, not only for all of his professional accomplishments but also by those who know him as being a true gentleman of our profession: generous with his time, wisdom and encouragement of others, a great listener, communicator and teacher, and a man of extraordinary integrity. I have never seen David so happy and overcome with emotion as during his acceptance of the medal. After the formal presentation there was an incredible outpouring of congratulations and good wishes from all of his colleagues at the ceremony, especially those he had mentored over many years at the RVC.

Perhaps the late Didier Carlotti summed up David's spirit and love of the specialty best as 'one of the group of friends, who used to travel across Europe to meet and share their passion, veterinary dermatology. They thought that friendship and brotherhood would sublimate their profound need to improve, to advance, and to promote and develop their discipline. They had to build up something, not for themselves, but indeed for their Profession and the Service to the Public'.

Thanks so much David! We look forward to sharing your passion for the worldwide advancement of veterinary dermatology for many years to come.

Kenneth W. Kwochka
President, WAVD

Hugo Schindelka Medal Presentation. Left to right: Kenneth Kwochka (WAVD President), David Lloyd (recipient), Richard Halliwell (Schindelka Committee Chair).

Part 1

Genetics and Genomics of the Skin and Skin Diseases

Endorsed by WAVD WORLD ASSOCIATION FOR VETERINARY DERMATOLOGY

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Genetic testing in veterinary dermatology

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Background – Molecular genetics has made significant advances in the analysis of hereditary dermatoses during the last several years.

Objectives – To provide an update on currently available genetic tests for skin diseases of dogs, cats and horses, and to aid the veterinary clinician in the appropriate selection and applications of genetic tests.

Methods – The scientific literature on the topic was critically reviewed. The list of known causative variants for genodermatoses and hair morphology traits was compiled by searching the Online Mendelian Inheritance in Animals (OMIA) database.

Results – Genetic testing has become an important diagnostic method in veterinary medicine. Genetic tests can help to establish the correct diagnosis in some diseases with relatively nonspecific signs. Genetic tests are also essential for sustainable breeding programmes and to help minimize the frequency of animals with hereditary diseases. Advances in genetic methodology and bioinformatics already allow genome-wide screening for potential disease causing mutations for research purposes. It is anticipated that this will become a routine process in clinical practice in the future.

Conclusion and clinical importance – As specific DNA tests and broad genome-wide analyses come into more common use, it is critical that clinicians understand the proper application and interpretation of these test results.

Introduction

Any trait exhibited by an organism including dermatological conditions is controlled by genetic factors and/or the environment ("nature or nurture"). Some traits are determined exclusively by the environment (e.g. a burn), whereas others are determined exclusively by genetic factors (e.g. ectodermal dysplasia). Most skin problems are influenced by a combination of genetic and environmental factors and consequently termed multifactorial or complex diseases (e.g. atopic dermatitis). Traits that are controlled exclusively by genetics very often show a monogenic mode of inheritance, which means that the genotype at a single gene determines whether the trait is expressed or not. Diseases of the skin that are largely controlled by genetics and typically follow a monogenic mode of inheritance are called genodermatoses. Genetic tests are currently most relevant for genodermatoses, but are likely to become increasingly important for complex diseases in the future. The following review summarizes the key principles of genetic testing for skin diseases. A conceptually similar, but more extensive review on genetic tests for neurological diseases contains additional information on this topic.[1]

Characteristics of hereditary diseases

In order to select appropriate DNA testing, the clinician must first recognize when the clinical signs suggest a hereditary disease. Each hereditary disease is unique, but some general features of a disease will raise suspicion of a hereditary cause. The hallmark of genodermatoses is familial clustering of cases. A typical example would be a litter of eight dogs with two affected and six nonaffected puppies. High incidence of a disease in one particular breed also suggests a hereditary basis for the disease. It does not necessarily reflect poor breeding practices. Inbreeding does not cause genetic disease *per se*, but makes the occurrence of recessive traits more likely. This could be a desirable trait that the breeder is trying to select for or an undesirable trait such as a genodermatosis. A popular sire inevitably will be a silent carrier of

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Conflict of Interest: The University of Bern holds a patent on genetic testing for hereditary nasal parakeratosis in Labrador retrievers. Royalties from this patent are paid to the University of Bern and Tosso Leeb

undesirable alleles as well as the desirable ones that made the animal popular. Unfortunately, this popular sire will not exclusively transmit the desirable alleles, but also his less favourable alleles to many offspring. However, it may take several generations before a recessive genetic disease is expressed phenotypically and recognized.

If a patient presents with a possible hereditary disease, references for breed associated diseases should be consulted.[2–4] If the disease has been associated with that breed, additional examination of the literature would be needed to determine how strong the evidence is for a hereditary basis and whether or not a causative variant has been identified. Thereafter, appropriate DNA testing can be selected if available.

Many hereditary diseases are congenital or have an early age of onset. Development of a complex organ such as the skin requires the precise coordination of many processes which are regulated by different genes. If one of these processes is altered by a genetic variation, then development may not proceed normally. The effects often will be apparent in the neonate. Unfortunately, neonatal diseases have received relatively little attention in veterinary medicine. Some breeders are unwilling to invest in veterinary care for neonates and often view losses or small litter size as something to be accepted. Neonatal diseases, however, can provide an ideal subject for gene mapping studies because the phenotype often is clearly discernible when affected and nonaffected littermates can be compared. Additionally, the entire family normally is available for DNA sampling when a phenotype is recognized in very young animals. Eliminating the disease not only prevents animal suffering, but can also decrease financial losses for the breeder.

Other hereditary diseases may have a more delayed onset of signs. Disorders of keratinocyte differentiation, such as hereditary footpad hyperkeratosis in the Kromfohrländer and Irish terrier, become clinically manifest only at a few months of age.[5] Renal cystadenocarcinoma and nodular dermatofibrosis in German shepherd dogs becomes clinically manifest in middle aged dogs.[6] It has to be kept in mind that the age of onset is independent from the question of whether a disease is heritable or not.

Advances in genetic research have made it possible to identify the causative variants responsible for many of these diseases and the numbers of characterized diseases are growing rapidly. To utilize DNA testing effectively, however, the clinician must be able to select the test and interpret results appropriately. This requires an understanding of the terminology used, the types of tests available and the potential pitfalls of DNA testing.

Genodermatoses with known causative genetic causes

In human medicine, more than 500 distinct genodermatoses and thousands of genetic variants in the more than 200 genes which cause these genodermatoses have been identified.[7,8] In veterinary medicine, the number of known causative genetic variants for genodermatoses is still relatively modest. Currently, 24 causative genetic variants for skin related traits are known in dogs (Table 1), eight in cats (Table 2) and five in horses (Table 3). The tables in this review include some morphological characteristics that are considered to be normal variation in domestic animals, but would be considered a genodermatosis in humans; for example, the ectodermal dysplasia seen in several hairless dog breeds.[9,10] Another breed defining characteristic, the ridge in Rhodesian and Thai ridgebacks, is now clearly recognized as being associated with dermoid sinuses. Although the ridge is inherited as a fully dominant trait with no phenotypic difference between homozygous mutant and heterozygous dogs, dermoid sinuses are much more frequent in homozygous mutant dogs.[11] Thus, a pragmatic approach for maintaining the ridge while simultaneously reducing the frequency of dermoid sinuses is the targeted breeding of heterozygous ridgebacks.

The list of known causative gene variants includes the *MLPH* variant for dilute coat colour in dogs (Table 1). Dilute coat colour is strongly associated with colour dilution alopecia in some breeds, such as the Doberman pinscher and the large Münsterländer.[12–14] Oculocutaneous albinism in the Doberman pinscher (white Doberman) is also included, because this coat colour is associated with an increased prevalence of cutaneous melanocytic neoplasms.[15]

Some other known coat colour variants in domestic animals are not included in Table 1, because they are not known to be correlated with skin diseases. However, in humans, many genetic variants leading to piebaldism or white spotting phenotypes would be considered pathogenic, whereas comparable alleles are positively selected for by the breeders in dogs,[16] cats,[17] horses[18,19] and many other domestic animal species. Some of the white spotting alleles are associated with an increased risk for deafness[19] and/or visual defects,[20] and some of them are lethal in the homozygous state.[18,19]

Semidominant variants in the *PMEL* gene are responsible for the merle coat colour in dogs[21] and the silver coat colour in horses,[22] but also cause severe eye defects in the homozygous state. Thus, selective breeding of animals for several special coat colours must be attempted with extra caution.

Genetic tests as a diagnostic tool in veterinary dermatology

A number of genodermatoses have now been associated with specific variants in domestic animals (Tables 1–3) and the number undoubtedly will increase as gene discovery becomes more and more efficient. DNA tests can be used like other diagnostic tests to help establish or eliminate differential diagnoses for a particular presenting case.

For example, if a Labrador retriever with crusts and possibly infected fissures of the nasal planum is presented, the genetic test for hereditary nasal parakeratosis (HNPK) is clearly indicated.[23] If this test is positive, the diagnosis is established and more invasive diagnostic procedures, such as taking a biopsy from the nose, can be avoided. Thus, it is recommended that clinical dermatologists should stay up-to-date with the ever expanding list of known causative genetic variants and available genetic tests.

Table 1. Genodermatoses and hair morphology traits with known causative genetic variants in dogs

Phenotype	Gene	Variant*	Breed	Inheritance	OMIA†	Reference
Dermoid sinus	*FGF3, FGF4, FGF19*	133 kb genomic duplication	Rhodesian ridgeback; Thai ridgeback	AR (complex)	000272-9615	11
Dilute coat colour (predisposing risk factor for colour dilution alopecia)	*MLPH*	c.-22G>A; r.spl (?)	Many breeds	AR (complex)	000031-9615	12
Ectodermal dysplasia	*FOXI3*	c.57_63dup7; p.A23 fs*219	Chinese crested; Mexican hairless dog; Peruvian hairless dog	ASD	000323-9615	9
Ectodermal dysplasia, anhidrotic	*EDA*	c.910-1G>A; r.spl	German shepherd dog	XR	000543-9615	27
Ectodermal dysplasia/skin fragility syndrome	*PKP1*	c.202+1G>C; r.spl	Chesapeake Bay retriever	AR	001864-9615	28
Epidermolysis bullosa, dystrophic	*COL7A1*	c.5716G>A; p. G1906S	Golden retriever	AR	000341-9615	29
Epidermolysis bullosa, junctional	*LAMA3*	6.5 kb insertion	German pointer	AR	001677-9615	30
Excessive skin and periodic fever	*HAS2*	16.5 kb genomic duplication	Shar-pei	ASD	001561-9615	31
Footpad hyperkeratosis	*FAM83G*	c.155G>C; p.R52P	Irish terrier; Kromfohrländer	AR	001327-9615	5
Hair morphology: Curly hair	*KRT71*	c.451C>T; p. R151W	Many breeds	AR (?)	000245-9615	32
Hair morphology: Furnishings (wire hair)	*RSPO2*	167 bp insertion into 3′-UTR	Many breeds	AD	001531-9615	32
Hair morphology: Long hair	*FGF5*	c.284G>T; p.C95F	Many breeds	AR	000439-9615	33
Hair morphology: Long hair	*FGF5*	c.556_571del16; p. A186Tfs*69	Eurasier	AR	000439-9615	34
Hair morphology: Long hair	*FGF5*	c.559_560dupGG; p.R188Afs*73	Afghan hound	AR	000439-9615	34
Hair morphology: Long hair	*FGF5*	c.578C>T; p.A193V	Akita Inu; Samoyed; Siberian husky	AR	000439-9615	34
Hair morphology: Long hair	*FGF5*	c.362-11T>A; r.spl	Afghan hound	AR	000439-9615	34
Hyperkeratosis, epidermolytic	*KRT10*	c.1125+1G>T; r.spl	Norfolk terrier	AR	001415-9615	35
Ichthyosis	*PNPLA1*	c.1445_1447delins TACTACTA; p. N482Ifs*11	Golden retriever	AR	001588-9615	24
Ichthyosis	*NIPAL4*	variant identified, but not yet published	American bulldog	AR	001980-9615	36
Ichthyosis	*SLC27A4*	c.1250G>A; p. Arg417Gln/r.spl	Great dane	AR	001973-9615	37
Ichthyosis	*TGM1*	1980 bp LINE-1 insertion	Jack Russell terrier	AR	000546-9615	38
Keratoconjunctivitis sicca and ichthyosiform dermatosis	*FAM83H*	c.977delC; p. P326Hfs*258	Cavalier King Charles spaniel	AR	001683-9615	25
Musladin-Lueke syndrome (geleophysic dysplasia)	*ADAMTSL2*	c.661C>T; p.R221C	Beagle	AR	001509-9615	39
Nasal parakeratosis	*SUV39H2*	c.972T>G; p.N324K	Labrador retriever	AR	001373-9615	23
Oculocutaneous albinism (predisposing risk factor for melanocytic neoplasms)	*SLC45A2*	4.1 kb deletion	Doberman	AR	001821-9615	15
Renal cystadenocarcinoma and nodular dermatofibrosis	*FLCN*	c.764A>G; p.H255R	German shepherd dog	AD	001335-9615	40

AD, autosomal dominant; AR, autosomal recessive; ASD, autosomal semi-dominant; XR, X-chromosomal recessive.

*A detailed description of genetic variant nomenclature can be found at http://www.hgvs.org/mutnomen/. For some large genomic insertions a simplified variant designation is given.

†Online Mendelian Inheritance in Animals, http://omia.angis.org.au/.

In humans, there is a high degree of genetic heterogeneity. Consequently, unrelated patients with the same genodermatosis due to the altered function of a single gene are likely to have different independent variants in the causative gene. Genodermatoses in purebred animals are much less heterogeneous than in humans. Thus, for many hereditary diseases we find that all affected individuals of one breed carry the same deleterious genetic variant. However, this is not an absolute rule and it should be kept in mind that the genetic tests currently offered typically interrogate only a single position in the genome. Therefore, a positive test result clearly establishes the diagnosis, whereas a negative test result only excludes one particular genetic defect, but not other unknown vari-

Table 2. Genodermatoses and hair morphology traits with known causative genetic variants in cats

Phenotype	Gene	Variant*	Breed	Inheritance	OMIA†	Reference
Hairlessness with short life expectancy	*FOXN1*	c.1030_1033delCTGT; p.L344Gfs*203	Birman	AR	001949-9685	41
Hairlessness	*KRT71*	c.816+1G>A; r.[816+1_816+43ins; 816+1g>u]	Sphynx	AR	001583-9685	42
Hair morphology: Curly hair (rex phenotype)	*KRT71*	c.1108-4_1184del81ins AGTTGGAG; r.1108_1221del	Devon rex	AR	001581-9685	42
Hair morphology: Curly hair (rex phenotype)	*KRT71*	c.445-1G>C; r.445_464del	Selkirk rex	AD	001712-9685	43
Hair morphology: Curly hair (rex phenotype)	*LPAR6*	c.250_253_delTTTG; pF84Efs*9	Cornish rex; German rex	AR	001684-9685	44
Hair morphology: Long hair	*FGF5*	c.356insT; p.M119Ifs*43	Maine coon; ragdoll	AR	000439-9685	45
Hair morphology: Long hair	*FGF5*	c.406C>T; p.R136*	Norwegian forest	AR	000439-9685	45
Hair morphology: Long hair	*FGF5*	c.474delT; p.F158Lfs*104	Ragdoll	AR	000439-9685	45
Hair morphology: Long hair	*FGF5*	c.475A>C; p.T159P	Many breeds	AR	000439-9685	45

AD, autosomal dominant; AR, autosomal recessive.

*A detailed description of genetic variant nomenclature can be found at http://www.hgvs.org/mutnomen/.

†Online Mendelian Inheritance in Animals, http://omia.angis.org.au/.

Table 3. Genodermatoses with known causative genetic variants in horses

Phenotype	Gene	Variant*	Breed	Inheritance	OMIA†	Reference
Ehlers–Danlos syndrome ("HERDA")	*PPIB*	c.115G>A; p.39G>R	Quarter horse	AR	000327-9796	46
Epidermolysis bullosa, junctional	*LAMA3*	6.6 kb genomic deletion	American saddlebred	AR	001677-9796	47
Epidermolysis bullosa, junctional	*LAMC2*	c.1368C[5]>[6]; p.R458Pfs*27	Belgian draft horse; Trait Breton; Trait Comtois	AR	001678-9796	48
Hoof wall separation syndrome	*SERPINB11*	c.504_505insC; p.T169Hfs*3	Connemara pony	AR	001897-9796	26
Incontinentia pigmenti	*IKBKG*	c.184C>T; p.R62*	Quarter horse; warmblood	XSD	001899-9796	49

AR, autosomal dominant; XSD, X-chromosomal semi-dominant.

*A detailed description of genetic variant nomenclature can be found at http://www.hgvs.org/mutnomen/. For a large genomic rearrangement a simplified variant designation is given.

†Online Mendelian Inheritance in Animals, http://omia.angis.org.au/.

ants which may very well be located in the same gene. Negative genetic test results therefore must be interpreted with care. For the same reason, if a genetic test has been validated in a particular breed, it should not be generally assumed that the test will also work in other breeds. Again, a positive test result is diagnostic, but a negative test result does not eliminate the possibility that a different genetic variant in the same gene may be causative for the disease in this different breed. In cases in which genetic testing is negative, but a hereditary disease is nevertheless suspected, it may be valuable to consult with a veterinary geneticist, ideally the one who was involved in the identification of the first causative variant.

Research perspectives for genetic conditions in dermatology

Although an individual genetic disease may be rare and can be expected to become more uncommon as DNA testing is accepted by breeders, identifying the causative mutation can also shed light on the pathogenesis of more common, acquired diseases. It may also provide new insights into skin biology and, potentially, even skin care, which may be relevant for therapy. The recognition of the gene responsible for a hereditary disease may identify a molecular pathway that is important to the normal function and homeostasis of the skin. Several mutant genes of this type have been identified in domestic animals. The *PNPLA* gene mutation responsible for ichthyosis in golden retrievers was first discovered in dogs and only later was it discovered that genetic variants in this gene are also responsible for a subset of the human ichthyosis cases.[24] Other examples of gene functions that were initially discovered through the study of dog mutants include *FAM83G* (proliferation of the palmoplantar epidermis and hair morphology),[5] *FAM83H* (hair morphology),[25] *FOXI3* (ectodermal development)[9] and *SUV39H2* (differentiation of the nasal epidermis).[23] The identification of the causative variant in the Connemara pony hoof wall separation

defect established that *SERPINB11* has an essential function for hoof integrity, which may actually be a horse specific function of this particular gene.[26]

It has now become technically feasible to sequence the entire genome of a patient at affordable costs. Together with other genetic tools this greatly facilitates the identification of the causative genetic variants underlying heritable phenotypes. This required relatively large sample cohorts in the past, but technological advances now allow for the investigation of single families or even isolated cases. Thus, it is strongly recommended that a veterinary geneticist be consulted if any new genodermatosis is suspected based on clinical observations.

Conclusion

Advances in genomic research will continue to change the way veterinary medicine is practiced. As with any advance, the implementation into routine clinical practice of genomics and personalized medicine will likely require time and may still pose some unforeseen difficulties. Understanding the basic principles of genomics as they apply to DNA testing will be essential for the practicing veterinarian to fully capitalize on these advances and avoid potential pitfalls.

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Epidermolysis bullosa simplex in sibling Eurasier dogs is caused by a *PLEC* non-sense variant

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Background – Plectin, a large linker protein found in many tissues, acts to connect components of the cytoskeleton to each other. In the epidermis, plectin binds keratin intermediate filaments to hemidesmosomes. A deficiency of plectin in the skin leads to blister formation in the basal layer and the disease epidermolysis bullosa simplex (EBS).

Hypothesis/Objectives – To describe a novel blistering disease that arose spontaneously in a litter of puppies.

Animals – Two female and one male 20-day-old Eurasier puppies, from a litter of six, were presented for evaluation of failure to thrive and then euthanized due to poor prognosis. The puppies had ulcers on the lips, tongue, nasal planum, paw pads and abdomen.

Results – Immunolabelling on frozen skin for basement membrane proteins revealed patchy and weak to absent staining for plectin as compared with strong linear staining in normal dogs. Ultrastructurally, hemidesmosomes were irregularly shaped and had loss of distinction between inner and outer plaques. Pedigree analysis supported an autosomal recessive mode of inheritance. A premature stop codon was discovered in exon 27 of *PLEC* that resulted in the production of a severely truncated protein.

Conclusion – The study describes the first documented spontaneous EBS associated with a *PLEC* variant in domestic animals.

Introduction

Epidermolysis bullosa (EB) is the name given to a group of blistering diseases that manifest as loss of epidermal–dermal integrity.[1,2] The phenotypic changes are due to abnormalities in specific structural proteins within the epidermis and basement membrane zone. Characterization of EB is further subdivided into four major types based on the location of the subcellular defect. EB simplex (EBS) is the most superficial form and involves proteins in the cytoskeleton of basal or suprabasal keratinocytes. In junctional EB (JEB), blisters arise in the lamina lucida, whereas in dystrophic EB (DEB) the defect occurs in the superficial dermis at the level of anchoring fibrils. Kindler syndrome is a mixed pattern that has not been described in domestic animals.[1,2] Light microscopy is generally unable to differentiate the subtypes of EB and some types of EB have undergone extensive reclassification. The current approach to classification of EB in humans is an "onion skinning" method based on (i) subcellular location of the blister, (ii) clinical features, (iii) heritability and (iv) identification of the gene involved by immunohistochemical and mutational analysis.[2]

In EBS, the cleavage plane lies within the epidermis and may involve basal keratinocytes (basal EBS) or keratinocytes within the middle to upper layers of the epidermis (suprabasal EBS). Proteins involved in basal EBS include keratins 5 and 14, plectin, BPAG1e (BP230), exophilin 5 and kindling 1. Suprabasal EBS may involve transglutaminase 5 in the upper epidermis, or plakoglobin, plakophilin 1 and desmoplakin in the middle epidermis. The clinical subtypes relate to extent of lesions (e.g. generalized severe EBS versus localized EBS) or the presence of concurrent conditions (e.g. EBS with mottled pigmentation, EBS with muscular dystrophy).[2,4–6]

In humans, three subsets of plectin-associated EBS have been identified: EBS with muscular dystrophy (EBS-MD OMIM # 226670), EBS with pyloric atresia (EBS-PA OMIM #612138) and EBS-Ogna. OMIM #131950). EBS-MD and EBS-PA are autosomal recessive and have skin lesions with abnormalities in other organs. EBS-Ogna is autosomal dominant and characterized by relatively mild blistering without lesions in other organs.[4–6]

Plectin, which is encoded by *PLEC,* is a large protein found in many tissues (skin, bone, muscle and nervous

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system); it links components of the cytoskeleton (e.g. actin microfilaments, microtubules, intermediate filaments) to the cell membrane.[5,7,8] In basal keratinocytes, plectin and BPAG1e are the main components of the inner plaque of hemidesmosomes. Although all major forms of EB have been identified in domestic animals,[9] this study documents the first spontaneous EBS associated with deleterious variant of plectin.

Materials and methods

Two female and one male 20-day-old Eurasier puppies, from a litter of six with an asymptomatic dam and sire, presented to the Matthew J. Ryan Veterinary Hospital of the University of Pennsylvania for failure to thrive. The puppies were underweight and approximately 50% smaller than the normal littermates. Physical examination revealed widespread ulcers on the lips, tongue, nasal planum, paw pads and abdomen with a positive Nikolskiy sign (Figure 1). The puppies were diagnosed with probable JEB and were humanely euthanized due to poor prognosis. At the time of death, peripheral blood was obtained for DNA analysis and skin samples were fixed in modified Karnosky's fixative for ultrastructural analysis and snap frozen in liquid nitrogen for immunostaining and RNA analysis.

Skin samples for conventional transmission EM (TEM) were processed by standard techniques to evaluate the basal layer and basement membrane zone (BMZ).[10]

Immunostaining for basement membrane proteins was performed to identify a defect in protein expression. Briefly, five micrometer frozen sections were cut from perilesional skin of the oral cavity (tongue or lip), pawpad and haired skin of the three EB-affected dogs. Sections were stained with a panel of antibodies specific for basement membrane proteins (BPAG1e, integrin alpha6 and beta4, collagen XVII, laminin 332 and collagen VII) as described previously[11] with the addition of a rabbit polyclonal plectin antibody at dilution 1:100 (Table S1). All samples were stained for each individual protein on the same day to avoid any variability between assays. The pattern and intensity of staining were compared to those of normal controls.

Genomic DNA was extracted from EDTA blood obtained from all members of the family. Because there is no curated canine *PLEC* transcript or gene reference sequence available (RefSeq data from the National Center for Biotechnology Information, US National Library of Medicine, Bethesda, MD, USA), exons were determined by examination of the CanFam 3.1 reference genome assembly [NCBI assembly/GCF_000002285.3 (Broad CanFam3.1)] using the UCSC Genome Browser (http://genome.ucsc.edu/), including the Broad Institute CanFam3 Improved Annotation Data v1, which contains additional SNP and RNAseq data, including RNAseq from skin.

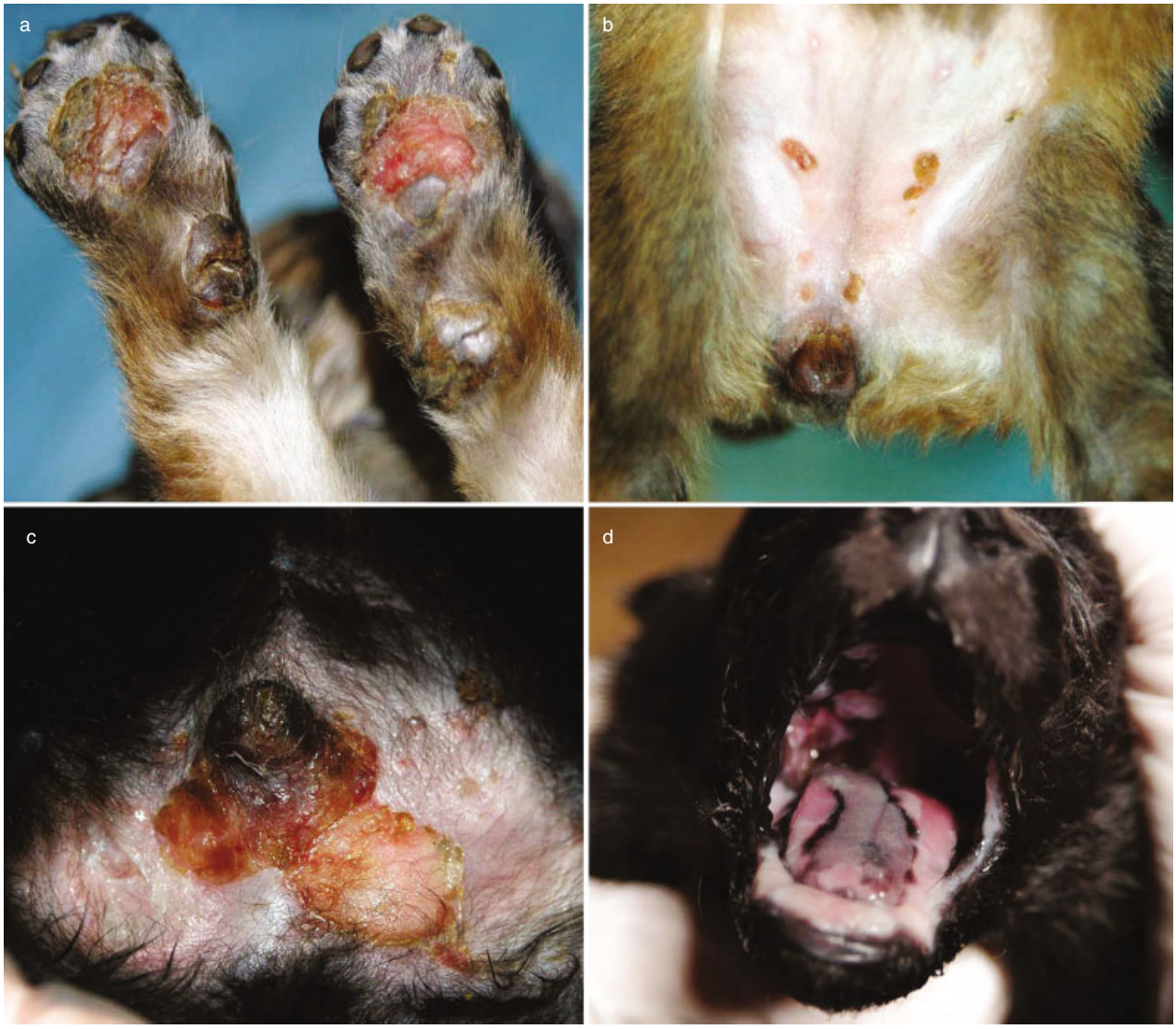

Figure 1. Clinical images; Male and female 20-day-old Eurasier puppies with epidermolysis bullosa simplex. (a) Large ulcers on the paw pads, (b) inguinum and vulva, (c) prepuce and peripreputial skin, and (d) sloughing of the oral mucosa on the tongue.

Exons and the intron–exon boundaries of the canine *PLEC* gene were sequenced.

The human *PLEC* gene encodes several isoforms that differ primarily by the use of different first exons, which are followed by the same 31 additional exons to encode a protein of 4,684 amino acids for the most common isoform (RefSeq NM_201380). The canine orthologue of this transcript is encoded by 32 exons and codes for a protein of 4,686 amino acids that is 92.6% identical to the human protein. Primers (Table S2) for amplification of the canine exons were designed using commercially available software (DNAStar Inc.; Madison, WI, USA). Sequence data were analysed using Lasergene software (DNAStar Inc.) and the sequences compared to the respective canine *PLEC* region from the CanFam 3.1 reference sequence.

Results

Postmortem examination abnormalities were confined to the haired and nonhaired skin and oral cavity; significant lesions were not apparent in other organs including the gastrointestinal tract and skeletal muscle. Microscopic examination of lesions skin revealed clefts, vesicles and broad areas of epithelial detachment in both the haired skin and mucous membranes (lips, tongue, oral cavity, genitalia) with ulcers and fibrinosuppurative inflammation (Figure 2a). Periodic acid Schiff staining revealed weakly positive stain uptake on the floor of the blisters (Figure 2b).

Transmission EM showed the lamina densa on the floor of the cleavage. Hemidesmosomes were located on the roof of the cleavage. The hemidesmosomes had an electron dense conical shape that attached to thickened keratin intermediate filaments. A loss of distinction was seen between inner and outer plaques (Figure 3).

Indirect immunofluorescence for basement membrane proteins revealed patchy and weak to absent plectin staining as compared with strong linear staining in normal dogs (Figure 4); other stains were unremarkable. A microscopic cleft was seen in biopsies from two of the three Eurasier puppies and, when present, cleavage was located above the stains for laminin 332 and collagen VII (not shown). These anomalies, which were restricted to sections stained for plectin, suggested that the *PLEC* gene encoding plectin might harbour a DNA variant leading to a defective expression of this protein.

A homozygous non-sense variant was identified in the affected puppies. The variant was located in exon 27 and predicted to truncate more than 70% of the open reading frame. A restriction fragment length polymorphism (RFLP) assay was developed to screen for the variant. DNA was amplified and digested with Bmt*I* restriction enzyme (Table S3), which digests DNA when the disease-associated allele is present. The parents of the affected puppies were heterozygous for this non-sense variant and a clinically normal littermate was homozygous for the wild-type sequence (Figure 5a).

In order to verify that the variant found was not a polymorphism found in the general dog population, DNA was sampled from five different breeds (German shorthair pointer, American bulldog, Irish wolfhound, bull terrier and mixed breed; 25 dogs each). All 125 dogs were homozygous for the reference allele (G/G). Several silent and missense variants in *PLEC* were also detected in DNA from the affected animal; many have been previously identified (Table S3).

Discussion

Plectin is an ubiquitous linker protein that serves to connect components of the cellular cytoskeleton to proteins in the skin, nervous system and skeletal muscle. The protein comprises four major domains: the amino-terminal or calponin homology domain, the plakin domain, the rod domain and the carboxy terminal domain;[4–6,8,12] the different isoforms (i.e. 1a–g) correlate with tissue specificity (e.g. 1a and c for epidermis; 1d for skeletal muscle).[4–6] Plectin 1a is the only isoform known to bind to the beta unit of the integrin α6β4 via the plakin domain, adjacent to the amino terminus, and to keratin intermediate filaments via its carboxy terminal domain. Therefore, plectin serves as a vital structural component of the BMZ; as a bridge between the inner cytoskeleton to the basal lamina.[5,6]

The importance of plectin in the BMZ was illustrated in the three Eurasier dogs in which a homozygous G to A variant in the *PLEC* gene resulted in the conversion of a tryptophan to a premature stop codon in exon 27. This resulted in a truncated 1,315 amino acid protein (normal 4,686 amino acids) and thus the loss of a large portion of

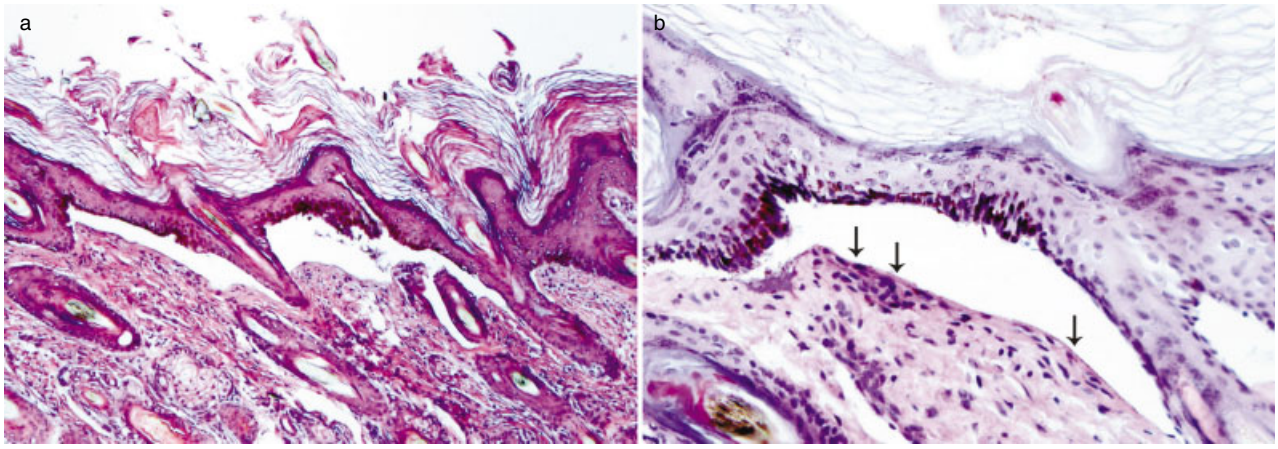

Figure 2. Photomicrographs of haired skin from Eurasier dog with epidermolysis bullosa simplex. (a) Note the large subepidermal vesicle. Haematoxylin and eosin 4×. (b) Weakly positive periodic acid Schiff staining (arrow) on the floor of the blister. 10×.

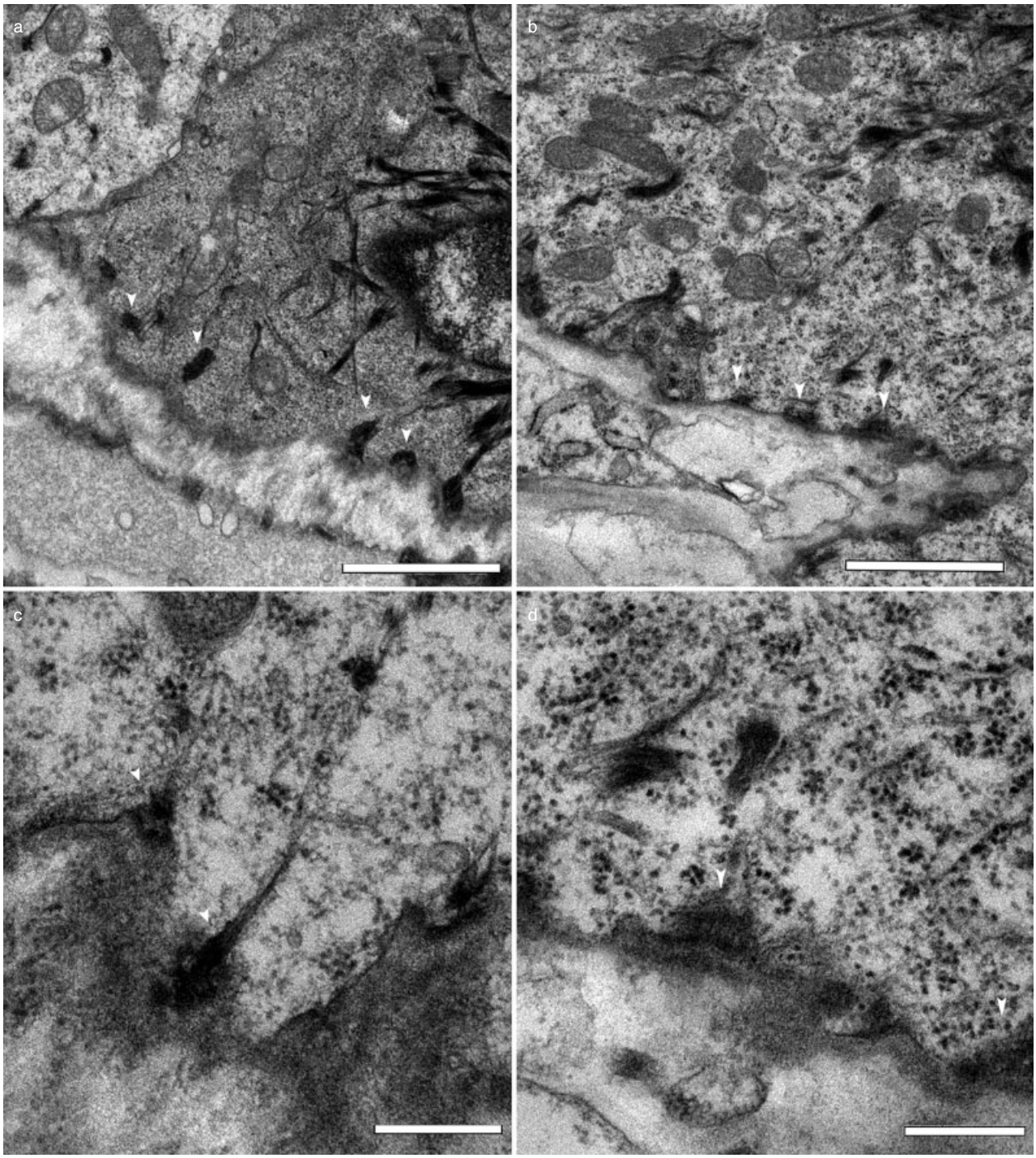

Figure 3. Electron microscopy of skin from Eurasier dog with epidermolysis bullosa simplex. (a) Eurasier puppy with separation in the basement membrane zone. Note the electron-dense hemidesmosomes (arrowheads) as compared with hemidesmosome in an aged matched control dog (b). Bar = 2 μm. (c) At higher magnification the hemidesmosomes are cone shaped (arrowheads) with loss of distinction between inner and outer plaques. (d) Normal hemidesmosomes from an age-matched control dog showing distinct inner and outer plaques (arrowheads). Bar = 0.5 μm

the rod domain and, more importantly, the C terminus that would bind to the keratin intermediate filaments. In this family of dogs, review of the pedigree, together with the mutational analysis, confirmed an autosomal recessive mode of inheritance.

Autosomal recessive, basal forms of EBS in humans include EBS with muscular dystrophy (EBS-MD) and EBS with pyloric atresia (EBS-PA). *KRT5*, *KRT14*, *BPAG1* and *PLEC* are the genes most commonly affected in cases of basal EBS in humans; variants in *PLEC* only account for about 8% of the cases.[13] A number of variants in human *PLEC* have been deemed responsible for EBS and the location of the variant will dictate the severity of disease.[14,15] EBS-MD is characterized by mild to subtle blistering and nail dystrophy early in life with variable onset muscular weakness (i.e. congenital to as late as the 4th

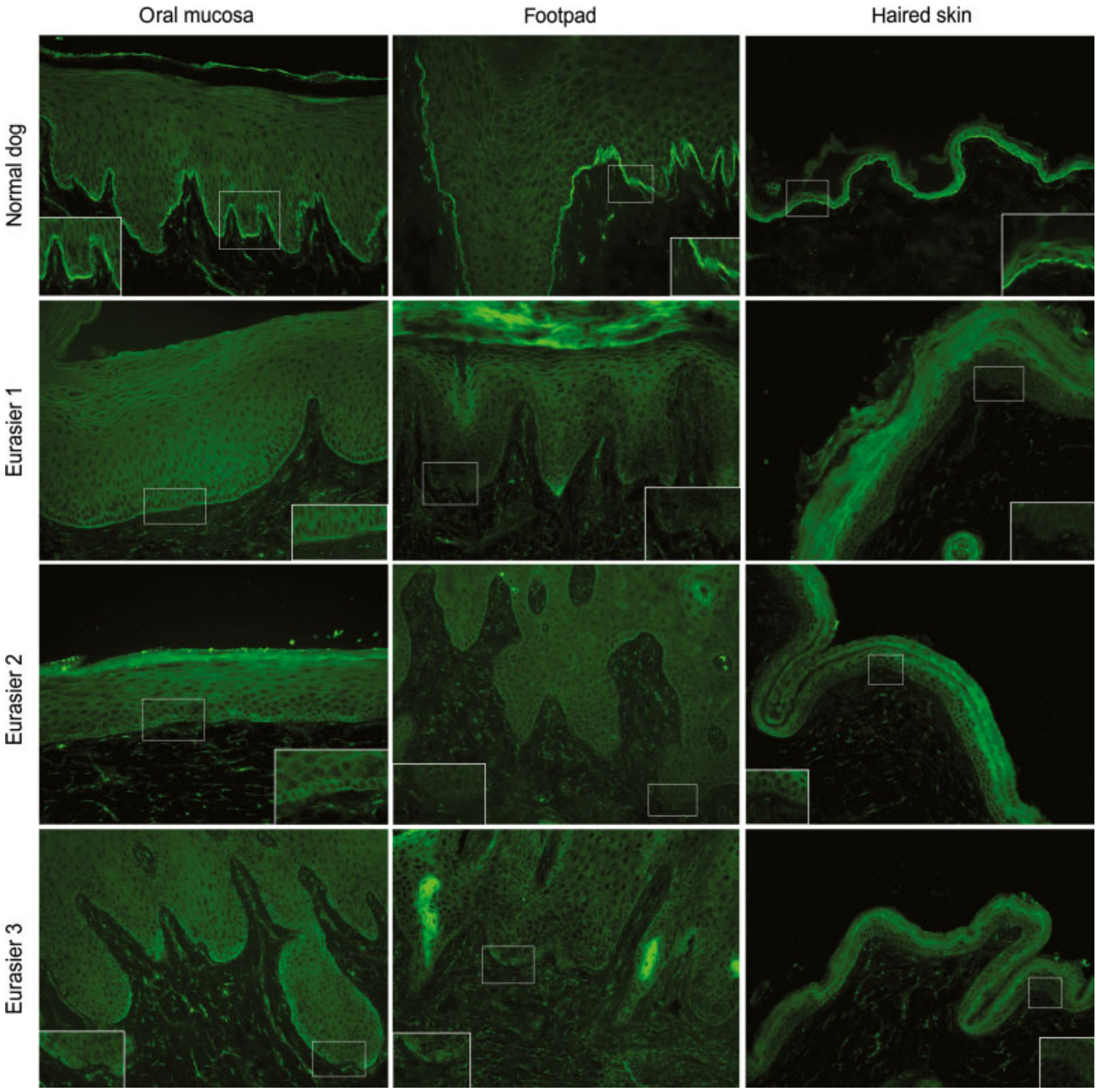

Figure 4. Indirect immunofluorescence staining for plectin in epidermolysis bullosa (EB). Samples from the oral cavity, pawpad and haired skin from a normal dog and three EB-affected Eurasier puppies were immunostained for plectin on the same day. Whereas the staining for plectin was strong, linear and continuous at the basement membrane zone, that of the three Eurasier puppies was very faint and often patchy or absent. 20×

decade). EBS-PA has a more severe congenital phenotype (often lethal) with severe skin blistering and pyloric or duodenal atresia. It has been hypothesized that the pyloric stenosis is related to localized blistering and chronic inflammation.[12] Like the Eurasier puppies, the severe EBS-PA phenotype may result from plectin variants that affect the rod and carboxy domain and thereby impair binding to keratin and possibly to integrin β4 as an alternative binding site.[4,5]

In the affected dogs, the hemidesmosomes were abnormally electron dense and globular to ovoid with thickening of the connecting tonofilaments. The thickening of keratin may represent a compensatory change due to the truncated protein and failure to cross-link. Furthermore, immunofluorescence demonstrated a marked decrease to absence in plectin staining. This finding led to the discovery of the candidate gene. However, based on the C-terminal location of the epitope in the polyclonal antibody, one would predict a complete lack of plectin staining. The light patchy staining is most likely explained by cross-reacting amino acid sequence homology with other basement membrane proteins.

Based on light microscopy and ultrastructural clefting, EB in this group of Eurasier puppies was initially classified as JEB, and the more severe form, Herlitz (now termed JEB generalized severe). Identification of the *PLEC* variant led to the appropriate categorization as basal EBS: (i) the subcellular location of the defect lies in the basal keratinocyte; (ii) clinical features were generalized; (iii) the disorder was shown to have an autosomal recessive

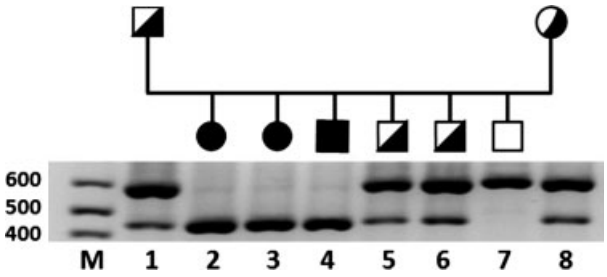

Figure 5. Pedigree and restriction fragment length polymorphism (RFLP) analysis. A PCR product of 606 bp was generated covering portions of exon 26 and 27 that contain the G>A non-sense polymorphism. The product is cleaved by Btm*I* in the presence of the variant (177 and 429 bp, respectively) but not in the wild-type sequence. Squares are males and circles females. Filled in symbols are affected animals (puppies 2, 3 and 4); half-filled symbols represent heterozygotes (sire 1 and dam 8; puppies 5 and 6); and empty symbols are homozygote normal animals (puppy 7). The gel depicts a homozygous wild-type, uncut band (7), homozygous affected, cut bands (2, 3 and 4), and heterozygotes, cut and uncut bands (1, 5, 6 and 8). The 177 bp fragment is not shown. M is100 bp ladder.

mode of inheritance; and (iv) the variant was discovered in *PLEC* and resulted in perturbation of the target protein plectin. This form of EBS is not characterized by cytolysis of basal keratinocytes as seen in EBS due to variants of keratin 5 and 14.[2] Light microscopy is unable to distinguish severe JEB from basal plectin-associated EBS.

The Eurasier puppies had severe lesions confined to the skin and oral cavity as documented on complete postmortem examination. Weakening of the dermo-epidermal integrity lead to blistering and skin sloughing in areas prone to frictional trauma (e.g. oral cavity, genitalia, paw pads). Sloughing of the oral mucosa may have led to nutritional deprivation as evidenced by the small size as compared to the littermates. It is unknown if the dogs eventually would have developed either PA or MD, as they were humanely euthanized at 20 days of age.

Treatment of the various forms of EB is similar: wound care, preventing and treating infection, as well as supportive care including pain management and nutritional support. A few clinical trials have been performed or are underway in humans.[3] This is the first canine model of EBS in which a variant in *PLEC* has been demonstrated. Studies herein may advance the understanding of *PLEC* and EBS pathogenesis and provide an avenue to explore new therapies such as topical protein replacement, viral vector gene therapy, small interfering (si)RNAs or gene editing by Clustered Regularly Interspaced Short Palindromic Repeats (referred to as CRISPR).

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Supporting Information

Additional Supporting Information may be found in the online version of this text at: http://onlinelibrary.wiley.com/doi/10.1111/vde.12394/full

Table S1. Antibodies used for indirect immunofluorescence for basement membrane proteins.
Table S2. Primers and restriction fragment lengths for detection of disease-associated *PLEC* non-sense variant.
Table S3. Single nucleotide polymorphisms identified in *PLEC* exons by sequencing DNA from an affected Eurasier dog.

Part 2

The Skin as an Immune Organ – Allergy

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2.1

A review of the roles of keratinocyte-derived cytokines and chemokines in the pathogenesis of atopic dermatitis in humans and dogs

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Background – Dysfunction of the physical and chemical barriers of the skin may play roles in the pathogenesis of atopic dermatitis (AD) by facilitating penetration of antigens through the skin and consequently evoking aberrant immune reactions. It is now emerging that keratinocytes are actively involved in cutaneous immune reactions by producing various soluble factors initiated by inflammatory stimuli, including mechanical injury or activation of Toll-like receptors and protease-activated receptors. Among the soluble factors, keratinocyte-derived cytokines and chemokines skew Type 2 helper T (Th2) cell-dominant immune reactions, with the recruitment of Th2 cells.

Objective – To review the roles of keratinocyte-derived cytokines and chemokines in the pathogenesis of AD in humans and dogs.

Conclusion and clinical importance – Keratinocyte-derived cytokines such as thymus and activation-regulated chemokine, granulocyte-macrophage colony stimulating factor, thymic stromal lymphopoietin and interleukin-33 are involved in the pathogenesis of human AD and possibly in canine AD. These cytokines and chemokines may possibly be used as subjective clinical markers and therapeutic targets for both human and canine AD.

Introduction

Canine atopic dermatitis (cAD) shares many clinical characteristics with its counterpart in humans, such as the presence of genetic predisposition, the early age of onset, the predilection sites of the affected skin, the association of epidermal barrier defects, the frequent colonization by *Staphylococcus* spp. and the elevated serum Immunoglobulin E (IgE) against environmental allergens.[1] Accumulated evidence suggests that cAD is generally considered to be a Type 2 helper T (Th2)-associated inflammatory disease, as well as human atopic dermatitis (AD).[2–8] In human AD, keratinocytes have been shown to produce various soluble factors inducing Th2-associated inflammation in response to a variety of stimuli such as mechanical injury, allergens and bacteria. Thymic stromal lymphopoietin (TSLP) produced by keratinocytes acts as a master switch for the allergic inflammation by eliciting the differentiation of naïve T cells into Th2 cells via dendritic cells.[9] Furthermore, keratinocytes enhance the production of Th2 cytokines including IL-5 and IL-13 from Th2 cells by producing IL-33.[10] On the basis of these studies, keratinocyte-derived cytokines and chemokines are critical to skew the Th2 cell-dominant immune reaction with a recruitment of Th2 cells in the pathogenesis of AD. Therefore, findings focused on the biological immune function of keratinocytes may provide new paradigms for the treatment of both human and canine AD.

In this review, to understand the current concept on the role of keratinocyte-derived cytokines and chemokines in the immunopathogenesis of AD, we summarize studies describing human AD followed by the findings in cAD.

Filaggrin

Humans

The skin comprises three different structures, the epidermis, dermis and panniculus adiposus, and protects the body by providing a physical, chemical and immunological barrier. The stratum corneum, the outermost layer of the epidermis, is formed by corneocytes that have been derived from apoptosis of keratinocytes. Keratin and lipids in the stratum corneum play important roles in this first physical barrier. Keratin is bundled by filaggrin to form a dense protein–lipid matrix.[11] It has been shown that skin barrier dysfunction due to mutations in filaggrin is a major pathogenic factor of human AD.[12]

Dogs

In dogs with cAD, decreased ceramide content[13–15] and abnormal stratum corneum ultrastructure[16,17] suggest that skin barrier dysfunction is similar to that of humans with AD. Previous studies have indicated a possible association of filaggrin with skin barrier dysfunction in dogs

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with AD;[18–20] however, direct evidence remains to be demonstrated.

Tight junctions

Humans

Tight junction proteins, such as claudins and occludins, regulate the attachment of keratinocytes and the transition of molecules by forming the second physical barrier. In humans with AD, expression of claudin-1 was shown to be reduced and inversely correlated with Th2 biomarkers.[21] Furthermore, identification of *claudin-1* SNPs revealed its association with AD in North American populations.[21] These results suggest that impairments in tight junctions due to a reduction of claudin-1 contribute to dysfunction of the second physical barrier in humans with AD.

Dogs

In an experimental cAD model it was shown that the expression level of claudin-1 was reduced in nonlesional skin compared with normal skin.[22] In another study of cAD, immunohistochemical analyses demonstrated decreased expression in zonula occludens-1 and occludin, but not claudin-1.[23] Although there have been no studies investigating the expression levels of proteins at tight junctions in naturally occurring cAD, previous results from experimental cAD models suggest that dysfunction of the second physical barrier may also exist in cAD, similar to human AD.

Antimicrobial peptides

Humans

Antimicrobial peptides derived from keratinocytes, such as cathelicidins, defensins and S100, play important roles in the chemical barrier function against various types of microorganisms. An immunohistochemical study demonstrated that expression of cathelicidins and beta-defensin-2 was decreased in both acute and chronic skin lesions of human AD compared with those in psoriasis.[24] In the same study, however, there were no significant differences on the expression of cathelicidins and beta-defensin-2 between AD and healthy.[24] A study using quantitative reverse transcription PCR (RT-qPCR) reported lower transcription levels of *beta-defensin-2* in humans with AD compared with patients with psoriasis.[25] The lower expression of antimicrobial peptides reported in these studies suggests that a deficiency in the expression of antimicrobial peptides may account for the susceptibility of skin infection by *Staphylococcus aureus* in humans with AD.

Dogs

Because recurrent staphylococcal skin infection or colonization is common in cAD, it is plausible that the reduced expression of antimicrobial peptides may also be involved in the pathogenesis of cAD. An initial study using RT-qPCR showed that transcription levels of *beta-defensins-1, -2, -3* and *cathelicidin* in cAD were higher than those in healthy dogs.[26] Another RT-qPCR study demonstrated that transcription levels of *beta-defensins-1, -103* and *-122* in skin lesions of cAD were lower than those in normal skin.[27] However, in the same study, there were no differences in transcription levels of *beta-defensins-1, -103* or *-122* observed between cAD and other inflammatory skin diseases, suggesting that the reduced transcription may be attributed to inflammation, but does not predispose patients to bacterial colonization or infection.[27] Finally, a similar study reported that no significant differences were found in the transcription levels of *beta-defensins-1* and *-103* in healthy, noninfected atopic or infected atopic skin.[28] Interestingly, another study has reported significantly higher transcription of *beta-defensin-103* in dogs with cAD than healthy dogs.[29] Discrepancies in canine results suggest that further studies, such as immunohistochemical analyses, are necessary to clarify whether the role of antimicrobial peptides in cAD differs from that in human AD.

Cutaneous immune reactions

Dysfunction of the physical and chemical barriers in the skin facilitates skin penetration of antigens, evoking local immune reaction. In the skin, dendritic cells and keratinocytes are strongly associated with cutaneous immune reaction. Dendritic cells are antigen-presenting cells that activate T cells, several types of which can be found in the skin, classified by the expression of specific surface molecules. It is known that dendritic cells in the skin play essential roles in polarizing helper T (Th) cells to either Type 1 helper T (Th1) or Type 2 helper T (Th2) cells. For polarizing the appropriate subset of Th cells, dendritic cells need to be activated by cytokines and chemokines derived from keratinocytes. Keratinocytes express various types of receptors to sense invading pathogens, which include Toll-like receptors (TLRs) and protease-activated receptors (PARs). Human keratinocytes express all TLRs except for TLR-7 and -8,[30–34] indicating an ability to recognize constituents of microorganisms, such as bacterial lipopeptides, peptidoglycan and flagellin, lipopolysaccharides (LPS), single- and double-stranded RNA and unmethylated cytosine-phosphate-guanine (CpG) oligonucleotides of bacterial DNA. In dogs, canine keratinocyte progenitor cell line (CPEK) was shown to induce transcription of *tlr-1, tlr-2, tlr-4* and *tlr-6*.[35] PARs belong to a subfamily of G protein-coupled, 7-transmembrane domain receptors, activated by specific proteolytic cleavage of their extracellular amino termini by proteases.[36,37] Among PAR-1 to -5, PAR-2 has been shown to be expressed in the keratinocytes of humans,[38] mice[39] and dogs.[40,41] Recent studies in humans and dogs have indicated that activation of TLRs and PAR-2 in keratinocytes induces the production of cytokines and chemokines necessary for initiating and maintaining allergic inflammation.

Keratinocyte-derived cytokines and chemokines

Pro-inflammatory cytokines

It has been known that keratinocytes produce a variety of cytokines; among them, pro-inflammatory cytokines, such as IL-1, IL-6 and TNF, have been studied extensively

in humans. Human keratinocytes produce both IL-1alpha and IL-1beta; however, only the active form of IL-1alpha can be detected in culture, attributed to the lack of IL-1 convertase that cleaves the IL-1beta precursor in keratinocytes.[42,43] The biological activities of both IL-1alpha and IL-1beta are similar, including stimulation of acute-phase proteins, cytokine production, cellular adhesion, chemotaxis and T and B cell proliferation.[44] Keratinocytes express the IL-1 receptor on their cell surface,[45] and thus may respond to IL-1 in an autocrine manner.[46,47] In dogs, neither the gene nor protein expression levels of IL-1 have been evaluated in keratinocytes.

IL-6 was shown to be expressed in keratinocytes of humans with psoriasis.[48] Although IL-6 was first identified as a cytokine involved in the activation of lymphocytes, it has also been shown to be involved in vascular disease, lipid metabolism, insulin resistance, mitochondrial activities, the neuroendocrine system and neuropsychological behaviour.[49] Together with TGF-beta, IL-6 is also known to be a potent inducer of Th17 differentiation from naïve T cells.[50] IL-1beta and TNF are major activators of IL-6 expression, and other pathways, such as TLRs, prostaglandins, adipokines, stress responses and other cytokines, also promote IL-6 production.[49] In cultured human keratinocytes, the expression of IL-6 receptor was observed in monolayer cells and the deeper cells of stratified keratinocytes, but not in the differentiated cells of the upper layers.[51] In dogs, *IL-6* transcription was reported to be increased in cultured primary keratinocytes stimulated with synthetic dsDNA.[52]

TNF-alpha was shown to be produced by human keratinocytes stimulated with LPS or ultraviolet light.[53] The main biological function of TNF-alpha is its cytotoxic effect by inducing apoptosis in tumour cells.[44] TNF-alpha also mediates inflammation and immune response by inducing cytokine secretion by a variety of different cells.[54] Human keratinocytes were shown to express TNF receptor 1,[55] which is the main mediator of skin inflammation induced by TNF-alpha.[56] Microarray analysis demonstrated that treatment of human keratinocytes with TNF-alpha induced transcription of genes associated with not only immune and inflammatory responses, but also with tissue remodelling, cell motility, cell cycle regulation and apoptosis, suggesting that TNF-alpha has a multifunctional effect on keratinocytes.[57] In dogs, production of TNF-alpha was observed in keratinocytes stimulated with IFN-gamma and LPS.[58] One study has reported that activation via PAR-2 increased the transcription of *tnf-alpha* in CPEK.[40] TNF-alpha was also shown to induce transcription of CC chemokine ligand (CCL) 17 and CCL28 in CPEK, suggesting that canine keratinocytes likely express TNF receptor, similar to human keratinocytes.[59]

Chemokines

Cellular trafficking is strictly regulated by interactions between chemokines and chemokine receptors. Accumulating evidence indicates that subsets of Th cells selectively express chemokine receptors, such as CXC chemokine receptor (CXCR) 3 in Th1, CC chemokine receptor (CCR) 4 in Th2 or CCR6 in Th17 cells.[60] In dogs, such distinct subsets of Th cells have not been reported; however, one study has demonstrated that CCR4 was selectively expressed in canine Th2 cells.[2] In both humans[61] and dogs,[62] atopic skin lesions in the acute phase are characterized by Th2 cell-dominant inflammation, suggesting that CCR4 plays a significant role in the trafficking of Th2 cells to lesional skin. Several studies in humans have demonstrated the infiltration of $CCR4^+$ cells in the lesional skin of AD patients.[63–65] In dogs, *ccr4* was shown to be preferentially detected in lesional AD skin.[5] In peripheral blood, the number of $CCR4^+$ cells have been shown to be increased in both humans[64] and dogs[4] with AD. Thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL20) are known to be biological ligands of CCR4.[66,67] Keratinocytes were first identified as the major cellular source of TARC/CCL17 in the lesional skin of humans[65] and dogs.[6] Apart from TARC/CCL17, human keratinocytes produce a number of CC and CXC chemokines.[68–70] The current understanding of the cytokine profile in AD lesions is that during the advancement toward the chronic state, T-cell subsets change from Th2 to a mix of Th1, Th2, Th17 and Th22, suggesting that a more complex chemokine network may be involved in the pathogenesis of AD.[61]

Keratinocyte-derived chemokines and cytokines that are directly associated with the pathogenesis of AD

Thymus and activation-regulated chemokine (TARC)

Thymus and activation-regulated chemokine (TARC/CCL17) is one of the most extensively investigated chemokines associated with cAD. TARC/CCL17 was cloned from humans in 1996[71] and from dogs in 2001, which shows 77.5% amino acid sequence similarity with the human orthologue.[72] In both humans and dogs, TARC/CCL17 has been shown to be a functional ligand for CCR4, which is selectively expressed in Th2 cells.[2,6,66,71,73] An initial study in dogs with cAD demonstrated the preferential transcription of *tarc/ccl17* in atopic skin lesions, but not in nonlesional and healthy skin.[3] In dogs that were epicutaneously sensitized with an allergen, *tarc/ccl17* transcription was induced, with the highest increase in transcription level among the cytokines and chemokines investigated.[74] It has been shown that prednisolone or anti-IgE suppresses transcription of *tarc/ccl17* in experimentally induced late phase reactions.[75] In human AD, TARC/CCL17 production was observed in keratinocytes, vascular endothelial cells, dendritic cells and T cells.[65,76] An *in vitro* study suggested that dermal fibroblasts, but not epidermal keratinocytes, may be the major cellular source of TARC/CCL17 in AD lesional skin, and this production was shown not to be inhibited by dexamethasone or tacrolimus.[77]

In cAD, an immunohistochemical study using anti-canine TARC/CCL17 monoclonal antibody revealed positive staining in the epidermal keratinocytes of lesional skin.[6] In humans, a transformed keratinocyte cell line (HaCaT), but not a normal epidermal keratinocyte cell line (NHEK), was shown to transcribe *tarc/ccl17*.[78] In contrast, both normal canine epidermal keratinocytes (NCEK) and CPEK were observed to constitutively transcribe *tarc/ccl17*.[59] For the initiation or increase of *tarc/ccl17*

transcription, HaCaT cells required activation by either TNF-alpha or IFN-gamma,[78] whereas CPEK required stimulation of TNF-alpha, but not IFN-gamma or IL-1beta.[59] In CPEK, it was shown that TNF-alpha-induced *tarc/ccl17* transcription was positively regulated by mitogen-activated protein kinase p38 (p38), but was negatively regulated by extracellular signal-regulated kinase (ERK).[79]

A study demonstrated that activation of PAR-2 by an agonist peptide increased the transcription level of *tarc/ccl17*.[40] Thus, exogenous proteases from mite allergens may directly induce TARC/CCL17 production in keratinocytes via PAR-2. Numerous clinical studies in humans have demonstrated that plasma or serum TARC/CCL17 level is correlated with disease severity, and has been used as a useful biomarker in AD.[76,80–84] At present, a clinical study of plasma and serum TARC/CCL17 levels is ongoing in Japan to investigate its use as a potential biomarker for cAD.

Granulocyte-macrophage colony-stimulating factor (GM-CSF)

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is known classically as a pivotal cytokine able to stimulate the proliferation of granulocytes and macrophages.[85] GM-CSF is produced by various types of cells, including activated T cells, B cells, NK cells, monocytes/macrophages, endothelial cells, fibroblasts and epithelial cells.[86] Among the epithelial cells, keratinocytes are included in the group of cells that not only produce GM-CSF,[87] but also express its receptor.[88] The current understanding of GM-CSF is focused on its pro-inflammatory functions in autoimmune and inflammatory diseases, particularly Th17 associated diseases.[89] The association of GM-CSF in the pathogenesis of AD has been investigated in mice and humans.

In mice, GM-CSF inhibits IL-12 production in Langerhans cells, suggesting its important role in the induction of the Th2-dominated immune responses in AD.[90,91] In humans, a greater number of *gm-csf*-transcribing cells were detected by *in situ* hybridization in AD lesions.[91] In dogs, mRNA transcription and protein expression profiles in cAD lesions are lacking. *In vivo*, production of GM-CSF in keratinocytes of humans with AD was higher than that of healthy controls.[92] It was also shown that keratinocytes from nonlesional skin of AD subjects exhibited increased spontaneous and PMA-stimulated production of GM-CSF.[92] In dogs, CPEK were found to produce GM-CSF upon stimulation by a house dust mite (HDM) allergen, *Dermatophagoides farinae* (Der f).[93] The GM-CSF production was suppressed by addition of a cysteine protease inhibitor, suggesting that its production was most likely mediated via PAR-2 by Der f 1, which is one of the major allergens and a cysteine protease.[93] Similar to Der f 1, papain, a naturally derived cysteine protease, also induced GM-CSF production with translocation of nuclear factor of activated T cells (NFAT) in CPEK, and production was partially inhibited by ciclosporin.[94] These results suggest that GM-CSF production in CPEK activated via PAR-2 may be regulated not only by NFAT, but also by another transcription factor, such as nuclear factor-kB (NF-kB) or activator protein-1 (AP-1). A recent study demonstrated that IL-17A, a typical Th17 cytokine, also induced the transcription of *gm-csf* in CPEK.[95] A number of clinical trials targeting GM-CSF are underway in humans with rheumatoid arthritis, multiple sclerosis, asthma and plaque psoriasis, but not AD.[86]

Thymic stromal lymphopoietin (TSLP)

Thymic stromal lymphopoietin (TSLP) was first identified as a stimulator of B cell development in the culture supernatant of a murine thymic stromal cell line.[96] Northern and RT-PCR analyses in various tissues revealed the transcription of *tslp* in the spleen, thymus, kidney, lung and bone marrow of normal mice.[97] Following this, the human orthologue was cloned and its transcription was detected in the heart, liver, testis and prostate, with lower expression in the lung, skeletal muscle, kidney, spleen, ovary, small intestine and colon, indicating a more widespread tissue distribution pattern than murine *tslp*.[98] To date, the partial, but not full, length of canine *tslp* has been isolated.[99] The receptor of TSLP (TSLPR) was identified in mice and humans, revealing a heterodimeric receptor that consists of IL-7 receptor alpha-chain (IL-7R alpha) and TSLPR alpha chain 1.[100,101] To date, TSLPR has not been cloned in dogs. The functional TSLPR is expressed mainly in hematopoietic cells, liver, brain, skeletal muscle, kidney, spleen and thymus.[102]

Among the haematopoietic cells with TSLP expression, dendritic cells, CD4 and CD8 T cells, B cells, mast cells, basophils, eosinophils and NKT cells are capable of responding to TSLP.[103] TSLP is involved in a number of biological functions, including maturation of dendritic cells, expansion of T and B cells, and activation of innate immune cells; it is associated with a growing number of different disorders, including allergic inflammation, infection, cancer and autoimmunity.[103] TSLP is associated with the pathogenesis of human AD. Immunohistochemical analyses with anti-TSLP monoclonal antibody demonstrated high expression of TSLP in keratinocytes of the uppermost layer of the epidermis in acute and chronic AD.[104] Another study indicated that the expression level of TSLP in the stratum corneum was increased in AD compared with healthy subjects, and was correlated with dry skin score and stratum corneum hydration.[105] TSLP produced by keratinocytes activates $CD11c^+$ dendritic cells and induces production of TARC/CCL17 and MDC/CCL22.[104] Dendritic cells activated by TSLP-primed naïve T cells produce Th2 cytokines, such as IL-4, IL-5 and IL-13.[104] In $TSLPR^{-/-}$ mice, allergic inflammation elicited by epicutaneous immunization with ovalbumin was severely attenuated, which was attributed to decreased infiltration of eosinophils and decreased local expression of Th2 cytokines.[106] Moreover, overexpression of skin-specific TSLP induced skin lesions with an increasing number of Th2 cells in the dermis, and elevated serum IgE levels.[107] These results indicate that TSLP-stimulated dendritic cells prime CD4 T cells with characteristic features of Th2 cells, leading to the development of skin lesions. Various environmental and endogenous stimuli induce TSLP production in the skin.[108,109] TSLP production by mechanical injury in mice has been suggested to be due to inflammatory cytokines that were produced in the skin lesion.[109] In mice with a mutation in *filaggrin*, activation of PAR-2 induced TSLP production in keratinocytes.[108]

Furthermore, AD-like skin lesion development by epicutaneous application of mite extracts was shown to be improved by a PAR-2 antagonist.[108] Binding of TSLP to TSLPR in $CD11c^+$ dendritic cells in humans was shown to activate multiple STAT proteins, such as STAT1, 3, 4 and 5, as well as JAKs 1 and 2.[110] Although this signalling pathway in keratinocytes has yet to be elucidated, it is plausible that it is similar in both dendritic cells and keratinocytes.

In 2013, a partial canine *tslp* cDNA was cloned and characterized. The predicted amino acid sequence deduced from the canine *tslp* cDNA shares 60.8% identity with human TSLP.[99] Although the cDNA identified in the study was a partial transcript, the deduced amino acid sequence ended with a stop codon, suggesting that the partial cDNA encoded the signal peptide and mature canine TSLP.[99] Subsequently, transcription analysis with RT-qPCR in lesional and nonlesional skin of cAD indicated that the transcription level of *tslp* was significantly higher in both lesional and nonlesional skin of cAD, compared with control dogs, but there was no significant difference between the lesional and nonlesional samples.[99] In primary canine keratinocyte culture, HDM allergen extracts or ligands of TLR3 and TLR4 were shown to induce *tslp* transcription.[99] Another study showed that synthetic triacylated lipopeptide (TLR1/2 ligand), a cell wall component of *Staphylococcus* spp., induced the transcription of *tslp*, which was completely suppressed by knockdown of TLR2, suggesting that *Staphylococcus* spp. may promote Th2 responses through TLR2-mediated TSLP production in canine keratinocytes, worsening the allergic inflammation in cAD.[111] Immunohistochemical analyses with human TSLP monoclonal antibody demonstrated that the staining intensity of TSLP did not significantly differ between atopic and normal dogs, although the staining pattern was different.[112] These results suggest that canine TSLP is most likely involved in the pathogenesis of cAD, similar to that in human AD.

The potential benefits of anti-TSLP antibodies have been investigated in various murine models of atopic diseases, such as eosinophilic oesophagitis, AD and asthma. It was demonstrated that anti-TSLP antibody inhibited the development of oesophageal eosinophilia and food impaction in a murine model of eosinophilic oesophagitis.[113] In a murine model of AD, treatment with anti-TSLP antibody inhibited IgE-mediated AD-like skin lesions and IL-17A production in the lymph nodes.[114] In a chronic HDM-induced asthma model, an anti-TSLP antibody reversed airway inflammation, prevented structural alterations, and decreased airway hyper-responsiveness to methacholine and TGF-beta1 level.[115] Finally, a study in humans with asthma showed that a fully human anti-TSLP monoclonal antibody reduced allergen-induced bronchoconstriction and indexes of airway inflammation.[116] Accumulating basic and clinical research indicate that TSLP is a promising pharmacological target for the treatment of allergic diseases.

Interleukin-33: IL-33

Interleukin-33 (IL-33) was designated a cytokine in the IL-1 family, which includes IL-1alpha/beta, IL-1Ra and IL-18, by a computational structural database search.[10] It was later clarified that IL-33 had been previously identified as a gene upregulated in the vasospastic cerebral arteries of dogs with experimentally induced subarachnoid haemorrhage,[117] and as a nuclear factor from high endothelial venules (NF-HEV) expressed in human secondary lymphoid tissue.[118] The amino acid sequence of canine IL-33 shares 61% identity with human IL-33.[117] Analysis of mouse cDNA libraries by RT-qPCR detected murine *il-33* in various organs, such as the stomach, colon, spleen, pancreas, kidney, heart, lung, lymph nodes, Peyers patches, thymus, spinal cord, brain and skin.[10] Furthermore, human *il-33* was found transcribed in the tonsils, Peyers's patches, lymph nodes and brain.[118,119] At the protein level, the expression of human IL-33 was observed in the stomach, kidney, lung, liver, fallopian tubes, prostate, skeletal muscle, tonsils, brain and skin.[119,120] In dogs, mRNA or protein for IL-33 was detected in the arteries,[117] heart[121] and skin.[122]

The receptor for IL-33 (IL-33R) was identified as a heterodimeric receptor complex consisting of transmembrane growth stimulation expressed gene (ST2), which was originally discovered as an orphan receptor,[123] and IL-1R accessory protein (IL-1RAcP).[124] An alternative transcript from the *ST2* gene locus codes for a soluble ST2 (sST2), which binds to and acts as a natural antagonist of IL-33.[125] To date, the canine IL-33R complex has not been cloned. IL-33R is ubiquitously expressed not only in haematopoietic cells, including mast cells, basophils, eosinophils, Th2 cells, macrophages, dendritic cells, NK cells, NKT cells and type 2 innate lymphoid cells (ILC2), but also in structural cells, including endothelial cells, epithelial cells and fibroblasts.[126] In particular, the expression of IL-33R was found to be high in Th2 cells,[127] mast cells[128] and ILC2.[129] IL-33 induces the development of Th2-associated inflammation in asthma and AD by promoting the production of Th2 cytokines and survival of mast cells and eosinophils.[130] Meanwhile, IL-33 has been shown to have various protective effects in helminth infections, atherosclerosis, obesity and type-2 diabetes.[130] Genetic polymorphisms in the *ST2* region have been associated with AD in humans.[131] Furthermore, the transcription level of *il-33* in lesional skin of human AD was shown to be higher compared with that in nonlesional skin.[132] Immunohistochemical analyses revealed an increased expression of IL-33 in suprabasal keratinocytes and endothelial cells in human AD.[133] Additionally, a number of IL-33R-expressing cells were observed in the dermis and epidermis of AD patients.[133] IL-33 enhances the production of IL-5 and IL-13, but not IL-4, by Th2 cells,[10] mast cells[128] and ILC2,[129] whereas human basophils stimulated with IL-33 were shown to produce IL-4 as well as IL-5 and IL-13.[134] In addition to Th2 cytokines, human mast cells stimulated with IL-33 were found to produce various cytokines and chemokines, including TNF-alpha, GM-CSF, IL-1beta, IL-3, IL-6, IL-10, CXC chemokine ligand (CXCL) 2, CXCL8, CCL1, CCL2 CCL3, CCL17, prostaglandin D2 and leukotriene B4.[128,135]

The expression of TSLP in normal human epidermal keratinocytes (NHEKs) was upregulated by stimulation with IL-33 in a dose-dependent manner.[136] It was demonstrated that the development of AD-like lesions in transgenic mice expressing IL-33 was driven by a keratin 14 promoter in keratinocytes.[137] IL-33 can be released from keratinocytes by several factors, such as pro-inflammatory cytokines,

mechanical injury, allergens and bacteria. Stimulation with TNF-alpha and IFN-gamma upregulated expression of IL-33 in both HaCaT[133] and cultured primary keratinocytes.[138] Furthermore, upregulation of *il-33* was observed in human skin after tape stripping[139] and in the skin of filaggrin-deficient mice,[133] suggesting that barrier dysfunction leads to the expression of IL-33. In humans with AD, RT-qPCR analyses revealed the upregulation of *il-33* and *st2* in the skin after patch testing with HDM and staphylococcal enterotoxin.[133] Binding of IL-33 to the IL-33R complex recruited the adaptor molecule myeloid differentiation primary-response protein 88 (MyD88), IL-1R-associated kinase 1 (IRAK1) and IRAK4,[10] consequently activating signalling pathways such as ERK, p38, JNK and NF-κB in mast cells[140] and T cells.[141] In keratinocytes, IL-33 induced the expression of TSLP through an early growth response protein 1 (Egr-1) dependent mechanism via ERK, JNK and p38.[136]

A microarray analysis in acute cAD skin lesions revealed that the transcription of *il-33* was approximately three times higher than that in healthy dogs.[142] Transcription analyses by microarray and RT-qPCR demonstrated the increased transcription of *il-33* in the skin of dogs sensitized to HDM allergens.[122] These results suggest that canine IL-33 is likely involved in the pathogenesis of cAD, similar to human AD.

The potential benefits of an anti-IL-33 monoclonal antibody have been investigated in murine models of allergic diseases. Treatment with an anti-IL-33 monoclonal antibody inhibited allergen-induced eosinophilic airway inflammation, mucus hypersecretion and production of Th2-type cytokines in a murine asthma model.[143] Administration of an anti-IL-33 antibody to an ovalbumin-induced allergic rhinitis model attenuated the frequency of nose scratching, serum IgE increases and eosinophil infiltration into airway tissues.[144] In humans with AD, serum IL-33 levels were correlated with skin severity, suggesting its potential as a biomarker.[145] These studies suggest that the IL-33/IL-33R pathway has potential as a therapeutic target and as a novel biomarker for AD.

Conclusion and future direction

Accumulated evidence demonstrates that keratinocyte-derived cytokines and chemokines, such as TARC/CCL17, GM-CSF, TSLP and IL-33, are involved in the pathogenesis of human AD and possibly in cAD. Thus, these cytokines and chemokines may possibly be used as subjective clinical markers and therapeutic targets for both human and canine AD. Therefore, prospective studies should focus more on the translation of such findings in fundamental research into dermatological practice.

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The future of immunotherapy for canine atopic dermatitis: a review

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Allergen specific immunotherapy (ASIT) is a foundation treatment for canine atopic dermatitis (CAD), though few critical studies have documented its effectiveness as a disease-modifying treatment in dogs. The mechanisms by which ASIT works in dogs have not been elucidated, although they are likely to parallel those known for humans. Current ASIT approaches in CAD focus on either subcutaneous or sublingual administration. Greater knowledge of major allergens in dogs, ideal dosage regimes and details of allergen admixture are likely to lead to better efficacy in CAD. Evaluation of biomarkers for successful therapy may also be of benefit.

Potentially important advances in human medicine, that have yet to be explored in dogs, include use of modified allergen preparations such as allergoids, recombinant major allergens or allergen peptides; modification with adjuvants; or packaging of the above in virus-like particles. Co-administration of immunomodulators such as CpG oligodeoxynucleotides or specific monoclonal antibodies might direct the immune response in the desired direction while calming the "cytokine storm" of active disease.

Initial trials of alternative routes of administration such as intralymphatic immunotherapy have yielded exciting results in humans, and continuing study in dogs is underway. Progress in ASIT of human food allergy may provide clues that will assist with improved diagnosis and patient management of CAD. Importantly, further study must be undertaken to clarify the conditions under which ASIT is a valuable treatment modality for dogs.

Introduction: why allergen-specific immunotherapy?

In 1941, Wittich published the first report of using allergen-specific immunotherapy (ASIT) for the successful treatment of allergy in a dog.[1] Seventy five years later, it is appropriate to examine how far we have come and to where we might travel in the future, with regard to this important and foundational treatment for canine atopic dermatitis (CAD).

A significant number of studies in people, and a slowly increasing number in dogs, have documented the effectiveness of ASIT for allergic diseases. It is the only current treatment for allergy that can modify, or reverse, at least part of the pathogenesis of this condition, both alleviating clinical signs and preventing progression of disease.[2] This modification is accomplished without the possible long-term adverse effects of a lifetime of drug treatment, with minimal adverse effects, and with the potential of long-lasting effectiveness. Yet, there are a great many unresolved questions about ASIT, especially with regard to defining more clearly its efficacy in animals, and answering these is likely to result in even greater therapeutic efficacy in the future.

One key question revolves around differences in atopic dermatitis (AD) and other allergic syndromes such as respiratory disease. The bulk of evidence for the effectiveness of ASIT in humans is for the management of allergic rhinitis and asthma; in fact, some authorities have considered its usefulness in human AD to be questionable. On the one hand, this position has been reinforced by systematic reviews concluding that that the strength of recommendation for use of ASIT in human AD is weak;[3] on the other, some clinical trials and reports of patient experience have been highly encouraging, even in refractory cases.[4–6]

In dogs, this controversy seemingly never arose, perhaps due to early reports of success in CAD; as even in a placebo-controlled trial, results provided initial reason for optimism in allergic skin disease.[7–9] Based on these and other studies, "response rates" (typically quoted as percentage of dogs that experience at least 50% improvement in clinical signs) are typically quoted as 60–70%. Although not perfect, ASIT has a clear role as a useful and important part of multimodal therapy of CAD.

Mechanisms: the known and the unknown

It is important to briefly review what is known about how ASIT works, to understand how proposed advances may exert their effects. The mechanisms by which ASIT works in dogs have not been completely elucidated, although they are likely to parallel those known in humans: early reduction in effector cell activity (eosinophils, basophils, mast cells) followed by a long-term immunologic shift from a T helper 2 (Th2) cell to a T helper 1 (Th1) cell response and development of immunological tolerance.[10–12] These shifts are accompanied by an increase in forkhead box P3 (FOXP3) + regulatory T (Treg)

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cells, and an increase in cytokines such as transforming growth factor beta (TGFß) and interleukin (IL)-10. As a result, there is an increase in allergen-specific immunoglobulin (Ig)G, especially IgG4 and, with extended treatment, a decrease in allergen-specific IgE. In dogs, although much less is known, a shift to a Th1 cell response, increases in IgG levels, the appearance of more Treg cells and increases in IL-10 levels have all been demonstrated, thus establishing the parallels to ASIT in humans.[13–16]

With sublingual administration, there is the additional effect brought about by oromucosal dendritic cells, frequently discussed for immunotherapy in people but unexplored in dogs. As with other dendritic cells, oromucosal dendritic cells are responsible for allergen uptake, processing and presentation to T-lymphocytes. They are abundant in the oral mucosa and have unique functional characteristics, being the key cell in induction of oral tolerance, the immunological function by which the immune system is set to a default of nonreactivity to substances placed in the mouth (e.g. foodstuffs).[17,18] Physician allergists take advantage of this normal, homeostatic process when attempting to desensitize a patient via the oromucosal route.

Review of current approaches to ASIT in dogs: how well does it work and can we do better?

Current ASIT approaches in CAD focus on either subcutaneous (SCIT) or sublingual (SLIT) administration. Injectable immunotherapy has been conducted for decades and SLIT is a newer modality that has only recently become available for dogs in some countries. There are aspects of both methods that are poorly understood and elucidating these may facilitate improvements in therapy.

With regard to SCIT for dogs, two methods have evolved, predominantly based on availability and regulatory approvals in North America versus Europe. All SCIT in North America is undertaken with aqueous, saline-phenol preserved extracts provided by several manufacturers. Typically, two- or three-vial sets of increasing concentration are used, beginning with frequent injections of dilute extract and progressing to less frequent injections of concentrated extract as maintenance treatment. In Europe, use of alum-precipitated allergen extracts has been more common. Here, adsorption of the allergen molecules to an aluminium hydroxide adjuvant provides a slower-release formulation, which has the advantage of less frequent injections. However, concern is being increasingly raised regarding the possible adverse effects of chronic aluminium exposure, and the fate of aluminium-based adjuvants widely used in vaccine products is uncertain.[19]

Administration of SLIT has been a popular method of human ASIT for many years in European countries, in some regions eclipsing the use of SCIT. Most studies evaluating the efficacy of SLIT are therefore European. In the past, these studies have yielded conflicting results, which is predictable given that significantly different dosage and administration protocols have been used. More recently the World Allergy Organization has published analyses and position papers which conclude that SLIT is an effective treatment with a favourable safety profile compared with SCIT.[20] The lower popularity of SLIT in the United States reflects that registered products have not been available until very recently and, with these, its use is increasing in human medicine. In dogs, use of SLIT has only recently been reported.[21]

There are numerous well-controlled studies of ASIT in human allergic disease that demonstrate efficacy, and critical reviews and meta-analyses that support this conclusion.[22] By contrast, in CAD very few studies have been published, and they are often small, uncontrolled and/or have confounding variables that make interpretation of results difficult. In a blind, placebo-controlled trial of 51 dogs, a response rate of 59% in allergen-treated dogs versus 21% in the placebo-treated group was reported.[7] More representative is another retrospective, uncontrolled study, where 60% of owners administering injections reported at least 50% improvement in their dogs. It should be noted that of the dogs receiving injections, 65% still required additional medication for control – which brings into question the ability of the owner to accurately judge the effectiveness of the ASIT itself.[8] Practice guidelines for treating CAD, employing a strength-of-recommendation taxonomy (SORT), continue to rate the quality of published evidence for efficacy of ASIT as limited, and at best based on inconsistent or limited quality patient-oriented evidence.[23]

With such limited evidence of efficacy, why then has a treatment so commonly used and recommended not been subjected to more frequent and rigorous study in dogs? Part of the reason, no doubt, is the high cost and time requirement for performing large, controlled studies of a complex disease that may require a year or more to respond to the intervention. Another part of the reason may be related to regulatory issues. Biological products (including allergenic extracts) are often subject to wholly different regulatory requirements than drugs, although the situation varies substantially between countries. In the United States, for example, veterinary biologicals are overseen by the US Department of Agriculture (USDA) under a different set of laws than drugs, which are overseen by the US Food and Drug Administration (FDA).[24] Historically, biologicals have been mainly evaluated for qualities such as safety, purity, sterility and consistency in manufacturing, and current veterinary allergen extracts received USDA licensure decades ago based on those standards. Dose-determination studies, short- and long-term safety analysis, and rigorous, large-scale controlled clinical trials in affected patients, as would be required for an FDA-regulated drug product, were not done – they were not required. This stands in contrast to allergenic extracts for human use, which *are* FDA-regulated and, therefore, were subjected to much more stringent standards and rigorous proof of efficacy.

Beyond consideration of efficacy, it is clear that ASIT protocols for dogs lack standardization and are subject to substantial variation. Veterinary clinicians use different allergen dosage regimes with unstandardized allergen extracts that vary in composition and potency between manufacturers and from batch to batch, using different

schedules of administration, differing concurrent treatments and with different 'rules' about how to mix extracts together. Given this, there are many steps that could theoretically be taken to examine and compare these procedures, and more carefully optimize protocols to produce maximal patient efficacy. Many of these may be completely impractical, given the difficulty and expense of conducting large-scale clinical trials. However, some important measures may be possible to accomplish with coordinated effort.

An important example would be to establish the major allergenic epitopes for dogs, especially for common allergens, and use this information to provide some measure of standardization to allergen extracts.[25] This information would permit standardized dosing, preparation of recombinant allergens, determination of T-cell epitopes, use of peptide immunotherapy and other advances. This has certainly been the course in human ASIT, as guidelines from the European Medicines Agency and other regulatory bodies – which increasingly mandate standardization of extracts – have prompted more clinical studies on optimal dose-finding for both safety and efficacy. The body of clinical evidence will increase and lead to more products with documented efficacy and safety. In veterinary medicine, central to the above is answering the question "what is the optimal dose of allergen extract in dogs?" Dose-dependency of ASIT efficacy has been long-observed in human trials and recent mouse models.[26,27] Not only the absolute dose, but also the dosing intervals have measurable effects on efficacy under some circumstances. Unfortunately, these effects remain unstudied for ASIT in animals.

Extending these thoughts further, what will be the role of rush immunotherapy in CAD in the future? At present, it appears that rush protocols are used by comparatively few veterinary clinicians, although they are the one specific administration schedule that has been examined in dogs and found to be equally effective to a conventional injection protocol.[28] Rush immunotherapy has a clear advantage of limiting the number of injections that an owner must give at home when initiating ASIT, although safety concerns are higher as well as the cost for a brief hospitalization under observation.

Likewise, it is hoped that veterinary clinicians will be able to employ a more scientific approach to allergen prescription formulation. With the marked variation among clinicians in how, and how many, extracts of different types are mixed together, it is not surprising that there is a variable outcome. Should the number of extracts in a mixture be limited from eight to ten to 12 or unlimited? Should protease-containing extracts such as moulds be admixed, or administered only by separate injection? Should the mixture be based upon results of intradermal testing or serological testing or both? Or made uniform for each dog, based on what allergens are predominant in a region? In humans, these debates are controversial, although recent large-scale studies have provided some guidance. Evidence suggests that in polysensitized humans, treatment with a single, dominant allergen is as effective as multi-allergen ASIT, even though polysensitizaton is more prevalent.[29,30] This "less is more" approach – prevalent in Europe – is unpopular in the United States among physician allergists. The evidence for limiting the number of allergen extracts used in treatment is in contrast to protocols used by most veterinary clinicians and, if applicable to dogs, will require a modification to our thinking. Authoritative guidelines specify that mould extracts should not be mixed in the same vial as pollens, as there is clear evidence that pollen allergens will be degraded by mould proteases during storage.[31] Despite similar (though less) evidence in dogs,[32] many veterinary clinicians continue to recommend this practice. Further research is clearly indicated to answer this question, among many others.

A central problem in answering many of these questions is that most studies in veterinary medicine evaluating the parameters of successful ASIT have been clinical trials. In conducting such trials, there is significant difficulty in obtaining reproducible, objective data based on validated scoring systems; the patient variability that confounds analysis; the pervasive and prominent placebo effect; and the very large number of dogs necessary to study effect sizes that may be small, but important. Turning again to the human ASIT experience, studies rely not only on clinical criteria, but also on biomarkers of successful treatment. Most studies report not only subjective patient response, but also objective changes in parameters such as ventilatory mechanics and changes in specific immunoglobulin levels. Objective changes that could be measured are notably under-researched in veterinary medicine. The initial proof-of-concept demonstration that accelerometer based "activity monitors" could provide objective measures of pruritus in dogs has not yet been adopted in clinical trials, although advances in this technology may facilitate such in the future.[33] Over 10 years ago, it was demonstrated that total serum IgG1 concentrations increased with successful ASIT in dogs,[13] and the IgG response of dogs during dust mite ASIT has been studied in even more detail.[15] The nature and functional characteristics of canine IgG subclasses have recently been described in much greater detail.[34] Significant increases in Treg cells and IL-10 have been demonstrated in dogs undergoing successful ASIT.[16] Yet, none of these findings has been extended or explored to the extent that these or other biomarkers could be useful, objective and perhaps more rapid measures of ASIT success with which to study different interventions.

New approaches: animal models are shedding light

Canine AD appears to have marked similarity to human and murine models of AD, and new research findings in people may bring forth ideas about useful management strategies for dogs. Important advances in human medicine that have yet to be explored in dogs include use of modified allergen preparations such as allergoids, recombinant major allergens, or allergen peptides. Enhancing the effect of allergens using adjuvant-like manipulations such as IL-10 inducers, packaging in virus-like particles (VLPs), or in the case of SLIT, mucoadhesive polymers holds promise. Co-administration of immunomodulators such as CpG oligodeoxynucleotides or specific monoclonal antibodies could direct the

immune response in the desired, nonallergic direction while calming the "cytokine storm" of active disease, thus allowing ASIT to work more effectively. Combining any of the above therapies with new methods of ASIT administration such as intralymphatic injection could be potentially effective for dogs.

Modified allergen preparations have substantial potential for the enhancement of ASIT. Allergoids are the simplest of these modifications in concept: native allergen is chemically treated to either polymerize it or, in some cases, render it monomeric. The resulting allergoids retain immunological activity, yet have lower ability to activate mast cells and other potential mediators of immediate-type hypersensitivity reactions.[35] Their major advantage in human medicine is safety, with less chance of a life-threatening anaphylactic reaction. Because these reactions are relatively uncommon and typically not life-threatening, allergoids may be a less exciting concept in dogs.

Conversely, recombinant allergens and allergen peptides may have greater potential in veterinary dermatology. Recombinant allergens, where an entire major allergen molecule such as Der f 1 is produced synthetically, have their major advantage in purity and consistency resulting in precise and uniform dosing. Of course, a significant issue here is that most individuals, canine or otherwise, are sensitized against a number of major allergens of each substance, such that a very large number of recombinant products might need to be manufactured and tested; not to mention the need for determining the best way to combine them. Peptide immunotherapy carries this principle a step further; major allergens are examined to find specific, even smaller amino acid sequences within the allergen to which T-lymphocytes may react ("T-cell epitopes"), yet are far too small to trigger anaphylaxis. A combination of these peptides can be created that equals the full immunological potency of the native allergen, without the risk of adverse reaction. Peptide allergen desensitization is currently at its most impressive in human cat dander allergy, where initial trials demonstrated a beneficial and long-lasting effect (years) after administering only four doses over a few months.[36,37]

Beyond modifying the specific allergen molecule; nonspecific enhancement of its desensitizing effect is another active area of research. Adjuvanting the allergen to enhance its delivery such as packaging in VLPs prior to injection, or in a mucoadhesive polymer to prolong oromucosal contact in SLIT, is promising.[38–41] Immunomodulating substances that direct the immune system towards a Th1 cell response and/or tolerance – again as a nonspecific action that could be administered along with any specific allergen – have been studied, but principally in mouse models.[42,43] Administration of CpG oligodeoxynucleotides either as sole therapy or along with specific allergen may have benefit, including in dogs.[40,44]

New routes of administration of the desensitizing allergen(s) have dazzling potential for future success. Particularly notable in human allergic patients is the recent use of epicutaneous or intralymphatic administration. Epicutaneous immunotherapy can be accomplished with synthetic allergen molecules that are modified such that they cause no cutaneous or systemic reaction, yet trigger a profound desensitizing effect as demonstrated in murine models.[45] Clinical trials of intralymphatic ASIT in human allergic disease are impressive; a few, very small doses of allergen painlessly injected into lymph nodes can produce a dramatic and long lasting therapeutic effect with complete safety (see review by Senti *et al.*[46]). Preliminary studies in dogs have been reported.[47–49]

Allergen-specific immunotherapy for food allergy is an exciting topic in human medicine, in part due to the social difficulties and potentially lethal consequences of this condition in people. In the past, ASIT for food allergens such as peanut or shellfish was generally considered prohibitive due to the risk to the patient. Molecular diagnostics (i.e. identifying the specific epitopes to which the individual patient reacts), modified treatment allergens and alternate methods of administration have now made this ASIT feasible. Large-scale trials of sublingual immunotherapy, in particular, have shown success with peanut allergy.[50,51] Although food allergy is more readily managed and of less serious consequence in dogs than in humans, the progress with ASIT for the management of food allergy in people may assist with better diagnosis and patient management of canine AD.

One cannot discuss the future of ASIT in dogs without considering future treatments that may render ASIT archaic or unnecessary. Highly effective drugs such as ciclosporin or oclacitinib,[23] or biologicals such as anti-IL-31 therapeutic monoclonal antibody,[52] may obviate some of the "need" for ASIT, as the former treatments may control clinical signs very well over long periods of time. However, they are still treatments that require lifetime administration; they only reduce clinical signs rather than reversing pathogenesis; their long-term safety is not always established; and they carry no hope of permanent "cure" as can sometimes be achieved with ASIT. Therefore, this author believes that there will always be a place for ASIT in our armamentarium for CAD.

There are many lines of investigation of ASIT that are yielding exciting results in humans and bode well for the future of this fundamental mode of treatment in animals. Importantly, ASIT has historically been viewed by some general-practice veterinarians as a "last-resort" treatment – after all other efforts have been exhausted, the pet is finally referred for evaluation for "allergy shots." At this point, the patient often has chronic and unremitting disease that is challenging for any treatment. If the veterinary profession encourages greater knowledge on how and when to use ASIT, and fosters improvement in our methods and technology, one hopes that veterinary clinicians and clients will consider ASIT as an early choice for treatment of AD, rather than as a last resort; modifying both clinical signs and the course of the disease over the patient's lifetime should be a primary and early goal. As new, more effective, and safer drug and biological treatments are developed and become more commonplace, we must also continually ask where ASIT fits in, in the world of multimodal allergy treatment.

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2.3

Co-sensitization and cross-reactivity between related and unrelated food allergens in dogs – a serological study

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Background – Knowledge of cross-reactivity between foods is useful so that potentially cross-reactive allergens can be avoided in diet trials.

Hypothesis/Objectives – To evaluate allergenic cross-reactivity in related foods.

Animals – Sera from 469 dogs with suspected adverse food reactions.

Methods – An IgE-based serological assay using 19 food allergens was performed in 469 dogs. Pairwise comparisons were used to calculate the odds ratios (ORs) for each food pair, with significance at $P < 0.0002$ by Holm–Bonferroni correction, both in all 469 dogs and in the 261 of 469 dogs with at least one positive reaction. One-way ANOVA with Tukey's post hoc tests (significance at $P < 0.05$) were used to test for differences between mean logE ORs in different food groups. Inhibition enzyme-linked immunosorbent assays (ELISAs) were performed to assess allergenic cross-reactivity between beef, lamb and cow's milk.

Results – Significant associations were observed between both related and unrelated food pairs. Associations were, however, more frequent and stronger among related than unrelated foods. In all 469 dogs, 38 of 43 related food pairs were significantly associated [mean (SD) logE OR 3.4 (0.9)] compared with 79 of 128 unrelated pairs [2.7 (1.0)], $P < 0.0002$. In positive dogs, 32 of 43 related pairs were significantly associated [2.7 [1.0)] compared with 49 of 128 unrelated pairs [1.8 (1.0)], $P < 0.0002$. Inhibition ELISAs confirmed the presence of cross-reactive IgE-binding epitopes in beef, lamb and cow's milk.

Conclusions and clinical importance – The results suggest that related and potentially cross-reactive foods should be avoided in elimination diets.

Introduction

Cutaneous adverse food reactions (AFRs) are a common cause of nonseasonal pruritus in dogs and can result from both immunological and nonimmunological reactions. In a recent study of 259 dogs with allergic dermatitis where flea bite hypersensitivity was excluded, 70.7% were diagnosed with aeroallergen-induced canine atopic dermatitis (CAD), 25.1% with an AFR and 4.2% with both conditions.[1] The clinical signs of CAD and AFRs in dogs can be indistinguishable, making it challenging to achieve a precise diagnosis.

The immunological mechanisms involved in cutaneous AFRs are complex and poorly understood. Early studies did not support a role for IgE in the pathogenesis,[2,3] but the diagnosis of AFR in these dogs was based upon a 3 week elimination diet trial as opposed to the eight to 10 weeks now considered mandatory.[4] Another study demonstrated good correlation between dietary provocation and levels of allergen-specific IgE and immunoblots.[5] Additionally, results of lymphocyte blastogenesis and patch testing in dogs with AFR have suggested a role for cell-mediated hypersensitivity.[6–8] Cell-mediated reactivity is likely to be accompanied to some extent by antibody production and the negative predictive value of allergen-specific IgE and IgG is reportedly 80.7% and 83.7%, respectively.[8]

Despite these immunological correlations, the "gold standard" for the diagnosis of an AFR is an elimination diet trial followed by dietary challenges. Dietary selection, however, remains controversial. Single antigen, novel protein commercial diets can be unreliable due to contamination with foreign proteins.[9,10] Hydrolysed diets are often effective for the diagnosis of cutaneous AFR but not completely reliable, possibly due to the presence of higher molecular weight, nonhydrolysed components.[11–13] Some veterinarians recommend a home-prepared single novel antigen diet for the diagnosis of AFRs

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before evaluating balanced commercial diets for maintenance. The food allergens that most commonly elicit clinically relevant reactions in dogs are beef, dairy products, chicken, wheat, eggs, soy and lamb.[14,15] Some dogs are multisensitive,[16] either from co-sensitization or cross-reactivity. One study of 10 sera collected from dogs with confirmed AFRs demonstrated IgE reactivity to beef, lamb and milk antigens with antibodies that recognized bovine IgG in beef and cow's milk, and that cross-reacted with ovine IgG in lamb extract.[5] Knowledge of allergenic cross-reactivity in foods could therefore be useful when assessing the likelihood of an AFR. Clearly, more extensive studies are required to identify potentially cross-reacting antigens in dogs with confirmed AFRs.

This study analysed food allergen-specific IgE reactivity in 469 sera from dogs with suspected allergic skin or gastrointestinal disease. Pairwise comparisons were undertaken to assess the significance of associations in the patterns of allergen recognition. Inhibition enzyme-linked immunosorbent assays (ELISAs) using sera reactive to beef, lamb and cow's milk were then performed to ascertain the prevalence and extent of cross-reactivity. The results could assist in the successful diagnosis and management of canine cutaneous AFRs.

Methods

Pairwise comparisons

Data from a commercial food allergen-specific IgE ELISA (Avacta Animal Health; Wetherby, UK) using serum from 469 dogs with suspected AFRs, submitted between January to April 2015, were evaluated retrospectively. Foods were categorized into taxonomically related groups: mammalian (beef, pork, lamb, venison, rabbit and cow's milk); avian (chicken, turkey, duck and whole hen's egg); fish (salmon and white fish); and plants (wheat, barley, soybean, potato, rice, corn and oat). Contingency tables for all the foods within each related group and between the foods from the unrelated groups were created to enable pairwise comparisons (n = 171) of positive (+) and negative (−) IgE reactions. The number of concordant results (+/+ or −/−) and discordant results (+/− or −/+) among food allergen pairs were used to calculate the odds ratios (ORs), 95% confidence intervals (CIs) and statistical significance. To control the false discovery rate, the level of significance was set at $P < 0.0002$ using a sequential Bonferroni technique[17,18]. Pairwise tests were run both on all dogs (n = 469) and dogs with at least one positive IgE reaction (n = 261). The ORs for each food allergen pair were grouped into taxonomically related foods with a statistically significant association (associated-related foods), related foods with a nonstatistically significant association (nonassociated-related) and unrelated foods. The natural logarithm (base e) was used to transform the OR data into a normal distribution for further analysis.[18] One-way ANOVA with Tukey's *post hoc* tests were used to test for differences between mean logE ORs in the associated-related, nonassociated-related and unrelated food groups. A level of $P < 0.05$ was considered significant. All analyses were performed using GraphPad Prism (GraphPad Software Inc.; San Diego, CA, USA).

ELISAs and inhibition ELISAs

Sera (n = 115) with a positive IgE reaction to at least two beef, lamb and cow's milk allergens were identified by ELISAs, performed as described previously.[8] Briefly, beef, lamb and cow's milk extracts (Greer Labs Inc.; Lenoir, NC, USA) were coated at 5 μg/mL. Sera were assayed at a dilution of 1/10 and alkaline phosphatase (AP)-conjugated anti-dog IgE (clone 5.91, North Carolina State University; Raleigh, NC, USA) was used at 0.5 μg/mL. Plates were read on a microplate reader (Tecan; Männedorf, Switzerland) at an absorbance of 405 nm (optical density, OD). A standard curve was prepared by assaying serial three-fold dilutions of a dog serum with high levels of beef-specific IgE, as determined by previous ELISAs. Undiluted, this serum was assigned a value of 500 arbitrary units (AU). After subtraction of the buffer-only (no-serum) control well ODs, mean standard ODs were fitted to a four-parameter sigmoidal standard curve (GraphPad Prism). Levels of specific IgE were determined from their mean duplicate ODs by interpolation from the resulting standard curve. A positive threshold of 5 AU was determined by interpolating the mean ODs + 3 standard deviations of *in vitro* allergen-specific IgE-negative sera (i.e. sera with ODs <0.25, 405 nm, to beef, lamb and cow's milk; n = 124) from the standard curve. Spearman's rank correlation was used to analyse AU levels for IgE binding to beef, lamb and cow's milk (GraphPad Prism).

Inhibition assays were performed using three related mammalian food allergens (beef, lamb and cow's milk) and one unrelated avian food allergen (turkey). To ensure that inhibitions were performed with comparable antibody titres, sera that gave ODs of 1.4 ± 0.7 (target OD ± allowed tolerance) upon 1/10 dilution were selected from previous noninhibited ELISAs. Sera (n = 45) were diluted 1/10 with 1, 5, 25, 625 and 3125 μg/mL solutions of beef, lamb, cow's milk and turkey extracts, and with assay buffer alone as a negative control. Serum/food allergen and serum/buffer mixtures were pre-incubated overnight at 2–8°C prior to measurement of IgE binding to beef, lamb and cow's milk allergen coats (all 5 μg/mL) by ELISA, as described above. After subtraction of buffer-only ODs, inhibition was calculated for each sample as follows: % inhibition = $100 - [(OD_{\text{serum with inhibitor}}/OD_{\text{serum without inhibitor}}) \times 100]$. The concentration of food allergen required to achieve 50% inhibition was interpolated from the sigmoid dose–response curves. Samples whose dose–response curves had an accuracy outside 80–120% of expected at concentrations effecting 50% inhibition were excluded from further analysis.

Food allergen concentrations for inhibition were determined from preliminary experiments using four dog sera, which showed that a concentration of up to 3125 μg/mL was required to effect maximum inhibition (>90%) on homologous coats.

Results

Incidence of reactions

Overall, 261 of 469 dogs (56%) had positive IgE reactions (i.e. >5 AU) to at least one of the 19 foods (Table 1). The frequency of positive IgE reactions to the 19 food allergens is shown in Figure 1. The median number of IgE food reactions per dog was four, with 26% of dogs reacting to a single food allergen and 74% reacting to two or more food allergens (Figure 2).

Pairwise comparisons

The results of pairwise analyses in all 469 dogs are given in Table 2. Pairwise comparisons were also used to calculate the ORs of the reactions to each food allergen pair in those dogs with at least one food IgE reaction (positive reactors; Table 3).

All dogs (n = 469)

Table 1. Incidence of IgE reactions to food allergens

IgE reaction	Number of dogs (n = 469)	% dogs
Positive to at least one food	261	56%
Positive to more than one food	194	41%
Negative to all foods	208	44%

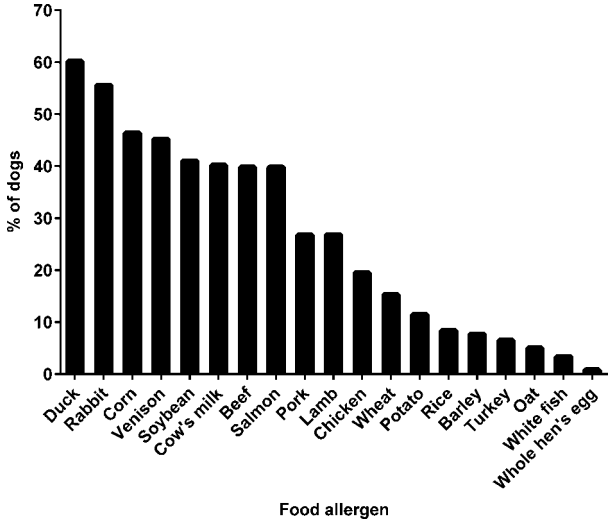

Figure 1. Percentage frequency of serum IgE reactions to food allergens.

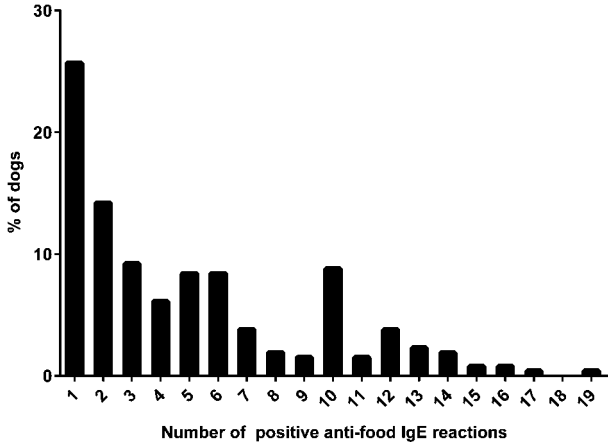

Figure 2. The frequency of IgE reactions to one or more food allergens (range 1–19 allergens) in 469 dogs with suspected adverse food reactions (AFRs).

With the exception of egg, white fish and potato, at least 50% of the comparisons for each allergen were significantly associated, with mean logE ORs ranging from 1.3 (CI 0.6–3.0) to 215.3 (CI 27.8–1668); $P = 0.6$ to $P < 0.0001$ (Table 2). In these dogs, 38 of 43 related food pairs were significantly associated compared with 79 of 128 unrelated pairs ($P < 0.0002$). The mean (SD) logE OR of these associated-related pairs [3.4 (0.9)] was significantly greater than unrelated pairs [2.7 (1.0)], $P < 0.05$ (Table 4).

Positive reactors (n = 261)

For all foods except egg, white fish, potato, rabbit, duck, turkey, barley, rice and oats, at least 50% of the comparisons for each allergen were significantly associated, with mean logE ORs ranging from 0.5 (CI 0.2–1.1) to 213.6 (CI 12.5–3636); $P = 1.0$ to $P < 0.0001$ (Table 3). Of the related allergen pairs, 32 of 43 were significantly associated compared with 49 of 128 unrelated pairs ($P < 0.0002$). The mean (SD) logE OR of these associated-related pairs [2.7 (1.0)] was significantly greater than all unrelated pairs [1.8 (1.0)], $P < 0.05$ (Table 4) and unrelated pairs with a statistically significant association [2.3 (0.8)], $P = 0.035$.

ELISAs and inhibition ELISAs

ELISA AU scores to beef and lamb were very strongly correlated ($r = 0.93$); 75 of 90 (83.3%) beef-positive sera were also lamb-positive and 75 of 81 (92.6%) of lamb-positive sera were positive to beef. AUs to lamb and milk were also strongly correlated ($r = 0.62$); 57 of 81 (70.4%) of lamb-positive sera were milk-positive and 57 of 85 (67.1%) milk-positive sera were positive to lamb. A moderate correlation between beef and milk was observed ($r = 0.59$); 62 of 90 (68.9%) beef-positive sera were also positive to milk and 62 of 85 (72.9%) of milk-positive sera were positive to beef.

Pre-incubation of sera with homologous allergen inhibitor resulted in almost complete (>88%) inhibition at the maximal inhibitory concentration of 3125 μg/mL. The mean concentration of inhibitor required for 50% inhibition of IgE binding to homologous allergen coats was 5.6 μg/mL (range <1–60.2 μg/mL), 27.8 μg/mL (range <1–371.7 μg/mL) and 5.3 μg/mL (range <1–34.4 μg/mL) for beef, lamb and milk, respectively.

Pre-incubation of sera with beef, lamb and milk resulted in 50% inhibition of IgE binding to mammalian protein substrates in the majority of sera (Figure 3). With beef as the coat, 50% inhibition was achieved in 27 of 27 sera by pre-incubation with lamb (mean concentration required for 50% inhibition 457.7 μg/mL, range 26–2016.4 μg/mL) and in 16 of 20 sera by milk (mean concentration required for 50% inhibition 666.5 μg/mL, range 1–2998.7 μg/mL). With lamb as the coat, 50% inhibition was effected in 19 of 19 sera by beef (mean concentration required for 50% inhibition 262.3 μg/mL, range 1–1786.6 μg/mL) and in 17 of 23 sera by milk (mean concentration required for 50% inhibition 419.3 μg/mL, range 1–1427 μg/mL). With milk as the coat, 10 of 19 sera were inhibited by lamb (mean concentration required for 50% inhibition 883.5 μg/mL, range 1–2785.1 μg/mL) and 14 of 16 by beef (mean concentration for 50% inhibition 616.4 μg/mL, range 1–2837.4 μg/mL). In contrast, pre-incubation of sera with turkey effected little to no inhibition of IgE binding to beef, lamb and cow's milk coats.

Discussion

In this study, we investigated whether serum IgE reactions were associated more frequently among related food allergens than unrelated food allergens, and whether such associations were the result of cross-reactivity. Pairwise comparisons showed that dogs with a positive IgE reaction to one food within a related group were significantly more likely to have a positive IgE reaction to another food within the same group than to an unrelated food. Moreover, IgE scores to beef, lamb and cow's milk were strongly correlated. However, another approach, using ELISA inhibition, was necessary to ascertain if associations between related food allergens were the result of immunological cross-reactivity.

Although the majority of the 469 dogs were positive to at least one food allergen, approximately 40% of the dogs

Table 2. Odds ratios (ORs, 95% confidence intervals) of food allergen pairs in all dogs (n = 469 dogs).

	Pork	Lamb	Venison	Rabbit	Milk	Chicken	Turkey	Duck	Egg	Salmon	White fish	Wheat	Soybean	Barley	Rice	Potato	Corn	Oat
Beef	32.8* (16.7–64.5)	11.3* (6.4–19.9)	19.4* (11.4–33.0)	13.3* (8.0–22.4)	9.1* (5.5–14.9)	12.3* (6.4–23.0)	18.8* (5.3–66.7)	45.0* (22.0–91.0)	17.8 (0.8–374.0)	34.7* (18.0–66.0)	30.4 (3.7–245.0)	5.8* (3.0–11.4)	8.1* (5.0–13.3)	5.8 (2.3–14.7)	3.8 (1.6–9.1)	2.5 (1.2–5.4)	5.1* (3.2–8.1)	3.1 (1.0–9.5)
Pork		30.0* (15.7–57.0)	31.0* (15.3–62.3)	142.2* (34.1–592.9)	15.6* (8.7–28.3)	29.0* (14.4–59.5)	54.1* (12.1–243.0)	243.0* (33.0–1780.0)	29.0 (1.3–614.0)	32.8* (16.7–65.0)	123.0 (7.1–2147.0)	12.5* (6.2–25.0)	10.6* (6.1–19.0)	16.4* (6.1–44.0)	12.2* (5.2–21.0)	3.2 (1.4–7.0)	18.5* (9.9–34.0)	37.0* (8.1–171.0)
Lamb			40.1* (19.1–86.4)	142.2* (34.1–593.0)	14.3* (8.0–25.6)	65.0* (28.0–146.0)	54.0* (12.0–243.0)	98.0* (30.0–320.0)	29.0 (1.4–614.0)	48.0* (23.0–101.0)	109.0 (6.3–1898.0)	8.5* (4.3–17.0)	11.5* (6.5–20.0)	10.1* (3.9–25.0)	5.3 (2.2–13.0)	2.7 (0.9–5.1)	13.8* (7.6–25.0)	10.1 (3.2–32.0)
Venison				29.2* (16.6–51.1)	13.2* (8.0–21.9)	29.0* (13.0–67.0)	25.0* (5.7–113.0)	26.0* (15.0–46.0)	15.0 (0.7–316.0)	26.0* (15.0–46.0)	61.0 (3.5–1056.0)	8.7* (4.3–18.0)	10.2* (6.3–16.7)	7.7* (2.9–20.0)	4.7 (1.9–11.3)	1.3 (0.6–3.0)	9.6* (5.9–15.0)	7.1 (2.1–23.0)
Rabbit					9.3* (5.7–15.1)	38.0* (13.0–109.0)	40.0 (5.2–305.0)	27.0* (16.0–46.0)	11.3 (0.5–237.0)	29.0* (16.0–54.0)	45.0 (2.6–781.0)	11.0* (5.1–25.0)	10.4* (6.3–17.0)	7.3 (2.6–21.0)	6.5* (2.5–17.0)	2.1 (1.1–4.5)	7.6* (4.8–12.0)	29.0 (3.8–226.0)
Milk						22.0* (11.1–23.0)	30.0* (6.7–134.0)	23.0* (11.2–48.0)	3.5 (0.2–56.0)	15.5* (9.2–27.0)	71.0 (4.1–1244.0)	10.1* (5.2–21.0)	12.4* (7.5–21.0)	11.7* (4.1–33.0)	8.5* (3.4–21.0)	1.5 (0.7–3.4)	13.1* (7.9–21.0)	21.0* (4.6–97.0)
Chicken							215.3* (27.8–1668.0)	25.5* (9.9–65.6)	17.0 (1.5–191.0)	91.0* (27.0–303.0)	77.0* (9.5–635.0)	6.6* (3.2–13.0)	13.2* (6.7–25.0)	8.0* (3.1–20.0)	6.8* (2.7–16.0)	2.7 (1.1–6.7)	23.4* (10.6–52.0)	15.4* (4.8–49.0)
Turkey								35.3* (4.6–268.0)	60.1* (5.2–700)	66.0* (8.7–505.0)	47.0* (11.1–195.0)	15.2* (5.5–42.0)	29.3* (6.6–130.0)	17.0* (5.5–52.0)	7.4 (2.2–25.0)	2.0 (0.4–9.2)	52.0* (6.9–403.0)	23.0* (6.5–81.0)
Duck									4.0 (0.4–44.6)	39.0* (20.0–78.0)	40.0 (2.3–691.0)	11.6* (5.0–27.0)	12.5* (7.5–21.0)	8.7* (2.9–26.0)	7.5* (2.7–20.0)	2.8 (1.3–5.9)	9.0* (5.7–14.0)	25.0 (3.3–199.0)
Egg										17.8 (0.8–374.0)	57.0 (3.3–1000.0)	10.9 (0.7–178.0)	3.4 (0.2–55.0)	23.5 (1.4–391.0)	21.2 (1.3–351.0)	15.0 (0.9–247.0)	14.6 (0.7–305.0)	37.9 (2.2–642.0)
Salmon											34.5* (4.3–275.0)	10.8* (5.3–22.0)	19.7* (11.5–33.0)	7.3* (2.8–16.0)	5.7* (2.3–13.6)	1.8 (0.8–4.0)	11.8* (7.2–19.0)	12.8 (3.5–47.0)
White fish												15.0* (3.9–59.0)	70.0 (4.2–1211.0)	22.2* (5.4–91.0)	11.6 (2.7–50.0)	1.9 (0.2–15.0)	58.0 (3.4–1019.0)	40.0* (9.2–174.0)
Wheat													45.4* (15.6–131)	174.0* (38.0–800.0)	18.0* (7.1–45.0)	16.6* (7.3–37.7)	36.4* (12.6–105.0)	47.3* (12.4–181.0)
Soybean														22.6* (6.5–78.8)	8.3* (3.3–20.9)	3.3 (1.5–9.9)	16.1* (9.6–27.0)	20.6* (4.5–94.6)
Barley															90.8* (29.3–281.0)	35.9* (13.0–98.0)	140.0 (8.4–2347.0)	148.0* (35.0–623.0)
Rice																17.8* (6.8–46.0)	34.0* (7.8–149.0)	77.0* (20.1–281.0)
Potato																	9.4* (4.1–21.0)	15.4* (4.8–49.0)
Corn																		86.7 (5.1–1170.0)

*Significantly associated ($P < 0.0002$).

had no IgE reaction to any of the 19 foods tested. None of the dogs in this study had a definitive diagnosis of AFR. Therefore, we do not know if these dogs were suffering from conditions other than an AFR, had non-IgE-mediated AFRs, or had AFRs to food(s) other than those included here. To eliminate the possibility that these dogs could have skewed the results, pairwise comparisons were repeated on data from dogs with at least one positive IgE reaction. The overall outcome was the same: a significant association was more likely between related food pairs than unrelated foods.

Significant associations between unrelated food allergens were observed frequently. However, it is notable that ORs for these associated-unrelated foods were significantly lower than associated-related foods. Pairwise analyses do not differentiate between cross-reactivity and co-sensitization, and we did not confirm the nature of these associations by ELISA inhibition. Having ascertained that significant associations existed between related foods, the presence of serum IgE antibodies cross-reacting with different mammalian species was assessed by inhibition ELISAs. The food extracts were used at concentrations over 600 times greater than those used for coating, thus effecting maximum homologous inhibition of IgE binding and ensuring that any cross-reactive IgE antibodies with low affinity for an extract could be identified. Considerable heterologous inhibition of IgE binding was observed with beef and lamb. Milk had less effect on IgE binding to beef and lamb, with fewer dogs reaching 50% inhibition, even at high inhibitor

Table 3. Odds ratios (ORs, 95% confidence intervals) of food allergen pairs in positive reactors (n = 261 dogs)

	Pork	Lamb	Venison	Rabbit	Milk	Chicken	Turkey	Duck	Egg	Salmon	White fish	Wheat	Soybean	Barley	Rice	Potato	Corn	Oat
Beef	13.4* (6.8–26.7)	4.4* (2.5–7.9)	6.9* (3.9–11.9)	4.03* (2.3–6.9)	3.2* (1.9–5.3)	5.0* (2.6–9.8)	8.0 (2.2–28.6)	14.0* (6.8–29.0)	7.7 (0.4–161.8)	12.5* (6.4–24.3)	13 (1.6–105.6)	2.3 (1.2–4.6)	2.8* (1.7–4.6)	2.4 (1.0–6.2)	1.6 (0.7–3.8)	1.0 (0.5–2.2)	1.5 (0.9–2.5)	1.3 (0.4–4.0)
Pork		13.3* (6.9–25.6)	12.0* (5.9–24.5)	50.3* (12.0–211.6)	6.2* (3.4–11.3*	14.2* (6.9–29.1)	25.8* (5.7–116.2)	80.8* (11.0–593.8)	14.0 (0.7–295.0)	13.4* (6.8–26.7)	59.2* (3.4–1032)	5.7* (2.8–11.6)	4.1* (2.3–7.3)	7.7* (2.8–21.0)	5.7* (2.3–14.3)	1.4 (0.6–3.2)	6.9* (3.6–13.2)	17.6* (3.8–81.8)
Lamb			15.9* (7.4–34.3)	50.3* (12.0–211.6)	5.6* (3.1–10.2)	30.3* (13.3–69.1)	25.8* (5.7–116.2)	25.1* (7.6–82.5)	14.0 (0.7–295.0)	20.1* (9.5–42.4)	59.2 (3.4–1032)	3.9 (1.9–7.8)	4.5* (2.5–8.0)	4.7 (1.8–12.1)	2.5 (1.0–6.0)	1.0 (0.4–2.3)	5.1* (2.8–9.5)	4.8 (1.5–15.2)
Venison				9.1* (5.1–16.4)	4.5* (2.7–7.6)	11.6* (5.0–26.9)	10.3 (2.3–45.9)	7.6* (4.2–13.8)	6.2 (0.3–129.6)	9.6* (5.4–17.0)	24.9 (1.4–432.8)	3.4 (1.6–7.0)	3.4* (2.0–5.7)	3.1 (1.1–8.3)	1.8 (0.8–4.5)	0.5 (0.2–1.1)	3.1 (1.9–5.0)	2.9 (0.9–9.6)
Rabbit					2.7 (1.6–4.5)	13.4* (4.7–38.6)	14.3 (1.9–109.3)	7.3* (4.2–12.8)	4.1 (0.2–85.5)	9.6* (5.2–18.0)	16.2 (0.9–281.8)	3.8 (1.7–8.7)	3.0* (1.8–5.1)	2.6 (0.9–7.3)	2.3 (0.9–6.0)	0.7 (0.3–1.4)	2.0 (1.2–3.3)	10.4 (1.3–81.1)
Milk						9.4* (4.4–19.8)	12.8* (2.9–57.5)	2.8 (1.6–4.8)	1.5 (0.1–24.1)	5.7* (3.3–9.8)	30.9 (1.7–535.8)	4.4* (2.1–9.1)	4.4* (2.6–7.5)	5.0 (1.8–14.3)	3.5 (1.4–9.0)	0.6 (0.3–1.0)	4.4* (2.6–7.5)	9.0 (2.0–41.6)
Chicken							213.6* (12.5–3636)	8.2* (3.1–21.5)	21.3 (1.0–450.3)	19.2* (7.8–47.4)	38.9* (4.7–319.2)*	3.1 (1.5–6.4)	5.3* (2.9–10.4)	3.9 (1.5–9.9)	3.2 (1.3–8.1)	1.3 (0.5–3.2)	9.1* (4.1–20.4)	7.6 (2.4–24.5)
Turkey								11.7 (1.5–89.6)	78.9 (3.6–1716)	28.4* (3.7–217.6)	25.0* (5.9–105.2)	7.7 (2.8–21.5)	12.4* (2.8–55.4)	9.0 (2.9–27.8)	3.9 (1.1–13.1)	1.0 (0.2–4.7)	21.2* (2.8–162.3)	12.3 (3.5–43.3)
Duck									3.4 (0.2–70.8)	12.3* (6.1–24.8)	13.4 (0.8–232.4)	3.7 (1.6–8.7)	3.5* (2.0–6.0)	2.8 (0.9–8.7)	2.4 (0.9–6.7)	0.9 (0.4–1.8)	2.2 (1.3–3.7)	8.5 (1.1–66.6)
Egg										7.7 (0.4–161.8)	31.4 (1.8–548.1)	5.6 (0.3–92.2)	1.4 (0.1–23.4)	12.6 (0.8–210.1)	11.3 (0.7–187.9)	7.9 (0.5–130.3)	5.9 (0.3–123.7)	20.6 (1.2–349.6)
Salmon											31.3 (1.8–544.9)	4.5* (2.1–9.2)	7.2* (4.1–12.6)	3.1 (1.2–8.0)	2.3 (1.0–5.7)	0.7 (0.3–1.6)	4.0* (2.4–6.7)	5.5 (1.5–20.4)
White fish												7.8 (2.0–30.3)	29.8 (1.7–518.2)	11.8 (2.9–48.3)	6.1 (1.4–26.5)	1.0 (0.1–8.0)	23.7 (1.4–412.4)	21.6 (4.9–94.3)
Wheat													19.0* (6.5–55.5)	89.6* (19.5–411.9)	9.0* (3.6–22.9)	8.2* (3.6–18.9)	14.4* (4.9–41.9)	24.2* (6.3–93.0)
Soybean														9.5* (2.7–33.4)	3.4 (1.3–8.7)	1.3 (0.6–2.8)	5.5* (3.2–9.4)*	8.8 (1.9–40.2)
Barley															47.9* (15.4–148.9)	18.6* (6.7–51.3)	56.8* (3.4–950.0)	79.3* (18.9–334.0)
Rice																9.1* (3.5–23.7)	13.7* (3.1–59.84)	40.7* (11.0–149.8)
Potato																	3.7 (1.6–8.6)	8.0 (2.5–25.8)
Corn																		35.0* (2.1–595.1)

*Significantly associated ($P < 0.0002$).

Table 4. LogE odds ratios (ORs) in all dogs and dogs with at least one positive reaction (positive reactors)

	LogE ORs					
	All dogs (469 dogs)			Positive reactors (261 dogs)		
	Associated-related	Nonassociated-related	Unrelated	Associated-related	Nonassociated-related	Unrelated
Mean	3.4*	3.0	2.7*	2.7†	2.1	1.8†
SD	0.9	1.7	1.0	1.0	1.2	1.0
n	38	5	128	32	11	128

SD, standard deviation; n, number of pairwise comparisons.

*Mean logE ORs of associated-related food pairs and unrelated food pairs that were significantly different in all dogs. ($P < 0.05$).

†Mean logE ORs of associated-related foods and unrelated foods that were significantly different in positive reactors. ($P < 0.05$). Mean logE ORs of associated-related food pairs and nonassociated-related or unrelated food pairs were not statistically different ($P > 0.05$).

concentrations. Furthermore, higher mean concentrations of beef and lamb were required to achieve 50% inhibition of IgE binding to milk. Lower heterologous inhibition of IgE binding to beef and lamb by milk could be due to a lack of cross-reactive proteins in the milk extract and/or lower IgE binding affinity to milk proteins compared with beef and lamb. Inhibition data were thus consistent with the presence of cross-reactive IgE binding epitopes in beef and lamb, and to a lesser extent, cow's milk. These IgE binding epitopes were largely absent in turkey. Previous studies have identified a number of common mammalian food allergens. In humans,

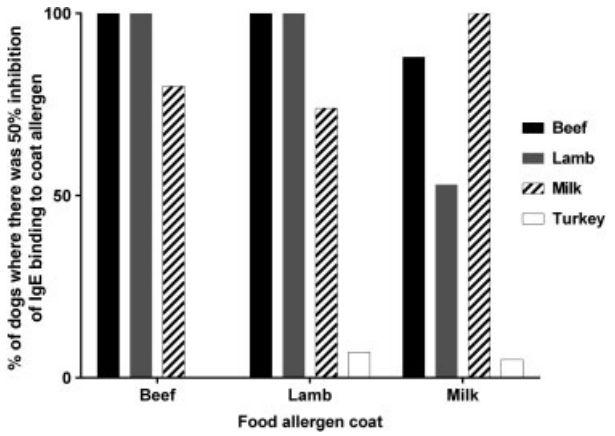

Figure 3. Percentage of dogs where there was 50% inhibition of IgE binding to beef, lamb and cow's milk allergen coats (5 μg/mL) following preincubation of sera with beef, lamb, cow's milk and turkey inhibitors (3125 μg/mL).

various allergenic beef proteins have been identified including bovine serum albumin (BSA, Bos d 6), actin and IgG (Bos d 7).[19] IgG and muscle phosphoglucomutase were also found to be major cross-reactive allergens in dogs with specific IgE against beef, cow's milk and, importantly, lamb,[5] which was, until fairly recently, commonly used in limited antigen diets.

The likelihood of cross-reactivity is increased amongst closely related foods, particularly if amino acid sequence homology is greater than 70%.[20] Beef, lamb and cow's milk are derived from the same biological family (Bovidae) and share a recent common ancestor. As a consequence they are more likely to have similar antigens, leading to increased cross-reactivity. In contrast, turkey shared a common ancestor with mammals more than 100 million years ago and cross-reactivity with mammalian proteins is unlikely. Cross-reactivity has, however, been described between phylogenetically distinct species,[21] particularly among highly conserved proteins. For example, serum albumins have highly conserved amino acid sequences and three-dimensional structures amongst a diverse range of species. Turkey serum albumin displays moderate (~50%) sequence identity and similarity to BSA,[22] although this is below the 70% identity threshold believed to be required for immunological cross-reactivity. In humans, the clinical significance of allergenic cross-reactivity has been variable. A number of studies in patients with cow's milk allergy demonstrated that between 60 and 87.5% had specific IgE to beef.[23,24] However, a review of studies in infants with cow's milk allergy found that only 13–20% were also beef allergic; interestingly, in their own double-blind, placebo-controlled food challenge study, the authors found up to 92.9% of children with beef allergy had concomitant milk allergy.[25] It is not known how many dogs in the present study had confirmed cutaneous AFR. Therefore, despite the demonstration of shared IgE-binding epitopes between related foods in some of the dogs, their clinical relevance is unknown.

In conclusion, this study demonstrated that associations in IgE reactivity between the related mammalian foods beef, lamb and cow's milk were, in some cases, due to the presence of cross-reactive epitopes. The observation of single IgE reactions to an allergen within a related food group known to share IgE-binding epitopes, however, suggests that there may be considerable heterogeneity in food-specific IgE reactions among dogs and that extrapolating cross-reactivity data to the dietary management of AFRs is not straightforward. ELISAs for food allergen-specific IgE could be helpful in identifying potential allergenic reactivity against novel proteins, and have been used to guide the selection of foods for inclusion in elimination diet trials based on a lack of IgE reaction to those foods.[8] Such tests cannot, however, definitively predict a clinical reaction or exclude the possibility that novel proteins could induce an allergic reaction in susceptible individuals. Thus, although it would be prudent to avoid cross-reactive and/or closely related foods in the selection of foods for elimination diet trials, further research is required to determine which, if any, cross-reactive food allergens mediate clinically relevant reactions in allergic dogs.

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Triggers, risk factors and clinico-pathological features of urticaria in dogs – a prospective observational study of 24 cases

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Background – Urticaria and anaphylaxis are frequently encountered in veterinary practice, but little is known about the causes and relative frequencies of these reactions.

Hypothesis/Objectives – This study was designed to improve current knowledge on the triggers, risk factors and clinico-pathological features of urticaria.

Animals – Twenty four dogs with signs of urticaria with or without anaphylaxis.

Methods – The study included dogs with cutaneous immediate-type hypersensitivity reactions. The cases were grouped by clinical severity into either an urticaria or an anaphylaxis group. All treatments and diagnostic tests (haematology, biochemical profile, allergy investigation) were recorded. A causality algorithm for urticaria and anaphylaxis (ALUA) was designed to determine the probability of the identified triggers and cofactors. Disease incidence, breed, age and gender predispositions were evaluated statistically.

Results – Sixteen of 24 urticaria cases were associated with anaphylaxis whilst 8 of 24 were confined to the skin. The annual hospital incidence was 0.12%. Females seemed to be over-represented (2.4:1) and most of the dog breeds were pure breed (22 of 24), with Rhodesian ridgeback, boxer, beagle, Jack Russell terrier, French bulldog and Vizslas over-represented. In addition to skin lesions, the most frequently and severely affected organ systems were the gastrointestinal and cardiovascular systems. The predominant blood abnormalities were elevated lipase and alanine aminotransferase values. Insects, food and drugs were the most commonly identified triggers.

Conclusions – To the best of our knowledge, this is the first study describing the trigger factors and clinico-pathological features of dogs with urticaria in veterinary medicine. Insects, food and drugs were the most frequently detected triggers.

Introduction

Urticaria is a disease characterized by the sudden development of wheals (hives), angioedema or both.[1–4] A wheal is a transient elevation of the skin due to dermal oedema.[5] Angioedema is characterized by a sudden, pronounced erythematous swelling of the lower dermis and subcutis with frequent involvement of the lower extremities and head, with or without associated wheals.[6] Pruritus may or may not be present.[7]

Urticaria is, along with flushing and acute pruritus, one of the typical cutaneous signs of an anaphylactic reaction. These signs may occur alone or in combination with other signs of anaphylaxis. Anaphylaxis is a serious, generalized or systemic, allergic or hypersensitivity reaction with sudden onset (minutes to a few hours) that can be life threatening or even fatal.[8–12]

The target organs in humans often include the respiratory tract (70% of episodes) and less frequently the gastrointestinal (GI) organs (30–45% of episodes), heart and vasculature (10–45% of episodes) and central nervous system (CNS) (10–15% of episodes).[8] It is generally agreed that anaphylaxis may occur with or without cutaneous signs, even though these latter signs are frequent and occur in 80–100% of cases.[8,11,13] In dogs, the main shock organ is the liver leading to GI signs such as vomiting and diarrhoea (over 90%).[14–18] Some GI changes may be due to direct histamine release from the intestine, but are mostly associated with mediator release directly from the liver, as shown in a study with dehepatized dogs.[15,19] The cardiovascular signs are most often secondary to liver changes (hepatic arterial vasodilation and increase in hepatic portal vascular resistance).[17,18] In two studies, other affected organs in dogs were the skin (ranging from 57 to 68%) and, less frequently, the respiratory and neurological systems.[18,20] Interestingly, however, the highest mast cell density and histamine content after experimental anaphylaxis induction was recorded in the

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liver, skin and in particular the ear pinnae, which suggests that the skin should often, if not always, be affected, as is the case in humans.[15] This conflicting interpretation may be due, at least in part, to the large number of differential diagnoses for anaphylaxis in the absence of skin signs.

In urticaria and anaphylaxis, activated target cells (mast cells and basophils) release histamine and other mediators (platelet-activating factor, cytokines) provoking vasodilatation, plasma extravasation and sensory nerve activation, as well as recruiting inflammatory cells to urticarial lesions.[7] The activating signals are of immunological and nonimmunological nature. The immunological reactions are mainly immunoglobulin (Ig) E driven, although IgG or immune complexes may also be involved, with triggers such as allergens, infectious agents (bacteria, parasites) or autoantibodies. Nonimmunological urticaria and anaphylaxis (WHO recommendation: "the term 'anaphylactoid' should be avoided") are caused by a heterogeneous group of mechanisms, such as heat, pressure, cold and vibration.[21]

The diagnosis of urticaria usually is straightforward in dogs and humans due to its distinct clinico-historical pattern and scarcity of differential diagnoses.[1–4] In humans, urticaria is currently classified as acute (<6 weeks duration) and chronic (>6 weeks duration). In contrast to veterinary medicine, the clinical criteria for diagnosing and grading anaphylaxis are well established in human medicine, but still under continuous debate.[11,22] As mentioned above, this is largely due to the huge number of possible differential diagnoses, especially when skin signs are lacking. In this latter situation, anaphylaxis should be differentiated from all other causes of acute diarrhoea or vomiting, respiratory distress, collapse, heart failure or sudden death.

A search for a specific trigger is recommended in every patient with allergic acute urticaria and all chronic urticaria cases.[7] In veterinary medicine, the following diagnostic tests in addition to a thorough history have been recommended: faecal flotation, diet trial, intradermal tests and/or IgE serology (mostly for insects), ice cube test and skin biopsy with histological examination.[1] The most important triggers of anaphylaxis and acute urticarial episodes in humans are food, stinging insects, drugs, latex and exercise.[10,12,23] Reported risk factors and co-factors include age, allergen amount and type (e.g. peanut), atopy, premenstrual status, stress, concomitant medications (e.g. ACE inhibitors, beta-blockers) or diseases (e.g. mastocytosis, asthma).[10,23,24] In dogs, the triggers of urticaria and/or anaphylaxis that are supported by original studies or case reports are vaccines,[25] anaesthetics,[26,27] food,[28,29] venoms,[30–32] drugs,[1,17] glucocorticoids,[33] transfusions,[34] plants[35] and radiocontrast media.[36] Some rare causes such as heat, exercise, sunlight, dermatographism, estrous, intestinal parasites, allergen-specific immunotherapy and intradermal testing are only anecdotally reported in veterinary textbooks.[1–3] Little is known about risk or cofactors in veterinary medicine.[1,3]

The therapeutic approach is universal and based on the same principles as in other mast cell dependent diseases in the field of allergy: (i) cause elimination/avoidance, (ii) symptomatic pharmacological treatment: by reducing mast cell mediator release and/or the effect of these mediators at the target organ and (iii) inducing tolerance. In dogs, clinical signs of urticaria may resolve spontaneously within 24–48 h, but symptomatically a short course of glucocorticoids and concurrent antihistamines may be considered.[1] The current recommendations for treatment of anaphylaxis depend on the disease severity and include epinephrine, fluid therapy, oxygen, glucocorticoids, antihistamines and bronchodilators.[5,12,17,37,38]

The purpose of this prospective study was to identify the incidence, aetiology, clinical features, and the therapeutic and diagnostic approach of dogs presenting with urticaria (with/without other signs of anaphylaxis).

Materials and methods

Study design and setting

This prospective study included dogs submitted to the Department of the Clinic for Small Animal Internal Medicine and Anesthesiology, Vetsuisse Faculty, Zurich, during a 1 year period (June 2014–2015).

Animal selection and definitions

Dogs with clinical cutaneous signs of immediate-type hypersensitivity reactions, such as urticaria, were included. Urticaria was defined as an acute onset of multiple wheals and/or angioedema.[7] Wheals were defined as well-circumscribed, raised lesions caused by oedema within the dermis.[7] An erythematous swelling of the lower dermis and subcutis was defined as angioedema. The diagnosis was confirmed after exclusion of other differential diagnoses (Table 1).

In some cases, clinical cutaneous signs occurred in combination with systemic signs of immediate-type hypersensitivity reactions. Anaphylaxis refers to a severe, potentially fatal, systemic reaction that occurs suddenly after contact with an allergy-causing substance.[11] Clinical criteria for anaphylaxis were adapted from human medicine.[8,11,12,21,39] A dog was diagnosed with anaphylaxis if, in addition to urticaria, there were signs of systemic involvement:

1. Gastrointestinal: vomiting/nausea, diarrhoea
2. Cardiovascular: tachy- or bradycardia, hypotension, pale mucous membranes, cardiac arrest
3. Respiratory: dyspnoea, tachypnoea cyanosis, respiratory arrest

Patients management and data collection

Data were collected during two consecutive time periods. Immediate data collection included signalment, history and a thorough clinical examination. In the case of anaphylaxis and with owner consent, patients were immediately hospitalized and treated accordingly. Dogs without signs of anaphylaxis were followed on an outpatient basis. Some cases required additional clinico-pathological work-up, such as haematology, biochemical profile, blood pressure measurement, ultrasound, radiographs and/or skin biopsies. Records were taken of the final outcome and all diagnostic and therapeutic procedures. In addition, the history of suspected triggers and co-factors was obtained as soon possible. Follow-up data included information regarding circumstances prior to or around the event: food consumption, utilization of drugs, observation of insect bites or stings, time of day at onset (morning, during the day, night), location (garden, during

Table 1. Differential diagnoses for wheals and angioedema

Wheals	Angioedema
Bacterial folliculitis	Juvenile cellulitis
Vasculitis	Infectious cellulitis
Erythema multiforme	Mast cell tumour
Cutaneous lymphoma	Cutaneous lymphoma
Mastocytosis	Lymphoedema
Amyloidosis	

walk, at home), exercise, stressful events, presence of estrous or pregnancy, association with physical factors (cold, heat, excessive sun, exposure, swimming, bathing).

If indicated and allowed by the owner, additional allergen tests were performed to attempt to confirm the trigger and immunological background (at least 2–4 weeks after the event). Depending on the suspected cause, skin testing (prick and intradermal) for hymenoptera, nuts and aeroallergens, *in vitro* testing to food allergens (Cyno-DIAL© Western blot-based test, Gallileo Diagnostic by Genclis; Vandoeuvre-lès-Nancy, France)[40] and aeroallergens (HESKA; Fribourg, Switzerland), elimination diet, ice cube test[41], autologous serum test[4] and dermatographism[42] were performed.

Outcome measurements and statistical analysis

Demographics and incidence

Breed, gender and age of the included population were compared to the data in the Swiss dogs registration system (ANIS) and to the data of our institution. In addition, incidence was computed by comparison to the overall number of consultations in our institution during the same time period.

Clinical signs and grading

An attempt to grade the severity of the reaction was also made (Table 2). The classification was based mainly on the affected organs and overall effect on the general condition of the dogs. Grades 0, 1, 2 and 3 refer to urticaria without anaphylaxis, mild, moderate and severe anaphylaxis, respectively.

Assessment of the causality

Assessment of the causality of allergic events is important not only in the management of patients who have experienced such events, but also for prevention of recurrence. In order to objectively determine the probability of the identified triggers, a causality algorithm for urticaria and anaphylaxis (ALUA) with a score range from 0 to 22 was designed. The principles of the Alden and Naranjo scales were followed and modified accordingly (Table 3).[43,44] A cause was considered very likely or likely with ALUA values >10 and 5–10, respectively. A trigger was considered possible, but could not be confirmed, if the ALUA was <5.

Correlations

An attempt to establish correlations between overall disease severity and demographic data (age, gender, breed), trigger type, severity of skin changes (wheals versus angioedema versus both) and blood changes was made.

Statistical analysis

Numerical data such as age, weight and hospitalisation duration, were expressed as the mean, with minimal and maximal ranges. Possible age, gender and breed associations were calculated using a Fischer two-tailed exact test. Associations between different descriptive data sets were made by chi-square with Yate's correction. All analyses were made using SPSS software v22.0 (IBM Corp; Armonk, NY, USA). Statistical significance was defined as $P < 0.05$.

Results

Incidence and demographics

Among all 20,000 hospital visits during the assessment period, 24 cases of urticaria were included in this study. The annual hospital incidence was 0.12% or 12 cases per 10,000 patients. Table 4 presents the demographic data. The average age was 4 years (0.7–11 years) and weight 21 kg (5–47.8 kg). Females outnumbered males by a ratio of 2.4:1 ($P = 0.06$).

Twenty two dogs were purebred and the following breeds appeared represented in comparison with the individual breed frequency within the Animal Identity Service Register (ANIS) Swiss reference population of the Animal Identity Service (ANIS): Rhodesian ridgeback (3 of 24, $P = 0.0002$), boxer (3 of 24, $P = 0.002$), beagle (2 of 24, $P = 0.02$), Jack Russell terrier (2 of 24, $P > 0.05$), French bulldogs (2 of 24, $P = 0.06$) and Vizsla (2 of 24, P = 0.003) appeared. There was no correlation between age, breed, gender and the disease severity.

Seasonality

Eleven cases were diagnosed in spring, whereas two, seven and four were seen in summer, autumn and winter, respectively. Spring seemed mainly correlated with insect bites, because 5 of 11 cases were likely to be due to insects.

Clinical signs and grading

Eight of 24 (33%) cases were considered as urticaria without anaphylaxis (grade 0) and 16 of 24 dogs (67%) urticaria with anaphylaxis. Four of the anaphylaxis group

Table 2. Grading of disease severity in dogs with signs of urticaria and/or anaphylaxis (as adapted[10,18,38])

	Urticaria	Anaphylaxis		
Organ	Grade			
	0 (Anaphylaxis absent)	1 (Mild)	2 (Moderate)	3 (Severe)
		≤ 1 sign from two different organ systems each	≤ 1 sign from two different organ systems each	≤ 1 sign from two different organ systems each
Skin	Wheals and/or angioedema	Wheals, angioedema, flushing, pruritus	Wheals, angioedema, flushing, pruritus	Wheals, angioedema, flushing, pruritus
Gastrointestinal system	None	Single episode of vomiting/ diarrhoea	Abdominal pain Persistent vomiting Persistent diarrhoea	Abdominal pain Persistent vomiting Persistent diarrhoea
Cardiovascular system	None	None	Tachycardia Pale mucous membranes	Collapse Bradycardia Hypotension Cardiac arrest
Respiratory system	None	None	Dyspnoea Tachypnoea Panting	Cyanosis Bradypnea Respiratory arrest

Table 3. Canine urticaria and anaphylaxis causality score (adapted from Alden and Naranjo[43,44])

	Criterion	Values	Final ALUA score
Trigger			
Food	Observed unusual food intake:		0 to 22
	< 3 h prior to onset	4 points	
	3-24 h prior to onset	2 points	
	Presence of IgE [Western blot, Skin prick test (SPT), Intradermal test (IDT)]	2 × points allocated above	
	Positive challenge	10 points	
Drug	Observed drug administration:		0 to 22
	3 h prior to onset	4 points	
	3-24 h prior to onset	2 points	
	Presence of IgE (SPT, IDT)	2 × points allocated above	
	Positive challenge or the drug present in the body at onset	10 points	
Insect	Observed insect bite or sting	6 points	0 to 22
	Suspected insect bite or sting	3 points	
	Presence of IgE (IgE serology, SPT, IDT)	2 × points allocated above	
	Previous similar observations	4 points	
Cold	Observed exposure to cold in the last 1-3 h prior to onset	4 points	0 to 16
	Positive ice cube test	2 × points allocated above	
	Previous similar observations	4 points	
Cofactors			
Exercise or stress	Onset during exercise or stressful event	4 points	0 to 8
	Previous similar observations	4 points	
Pregnancy or estrous	Onset during pregnancy or estrous	4 points	0 to 8
	Previous similar observations	4 points	
Illness	Presence of mast cell tumour or mastocytosis	5 points	0 to 5
	Presence of disease affecting the immune system or known to enhance IgE production	2 points	
	Presence of other disease	1 point	

ALUA, algorithm for urticaria and anaphylaxis.

Table 4. Demographics of 24 dogs with urticaria

Variable	Parameter
Age	
Average age in years (minimum, maximum)	4.0 (0.7, 11)
Age range, no. (%)	
0–4 years	12 of 24 (50)
5–8 years	11 of 24 (45.8)
>9 years	1 of 24 (4.2)
Average weight in kg (minimum, maximum)	20.9 (5, 47.8)
Gender, no. (%)	
Female	17 of 24 (70.8)
Male	7 of 24 (29.2)
Breed, no. (%)	
Pure breed	22 of 24 (91.7)
Rhodesian ridgeback, boxer	3 of 24 (12.5)
Beagle, Vizsla, Jack Russell terrier	2 of 24 (8.3)

were considered mild, nine as moderate and three as severe (Table S1). The first clinical signs observed by the owner in the anaphylaxis group were the urticaria/angioedema in 8 of 16 (50% cases) and vomiting/diarrhoea in 9 of 16 cases. In three dogs with anaphylaxis, the GI and skin signs appeared simultaneously. In two of three dogs with severe anaphylaxis, the first sign was collapse; later on, all three developed angioedema and one of these also wheals. Gastrointestinal signs were present very frequently (14 of 16) and occurred several times. In the mild group, four of five showed just one episode of vomiting or diarrhoea. Almost all shock organs were affected in the moderate and severe groups (Table S2) and most of the signs related to the liver/gastrointestinal, cardiovascular and neurological systems. No severe respiratory signs were encountered; with tachypnoea as the only feature (6 of 16). Neurological signs included only changes in mentation (10 of 16 cases), with 9 of these 10 being dull and the remaining one stuporous. Six of the 16 dogs showed body temperature changes by demonstrating hypothermia and hyperthermia (three cases each).

As far as the skin changes were concerned, wheals were observed in 16 of 24 (67%) dogs (7 urticaria, 9 anaphylaxis) and angioedema in 17 of 24 dogs (4 urticaria, 13 anaphylaxis). Angioedema occurred concomitantly with wheals in 9 of 16 cases (3 urticaria, 6 anaphylaxis).

The overall severity of the case did not seem to be related to the severity of skin signs (Table S2), except for some tendencies related to angioedema and erythema. Isolated angioedema was more often observed in anaphylaxis cases (1 urticaria, 7 anaphylaxis) and never related to food, but most often to insect bites (n = 4), drugs (n = 1) or unknown causes (n = 2).

Erythema (pinnal and/or on other body regions) was observed in 13 of 24 cases (3 urticaria, 10 anaphylaxis). Pinnal erythema was seen altogether in seven cases (1 urticaria, 6 anaphylaxis) and was graded as mild in the urticaria group and as moderate or severe in the anaphylaxis group.

It was interesting to note that the majority of cases with reactions to insects were associated with angioedema (n = 5) whereas this change was present only in two cases of food allergy, both involving nuts. In addition, reactions to insects were more often associated with anaphylaxis (3 urticaria, 6 anaphylaxis).

Laboratory abnormalities

As expected, diagnostic investigations were most frequently carried out in anaphylaxis cases. These included haematology and biochemistry in 14 of 16 (87%) and three of eight (37%) urticaria and anaphylaxis cases, respectively. Abdominal and cardiac ultrasound examinations were performed in five and one dog, respectively, and were unremarkable. Haematology revealed leukocytosis in six cases (three urticaria, three anaphylaxis) and leukopenia in one moderate anaphylaxis case. In the urticaria group, no abnormalities were detected. In the anaphylaxis group, elevated lipase (range 109–1480 U/L, mean 521.4 U/L, reference range 24–108 U/L) was observed in seven cases (one mild, five moderate, one severe). Alanine aminotransferase (ALT) was elevated in five cases (range 106–617 U/L, mean 263.6 U/L, reference range 20–93 U/L) from the moderate and severe anaphylaxis groups. Bilirubin and creatinine kinase were elevated in only two cases. Only one of the aforementioned dogs received glucocorticoids before the examination. No correlation between elevated lipase and the specific trigger could be found (four food, two insect, one drug). Interestingly, ALT was elevated only in hymenoptera-triggered cases.

Treatment and outcome

Interestingly, none of the cases died and the use of epinephrine was never considered necessary. The treatment of the great majority of cases was based on the use of H1 and H2 antagonists and/or glucocorticoids associated with intravenous fluid therapy (Table S3). All moderate and severe cases were hospitalized for 1.5 days on average, with the shortest hospitalization duration 4 h and the longest 4 days.

Causality (and follow-up)

Table 5 details the triggers, comorbidities and other possible risk or cofactors**.** The cause was identified in 17 cases and was considered very likely (ALUA > 10) in 12 (50%) and likely (ALUA 5–10) in five cases (29%). These cases consisted of reactions to insect bites or stings (n = 9), foods (n = 5), drugs (n = 2) and cold (n = 1). The triggers were confirmed either with allergen tests or by observation following provocation, in 10 and seven cases, respectively. The cause could not be determined in seven cases (ALUA < 5) and the suspicion was directed mainly to foods.

Even though causality or association could not be proven, it was interesting to note that 11 cases presented with signs of atopic dermatitis (AD) and/or known food allergy history and that two dogs were treated with phenobarbital for epilepsy. One dog (number 8) was known to be food allergic but was also treated with multiple drugs, especially prednisolone and azathioprine for a concurrent autoimmune thrombocytopenia. Exercise immediately before the onset of the reaction was mentioned in the history of five dogs; stress and estrous were observed in one case each.

Six dogs were reported to have reacted to the same allergen in the past (4 urticaria, 2 anaphylaxis), with no correlation to disease severity.

Most of the reactions occurred during daytime; only three were observed during the night and these were the animals with no trigger identified. All animals that

Table 5. Triggers, comorbidities and risk/cofactors for 24 dogs with urticaria

	Causal group	Cause	ALUA score	Trigger probability	Previous episodes of urticaria	Concurent allergic disease	Concurrent other disease	Concurrent treatment	Concurrent exercise, stress, estrous or gravidity
1	Insect	Mosquito	6	Likely	Yes				
2	Food	Unknown	<5	Possible	Yes	AD			Estrous
3	Physical	Cold	8	Likely	Yes	FA			Exercise
4	Food	Potatoes	16	Very likely		AD,FA			
5	Insect	Wasp	18	Very likely			Incontinence	Estriol	
6	Drug	Anaesthetic	12	Very likely		AD		Prednisolone	
7	Insect	Bee	6	Likely					
8	Food	Rice, beef	22	Very likely	Yes	FA	IMTP	Prednisolone Azathioprin	Stress
9	Food	Unknown	<5	Possible			Epilepsy	Phenobarbital	Exercise
10	Food	Unknown	<5	Possible					
11	Food	Unknown	<5	Possible			Epilepsy	Phenobarbital	Exercise
12	Food	Cheese	12	Very likely					
13	Food	Unknown	<5	Possible		AD, FA			
14	Food	Unknown	<5	Possible					
15	Insect	Bee	6	Likely					
16	Food	Walnut	6	Likely					
17	Insect	Unknown	<5	Possible		AD			Exercise
18	Drug	ILIT	12	Very likely		AD			
19	Insect	Bee	18	Very likely					
20	Insect	Bee	18	Very likely		AD			
21	Food	Nuts	6	Likely					
22	Insect	Bee	18	Very likely		AD			
23	Insect	Bee	22	Very likely	Yes				
24	Insect	Bee	16	Very likely	Yes				Exercise

Abbreviations: ALUA, algorithm for urticaria and anaphylaxis; AD, atopic dermatitis; FA, food allergy; ILIT, intralymphatic immunotherapy; IMTP, immune-mediated thrombocytopenia. No shading – urticaria without anaphylaxis; Mild grey shading - mild anaphylaxis; Grey - moderate anaphylaxis; Strong grey shading - severe anaphylaxis..

developed the first signs while being outside the house were reacting to insects. Two animals reacted to medication while in our clinic (anaesthesia and allergen-specific immunotherapy). In animals with reactions occurring while at home, the trigger was food or not identifiable.

Discussion

We describe herein 24 cases of urticaria with 16 of 24 of these dogs showing signs of anaphylaxis. The cause was identified in 17 of 24 and consisted predominantly of reactions to insect bites or stings (7 of 17), foods (5 of 17) and drugs (2 of 17), as reported in the human literature.[45,46] Only one dog had recurrent episodes of urticaria (chronic urticaria) likely due to cold, occurring only during winter, confirming previous case reports.[47] The cause could not be determined in seven cases, a similar rate to human studies.[8] The triggers were confirmed either with compatible allergen tests or by observation following provocation. In order to determine the probability of various causes (very likely, likely, possible), an algorithm (ALUA) was created, utilizing the following criteria: observation of allergen exposure by owner or veterinarian, immunological evidence and positive rechallenge (Table 3). The authors would like to emphasize that this algorithm needs further validation in a larger study population.

This report also demonstrates that dogs with a history of AD seem to be at increased risk of developing urticaria or anaphylaxis regardless of cause (11 of 24 dogs). Although the worldwide or country-specific prevalence of atopy in dogs has not been determined, in humans it can reach up to 25%.[48] Exercise immediately before or during the onset of the reaction was mentioned in the history of five dogs and in three of them the cause could not be identified, but food was suspected. These cases may represent a variant of exercise-induced anaphylaxis (with or without food association), which is a well-recognized entity in humans that remains to be further investigated.[49] With regard to the idiopathic cases, another possible explanation to pursue in further studies based upon the human literature is the delayed anaphylaxis to red meat, provoked by the cross-reactive allergen galactose alpha-1,3-Galactose (alpha-gal) present in ticks.[50]

Currently, no data on the incidence of urticaria or anaphylaxis in veterinary medicine exists.[37] The European Academy of Allergy and Clinical Immunology (EAACI) Taskforce on Anaphylaxis has published, amongst others, prevalence data in people in Europe, which is estimated to be at 0.3%,[10] whereas global data for urticaria are unavailable.[7] Herein we show an overall incidence of urticaria with or without anaphylaxis of 0.12% or 12 cases per 10,000 dogs in a veterinary teaching hospital in Switzerland. The high proportion of dogs with anaphylaxis in this study (16 of 24 or 67%) can be explained by the hospital's referral nature; it could also be partly attributed to the precise clinico-pathological investigation, anaphylaxis grading and case recording.

Small-size breed dogs seem to be more often associated with anaphylactic reactions, which is consistent with our findings (Jack Russell terriers, French bulldog).[18,20,51] The rate of adverse effects due to vaccination in dogs of <10 kg is doubled;[51] therefore, not only the type of allergen, but also its relative amount seem to play an important role in the pathogenesis of allergic reactions. Among medium- to large-size breeds, boxers, Labrador retrievers and golden retrievers are more frequently associated with anaphylaxis.[18,51] Boxer dogs, Rhodesian ridgeback dogs and Vizslas were significantly over-represented in our population, when compared with the whole Swiss dog population, suggesting the animal's genetic background as an additional pathogenetic player in the development of urticaria and anaphylaxis. In humans, epidemiological data and clinical observations suggest that gender plays a role in the development and manifestation of IgE-dependent allergic diseases,[52] often with a female preponderance in anaphylaxis cases.[45] Research in a mouse model showed that estrogen is not a main trigger of anaphylaxis but, importantly, increases the severity of anaphylaxis through its action on endothelial cells.[53] Our study population consisted of more female then male dogs, although not statistically significantly. Interestingly, one case repeatedly showed urticaria only while in estrous. An association with progesterone can only be speculated, on the basis of existing human data;[54] such cases should be followed and further worked-up.

This study showed that the liver and GI tract are the main shock organs in dogs, and this would contribute in a major way to the severity of the clinical signs with anaphylaxis. Fourteen of 16 dogs showed either vomiting or diarrhoea and also elevated ALT values, confirming findings of other studies.[14,15,17,18] Serum lipase was also elevated in 8 of 16 cases, pointing toward a pancreatic disease, in part probably causing some of the observed clinical signs. This was not reported previously in dogs with anaphylaxis. In humans, mast cells are hypothesized to play a role in acute pancreatitis,[55] which could explain these findings.

Eight of 16 dogs dogs showed cardiovascular compromise, which is consistent with the human literature[8] and slightly less than in two previous studies in dogs.[18,20] This can be explained by the fact that previous studies focused mainly on anaphylaxis and a potential selection bias could explain this discrepancy. Neurological and respiratory signs were infrequent findings and included only changes in mentation, in agreement with other reports.[18,20]

Although our study population was selected by skin lesions, it is interesting to observe that skin seemed to be the first organ affected in at least 50% of all anaphylaxis cases. This could be an underestimation, as these data were obtained through owner observation. When different skin lesions (erythema, wheals and angioedema) were correlated with disease groups, urticaria versus urticaria with anaphylaxis, then erythema was observed more frequently in the latter group (3 versus 10). Pinnal erythema was involved in seven of these cases, mostly in the anaphylaxis group, confirming previous findings.[15] The low frequency of skin involvement from previous studies could be partly explained by the lack of precise dermatological observations in addition to the different clinical criteria for our study[18,20] Although not statistically significant, there was a tendency for dogs with anaphylaxis to experience more frequent angioedema without wheals. This is very likely due to the fact that these same

dogs were stung by insects, which most commonly causes deep tissue oedema.[30] Surprisingly, although angioedema was often encountered on the head region, none of our cases encountered laryngeal or other respiratory involvement as reported elsewere.[56] It is clear that the severity of skin lesions did not affect the patient's outcomes, but these typical lesions (wheals, angioedema and flushing) seem to be an excellent marker for anaphylaxis, as shown in human medicine.[10]

Although epinephrine is the mainstay of anaphylaxis therapy, its use was not deemed necessary in dogs in this study. One explanation is that the dogs recovered due to endogenous epinephrine release helping to auto-reverse the effect of biogenic amines and due to appropriate supportive treatment.[14] The main factor explaining the reluctance to use epinephrine is the fear of adverse effects, a continuous dilemma in human medicine.[38] Intramuscular epinephrine shows an excellent safety profile and there are no absolute contraindications for its use. It should therefore be considered for all grades of anaphylaxis, even with those with clinical features likely to evolve due to anaphylaxis.[10] Veterinary medical publications do not list reports of life-threatening adverse effects.[14,17,37] Grading anaphylaxis is very important in the light of therapeutic interventions, but the authors would like to emphasize here that dogs may pass through various stages of disease such that assigning each a set grade may not be appropriate. An example is chronic urticaria, a "non-anaphylactic" allergic disease, with enormous life-threatening potential.[57]

In humans, an observation period of 6–8 h for patients with respiratory symptoms and 12–24 h for patients with hypotension and collapse is recommended due to possible biphasic reactions, but should be assessed on a case-by-case basis.[11,12] An anaphylaxis veterinary review recommended 3 days, but this was not based on clinical data from studies.[37] In our study, dogs with anaphylaxis were hospitalized for 1.5 days on average and no biphasic reactions were observed, as reported previously.[58] Therefore, this duration seems to be appropriate and safe.

To the best of our knowledge, this is the first study describing the hospital incidence, triggering factors and clinico-pathological features of dogs with urticaria, angioedema and anaphylaxis in veterinary medicine. Insects, food and drugs were the leading triggers, resembling what is described in human medicine. As genetic predisposition, environmental factors and novel cross-reactions seem to interact to orchestrate the presentation of these reactions, much remains to be elucidated.

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Supporting Information

Additional Supporting Information may be found in the online version of this text at: http://onlinelibrary.wiley.com/doi/10.1111/vde.12342/full

Table S1. Additional data on first organ affected and gastrointestinal, cardiovascular, respiratory and neurological features, hydration status, body temperature.

Table S2. Dermatological and mucocutaneus signs in 24 dogs with urticaria.

Table S3. Information regarding hospitalization duration and treatment before admission, in clinic and after discharge.

Part 3

New Trends in Therapy

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The selective glucocorticoid receptor agonist mapracorat displays a favourable safety–efficacy ratio for the topical treatment of inflammatory skin diseases in dogs

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Background – Mapracorat is a nonsteroidal Selective Glucocorticoid Receptor Agonist (SEGRA) that is presumed to have a better therapeutic index compared to classical glucocorticoids.

Objectives – To compare the efficacy and safety of mapracorat with classical glucocorticoids used for the treatment of allergic skin diseases in dogs.

Animals – Six laboratory beagles.

Methods – The effect of mapracorat on lipopolysaccharide-induced TNFα secretion from canine peripheral blood derived mononuclear cells (PBMC) was tested. *In vivo*, mapracorat was compared to triamcinolone acetonide using a skin inflammation model. Skin fold thickness was determined after daily administration of mapracorat and triamcinolone acetonide over 14 days.

Results – Mapracorat concentration dependently inhibited TNFα secretion from activated canine PBMC with a half maximal inhibitory concentration (IC_{50}) value of approximately 0.2 nmol/L. Intradermal injection of compound 48/80 (50 μg in 50 μL saline) resulted in a clear wheal and flare reaction over the 60 min observation period. Topical pre-treatment with mapracorat (0.1%) and triamcinolone acetonide (0.015%) led to significant reduction in the wheal and flare responses compared to vehicle (acetone) treated areas. However, once daily topical administration of triamcinolone acetonide significantly reduced skin fold thickness from day 8 to 14, whereas no such reduction was observed for mapracorat.

Conclusion – These results demonstrate that mapracorat has comparable anti-inflammatory efficacy to classical steroidal glucocorticoids under these experimental settings and maintenance of skin fold thickness indicates a better safety profile compared to triamcinolone acetonide at equipotent concentrations. This profile further suggests that SEGRAs show promise in the management of inflammatory and pruritic skin diseases in dogs.

Introduction

Canine atopic dermatitis (CAD) is defined as a genetically predisposed inflammatory and pruritic skin disease with characteristic clinical features. In most cases CAD is associated with IgE antibodies directed at environmental allergens.[1] Dogs are frequently treated with immune modulators such as glucocorticoids and the calcineurin-inhibitor ciclosporin; the Janus kinase inhibitor oclacitinib has been shown to have comparable efficacy to ciclosporin.[2]

Glucocorticoids are used frequently in the treatment of pruritus and CAD as they are effective and inexpensive. They are, however, associated with adverse effects including polyphagia and polyuria, diabetes mellitus, growth retardation, enhanced risk of infections and – especially when administered by the topical route – the potential for cutaneous atrophy.[3,4] Many efforts have been made in recent years to identify specific glucocorticoid receptor ligands with a dissociated profile: maintenance of the potent anti-inflammatory effects but reduced potential for adverse effects.[5] Common adverse effects such as diabetes mellitus, dyslipidaemia, glaucoma induction, muscle atrophy and skin thinning are partly or even mainly dependent on glucocorticoid

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receptor mediated activation of gene expression (transactivation). By contrast, many of the immunosuppressive and anti-inflammatory effects of glucocorticoids are due to glucocorticoid receptor mediated suppression of cytokine and chemokine synthesis, and expression of adhesion molecules and enzymes. This phenomenon is referred to as transrepression.[5] In best cases, dissociated glucocorticoid receptor ligands such as the nonsteroidal family of Selective Glucocorticoid Receptor Agonists (SEGRA) show less transactivation compared to classical glucocorticoids but similar transrepression potential. One SEGRA, for which pre-clinical data exist in dermatology and ophthalmology, is mapracorat (ZK 245186). Mapracorat showed similar efficacy but a better safety profile in several rodent models of dermatitis compared to classical glucocorticoids.[6] Mapracorat was also tested in a rabbit dry eye model, where it had comparable anti-inflammatory potential to dexamethasone and displayed fewer adverse effects (intraocular pressure and muscle wasting).[7] Currently, clinical trials for the topical treatment of atopic dermatitis in humans are underway (https://clinicaltrials.gov/ct2/show/NCT01359787).

The aims of the present study were to evaluate the immunomodulatory potential of the nonsteroidal SEGRA mapracorat in dogs and to assess its potential for promoting skin atrophy as compared to the topical glucocorticoid triamcinolone acetonide.

Material and methods

Animals

All procedures involving animals were carried out in agreement with the current version of the German Law on the Protection of Animals. The animal experiments were registered by Bezirksregierung Hannover, Germany (AZ 33.9-42502-04-11/0636).

Six laboratory beagles (three male, three female, aged between 3 and 5 years, weighing between 8.8 and 16.7 kg) were group housed in rooms at 20–22°C. Water was offered *ad libitum* and a standard diet (Altromin; Lage/Lippe, Germany) was offered twice daily. All dogs were neutered, vaccinated (Virbagen SHAP, LT; Virbac Tierarzneimittel GmbH; Bad Oldesloe, Germany) and regularly dewormed (Milbemax, Novartis Tiergesundheit GmbH; München, Germany). The dogs had access to a courtyard and were taken for a walk once daily for approximately 30 min. The dogs did not receive any medication within 30 days before blood samples were taken or *in vivo* experiments were performed.

Female BALB/c mice (Charles River; Sulzfeld, Germany), aged between 8 and 12 weeks, were housed in groups at 22°C with a 12 h light/dark cycle. Water and a standard diet (Altromin) were available *ad libitum*.

Generation of canine peripheral blood derived mononuclear cells (PBMC)

Blood samples (8 to 9 ml) were taken from the jugular vein by means of a vacuum container with heparin (BD Vacutainer, NH 170 I.U; Plymouth, UK). The following steps were carried out in a class 1 safety cabinet. In an appropriate plastic tube, 9 ml blood were diluted with approximately 10 ml phosphate buffered saline (PBS) and underlain with 15 ml histopaque 1077 (Sigma; Steinheim, Germany). Samples were centrifuged at 1200 ***g***, 19°C for 35 min with the brakes turned off. The peripheral blood mononuclear cells (PBMC) were removed from the interface between the layers and cells were washed two times by the addition of phosphate buffered saline (PBS) (1000 ***g*** for 10 min, 4°C, brakes activated). Following the final washing step, the cell pellet was resuspended in an appropriate volume of tissue culture medium (RPMI 1640 + 10% fetal calf serum, both Biochrom; Berlin, Germany) and viable cells were counted with trypan blue staining. The cells were used directly for the experiments.

Generation of murine bone marrow derived dendritic cells (BMDC)

Large numbers of highly pure BMDC were generated according to a standard protocol.[8] Briefly, bone marrow was flushed from femurs of the hind limbs of drug-naïve mice with ice cold PBS and taken into RPMI 1640 + 10% fetal calf serum (Biochrom) with 50 μM 2-mercaptoethanol (Sigma) added. Cells, 2 x 10^6, were seeded in 10 ml medium on a petri dish (Cell$^+$, Sarstedt; Nümbrecht, Germany). The medium contained 20 ng/ml granulocyte macrophage colony stimulating factor (GM-CSF) (R&D systems; Wiesbaden, Germany). On Day 3, 10 mL fresh medium supplemented with 200 ng GM-CSF was added. At days 6 and 8, 50% of the medium was collected, centrifuged and the cell pellet re-suspended in 10 mL fresh medium containing 200 ng GM-CSF. Flow cytometry analysis of the Day 10 cell suspension demonstrated a high yield of CD 11c and major histocompatibility complex class II positive cells.[8]

In vitro test incubation protocol

The compound stock solutions of mapracorat in acetone (Bayer Animal Health GmbH; Leverkusen, Germany) and mometasone furoate [10 mg/mL in dimethyl sulfoxide (DMSO), Sigma] were serially diluted to the final concentrations tested; the vehicle was spiked with the highest possible DMSO concentration used for the drugs (0.001%). The mapracorat concentration ranged from 0.1 to 100 nmol/L and mometasone furoate from 0.01 to 1 nmol/L. Compound (mometasone furoate) and concentration selection were performed according to published data.[6] For PBMC, 200,000 cells in 200 μL were treated with each concentration at least in duplicate in three to four independent settings (i.e. cells from the blood of three to four different dogs). For murine BMDC, 200,000 cells in 200 μL were treated with each concentration at least in duplicate in two independent settings (i.e. cells from the bone marrow of two mice).

A pilot study was conducted to determine the best conditions for *in vitro* stimulation of canine PBMC. From this study it was concluded that 1 μg/mL lipopolysaccharide (LPS) was better than 10 μg/mL LPS. After 30 min of pre-incubation with the test compounds, cells were stimulated with 1 μg/mL LPS (from *E. coli*, O127:B8, Sigma). Twenty four hours after LPS stimulation cells were centrifuged (500 ***g***, 10 min, 4°C) and the cell culture supernatant was frozen at −20°C until determination of TNFα (canine and murine Duoset ELISA, R&D systems; Wiesbaden, Germany).

In vivo experiments: Topical application of glucocorticoid agonists, injection of compound 48/80 and determination of wheal reaction as well as erythema

The skin of the abdomen (mainly nonpigmented) was shaved with an electric hair clipper at three different sites of about 100 cm^2 each. At least 15 cm space was left between each application site. All dogs received vehicle (500 μL acetone), mapracorat (500 μL 0.1% in acetone) and triamcinolone acetonide (500 μL 0.015% in acetone, Sigma) at one of the shaved areas one day after skin preparation. The doses were chosen according to a licensed product (Genesis® spray, Virbac; Fort Worth, TX, USA, 0.015% triamcinolone acetonide) or *in vivo* data in mice (80% reduction of ear swelling in a mouse model of allergic dermatitis with a 0.1% solution for mapracorat).[6] The topical administration was repeated 24 h after first application and again at 48 and 72 h. The administration of test compounds was performed in a blinded fashion (substance "A" to "C") and were varied from dog to dog to be equally distributed across the test items. One hour after the last (fourth) application of substances, compound 48/80 (50 μg/50 μL) was injected intradermally (30 gauge needle) into the centre of each of the three shaved areas. Wheal and erythema reactions were assessed by means of planimetry at 10, 30 and 60 min after injection of compound 48/80.

Determination of skin thinning potential

The same dogs were used for this experimental protocol. They had not received any medication or other experimental treatment within 30 days prior to this part of the study. The skin of the lateral thorax (left and right side) and flank (left and right side) were shaved with an electric hair clipper and the skin thickness was determined by means of a cutimeter (model 7309, Mitotoyo; Neuss, Germany). For determination of the baseline value, the mean of three independent thickness measurements (performed on days –3, –2 and –1) was used. All dogs received vehicle (50 µL acetone), mapracorat (50 µL 0.1% in acetone) and triamcinolone acetonide (50 µL 0.015% in acetone), once daily over 14 days on an area of approximately 9 cm^2. The treatment areas were varied from dog to dog to be distributed equally across the test items. However, the skin areas allocated to the respective treatments remained the same in each individual dog over the entire treatment phase. The skin fold thickness was determined three times a week for each area, directly before the next scheduled application of test item. The skin was shaved again 11 days after first treatment. This had no impact on determination of skin fold thickness (evaluated before and after shaving).

Statistical evaluation

For the *in vitro* experiments, results are presented as mean % inhibition of two determinations at each tested concentration, each investigated in duplicate or triplicate. The concentrations which led to 50% inhibition (IC_{50}s) were determined directly from the graphs.

For the *in vivo* compound 48/80 study, time courses are presented as mean (±SEM). For statistical analysis of wheal and erythema areas, a two-way repeated measures ANOVA with the fixed factors pre-treatment, time and the pre-treatment by time interaction was performed. Analysis was performed with SigmaPlot (version 12.5, Systat Software Inc.; San Jose, CA, USA) followed by a post hoc test (All Pairwise Multiple Comparison Procedure, Holm–Sidak method) when the difference in the mean values among the different levels of pre-treatment or time was greater than would be expected by chance.

Skin thickness results are presented as mean (±SEM) for the determination of skin fold thickness by means of a cutimeter. The different treatment groups were checked at all time points for significant differences against vehicle treatment by means of a one-way ANOVA followed by a post hoc test (Dunnett's). *P* values <0.05 were considered to be statistically significant for all comparisons.

Results

Modulation of TNFα secretion of murine BMDC

A mean concentration of TNFα of 7,500–9,500 pg/mL was recovered in the supernatant of vehicle-treated murine BMDC 24 h after LPS stimulation (these values had

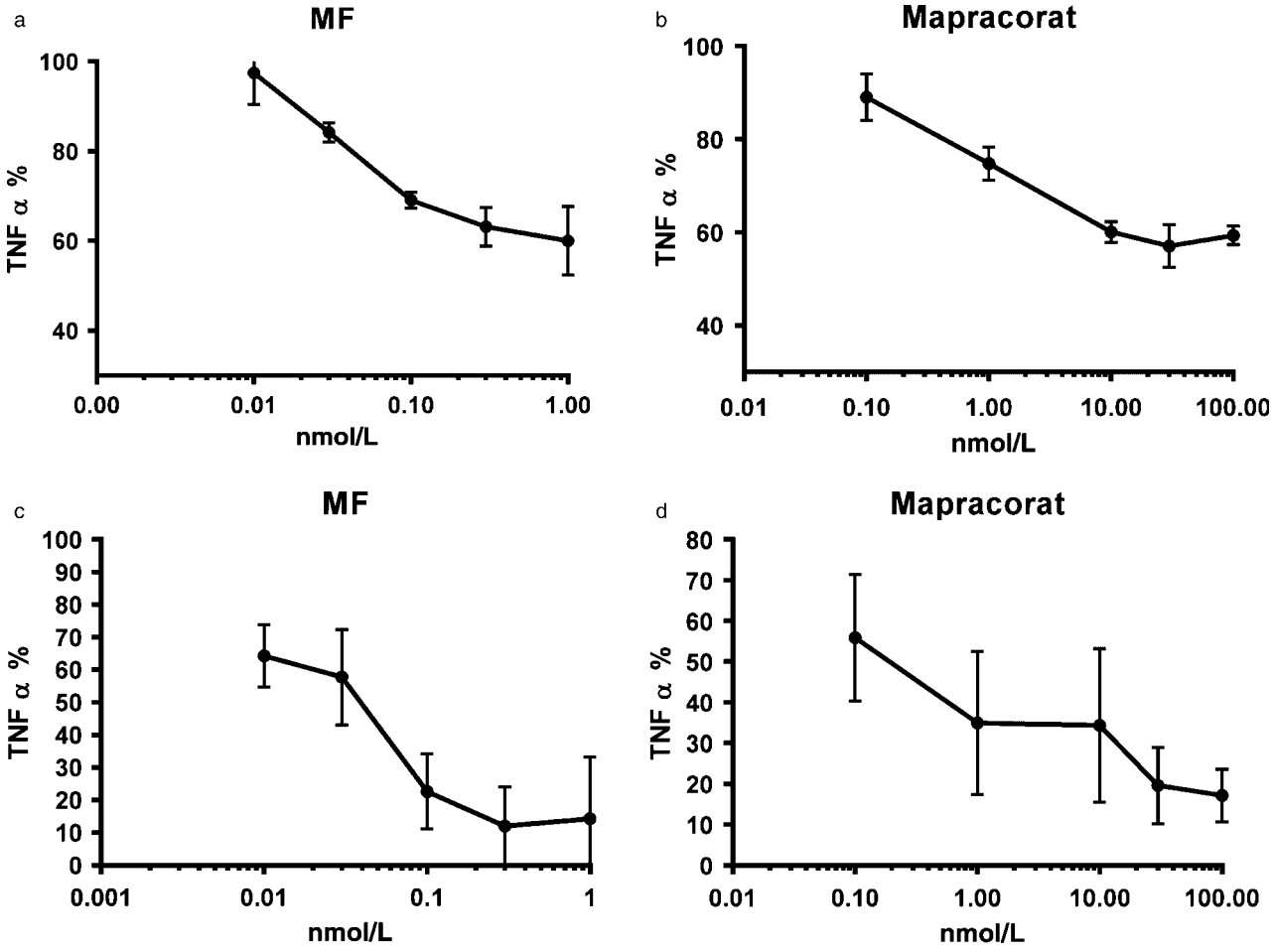

Figure 1. Inhibition of lipopolysaccharide (LPS)-induced TNFα secretion from murine bone marrow derived dendritic cells (a and b) or canine peripheral blood derived mononuclear cells (c and d) by increasing concentrations of mapracorat and mometasone furoate. Cells were incubated with different concentrations of mapracorat (0.1–100 nmol/L) and mometasone furoate (MF) (0.01–1 nmol/L) and stimulated with LPS 30 min later. Supernatants were collected 24 h after LPS stimulation. Vehicle (dimethyl sulfoxide) treated and LPS stimulated cells were taken as maximum induction of TNFα secretion (100%).

been set as 100%; see Figure 1). Mapracorat and mometasone furoate concentration-dependently inhibited the LPS-induced TNFα secretion. However, the maximal effect achieved by all tested glucocorticoid agonists was approximately 40% inhibition (Figure 1); this value was set as the maximal effect. IC_{50}s determined directly from graphs were approximately 0.5 nmol/L for mapracorat and 0.05 nmol/L for mometasone furoate.

Modulation of TNFα secretion of canine PBMC

A mean concentration of TNFα of 100–250 pg/mL was determined in the supernatant of vehicle-treated canine PBMC 24 h after LPS stimulation (these values had been set as 100%; see Figure 1), whereas the TNFα concentration of non-LPS stimulated PBMC was below the limit of detection. Mapracorat and mometasone furoate concentration-dependently inhibited the TNFα secretion (Figure 1c and d). IC_{50}s determined directly from graphs were approximately 0.2 nmol/L for mapracorat and 0.04 nmol/L for mometasone furoate.

Compound 48/80 induced inflammation and oedema

Intradermal injection of compound 48/80 resulted in a significant wheal and erythema reaction over the 60 min observation period in vehicle-treated areas (Factor Time $P < 0.001$; Figure 2). There was a significant overall effect of pre-treatment on this reaction ($P = 0.005$ for wheal and 0.012 for erythema, respectively). Post hoc testing confirmed that mapracorat and triamcinolone acetonide significantly reduced wheal and erythema reaction to compound 48/80 compared to sites pre-treated with vehicle. Pairwise comparisons further revealed that the SEGRA compound mapracorat and the classical glucocorticoid did not differ in their efficacy and potency, neither overall nor at any specific observation time point.

Skin thickness evaluation

Topical treatment with vehicle or mapracorat (0.1%) had no obvious impact on skin fold thickness within the 14 days of consecutive treatment. However, triamcinolone acetonide (0.015%) reduced skin fold thickness significantly from Day 7 onwards (Figure 3).

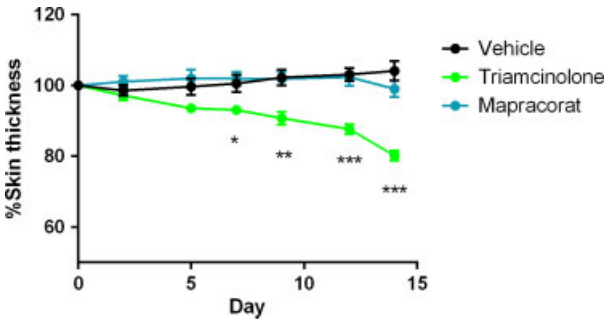

Figure 3. Changes in skin fold thickness following daily topical administration of vehicle (acetone), triamcinolone acetonide (0.015%) or mapracorat (0.1%) over 14 days after daily application to different skin sites of six beagle dogs. The baseline value is the mean of three skin fold thickness determinations performed directly before the start of the study, normalized to 100%. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to vehicle.

Discussion

This study has demonstrated that mapracorat exerts *in vitro* inhibitory effects on activated canine and murine immune cells with comparable efficacy to mometasone furoate. Mapracorat has also been shown to have anti-inflammatory efficacy similar to topical triamcinolone acetonide – with less thinning of the skin – at equipotent concentrations. The better safety profile might be explained by the dissociative profile of the SEGRA. Whereas transrepression and reduction of the expression of enzymes involved in inflammation are comparable between SEGRAs and classical glucocorticoids, the transactivation potential of SEGRAs is reduced.[5] Several adverse effects such as glaucoma induction, muscle atrophy and skin atrophy previously have been shown to be less pronounced with SEGRAs.[6,7] As such, this new class of glucocorticoid agonists holds promise for long-term treatment of atopic dermatitis patients.

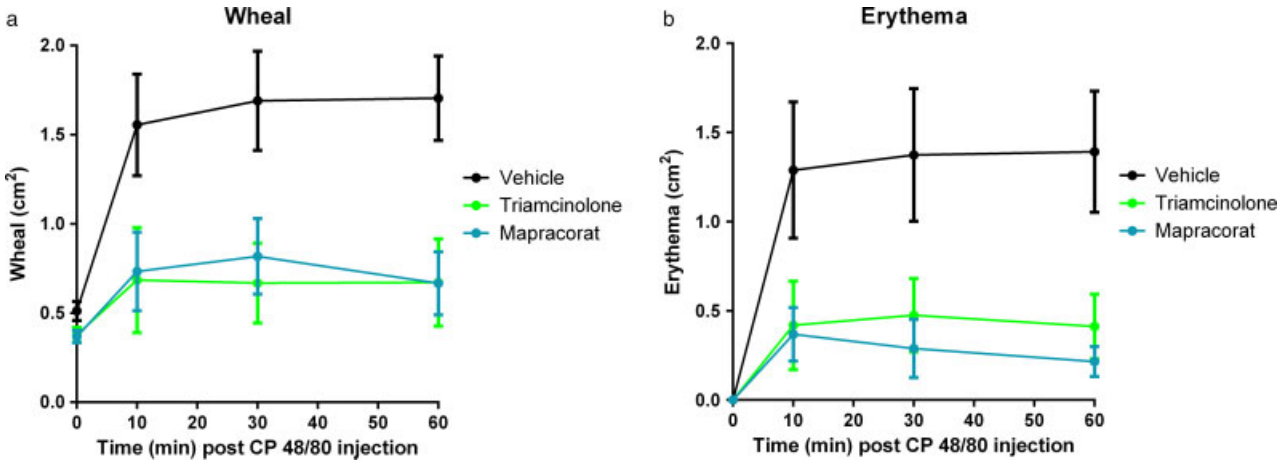

Figure 2. Time course (mean ± SEM; cm^2) of wheal (a) and erythema (b) after intradermal injection of compound 48/80 (CP 48/80). Six dogs were treated topically with acetone, triamcinolone acetonide or mapracorat, four times before administration of compound 48/80. The differences between vehicle (acetone) treated and mapracorat or triamcinolone acetonide treated areas were statistically significant ($P < 0.05$) at all time points (10 min, 30 min and 60 min after administration of compound 48/80).

Results of this study, which show a concentration-dependent inhibition of TNFα secretion from activated canine PBMC and murine BMDC *in vitro* that is similar in efficacy to mometasone furoate, is consistent with reports from prior studies that used similar cell culture systems.[6] However, the potency of mapracorat was higher in the present study.

The observation that glucocorticoids mediate only a partial inhibition of LPS-induced cytokine release from murine BMDC is corroborated by former findings,[9] where the highly potent topical steroid (WHO class 1) diflorasone diacetate was tested in the same system, leading to similar maximal inhibitory potential.

The *in vitro* results of the present study indicate that the novel SEGRA mapracorat is able to stimulate canine glucocorticoid receptors as effectively as murine glucocorticoid receptors. Hence, it can be reasonably assumed that mapracorat will display similar anti-inflammatory/immunomodulatory potential in dogs as that observed in mice[6] if provided in a suitable formulation for topical administration.

The *in vivo* inflammation study indicated that the intracutaneous compound 48/80 injection model is a robust, reproducible and fast system with which to study skin inflammation in dogs and to test the possible anti-inflammatory effects of steroidal and nonsteroidal glucocorticoid receptor agonists. Compound 48/80 is a basic secretagogue which induces mast cell degranulation followed by liberation of histamine[10] and other pro-inflammatory mediators such as cytokines,[11] proteases[12] and prostaglandins.[10] A recent study indicated that compound 48/80-induced mast cell degranulation is mediated by activation of the Mas-related G protein-coupled receptor X2 (MrgprX2) in humans and mice.[13] Corroborating studies in dogs have not yet been published, although mRNA of MrgprX2 has been identified in canine mastocytoma cells in pilot studies conducted by the authors (unpublished data).

The intradermal injection compound 48/80 has been studied as a positive control for the intradermal allergen test in dogs[14] and also as a test system to evaluate glucocorticoid action in an immediate type hypersensitivity model.[15] It was shown that a 0.015% topical triamcinolone acetonide solution inhibited the "reaction area" induced by compound 48/80.[15] We applied the topical test solutions (triamcinolone acetonide and mapracorat) four times in order to obtain a reliable anti-inflammatory response. A pilot study had determined that two applications (24 h and 1 h prior to compound 48/80 challenge) resulted only in marginal reduction of erythema and wheal reaction for both compounds (data not shown). Although compound 48/80 provoked distinct wheal and flare reactions, it did not induce pruritic behaviours in the dogs tested. Therefore, we were unable to evaluate the topical test solutions for any anti-pruritic effects. Of note, the inflammatory response to compound 48/80 in vehicle treated areas was visible for at least 2 weeks, so it should be kept in mind that long inter-study periods might be necessary for a full reconstitution of degranulated mast cells in skin when using this compound *in vivo*.

Given that cutaneous atrophy during use of topical glucocorticoids is a major concern,[16] we addressed this potential adverse effect in a separate experiment. The progressive reduction of skin fold thickness observed with triamcinolone acetonide, which had already achieved statistical significance after 7 days of treatment, is consistent with a study in human subjects where daily administration of 0.01% triamcinolone acetonide led to a reduction of skin thickness by roughly 10% within 10 days.[4] A similar approach has been used in mice where skin thickness was observed over a period of 19 days, where mapracorat led to a significant reduction in skin fold thickness, although the effect was less pronounced than with an equipotent concentration of mometasone furoate.[6]

Results of the study reported here have demonstrated the safety advantage of the SEGRA mapracorat over a conventional topical steroid when applied in the same vehicle formulation. However, bioavailability of topically administered drugs is strongly dependent on the physicochemical characteristics of the pharmaceutical formulation, so results of this study must be interpreted with care. The true safety advantage of a SEGRA for veterinary use over licensed benchmark steroidal glucocorticoid should be determined using both the respective final formulations and the therapeutically recommended dose volumes.

In conclusion, the experiments described here demonstrate that the SEGRA mapracorat exerts *in vitro* inhibitory effects on activated canine immune cells with comparable efficacy to mometasone furoate and provides comparable *in vivo* anti-inflammatory activity to topical triamcinolone acetonide with less cutaneous atrophy at equipotent concentrations. Future studies should assess the clinical efficacy and safety profiles of mapracorat in controlled clinical trials of dogs with atopic dermatitis using an approved pharmaceutical formulation.

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Therapeutic anti-IgE monoclonal antibody single chain variable fragment (scFv) safety and immunomodulatory effects after one time injection in four dogs

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Background – The therapeutic monoclonal antibody omalizumab that is specific for IgE has proven to be an effective addition to the treatment of allergic disease in humans.

Hypothesis/Objectives – The aims of this study were to demonstrate the safety and immunomodulating effects of a single injection of a monoclonal antibody single chain variable fragments (scFv) specific for canine IgE in normal dogs.

Animals – Three normal dogs were bled for EDTA whole blood samples for 112 days post-injection (dpi). A fourth dog was monitored for 28 days.

Methods – Anti-IgE scFv was pegylated to minimize scFv dimerization. Four normal dogs were injected once subcutaneously with anti-IgE scFv at 1 mg/kg. Flow cytometry was performed on whole blood. Plasma levels of IgE were measured by ELISA.

Results – None of the four dogs showed signs of anaphylaxis. All dogs demonstrated decreases in IgE(+) cells in lymphocyte-gated events by 14 dpi. Dogs C and D returned to pre-injection levels by 21 days, whereas dogs A and B remained below pre-injection levels until Day 112. Similar differences were seen in IgE-bearing granulocyte-gated cells. Free plasma IgE decreased below pre-injection levels by 47% in Dog A and by 52% in Dog B at 112 days. Dogs C and D did not change by more than 32% from preinjection levels.

Conclusion – A single injection of monomeric, pegylated scFv with high affinity for dog IgE was demonstrated to be safe. Marked reduction in IgE-bearing lymphocytes and granulocytes accompanied by reduced "free" plasma IgE level in two of four dogs is analogous to omalizumab in humans.

Introduction

Allergic disease pathogenesis is initiated primarily by allergen-specific immunoglobulin class IgE. IgE is strongly bound by the high affinity IgE epsilon receptor I (FcεRI) found on the surface of inflammatory cells such as mast cells and basophils,[1] and on keratinocytes, monocytes and dendritic cells.[2] When IgE that is bound to these cells is cross-linked by multivalent allergens, cytokines and inflammatory mediators are generated with results ranging from acute systemic anaphylaxis to dermal pruritic inflammation to immunomodulation.[3]

The efficacy and safety of monoclonal antibodies with specificity for IgE epitopes located in the binding site that interacts with FcεRI have been the basis for the highly successful therapeutic humanized IgG monoclonal antibody omalizumab, marketed as Xolair®.[4–6] Because of this specificity, omalizumab does not cross-link IgE bound to cells bearing FcεRI and thus does not stimulate the release of inflammatory mediators such as histamine from mast cells or basophils.

Recent studies of the complex molecular conformational changes in IgE that are associated with the high affinity binding to FcεRI indicate that other sites outside the direct binding site contribute to IgE high affinity binding by FcεRI. Thus, potentially therapeutic antibody binding to IgE sites distant from the FcεRI binding site may diminish IgE affinity for mast cells and basophils. However, the consequences of bivalent antibody binding limit the use of intact immunoglobulin and require a monovalent form of the potentially therapeutic antibody.

The explanation of therapeutic efficacy by a simplistic mechanism of antibody binding to IgE at a site that blocks interaction with FcεRI is currently accepted for omalizumab.[7] However, there are recent reports which suggest that binding of omalizumab to IgE expressed on the surface of B cells committed to production of IgE results in inhibition of the production of IgE *in vitro*.[7,8] Production of IgE is regulated by cytokine-driven switching of B cells to IgE commitment and by IgE feedback through binding to low affinity receptor for IgE, CD23/FcεRII.[9] The

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question of whether monovalent binding of IgE on B lymphocytes also reduces IgE production has not been addressed.

If it can be demonstrated that monovalent antibodies directed against epitopes on IgE other than those at the FcεRI binding site can be safely administered at therapeutic doses, then new antibodies with specificities for novel epitopes on IgE may be tested *in vivo* for empirical evidence for potential efficacy.

Single chain variable fragments (scFv) of monoclonal antibodies can be generated from the sequences of the heavy and light chain variable regions linked by a short bridge of four glycine and one serine sequence repeats.[10] The scFv are monovalent but often show a low level of aggregation at high concentrations.[11] Polyethylene glycol (PEG) has been used for many years to reduce the aggregation and antigenicity of therapeutic peptides.[12]

The aim of this study was to determine if monovalent forms of antibodies against IgE epitopes distant from epitopes that directly bind FcεRI could be developed to empirically test for immunomodulatory effects. In the approach used here, short chain PEG was covalently bound to lysine amino acids in the scFv.

Two different detection protocols were used in this study for the measurement of plasma IgE levels to minimize possible confounding effects of various plasma proteins that bind IgE, such as secreted FcεRI alpha chain,[13] CD23[13] and autologous autoimmune IgG anti-IgE.[14] Thus, biotinylated human recombinant FcεRI alpha chain (R&D Systems; Minneapolis, MN, USA) and biotinylated scFv were used to detect plasma IgE, with the former being conventionally referred to in human studies with omalizumab as detecting "free" IgE (not complexed with omalizumab or FcεRI alpha chain), and the latter being referred to as "total" IgE based on scFv binding at a site not blocked by the FcεRI alpha chain. Absolute values of IgE Ig/ml are reported based on standard curves using affinity purified monoclonal canine IgE; however, the use of low pH elution of the IgE standard from affinity matrix reduces the signal values from biotinylated FcεRI alpha chain detection, which precludes direct comparison of the plasma values generated by the two different protocols.

Materials and methods

Study dogs

The protocol of this study was approved by the North Carolina State University (NCSU), Institutional Animal Care and Use Committee. Mature, mixed breed dogs were randomly sourced by the Laboratory Animal Resources at North Carolina State University from animal shelters as healthy dogs and maintained with standard vaccination for distempter and rabies, and deworming in indoor runs for at least 6 months before this study. Dogs A and B were neutered females and dogs C and D were intact males. Three dogs were available for 4 months and a fourth dog for 1 month to determine the response to a single subcutaneous injection of pegylated scFv anti-IgE.

Generation of scFv anti-IgE

The heavy and light chain variable regions of a mouse monoclonal antibody (mAb 5.91) with high affinity binding to an epitope in the C2 domain of the epsilon chain of canine IgE were sequenced (Creative Biolabs; Shirley, NY, USA) and a scFv DNA sequence created that contained a linkage between the carboxy terminal of the heavy chain and the amino terminal of the light chain using three repeats of glycine 4 serine 1 (GenScript; Piscataway, NJ, USA). This sequence was incorporated into the vector pcDNA3.4TOPO (Thermo Fisher Scientific; Rockford, IL, USA) for transfection of Expi293F cells by the ExpiFectamine 293 transfection kit (Thermo Fisher Scientific). The scFv secreted by Expi293F cells in culture for 7 days was purified by affinity chromatograpy with HiTrap protein L agarose beads (GE Healthcare; Pittsburgh, PA, USA). Affinity pure scFv was pegylated with SAT(PEG)4 (Thermo Fisher Scientific) at lysine primary amine moieties, and concentrated in phosphate buffered saline, pH 8.0 (PBS) to 2 mg/ml using 9K molecular weight cut-off filtration (Thermo Fisher Scientific). All procedures were conducted with endotoxin-free water and buffers with the resulting concentrated pegylated scFv containing less than 0.25 EU/ml of endotoxin.

Gel electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) of scFv and pegylated scFv were conducted under nonreducing conditions using 4–16% polyacrylamide gel (Thermo Fisher Scientific). Native gel electrophoresis samples were not heated to allow detection of noncovalent aggregation of scFv. After running, gels were heated and stained with Coomassie Blue to show protein bands.

scFv binding to IgE *in vitro*

ELISA was used to compare scFv with the original mAb 5.91 binding to IgE. Microtitre plates (Thermo Fisher Scientific) were coated with a canine monoclonal IgE, generated from mouse × dog heterohybridoma cell line 2.39, overnight at pH 9.0 in 0.05M sodium carbonate buffer, washed in PBS with 0.05% tween 20 (PBST) and blocked with 1% bovine serum albulmin (BSA). After 2 h, plates were washed and dilutions of biotinylated scFv or mAb 5.91 were added and incubated for a further 2 h. Streptavidin-HRP was added after washing followed by a final wash and addition of ABTS. Absorbance at 450 nm was read after 1 h.

Measurement of plasma IgE

ELISA was used to measure IgE in plasma by two different methods to determine total and "free" IgE levels. Both measurements used the same coating of microtitre plates with 10 Ig/ml of rabbit IgG anti-IgE that was affinity-purified by canine IgE linked to agarose beads. Blocking with 4% heat inactivated fetal bovine serum (FBS), and washing was as described above. Detection of IgE after incubation with appropriate dilutions of plasma on coated plates was with biotinylated human recombinant FcεRI alpha chain to measure "free" canine IgE, and with biotinylated scFv to measure total IgE. Standard curves for concentration were generated for each plate by using serial dilutions of canine monoclonal IgE in place of dog plasma samples.

SPOTS ELISA

The cDNA-derived amino acid sequence for canine IgE heavy chain constant region, or epsilon chain, with accession number AAB72882 was used to produce a matrix sequence of 13 amino acid long peptides offset by three amino acids representing the entire epsilon chain as 139 spots on a cellulose membrane (JPT Peptide Technologies GmbH; Berlin, Germany). Biotinylated mAb 5.91 and scFv were tested for binding to the membrane spots as described by the manufacturer.

Flow cytometry

Five millilitres of whole blood from each dog was collected into EDTA and centrifuged at 400 ***g*** for 20 min. Plasma was harvested for analysis of total and free IgE. The packed cells were washed with HBSS-0.5 mM EDTA and cells were suspended back to the original volume. One hundred microlitres of washed cells were added to each polystyrene tube for flow cytometry analysis and incubated for 3 min with 3 ml of 4.1 mM lactic acid, pH 3.9 for IgE stripping or with HBSS-0.5 mM EDTA, respectively. Cells were centrifuged and washed once with FBS staining buffer containing 0.1% NaN_3 and suspended in 100 Il of staining buffer for labelling with allophycocyanin (APC) conjugated anti-canine IgE antibodies (scFv, mAb 5.91) and anti-CD21

(AbD Serotec; Raleigh, NC, USA) to detect bound and expressed IgE, and anti-PEG (GenScript; Piscataway, NJ, USA) to detect pegylated scFv. Cells were incubated with labeled antibodies for 1 h at 4°C with gentle shaking. After incubation, red blood cells were lysed using 1-Step Fix/Lyse Solution (eBioscience; San Diego, CA, USA). Samples were then analysed on a Becton Dickinson LSRII system using FCS Express 4 Flow (Denovo Analysis software; Glendale, CA, USA). Cell populations (granulocytes, monocytes and lymphocytes) were identified by gating on forward (FSC-A) and side angle (SSC-A) light scatter. The total numbers of labelled granulocytes, monocytes and lymphocytes in specific gated regions were recorded and those labelled with anti-IgE were expressed as a percentage of the total gated population.

Results

scFv characterization

scFv isolated from Expi293F cell culture supernatants after 7 days by protein L affinity chromatography and concentrated to 2 mg/ml showed slight opacity that clarified in buffers above pH 9.0. On nondenaturing, native PAGE of scFv, a strong band was visible at the expected molecular weight of the monomer, 27 kDa, as well as a weak band at 54 kDa (Figure 1). In order to eliminate aggregation and minimize dimerization affinity, purified scFv was pegylated at multiple primary amine groups with $SAT(PEG)_4$ (Thermo Fisher Scientific) creating scFv-PEG4-S-acetyl. The pegylated scFv showed minimal dimeric form on native PAGE (Figure 1).

Binding of scFv to canine IgE was compared to the intact IgG_{2b} mAb 5.91 from which it was derived. The endpoint molar concentration for signal on ELISA plates coated with 10 Ig/ml of IgE was 2.0×10^{-12} M for biotinylated mAb 5.91 and 2.6×10^{-9} M for biotinylated scFv.

Biotinylated mAb 5.91 and scFv bound the same IgE epsilon 13mer peptide sequences as demonstrated on SPOTS ELISA (data not shown). Two adjacent spots were strongly positive, representing a shared amino acid sequence of QKATNIFPYTAPG which is located near the amino terminus of the C2 domain of the IgE epsilon chain (Figure 2).

Clinical response to scFv injection

Subcutaneous injection of pegylated scFv at 2 mg/ml in volumes required to deliver doses of 1 mg/kg to dogs weighing 10–12 kg showed no change in behaviour or vital signs in any of the four dogs during continuous observation for 1 h, 60 min intervals for 8 h and at 24 h after injection. Observation measurements included respiratory rate, heart rate, mucous membrane reperfusion and dermal hyperaemia. No defaecation was observed within 1 h of injection, nor was vomiting observed during the 24 h after injection. No reaction was observed at the injection site of any of the dogs.

Plasma IgE levels

Figure 3 shows a sustained, long-term reduction in plasma IgE over 112 days after a single injection of pegylated scFv in three (dogs A–C) of the four dogs. The most notable differences in IgE values seen in comparing the two different detection protocols for each dog were during the first 28 days, after which the sustained reduction pattern for each dog was similar for both protocols. Dog D showed no reduction during the 28 days post-injection (dpi) period it was available for sampling.

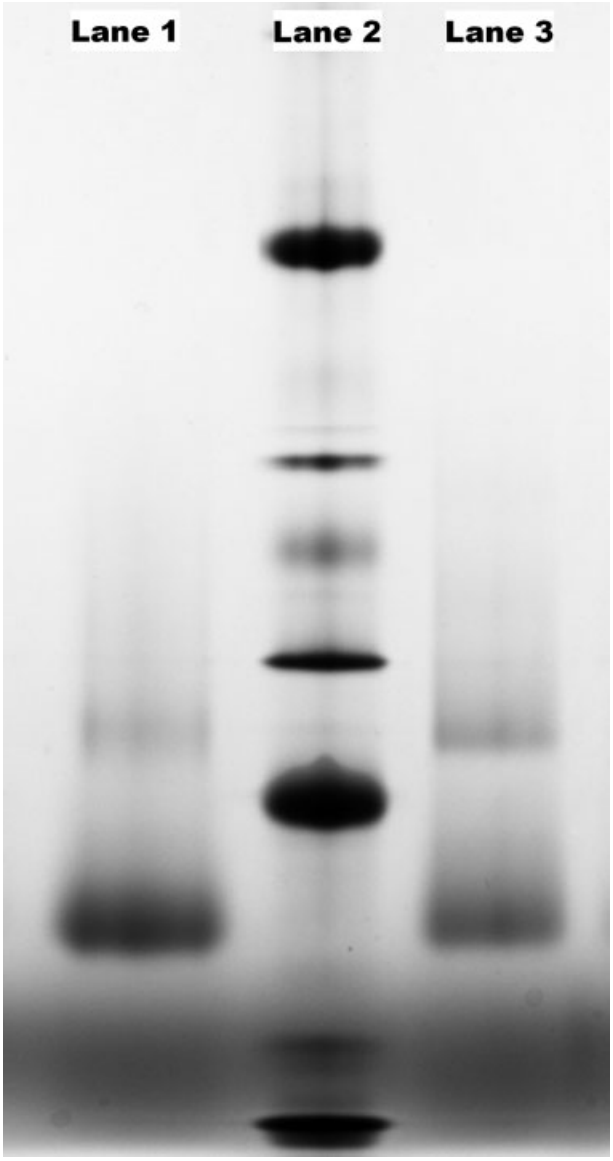

Figure 1. Native polyacrylamide gel electrophoresis (PAGE) of single chain variable fragments (scFv) and pegylated scFv showing minimal dimerization following pegylation. Molecular weight marker is shown in lane 2. Both scFv and pegylated scFv demonstrate dense bands at approximately 27 kDa, whereas pegylated scFv in lane 1 has reduced staining of the band at 54 kDa compared to scFv in lane 3.

Whole blood leucocyte surface IgE

Whole blood leucocytes were gated into granulocyte, monocyte and lymphocyte populations based on FSC-A and SSC-A scatter, as shown in Figure 4. The numbers of cells in these populations were within normal values for all dogs and the populations in each dog fluctuated very little over the course of the study. Responses to injection of pegylated scFv did not include changes in gated population numbers. This allowed comparison of percentages of APC-scFv staining cells within gated populations to be reported.

Detection of IgE on blood cells by flow cytometry was carried out with APC-labelled scFv instead of mAb

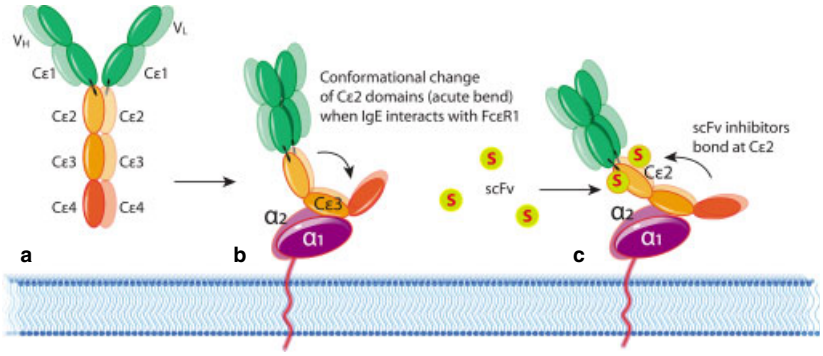

Figure 2. Schematic diagram showing the domain structure of IgE (a) and the conformational change that occurs when the second alpha chain domain of high affinity IgE epsilon receptor I (FcεRI) binds to the C3 domain of IgE (b). Pegylated single chain variable fragments (scFv), represented by a red "S", binds to an epitope near the amino terminus of the C2 domain of IgE, distant from the FcεRI binding site (c), potentially interfering with the bent conformation of IgE.

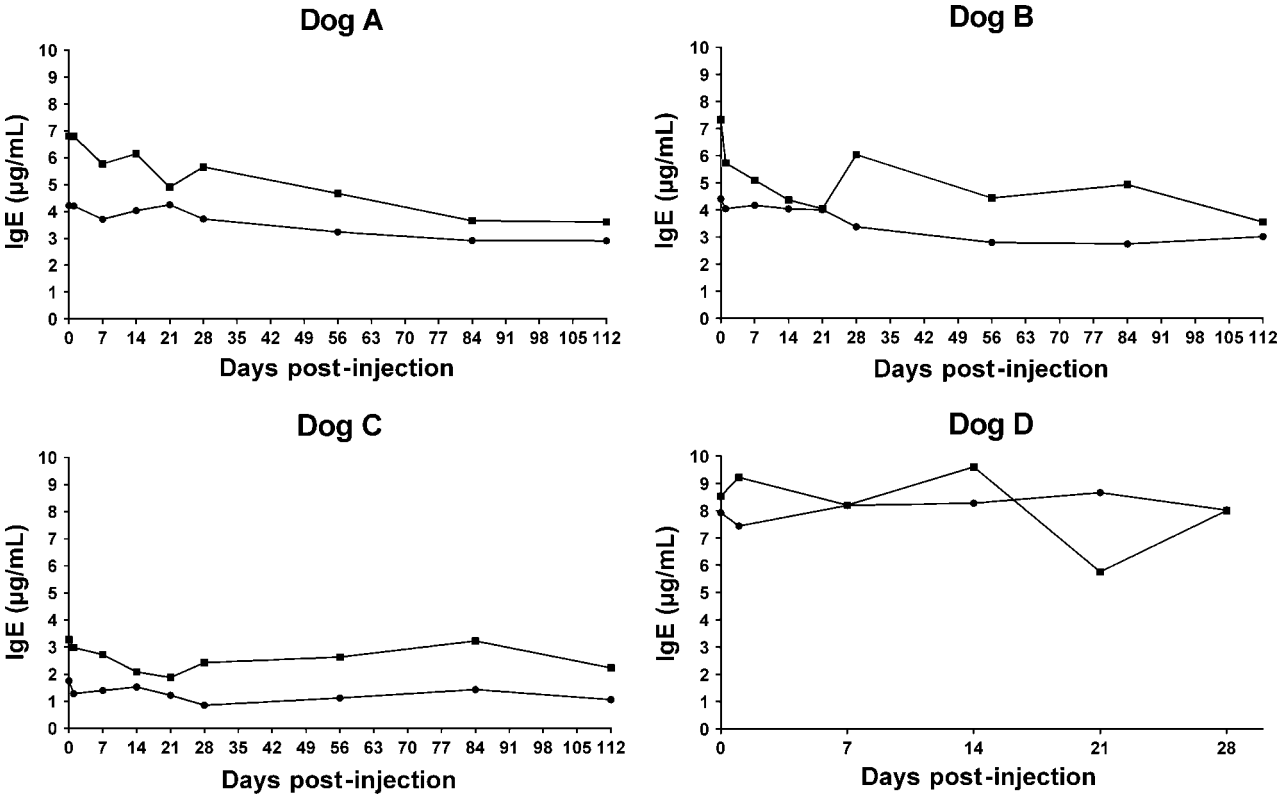

Figure 3. IgE plasma levels after a single injection of pegylated single chain variable fragments (scFv) in four dogs. Closed squares represent "free" IgE, whereas closed circles represent total IgE. Modest, but prolonged, reduction in plasma IgE was observed in three normal dogs (a–c) but not in dog D.

5.91, because it was shown that APC-scFv positive staining cell populations were more distinctly separated from negative populations (Figure 5) for each dog. No pegylated scFv could be detected with anti-PEG antibodies by flow cytometry of blood cells 24 h after injection of pegylated scFv (results not shown) which eliminated the possibility of pegylated scFv blocking APC-scFv binding to cell surface IgE in flow cytometry measurements.

Because APC-scFv binds IgE that is bound by cell surface FcεRI, as would be expected for monocytes and leucocytes, as well as IgE expressed by B lymphocytes committed to IgE production, lactic acid treatment of blood samples, as previously reported,[15] was attempted to distinguish bound and expressed IgE. Comparison of nontreated and lactic acid treated samples showed no consistent difference in APC-scFv positive cell numbers in any of the gated populations (Figures 6, 8 and 9), nor any loss of total cell numbers.

The most consistent changes in IgE(+) cell numbers were in the lymphocyte gate where all dogs showed reductions by 14 dpi (Figure 6). Notably, dogs A and B maintained low numbers of IgE(+) lymphocytes, whereas dogs C and D IgE(+) lymphocytes returned quickly to pre-injection levels. This decrease in IgE(+) lymphocytes was not associated with any decrease in CD21(+) B cells. Indeed, there appeared to be an increase in CD21(+)

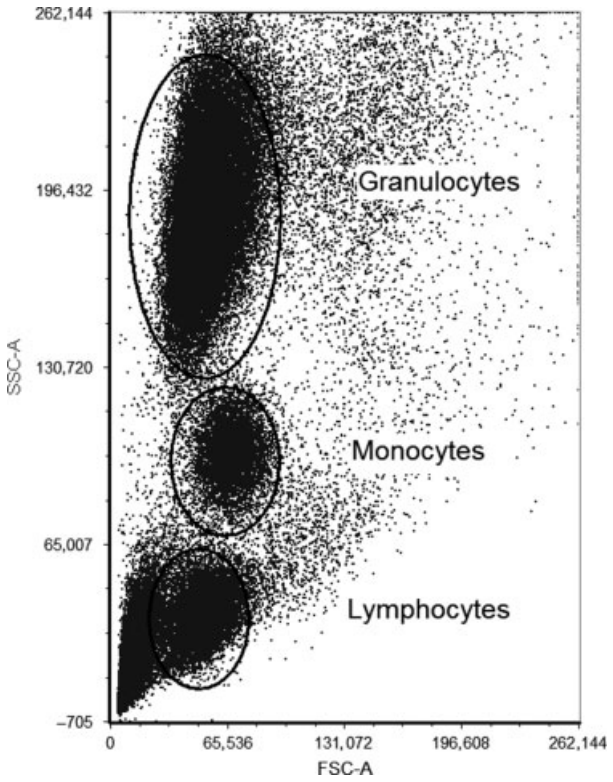

Figure 4. Typical dot plot showing the gating used to select cell populations containing granulocytes, monocytes and lymphocytes based on side scatter (SSC-A) and forward scatter (FSC-A) light.

lymphocytes following injection of scFv in all four dogs (Figure 7).

The more dramatic responses of dogs A and B in reduction of IgE(+) lymphocytes compared to dogs C and D was also reflected in changes in IgE(+) granulocytes. Dogs A and B showed rapid and sustained loss of APC-scFv staining of cells in the granulocyte gate; however, dog C showed varying changes with both increased and decreased IgE(+) cell numbers, and dog D showed a consistently higher level of IgE(+) granulocytes (Figure 8). APC-scFv staining of cells in the monocyte gate did not show any consistent change following scFv injection (Figure 9).

Discussion

This preliminary report of safety and immune modulation in four dogs injected with potentially therapeutic levels of a scFv specific for IgE indicates that the development of anti-IgE therapies need not be constrained to targeting IgE epitopes directly interacting with FcεRI. The scFv generated from the sequence of mAb 5.91 retained the original mAb specificity for an epitope in the C2 domain of IgE that was accessible whether or not IgE was bound to FcεRI.

The major barrier to targeting epitopes on IgE that are not in the FcεRI binding site is the risk of anaphylactic response resulting from crosslinking of IgE bound to basophils and mast cells. Testing mAb 5.91 by whole blood *in vitro* release of histamine and by intradermal injection showed no detectable histamine release;

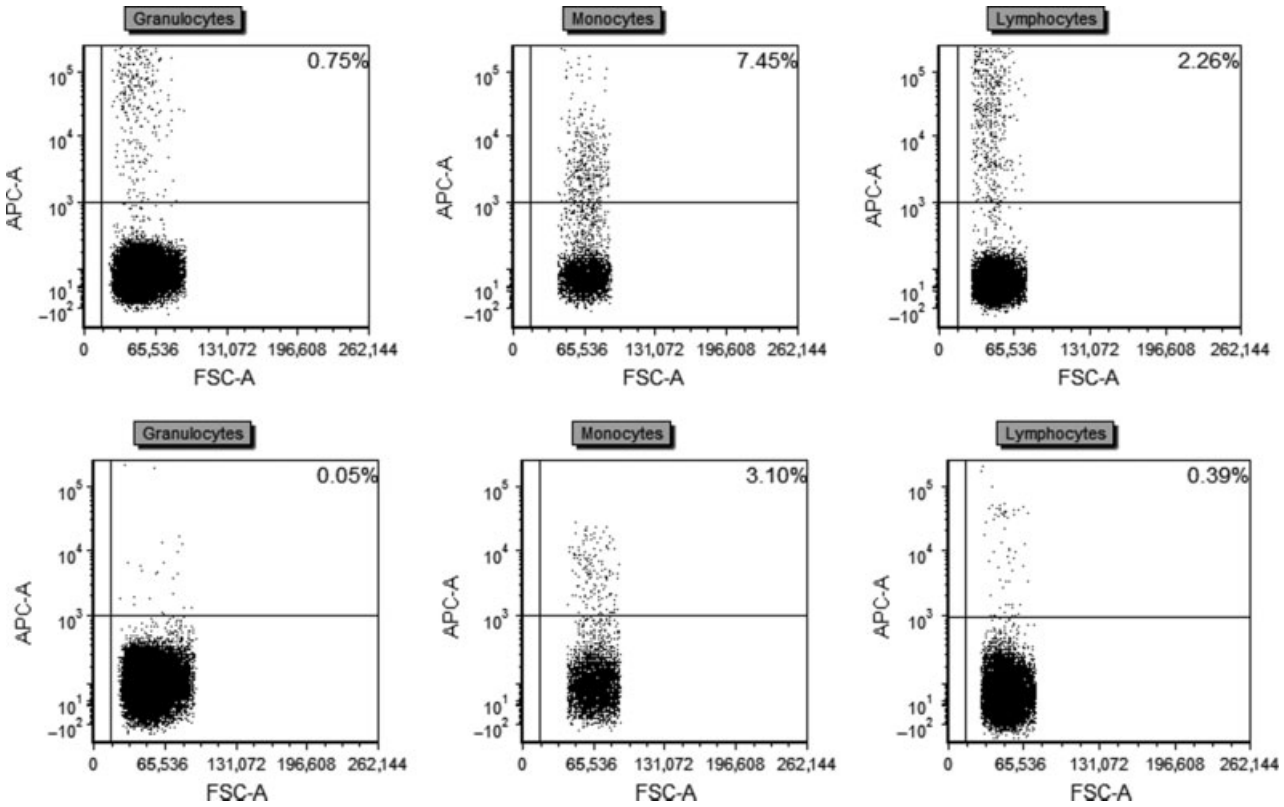

Figure 5. Representative dot plot demonstrating higher sensitivity and greater separation of positive staining cells from negative cells. Blood sample from Dog C week 1 showing the three gated populations stained with APC-scFv (allophycocyanin-single chain variable fragments) in the upper row compared to APC-mAb 5.91 stained cells in the lower row.

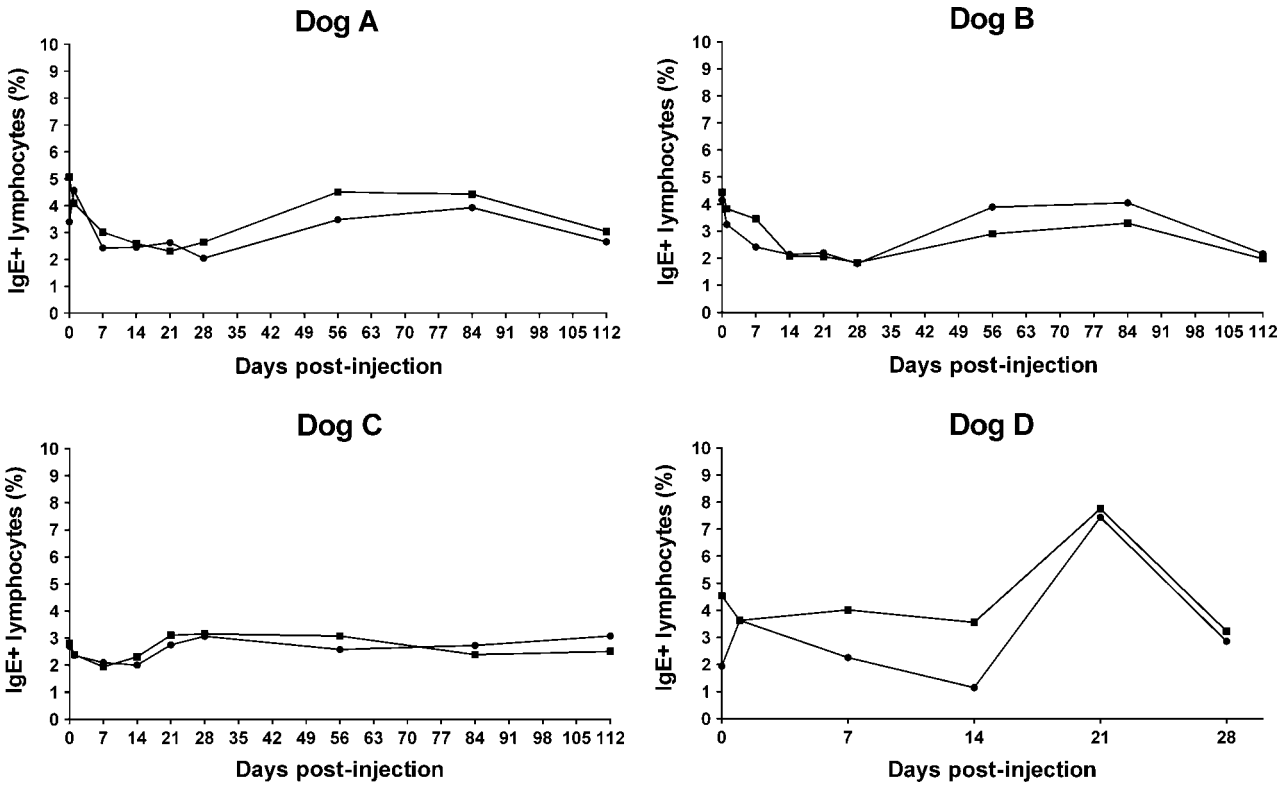

Figure 6. Changes in IgE(+) lymphocytes following injection of pegylated single chain variable fragments (scFv). The percentage of lymphocyte gated cells staining with APC (allophycocyanin)-scFv show early reduction in all dogs that is sustained in dogs A and B.

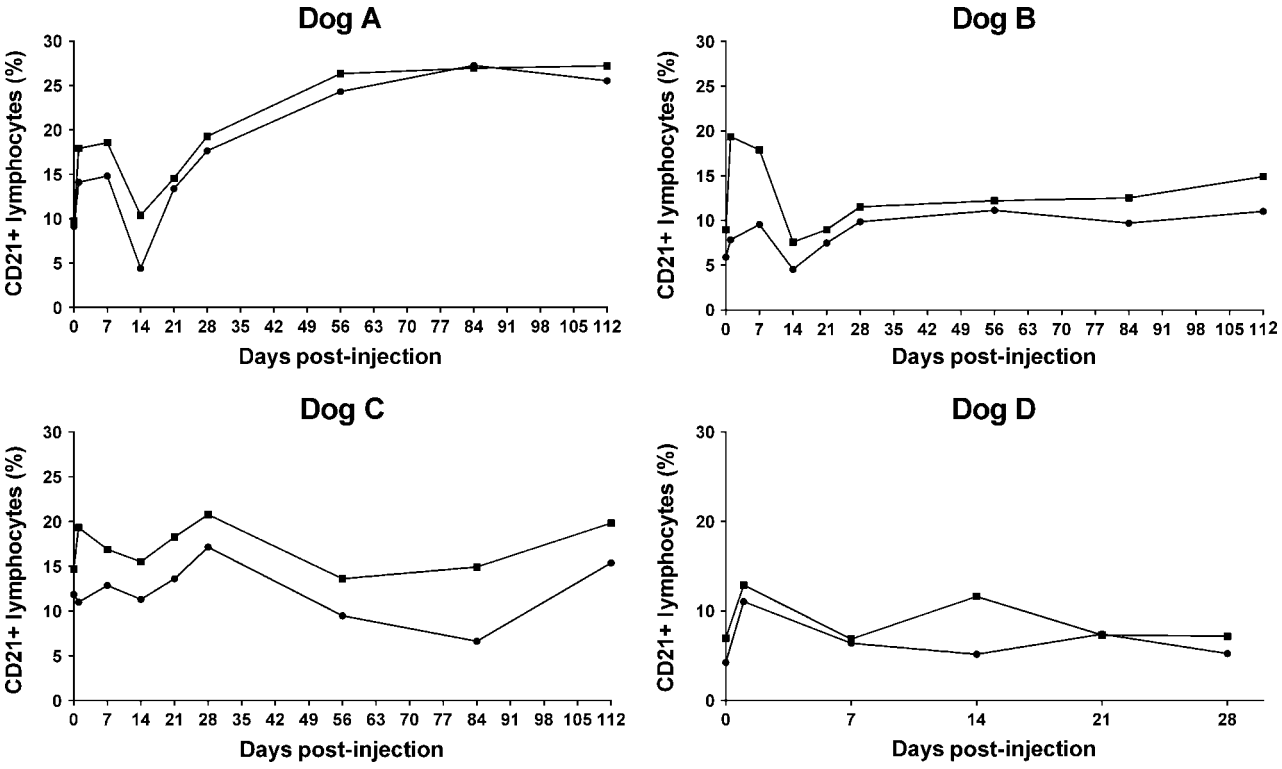

Figure 7. Changes in CD21 (+) lymphocytes following injection of pegylated single chain variable fragments (scFv). The percentage of lymphocyte gated cells staining with APC (allophycocyanin)-anti-CD21 show an early increase in this B cell population and no loss of B cells following scFv injection.

however, a single subcutaneous injection trial at a potentially therapeutic level (1 mg/kg) showed clinical signs of anaphylaxis which were reversible with diphenhydramine (unpublished observation: Bruce Hammerberg BH). This barrier was circumvented by creating the monovalent scFv of mAb 5.91 and pegylating scFv to minimize the

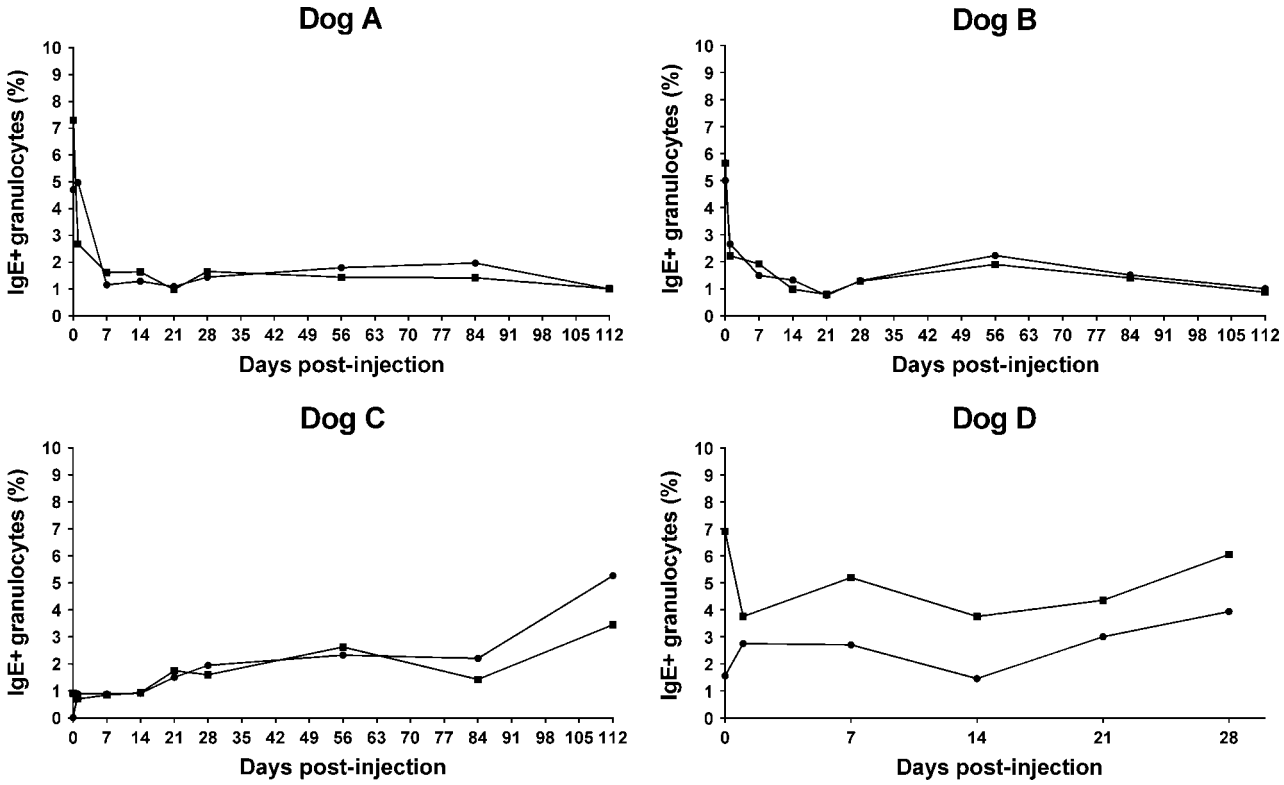

Figure 8. Changes in IgE(+) granulocytes following injection of pegylated single chain variable fragments (scFv). The percentage of granulocyte gated cells staining with APC (allophycocyanin)-scFv demonstrate highly variable responses between dogs with dogs A and B showing rapid and marked declines in APC-scFv staining.

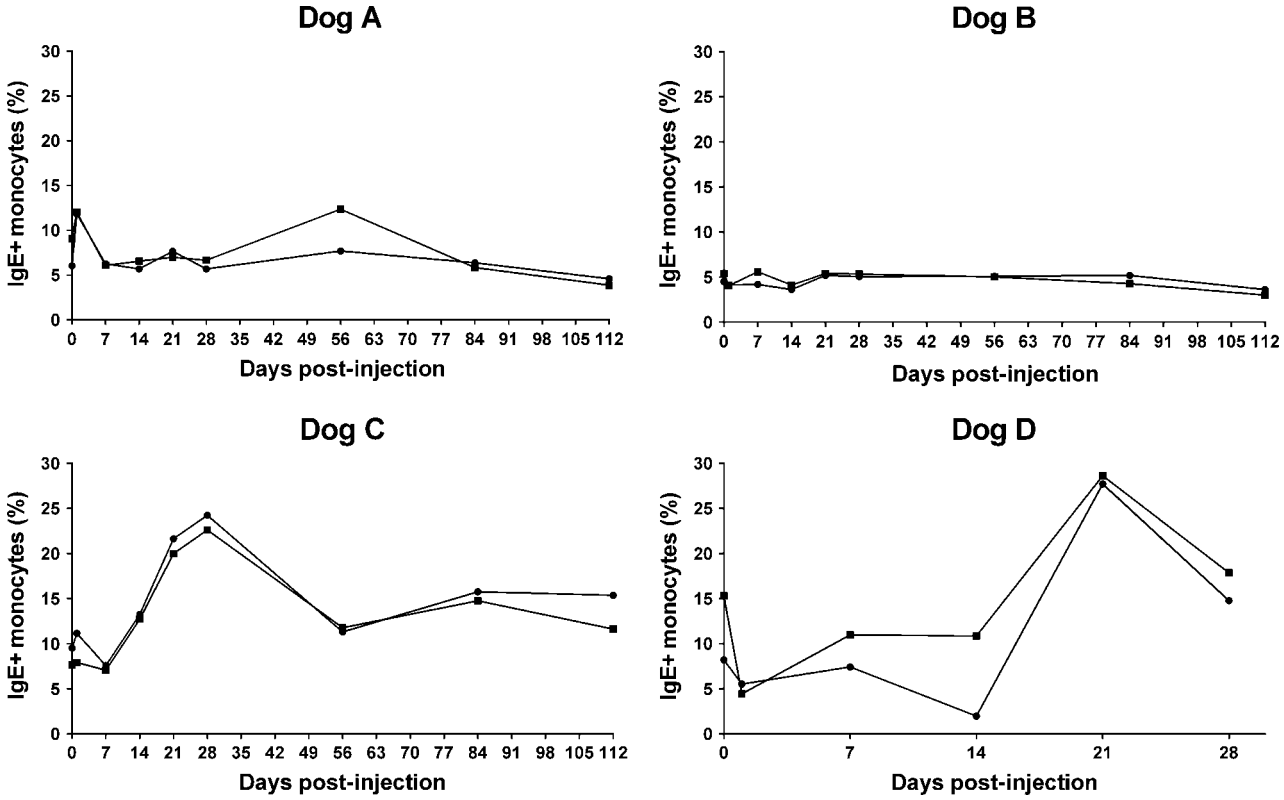

Figure 9. Changes in IgE(+) monocytes following injectcion of pegylated single chain variable fragments (scFv). The percentage of monocyte gated cells staining with APC (allophycocyanin)-scFv demonstrate no consistent pattern of response to pegylated scFv.

dimerization that is often reported for scFv.[10] The scFv reported here retains the murine framework sequences for heavy and light chain variable regions. The SAT(PEG)$_4$ reagent contains an N-hydroxysuccinimide ester that reacts with primary amine groups on lysine. There are 14 lysine amino acids in the scFv sequence, all but three of which are within the framework regions, and this may contribute to reduced antigenicity for scFv, which will be evaluated in future studies of repeated injections. It may be necessary for safety in prolonged repeated therapeutic injections to prevent anti-mouse IgG antibody development in treated subjects by caninizing the framework regions of the scFv.

Measurement of circulating IgE has been used as a marker for anti-IgE therapy, other than improvement in clinical signs, for the development and therapeutic use of omalizumab in humans.[7] A mechanism for the rapid reduction in circulating IgE seen in humans injected with omalizumab is proposed to be complex formation by the humanized complement-fixing IgG1 anti-IgE with IgE and its subsequent clearance by phagocytic cells. This could not be the mechanism for the more gradual reduction in circulating IgE shown after pegylated scFv injection in dogs due to the lack of crosslinking or complement-fixing ability by pegylated scFv. A more likely mechanism may be associated with the decrease in IgE expressing circulating lymphocytes following the pegylated scFv injection shown in this report. It has been reported recently that omalizumab reduces IgE production by human tonsil-derived B lymphocytes *in vitro*.[8] The precise mechanism whereby monovalent or bivalent antibodies against IgE expressed on B lymphocytes cause a reduction in IgE production remains to be discovered.[8]

A limited number of cells in the granulocyte gate, likely to be eosinophils[16,17] and basophils,[15] showed APC-scFv staining; however, only dogs A and B demonstrated a consistent, long-term reduction in IgE(+) cells following pegylated scFv injection. Eosinophil counts were not done on the serial samples.

The attempt to differentiate detection of IgE expressed on cells from receptor bound IgE – the former expected to be in granulocyte and monocyte gates, the latter expected to be in the lymphocyte gate – was not successful using lactic acid stripping. The failure of lactic acid stripping to remove IgE from granulocytes and monocytes suggests that conformational changes in IgE and/or FcεRI induced by low pH have less effect on their binding in the dog than in humans or mice.

The evidence presented here for the safety of monovalent anti-IgE forms specific for IgE epitope sites distant from the FcεRI binding site, that are modified to assure the maintenance of monovalent form at concentrations used for therapeutic doses, should encourage future studies with these types of antibodies to verify safety and to test for immunomodulatory efficacy of antibodies specific for novel IgE epitopes. Because of the complexity of possible mechanisms for immunomodulation that is clinically relevant, empirical studies testing for clinical efficacy in allergic dogs may be more effective in identifying therapeutic antibodies than pre-screening candidate antibodies based solely upon reduction of circulating IgE levels.

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Treatment of itch in dogs: a mechanistic approach

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The mechanisms that underlie itch can be studied from anatomic or etiological viewpoints. Anatomically, the pathogenesis of itch begins with mediators released by epidermal and dermal cells, which bind to receptors stimulating peripheral nerve afferents with cell bodies in dorsal root ganglia. After at least two neuronal synapses, pruritogenic itch signals are transmitted to the brain via spinothalamic tract neurons. Etiologically, pruritus is usually subdivided in dermatological, systemic, neurological, psychogenic, mixed or undetermined origins. Similarly to the various angles under which the pathogenesis of itch can be looked at, antipruritic drugs can be categorized based on their anatomic targets and/or their etiological indications. The antipruritic drugs that have targets in the skin usually broadly suppress cells releasing pruritogenic mediators, for example glucocorticoids, calcineurin inhibitors (e.g. ciclosporin or tacrolimus), cytotoxic drugs (e.g. azathioprine, methotrexate or mycophenolate), phosphodiesterase inhibitors or autacoid local injury antagonist (ALIAmides) such as palmitoylethanolamide/palmidrol. Pruritogens can also be targeted more specifically, for example with therapeutic monoclonal antibodies such as the newly developed anti-IL-31 Canine Atopic Dermatitis Immunotherapeutic, also known as Lokivetmab. Peripheral receptors to which pruritogens bind can be targeted broadly, for example with Janus kinase inhibitors (e.g. oclacitinib). On another hand, receptors can also be targeted in a specific fashion, for example with histamine 1 receptor antihistamines, transient receptor potential (TRP) agonists or topical anesthetics binding to voltage-gated sodium channels. The treatment of central itch impulses involves mu-opioid receptor antagonists such as naltrexone, the gamma-aminobutyric acid analogs (GABAergics) gabapentin and pregabalin, tricyclic antidepressants (TCAs, e.g. clomipramine and amitriptyline) or selective serotonin-reuptake inhibitors (SSRIs) such as fluoxetine. Looking at an etiological treatment of pruritus, the most prominent dermatological itch is usually relieved by most drugs targeting cells releasing mediators, the pruritogens themselves, or receptors on peripheral nerves. Neuropathic itch might respond to topical anesthetics, TRPV1 or TRPM8 ligands, GABAergics, TCAs or SSRIs at increasing doses, while psychogenic itch is best treated with SSRIs and TCAs. By combining knowledge on the pathogenesis of the pruritic disease to be treated, the itch pathways and the mechanism of action of available antipruritic drugs an effective relief of itch is usually achievable.

Introduction

In a recent epidemiological study, pruritus (itch) was found to represent the most common clinical sign (>30% of cases) exhibited by dogs presented to veterinarians for skin diseases, the latter being the number one cause (>20% of cases) motivating owners to bring their dogs to veterinarians outside of preventive healthcare.[1] Pruritic skin diseases can affect the quality of life of dogs and their owners.[2,3] This high prevalence and impact on animals should entice veterinarians to become familiar with both the mechanism of itch and the various options available to treat it.

A mechanistic classification of itch: anatomic or etiological?

In animals as in humans, the mechanisms that underlie pruritus can be studied from both anatomic or etiological viewpoints.

Anatomically, the pathogenesis of itch begins with mediators released by epidermal and dermal cells, which bind to receptors stimulating peripheral nerve afferents with cell bodies in dorsal root ganglia. After at least two neuronal synapses, pruritogenic itch signals are transmitted to the brain via spinothalamic tract neurons. Readers are directed to a recent review that summarizes the latest knowledge on skin innervation and pruritogenic mediators, receptors and pathways;[4] consequently, this information will not be repeated herein.

The 2007 etiological classification of pruritus proposed by members of the International Forum for the Study of Itch (IFSI)[5] was recently adapted to dogs:[6] the etiology of canine pruritus can be subdivided in one of six categories (Table 1). Most forms of canine itch are likely to belong to

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Table 1. Proposed classification of itch in dogs.

Category	Diseases in dogs
I. Dermatological	*Arising from 'diseases of the skin*': e.g. ectoparasites (e.g. scabies, fleas, etc.); allergies (e.g. atopic dermatitis, urticaria, flea allergy dermatitis, etc.); infections (staphylococcal folliculitis, *Malassezia* dermatitis, etc.); neoplastic (e.g. epitheliotropic T-cell lymphoma, mast cell tumor, etc.)
II. Systemic	*Arising from 'diseases of organs other than the skin*': none recognized so far in dogs
III. Neurological	*Arising from 'diseases or disorders of the central or peripheral nervous system*': e.g. syringomyelia (Chiari-like malformation), acral mutilation syndrome, etc.
IV. Psychogenic- psychosomatic	e.g. acral lick dermatitis, tail chasing, etc.
V. Mixed	*Overlapping and coexistence of several diseases:* e.g. atopic dermatitis with staphylococcal folliculitis, etc.
VI. Other	*Undetermined origin*

Source: Olivry and Baumer 2015.[6] Reproduced with permission of Springer.

the first category (i.e. 'dermatological itch'), with pruritus occurring in association with, or secondary to, cutaneous inflammation; a characteristic example of dermatological itch is that present in dogs with atopic dermatitis (AD). In this disease, erythema and pruritus scores appear to be correlated in most dogs, but there are animals with high pruritus and low erythema values or vice versa.[7] The latter could herald subclinical dermal inflammation, however.

Whereas most other forms of itch are shared between species, an important difference between dogs and humans is the lack of recognized systemic itch associated with chronic liver or kidney disease in animals. There is yet no logical explanation for this discrepancy.

Similarly to the various angles under which the pathogenesis of itch can be looked at, antipruritic drugs can be categorized based on their anatomic targets and/or their etiological indications. The paragraphs below are restricted to pharmacological interventions for which the use in dogs has already been documented.

Anatomic classification of antipruritic drugs

Targeting of peripheral cells and pruritogenic mediators

Drugs with broad targets

Targeting itch – or, for that matter, inflammation – in a broad fashion brings the advantage of a high sensitivity and a low specificity of action that will affect mediators and cells widely. Such properties will ensure a high probability of treatment effect in cases with variable mechanism and/or stage of lesion formation. Conversely, a broad mechanism of action is likely to be more propitious to the development of adverse events due to their bystander effect on cells not involved in the pathology being treated.

Glucocorticoids. Glucocorticoids, either applied topically or administered systemically, exhibit a strong antipruritic effect that stems from their wide targeting of cells expressing glucocorticoid receptors. Their anti-itch properties most likely occur secondarily to the reduction of cutaneous inflammation. There are only rare studies on the effect of glucocorticoids on pruritogenic mediators in dogs. Recently, the strong pruritogen IL-31 was found to induce pruritus when injected into dogs.[8] Prednisolone given 1 h before IL-31 injection to dogs did not reduce the induced pruritus, but it did so when given 10–12 h before injection.[9] Because of their presumed antipruritic mode of action, glucocorticoids are likely to be of highest value in dogs with inflammatory skin diseases such as AD;[10] details on their use, for example in canine AD, can be found in recent reviews and practice guidelines.[11,12]

Calcineurin inhibitors. The calcineurin inhibitors ciclosporin and tacrolimus act mainly (but not exclusively) as T-cell inhibitors. By blocking the translocation of the nuclear factor of activated T cells (NFAT) transcription factor, the secretion of cytokines such as IL-2 and interferon-gamma is reduced in T cells. A direct effect of ciclosporin on canine T cells reveals that oral doses of this drug (5–10 mg/kg/day) reduce the expression of IL-2 and interferon-gamma in *in vitro*-stimulated T cells purified from peripheral blood mononuclear cells of treated dogs.[13]

Specific studies on the influence of ciclosporin on pruritogens have not yet been performed in dogs. As ciclosporin treatment in atopic humans is accompanied by a decrease of serum levels of IL-31,[14] the effect of ciclosporin on canine serum IL-31 or on activated T cells and mast cells should be investigated.

In addition to its effect on T cells, the benefit of ciclosporin in AD and its itch could also be due to its targeting of other cells that express the same transcription factor, such as dendritic cells, eosinophils, mast cells and keratinocytes.[15] For example, protease-induced secretion of granulocyte macrophage colony-stimulating factor (GM-CSF) was reduced by ciclosporin in a canine keratinocyte progenitor cell line.[16] Also synthesis of the lipopolysaccharide-induced prostaglandin E_2 (PGE_2) was significantly reduced by ciclosporin in primary canine keratinocytes.[17]

In addition to their effect on inflammatory cells, calcineurin inhibitors exhibit an antipruritic effect that likely involves more than inflammation reduction, as there is increasing evidence of a direct effect of this class of drugs on several pruritogenic nerve receptors. Both oral ciclosporin and topical tacrolimus have long been known to be effective anti-allergic drugs in dogs with AD; details on the use of these drugs for this indication can be found in recent reviews.[12,18,19] At this time, however, the effect of 0.1% tacrolimus ointment on the itch of canine AD has only been assessed in one small randomized controlled trial (RCT), which reported only partial improvement in pruritus scores (41% of dogs improving more than 50%); the effect was (as expected) highest in localized disease manifestations.[20]

Cytotoxic drugs. To inhibit cells (e.g. T cells) that drive the inflammation and the associated itch that occurs in the skin of human patients with AD or other inflammatory skin diseases, cytotoxic drugs such as azathioprine,

mycophenolate mofetil or methotrexate are being used with increasing frequency.[10]

Information on the use of cytotoxic immunosuppressants in dogs with pruritus is limited. A small trial tested the efficacy azathioprine in dogs with AD, and results were heterogeneous and not suggestive of a strong and consistent benefit on either skin lesions or pruritus.[21] An abstract reported that methotrexate, administered once weekly at variable dosages, induced a reduction in pruritus and skin lesions scores in dogs with AD.[22] Results of an open study and an RCT both suggested that the methotrexate analog LD-aminopterin (aminotrexate) had a noticeable, yet partial and variable, effect on both skin lesions and pruritus in dogs with AD.[23,24]

Phosphodiesterase inhibitors. Phosphodiesterase 4 (PDE4) inhibitors show a broad anti-inflammatory effect by increasing the second messenger cyclic AMP in immune cells, as well as keratinocytes. This anti-inflammatory action of topical and oral PDE4 inhibitors has been confirmed recently in clinical trials enrolling children and adults with AD. In these studies, PDE4 inhibitors exhibit a moderate antipruritic effect.[25–28] Although the decrease in pruritus is accompanied by a concurrent reduction of inflammation, their anti-itch effect might not solely be due to anti-inflammatory properties. Indeed, a PDE4 inhibitor has been shown recently to directly modify the sensory response by increasing cyclic AMP in neurons.[29]

In a small RCT, dogs with AD were treated with the PDE4 inhibitor arofylline, with most dogs (70%) exhibiting a halving in their pruritus scores after 4 weeks of treatment.[30] Unfortunately, vomiting was a prominent adverse event limiting the benefit of this oral intervention.[18,30]

ALIAmides. The cannabinoid receptors CB1 and CB2 are expressed on sensory nerve fibers, keratinocytes and mast cells. Treatment with topical cannabinoid receptor agonists reduces histamine-induced itch and vasodilation in healthy humans. In mice, cannabinoid antagonists induce a dose-dependent itch.

Palmitoylethanolamide (PEA; palmidrol) is an endogenous arachidonic acid-derived fatty acid amide that targets mainly the peroxisome proliferator-activated receptor (PPAR)-alpha; it has a cannabinoid-like effect but does not seem to bind directly to CB1 or CB2. PEA was shown also to activate and then desensitize the transient receptor potential (TRP) vanilloid 1 (TRPV1) channel, one of the principal neuronal receptors involved in itch transmission.[31] The mechanism of action of PEA has been described as autacoid local injury antagonism (ALIA) and under this denomination it is an autacoid that is producing regulatory molecules locally.

PEA cultured with canine mast cells reduces the anti-IgE-induced release of histamine, prostaglandin D2 (PGD2) and tumor necrosis factor (TNF)-alpha.[32] While an experiment confirmed that the oral administration of PEA to *Ascaris*-hypersensitive beagle dogs significantly reduced (about 30% in average) mast cell degranulation-induced wheals,[33] a small pilot study did not find significant differences in treatment effect between PEA and placebo interventions in reducing allergen-induced skin lesions in an experimental model of canine AD.[34]

Recently, PEA was tested as an anti-allergic intervention in dogs with mild-to-moderate nonseasonal AD and mild-to-severe pruritus levels.[35] The tested formulation of PEA was found to significantly reduce pruritus in dogs with each category of itch severity. After 2 months of treatment, levels of pruritus manifestations and skin lesions similar to those of normal dogs were achieved in 30 and 62% of 122 dogs, respectively.[35] These encouraging results need to be confirmed in a well-designed RCT that incorporates PEA into current treatment regimens.[12]

Drugs with narrow targets. With a narrow target specificity comes the clear advantage of inhibiting the action of single pruritogens without negatively affecting others; this theoretically yields a higher efficacy/risk ratio by reducing adverse events. However, interventions aimed at restricted targets are unlikely to be of strong benefit whenever itch and inflammation are due to a myriad of mediators and cells, unless the target clearly initiates a pruritus and/or inflammation cascade.

Biologics. The caninized (i.e. made in canine sequence) monoclonal antibody Lokivetmab has been developed recently to target the strongly pruritogenic cytokine IL-31.[36] When injected into dogs with nonseasonal moderate-to-severe AD, Lokivetmab exhibits a strong antipruritic effect and a moderate one to reduce skin lesions; it also appears very safe.[36–38] In contrast to these positive results of IL-31 inhibition, one subcutaneous injection of a caninized monoclonal antibody targeting the pruritogenic cytokine nerve growth factor (NGF) did not appear to prevent the recurrence of pruritus flares after discontinuation of glucocorticoids in a pilot trial of five dogs with AD.[39] This lack of effect occurred in spite of this cytokine being transcribed early and expressed at high levels in dogs with AD and after allergen challenge in sensitized dogs.[39,40]

Targeting of peripheral cellular receptors

Drugs with broad targets

Janus kinase inhibitors. Janus kinase (JAK) inhibitors encompass a group of small molecules that inhibit one or more enzymes involved in cellular signal transduction after binding of cytokines to their receptor; as a result, the effect of JAK inhibitors varies depending on which of the four JAKs (JAK1–3, TYK2) are being affected. Oclacitinib is a JAK1-predominant inhibitor that principally blocks the signaling of cytokines involved in allergy, inflammation and itch (e.g. IL-2, IL-4, IL-6, IL-13, IL31, etc.).[41]

After several RCTs documented the rapid antipruritic efficacy of oclacitinib in dogs with allergic skin diseases and AD, this drug is currently recommended for treatment of AD and its pruritus in dogs.[12] The antipruritic speed of action of oclacitinib is comparable to that of prednisolone,[42] and faster than that of ciclosporin.[43]

Drugs with narrow targets

Biologics. Both recombinant canine interferon-gamma and feline interferon-omega, by binding to their respective receptors, have been reviewed to be effective antipruritic biologics in dogs with AD.[12] Their hypothetical mode of

action – yet still unproven – might be due to the inhibition of type 2 pro-allergic cytokines such as IL-4 and IL-13.

Histamine 1 receptor antihistamines. The frequently used histamine 1 receptor (H1R) antihistamines are competitive inhibitors at the H1 receptor,[44] one of the four known receptors for histamine. These four G-protein-coupled receptors are widely distributed in the body; the H1R is mainly found on endothelial cells, smooth muscle cells and immune cells. The activation of H1R plays a central role in the pathophysiology of immediate-type hypersensitivity reactions; the H2R is involved in gastric acid production, whereas the H3R is mainly found in the central and peripheral nervous systems. Interestingly, the H4R is mainly expressed on hematopoetic cells (monocytes, dendritic cells, Langerhans cells, T lymphocytes, eosinophils, basophils, mast cells) and on sensory neurons. This particular distribution indicates a significant role in allergy and inflammation.[45] Importantly, histamine-induced pruritus in mice seems to be mediated via the histamine H1 and H4 receptors, while the H3R appears to have a negative regulatory role.[46] Typical H1 antihistamines like diphenhydramine, cetirizine, loratadine and hydroxyzine show no significant inhibitory action on the H4R.[47] Antihistamines that cross the blood–brain barrier (e.g. 'sedating' or 'first-generation' antihistamines like diphenhydramine) are believed to be more antipruritic than those that lack – or have a vastly reduced – central action (for example, 'low-sedation' or 'second-generation' cetirizine or loratadine).

Antihistamines targeting H1Rs have been used for decades for treatment of AD in humans. In spite of this widespread usage, two recent guidelines concluded that there was a lack of evidence suggesting the benefit of H1R antihistamines as effective antipruritic drugs in human AD.[48,49] Consequently, oral or topical H1R antihistamines are not recommended for treatment of human AD, except when the short-term and intermittent use of sedating antihistamines is needed in case of itch-associated insomnia.[48] The use of H2R antihistamines for AD and its itch is similarly not recommended.[49]

Several clinical trials have tested the efficacy of both first- and second-generation H1R antihistamines in dogs with AD (reviewed in references 11,18,19). In general, most RCTs did not document a strong antipruritic benefit of this class of drugs.[11,18,19,50] In spite of the lack of consistently supportive trial results, clinicians anecdotally report a low-to-medium efficacy of H1R antihistamines to control pruritus in dogs with AD.[51] After the publication of small yet somewhat positive clinical trials, the latest update of the consensus practice guidelines for treatment of canine AD upgraded its recommendations for H1R antihistamine as drugs having a modest and variable antipruritic efficacy in dogs with this disease.[12]

TRP agonists. Capsaicin is a vanillylamide and the main capsaicinoid derived from hot chili peppers of the *Capsicum* genus. The main action of capsaicin results from its binding to TRPV1, an ion channel. The binding of capsaicin to TRPV1 results in calcium and sodium influx, nerve depolarization and substance P release, which causes an initial intense burning and stinging perceived as pain or itch. Repeated applications of capsaicin leads to long-lasting nerve desensitization, by exhaustion of substance P nerve reserves, with ensuing decreases in pain and itch. As a result, repeated applications of capsaicin can block peripheral nerve transmission of itch, thereby 'numbing' peripheral nerve pathways. At high concentrations (e.g. in 8% patches), capsaicin also causes reversible peripheral nerve-ending degeneration.[52,53]

Topically, capsaicin has been reported to be helpful for treatment of humans with localized itch, such as that associated with the chronic sensory neuropathy notalgia paresthetica,[54] but also that seen with prurigo nodularis, aquagenic pruritus and idiopathic pruritus.[53] The higher the initial concentration of capsaicin and the more frequent the applications, the earlier nerve desensitization appears and the antipruritic effect occurs. Once pruritus control is obtained, the frequency of application of capsaicin can be reduced. To enhance patient compliance, topical anesthetics can be applied 20 min before capsaicin application, but a concentration of 0.025% is generally tolerated by most patients.[53]

There is only one RCT reporting the effect of topical capsaicin for treatment of itch associated with an animal skin disease.[55] Twelve dogs with AD were treated with either a 0.025% capsaicin lotion or placebo onto lesional areas, twice daily for 6 weeks.[55] After a wash-out of 4 weeks, treatments were reversed. After 6 weeks of capsaicin, the owner pruritus scores, but not those assessed by investigators, were significantly lower than those after placebo application. The use of capsaicin in dogs with neuropathic itch deserves further evaluation.

Voltage-gated sodium channel blockers. Topical anesthetics block voltage-gated sodium channels in the cell membrane of postsynaptic neurons, thereby inhibiting the generation of electric potentials. Topical anesthetics have been shown to exhibit antipruritic properties.[56] An RCT confirmed the efficacy of a 1% pramoxine (pramocaine) lotion in human patients with uremic itch.[57]

There is only one small open trial evaluating the antipruritic effect of two pramoxine-containing cream rinses in dogs with AD.[58] Atopic dogs were bathed in baby shampoo, then one of the pramoxine-containing (leave-on) rinses was applied to the coat twice weekly for 2 weeks, after which the other formulation was used similarly for another 2 weeks. A satisfactory reduction in pruritus manifestation was observed in fewer than 20–30% of dogs with either formulation, and the antipruritic effect was estimated to last 48 h.[58]

Targeting of the distal pruritogenic neural pathways

Drugs with narrow targets

Mu-opioid receptors antagonists. Endogenous opioids (e.g. dynorphins, enkephalins, endorphins, endomorphins, nociceptin) and opiates (e.g. morphine) bind to one of four opioid G-protein-coupled receptors (OP1–4), also known as the delta (DOR), kappa (KOR), mu (MOR) or nociceptin receptors, respectively.[59] Mu-opioid receptor agonists induce mast cell histamine release. Epidermal keratinocytes and peripheral sensory neurons express both MORs and KORs. Recent data suggest that stimulation of MORs induces itch while that of KORs antagonizes it.

These results are concordant with the observation that KOR stimulation inhibits MOR effects in both peripheral and central nervous systems.

Several randomized controlled trials have reported a significant antipruritic effect of the MOR antagonists naloxone, naltrexone and nalmefene in humans with cholestatic pruritus, chronic urticaria and AD.[59,60] Interestingly, the topical application of MOR antagonists reduces itch in humans with AD, suggesting a peripheral effect rather than, or in addition to, a central effect. Nalfurafine, a KOR agonist, has been shown to be effective in reducing uremic pruritus.[61,62]

Only scant information exists on the use of MOR antagonists as antipruritic drugs in dogs: there are two small reports of canine acral lick dermatitis positively responding to either naltrexone[63,64] or nalmefene.[63]

Gamma-aminobutyric acid analogs. Gabapentin and pregabalin are both gamma-aminobutyric acid (GABA) structural analogs (GABAergics) that are approved for the treatment of epilepsy and for neuropathic pain in humans. These drugs elicit their pharmacological effect by binding to the alpha-2-delta subunit of voltage-dependent calcium channels, mainly at the spinal cord level. This binding inhibits presynaptic calcium influx and decreases glutamate release and associated synaptic transmission. Furthermore, these molecules also inhibit the inflammation-induced release of substance P and calcitonin-gene-related peptide (CGRP).

There is anecdotal evidence of the antipruritic efficacy of gabapentin and/or pregabalin in humans with the neuropathic pruritus associated with cutaneous dysesthesias.[65]

Oral gabapentin and pregabalin were shown to induce the complete cessation of scratching in only one of five cavalier King Charles spaniels with Chiari-like malformation and associated syringomyelia.[66] Evidence for the antipruritic efficacy of gabapentin and pregabalin in other canine skin diseases or in other forms of neuropathic pruritus is currently lacking.

Antidepressants. Antidepressants generally inhibit the serotonin (SERT) and/or the norepinephrine (NET) (reuptake) transporters in a selective or nonselective manner. Tricyclic antidepressants (TCAs) can also be antagonists or inverse agonists of serotonin, adrenergic or histamine receptors, among others.

The selective serotonin-reuptake inhibitors (SSRIs) fluoxetine, fluvoxamine, paroxetine and sertraline have been shown to be effective in relieving itch, albeit partially, in humans with various subsets of chronic and refractory inflammatory (including that of AD), systemic and psychogenic itch.[4,67,68]

Similarly, TCAs such as amitriptyline and doxepin appear to have some antipruritic efficacy in a subset of patients with dermatologic, systemic, neuropathic and psychogenic itch.[4,68] A 5% topical doxepin cream relieves AD-associated itch.[69,70]

In contrast, there are only a few reports of the use of SSRIs and TCAs to treat pruritic diseases in dogs. In dogs, as in humans, dosages should be increased until the desired benefit occurs or unacceptable treatment-induced side effects develop.[71–79]

At this time, the beneficial effect of the SSRI fluoxetine appears highest to alleviate licking behavior in the obsessive–compulsive – and presumably inflammatory pruritic and/or painful – disorder acral lick dermatitis.[74–76,78,80] In contrast, fluoxetine appeared no better than placebo in relieving canine atopic itch.[79]

In a small open trial, dogs with pruritus (one third of them with AD) were treated with the TCA doxepin, orally, without any apparent benefit.[71] In another open study, pruritic dogs, half with AD, were given oral amitriptyline with a good-to-excellent response

Table 2. Etiological targeting of itch by antipruritic drugs in dogs.

Category	Antipruritic drugs of choice	Suggested initial dosages
I. Dermatological	Glucocorticoids:	
	Oral (e.g. prednisolone)	0.5 mg/kg once to twice daily (PO)
	Topical (various)	variable (TO)
	Calcineurin inhibitors:	
	Ciclosporin, oral	5.0 mg/kg/day (PO)
	Tacrolimus, topical	0.1% twice daily (TO)
	JAK inhibitors:	
	Oclacitinib	0.4–0.6 mg/kg twice daily (PO)
	Biologics:	
	Recombinant canine interferon-gamma	5000 units/kg three times weekly (SQ)
	Lokivetmab (caninized anti-dog IL-31 monoclonal antibody)	1.0–2.0 mg/kg once monthly (SQ)
II. Systemic	*Not recognized in dogs at this time*	
III. Neurological	*Anecdotal and likely variable therapy, most likely involving GABAergics, topical anesthetics or TRP ligands and, perhaps, oral SSRIs and TCAs*	Effective dosages are mostly unknown
IV. Psychogenic-psychosomatic	Selective serotonin-reuptake inhibitors:	
	Fluoxetine	1.0 mg/kg once daily (PO)
	Tricyclic antidepressants:	
	Clomipramine	3.0 mg/kg once to twice daily (PO)
V. Mixed	*Unknown and likely variable*	
VI. Other	*Unknown and likely variable*	

Note: the dosages mentioned in this table are those for which there is randomized-control-trial-grade evidence of consistent and clinically relevant antipruritic efficacy. Abbreviations: GABA: gamma-aminobutyric acid; JAK: Janus kinase; PO: orally; SQ: subcutaneously; SSRIs: selective serotonin-reuptake inhibitors; TCAs: tricyclic antidepressants; TO: topically, TRP: transient receptor potential.

seen in only one third of patients.[73] In several case series and one small trial, there was general agreement on the usefulness of the TCA clomipramine to decrease lick behavior in dogs with acral lick dermatitis.[72,75,77]

Etiological classification of antipruritic drugs

The etiological classification proposed for canine itch (Table 1) offers an opportunity to have an antipruritic strategy targeting the various causes of itch (Table 2).

While the most prominent dermatological itch is usually relieved by most drugs targeting cells releasing mediators, the pruritogens themselves or receptors on peripheral nerves, neuropathic itch might better respond to topical anesthetics, TRPV1 or TRPM8 agonists, GABAergics at increasing doses or antidepressants. Canine psychogenic itch has been shown to respond to SSRIs and antidepressants.

In conclusion, by combining knowledge on the pathogenesis of the pruritic disease to be treated, the itch pathways and the mechanism of action of available antipruritic drugs, an effective relief of itch is likely achievable. The development of drugs targeting recently discovered (or newly important) mediators or receptors involved in mediating itch in humans and dogs will likely provide novel avenues to treat this common symptom that affects the quality of life of pets and their owners.

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Part 4

The Living Skin – Skin Biology and the Cutaneous Ecosystem

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The cutaneous ecosystem: the roles of the skin microbiome in health and its association with inflammatory skin conditions in humans and animals

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Background – Inhabiting a sterile world is no longer an acceptable or desirable concept. Recent studies developed in the microbiome field have unveiled complex microbial populations inhabiting the skin, digestive, respiratory and reproductive tracts. Microbiome studies have opened new venues to explore the human and animal second genome, its functions and its importance in maintaining health.

Skin microbiome in health – The composition of the skin microbiome varies across different body sites and across individuals, being influenced by different host habits, including for instance age, sex, diet, hygiene and lifestyle. Exposure to a diverse skin microbiome is now considered to be a key component in immune regulation, and imbalances in these microbial populations are being associated with human and animal skin inflammatory disorders.

Skin microbiome in inflammatory skin conditions – We have learned that in several skin conditions, there is a significant alteration in the diversity and composition of the microbiota colonizing the skin. For instance, in human and animal patients with atopic dermatitis, dysbiosis of the skin microbiota results in lower diversity of microbial populations. Whether these altered microbial populations are the cause or the effect of inflammatory skin conditions seen in humans and animals are still under investigation, but there is no doubt that the microbiome has an important role in maintaining skin health.

Summary – This review focuses on the most current studies describing the skin microbiome in humans and animals, its role in modulating the immune system, and its association with human and animal skin diseases.

Introduction

Several studies published in the last 20 years have shown that complex communities of microbes, known as the microbiome, inhabit the different surfaces of the human and animal body. These communities often have a commensal relationship with the host and recent studies have also shown the host to be dependent on these communities.[1–9] Microbes are no longer seen only as the "bad guys"; they are no longer our main enemies. Certainly, we know this is not true for all microbes, but we have now learned that the vast majority of micro-organisms inhabiting our bodies are actually beneficial.[1,8,10] These commensal microbial communities act as our assistants, competing with pathogenic microbes for nutrients, producing numerous metabolites and modulating our immune system, allowing human and animal bodies to thrive. Due to these new concepts gathered based on research performed in the last few years, most of us no longer wish to inhabit a sterile world.

Lederberg first coined the term microbiome in 2000,[11] when describing a more "ecologically-informed metaphor" to better understand and describe the relationship between humans and microbes. The term microbiome now encompasses the whole range of micro-organisms, including bacteria, archeae, fungi, viruses and parasites, their genes and metabolites, and the environmental conditions within a habitat (see Box 1).[12] The term microbiota is more limited and refers to the collection of micro-organisms in a defined environment and is now preferred instead of microflora, which refers to microscopic plants. Most microbiome studies to date have focused on sequencing the bacterial *16S* rRNA gene and fungal *18S* rRNA or ITS regions (Box 2). Recent studies have also used shotgun sequencing to evaluate the entire genomic DNA in a given sample, thus sequencing the plethora of microbes and their microbial genes. The value of these studies have been their ability to survey the functional potential of microbial communities.[13–15] By utilizing different molecular tools, and with the advancement of bioinformatics, microbial ecologists are able to classify micro-organisms taxonomically and analyse data using different diversity metrics to evaluate changes in diversity within and between communities (Box 1).[8,16,17]

It is estimated that the number of microbial cells colonizing the human body are 10 times more numerous than the number of human cells.[18] These microbes not only

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Box 1. Definition of microbiome nomenclature, diversity analysis and taxa[12,96,97]

Microbiome	All microbes in a habitat, their genes and metabolites
Microbiota	Collection of micro-organisms in a defined microenvironment Microbiota characterization is often based on conserved genes found on bacteria and fungi
Metagenome	Collection of genes and genomes from members of a microbiota obtained with shotgun sequencing
Operational Taxonomic Unit (OTU)	OTUs often correlate with microbial genus or species. The term OTU is used in microbiome diversity analysis, instead of species, as some genome sequences are not available in microbial databases
Alpha diversity (within sample)	Measures used to calculate number (richness) and distribution (evenness) of taxa within a sample *Richness metrics* *Observed OTUs*: number of observed species identified within a sample *Chao1* – estimated OTUs that would be found if population was fully sampled *Diversity metrics* *Shannon diversity*: considers evenness and abundances of different OTUs
Beta diversity (between samples)	Measures used to estimate shared OTUs (microbial species) between different samples and/or subjects. Data are often presented as PCoA plots. These can be phylogenetic based, which take into account microbial evolution, or OTU based *Phylogenetic based* *Unifrac distance metric*: analysis based on relative phylogenetic distances between observed organisms in a community *Non phylogenetic/OTU based* *Bray-Curtis index*: dissimilarity between two communities based on species abundance (community structure) *Jaccard index*: dissimilarity based on presence or absence of species (community membership)
Taxonomic microbial composition	Relative abundances of different members within the microbiota. Often presented as graphs, tables or heatmaps describing different taxonomic levels for microbes of interest

Box 2. Sequencing methods and targeted microbial genes used in microbiome studies[12,14,98]

Next generation sequencing (NGS)	Amplicon sequences from targeted amplified DNA clones and nontargeted DNA (whole genome and shotgun sequencing) are obtained with NGS methods. Multiple samples can be sequenced in a single run. Most common platforms used in NGS include the Roche 454 pyrosequencing and Illumina
Whole genome shotgun sequencing	Shotgun sequencing-based methods randomly sequence small sheared DNA fragments from whole genomes in a sample. These small fragments are assembled into continuous longer sequences. In microbiome studies, this method is used to characterize any genes that are sequenced in a nontargeted manner from host and micro-organisms, allowing phylogenetic characterization and identification of microbial genes
Bacterial *16S* rRNA	The transcribed form of the 16S ribosomal subunit gene (16S rRNA) gene is universal among prokaryotes. Highly conserved regions within this gene are followed by hypervariable regions. Sequences obtained from this gene allow phylogenetic characterization of bacterial communities
Fungal *18S* rRNA, *28S* rRNA, ITS	Similar to prokaryotes, eukaryotes also have conserved regions within their genome. For fungi, the *18S* rRNA, *28S* rRNA and the internal transcribed spacer (ITS) regions are genes of choice used in NGS studies. Although these genes allow characterization of fungal communities, fungal databases are still incomplete, offering limited characterization of obtained sequences
Virome	Assemblage of viral communities within a sample, including bacteriophages, single-stranded and double-stranded DNA and RNA viruses. Different from bacterial *16S* rRNA, viruses lack conserved regions in their genome. Development of viral databases are challenging due to marked viral genomic variability and rapid evolution of viruses

colonize the body, but also they carry important functional genes, responsible for synthesizing numerous metabolites, which can influence host health.[10] These diverse microbial populations inhabit the skin, digestive, respiratory and reproductive tracts within the human and animal bodies, but are variable across body sites and individuals.[10,19] The vast majority of the microbiome studies to date have focused on describing the gastrointestinal microbiome, with the cutaneous microbiome gaining more attention in the past 10 years. These culture-independent studies have revealed that the skin and other body surfaces are colonized with a larger number of microbes than had been described previously based only on culture methods. Certainly, microbiome studies have some pitfalls as they account primarily for the presence of fragments from microbial genes, without considering whether these organisms are alive or dead, but they do allow us to categorize the history of the micro-organisms inhabiting these different body surfaces.[14] It is estimated that the great majority of microbes identified with next

generation sequencing (NGS) studies (Box 2) are likely to be inactive or dead.[20,21] Furthermore, part of the sequenced microbes might be transient in the human and animal body,[22] and phylogenetic differences can be seen depending on the use of different methods, and depending on the selected microbial genomic regions that are amplified in these studies.[23,24]

These new microbiome concepts have opened avenues to explore the human and animal second genome, its functions and its importance in maintaining health, and how imbalances in these communities are associated with and possibly result in development of disease in the host. This review paper will focus on describing recent studies revealing the "invisible jungle" inhabiting human and animal skin based on NGS, and its relationship with skin disorders.

The human skin microbiome varies across individuals and body sites

The skin microbiome is unique, it varies across body sites and a remarkable variability is seen across different individuals.[2,14,15,22,25–29] Age, sex, diet, hygiene, lifestyle and the environment influence the composition of the skin microbiome.[15,22,25–28,30–32] It is estimated that the human skin is inhabited by approximately one million bacteria/cm^2.[29] Using direct shotgun sequencing to analyse various complex whole genomes from different microbes and viruses, it was found that the human skin is inhabited by highly diverse communities divided between a predominantly bacterial population, and relatively lower abundances of viruses and fungi.[15]

The human skin can be divided into dry, sebaceous and moist micro-environments based on different physiological niches.[27,28] These different skin micro-environments are predominantly inhabited by the bacterial phyla Actinobacteria and Firmicutes, with lower proportions of Bacteroidetes and Proteobacteria (Table 1).[22,27–29] The dry areas are considered to have the higher bacterial diversity, and these are more evenly colonized with bacteria within the four main phyla found in the skin. The sebaceous areas have lower diversity, being colonized primarily by Actinobacteria, and the genus *Propionibacterium*. The moist areas are colonized predominantly by *Corynebacterium* spp. and *Staphylococcus* spp., within the phyla Actinobacteria and Firmicutes, respectively. Significant variation is observed across individuals; however, certain skin sites, such as the antecubital fossa, back, nare and plantar heel are more similar across different individuals.

Table 1. Bacterial microbiota diversity and composition across microenvironments and skin sites in humans and animals

Host	Skin sites and physiology	Alpha diversity	Beta diversity	Microbial composition	Ref
	Dry	High	Depends on skin sites, with dry areas having more interpersonal variation than moist and sebaceous	Dry – evenly distributed among four main phyla: Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes	27–29
	Moist	Low		Moist –colonized predominantly by *Staphylococcus* and *Corynebacterium* spp.	
	Sebaceous	Lower		Sebaceous –colonized predominantly by *Propionibacterium* spp.	
	Mucosal surfaces/ mucocutaneous junctions	Overall low Nostril is site with lower diversity	More likely to share microbiota across body sites	Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes *Moraxella* spp. predominate in the nostril Proteobacteria and Bacteroidetes predominate in the lip comissure	54
	Haired skin	Overall high Dorsal nose is site with higher diversity	High variability across individuals and body sites	Proteobacteria most abundant phyla, followed by Firmicutes, Actinobacteria and Bacteroidetes	
		Overall high alpha diversity	Shared across different body sites	Proteobacteria, Bacteroidetes and Actinobacteria High relative abundances of bacteria found in the oral cavity	62
	Interdigital skin	High		Firmicutes, Spirochaetae, Bacteroidetes and Actinobacteria High abundances of *Treponema* sp. also found in healthy animals	56–58
	Interdigital skin	High		Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria *Dichelobacter nododus* also found in healthy skin	59
	Nostril	High	Community membership differences between conventional and liquid-fed	Proteobacteria, with the genus *Moraxella* predominate in most samples	65,66
	Ear pinna	High		Firmicutes, with the genera *Streptococcus* and *Lactobacillus* being the most abundant	
	Dorsal and ventral skin	Higher in wild than captive toads	Varies by amphibian species and wetland sites, and wild toads are colonized with complex communities	Proteobacteria, Bacteroidetes, Firmicutes and Sphingobacteria	60,61

The skin microbiome also extends beyond the skin surfaces and hair follicles. Using fluorescent *in situ* hybridization to demonstrate fragments of bacteria, and by sequencing the bacterial *16S* rRNA gene, it has been demonstrated that bacteria/bacterial products are present within the deep dermis and subcutaneous tissues, skin compartments previously thought to be sterile.[33] Although this is certainly a remarkable and intriguing finding, whether or not these actually represent viable bacteria is still unknown.

The composition of the skin microbiome seems to evolve with age.[25,31] Newborn infants are colonized with relatively high abundances of *Staphylococcus* and the skin microbiota becomes more diverse within the first year of life.[25] The composition of the skin microbiota shifts during adolescence. Within the nares, children have relatively high abundances of the phylum Proteobacteria shifting to higher relative proportions of the phylum Actinobacteria, due to increases in Corynebacteriaceae and Propionibacteriaceae in adolescents.[30] It was shown that adults have a more diverse microbiota compared to children and the elderly.[34]

Although gender would be expected to influence the diversity and composition of the skin microbiota, most studies to date have found very little evidence demonstrating gender differences.[22,27,35] Only a few studies have shown gender differences and these included the palm of the hands, where women were shown to have more diverse hand microbiota than men;[36] and the glabella, which was found to have significant differences in richness, as well as composition of the microbiota.[34] The gender differences observed in these two sites have been proposed to be associated with use of cosmetics and hygiene products. In fact, more and more scientific evidence supports the idea that our skin microbiota is influenced directly by our hygiene and use of cosmetics. A recent and quite impressive study describing the cartography of more than 400 skin sites of the human body evaluated the microbiota by sequencing the *16S* rRNA gene and correlated it with molecular profiles using mass spectrophotometry from each of these skin sites.[37] In this study, it was found that most of the metabolites identified in these different body regions originated from skin cells and microbes, but also from the use of hygiene and cosmetic products. The use of deodorants and antiperspirants, two of the most commonly used cosmetic products in the world, also have been associated with increased diversity in the axillary microbiota, and selection of increased proportions of *Staphylococcus* spp. and the malodorous bacteria in the genus *Corynebacterium*.[38]

Besides a vast bacterial population colonizing the skin surfaces of humans, fungi and DNA viruses also are found on human skin, although the relative fungal proportions compared to other microbes are relatively low.[15] Based on shotgun sequencing, fungi were found to comprise less than 1% of the microbiota in most body sites, except for the region around the ears and forehead, which had relatively higher abundances.[15] In all of these skin regions, the main fungi observed throughout the human body were *Malassezia* spp., and most commonly *M. restricta*, *M. globosa* and *M. sympodialis* (Table 2).[15,39] The genus *Malassezia* comprised more than 90% of the relative abundances of fungi found in the human skin. One of the few exceptions was observed in the feet, which are colonized with much lower proportions of the genus *Malassezia*, and much more diverse fungal communities. This is no surprise, given the close contact to the floor and different environmental surfaces.

Genomic studies based on shotgun sequencing have further been able to identify a vast viral population in the skin: the skin virome.[15,40,41] Similar to what was observed with the bacterial and fungal microbiota, marked interpersonal variation was observed in the skin virome (Box 2). Most of the skin virome was composed of bacteriophages, which are DNA viruses targeting bacteria. Of these, *Propionibacterium* and *Staphylococcus* bacteriophages were predominant in most skin sites. A few other viruses such as papillomavirus, polyomavirus and poxvirus were identified despite the fact the individuals had no clinical lesions. Even with development and improvement of these genomic studies, a large abundance of viral DNA found with shotgun sequencing could not be annotated, because viruses do not have conserved regions in their genome, as observed with rRNA genes in bacteria and fungi, and viral databases are largely incomplete. Likewise, there still remains a large number of RNA

Table 2. Fungal mycobiota diversity and composition across microenvironments and skin sites in humans and companion animals

Host	Micro-environment/ Skin sites	Alpha diversity	Beta diversity	Mycobiota composition	Ref
	Most skin sites	Low	High sharing across body sites in the same individual	Composed predominantly of *Malassezia* sp.	[15,39]
	Feet	High	Low sharing across individuals	More diverse and composed of *Malassezia* spp., followed by *Aspergillus*, *Cryptococcus*, *Rhodotorula* and *Epicoccum*	
	Mucosal surfaces/ mucocutaneous junctions	Overall lower	High sharing across body sites in the same individual	Composed mainly of environmental fungi within the phylum Ascomycota, including *Alternaria*, *Cladosporium* and *Epicoccum*, with lower abundance of the Basidiomycota *Cryptococcus* and *Malassezia*	[63]
	Haired skin	Overall higher	High sharing across body sites in the same individual	Composed mainly of environmental fungi within the phylum Ascomycota, including *Cladosporium* and *Alternaria* and *Epicoccum*, with lower abundance of the Basidiomycota *Cryptococcus*	[64]
	Mucosal surfaces	Lower			
	Oral	Overall high			
	Sebaceous	Overall high			

viruses to be investigated further, because current skin microbiome studies have described only DNA viruses.

The skin microbiome is influenced by the environment and cohabitation

Of all systems in the human and animal body, the integumentary system is the one with closest contact and direct exposure to the environment. Given its close contact with the outside world, the integumentary system and its microbiota are likely to be one of the main body systems influenced by the environment. Indeed, several studies have shown that this might be one of the reasons for such high variability and diversity one sees in the skin microbiome. For instance, urbanization of the human population has been one significant factor altering the skin microbiota.[34,42] This was demonstrated in one study which found that indigenous Amerindian communities who had no contact with people from the West, have a more diverse skin microbiota than similar indigenous communities who are transitioning to a Westernized lifestyle, and an even more diverse microbiota than the skin from people in the United States.[43] Similarly, the faecal microbiome was also found to be more diverse in these indigenous populations. Furthermore, their skin microbiota was more likely be similar among their group, and differed significantly from the other indigenous communities, and individuals living in the United States. Interestingly, even though these individuals have never received antimicrobial therapies, the study found antimicrobial resistance genes in the samples from their populations. Other studies also have shown similar differences in diversity between individuals that live in rural and urban areas, with individuals living in rural areas having greater diversity[42] and variability in their skin microbiota compared to those living in urban areas.[33]

Studies looking at cohabitation have found that individuals living in the same household are more likely to share similar skin microbiota.[44,45] One of these studies also found that dog ownership influenced the diversity of the microbiota and shared microbiota in adults, but not in children.[45] The same study did not find any effect on microbial diversity in the skin of individuals that cohabited with cats. Another study evaluating the nares and oral cavity of humans, dogs and cats, found that humans inhabiting households with pets had a more diverse microbiota than those that did not cohabit with pets.[43] Pets in households have further been shown to influence diversity and composition of the house environment microbiota.[46–48] These households are particularly influenced by dog ownership, due to the dog's resident microbiota and by bringing microbes from outdoors into the indoor environment.[47,49] These studies support microbial sharing between pets and humans, and possibly increased cutaneous microbial diversity associated with pet exposure. Pet ownership also has been associated with fewer cases of allergies in children,[50–53] which has been proposed to be due to exposure to diverse microbial communities brought by pets into the indoor environment. There is a certainly need for additional studies to be developed in this area to better evaluate the role of pets in prevention or development of allergies in people.

The skin of animals are inhabited by an even more diverse microbiome than seen in humans

Very few NGS studies have been published to date describing the skin microbiota in animals and these have included only limited numbers of animals, rendering them rather descriptive currently. Of these, a few studies have described the bacterial skin microbiota in dogs,[45,54,55] the nasal cavities of cats,[44] and the feet of cattle[56–58] and sheep (Table 1).[59] In exotic animal species, the skin microbiota in amphibians has been characterized,[60,61] given the marked concern with fungal infections decimating several amphibian species due to severe chytrid infections.

Companion animals

The few studies describing the canine skin microbiota have shown that canine skin is inhabited by a diverse microbiota that is even more diverse than seen in human skin.[45,54,55] Based on NGS studies, the main bacterial phyla found across different body sites in dogs include Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes and Fusobacteria (Table 2). Dogs have marked variability in their microbiota and numerous bacteria colonizing their skin have been identified as environmental microbes.

In contrast to humans, the skin of dogs, and other animals, is mostly covered with dense fur. Apocrine glands are distributed throughout their bodies, whereas eccrine glands that produce sweat are found only in their feet. They also have more uniform distribution of their sebaceous glands. Given all of these physiological differences, dividing the skin of dogs between dry, moist and sebaceous microenvironments is not really feasible. A study including 12 dogs investigated microbiota differences between skin sites, and found that the mucosal surfaces (i.e. conjunctiva, lips and nostrils) were colonized with less diverse bacterial populations compared to haired skin sites (i.e. axilla, groin, dorsum, ear pinna, dorsal aspect of the nose).[54] Of all these sites, the dorsal aspect of the nose was more diverse, on average. This is an interesting finding, which fits well with dog behaviour and their habits of having their nose in close contact with different surfaces. Their nostrils are colonized primarily with the bacterial genus *Moraxella*. Significant variability was found across different body sites in dogs and, similar to humans, some skin regions were more similar across different dogs, than across different body sites from the same dog. Dogs also share their microbiota and, similar to humans, dogs that cohabit the same household are more likely to share their microbiota than dogs inhabiting different households.[45] Although their skin bacterial microbiota was highly variable across different body sites, predicted metabolic profiles produced by their cutaneous microbiota was fairly similar across different body sites (A. Rodrigues Hoffmann, unpublished data,), a characteristic that has been demonstrated previously in humans.[1]

Based on preliminary studies describing the skin microbiota in 11 healthy cats, the feline skin microbiota is highly diverse, as observed in the skin of dogs, and some of the most common bacterial phyla observed on feline skin were Proteobacteria, Bacteroidetes, Firmicutes and

Actinobacteria (Table 1).[62] Interestingly, their skin had relatively more abundant Bacteroidetes across different body sites than seen in the skin of dogs, and the main families seen in this phylum were Porphyromonadaceae and Paraprevotellaceae, some of the most common bacterial families found in the oral cavity.[62] These findings are likely related to their grooming habits.

A recent study also has characterized the fungal microbiota (mycobiota) inhabiting the skin of 10 healthy dogs across different body sites (Table 2).[63] The study described the mycobiota of dogs as being highly diverse and, similar to the bacterial microbiota, was more diverse than observed in human skin. The canine mycobiota was more likely to be similar across different body sites within the same dog, than at the same body site between different dogs. Based on NGS studies, the skin of dogs was colonized predominantly with fungi within the phyla Ascomycota and Basidiomycota. Within the phylum Ascomycota, *Alternaria*, *Cladosporium* and *Epicoccum* were the most abundant genera found on the skin of dogs. Although *Malassezia* is one of the most common fungal genuses cultured from the skin of dogs, overall low abundances were identified in this dog study population. However, a few dogs in the study also presented relatively higher abundances of *Malassezia* spp. in some skin sites. The feline mycobiota evaluated in 11 healthy cats also was highly diverse and, similar to dogs, the main fungi found on the skin of these cats were within the phyla Ascomycota, including the genera *Cladosporium* and *Alternaria*, and the phyla Basiodiomycota (Table 2).[64] The fungi found in the skin of companion animals are ubiquitous fungi likely acquired from their environment and additional studies including larger number of animals should further investigate whether these are transient microbes, or are truly indigenous mycobiota. In both cases, carriage of different microbes in the skin of companion animals could possibly impact exposure and sensitization of humans in early life, which may explain the association of lower cases of allergies in individuals cohabiting with pets.

Large animals

Fewer studies have described the skin microbiome using NGS in large animals,[57–59] and no studies to date have characterized the skin microbiome inhabiting different skin surfaces in large animals. In ruminants, most attention has been given to describing the microbiome of digits (Table 1), due to the high prevalence of digital dermatitis.[56–58] The digits of bovine are highly diverse and Firmicutes, Spirochaetae, Bacteroidetes and Actinobacteria are the most predominant phyla. The digits of sheep have high abundances of Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria, with high abundances of the genus *Peptostreptococcus*.[59] Furthermore, *Dichelobacter nodosus*, a common cause of ovine footrot was amplified from healthy skin; however, it required the use of real-time PCR targeting and amplifying specific regions in this bacterium, due to mismatches in the *16S* rRNA gene.

The skin microbiota in ear and nostril regions in pigs have been described previously.[65,66] In the study that evaluated the ear,[65] pigs were followed over time, and neonatal pigs had higher diversity of their microbiota compared to 21-week-old pigs. The most common bacterial genera colonizing the ear were *Streptococcus* and *Lactobacillus*. Within the nasal cavity,[64] the predominant phylum was Proteobacteria, with the genus *Moraxella* accounting for more than one third of all sequences (Table 1). The community membership in the nasal micro biota was different between pigs from conventional antibiotic-free farms and pigs from liquid-fed farms routinely treated with tylosin; however, no differences were observed between MRSA carriage.

Exotic species

In exotic species, most NGS studies have focused on characterizing the skin microbiota of amphibians.[60,61] The most common phylotypes colonizing their skin include Bacteroidetes, Proteobacteria, Firmicutes and Sphingobacteria (Table 1). One study found that different amphibian species were strong predictors of microbial community composition and wetland sites explained significant variations across the same species of amphibians.[61] Furthermore, another study found wild toads had greater bacterial richness and diversity than captive toads.[60]

A study from Australia evaluated the skin, oral cavity and faecal microbiota of the Tasmanian devil, and found that the faecal microbiota was more diverse compared to the skin and oral cavities.[67] Their skin was colonized mainly with bacteria in the phyla Firmicutes followed by Proteobacteria.

Microbial dysbiosis is associated with inflammatory skin conditions

Most focus in the microbiome field has been given to gastrointestinal inflammatory conditions and there are sufficient data to support the gastrointestinal microbiome being responsible for causing certain gastroenteritis in humans and animals. Studies evaluating the skin microbiome in inflammatory skin conditions have been limited and rather descriptive, and it is still unclear whether microbial dysbiosis is the cause or the result of inflammatory skin conditions in humans and animals. Despite the limited number of studies published to date, exposure to a diverse skin microbiome is now considered to be a key component in immune regulation; cutaneous dysbiosis, which is defined as imbalances in the composition of microbial populations, are associated with human and animal inflammatory skin disorders.[9,68–74]

It has been demonstrated that the commensal skin microbiota directly influences skin immunity.[75] In this study, *Staphylococcus epidermidis* enhanced innate immunity and limited pathogen invasion, by inducing specialized T cells to move to the epidermis, which occurs in coordination with dendritic cells residing in the skin. In this experimental model, no inflammatory skin responses were associated with *S. epidermidis* exposure. However, when the skin encountered a new commensal, there were increases in IL17A+ T cells and induction of the cytokine IL-17A. This mechanism was mediated by the skin commensal *S. epidermidis*. The study showed that the skin is a dynamic environment, with its immune system responding to alterations in its commensal microbiota. It appears

that even *Staphylococcus aureus* might be capable of modulating the immune system, given that *S. aureus* carriers tend to have better outcomes when developing bacteraemia, when compared to noncarriers.[76] This suggests that *S. aureus* colonization might also be able to "prime" the immune system of carrier individuals.

A recent study has shown that neonatal exposure to commensal bacteria is required to establish immune tolerance to these commensal microbes. Using a murine model, it has been demonstrated that *S. epidermidis* colonization in neonatal life was responsible for activating a wave of regulatory T cells, which resulted in tolerance to commensal microbes.[77] This study as well as evidence from other published studies,[72,78,79] suggest that some chronic skin disorders could be a result of exaggerated skin response to the commensal skin microbiota. Furthermore, given the rise of autoimmune diseases in urbanized populations, it is been proposed that reduced exposure to commensal microbes, due to increased cleanliness in urban areas, has resulted in augmentation of immune responses later in life, further leading to development of hypersensitivities. We have now learned that in several skin conditions, there is a significant alteration in the diversity and composition of the microbiota colonizing the skin.

Human atopic dermatitis

Atopic dermatitis (AD) is the most common chronic inflammatory skin disorder diagnosed in humans, being especially common in children. AD incidence has increased worldwide over the last decades.[80] Patients with AD have intermittent lesions characterized by dry, erythematous and pruritic skin. A population of patients with AD have altered skin barrier function, due to mutations in the filaggrin genes, which results in disruption of stratum corneum, allowing penetration of foreign antigens and hypersensitization to occur.[81] In a longitudinal study, it was shown that lesional skin of children during AD flares has lower microbiota diversity, which corresponded to significant increases in the relative proportions of *S. aureus*.[68] In that study, relative increases in the proportions of *S. aureus* were seen before development of lesions and the diversity of the microbiota was restored even before recovery of skin lesions. In children that received intermittent treatment, the diversity of the microbiota and relative proportions of *S. aureus* were maintained similar to nonaffected skin. It is possible that these increases in *S. aureus* abundances are related to permissiveness of an altered skin barrier to colonization by *S. aureus*. This hypothesis is supported, at least in part, by a study describing that after tape stripping and following removal of the superficial skin layers, fewer bacterial genera than seen in intact stratum corneum, primarily *Staphylococcus* and *Propionibacterium*, recolonized the deeper stratum corneum.[35]

Certain human primary immunodeficiencies (PID) are characterized by AD-like skin disease. These individuals have increased skin permissiveness and dysbiosis of their skin microbiota, characterized by lower specificity and temporal stability. In these PID conditions, disease severity has been correlated with increases in *Staphylococcus* spp. and *Corynebacterium* spp., as well as other less abundant taxa.[82]

More and more scientific evidence, based on natural and experimental studies, demonstrates that *S. aureus* is a key player in the development and increased severity of skin lesions in AD. A mouse model was developed with ADAM17-deficiency which developed eczema and microbial dysbiosis, similar to AD in humans.[83] In this experimental study, it was not only demonstrated that *S. aureus* lead to eczema formation, but also showed subsequent *Corynebacterium bovis* colonization induced T helper 2 cell responses. The findings in this experimental mouse study are the first to suggest microbial dysbiosis might be capable of driving eczematous lesions and show evidence to support that the skin microbiota is capable of causing skin lesions.

In AD and PID patients with AD-like lesions, it has been described that the diversity of the mycobiota increases with development of skin lesions.[82,84] This is in contrast to what is observed in the bacterial microbiota, wherein diversity tends to decrease with development of skin lesions.[68] Relative increases in opportunistic fungi, including those in the genera *Candida* and *Aspergillus*, were observed in AD patients.[82] Another study found that *Candida albicans*, *Cryptococcus diffluens*, *Cryptococcus liquefaciens, Cladosporium* spp. and *Toxicocladosporium irritans* were the predominant fungal taxa in AD patients.[84]

In both healthy and AD individuals, *Malassezia* spp. predominate in the skin. Some studies have reported that AD individuals have higher abundances of *M. sympodialis* than observed in healthy individuals.[85] Other studies have found different *Malassezia* spp. to be increased in AD patients. These findings highlight some of the problems of skin microbiome research, and although it still not well defined whether certain Malassezia species are associated with skin lesions, it is a consensus that *Malassezia* exacerbate AD skin lesions.[86]

Dysbiosis of the skin microbiome is associated with skin diseases in animals

Atopic dermatitis and allergic skin diseases in companion animals

Similar to humans, dogs naturally develop AD with chronic skin lesions, being characterized by erythematous macules and patches with intense pruritus primarily involving the face, axilla, inguinal region and feet.[87] Canine AD is characterized by a hypersensitivity reaction with production of IgE antibodies against environmental allergens, such as house dust mites (HDM), pollens and moulds. Occasionally, dogs also may develop hypersensitivity against *S. pseudintermedius* or *M. pachydermatis*.[88] The skin lesions can be exacerbated by bacterial and/or fungal infections (most commonly *S. pseudintermedius* infection),[89] which result in development of papules, pustules and crusts.[88] Only a limited number of studies including very few animals have been reported to date to investigate the role of the microbiota in skin diseases in companion animals and these have been mostly descriptive (Table 3). In a preliminary study including six allergic/atopic dogs during remission of skin lesions, the skin microbiota of these dogs was evaluated and compared with healthy dogs.[54] The allergic dogs had lower richness of their skin microbiota and were colonized with

Table 3. Bacterial microbiota and fungal mycobiota diversity and composition in common inflammatory skin diseases in animals

Host	Skin condition	Bacterial diversity	Bacterial composition	Fungal diversity	Fungal composition	Ref
	Atopic dermatitis (AD)	Low in lesional skin and AD versus healthy	Lesional skin has abundant *Staphylococcus aureus*	Low, with shared mycobiota among AD	Different proportions of fungal taxa predominate in AD versus healthy dogs	54,63,90
	Allergic skin disease	Not altered	Different proportions of fungal taxa predominate in allergic versus healthy cats	Not altered	Increased Agaricomycetes and Sordariomycetes, lower *Epicoccum* proportions compared to healthy	62
	Digital dermatitis (DD)	High	Increased Spirochaetes, Bacteroidetes and Proteobacteria in DD *Treponema* spp. and *Fusobacterium necrophorum* predominate, especially in deeper lesions			56–58, 92, 93
	Footrot	Increased richness and diversity in acute footrot	Increased *Corynebacterium* spp. and *Staphylococcus* spp. in footrot Higher abundances of *Dichelobacter nodosus* in footrot			59, 94
	Chytrid infection	High	Composition is driven by chytrid infections			60, 61, 95

different abundances of bacterial populations than the healthy dogs. A longitudinal study including 14 dogs showed that the skin of AD dogs had increased relative abundances of *S. pseudintermedius* during development of skin lesions, which resulted in reduced diversity of the microbiota, similar to that described previously in humans.[90] Bacterial diversity also correlated with transepidermal water loss and pH changes. Following treatment, remission of lesions correlated with increases and restored microbial diversity and lower *Staphylococcus* spp. relative abundances, similar to nonlesional skin.

The skin microbiota also was longitudinally evaluated in a canine model of AD using HDM challenges.[91] This experimental study included eight dogs that were hypersensitized with HDM and when challenged on the right inguinal area with HDM, they developed focal skin lesions. Samples were collected prior to and after challenges from the affected and contralateral areas, and changes in the microbiota were analysed longitudinally. No changes in richness or diversity were observed for the different timepoints evaluated; however, several bacterial phylotypes increased during development and following remission of skin lesion. These included increases in the family Corynebacteriaceae shortly after development of skin lesions, and Staphylococcaceae proportions, confirmed with real-time PCR to be due to *S. pseudintermedius*, were significantly more abundant in lesional skin for more than two weeks after remission of lesions.

These preliminary studies described above show microbial dysbiosis in canine AD, associated with increases in *S. pseudintermedius*, and demonstrates the need for additional studies to be developed in this area.

Microbial dysbiosis in canine AD occurs not only associated with the bacterial microbiota, but also with the mycobiota. A study including a population of eight allergic/atopic dogs described that their skin had lower diversity of the mycobiota.[63] This difference is in contrast to what is described in human AD, which present increased fungal diversity.[82] We further evaluated the mycobiota in cats with allergic dermatitis and, despite the fact that allergic cats had dysbiosis of their skin mycobiota, there were no differences in diversity between allergic and healthy cats.[64] One possible explanation for these differences between human and canine skin rely on the fact that the fungal skin mycobiota in humans is colonized primarily by the genus *Malassezia* and is less diverse to begin with,[82] whereas the skin of healthy dogs is characterized by more diverse fungal populations.[63] It was hypothesized that the canine mycobiota shifts in disease states, lowering fungal diversity and allowing certain fungal populations to predominate in skin lesions. Furthermore, the skin of atopic dogs also is described to be associated with altered skin barrier, such as caused by fillagrin mutations, whereas similar alterations in skin barriers have not been described or confirmed in allergic cats, which might explain why no alterations were seen in diversity in the feline allergic skin disease.[64]

Pododermatitis in ruminants

A significant proportion of cases of lameness observed in cattle are due to skin lesions involving the digits and resulting in digital dermatitis (DD). DD is seen most often in dairy cattle although less frequently it also occurs in beef cattle. DD is a polymicrobial disease and the most common bacteria associated with DD include multiple *Treponema* species.[92] Bacterial *16S* rRNA gene studies reveal that these lesions have remarkable diversity (Table 3). In DD lesions, Firmicutes predominated in superficial and intermediate lesions, whereas Treponema dominated the deeper layers of the DD lesions.[56] Another study based on *16S* rRNA gene and fluorescent *in situ* hybridization (FISH), showed approximately that 50% of the sequences were *Treponema*-like, 25% were of

Fusobacterium necrophorum and the remaining were composed of other bacterial species.[93]

In a metagenomic study, using shotgun sequencing, it was reported that cattle with DD had increased relative abundances of Spirochaetes, Bacteroidetes and Proteobacteria, in contrast to healthy feet which were colonized predominantly by Firmicutes and Actinobacteria.[57] *Treponema denticola* and *T. vincentii* were the predominant bacterial species identified in both active and inactive DD. They further described the functional composition of the microbiome and higher abundance of genes associated with resistance to copper and zinc, products commonly used in footbaths to prevent DD, and genes associated with antibiotic resistance, were observed in cattle with DD. In another study it was shown that distinct *Treponema* phylotypes colonized the skin of active (ulcerative) versus inactive (healing) DD.[58] It was suggested that the gut might be an important reservoir for these *Treponema* species, as these were found ubiquitously in the ruminal and faecal microbiomes; however, other studies have failed to identify similar *Treponema* species in the gut and feet of cattle with DD.[92] These studies have now shown that bovine DD is a polymicrobial disease, making this an excellent model to investigate the role of the microbiome in these polymicrobial infections.

Another example of a polymicrobial disease is footrot in sheep and goats. The causative agents for this disease are *Dichelobacter nodosus* and *Fusobacterium necrophorum*.[94] Several bacterial taxa have been associated with this disease and, based on a *16S* rRNA gene study,[59] microbial diversity and richness was greater in tissues from sheep with interdigital dermatitis, the first clinical sign in footrot lesions, than in healthy interdigital areas, or those with a chronic form of footrot (Table 3). This chronic form is known as virulent footrot and results in separation of the hoof horn from sensitive tissue. The study demonstrated that the genus *Corynebacterium* was associated with interdigital dermatitis and the genus *Staphylococcus* with virulent footrot. Additionally, a specific real-time PCR assay for *D. nodosus* demonstrated sheep with interdigital dermatitis had significantly higher numbers of *D. nodosus* than sheep with healthy digits or virulent footrot.

Skin infections in amphibians

The chytrid fungus *Batrachochytrium dendrobatidis* (Bd) has been studied extensively due to severe cutaneous infections resulting in marked mortality in several amphibian species and raising serious concerns especially to endangered amphibian species. Due to its severity, a few studies were developed to better understand the interactions between this fungus with the skin microbiota.[60,95] One study[95] demonstrated that Bd infection caused significant changes to the bacterial microbiota inhabiting the skin (Table 3). The fungus Bd was capable of driving changes in the cutaneous bacterial communities, with strong correlation between chytrid infection load and bacterial community composition. Despite this strong correlation, 100% mortality was observed in postmetamorphic frogs, and there was no association between survival and bacterial microbiota. The findings of that study indicated a significant and relatively predictable interaction between the skin microbiota and Bd infections.

Conclusions

The studies described above are some examples supporting the hypothesis that the skin microbiome of humans and animals play a significant role in maintaining skin health. The skin microbiome is vastly diverse, and remarkable variation between and across individuals are observed in humans and the different animal species studied to date. Despite these variations, such studies have demonstrated that imbalances in these microbial populations can contribute to development and/or severity of skin lesions. Moreover, recent studies have demonstrated that interactions between the skin microbiome and the immune system can maintain a healthy skin versus the establishment of a disease status. New mechanisms used by these commensal microbes are now being described, explaining the interplay between the microbiome and the immune system.

The microbiome is certainly an exciting area and there is still much more to be done in this field, as we are only starting to unveil the cutaneous ecosystem. Additional studies including larger number of animals investigating the skin microbiome composition in different skin diseases are needed. Future studies should focus on functional aspects of the microbiota, including evaluation of metabolomics, transcriptomics and proteomics. There is a need to better understand the relationships between microbes and the host immune system, and to determine if and how the microbiome can cause skin lesions, or alter its severity. Furthermore, studies looking at development of new drugs to treat different skin conditions should include evaluation of the microbiome as an additional resource to monitor treatment outcomes. We foresee that in the future NGS and microbiome studies could be further used in assisting in diagnostic tests for skin infections and identification of novel pathogens. It is also highly possible that the understanding of these mechanisms and with advancement of these technologies, we will be able to modulate the microbiome in favour of its host by augmenting beneficial microbes. Together with other disciplines, microbiome studies will allow us to better understand the complexity of the human and animal body functions, how the skin and other organs respond to exposure to different microbes, as well as pathogenesis of infectious and inflammatory diseases.

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4.2

Characterization of the cutaneous mycobiota in healthy and allergic cats using next generation sequencing

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Background – Next generation sequencing (NGS) studies have demonstrated a diverse skin-associated microbiota and microbial dysbiosis associated with atopic dermatitis in people and in dogs. The skin of cats has yet to be investigated using NGS techniques.

Hypothesis/Objectives – We hypothesized that the fungal microbiota of healthy feline skin would be similar to that of dogs, with a predominance of environmental fungi, and that fungal dysbiosis would be present on the skin of allergic cats.

Animals – Eleven healthy cats and nine cats diagnosed with one or more cutaneous hypersensitivity disorders, including flea bite, food-induced and nonflea nonfood-induced hypersensitivity.

Methods – Healthy cats were sampled at twelve body sites and allergic cats at six sites. DNA was isolated and Illumina sequencing was performed targeting the internal transcribed spacer region of fungi. Sequences were processed using the bioinformatics software QIIME.

Results – The most abundant fungal sequences from the skin of all cats were classified as *Cladosporium* and *Alternaria*. The mucosal sites, including nostril, conjunctiva and reproductive tracts, had the fewest number of fungi, whereas the pre-aural space had the most. Allergic feline skin had significantly greater amounts of Agaricomycetes and Sordariomycetes, and significantly less *Epicoccum* compared to healthy feline skin.

Conclusions – The skin of healthy cats appears to have a more diverse fungal microbiota compared to previous studies, and a fungal dysbiosis is noted in the skin of allergic cats. Future studies assessing the temporal stability of the skin microbiota in cats will be useful in determining whether the microbiota sequenced using NGS are colonizers or transient microbes.

Introduction

Next generation sequencing (NGS) techniques have provided a methodology to characterize host-associated microbial communities (microbiota) more comprehensively and have consequently revealed a much more diverse microbiota than was previously thought to exist.[1] In humans, NGS studies have shown that skin-associated bacterial microbiota are distributed according to physiological niches,[2] such as dry, moist and sebaceous skin microenvironments,[3] whereas the distribution of the fungal microbiota (mycobiota) is more dependent upon body site location such as core body versus feet.[4] In contrast to what is observed in humans, the bacterial microbiota of canine skin are influenced by body site rather than physiological niches.[5] The mycobiota are more likely to be distributed evenly across body sites within a dog and significant differences in mycobiota are observed between dogs.[6]

The specific bacterial and fungal taxa of canine skin differ from those of human skin. Canine skin is dominated by bacteria in the phyla Proteobacteria, Firmicutes and Actinobacteria,[5] and environmental fungi such as *Alternaria* and *Cladosporium*,[6] whereas human skin is colonized more abundantly by bacteria in the phyla Actinobacteria and Firmicutes,[3] and the fungal genus *Malassezia*.[4] Hygiene practices and environmental exposures are thought to contribute to the differences in diversity and taxa between host species,[6] although studies are still required to better investigate their influence on the microbiome. The microbial communities present on feline skin have only been investigated using culture dependent methods.[7–16] The results of these studies are variable and fungal genera commonly isolated include *Penicillium*, *Cladosporium*, *Aspergillus*, *Alternaria* and *Malassezia*.

Bacterial and fungal dysbiosis (alteration to the normal microbiota) has been identified in human atopic dermatitis (AD)[17,18] and canine allergic dermatitis.[5,6] The lesional skin of atopic human patients exhibits reduced bacterial diversity with proportionate increases in *Staphylococcus*

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species[17] and increased fungal diversity.[18] By contract nonlesional skin of allergic dogs possessed reduced diversity of both bacterial and fungal microbiota.[5,6]

Cats suffer from an allergic dermatitis sometimes resembling human and canine AD, referred to as nonflea nonfood-induced hypersensitivity dermatitis (NFNFIHD), which suggests that environmental allergens are triggers for these cases.[19] However, the pathogenesis of NFNFIHD is incompletely understood and lacks some of the defining characteristics of human and canine AD.[20,21] These include absence of a proven genetic predisposition for any subgroup of NFNFIHD (with the exception of a report of three affected littermates),[22] clinical presentation[23] and uncertainty as to whether the skin barrier is impaired in NFNFIHD. Furthermore, there have been variable reports on the role of allergen-specific IgE in cats with NFNFIHD.[24,25] The skin microbiota of cats with NFNFIHD has yet to be investigated with either NGS or culture-dependent methods. Only a single study using cytological examination of tape strips demonstrated an overgrowth of *Malassezia* in allergic cats compared to control cats.[13]

The goals of this study were to characterize the mycobiota of feline skin using NGS and to determine whether alterations to the mycobiota exist in feline allergic skin diseases. It was hypothesized that the mycobiota of feline skin would be similar to canine skin and that fungal dysbiosis would also exist in feline allergic dermatitis. Similar to previous studies, the influence of individual characteristics such as the type of body site (haired, moist, oral, sebaceous) and body site location were assessed. Overall fungal diversity and relative abundance of select taxa (i.e. the amount of a fungal taxon sequenced in a sample relative to the total amount of fungal DNA sequenced for that sample) were compared between healthy and allergic feline skin.

Materials and methods

Subject recruitment

All samples for this study were collected following a protocol approved by the College of Veterinary Medicine, Texas A&M University, Institutional Animal Care and Use Committee. Eleven cats (numbered C1–C11) were enrolled in this study on the basis of no current or prior dermatological conditions and were assigned to the healthy group (Table 1). There were five castrated males and six spayed females ranging in age from two to 17 years. Six cats were domestic short hair, two were domestic medium hair and three were domestic long hair cats.

Nine cats (C12–C20) were included in the allergic group (Tables 1 and 2). This group ranged in age from 4 to 11 years of age and included four castrated males and five spayed females. There were six domestic shorthairs, two Siamese and one Persian cat represented. All were diagnosed with a hypersensitivity dermatitis (HD) after exclusion of other pruritic dermatoses such as ectoparasitism and bacterial or fungal infections.

The classification of HD for each cat is presented in Table 2: flea bite hypersensitivity (FBH, $n = 8$), food-induced hypersensitivity dermatitis (FIHD, $n = 1$) and nonflea nonfood-induced hypersensitivity dermatitis (NFNFIHD, $n = 4$). Four cats had received a diagnosis of more than one type of HD and one cat, which had failed to respond to an appropriate trial with a flea preventative, but which had not completed a dietary elimination trial at the time of sampling, was classified as having nonflea bite hypersensitivity (NFBH). The age of onset ranged from three to six years of age, although two cats had experienced a gradual progression of clinical signs with the exact age of onset unknown. Seven of ten cats had no seasonal exacerbations of clinical signs, whereas one had flares during the summer only and two experienced flares during the spring and summer. The most common clinical signs included pruritus and alopecia. There was a wide range of lesion distribution (Table 2). Six cats had documented steroid administration, but only two (C14 and C15) were receiving steroids at the time of sample collection. Additionally, three cats were receiving therapies including oral ciclosporin (C15 and C16), sublingual immunotherapy (C17) and oral antihistamines (C16).

Exclusion criteria included exposure to systemic antimicrobial drugs within the six months (healthy control group) or 30 days (HD group) prior to sampling. Bathing was not allowed during the week prior to sampling. None of the cats exhibited any signs of secondary bacterial or fungal infections at the time of collection.

Sample collection and DNA extraction

Twelve body sites were sampled on healthy cats including the axilla, chin, conjunctiva, dorsal nose, dorsum, ear canal, groin, interdigital space, nostril, oral cavity, preaural space and vulva or prepuce. Six sites commonly affected by HD were sampled from the allergic group, including the axilla, ear canal, dorsum, groin, interdigital space and nostril. Samples were collected by rubbing sterile skin swabs against skin; DNA was extracted and stored as previously described.[6]

ITS sequencing and sequence analysis

Illumina sequencing (Illumina Inc.; San Diego CA, USA) of all samples was performed on an Illumina MiSeq instrument at the University of Minnesota Genomics Center using ITS1F (5′- CTTGGTCATTTAGA GGAAGTAA-3′) and ITS2R (5′- GCTGCGTTCTTCATCGATGC-3′) primers that amplified the internal transcribed spacer (ITS-1) region, a noncoding segment of genome found within the ribosomal genes of all eukaryotes. Sequences from only the forward reads were then processed in the open source bioinformatics software Quantitative Insights into Microbial Ecology, QIIME.[26] Quality filtering was performed and operational taxonomic units (OTUs; group of similar sequences that represents a taxonomic unit of a fungal species or genus) generated using the open reference picking command and the ITS sequence database.[4] Taxonomic assignments were made with a formatted version of the ITS taxonomy file.[4] OTU tables were rarefied at 3,100 sequences for healthy only samples, 5,000 for allergic only samples and 3,300 for the table including only the six sites sampled in both healthy and allergic cats.

Alpha diversity was measured using Chao1, observed OTUs and Shannon metrics. To determine whether fungal richness and diversity of skin microbiota were different between cats, body sites or type of body site, the alpha diversity measures for each metric were analysed across all body sites within a cat ("Cat"), across all cats at one body site ("Body Site") or for all body sites within a particular type of body site ["Skin Type" included haired (axilla, dorsal nose, dorsum, ear canal, groin, interdigital space, preaural space), mucosal (conjunctiva, nostril, prepuce and vulva), oral (cavity) and sebaceous (chin)]. Beta diversity was measured using weighted Jaccard, Bray Curtis and Pearson metrics. These calculations are performed for each possible pair of samples and the distance matrix generated was then used to create 3D PCoA plots. Analysis of similarities (ANOSIM) was performed on the distance matrices to determine statistical significance of a factor (cat, body site, skin type) on the dissimilarity between samples.

Statistical analysis

All statistical analyses were completed as described previously,[6] except that distance matrices and relative abundance tables were generated in QIIME. The relative abundance tables were combined for all taxonomic levels (Phylum, Class, Order, Family and Genus) and filtered to include taxa present at greater than 1% in at least three samples for allergic cats, or five samples for healthy cats. Using the statistical software JMP Pro 11, (SAS Institute, Inc.; Cary, NC, USA) data were tested for normality and Kruskal–Wallis tests were performed to determine whether the mean value (relative abundance or

Table 1. Signalment and medical histories of twenty cats enrolled in the study

Cat number	Health status	Breed	Age	Sex	Fleas	Time indoors	Indoor environment	Outdoor environment	Previous antibiotic usage
C1	Healthy	DLH	5	MC	Y	100	n/a	n/a	N
C2	Healthy	DSH	2	FS	N	100	TFB	n/a	N
C3	Healthy	DSH	13	MC	N	100	CTFB	n/a	N
C4	Healthy	DSH	7	MC	N	70	TFB	TGW	N
C5	Healthy	DMH	4.5	FS	N	99	CTFB	TGW	N
C6	Healthy	DSH	7	FS	N	100	TFB	n/a	N
C7	Healthy	DSH	9.5	FS	N	50	B	TGW	N
C8	Healthy	DLH	13	FS	N	100	CTFB	n/a	N
C9	Healthy	DLH	15	FS	Y	0	n/a	TGW	N
C10	Healthy	DMH	6	MC	N	100	CTFB	n/a	N
C11	Healthy	DSH	17	MC	N	100	CTF	n/a	N
C12	Allergic	DSH	9	MC	N	100	TFB	n/a	N
C13	Allergic	Sia	8	MC	N	100	TFB	n/a	N
C14	Allergic	DSH	11	MC	Y	95	CFB	TGW	N
C15	Allergic	Sia	9	FS	N	100	TFB	n/a	N
C16	Allergic	DSH	5	FS	N	60	CTFB	TGW	Y
C17	Allergic	DSH	9	FS	N	100	CTFB	n/a	N
C18	Allergic	Per	4	MC	Y	100	CTB	n/a	Y
C19	Allergic	DSH	7	FS	N	100	CTFB	n/a	N
C20	Allergic	DSH	8	FS	Y	95	TFB	TGW	N

Fleas and ear problems were part of the medical history and not present at the time of sample collection.
Signalment: DLH domestic long hair, DMH domestic medium hair, DSH domestic short hair, Per Persian, Sia Siamese, MC castrated male, FS spayed female. Indoor environment: C carpet, T tile, F furniture, B bedding. Outdoor environment: T trees, G grass, W weeds. Y yes, N No.

Table 2. Hypersensitivity classification, age of onset, seasonality, clinical signs and distribution, and treatments for nine allergic cats

Cat number	Breed	Age	Type of HD	Age of onset	Seasonality	Clinical signs	Lesion distribution	Ear problems	Allergy treatments	Steroids
C12	DSH	9	FBH	6	N	Pruritus, self-induced alopecia	Limbs	N	N	N
C13	Sia	8	FBH	6	N	Pruritus, self-induced alopecia	Dorsum	N	N	N
C14	DSH	11	FBH	G	Summer	Pruritus, self-induced alopecia, crusting	Rump, tail, ears, ventral abdomen	Y	N	Y
C15	Sia	9	FBH, FIHD, NFNFIHD	3	Spring, summer	Pruritus, cervicofacial, self-induced alopecia	Face, neck, ears	Y	Ciclosporin	Y
C16	DSH	5	FBH, NFNFIHD	4	N	Pruritus, self-induced alopecia, excoriations	Chest, ventral abdomen, dorsum, tail, limbs	Y	Ciclosporin, antihistamines	Y
C17	DSH	9	FBH, NFNFIHD	6	N	Pruritus, cervicofacial dermatitis, self-induced alopecia, eosinophilic plaques	Face, ventral abdomen, limbs	N	Sublingual immunotherapy	Y
C18	Per	4	FBH, NFBH	3	N	Pruritus, self-induced alopecia	Ears, ventral abdomen, rump, tail, limbs	Y	N	N
C19	DSH	7	NFNFIHD	6	N	Pruritus, cervicofacial	Face, ears	N	N	Y
C20	DSH	8	FBH	G	N	Pruritus, self-induced alopecia	Ventral abdomen, limbs	N	N	N

Allergen treatments were concurrent. All cats with a Y in the steroids column had previously received steroids, except for C14 and C15 that were receiving steroids at the time of sampling. C18 was diagnosed with a dermatophyte infection, treated with lime sulfur dips and lesions resolved 3 months prior to sample collection.
DSH domestic short hair, Per Persian, Sia Siamese, FBH flea bite hypersensitivity, FIHD food-induced hypersensitivity dermatitis, NFNFIHD nonflea nonfood-induced hypersensitivity dermatitis, NFBH nonflea bite hypersensitivity, G gradual, Y yes, N no.

alpha diversity) of at least one cat or body site was significantly different from all others ($P < 0.05$). When significant, a Steel–Dwass all pairs test was performed to identify the cat(s) or body site(s) with significant differences. A Wilcoxon–Mann–Whitney *U*-test was performed to determine significant differences between health statuses.

In order to determine whether the beta diversity of samples was significantly influenced by cat, body site, skin type, steroids or health

status, the analysis of similarities (ANOSIM) function in the statistical software PRIMER 6 (PRIMER-E Ltd.; Luton, UK) was performed on the distance matrices generated in QIIME using the Jaccard, Bray Curtis and Pearson metrics. *R* values were calculated for each pairwise comparison between groups (significant comparisons summarized in Table S2) and a global *R* statistic was calculated for the factor under study (cat, body site, skin type) (ANOSIM, PRIMER 6). The combined and filtered relative abundance tables were also used in linear discriminant analysis (LDA) effect size (LEfSe)[27] to determine significant differences between cats, body sites or health statuses. All *P*-values were corrected for multiple comparisons using the Benjamini and Hochberg false discovery rate.[28]

Results

One hundred and thirty two samples were collected from healthy cats and 54 from allergic cats. Due to low numbers of sequences (less than 3,000), 24 samples from healthy cats and 15 from allergic cats were removed from downstream analyses. Following quality processing, the total number of sequences from the remaining healthy samples was 7,249,611 with a median of 42,742 sequences per sample. The total number of sequences from allergic samples was 2,521,229 with a median of 49,684 sequences per sample.

Skin fungal diversity analyses of healthy cats

The alpha diversity (diversity within a sample) of fungi sampled from feline skin was estimated using three different alpha diversity metrics: the observed OTUs estimator measures the number of OTUs per sample, which is thought to be a close representation of the number of fungal species present (i.e. fungal richness); the Chao1 estimator is a richness estimator that accounts for sequencing depth (likelihood OTUs were not identified in acquired sequencing data); and the Shannon Index is a diversity measure that accounts for OTU abundance and evenness. All median alpha diversity measurements can be found in Table S1. For healthy cats there was a significant difference in fungal richness and diversity between cats (Observed OTUs, $P < 0.001$; Shannon, $P = 0.022$) and body sites (Observed OTUs, $P = 0.044$; Shannon, $P < 0.0001$). Specifically, the skin of C9 harboured a more rich and diverse mycobiota than the other cats (Figure 1). The conjunctiva and reproductive tract sites of healthy cats were the least diverse body sites, whereas the preaural space was the most rich and diverse (Figure 1). Fungal diversity was also significantly different between skin types (Shannon, $P < 0.0001$), with the mucosal sites (including conjunctiva, nostril and reproductive tract sites) being significantly less diverse than oral, sebaceous (chin) and haired sites (Figure 1).

The beta diversity (diversity between samples) of feline skin mycobiota was estimated using three different non-phylogenetic based metrics: the Jaccard estimator is calculated by comparing the presence of shared fungal taxa between samples, whereas the Bray Curtis and Pearson estimators further account for differences in amounts of fungal taxa between samples. The results of performing ANOSIM on the distance matrices generated by all three metrics produced comparable results, as demonstrated in

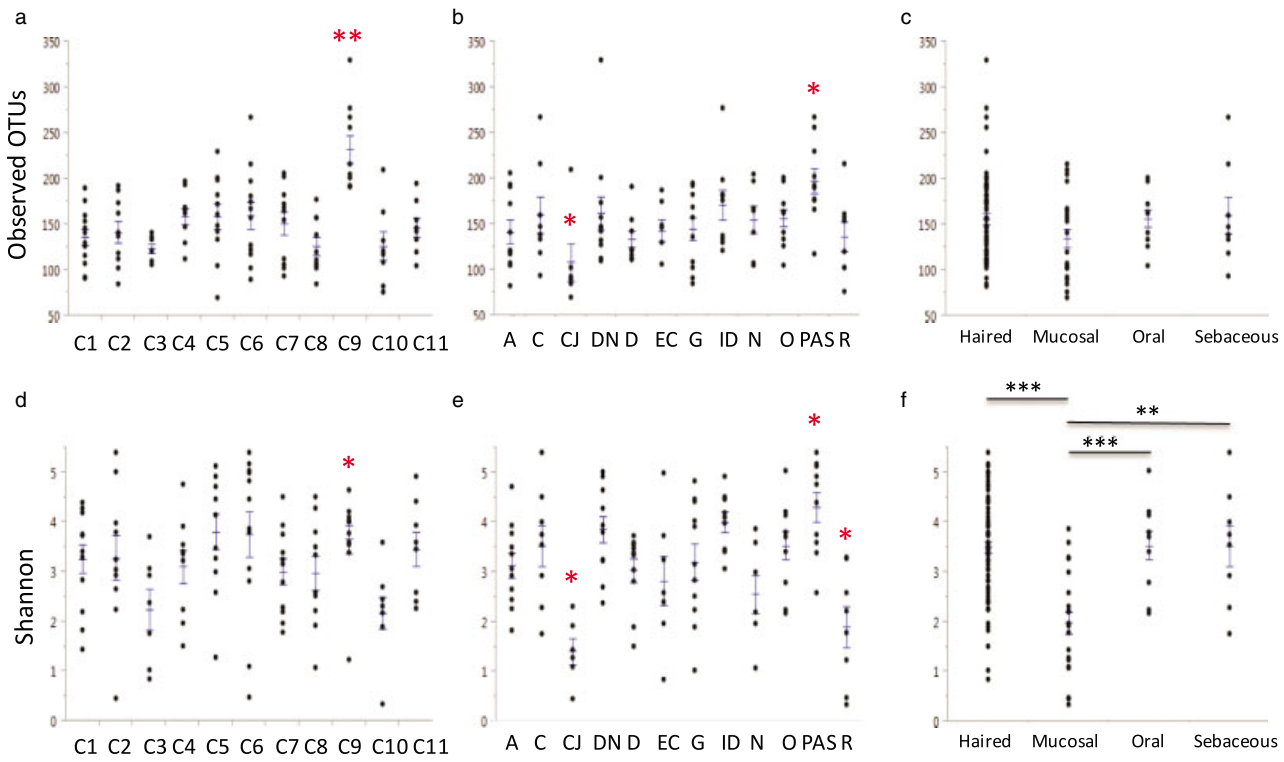

Figure 1. Alpha diversity of healthy cats. (a–c) Alpha diversity estimated with observed OTU's and samples grouped by (a) cat, (b) body site and (c) skin type. (d–f) Alpha diversity estimated with Shannon diversity metric and samples grouped by (d) cat, (e) body site and (f) skin type. Means and mean error bars are plotted in blue for each group. Groups with a mean significantly different from other means are denoted by asterisks, with associated *P*-values (Steel–Dwass multiple comparisons test, of *<0.05, **<0.01, ***<0.001. A axilla, C chin, CJ conjunctiva, DN dorsal nose, D dorsum, EC ear canal, G groin, ID interdigital space, N nostril, O oral, PAS preaural space, R reproductive tract. Haired (axilla, dorsal nose, dorsum, ear canal, groin, interdigital space, preaural space), mucosal (conjunctiva, nostril, prepuce and vulva), oral (cavity) and sebaceous (chin).

Figure 2. The *R* statistic indicated the effect that a variable has on the dissimilarity between samples. This value ranges from zero to one, with an *R* value of one indicating complete dissimilarity between two groups within a factor (e.g. axilla and groin are the groups, body site was the factor). An *R* value of one would indicate that the factor has a very strong influence on the presence and/or abundance of mycobiota. Some clustering of healthy cat samples (n = 108) by cat was observed in the PCoA plot of the Bray Curtis pairwise distances between healthy cats, indicating similarity of fungal communities in the sites that cluster together (Figure 2; ANOSIM, R = 0.324, P = 0.001). Nineteen of the pairwise comparisons between cats were significantly different, with an average *R* value of 0.215 and *P*-values ranging from 0.003 to 0.038 (Table S2). Clustering was less apparent by skin type (Figure 2; ANOSIM global R = 0.208; P = 0.002) and absent by body site (Figure 2; ANOSIM global R = 0.083; P = 0.001).

Skin fungal taxonomic composition of healthy cats

The most abundant fungal phylum identified was Ascomycota, accounting for 79% of fungal sequences from healthy cats; the most abundant class within this phylum was Dothideomycetes, accounting for 48% of the sequences. The three most abundant genera within this class were *Cladosporium*, *Alternaria* and *Epicoccum* (Figure 3). There was also a remarkable proportion of fungal sequences (21%) that were classified within the Ascomycota phylum but could not be classified further (Figures 3 and 4; Other Ascomycota). The most abundant genus within the Basidiomycota phylum was *Cryptococcus*. Although these were the most abundant taxa across healthy sites sampled, a high degree of variability between samples was noted, as presented in the taxa plots of Figure 4. *Malassezia* was sequenced from 30% of healthy cat samples (n = 35) but was present at greater than 1% relative abundance in only 5% (n = 6) of samples (Figure S1). The median relative abundance of unassigned sequences was 6%; however, there were several samples that had greater than 50% unassigned sequences. Due to the fact that fungal databases are still undergoing curation, these sequences may be assigned to fungal taxa in future studies.

Two types of statistical testing, Kruskal–Wallis and LEfSe, were performed to determine whether specific taxa (phylum, class, order, family or genus levels) were differentially abundant between cats or body sites. Kruskal–Wallis testing performed in JMP revealed that the relative abundances of 53 taxa were significantly different between cats (Table S3; FDR adjusted $P < 0.05$); only two taxa were significantly different between body

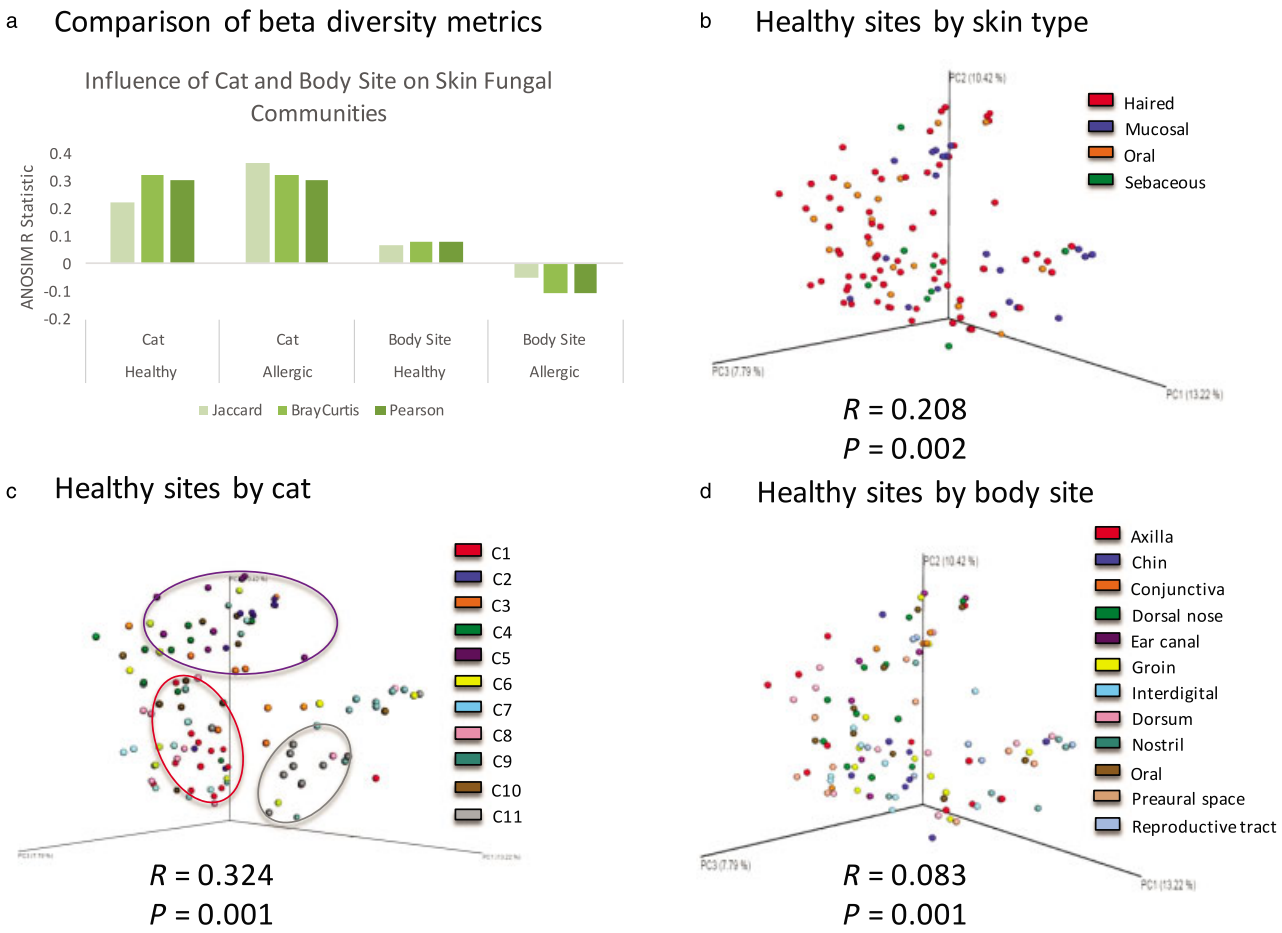

Figure 2. Beta diversity of healthy cats. (a) Comparison of ANOSIM global R statistic between three metrics, Jaccard, Bray Curtis and Pearson, for the factors of cat and body site in both health status groups. (b–d) PCoA plot of Bray Curtis pairwise distances for healthy cat samples, with associated ANOSIM global *R* statistic and *P*-value; coloured by (b) skin type, (c) cat and (d) body site. Haired (axilla, dorsal nose, dorsum, ear canal, groin, interdigital space, preaural space), mucosal (conjunctiva, nostril, prepuce and vulva), oral (cavity) and sebaceous (chin).

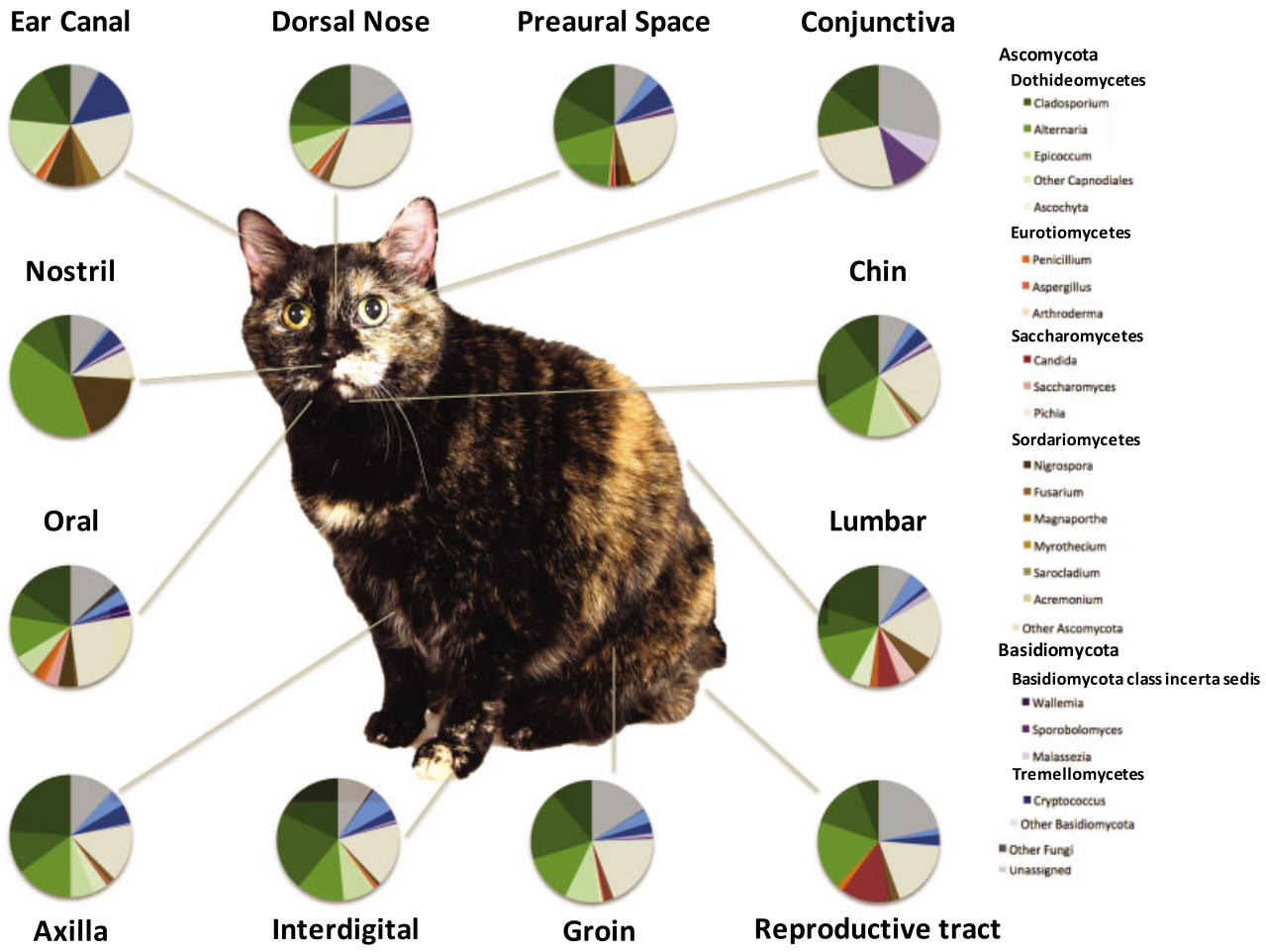

Figure 3. Fungal taxonomic composition of healthy cat body sites. The relative abundances of the most common taxa were averaged by body site and are represented by pie charts.

sites and eight taxa were different between skin type. The relative abundance of the three most abundant fungal genera on the skin of healthy cats, *Cladosporium*, *Alternaria* and *Epicoccum*, were significantly different between cats (Table S3). LEfSe analysis did not identify any significant differences in fungal taxa between healthy cats or body site.

Skin fungal diversity analyses of allergic cats

Alpha diversity was estimated for allergic samples with the Chao1, Observed OTUs (fungal richness) and Shannon (fungal diversity) metrics, and all median values are reported in Table S4. No significant differences in fungal richness or diversity between allergic cats, nor between allergic body sites (Figure S2) were identified with Kruskal–Wallis tests. Similar to healthy cats, allergic cats possessed reduced fungal diversity at mucosal sites (conjunctiva, nostril and reproductive; Figure S2; Kruskal–Wallis, $P < 0.05$). No differences in fungal richness nor diversity were identified between allergic cats that had received or were currently receiving steroids and allergic cats that had never received steroids (Figure S2).

The beta diversity of allergic cat samples ($n = 43$) were calculated using the weighted Jaccard, Bray Curtis and Pearson metrics, to determine if there were any differences between cats, body sites, skin type and steroid usage. PCoA plots revealed some clustering of sites by cat (Figure S3; ANOSIM, $R = 0.324$, $P = 0.001$) but no clustering by body site. Although the ANOSIM R statistic was low for steroid usage ($R = 0.100$, $P = 0.020$), sample clustering was visually apparent in the PCoA plot of Bray Curtis pairwise distances between allergic cat samples. Skin type did not have a major effect on differences in beta diversity between allergic samples (Figure S3; ANOSIM, $R = 0.208$, $P = 0.047$). ANOSIM performed on the Bray Curtis distance matrix for allergic cat samples revealed that the beta diversities of six pairs of cats were significantly different, with an average R value of 0.370 and FDR adjusted P-values of 0.041 (Table S2). No pairwise comparisons of allergic body sites were significantly different for any beta diversity metric.

Skin fungal taxonomic composition of allergic cats

The most abundant fungal phylum sequenced from the skin of allergic cats was Ascomycota, accounting for 77% of all sequences, and the most abundant class within this phylum was Dothideomycetes, accounting for 34% of sequences (Figures 3 and 4). The three most abundant Ascomycete genera were *Cladosporium, Alternaria* and *Nigrospora.* The most abundant Basidiomycete genus was *Cryptococcus. Malassezia* was sequenced from 21% of allergic cat samples ($n = 8$) but was present at greater

than 1% relative abundance in only one sample (Figure S1).

Kruskal–Wallis tests identified six taxa that were differentially abundant between allergic cats, but no taxa were identified as significantly different between body sites (Table S5). Two of the genera that were significantly different between cats were *Arthroderma* (sexual stage of *Microsporum,* causative agent for dermatophytosis) and *Fusarium* (Figure S4). *Arthroderma* and *Fusarium* were more abundant on C18 compared to other cats. These results were further corroborated in LEfSe analysis that revealed *Fusarium* as a taxon significantly more abundant on C18 compared to all other cats (Figure S5; LDA score of 5). LEfSe analysis also showed that an unclassified *Tremellales* genus, phylum basidiomycete, was more abundant on the dorsum of allergic cats than on other body sites of allergic cats (Figure S5; LDA score of 4.5).

Comparison of skin-associated fungi between healthy and allergic cats

For the comparison of fungi colonizing the skin of healthy cats to that of allergic cats, only the six shared sites (axilla, dorsum, ear canal, groin, interdigital space and nostril) were included in the following analyses. For these sites, the estimated alpha diversities were not significantly different between the two groups (Figure S6 and Table S6) and neither were the estimated beta diversities influenced by health status overall (Table S7). However, the Jaccard pairwise comparisons at two sites were significantly affected by health status: axilla (ANOSIM, $R = 0.378$, FDR adjusted $P = 0.03$) and interdigital space (ANOSIM, $R = 0.255$, FDR adjusted $P = 0.036$). Clustering by health status can be observed for most samples at these two sites in PCoA plots of the Jaccard pairwise distances (Figure 5).

The Kruskal–Wallis tests revealed that nine taxa were significantly different between groups including the genus *Epicoccum* and nonclassified Capnodiales order (Table S8), which were also identified as significantly more abundant in the healthy group by LEfSe analysis (Figure 6; LDA score of 4 to 5). The classes Agaricomycetes and Sordariomycetes were also identified as significantly different between groups (Table S8) and LEfSe analysis showed these classes to be significantly more abundant in the allergic group (Figure 6; LDA score of −3 to −4). Figure 3 visually demonstrates differences in averages of fungal taxa between healthy and allergic groups at each of the six sites common to the two groups.

Discussion

This study has demonstrated that fungi colonizing the skin of cats tend to be similar across the entire body of the cat,

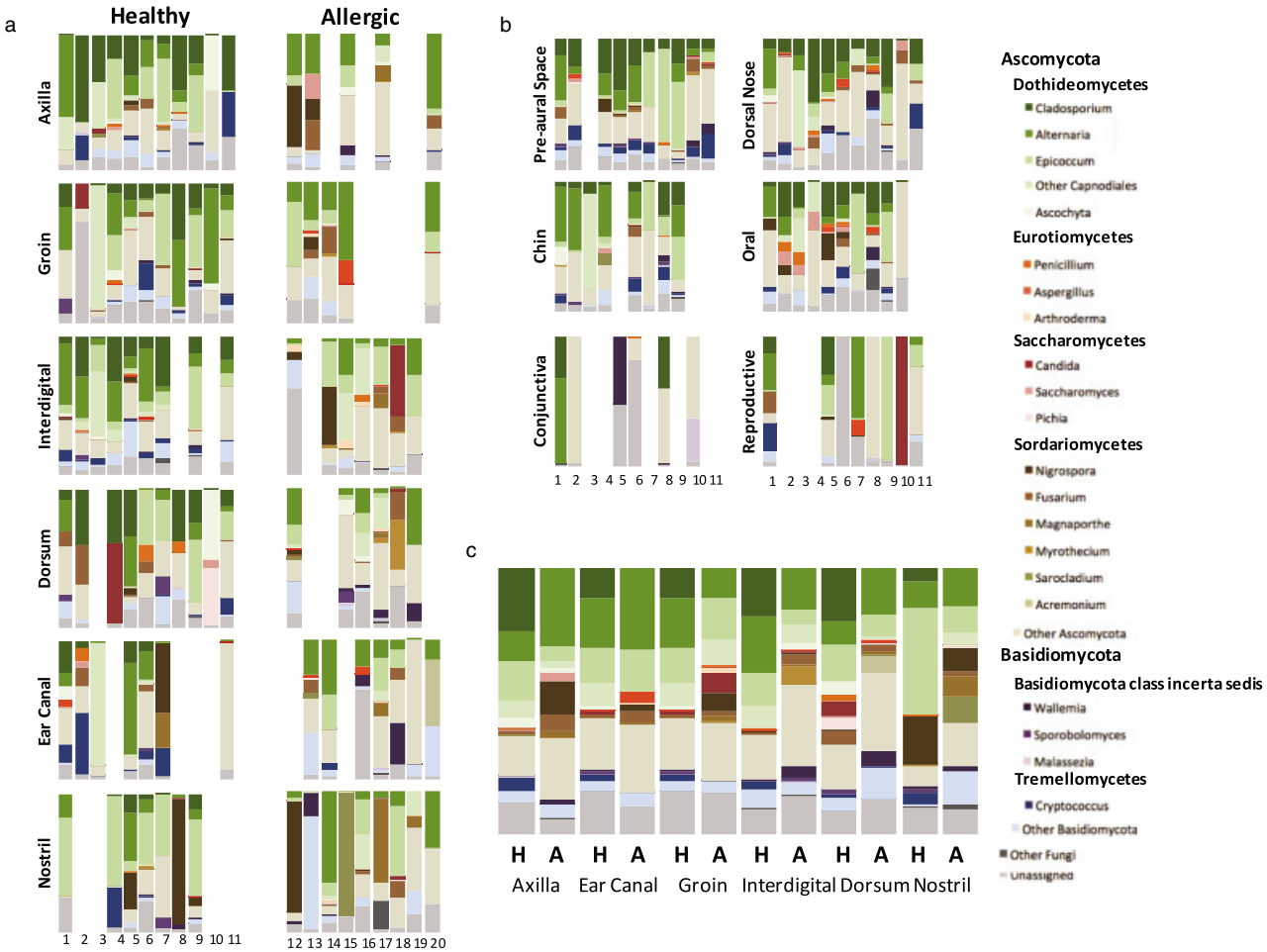

Figure 4. Fungal taxonomic composition of healthy and allergic feline skin. (a–b) Relative abundance of fungal taxa are presented for each sample and coloured by fungal genus. (c) Comparison of most abundant fungal taxa between healthy and allergic skin, averaged for each of six sites.

with differences observed between cats. It is possible that the grooming habits of cats may influence the dissemination of mycobiota across the entire body. This study also identified reduced diversity at mucosal sites and a predominance of Dothideomycetes (*Cladosporium, Alternaria* and *Epiccocum*) similar to what has been reported for canine skin.[6] Although it is not possible to compare the results of two NGS studies quantitatively, the qualitative diversity of fungi sequenced from feline skin appears to be comparable to that of canine skin[6] and much more diverse than what has been found on the human body (with the exception of pedal sites[4]). A previous study suggested that outdoor exposure might explain the predominance of environmental fungi sequenced from the skin of dogs;[6] however, the same taxa of fungi were also abundant on these cats, many of which (13 of 20) were housed strictly indoors. Further studies are warranted to evaluate how outdoor exposure might influence the carriage of fungi on the skin of companion animals.

Aside from the influences on diversity of fungi inhabiting the skin of people and animals, many questions remain regarding the temporal stability of these fungi on animal skin. One of the cats in this study was diagnosed with dermatophytosis a few months prior to collection of samples. The skin lesions in this cat resolved with application of lime sulfur dips and no clinical signs were observed at the time of sample collection. Statistical analysis of the relative abundances of fungi sequenced from the skin of

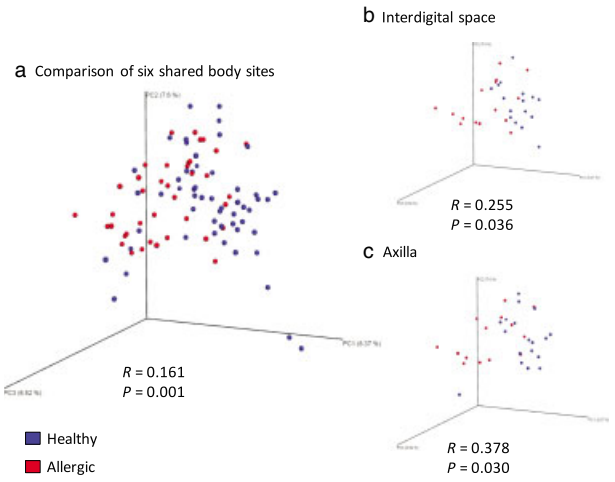

Figure 5. Comparison of beta diversity between healthy and allergic skin. PCoA plots of Jaccard pairwise distances for healthy and allergic feline skin samples, with associated ANOSIM global *R* statistic and associated *P*-value for (a) six sites, (b) only the interdigital spaces and (c) only the axillae. Coloured by health status.

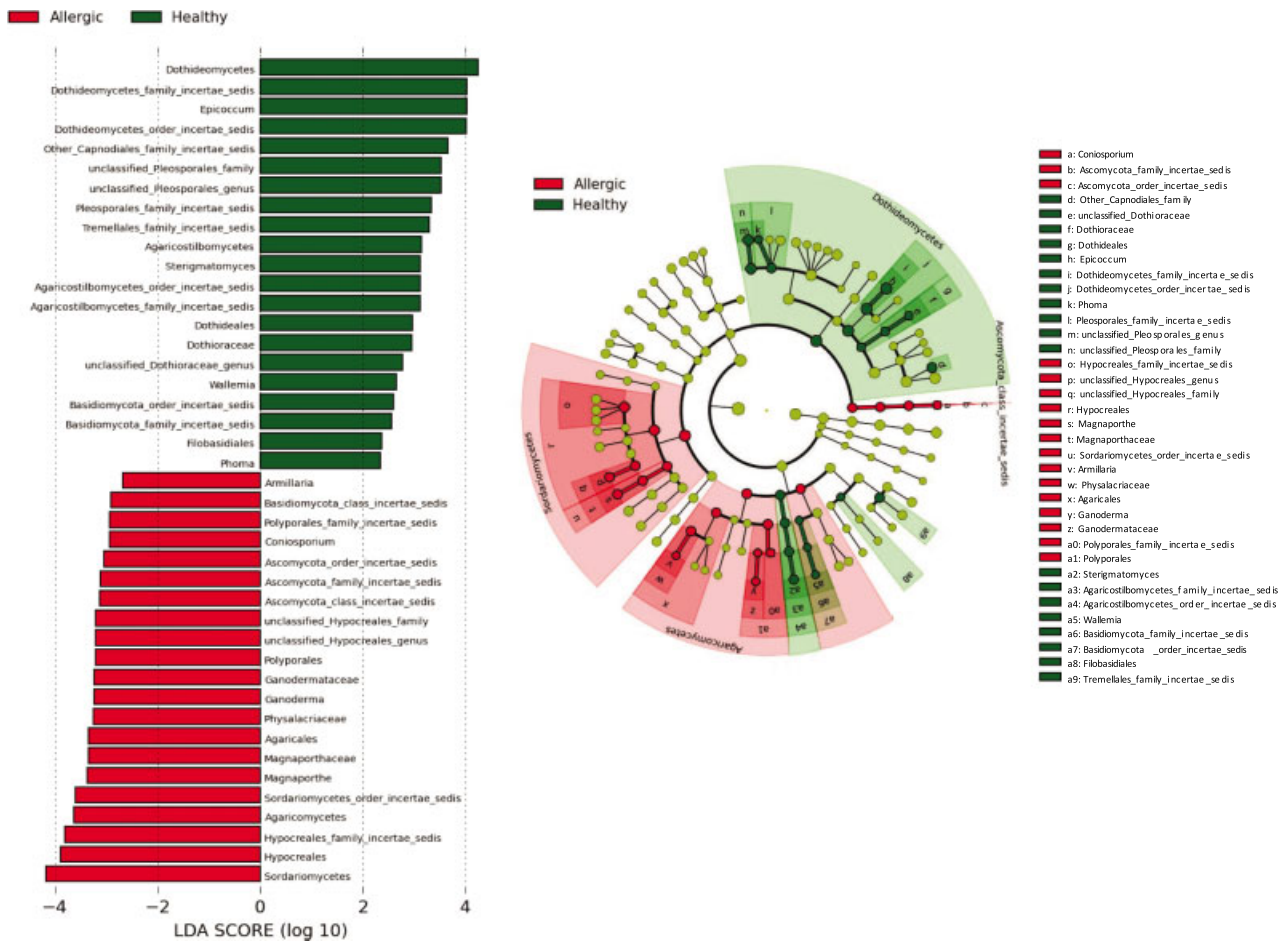

Figure 6. Linear discriminant analysis (LDA) effect size (LEfSe) analysis of healthy and allergic cats. Fungal taxa that are significantly increased or decreased in healthy or allergic skin are presented in two forms: as bar blots showing the LDA score and as a cladogram demonstrated the phylogenetic relationships. Taxa are coloured according to the health status group in which they are increased in abundance.

this cat compared to the skin of other cats revealed significantly higher amounts of the fungus *Arthroderma*, which is the sexual stage of *Microsporum*, one of the causative agents of dermatophytosis. Although this finding was isolated to one cat, the clinical history of this case suggests the possibility that dermatophytosis could have a long-standing effect on the skin mycobiota across the entire cat. This finding also raises continued concern regarding a potential carrier state for dermatophytosis in cats[29] and demonstrates the ability of NGS to detect this state in the absence of clinical signs. Additional studies including increased numbers of animals would certainly be required in order to confirm long-term alterations to the cutaneous fungal microbiota and a carrier state following resolution of lesions. Interestingly, this cat (C18) also had a significant increase in *Fusarium* DNA across all of its body sites, compared to other cats. The potential relationship between colonization of *Fusarium* and *Arthroderma* may be of interest for future studies.

Malassezia has been implicated as a significant allergen in human and canine AD,[30–32] whereas it has yet to be associated with feline HD. Several studies have cultured *Malassezia* spp. from the skin of healthy cats[9,33] and cats with otitis.[8,10,11,14] In one of these studies, *Malassezia* was cultured from approximately 40% of healthy cats.[8] In the present study, *Malassezia* DNA was sequenced from around 30% of healthy cat samples, but at a low abundance relative to all fungi sequenced. There also have been documented breed differences in the type and amount of *Malassezia* colonization of feline skin; in a study including 73 cats, *Malassezia* was isolated from 90% of Devon Rex cats, 39% of Cornish Rex cats and 50% of domestic short hair cats.[33] Another study identified an overgrowth of *Malassezia* spp. from the skin of allergic cats using cytological examination of tape strips.[13] We were not able to replicate these findings in the current study; *Malassezia* was sequenced from 21% of allergic cats and no significant difference in abundance of *Malassezia* was identified between groups. A previous NGS study of healthy and allergic canine skin also reported an unexpectedly low abundance of *Malassezia*.[6] Future studies including additional methodologies may be required to confirm the relative abundances of *Malassezia* spp. on the skin of companion animals and whether there exists any increased relative abundances of *Malassezia* on the skin of allergic animals.

The allergic cats enrolled in this study were diagnosed with a range of HD lesions. Lesion distributions varied amongst study participants, but in accordance with typical cutaneous reaction patterns associated with these types of HD. However, there were still some significant changes to the mycobiota of their skin as a group, namely the increase or decrease of particular fungal taxa. Fungal dysbiosis has also been identified in both canine and human AD,[6,18] and fungal richness and diversity have differed between species (increased diversity in human AD patients and reduced richness in allergic dogs). Unlike in dogs with allergic dermatitis,[6] there was not an overall reduction in fungal diversity in the allergic cat group. Some factors that might explain this finding include the differences in distribution and phenotypic presentation of lesions between canine AD and NFNFIHD in cats,[34] or differences in immune regulation of the skin in these two species. Another possible explanation could be a lack of skin barrier impairment in allergic cats, as is often described in atopic dogs[35] and people.[36] There have yet to be any studies to provide evidence for or against impairment of the skin barrier in allergic cats, nor have there been any studies comparing transepidermal water loss between healthy and allergic cats.

A complex dialogue between skin microbiota and host immune systems is known to occur.[37,38] For instance, the host commensal microbiota is capable of inducing expression of antimicrobial peptides,[39] which can then alter or modulate the presence and abundance of certain skin microbes. There is still debate as to whether the microbial dysbiosis observed in inflammatory skin disorders is a cause or effect of immune dysfunction. Regardless, microbial dysbiosis identified in canine allergic dermatitis and the results of this study in allergic cats suggest that there is some alteration to this dialogue between host and commensal microbiota in allergic dermatitis of companion animals.

In summary, NGS performed on skin swab samples of healthy and allergic feline skin identified a diverse mycobiota with a predominance of environmental fungi such as *Cladosporium* and *Alternaria*. These findings correlate well with what has been shown through culture-dependent studies of feline skin[7,8,12,15,16] and NGS studies of canine skin.[6] Further studies with larger numbers of animals are needed to confirm the present findings, and to evaluate the role of the environment on the skin microbiota. Investigation into the immune regulation of feline skin, and pathogenesis of feline NFNFIHD might help to explain the differences identified in this study compared to that of allergic dogs.

Acknowledgements

We would like to acknowledge Amanda Friedeck from Texas A&M Small Animal Hospital for assisting with skin swab collections, Kim Wahl for assistance with the DNA extractions and Tim Stephens for the photograph of Fleabag, the cat depicted in Figure 4 (both from the College of Veterinary Medicine and Biomedical Sciences at Texas A&M). The owners of the cats enrolled in this study are thanked for their time and willingness to collaborate with this study.

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Supporting Information

Additional Supporting Information may be found in the online version of this text at: http://onlinelibrary.wiley.com/doi/10.1111/vde.12373/full

Figure S1. Relative abundance of *Malassezia* in healthy and allergic feline skin samples. The relative abundance of *Malassezia* is plotted for each skin sample from healthy and allergic cats. A axilla, C chin, CJ conjunctiva, DN dorsal nose, D dorsum, EC ear canal, G groin, ID interdigital space, N nostril, O oral, PAS preaural space, R reproductive tract.

Figure S2. Alpha diversity of allergic cats. Alpha diversity estimated with Shannon diversity metric and samples grouped by (a) cat, (b) body site, (c) skin type and (d) steroid usage. Means and mean error bars are plotted in blue for each group. Groups with a mean significantly different from other means are denoted by asterisks, with associated *P*-values (Steel–Dwass multiple comparisons test, of *<0.05, **<0.01, ***<0.001.

Figure S3. Beta diversity of allergic cats. PCoA plot of Bray Curtis pairwise distances for healthy cat samples, with associated ANOSIM global *R* statistic and *P*-value; coloured by (a) cat, (b) body site, (c) steroids usage and (d) skin type.

Figure S4. Relative abundance of *Arthroderma* and *Fusarium* in allergic feline skin samples. The relative abundance of (a) *Arthroderma* and (b) *Fusarium* is plotted for each skin sample from allergic cats.

Figure S5. LDA effect size (LEfSe) analysis of allergic cats. Fungal taxa that are significantly increased or decreased in allergic (a–b) cats or (c–d) body sites are presented in two forms: as bar plots showing the LDA score and as a cladogram demonstrating the phylogenetic relationships. Taxa are coloured according to cat or body site in which they are increased in abundance.

Figure S6. Comparison of alpha diversity between healthy and allergic feline skin for six sites. Alpha diversity estimated with Shannon diversity metric and samples grouped by (a) body site and health status, and (b) health status only. Means and mean error bars are plotted in blue for each group. Means were not significantly different for any group.

Table S1. Alpha diversity median for healthy cats. The median alpha diversity was calculated for each body site, cat and skin type in the group of healthy cats using the three metrics: Chao1, observed OTUs and Shannon.

Table S2. Average *R* statistic and range of *P*-values for significant pairwise comparisons. The average *R* statistic and *P*-values were reported for only the significant ($P < 0.05$) pairwise comparisons between cats and between skin type groups. All *P*-values were adjusted for multiple comparisons.

Table S3. Fungal taxa from filtered relative abundance table for healthy cat samples. The average relative abundances of fungal taxa sequenced from healthy cats are reported. Results from testing that the relative abundance of each taxon was significantly different for at least one body site or cat are included on respective rows.

Table S4. Alpha diversity median for allergic cats. The median alpha diversity was calculated for each body site, cat, skin type and steroid usage in the group of allergic cats using the three metrics: Chao1, observed OTUs and Shannon.

Table S5. Fungal taxa from filtered relative abundance table for allergic cat samples. The average relative abundances of fungal taxa sequenced from allergic cats are reported. Results from testing that the relative abundance of each taxon was significantly different for at least one body site or cat are included on respective rows.

Table S6. Alpha diversity median for health status. The median alpha diversity was calculated for health status group using the three metrics: Chao1, observed OTUs and Shannon.

Table S7. Global *R* statistics for beta diversity analysis for healthy and allergic cats. Beta diversity of samples was calculated using Jaccard, Bray Curtis and Pearson metrics. ANOSIM was performed on all three metrics to determine significant differences in fungal communities between healthy and allergic cats using the factors Cat, Body Site and Skin Type. The global *R* value is representative of all members within a factor and is distinct from previously reported pairwise comparisons.

Table S8. Fungal taxa from filtered relative abundance table for shared skin sites for healthy and allergic cats. The average relative abundances of fungal taxa sequenced from only the sites that were sampled in both healthy and allergic cats are reported. Results from testing that the relative abundance of each taxon was significantly different for at least one body site or cat are included on respective rows.

Part 5

Infectious Diseases

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5.1

Bacterial resistance to antimicrobial agents and its impact on veterinary and human medicine

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Background – Antimicrobial resistance has become a major challenge in veterinary medicine, particularly in the context of bacterial pathogens that play a role in both humans and animals.

Objectives – This review serves as an update on acquired resistance mechanisms in bacterial pathogens of human and animal origin, including examples of transfer of resistant pathogens between hosts and of resistance genes between bacteria.

Results – Acquired resistance is based on resistance-mediating mutations or on mobile resistance genes. Although mutations are transferred vertically, mobile resistance genes are also transferred horizontally (by transformation, transduction or conjugation/mobilization), contributing to the dissemination of resistance. Mobile genes specifying any of the three major resistance mechanisms – enzymatic inactivation, reduced intracellular accumulation or modification of the cellular target sites – have been found in a variety of bacteria that may be isolated from animals. Such resistance genes are associated with plasmids, transposons, gene cassettes, integrative and conjugative elements or other mobile elements. Bacteria, including zoonotic pathogens, can be exchanged between animals and humans mainly via direct contact, but also via dust, aerosols or foods. Proof of the direction of transfer of resistant bacteria can be difficult and depends on the location of resistance genes or mutations in the chromosomal DNA or on a mobile element.

Conclusion – The wide variety in resistance and resistance transfer mechanisms will continue to ensure the success of bacterial pathogens in the future. Our strategies to counteract resistance and preserve the efficacy of antimicrobial agents need to be equally diverse and resourceful.

Introduction

Antimicrobial agents are used extensively in aquaculture, horticulture, and to treat bacterial infections in humans and animals. Due to this extensive use, antimicrobial resistance has become a significant problem in both human and veterinary medicine, mediated by a multitude of mechanisms.[1,2] Although the presence of resistance genes in bacteria is not a new phenomenon – as recently highlighted in a study describing resistance genes in bacterial DNA from permafrost soil samples[3] – what is new is the selective pressure exerted on bacterial pathogens through antibacterial use. Since the 1950s, the selective pressure imposed on bacteria by the use of antimicrobial agents for various clinical and nonclinical purposes has increased dramatically. As a consequence, bacteria have developed and refined various ways and means to resist or escape the inhibitory effects of the antimicrobial agents.[1,2] In addition, certain bacterial pathogens have managed to accumulate or develop resistances to multiple classes of antimicrobial agents at the same time. Such multidrug-resistant, extensively resistant or even pandrug resistant pathogens[4] typically succeed in human and veterinary healthcare establishments or in patients repeatedly requiring antibacterial therapy. Risk groups include dogs with recurrent pyoderma. Such patterns of resistance may seriously compromise the prognosis of infected patients. As a result, for the first time in decades, the prognosis for patients with infections caused by multidrug-resistant bacteria has been seriously compromised by the lack of effective antimicrobial agents. This development has threatened the advancement of modern medicine.[5]

Antimicrobial resistance

A bacterium is defined as being clinically resistant to an antimicrobial agent when the drug – after recommended dosing – does not reach a concentration at the site of infection that is able to effectively inhibit the growth of the bacterium or to kill it.[6] This definition takes into account the pharmacological parameters relevant for systemic therapy of the antimicrobial agent in the patient species concerned. It also considers the minimum inhibitory concentration (MIC) of the causative bacteria to the

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antimicrobial agent applied. These factors, along with the results of clinical efficacy studies, play key roles in the definition of clinical breakpoints.[6] Such clinical breakpoints are available for humans and various animal species as recommended by the Clinical and Laboratory Standards Institute (CLSI) and usually are applicable for a specific combination of host species/target bacterium/antimicrobial agent/disease condition, such as dog/*Staphylococcus* spp./tetracycline/skin and soft tissue infections.[7,8] In general, these breakpoints were derived from microbiological, pharmacokinetic (using accepted clinical doses) and pharmacodynamic data. In the veterinary field, clinical breakpoints applicable for bacteria involved in skin and soft tissue infections are available for the canine, feline and equine bacteria shown in Table 1.

In general, antimicrobial resistance in bacteria can be either intrinsic or acquired. Intrinsic resistance is a bacterial genus- or species-specific characteristic and is often based on either the absence or inaccessibility of the target structures in the respective bacteria,[1] for example, resistance to β-lactam antibiotics and glycopeptides in cell wall-free bacteria such as *Mycoplasma* spp. or vancomycin resistance in Gram-negative bacteria due to the inability of vancomycin to penetrate the outer membrane. It can also be due to the presence of export systems or the production of species-specific inactivating enzymes in certain bacteria,[1] such as the AcrAB-TolC system and the production of AmpC β-lactamase in *Escherichia coli*. In addition, some bacteria, such as enterococci, are not dependent on a functional folate synthesis pathway, but instead can use exogenous folates. As a consequence, they are intrinsically resistant to folate pathway inhibitors, such as trimethoprim and sulfonamides.[9] In contrast, acquired resistance is a strain-specific property which can be based on a wide variety of resistance mechanisms present in the different bacteria.[1] Such acquired resistance mechanisms can be due to mutations of cellular genes or to the acquisition of novel/foreign genes, commonly referred to as resistance genes. The following basic considerations are important in the context of acquired resistance genes:

1. Acquired resistance genes can confer resistance to an entire class of antimicrobial agents or can be specific for only a single member of an antimicrobial class.
2. Certain acquired resistance genes can confer resistance to members of different classes of antimicrobial agents.
3. Acquired resistance to a specific class of antimicrobial agents can be due to several different resistance mechanisms.

Table 1. Clinical and Laboratory Standards Institute-approved veterinary-specific clinical breakpoints available for skin, soft tissue and wound infections in dogs, cats and horses[8]

Animal species	Target bacteria	Antimicrobial agent	Clinical breakpoints (mg/L)*		
			S	I	R
Dog	*Escherichia coli*	Ampicillin	≤0.25	0.5	≥1
	Staphylococcus pseudintermedius	Ampicillin	≤0.25	—	≥0.5
	Streptococcus spp., *Streptococcus canis* (group G, β-haemolytic group)	Ampicillin	≤0.25	—	—
	E. coli, *Staphylococcus* spp.	Amoxicillin-clavulanate	≤0.25/0.12	0.5/0.25	≥1/0.5
	E. coli,† *S. aureus*, *S. pseudintermedius*, *Streptococcus* spp. (β-haemolytic group)	Cephalothin	≤2	4	≥8
	E. coli, *Pasteurella multocida*, *S. aureus*, *S. pseudintermedius*, *Streptococcus* spp. (β-haemolytic group)	Cefazolin	≤2	4	≥8
	E. coli, *Streptococcus* spp. (β-haemolytic group)	Cefalexin‡	≤2	4	≥8
	S. aureus, *S. pseudintermedius*	Cefalexin‡	≤2	—	≥4
	E. coli, *Proteus mirabilis*, *P. multocida*, *S. aureus*, *S. pseudintermedius*, *S. canis* (group G, β-haemolytic group)	Cefpodoxime	≤2	4	≥8
	Enterobacteriaceae, *Staphylococcus* spp., *Streptococcus* spp.	Difloxacin	≤0.5	1–2	≥4
	Enterobacteriaceae, *Staphylococcus* spp., *Streptococcus* spp.	Enrofloxacin	≤0.5	1–2	≥4
	Enterobacteriaceae, *Staphylococcus* spp., *Streptococcus* spp.	Marbofloxacin	≤1	2	≥4
	Enterobacteriaceae, *Staphylococcus* spp., *Streptococcus* spp.	Orbifloxacin	≤1	2–4	≥8
	E. coli, *S. pseudintermedius*	Pradofloxacin	≤0.25	0.5–1	≥2
	Staphylococcus spp., *Streptococcus* spp. (β-haemolytic group)	Clindamycin	≤0.5	1–2	≥4
	S. pseudintermedius	Doxycycline	≤0.12	0.25	≥0.5
	Staphylococcus spp.	Tetracycline	≤0.25	0.5	≥1
Cats	*E. coli*, *Staphylococcus* spp., *Streptococcus* spp.	Amoxicillin-clavulanate	≤0.25/0.12	0.5/0.25	≥1/0.5
	Enterobacteriaceae, *Pseudomonas aeruginosa*, *Staphylococcus* spp., *Streptococcus* spp.	Enrofloxacin	≤0.5	1–2	≥4
	Enterobacteriaceae, *Staphylococcus* spp., *Streptococcus* spp.	Marbofloxacin	≤1	2	≥4
	Enterobacteriaceae, *Staphylococcus* spp., *Streptococcus* spp.	Orbifloxacin	≤1	2–4	≥8
	E. coli, *S. aureus*, *S. pseudintermedius*, *Staphylococcus felis*	Pradofloxacin	≤0.25	0.5–1	≥2
	P. multocida, *S. canis*	Pradofloxacin	≤0.25	—	—
Horse	*Staphylococcus* spp., *Streptococcus* spp.	Penicillin G	≤0.5	1	≥2

*S susceptible, I intermediate, R resistant.

†During the June 2015 meeting of the Veterinary Antimicrobial Susceptibility Testing subcommittee of the Clinical and Laboratory Standards Institute (CLSI), the following statement was approved "Cephalothin is used to predict results for all first-generation cephalosporins except cefazolin and cefalexin."

‡These cefalexin breakpoints were approved by the Veterinary Antimicrobial Susceptibility Testing subcommittee of the CLSI (June 2015); however, they are not considered final until published in the next edition of the VET01 supplement.

4 The same acquired resistance mechanism can be encoded by different genes.
5 Different acquired resistance mechanisms and resistance genes can be present at the same time.
6 Definitions of multidrug-resistance vary but a bacterium is typically referred to as multidrug-resistant if it shows acquired resistance to members of at least three classes of antimicrobial agents.

Resistance mechanisms and associated resistance genes

Acquired resistance mechanisms can be divided into one of the three major categories: (i) enzymatic modification or inactivation of antimicrobial agents, (ii) reduced intracellular accumulation of antimicrobial agents or (iii) alterations at the target sites of the antimicrobial agents.[1,2]

Enzymatic modification or inactivation of antimicrobial agents is widespread among Gram-positive and Gram-negative bacteria (Table 2). In the case of enzymatic modification, bacteria produce enzymes that chemically modify the drug molecule by the attachment of acetyl, adenyl or phosphate groups to specific sites of the antimicrobial molecule. Such modified antimicrobial molecules can no longer bind to their target site and consequently cannot maintain antimicrobial activity. This mechanism is commonly used for the enzymatic inactivation of nonfluorinated phenicols, such as chloramphenicol, by acetylation,[10] or of aminoglycosides by acetylation, adenylation or phosphorylation.[11] Other enzymatic inactivation processes include the phosphorylation of macrolides, nucleotidylation of lincosamides, and acetylation of streptogramin A antibiotics.

In the case of enzymatic inactivation, bacteria produce enzymes that bind directly to the antimicrobial molecule and disintegrate it. This is commonly done by hydrolytic cleavage of specific bonds within the antimicrobial molecule. Such cleaved antimicrobial molecules also do not exhibit antimicrobial activity. Examples of this mode of enzymatic inactivation are the β-lactamases, which occur in Gram-positive and Gram-negative bacteria and, depending on the type of β-lactamase, may exhibit a more or less expanded substrate spectrum that can include penicillins, cephalosporins, monobactams and/or even carbapenems.[12,13] Other examples are esterases which confer macrolide resistance or lactone hydrolases which inactivate streptogramin B compounds.[14]

Reduced intracellular accumulation of antimicrobial agents can be achieved in two ways: reduced influx or enhanced efflux (Table 3). It is known that certain outer membrane proteins (OMPs), so-called porins, represent an entry point for antimicrobial agents to enter the bacterial cell. As such, OmpF is involved in the uptake of tetracyclines, β-lactams and chloramphenicol in *E. coli*, whereas OmpD is involved in the uptake of carbapenems in *Pseudomonas aeruginosa*.[1] Reduced influx of antimicrobial agents is usually the consequence of downregulation, structural modification or even functional deletion of the genes coding for these porins. In such cases, the outer membrane of Gram-negative bacteria can represent a permeability barrier for antimicrobial agents.

By contrast, increased efflux describes a way by which incoming antimicrobial agents are actively pumped out of the bacterial cell. This can be achieved by multidrug transporters or specific transporters.[1,2] Multidrug transporters are present in virtually every bacterium and are mainly responsible for the transport of toxic substances from the cell metabolism. However, studies have shown that some multidrug transporters can also export antimicrobial agents. Most of them belong to the resistance-nodulation-cell division (RND) family. RND transporters mainly occur in Gram-negative bacteria and are composed of a cytoplasmatic and a periplasmatic component which can interact with different outer membrane components. Examples are AcrAB-TolC transporter in *E. coli* and *Salmonella enterica* or the MexAB-OprM transporter in *P. aeruginosa* which can export chloramphenicol,

Table 2. Examples of resistance to antimicrobials by enzymatic modification or inactivation (modified from[1])

Resistance mechanism	Resistance gene(s)	Gene product	Resistance phenotype	Bacteria involved	Location of the resistance gene
Chemical modification	*aac*, *aad* (*ant*), *aph*	Acetyl-, adenyl-, phosphotransferases	Aminoglycosides	Various Gram+, Gram– aerobic bacteria	T, GC, P, C
	aad (*ant*)	Adenyltransferases	Aminocyclitols	Various Gram+, Gram– aerobic bacteria	T, GC, P, C
	catA, *catB*	Acetyltransferases	Chloramphenicol	Various Gram+, Gram– aerobic, anaerobic bacteria	P, T, GC, C
	vat(A-E)	Acetyltransferases	Streptogramin A	*Staphylococcus, Enterococcus*	P, C
	mph(A-E)	Phosphotransferases	Macrolides	*Escherichia*, *Shigella*, *Staphylococcus*	P, T, C
	lnu(A), *lnu*(B)	Nucleotidyltransferases	Lincosamides	*Staphylococcus*	P
	tet(X), *tet*(37)	Oxidoreductases	Tetracyclines	*Bacteroides*	T, P
Hydrolytic cleavage	*blaZ*, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, etc.	β-Lactamases	β-Lactam antibiotics	Various Gram+, Gram–aerobic, anaerobic bacteria	P, T, GC, C
	ere(A), *ere*(B)	Esterase	Macrolides	*E. coli*, *Staphylococcus*	P, GC
	vgb(A), *vgb*(B)	Lactone hydrolases	Streptogramin B	*Staphylococcus*	P

P plasmid, T transposon, GC gene cassette, C chromosomal DNA.

fluoroquinolones, tetracyclines, β-lactams and macrolides among others.[1,15] It should be noted that multidrug-transporters increase the MICs for their substrates, but not necessarily to a level that correlates with clinical resistance.

Specific transporters involved in antimicrobial resistance commonly belong to the following families: (i) major facilitator superfamily (MFS), (ii) ATP-binding cassette (ABC) family or (iii) multidrug and toxic-compound extrusion (MATE) family.[15,16] MFS transporters often consist of 12–14 transmembrane segments, exchange a drug molecule against a proton and use the proton-motive force of the membrane as an energy source for the translocation. Examples of MFS transporters are the tetracycline transporters Tet(K) and Tet(L) in Gram-positive bacteria and Tet (A-E, G, H) in Gram-negative bacteria as well as the phenicol transporters FexA in Gram-positive bacteria and FloR, CmlA and CmlB in Gram-negative bacteria.[17,18] ABC transporters use the energy of ATP hydrolysis for the translocation of substrates across biological membranes. They represent a highly diverse class of transporters which are not only involved in antimicrobial resistance, but also in the uptake of nutrients and the secretion of proteins among other functions.[19] ABC transporters involved in antimicrobial resistance are seen mainly in staphylococci and enterococci. Examples are the transporters Vga(A), Vga(C), Vga(E), Lsa(E) and Sal(A) conferring combined resistance to lincosamides, pleuromutilins and streptogramin A antibiotics or Msr(A) involved in resistance to macrolides and streptogramin B antibiotics.[20,21] MATE proteins are also located in the cytoplasmatic membrane and act in a similar way to MFS transporters. However, in contrast to MFS proteins, they are rarely involved in antimicrobial resistance. Examples of MATE proteins that export antimicrobial agents are NorM (hydrophilic fluoroquinolones) from *Vibrio parahaemolyticus* and MepA (glycylcyclines) from *Staphylococcus aureus*.[15,16]

Alterations at the target sites of the antimicrobial agents represent the third and most variable group of resistance mechanisms (Table 4). These include mutational and chemical modifications, protection of the target sites, the replacement of sensitive targets by functionally analogous but insensitive ones, and overproduction of sensitive targets.[22]

Mutational alterations of the target sites are best known for (fluoro)quinolone resistance in various Gram-positive and Gram-negative bacteria. Within the genes for DNA gyrase (topoisomerase II and topoisomerase IV), a specific region known as the quinolone-resistance

Table 3. Examples of resistance to antimicrobials by decreased intracellular drug accumulation (modified from[1])

Resistance mechanism	Resistance gene(s)	Gene product	Resistance phenotype	Bacteria involved	Location of the resistance gene
Efflux via multidrug transporters	*mexA-mexB-oprM*, *acrA-acrB-tolC*	Multidrug efflux in combination with specific OMP's	Chloramphenicol, β-lactams, macrolides, fluoroquinolones, tetracyclines	*Pseudomonas*, *E. coli*, *Salmonella*	C
	emrE	4-TMS multidrug efflux protein	Tetracyclines, nucleic acid binding compounds	*E. coli*	C
	blt, *norA*	12-TMS multidrug efflux protein of the major facilitator superfamily	Chloramphenicol, fluoroquinolones, nucleic acid binding compounds	*Bacillus, Staphylococcus*	C
Efflux via specific transporters	*tet*(A-E, G, H, I, J, K, L, Z), *tetA*(P), *tet*(30)	12-, 14-TMS efflux system of the major facilitator superfamily	Tetracyclines	Various Gram+ and Gram– bacteria	P, T, C
	floR	12-TMS efflux system of the major facilitator superfamily	Phenicols	Various Gram– bacteria	T, P, C
	cmlA, *cmlB*	12-TMS efflux system of the major facilitator superfamily	Chloramphenicol	Various Gram– bacteria	T, P, GC, C
	fexA	14-TMS efflux system of the major facilitator superfamily	Phenicols	*Staphylococcus*	T, P, C
	mef(A)	Efflux system of the major facilitator superfamily	14-, 15-Membered macrolides	*Streptococcus*, other Gram+ bacteria	T, P, C
	msr(A)	Efflux system of the ABC transporter family	Macrolides and streptogramin B	*Staphylococcus*	P
	vga(A), *vga*(C), *vga*(E), *lsa*(E), *sal*(A)	Efflux system of the ABC transporter family	Pleuromutilins lincosamides, streptogramin A	*Staphylococcus*, *Enterococcus*	P
	optrA	Efflux system of the ABC transporter family	Phenicols, linezolid, tedizolid	*Enterococcus, Staphylococcus*	P, C

P plasmid, T transposon,GC gene cassette, C chromosomal DNA, TMS transmembrane segments.

Table 4. Examples of resistance to antimicrobials by target site alteration (modified from[1])

Resistance mechanism	Resistance gene(s)	Gene product	Resistance phenotype	Bacteria involved	Location of the resistance gene
Methylation of the target site	*erm*(A-46)	rRNA methylase	Macrolides, lincosamides, streptogramin B	Various Gram+ and Gram– bacteria	P, T, C
Methylation of the target site	*cfr, cfrB*	rRNA methylase	Phenicols, lincosamides, linezolid, pleuromutilins, streptogramin A	Various Gram+ and Gram– bacteria	P, C
Protection of the target site	*tet*(M, O, P, Q, S, T)	Ribosome protective proteins	Tetracyclines	Various Gram+ and Gram– bacteria	T, P, C
	fusB	Ribosome protective protein	Fusidic acid	*Staphylococcus*	P
Replacement of a sensitive target by an alternative drug-resistant target	*mecA, mecC*	Penicillin-binding proteins with altered substrate specificity	Penicillins, cephalosporins, carbapenems, monobactams	*Staphylococcus*	C
	sul1, sul2, sul3	Sulfonamide-insensitive dihydropteroate synthase	Sulfonamides	Various Gram– bacteria	P, I
	dfrA, dfrB, dfrG, dfrK	Trimethoprim-insensitive dihydrofolate reductase	Trimethoprim	Various Gram+ and Gram– bacteria	P, GC, T, C
	mupA, ileS2	Mupirocin-insensitive isoleucyl-tRNA synthase	Mupirocin	*Staphylococcus*	P
	vanA-E	Alternative peptidoglycan precursors	Glycopeptides	*Enterococcus*, *Staphylococcus*	T, P, C
Mutational modification of the target site	—	Mutations in the genes for topoisomerase II and IV	Fluoroquinolones	Various Gram+ and Gram– bacteria	C
	—	Mutation in the gene for ribosomal protein S12	Streptomycin	Several Gram+ and Gram– bacteria	C
	—	Mutation in the gene for the ribosomal protein L3	Tiamulin	*E. coli*	C
	—	Mutation in the 16S rRNA	Tetracyclines	*Propionibacterium*	C
	—	Mutations in the 23S rRNA	Oxazolidinones	*Staphylococcus*	C
	—	Mutation in the *fusA* gene	Fusidic acid	*Staphylococcus*	C
Mutational modification of regulatory elements	—	Mutations in the *marRAB soxR* or *acrR* genes	Fluoroquinolones	*E. coli*	C

P plasmid, T transposon, GC gene cassette, C chromosomal DNA, I integron.

determining region (QRDR) has been defined where mutations accounting for (fluoro)quinolone resistance are located. Resistance to (fluoro)quinolones usually occurs in a step-wise manner by which the MIC is increased with each additional mutation.[23,24] Such a step-wise increase in resistance illustrates well the advantage of using mutant prevention concentrations (MPCs) as a measure for antimicrobial potency rather than MICs.[25] Because two mutations are required for full (fluoro)quinolone resistance to occur, and with mutations occurring randomly, the likelihood that bacteria with double mutations will persist after treatment is low and measurable only in a large population of cells (i.e. in large numbers of colony forming units in the laboratory). To date, MPC measurement has not been applied routinely in clinical microbiology laboratories, possibly hampered by practical constraints.[26]

Mutations in the gene *fusA*, which encodes the elongation factor G (EF-G), have been found to account for resistance to fusidic acid in *S. aureus* as well as in meticillin-susceptible (MSSP) and meticillin-resistant *Staphylococcus pseudintermedius* (MRSP).[27,28] Mutations in 16S ribosomal RNA (rRNA) have been described to account for resistance to streptomycin in *Mycobacterium tuberculosis*, to tetracyclines in *Propionibacterium acnes* and to spectinomycin resistance in *Pasteurella multocida*.[1,29] Mutations in 23S rRNA are known to cause macrolide resistance in various bacteria including *Mycobacterium* spp., *Brachyspira hyodysenteriae*, *Campylobacter coli*, *Campylobacter jejuni*, *Haemophilus influenzae* and *Streptococcus* spp. among others.[1] In addition, mutations in the genes for specific ribosomal proteins are associated with resistance to streptomycin and spectinomycin.[1,29] Mutations in the gene *rpoB*, which codes for the β-subunit of the enzyme RNA polymerase, have been described recently to cause high-level rifampicin resistance in *Rhodococcus equi* and in MRSP.[30,31]

Chemical modification of the target site by methylation has proved to be an effective way to confer combined resistance to macrolides, lincosamides and streptogramin B antimicrobial agents. The corresponding Erm methylases, which target the adenine residue at position 2058 in 23S rRNA, are widely distributed among Gram-positive and Gram-negative bacteria.[32] To date, 46 different Erm methylases have been differentiated.[33] Methylation of the adenine residue at position 2503, which is located in the overlapping binding region of phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A antibiotics, results in resistance to these five classes of antimicrobial agents.[34] The corresponding methylase gene, *cfr*, has been detected in various *Staphylococcus* spp., *Enterococcus* spp., *Bacillus* spp., *Macrococcus caseolyticus*, *Jeotgalicoccus pinnepedialis*, *Streptococcus suis*, *E. coli* and *Proteus vulgaris*.[20,35] Recently, the gene *cfrB*, which confers the same resistance phenotype but is <80%

identical to *cfr*, has been detected in *Enterococcus* spp. and *Clostridium difficile* isolates.[17,33]

Protection of the ribosomal target site has been noted in tetracycline resistance. So far, 12 ribosome protective proteins are known which show similarities to elongation factor EF-G. These proteins bind to the ribosome, do not interfere with protein synthesis, but protect the ribosome from the inhibitory effects of tetracyclines.[36,37] The gene *fusB* also codes for an EF-G-binding protein that protects the staphylococcal ribosomes from inhibition by fusidic acid.[27]

The replacement of a sensitive target by an alternative drug-resistant target is well known in sulfonamide and trimethoprim resistance. The sulfonamide resistance genes *sul1*, *sul2* or *sul3*, which code for sulfonamide-insensitive dihydropteroate synthases, are widespread in Gram-negative bacteria.[1,2] Gram-negative and Gram-positive bacteria have acquired various *dfr* genes which code for trimethoprim-insensitive dihydrofolate reductases.[1,2] In addition, the genes *mecA* and *mecC*, present in various *Staphylococcus* spp., code for alternative penicillin-binding proteins which exhibit a substantially reduced affinity to virtually all β-lactam antimicrobial agents. Moreover, the genes *vanA–vanE* code for alternative D-Ala–D-Lac or D-Ala–D-Ser peptidoglycan precursors that render the respective bacteria resistant to glycopeptides, which also act at the level of cell wall synthesis.[1,2,38]

Sulfonamide resistance via the hyper-production of *p*-aminobenzoic acid has been observed in isolates of the genera *Staphylococcus* and *Neisseria*. Likewise, promoter mutations resulting in the overproduction of a trimethoprim-susceptible dihydrofolate reductase have been described to account for trimethoprim resistance in *E. coli* and *Haemophilus influenzae*.[22]

Additional discussions of MIC distributions, as well as resistance genes and the mechanisms specified by them in bacteria involved in skin and soft tissue infections of animals, including staphylococci, streptococci and Gram negative bacteria, are available in other articles and book chapters.[39–54]

Horizontal gene transfer and mobile genetic elements involved

As resistance-mediating mutations usually are located in essential chromosomal genes or in the 16S and 23S rRNA, they can only be transferred vertically during cell division.[1,2] It is important that such mutations should not negatively affect the fitness of the bacteria. In contrast, mobile resistance genes are transferred vertically and horizontally, and thereby contribute to the dissemination of resistance properties.[1,2,55] Horizontal gene transfer (HGT) from the donor cell occurs via transformation, transduction or conjugation/mobilization and may include recipient cells of the same species, the same genus but also of different species and genera.

Transformation describes the transfer of "naked" DNA. It is the usual way used to transfer DNA under *in vitro* conditions. Although it also occurs in nature, it is believed to play a minor role in the transfer of DNA under natural conditions.[1,2,55]

Transduction describes the transfer of DNA via bacteriophages. Limitations to transduction are (i) the size of the head of the transducing phages into which plasmids or other DNA elements are packaged and (ii) the requirement for receptors on the recipient cell to which the transducing phage can attach. Thus, only a limited amount of DNA, approximately 45 kb for staphylococci, can be transduced and transduction occurs mainly between members of the same or closely related bacterial species.[1,2,55]

Conjugation, however, can also occur between bacteria of different species and genera. It describes the self-transfer of a conjugative element from a donor to a recipient cell. Plasmids and transposons can be conjugative, whereas integrative and conjugative elements (ICEs) are by definition always conjugative. The conjugative element harbours a *tra* gene complex which specifies the transfer apparatus. If a conjugative element provides its transfer apparatus to nonconjugative elements, mainly plasmids that co-reside in the same donor cell, such nonconjugative plasmids can move over to the recipient cell. This process is referred to as *mobilization*. Conjugation and mobilization of various mobile genetic elements are believed to play key roles in the dissemination of antimicrobial resistance in bacteria.[1,2,55] Furthermore, dissemination is thought to be particularly efficient amongst bacteria of the same species or clonal lineage. Barriers to HGT gene transfer, which protect bacteria against "foreign" DNA from other bacterial species or lineages, have been identified and are now widely described in many bacterial species.[56] Barrier systems described in staphylococci, including *S. pseudintermedius*, include restriction-modification systems, competence genes and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems, and these have been linked to the successful spread of certain lineages and their ability to protect themselves from foreign DNA.[57] However, their role in preventing acquisition of resistance genes, at least in *S. pseudintermedius*, is questionable based on finding them distributed randomly amongst multidrug-resistant and -susceptible isolates.[28]

There are several mobile genetic elements (MGEs) which can harbour antimicrobial resistance genes and which are essential to horizontal gene transfer. All of them are double-stranded DNA molecules. *Plasmids* are the most abundant MGEs. They can vary distinctly in their sizes between < 2 kb and > 200 kb. Plasmids replicate autonomously and independently from the chromosomal DNA. They can carry antimicrobial resistance genes, heavy metal resistance genes, virulence genes and genes for a number of other properties, including metabolic functions. Plasmids can harbour transposons and gene cassettes/integrons.

Transposons differ distinctly in size and structure. In contrast to plasmids, they are replication-deficient and as such must integrate for their replication either into plasmids or the chromosomal DNA. They move by transposition, either into specific sites or into various sites in plasmids or in the chromosomal DNA. The importance of large transposons in the emergence of the extremely drug resistant phenotypes was recently highlighted by the identification of a Tn*5405*-like element carrying up to

five antimicrobial resistance genes in all of 11 fully sequenced multidrug-resistant MRSP isolates of four different lineages.[28]

Gene cassettes are the smallest MGEs which commonly carry only one gene, mostly an antimicrobial resistance gene, and a recombination site, known as the 59-base element. They can neither replicate nor transpose. They move by site-specific recombination and are commonly found in integrons. The integrase of the integron catalyses the integration and excision of the gene cassette using the 59-base element. As gene cassettes usually do not have an own promoter, the cassette-borne gene is transcribed from a promoter in the 5′-conserved region of the integron. Gene cassettes are rarely found at secondary sites outside of an integron.[1,2,55]

Integrative and conjugative elements (ICEs) are large elements of >20 kb which integrate site-specifically into the chromosomal DNA. They can excise from the chromosomal DNA, form a circular intermediate and transfer themselves via a replicative cycle into new host cells where they integrate again into the chromosomal DNA. In terms of antimicrobial multidrug-resistance, the SXT element of *Vibrio cholerae* and the ICE*Pmu1* from *P. multocida* are well-studied ICEs.[58–60] The latter has been shown to carry and transfer a total of 12 different antimicrobial resistance genes conferring resistance to eight classes of antimicrobial agents.[59,60] Other elements that integrate site-specifically into the chromosomal DNA of the respective bacteria include the various different types of the SCC*mec* elements in staphylococci, as well as the numerous variants of the integrative and mobilizable *Salmonella* genomic islands SGI1, SGI2 and PGI1 in *S. enterica* and *Proteus mirabilis*.[1,2,38,61–63] Why the composition and predominant types of MGEs vary between species (e.g. plasmids predominate in *S. aureus* whereas transposons are more frequently described in *S. pseudintermedius*), remains to be answered.[28,40,57]

Consequences of the use of antimicrobial agents

Whenever antimicrobial agents are applied to either humans or animals, a selective pressure is set under which susceptible bacteria are inhibited in their growth or killed, whereas resistant bacteria can propagate at the expense of the susceptible bacteria.[64,65] Antimicrobial agents do not differentiate between beneficial and pathogenic bacteria. They inhibit or kill all those bacteria for which MICs are at or below the antimicrobial concentration in the respective body compartment. As a consequence, the proportion of resistant bacteria increases during antimicrobial therapy and the composition of the microbiota is altered. This is true for virtually every antimicrobial agent and every human or animal host. Under the selective pressure imposed by the use of antimicrobial agents, antimicrobial resistance genes can also be disseminated between different bacteria within the same host.[1,64,65] However, when resistant bacteria are transferred between humans or between animals, they can also exchange their resistance genes with bacteria already resident in or on the new host.[64,65]

There are three basic requirements that favour the exchange of resistance genes: (i) close spatial contact between the exchange partners (which is present in the polymicrobial environments of the respiratory and intestinal tracts and also on the skin); (ii) location of the resistance genes on MGEs (which is given by the fact that most resistance genes are located on plasmids, transposons, gene cassettes and ICEs) and (iii) a selective pressure (which is provided by the application of antimicrobial agents).[55] Exchange via horizontal gene transfer may involve obligatory and facultatively pathogenic bacteria as well as the commensal microbiota. If a multidrug-resistance MGE is transferred to new bacterial host and this host cell gains all the resistance genes associated with the MGE, the selective pressure imposed by the use of a single antimicrobial agent will ensure that the new host cell does not lose the multidrug-resistance MGE.[64,65] This means that the co-location of resistance genes furthers their co-selection and persistence even if no direct selective pressure is present. Thus, measures such as the voluntary withdrawal or even the ban of the use of an antimicrobial agent will not necessarily lead to a decrease in resistance. To better understand processes such as co-selection and persistence, and to judge the efficacy of the aforementioned measures, in-depth knowledge of the genetics of antimicrobial resistance is indispensable.

Exchange of resistant bacteria between animals and humans

As shown in Figure 1, the application of antimicrobial agents in human medicine as well as in veterinary medicine and food animal production can lead to the evolution and dissemination of resistant bacteria among humans and animals, respectively.[65] Depending on the virulence of the resistant bacteria, they may cause clinical diseases with limited treatment options. Transfer of bacteria – including resistant strains – can be exchanged between humans and animals in both directions by either contact, inhalation of dust and aerosols that contain bacteria, or via the food chain.[65]

Direct contact is likely the quickest and easiest way by which bacteria are transferred in either direction between humans and animals, particularly for those such as staphylococci which reside on body surfaces. Anyone who shares close contact with pets or companion animals may be affected.[66] In this regard, it is important to consider the current role of dogs and cats as actual family members in many households in industrialized countries. A study published in 2014 revealed the presence of approximately 11.5 million cats, 6.9 million dogs, 6.1 million other pet animals (e.g. rabbits, guinea pigs, hamsters) and 3.4 million pet birds in German households.[67] Pet owners often have extensive contact with their pets, especially to cats and dogs which may be allowed lick their owners' faces and hands or to sleep in their owners' beds.[67,68] Based on this close contact, a transfer of bacteria between pets and people is unavoidable and not surprising.[66,69–72] As "family members", cats, dogs and other pet animals often enjoy not only an extensive support in terms of food supply and housing, but also broad

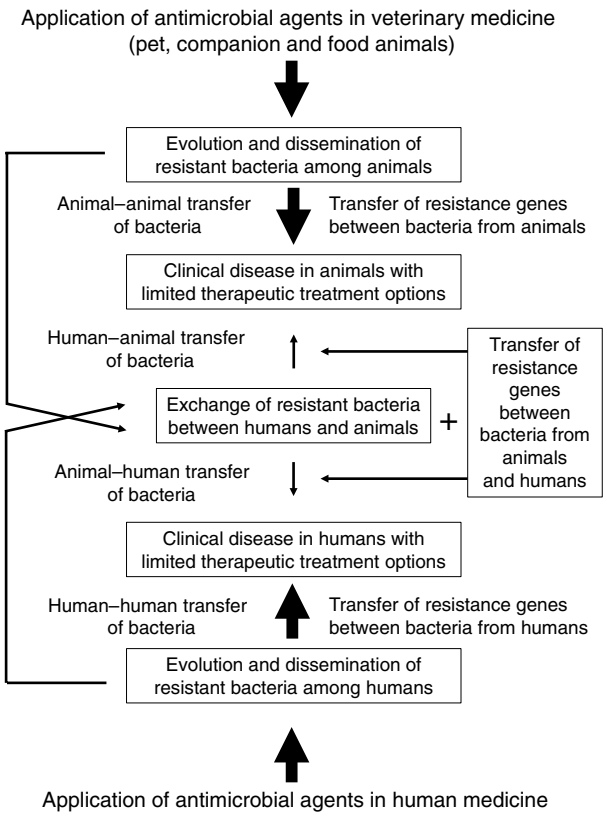

Figure 1. Schematic presentation of the dissemination of resistant bacteria and resistance genes among different hosts with particular reference to the exchange between humans and animals. The thickness of the different arrows shall indicate the likelihood of the various transfer ways.

medical care. In Germany, pet owners spent almost €4.8 billion for pet supplies in 2013, of which €3.75 billion accounted for pet food and €1.05 billion for equipment.[67] For medical care of their pets, Germans spent approximately €2.1 billion in 2013.[67] These data clearly show that pet owners have considerable interest in maintaining the health of their pets. As many infectious diseases in cats and dogs are caused by bacteria, particularly those infecting the skin of dogs,[73] this also involves the application of antimicrobial agents. A wide range of antimicrobial agents has been licensed for use in cats and dogs. In addition, antimicrobial agents approved for use in human medicine may also be applied to nonfood-producing animals under the Animal Medicinal Drug Use Clarification Act (AMDUCA) in the USA or similar regulations in other countries.[74] Although such applications should be kept to a minimum, it means that antimicrobial agents of last resort in human medicine, such as carbapenems, glycopeptides, oxazolidinones or lipopeptides, may be used in small animal medicine. However, no data are available to allow quantification of the use of these last resort agents for cats and dogs.

Animal transmission to companion animal owners

There have been numerous examples of the transfer of resistant bacteria, especially staphylococci and *E. coli*, between pets and people, beginning with the landmark report of the possible zoonotic spread of MRSA by a cat to hospitalized people.[75] Reports of interspecies transmission of MRSA include: livestock-associated (LA-) MRSA ST398-t034 transferred from a colonized veterinarian to his dog,[70] healthcare-associated MRSA ST225-t014 transferred from a family member (who suffered from an infected decubitus ulcer) to the family dog,[70] MRSA ST80-t131 isolated from a woman who suffered from multiple recurrent skin abscesses and her husband, children and a cat living in the same household (where the patient's disease resolved completely after topical decolonization of all family members including the MRSA-positive cat),[76] and the likely horse-to-human transmission of a LA-MRSA ST398-t011.[77] MRSA colonization of persons in contact with infected or colonized horses has been reported from the investigation of several outbreaks.[78] Aside from MRSA, indistinguishable isolates of *S. pseudintermedius* ST33 have been reported from a dog and its owner.[69]

Typically, such reports are based on evidence from genetic typing studies which identify indistinguishable isolates from animals and in-contact humans. However, the direction of inter-host transmission can rarely be proven definitively, but rather, is often deduced from epidemiological characteristics. Even an MRSA outbreak investigation in a small animal hospital using whole genome sequencing of multiple isolates from each sample had to conclude that directions of transmission could only be suspected.[79] For MRSA isolated from dogs and cats, for example, a predominantly human-to-animal direction of transmission is assumed because most isolates belong to MRSA clonal lineages that are also prevalent in human healthcare facilities and thus likely represent a "spill-over" to pets.[69,70,80,81]

Evidence for transmission of Gram-negative pathogens between animals and humans is only just beginning to emerge, but already includes some highly drug-resistant nosocomial pathogens, such as *E. coli* ST410 and other multidrug-resistant Extended Spectrum Beta Lactamase-producing (ESBL) *E. coli*.[82–84] *Escherichia coli* isolates, which belonged to the same phylogenetic group (B2 or D) and exhibited the same Amplified Fragment Length Polymorphism patterns, were detected among family members and their dogs.[68]

People with occupational contact with animals

In addition to pet and companion animal owners, people who have occupational contact with animals also are at risk for acquisition of bacteria from animals. Notably, these include veterinarians, but also veterinary students, farmers, abattoir workers and other animal caretakers. These people often work in an environment where they care for sick animals and in which antimicrobial agents are applied. Besides direct contact with animals, dust and aerosols, especially on farms and in abattoirs, may also play a role as vehicles that transport resistant bacteria and are inhaled by animals and humans.

There are a number of published reports which suggest occupational transmission in various settings. In a small

animal clinic, multidrug-resistant *Staphylococcus epidermidis* ST5 was shown to be present at various locations in the stationary area and the quarantine ward, as well as in feline patients and in the nose of one veterinary nurse.[85] A study from Australia revealed that veterinarians often carry multidrug-resistant MRSA isolates.[86] A study conducted in Germany showed that 97 (85.8%) of 113 swine farmers but only five (4.3%) of their 116 family members were positive for LA-MRSA.[87] Likewise, 22 (44.9%) of 49 swine veterinarians but only four (9.1%) of their 44 family members were positive for LA-MRSA in another report.[87] These observations suggest that the human-to-human transfer of LA-MRSA occurs distinctly more rarely than the animal-to-human transfer. A study involving 26 dairy farms in the Netherlands revealed that the same LA-MRSA types, based on pulsed-field gel electrophoresis (PFGE) type, *spa* type and resistance patterns, were detected not only among dairy cattle and their contact personnel (e.g. milkers), but occasionally also among other animals living on the same farm.[88]

LA-MRSA isolates with the molecular characteristics ST398-t011-dt11a and ST9-t1430-dt10a, both with very similar PFGE patterns and resistance phenotypes, were detected among poultry and workers in a Dutch poultry abattoir.[89] The analysis of turkey flocks and their carers revealed that almost 60% of the farm personnel were colonized by LA-MRSA that exhibited the same *spa* type and SCC*mec* type as the turkeys.[90] A study on the transmission of LA-MRSA on broiler farms in the Netherlands revealed the presence of MRSA ST398-t034-dt10q with indistinguishable PFGE and resistance patterns among the broilers, dust samples from the broiler house and the farmer.[91] The emission of bacteria from pig fattening and broiler chicken farms to the surrounding area was confirmed by the detection of ESBL-/AmpC-producing *E. coli* in air samples from inside as well as outside the farm buildings.[92,93] Another study showed that food animal transport in open crates resulted in the dissemination of bacteria, including resistant enterococci, into the environment.[94] In addition, indirect transmission via insects or rats can occur on farms.[95,96]

Transmission via the food chain

Transfer of resistant bacteria via the food chain usually occurs by ingestion of raw or insufficiently heated, contaminated food. In this regard, it is worth noting that (i) the number of ingested bacteria must be sufficiently high to survive the passage through the acidic environment in the stomach, which varies according to the type of foodborne pathogen and (ii) the virulence of most food-borne pathogens is more relevant than their antimicrobial resistance due to the fact that antimicrobial agents are not recommended for use in uncomplicated self-limiting cases of intestinal infections.[97] However, when resistant bacteria are ingested, they may transfer antimicrobial resistance genes to members of the intestinal microbiota of the host. Unfortunately, there are little if no data which provide reliable information about the extent at which bacteria transfer their resistance genes during transient colonization of a new host.

Proof of transfer of resistant bacteria and resistance genes

In view of the many opportunities for exchange of resistant bacteria and resistance genes amongst human and animal hosts and the respective selection pressures, a key question is: what proportion of resistance problems in human medicine is caused by bacteria of animal origin? One study has assessed the impact of antimicrobial resistance in different bacterial species and of the contribution of animal sources to resistance in human infections.[98] Based on the results of a questionnaire sent to recognized experts in the UK and elsewhere, the authors concluded that bacteria from animal sources, mainly nontyphoid *Salmonella enterica* serovars, *E. coli* O157, *Campylobacter* spp. and vancomycin-resistant enterococci, might account for 3.88% of the human antibiotic resistance problem.[98] It should be noted that this survey was conducted at a time when LA-MRSA and ESBL-producing *E. coli* were not yet recognized as emerging zoonotic problems.[99] Nevertheless, this survey suggested strongly that most of the resistance problems encountered in human medicine as well as in veterinary medicine are self-made problems in either sector. Only a minority results from the transfer of zoonotic bacteria.

A study on zoonotic MRSA colonization and infection in Germany showed that zoonotic transmission of LA-MRSA CC398 from livestock to humans occurs predominantly in people with occupational livestock contact, whereas dissemination in the general population is limited so far.[100] LA-MRSA CC398 currently causes about 2% of all human MRSA infections in Germany, but up to 10% in regions characterized by a high density of livestock farming.[100] Likewise, a study investigating 629 ESBL-producing *E. coli* from people in the Netherlands, Germany and UK, which were collected during the years 2005-2009 and examined by DNA microarray and multilocus sequence typing (MLST), showed that the majority of the human isolates differed distinctly from isolates of animal origin due to diversity in virulence and antimicrobial resistance genes.[101] It was concluded that attempts to minimize the human-to-human transfer of ESBL-producing *E. coli* are essential to limit the dissemination of these bacteria among humans. ESBL-producing *E. coli* from animals may play a role as a reservoir of virulence and antimicrobial resistance genes rather than directly causing infections in humans.[101]

The methodological attempts to prove the transfer of resistant bacteria or resistance genes strongly depend on the location of the resistance gene. For bacteria such as MRSA, where the meticillin resistance genes *mecA* or *mecC* are located on a chromosomally integrated SCC*mec* cassette, molecular strain typing methods can be applied. These include pattern-based techniques, such as PFGE, or sequence-based methods such as MLST, single locus sequence typing via *spa* and *dru* typing, as well as multiple loci VNTR analysis (MLVA).[102,103] In addition, the presence of the relevant resistance genes can be

detected by PCR. Whole-genome sequencing with subsequent SNP analysis can also be used as the ultimate proof.[81,104] The results of these methods can enable definite proof of clonality and transference of resistance genes.

If a resistance gene is located on a MGE (e.g. plasmid-borne ESBL genes in *E. coli*) strain typing methods like PFGE, MLST or PCR-directed typing methods can still be applied. In addition, it is necessary also to characterize the resistance plasmid in question (e.g. by pMLST, replicon typing, restriction analysis or even whole plasmid sequencing).[105] In the transfer of resistance plasmids, different scenarios are conceivable. *Scenario 1* describes a situation where the transferred strain and its resistance plasmid multiply stably in the new host. In such a case, the aforementioned methods enable the verification of the transferred strain and the resistance plasmid.[106] In *scenario 2*, the transferred strain cannot replicate in the new host, but transfers its resistance plasmid to bacteria of the new host. In this case, the transferred strain is not detectable any more, but the resistance plasmid may be detected in the new host bacteria. *Scenario 3* describes a situation in which the transferred strain cannot replicate in the new host and the transferred plasmid cannot replicate in the new host bacteria but undergoes recombination with plasmids already residing in these new host bacteria. In this case, neither the original bacterial strain nor the original plasmid are detectable and the confirmation of transfer is not possible.

Another problem is the confirmation of the direction of transfer. In staphylococci, for instance, structurally closely related small mobilizable plasmids that carry the tetracycline resistance gene *tet*(K), the chloramphenicol resistance gene cat_{pC221} or the MLS_B resistance gene *erm*(C) are prevalent in various staphylococcal species from both humans and animals.[107–109] Because tetracyclines, chloramphenicol and macrolides have been used in human and veterinary medicine for more than 60 years, it is impossible to determine in retrospect where and when these resistance genes first developed and which transfer events across species and host boundaries have taken place since then. In contrast, the recently identified phenicol and oxazolidinone resistance gene *optrA* is likely to have developed in enterococci of animal origin in China under the selective pressure imposed by the use of florfenicol in livestock animals.[110] Chloramphenicol was banned from use in food producing animals in China in 2002, whereas florfenicol was licensed in 1999 for animals only and has been used widely since then.[110] The first *optrA*-carrying *E. faecium* isolate of human origin orginated in 2005. This happened two years before linezolid, the sole commercially available oxazolidinone in China, was approved for use in human medicine in 2007.

The future of antibacterial therapy

For surface and superficial skin infections, and otitis involving multidrug-resistant bacteria, topical antimicrobial therapy is likely to remain effective in the future because very high concentrations of the drug, easily exceeding MICs, can be achieved at the site of infection.[111,112] However, for deep infections or those requiring systemic therapy, new classes of antimicrobial agents are unlikely to be approved for veterinary medicine. All new classes of antimicrobial agents will first be tested for their suitability as therapeutics in human medicine. Only if a new class of antimicrobial agents is unsuitable for use in humans based on its pharmacological parameters, toxicity or adverse effects, may it be considered for veterinary applications. The antimicrobial agents approved for veterinary use during the last 15 years are all derivatives of already known substances. Thus, pradofloxacin is a fluoroquinolone with improved activity against canine and feline bacterial pathogens. Tulathromycin, tildipirosin and gamithromycin are macrolides for the control of bovine and porcine respiratory tract infections. Finally, florfenicol is a fluorinated phenicol with activity against chloramphenicol-resistant bacteria in which resistance is based on a chloramphenicol acetyltransferase. Florfenicol is an example where the detailed knowledge about the resistance mechanism has led to the development of a molecule which is resistant to enzymatic inactivation by acetylation.[10] However, soon after the introduction of florfenicol into clinical veterinary use, genes specifying other phenicol resistance mechanisms, which also confer resistance to florfenicol, have emerged.[10,17]

It is our responsibility to use the available antimicrobial agents wisely and try to preserve their activity for as long as possible. This needs to include following pharmacokinetic and pharmacodynamic data (and creating such data where they are not yet available) for agents that are not licensed for use in pets. One example is use of the published recommendations on minocycline.[113] Most importantly, prudent use guidelines must be followed alongside the well-proven (but still too frequently neglected) concepts of rigorous hygiene measures. Moreover, improved microbiological diagnostics, which also include harmonized protocols for antimicrobial susceptibility testing of the various veterinary bacterial pathogens and additional veterinary-specific clinical breakpoints, especially for bacteria of poultry and fish origin, are urgently needed.

In summary, a multifaceted holistic approach which takes into account education as well as antimicrobial stewardship, is required:[114]

Education of the public in addition to prescribers of antimicrobial drugs is needed. Understanding how antimicrobial agents work and under which conditions antimicrobial resistance develops and spreads promotes the awareness needed to implement measures that counteract resistance development. Examples of such educational measures are the pan-European e-Bug program,[115,116] the "Get smart" program of US Centers for Disease Control and Prevention,[117] and antibiotic awareness days promoted in Europe and Canada.[118,119]

The search for new antimicrobial agents – natural and synthetic – should be stimulated by making the development of new agents more attractive to the pharmaceutical industry (e.g. by expanding the time of patent protection or lowering the administrative hurdles in the approval process). Public–private partnerships, which take the development of new antimicrobial agents forward, should be encouraged. As mentioned for florfenicol, more efforts also should be made to develop

chemical modifications which provide antimicrobial derivatives that evade known resistance mechanisms.

Revival of "old" antimicrobial agents, including those discarded, not fully developed or even rejected, should be re-investigated. Combinations of antimicrobial agents with an inhibitor (e.g. an efflux inhibitor) should be explored for their ability to restore the activity of old antimicrobial agents.[120]

Control of the use of antimicrobial agents: As the selective pressure imposed by the use of antimicrobial agents is a major driving force in the development of antimicrobial resistance, the nontherapeutic use of antimicrobial agents, for example, as growth promoters, must be discontinued worldwide. Antimicrobial agents in humans and animals should be made available by prescription only. Over-the-counter sales of antimicrobial agents should be forbidden worldwide. Monitoring of the consumption of antimicrobial agents in both human and veterinary medicine, including antimicrobial use in small animal practice, should be implemented.

Alternatives to antimicrobial agents: Novel nonantibiotic approaches for prevention of and protection against infectious diseases should be explored.[121] These include the development of vaccines (especially for animal diseases), phage therapy[122,123] and phage lysin therapy,[124–126] adjuvants, antivirulence therapies (including synthetic polypeptides that neutralize bacterial pathogenicity factors),[127] pre- and probiotics, immunostimulants, antimicrobial peptides (such as cathelicidins, defensins and dermicins),[128,129] anti-biofilm therapies[130–132] and reprogrammed nucleases that target antimicrobial resistance genes.[133]

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Bartonellosis, One Health and all creatures great and small

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Background – Bartonellosis is a zoonotic infectious disease of worldwide distribution, caused by an expanding number of recently discovered *Bartonella* spp.

Objectives – This review serves as an update on comparative medical aspects of this disease, including the epidemiology, pathogenesis, clinical diagnosis, treatment and challenges.

Results – Of comparative medical importance, *Bartonella* spp. are transmitted by several arthropod vectors, including fleas, keds, lice, sand flies, ticks and, potentially, mites and spiders. Prior to 1990, there was only one named *Bartonella* species (*B. bacilliformis*), whereas there are now over 36, of which 17 have been associated with an expanding spectrum of animal and human diseases. Recent advances in diagnostic techniques have facilitated documentation of chronic bloodstream and dermatological infections with *Bartonella* spp. in healthy and sick animals, in human blood donors, and in immunocompetent and immunocompromised human patients. The field of *Bartonella* research remains in its infancy and is rich in questions, for which patient relevant answers are badly needed. Directed *Bartonella* research could substantially reduce a spectrum of chronic and debilitating animal and human diseases, and thereby reduce suffering throughout the world.

Conclusion – A One Health approach to this emerging infectious disease is clearly needed to define disease manifestations, to establish the comparative infectious disease pathogenesis of this stealth pathogen, to validate effective treatment regimens and to prevent zoonotic disease transmission.

Preface

James Herriot published "All Creatures Great and Small" in 1972, 2 years before I graduated from the University of Georgia, College of Veterinary Medicine with the degree Doctor of Veterinary Medicine. Herriot embodied the "One Health Philosophy" by providing the best medical care possible to all creatures, regardless of their size, disposition, societal stature or economic worth. As a veterinarian, Herriot was very familiar with zoonotic diseases, such as brucellosis, rabies, tuberculosis, toxoplasmosis and others that were prevalent at the time. He also understood the critical role of genetics, nutrition and environmental toxins (naturally occurring and man-made), as mediators of infectious and noninfectious disease expression. Importantly, it would be another 20 years before Herriot, I and most other health care providers would come to know of the existence of the genus *Bartonella*.

Currently, resurgent efforts by groups around the world are promoting the importance of the "One Health Philosophy", which involves coordinated interaction among animal, human and environmental health professionals. I am sure that James Herriot would applaud these efforts and revel in the contemporary success stories. As one example, recently the World Organisation for Animal Health (see http://www.oie.int/) in conjunction with the World Small Animal Veterinary Association issued a One Health proclamation to eliminate rabies, which claims the lives of nearly 60,000 people each year throughout the world. On 3 November 2016, the first Global One Health Day was celebrated, emphasizing the connection between the health of animals, people and the environment.

Introduction

The genus *Bartonella* and the disease bartonellosis represent one of the more important contemporary One Health challenges of modern times. Bartonellosis also offers an opportunity to demonstrate the societal benefits of a One Health Approach to disease prevention in general and to enhance the comparative medical understanding of this emerging infectious disease.

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Conflict of Interest: In conjunction with Sushama Sontakke and North Carolina State University, Ed Breitschwerdt holds U.S. Patent No. 7,115,385; Media and Methods for cultivation of micro-organisms, which was issued 3 October 2006. He is the chief scientific officer for Galaxy Diagnostics, a company that provides serological and microbiological diagnostic testing for the detection of *Bartonella* species infection in animals and human patients.

Coordinated efforts are needed to clarify the medical importance of *Bartonella* spp. (currently 36 named and 17 Candidatus species) as a cause of disease in animals and humans. This review will focus on the complex interplay between bartonellosis, One Health, and all creatures great and small.

Bartonella species are fastidious Gram-negative bacteria that are highly adapted to a mammalian reservoir host within which the bacteria usually cause a long lasting intra-erythrocytic bacteraemia and endotheliotropic infection, often not in association with concurrent disease.[1–3] These facts are of particular importance to veterinarians, physicians and other healthcare professionals, because an increasing number of animal reservoir hosts have been identified for various *Bartonella* species (Table 1). Among numerous other examples, *Bartonella henselae* has co-evolved with cats, *Bartonella vinsonii* subsp. *berkhoffii* and *Bartonella rochalimae* have co-evolved with wild canines, *Candidatus Bartonella melophagi* has co-evolved with sheep, and *Bartonella bovis* has co-evolved with cattle. Importantly, as new *Bartonella* spp. are discovered, the list of reservoir-adapted *Bartonella* species, including a large number of rodent and bat species, continues to grow exponentially.[2,3] Coevolution has created a hidden, large and constant source of infection for accidental hosts in natural environments.[4–6]

In the natural reservoir host, chronic bacteraemia with a *Bartonella* species can frequently be detected by blood culture or PCR in outwardly healthy animals.[2,3] Infection with *B. henselae* and *Bartonella clarridgeiae,* species associated with cats and their fleas, has been reported in healthy Brazilian blood donors, suggesting that asymptomatic infection occurs in humans as well as cats, dogs and other animals.[5,7] In contrast to the reservoir-adapted host, the microbiological detection of a *Bartonella* spp. in a nonreservoir adapted host can be extremely difficult.[8–11] Most, although not all, diseases caused by *Bartonella* spp. occur in accidental hosts and these organisms are being

Table 1. *Bartonella* species or subspecies, primary reservoir hosts, confirmed or potential vectors, and infection in accidental hosts. Details are provided in references 2, 3, 15 and 49.

Bartonella species	Main reservoir	Potential vectors	Accidental hosts
B. acomydis	Mice (Acomys russatus)	Unknown	
B. alsatica	Rabbits (*Oryctolagus cuniculus*)	Fleas, ticks	Humans
Candidatus B. antechini	Yellow-footed antechinus (*Antechinus flavipes)*	Fleas, ticks	
B. bacilliformis	Humans	Fleas, sandflies	
B. birtlesii	Wood mice (*Apodemus* spp.)	Fleas	
B. bovis (weissii)	Domestic cattle (*Bos taurus*)	Biting flies, ticks	Cats, Dogs Humans,
B. callosciuri	Squirrel (Callosciurus notatus)	Unknown	
B. capreoli	Roe deer (*Capreolus capreolus*)	Biting flies, ticks	
B. chomelii	Domestic cattle (*Bos taurus*)	Biting flies, ticks	
B. clarridgeiae	Cats (*Felis catus*)	Fleas, ticks	Humans, Dogs
B. doshiae	Meadow voles (*Microtus agrestis*), Rats (*Rattus* spp)	Fleas	Humans
B. elizabethae	Rats (*Rattus norvegicus*)	Fleas	Humans, Dogs
B. florenciae	Shrew (*Crocidura russula*)	Unknown	
B. grahamii	Voles (*Clethrionomys* spp.), mice (*Apodemus* spp)	Fleas	Humans
B. henselae	Cats (*Felis catus*), dogs (*Canis familiaris)*	Fleas, ticks	Humans, Dogs
B. japonica	Mice (*Apodemus argenteus*)	Lice (*Hoplopiura affinis)*	
B. koehlerae	Cats (*Felis catus*), gerbils (*Meriones lybicus*)	Fleas	Humans
B. mayotimonesis	Daubenton's bat (*Myotis daubentonii*)	Bat flies, fleas	Humans
*B. melophagi**	Sheep (*Ovis* spp.)	Sheep keds	Humans
Candidatus B. merieuxii	Dogs (*Canis familiaris*)	Fleas	
B. pachyuromydis	Mice (Pachyuromys duprasi)		
B. peromysci	Field mice (*Peromyscus* spp.)	Fleas	
B. queenslandensis	Rats (*Rattus* spp)	Fleas	
B. quintana	Humans, gerbils (*Meriones lybicus*)	Human body lice, fleas	Cats, Dogs
"B. rattimassiliensis"	Rats (*Rattus* spp)	Fleas	
B. rattaustraliani	Rats (*Rattus* spp)	Fleas	
"B. rochlimaea"	Dogs (*Canis familiaris*)	Sandflies	Humans
B. schoenbuchensis	Roe deer (*Capreolus capreolus*)	Deer keds, biting flies, ticks	Humans
B. senegalensis	Burrowing rodents	Soft tick *Ornithodoros sonrai*	
B. silvatica	Mice (*Apodemus speciosus*)		
B. talpae	Moles (*Talpa europaea*)	Fleas	
"B. tamiae"	Rat (*Rattus* spp.)	Fleas	Humans
B. taylorii	Mice (*Apodemus* spp.), gerbils (Meriones lybicus), voles (*Clethrionomys s*pp.)	Fleas	
B. tribocorum	Rats (*Rattus* spp), mice (*Apodemus* spp.)	Fleas	
B. vinsonii subsp. *arupensis*	White-footed mice (*Peromyscus leucopus*)	Fleas, ticks	Humans
B. vinsonii subsp. *berkhoffii*	Coyotes (*Canis latrans*), dogs (*Canis familiaris*), foxes (*Urocyon* spp.*)*	Ticks	Humans
B. vinsonii subsp. *vinsonii*	Meadow voles (*Microtus pennsylvanicus*)	Ear mites	
"B. volans"	Southern flying squirrels (*Glaucomys volans*)	Fleas	Humans
"B. washoensis"	California ground squirrel (*Spermophilus beecheyi*), rabbits (*Oryctolagus cuniculus*)	Fleas, ticks	Humans, Dogs

increasingly implicated as a cause of zoonotic infections.[8–11] Mechanisms that facilitate persistent *Bartonella* bacteraemia in mammals remain incompletely understood. Intra-endothelial and intra-erythrocytic location of these bacteria represents a unique strategy for bacterial persistence.[2,6] Nonhaemolytic intracellular colonization of erythrocytes and invasion of endothelial cells preserves these organisms for efficient vector transmission, facilitates movement throughout the vascular system, protects *Bartonella* from the host immune response and potentially contributes to diminished antimicrobial efficacy. In addition to erythrocytic and endotheliotropic cell invasion, *in vitro* studies indicate that *Bartonella* spp. can infect dendritic cells, microglial cells, pericytes, monocytes and CD34 + bone marrow progenitor cells.[6] Thus in the context of disease pathogenesis, these bacteria can infect numerous cell types, can access any location within the body via small blood vessels and, potentially, through lymphatics (which has not been studied to date), can promote endothelial cell proliferation and can slow endothelial cell death by inhibition of apoptotic pathways. These and other factors make *Bartonella* the perfect stealth pathogen, defined as an organism that can induce persistent infection in association with immune evasion and fluctuating symptoms varying in type and severity.[2,3,6]

One Health and the medical aspects of *Bartonella* epidemiology

As a genus, *Bartonella* epidemiology continues to evolve and change as reflected in trends between earlier and more recent reviews.[1–3,12–15] *Bartonella henselae, B. vinsonii* subsp. *berkhoffii* and *Bartonella koehlerae* appear to be the most medically important species to infect cats, dogs, horses and potentially humans. Other *Bartonella* spp. have been less frequently reported in association with various disease processes in animals and humans (Table 1). *Bartonella bacilliformis* and *Bartonella quintana* are also important human pathogens, for which primates are considered the reservoir hosts.[12–14] As an important example of an evolution in ecological understanding, *B. henselae* was initially isolated from a HIV-infected human and subsequently from cats around the world.[1–3] Subsequently, *B. henselae* bacteraemia has been documented in cows, dogs, horses, marine mammals, small terrestrial mammals and sea turtles, making the epidemiology of this single medically important species much more complex than initially anticipated.[3,15]

In contrast to the initial *B. henselae* isolation from an immunocompromised human, *B. vinsonii* subsp. *berkhoffii* was initially isolated from a dog with endocarditis. Subsequently this subspecies has been isolated or DNA PCR-amplified from cats, coyotes, deer, fox, horses and human patients.[12,15] As described below, successful microbiological isolation of *Bartonella* species is difficult. Long-term administration of immunosuppressive corticosteroids for a presumptive diagnosis of systemic lupus erythematosus with cutaneous vasculitis may have facilitated the isolation of this original type strain of *B. vinsonii* subsp. *berkhoffii* (the first *Bartonella* sp. reported to infect a dog). Immunosuppression may also have contributed to the location of these bacteria on the aortic and mitral valves resulting in vegetative valvular endocarditis, as has been reported in immunosuppressed human patients.[3,15] *Bartonella vinsonii* subsp. *berkhoffii* seroprevalence in 1,920 sick dogs from North Carolina (NC) or surrounding states in the United States was evaluated at a tertiary care veterinary teaching hospital.[16] Using a reciprocal titre of >32, 3.6% of sick dogs were *B. vinsonii* subsp. *berkhoffii* seroreactive. Risk factors associated with seroreactivity included: heavy tick exposure [Odds ratio (OR) 14.2], cattle exposure (OR 9.3), a rural versus urban environment (OR 7.1) and heavy flea exposure (OR 5.6). These data supported the hypothesis that exposure to *B. vinsonii* subsp. *berkhoffii* was more likely to occur in dogs in rural environments that had arthropod infestations and were allowed to roam freely.

In support of the potential for tick transmission of *B. vinsonii* subsp. *berkhoffii*, 36% of serum samples derived from dogs naturally infected with *Ehrlichia canis* were seroreactive to *B. vinsonii* subsp. *berkhoffii* antigens.[16,17] In contrast, sera from dogs experimentally infected with *Rickettsia rickettsii* or *E. canis*, two closely related alpha proteobacteria species, were not cross-reactive to *Bartonella* antigens. As *E. canis* is thought to be transmitted solely by *Rhipicephalus sanguineous*, natural transmission of *B. vinsonii* subsp. *berkhoffii* by this tick was suspected. As reviewed previously, the possibility of tick transmission was further supported by additional studies involving *Ehrlichia* spp. co-exposures from the same geographical region (NC), in which seroreactivity to *E. canis* and *B. vinsonii* subsp. *berkhoffii* antigens was 30% and 89%, respectively.[3] Similarly, *B. vinsonii* subsp. *berkhoffii* seroprevalence was 10% (four of 40 dogs) in dogs with suspected tick-borne illness from Israel, 36% in dogs with fever and thrombocytopenia from Thailand, and 6.6% of stray and rural dogs in Turkey.[3]

Further studies from around the world have emphasized co-exposures and co-infections with vector-borne organisms (particularly fleas and ticks) in pets and people. As one example involving dogs, a vector-borne disease surveillance study that spanned 2004–2010, (N = 14,496 serum samples) submitted to the North Carolina State University, College of Veterinary Medicine, Vector Borne Disease Diagnostic Laboratory for diagnostic purposes were tested using immunofluorescent antibody (IFA) and enzyme linked immunosorbent assay (ELISA) assays. Seroreactivity to *R. rickettsii*, *Borrelia burgdorferi, Ehrlichia* spp., *B. henselae*, *Anaplasma* spp., *B. vinsonii* subsp. *berkhoffii*, *Babesia canis* and *Dirofilaria immitis* was 10.4%, 5.2%, 4.3%, 3.8%, 1.9%, 1.5%, 1.1% and 0.8%, respectively.[18] In contrast, a study from Algeria involving stray and pet dogs, the *Anaplasma* spp., *Borrelia* spp., *E. canis, B. henselae* and *B. vinsonii* subsp. *berkhoffii* seroprevalences were 47.7%. 37.6%, 30.0%, 32.4% and 27%, respectively.[19] Although seroepidemiological data continue to support tick transmission of *B. vinsonii* subsp. *berkhoffii* and potentially other *Bartonella* spp. to dogs, the mode of transmission has not been proven for this subspecies. Importantly, evolving evidence supports the possibility that *Ixodes* spp. are transmitting *B. henselae,* in addition to *Borrelia, Anaplasma* and *Babesia* spp. to animals and humans throughout the Northern hemisphere.[20]

Studies from Hawaii, USA, UK and Japan were among the first to document *B. henselae* seroprevalences of 6.5% (two of 31), 3.0% (three of 100) and 7.7% (four of 52) in dogs, respectively.[1,3] In the context of clinical diagnosis, *B. henselae* is the most common *Bartonella* species found in the blood of sick dogs using the *Bartonella alpha-Proteobacteria* growth medium (BAPGM) enrichment blood culture platform; however, most sick bacteraemia dogs, despite a history of chronic illness, do not have detectable *B. henselae* antibodies by immunofluorescent antibody testing, for reasons that remain unclear.[21] As discussed under the diagnosis section, seronegative bacteraemia may result in a substantial underestimation of *Bartonella* seroprevalence in animal and human studies.

In cats, *B. henselae, B. clarridgeiae* and *B. koehlerae* DNA have been amplified or the organisms have been isolated most frequently from clinically healthy cats that have experienced flea infestations.[1–3] Flea-associated transmission of *B. henselae* by *Ctenocephalides felis* amongst cats has been documented in laboratory studies.[22] In a laboratory transmission study, *C. felis* also transmitted *B. henselae* to dogs (Lappin M and Breitschwerdt E, unpublished data). Clinical, epidemiological and laboratory studies support the transmission of *B. koehlerae, B. clarridgeiae, B. quintana* and potentially *B. vinsonii* subsp. *berkhoffii* by *C. felis*, and potentially other flea species such as *Pulex* spp.[23–26] Although the source of feline infection was not determined, a feral cat transmitted *B. quintana* to a human by scratch or bite. Subsequently, the feral cat was shown to be *B. quintana* bacteraemic.[27] Historically, *B. quintana,* the cause of Trench Fever in World War I and more recently Urban Trench Fever throughout the world, was thought to be transmitted solely by the human body louse (*Pediculus humanus*) and humans were considered the sole reservoir hosts. It now appears that cats can be a reservoir host and that fleas may be the source of infection for cats and potentially humans.[25,27] As described above for *B. henselae* and *B. vinsonii* subsp. *berkhoffii,* epidemiological understanding of *B. quintana* continues to change in complexity as additional potential vectors and reservoir hosts are identified.

In flea endemic areas, *Bartonella* spp. seroprevalence rates in cats can be greater than 90% and bacteraemia rates can be greater than 50%.[1–3] In a study from the United States involving PCR of blood from feral cats and raccoons, the prevalence of *Bartonella* bacteraemia was nearly identical, 48% in cats and 43% in raccoons.[28] Also, rat mite (*Ornithonyssus bacoti*) transmission of *B. henselae* to a woman and her pet dogs was implicated after removal of raccoons from under a house in New York, USA.[29] Thus, cats (both pet and feral), raccoons, mongoose and potentially other animals can serve as a source of *B. henselae* infection for humans and other animals, such as pet dogs. In addition to fleas, ticks, mites and spiders may also contribute to the transmission of *B. henselae.*

Fleas and flea infestations in households and on pets are of increasing zoonotic and medical importance to dermatologists, parasitologists, physicians, veterinarians and other health professionals. Collectively, recent studies emphasize an underappreciated role for mammals other than cats as reservoir hosts, for vectors other than fleas, lice and sandflies as a source of transmission, and for potential *C. felis* (and potentially other flea species) to be vector competent for the transmission of several *Bartonella* spp. to cats, dogs and humans. It is increasingly clear that the ecology and epidemiology of bartonellosis in many environmental settings is much more complex and dynamic due to the large number of *Bartonella* species, the large number of reservoir hosts and vectors, and the numerous modes of transmission, than was appreciated a decade ago.[30]

Pathogenesis and pathology

Despite substantial efforts by researchers around the world, relevant animal models to study disease pathogenesis have not been forthcoming. Because *Bartonella* spp. can cause chronic intra-erythrocytic and endotheliotropic infections in cats, dogs, humans and numerous other animal species that can span weeks, months or potentially years in duration, characterizing the pathogenesis of naturally occurring disease remains challenging.[1–3,15] Similar to other highly adapted intracellular vector-transmitted pathogens, the factors that ultimately result in disease manifestations are yet to be determined, but are most likely multifactorial and include virulence differences among *Bartonella* species and strains, differences in the host immune response and other epiphenomena such as co-infection, immunosuppression, concurrent noninfectious diseases and malnutrition.

Because bartonellosis is characterized by persistent intravascular infection, subsets of animal and human patients develop endocarditis, myocarditis or various forms of vascular pathology (Table 2). In addition, persistent infection may predispose to autoimmune (e.g. immune-mediated anaemia or immune-mediated thrombocytopenia) and immune-mediated (leukocytoclastic vasculitis or immune complex glomerulonephritis) manifestations as a component of the disease pathogenesis.[3,15] Similar to what is known for babesiosis, a tick-borne intra-erythrocytic pathogen, stress, hard work, parturition, concurrent or sequential infection with other vector-borne organisms or therapeutic immunosuppression may contribute to the development of pathology in an individual who was previously in a state of premunition (term denoting "infection immunity" and reflecting an immunological balance between the infectious agent and the host immune response) with the bacteria.

Although minimally studied to date, persistent infection with a *Bartonella* spp. in a sick individual may ultimately result in organism-induced immunosuppression. Experimental inoculation of dogs with culture-grown *B. vinsonii* subsp. *berkhoffii* resulted in impaired phagocytosis by monocytes, sustained suppression of peripheral blood CD8 + lymphocytes accompanied by an altered cell surface phenotype, an increase in CD4 + lymphocytes in the peripheral lymph nodes and potentially impaired B cell antigen presentation.[31] Thus, bacteria-induced immunosuppression could predispose dogs to secondary infections, further contributing to the wide array of clinical manifestations that occur in naturally infected dogs. Cats

Table 2. *Bartonella*-associated endocarditis, myocarditis and vascular pathology. Details are provided in references 12–15.

	Host(s)
Verruga peruana	
B. bacilliformis	Human
Candidatus B. ancashi	Human
Bacillary angiomatosis	
B. henselae	Human
B. quintana	Human
B. vinsonii subsp. *berkhoffii*	Dog
Bacillary peliosis	
B. henselae	Dog, human
Endocarditis	
B. alsatica	Human
B. bacilliformis	Sea otter*
B. bovis	Cattle
B. clarridgeiae	Dog
B. elizabethae	Human
B. henselae	Human, cat, dog, coyote,* sea otter*
B. koehlerae	Human, dog
B. quintana	Human, dog
B. rochalimae	Dog, coyote*
B. vinsonii subsp. *arupensis*	Human
B. vinsonii subsp. *berkhoffii*	Human, dog, coyote*
B. washoensis	Human, dog
Candidatus B. mayotimonensis	Human
Bartonella spp. JM-1	Sea otter*
Myocarditis	
B. henselae	Human, cat
B. vinsonii subsp. *vinsonii*	Dog
B. washoensis	Human
Aneurysm	
B. henselae	Human
B. quintana	Human
Vasculitis and/or thrombosis	
B. henselae	Human, dog
B. quintana	Human
Haemangiosarcoma	
B. vinsonii subsp. *berkhoffii*	Dog
Haemangiopericytoma	
B. henselae	Dog, horse
B. vinsonii subsp. *berkhoffii*	Dog, horse, red wolf
Epithelioid haemangioendothelioma	
B. vinsonii subsp. *berkhoffii*	Human
B. henselae	Human
B. koehlerae	Human
Systemic reactive angioendotheliomatosis	
B. henselae	Cat, cattle
B. koehlerae	Cat
B. vinsonii subsp. *vinsonii*	Cat

* *Bartonella* DNA was amplified from nondiseased mitral and aortic valves of coyotes and sea otters.

infected with *Bartonella* spp. are commonly co-infected with haemoplasmas and at times, more than one *Bartonella* sp.[3] However, whether co-infections magnify disease manifestations in cats is unclear, and in most epidemiological studies co-infection with a haemotropic *Mycoplasma* sp., feline immunodeficiency virus (FIV) and *Bartonella* did not appear to potentiate illness.[1,3] In the context of comparative medicine and a One Health approach to bartonellosis, co-infections with haemotropic *Mycoplasma* spp. and *B. henselae* have been recently reported in humans.[32]

The microbiological and molecular pathogenesis of *Bartonella*-induced vasoproliferative lesions have been studied in detail and readers are referred to reviews.[6,14,33] Although persistent bacteraemia most likely plays a role, the cellular and immunopathogenesis of other *Bartonella*-associated pathological lesions, including endocarditis, myocarditis, vasculitis and granulomatous tissue inflammation (lymphadenitis, hepatitis, panniculitis, splenitis) has not been studied to any extent.

Reviews have emphasized an emerging role for a dermal niche, as well as the previously described vascular niche, as an important pathogenic component of the pathophysiology of *Bartonella* infections.[6] Once transmitted to the host by inoculation of contaminated arthropod faeces (fleas, mites and lice) or potentially by a bite (tick or sandfly), the bacterium penetrates the dermal barrier, most often in conjunction with scratching or through a pre-existing abrasion. Subsequently, *Bartonella* are thought to infect dermal dendritic cells, which then transport (macrophages serving as a Trojan horse) bacteria to the endothelium to create a vascular niche. The endothelial or vascular niche provides the bacterium with a means of seeding the blood with organisms on a sporadic basis, resulting in infection of CD34 + cells in the bone marrow, as well as circulating erythrocytes and monocytes. The clinical relevance of the dermal niche for human dermatologists and other clinicians is only just now being considered on a research basis. Previous comparative infectious disease observations suggest that the dermal niche may be involved in panniculitis, cutaneous vasculitis and cutaneous vasoproliferative lesions such as bacillary angiomatosis, across animal species.[34–37]

In the context of comparative medicine, *B. henselae* has been reported in dogs and humans with granulomatous lymphadenopathy, granulomatous hepatitis and fever of unknown origin; it appeared to cause steatitis and prostatitis in a dog following flea transmission.[14,38,39] It is also possible that a subset of striae lesions, particularly in children, are caused by *Bartonella* spp. infections.[9] *Bartonella henselae* DNA was amplified from four of 29 (13.8%) paraffin-embedded canine histiocytoma tissues; however, the prevalence did not vary statistically from the control group.[40] Subsequent investigations have determined that bacteria DNA is rapidly degraded in formalin-fixed paraffin embedded skin (J.S. Pendergraft, N. Balakrishnan, E.B. Breitschwerdt, unpublished data), a factor that may have influenced the histiocytoma study conclusions. Thus, frozen, rather than formalin-fixed, paraffin-embedded dermal biopsy tissue would be recommended for diagnostic and investigational purposes when attempting to determine the presence or absence of *Bartonella* species DNA. Because aseptic measures to completely remove rapid-growing dermal bacteria such as *Staphylococcus* and *Corynebacteria,* are unlikely to be effective, using an enrichment skin culture approach as described below for blood and other aseptically obtained effusion or tissue samples is unlikely to be successful. For human and veterinary clinicians, determining if the dermal niche of *Bartonella* spp. is involved in the pathogenesis of striae in children, panniculitis or "flea allergy dermatitis" in dogs, may represent relevant research questions. Also in the context of dermatology, *Bartonella* DNA was amplified from house dust mites, a known cause of cutaneous allergic reactions.[41,42]

The spectrum of disease manifestations in animals and humans with serological, culture or PCR evidence of bartonellosis varies substantially. Reasons for variations in symptomatology as well as the varied pathology most likely relate to differences in organism virulence, which has now been documented for *B. henselae* strains, variation in the genetically mediated host immune response following infection, the duration of infection, inadequate or excessive nutrition, co-infection with other bacterial, viral or protozoal organisms, and noninfectious co-morbidities. In addition to complicating diagnostic confirmation of *Bartonella* spp. infections, these factors also complicate efforts to prove disease causation. From an evolutionary perspective, it is obvious that vectors, vector-borne organisms, and animal and human hosts have developed a highly adapted form of interaction over millions of years of coexistence. In general, vectors need blood for nutrition; some bacterial, rickettsial, protozoal and viral organisms need an intracellular environment to survive and, immunologically, many animal hosts appear to be able to support chronic infection with one or more vector-borne organisms for months to years without obvious deleterious effects. These factors serve to illustrate the potential difficulty in establishing disease causation in cats, dogs or people infected with a *Bartonella* spp. or when co-infected with multiple vector-borne pathogens.

We have proposed an addition to Koch's postulates entitled the Postulate of Comparative Infectious Disease Causation.[14] In satisfying this postulate we have stated that: "causation can be established if the same infectious agent (or combination of agents) are isolated or organism specific DNA sequences are amplified from a naturally occurring pathological entity found in at least three different mammalian genera". Based upon this postulate, *Bartonella* spp. appear to be able to cause endocarditis, granulomatous inflammatory diseases, particularly involving the heart, liver, lymph nodes and spleen, persistent intravascular infections, and the induction of vasoproliferative tumours. In the context of comparative infectious disease causation, *B. henselae* was amplified and sequenced from the liver of a dog with peliosis hepatitis, a unique pathological lesion initially reported in *B. henselae* infected HIV patients and from another dog with granulomatous hepatitis, a histopathological lesion reported with some frequency in *B. henselae*-infected children and adults.[3,14] *Bartonella clarridgeiae* DNA was amplified and sequenced from the liver of a Doberman dog with copper storage disease and from the aortic valve of a dog with vegetative valvular endocarditis.[2,3] *Bartonella elizabethae*, a species that infects rodents, was found in a dog that had shown chronic weight loss culminating in sudden unexplained death and from a human endocarditis patient.[2,3] Based upon a large seroepidemiological, controlled study from the University of California (Davis), dogs that were seroreactive to either *B. henselae, B. clarridgeiae* or *B. vinsonii* subsp. *berkhoffii* were referred for evaluation of lameness, neutrophilic polyarthritis, nasal discharge, epistaxis or splenomegaly.[13] *Bartonella henselae* and *B. vinsonii* subsp. *berkhoffii* were isolated from the synovial fluid of a dog with polyarthritis[43] and from the blood of dogs with epistaxis.[44] Based upon clinical observations, it seems likely that the spleen plays an important immunomodulatory role in controlling persistent *Bartonella* spp. bacteraemia in animals and people, as occurs with other intra-erythrocytic infections.[11,35] The extent to which *Bartonella* spp. induce splenic pathology in animals and humans deserves additional research consideration.[11,45]

Clinical findings

The complete spectrum of disease associated with *Bartonella* infection in cats, dogs, humans and most other animal species is currently unknown. Endocarditis, has been reported in cats, cows, dogs and humans, infected with a spectrum of reservoir-adapted *Bartonella* spp (Table 2).[2] In dogs, intermittent lameness, bone pain, epistasis or fever of unknown origin have preceded the diagnosis of endocarditis by several months, whereas other dogs will present with an acute history of cardiopulmonary decompensation (ARDS or acute respiratory distress syndrome) without a history of premonitory signs. Cardiac arrhythmias secondary to myocarditis can be detected in cats and dogs without echocardiographic evidence of endocarditis.[46] Granulomatous lymphadenitis has been associated with *B. vinsonii* subsp. *berkhoffii* and *B. henselae* in dogs.[14,38] In dogs, *Bartonella* species appear to contribute to the development of dermatological lesions indicative of a cutaneous vasculitis, panniculitis, as well as anterior uveitis, polyarthritis, meningoencephalitis and immune-mediated haemolytic anaemia.[3,13,47] Additional research efforts, using carefully designed case-controlled studies, are necessary to establish the frequency and extent to which *Bartonella* spp. contribute to dermatological, ocular, orthopaedic, neurological or haematological abnormalities in dogs (and humans).

Clinically, many disease manifestations also have been attributed to *Bartonella* spp. infections in cats.[1–3] However, it is very difficult to prove disease associations in cats in the field because of the high prevalence rates in nonclinical carriers. In research cats that are infected by exposure to *C. felis*, fever, endocarditis and myocarditis are the most common disease manifestations.[1,3,46,48] As discussed for dogs, additional case-controlled, prospective studies are needed in cats.[48]

Due to the spectrum of clinical and pathological abnormalities that have been associated with *Bartonella* spp. infections, the disease bartonellosis should be included in the differential diagnosis for many patients, particularly those with nonspecific clinical signs, poor responses to symptomatic or short duration antibiotic therapy, deterioration in clinical status following immunosuppression, or when historically the pet was found or rescued as a stray (animals that often have extensive arthropod exposures prior to rescue). As cats and dogs with prior exposure to fleas, ticks and other arthropod vectors are at risk for acquiring *Bartonella* infections, veterinarians should always obtain a comprehensive vector exposure history for sick animals and should determine client compliance if acaricides have been used to prevent arthropod infestations. The fact that a product was dispensed on a routine basis, does not mean that the product was administered or used in the manner recommended by the manufacturer.

The numerous species within the genus *Bartonella*, antigenic and virulence differences among strains, species and subspecies, the diverse cell tropism of these bacteria, their ability to induce persistent occult endotheliotropic and intravascular infections in both reservoir and nonreservoir hosts, and the extraordinarily low levels of relapsing bacteraemia found in accidentally infected, nonreservoir hosts, all contribute to the clinical, microbiological and pathological complexities associated with the diagnosis of bartonellosis in cats, dogs, horses, humans and all other "creatures great and small".[49] Following what in most instances appears to be a persistent dermatological and blood-borne infection, dogs and human patients develop similar disease manifestations (Table 3). Endocarditis, myocarditis, pericarditis, peliosis hepatitis, bacillary angiomatosis, systemic angiomatosis, granulomatous inflammatory lesions in various tissues, lymphadenitis, vasculitis, thromboembolism, cutaneous panniculitis, anterior uveitis, lameness/polyarthritis, splenomegaly and meningoencephalitis have been reported in both dogs and humans, making dogs a useful naturally occurring infectious disease model for human Bartonellosis and vice versa (namely One Health).[15]

Due to the relatively small number of PCR- or culture-confirmed cases overall, few studies have addressed clinicopathological differences induced by different *Bartonella* spp. in cats, dogs, horses or humans. Thrombocytopenia, anaemia, which can be secondary immune-mediated, and neutropenia or neutrophilic leucocytosis are the haematological abnormalities reported in dogs that are *Bartonella* seroreactive or BAPGM enrichment blood culture/PCR positive.[3,21,47] Thrombocytopenia is found in approximately half and eosinophilia in approximately one third of infected dogs, and monocytosis frequently occurs in association with *Bartonella* endocarditis.[2,3] Haematological abnormalities have been rarely reported in cats, but similar to dogs, a subset of *Bartonella*-infected cats are neutropenic or mildly thromobocytopenic.[48]

Serum biochemical abnormalities are usually very mild or nonexistent in healthy and sick bacteraemic cats and dogs. In cats, *Bartonella* spp. antibodies have correlated with polyclonal hyperglobulinaemia and hypoglycaemia.[50] Hyperinsulinemic hypoglycaemia syndrome has been reported in two dogs infected with a *Bartonella* sp.[51] Monoclonal gammopathies have been reported in association with bartonellosis in cats, dogs and humans.[52,53] In a study that compared *Bartonella* bacteraemic dogs to non-*Bartonella* bacteraemic dogs, suspected of a vector-borne infection, hypogammaglobulinemia was the only statistically significant laboratory finding that discriminated between the two groups.[47] Similar to cats and humans, *Bartonella* bacteraemia can be detected in healthy dogs being screened as blood donors.[54] Because *Bartonella* spp. occur as co-infections with other vector-borne organisms, comprehensive serology and PCR testing should be considered when pursuing a diagnosis of vector-borne illness.[52,55–57]

Table 3. Comparative pathological and haematological abnormalities associated with human and canine bartonellosis. Causation has not been clearly established for all of these entities in either species. Details are provided in references 3, 12, 14 and 15.

Abnormality	Human	Dog
Peliosis hepatis	+	+
Bacillary angiomatosis	+	+
Endocarditis	+	+
Myocarditis	+	+
Granulomatous		
lymphadenitis	+	+
hepatitis	+	+
panniculitis	+	+
Anterior uveitis	+	+
Encephalitis	+	+
Thrombocytopenia	+	+
Haemolytic anaemia	+	+

Serology, isolation and molecular detection of *Bartonella* species

Achieving an accurate microbiological diagnosis of bartonellosis can be extremely challenging, particularly in patients with chronic, long standing infections.[49,57] Conventional bacterial isolation techniques, ELISA, Western Blot or IFA detection of *Bartonella* spp. antibodies, and PCR amplification of *Bartonella* spp. DNA directly from patient samples all have diagnostic limitations.[49,56] Due to the fastidious growth requirements of this genus of bacteria, PCR amplification of organism-specific gene fragments is often used diagnostically, particularly when isolation was not successful and serology was negative (a frequent occurrence in bacteraemic dogs and humans). Direct culture of blood and other specimens (cerebrospinal fluid, joint fluid or cavitary effusions) onto blood agar plates from sick dogs or human patients has proven to be diagnostically insensitive.[15,49] Similar to microbiological culture onto blood agar plates, the sensitivity of PCR amplification of *Bartonella* spp. DNA directly from patient samples also is insensitive for the documentation of active infections. Therefore, our research found that enrichment culture of clinical specimens in an optimized, insect cell culture growth medium, prior to PCR testing, substantially increased the sensitivity of detecting infection and diagnosing bartonellosis, see below for more details.[21,49,58–61]

As with other infectious diseases, seroconversion (at least a four-fold rise in antibody titre over a 2–3 week period) can be used to confirm an acute case of bartonellosis. For reasons that remain unclear, antibody reactivity is detected in only 50% of dogs infected with *B. vinsonii* subsp. *berkhoffii* and 25% of dogs infected with *B. henselae*.[21] Research addressing these discrepancies is underway. From a zoonotic and comparative infectious diseases perspective, most dogs, some cats and many *Bartonella* bacteraemic human patients do not have antibodies against the infecting *Bartonella* sp. found in their blood or tissues.[21,48,58,59] Therefore, antibody testing remains highly insensitive. Serology can be used to support a clinical diagnosis; however, the presence of antibodies can only be used to infer prior exposure to a *Bartonella* sp. A seroreactive dog may be actively infected, but studies to characterize the association between serology and active infection in dogs are lacking. Based upon our testing, *B. henselae* and *B. vinsonii*

subsp. *berkhoffii* antibodies are infrequently detected in healthy or sick, well cared for dogs[16,18,54] For this reason, treatment of seroreactive, sick dogs or dogs from which any *Bartonella* spp. is isolated or DNA is detected in blood or tissue samples would be recommended.

Ideally, the microbiological diagnosis of all *Bartonella* infections should be confirmed by culturing the organism from blood, cerebrospinal fluid, joint fluid or aseptically obtained tissues (lymph node, spleen, heart valve) or by PCR amplifying Bartonella DNA directly from diseased tissues, such as skin. When testing cat blood samples, *B. henselae* and *B. clarridgeiae* can often be isolated effectively using agar plates, however, isolation of these same *Bartonella* spp. from dog, horse or human blood samples using an identical isolation approach is very insensitive.[58] Since 2005, we have used BAPGM: this is a novel, chemically modified, insect-based liquid culture medium to support the growth of *Bartonella* species.[58,61] This medium also facilitates documentation of co-infections with more than one *Bartonella* species or with other bacteria.[21,59–61] Obviously, the relative sensitivity of diagnostic methods used to detect *Bartonella* species infection greatly influences the results and interpretation of epidemiological studies, an investigator's ability to establish disease causation, or a clinician's ability to achieve a diagnosis and initiate appropriate treatment. The BAPGM platform combines enrichment culture of a clinical specimen in the liquid growth medium for a minimum of 7 days, followed by a highly sensitive PCR assay designed to amplify all known *Bartonella* species. The combined BAPGM enrichment culture/PCR assay has become the main testing platform utilized by the Intracellular Pathogen Research Laboratory (IPRL), North Carolina State University, USA, for research studies and is available from Galaxy Diagnostics, Inc. (contact@galaxydx.com; Morrisville, NC, USA) to document *Bartonella* infection in animals and immunocompetent or immunocompromised human patients. When compared with more traditional diagnostic methods, this combinational approach has facilitated the detection of canine infections with six *Bartonella* sp. (*B. henselae*, *B. koehlerae, B. quintana*, *B. vinsonii berkhoffii*, *B. bovis* and *Bartonella volans*-like), but of perhaps greater comparative microbiological importance, this approach has resulted in the successful isolation of *B. henselae* (among the only canine-derived isolates to date) from sick dogs.[15,21]

Therapy

To date, an optimal protocol has not been established for the treatment of chronic bartonellosis in cats, dogs or people.[3,15,62–64] Similar to other vector-borne infections, such as anaplasmosis, borreliosis and ehrlichiosis, many acutely infected individuals may eliminate *Bartonella* spp. immunologically at the time of initial infection, without the need for antibiotic administration. If bacteraemia becomes persistent, a long duration of antibiotic administration (4–6 weeks) may be necessary to eliminate a chronic infection regardless of the antibiotic(s) used for treatment. Due to the rapid development of antimicrobial resistance to macrolides (azithromycin), the author no longer recommends this class of antibiotics as sole therapy for treating *Bartonella* infections.[63] Fluoroquinolones in combination with doxycycline are currently being used by the author to treat clinical cases of bartonellosis, while exploring efficacy through experimental infection studies.[62,63] Despite clinical improvement or resolution of disease manifestations, doxycycline alone does not appear to eliminate *B. vinsonii* subsp. *berkhoffii, B. henselae* or *B. clarridgeiae* bacteraemia in cats, dogs or other animal species.[15,62,63] In human endocarditis patients, administration of aminoglycosides at the time of initial diagnosis improves prognosis and decreases morbidity and hospitalization duration.[65]

If seroreactive at the time of initial diagnosis, serum antibody titres decrease rapidly (3–6 months) and are generally no longer detectable in cats and dogs that recover following antimicrobial therapy.[3] Interestingly, seronegative animals with a history of chronic illnesses (presumably chronic *Bartonella* infection) can seroconvert within days to weeks after initiating antibiotic treatment, thereby providing support for a diagnosis of bartonellosis.[3,48] Therefore, post-treatment serology may be a useful adjunct to BAPGM enrichment PCR to determine if a patient is infected (i.e. patients initially seronegative and PCR negative in blood) or if therapeutic elimination of *Bartonella* infections has been achieved (initially seroreactive regardless of PCR status). Whether there is clinical benefit to follow serological or molecular assay results in cats has not been widely studied, but most treated cats do not become seronegative in the short term. Bacteraemia can resolve after treatment or resolve spontaneously in some cats, whereas other cats remain bacteraemic despite 4–6 weeks of antibiotic (documented for several antibiotic regimens) administration, and despite resolution of clinical abnormalities (such as lethargy, inappetence and fever).[48]

Prevention

There is increasing evidence that *Bartonella* species can be transmitted by fleas and ticks to cats, dogs and potentially to human beings.[15,20,66–68] Therefore, minimizing or eliminating flea and tick exposure is perhaps of greater veterinary and public health importance today, than during any previous time in history. After obtaining a blood meal from a *B. henselae*-infected cat, bacteria numbers increase within the flea's intestinal tract and the bacteria remain viable in flea faeces for at least 9 days.[24] Based upon experimental co-housing experiments, direct transmission from *B. henselae* bacteraemic cats to noninfected cats does not occur in the absence of fleas.[6,69,70] This suggests that human contact with cat saliva by licking, biting or scratches is an unlikely source of human infection, unless the claws or saliva are contaminated with viable *B. henselae* flea faeces. Thus, the best current preventive strategy to avoid *Bartonella* spp. infection in pets and their owners is the routine use of acaricide products to avoid flea infestations from occurring in the cat's environment. This conclusion is further supported by a PCR study that amplified *B. henselae* DNA from cat nail bed clippings or saliva, only if the cat was simultaneously infested with fleas.[71] Dogs have occasionally been implicated in the bite transmission of *B. henselae* to

humans and DNA of several *Bartonella* spp. has been amplified from dog saliva specimens[72] In addition, there was a statistical correlation between dog bites in China and seroreactivity to *Bartonella* spp. antigens.[73] In the United States, an estimated 12,000 outpatients are diagnosed with Cat Scratch Disease (CSD is an acute prototypical *B. henselae* human disease presentation) each year, with 500 patients hospitalized.[74] Inpatients were significantly more likely than outpatients to be male and were 50–64 years of age, suggesting the possibility of more severe disease in older patients. Clearly, additional research is needed to assess the risks associated with vector exposure, contact with flea faeces through inhalation or wounds, and pet bites or scratches.

Veterinary clinicians are frequently consulted about flea infestations and flea allergy dermatitis, and therefore understanding the environmental and biological interactions between cat fleas and *Bartonella* spp. is of clinical importance and facilitates zoonotic disease prevention education. In the context of One Health, veterinarians play a critically important public health role in prevention of zoonotic disease transmission through education and communication with clients, physicians and other health professional colleagues. When rigorous flea and tick control measures are instituted, it is highly probable that transmission of *Bartonella* species to pets and their owners will be greatly reduced or eliminated.[6,69,70] In the context of cats, fleas and CSD, flea-infested cats around the world play an important role in scratch transmission of *B. henselae* (and potentially other *Bartonella* spp. as described above), which is the primary or sole bacterial cause of CSD.[3,74]

Public and occupational health considerations

There is increasing evidence to support a potentially important role for *Bartonella* species as a cause of a spectrum of chronic disease manifestations in human patients.[2–4,15,72] Currently, arthropod exposure and animal contact represent the most important risk factors for a human being acquiring an infection with a *Bartonella* spp. Due to extensive contact with a variety of animal species and at times their associated arthropod infestations, veterinary and other animal workers appear to be at occupational risk for bartonellosis because of frequent exposure to *Bartonella* spp.. As a cosnequence, these individuals should exercise increased precautions to avoid arthropod bites, arthropod faeces (i.e. fleas and lice), animal bites or scratches and direct contact with bodily fluids from sick animals.[10,15,59,67–69,75] As *Bartonella* spp. have been isolated from cat, dog or human blood, cerebrospinal fluid, joint fluid, aqueous fluid, seroma fluid and from pleural, pericardial and abdominal effusions, a substantial number of diagnostic biological samples collected on a daily basis in veterinary practices could contain viable bacteria. The increasing number of defined *Bartonella* spp., in conjunction with the high level of bacteraemia found in reservoir-adapted hosts, which represent the veterinary patient population, ensures that all veterinary professionals will experience frequent and repeated exposure to animals harbouring these bacteria. Therefore, personal protective equipment, frequent hand washing, and avoiding cuts and needle sticks have become more important as our knowledge of this genus has improved and various modes of transmission, including needle sticks, have been defined.[76]

Physicians should be educated as to the large number of *Bartonella* spp. in nature, the extensive spectrum of animal reservoir hosts, and the diversity of confirmed and potential arthropod vectors, current limitations associated with diagnosis and treatment efficacy, and the ecological and the medical complexities induced by these highly evolved intravascular, endotheliotropic bacteria. Also, based upon recent microbiological findings, physicians and microbiologists should also be aware of the potential for perinatal and familial infections with one or more *Bartonella* species.[8,77–80] In family clusters reported to date, the route(s) of transmission to or among family members remains unclear and the mode(s) of transmission may vary among and within families depending on environmental, social, microbiological and epidemiological factors. Importantly, pets can serve as sentinels for human exposure and humans can serve as sentinels for pet vector infestations, potentially resulting in longstanding *Bartonella* spp. bacteraemia and complex disease manifestations in both pets and owners.[29,30] In conclusion, a One Health approach to the genus *Bartonella* and the diseases collectively called bartonellosis, should improve animal and human health throughout the world.

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5.3

Clinical, microscopic and microbial characterization of exfoliative superficial pyoderma-associated epidermal collarettes in dogs

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Background – The microscopic and microbial features of the spreading epidermal collarettes of canine exfoliative superficial pyodermas are poorly characterized.

Objectives – To characterize the clinical, cytological, microbial and histopathological features of epidermal collarettes in five dogs.

Results – Cytology from the margins of collarettes identified neutrophils, extracellular and intracellular cocci within neutrophils but no acantholytic keratinocytes. Phenotypic and genotypic analyses identified all bacterial isolates from the centre and margin of five epidermal collarettes as *Staphylococcus pseudintermedius*. PCRs of collarette-associated *Staphylococcus* strains did not amplify genes encoding for the known exfoliative toxins *expA* and *expB,* whereas the predicted *siet* and *speta* amplification products were detected in all isolates.

Microscopically, epidermal collarettes consisted of interfollicular, epidermal spongiotic pustules. Advancing edges of lesions consisted of peripheral intracorneal clefts in the deep stratum disjunctum above an intact stratum compactum; they contained lytic neutrophil debris, bacterial cocci and fluid, but no acantholytic keratinocytes. This intracorneal location of bacteria was confirmed using Gram stains and fluorescent *in situ* hybridization with eubacterial- and *Staphylococcus*-specific probes. The indirect immunofluorescence staining patterns of desmoglein-1, desmocollin-1, claudin-1, E-cadherin and corneodesmosin were discontinuous and patchy in areas of spongiotic pustules, whereas only that of corneodesmosin was weaker and patchy in advancing collarette edges.

Conclusion – Epidermal collarettes represent unique clinical and histological lesions of exfoliative superficial pyodermas that are distinct from those of impetigo and superficial bacterial folliculitis. The characterization of possible causative staphylococcal exfoliatin proteases and the role of corneodesmosin in collarette pathogenesis deserve further investigation.

Introduction

Staphylococcus (*S.*) *aureus* is an important pathogen of humans that causes – in addition to skin diseases of other phenotypes –two blistering dermatoses: bullous impetigo (BI) and the staphylococcal scalded skin syndrome (SSSS).[1] Both conditions share a common pathogenesis that involves the proteolytic cleavage of the extracellular segment of human desmoglein 1 (DSG1) by *S. aureus* exfoliatin toxins A, B or D (ETA, ETB, ETD); these entities differ mainly in the site of toxin secretion and extent of skin damage.[2–4] In BI, a common skin infection of children, the local production of exfoliatin toxin produces small vesicles that enlarge rapidly into superficial flaccid bullae filled with a cloudy fluid and surrounded by an erythematous rim. These bullae rupture easily, leaving shiny erosions with scale-crusts.[1] The SSSS is a generalized exfoliative dermatosis that most commonly affects newborns, young children or sometimes adults with immunosuppression or renal failure.[1] In this syndrome, an extracutaneous infection (of the pharynx, umbilicus, nose, ear or conjunctiva) with exfoliatin-producing staphylococci results in high levels of these toxins in the circulation, and this leads to sloughing of the epidermis that exfoliates in large sheets overlying widespread erosions.[1] Histologically, both diseases share a similar intraepidermal cleavage beneath or within the stratum granulosum, with the difference being that the remainder of epidermis in SSSS appears normal, whereas in BI blisters are filled with neutrophils and inflammation is present in the dermis.[5]

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In dogs, there is increasing evidence suggesting that some lesions of superficial bacterial skin infections are caused by *S. pseudintermedius* producing exfoliative toxins. Although a first attempt failed to detect *in vitro* exfoliative toxin production from staphylococci historically classified as *S. intermedius* in dogs,[6] a 30 kDa *S. intermedius* exfoliatin toxin (SIET) has been isolated.[7] Subcutaneous injections of purified SIET into the skin of two dogs reportedly induced lesions with a positive Nikolskiy sign and secondary crusting after 40 h, but the level of epidermal cleavage was not described or histologically confirmed.[7] In contrast to these findings, recombinant SIET has been shown not to cause intraepidermal splitting when injected in canine skin; SIET also was found to be unable to digest canine DSG1 and DSG3.[8] At this time, whether or not SIET is relevant to the pathogenesis of canine exfoliative superficial pyoderma (ESP) remains unknown.

A novel exfoliative toxin (EXI with "I" for "*intermedius*") was identified after screening 43 strains of *S. pseudintermedius* isolated from dogs with uncharacterized pyoderma using PCR with degenerate primers for consensus sequences of the exfoliative toxins of *S. aureus* (ETA, ETB, ETD) and *S. hyicus* (SHETB).[9] Thereafter, EXI-negative *S. pseudintermedius* strains were isolated from a pustule of a miniature dachshund diagnosed with BI, and were found to have a gene encoding another protein that resembled EXI.[10] The *S. pseudintermedius* exfoliative toxin EXI was then renamed EXPA (*ex*foliatin of *S. pseudintermedius* type *A*) and the novel protein was named EXPB (*ex*foliatin of *S. pseudintermedius* type *B*). Results from this study also showed that both EXPA and EXPB could digest canine DSG1 and cause subcorneal splits in the epidermis when injected in mice, characteristics that are likely relevant to the pathogenesis of intraepidermal splitting in canine impetigo.[8]

Whereas the clinical and histological characteristics of impetigo and superficial bacterial folliculitis have long been described in dogs,[11] those of exfoliative forms of superficial staphylococcal infections have not been studied in depth. The clinical lesions of epidermal collarettes – expanding coalescing erythematous rings with peripheral peeling – were first described as a lesional stage of superficial bacterial folliculitis.[12] The term "superficial spreading pyoderma" was used to describe a condition in a border collie with chronic, recurrent epidermal collarettes on the ventral abdomen with microscopic examination revealing superficial epidermal spongiotic pustules with lifting of the stratum corneum.[13] It was later proposed that this condition be renamed "exfoliative superficial pyoderma" to better account for the exfoliative superficial feature of this entity and to differentiate it from a worsening bacterial folliculitis that would also qualify for the same "superficial spreading pyoderma" denomination.[14] However, despite proposed changes in disease terminology, the characteristics of epidermal collarettes remain poorly characterized and it is still classified under the umbrella term of "superficial bacterial folliculitis".[15]

In order to test the hypothesis that ESP-associated epidermal collarettes represent a unique clinical phenotype, potentially due to superficial epidermal splitting by protease(s) that cleave superficially expressed keratinocyte adhesion molecule(s), our objectives were to characterize the clinical, cytological, microbial and histopathological features of epidermal collarettes that occur in canine ESP. Furthermore, the expression patterns of the principal superficial epidermal desmosomal and nondesmosomal adhesion proteins were evaluated in biopsies collected at the advancing edges of epidermal collarettes to detect any evidence of suspected toxin-related adhesion protein digestion.

Material and methods

Study participants

Dogs of any breed, body weight and sex with ESP-associated epidermal collarettes were selected for the study. Dogs with different stages (early, expanding) of epidermal collarettes were screened and dogs were selected if they that had at least one actively expanding epidermal collarette, which developed within the preceding 2 days with an erythematous rim. Dogs with the more widespread phenotype of ESP,[11] which is characterized by regional and/or generalized erythema with scaling composed of large sheets of stratum corneum, were excluded. To be selected, dogs were not being currently treated with antibacterial shampoos, systemic and/or topical antibacterial agents. Withdrawal times from anti-inflammatory medications were 4 weeks for topical (skin and ear) and oral glucocorticoids and 8 weeks for injectable glucocorticoids and oral ciclosporin. The study was preapproved by the Institutional Animal Care and Use Committee, and all owners were provided a written informed consent.

Skin cytology, bacterial cultures and susceptibility testing

Two samples were obtained using a nonsterile cotton-tipped applicator from the leading edge (margin) and the centre of expanding epidermal collarettes. All slides were heat-fixed and stained with Diff–Quick (Fischer Scientific; Pittsburgh, PA, USA) and Gram (Becton-Dickinson and Company; Sparks, MD, USA) stains. Each slide was evaluated in its entirety, by the same investigator (FB), using a subjective, semiquantitative method at high power (×100). The presence of neutrophilic granulocytes, lymphocytes, nonlymphocyte mononuclear cells (Langerhans cells, macrophages), eosinophils, cocci bacteria (free or intracytoplasmic in neutrophils), other bacteria and acantholytic keratinocytes was graded 0–4+. The Gram-stained slides were evaluated for the presence and types of bacteria, and these were graded as above. Sterile culture swabs were taken from sites different from those of cytology at the leading edge and the centre of expanding epidermal collarettes. The swabs were inoculated onto Columbia agar with 5% sheep blood (Fischer Scientific) using the four quadrants technique and incubated at 35°C for 18–24 h. All isolates were identified phenotypically, biochemically and through PCR amplification of *hsp60* and *nuc* gene, as described previously.[16,17] Antimicrobial susceptibility by the broth microdilution method was performed using an automated system (Sensititre, Trek Diagnostic Systems; Cleveland, OH, USA) according to the Clinical and Laboratory Standards Institute guidelines for minimum inhibitory concentration testing.[18] Meticillin resistance was confirmed by PCR amplification of *mecA*, the gene conferring meticillin-resistance.[19] All *S. pseudintermedius* isolates were stored frozen (−80°C) in tryptic soy broth containing 15% sterile glycerol until further testing was performed.

Genotypic relatedness of isolated staphylococcal strains from epidermal collarettes

Preparation of bacterial genomic DNA, digestion by *Sma*I and pulse field gel electrophoresis (PFGE) were performed according to methods modified from the Centers for Disease Control (Atlanta, GA, USA) methods.[20] Similarity coefficients were calculated and dendrograms constructed using the Dice coefficient and unweighted pair group

method with arithmetic means, respectively, with an optimization value of 1.0% and a position tolerance of 0.65%. Isolates were considered to be of similar genotypes if PFGE band patterns were identical. Genotypes with grouping above 80% on the dendrogram (BioNumerics 6.0, Applied Maths Inc.; Austin, TX, USA) were considered members of the same genotype group or cluster of similarity.[21]

Detection of *S. pseudintermedius* exfoliative toxin genes

The presence of *S. pseudintermedius* exfoliative toxin genes *expa*, *expb* and *siet* in the isolated staphylococcal strains was determined by PCR using previously described primers.[8,9] In addition, bacterial strains were analysed for the presence of a novel suspected *S. pseudintermedius* exfoliative toxin SPETA using the published sequence (Table S1 in Supportng information).[22] Ten frozen *S. pseudintermedius* isolates collected from pustules of dogs with superficial bacterial folliculitis served as controls, as previously suggested.[10] Frozen isolates were revived from the glycerol stock, thawed and inoculated into Columbia agar with 5% sheep blood agar and incubated at 35°C for 18–24 h. Template DNA was prepared by a simple and rapid boiling procedure.[23] The PCR assays were performed as described previously;[9] annealing temperatures were chosen depending on the primer set used (Table S1 in Supporting information). All PCR products were resolved by electrophoresis through a 1.2% (w/v) agarose gel, and visualized by the application of the SYBR safe DNA gel stain (Fischer Scientific). The PCR products of predicted size from positive isolates were purified using QIAquick PCR Purification Kit (Qiagen Inc.; Valencia, CA, USA) and sequenced. The sequences were aligned using the ABI Sequencing analysis software, with contiguous sequences matched to the GenBank database using the Basic Local Alignment Search Tool (BLAST) and positively identified if there was ≥98% sequence similarity with a reference sequence.

Biopsy samples for histopathology, fluorescent *in situ* hybridization (FISH) and immunomapping

All dogs were sedated with medetomidine (Domitor, Zoetis; Florham Park, NJ, USA) intravenously. An injection of 1 mL of lidocaine hydrochloride 2% (Hospira Inc., Lake Forest, IL, USA) was administered subcutaneously to provide additional local anaesthesia. One 8 mm skin biopsy sample was taken from the leading edge of epidermal collarettes and bisected: one half was placed in 10% neutral buffered formalin for paraffin embedding and routine histopathology, whereas the second half was immediately frozen in liquid nitrogen, and then stored at –80°C for subsequent adhesion molecule immunomapping. If a small, presumed early, epidermal collarette was present together with expanding collarettes, then an additional 8 mm biopsy of the early lesion was taken and processed in a similar way. Additionally, as a control for immunomapping, an 8 mm skin sample was collected from normal skin at least 10 cm away from any lesion.

For histopathology, formalin fixed, paraffin-embedded skin biopsy samples were routinely processed, sectioned to five micrometres and stained with haematoxylin and eosin (H&E) or Gram stain. Five histological step-sections were evaluated for each sample (four H&E and one Gram stain). Histological preparations were subjectively evaluated by two of the authors (KL and FB) who were both blinded to the type of gross lesion sampled and to the presence or absence of bacteria identified by cytology and/or culture. The presence and/or severity of histopathological changes were scored as absent, mild, moderate or marked. Clefts in the epidermis were evaluated for the level of epidermal separation and for cleft contents (bacteria, acantholytic keratinocytes, inflammatory cells and fluid); individualized corneocytes were also scored in clefts and crusts. The epidermis and dermis were evaluated for injury and inflammation, with attention to the absence or presence of any changes in relation to the advancing, superficial and deep peripheral margin of epidermal clefts. This included the pattern of cornification above and below clefts, as well as the integrity of stratum disjunctum and compactum. The presence of epidermal pustules, including spongiotic or acantholytic type, was recorded. Hair follicles were assessed for the changes identified in the epidermis, as well as for extension of epidermal clefts into infundibula, folliculitis, follicular atrophy and the stages of the follicle growth cycle present.

In order to further confirm the location of bacteria within the tissue, formalin-fixed, paraffin-embedded histopathological sections (5 Im) from the sampled epidermal collarettes were evaluated with FISH using eubacterial (EUB338; GCTGCCTCCCGTAGGAGT) and *Staphylococcus* spp. (TCCTCCATATCTCTGCGC) probes, as described previously.[24]

Finally, indirect immunofluorescence (IF) with monoclonal or polyclonal antibodies specific for the corneodesmosome protein CDSN, the desmosomal (and corneodesmosomal) proteins DSG1 and desmocollin 1 (DSC1), the tight junction transmembrane adhesion molecule claudin 1 (CLDN1) and the adherens junction protein E-cadherin (cadherin-1, CDH1) were performed on the frozen 5 Im thick sections of each skin biopsy samples collected from dogs with epidermal collarettes, as described previously.[25] Additionally, immunomapping was performed on the punch biopsy samples collected from skin of normal appearance of each dog as controls. Furthermore, IF of frozen sections was performed using the serum of a human patient with endemic pemphigus foliaceus who had high levels of autoantibodies against the extracellular aminoterminus of DSG1 (Zhi Liu, University of North Carolina Chapel Hill). The pattern of fluorescence was recorded as described previously[25] and any deviation from the normal pattern was described subjectively.

Results

Study subjects and skin cytology

Five dogs of different breed, age and weight met the inclusion criteria (Table 1). All dogs presented with widespread expanding epidermal collarettes (Figure 1), ranging in size from 1.5 to 4 cm and which were distributed on the lateral thorax, axilla, ventral abdomen and inguinal areas. In three dogs (cases 1, 2 and 5), additional lesions of early collarettes were present (Figure 1b). All collarettes sampled had an active erythematous rim with a peripheral scaling/crusting (Figure 1). Focal, follicular-centred papules, pustules and crusts, representing typical clinical lesions of superficial bacterial folliculitis, were not observed in any dogs. Detailed cytology results are presented in Table 1. Neutrophils, as well as extracellular and intracellular cocci within neutrophils, were identified from marginal cytology samples in all dogs. Acantholytic keratinocytes were not seen, but occasional non-acantholytic keratinocytes were found in marginal samples of dogs 2 and 3. Gram stains of cytology swab samples taken from the margin of epidermal collarettes revealed Gram-positive cocci in all dogs.

Bacterial culture, susceptibility testing and genotypic relatedness

Phenotypic, biochemical and genotypic analysis identified all bacterial isolates from the centre and margin of epidermal collarettes as *S. pseudintermedius*. The degree of bacterial growth between marginal and centre samples differed in three dogs (case 1, 4 and 5), being more abundant when sampled at the margins of epidermal collarette (Table S2 in Supportng information). There were no differences in the antimicrobial susceptibility profiles between bacterial isolates collected from the margin or the centre of an epidermal collarette from the same dog (Table S2 in Supporting information). Meticillin resistance was determined in isolates from two dogs and the presence of

Table 1. Signalment, skin cytology results and sampling sites (number of biopsies) for adhesion molecule immunomapping of five dogs with epidermal collarettes.

		Skin cytology				Immunomapping of adhesion molecules		
		Diff-Quick stain		Gram stain				
Case number	Breed, age, sex	Margin	Centre	Margin	Centre	Margin of expanding collarette	Early collarette	Normal skin
Case 1	Dachshund, 1.5 years, mc	2+ neutrophils, 1+ cocci (intra- and extracellular)	None	2+ cocci	–	+	+	+
Case 2	Hound, 10 years, mc	2+ neutrophils, 2+ cocci (intra- and extracellular), 1+ non-acantholytic keratinocytes	None	2+ cocci	–	++	–	+
Case 3	American Staffordshire terrier, 5 years, mc	1+ neutrophils, 1+ cocci (intra- and extracellular), 1+ non-acantholytic keratinocytes	1+ non-acantholytic keratinocytes	1+ cocci	–	+	+	+
Case 4	Golden retriever, 5 years, mc	1+ neutrophils, 1+ intracellular cocci	None	1+ cocci	–	+	–	+
Case 5	Irish setter, 9 years, mc	1+ neutrophils, 1+ cocci (intra and extracellular)	None	1+ cocci	–	+	+	–

mc, male castrated.
The Diff-Quik® cytology findings using subjective semiquantitative method (0–4+) and presence of bacteria on Gram stains from margin and centre of epidermal collarettes are described.

mecA gene was confirmed in these two isolates (cases 1 and 5). Marginal and centre-collected ESP bacterial isolates from three dogs (cases 1, 4 and 5) were defined as multidrug resistant according to definitions proposed for *S. aureus*.[26] Six bacterial isolates collected at the margin and centre of epidermal collarettes from three dogs (cases 2, 3 and 5) were available for PFGE analysis; the remaining four bacterial isolates (cases 1 and 4) were not available for testing. According to PFGE analysis, marginal and centre-collected ESP bacterial isolates from the same dog had identical band patterns suggesting that they were identical isolates. Furthermore, isolates from cases 2 and 3 belonged to the same genotype (Figure S1 in Supporting information).

Detection of *S. pseudintermedius* exfoliative toxins

Six bacterial isolates from three dogs (case 2, 3 and 5) were available for PCR analysis. The analysis of these isolates showed an absence of exfoliative toxin genes *expa* and *expb*, whereas amplicons corresponding to the predicted sizes of *siet* and *speta* products were present in all isolates. These exfoliative toxin genes were also detectable in the ten control *Staphylococcus* isolates from superficial bacterial folliculitis: *expa* (3/10; 30%), *expb* (1/10; 10%), *siet* (8/10; 80%) and *speta* (9/10; 90%). Sequencing of the amplicons from epidermal collarette and control isolates of exfoliative toxin genes *expa*, *expb*, *siet* and *speta* confirmed the 100% sequence identity when compared with the corresponding published gene sequence.

Histopathology

A total of nine skin lesions (six expanding and three early collarettes) from five dogs were evaluated in 45 histological sections. At the outer leading edge of epidermal collarettes, large laminar epidermal clefts (eight of nine; 89%) occurred in the deep stratum disjunctum, just above the stratum compactum (Figures 2 and 3). The roof of clefts had a normal basket-weave pattern of cornification (eight of eight; 100%) that centrally often appeared thinner than normal due to the release of individualized corneocytes from its inner surface. At the cleft base, a thin layer of stratum disjunctum was present or, more often, the exposed surface of the stratum compactum was observed, which was mostly intact. Near the leading cleft margin, the stratum compactum was disrupted multifocally by discrete small areas of superficial corneocyte swelling and pallor (six of eight; 75%), whereas occasional full-thickness loss of stratum compactum was observed for only a few corneocytes. In more central areas, along the cleft base, very small parakeratotic foci (seven of eight; 88%) and/or mild lamination and thickening of the stratum compactum were seen.

The epidermis underlying the leading edge of clefts was minimally altered, and only mild epidermal hyperplasia and occasional individual lymphocytic exocytosis occurred (Figure 2a). The epidermis just behind the peripheral cleft margin contained multifocal, discrete, epidermal spongiotic foci with neutrophil exocytosis (five of eight; 63%) that progressed to spongiotic pustules (Figure 2b,c). Above these spongiotic foci, the stratum compactum disintegrated into individual corneocytes, and neutrophils transmigrated to the cleft lumen and spilled onto the cleft floor along with fluid (Figure 2c). More centrally, small crusts formed on the cleft floor from resolving neutrophilic spongiotic pustules (seven of eight; 88%), often still under an intact cleft roof formed by the upper stratum disjunctum layers. Interestingly, acantholytic keratinocytes were not observed (zero of eight; 0%) and acantholytic epidermal pustules were not a feature in any skin sections.

The lumen of stratum corneum clefts at the expanding collarette margin contained either only bacteria and fluid, or lytic neutrophil debris and fluid with bacteria (Figure 3a, b). Cocci were present individually and/or in clusters in clefts (seven of eight; 88%) (Figure 3b), either free or closely apposed to the cleft base, cleft roof or individualized

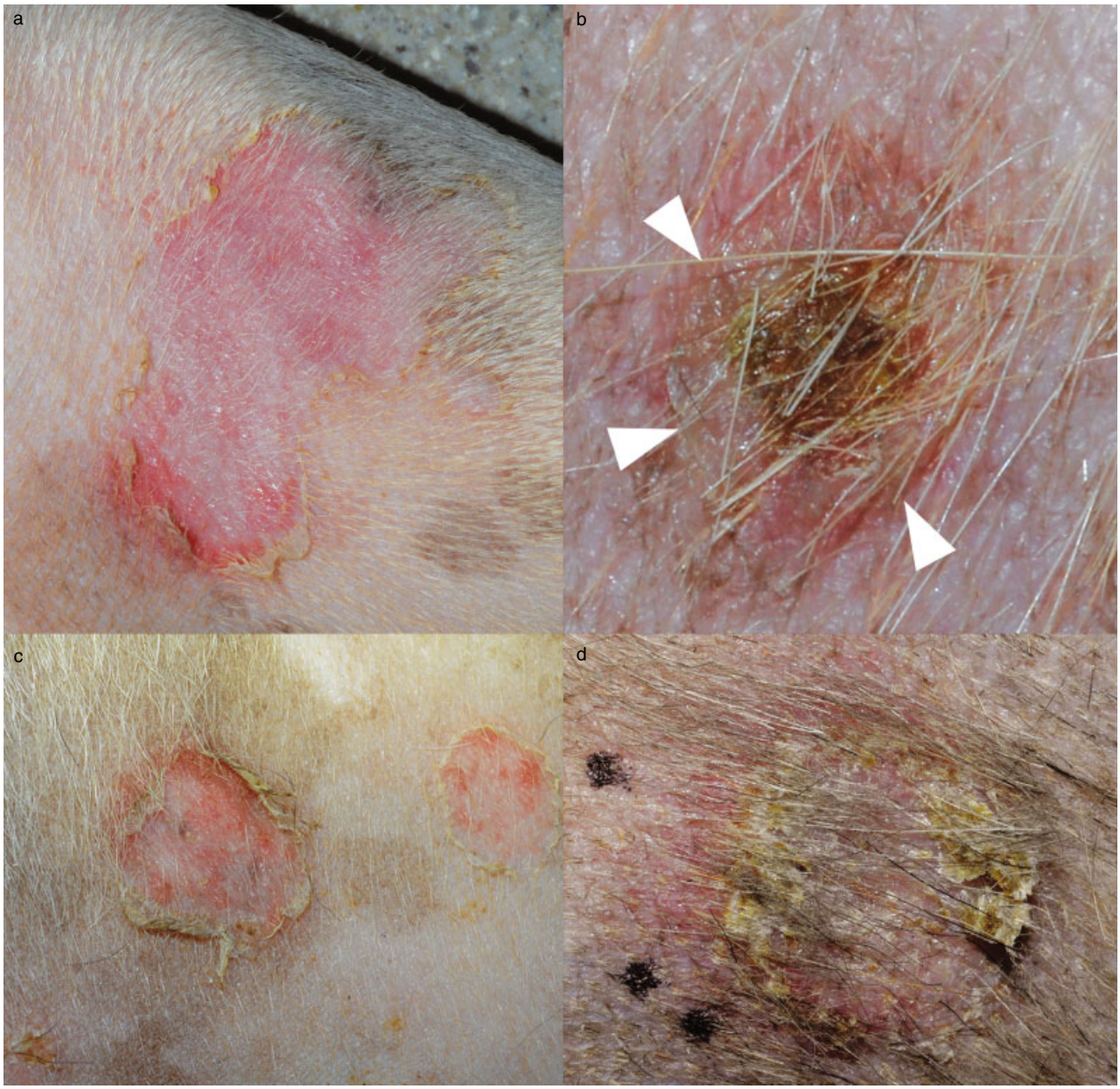

Figure 1. Canine epidermal collarettes – clinical lesions. (a,c,d) Annular to polycyclic epidermal collarettes with erythema, lifting and peeling of stratum corneum, and partial alopecia confined to the centre of the lesions (a,c – Case 3; d – Case 1). (b) Early epidermal collarettes were characterized by central flattened crust with erythematous leading edge and lifting of peripheral stratum corneum (arrowheads) (Case 3).

corneocytes. Gram stains and FISH (Figure 4), the latter performed with both eubacterial and *Staphylococcus* probes, confirmed *Staphylococci* in the leading cleft. Gram-positive cocci were also observed in spongiotic foci and focal crusts (six of eight; 75%) and sometimes closely apposed to the cleft base or roof, more centrally. A few clefts had no visible content at their margin, perhaps lost from aging of lesions or from tissue processing.

In the dermis, superficial perivascular dermatitis (nine of nine; 100%) was generally moderate centrally, mild or absent under the most peripheral epidermal cleft and included lymphocytes, plasma cells, neutrophils and eosinophils. Neutrophils were most numerous under neutrophilic spongiotic foci in the epidermis. Neutrophils were not observed in the epidermis below the leading edge of the epidermal cleft (eight of eight; 100%) and generally did not extend in the dermis beyond the margin of the epidermal cleft. A mild eosinophilic dermal infiltrate was common (nine of nine; 100%) and mild exocytosis and mild eosinophilic micropustules were occasional in the epidermis of more central collarette areas of biopsies. Dermal oedema, fibrin exudation and haemorrhage were most often mild or absent in the superficial dermis. Hair follicles were generally not affected and extension of epidermal clefts into hair follicle infundibula (two of nine; 22%) was uncommon and mild. Neutrophilic folliculitis (zero of nine; 0%) was not observed. A single follicle in the central collarette area had mild luminal eosinophilic folliculitis (one of nine; 11%). Biopsy sections contained few hair follicles and typically occasional mildly atrophic follicles, lacking a hair shaft, were present with a few anagen and/or telogen follicles.

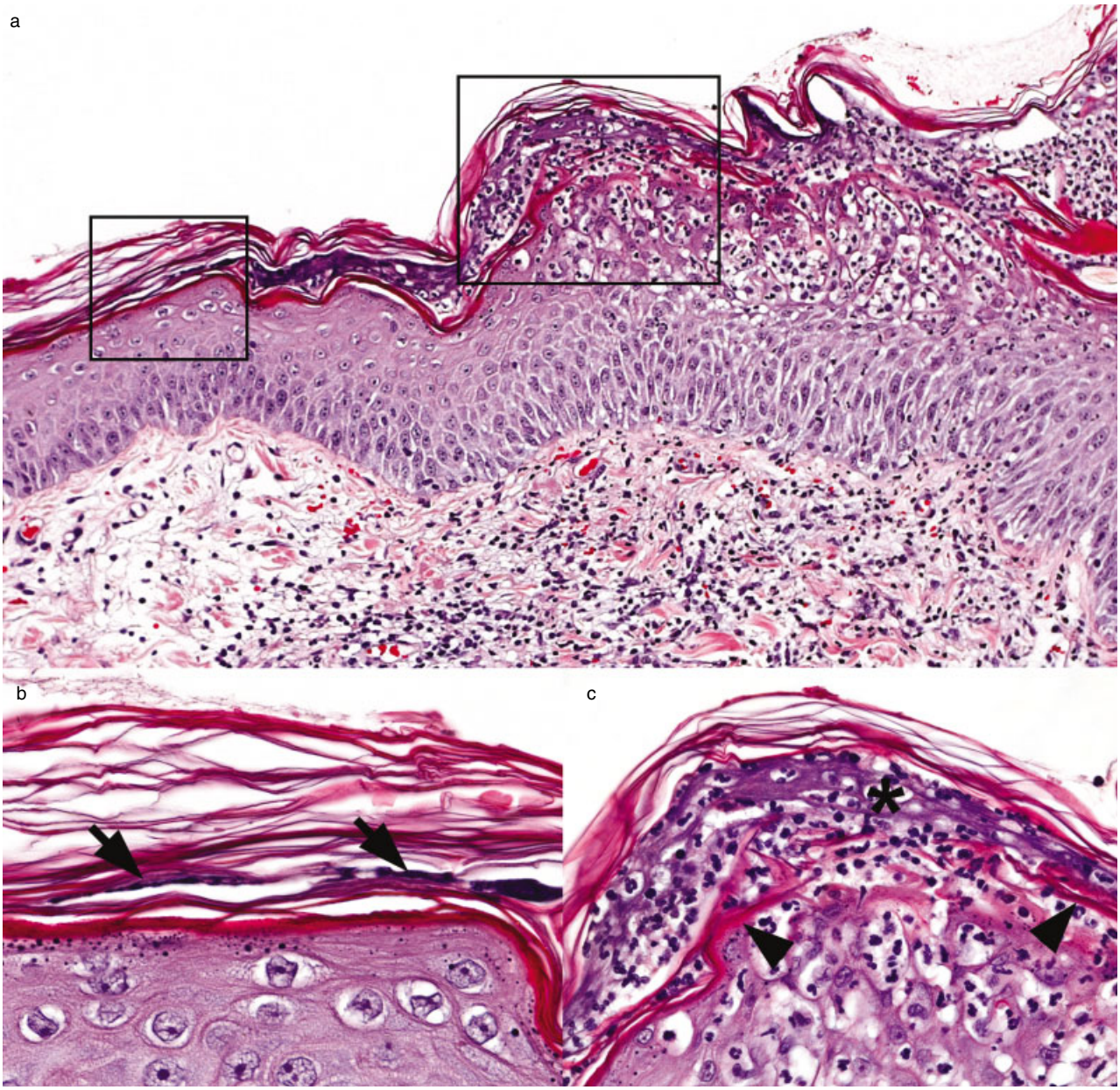

Figure 2. Exfoliative superficial pyoderma (ESP) in a dog. Photomicrograph of Case 4. (a) The peripheral spreading margin of an ESP skin lesion (small box) is an intracorneal cleft with no associated inflammation in the epidermis below. Spongiotic neutrophilic pustules (large box) are present just behind the leading margin of the cleft. (b) A high magnification photomicrograph from the small box insert in image (a) reveals that intracorneal clefts (arrows) spread initially in the deep stratum disjunctum, just a few layers above the stratum compactum. (c) A high magnification photomicrograph from the large box insert in image (a) captures a portion of a spongiotic pustule where it disrupts the stratum compactum (arrowheads) and provides fluid and neutrophils (asterisk) to the intracorneal cleft. Corneocytes of the stratum compactum appear individualized with neutrophils at the site of disruption. Haematoxylin and eosin. 20×

Immunomapping of adhesion molecules

Immunomapping was performed on three biopsies collected from early collarettes and seven biopsies from larger expanding lesions with staining of normal skin from each dog serving as a control. The expression patterns of DSG1, DSC1, CLDN1, CDH1 and CDSN were assessed for their continuity on the entire epidermis of each section with detailed examination of advancing intracorneal cleavage areas. The staining patterns of DSG1, DSC1, CLDN1, CDH1 and CDSN in control canine skin samples (normal skin) were identical to previously reported patterns for normal canine skin (data not shown).[25] The skin tissues samples from the early and expanding epidermal collarettes contained normal (i.e. basket-weave) and stratum corneum separation sites, as they were taken at the margin of the lesions. The immunostainings for DSG1, DSC1, CLDN1, CDH1 and CDSN were discontinuous and patchy in areas of spongiotic pustules, whereas only that of CDSN was weaker and patchy in advancing collarette edges (Figure 5).

Discussion

Two often overlapping phenotypes of ESP are proposed to exist in dogs: the first, which is more common, is characterized by skin lesions of rapidly expanding epidermal

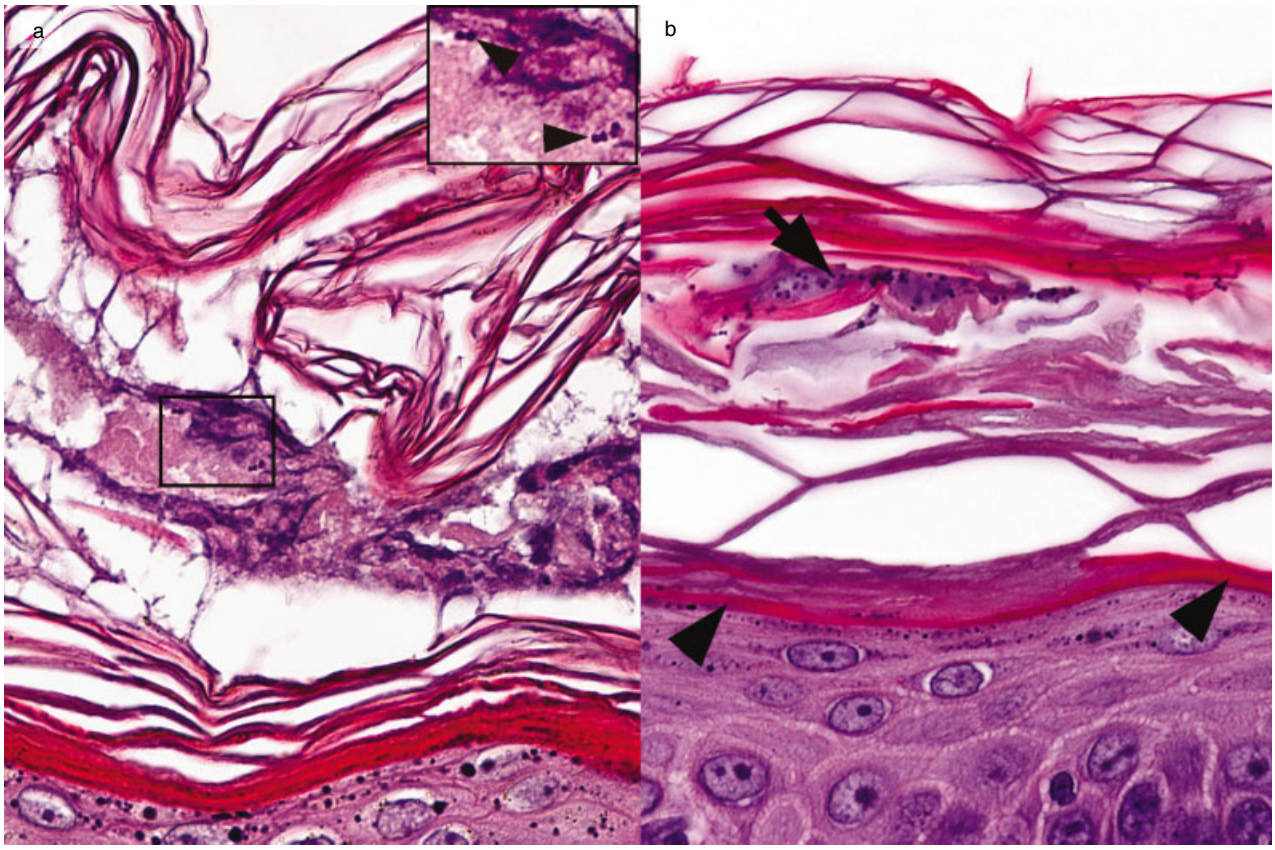

Figure 3. Exfoliative superficial pyoderma (ESP) in a dog. Photomicrographs of Case 2 (a) and Case 3 (b). (a) A peripheral intracorneal cleft in the deep stratum disjunctum contains bacteria (box insert, arrowheads) floating in fluid and lytic neutrophilic debris. (b) Some peripheral clefts contained bacteria (arrow) and only a scant amount of fluid. Below some clefts, thinning and discontinuity of the stratum compactum (arrowheads) is associated with swelling, pallor and separation of corneocytes (between arrow heads). Haematoxylin and eosin. 60×

collarettes that can coalesce to cover large areas with a polycyclic pattern, whereas the second type consists of an acute onset of regional or generalized erythema with overlying scaling composed of large sheets of stratum corneum.[11,14] The present study establishes the clinical, cytological, microbial and histopathological features of ESP-associated epidermal collarettes in five dogs. In these dogs, epidermal collarettes featured an erythematous leading edge where the stratum corneum lifted and peeled in a round, oval to polycyclic pattern with partial alopecia confined to the centre of the lesions. Hyperpigmentation, presumed post inflammatory, in the centre of epidermal collarettes is sometimes a sequel of aging lesions, but it was not seen in this study due to their acute onset, which was a criterion for inclusion. Although concurrent signs of bacterial folliculitis were not detected in any dogs, ESP-associated epidermal collarettes anecdotally have been mentioned to co-exist in some dogs with superficial bacterial folliculitis.[13]

The phenotypic and genotypic analysis of bacterial cultures from the leading edge and centre of ESP-associated epidermal collarettes identified *S. pseudintermedius* as the main causative agent of epidermal collarettes in dogs, which is similar to previous results.[13,27] The identical antibiotic susceptibility pattern and PFGE results of bacterial isolates collected from leading edges and centres of epidermal collarettes suggested that each lesion was caused by a single strain of *S. pseudintermedius*. The sampling method used in this study yielded higher bacterial growth on blood agar when sterile swabs were rolled gently over erosions underneath the stratum corneum of clefts at the leading edge than from sampling the centre of collarettes. This finding corresponds to the detection of a high number of bacteria in the superficial stratum corneum at the leading edge of skin sections using cytology, Gram stains and FISH. The appropriateness of sampling methods for ESP-associated epidermal collarettes has received only limited scrutiny. One study recommended no surface disinfection before bacterial isolation and rolling a sterile swab three to four times across the epidermal collarettes, which resulted in four negative bacterial cultures from 22 dogs with epidermal collarettes.[27] In contrast, the results of our study, which included the identification of bacteria in skin sections using routine and specialized histological techniques, support a sampling method underneath the scales/crusts at their leading edge for future studies on epidermal collarettes. Furthermore, given the findings reported in an abstract that different staphylococcal strains may be associated with superficial pyoderma lesions in the same dog,[28] an investigation using PFGE analysis is necessary to compare the genetic relatedness of isolates across multiple epidermal collarettes in order to understand if all epidermal collarettes on the same animal are caused by the same bacterial strain. This information about clonal relationship may have a crucial impact for therapeutic effectiveness, because any possible variability in the staphylococcal strains between epidermal collarettes

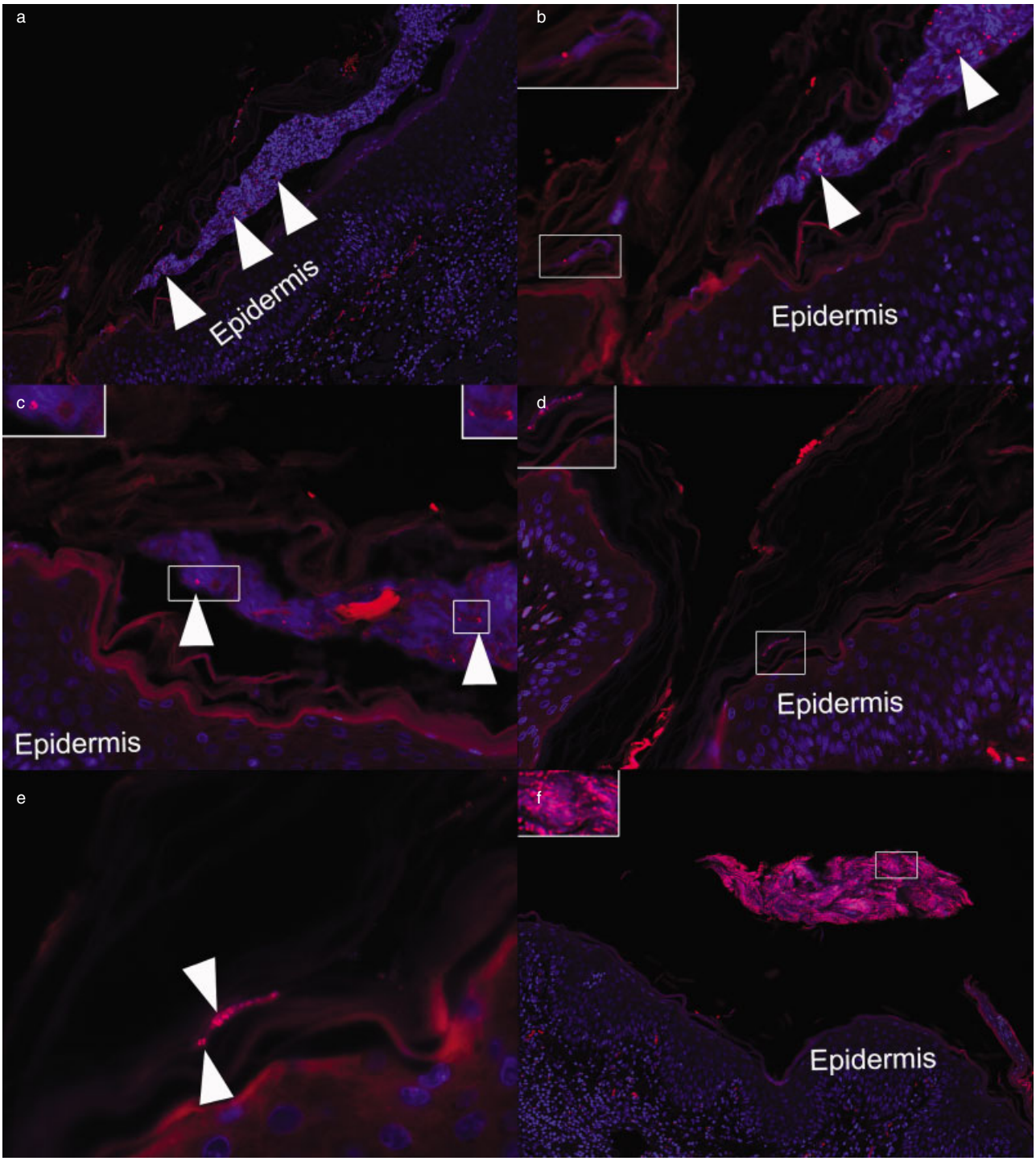

Figure 4. Fluorescence *in situ* hybridization (FISH) of paraffin-embedded skin sections from collected epidermal collarettes using *Staphylococcus* spp.-specific probe. (a,b,c) At the leading edge of expanding and early epidermal collarettes within the stratum corneum, large epidermal clefts contain numerous FISH-labelled coccoid bacteria (arrowheads), visible as red organisms within the blue staining lytic neutrophil debris and fluid. Insets: higher magnification identifies single or paired FISH-positive coccoid bacteria (Case 3). (d) A cluster of FISH-labelled coccoid bacteria (arrowheads) without lytic neutrophil debris and fluid is seen at the leading edge of expanding epidermal collarette. Inset: higher magnification identifies bacteria (Case 5). (e) Examination of the same skin section under oil clearly distinguished clusters of paired FISH-labelled staphylococci (arrowheads) within the stratum corneum cleft (Case 5). (f) Numerous FISH-positive cocci apposed at the epidermal crust in the centre of skin sections (Case 5). Fluorescence *in situ* hybridization using *Staphylococcus* spp.-specific 16SrRNA red-labelled probe; DAPI-stained nuclei are blue, a,d,f: 10×, c,d: 20×; e: 63×.

potentially could result in variable antibiotic susceptibility patterns.

It has been hypothesized that ESP-associated epidermal collarettes could be caused by exfoliative toxins of *S. pseudintermedius* similar to those of *S. hyicus* in piglets.[13] In our study, PCR results for collarette-associated *Staphylococcus* strains showed a lack of amplification of exfoliative toxin genes *expa* and *expb,* whereas amplicons corresponding to predicted sizes of *siet* and *speta* products were present in all isolates. Our results

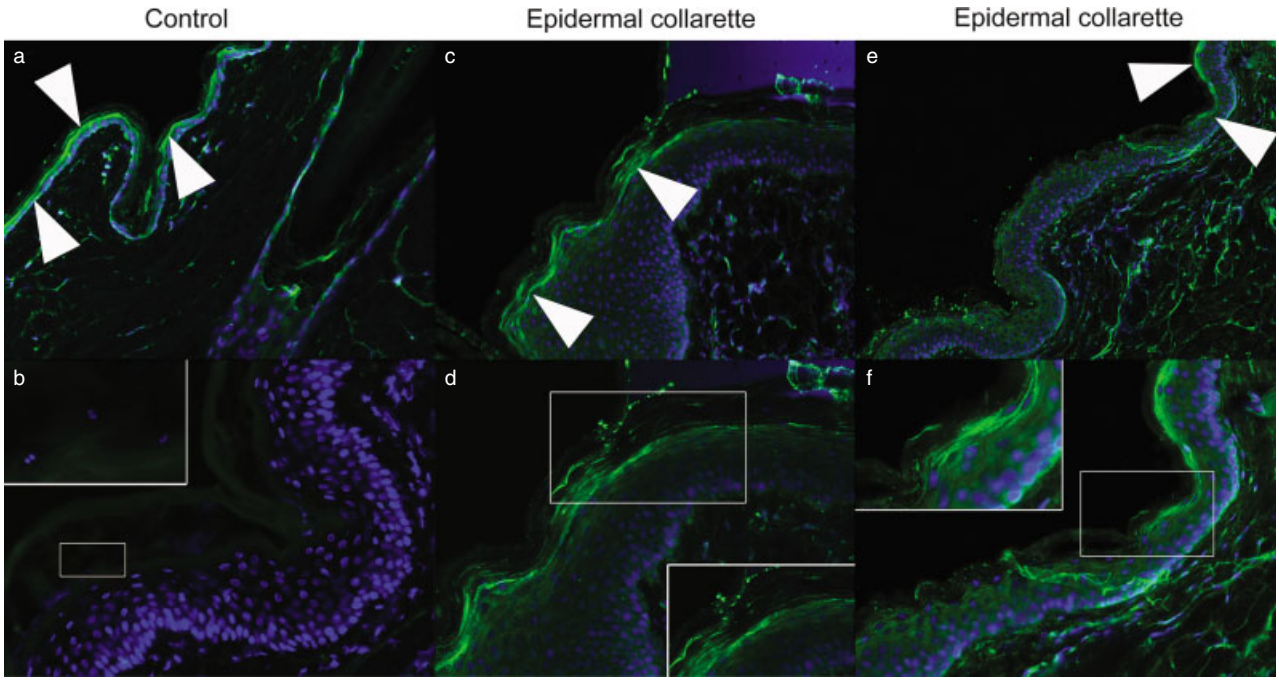

Figure 5. Immunostaining of frozen skin sections for corneodesmosin (CDSN). (a) The expression of CDSN can be seen as a thick line (arrowheads) in the lower stratum corneum of positive control unaffected skin sections (Case 3). (b) Negative control immunostaining reveals blue DAPI positive-stained paired coccoid bacteria within the cleft of stratum corneum (Case 5). (c,d) The immunostaining of epidermal collarettes for CDSN is discontinuous in areas of spongiotic pustules, whereas in leading collarette edges CDSN staining was weaker and patchy (bottom centre). In contrast, a normal CDSN immunostaining pattern was found beyond the collarette's leading edge (arrowheads) (Case 5). (e,f) Abrupt interruption of normal CDSN immunostaining (arrowheads) was found in the leading collarette edge (Case 3). Immunofluorescence for CDSN with Alexa 488 secondary antibody (green). DAPI-stained nuclei are blue. a,c,e: 10×, b,d,f: 20×.

are consistent with a previous study that reported only one isolate of EXPB-producing *S. pseudintermedius* among 13 isolates from dogs with scales/epidermal collarettes.[10] In the present study, the prevalence of *expa* and *expb* amplicons in control strains from bacterial folliculitis ranged from 10 to 30%, as described previously.[10] Although several collarette- and superficial folliculitis-associated *Staphylococcus* strains were positive for the proposed exfoliative toxins SIET and SPETA in our study, their role in the pathogenesis of epidermal collarettes remains unknown. The putative toxin SIET shares no homology in amino acid sequence with other known exfoliative toxins and it does not contain the typical catalytic triad, the active site of serine exfoliatin proteases.[7,29] Further investigation revealed a lack of epidermal changes after intradermal injection of recombinant SIET into canine skin,[8] emphasizing the controversy existing on the true role of SIET in canine pyoderma. A novel toxin, designated SPETA, was discovered after whole-genome sequencing of *S. pseudintermedius* ED99;[22] an exfoliative role for this toxin was presumed based on the high amino acid similarity to the exfoliatin SHETA from *S. hyicus*. Because the functionality testing of recombinant SPETA has not been reported and as acantholytic keratinocytes were not seen in any skin sections in our study, future studies are warranted to determine if SPETA is a truly exfoliative toxin.

Microscopic examination of the leading edge of epidermal collarette-associated ESP skin lesions identified early epidermal separation in the deep stratum disjunctum, a few corneocyte layers above the stratum compactum, and it was similar to images reported previously.[11,13] Epidermal clefts appeared to expand through minimally altered epidermis, consistent with the content of the cleft itself causing the peripheral extension of epidermal separation. Gram-positive cocci, lytic neutrophils and small amounts of fluid were all found in the leading cleft; any combination of these could promote epidermal separation. Although historically regarded as nonmotile organism, *S. aureus* aggregates have recently been shown to have active spreading behaviour under certain conditions, which resembles the so called bacterial gliding motility.[30] This may explain cleft extension in several skin sections where only clusters of cocci bacteria without neutrophils were revealed.

Microscopic spongiotic pustules were located from centrally to the leading cleft edge, and these were therefore unlikely to directly induce epidermal separation and cleft extension; however, they could contribute to the extension of epidermal clefts by supplying fluid and/or neutrophils to the leading cleft edge. Interestingly, presumed older epidermal clefts usually were observed at the interface of the stratum disjunctum and the stratum compactum, a slightly deeper epidermal position than early clefts at the very margin of lesions. Additionally, thinning of the cleft roof was observed. Both of these observations were attributed to lesion aging and keratinocyte desquamation from the exposed inner surfaces of the cleft. The common occurrence of individualized corneocytes in the cleft lumen supports this possibility. Individualized corneocytes were also observed at foci of disintegrating stratum compactum, just above spongiotic pustules, which suggests a possible role for neutrophil enzymes, and/or fluid mediators in corneocyte separation.

Neither keratinocyte acantholysis nor the formation of acantholytic pustules were observed in ESP-associated epidermal collarettes, despite the close proximity of cocci to breaks in the stratum compactum over spongiotic pustules and exposed stratum granulosum keratinocytes. Therefore, based on morphology, *S. pseudintermedius* involved in epidermal collarettes should not be expected to produce DSG1-digesting exfoliative toxins thought to induce keratinocyte acantholysis and pustules in other forms of superficial pyoderma such as impetigo.[8] In the current study, dogs lacked clinical evidence of bacterial folliculitis. Histologically, epidermal clefts did not involve hair follicles significantly and neutrophilic luminal folliculitis was not observed. Therefore, ESP lesions can occur in the absence of bacterial folliculitis and folliculitis is unlikely to be a key mechanism underlying ESP epidermal collarette lesion induction or spread, an association that had been suggested – but was not proven – in previous reports.[13] In our study, mild follicular atrophy/inactivity with hair shaft shedding might be the mechanism for alopecia described for some ESP lesions;[11] however, changes were mild in this study and biopsies did not include the centres of lesions and therefore may not fully include typical follicular changes. Another explanation for alopecia may include the digestive activity of bacterial and/or neutrophilic proteases targeting the adhesion molecules between hair shafts and hair follicles. Indeed, the loss of CDSN normally present in the hair follicle inner root sheath leads to alopecia in mice.[31]

Indirect immunofluorescence revealed only anomalies in CDSN staining at the leading edge of epidermal collarettes in our dogs. Interestingly, an extracellular *S. aureus* enzyme, commonly referred to as the V8 protease, impairs the epidermal barrier permeability and causes stratum corneum structural disturbance in mice.[32] Analysis of the stratum corneum by transmission electron microscopy revealed that the V8 protease induced the loss of corneodesmosome integrity and resulted in disturbance of corneocyte cohesion.[32] As *S. aureus* V8 protease shows a sequence similarity to that of other "classic" exfoliative toxins, the epidermal barrier disruption after V8 protease application to the skin could be due to its enzymatic cleavage of corneodesmosomes in stratum corneum. The exfoliative toxin EXPA from *S. pseudintermedius* shares homology (33% amino acid identity) to the staphylococcal V8 protease;[9] however, recent whole-genome sequencing of a single strain of *S. pseudintermedius* ED99 isolated from superficial bacterial pyoderma lesions (likely to be pustules) did not reveal the presence of a V8 protease as in *S. aureus*.[22] Further studies involving whole-genome sequencing of collarette-associated *Staphylococcus* strains and sequence alignment to previously published exfoliative toxins, including V8 protease, are needed to reveal new proteases that might be involved in the pathogenesis of epidermal collarettes in dogs.

In conclusion, the results of this study indicate that epidermal collarettes represent unique clinical and histological lesions of ESP that are distinct from those of impetigo and superficial bacterial folliculitis. Strains of *S. pseudintermedius* – presumably secreting yet unknown toxin(s) – with or without lytic neutrophils spread in fluid and disrupt corneodesmosomes in the deep stratum disjunctum, forming an epidermal cleft where the intact stratum corneum roof maintains hydration at the lesion margin. Desquamation of the cleft base, superficial injury to the stratum compactum and bacterial mediators induce the formation of peripheral neutrophilic spongiotic pustules that rupture through the stratum compactum to the cleft lumen and provide more fluid and neutrophils to the cleft, thereby enhancing the bacterial mediated extension of the cleft in a cyclical process. Centrally within the collarettes, rupture and opening of the epidermal cleft, along with epidermal barrier reparative responses, and upregulation of innate defence mechanisms, promote the drying and resolution of skin lesions. The mechanism underlying exfoliation and spreading of epidermal collarettes does not appear to be associated with EXPA, EXPB, SIET and SPETA exfoliative toxins of *S. pseudintermedius*; another mechanism that involves CDSN proteolysis and corneodesmosome separation is suspected to contribute to the exfoliation.

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Supporting Information

Additional Supporting Information may be found in the online version of this text at: http://onlinelibrary.wiley.com/doi/10.1111/vde.12352/full

Figure S1. Pulse field gel electrophoresis results reveal identical band patterns of marginal and centre-collected epidermal collarette bacterial isolates from the same dog.
Table S1. Exfoliative toxin primers used in this study.
Table S2. Bacterial culture and antimicrobial susceptibility results from epidermal collarettes.

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Molecular and epidemiological characterization of canine *Pseudomonas* otitis using a prospective case-control study design

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Background – *Pseudomonas aeruginosa* is an opportunistic pathogen of the canine ear canal and occupies aquatic habitats in the environment. Nosocomial and zoonotic transmission of *P. aeruginosa* have been documented, including clonal outbreaks.

Hypothesis/Objectives – The primary objective of this study was to assess various environmental exposures as potential risk factors for canine *Pseudomonas* otitis. It was hypothesized that isolates derived from infected ears would be clonal to isolates derived from household water sources and the mouths of human and animal companions of the study subjects.

Animals – Seventy seven privately owned dogs with otitis were enrolled, along with their human and animal household companions, in a case-control design.

Methods – Data on potential risk factors for *Pseudomonas* otitis were collected. Oral cavities of all study subjects, their human and animal companions, and household water sources were sampled. Pulsed field gel electrophoresis was used to estimate clonal relatedness of *P. aeruginosa* isolates.

Results – In a multivariate model, visiting a dog park was associated with 77% increased odds of case status ($P = 0.048$). Strains clonal to the infection isolates were obtained from subjects' mouths ($n = 18$), companion pets' mouths ($n = 5$), pet owners' mouths ($n = 2$), water bowls ($n = 7$) and water taps ($n = 2$). Clonally related *P. aeruginosa* isolates were obtained from dogs that had no clear epidemiological link.

Conclusions and clinical importance – Genetic homology between otic and environmental isolates is consistent with a waterborne source for some dogs, and cross-contamination with other human and animal members within some households.

Introduction

Pseudomonas aeruginosa is a common cause of treatment-resistant otitis externa and media in dogs, which results in significant morbidity and medical expenditure.[1,2] Clinical observation supports an opportunistic role for *P. aeruginosa,* which overgrows in diseased ear canals when the normal flora is inhibited by antimicrobial drugs.[2] *Pseudomonas aeruginosa* is not a normal inhabitant of the healthy canine ear canal,[3–5] nor is it an obligate animal pathogen.[6] This disconnect suggests that dogs are likely to acquire the organism through transmission from external reservoirs, but the location of these putative reservoirs remains largely unknown.

The most common environmental habitat for *P. aeruginosa* is water; it is able to live and multiply in a wide variety of aquatic environments, such as rivers, seawater, bottled drinking water, tap water, household taps and saunas.[6] Nosocomial transmission is well documented in human healthcare settings,[7–9] and evidence has accrued to support a role for aquatic sourced nosocomial transmission of this pathogen to dogs. Examples include cases of bacterial pyoderma caused by bathing dogs with contaminated shampoo products.[10,11] In many cases, these products had been diluted with tap water and stored for repetitive use.[10,11] *Pseudomonas aeruginosa* may also contaminate otoscope cones[8,12,13] and ear bulb syringes.[14] Cross-species transmission of *P. aeruginosa* also appears to be possible. In a microbiological survey of healthy captive snakes, clonal strains were isolated from snake faeces, their prey and water sources, other animals

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in the environment, and from the mouths of their human handlers.[15]

Molecular characterization of isolates from human infections show that both unique strains and highly successful, widely disseminated clones have been identified within the *P. aeruginosa* populations studied.[16] More limited data are available regarding clonal relationships between *P. aeruginosa* isolates derived from domestic animals, but genetic heterogeneity among geographically clustered ruminant mastitis isolates and canine soft tissue infection isolates has been described.[17,18] Genetic analysis of strains derived from diverse sources (human, animal and environmental) report a largely nonclonal population structure with frequent genetic recombination.[6] There is extensive overlap in the genomes of strains isolated from environmental and human sources,[6,19] and wild type environmental isolates express an extensive set of virulence genes that may promote pathogenicity to animals.[20]

The objectives of the observational study reported here were to survey a population of dogs with bacterial otitis externa using a case-control design, in order to assess various environmental and nosocomial exposures as potential risk factors for acquisition of *P. aeruginosa*. It was hypothesized that *P. aeruginosa* isolates derived from infected ears would be clonal to isolates derived from household water sources and the mouths of human and animal (dog and cat) companions of the study subjects. Genetic homology with otic isolates could therefore indicate a household water source and cross-contamination to other human and animal members of these households. By contrast, it was hypothesized that strains would be heterogeneous when compared between participating households. It was also hypothesized that otic isolates – which had undergone direct exposure to antimicrobial drugs – would express more extensive antimicrobial resistance patterns than genetically related environmental isolates.

Materials and methods

Power and sample size determination

Preliminary data from a nontargeted study**,** shown in Table S1, suggested a 50% difference in exposure to environmental *P. aeruginosa* among positive animals compared to negative animals. Therefore, we conservatively predicted a 40% prevalence difference for *P. aeruginosa* isolation from aqueous environmental-source swabs (water bowls, taps, fish tanks and aquatic reptile tanks) in case households compared to control households. No data were available to predict human or pet oral carriage in the context of a companion pet with otic infection. Based upon data reported from our clinical microbiology laboratory over the prior 2 year period, we assumed that 50% of canine ears cytologically positive for rod-shaped bacteria would yield *P. aeruginosa* upon aerobic culture. Hence, we estimated that a sample size of 80 households (1:1 case/control ratio) would provide 80% power given an alpha of 0.05.

Regulatory oversight of research procedures

This protocol was approved by the Institutional Animal Care and Use Committee (IACUC) for the use of client-owned animals. Approval for human sampling and use of survey data that contained personal identifiers was granted by the Institutional Review Board (IRB) for biomedical research. Pet owners provided written informed consent for use of their personal data and processing of their oral swabs. They provided separate written informed consent to use swab samples collected from the pet with otitis and from all other dogs and cats that resided in their homes.

Study population, recruitment and specimen collection

Dogs and their owners were recruited from a population referred for specialty care to a university teaching hospital and an affiliated dermatology referral practice between June 2013 and December 2014. Any dog with clinical signs consistent with otitis and which had rod-shaped bacteria present on cytological evaluation of ear exudate (either bilateral or unilateral) was invited to participate. Mixed populations of bacteria (e.g. rods and cocci) with or without yeast were permitted so long as some rod-shaped bacteria were present. The rapid staining technique used for cytological evaluation by the investigators was a modified Giemsa stain (Differential Quik Stain Kit, Polysciences Inc.; Warrington, PA, USA). Gram stains were not used during recruitment in order to simulate the standard of care practiced by most veterinarians in North America. There were no exclusions for prior or current therapies. Once the pet owner had provided informed consent, sterile swabs (Bacti swab, Remel; Lenexa, KS, USA) were used to collect otic exudate for standard aerobic culture processing. Both canals were sampled for culture regardless of cytological results, to rule out subclinical *P. aeruginosa* carriage in a cytology negative ear. The oral cavities of all participating dogs with otitis were also swabbed by the investigators at the time of this visit.

At the time of the clinic visit, pet owners were instructed on proper techniques for swabbing the targeted environmental sites within their homes as well as their own oral cavities (buccal and sublingual spaces) and the oral cavities of other dogs and cats that resided in the household. Environmental sites sampled included the inside edge of the pet's emptied water bowl, the most proximal internal surface (reachable by the swab) of the water tap used to fill the pet's bowl, any aquarium or cage used to house fish or reptiles (above the water line of a tank or the floor of a cage), the internal wall of any swimming pool (just above the water line) and the internal surface of any applicator tip or lid from an otic medication or ear cleanser solution bottle that had been used to treat the affected pet.

Swabs and shipping materials were provided to participants in a prepaid envelope which complied with Federal regulations for shipment of clinical specimens. Samples were processed for aerobic culture by the microbiology laboratory within 5 days of shipment. Enrolled households were excluded if the pet owner failed to return the environmental and oral swabs as requested, which were required to include (at a minimum) swabs of the pet owner's mouth, the study subject's water bowl and the tap used to fill the water bowl. Swabs and questionnaires were coded for linkage to the pet's medical record, and results of otic cultures were used to make clinical treatment decisions when applicable. Due to privacy regulations governing research on human subjects, pet owners were not advised of the results derived from environmental samples or from their mouths.

Survey instrument

A 22 question survey was reviewed with the participant at the time of consent. Data were collected with regard to several potential risk factors for community based acquisition of *P. aeruginosa* by the dog, such as swimming in treated or untreated water sources, visits to community dog parks or boarding at group facilities. Lifestyle was defined by residence in an urban, suburban or rural setting. To assess the potential for acquisition of a hospital-associated strain of *P. aeruginosa* by the pet owner, participants were asked about any recent surgery, hospitalization, emergency room visits, repeated visits to outpatient treatment facilities or employment in human healthcare practice by any household member. The survey also captured data regarding pet related factors such as age, breed, sex, living conditions (indoor/outdoor/mixed) and the presence of other pets in the household (including aquatic species, small mammals, birds and reptiles).

The nature of owner–pet contact was defined by assigning scores for human–animal interactions. Briefly, points were allocated for the frequency by which the pet licked the human participant, whether the pet slept in/on their owner's bed and whether the owner defined him/herself as the primary caregiver for the dog's otic therapies. The contact score was dichotomized for statistical analysis as either "close" or "casual" contact, as described previously.[21]

Attending veterinarians were asked to provide the most likely primary cause for each dog's otitis. These were categorized as either allergic, endocrinopathic, conformational or immune-compromised aetiologies. Severity and duration of ear disease was not defined for participants, but the structure of pinnae (erect versus pendulous) was recorded. All antimicrobial therapy of human and animal participants within the 90 days that preceded study enrolment was documented, as was the use of steroids and other immune modulators in the canine participants. All routes of exposure to drugs (topical, oral and parenteral) were considered.

Bacterial cultures

Pet-source clinical samples

Bacterial isolates from ear exudate samples were processed using standard protocols[22] for bacterial speciation and antimicrobial susceptibility testing in an automated system (Microscan, Dade Behring, Irvine, CA, USA). Isolates confirmed to be *Pseudomonas aeruginosa* were cryopreserved in storage tubes (Microbank, Pro-Lab Diagnostics; Austin, TX, USA) at −70°C. Pets negative by aerobic culture for *P. aeruginosa* served as the control group for a case-control analysis of risk factors.

Human-sourced and environmental samples

Environmental and oral cavity swabs were plated to Pseudomonas Isolation Agar (PIA, Remel; Lenexa, KS), which is used for the selective isolation of *P. aeruginosa* and for differentiating *P. aeruginosa* from other pseudomonads based on pigment formation. Isolates with morphology typical of *Pseudomonas* spp. were biochemically identified as *P. aeruginosa* and antibiotic susceptibility results were generated by the automated system referenced above. Selective agar was used for isolation of the organism from environmental and oral sites because many competing bacterial species and low colony forming units (CFUs) of the target organism were expected to be present outside the setting of a monotypic infection. Isolates were stored in cryopreservation tubes and coded for storage along with the corresponding clinical isolate(s) obtained from the participating pet.

Molecular testing of bacterial isolates

Pulsed field gel electrophoresis (PFGE) was performed on all *P. aeruginosa* isolates from each participating household unit, as described previously.[23] BioNumerics software v5.1 (Applied Maths; Kortrijk, Belgium) was used to identify percentage similarities on a dendrogram derived from the unweighted pair group method using arithmetic averages and based on the Dice coefficient of similarity. A similarity of 85% was the cut-off selected to define pulsed-field profile (PFP) clusters in this study which was similar to an 87% cut-off described in a previous study with *P. aeruginosa*.[23] Strains with 100% similarity were classified as "indistinguishable" (i.e. clones), whereas strains that exhibited two to three band differences – as may occur from a single genetic event such as a DNA deletion, insertion or point mutation – were classified as "closely related" (i.e. members of the same clonal cluster).[24]

Statistical analysis

Descriptive statistical procedures and regression modelling for risk factor analysis was performed with Stata 13 (Stata Corp.; College Station, TX, USA). Chi-square analysis was used to detect differences between cases and controls, and to assess for differences in antimicrobial resistance patterns between otic and environmental clones. Testing of associations for putative risk factors utilized Poisson models with robust estimation of standard errors as described previously.[25,26] Variables were included in the multivariable regression model if they were significant at the $P < 0.1$ level in bi-variable analysis. Odds ratios (ORs) estimated from logistic regression models and prevalence ratios (PRs) estimated from Poisson models were evaluated *a priori*. Where inference was similar between the two options, we report PRs preferentially as these are less susceptible than ORs to overestimation of effect size.[25] Reference groups were assigned to the largest stratum.

Results

Study population

Eighty six dogs that were presented with the complaint of chronic or recurrent otitis were consented and enrolled in the study, along with their owners and animal companions (canine and feline). Of these 86 household units, 89.5% returned swabs from the required sites, for a total study population of 77 dogs. There were 38 cases (canine subject with culture-confirmed otic infection by *P. aeruginosa*) and 39 controls (canine subject with culture-confirmed otic infection by bacteria other than *P. aeruginosa*). In addition, 32 households returned oral cavity swabs from other companion pets (dogs and cats), 17 returned swabs from otic medication bottles and two returned swabs from fish tanks (Table 1). Ten of 32 (31%) companion pet samples were positive for growth of *P. aeruginosa*. All swabs from fish tanks and otic medication bottles were negative.

None of the ear canals that were negative for rod-shaped bacteria upon cytological examination produced a positive *P. aeruginosa* culture. However, reference ears from which rod-shaped bacteria were visualized by cytology often yielded bacillary species other than *P. aeruginosa* upon aerobic culture (37 of 77 subjects; 48%), and two reference ears were positive only for growth of Gram-positive bacteria (complete data not shown). By contrast, *Staphylococcus spp.* were commonly isolated from ears that were cytology negative for cocci (12 of 39 subjects, 31%; data not shown). Of the 77 subjects with complete data, 45 (60%) were considered to have matching cytology and culture results (i.e. when rod-shaped bacteria were observed by cytology, a Gram-negative bacillus was isolated by culture).

Epidemiological analyses

Results of unadjusted analyses of demographic and lifestyle data are presented in Table 2. Cases were more likely than controls to have had close human contact, to have visited dog parks or to have swam in a pool prior to development of otitis. Case households were less likely to report presence of a child or baby in the home (ages 3 to 10 year); these comparisons met the $P < 0.10$ criterion for inclusion in the final adjusted model. Case dogs were significantly more likely than control dogs to be *Pseudomonas* culture-positive from an oral swab ($p < 0.001$), and were somewhat but not significantly more likely to be associated with *Pseudomonas* contamination of a water bowl in the home. Water taps and animal companions were equally likely to be *Pseudomonas*-positive among case and control dog households.

In both unadjusted and multivariable models adjusted for demographic characteristics (Table 3), an owner report of the dog swimming in a pool and the

dog being *Pseudomonas*-positive on oral swab were significantly associated with case status. In addition, having one additional positive household source (bowl, tap, other pet or human in home) was associated with a 68% increased prevalence of *Pseudomonas* otitis as opposed to non-*Pseudomonas* otitis. However, this was strongly co-linear with a positive oral cavity (10 of 11 case dogs with one home site positive were also positive from the oral cavity). In a final multivariable model using a complete set of data on 73 dogs, a positive oral site was associated with a two-fold increased prevalence (PR 2.09; 95% CI: 1.17, 3.74; $P = 0.013$), and history of visiting a dog park was associated with an independent 77% increase in prevalence of being a positive for *Pseudomonas* (PR 1.77; 95% CI: 1.01, 3.10, $P = 0.046$), controlling for the number of positive home sites, close contact with people in the home, swimming in a pool, sex, older age, mixed breed and neuter status. None of the latter variables were significant in the final adjusted model, although power was limited to detect weaker associations.

Molecular typing analyses

Clonal relationships between the *P. aeruginosa* infection isolates and other animal, human or household environmental *P. aeruginosa* isolates are provided in Table 1. These data show that, among the 26 case dogs with at least one other *P. aeruginosa* isolate available from household samples, at least one genotypically identical household isolate was identified for 23 case households (88.5%). Nonmatching isolates were identified for two case households (7.5%) and isolates from one household were lost prior to processing (Table 1). Although the dog owner rarely was *Pseudomonas*-positive, with rates that ranged from 3% (one of 39 control homes) to 5% (two of 38 case homes), the isolates obtained from both people in *Pseudomonas* case homes were genetic matches to their respective dogs' infection isolates.

Matching *P. aeruginosa* strains were also isolated from oral or environmental sites in four control households (Table 1). In households 29, 45 and 51, clonal strains were isolated from the control subject's mouth and the water bowl, and in household 29 the clone was also isolated from a canine companion's mouth. In Household 69, clonal strains were isolated from the mouth of a canine companion (but not the control subject) and the water bowl that the two dogs shared.

Clonality of isolates between homes

As defined by a strain Dice similarity of 85%, there were 17 clonal groups (>1 isolate per group) represented within the 117 isolates that were typable by PFGE (Figure S1). A range of one to nine households were represented within each clonal group. There were four singlet isolates which did not match any others. Although geospatial modelling was not performed to analyse physical distance between the households represented within each clonal group, none of the households within any clonal group shared the same USA zip code, nor did any dogs within these grouped households share a primary care veterinary practice in common.

Discussion

The genotypic results support the hypothesis that *P. aeruginosa* isolates derived from the infected ears of dogs are commonly clonal to isolates derived from household water sources and the mouths of their human and animal companions (true for at least one of these sites for 88.5% of case households). A high proportion of case dogs with a typable otic isolate had clonal *P. aeruginosa* strains isolated from their own oral cavities (21 of 25; 84%). In seven of these 21 households, the water bowls used by the case dogs were positive for the same *Pseudomonas* clone, and in four households an animal companion that shared the bowl was also positive. However, the directionality of transfer (and hence causation) cannot be deduced from these cases due to the cross-sectional study design. Contamination of the subject's mouth and water bowl could be explained by auto-inoculation from the ear rather than an aquatic reservoir. The oro-pharynx communicates with the tympanic cavity via the auditory tube, and although we did not confirm tympanic status in all subjects, the prevalence of otitis media in dogs with *Pseudomonas* otitis is predictably high.[27] Alternatively, contamination of the pinna/face with transfer of bacteria to the oral cavity and environment could also serve as a route of transmission. Together, these data suggest that fomites, which may be contaminated by the dogs or other household inhabitants, are potential reservoirs to recycle *P. aeruginosa* within the home. Cross-contamination between animal and human housemates and their shared objects is perhaps unavoidable to some degree, but highlights the importance of personal and environmental hygiene in the context of clinical management of disease.

Water taps used to fill the dogs' water bowls were selected as the most informative sites to determine if primary aquatic sources (not simply reservoirs) of *P. aeruginosa* exist within the home. Two households had positive water taps with *P. aeruginosa* isolates identical to otic infection isolates, and one of these also had a positive water bowl. The owners of these dogs confirmed that they did not have direct access to the taps and that the internal surfaces were not human hand touch-points. Therefore, these cases provide the best evidence – within the design of this particular study – that a household water source could be the origin of an infection strain for some dogs. Likewise, an investigation of a small *P. aeruginosa* outbreak in a human medical ward showed that clinical isolates recovered from several lung transplant recipients matched isolates acquired from sink drains in the ward.[9] Regrettably, isolates from one potentially informative case (Household 14), where the tap and bowl were each culture positive, were unavailable for PFGE testing due to loss of the organisms after initial isolation.

Based on prior reports,[17,18] it was hypothesized that *P. aeruginosa* infection and environmental strains would be heterogeneous when compared across participating households. This was true to some degree, because 17 clonal groups were represented within the 117 isolates that were typable by PFGE (Supporting Information Figure S1). However, some clones were present within disparate households that had no clear epidemiological link, which suggests that some successful

Table 1. Households from which at least one *Pseudomonas aeruginosa* isolate was obtained and available for pulsed-field gel electrophoresis

Household	Case/Control	Reference ear isolate	Contralateral ear isolate	Mouth	Bowl	Tap	Person (mouth)	Other pet (mouth)
1	Case	*P. aeruginosa*	N/A	100%	100%	100%	Neg	Neg
4	Case	*P. aeruginosa*	N/A	Neg	N/A	N/A	N/A	N/A
5	Control	*E. coli*	N/A	Neg	Neg	Pos	Neg	Neg
6	Control	*Proteus*	N/A	Neg	Neg	Pos	Pos*	N/A
9	Case	*P. aeruginosa*	N/A	100%	100%	Neg	Neg	N/A
11	Case	*P. aeruginosa*	N/A	Neg	Neg	Neg	Neg	N/A
12	Case	*P. aeruginosa*†	MSSP	90%±	Neg	90%±	Neg	Neg
13	Control	Diptheroids	MRSP	Pos	Neg	Neg	Neg	N/A
14	Case	*P. aeruginosa**	N/A	Neg	Pos*	Pos	Neg	Neg
15	Case	*P. aeruginosa*	No Growth	100%	Neg	Neg	Neg	85%D
17	Control	*Enterococcus*	*Enterococcus*	Pos	Neg	Neg	Neg	Neg
19	Control	*Proteus*	*Proteus*	Neg	98%±	<80%	Neg	Neg
21	Case	*P. aeruginosa*	*Acinetobacter*	Neg	Neg	Neg	Neg	Neg
22	Case	*P. aeruginosa*	N/A	<80%	Neg	<80%	Neg	Neg
23	Case	*P. aeruginosa*†	*Bacillus*	85%	Neg	Neg	Neg	85%D
24	Case	*P. aeruginosa*	N/A	Neg	Neg	Neg	Neg	N/A
25	Control	*Proteus*	*Proteus*	<80%	<80%	Neg	Neg	Pos*
26	Case	*P. aeruginosa*	*P. aeruginosa*†	95%	Neg	Neg	Neg	N/A
27	Case	*P. aeruginosa*	Posδ	100%	Posδ	Neg	Neg	84%c
29	Control	*Proteus*	No Growth	100%	100%	Neg	Neg	100%D
30	Case	*P. aeruginosa*	96%	Neg	Neg	Neg	Neg	Neg
33	Case	*P. aeruginosa*	N/A	96%	Neg	Neg	Neg	N/A
35	Case	*P. aeruginosa*	N/A	100%	100%	Neg	Neg	100%D
36	Case	*P. aeruginosa*	97%	100%	Neg	Neg	Neg	Neg
37	Case	*P. aeruginosa*	95%	100%	Neg	Neg	Neg	Neg
39	Case	*P. aeruginosa*	100%	100%	100%	Neg	Neg	Neg
41	Case	*P. aeruginosa*	<80%	100%	Neg	Neg	Neg	100%D2
43	Case	*P. aeruginosa*	100%	Neg	Neg	100%	Neg	N/A
44δ	Control	*Proteus*	*Proteus*	<80%	<80%	<80%	Neg	<80%C
45	Control	*E. coli*	*E. coli*	100%	100%	<80%	Neg	N/A
47	Case	*P. aeruginosa*	100%	Neg	Neg	Neg	Neg	Neg
48	Control	*Bacillus*	MRSP	Neg	Neg	Neg	Neg	Pos
49	Case	*P. aeruginosa*	N/A	Neg	Neg	Neg	Neg	N/A
51	Control	Diptheroids	N/A	99%	99%	Neg	Neg	Neg
52	Control	*Proteus*	N/A	Neg	Neg	Pos	Neg	Neg
54	Case	*P. aeruginosa*	100%†	100%	100%	Neg	Neg	N/A
55	Case	*P. aeruginosa*	100%	100%	Neg	Neg	Neg	N/A
58	Case	*P. aeruginosa*	N/A	Neg	Neg	Neg	Neg	N/A
59	Case	*P. aeruginosa*	N/A	Neg	Neg	Neg	Neg	Neg
60	Case	*P. aeruginosa*	N/A	100%	95%	Neg	Neg	N/A
65	Case	*P. aeruginosa*	*E. coli*	100%	100%	Neg	Neg	N/A
66	Case	*P. aeruginosa*	100%	97%	Neg	Neg	Neg	Neg
67	Control	MSSP	No Growth	Pos	Neg	Neg	Neg	Neg
68	Case	*P. aeruginosa*	Neg	Neg	Neg	Neg	Neg	Neg
69	Control	*Proteus, E. coli*	N/A	Neg	100%	Neg	Neg	100%D
72	Case	*P. aeruginosa*	N/A	<80%	Neg	Pos†	Neg	N/A
76	Case	*P. aeruginosa*	N/A	Neg	<80%	Neg	90%	N/A
77	Case	*P. aeruginosa*	N/A	Neg	Neg	Neg	Neg	N/A
81	Case	*P. aeruginosa*	100%	100%	Neg	Neg	Neg	Neg
83	Case	*P. aeruginosa*	N/A	100%	100%	Neg	100%	Neg
90	Case	*P. aeruginosa*	100%	100%	Neg	Neg	Neg	Neg
91	Case	*P. aeruginosa*	*Proteus*	Neg	Neg	Neg	Neg	N/A

N/A, not applicable; Neg, no *P. aeruginosa* isolated from sample.

Percentages represent the proportional matches (clonal relationships) between *P. aeruginosa* strains isolated from various sources within a household, as determined by pulsed field gel electrophoresis (PFGE). For case households, the reference strain is the otic isolate unless noted otherwise. Singlets are designated only as "positive" (Pos). When an isolate was not assigned a percentage for clonality, the following codes indicate the reason:

*Pos isolate not available for PFGE testing.

†Pos isolate not susceptible to *Spe* 1 digestion.

δPos isolate not clonally related to the reference isolate.

MRSP, meticillin-resistant *Staphylococcus pseudintermedius.*

MSSP, meticillin-susceptible *Staphylococcus pseudintermedius.*

±Positive environmental samples; clonal match to each other.

DDog.

D2Two dogs.

CCat.

Table 2. Demographic and household characteristics of dogs with otitis enrolled in the study [data given as *n* (%)]

	Pseudomonas case (n = 38)	Non-*Pseudomonas* control (n = 39)	v^2 *P*-value
Female sex	18 (47%)	24 (62%)	0.21
Neutered	35 (92%)	34 (87%)	0.48
Older dog (over 7 years old)	15 (39%)	22 (56%)	0.14
Mixed breed	5 (13%)	11 (28%)	0.11
Erect pinnae	3 (8%)	5 (13%)	0.48
Allergic disease	33 (87%)	36 (92%)	0.44
Recent antimicrobials	27 (71%)	29 (74%)	0.75
Recent steroids	28 (74%)	29 (74%)	0.95
Outdoor dog *	6 (16%)†	5 (13%)	0.68
Other pet(s) in the home‡			
No other pets	10 (28%)	15 (40%)	
1 other pet	11 (30%)	13 (34%)	
2+ other pets	15 (42%)	10 (26%)	0.16**
4+ people in the home §	15 (43%)	19 (50%)	0.54
Child or baby in home §	2 (6%)	9 (24%)	0.05
Close human contact §	22 (63%)	16 (42%)	0.08
Home location			
Urban	6 (16%)†	7 (18%)	0.86
Suburban (ref)	25 (66%)†	26 (67%)	ref
Rural	6 (16%)†	6 (15%)	0.95
Visits dog parks	10 (28%)¶	4 (10%)	0.06
Swims in a pool	6 (16%)†	1 (3%)	0.07
Swims in a pond	7 (19%)†	8 (21%)	0.86
Pseudomonas positive Dog's mouth	22 (58%)	8 (21%)	0.001
Dog's bowl	11 (29%)	7 (18%)	0.26
House tap	5 (13%)	6 (15%)	0.78
Other pet	5 (13%)	5 (13%)	0.97
Human caregiver	2 (5%)	1 (3%)	0.55

*Outdoor dog includes any dog not exclusively indoor.
†n = 37 among cases.
‡n = 36 among cases and n = 38 among controls.
§n = 35 among cases and n = 38 among controls.
¶n = 36 among cases.
** *P*-value for trend.

strains are circulating within our regional community. Within the larger clonal groups, both case and control households were represented. Although clonal proliferation is not as striking as has been reported for meticillin-resistant *Staphylococcus* spp.,[28,29] laboratory surveillance[30] for evidence of virulent *P. aeruginosa* outbreak strains is warranted.

One limitation of PFGE was the failure of the *Spe*1 enzyme to digest all available *P. aeruginosa* isolates for analysis. PFGE was chosen because it is a highly discriminatory and successful technique to estimate clonal relationships within *P. aeruginosa* collections,[23] has the greatest predictive value of labelling strains as clonal,[30,31] and is more economically efficient than gene sequencing techniques. The failure of enzyme digestion presented by some isolates in this study is related to extensive methylation of the bacterial genome and has been reported for *P. aeruginosa* previously.[23]

It was also hypothesized that *P. aeruginosa* otic isolates that had undergone direct exposure to antimicrobial drugs would express more extensive antimicrobial resistance patterns than genetically related environmental isolates. No statistically significant differences were detected within household units, but the study was likely underpowered for such evaluations. There were several households in which the otic isolate exhibited a one dilution difference in minimum inhibitory concentration (MIC) for a particular antimicrobial than the environmental clone(s) (Figure S1). However, genetic analysis was not conducted to detect antimicrobial resistance genes and a one dilution difference in antimicrobial susceptibility may not be indicative of genotypic variation.[32]

In addition to evaluating the genotypic relatedness of *P. aeruginosa* strains within and across households, a primary objective of this case-control study was to assess various environmental and nosocomial exposures as potential risk factors for acquisition of *P. aeruginosa* otitis. In a multivariable model, history of visiting a dog park or swimming in a pool was independently associated with a 64% increase in prevalence of being a *Pseudomonas* case, but statistical significance was borderline (P = 0.05). All other external factors were either nonsignificant in the multivariable analysis or potentially confounded by plausible biological relationships (e.g. strong statistical collinearity between a positive oral culture and positivity of another household site such as the water bowl).

Failure to find significant associations between certain environmental exposures and case status could be related to a lack of statistical power. The prevalence of household contamination by *P. aeruginosa* was much

Table 3. Prevalence Ratio (PR) and 95% Confidence Interval (95% CI) estimates for factors associated with *P. aeruginosa* otitis cases compared to non-*Pseudomonas* otitis controls [data given as *n* (%)]

	Unadjusted PR (95% CI)	Adjusted PR† (95% CI)
Erect pinnae	0.74 (0.29, 1.87)	0.93 (0.38, 2.24)
Recent antimicrobials	0.92 (0.56, 1.51)	0.92 (0.56, 1.51)
Recent steroids	0.98 (0.57, 1.64)	1.04 (0.61, 1.77)
Outdoor dog‡	1.14 (0.63, 2.08)	1.14 (0.71, 1.83)
Other pet(s) in the home§		
No other pets (ref)	ref	ref
1 other pet	1.15 (0.60, 2.20)	1.05 (0.54, 2.06)
2 + other pets	1.50 (0.84, 2.68)	1.39 (0.78, 2.46)
Four or more people in the home¶	0.86 (0.53, 1.40)	0.88 (0.54, 1.42)
Child or baby in home, *n* (%)¶	0.34 (0.09, 1.23)*	0.36 (0.10, 1.25)
Close human contact¶	1.56 (0.93, 2.60)*	1.61 (0.99, 2.63)*
Home location‡		
Urban	0.94 (0.49, 1.81)	0.94 (0.49, 1.80)
Suburban (ref)	ref	ref
Rural	1.02 (0.54, 1.93)	1.01 (0.55, 1.84)
Visits dog parks††	1.68 (1.07, 2.61)**	1.47 (0.92, 2.37)*
Swims in a pool‡	1.91 (1.28, 2.85)***	1.80 (1.17, 2.76)***
Swims in a pond‡	0.95 (0.52, 1.73)	1.04 (0.55, 1.96)
Dog's mouth *Pseudomonas* positive	2.28 (1.43, 3.63)***	2.21 (1.39, 3.51)***
Household source *Pseudomonas* positive‡‡		
No sites positive (ref)	ref	ref
One site positive	1.71 (1.09, 2.68)**	1.68 (1.10, 2.59)**
Two or three sites positive	1.08 (0.55, 2.11)	1.03 (0.56, 1.88)

Ref, reference group.
†Adjusted for sex, age, neutering status and mixed versus purebred.
‡n = 37 among cases.
§n = 36 among cases, n = 38 among controls.
¶n = 35 among cases, n = 38 among controls.
††n = 36 among cases.
‡‡Household sources: bowl, household tap, other pet or human in home.
*$P \leq 0.10$; **$P \leq 0.05$; ***$P \leq 0.01$.

lower in the present study than in the pilot study that informed the sample size calculations. There are several potential explanations for this difference. Although the human, animal and environmental sampling techniques and microbiological processing algorithms were the same, the present study relied on pet owners to collect and ship the samples, and the postal service to deliver them to the laboratory. In the pilot study, samples were collected by a single investigator and plated to culture media, typically within 72 hours. The mean lag time between sampling and inoculation to culture media was likely longer for the present study (complete data not available) and this may have affected bacterial isolation rates.

Furthermore, the geographical distribution of pets in the pilot study, in which 25% were from suburban areas, was different than that of this evaluation, in which 66% of dogs were from suburban areas. Rural home location in the pilot evaluation was nonsignificantly associated with seven-fold higher odds of home environmental exposure to *P. aeruginosa* among dogs (P = 0.14, unpublished data).

Finally, the pilot study cohort included dogs without *Pseudomonas* associated disease and these dogs rarely had contact with veterinary clinical settings. The study reported here exclusively included dogs referred to referral veterinarians for evaluation of otic disease. Therefore, untested nosocomial risk factors may have played an epidemiological role in the present case-control study cohort. Nosocomial transmission of *P. aeruginosa* has been well documented in human hospitals[7] and veterinary studies have suggested that a medical device may serve as a nidus in veterinary clinical settings.[12,13] In addition, progression from exposure to disease may entail individual risk factors that were not captured by the health and environmental exposures data collected for this study.

An alternativee explanation for lack of statistical associations is that the risk factors for *Pseudomonas* otitis may not differ from risk factors for other types of bacterial otitis (which served as the control group). *Pseudomonas* infections often arise in the setting of chronic otic disease and after therapies for other types of fungal or bacterial organisms have been applied. Therefore, most *Pseudomonas* infections do not arise *de novo*. There is also some potential for mis-classification of cases and controls to have occurred in this study. Culture results did not always match the cytological observations (i.e. no bacillary bacteria were grown in some of the control cases, despite rods having been present using cytology).

In conclusion, the genetic homology between otic and environmental isolates identified by this study is consistent with a waterborne source of *P. aeruginosa* for some dogs, and cross-contamination with other human and animal members within some households. However, the cross-sectional design of the study precludes inference of directionality of transmission in most cases. Concordance of strain types across households that have no clear epidemiological link suggests that proliferation of some successful *P. aeruginosa* strains has occurred within the study's catchment area. Clear epidemiological risk factors for development of *Pseudomonas* otitis were not identified, which may either be reflective of an underpowered

study, or an indication of shared risk factors for otic infection within the case and control groups.

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Supporting Information

Additional Supporting Information may be found in the online version of this text at: http://onlinelibrary.wiley.com/doi/10.1111/vde.12347/full

Figure S1. Dendrogram of pulsed-field gel electrophoresis and heat map of antibiogram profiles of *P. aeruginosa* isolates.

Table S1. Data, used as the basis for sample size calculations, on *Pseudomonas* spp. and *P. aeruginosa* recovery from households and pets sampled at a three-month interval in a pilot study[a] involving convenience enrollment of homes participating in a study of *Staphylococcus* spp. in people

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5.5

Staphylococcus aureus penetrate the interkeratinocyte spaces created by skin-infiltrating neutrophils in a mouse model of impetigo

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Background – Impetigo is a bacterial skin disease characterized by intraepidermal neutrophilic pustules. Previous studies have demonstrated that exfoliative toxin producing staphylococci are isolated in the cutaneous lesions of human and canine impetigo. However, the mechanisms of intraepidermal splitting in impetigo remain poorly understood.

Objective – To determine how staphylococci penetrate the living epidermis and create intraepidermal pustules *in vivo* using a mouse model of impetigo.

Methods – Three *Staphylococcus aureus* strains harbouring the *etb* gene and three *et* gene negative strains were epicutaneously inoculated onto tape-stripped mouse skin. The skin samples were subjected to time course histopathological and immunofluorescence analyses to detect intraepidermal neutrophils and infiltrating staphylococci. To determine the role of neutrophils on intraepidermal bacterial invasion, cyclophosphamide (CPA) was injected intraperitoneally into the mice to cause leucopenia before the inoculation of *etb* gene positive strains.

Results – In mice inoculated with *etb* gene positive *S. aureus*, intraepidermal pustules resembling impetigo were detected as early as 4 h post-inoculation (hpi). Neutrophils in the epidermis were detected from 4 hpi, whereas intraepidermal staphylococci was detected from 6 hpi. The dimensions of the intraepidermal clefts created in mice inoculated with *etb* gene positive strains at 6 hpi were significantly larger than those in mice inoculated with *et* gene negative strains. In CPA treated mice, staphylococci or neutrophils were not detected in the deep epidermis until 6 hpi.

Conclusion – Our findings indicate that intraepidermal neutrophils play an important role in *S. aureus* invasion into the living epidermis in a mouse model of impetigo.

Introduction

Impetigo is a bacterial skin infection that affects mammals including humans and dogs. Human bullous impetigo is a contagious skin disease of infants characterized by cutaneous bullae with a definable boundary.[1] Canine impetigo is characterized by nonfollicular intraepidermal pustules that generally affect sparsely haired areas of the skin.[2] *Staphylococcus aureus* is known as the causative pathogen in human bullous impetigo, whereas *Staphylococcus pseudintermedius* frequently is isolated from the skin lesions of canine impetigo.[1,3]

The mechanism of intraepidermal splitting in impetigo can be explained by selective digestion of desmoglein 1 (Dsg1), a keratinocyte cell–cell adhesion molecule, by staphylococcal exfoliative toxins (ETs).[4] The ETs dissociate keratinocytes in the stratum spinosum and granulosum, in which loss of adhesive function by Dsg1 cannot be compensated for by other Dsgs. To date, three types of *S. aureus* ETs (ETA, ETB and ETD) and two types of *S. pseudintermedius* ETs (ExpA and ExpB) have been isolated.[4–6] In human medicine, it has been reported that ETB-producing *S. aureus* is the predominant isoform in generalized staphylococcal scalded skin syndrome (SSSS), whereas no serotype preference for ETs was recognized in bullous impetigo.[7] ETD-producing *S. aureus* is isolated from a broad spectrum of skin diseases including cutaneous abscess and furuncles.[7] All of the three *S. aureus* ETs selectively digest human and murine Dsg1, and cause superficial epidermal splitting when they are injected into neonatal mice.[8–10] In addition, it has been

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reported that *S. aureus* ETA, ETB and ETD share similar enzymatic efficiency and specificity to cleave Dsg1.[11] Thus, the three *S. aureus* ETs may disrupt the epidermal barrier in an identical manner and be associated with bacterial cutaneous invasion.

It has been reported that ETs do not penetrate tight junctions (TJs), which constitute strong barriers in the uppermost stratum granulosum.[12] This finding raises a counterargument to the hypothesis that ETs facilitate penetration of staphylococci to the living epidermis, although ET-producing staphylococci are detected in the intraepidermal pustules in human and canine impetigo. Therefore, the exact mechanism of intraepidermal invasion by ET-producing staphylococci in impetigo has not yet been elucidated. The aim of this study was to investigate the detailed mechanisms of staphylococcal invasion into living epidermis in a mouse model of impetigo.

Materials and methods

Staphylococcus aureus strains and culture conditions

Three *S. aureus* strains harbouring the *etb* gene (TY825,[13] N1 and N2) as well as three strains negative for the three *et* genes (N3, N4 and N5) were used in this study. Identification of *S. aureus* was confirmed by a coagulase test and multiplex PCR was used to identify staphylococcal species.[14] The detection of *et* genes in *S. aureus* was carried out by multiplex PCR as previously reported.[15] *Staphylococcus aureus* strains TY34, harbouring the *eta* gene, and TY114, harbouring the *etd* gene, were used as controls in PCR to detect the corresponding *et* genes.[10,15]

The *S. aureus* strains were cultured in Luria–Bertani medium at 37°C for 12 h, washed three times in phosphate buffered saline (PBS) and then adjusted to a concentration of 1.0×10^9 colony forming units (CFU)/mL in PBS before use. All experiments using *S. aureus* strains were approved by the Research Committee for Specific Biological Safety Management at Tokyo University of Agriculture and Technology.

Mouse model of impetigo

Six-week-old, female BALB/c Cr Slc mice (Sankyo Labo Service; Tokyo, Japan) were housed in microisolator cages and were given food and water *ad libitum*. A mouse model of impetigo was established by partial removal of the stratum corneum (SC) of the inner pinna by tape stripping using Scotch™ tape (3M Japan Ltd.; Tokyo, Japan) for seven times. An inoculum of 2.0×10^8 CFU *S. aureus* was applied to 1 cm^2 pieces of medical gauze, which were placed on the tape-stripped skin and overwrapped with polyurethane film (Nichiban; Tokyo, Japan). Gauze containing PBS alone was placed on another side of the pinna as a negative control. In some experiments, mice were injected intraperitoneally with cyclophosphamide (CPA, Endoxan™, Shionogi Co.; Osaka, Japan) at 2 mg per mouse for five consecutive days to render them leucopenic before the epicutaneous inoculation of *etb* gene positive strains. At 0.5, 2, 4 and 6 h post-inoculation (hpi), the gauze were removed and the pinna were collected from mice after euthanasia by cervical dislocation. All animal experiments were ethically approved by the Animal Research Committee at Tokyo University of Agriculture and Technology.

Histopathological analysis

Pinna samples were fixed in 10% neutral buffered formalin and then paraffin-embedded. Serially sectioned skin samples were deparaffinized and subjected to staining with haematoxylin and eosin (H&E) or Gram stain followed by counterstaining of nuclei with haematoxylin. All histopathological specimens were examined under an optical microscope (BX43F, Olympus; Tokyo, Japan). The images were captured using a digital camera (DP73; Olympus) and imaging software (CellSens Standard; Olympus).

Immunofluorescence staining

Formalin-fixed, paraffin-embedded sections were deparaffinized and pretreated in 10 mM Tris/EDTA, pH 9.0, using the microwave method for antigen retrieval. The slides were incubated in blocking buffer (5% goat serum, 3% skim milk and 0.2% Tween 20 in PBS). The following primary antibodies were used: anti-*Staphylococcus* rabbit polyclonal antisera (1:2560, clone CH91; courtesy of Makoto Haritani, National Institute of Animal Health, Ibaraki, Japan)[16] and anti-keratin type I & II mouse monoclonal antibodies (1:200, clone AE1 + AE3; Progen Biotechnik GmbH; Heidelberg, Germany). The sections subsequently were incubated with Alexa Fluor™ 546-conjugated goat anti-rabbit IgG and Alexa Fluor™ 488 goat anti-mouse IgG (Life Technologies; Carlsbad, CA, USA). Nuclei were counterstained with Hoechst 33258 (Life Technologies). All sections were examined under a fluorescence microscope (Olympus). The images were captured by digital camera and imaging software (Olympus).

Statistical analysis

Kruskal–Wallis one-way ANOVA was used to compare the frequency or dimensions of intraepidermal pustules as well as the frequency of intraepidermal staphylococci among different time points. Wilcoxon–Mann–Whitney U-test was used to compare the number of intraepidermal clefts in mice inoculated with *etb* gene positive strains and those in mice inoculated with *et* gene negative strains, as well as the number of intraepidermal bacteria in CPA treated and nontreated mice. All statistical analyses were performed using the GraphPad Prism 6 software (GraphPad Software; La Jolla, CA, USA).

Results

Staphylococcus aureus harbouring the *etb* gene induced intraepidermal pustules resembling impetigo in mouse skin

We first examined whether intraepidermal pustules resembling impetigo were created by epicutaneous inoculation of *S. aureus* harbouring the *etb* gene. Three *S. aureus* strains were inoculated to six mice (two mice per strain) and 18 skin sections obtained from those mice (three sections per mouse) were subjected to histopathological and immunofluorescence analyses. Staining with H&E revealed intraepidermal pustules containing neutrophils in 18 of 18 skin sections obtained 6 hpi (Figure 1). By contrast, these histopathological changes were not evident in the skin treated with PBS alone (data not shown). Gram-positive cocci were detected in intraepidermal pustules (Figure 1). Immunofluorescence analysis revealed staphylococci in the intraepidermal clefts in all 18 sections analysed (Figure 1). These findings indicated that intraepidermal pustules containing staphylococci resembling impetigo were successfully created on tape-stripped mouse skin inoculated with *etb* gene positive *S. aureus*.

Staphylococci invaded the intraepidermal clefts after neutrophils infiltrated the epidermis

In order to determine how *S. aureus* harbouring the *etb* gene invaded the epidermis, time course histopathological and immunofluorescence analyses

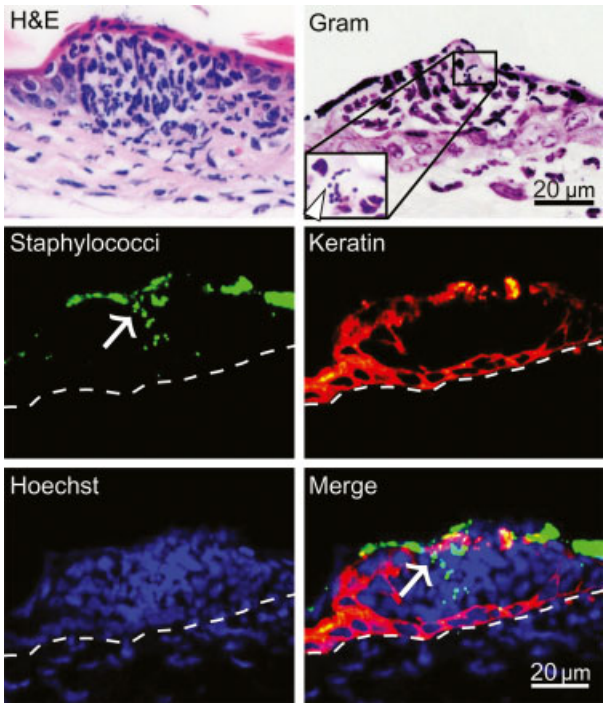

Figure 1. Analysis of the intraepithelial pustules containing staphylococci created in a mouse model of impetigo, 6 h post-inoculation with *Staphylococcus aureus* TY825. Neutrophils, Gram-positive cocci (arrowhead) and staphylococci (arrow) were observed in the intraepidermal clefts. Dotted lines indicate the basement membrane. H&E: Haematoxylin and eosin, Gram: Gram staining, Staphylococci immunofluorescence for Staphylococci, Keratin: immunofluorescence for keratins I & II, Hoechst: a nuclear counterstain that mainly shows neutrophils and keratinocytes, Merge: immunofluorescence for staphylococci, keratins I & II and Hoechst combined.

using the skin obtained from the mouse model of impetigo were performed. Three *etb* gene positive strains were inoculated into six mice (two mice per strain) and 18 skin sections obtained from these mice (three sections per mouse) at each time course were analysed. Intraepidermal neutrophilic pustules were not detected in any of the 18 sections until 2 hpi, appeared in 14 of 18 sections at 4 hpi, and in 18 of 18 sections at 6 hpi (Figure 2a).

Immunofluorescence analysis was performed to confirm the presence of staphylococci in the epidermis. Intraepidermal staphylococci were not detected in the epidermis in any of the 18 sections until 4 hpi, whereas they were found to be abundant on the surface of the epidermis (Figure 2a). By contrast, staphylococci in the interkeratinocyte spaces were recognized in 18 of 18 sections at 6 hpi (Figure 2a). In some sections, immunolabelling for staphylococci overlapped with that for keratin in the epidermis (data not shown).

Figure 2b summarizes the data collected by image analyses of the intraepidermal pustules, as well as staphylococci recognized in the skin sections analysed. The number of intraepidermal pustules per 1 cm of the basement membrane 6 hpi (2.08 $\pm$ 0.64 per cm) was significantly higher than that at 4 hpi (0.66 $\pm$ 0.51 per cm; $P < 0.001$). Similarly, the dimensions of the interkeratinocyte spaces, as determined by immunofluorescence analysis, at 6 hpi (391.70 $\pm$ 131.97 μm^2) were significantly larger than those observed at 4 hpi (142.27 $\pm$ 26.98 μm^2; $P < 0.001$). The number of staphylococci in the interkeratinocyte spaces per 1 mm of the basement membrane at 6 hpi was 2.07 $\pm$ 0.90 per mm. These findings indicated that *S. aureus* harbouring the *etb* gene had invaded the interkeratinocyte clefts after neutrophils infiltrated the epidermis in this mouse model of impetigo. In addition, the dimensions of the interkeratinocyte spaces in mice inoculated with *etb*-gene positive strains increased over the time course analysed.

Intraepidermal clefts created in mice inoculated with *etb* gene positive *S. aureus* were larger than those inoculated with et gene negative strains

In order to determine the role of ETs in staphylococcal intraepidermal invasion, three strains of *S. aureus* positive for the *etb* gene (TY825, N1 and N2), as well as three strains negative for the three known *et* genes (N3, N4 or N5), were inoculated epicutaneously into mouse skin and subjected to immunofluorescence analysis. The presence of the *etb* gene in *S. aureus* strains was confirmed by PCR analysis (Figure S1). The six *etb* gene positive strains were inoculated into 18 mice (three mice per strain), and 54 skin sections obtained from those mice (three sections per mouse) were analysed.

After inoculation with *etb* gene positive strains, Interkeratinocyte spaces were evident in 14 of 18 sections at 4 hpi and 18 of 18 sections at 6 hpi. By contrast, when *et* gene negative strains were inoculated, interkeratinocyte spaces were evident in 12 of 18 sections at 4 hpi and 18 of 18 sections at 6 hpi. Both *etb* gene positive strains and *et* gene negative strains induced interkeratinocyte spaces 6 hpi (Figure 3a). Image analysis of the sections obtained 6 hpi revealed that there were no significant differences in the number of interkeratinocyte spaces per 1 cm of basement membrane in mice inoculated with the six strains ($P > 0.05$; Figure 3b). However, the dimensions of the interkeratinocyte spaces created in mice inoculated with three *etb* gene positive strains were significantly larger than those in mice inoculated three *et* gene negative strains ($P < 0.001$; Figure 3b). There were no significant differences in the number of staphylococci in the interkeratinocyte spaces resulting from inoculation with six strains ($P > 0.05$; Figure 3b). These findings suggest that ETB is not crucial for staphylococcal epidermal invasion, although it may contribute to increasing the size of intraepidermal pustules once staphylococci penetrate the epidermis.

Intraepidermal neutrophils are crucial for staphylococcal epidermal invasion

Because the epidermal invasion of staphylococci was recognized after intraepidermal neutrophilic infiltration, we hypothesized that neutrophils may play an important role in staphylococci epidermal invasion. To elucidate this, mice ($n = 6$) were injected intraperitoneally with CPA that causes leucopenia, followed by inoculation with three *etb* gene positive strains (two mice per strain) as described above. Control mice did not receive CPA before inoculation. A total of 36 skin sections (three sections per

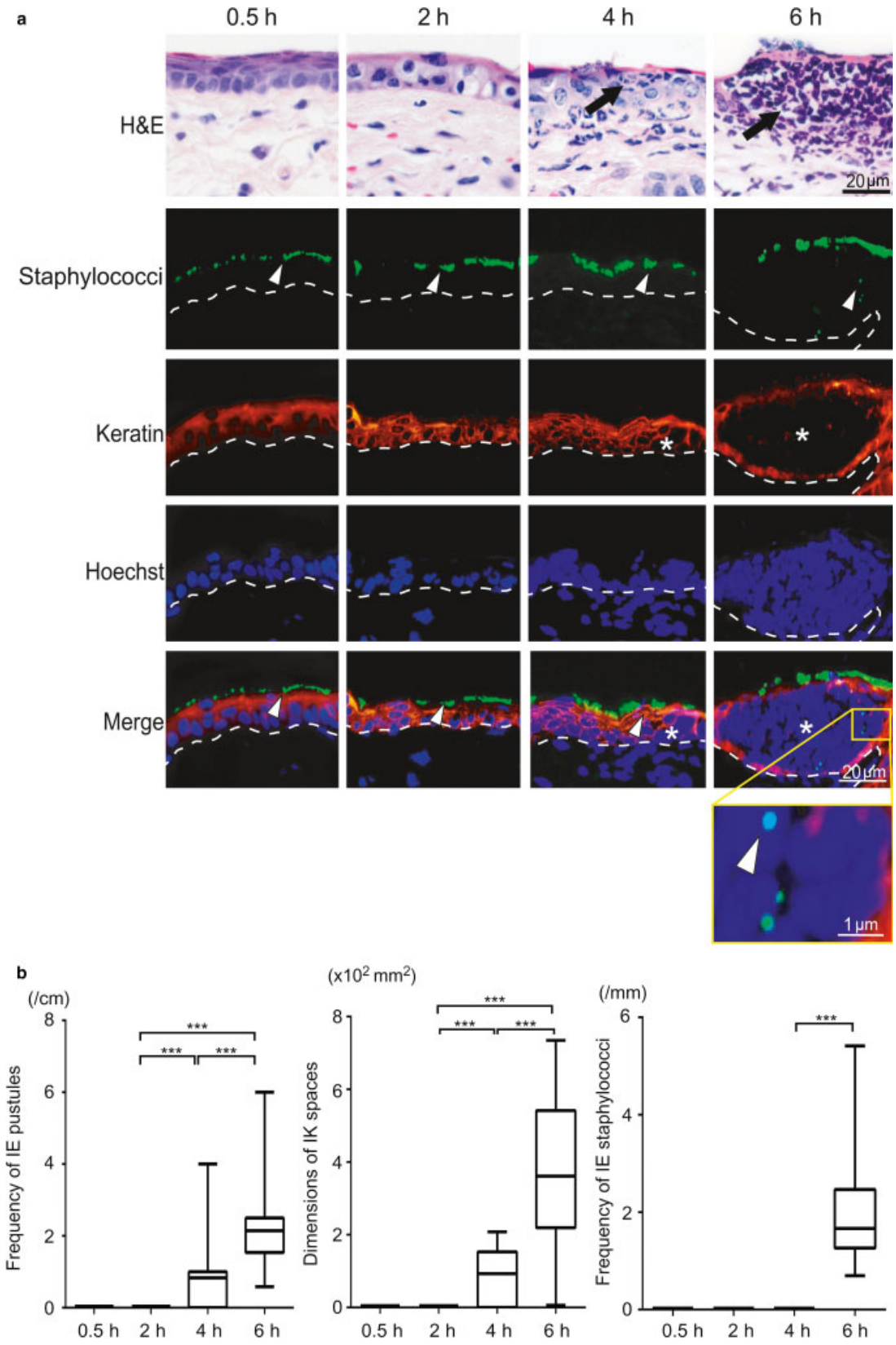

Figure 2. Detection of intraepidermal staphylococci and neutrophils in a mouse model of impetigo. (a) Time course for histopathological and immunofluorescence analyses. Haematoxylin and eosin (H&E) stain revealed that intraepidermal neutrophils (arrows) were detected as early as 4 h post-inoculation (hpi) (epicutaneous) of an *etb* gene positive strain (TY825). Staphylococci (arrowheads) were detected exclusively on the surface of the epidermis up to 4 hpi, but appeared in the interkeratinocyte spaces 6 hpi. Asterisks indicate the interkeratinocyte spaces. Dotted lines indicate the basement membrane. Staphylococci: immunofluorescence for staphylococci, Keratin: immunofluorescence for keratins I & II, Hoechst: a nuclear counterstain that mainly shows neutrophils and keratinocytes, Merge: immunofluorescence for staphylococci, keratins I & II and Hoechst combined. (b) Frequency of intraepidermal (IE) pustules, the dimensions of interkeratinocyte (IK) spaces and the frequency of IE staphylococci. ***$P < 0.001$.

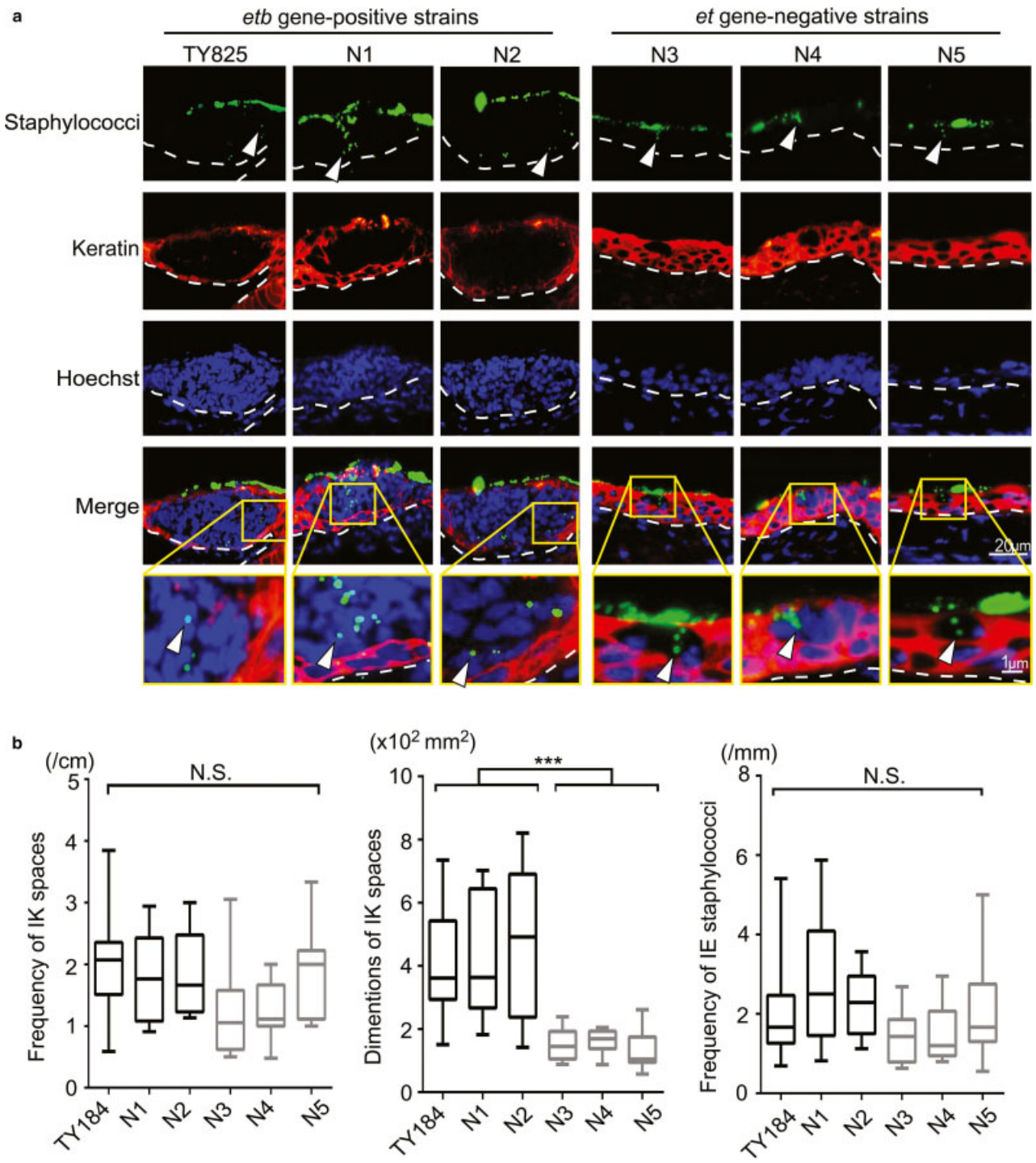

Figure 3. Intraepidermal clefts in mice inoculated with *Staphylococcus aureus* harbouring the *etb* gene compared with mice inoculated with strains that were negative for the *et* gene. Mouse skin was inoculated epicutaneously with *S. aureus* harbouring the *etb* gene (TY825, N1 and N2) or *S. aureus* lacking *et* gene (N3, N4 or N5) and was then analysed 6 h post-inoculation. (a) Immunofluorescence analysis revealed staphylococci (arrowhead) in the interkeratinocyte spaces after inoculation with all six *S. aureus* strains. Dotted lines indicate the basement membrane. Staphylococci: immunofluorescence for staphylococci, Keratin: immunofluorescence for keratins I & II, Hoechst: a nuclear counterstain that mainly shows neutrophils and keratinocytes, Merge: immunofluorescence for staphylococci, keratins I & II and Hoechst combined. (b) Frequency and dimensions of the interkeratinocyte (IK) spaces, and the frequency of intraepidermal (IE) staphylococci. Note that the dimensions of the IK spaces in mice inoculated with the three *etb* gene positive strains were larger than those in mice inoculated with the three *et* gene negative strains. ***$P < 0.001$. N.S.: not significant.

mouse) obtained from three CPA-treated mice and three control mice were subjected to histopathological and immunofluorescence analyses.

In the control mice, neutrophilic epidermal pustules containing cocci were detected in all 18 sections analysed (Figure 4). Conversely, in CPA-treated mice, neutrophils

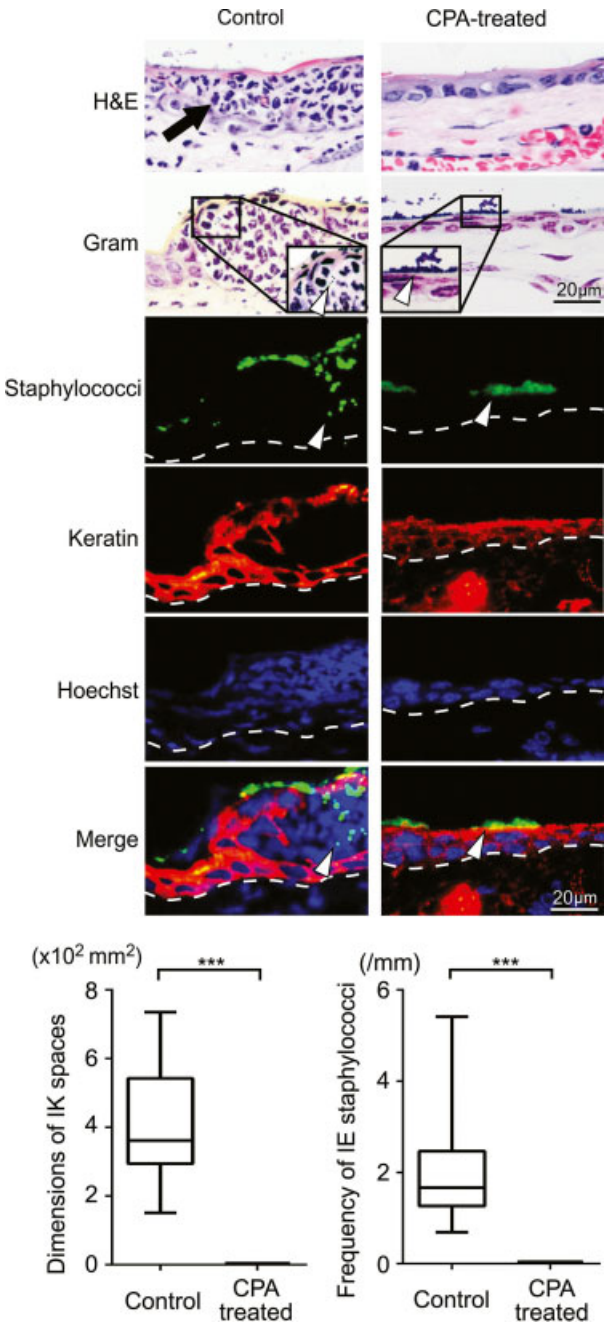

Figure 4. Intraepidermal pustules containing staphylococci in cyclophosphamide (CPA) treated mice. CPA was injected intraperitoneally into mice before epicutaneous inoculation of *Staphylococcus aureus* harbouring the *etb* gene (TY825). Control mice did not receive any medication before the inoculation. Skin samples were collected 6 h post-inoculation. Note that intraepidermal neutrophils (arrow) and cocci or staphylococci (arrowheads) were detected in control mice, but not in CPA-treated mice. H&E: Haematoxylin and eosin, Gram: Gram staining, Staphylococci: immunofluorescence for staphylococci, Keratin: immunofluorescence for keratins I & II, Hoechst: a nuclear counterstain that mainly shows neutrophils and keratinocytes. Merge: immunofluorescence for staphylococci, keratins I & II and Hoechst combined, Dotted lines indicate the basement membranes. ***$P < 0.001$.

were not detected in the deep epidermis or dermis at 6 hpi in any of the 18 sections analysed (Figure 4). Staphylococci were present on the surface of the epidermis in all 18 sections (Figure 4). These findings suggest that neutrophils migrating to the epidermis are crucial for staphylococcal invasion of the living epidermis in the mouse model of impetigo.

Discussion

In this study, we established a mouse model of impetigo by epicutaneous inoculation of *S. aureus* harbouring the *etb* gene onto tape-stripped skin. In this model, staphylococci invaded the epidermis after neutrophils infiltrated the skin 6 hpi. Interkeratinocyte spaces created in mice inoculated with the *etb* gene positive strains were larger than those in mice inoculated with *et* gene negative strains. Interestingly, staphylococci did not invade the epidermis until 6 hpi if neutrophilic infiltration was blocked by injection of CPA. Because all of the three *S. aureus* ETs share similar enzymatic efficiency and specificity, we speculate that similar findings may be found when *eta* or *etd* gene positive strains are inoculated onto the skin. Moreover, as *Staphylococcus hyicus* ETs and *S. pseudintermedius* ETs share similar amino acid sequences and enzymatic activities with *S. aureus* ETs, our findings also may be implicated in the pathogenesis of swine exudative epidermitis and canine impetigo.

Our findings suggest that neutrophils migrated to the epidermis are crucial for staphylococcal percutaneous invasion. However, the exact mechanisms by which neutrophils facilitate staphylococcal invasion are not yet well understood. A previous study revealed that neutrophils transmigrate across TJs by interactions between junctional adhesion molecule-like protein expressed on neutrophils and its receptor that is specifically expressed at TJs in cultured epithelial cells.[17] Therefore, the authors hypothesized that skin infiltrating neutrophils affect TJ proteins in response to *S. aureus*, causing the opening of epidermal TJs. Although the opening of epidermal TJs might facilitate percutaneous migration of neutrophils towards the SC and may play a role in antimicrobial activity, it may also cause percutaneous invasion of bacteria to some extent, especially if staphylococci are abundant in the deep SC. There is substantial evidence for the molecular mechanisms of neutrophilic migration in response to bacteria. It has been reported previously that the interaction between *S. aureus* cell wall components, peptidoglycan and lipoprotein, and toll-like receptor 2 (TLR2) expressed on keratinocyte membranes, promotes neutrophilic migration towards cultured keratinocytes when they were co-cultured.[18,19] Other studies revealed that binding of bacterial soluble enzymes, trypsin and trypsin-like enzymes to proteinase activated receptor 2 (PAR2) expressed on keratinocyte cell surfaces causes migration of neutrophils towards cultured keratinocytes.[20–22] Further studies are expected to elucidate the exact molecular mechanisms behind the neutrophilic infiltration mediated by staphylococci and the opening of TJs in the epidermis in the mouse model.

Another hypothesis might be that *S. aureus* itself degrades TJ proteins as described previously and promotes bacterial cutaneous invasion.[23] However, our study revealed that *S. aureus* did not invade the deep epidermis unless neutrophils infiltrated the epidermis. From this finding, we suspect that *S. aureus* itself may not

have a significant impact on TJ function in the early phase of cutaneous infection. We also revealed that both *etb* gene positive strains and *et* gene negative strains invaded the epidermis equally. Thus, we suspect that ETs may not contribute directly to degradation of TJs. Instead, we suggest that ETs contribute to the expansion of intraepidermal clefts once ET-producing staphylococci have invaded the epidermis. Meanwhile, previous *in vitro* studies revealed that staphylococci invade the cytoplasm of keratinocytes.[24–27] Our data also implicated that staphylococci appeared to be in the cytoplasm of keratinocytes in the mouse model of impetigo. Further studies using electron microscopic analysis will be needed to confirm the route of staphylococcal intraepidermal penetration in this mouse model.

The production of intraepidermal pustules in response to epicutaneous inoculation of *S. aureus* has been reported in previous studies.[28,29] Another study reported that bacteria invaded the epidermis as early as 3 hpi in CPA-treated mice.[25] One study reported that bacteria invaded the epidermis as early as 6 hpi of *et* gene negative *S. aureus* into control mice and that similar changes were observed in CPA-treated mice.[29] These findings are inconsistent with our findings in which intraepidermal bacterial invasion in CPA-treated mice was not detected until 6 hpi. The exact reasons for this discrepancy are unknown. In an earlier study, bacteria were inoculated onto haired skin after the skin had been shaved by razor blade and further abraded by sand paper.[28] Thus, it is possible that bacteria invaded the epidermis as a result of mechanical damage of the SC. In another study, the skin had been shaved by an electric razor before tape stripping, and that the technique was found to cause only minimal damage to the epidermis.[29] On reviewing a figure from the latter study, the SC appeared to be entirely absent from the skin surfaces, especially in the infected sites.[29] In our study, we selected the inner pinnae as the site of inoculation as this site has sparse hairs and therefore does not require shaving. In addition, our tape-stripping technique did not completely remove the SC in any of serial sections as determined by histopathological analysis (data not shown). Thus, it is unlikely that our technique allowed for bacterial penetration through cutaneous microwounds or the skin surfaces in which the SC had been removed completely. Furthermore, we found that *S. aureus* harbouring the *etb* gene could not penetrate intact SC (i.e. skin that was not subjected to tape stripping) until 6 hpi (data not shown), although staphylococci could penetrate the SC in spontaneous cases of impetigo. The exact mechanisms of staphylococci penetration through the SC remain to be elucidated.

Although CPA also affects leucocytes other than neutrophils (e.g. macrophages and lymphocytes), the effect of those cells on the blockage of staphylococcal invasion might be minimal, because the number of those cells in the inoculated skin were low compared with neutrophils. We did not analyse the skin at later time points (e.g. 12 or 24 hpi) in detail because we found that keratinocyte degeneration in the superficial epidermis was remarkable after 6 hpi. Such skin sections would not be ideal to investigate the mechanisms of bacterial invasion in the early stage of the cutaneous infection.

In summary, the current study indicates that *S. aureus* invade the interkeratinocyte spaces created by neutrophils. In addition, once *S. aureus* invade the interkeratinocyte spaces, ETs may expand the space and create pustules similar to those observed in human and canine impetigo. These findings provide important insights into the mechanisms of pustule formation in impetigo. The detailed molecular interactions that underlie neutrophilic migration to the epidermis, as well as epidermal invasion of staphylococci, require further investigation.

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Supporting Information

Additional Supporting Information may be found in the online version of this text at: http://onlinelibrary.wiley.com/doi/10.1111/vde.12398/full

Figure S1. Detection of *eta*, *etb*, *etd* and ribosomal 16S genes in *S. aureus* strains by PCR.

Part 6

New Diagnostic Approaches

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6.1

Dermatopathology – the link between ancillary techniques and clinical lesions

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Background – Histopathology has been essential in advancing our knowledge in veterinary dermatology. However, morphological features and histological patterns cannot always reveal an aetiological diagnosis.

Objectives – Several ancillary techniques can assist in achieving an aetiological diagnosis. Some of these techniques have found their way into routine diagnostic dermatopathology, whereas others are still mainly used in research. This review discusses the utility, strengths, advantages/disadvantages and challenges associated with each technique.

Methods – Digital microscopy, immunohistochemistry, immunofluorescence, salt-split skin, Western blots, electron microscopy, PCR, *in situ* hybridization, tissue microarrays, next-generation sequencing, DNA microarrays and laser microdissection are discussed.

Conclusions – It is crucial to understand the limitations of each technique and to correlate the results both with pathological findings and the clinical presentation. As such, dermatopathology will remain the important link between benchtop science, available results from ancillary techniques and clinical veterinary dermatology.

Introduction

Histopathology has made significant contributions to vastly advance our knowledge in veterinary dermatology. We routinely use morphological features and histological patterns in association with clinical presentation to achieve a diagnosis. Despite this increased understanding, more frequently than we would like, histopathology alone does not reveal an aetiological diagnosis. As a consequence, the dermatopathologist is confronted with the need to use additional techniques to further characterize a particular lesion in hope of identifying its exact aetiology.

Over the years, many of these techniques have been incorporated in numerous published studies of various skin diseases and the results have been interpreted carefully in tandem with the morphological changes seen in the skin lesions. Although not readily available for each difficult case, these studies have improved our understanding of the aetiology and pathomechanisms of many skin diseases. This acquired knowledge assists the diagnostic dermatopathologist to draw better conclusions from basic histopathology seen on H&E stained sections.

This review will discuss the utility and potential of digital microscopy, immunohistochemistry, immunofluorescence, Western blot, electron microscopy, PCR, *in situ* hybridization, tissue microarray and DNA microarray, next-generation sequencing (NGS) and laser microdissection. As it is crucial to understand the challenges and limitations of each technique, both advantages and disadvantages are discussed in this review and examples of how our understanding of skin diseases have been expanded by each technique are given.

Digital microscopy

Instead of using the original glass slide with the actual sample, many diagnostic laboratories scan each slide and provide digital scans to the pathologist for evaluation.[1]

Method

Entire slides are scanned at different magnifications, typically an overview scan (2×) and a scan at higher magnification (40×). These are uploaded on a server and the scanned images accessed over the internet. The customized software allows the scan to be moved around in a similar way to moving a glass slide on the microscope stage. Instead of changing objectives, zooming-in and -out reveals additional details of the lesion.

Advantage

Because digitized cases can be shared easily between pathologists, second opinions can be provided in a very timely manner. Pictures are obtained from the scanned slides and either sent along with the pathology report or used for publication. Teaching from a scanned slide ensures that all students are looking at exactly the same lesion; this may not always be the case with serial sections, which may exhibit differences in morphological features between different slides from the same biopsy sample.[2] Moreover, limited numbers of serial sections

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can be produced from small needle or punch biopsy specimens.[3] The computer software also allows quantitative evaluations of various features on images from the tissue sections. For example, immunohistological stains can be evaluated with regards to percentages of positively staining cell populations within a lesion.

Disadvantages and challenges

Each file is on average between 1 and 5 GB, but may go all the way up to 10 GB. Hence, storage of these files requires a large amount of available memory on the receiving computer to evaluate each scanned slide successfully. High quality thin histological sections are crucial to produce high quality full slide scans.[4,5] Despite meticulous scanning of a sample, certain features such as granules, small inclusion bodies and small pathogens are not necessarily obvious on scanned slides (personal observation). In such instances, the pathologist needs to go back to the actual glass slide or request reimaging of the glass slide by alternative modalities, such as Z-stack images.[6]

Immunohistochemistry

The use of antibodies in diagnostic pathology has become an indispensable technique that is routinely used by many diagnostic laboratories and pathologists.[7] Pathologists often have to rely on the immunophenotype of a neoplastic cell population to identify its origin.[8–10] However, antibodies are specific for an antigen in a specific species. Prior to using an antibody in a different species, it has to be evaluated carefully for its potential for correct cross-reactivity in this new species. Cross reactivity is more likely in highly conserved antigens such as CD3.

Anatomic pathologists also use immunohistochemistry (IHC) to highlight certain tissue structures and the location of morphological changes in relation to those particular structures. This approach has become very helpful in differentiating various diseases affecting the basement membrane zone (BMZ).[11] Moreover, many infectious pathogens, in particular viruses, are too small to appreciate in routine histopathology. Immunohistochemistry has proven to be very helpful in identifying such pathogens within affected tissues.[7,12,13]

It is important to keep in mind that specimen fixation can affect the potential of an antibody to bind to its epitope (see below). Moreover, it cannot be stressed enough that IHC results have to be viewed together with the actual morphological lesion on the H&E section. It is imperative to know the antigen of interest and its correct location within the cell or tissue in order to evaluate appropriate binding of an antibody, as occasionally irrelevant or spurious staining can occur; this is quite often observed with the commonly used anti-CD79a antibody (personal observation).[14]

Method

Primary antibodies specific for certain epitopes are applied to histology sections or cytology samples. Unbound antibodies are washed off, whereas bound antibodies are identified by a second anti-immunoglobulin antibody. This secondary antibody is specific for the species in which the primary antibody has been produced. For example: if the primary antibody is a mouse–anti-dog leukocyte marker, the secondary antibody will be an anti-mouse IgG. This secondary antibody is conjugated to an enzyme and detection of antibody binding is based on the enzymatic reaction on a chromogen, resulting in a colour reaction occurring wherever the tagged antibody binds. For example: streptavidin-hydrogen peroxidase (HRP) binds to biotin on secondary antibodies and HRP will initiate the enzymatic reaction on the chromogenic.[7] The costs of IHC lay primarily in the purchase of the antibodies, some of which have a limited lifespan. Hence, most diagnostic laboratories limit their choice of antibodies to the most commonly used ones.

Advantage

Typically IHC is used for immunophenotypic characterization of tumour cells, identification of particular tissue structures or infectious pathogens in both diagnostic dermatopathology and research. Immunohistochemistry can be applied on aspirates, tissue imprints or snap-frozen tissue sections, and some antibodies also recognize antigens in formalin-fixed paraffin-embedded (FFPE) tissue sections. Most diagnostic laboratories limit their IHC to antibodies that can be used on FFPE tissues.

Disadvantages and challenges

Formalin fixation creates crosslinks, which potentially obscure the specific epitopes recognized by a particular antibody.[14,15] Hence, a limited number of antibodies are currently available for use in formalin-fixed tissues. Typically, an antigen retrieval procedure (enzymatic or heating with citric acid) is required to break the crosslinks in order to make epitopes available for the binding of an antibody.[15,16] False negative results may occur on inappropriately fixed tissues because subsequent processing of tissue sections through the alcohol series can destroy insufficiently fixed antigens.[14,17] False positive results may occur as antibodies can bind nonspecifically, in particular to partially damaged tissue. This is readily evident in areas of necrosis. If the procedure is done manually, immunohistochemistry is a rather labour-intensive procedure; automatic immunostainers are available, but costly.

Examples of contributions to dermatology

There are innumerous examples of the use of antibodies to immunophenotype neoplastic processes, including leukocytic tumours (Figure 1), cutaneous glomus tumours and amelanotic melanomas.[8–10,18,19] As intranuclear inclusion bodies are not always evident on H&E, identification of papilloma virus typically is dependent on using the cross-reactive anti-bovine papilloma virus (BPV)-2 antibody.[12] With acute and subacute distemper, canine distemper virus can be identified within keratinocytes of haired skin, footpads and nasal mucosa.[20] Identifying the location of laminin or collagen IV in lesions of subepidermal vesiculation assists the differentiation between bullous pemphigoid (BP), epidermolysis bullosa aquisita (EBA) and linear IgA disease.[11]

Immunofluorescence

Similar to immunohistology, immunofluorescence (IF) is used to identify proteins with the help of specific

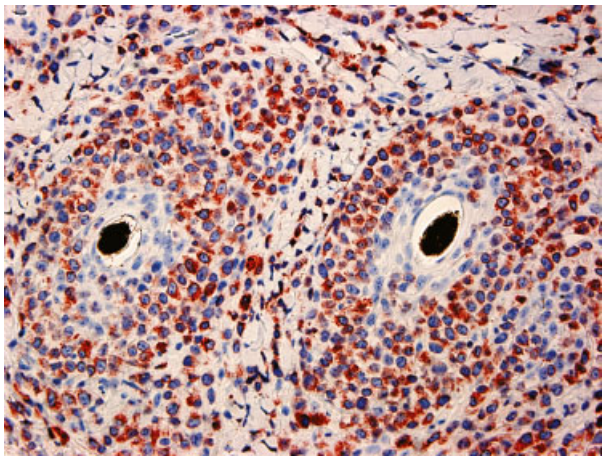

Figure 1. Immunohistochemistry. Cutaneous epitheliotropic T cell lymphoma in a horse. The monomorphous round cell infiltrate within the follicular epithelium is composed of CD3 positive T cells. Cross-reacting anti-human-CD3ε (CD3-12, Serotech; Oxford, UK), Chromogen: 3-amino-9-ethylcarbazole (AEC, Dako; Carpinteria, CA, USA). 40×

antibodies coupled with a fluorophore.[21] Each fluorophore requires a light source of specific wavelength to initiate emission of fluorescence. Examples are fluorescein isothiocyanate (FITC), Texas red (TR) or phycoerythrin (PE). Immunofluorescence is usually used on snap-frozen tissues or on cytology specimens.

Method

Both direct immunofluorescence (DIF) and indirect immunofluorescence (IIF) are used in dermatopathology, in particular in diagnosing autoimmune diseases. Typically, DIF is used to evaluate skin lesions for the presence of intralesional immunoglobulins and/or complement factors using fluorescent-coupled anti-Ig or anti-complement factor antibodies.[22] Alternatively, with IIF sera are applied to a healthy skin specimen (usually lip or tongue) in order to identify tissue-specific circulating autoantibodies.[23] In a second step, a fluorophore-coupled anti-species immunoglobulin heavy chain antibody is applied to identify location of bound primary antibodies. Similar to IHC, costs of IF are mostly associated with the purchase of the antibodies and an immunofluorescence microscope.

Advantage

Immunofluorescence is often used for double labelling of samples for the presence of two different proteins.[21,23] By exposing the sample to light that excites both fluorophores used, the expression of both proteins can be evaluated simultaneously. For example, FITC labelled proteins will emit a green light, whereas TR labelled proteins will emit a red light, which makes it visually advantageous to distinguish the individual protein expressions. If both proteins are expressed in the exact same location (overlapping), the merge of green and red lights will result in a yellow light emission.

Disadvantages and challenges

Auto-fluorescence is a common challenge with IF on formalin-fixed tissues. Hence, IF is usually performed on frozen sections and is therefore not a common procedure in routine diagnostic dermatopathology. Typically it is performed manually, which is a rather labour-intensive procedure. Also IF stained slides need to be stored in the dark, preferentially at cool temperatures, because fluorophores tend to fade. Hence, IF stained slides cannot be stored for a prolonged period of time. Moreover, certain fluorophores fade very rapidly upon microscopic evaluation. It is therefore very important to document reaction quickly by capturing images.

Examples of contributions to dermatology

Both BP and EBA are characterized by anti-basement membrane antibody that can be identified by both DIF and IIF (Figure 2).[24,25] With salt-split skin samples, BP is mostly associated with immunoglobulin deposition at the roof of the split (i.e. above the lamina lucida; see Salt-split skin section below).[26] The location of the positive reaction reflects the presence of autoantibodies against collagen XVII in BP, which is a protein located above the lamina lucida. In contrast, with EBA positive IIF is mostly at the bottom of the cleft in salt-split skin because circulating autoantibodies are directed against collagen VII, a protein located below the lamina lucida.[25] Dogs with pemphigus foliaceus (PF) have circulating antibodies against desmocollin-1 (DSC1);[27] this has been demonstrated with IIF by applying sera from dogs with PF to canine transfected DSC1 293Tcells.

Salt-split skin (SSS)

The BMZ is a complex structure with many different glycoproteins, several of which are targets for different autoimmune diseases.[25,26,28] Antibody binding to any of these BMZ proteins results in dermo-epidermal separation and identifies it microscopically as a sub-epidermal blistering disease. However, light microscopy does not allow identification of the exact location of the separation within the BMZ.

Salt-spilt skin (SSS) samples consistently reveal a split within the lamina lucida, leaving the hemidesmosomes attached to the epidermis, whereas the lamina densa region of the BMZ will stay attached to the dermis.

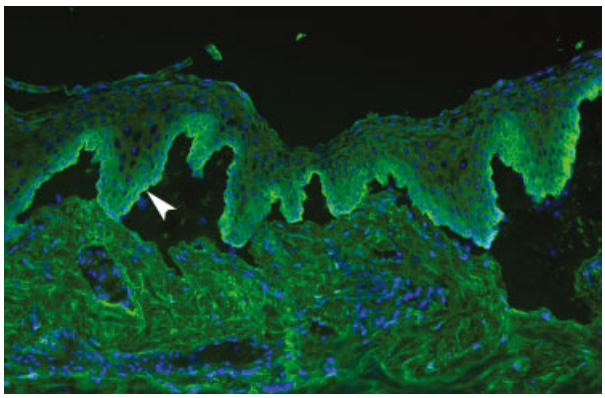

Figure 2. Indirect immunofluorescence. IgG binding along the roof of salt split skin (white arrowhead) is consistent with an autoimmune subepidermal blistering dermatosis of the pemphigoid group. Fluorescein isothiocyanate (FITC)-coupled anti-dog IgG. 10×; Courtesy of Thiery Olivry, North Carolina State University.

Method

Typically, small samples of normal lip or tongue are immersed in 1 M NaCl for 24 h.[25] Subsequently the samples are either embedded in Tissue-Tek® O.C.T compound (Sakura Finetek; Torrance, CA, USA) and snap frozen for either IHC or IIF. The samples can then be exposed to a patient's serum to evaluate for presence of circulating autoantibodies.[25] Alternatively, samples are prepared in 1 M NaCl with 5 mM ethylenediamine tetraacetic acid (EDTA), 50 mM phenylmethane sulfonylfluoride (PMSF) and 50 mM *N*-ethylmaleimide (NEM) at 4% for 96 hours with stirring. Samples are fixed in 2% glutaraldehyde and 1% osmium tetroxide and processed for electron microscopy (EM).[26] Patients' sera can be kept frozen at −20°C or −80°C for a prolonged period of time.

Advantage

Frozen SSS lip or tongue can be stored at −80°C. Sections from these blocks can be used over a long period of time. It offers a first step to differentiate autoimmune diseases targeting the BMZ.[29]

Disadvantages and challenges

Processing of frozen tissue is still not a routine technique performed in veterinary diagnostic settings. It also requires that clinicians collect patients' sera before immunosuppressive therapy is initiated.

Examples of contributions to dermatology

SSS has been used to further characterize and differentiate pathomechanisms involved in BP, EBA and mucous membrane pemphigoid (MMP). As mentioned above, IIF using sera from patients with BP and MMP (Figure 2) mostly reveal a positive staining of the top portion of the SSS sample, because circulating antibodies in these diseases target the NC16A ectodomain of collagen XVII (BP180, BPAG2).[26,28,30] Alternatively, circulating antibodies in sera of dogs with EBA have antibodies targeting the NC12 domain of collagen VII; consequently, IIF will reveal a positive linear staining along the bottom portion of the SSS.

Electron microscopy

Ultrastructural evaluation of tissues and cell cultures has been used since 1939. However, other techniques such as PCR have often replaced the need for access to EM in daily diagnostic pathology.

Method

Samples need to be fixed in a special fixative such as 2% glutaraldehyde and 1% osmium tetroxide. Previously formalin-fixed tissues can be used also; however, the quality of the samples may be somewhat impaired.[31]

With scanning electron microscope (SEM), a focused beam of electrons interacts with atoms in the sample and is used to identify surface topography and composition of a sample. Resolution >1 nm can be achieved. Specimens can be observed in high vacuum, in low vacuum, in wet conditions (in environmental SEM), and at a wide range of cryogenic or elevated temperatures.

With transmission electron microscopy (TEM), a beam of electrons is transmitted through an ultra thin tissue section. As the electrons interact with the specimen, an image is formed, which subsequently is enlarged and visualized on a fluorescent screen or on photographic film. The small samples are put on a fine metallic grid and are evaluated within a vacuum.

Advantages

Electron microscopy allows identification of pathogens (Figure 3) as well as cellular and extracellular matrix changes in the context of the structures of the surrounding tissues. It is a powerful tool to identify new pathogens and their exact location within cellular structures. Conjugating antibodies with gold beads (immuno-gold) have been used to identify a specific protein within extracellular matrix or cellular compartments.[25,32]

Disadvantages and challenges

The maintenance of an EM facility is costly and requires trained staff to run it. Hence, most diagnostic laboratories do not include an EM service and many anatomical pathology departments at universities no longer have their own EM facility. Therefore, samples will typically need to be sent out to laboratories with EM capabilities for further evaluation.

Examples of contributions to dermatology

Electron microscopy enabled the identification of a reduction of anchoring fibrils in the basement membrane of a young dog with subepidermal blistering disease consistent with dystrophic epidermolysis bullosa (DEB).[33] Although periodicity of collagen fibres was within normal limits, horses with hereditary equine regional dermal asthenia (HERDA) had a higher variability of the cross-sections of the collagen fibrils when compared to healthy horses.[34] Ultrastructurally, fibroblasts of shar-pei dogs with hereditary cutaneous hyaluronosis (mucinosis) differ

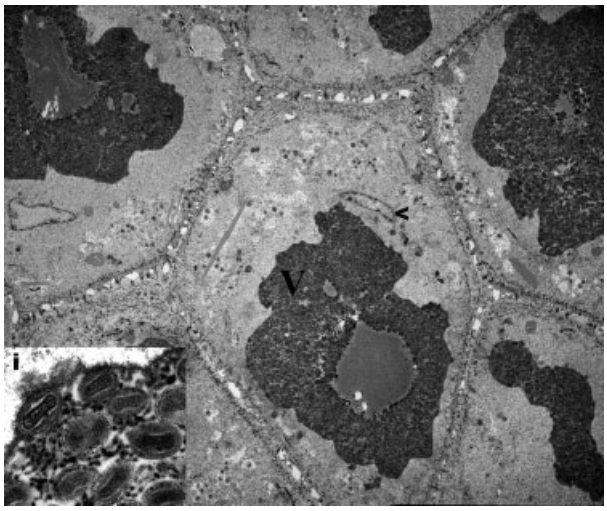

Figure 3. Transmission electron microscopy of pox virus in a finch. Numerous virions (V) are aggregated in the cytoplasm close to the nucleus (<). The insert (i) shows the very large virions, 200+ nm. 2690 × 2250. Courtesy of Patricia Pesavento, University California Davis and Wednesday Slide Conference (19-2007) of the Joint Pathology Center.

from healthy dogs; cellular protrusions were noted as well as an increase in hyaluronic acid content in several subcellular structures.[35] Innumerous viral infections have first been identified with the help of EM. For example, a new poxvirus was identified in skin lesions of two human patients;[36] subsequent DNA analysis revealed a new poxvirus, which could not be classified as either *Parapox* genus or *Molluscivirus* genus.[36] Alternatively, EM also assists in ruling out the presence of a viral infection as shown in pododermatitis in flamingos.[37]

Western blot

Protein separation by SDS–PAGE and subsequent Western blot is used by many laboratories to investigate and demonstrate the expression of proteins in a lysate.[38] Alternatively, with access to purified proteins, the presence of specific antibodies to certain proteins can be identified in a patient's serum.[27,39]

Method

Proteins in a lysate are separated by electrophoresis based on their molecular weight (SDS–PAGE); often the proteins are linearized as electrophoresis is performed under reducing conditions.[40] Simultaneously, proteins of known molecular weights are added as molecular weight markers. The proteins subsequently are transferred from the gel to a highly hydrophobic membrane (nitrocellulose or polyvinylidene difluoride (PVDF), charged nylon).[41] The membrane is exposed to specific antibodies. Various techniques are used to detect the presence of bound antibodies directly on the membrane or, alternatively, on radiographic film using enhanced chemiluminescence.[42]

Advantages

Western blots allow the identification of molecular weights of proteins and glycoproteins in tissue extracts of the skin. Western blot is a reliable method to evaluate antibodies for specificity and correct cross-reactivity. It is a simple and rather inexpensive method that tends to be easily interpretable.

Disadvantages and challenges

This technique requires access to specific antibodies to identify the protein in question, or alternatively, purified proteins to allow detection of circulating antibodies within the patient's serum. Moreover, it is a rather labour-intensive technique. Hence, Western blots are limited mostly to research settings in veterinary medicine. Several factors may impair the accuracy of Western blots.[38] Occasionally, several bands reflecting proteins of different molecular weights are detected with a particular antibody. This could be the result of allelic differences of a protein, different isoforms or various glycosylation of proteins. Typically the electrophoretic separations of proteins are run under denaturing conditions (SDS–PAGE), which could change the capability of antibodies to identify the appropriate epitope. Moreover, the blotting may not be reproducible for each lysate, because transfer to the membrane may vary between runs. A further challenge is the quantification of the detected protein.

Examples of contributions to dermatology

Dogs with BP have circulating antibodies that recognize a 180 kD hemidesmosome associated glycoprotein, subsequently identified as NC16A of collagen XVII.[24] Circulating antibodies in dogs directed against the major *Microsporum* proteins have been identified.[43] Western blot analysis identified a long interspersed nucleotide element (LINE-1) insertion in the transglutaminase-1 gene as the cause of decreased levels of TGM-1 in Jack Russell terriers with ichthyosis.[44]

PCR and quantitative PCR

PCR is the most commonly used technique for the detection of DNA/RNA in biological samples.[45] It allows amplification of very small amounts of target DNA or RNA. First described in 1983, PCR has become well established in many diagnostic laboratory settings.[46]

Methods

RNA is quickly degraded by universally present RNAses. Therefore, sample RNA is first transcribed to complementary DNA (cDNA) to be used as template for PCR. High temperatures separate DNA strands, which then become available as templates for the synthesis of a new complementary strand of DNA. Upon cooling down of the reaction, small complementary DNA fragments (referred to as primers) anneal with the template and the rest of the complementary DNA strand is formed by adding nucleotides with the help of the polymerase activity. Cyclic changes of temperatures result in exponential amplification of the target DNA of interest. The size of the amplicons is evaluated by electrophoresis and its actual composition is evaluated by sequencing. The success of PCR is dependent on the quality of the starting DNA/RNA in the sample that is being evaluated.

Real-time quantitative PCR (RT-qPCR) has become the gold standard for detection and quantification of nucleic acids from multiple sources. There are different systems of qPCR. One method includes running a dilution series of known independently quantified standard templates parallel to the unknown sample.[47,48] A DNA-binding dye such as cyber green binds to all double-stranded (ds) DNA in PCR, causing fluorescence of the dye. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity measured at each cycle. Alternatively, qPCR is achieved by the TaqMan technique, which relies on the 5′–3′ exonuclease activity of Taq polymerase to cleave a dual-fluorophore labelled probe during hybridization to the complementary target sequence.[49] Quantification relies on a fluorescent signal produced with each cycle of the PCR reaction.[49,50] It is often used to identify a viral load in a lesion or to identify levels of mRNA for various intralesional growth factors or cytokines.[45]

Advantages

It is a very time efficient, inexpensive and sensitive technique that requires very little starting material. DNA or RNA can be extracted from either fresh or frozen material and with variable success from FFPE tissues. Culturing of certain pathogens is labour-intensive and requires special

techniques; PCR may give a much faster result. PCR is also used to further specify cultured pathogens. Another common use for PCR is the identification of mutations, as for example seen with neoplastic cell populations.[51]

Disadvantages and challenges

PCR is a very sensitive technique. On the one hand, false positive results due to cross-contamination are a major challenge. This has been clearly documented by finding papilloma virus in healthy canine skin.[52] Moreover, it is important to prove causality of a particular pathogen identified by PCR. This often requires application of additional techniques such as immunohistology or *in situ* hybridization.[53,54] On the other hand, false negative results may occur due to low quality DNA/RNA in a sample. Moreover, prolonged or inappropriate formalin fixation can interfere with the successful amplification of DNA or RNA, particularly when the amplified segment is long. Furthermore, false negative results can occur in the presence of adverse factors such as contamination, inhibition of the amplification reaction or problems during nucleic acid extraction.[55]

Examples of contributions to dermatology

PCR is used routinely for the detection of pathogens, in particular those infectious agents that are difficult to culture including mycobacteria, fungi, protozoa and viruses.[56–58] Truncated LINE-1 inserts were identified associated with transmissible venereal tumours of dogs.[51] The 1378 bp LINE-1 insert can be identified by PCR and, hence, confirm the diagnosis of metastatic neoplastic cells.[51] Clonality testing or PCR for antigen receptor rearrangement (PARR) and subsequent electrophoresis is used routinely now to differentiate between lymphomas and an inflammatory lymphoid rich process (Figure 4). Clonal rearrangement of T cell receptor gamma (TCR-γ) or immunoglobulin heavy chain (IgH) supports the diagnosis of T cell lymphoma or B cell lymphoma, respectively, whereas polyclonal rearrangements are more consistent with an inflammatory process.[59–64] It is important to interpret the clonality results in association with morphological and immunophenotypic features and clinical presentation, because occasionally clonal populations can be found in reactive processes. This has been seen in regressing histiocytomas, which may exhibit the presence of a clonal CD8+ T cell population (personal observation). In addition, if there is a prominent reactive lymphoid infiltrate associated with the neoplastic lymphocytes, the clone may not be evident within the polyclonal background.

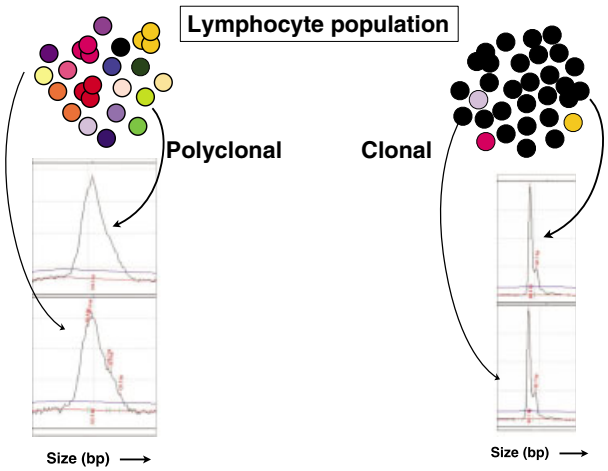

Figure 4. Clonality testing, also referred to as PCR for antigen receptor rearrangement (PARR) for (TCR-γ) and IgH. (a) A polyclonal population is characterized by lymphocytes with different antigen receptor rearrangements (length as well as sequence). This represents a reactive population typically seen with inflammation. A polyclonal result in the setting of a neoplastic process – referred to as a "false-negative" result – can occur due to: primers cannot bind as neoplastic cells have mutations in the primer binding site, a small neoplastic population (<5% of cells) may be obscured by a reactive lymphoid population or available primers may not cover the entire locus. (b) A neoplastic cell population is developing from a clone and has an identical length and sequence of the antigen receptor rearrangement. However, occasional clonal populations can be seen with benign expansions; examples include lymphocytic infiltrate in canine regressing histiocytomas, reactive T cell populations in equine T cell rich B cell lymphomas, ehrlichiosis and drug hypersensitivities in dogs. Courtesy of Peter F. Moore, University California Davis.

In situ hybridization

Small complementary molecular probes are used to detect sequences of certain chromosomal regions or genes. This can be applied for identification of host or pathogen DNA/RNA in tissues and cytology specimens.[65]

Methods

Variably labelled small complementary DNA or RNA probes can be applied to fresh snap-frozen tissues, FFPE tissues, smears or microarrays. The complementary molecular probes are either radiolabelled or conjugated to fluorescent compounds (fluorescence *in situ* hybridization; FISH), horseradish peroxidase or digoxigenin.[54,65,66] The detection system depends on the label used [digoxigenin: 4-nitroblue tetrazolium chloride (NBT)/15-bromo-4-chloro- 3-indolyl-phosphate (BCIP); fluorophore: light of appropriate wavelength; horseradish peroxidase-based signal: using a chromogenic such as 3,30-diaminobenzidine (DAB). Occasionally, sequence amplification techniques need to be applied to increase the chance of a signal detection from a rare and or small molecule, as for example micro RNA (miRNA)].[65]

Advantages

In situ hybridization (ISH) can be applied to cytology specimens (aspirates and samples from cultures), snap-frozen sections and FFPE tissue sections. In tissue sections, the positive results can be visualized in the context of the morphological lesions. Hence, ISH avoids false positive results as can be seen with PCR.[54] Moreover, ISH and IHC can be applied simultaneously on a single sample.[67,68] This enables direct visualization of a pathogen or genetic mutation within a particular cell subpopulation within a heterogeneous cell infiltrate. As pathogens can be readily identified within formalin-fixed tissues, it eliminates the need of access to fresh tissue for a variety of culture techniques. Confirmation of direct causation of a particular pathogen in a skin lesion can be confirmed by applying ISH on a transitional zone from lesional to

nonlesional skin; the former presents a positive result, whereas the latter should be negative.[54]

Disadvantages and challenges

ISH is a rather expensive technique, and although quite common in human dermatopathology, it is still not well-established in daily veterinary diagnostic dermatopathology. It has to be emphasized that false positive or negative results can be ruled out only by careful interpretation of ISH results in tandem with the morphological features of the sample. For detection of genetic mutations, the probe has to target the specific location of interest to differentiate between affected and nonaffected samples.

Examples of contributions to dermatology

A close relationship of human and equine *Molluscum contagiosum* virus has been suggested based on the identification of the virus in equine lesions using two human *Bam*HI-restricted fragments of human *M. contagiosum* type I DNA.[69] The pathological effect of equine Herpesvirus-5 on keratinocytes has been documented where the virus in keratinocytes exhibited cytotoxic effects.[70] As in many other species, PCR identifies papilloma virus in normal skin and BPV has been claimed to be associated with various non-neoplastic skin lesions in horses.[71–73] However, ISH offered proof that the presence of BPV-1 and BPV-2 are very restricted to lesions of equine sarcoid, whereas adjacent keratinocytes and dermal tissues were negative (Figure 5).[54] Moreover, ISH demonstrated that equine penile squamous cell carcinomas can be either papilloma virus induced or the result of solar exposure;[74] however, none of these cases had evidence of a combination of both.

Tissue microarrays

Tissue microarrays are blocks composed of numerous different small tissue samples, on average 0.6 mm in diameter, which subsequently can be evaluated simultaneously by different techniques, including IHC and ISH.[75–77]

Methods

Blocks can be prepared manually or obtained from various manufacturers.[77] Sections from these blocks are transferred to glass slides and subsequently processed in a similar way to regular paraffin sections (Figure 6).

Advantages

It allows simultaneous standardized screening of a larger number of tissue samples by ISH or IHC.

Disadvantages and challenges

The construction of tissue microarray is labour-intensive. Each actual tissue sample is very small and may not be truly representative of the entire lesion. Hence, careful selection of location for collecting the 0.6 mm sample from a particular lesion is important.

Examples of contributions to dermatology

New tissue reagents can be evaluated easily, using a microarray of normal and diseased tissues. Tissue microarrays are therefore particularly helpful in laboratory quality assurance controls. They are also used in a research setting focused on screening a large number of samples by ISH or IHC.[78,79] Alternatively, they can be helpful for training programmes.

Gene microarray or genome-wide association studies (GWAS)

Gene microarrays or GWAS are also referred to as DNA or genome chips. They facilitate screening of the entire genome within a certain population in one single test.[80] Systematic analysis of the entire genome includes noncoding areas (introns) as well as coding areas (exons), screening for mutations and polymorphism in biological samples. The technique of microarrays allows detection

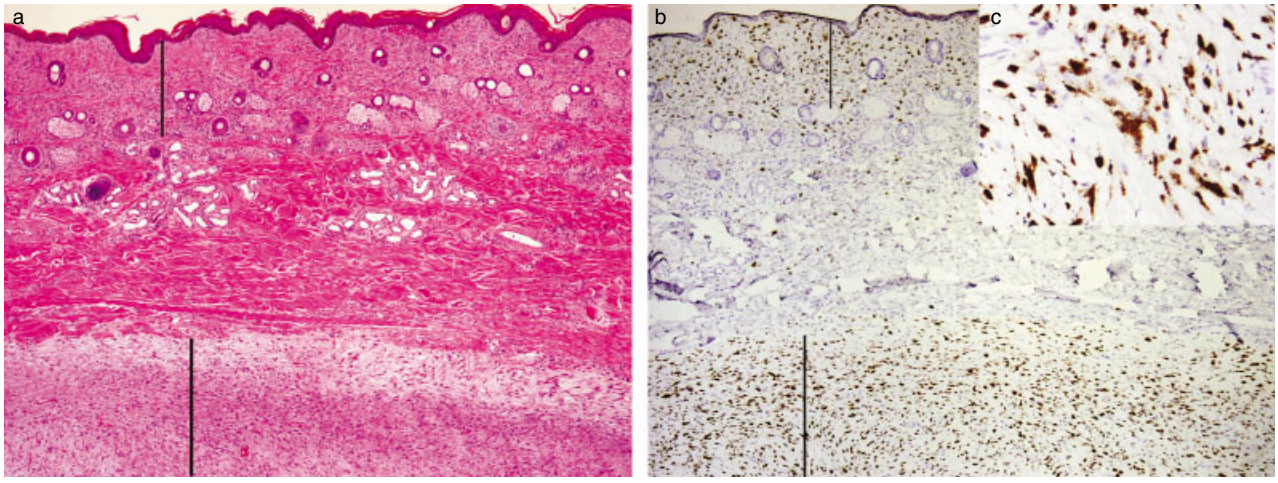

Figure 5. *In situ* hybridization (ISH) for bovine papilloma virus (BPV) in an atypical equine sarcoid. (a) The proliferating spindle cell population in the superficial and deeper portion of the dermis (black bars) are separated by a band of normal dermal collagen (Haematoxylin and eosin, 2×). (b) Hybridization to BPV is detectable within the nuclei of the proliferating fibroblasts in the superficial and deeper portion of the dermis (black bars), but not within the normal dermal collagen (2×). (c) Presence of BPV within proliferating fibroblasts (10×). ISH probes complementary to regions of the E5, E6 and E7 coding regions for BPV1 (×02346.1) and BPV2 (M20219.1); horseradish peroxidase-based signal amplification system; 3,30-diaminobenzidine (DAB; Advanced Cell Diagnostics, Newark, CA, USA); haematoxylin counter stain.

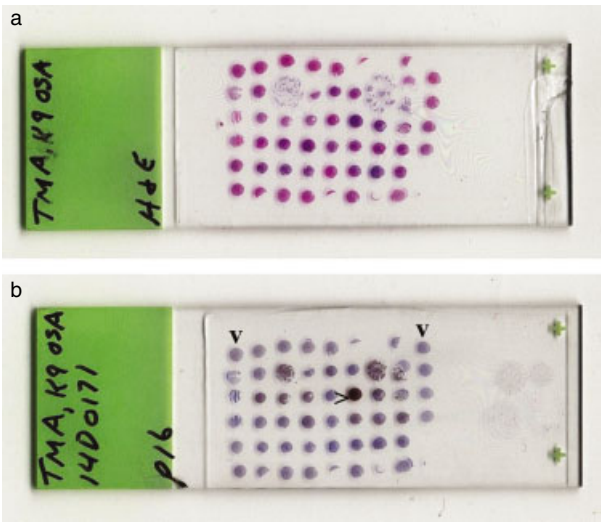

Figure 6. Tissue microarray: small tissue samples of multiple previously examined tissue samples are collected and embedded in one single block. H&E stained core samples (a) from canine cell rich areas of osteosarcomas and additional positive control tissues were subsequently evaluated for expression of p16, a factor expressed in glioblastomas and presumed to be involved in the development of canine osteosarcomas (b) using cross-reacting anti-human p16 antibody (Santa Cruz Biotechnology; Dallas, TX, USA). Having numerous different samples on one single slide allows inclusion of positive and negative controls within the same slide. The outer rim of cores are negative control tissues of brain (v). A randomly placed positive control core of confirmed p16 positive canine glioblastoma multiforme is added (>). Courtesy of Mai Mok, University California Davis.

of single nucleotide polymorphisms (SNPs) within a population of a particular species. Expected incidence is of about one SNP for every 1000 bp in the human genome.[81] Its incorporation in dermatology and dermatopathology will enhance our understanding of skin diseases tremendously.[82]

Methods

The procedure involves immobilization of DNA fragments of known sequences to a solid platform such as glass or nylon.[81] Due to its fast degradation, RNA is first transcribed to cDNA. The cDNA microarrays (variable length of fragments) are produced by high speed robotics. Alternatively, oligonucleotide microarrays (usually 20–80-mer) are used. The sample to be tested is complementary, usually fluorescent-labelled DNA that will hybridize to the DNA on the solid platform. Hybridized spots are detected by digital imaging and analysed by special software.[80] RNA and DNA can be extracted from fresh frozen tissue or blood. In some instances FFPE tissues have been used.

Advantages

This comparative gene analysis detects differences in gene expression between normal and disease state tissues.[82] It can identify altered expression of multiple genes simultaneously. This technique usually is applied to study the genetic background of a disease for which a candidate gene has not yet been identified. The potential applications include prediction of behaviour of specific neoplastic processes and their chemosensitivity, profiling of inflammatory skin diseases, the mutational analysis of genodermatoses and polymorphism screening.[83]

Disadvantages and challenges

GWAS requires prior knowledge of a sequence and thus cannot detect novel genes or unknown gene sequences that may be involved. Gene microarrays are still expensive and labour-intensive. Moreover, the genome of many species has still not been completely sequenced and many genes in domestic animals have not been annotated correctly, which makes the interpretation of data achieved by microarrays difficult. DNA microarrays are therefore still limited to a few species and mostly used in a research setting.

Examples of contributions to dermatology

This technique has been introduced to evaluate gene profiles for the development and progression of tumours, in particular melanomas in humans. For example, thrombospondin-2 and desmoglein-2 are enhanced in aggressive melanomas in humans.[83] More sophisticated microarrays are generated to evaluate SNPs on a larger scale to evaluate for mutations (insertion and deletions) as the underlying cause for a particular skin disease. Neoplastic T cells in humans with Sézary's syndrome express PLS3 or plastin-T, an actin-binding protein that is not observed in normal lymphocytes.[84] The significance of this expression is not known, but screening for the presence of this protein assists clinical follow-up for residual disease. Although not readily available in a diagnostic setting, cDNA microarrays will shape the diagnostic approach to skin diseases in the future.

Next-generation sequencing (NGS)

Next-generation sequencing allows fast and comprehensive sequencing of the entire genome of an individual. It can be performed on the entire genome, which includes noncoding areas of the DNA (introns), coding areas of the DNA (exons) and mitochondrial DNA, or limited to coding areas of the genome. The former is referred to as whole-genome sequencing (WGS), whereas the latter is referred to as whole-exome sequencing (WES).[85,86] The exome involves roughly 1.5% of the human genome, encompassing most exons of about 20,000 human genes; WES is therefore less labour-intensive than WGS.[87] WES involves enrichment procedures, such as exome enrichment and, thus, RNA sequencing, also called whole transcriptome analysis or whole transcriptome shotgun sequencing (WTSS), may often be used as an alternative approach to detect variability in protein-coding regions.[88] However, it is important to recognize that the majority of genetic diseases in humans are due to mutations and SNPs within the noncoding regions of the genome; identification of these mutations requires WGS.[89] Like every other ancillary testing method, it is crucial to demonstrate the causality of such mutations.

Methods

Originally sequencing was performed by labour-intensive selective incorporation of chain-terminating fluorescent labelled dideoxynucleotides by DNA polymerase during

in vitro DNA replication followed by capillary electrophoresis, referred to as Sanger's chain termination sequencing.[90] NGS is a rapid, automated sequencing process performed by either DNA polymerase- or DNA ligase-dependent methods.[86,91,92]

Advantages

Of particular note is that WES and WTSS are relatively fast procedures, which allow identification of genetically based diseases that tend to have a rather phenotypically heterogeneous clinical presentation. In contrast to gene microarrays, these allow the identification of novel genes and absolute gene quantification, as well as the determination of alternatively spliced gene transcripts, which may be important in disease biology.

Disadvantages and challenges

NGS is still expensive and requires expertise in handling large data outcome and thus is currently still mostly limited to research settings. However, it has found its way into diagnostics in human medicine.[85,86] In veterinary medicine, the genomes of many species have still not been sequenced, which limits the use of this method. In a similar manner to the challenge with gene microarrays, interpretation of data can be a challenge due to incorrect annotation of many genes in domestic animals.

Examples of contributions to human dermatology

Two cases of X-linked hypohidrotic ectodermal dysplasia were confirmed by WES.[85] The diagnosis of acrodermatitis enteropathica and localized DEB in siblings was achieved by WES, as clinical presentation of these diseases were not straightforward.[85]

Laser capture microdissection

Laser capture microdissection (LCM) is a very powerful tool with which to isolate cells from a heterogeneous cell population in culture or on histological slides. The cells can be visualized and photographed before collection. The collection process does not disrupt the phenotypic characteristics or molecular state of the cells. Therefore DNA, RNA and protein can be extracted from collected cells (Figure 7).

Methods

LCM is performed with direct microscopic visualization. There are two basic methods used to date: infrared (IR) and ultraviolet (UV) light.[93,94] They both allow direct microscopic selection of tissues/cells in culture and capture into a collection tube. A UV light beam excises the selected cells and a light catapult transports the cells into a collection tube.[93] Alternatively, an IR laser is used to melt a thermoplastic membrane attached to a cap that overlies the cells of interest.[94]

Advantages

LCM allows selection of cells from cytological specimens, frozen sections and FFPE tissue sections, as well as cell cultures. Tissue sections can be stained with H&E, immunohistochemistry or FISH, before being moved to the dissection chamber, which ensures identification of cell populations of interest. Potentially various subpopulations of cells can be collected from one original specimen and subsequently evaluated separately. The cells left behind remain unaltered in the tissue.

Disadvantages and challenges

The method is very expensive and the collection of the samples is a time consuming process. Therefore, LCM is still used mostly in research rather than diagnostic dermatopathology.

Examples from the literature

Patients with psoriasiform arthritis have altered miRNA expression in Th17 cells.[95] LCM was used to identify

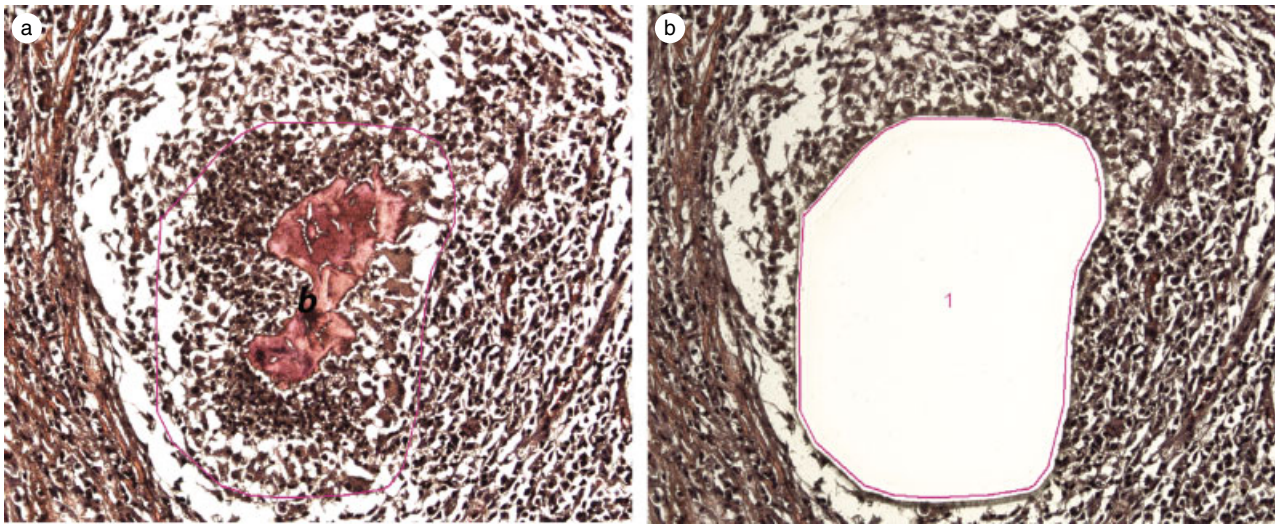

Figure 7. Laser capture microdissection (LCM) of an example of Bullwinkle disease in a deer.[101] The detection of pathogens in tissues by PCR is often confounded by the presence of normal flora, or postmortem contaminants. (a) A specific colony of bacteria (*b*) was isolated and removed by LCM to minimize contamination from other bacteria that may have contaminated and proliferated in tissues after death. PCR of the isolated colonies confirmed the presence of a novel *Mannheimia* spp. infection, that was not evident in areas of the tissue, which lacked visible colonies of bacteria. (b) LCM leaves the remaining tissue unaltered and available for additional evaluations. Courtesy of Kevin Keel, University California Davis.

altered compartmentalization of miRNAs in epidermal and dermal cells of patients with psoriasis.[96] Transition from precancerous to neoplastic cells can be confirmed within a single tissue sample, as has been shown in people with nevi cells transitioning into melanoma.[97] LCM also allows evaluation of extracellular matrix proteins; for example, it was reported that decorin, a major protein involved in regulation of collagen fibre diameter, is expressed in reticular dermis, but absent in papillary dermis.[98] The difference in decorin mRNA expression may contribute to the age and UV irradiation induced decrease of collagen I and IV by affecting bundle diameter in the superficial dermis. It also has been shown that the immunosurveillance for herpes simplex is due largely to the persistence of CD8αα+ T cells in the dermo-epidermal junction; these cytotoxic T cells lack expression of chemokines to egress and recirculate.[99] Lastly, LCM was used to collect lesional bacterial colonies subsequently characterized as *Streptomyces* species in mycetoma-like lesions in cats.[100]

Conclusions

The techniques reviewed in this paper have greatly improved our understanding of skin diseases. However, many are labour-intensive and costly, and consequently they are more frequently used in a research setting. Some techniques, including PCR and IHC, are more commonly offered in diagnostic laboratory settings, whereas others such as clonality and ISH are still limited to a few diagnostic laboratories.

The summary of the clinical presentation and morphological features of a lesion guides the clinician and pathologist in their choice of appropriate additional techniques that should be considered to achieve the aetiological diagnosis. It is crucial to know the limitations of each technique to avoid overinterpretation of the results. Moreover, it is imperative that the results of each additional test are not interpreted in a vacuum: each technique contributes a piece to solving the puzzle. Each result has to be viewed in association with the histological characteristics of the lesions as well as clinical presentation of the patient to ensure correct interpretation and ultimately the most appropriate treatment. With that knowledge, dermatopathology will always remain an important link between benchtop science, new techniques and clinical veterinary dermatology.

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Diagnostic microbiology in veterinary dermatology: present and future

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Background – The microbiology laboratory can be perceived as a service provider rather than an integral part of the healthcare team.

Objectives – The aim of this review is to discuss the current challenges of providing a state-of-the-art diagnostic veterinary microbiology service including the identification (ID) and antimicrobial susceptibility testing (AST) of key pathogens in veterinary dermatology.

Methods – The Study Group for Veterinary Microbiology (ESGVM) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) identified scientific, technological, educational and regulatory issues impacting the predictive value of AST and the quality of the service offered by microbiology laboratories.

Results – The advent of mass spectrometry has significantly reduced the time required for ID of key pathogens such as *Staphylococcus pseudintermedius*. However, the turnaround time for validated AST methods has remained unchanged for many years. Beyond scientific and technological constraints, AST methods are not harmonized and clinical breakpoints for some antimicrobial drugs are either missing or inadequate. Small laboratories, including in-clinic laboratories, are usually not adequately equipped to run up-to-date clinical microbiologic diagnostic tests.

Conclusions and clinical importance – ESGVM recommends the use of laboratories employing mass spectrometry for ID and broth micro-dilution for AST, and offering assistance by expert microbiologists on pre- and post-analytical issues. Setting general standards for veterinary clinical microbiology, promoting antimicrobial stewardship, and the development of new, validated and rapid diagnostic methods, especially for AST, are among the missions of ESGVM.

Introduction

In veterinary medicine, the microbiology laboratory is perceived as a service provider rather than an integral part of the healthcare team, resulting in limited interaction between microbiologists and clinicians. This differs from human medicine, where microbiologists interact with infectious disease specialists to provide advice on antimicrobial therapy, infection control, antimicrobial stewardship practices, antimicrobial resistance trends and compliance with antimicrobial guidelines. The use of diagnostic microbiology is comparatively lower than in human medicine, although differences exist between countries and veterinary practices.[1] This difference is attributable to structural, economic and cultural factors that differentiate the veterinary healthcare system from the human counterpart. The limited utilization of microbiology tests in veterinary practice has negative consequences on the costs, with these being as much as three times higher than the costs of comparable tests in the human healthcare sector. Formal antimicrobial stewardship programmes, which traditionally involve microbiology laboratories in human hospitals, are rarely implemented by veterinary clinics.[2] Antimicrobials are mainly used empirically and the use of antimicrobial susceptibility testing (AST) is generally limited to difficult cases with poor response to initial therapy.[1] This trend is unfortunate given the current concerns regarding antimicrobial use and emergence of multidrug-resistant bacteria in animals, including companion animals.[3] Use of culture and AST to guide antimicrobial choice is recommended

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by numerous guidelines on responsible antimicrobial use developed by governmental, animal health and veterinary organizations, including the European Commission,[4] the World Organization of Animal Health (OIE)[5] and the American Veterinary Medical Association (AVMA).[6] As demonstrated in human medicine, implementation of antimicrobial stewardship at the clinic level has positive consequences on appropriate antimicrobial use, control of antimicrobial resistance and patient care.[7]

Quality and quality control are important in clinical microbiology. International standards[8,9] and manuals[10,11] for clinical microbiology are available but their use is, for the most part, voluntary, although some guidelines have been adopted by accrediting organizations as part of their accreditation requirements. Uniform guidelines for best practice are not widely available for veterinary clinical microbiological laboratories; in general, accredited laboratories have implemented the guidelines for human clinical microbiology laboratories. Furthermore, there is an increasing trend for veterinary clinics to perform in-house microbiology. Despite the advantages of reduced turnaround time and costs, there are also disadvantages and risks associated with this practice. The microbiological expertise required to accurately perform and interpret the diagnostic tests, as well as to perform routine quality control and manage the biohazard risks, are lacking in most in-clinic and small diagnostic laboratories.

The aim of the Study Group of Veterinary Microbiology (ESGVM), established within the European Society for Clinical Microbiology and Infectious Diseases (ESCMID), is to promote state-of-the-art veterinary clinical microbiology. This review highlights some of the current challenges in veterinary microbiology and outlines the quality standards required with particular reference to veterinary dermatology.

State-of-the-art methodologies

Microbe identification

Classic culture-based methods have been the mainstay of clinical microbiology for the past century. Automated systems are being implemented, but to date most of these technologies rely on pure culture of the micro-organism. Identification (ID) of the micro-organism is an important prerequisite before AST to distinguish between potentially pathogenic micro-organisms and possible contaminants from the commensal microbiota on nonsterile body sites. Microbial ID has traditionally been performed by testing biochemical properties of the micro-organism. A step forward was achieved with the development of standardized commercial test systems (e.g. API® or rapID™), which have gradually replaced the use of in-house tube tests, enabling diagnostic laboratories to use a validated manual system without expensive hardware. The next step was to offer these tests in more or less automated versions to avoid subjective interpretation (e.g. VITEK® Systems, BD Phoenix™ Automated Systems, TREK Sensititre® Diagnostic Systems). The quality of these systems in veterinary microbiology is strongly dependent on the databases used. Species found commonly in human microbiology, such as *Pseudomonas aeruginosa*, are well represented within the databases of these ID systems and therefore reliably identified. However, some species of veterinary relevance, including *Staphylococcus pseudintermedius* and *Staphylococcus felis*, are very difficult to reliably identify and differentiate from closely related staphylococci. Additionally, as the biochemical activity of a strain depends on growth, micro-organisms that do not grow in these systems cannot be identified (e.g. some members of *Pasteurellaceae*) and the ID may not be reliable for some micro-organisms (e.g. *Malassezia*) if the patient is under treatment with antimicrobials at the time of specimen collection.

New technologies have been introduced in recent years to overcome the disadvantages of biochemical ID. One technology that has gained increasing attention in veterinary microbiology is MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry (MS) (Figure 1). This technique identifies any culturable bacteria within minutes and has low running costs.[12,13] For most fungi a somewhat more complex sample preparation is necessary, but even dermatophytes can be identified with this method within 2 h. Again, identification depends on database entries, but the ability to discriminate between different bacteria is generally very good for most species. In general, the available databases are much broader than any of the former biochemistry based databases, but still some veterinary specific entries are lacking. The databases are updated

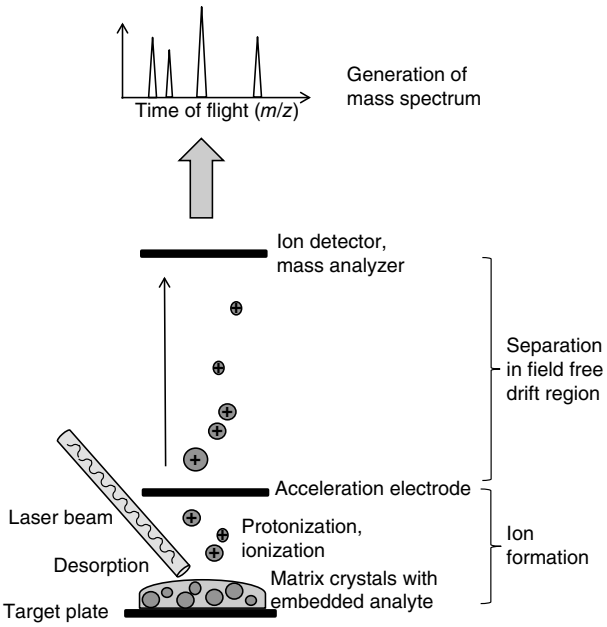

Figure 1. Principle of the MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) MS process. For most bacteria a simple direct smear preparation onto a target plate is covered by a matrix solution to enable the generation of ions by a laser. These ions, derived mainly from the highly abundant proteins of the micro-organism, are then accelerated and travel through a predefined distance in a vacuum tube (field free drift range). The time delay of their journey until the ions reach a detector is measured and displayed according to the mass of the ions as a characteristic pattern of the proteins (spectrum) detected in the micro-organism. Identification is then derived from comparison of the protein profile to database matches.

regularly and each laboratory can add entries to the database. This approach has been shown to be successful for the *Staphylococcus intermedius* group (SIG), which is of special importance in the field of dermatology.[14] Of course, a prerequisite for database expansions are strict protocols for quality control that must be followed to ensure highly reliable entries. In general, confirmation of the respective strains by sequencing before addition to the database is necessary. In human medicine, MALDI-TOF MS is used for direct ID of bacteria in blood cultures.[15] Similar applications for direct ID in veterinary clinical specimens have not yet been developed. The main disadvantage of this technology is the high cost for purchasing and servicing the instrument, which makes it unaffordable by small diagnostic laboratories. However, the actual cost of the test is extremely low and alliance between laboratories may be used to make this technology accessible without every laboratory buying the instrument.

Another technology, DNA sequencing, is widely used as a research tool to investigate bacterial evolution and molecular epidemiology; at the time of writing this is not frequently employed in routine clinical microbiology. Recently, more advanced sequence-based techniques have become available.[16] Isolated and purified microorganisms can be identified by Whole Genome Sequencing (WGS) over 24 h,[17] and publicly available web tools are available for multi-locus sequence typing (MLST) and ID of acquired antimicrobial resistance genes using raw WGS data.[18,19] Direct sequencing of DNA extracted from clinical specimens enables bacteria ID in polymicrobial samples and reduces diagnostic times to 24 h.[20] DNA sequencing technologies are rapidly evolving and becoming more affordable, but widespread implementation in veterinary microbiology laboratories in the near future probably is limited to larger laboratories.

Antimicrobial susceptibility testing

Broth micro-dilution and disk diffusion are the most widely used methods for AST. Broth micro-dilution is the gold standard method for AST and the only method for which an internationally accepted ISO standard exists (ISO 20776-1, 2006).[9] The principle of this method is simple. Broth suspensions containing the test strain are added to wells containing two-fold dilutions of antimicrobials. Upon incubation, the minimum inhibitory concentration (MIC) is read for each antimicrobial as the lowest concentration inhibiting visible bacterial growth, and used for interpretation of susceptibility. The method can be highly automated and is generally performed using commercial panels with a fixed composition of antimicrobials. Disk diffusion, also known as the Kirby–Bauer method, is performed by streaking broth containing the test strain on an agar plate followed by applying antimicrobial-impregnated disks. Upon incubation, inhibited bacterial growth around each disc is measured as a zone diameter and used for interpretation of susceptibility. This method is cheaper and more flexible than broth micro-dilution, as the user can easily change the antimicrobials between tests. It is, however, less robust and reproducible, and semi-quantitative in nature as it only indicates whether the test strain is susceptible (S), intermediate (I) or resistant (R). Laboratories have to select the most appropriate antimicrobials for routine AST based on bacterial species, breakpoint availability, animal species, infection site and available guidelines. The major shortcoming of both methods is turnaround time (approximately 48 h) from culture of the clinical specimen to reporting of the results. Both methods must be performed following quality standards (e.g. inoculum density and size, media, incubation conditions, etc.) that are set by two international committees; namely the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI), and various national committees. To date, only CLSI provides clinical breakpoints and interpretive criteria for veterinary pathogens.[21] A veterinary subcommittee of EUCAST (VetCAST) recently has been established with the purpose of harmonizing AST in Europe as well as on a global scale (http://www.eucast.org/organization/subcommittees/vetcast/).

Alternative technologies are currently being evaluated to reduce the turnaround time of AST. Real-time PCR assays have been developed for rapid detection of resistant bacteria of high clinical relevance such as meticillin-resistant *Staphylococcus aureus* (MRSA) directly from specimens.[22] MALDI-TOF MS can be employed for rapid detection of extended-spectrum beta-lactamase (ESBL)-producing bacteria in blood cultures through quantification of β-lactam degradation products.[23] Flow cytometry is a method used for detection of morphological and metabolic changes of cells, for example upon antimicrobial exposure. This method has been tested for rapid AST of various organisms, and one study demonstrated the potential for detecting ESBL in 3 h from pure bacterial cultures.[24]

WGS is not yet as rapid as these two other methods but offers the advantage of enabling screening of all known resistance genes by a single analysis, and it requires little hands-on time. WGS provides information on the presence of resistance genes, allowing prediction of antimicrobial susceptibility. High (99.7%) accordance between pheno- and genotypic resistance was demonstrated between 200 bacterial isolates belonging to four different species,[19] and the same predicted susceptibility profiles have been obtained using direct sequencing on clinical specimens and sequencing of single isolates.[20] The disadvantage of WGS is that it fails to reveal as yet undescribed resistance genotypes, and the actual phenotype may not always be deduced from sequencing data. For example, detection of nonfunctional pseudogenes or repressed efflux systems may lead to false positive (R) results.

Point-of-Care testing

Point-of-Care (PoC) tests are diagnostic tests that can be performed with the patient, therefore reducing turnaround time. The tests are based on different technologies, predominantly immunochromatography, agglutination assays and real-time PCR.[25] A rapid immunoassay for PoC detection of urinary tract infection in dogs (RapidBac™ Vet; http://www.rapidbacvet.com/) has a high sensitivity (97.4%) and specificity (98.8%) for identification of clinical bacteriuria.[26] A limited number of commercial PoC tests

are available for on-site AST in veterinary clinics. A simple diagnostic system (Speed-Biogram™; https://www.bvt.fr/en/home/diagnostic-solutions/pour-le-veterinaire-praticien/infectious-diseases/main/gamme-speed/speed-biogram-1.html) has become available and can perform simultaneous ID and AST on cutaneous and ear specimens within 24–48 h. The main disadvantage is that the inoculum might be polymicrobial and cannot be standardized, leading to possible false resistance or false susceptibility reporting, which may also arise with disk diffusion testing.

Direct AST of clinical specimens (e.g. urine), without prior isolation of bacterial colonies, has the advantage of making results available earlier but this is controversial because of concerns regarding its accuracy. A human study demonstrated a 93% agreement between direct and conventional AST.[27] The highest percentage of discordance (13%) was observed for β-lactam antimicrobial drugs such as amoxicillin clavulanate and cephalosporins. Similar results have been reported for another PoC test designed for direct ID and AST of uropathogens (Flexicult® Vet; http://www.ssidiagnostica.dk/da/Produkter/Substrater/Flexicult-Vet-URINKIT).[28] In human medicine, direct AST is recommended only for critically ill patients and does not replace conventional AST, which is additionally performed to confirm the preliminary results obtained by direct AST.[26] Accordingly, ESGVM recommends that samples testing positive and strains testing resistant by PoC tests are sent to accredited laboratories for AST by validated methods. In some countries (e.g. France), PoC tests are not permitted for AST of critical antimicrobial drugs (e.g. fluoroquinolones and higher generation cephalosporins) due to test limitations. Conversely, PoC tests may be useful for rapid detection of negative samples and susceptible strains, avoiding the time and the cost of laboratory analysis.

Current challenges in veterinary diagnostic microbiology

Specimen management

Improper specimen management impacts on both the diagnosis and outcome of therapy.[29] Microbiology laboratories should provide information to ensure the appropriate selection, collection, storage and transportation of clinical specimens. National and international guidelines provide detailed information on the best sample type, sampling technique and transport conditions for bacterial infections. For superficial bacterial folliculitis, pustular contents and papule biopsies are optimum. Swabs of crusts and epidermal collarettes result in a higher risk of contamination with commensal skin surface bacteria.[30] For wound infections, the type of specimen and sampling technique depend on the wound type.[30] In general, biopsy samples obtained after initial debridement and cleansing are the most useful for determining the microbial load and the presence of relevant pathogens. Fluid samples obtained by aseptic needle aspiration may be used for cavity wounds (e.g. pressure sores) and cutaneous abscesses. The value of wound swabs even after cleansing a wound prior to sampling is questionable.[31] Visible contamination, however, should be removed before a sample is collected.

Usually a single lesion is sampled and relatively few colonies are used by the laboratory for both ID and AST. Recent studies have demonstrated, however, that multiple strains with distinct antimicrobial resistance profiles may occur in the same lesion or in different lesions from the same patient.[32,33] Further evaluation to assess the magnitude and clinical significance of this phenomenon is indicated. In theory, the involvement of multiple strains from canine skin infections is plausible given the frequent carriage of multiple *S. pseudintermedius* strains in dogs.[34] Primary isolation using commercial selective agar plates may be performed in addition to nonselective isolation on blood agar to facilitate detection of meticillin-resistant staphylococci occurring at low numbers in mixed cultures. Unless anaerobic bacteria are being investigated (e.g. deep wound infections), storage and transportation of dermatological specimens does not present any specific challenges, because the main pathogens involved (Table 1) can survive for several days in transport media. Nevertheless, sample pickup by courier and overnight transport offer the advantage of reducing the overall turnaround time.

Pathogen identification

Bacterial species relevant for common disease conditions in veterinary dermatology are listed in Table 1. Staphylococci are the most frequent bacterial pathogens associated with skin and soft tissue infections. Historically,

Table 1. Performance of biochemistry, including manual and automated methods, and MALDI-TOF MS for species identification of micro-organisms of recognized clinical relevance in veterinary dermatology

Micro-organism	Biochemistry	MALDI-TOF MS
Staphylococcus pseudintermedius	Inadequate	Inadequate with standard database Excellent with extended database
Staphylococcus schleiferi	Inadequate	Good (no distinction between subspecies)
Staphylococcus aureus	Good	Excellent
Staphylococcus felis	Inadequate	Good
ß-haemolytic streptococci	Good	Good at species level Inadequate at subspecies level (excellent with extended database)
Pseudomonas aeruginosa	Good	Excellent
Proteus spp.	Good	Excellent
Dermatophytes	Good	Good (*M. canis*: excellent; *Trichophyton* spp.: genus level only)
Malassezia spp.	Inadequate	Good
Candida spp.	Inadequate	Good

MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry (MS).

animal pathogenic staphylococci have been associated with coagulase-positive staphylocci (CoPS), whereas CoNS generally have been regarded as bacteria with low pathogenic potential. Before the description of *S. intermedius* in 1976,[35] all CoPS isolated from animals were (mis)identified as *S. aureus*. Subsequently, *S. intermedius* was differentiated into three distinct species: *S. intermedius*, *S. delphini* and *S. pseudintermedius* (referred to as the SIG group).[36] The latter species is the normal commensal and opportunistic pathogen of the dog, even though infections also are reported in cats and less frequently in other hosts, including humans.[37] *Staphylococcus pseudintermedius* cannot be easily distinguished from the other members of the SIG group by phenotypic methods and its speciation requires PCR-based tests or MALDI-TOF MS, provided that the database has been specifically refined for identification of this species (see above).

CoNS are commensal organisms with a relatively high rate of meticillin-resistance in companion animals.[38] CoNS have been regarded as "contaminants" and either not reported or speciated except when isolated in pure culture from hospital-acquired infections associated with surgery or invasive procedures. The recognition of *S. schleiferi*[39,40] as a canine pathogen underpins the importance of identifying CoNS species as the coagulase activity of this species and subspecies (subspp. *schleiferi* and *coagulans*) is variable. MALDI-TOF MS is superior to other methods for the identification of this group of staphylococci.[41] ESGVM recommends that AST profiles for *S. schleiferi* and other CoNS should only reported when the organisms are isolated in pure culture from sterile sites or from intact primary skin lesions sampled under strict aseptic conditions.

Polymicrobial cultures are common for otitis and wound infections, and can occur from skin samples. In these cases, the relevance of the culture result and the selection of the isolate for AST need to be determined. The current recommendation for human wound infections is that growth of potential pathogens should be reported, preferably semi-quantitatively.[30] AST should be performed when a pathogen is isolated in pure culture or in abundance with minimal involvement of other micro-organisms. Antimicrobial therapy should target the micro-organism with greatest pathogenic potential. Indiscriminate reporting of AST profiles for micro-organisms of minimal clinical relevance is discouraged to avoid unnecessary use of broad-spectrum antimicrobial drugs to cover the composite AST profile of multiple isolates.

Lack or inadequacy of clinical breakpoints

A clinical breakpoint (CBP) is the critical MIC (or the corresponding interpretive inhibition zone diameter for disk diffusion) selected by *ad hoc* international (e.g. CLSI or EUCAST) or national (e.g. US Food and Drug Administration) committees to categorize a bacterial strain as susceptible (S), intermediate (I) or resistant (R). CBPs are typically established on the basis of microbiological, pharmacokinetic (PK), pharmacodynamic (PD) and clinical outcome data.[42] The purpose of CBPs is to assist clinicians to select appropriate drugs for therapy. *In vitro* AST does not, however, consider other factors that affect the outcome of antimicrobial therapy, such as host immune status, co-morbidities, strain virulence and compliance. By definition, a strain is reported susceptible to a drug when the standard dosage regimen is associated with a high likelihood of therapeutic success (approximately 90% according to human studies). The resistant category does not unequivocally predict treatment failure but a reduction of therapeutic success with a cure rate up to 60%. This is referred to as the 90–60% rule in human medicine.[43,44] The clinical predictive value of AST is further impacted in veterinary medicine by the lack, or inadequacy, of available breakpoints. For example, breakpoints are unavailable for several antibiotics suitable for the treatment of skin infections in cats (Table 2). In those cases a CBP from dogs would typically be used. For bacteria or infections without any veterinary CBP, a human-derived CBP may be employed. This is the case for sulphonamides/trimethoprim and antibiotics such as chloramphenicol or rifampicin used for treatment of MRSA and meticillin-resistant *S. pseudintermedius* (MRSP) infections (Table 2). Cefovecin is a veterinary drug for which no CBP exist, hence the *in vivo* efficacy of this drug is difficult to predict by AST. Clearly, the predictive value of AST can be severely impacted by the use of inadequate CBPs, because a human CBP reflects the dosage regimen and the PK of the drug in humans, and both dosage regimen and drug disposition exhibit large differences between animal species. Reliable CBPs require animal species-specific determinations and there is an urgent need for animal-specific CBPs.

CBPs are dosage regimen-dependent because they are set by PK/PD analysis according to a specific dosage. Thus, a CBP set for a drug administered twice a day may not be appropriate if the same drug is administered three times a day. For example, amoxicillin clavulanate has a set breakpoint according to a defined dosage regimen [11 mg/kg per os (PO) twice daily],[21] even though an increased dose according to label recommendations (12.5–25 mg/kg PO twice daily) can be used and three doses a day are recommended by international guidelines for treatment of urinary tract infections.[45] Similarly, for time-dependent drugs such as the β-lactams, CBPs are heavily influenced by drug formulation. For example, a CBP that is valid for oral tablets may not be valid for the same drug administered by a long-acting intravenous formulation, even if the total dose is the same. To overcome this, several CBPs should be determined for a given substance depending on dose and formulation. However, this approach would be very difficult to manage for diagnostic companies and microbiology laboratories, because commercial systems for AST should be implemented and validated for each CBP.

Currently no CBPs are available for topical antimicrobial therapy, which is often used as a sole treatment in veterinary dermatology, especially for management of otitis externa. The relevance of AST for guiding topical antimicrobial therapy is questionable because CBPs are set for systemic therapy, and the drug concentrations achieved in serum by systemic administration are markedly lower than those obtained by the topical route. Such concentrations may exceed the MICs of skin pathogens greater than 100,000 fold (Table 3). These data suggest that infections

Table 2. Bacteria for which host- and infection-specific clinical breakpoints exist in veterinary dermatology according to Clinical Laboratory Standards Committee (CLSI).[21] Drugs for which only human-derived breakpoints are available are highlighted in bold

	Animal/bacterial combinations for which clinical breakpoints for systemic treatment of skin infections exist	
Antibiotic	Dogs	Cats
Amoxicillin-clavulanic acid	*Escherichia coli*, *Staphylococcus* spp.	*E. coli*, *Staphylococcus* spp., *Streptococcus* spp., *Pasteurella* spp.
Ampicillin	*E. coli*, *Streptococcus canis*, *Staphylococcus pseudintermedius*	None*
Cefalothin	*E. coli*, *Staphylococcus aureus*, *S. pseudintermedius*, *Streptococcus* spp.	None*
Cefazolin	*E. coli*, *S. aureus*, *S. pseudintermedius*, *Pasteurella multocida*, *Streptococcus* spp.	None*
Cefovecin	None	None
Cefpodoxime	*E. coli*, *S. aureus*, *S. pseudintermedius*, *Pasteurella multocida*, *Proteus mirabilis*, *Streptococcus* spp.	None*
Chloramphenicol	None*	None*
Clindamycin	*Staphylococcus* spp., *Streptococcus* spp.	None*
Difloxacine	Enterobacteriaceae, *Staphylococcus* spp.	None*
Doxycycline	*Staphylococcus pseudintermedius*	None*
Enrofloxacin	Enterobacteriaceae, *Staphylococcus* spp.	None‡
Gentamicin	None†	None*
Marbofloxacin	Enterobacteriaceae, *Staphylococcus* spp.	None‡
Orbifloxacin	Enterobacteriaceae, *Staphylococcus* spp.	None‡
Pradofloxacin	*E. coli*, *S. pseudintermedius*	*E. coli*, *S. pseudintermedius*, *Staphylococcus felis*, *Staphylococcus aureus*, *S. canis*, *Pasteurella* spp.
Rifampicin	None*	None*
Trimethoprim-sulfamethoxazole	None*	None*
Tetracycline	*Staphylococcus* spp.	None*
Ticarcillin ± clavulanic acid	None*	None*

*Breakpoints (BP) from human medicine or another animal species are used instead.
†A generic BP exists for Enterobacteriaceae and *Pseudomonas* spp. in dogs, but this is not specific to any infection type.
‡A generic BP exists for skin and soft tissue infections in cats, but this is not specific to any bacterial species.

Table 3. Examples of antimicrobial concentrations in veterinary products for topical use and minimum inhibitory concentrations (MICs)

Active compound	Examples of topical products containing compound	Concentration in commercial product (mg/L)*	Reported MIC ranges (mg/L)	Reported MIC90 (mg/L)	References for MIC ranges
Gentamicin	Otomax Vet/EasOtic®	4,119/2,348	*Pseudomonas aeruginosa*: 0.25–16	8	54
Miconazole	EasOtic®/Surolan® Vet	13,100/19,970	Coagulase-positive staphylococci: 1–8	NA	55
Polymyxin B	Surolan® Vet	654	Coagulase-positive staphylococci: 0.25–64	NA	55
Fusidic acid	Canaural®	4,150	Coagulase-positive staphylococci: 0.06–1,024	0.5–4	56
Framycetin†	Canaural®	4,300	Coagulase-positive staphylococci: ≤0.5–64	NA	55
			P. aeruginosa: 8–1,024	128–256	57
Mupirocin	Muricin®	20,000	*Staphylococcus pseudintermedius*: ≤0.03 to >1,024	NA	58
			Coagulase-positive staphylococci: 0.06–16	0.125–1	56
Enrofloxacin	Baytril® Otic	5,000	*P. aeruginosa*: 0.015–32	32	54
			P. aeruginosa: 0.125 to >64	NA	59
Florfenicol	Osurnia®	10,000	*Escherichia coli*: 1->64	16	60
			S. pseudintermedius: 0.25–32	8	
			Staphylococcus spp: 2–32	8	
			Streptococcus spp.: 0.5->128	2->128	
			Proteus spp.: 4–16	8	
			Enterococcus spp.: 1–8	8	
			Pseudomonas spp.: >64	1,024	

NA data not available.
*The concentrations stated for Canaural® and Muricin® represent mg/kg instead of mg/L.
†Framycetin is a synonym for neomycin B and MIC data are reported here for neomycin.

caused by strains categorized as resistant by AST can be treated successfully by topical therapy. However, this hypothesis has not been validated clinically or experimentally and needs to be supported by scientific evidence in order to be translated into guidelines for antimicrobial use.

Detection of meticillin resistance in staphylococci

According to the MRSA expert rule, a *S. aureus* strain found to be meticillin-resistant, as determined by oxacillin, cefoxitin, or detection of *mecA* or its product PBP2a, should be reported as resistant to all β-lactams, except those that have been specifically licensed to treat MRSA infections (e.g. ceftaroline and ceftobiprole, which are not licensed for veterinary use).[46] This rule was established based on clinical and microbiological evidence that MRSA strains display cross-resistance to β-lactams used in clinical practice for treatment of human staphylococcal infections. This rule has been translated to veterinary medicine without any clinical and/or microbiological evidence that MRSP and meticillin-resistant *S. schleiferi* (MRSS) display cross-resistance to the β-lactams used in veterinary dermatology. Various factors suggest that this rule may lead to reporting of false resistance to these β-lactams in strains expressing low-level meticillin resistance. A considerable proportion of MRSP strains display oxacillin MICs (0.5–4 μg/mL) that are significantly (2–8-fold) lower than the resistance breakpoint for MRSA detection ($R \geq 4$ μg/mL).[47] This is why, similarly to CoNS, the resistance breakpoint set for MRSP detection is considerably lower compared to MRSA ($R \geq 0.5$ μg/mL).[21] Cefalexin is one of the most active cephalosporins against staphylococci and has been associated with good clinical cure rates (90–100%) for uncomplicated MRSA skin infections in humans.[48,49] Studies have demonstrated that cephalosporin resistance in CoNS, which display levels of meticillin resistance comparable to those in MRSP, is dependent on the degree of meticillin resistance expressed by the strain.[50] Lastly, amoxicillin and ampicillin have been reported to have relatively good affinity for PBP2a, and older *in vivo* studies claimed anti-MRSA efficacy of high doses of aminopenicillins combined with β-lactamase inhibitors for treatment of skin and soft tissue infections, and urinary tract infections.[51]

Research to provide evidence to support this expert rule in veterinary medicine is indicated. In the interim, the authors recommend that any oxacillin-resistant staphylococci should be reported as resistant to all β-lactams licensed for veterinary use. However, if therapy with amoxicillin clavulanate or cefalexin has been initiated and the causative strain has a low MIC of oxacillin, we recommend evaluating the clinical outcome of therapy before changing antimicrobial prescription. As already mentioned, AST has a limited predictive value for infections caused by strains reported as resistant.[44]

Although the cefoxitin disk test is generally recognized as reliable for MRSA detection, a recent study has shown that cefoxitin may not be a good surrogate for MRSP detection by disk diffusion.[47] In the absence of an internationally recognized cefoxitin breakpoint clearly differentiating *mecA*-positive from *mecA*-negative isolates of *S. pseudintermedius*, we recommend that laboratories use oxacillin disk or MIC tests for detection of meticillin resistance in this and other staphylococcal species, other than *S. aureus*.

Result reporting

Reporting of polymicrobial skin and wound culture results is a challenge, especially when samples derive from contaminated sites. In these cases, the dominant colony type(s) associated with micro-organisms of clinical relevance should be selected or the report should outline that an unspecific mixed growth with limited or no clinical relevance was detected. Samples from ears also tend to be polymicrobial. For these samples, the same principle of reporting the dominant colony type should be used, but additional factors complicate the decision of selection for subculture and AST: (i) relatively few bacterial species (*Proteus* spp. and *Pseudomonas aeruginosa*) are obligate pathogens of canine ears, whereas other species also occur in healthy dogs, hence the latter would only be relevant in case of pure or almost pure culture; (ii) *Corynebacterium auriscanis* should not be selected for AST as it seems clinically irrelevant and there is no CBP for this species.[52,53] Clinicians should consider the limited value of AST for topical therapy when sampling ear infections and when interpreting results obtained from diagnostic laboratories that indiscriminately report any type of growth.

Various measures such as selective or cascade reporting of AST results can be used by the microbiology laboratory to guide rational choice of antimicrobials. This approach is used extensively in human hospitals to encourage use of first-line drugs. The practice of not reporting the results for selected agents is regarded as selective reporting. For example, AST data should not be reported for critically important drugs that are not licensed for veterinary use (e.g. imipenem, vancomycin and linezolid), even if these drugs are included in the antimicrobial panel as last-resort agents for surveillance purposes. Cascade reporting is the practice of reporting the AST result for only one drug that tests susceptible within a certain class (e.g. gentamicin within the aminoglycosides) to reduce the use of more expensive and/or broader spectrum drugs of the same class (e.g. amikacin). In the absence of guidelines for selective or cascade reporting, decisions should be made in consultation with an infectious disease specialist. Linking the clinic to the laboratory information management system to enable data exchange and implementation of antimicrobial stewardship programmes would be optimal.[7] A variety of software programmes are available on the market for effective management of veterinary practices but they are not designed to interact with the laboratory or are difficult to implement. It is desirable for manufacturing companies to improve veterinary practice management software in order to facilitate antimicrobial stewardship.

Conclusions

The microbiology laboratory should play an important role in the diagnosis of infectious diseases by providing key support to various steps of the diagnostic process, from specimen collection and transportation to

interpretation of AST results. The laboratory's role and responsibilities should extend beyond correct specimen testing and reporting of results, and include guidance in both the pre- and postanalytical phases of the diagnostic process. Furthermore, a good microbiology service is essential for implementation of antimicrobial stewardship programmes in veterinary practice.

The advent of MALDI-TOF MS in clinical microbiology has significantly reduced the time required for bacterial ID and facilitated ID of veterinary pathogens that previously could not be identified. The concomitant developments in genome sequencing technologies are improving our understanding of the taxonomy, ecology and population structure of key pathogens in veterinary dermatology such as *S. pseudintermedius* and *S. schleiferi*. Despite these technological advances, veterinary diagnostic microbiology is still based predominantly on traditional culture methods, and the turnaround time for AST has essentially remained unchanged for many years. Methods for AST are not yet harmonized and clinical breakpoints for important drug–pathogen combinations are either missing or inadequate. Small veterinary microbiology laboratories, including in-clinic laboratories, often neither have the infrastructure nor the expertise required to run up-to-date clinical microbiology, and adequate postgraduate training in veterinary clinical microbiology is not available in most countries.

ESGVM recommends that diagnostic microbiology laboratories are selected by veterinary practitioners taking into consideration the following factors:

- Guidance for optimal specimen management (i.e. selection, collection, storage and transportation of clinical specimens).
- State-of-the-art methods for ID (MALDI-TOF- MS) and AST (MIC determination by broth micro-dilution).
- Implementation of transparent and ongoing quality assurance measures, preferably by accredited laboratories
- Availability of skilled microbiologists for case-based expert advice and data interpretation.

Other factors include the availability of a courier system for overnight delivery of specimens to the laboratory, and access to data for passive epidemiological surveillance and implementation of antimicrobial stewardship programmes at the clinic level. Certification of veterinary microbiologists at a national or, preferably, international level should be a prerequisite. National accreditation, such as according to ISO standards, should be obtained to ensure minimum quality and safety standards.

ESGVM supports the development of PoC tests that could rationalize antimicrobial use in veterinary practice, provided that (i) the performance of the test has been evaluated scientifically, (ii) clinical staff are adequately trained to interpret the results and (iii) clinics meet the minimal requirements for handling microbiological specimens (biosafety level 1). There is concern about direct AST replacing conventional AST due to the potential for error and the subsequent selection of a drug that is not effective.

ESGVM has a mission to set standards for veterinary clinical microbiology, including methods and training, and the promotion of antimicrobial stewardship and constructive interaction between microbiologists and clinicians. The group promotes diagnostic microbiology in veterinary practice by standardizing procedures and by educating veterinarians about the key role played by microbiology laboratories in antimicrobial stewardship and patient care. ESGVM strongly supports (i) global harmonization of methods and setting of infection-, animal- and bacterial-specific CBPs for AST of veterinary pathogens; (ii) postgraduate education and board certification of specialists in veterinary clinical microbiology and antimicrobial stewardship; (iii) official licensing of veterinary diagnostic microbiology laboratories and quality assurance to guarantee the minimum quality and biosafety standards required to perform veterinary microbiology; and (iv) development of new diagnostic tests providing veterinarians with rapid and reliable results at reasonable cost.

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6.3

Comparison of two *in vitro* antifungal sensitivity tests and monitoring during therapy of *Sporothrix schenckii sensu stricto* in Malaysian cats

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Background – Feline sporotrichosis is common in Malaysia. Thermosensitivity and effects of azole treatment on fungal susceptibility are unknown.

Objectives – To evaluate thermotolerance and antifungal susceptibility of feline Malaysian *Sporothrix* isolates, compare microdilution (MD) and E-test results, and investigate changes in susceptibility during azole therapy.

Methods – *Sporothrix schenckii sensu stricto* was isolated from 44 cats. Thermotolerance was determined via culture at 37°C for 7 days. Susceptibility to itraconazole (ITZ), ketoconazole (KTZ) and terbinafine (TRB) was assessed in 40 isolates by MD; to amphotericin B (AMB), KTZ, ITZ, fluconazole (FLC) and posaconazole (POS) by E-test. Results were statistically compared by Pearson's Product Moment. In eight ketoconazole treated cats, susceptibility testing to itraconazole and ketoconazole was repeated every two months for six months.

Results – Thermotolerance was observed in 36 of 44 (82%) isolates. Assuming that isolates growing at antifungal concentrations ≥4 mg/mL were resistant, all were resistant on E-test to FLC and AMB, 11 (28%) to POS, 6 (15%) to ITZ and 1 (3%) to KTZ. On MD, 27 of 40 (68%) were resistant to TRB, 2 (5%) to ITZ and 3 (8%) to KTZ. There was no correlation between E-test and MD results (KTZ $r = 0.10$, $P = 0.54$, and ITZ $r = 0.11$, $P = 0.48$). MD values for ITZ and KTZ did not exceed 4 mg/L during KTZ therapy.

Conclusion – The majority of feline isolates in Malaysia are thermosensitive. Lack of correlation between E-test and MD suggests that the E-test is unreliable to test antifungal susceptibility for *Sporothrix* spp. compared to MD. KTZ was the antifungal drug with the lowest MIC. Prolonged KTZ administration may not induce changes in antifungal susceptibility.

Introduction

Sporotrichosis is an important zoonotic fungal infection reported worldwide and a common cause of subcutaneous and systemic disease of humans, dogs and cats.[1] *Sporothrix* spp. are classified under the division Ascomycota, class Pyrenomycetes, order Ophiostomatales and family Ophiostomataceae, with no known sexual form.[2] Studies have revealed high genetic variability among *Sporothrix* isolates resulting in six species within the complex.[1] Five pathogenic species have been identified, namely *Sporothrix brasiliensis*, *S. schenckii sensu stricto*, *Sporothrix globosa*, *Sporothrix mexicana* and *Sporothrix luriei* (formerly *S. schenckii* var. *luriei*).[1] *Sporothrix brasiliensis*, *S. globosa*, *S. schenckii sensu stricto* and *S. luriei* have been reported as human and animal infectious agents.[3–5] *Sporothrix schenckii* is considered to be a cryptic species, defined as two or more distinct species that are erroneously classified under one name; surveys of DNA variation have enabled these species to be differentiated.[6] A study of infected cats in Malaysia suggested that a clonal strain of *S. schenckii sensu stricto* is the prevailing causative agent of feline sporotrichosis in Malaysia.[7]

The role of cats as carriers and zoonotic propagators of *Sporothrix* spp. is well documented; its manifestation as a clinical disease is dependent on the host immune competence and on the virulence of the particular strain of *S. schenckii*.[1,8,9] The latter is partially dependent on the particular isolates' thermotolerance. Isolates that were able to grow at 35°C but not at 37°C were not capable of causing lymphatic sporotrichosis, but only caused an infection at the site of inoculation (fixed cutaneous disease).[1]

Therapy of feline sporotrichosis in Malaysia is based on the use of azole compounds, such as ketoconazole (KTZ) or itraconazole (ITZ). Treatment can be challenging because of the risk of zoonosis to owners and veterinarians alike, and the prolonged period of treatment necessary to obtain clinical improvement or cure. Hence, questions arise on which azole compound is most suitable for Malaysian

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isolates and if the agent could develop low susceptibility or overt resistance during a long treatment period.

There are published guidelines from the Clinical Laboratory Standards Institute (CLSI) for the susceptibility testing of filamentous moulds using broth microdilution (MD).[10] However, due to its complexity, this procedure is not practical for routine laboratory use, and simpler and more affordable agar-based methods, such as the E-test and disc diffusion, have been used.[11] Results of these alternative techniques were compared for human dermatophyte isolates and benchmarked with those obtained by the CLSI MD reference method.[11] There was usually low agreement for the estimation of antimycotic susceptibility between these two methods.[11]

The Antifungal Susceptibility Testing of the European Committee for Antimicrobial Susceptibility Test (AFST-EUCAST) offers an alternative MD method which categorizes mould sensitivity as 'susceptible', 'intermediate' or 'resistant'.[12] EUCAST has determined the breakpoints for *Aspergillus* spp. and *Candida* spp., and not for *Sporothrix* spp. One study has described the susceptibility profile of *S. schenckii* isolates from humans in both growth phases, determined by microdilution (based on AFST-EUCAST) and compared them with those obtained by the E-test.[13] It was concluded that AFST-EUCAST method could not be recommended as a test to assess antifungal susceptibility for *S. schenckii* and that the E-test may be a better option, especially for *in vitro* susceptibility to amphotericin B (AMB) and fluconazole (FLC).

The aim of this study was to determine the thermotolerance pattern of Malaysian isolates of *S. schenckii* and identify the most suitable antifungal drug for their treatment by evaluating and comparing antimycotic susceptibility results obtained by the E-test and by MD following the CLSI guidelines. Repeated antifungal susceptibility testing was conducted to identify possible changes in susceptibility, during a six month period in eight cats receiving KTZ antifungal therapy based on the results of the E-test and of MD.

Materials and methods

Sample collection and identification

Samples from cats that showed cutaneous lesions with cytological evidence of sporotrichosis were collected with a sterile cotton swab from 2014–2015. The cats resided primarily in urban areas; other cats were from rural areas across various states of peninsular Malaysia (Perak, Selangor, Melaka, Johor, Kelantan and Terengganu). Samples were inoculated on Sabouraud's dextrose agar containing 0.1 mg/mL of chloramphenicol (Oxoid; Hampshire, England) and stored at room temperature. Each isolate was then identified based on its morphological characteristics on culture, and on sequence analyses of its calmodulin gene and rDNA Internal Transcribed Spacer (ITS) region.[7]

Thermal tolerance test

Isolates were cultured on potato dextrose agar at 37°C for 7 days; *S. brasiliensis* strain UNIFESP SS74 and *S. globosa* NUBS 14 strain isolated from feline sporotrichosis in Japan, were used as positive and negative controls, respectively.

Antifungal susceptibility tests by the broth microdilution assay

Broth MD assay was performed to assess the susceptibility according, in part, to CLSI guidelines.[10] Given the slow growth of *S. schenckii sensu stricto* in RPMI 1640 broth, we used an incubation time of 72 h (not 48) and a temperature of 30°C (instead of 35–37°C), as described previously.[22] Minimum inhibitory concentrations (MIC) of terbinafine (TRB), ITZ and KTZ were determined after 72 h incubation at 30°C against the hyphal phase of the fungus. TRB concentrations were 0.03125 to 6 mg/L in physiological saline. The MIC was defined as the lowest concentration showing 100% growth inhibition. All MD procedures were performed in two replicates, along two lanes in a 96-well microdilution plate to ensure accuracy of MD results. Assays in this study were done in duplicate to ensure accuracy of data in event of pipetting error, multiple skipped wells or contamination.

Antifungal susceptibility assessed by the E-test

The *in vitro* activity of AMB, KTZ, FLC, ITZ and posaconazole (POZ) against clinical isolates of *S. schenckii* was investigated by the use of E-test gradient strips obtained from AB BIODISK (Solna, Sweden). The E-test was performed according to the manufacturer's instructions, using 20 mL RPMI 1640 agar on 90 mm petri plates.[14] The MICs were determined after 72 h incubation at 32°C against the hyphal phase of the fungus. For quality control, the strain *Candida parapsilosis* ATCC 22019 was used in each experiment to check the accuracy of drug dilution.

Antifungal susceptibility monitoring

A cohort of eight cats with sporotrichosis confirmed on culture and PCR were sampled. Initial examinations included complete blood count, blood biochemistry and Feline Immunodeficiency Virus (FIV) and Feline Leukaemia virus (FeLV) status (Snap Combo FIV/FeLV IDEXX Laboratories, Westbrook, ME, USA). Two of eight cats were positive for FIV; the entire cohort did not have any systemic sign of illness. Cats were treated with daily oral KTZ (Pristinex, Xepa-Soul Pattinson; Melaka, Malaysia) for six months at 10 mg/kg. Three rounds of lesion sampling for antifungal susceptibility to ITZ and KTZ were conducted, using the broth MD assay and E-test, at two month intervals, for three cats.

As breakpoints have not been established in moulds including dimorphic fungi, claims of resistance could not be made so the authors adopted a low susceptibility for isolates. For analytical purposes, previous studies of isolates of *Aspergillus* spp., *Fusarium* spp., *Rhizopus* spp. and *S. schenckii* have considered a MIC of <1 mg/L as susceptible, MIC = 2 mg/L as intermediate and MIC >4 mg/L as putative resistant; for amphotericin B and azole drugs.[1] For the purposes of this study, we used the same nominal break points for assessing sensitivity to azole drugs and amphotericin.

Statistical analysis

The Pearson's Product Moment Correlation test was used to compare results of the E-test with those of the MD for KTZ and ITZ (Microsoft Office, Excel 2013).

Results

Forty four isolates were identified as *S. schenckii sensu stricto,* based on sequence analysis of the calmodulin gene and rDNA ITS region. For the study of thermal tolerance, *S. brasiliensis* grew well at 37°C but *S. globosa* did not. Only eight of the 44 strains (18%) showed modest growth at 37°C. Results of E-test and MD MIC of all isolates and their geometric mean are shown in Table 1. During estimation of MIC using MD, four isolates were discarded as they did not meet internal quality control requirements (did not grow in RPMI 1640 media). For all 40 isolates tested by MD, the dilution results for the two replicates showed no difference.

Presumed resistance was observed towards AMB (>32 mg/L), POS (>32 mg/L) and FLC (>256 mg/L) in

Table 1. Antifungal susceptibility and thermotolerance of *Sporothrix schenckii sensu stricto* isolates to antifungal agents based on E-test and microdilution

	E-Test					MD			
Case number	AMB	KTZ	ITZ	POS	FLC	TRB	ITZ	KTZ	Thermotolerance
1	6	0.25	1.5	1	>256	4	0.5	0.5	(–)
2	>32	0.75	6	>32	>256	2	2	1	(+)
3	6	0.064	1.5	1	>256	1	0.5	0.125	(–)
4	12	1	3	>32	>256	4	1	1	(–)
5	>32	1	6	>32	>256	4	2	0.5	(+)
6	24	1.5	2	>32	>256	2	2	0.5	(–)
7	>32	1	1.5	>32	>256	4	2	0.5	(–)
8	>32	0.75	2	>32	>256	4	2	0.5	(–)
9	24	1	8	>32	>256	1	2	0.25	(–)
10	4	1	6	8	>256	4	1	1	(–)
11	12	1	2	32	>256	4	2	1	(–)
12	6	1	2	24	>256	4	2	0.5	(–)
13	6	1	2	24	>256	8	4	2	(–)
14	12	0.38	3	2	>256	4	2	0.5	(–)
15	6	0.5	0.5	0.38	>256	4	2	0.5	(–)
16	32	4	3	1.5	>256	4	0.5	1	(–)
17	32	1.5	4	1	>256	4	1	1	(–)
18	32	1	2	1	>256	1	0.5	0.5	(+)
19	32	1.5	2	1.5	>256	2	0.5	0.5	(–)
20	0.38	0.19	0.094	0.75	>256	2	NG	NG	(–)
21	6	1.5	0.75	1	>256	2	NG	NG	(–)
22	0.5	0.094	0.125	0.38	>256	1	NG	NG	(+)
23	8	0.25	0.5	0.25	>256	4	4	2	(–)
24	8	0.25	0.75	0.19	>256	1	1	1	(–)
25	8	0.38	0.5	0.25	>256	2	1	1	(–)
26	4	0.25	0.75	0.38	>256	1	1	1	(–)
27	NG	NG	NG	NG	NG	NG	NG	NG	(–)
28	4	0.19	0.75	0.19	>256	1	1	1	(–)
29	>32	1.5	1.5	1.5	>256	2	2	0.5	(–)
30	>32	1.5	1.5	3	>256	1	1	1	(–)
31	32	2	1.5	3	>256	1	2	0.5	(–)
32	24	1.5	1	1.5	>256	8	1	2	(–)
33	32	1.5	1	1.5	>256	4	2	0.5	(–)
34	>32	1	1	2	>256	8	2	1	(–)
35	32	2	0.5	2	>256	4	0.5	1	(–)
36	>32	1	1.5	1.5	>256	4	1	2	(–)
37	12	0.75	1.5	1.5	>256	4	1	2	(–)
38	12	0.75	2	1	>256	8	1	1	(+)
39	>32	2	1.5	3	>256	4	2	2	(+)
40	4	1	1	3	>256	4	1	2	(+)
41	>32	0.75	0.75	3	>256	4	0.5	1	(–)
42	>32	0.75	1.5	2	>256	4	2	4	(–)
43	12	1.5	>32	2	>256	4	2	4	(–)
44	32	1.5	1.5	1.5	>256	4	2	4	(+)
Geometric Mean MIC	9.80	0.78	1.38	1.51		2.85	1.30	0.92	
Median MIC	14.90	1.03	1.94	3.71		3.42	1.51	1.20	

AMB Amphotericin B, KTZ Ketoconazole, ITZ Itraconazole, POS Posaconazole, FLC Fluconazole, TRB Terbinafine, MIC Minimum Inhibitory Concentration, NG no growth.

For AMB, FLC, ITZ, KTZ, POS the MIC of <1 mg/L was considered susceptible, MIC = 2 mg/L intermediate; MIC >4 mg/L was considered as putative resistant. For TRB the susceptible range was 0.031 to 6 mg/L.

most strains via the E-test. Based on MD, 27 of 40 isolates were above the MIC of >4 mg/L for TRB, three for KTZ and two for ITZ. There was a low and insignificant correlation between results of the E-test and those of the MD for KTZ and ITZ (KTZ $r = 0.09$ and $P = 0.53$ and ITZ $r = 0.11$ and $P = 0.48$). Despite the lack of correlation, both sets of data suggested that KTZ had the lowest MIC when compared to other antifungal agents.

Cats treated with 10 mg/kg oral daily KTZ for six months tolerated well the therapy without signs of anorexia. Of the eight cats enrolled into antifungal susceptibility monitoring, four cats survived up to six months with one cured at four months, whereas the other four died. Cure was defined as two consecutive negative cultures at monthly interval. The MD and E-test based MIC values for ITZ and KTZ did not exceed 4 mg/L throughout the susceptibility monitoring period (Table 2).

Discussion

To the best of the authors' knowledge this is the first evaluation of thermotolerance and antifungal sensitivity

Table 2. Minimum inhibitory concentration of itraconazole (ITZ) and ketoconazole (KTZ) during susceptibility monitoring

Sampling time point / Cat identity	0 months (onset of treatment)				2 months				4 months				6 months			
	MD KTZ	E-TEST	MD ITZ	E-TEST	MD KTZ	E-TEST	MD ITZ	E-TEST	MD KTZ	E-TEST	MD ITZ	E-TEST	MD KTZ	E-TEST	MD ITZ	E-TEST
D	1	0.25	1	0.75	1	0.38	1	0.5	1	2	2	2	1	1.5	1	1
C	1	0.38	1	0.5	1	0.25	1	0.75	0.5	2	2	2	1	1.5	1	1
S	1	0.19	1	0.75	1	1.9	1	0.75	0.5	3	1	2	UC	UC	UC	UC

All values are mg/L; UC unable to culture.
Cats were treated with KTZ for six months and clinical lesions sampled every two months on three occasions for susceptibility testing to ITZ and KTZ by broth microdilution assay (MD) and E-test.

profiles for feline Malaysian isolates of *S. schenckii sensu stricto*. It is also the first time that antifungal susceptibility monitoring has been conducted over time in treated animals. It is also the first evaluation and comparison between MD MIC determination following CLSI guidelines and the E-test, for the antimycotic susceptibility of *S. schenckii sensu stricto*.

Based on our results, similar to what has previously been described, we confirmed that the feline *Sporothrix* isolates are of the species *S. schenckii sensu stricto* of a single clonal strain.[7] The majority of the isolates were thermosensitive, with a minority (18%) having the potential ability for lymphatic spread.

Based on the results of *in vitro* testing KTZ would seem to be the most suitable antifungal for the treatment of feline sporotrichosis. When comparing our data with reports from Spain and Brazil, geographical differences appear in susceptibility patterns of *S. schenckii sensu stricto* based on MD MICs for human isolates.[15,16] Terbinafine was considered the most suitable antifungal for the treatment of human isolates of sporotrichosis in Spain and Brazil, compared to KTZ for our Malaysian feline isolates.

There seemed to be a general lack of correlation between the E-test and MD based on CLSI guidelines for the estimation of antifungal susceptibility for *S. schenckii* and our data support previous observations in two tests for dermatophytes.[11] This also is similar to a study that compared the AFST-EUCAST microdilution technique to the E-test.[13] This variability and lack of standardisation for susceptibility testing and breakpoints for the different testing methodologies are directly related to variability in the kingdom fungi. Not only do fungi differ greatly in terms of ideal temperature for growth and incubation period, but some, such as *S. schenckii,* are dimorphic moulds and exist in either mycelial (hyphal) or yeast form. This characteristic adds ambiguity as to which form is ideal to test susceptibility, one study reported that the mycelial phase of *S. schenckii* had higher MIC *in vitro* than the yeast.[13] For this reason we chose to test the hyphal phase of our isolates, in order to obtain more conservative results to be used in the clinical setting.

Generally, the results of MIC evaluation of Malaysian isolates of *S. schenckii sensu stricto* displayed low susceptibility towards all antifungals and this potentially poses problems for the choice of the best treatment. Furthermore, given prolonged treatment periods in severe cases of sporotrichosis, there could be some risk of developing low susceptibility or resistance to the antifungals used. Data from this study would suggest that, at least in a small group of KTZ-treated cats, this is not a significant factor, based on MD MIC values recorded over time. Susceptibility values recorded from MD were stable throughout the study, although those from the E-test were more variable.

In spite of KTZ being the antifungal with the lowest MIC value; treatment of feline sporotrichosis with this drug was unsuccessful in our cases, with only one of eight cats achieving clinical cure and four dying during the six months of study. The lack of clinical response to the treatment could be attributed to the particular virulence and/or the low susceptibility of Malaysian *Sporothrix* spp. isolates to antifungals, or to host immune dysfunction. Host immuno-incompetence may indeed play a decisive role in the clinical manifestation of *S. schenckii* infection, as clinical signs may range from single fixed cutaneous lesions to multifocal fatal systemic forms.[1]

Cats with sporotrichosis have been observed to have an inversion of the CD4/CD8 peripheral circulating lymphocyte ratio; infection was associated with greater percentages of CD8+ cells, and especially of $CD8^{low}$ cells, in cases where clinical signs were more severe.[17] This observation supports the view that the host immune response is a key factor that may hinder complete cure, despite correct medication and sensitivity of the microorganism to the antimicrobial therapy. Even so, it is assumed that co-infection with FIV and/or FeLV in cats with sporotrichosis has no significant effect on the final clinical manifestations or on the outcome of the disease.[18,19] From the eight cats in this study, two (cases C and S) were FIV positive and one of these (case S) was cured after 12 months of treatment (data not shown), whereas none were FeLV positive. Understanding of the immunological processes in Sporothrix infection in cats is limited, although the role of an intact and capable Th1 and Th17 cellular response has been documented in cats and mice.[20,21]

We used a modification of the CLSI guidelines for susceptibility testing, with an extended incubation time of 72 h and reduced incubation temperature of 30°C as previously described.[22] This is different from the methodology for susceptibility testing reported in another study which investigated the susceptibility of *S. brasiliensis*, where the incubation period was extended to 120 h at 35°C.[23] This variability in methodology reflects the genetic diversity that exist within the *Sporothrix* complex,

where there are different species (*S. schenckii sensu stricto* versus *S. brasiliensis*) and numerous other distinct individuals, each with distinct expression of virulence affecting final clinical manifestation and outcome.

Sequencing of the rDNA ITS is currently the most widely applied gene for routine identification of fungi, and has been recommended as a fungal barcoding gene.[24] For *S. schenckii sensu lato*, species distinction within the complex presently is based on calmodulin sequences.[3] Using these techniques and data deposited in Genbank, phylogenetic analysis of *S. schenckii sensu lato* has been reported.[25] In that study it was found that there was scarce genetic flow with low migration index and high ancestry coefficient between *S. brasiliensis* and *S. schenckii sensu stricto*; however, *S. globosa*, *S. mexicana* and *S. pallida* displayed highly differentiated species with no genetic exchange. In the present study the identification of a single clonal strain of *S. schenckii sensu stricto*, consisting of an independent genetic population from Malaysia lends support for the hypothesis of purifying selection followed by population expansion due to divergent evolutionary process from environmental and host selection pressure.

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The usefulness of dermoscopy in canine pattern alopecia: a descriptive study

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Background – Dermoscopic studies evaluating noninflammatory, nonpruritic progressive alopecia attributable to pattern alopecia are currently unavailable.

Hypothesis/objectives – To evaluate the dermoscopic features observed in healthy skin of short coated dogs and compare these findings with those observed in dogs affected by pattern alopecia diagnosed by clinical and dermatopathological examination.

Animals – Thirty male and female, healthy, breed matched, young adult, short coated dogs (controls) and 30 male and female, young adult, short coated dogs affected by pattern alopecia.

Methods – Dermoscopy was performed with a Fotofinder II videodermoscope equipped with software that allowed the measurement of structures visualized in magnified images (20×–40×–70×). Skin biopsy samples were obtained from the thorax and evaluated dermoscopically for dermoscopic–histological correlation in affected dogs.

Results – Dermoscopic findings in canine pattern alopecia were hair shaft thinning, circle hairs and follicular keratin plugs; in the affected sun exposed areas there was a honeycomb-like pattern of pigmentation. Arborizing red lines reflecting vascularization were classified as a nonspecific finding because they were also common in healthy dogs. Dermoscopic features correlated with histology for selected hair follicle abnormalities.

Conclusions and clinical importance – Although canine pattern alopecia is a visually striking disease, this study supports the value of dermoscopy for clinical examination and also opens promising perspectives for the identification of diagnostic dermoscopic patterns that may be useful for other skin disorders.

Introduction

Skin surface microscopy has been reported to date back to 1663, when Johan Kolhaus first looked at nail fold vessels with a microscope.[1] Nevertheless, it was only during the 20th Century that several diagnostic methods were developed utilizing surface microscopy. Currently, two techniques are used for *in vivo* diagnosis. The first is dermoscopy, originally used to observe and diagnose pigmented skin lesions such as melanocytic nevus and melanoma; the second is trichoscopy of the hair and scalp.[2–8] Trichoscopy has been used to visualize normal hairs and assess their number per follicular unit, to distinguish whether hair follicle openings are normal, empty, fibrotic or containing biological material as hyperkeratotic plugs, and to study the appearance of perifollicular epidermis and cutaneous microvessels.[9] Therefore, trichoscopy has proved relevant in the differentiation between cicatricial and noncicatricial alopecias. On the one hand, as a large group of disorders characterized by permanent destruction of hair follicles, cicatricial alopecia shows trichoscopic features such as loss of follicular ostia and presence of white patches corresponding to fibrous tracts that mark extinct hair follicles.[10] On the other, in all noncicatricial alopecias, such as alopecia areata and androgenetic alopecia (male and female pattern alopecia), suggestive trichoscopic findings are represented by specific hair shaft and follicular opening abnormalities.[11–13]

In spite of the widespread use in human medicine, to date only a few studies on the application of dermoscopy

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have been documented in veterinary medicine; specifically in feline dermatology.[14–16] Moreover, except for an unpublished abstract regarding the dermoscopic features in 35 dogs with juvenile-onset demodicosis and 35 breed- and age-matched dogs,[17] to the best of the authors' knowledge there is no dermoscopic study of canine non-inflammatory alopecia. Therefore, the purpose of this study was two fold. The first aim was to evaluate dermoscopic features observed in short coated healthy dogs and compare these findings with those observed in short coated dogs affected by pattern alopecia diagnosed by clinical and dermatopathological examination. The second aim was to validate the use of dermoscopy using histopathology as a reference standard in order to generate dermoscopic criteria that would be useful for the diagnosis of pattern alopecia.

Material and methods

Study population

A population of 30 healthy, short coated dogs was matched with 30 short coated dogs referred for noninflammatory, nonpruritic progressive alopecia attributable to pattern alopecia. Details about both groups are presented in Table 1.

Dogs were owned by dog breeders or clients; informed owner consent was obtained prior to any procedure. Dogs were selected on the basis of the following criteria: (i) no other clinical abnormalities at the time of physical examination; (ii) except for pattern alopecia, no evidence of additional skin lesions on dermatological examination; (iii) for intact female dogs, not being pregnant or lactating; and (iv) normal complete blood count and routine serum biochemical analysis.

Dermoscopic examination

A videodermoscope (Fotofinder® TeachScreen Systems software GmbH; Bad Birnbach, Germany) was used and six body sites including convex pinnae, periaural area, ventral neck, thorax, abdomen and caudal thighs were selected. Alcohol (Kodan® spray, Schulke & Mayr; Vienna, Austria) was applied as an interface solution to better observe surface and subsurface microscopic features.

In order to take a dermoscopic overview image of the selected cutaneous region, images at 20-fold and 40-fold magnification were observed initially. Then, as previously reported,[11] images at 70-fold magnification, which allows a high quality enlargement of 9 mm^2 of the skin area to the size of the computer screen, were used for statistical purposes. An area of 3.14 mm^2 was calculated on the selected 70-fold images by means of the FotoFinder® software; dogs with pattern alopecia and controls were compared for the following parameters: diameter and total number of hair tufts next to follicular ostia per examined area; total number of hairs per hair tuft plus the ratio between the number of secondary hairs/primary hair; and diameter of both primary and secondary hairs in each hair tuft. Hair follicle infundibula, perifollicular epidermis and vascular structures such as very small capillaries were also observed.

Table 1. Signalment, age, body weight and sex of selected dogs used for dermoscopy

Breed and number of dogs	Age (years)	Body weight (kg)	Sex
Controls			
Dachshund: 15	4.20 ± 0.77	8.09 ± 1.84	7F (2 FS); 8M
Italian greyhound: 10	3.50 ± 1.13	4.85 ± 0.63	6F (2 FS); 4M
Miniature pinscher: 5	2.00 ± 1.17	3.89 ± 0.70	2 F; 3M
Dogs with pattern alopecia			
Dachshund: 15	3.98 ± 1.21	7.25 ± 1.01	7F (2 FS); 8M
Italian greyhound: 10	3.80 ± 1.23	4.88 ± 1.31	6F (2 FS); 4M
Miniature pinscher: 5	2.1 ± 1.43	3.32 ± 1.19	4 F (2FS); 1MN

F, female; FS, spayed female; M, male; MN, neutered male; data are expressed as mean ± SD.

Dermoscopy versus histopathology

For dermoscopic–histopathological correlation, in 20 of the affected dogs a single skin biopsy taken from the thoracic skin area previously circled with a marker during dermoscopic examination was collected under local anaesthesia using a 4–6 mm skin biopsy punch. The biopsies were fixed in 10% neutral buffered formalin, trimmed, routinely processed and paraffin embedded. Transverse serial sections (4 μm thick) were obtained and stained with haematoxylin and eosin for histological examination. Histological images were observed under an Olympus BX51 photomicroscope equipped with an Olympus C-5060 Wide Zoom and DP software digital camera (Olympus; Tokyo, Japan) for computer-assisted image acquisition and analysis. The slides contained multiple transverse sections of the skin at different levels starting from the panniculus and ending with the stratum corneum. For hair follicle number assessment, transverse skin sections were examined at the level of the mid/lower isthmus. The total number of follicular units per examined area and number of total hairs per follicular unit were counted.

Other parameters assessed included: infundibular hyperkeratosis evaluated in the superficial slides at the level of the infundubulum in cross-section; vascularization scored in the same slides used to examine infundibular hyperkeratosis; and pigment clumping evaluated in overall sections and scored according to severity of clumping in bulbs and hair shafts. Infundibular hyperkeratosis was graded as − (absent), + (mild), ++ (moderate) or +++ (severe); vascularization and pigment was graded as − (absent), + (weak), ++ (evident) or +++ (prominent).

Statistical analyses

In order to assess whether dogs with and without pattern alopecia were correctly matched for age and body weight, the Mann–Whitney U-test was used; for sex and hair colour, Fisher's exact test and an $r \times c$ contingency table were used, respectively. For each of the three dog breeds investigated, dogs with pattern baldness and controls were compared for the measured parameters on the six selected body regions described above.

The analysis was performed using the Mann–Whitney test followed by Bonferroni correction. Furthermore, the same hair parameters were compared between regions within each dog breed for those with and without pattern alopecia, using the Friedman test followed by Dunn's multiple comparison test. To assess whether dermoscopic examination yielded similar results to histology, the Spearman's rank correlation coefficient was calculated between the total number of hair tufts next to follicular ostia per examined area based on the former method and the total number of follicular units per examined area counted with the latter. The same test was also used to verify whether the total number of hairs per hair tuft at dermoscopy correlated with the total number of hairs per follicular unit identified at histology. Significance was considered with $P < 0.05$. In addition, Cohen's kappa coefficient was used to assess whether there was agreement between the two methods in the analysis of infundibular hyperkeratosis, vascularization and pigment. κ values <0 indicated no agreement, 0–0.20 slight, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 substantial and 0.81–1 as almost perfect agreement. Software package was used for analysis (GraphPad Prism v.5.0, GraphPad Software; La Jolla, CA, USA).

Results

Group matching

Population characteristics did not differ statistically in any of the three breeds between dogs with pattern alopecia and controls, suggesting appropriate matching.

Dermoscopic features

In control dogs, hair shafts were grouped into follicular units consisting of thick hairs emerging independently from their follicular ostia and considered as primary hairs, and surrounded by a variable number of thinner hairs all protruding through a common external orifice and considered as secondary hairs (Figure 1a). Other observed features included: hair follicle openings that were not empty, fibrotic or filled with material such as keratotic plugs; no scaling on perifollicular and interfollicular skin surface; and thin arborizing red lines corresponding to vessels between follicular units. All of these findings are illustrated in Figure 1b. In dogs with dilute hair colour, pinpoint black spots were also observed on the interfollicular skin surface.

In dogs affected by pattern alopecia, the most common dermoscopic finding included primary and secondary hair shafts that were shorter and thinner than those of controls (Figure 1c–d). Other findings were: scattered circle hairs, plugging of the follicular infundibulum with yellow brown material and on periaural and caudal thigh regions, a honeycomb-like pigmented network. Scattered circle hairs, plugging of the follicular infundibulum with yellow brown material; on periaural and caudal thigh regions, a honeycomb-like pigmented network. As in control dogs, dogs with dilute hair colour showed interfollicular pinpoint black spots or, in some cases, larger black spots around hair follicle openings. Thin arborizing vessels regularly distributed between follicular units were also detected as in controls. These findings are illustrated in Figure 2 (a–f).

Dermoscopic parameters

Results are reported in Table S1.

Dachshunds

Comparing dachshunds with pattern alopecia and control dogs, the following significant differences were documented: (i) the median diameter of hair tufts next to follicular ostia was smaller in those with pattern alopecia than control dogs in the convex pinnae (0.05 mm versus 0.08 mm; $P < 0.001$), ventral neck (0.07 mm versus 0.08 mm; $P < 0.01$), chest (0.06 mm versus 0.08 mm; $P < 0.05$) and abdominal region (0.06 mm versus 0.08 mm; $P < 0.01$); and (ii) the median diameter of primary hairs was smaller in those with pattern alopecia in the ventral neck (0.03 mm versus 0.04 mm; $P < 0.05$) and chest (0.03 mm versus 0.04 mm; $P < 0.01$). In dachshunds with pattern alopecia, the periaural region had a higher median ratio of secondary hairs/primary hair [7 (range: 4–14)] than the abdominal region [5 (2–8); $P < 0.001$]. In control dogs, the periaural region had a smaller median diameter of hair tufts located next to follicular ostia [0.07 mm (0.04–0.08)] than the ventral neck [0.08 mm (0.07–0.11); $P < 0.01$], the chest [0.08 mm (0.06–0.11); $P < 0.01$] or abdominal region [0.08 mm (0.06–0.11); $P < 0.05$], whereas the periaural region had a smaller median diameter of primary hairs [0.03 mm

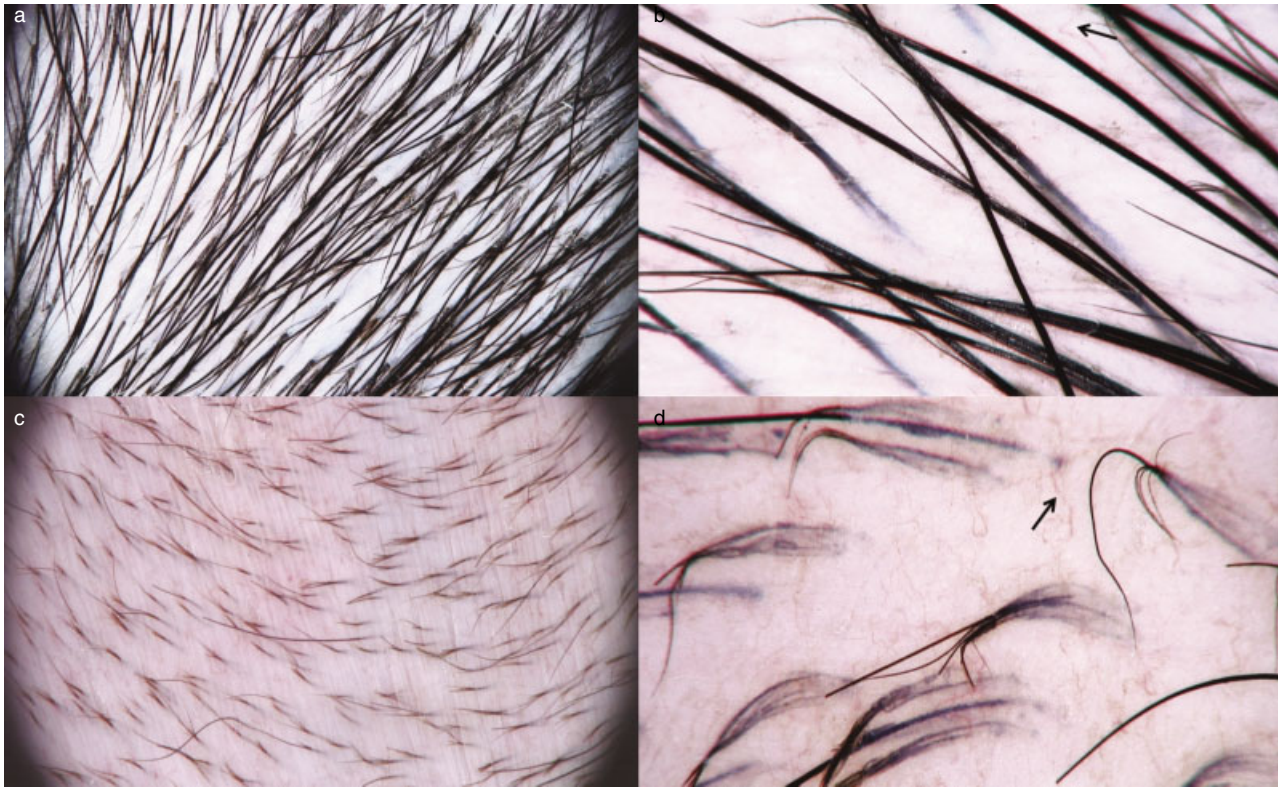

Figure 1. Representative hair features in control dogs and dogs affected by pattern alopecia. Control dogs: (a) primary thick hairs surrounded by thinner secondary hairs (20×); (b) normal hair follicle openings from which emerge thick primary hairs surrounded by thinner secondary hairs; between follicular units, presence of thin arborizing red lines corresponding to vessels and indicated (black arrow) (70×). Affected dogs: (c) diffuse hair thinning (20×); (d) both primary and secondary hairs thinner and shorter than in controls. Arborizing red lines are evident between follicular units (black arrow) (70×).

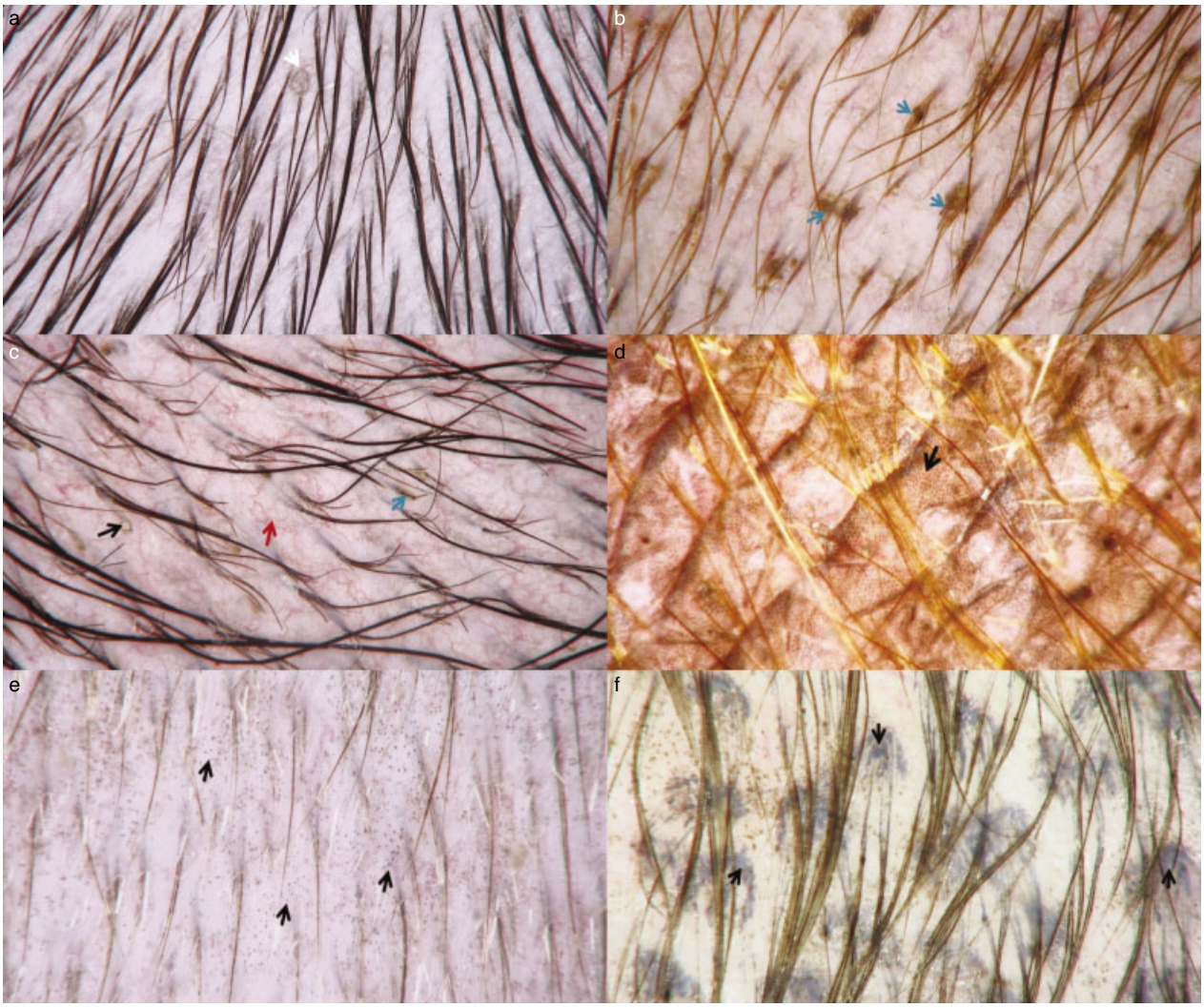

Figure 2. Representative follicular and interfollicular dermoscopic features in dogs with pattern alopecia. (a) Hair circles (white arrows) between miniaturized hairs (20×); (b) plugging of the follicular infundibulum with yellow brown material (blue arrows) (40×); (c) hair circles (black arrow), thin arborizing vessels (red arrow) and plugging of the follicular infundibulum (blue arrow) (70×); (d) honeycomb-like pattern (black arrows) on the caudal thigh (70×); (e) pinpoint black dots in a dog with diluted hair colour (black arrow) (70×); (f) large black dots around hair follicles in a dog with diluted hair colour (70×).

(0.02–0.03)] than either the ventral neck [0.04 mm (0.02–0.05); $P < 0.01$] or chest [0.04 mm (0.03–0.05); $P < 0.01$].

Italian greyhounds

Between Italian greyhounds with pattern alopecia and control dogs, the median diameter of hair tufts next to follicular ostia was smaller in those with pattern alopecia [0.05 mm (0.04–0.07)] than control dogs [0.07 mm 0.07–0.08); $P < 0.01$] in the ventral neck. No other differences were documented for the hair tuft parameters in any region.

Miniature pinschers

Between miniature pinschers with pattern alopecia and control dogs, the following significant differences were documented: (i) the median diameter of hair tufts next to follicular ostia was smaller in those with pattern alopecia than control dogs in the convex pinnae (0.05 mm versus 0.08 mm; $P < 0.001$), ventral neck (0.05 mm versus 0.08 mm; $P < 0.05$) and caudal thigh region (0.05 mm versus 0.07 mm; $P < 0.05$); and (ii) the median diameter of secondary hairs was smaller in those with pattern alopecia than control dogs in the convex pinnae (0.01 mm versus 0.02 mm; $P < 0.01$), ventral neck (0.01 mm versus 0.02 mm; $P < 0.001$) and chest region (0.01 mm versus 0.02 mm; $P < 0.01$).

In control dogs the convex pinnae had a higher median ratio [9 (8–11)] than either the chest [5 (5–6); $P < 0.05$] or caudal thigh [5 (4–6); $P < 0.01$].

Histological findings

In transverse histological sections taken from the thoracic region, hair follicles were characterized by a moderate to severe decrease in size without distortion or irregularity of their contour or reduction of the overall number of adnexal units. Infundibular hyperkeratosis and melanin clumping were also variably observed, whereas in some areas, vessels appeared more prominent but not increased in number.

Dermoscopy versus histopathology

Dermoscopic and histological findings are reported in Table S2. A very strong positive correlation was observed

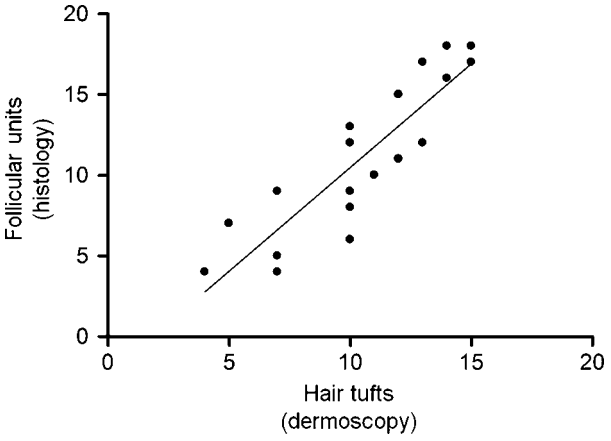

Figure 3. Correlation of the number of hair tufts located next to follicular ostia based on dermoscopy (*x*-axis) with number of follicular units counted with histology (*y*-axis). The regression line is shown.

for the total number of hair tufts next to follicular ostia based on dermoscopy and the total number of follicular units per examined area counted with histological examination (rho = 0.898; 95% CI = 0.750–0.961; $P < 0.001$). Also, the total count of hairs per hair tuft at dermoscopy correlated with the total number of hairs per follicular unit identified with histological examination (rho = 0.868; 95% CI = 0.683–0.948; $P < 0.001$) (Figure 3). A fair agreement was observed between dermoscopic and histopathological findings for the analysis of follicular hyperkeratosis ($\kappa = 0.333$; 95% CI = 0.013–0.679) with 12 of 20 (60%) agreements, vascularization ($\kappa = 0.200$; 95% CI = 0.120–0.520) with nine of 20 (45%) agreements and pigment ($\kappa = 0.294$; 95% CI=0.032–0.556) with 11 of 20 (55%) agreements.

Discussion

In this study dermoscopic findings in dogs affected by pattern alopecia have been characterized for the first time, highlighting the value of dermoscopy as an adjunctive technique for cutaneous clinical examination.

Canine pattern alopecia is a relatively common but poorly studied skin disorder somehow similar to, but also clearly different from, human androgenetic alopecia.[18] Fine hairs referred to as miniaturized hairs represent the hallmark clinical presentation of the disorder in people. However, to the best of the authors' knowledge, *in vivo* measurement of hair shaft thickness based on dermoscopy has not been performed before in dogs. In this study, the first hair parameter measured dermoscopically was the median hair tuft thickness diameter next to follicular ostia, which was shown to be smaller in all affected dogs compared with controls. This result is not surprising if we consider that the relative thinning of hairs is the most striking feature of the disease. Of note, however, differences between breeds and within the same breed were detected, dependent on other hair parameters accounted for. For example, in affected dachshunds the median ratio between the number of secondary hairs:primary hairs was shown to be higher in diseased animals than in controls in all the skin regions evaluated. The periaural region demonstrated the largest number of secondary hairs. Moreover, within the group of dachshund dog controls, the periaural region was demonstrated as having the smallest median diameter of primary hairs, indicating that thinning of hairs in this region may be considered as a normal feature in this breed. In Italian greyhounds, the ventral neck region was described as affected mainly by thinning hairs, and this finding indicates the relevance of this region in distinguishing affected from healthy dogs. In miniature pinschers, secondary hairs were smaller in affected dogs than in control dogs, mostly in the convex pinnae, ventral neck and chest areas, whereas in control dogs the median number of secondary hairs:primary hairs ratio was higher in the convex pinnae. All of these results taken together reveal that hair shaft thinning in canine pattern alopecia is a process that does not simultaneously affect all hairs of all regions, and that great variability exists between and within affected dog breeds. This variability may be the result of artificial selection pressure for extremely fine hair coats sought by breeders who often attempt to manipulate the appearance of a dog, thereby predisposing it to this presumptively genetic alopecia.[19]

In order to provide both qualitative and quantitative diagnostic follicular information, transverse sections of skin biopsy specimens were used in this study, as in human studies.[20,21] Some key information such as follicular counts was easily assessed and histological findings were shown to positively correlate with dermoscopic calculations of hair parameters. However, accurate determination of growth stages of the hair cycle was not possible on transverse sections due to the absence of the entire length of the hair follicle including site, shape and depth of the hair inferior portion and, specifically, of the bulb. Therefore, longitudinal sections may continue to provide the best morphological and spatial information to assess specific growth stages of the hair cycle in dogs.

In order to detect other dermoscopic features that could differentiate diseased dogs from controls, hair follicle openings, perifollicular and interfollicular skin surface, and vascular structures were examined dermoscopically and evaluated in conjunction with histological findings. Follicular ostia filled with light yellowish or brownish material were observed mostly in the ventral regions of dogs affected by pattern alopecia; histologically, this was related to a variable amount of keratin filling the follicular infudibulum. In humans, this dermoscopic finding, termed 'yellow dot', represents sebum mixed with variable amounts of keratin secreted by normal, active sebaceous glands through the miniaturized hair follicle.[7,9,11] Therefore, the result of this process is the accumulation of yellow material in the follicular infundibula. In spite of a fair agreement between dermoscopy and histopathology that might have been influenced by preparation of the biopsy site, our hypothesis is that a similar mechanism may occur in canine pattern alopecia.

Moreover, in some affected dogs, hairs with typical circular or spiraliform arrangement were observed dermoscopically, but no histopathological change was identified in relation to this dermoscopic feature. In humans, circle hairs are seen on the abdomen, buttocks, trunk and upper legs, as coiled hairs without any signs of follicular abnormality. Their pathogenesis is still unclear; some authors believe they correspond to remnants of the mammal undercoat, and others postulate that they are hairs with a smaller diameter making it difficult for the hairs to perforate the stratum corneum, resulting in coiling underneath the skin surface.[22,23] Based on this, our dermoscopic finding could provide an explanation, but further studies are necessary to better understand the pathogenesis of these hairs with this typical arrangement.

Agreement between dermoscopy and histopathology was demonstrated for pigment, mostly in dogs with dilute hair colour, probably as a result of abnormal melanin deposition in and around hair follicles. Pinpoint and large black spots corresponding to melanin clumping were dermoscopically observed on the interfollicular skin surface and this finding may open new insights into the application of dermoscopy for other skin disorders.

Finally, a honeycomb-like hyperpigmentation pattern, characterized by hyperchromic rings on the skin surface and resulting from solar exposure in thinning or completely balding areas, as demonstrated in humans, often coexisted as an additional feature in the periaural and caudal thigh regions.[10] However, these regions were not selected for histological correlation and this finding requires further studies in order to be better elucidated.

Cutaneous microvessels that arborize into thin red branches in a nonhomogeneous fashion were considered as nonspecific dermoscopic findings because they are also common in normal skin. Given that dermoscopy enables horizontal inspection of the skin, vessels that run parallel to the skin surface are visualized as lines, whereas those that run perpendicularly are generally viewed as dots, or even loops.[24,25] However, they are best evaluated when the pressure exerted by the dermoscope against the skin is low. High outside pressure may indeed reduce blood flow in cutaneous capillaries.[10] In this study, the lack of dermoscopic visualization of cutaneous blood vessels in some selected areas and the fair agreement demonstrated with histopathology may have resulted from excessive pressure applied to the skin with the dermoscopy instrument. Translucent ultrasound gel that allows gentler application of the lens against the skin in order to better visualize blood vessels could be used in future studies.

In summary, the results of this study suggest that although pattern alopecia is a visually striking disease, dermoscopy provides additional information beyond that obtained by evaluating the lesions through a dermatological examination. Besides hair thinning, new dermoscopic features have been identified offering the clinician a novel way in which to uncover clinical aspects of hair disorders. In the future, dermoscopy may be of benefit in further studies to differentiate various hair disease states.

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Supporting Information

Additional Supporting Information may be found in the online version of this text at: http://onlinelibrary.wiley.com/doi/10.1111/vde.12359/full

Table S1. Results of dermoscopic hair parameters in both affected and control dogs.
Table S2. Dermoscopic histological correlation of quantitative and qualitative parameters.

Part 7

Workshop Reports

Co-editors:
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Dr Kinga Gortel, Lake Country Veterinary Specialist Hospital, Lake Country, BC, Canada

Vaccines for canine leishmaniasis

C. Noli[1] (Chairperson) and A. Fondati[2] (Secretary)

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Introduction on vaccines in leishmaniasis (C. Noli)

Chiara Noli (*Italy*) welcomed the attendants to the workshop and opened it by briefly explaining that canine leishmaniasis (CanL) is a severe, chronic, vector-borne, protozoan disease which cannot be easily cured with current therapies. In endemic areas it is estimated that approximately 30% of dogs are seropositive and some will eventually become clinically ill. A vaccine capable of preventing the development of the disease after the animal has been infected would be highly desirable. It would protect vaccinated dogs and, through reducing disease incidence, also decrease infection rates in non-vaccinated dogs and humans. To be effective the vaccine should induce a strong and long-lasting Th1-mediated immune response. Different antigens and adjuvants, as well as different combinations of antigens and adjuvants, have been investigated during the past 15 years. They have led to the development of three generations of vaccines, based on antigen type:

1. attenuated or killed *Leishmania* parasites,
2. purified or recombinant *Leishmania* proteins,
3. DNA-encoding *Leishmania* or salivary sandfly proteins.

Different types of antigen-delivery system have also been investigated, including liposomes, nanomaterials and electroporation. However, due to difficulties in the production of *Leishmania* vaccines, only the following products have reached the market thus far: Leish-Tec® (Hertape Calier Saúde Animal S.A., Juatuba, Brazil) and Leishmune® (Fort Dodge Saúde Animal Ltd, Campinas, Brazil) in Latin America and CaniLeish® (Virbac S.A., Carros, France) in Europe.[a] Leishmune® has been removed from the market and only Leish-Tec® is available in Latin America. Unfortunately, none of these vaccines offers 100% protection against the disease. Based on published data, the overall absolute risk reduction in progression to symptomatic active infection for *Leishmania* vaccines ranges from 0.2 to 0.54 and the clinical efficacy ranges from 57 to 80%. CaniLeish® was released in Europe approximately 5 years ago and still there are many questions about its use. Hopefully, invited speakers will help to answer some of these questions, including, for example, the following.

1. When should we advise the owner to vaccinate his or her dog in endemic and non-endemic areas? (P. Bourdeau)
2. It is recommended by the CaniLeish® manufacturer that only healthy dogs, serologically negative for leishmaniasis should be vaccinated. How do we define a healthy dog? (M. Saridomichelakis)
3. Which cellular and humoral immune responses should we expect in a vaccinated dog? (L. Solano Gallego)
4. Is this vaccinated dog infected or ill? How can we confirm or rule out leishmaniasis in seropositive vaccinated dogs? (L. Ordeix)
5. *Leishmania* vaccine side effects: facts or urban legends? (L. Ferrer)

Chiara Noli thanked the attendees for participating and the Secretary (A. Fondati). She then introduced the next speaker.

Should my dog be vaccinated? Necessity of vaccination in endemic and non-endemic areas (P. Bourdeau)

Patrick Bourdeau (*France*) explained that the rationale for vaccination in CanL is based on the elicitation of a specific and protective Th1-mediated immune response. If infection occurs, the vaccine should protect against the disease or reduce its severity. In fact, the aim of the currently available vaccines is protection not against infection but against its consequences. For this reason, vaccination should be considered only in dogs already protected by insecticides having a strong, clearly demonstrated repellent activity against sandflies. Vaccinating such dogs would integrate the protective, albeit incomplete, effect of insecticides.

As for any vaccine, only *Leishmania* vaccines with efficacy recognized by the European Medicines Agency (EMA) should be used, manufacturer recommendations must be followed and the balance between benefit (efficacy) and risk (side effects) must be evaluated. When considering whether or not to vaccinate dogs, several factors have to be taken into account, including the duration of exposure to sandflies and the endemicity of the zone where the patient resides.

In some endemic (*syn*. enzootic) areas, exposure of dogs to infecting sandfly bites may occur up to 8 months per year. In these areas, aside from a few exceptions, promoting vaccination in the dog population is justified. On the other hand, non-endemic areas are characterized by the absence or a reduced number of vectors and a

low number of infected sick dogs. In these areas the risk of vectorial transmission of CanL is negligible and vaccination does not provide significant benefits. Special attention has to be paid to 'fringe' (*syn.* periendemic) areas such as northern Italy, northern Spain or south-western France, which are at the periphery of well-identified endemic zones. Here the disease is rapidly expanding through the increase of both sandfly populations and of dogs travelling to and from enzootic zones. In addition, *Leishmania* parasites are likely transferred from enzootic to 'fringe' areas by the movements of infected wild animals. Therefore, these areas should be considered equal to enzootic zones and vaccination should be recommended. In a collective prophylactic approach, if a high percentage of susceptible dogs were vaccinated, CanL expansion could be reduced. However, the global prophylactic benefit would be limited if wild animals play an important role in CanL expansion. For dogs travelling from non-endemic to endemic areas the choice to vaccinate will depend on duration and/or frequency of travels during the sandfly season. For short visits (e.g. 1 month), adequate protection can be afforded by insecticides alone and vaccination might be optional. On the other hand, vaccination should be considered with repeated or longer visits. It has been estimated by a mathematical model[1] that the combination of vaccination and insecticides in dogs imported from a non-endemic to an endemic area is more effective than either measure alone for preventing the development of active CanL infection.

Chiara Noli thanked Patrick Bourdeau for his contribution, opened the discussion and asked Patrick Bourdeau's advice about vaccination for those dogs living in non-endemic areas, such as Germany, spending, for instance, 1 week during the owner's vacation in a highly endemic area, such as Spain.

Patrick Bourdeau remarked that vaccination of dogs that are going to spend up to 2 months in endemic areas could be done but is probably not very useful. Two more aspects have to be taken into account before recommending vaccination: namely, financial aspects and the delay of vaccination effects. To be effective the vaccination protocol should be started many weeks before travelling. When financial constraints are a concern, insecticides should be the first choice. Nevertheless, vaccination should be promoted in endemic areas because, despite not being protective against infection, it is protective against disease. In addition, *Leishmania* vaccine reduces dogs' infectivity and, as a result, the risk of infection to other dogs living in the area.

Chiara Noli summarized the messages from the previous presentation. The use of insecticides with repellent activity should be mandatory for all dogs living in or travelling to endemic areas. Vaccination is recommended for dogs living in endemic regions and is also suggested for dogs that move from non-endemic to endemic regions for a long period of time. Provided that vaccination does not represent a financial problem, dogs spending short periods of time in endemic areas might also be vaccinated.

Patrick Bourdeau remarked that the combination of repellents and vaccine should also be promoted in dogs living in periendemic areas, in order to reduce the rapidity of infection and disease expansion.

Alessandra Fondati (*Italy*) asked if pyrethroids can be considered true repellents; in other words, whether or not pyrethroids prevent sandfly contact with the skin of treated dogs.

Patrick Bourdeau explained that pyrethroids do not possess a 'true' repellent activity; that is, they do not keep insects at a distance. Sandflies do contact the dogs' skin but they do not bite because they die rapidly or are repelled by contact with pyrethroids. Pure repellents have no residual effect and, so far, no marketed veterinary product has true repellent activity. While companies state that pyrethroids have an anti-feeding effect on sandflies, this lack of feeding is the result of a rapid killing effect combined with a repellent effect that prevents bites.

Laura Ordeix (*Spain*) asked for information about the length of the interval between the beginning of the vaccination protocol and travel to endemic regions.

Patrick Bourdeau replied that vaccine protection is complete 1 month after the last injection, when a Th1 cell response has developed, according to experimental studies. Therefore, the vaccination schedule should be completed 1 month before exposure.

Alessandra Fondati asked whether it would be appropriate to personalize vaccine recommendations depending, for example, on a dog's breed, age or lifestyle.

Patrick Bourdeau commented that data on the variability of vaccination efficacy with dog breeds are lacking. However, there is likely to be individual variation of vaccination responses.

Guadalupe Miró (*Spain*) pointed out that tailoring the vaccination to different factors, for example a dog's lifestyle, time of exposure to sandflies and other factors, is very important. Recommendations for a hunting dog should differ from those for a Chihuahua living in a carrying bag.

Chiara Noli introduced and welcomed the next speaker and highlighted that given the high prevalence of infection in endemic areas, deciding which dogs are truly healthy – that is, suitable for vaccination – can be challenging.

Can I vaccinate this dog? Is this dog healthy enough to be vaccinated? Criteria to decide if a dog is really healthy (M. Saridomichelakis)

Manolis Saridomichelakis (*Greece*) began by stating that at the time of vaccination against an infectious agent, it is usually necessary to ascertain that the dog is not infected by that agent. However, in endemic areas, many seemingly healthy dogs that are candidates for vaccination against CanL are actually infected by *Leishmania*

infantum. Subsequently, even if the vaccination schedule starts early in life, it is probable that a large number of infected dogs will be vaccinated against *L. infantum*. To vaccinate dogs, clinicians should confirm that they are clinically healthy (do not currently present mild CanL) and they are not progressing to develop the disease in the near future (i.e. they are not in the prepatent period of CanL). Careful physical examination is the first step to decide if a dog is really healthy and thus whether it can be vaccinated. It is advisable to pay special attention to detecting subtle clinical signs of CanL, including mild peripheral lymphadenomegaly, papular dermatitis or early exfoliative dermatitis. Documentation of seronegativity – that is, lack of *Leishmania*-specific circulating IgG antibodies – is also necessary before vaccination. Many infected, clinically healthy seropositive dogs are in the process of developing clinically overt CanL. It is known that in seropositive dogs histologic lesions of various tissues and organs, including skin, masticatory muscles, intestine, liver, joints and kidneys, develop before the ensuing clinical signs and laboratory abnormalities. It is worth highlighting that the results of serological tests, especially in dogs with low antibody levels, depend on the accuracy, sensitivity and specificity of the test. Consequently, some dogs in the process of developing the disease might test seronegative despite having low levels of circulating antibodies and be accidentally vaccinated. There are few data on the effect of vaccination in these dogs. The use of Leishmune® as immunotherapy in seropositive dogs with CanL was associated with some benefits without obvious side effects. Similar data are not available for CaniLeish®.

Chiara Noli thanked Manolis Saridomichelakis and commented that this presentation raised some questions. It is not known whether vaccination of an infected dog might help to clear the infection or, instead, might be harmful.

Alessandra Fondati asked for advice about vaccination in non-infected dogs suffering from concurrent cutaneous or internal organ disease.

Manolis Saridomichelakis replied that normally only healthy animals should be vaccinated, similar to other canine and feline vaccines. It is not known what will happen if sick dogs are vaccinated against CanL; additionally, the efficacy of the vaccination might be compromised. Side effects are not expected but no data are available.

Chiara Noli introduced the next speaker.

Which antibody and cellular immunity responses should be expected in a dog vaccinated against leishmaniasis? (L. Solano Gallego)

Laia Solano Gallego (*Spain*) explained that vaccines against canine *L. infantum* infection should elicit protective humoral and cellular immune responses. Limited knowledge is available about the complex dog–parasite interactions; however, it is well known that a predominant Th1-like response is capable of controlling *L. infantum* infection in dogs. Therefore, a vaccine should elicit a Th1-like response. Considerations regarding the immune response induced by the vaccination include antigen recognition, kinetics of antibody- and cell-mediated responses, differences in assay diagnostic performance and individual variability among dogs. Limited studies are available regarding the humoral and cellular immune responses after vaccination in dogs living in endemic areas. Antibodies reactive with *Leishmania* antigen have been reported in experimental healthy dogs to peak 2 weeks after completion of the primary vaccination course with CaniLeish®, which consists of three injections 3 weeks apart in dogs at least 6 months of age, and to persist for at least 4–6 months. Detection of antibodies persisting after vaccination likely depends on the sensitivity of the serological technique. The sensitivity of rapid serological tests is lower compared to that reported for quantitative techniques such as enzyme-linked immunosorbent assay (ELISA) and immunofluorescence antibody test (IFAT). These latter techniques are capable of detecting antibodies elicited by vaccination, whereas Speed Leish K® (Virbac BVT, La Seyne-sur-Mer, France), the test also used to screen dogs prior to vaccination, does not commonly detect these antibodies. No data are currently available regarding the antibody peak and persistence after the annual booster with CaniLeish®. The specific *L. infantum* T-cell response assessed by lymphocyte proliferation and/or interferon-γ production develops 3 weeks after the completion of the primary course and has at least 1 year's duration. There are limited studies on the Th1-cell-mediated immune response to vaccination. However, it might be hypothesized that in a large-scale study on dogs of various breeds, the T-cell-mediated immune response to vaccination would not be as homogenous as in experimental beagle dogs and that it might include non- or low-responders. Future research should be directed at the development of new techniques for the discrimination of humoral and cell-mediated immune responses due to vaccination from those resulting from natural infection. These tests would be essential from a diagnostic point of view and would allow for a better understanding of the effect of vaccinating dogs against leishmaniasis in endemic areas.

Chiara Noli thanked Laia Solano Gallego and asked the audience for comments or questions.

Manolis Saridomichelakis commented that our current knowledge on antibody kinetics after the primary course of vaccination is based on data from healthy laboratory animals living in a sandfly-free environment. It is likely that the situation will be different in dogs living in endemic areas and exposed to bites from infected and non-infected sandflies before and after the vaccination.

Laia Solano Gallego agreed and added that there are only limited studies on antibody kinetics after either a booster or the primary course of vaccination. Personal studies showed that in the majority of dogs the maximum peak of antibodies is reached 2 weeks after the third dose of a primary course of CaniLeish® and is followed by a decrease. Nevertheless, there are exceptions.

Chiara Noli asked if *Leishmania* parasites continuously inoculated by infecting sandflies in vaccinated dogs living in endemic areas might eventually act as a booster, keeping the antibody levels high.

Laia Solano Gallego responded that this is not known and might depend on the susceptibility of the dog. A very resistant dog might produce antibodies only after vaccination.

Chiara Noli introduced the next speaker.

Problems with confirming or ruling out leishmaniasis in vaccinated dogs (L. Ordeix)

Laura Ordeix (*Spain*) presented the preliminary results of a descriptive study on 17 dogs from an endemic area that developed leishmaniasis after CaniLeish® vaccination. Clinical and clinico-pathological alterations were similar to those observed in non-vaccinated dogs and included ulcerative and nodular cutaneous lesions, lymphadenomegaly, mild-to-moderate non-regenerative anaemia, hypergammaglobulinaemia, proteinuria and renal azotemia. Most dogs were diagnosed several months after vaccination, suggesting that they were previously infected. Diagnosing CanL in vaccinated dogs can be challenging as serological tests – that is, IFAT and ELISA – are not normally helpful because they detect vaccine-elicited antibodies. Demonstration of *Leishmania* in diseased tissues through cytology, histopathology, immunohistochemistry or molecular techniques is essential to confirm the disease in these patients. However, the detection of parasites in lymph nodes, bone marrow or blood does not allow one to directly correlate the infection with the cutaneous disease. Only a favourable response to antileishmanial treatment allows a causal role to be attributed to the parasite. After the presentation of two clinical cases, Laura Ordeix concluded that a history of *Leishmania* vaccination does not allow CanL to be ruled out, and that the disease should be included in the list of differential diagnoses for vaccinated dogs. Parasite detection in typical skin lesions through cytology, histology or immunohistochemistry confirms the disease. If the results are negative, RT-PCR should be performed on skin samples. Detection of the parasite or its DNA in organs other than the skin (e.g. blood, bone marrow or lymph node) demonstrates the infection but not the causal relationship between the parasite and skin lesions.

Chiara Noli asked about the possibility of a different clinical presentation of CanL in vaccinated versus unvaccinated dogs.

Laura Ordeix recalled that while no differences were observed in the preliminary study, this was an uncontrolled study.

Gaetano Oliva (*Italy*) stated that, according to the study he authored,[2] less severe renal damage was observed in vaccinated experimental beagle dogs compared to untreated controls.

Laia Solano Gallego added that in the preliminary study shown by Laura Ordeix, most dogs presented clinical signs of leishmaniasis very early after vaccination. This suggests that at least some dogs were vaccinated when already infected. Therefore it is more likely a failure to identify infected dogs, possibly related to the low sensitivity of diagnostic tests (e.g. rapid qualitative serological tests) rather than a failure of vaccine protection.

Chiara Noli remarked that this would also help to explain why no clinical differences between vaccinated dogs and dogs with natural infection were noticed in this preliminary study. On the other hand, Gaetano Oliva's study showed a difference because dogs were infected after vaccination. Chiara Noli then asked Laura Ordeix whether the results of the study she presented had been stratified according to the time span between vaccination and development of CanL.

Laura Ordeix responded that the number of dogs was too small to analyse results in this way.

Manolis Saridomichelakis asked if the vaccinated dogs that developed CanL had received immunosuppressive treatment for other diseases.

Laura Ordeix replied that no dogs were treated with immunosuppressive drugs.

Patrick Bourdeau commented that any infected dog can have *Leishmania* in the skin; thus the presence of parasites in the skin by itself does not demonstrate their causal role. In infected dogs *Leishmania* parasites circulate inside macrophages and they can concentrate in lesions primarily unrelated with leishmaniasis. The only way to attribute a causal role to parasites is the response to specific antileishmanial treatment.

Laura Ordeix partly disagreed with the comment from Patrick Bourdeau and explained that there might be a difference depending on the clinical picture of the dog. If *Leishmania* is demonstrated in the skin of a dog living in an endemic area and showing typical clinical signs and suggestive/compatible cytological and/or histopathological alterations, a causal role of parasites is likely. However, when dermatological signs are atypical and many other causes of skin disease have to be considered, response to antileishmanial treatment is more important for the diagnosis.

Guadalupe Miró shared that she saw two cases of lymphoma in dogs 2–3 years after vaccination and asked whether anyone had a similar experience, or if other diseases, apart from leishmaniasis, had been observed in vaccinated dogs.

Chiara Noli and the attendants did not report a similar experience. Nevertheless, Chiara Noli added that since vaccination for CanL is more widespread in Spain than in Italy, the odds that vaccinated dogs develop concurrent diseases is high.

Alessandra Fondati asked for suggestions on the most suitable tests to rule out *Leishmania* infection in clinically healthy recently vaccinated dogs, for example those that have to be treated with immunosuppressive therapy.

Laura Ordeix recommended blood work, looking for clinico-pathological alterations and both quantitative and qualitative serological tests if vaccination dates back to less than 6 months. Depending on the results, a very sensitive test to diagnose infection, for example RT-PCR on bone marrow, could be also performed.

Manolis Saridomichelakis stated that if *Leishmania* infection had to be excluded in every dog living in an endemic region and needing immunosuppressive therapy, then, considering the high prevalence of infected dogs and the chance that non-infected dogs will become infected during the course of the treatment, immunosuppressive drugs should not be used at all in these areas. Fortunately, it seems that only a minority of infected dogs under long-term immunosuppressive treatment develop CanL.

Chiara Noli thanked Laura Ordeix and introduced the next speaker.

Adverse reactions of the vaccine against CanL: facts or urban legends? (L. Ferrer)

Lluis Ferrer (*USA*) reported that his sources of information on adverse reactions to CaniLeish® were represented by pharmacovigilance data from the EMA and the French agency Agence Nationale de Sécurité du Médicament et des Produits de Santé (ASMPS), by a survey performed in seven large veterinary hospitals in highly endemic areas in Spain (including files from more than 4000 vaccinated dogs) and by data from the Virbac Pharmacovigilance Department. Information from social networking sites was also consulted. Data referred only to short-term adverse effects. Side effects reported in more than 10% of cases included local pain and injection-site inflammation, and fever. According to data from veterinary hospitals, the frequency of these side effects ranged from 5 to 25% of cases, whereas according to the EMA local reactions accounted for 0.079% of cases. They appeared to be more common in small dogs, were probably associated with the adjuvants and resolved spontaneously in 2–15 days. They could easily be prevented by administering non-steroidal anti-inflammatory drugs. Based on data from pharmacovigilance, uncommon side effects reported in 0.1–1% of dogs included anorexia, emesis, lethargy and localized-to-generalized transient urticarial reactions (which can be prevented by antihistamine administration). Very rare side effects described in fewer than 0.01% of dogs were ulcerative necrotizing dermatitis, panniculitis and vasculitis at the injection site. Severe and fatal anaphylactic reactions have thus far only been reported in social networks. In conclusion, side effects are similar to those reported for other vaccines. The only difference is the greater frequency of injection-site reactions, likely associated with the type of adjuvants included in the vaccines. Owners and veterinarians must be informed and the preventative use of anti-inflammatory drugs might be recommended. In any case, the reported short-term side effects should not be a reason to avoid vaccinating dogs. Long-term side effects, if any, are currently unknown.

Chiara Noli thanked Lluis Ferrer for reassurances about the lack of serious side effects of CaniLeish® and asked for questions or comments.

Guadalupe Miró pointed out that data from Virbac pharmacovigilance were valuable; however, they were lower than data obtained directly from practitioners and clinics because of the reluctance of veterinarians to officially report adverse reactions to drugs. The importance of pharmacovigilance should be stressed among veterinarians.

Chiara Noli thanked all the speakers, Alessandra Fondati and the audience for their attendance and contributions, and closed the workshop.

Note

[a]After the workshop took place (June 2016), a new vaccine called Letifend® (Laboratorios LETI, Madrid, Spain) was authorized in Europe.

References

1. EFSA Panel Animal Health and Welfare. Scientific opinion on canine leishmaniasis. *EFSA J* 2015; 13: 4075.
2. Oliva G, Nieto J, Foglia Manzillo V et al. A randomised, double-blind, controlled efficacy trial of the LiESP/QA-21 vaccine in naïve dogs exposed to two *Leishmania infantum* transmission seasons. *PLoS Negl Trop Dis* 2014; 8: e3408.

Pros and cons of oclacitinib therapy

W.S. Rosenkrantz[1] (Chairperson) and A.S. Bourgeois[2] (Secretary)

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Wayne Rosenkrantz (*USA*) opened the workshop by explaining the use of a polling-based programme called Turning Point Technology. This allows each individual audience member to answer questions anonymously and presents the collective results for discussion.

To demonstrate use, a general question was asked: *How familiar are you with Janus kinase (JAK) inhibitors?*

1 Never heard of them (2%)
2 I have a very basic understanding (17%)
3 Only heard about them due to the Apoquel® release (55%)
4 I am tired of hearing about them (26%)

Wayne Rosenkrantz recognized that most of the group were familiar with JAK inhibitors. The JAK enzymes are associated with cytokine receptors on the surface of cells. The Janus domain is composed of two domains located at the interface of the cell membrane. One domain stimulates kinase activity and one downregulates activity. These domains can induce a variety of immune reactions. Inhibiting JAK can reduce immune responses, particularly those associated with allergic reactions.

A schematic was shown illustrating the pathway of JAK activation and the interactions of cytokines with the receptors. Activation of JAK leads to phosphorylation of receptor chains and translocation to the nucleus. JAK-STAT (signal transducer and activator of transcription) inhibitors function to block this pathway. Oclacitinib (Apoquel®; Zoetis Inc., Parsippany, NJ, USA) inhibits primarily JAK-1 and has minimal effect on JAK-3. At recommended dosages there is no effect on JAK-2 or tyrosine kinase. Oclacitinib's primary mode of action in reducing itching is inhibiting production of interleukin (IL)-31. However, production of other cytokines (IL-2, 4, 6 and 13) is also affected. While the JAK-1 pathway is primarily affected, when dosed at 0.6 mg/kg twice daily, the peak concentration of the drug in some dogs may also inhibit JAK-2 pathways. This may be why, in rare instances, oclacitinib-treated dogs may exhibit transient leukopenia and neutropenia.

The audience was polled with a second question, *How would you classify your current usage of oclacitinib?*

1 Have never used it (18%)
2 Treated 25–50 cases (37%)
3 Treated 50–100 cases (8%)
4 Treated 100–200 cases (15%)
5 Treated more than 200 cases (22%)

Wayne Rosenkrantz recognized that the attendees have treated a significant number of cases. Only a small percentage of the audience had never used the drug and is likely participating in the workshop to obtain more information regarding how to use this drug.

The next question was *How effective is oclacitinib in your practice for controlling pruritus to an acceptable level [using a definition of 50% reduction of the original pruritus visual analogue scale (PVAS) scoring]?*

1 <40% (3%)
2 40–50% (6%)
3 50–60% (2%)
4 60–70% (21%)
5 >70% (68%)

Wayne Rosenkrantz noted that participants are finding good control of itching with use of oclacitinib when defined as a reduction of 50% from the original PVAS scoring.

The follow-up question was *How effective is oclacitinib in your practice for controlling pruritus to an acceptable level using the definition of <2 on PVAS?*

1 <40% (7%)
2 40–50% (0%)
3 50–60% (20%)
4 60–70% (27%)
5 >70% (46%)

Wayne Rosenkrantz expected a lower percentage of complete control.

The floor was then opened to feedback and questions from participants regarding clinical experience with oclacitinib improving pruritus.

Sue Paterson (*UK*) mentioned that she often finds good control of patient pruritus. However, she noted that patients frequently experience a loss of control due to *Malassezia* and bacterial overgrowth.

Valerie Fadok (*USA*) disclosed that she works for Zoetis Inc. She believes a confounding factor is whether dogs are on immunotherapy or not. In her experience, dogs on concurrent immunotherapy have a better response to oclacitinib.

Paul Bloom (*USA*) shared his clinic's results in 444 cases. There was a 22% failure rate defined as clients that did not feel dogs achieved a normal level of pruritus. When the dose was split throughout the day 1.5% of patients

improved. Clinical improvement was also dependent on the dose. If the higher dose of 0.6 mg/kg daily was used there was better control compared to dogs at the lower end of the dosage range (0.4 mg/kg daily).

Candace Sousa (*USA*) disclosed that she works for Zoetis Inc. She stated that the majority of cases studied in the USA were chronic atopic patients. This may be why success rates in dermatology referral practices are lower than in general practice. Acute or short-term use tends to show better success rates than chronic use or when disease has been present for several years.

Wayne Rosenkrantz stated that oclacitinib has a very rapid onset of activity compared to corticosteroids and that its effect is much more rapid than that of ciclosporin. There are several references supporting oclacitinib being highly effective in treating pruritus. The Cosgrove study[1] showed a greater than 50% reduction in pruritus within 90 days in 63.4% of cases. The range of dogs achieving a PVAS <2 was 38.5–48.5%. There was also a dramatic improvement in quality of life in many cases.

Wayne Rosenkrantz went on to discuss details from a random selection of 175 cases from an oclacitinib-treated population of more than 1000 cases at the Animal Dermatology Clinic's Tustin (California, USA) location (ADC-T). These cases had been on oclacitinib for a minimum of 6 months and did not include cases that dropped out due to perceived early failure or inconsistent use.

The study was a retrospective evaluation. The results showed that 56/175 (32%) had an excellent response, with owners considering treated dogs to be 'normal'. Some of these cases were on modified dosing protocols (such as 0.3 mg/kg twice daily). Some cases 11/56 (19.6%) were on concurrent immunotherapy. Moderate control was seen in 102/175 (58.3%) cases. Some patients in this group received modified or increased dose protocols. Concurrent corticosteroids with oclacitinib were used in 23/102 (22.5%) of the moderate responders. Within the moderate responder group, 24/102 (23.5%) were on concurrent immunotherapy. A smaller number of cases, 16/175 (9.1%), had limited to no response to oclacitinib and were transitioned to other treatment options after 6 months of treatment.

Photos were shown of dogs with various responses to Apoquel®.

The recommended dose of oclacitinib (0.4–0.6 mg/kg twice daily for 14 days and then reduced to once daily) was discussed. Options for dose adjustments were also further discussed. This led to a question to the participants regarding dosage adjustments: *When response is limited, what type of dose adjustments do you make?*

1. No adjustments; follow the recommended label dosing and give time (26%)
2. Divide daily dose into twice daily 25% of the time (19%)
3. Divide daily dose into twice daily 50% of the time (24%)
4. Need to keep at induction dose twice daily >10% of the time (19%)
5. Other modifications (12%)

Wayne Rosenkrantz recognized that many people use modified dosages and protocols. Further discussion on this followed.

Margreet Vroom (*The Netherlands*) stated that it is not always necessary to start with twice-daily oclacitinib dosing even for induction. She often starts with once-daily dosing initially and still sees a rapid response. She increases to twice-daily dosing after a few days if once daily is not effective. Owners also often make dose adjustments on their own, which can be worrisome.

Sophie Gilbert (*Canada*) mentioned that changing the time of day the drug is given can also be beneficial and should correspond to the time of the day that the dog exhibits the most intense pruritus. In addition, if patients are at the lower end of the dosing (0.4 mg/kg), increasing to the higher end (0.6 mg/kg) can help. Making changes may be limited by the availability of tablet sizes.

Jerome Ngo (*Belgium*) stated owners do not seem satisfied when dosing is changed from twice daily to once daily. He often starts at once-daily dosing to avoid this.

Robert Dixon (*Australia*) mentioned some dogs will do better receiving a higher dose in the morning and a lower dose in the evening. This could be due to the short half-life of the drug. This type of modification can be used to avoid using the full 0.6 mg/kg twice-daily dosing beyond the 14-day induction.

Wayne Rosenkrantz stated that he likes to try to start at 0.6 mg/kg twice daily for his patients and typically calculates the dosing to make sure patients start at the higher end of the dosing recommendations. He asked if the group uses the dosing chart or if they actually calculate the dosage based on body weight to get a more exact mg/kg dosing. About a third of the attendees stated they strictly use the provided dosing chart when starting dogs on oclacitinib. Approximately two-thirds calculate the dose themselves.

Ed Rosser (*USA*) stated he has tried many different modifications but in about 10% of his cases he cannot reduce below 0.6 mg/kg twice-daily dosing for maintenance.

Wayne Rosenkrantz next asked the following question: *What type of dose adjustments do you make in a patient with a good response to oclacitinib?*

1. Reduce to every other day dosing in <5% of the cases (48%)
2. Reduce to every other day dosing in 5–10% of the cases (25%)
3. Reduce to every other day dosing in 10–15% of the cases (5%)
4. Give only as needed (22%)

It appears that only a limited percentage of cases can successfully taper to dosing every other day. The audience was invited to share experiences regarding dose reductions.

Claudia Nett (*Switzerland*) only uses the oclacitinib as needed in patients. She finds there are dogs in which you can give the drug for 3–4 days in a row and then just use as needed.

Valerie Fadok mentioned that she gives oclacitinib as needed more in patients that are on immunotherapy. She uses the drug only when patients flare when they are on concurrent immunotherapy.

Wayne Rosenkrantz discussed his experience of dosing and frequency in 175 cases evaluated in a retrospective study at ADC-T: 77/175 (48%) could be maintained on once-daily dosing; 43/175 (24.5%) were maintained by splitting the 0.4–0.6 mg/kg dose into twice-daily increments; 19/175 (10.8%) received 0.4–0.6 mg/kg dosing in the morning and 0.2–0.3 mg/kg dosing in the evening; and 13/175 (7.4%) had to be maintained at 0.4–0.6 mg/kg twice daily. The latter is obviously off label and may increase the risk of side effects.

The next two questions related to comments that oclacitinib loses effectiveness over time. First, *How often do you see this?*

1 Not seen (31%)
2 10% of cases (26%)
3 20% of cases (26%)
4 30% of cases (6%)
5 Other (11%)

Second, *In those cases where it (oclacitinib) has lost its effectiveness, why did it occur?*

1 Compliance (2%)
2 Drug just quit working (12%)
3 Pyoderma (24%)
4 *Malassezia* (5%)
5 Fleas (4%)
6 Food allergy (5%)
7 Other (48%)

Wayne Rosenkrantz recognized that most of the audience agree that the drug did not just stop working; in most cases, some other confounding or flare factor is present. A picture was shown of a dog that had developed pyoderma while on Apoquel®. The pyoderma created the lack of control.

Sue Paterson asked for the audience's opinions regarding discontinuing oclacitinib while treating pyoderma.

Jackie Campbell (*USA*) stated it was important to manage all factors contributing to skin disease in a patient, including pyoderma. Adding antibiotics while continuing to treat the allergies with oclacitinib will help the patient. There are also certain times of the year when oclacitinib is needed more than others.

Ursula Mayer (*Germany*) agreed that successful treatment of a dog with allergies goes beyond just treating the pruritus. It includes reducing frequency of infections. If infections are continuing in allergy patients, then their allergies are not fully controlled with oclacitinib alone. Additional anti-allergic therapies may be needed.

Alberto Cordero (*Mexico*) mentioned that he does not think ear disease responds as well to oclacitinib when compared to other available medications such as corticosteroids.

Wayne Rosenkrantz stated the next topic would include adverse events and then asked a series of questions comparing oclacitinib to other therapies in regards to adverse events.

First, *What is your perception of adverse events with oclacitinib compared to ciclosporin?*

1 It is very low, less than ciclosporin (86%)
2 It is similar to ciclosporin (11%)
3 It is higher than ciclosporin (3%)

This is an expected finding due to the higher incidence of gastrointestinal side effects associated with ciclosporin.

Second, *What is your perception of adverse events with oclacitinib compared to glucocorticoids?*

1 It is very low, less than steroids (100%)
2 It is similar to steroids (0%)
3 It is higher than steroids (0%)

Finally, *What is your perception of adverse events with oclacitinib compared to allergen-specific immunotherapy (ASIT)?*

1 It is very low, less than ASIT (18%)
2 It is similar to ASIT (50%)
3 It is higher than ASIT (32%)

Wayne Rosenkrantz stated that it is a bit unusual to see such high numbers reporting adverse events in ASIT, which is generally considered the safest of all options. Regarding oclacitinib, when you look at the current literature the reports of adverse events are relatively low. Looking at the previously mentioned Cosgrove study,[1] urinary tract infections, pyoderma, otitis, weight gain, vomiting and diarrhoea were the most commonly noted adverse events. Less common adverse events reported in the literature or anecdotally include pneumonia, interdigital cysts, demodicosis, lethargy, tremors, haematological abnormalities, neoplasia, seizures and aggression.

Next, questions were used to survey the group on incidence of some of these adverse events.

What incidence of cystitis are you noticing?

1 Do not routinely check (53%)
2 <5% of cases (37%)
3 5–10% of cases (5%)
4 10–15% (0%)
5 >15% (5%)

Catherine Outerbridge (*USA*) wanted to further discuss reports of weight gain and reasons for it. She was wondering if it could be associated with the dogs that start out

extremely pruritic prior to using oclacitinib. Once the pruritus is improved, patients are not moving as much due to lack of scratching. She felt you could see weight gain in many of these patients no matter which therapy is used as long as the pruritus is controlled.

Wayne Rosenkrantz replied he does not necessarily think weight gain is related to reduced pruritic activity and plans to discuss other theories later in the workshop.

Ed Rosser mentioned complaints from clients regarding polyphagia.

Wayne Rosenkrantz asked participants to hold discussion of weight gain until after the discussion on cystitis and some of the other adverse effects.

Rod Rosychuk (*USA*) reported that a study of 43 dogs from Colorado State University on oclacitinib did not find any cases with cystitis.

Paul Bloom was curious about the definition of cystitis everybody was using. Specifically, he asked whether the patients were symptomatic or not when the term cystitis was being used.

Wayne Rosenkrantz stated that most doctors in his practices collect complete blood count (CBC), serum chemistry (CHEM) and urinalysis (UA) prior to starting therapy. Of the 175 cases followed in the ADC-T study, 11 dogs (6.3%) were diagnosed with cystitis. The presence of bacteria with white blood cells was the criterion used to diagnose cystitis. In addition, 24/175 (13.7%) developed 2+ proteinuria and 10/175 (5.7%) developed 3+ proteinuria. The dogs with 3+ proteinuria had a urine protein/creatinine (UPC) ratio performed and 3/10 (30%) of these cases had an elevated UPC ratio. Of these, one owner decided to discontinue therapy with oclacitinib and two owners decided to continue. All 10 dogs with 3+ proteinuria had negative urine cultures.

Sue Paterson commented that intertriginous dermatitis around the vulvar fold and preputial fold is a factor she believes is associated with a higher incidence of bacteriuria.

Paul Bloom was curious if the 6.3% of cystitis cases in the ADC-T study were symptomatic.

Wayne Rosenkrantz answered that some were; however, not all cases exhibited frequent urination or a stronger odour to their urine.

Paul Bloom wanted to know if there was follow-up with the cases mentioned having 3+ proteinuria in the ADC-T study.

Wayne Rosenkrantz stated cases were referred back to their primary care veterinarian and enalapril was prescribed for blood pressure issues in some cases. Only one-third of dogs with the proteinuria stayed on Apoquel® and the UPC ratio remained stable. He asked if anybody else had seen proteinuria associated with oclacitinib treatment.

Rod Rosychuk mentioned proteinuria was noted in some of the 43 cases at Colorado State University. However, one of the cases had lymphoma.

Valerie Fadok asked the internists how a 6% rate of occurrence would compare to normal age-matched dogs.

Rod Rosychuk stated it would be right where you would expect it.

Wayne Rosenkrantz then discussed the occurrence of pyoderma. In the same case review at ADC-T, 91/175 (52%) were treated two or more times for pyoderma during treatment with oclacitinib. It was recognized that 20/91 (21.9%) had a previous history of recurrent pyoderma. It is difficult to know whether oclacitinib contributed to the high incidence of pyoderma. Occurrence of otitis was also looked at and it also seemed high but no exact numbers were presented. He asked the audience their general impression of oclacitinib causing otitis.

Rod Rosychuk mentioned that the ears are examined on all patients at Colorado State University. He does not feel that cases with recurrent otitis are well controlled on oclacitinib. Concurrent topical therapy is often used for these cases.

Jon Plant (*USA*) commented that we need to be careful when we retrospectively look at some of the newer drugs because we often follow these cases much more closely compared to patients treated with other medications that have been available for years.

Wayne Rosenkrantz mentioned that it is hard to determine oclacitinib's ability to control otitis since this is such a common secondary issue with atopic dermatitis. However, it does not appear to work as well as other anti-inflammatory drugs for otitis cases.

Catherine Outerbridge agreed. She still uses topical corticosteroids for management of otitis in addition to oclacitinib. She believes oclacitinib is more of an antipruritic agent than an anti-inflammatory one.

Brett Wildermuth (*Germany*) has mixed opinions on the control of otitis with oclacitinib. Stenotic ears do not seem to improve as quickly with oclacitinib as with corticosteroids. However, some Labrador retrievers do seem to have less frequent episodes of *Malassezia* otitis while on oclacitinib.

Wayne Rosenkrantz agreed that he would use corticosteroids preferentially for proliferative ear disease.

Ed Rosser feels that pyoderma can worsen when oclacitinib is used concurrently for pruritus. Also, he has seen side effects such as borborgymus, bloating and abdominal discomfort in patients with a history of gastrointestinal disease.

Ursula Mayer commented that she does not think oclacitinib works well for treating interdigital granulomas.

Wayne Rosenkrantz agreed and stated that this was confirmed in some of the earlier studies and also in his clinical cases.

Margreet Vroom stated that she has two cases that became quite ill (lethargy, diarrhoea, etc.) on oclacitinib. These clinical signs resolved when the drug was discontinued and returned when the drug was restarted.

Paul Bloom has had dogs with a history of inflammatory bowel disease resolve their gastrointestinal disease when on oclacitinib.

Wayne Rosenkrantz mentioned that liver enzyme elevations were seen in his cases at ADC-T, with 47/175 (26.8%) having alkaline phosphatase (ALP) elevations. Most elevations were fairly mild. Some 23/175 (13.1%) of cases were on concurrent oral glucocorticoids and 12/23 (52.5%) of those cases had ALP elevations. When just evaluating the 47 cases of ALP elevations, 35 (74.4%) of the ALP elevations were not related to systemic steroid use. Also, 6/175 (3.4%) had alanine transaminase (ALT) elevations.

He reported that Allison Kirby at Animal Dermatology Clinic's Marina Del Rey location (California, USA) has had three dogs treated with oclacitinib diagnosed with biliary mucoceles. These patients started with elevated ALT and ALP values and then became extremely ill. All three cases were confirmed through abdominal ultrasound and had surgery. These cases have been reported to Zoetis Inc.

Many audience members agreed that they have seen liver enzyme elevations from oclacitinib. Nobody in the audience had seen a biliary mucocele as a possible side effect.

Rod Rosychuk asked if the dosing regimen was evaluated for these cases.

Wayne Rosenkrantz replied that there did not seem to be a correlation between the high dose (0.6 mg/kg daily) versus the lower end of the range (0.4 mg/kg daily).

Ed Rosser commented that he has five patients that have been monitored with ALP elevations from oclacitinib for over a year. Four of the cases had ALP in the 400 µg/dL range. One case had an ALP in the 900 µg/dL range. He monitors laboratory parameters every 3 months rather than every 6 months in these patients. These patients have no evidence of Cushing's disease. However, when an ALP panel was evaluated at the University of Illinois, 90% was the steroid-induced isoform.

Catherine Outerbridge was curious of the breed representations for the three previously diagnosed dogs with biliary mucocele. There are certain breeds that are predisposed.

Wayne Rosenkrantz did not have that information readily available but could easily look it up.

The next question was *How many cases of demodicosis have you seen related to oclacitinib?*

1 1 (69%)
2 2–3 (25%)
3 4–5 (0%)
4 5–10 (3%)
5 Greater than 10 (3%)

Candace Sousa mentioned there was no option to select zero cases in the above question.

Wayne Rosenkrantz recognized that it was an error on his part to not include zero. Attendees were asked to raise their hand if they had not seen any cases of demodicosis related to oclacitinib. Approximately 12/65 (18.5%) people raised their hands. In the ADC-T population, there were 6/175 (3.4%) that were diagnosed with demodicosis. However, 3/6 (50%) of those cases were treated with afoxolaner (Nexgard®; Merial Inc., Duluth, GA, USA) and continued with their oclacitinib, and the demodicosis has been in remission.

He shared a case example of one of these cases that continued on oclacitinib with afoxolaner. He asked if the audience had any comments.

Brett Wildermuth asked about the case example and if he was receiving once-daily or twice-daily dosing of oclacitinib.

Wayne Rosenkrantz stated that the case example was on once-daily dosing but was not sure of the exact dose although he typically tries to use the higher-end dosing at 0.6 mg/kg/day.

Margreet Vroom reported she uses oclacitinib in dogs that are pruritic even if demodectic mites are present.

Wayne Rosenkrantz asked how she was treating the *Demodex*.

Margreet Vroom stated that she treated with either afoxolaner or fluralaner (Bravecto®; Merck Animal Health, Summit, NJ, USA). She mentioned that many dogs develop demodicosis, so we cannot necessarily implicate oclacitinib as the cause.

Wayne Rosenkrantz recognized that the number of *Demodex* cases is low. However, it should be something we are aware of and should screen cases when lesions are compatible.

Ed Rosser stated that dogs he has seen with demodicosis from oclacitinib tend to be adult-onset and not juvenile.

Sue Paterson mentioned that she often starts patients on oclacitinib and fluralaner concurrently to prevent demodicosis.

Wayne Rosenkrantz then asked about weight gain. *Noticeable weight gain is seen in what percentage of your cases?*

1 None (30%)
2 <5% (45%)
3 5–10% (17%)
4 10–20% (5%)
5 >20% (3%)

Based on previous studies, weight gain seemed to occur more the longer dogs were on oclacitinib. In most studies a small percentage of treated dogs are affected (4%). Another study by Little and coworkers[2] reported 1.19% of dogs on ciclosporin and 3.35% of dogs on oclacitinib with weight gain.

In the Cosgrove study,[1] 10% average weight gain was seen; this is higher than other studies. Wayne Rosenkrantz has been quite interested in weight gain and has been looking into possible explanations. In most of his cases the dogs did not appear to be polyphagic. In the 175 cases evaluated at the ADC-T there was an average weight gain of 6.8%.

One theory discussed is the possibility of adipose tissue being affected by JAK-STAT inhibitors. There are dozens of adipose tissue cytokines and some use JAK receptors. JAK-STAT transcription factors impact various areas of adipocyte metabolism including insulin action, modulation of lipid stores, leptin levels and glucose homeostasis. Leptin regulates energy balance, inhibiting hunger. The JAK-STAT pathway is also involved in deposition of brown adipose tissue.

A study[3] revealed that some mice have a mutation in the leptin receptor gene that prevents activation of STAT-3; these mice become obese.

Valerie Fadok stated some studies have shown that blocking IL-6 leads to weight gain. This is being evaluated in human oncology patients. She agreed there are many adipose cytokines that use the JAK-STAT pathway.

Wayne Rosenkrantz next initiated a discussion regarding oclacitinib and neoplasia by asking the group *What are you telling your clients regarding oclacitinib and neoplasia?*

1 I do not tell them anything (20%)
2 There appears to be no known association with neoplasia (31%)
3 It is possible that it could create neoplasia (13%)
4 It is possible that it could exacerbate neoplasia (22%)
5 We just do not know so I would be careful using it (11%)
6 I would not use it in my dog (3%)

He went on to say that when reviewing the literature regarding JAK-STAT inhibitors and neoplasia in human medicine he found many reports showing benefits of using these drugs to treat neoplasia but very little discussion regarding it as a risk factor for neoplasia. One study[4] made the following summary statement, 'As Janus kinase inhibitors alter the immune response they increase the risk of serious infections. There is a possibility they may also increase the risk of cancer.'

Another study[5] evaluated tofacitinib, a JAK-STAT inhibitor used in humans for rheumatoid arthritis. This study evaluated 5671 cases and there was no increased risk or incidence of neoplasia in people treated with tofacitinib compared to other forms of therapy. The standardized incidence ratios for all malignancies and selected malignancies were within the expected range of patients with moderate-to-severe rheumatoid arthritis.

There has been some concern about papillomas associated with oclacitinib in dogs. Some members of the audience agreed they have seen this. Papillomas in pivotal laboratory studies in healthy dogs were not dose-related and were seen at one-, three- and five-times dosing.

Data shared in a previously sponsored Zoetis Inc. webinar by Cheryl London from the Ohio State University (USA) reviewed neoplasia cases from continuation studies. There were 239 dogs treated with oclacitinib for a mean of 372 days (range 1–610 days); of these, 16 dogs (6.6%), with an average age of 9 years, developed suspected or confirmed neoplasia. Twelve different types of tumour, including two grade II mast cell tumours (MCTs), were seen. Her conclusion was that the neoplasia rates in oclacitinib-treated dogs were not increased compared to an age-matched population.

Craig Griffin reviewed 179 cases seen at Animal Dermatology Clinic's San Diego location and four dogs (2.2%) developed masses that were not identified, three (1.7%) developed lymphoma and one (0.55%) developed prostate cancer. Again, these numbers are quite low and were seen in elderly dogs (range 8–16 years old).

Of the 175 cases at ADC-T, one dog (0.57%) developed a retroperitoneal hemangiosarcoma, one (0.57%) developed nerve sheath tumour, one (0.57%) developed a grade I MCT, one (0.57%) developed an undifferentiated lymphohistiocytic tumour, one (0.57%) developed lymphoma, one (0.57%) developed mammary carcinoma, one (0.57%) developed a ceruminous gland adenocarcinoma, one (0.57%) developed an adrenal adenoma and four dogs (2.28%) developed histiocytomas.

Wayne Rosencrantz concluded this topic discussion with some summary points given by Cheryl London from the Zoetis Inc. webinar: (1) when you review the incidence of neoplasia in the general population of dogs, cancer is the leading cause of death in dogs and 1/4 (25%) die of cancer, (2) the highest incidence is seen in dogs between 6 and 12 years of age with a peak of 10 years, (3) many breeds of dogs that are predisposed to neoplasia are also predisposed to atopic dermatitis and (4) controlled studies to date do not support a direct association with oclacitinib.

Despite this, there remain anecdotal reports on oncology and veterinary dermatology list serves regarding the concern of an increased risk for neoplasia.

Wayne Rosenkrantz next wanted to get feedback from the group regarding the types of monitoring of oclacitinib cases. Specifically, he does CBC, CHEM and UA prior to starting medication, 3 months after and then every 6 months on maintenance. There was some consensus on

similar monitoring from the group, although a small number of attendees did no monitoring.

The floor was then opened for final comments and feedback.

Ursula Mayer asked about experiences of oclacitinib use in cats.

Wayne Rosenkrantz stated he has treated 5 cats with oclacitinib at 1 mg/kg twice daily. One of the five had a complete response and two had a partial response. The partial responders also needed concurrent lower dose corticosteroids.

Many in the audience mentioned that their experiences were varied.

Ursula Mayer reported that she had three cats that had failed previous therapies but which had good results with oclacitinib.

Margreet Vroom had one of four cats respond well to oclacitinib.

Otto Fischer (*Austria*) was curious about combination therapy with oclacitinib and other medications.

Wayne Rosenkrantz mentioned several dogs that were on concurrent corticosteroids with oclacitinib in the ADC-T study were controlled with low doses of both medications. He has not noticed complications from using low doses of both medications, especially if just a short-term duration.

Catherine Outerbridge stated it is important to confirm no secondary pyoderma is present. She has experience with one diabetic cat that could be controlled with a lower dose of triamcinolone by using concurrent oclacitinib.

Otto Fischer asked if the audience would prefer oclacitinib or ciclosporin as first-line treatment. Due to access in his country, he has only had experience with oclacitinib for 2 weeks.

Wayne Rosenkrantz mentioned oclacitinib is excellent for acute flares in atopic dogs. Immunotherapy is still his drug of choice for long-term management. Immunotherapy is the safest therapy that can be used and has benefits even if it just reduces the dose of other immunosuppressive drugs.

Jerome Ngo asked about loss of treatment efficacy over time. He was wondering if it could be due to the accumulation of IL-31 due to blocking of the receptor.

Wayne Rosenkrantz mentioned that other complications such as pyoderma and other flare factors seem to be more of a problem. He questioned whether blocking IL-31 could possibly cause a rebound effect. He asked for opinions from members of the audience.

Valerie Fadok and **Candace Sousa** were not aware of data that supported an accumulation of IL-31.

Catherine Outerbridge thought that this could be possible. When owners stop oclacitinib on their own they often report that the pruritus seems worse than before treatment. Owners may have forgotten how severe the allergies were or the allergies may be getting worse since the treatment is just blocking the symptom of itching. Allergies do tend to get worse over time in some individuals. This is a reason why immunotherapy is still essential in the treatment of atopic dermatitis.

Candace Sousa mentioned a presentation that was going to be given by Rosanna Marsella at the current World Congress reporting that oclacitinib may have the ability to increase time to new sensitizations in atopic dogs.[6]

Wayne Rosenkrantz thanked everyone for their feedback and for attending the workshop.

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Resisting the resistance. Is there progress in maintaining antimicrobial efficacy?

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David Lloyd (*UK*) acknowledged Dermoscent for their sponsorship of this workshop, which consisted of five invited presentations.

Antimicrobial resistance: problem, prevalence and UK perspective (V.M. Schmidt)

Vanessa Schmidt (*UK*) discussed the major health threat caused by multiple drug resistance affecting both human and animal welfare, and the anticipation there will be limited future availability of new antibiotics, particularly in veterinary medicine. She briefly outlined antimicrobial treatment history, from the recognition of the importance of hand hygiene, to the recent golden era of antibiotic availability and efficacy, to the current 'period of disenchantment'; a time when antibiotics are starting to be of limited benefit due to increasing development of resistance.[1] She then discussed the main pathogens of concern in human medicine, including Gram-negative organisms, and compared that to current veterinary concerns. In her practice, Vanessa Schmidt sees methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) as the greatest concern, but also methicillin-resistant *Staphylococcus aureus* and *Staphylococcus schleiferi* (MRSA and MRSS). Multidrug resistance (MDR) is also emerging in Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas*, *Proteus*, *Klebsiella* and *Acinetobacter* spp. from cases of deep pyoderma. Multidrug-resistant enterococci are also becoming a concern.

Vanessa Schmidt emphasized that it is important for clinicians to have knowledge of the local and regional antimicrobial resistance situation to help them in empirical clinical decision-making and also to be aware of when an empirical choice would be less appropriate. She presented current data and regional patterns for antimicrobial resistance in the UK. Nationwide data can be accessed online on Pet Resist.[a] This website provides antimicrobial resistance data from laboratory submissions to IDEXX UK, shown plotted on a map based on where submissions originate. The data lack information indicating from which body regions samples were taken, and on the numbers of isolates the percentages are based upon, but still provide updated information on regional resistance patterns. Data from a study conducted in Liverpool were presented.[2] This laboratory-based study analysed *S. pseudintermedius* data from submissions to the university veterinary laboratory in Liverpool. Bacterial culture results were examined over time, and the site of infection was recorded. The data showed a significant increase in resistance over the past 5 years to clindamycin, gentamicin and fluoroquinolones, as well as MDR. Soft tissue- and skin-derived isolates were more resistant than isolates from urine. These results correlated with other studies.[3,4] Risk factors for MRSP have been reported to be chronic skin infections, multiple hospital visits and the use of broad-spectrum antibiotics.

Oxacillin (methicillin) resistance between 2004 and 2015 was also reviewed in Liverpool. Cases of MRSP, which were first seen in 2007, have since increased year by year. This correlates with international data. The methicillin-sensitive *S. pseudintermedius* (MSSP) strains showed a high level of clindamycin resistance. The level of resistance to clindamycin in this study was higher when compared to levels reported in other studies, and comparable to the prevalence in cases of chronic, rather than first-time, pyoderma cases. Rifampicin resistance was higher in MRSP strains compared to MSSP strains (27% compared with 7%) and high compared to other studies. MDR was seen in 20% of strains. Comparing MRSP data between countries, reports from Texas, USA, show 30% MRSP strains. In Europe, the situation varies between countries, from 6 to 27% in continental Europe and 0.7 to 5% in the UK. Data indicate an increase in MRSP strains cultured over time. For example, in the Netherlands, there was an increase from 0.9% in 2004 to 7% in data published in 2016.[5–13]

Vanessa Schmidt concluded that MDR strains were common in the population studied. It is increasingly important to obtain bacterial cultures before treatment. Risk factors for MDR need to be considered and reduced, and topical treatment should be used when possible. Regional levels of MDR prevalence should be monitored and regional recommendations for antimicrobial use should be established to help identify high-risk cases in hospitals, as well as to help establish hospital infection control policies.

Edmund Rosser (*USA*) commented that in his clinics it is common to see MDR cases, which is a concern. He uses daily chlorhexidine baths for treatment. He wonders whether extensively drug-resistant strains that are resistant to eight or more classes of antimicrobials have been seen, because he has started to see these.

Vanessa Schmidt and **Annette Loeffler** (*UK*) said they had not seen extensively drug-resistant strains in the UK.

Galia Sheinberg (*Mexico*) felt that in most of South and Central America, surveillance is very poor and the use of antibiotics still uncontrolled. People have access to antibiotics without prescriptions. She is starting to see some resistance in Mexico City that is very concerning. She felt they were 'in the dark' about the current resistance situation due to a lack of surveillance.

Elisa Bourguignon (*Brazil*) recently published a paper where she had isolated *Staphylococcus* from pyoderma in dogs. She had a small number of dogs and only 75 isolates but more than 90% of these had the *mecA* gene.[14]

David Lloyd asked how differentiation between first-opinion and referral cases in Britain had been done.

Vanessa Schmidt replied that it was difficult as all the cases they see in referral come from first opinion and they can come with infections at the time of referral. The laboratory in Liverpool receives samples from both, but mainly from referral sources. Examination of the data from all UK labs would be useful.

Andrew Hillier (*USA*) commented that referral data might not always be as useful. Antech laboratory had looked at their data that primarily originated from first-opinion practice. An interesting trend was seen, in that methicillin resistance in *S. pseudintermedius* had stabilized at around 30% of isolates. He found this stabilization intriguing, as use of antimicrobials has not changed much, and if anything ongoing or increased horizontal transfer could occur. Despite this, a plateau appears to have been reached for the past 4–5 years.

Yuchi Chen (*Australia*) lectured in multiple cities in China during May 2016. He found drug resistance quite alarming and felt that this is being ignored in developing countries. He felt the situation was deteriorating compared to 5 or 6 years ago. For example, the Chinese now have access to more drugs than before and limitations to their use are not in place. He thinks it is important to point out the public health concern in developing countries and to warn veterinarians that there is a risk of becoming carriers of resistant bacteria. He feels this would persuade them to take other factors than the price of the drug into account and to be more restrictive in the use of broad-spectrum antibiotics.

Genetic evidence of the development of multiresistance and consequences with regard to the use of antimicrobial agents (S. Schwarz)

Stefan Schwarz (*Germany*) first discussed the genetic background to MDR development. Resistance can occur through resistance-mediating mutations, resistance genes or a combination of both. Many antimicrobial resistance genes can be located together on the same mobile genetic elements (MGEs). As a consequence, co-selection of resistance genes, and persistence of genes, can occur even in the absence of direct selective pressure. There are many MGEs involved in multiresistance. One example is a plasmid found in livestock-associated MRSA that carries multiple different antimicrobial resistance genes including the *mec* gene, a biofilm gene cluster and metal-resistance genes. All these different genes can co-select for each other. Examples of MGEs spread widely among Gram-negative bacteria are multiresistance integrons. These are able to assemble different gene cassettes that carry antimicrobial resistance genes. Another alarming finding is the multiresistance-conferring integrative and conjugative element ICE*Pmu1* found in *Pasteurella multocida* from bovine respiratory infections. This MGE is large and is able to transfer itself from one cell to another. The MGE was found to carry 12 different antimicrobial resistance genes including resistance to virtually all antibiotics used to control bovine respiratory tract infections. Many other MGEs are also seen, such as conjugative transposons in *Salmonella*, the *Salmonella* genomic islands 1 and 2, and the SSC*mec* elements that carry not only the *mecA* or *mecC* gene but also other integrated plasmids or transposons. These multiresistance-mediating MGEs contribute to dissemination of resistance. When a MGE is transferred from one bacterium to another, all the resistance genes are transferred as well, which means bacteria gain multiresistance through a single genetic event. This transfer can occur across strain, species and even genus boundaries. Co-location of resistance genes supports co-selection and persistence, and understanding of the genetic background is therefore important.

Stefan Schwarz then presented data that showed what had occurred following national legal bans of use of certain antimicrobials. Ten years after chloramphenicol was banned in Europe for use in production animals, *Pasteurella* and *Mannheimia* isolates from cattle and swine were examined. Resistance to chloramphenicol remained high in the isolates.[15] Following genetic examination, it was found that a plasmid was present that carried a multiresistance gene cluster in which a chloramphenicol resistance gene was bracketed by sulfonamide- and streptomycin-resistance genes. Sulfonamides and streptomycin were still being used, and co-location of the resistance genes caused co-selection and persistence of the chloramphenicol-resistance gene. Another example described the results following the ban of avoparcin (glycopeptide) as a growth promoter. Glycopeptide resistance of *Enterococcus faecium* in broilers declined from 81 to 12% within 2 years. However, in pigs no change in resistance occurred.[16] This was likely caused by tylosin treatment in pigs and co-selection of a plasmid carrying the *vanA* gene cluster that also harboured the macrolide-resistance gene *erm*(B).

Resistance development following introduction of new antimicrobials on the market has also been studied. Florfenicol was introduced in 1994 for use in cattle in the EU. Two years later, the first resistance genes were identified in Gram-negative bacteria, and a year after that also in Gram-positive bacteria. Rapid resistance development has been seen occurring for most antimicrobials. The exception is vancomycin, as it took almost 15 years for

resistance to occur, possibly due to the selective and restrictive human use. Stefan Schwarz concluded that resistance is an evolutionary principle by which bacteria try to cope with changed environmental conditions. This cannot be stopped, but adhering to prudent use guidelines and decreasing selective pressure can slow resistance development.

Yuchi Chen commented that there are veterinarians in China who use vancomycin for their dermatology cases. In six of seven cities he had visited in May 2016 vancomycin had been used for routine dermatological cases.

Stefan Schwarz commented that this is a problem in China; he is himself a visiting professor in Beijing. The situation in other parts of the world is different. For example, in the EU vancomycin is not approved at all for use in animals. The drug can still be used in dogs and cats, but this is rare, at least in Germany.

Joseph Blondeau (*Canada*) commented that he thinks we need to be very careful with vancomycin. His current PhD student has studied vancomycin and MRSA from human blood culture isolates. He feels that susceptibility testing using standardized inocula of 10^5 CFU/mL is problematic, because it assumes that this is the only density of organisms that is important. If density is increased, strains of MRSA surviving in 32 or 64 μg/mL vancomycin have been seen in his laboratory. Out of 60–70 blood culture isolates of MRSA, 40–50% survived in the presence of 16–32 μg/mL vancomycin when tested at higher densities. From the clinical point of view, he feels that one could argue that some organisms are persisting in patients treated with vancomycin. This likely explains some of the lack of clinical improvement seen. He expressed the need to be careful and that a lack of vancomycin resistance based on minimum inhibitory concentration (MIC), or very limited vancomycin resistance, might not represent the full picture.

Stefan Schwarz commented that when they have vancomycin-resistant strains, they do genetic tests for *van* genes and re-test if they are negative.

David Lloyd asked for comments on Luca Guardabassi's lecture (see Chapter 6.2 in this volume), in which he discussed enhancing the activity of existing antimicrobials to make them more effective. Carprofen had been given as an example of an agent that could promote antibiotic potency and efficacy.

Stefan Schwarz replied that this was an interesting approach, but that it was not yet fully explored. The main problem he sees is, as stated in the lecture, that only the *tet*(K) gene is affected, but there are about 45 other *tet* genes. It is not known whether this approach works with other *tet* genes that also code for efflux pumps. He has not seen any data about other efflux genes affecting, for example, chloramphenicol resistance or macrolide resistance.

David Lloyd asked if these drugs, as has been suggested, would be unlikely to promote antimicrobial resistance as they are not directly antimicrobial.

Stefan Schwarz did not feel he could say anything about this based on the available data. **Joseph Blondeau** commented that he does not believe that this is true.

Kim Coyner (*USA*) commented that she felt we need to think about paradigm changing from inventing new antimicrobials that will eventually fail, to finding ways to modulate the immune system to fight the microbes. She asked if there is any work looking at genetic modification of pigs or cows to make them intrinsically resistant to some infections.

Stefan Schwarz replied that he has heard about approaches to developing, for example, pigs resistant to *E. coli* infection. He is not in favour of this approach as he thinks bacteria will modify pathogenicity more rapidly than people can generate genetically modified pigs.

Allan Bell (*New Zealand*) asked about the prospects of treating an infected patient with something that downregulates the resistance genes.

Stefan Schwarz replied that this it is one approach, and that we have to see how it works on a large scale. He feels there are too many different expression modes, so finding an agent that can downregulate all the resistance genes is unlikely.

Allan Bell commented that we only need to downregulate one resistance gene to treat a particular animal.

Stefan Schwarz replied that if it were one very important gene, for example *mec*A, beta-lactam activity could be restored. This would be interesting for certain conditions such as bovine mastitis, for which most antibiotics available belong to the beta-lactam class.

Mutant prevention concentration, MPC (J.M. Blondeau)

Joseph Blondeau opened his presentation by stating that he is not sure whether we know how to measure susceptibility versus resistance. He then explained how resistance and susceptibility are determined in laboratories today, and some of the problems he sees with this determination.

The standardized laboratory assessment of whether a strain is considered resistant or susceptible to a certain antimicrobial is by determining the minimal concentration of drug that inhibits visible growth of a standardized bacterial inoculum (10^5 CFU/mL). This inhibition does not necessarily mean the organism is killed, only that it shows inhibition of growth *in vitro* in the presence of an antibiotic. This concentration is called the minimum inhibitory concentration (MIC). The MIC is then assessed in the context of drug pharmacokinetics and pharmacodynamics, and a breakpoint is established for what is called susceptible and resistant. Bodies such as the Clinical Laboratory Standards Institute, USA, have over the years changed these breakpoints. These changes then suddenly alter the percentage of strains that are deemed resistant or susceptible to the drug. Infections can also carry markedly

higher bacterial densities than the concentrations tested for *in vitro*.[17–20] Work in recent years has aimed to assess the influence of the number of infectious organisms (density of the microbial population) on antimicrobial susceptibility and resistance. The argument is that if a patient has a low infectious burden, MIC drug concentrations probably over-estimate the drug concentration needed for bacterial eradication. Under-dosing in these situations is unlikely to occur. At the other end of the spectrum, when the bacterial density is high, it is possible that under-estimation of the drug dose needed for sufficient exposure to antimicrobials occurs. MIC is only relevant for systemic and oral treatment, not topical, but even with systemic treatment it does not fully represent drug body distribution. Higher concentrations can be achieved in certain body systems (e.g. urine) depending on the drug, whereas other regions obtain lower concentrations. This can be very confusing to clinicians.

Joseph Blondeau then explained the mutant prevention concentration (MPC), which was described in 1999. It was recognized that, upon exposure to fluoroquinolones, the majority of bacterial cells in a high-density population were inhibited by drug concentrations approximating the MIC. Some cells in the population, however, were able to continue to replicate. The drug concentration that inhibited these cells was called the MPC.[21] It was recognized that in the presence of quinolones, selection of first-step-resistant mutated strains, and genes that coded for either DNA gyrase or topoisomerase IV, occurred. The MPC prevented an organism with a first-step-resistance mutation from growing. MIC tests are based on populations of 10^5 CFU/mL but it is known that in infections with populations of 10^7–10^9 CFU/mL spontaneous resistance occurs in the population. These organisms might not be inhibited by the antimicrobial dosages used based on MIC drug concentrations. To inhibit these organisms, a higher drug concentration is required. When higher dosages that would inhibit these strains are not used, selection of resistant strains is allowed to occur. Susceptible strains are eliminated, and clinical improvement can occur, but the patient may become colonized with strains that are not inhibited by the drug. If the immune system is functioning properly, these cells should be cleared as well. However, in patients with compromised immune systems, or when suboptimal therapy occurred, these organisms could continue to proliferate. Should dosing be based on the MPC rather than the MIC, not only would the susceptible strains be eliminated, but the resistant ones as well. Selective pressure occurs in the mutant selection window (MSW) between MIC and MPC drug concentrations.[22]

A recent study by Joseph Blondeau's group looked at macrolides in human infectious diseases. Some 190 *Streptococcus pneumoniae* isolates were examined against three macrolides: azithromycin, clarithromycin and erythromycin.[23] The amount of time that the drug concentration remained in the MSW was assessed. Of these antibiotics, clarithromycin remained in the window for the shortest duration. It was therefore concluded that clarithromycin was least likely to select for resistant strains when treating infections caused by *S. pneumoniae*. Azithromycin drug concentration was estimated to remain within the MSW for prolonged periods of the dose and so azithromycin use was more likely to select for resistance in this particular organism. *In vivo* data by other groups supported this finding. One study showed that in areas of Canada where more azithromycin had been used, more macrolide resistance was seen compared to areas where more clarithromycin or erythromycin was used. This is consistent with data from other parts of the world.

Joseph Blondeau emphasized that two antimicrobials supported by clinical trial data to give similar outcomes might not be the same from a microbiological and pharmacological point of view. Prescribing practices should be tailored to the organism seen, and consider different antimicrobial properties, to optimize antimicrobial use. Drug dosages should be targeted to avoid drug concentrations remaining in the MSW for extended periods. An example is cefovecin. This drug reaches very good serum concentrations[24,25] but has high protein binding, which reduces the amount of free drug and thus antimicrobial activity. This gives rise to concerns, as the free drug concentration remains in the MSW for an extended period of time. The evidence for the MSW *in vitro* is today established. Some *in vivo* data are lacking, although there are data showing clinical failure of treatment, as well as selection of resistant strains, using concentrations in the mutant selection window. Animal models have also been published. A rabbit empyema model showed that using concentrations that stayed in the MSW for 40% of the time a 100% selection of mutant strains occurred.[26] Joseph Blondeau's group has established a rat *Pseudomonas* model showing data on selection of resistance that are not yet published.

He also discussed some recent worrying findings of vancomycin-resistant bacteria that lack the genes associated with vancomycin resistance. Despite the lack of genes associated with resistance, these strains are still persisting and even dividing in the presence of high concentrations of vancomycin. The bacteria were selected from high-density populations. This is, he feels, an additional aspect that shows that MICs do not give the complete picture of antimicrobial resistance or susceptibility.

Finally, Blondeau gave an example of how data could be used in the future to help prevent or reduce resistance development. Moxifloxacin, similar to pradofloxacin in veterinary medicine, is one of the key antimicrobials used in *S. pneumoniae* infections in human medicine. The drug has been on the market since 2000 in North America, and no changes to *S. pneumoniae* susceptibility to this antimicrobial have been seen. This indicates that the antimicrobial is adequately dosed for this particular organism and has the right mechanisms for treating this particular organism to reduce selective pressure and resistance development. However, resistance of other organisms to this antibiotic has occurred.

Resisting the resistance: topical antimicrobial treatment (A. Loeffler)

Anette Loeffler (*UK*) started her presentation by asking how many in the audience felt that they practise in a low-prevalence MRSP area. About one in seven of the workshop participants raised their hands. Anette Loeffler then discussed opportunities and challenges of topical

antimicrobial therapy, and outlined current evidence behind its use.

Topical treatment can replace systemic therapy in many cases of bacterial skin infection, particularly surface and superficial infections, and otitis. Use of topical treatment instead of systemic antimicrobials reduces selection pressure that drives emergence and spread of resistance. Anette Loeffler feels that at times she prescribes systemic antimicrobials when topical treatment could have sufficed to control infection. This mainly occurs because of the human factor; that is, owner convenience and compliance, whether perceived or actual. To highlight that there is plenty of room for improvement in our prescribing practices, Anette Loeffler quoted a recent publication in *Veterinary Record* of antimicrobial use in some European countries among equine practitioners.[27] The authors concluded that inappropriate antibiotic use was widespread. This included prescribing without a correct indication, and the use of third- and fourth-generation cephalosporins as first-line antimicrobials. She stated that we need to continuously remind ourselves that we still have many effective and safe antimicrobials available, and that we need to use them wisely for cases in which they are needed. Whether improvements in responsible antimicrobial prescribing practices in veterinary medicine are best achieved through voluntary actions by clinicians, or through legislation and restrictions, remains to be seen. In 2008, the UK Chief Medical Officer stated that there may need to be a ban on the use of cephalosporins and fluoroquinolones in animals. However, no changes in legislation have been introduced in the UK to date, unlike in some other European countries. In order to prevent general restrictions, voluntary limitations and evidence of responsible antimicrobial use should be shown. Anette Loeffler pointed out that restrictions could be helpful in convincing owners to wash their pets rather than giving a tablet. In countries where legal restrictions are not in place, adhering to guideline recommendations, and emphasizing the safety of topical therapy, its suitability for long-term use and its ability to treat the diseased organ directly can all aid the clinician when discussing treatment with owners.

Topical antibacterial therapy represents a great opportunity to reduce the need for systemic treatment, and recommendations for the use of topical treatment as first-line management are already included in many antimicrobial guidelines today. Anette Loeffler quoted the Federation of European Companion Animal Veterinary Associations (FECAVA) guidelines as a good example.[b] The guidelines list, among other indications, several common dermatological conditions for which systemic antimicrobial use is considered unnecessary today. Surface and superficial pyoderma, otitis and skin infections involving MDR staphylococci are the main conditions for which topical treatment is recommended.[28,29] A review published in *Veterinary Dermatology* in 2012 showed that good-quality studies are sparse and that there is a need for more randomized controlled trials using topical products.[30] However, the review found good evidence for efficacy of 2–4% chlorhexidine and benzoyl peroxide shampoos. Limited evidence exists for use of silver sulfadiazine and medical honey.

The textbook dogma that superficial pyoderma should be treated with 3 weeks of systemic antimicrobials with or without topical treatment was recently challenged. Anette Loeffler presented the results of a study that she had conducted using 2% chlorhexidine shampoo two to three times weekly as a sole antibacterial treatment for superficial pyoderma in dogs. The results showed that this treatment was clinically effective in 70% of dogs, providing evidence that superficial pyoderma can be treated with topical treatment alone, with additional systemic treatment only added in complicated cases.[31]

Finally, she discussed the evidence for resistance development to topically used agents. While there are reports in the human literature describing failure of protocols using topical therapy for MRSA decolonization, there is no evidence of true treatment failure of topically used antibacterial products. Recontamination or recolonization from different body sites or from the environment are likely. Several recent *in vitro* studies on animal isolates have shown that MICs for topical agents are low at this time. Furthermore, there is a plausible concept that topical treatment can overcome MICs by achieving high concentrations locally, and that susceptibility testing is therefore not relevant when topical treatment is used. There is very little evidence in the literature to demonstrate local drug concentrations achieved by topical therapy. Current work at the Royal Veterinary College, London, that includes studies into penetration of topical agents and association with MICs, might provide more data.

A controversial topic and question is whether topical treatment should be used to decolonize animal patients that are MDR carriers, for example prior to planned surgical procedures. Decolonization is currently not supported by any data showing a beneficial outcome. Future studies including effects on the microbiome and the interaction of microbial communities are needed to explore alternative approaches or preventative treatments. Anette Loeffler concluded that topical therapy, using the wide variety of products available, is worth the effort as it provides a great opportunity to reduce the need for systemic antimicrobials in the treatment of superficial skin and ear infections, particularly in the treatment of MDR infections.

Alternatives to conventional antimicrobial treatment. (S.L. Marks)

Stanley Marks (*USA*) discussed the risks associated with use and/or abuse of antimicrobial treatment. Early foetal exposure to antibiotics through the mother has been shown to increase risk of diabetes mellitus development in infants. It is important to be mindful of the risks of antimicrobial exposure during pregnancy and early life. In addition to the risk of antimicrobial resistance, he highlighted the risks of asthma, certain types of allergies, type I and type II diabetes mellitus and the risk of *Clostridium difficile* infection that can be life threatening.[32] The long-term sequelae of antimicrobial administration on the intestinal microbiome cannot be ignored, even when the drugs are discontinued. Work at Texas A&M College of Veterinary Medicine in research beagles has shown the effects of tylosin exposure on the gut microbiota.[33] In that

study, five healthy beagle dogs were administered tylosin once daily for 2 weeks. On day 28 (2 weeks following cessation of antimicrobial therapy), the phylogenetic composition of the microbiota was similar to day 0 in only 2/5 dogs. Changes in the intestinal microbiota can persist for months, or even longer, depending on the individual, duration of treatment and the drug used.

Stanley Marks then spoke about the use of probiotics and faecal microbiota transplantation (FMT). He considers the latter the ultimate probiotic. Probiotic effects depend on the strain(s) used, the viability of the strain(s), the number of bacteria or yeast administered in the probiotic, and the host disease. Probiotics by definition are live and viable micro-organisms that in sufficient numbers alter the microflora (by implantation or colonization) in a compartment of the host and exert a beneficial effect. A prebiotic is a poorly digestible carbohydrate source, typically an oligosaccharide, which is quite different in its mechanism of action to a probiotic. The prebiotics selectively stimulate the growth and/or activity of one or a limited number of bacteria that have already been established in the colon. In general, prebiotics might be less effective in animals or people in which the targets of the prebiotic (*Lactobacillus* spp. and *Bifidobacterium* spp.) are limited secondary to dysbiosis. When using probiotics, massive numbers of viable micro-organisms are added. Many die in the stomach, but a subset will survive and colonize the distal small bowel and colon.

There are four broad mechanisms of action for probiotics: (1) competitive exclusion of enteric pathogens; (2) direct antagonism by production of various antimicrobial metabolites, for example bacteriocins; (3) modulation of mucosal immunity, and (4) enhancement of epithelial barrier function. It is important to emphasize that probiotics, by their species and/or their strains, have differing mechanisms of action and/or effect. Studies evaluating the efficacy of probiotics in veterinary medicine have not always been well standardized, have been associated with relatively small numbers of study subjects and have not always included a control population. In contrast, a series of meta-analyses evaluating the efficacy of probiotics has been conducted in large numbers of human patients with different maladies including infectious diarrhoea, antibiotic-associated diarrhoea, inflammatory bowel disease (IBD) and atopic dermatitis, and the different probiotic strains have been ranked according to the degree of evidence supporting their benefit.[34] In veterinary medicine, probiotics have been best evaluated for the management of acute non-specific diarrhoea in dogs and cats. Rosanna Marsella and her colleagues have evaluated the effects of early exposure to *Lactobacillus rhamnosus* GG (LGG) in a canine model of atopic dermatitis, and have shown the long-term clinical and immunological effects of this intervention.[35,36] FortiFlora® (Nestle Purina PetCare, St Louis, MO, USA) contains *E. faecium* SF68, and is currently the most comprehensively studied probiotic in clinically affected dogs and cats. The product has been shown to shorten the duration of acute diarrhoea in canine and feline patients.[37,38]

The concurrent administration of a probiotic (*E. faecium* SF68) with an antibiotic has also been investigated, and results have shown that the probiotic can be effectively co-administered with metronidazole or amoxicillin/clavulanic acid in dogs and cats, respectively.[c,39] *E. faecium* SF68 was shown to survive effectively when co-administered with both antibiotics, and an improved response to the co-administered probiotic and antibiotics was recognized in diarrhoeic dogs and cats compared to that when the antibiotics were given alone. In an experimental model, mice were infected with *Giardia* trophozoites. *E. faecium* SF68 enhanced humoral and cellular immune responses to *Giardia*, and reduced subsequent shedding of trophozoites compared to a control group.[40] FMT is used for treatment of recurrent *C. difficile* infections in people with excellent efficacy and far better responses compared to conventional vancomycin treatment.[41] A current study at UC Davis is evaluating the effects of FMT in dogs with atopic dermatitis but it is too soon to determine the efficacy of this novel therapy for the management of atopic dermatitis in dogs.

Kimberly Coyner asked whether Stanley Marks recommends that all animals that are sent home on antibiotics are also given probiotics.

Stanley Marks replied that we unfortunately do not know which probiotics are killed by antibiotics because of the limited studies evaluating these effects that have been published to date. We know that metronidazole and amoxicillin/clavulanic acid do not kill *E. faecium* SF68; however, they might kill other probiotic strains. He does believe it is good practice to utilize the benefits of probiotics to minimize or prevent diarrhoea that can ensue from the administration of these antimicrobials. The metronidazole was given concurrently with the *E. faecium* SF68 whereas the amoxicillin/clavulanic acid was given 2 hours before the probiotic.

Notes

[a]www.petresist.com/

[b]www.fecava.org/sites/default/files/files/DSAVA_AntibioticGuidelines%20-%20v1-1_3(1).pdf

[c]Personal communication, M. Lappin (Colorado State University, USA).

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Update on the diagnosis and treatment of canine demodicosis

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Ivan Ravera (*Spain*) opened the workshop with a discussion of the number of *Demodex* species found in dogs. Historically, determining species in *Demodex* mites was based on morphological features and on which host harboured the species of mite. In the last few years, PCR techniques have been developed to specifically amplify *Demodex* DNA, and these have been utilized to distinguish species in several phylogenetic studies.[1–5] Two studies using 16S rDNA have determined that there are two different species on canine skin: *Demodex canis* and *Demodex injai*.[3,4] According to these studies, the short-bodied mite (that has at times been called *Demodex cornei*) is a morphological variant of *D. canis* with insufficient genetic difference to separate it from the latter. An earlier study did not support this finding but found *D. canis* and *D. cornei* to be different species.[5] A possible explanation for the discrepancy is that the earlier study used PCR primers from other arthropods and not from *Demodex*, whereas the two studies that found *D. canis* and *D. cornei* to be same species used *Demodex*-specific primers. *D. canis* and *D. injai* have distinct clinical presentations and are easily differentiated by size using light microscopy. In some countries, *Demodex*-specific PCR is offered for the diagnosis of clinical demodicosis.

Ralf Mueller (*Germany*) asked about the pros and cons of the use of PCR to detect *Demodex* mites in a clinical setting. Ivan Ravera did not think this test is useful for clinical practice. He believes that PCR cannot replace the standard methods (skin scrape, hair pluck, tape strip) for the diagnosis of demodicosis. *D. canis* was found in 100% of normal dogs using PCR, so a positive PCR test does not correlate with clinical disease. Lastly, when normal dogs were sampled at five different skin locations, approximately 18% of dogs were positive for *Demodex* mites, but if the number of sample sites was increased to 20, then 100% of dogs were positive for *Demodex* DNA. Thus before an animal is classified as negative the sample size must be increased. However, the positive aspect of PCR is that it is an extremely sensitive technique and can detect one mite in a litre of phosphate-buffered saline (PBS). PCR could be useful, for example, when establishing a new disease-associated mite, a new population distribution or phylogenetic distribution (e.g. incidence of *D. injai* in healthy dogs). This would require the use of species-specific primers.

Question from the audience: *If the technique is so sensitive that 100% of dogs are positive, how would you apply this technique to epidemiological studies?*

Ivan Ravera replied that it is useful to determine the incidence of specific species of mites. He has developed a technique (using different primers) to identify *D. injai* and this would allow the determination of the incidence of *D. injai* in a normal population. While the clinical presentation is quite unique nearly nothing is known about the mite and its carriage rates or other ecological factors.

Question from the audience: *So would this [using PCR to establish if the animal had D. canis or D. injai carriage] make a difference clinically?*

Ralf Mueller replied it would not, because the treatment for both mites is the same. However, *D. injai* is more difficult to find because the number of mites carried is lower, and occasionally there are cases that fit clinically (e.g. a terrier with a patch of greasy skin) in which the skin scrapes are negative. PCR might be considered, but to date there is no information on the prevalence of carriage in a normal population and therefore the relevance of a positive test is unknown.

Question from the audience: *How many samples were necessary to obtain a 100% carriage rate in normal dogs and were any specific locations chosen?*

Ivan Ravera replied that sampling at least 20 skin locations was needed. There was no preferred location; the unpublished observation was that facial and pedal samples have a higher incidence of mite carriage and would be the top choices when searching for *Demodex*.

Question from the audience: *With regards to the 100% incidence study, what population was selected: treated or untreated dogs?*

Ivan Ravera responded that all were healthy, privately owned dogs from their hospital population. Each of the 20 hair-pluck samples was individually tested, so in total there were 200–300 hairs that were collected and tested from each dog.

Question from the audience: *Have any studies been performed in animals that were treated and cured to see*

if they remained PCR-positive, despite the clinical resolution of disease?

Ivan Ravera responded such studies are still lacking.

Ralf Mueller replied that is a very interesting question. It is unknown whether treatment decreases the infestation level to the point where the body's own immune system is then able to maintain control, or whether all the mites are actually killed and the animals are subsequently completely free of *Demodex*.

New technique for in-house diagnosis of demodicosis (A. Pereira)

Alessandra Pereira (*Brazil*) presented data on a new technique for the in-house diagnosis of demodicosis.[6] Diagnosis is made by the identification of the *Demodex* mite or any of its life cycle stages. The mites have been traditionally collected using deep skin scrapings; however, trichograms have also been used as they tend to be less traumatic (for both the dog and owner). The new technique combines use of an acetate tape preparation with skin squeezing. For sampling she used a 10 cm piece of clear acetate sticky tape placed on the skin, with the area squeezed for 4–5 s. The tape was then removed and placed on a glass slide for observation under a light microscope. The results of a study comparing numbers of mites found with acetate tape preparation and with skin scrapes were presented (Table 1).

The acetate preparation is more sensitive and, in addition, is less traumatic for the dogs, and is safer than scrapes, particularly in sensitive areas such around the eyes, lips or interdigital spaces. It can be performed in uncooperative or aggressive dogs, is low-cost and easy to do.

Ralf Mueller asked how she squeezes the skin.

Alessandra Pereira responded that she places tape on the skin and then squeezes the skin using her fingers to apply pressure. It does not seem to matter if constant pressure is applied or if the skin is repeatedly squeezed.

Ralf Mueller inquired whether there is any difference in the sensitivity of this technique in dogs that are mildly affected as opposed to more severely affected cases.

Alessandra Pereira replied she has not found any difference with this technique regardless of the severity of the lesions.

Table 1. Distribution of the number of demodectic mites in samples obtained by acetate tape impression with skin squeezing and by deep skin scraping. Source: Pereira et al. 2012.[6] Reproduced with permission of John Wiley & Sons, Inc.

Mite stage	Acetate tape impression with squeezing	Skin scraping	P value
Eggs	108	34	0.283
Larvae	69	27	0.024
Nymphs	91	29	0.325
Adults	827	434	<0.001
Total	1095	524	<0.001

Question from the audience: *Has this technique been used in Scottish terriers and shar-peis?*

Alessandra Pereira responded that she has some experience with shar-peis but not with Scottish terriers, and has had positive samples from shar-peis.

Question from the audience: *Has this technique been used to diagnose D. injai?*

Alesssandra Pereira answered she has only had one case with *D. injai* and the technique was successful in finding mites.

Question from the audience: *If the tape sample is stained to look at normal cytology, would the Demodex mites be lost from the sample?*

Alessandra Pereira replied that she has not stained any of the tapes.

Ralf Mueller stated that it would be unlikely to lose the mites if the tape is only dipped in the stains and not the alcohol, as cells and bacteria stay on the tape.

Question from the audience: *Is this technique sensitive enough to monitor treatment as the number of mites decreases?*

Alessandra Pereira responded that she uses the technique for monitoring and it appears to be as sensitive as skin scrapings.

Question from the audience: *Is the hair clipped from over the sample site prior to application of the tape?*

Alessandra Pereira answered that generally the hair is not clipped; however, if the hair is very long, it can be carefully clipped with scissors. In most cases it is sufficient just to part the hair to allow application of the tape directly onto the skin. She noted that her research group is currently in the process of comparing the tape preparation technique with trichograms and the preliminary results show that the tape preparation is more sensitive. A free communication presented the previous day by Linda Vogelnest showed similar results.[7]

Use of doramectin in treatment of generalized demodicosis (M. Shipstone)

Michael Shipstone (*Australia*) presented on the use of doramectin to treat generalized demodicosis. Doramectin is a member of the avermectin group of compounds. It is available in two formulations, a 10 mg/mL injectable solution and a 5 mg/mL pour-on solution. There have been two papers detailing its use.[8,9] The first reported on a group of 23 dogs that were treated with weekly subcutaneous injections of 0.6 mg/kg with 100% remission after 8 weeks (5–20 weeks).[8] Ten remained in remission, five were lost to follow-up and seven required a second course of injections followed by monthly maintenance injections. The second paper was in a group of 29 dogs

treated with 0.6 mg/kg orally; 72% showed remission after 11 weeks (4–35 weeks).[9] He next discussed a paper in which 400 dogs with generalized demodicosis were treated with 0.6 mg/kg subcutaneous doramectin weekly.[10] There was no sex predisposition for affected dogs, but the majority were juvenile-onset (80–93%, depending on the age cut-off chosen for classification). Some 168 animals were excluded from data analysis because they did not meet all of the study criteria. Of the 232 that did, 220 (94.8%) achieved remission in a mean of 7.1 weeks (4–20 weeks). Of those in remission, 3/220 relapsed within 4 weeks, 10/232 failed treatment with no reduction in mite numbers observed and 2/232 showed adverse reactions. A total of 17/400 were diagnosed as adult-onset cases and 8 of these (47%) had an identifiable underlying disease process. Some 12/17 fulfilled the criteria for admission into the study and of these 8/12 (66.6%) achieved remission in an average of 7 weeks (6–8 weeks). If all cases were included (regardless of the number of scrapes performed) the overall clinical response was 86.3% (345/400). Overall, doramectin was found to be efficacious and well tolerated.

Ralf Mueller reported that a free communication presented at the same congress[11] comparing once-weekly subcutaneous injections with twice-weekly oral doramectin administration in a randomized trial found no difference in response rates between the two protocols.

Use of fluralaner for treatment of generalized demodicosis (J. Karas-Tecza)

Joanna Karas-Tecza (*Poland*) presented two studies on the use of fluralaner for generalized demodicosis. The first study used fluralaner in 162 dogs with generalized demodicosis. Fluralaner affects neurotransmission in the mites leading to paralysis and death. Remission was observed in all dogs within 3 months, although a number of those dogs were adult-onset. It is safe to use in breeding, pregnant and lactating dogs. Her next study evaluated use of fluralaner in 16 breeding bitches to prevent infection in the puppies. All of the breeding bitches in the study had had problems with generalized demodicosis in previous litters. In this trial all bitches were treated with 5 mg/kg fluralaner 10 days prior to scheduled mating. Fourteen bitches gave birth to litters that were clinically unaffected by demodicosis, although two puppies from one litter developed localized demodicosis.

Ralf Mueller commented that the second study is very interesting. At least in two dogs, the mites were not completely eliminated, otherwise there would have been no localized disease cases. He recommends a discussion as to whether it makes sense to use a product that will allow genetically deficient bitches to be bred and to produce more deficient puppies. This will lead to a situation where breeders can use these products to create a population of abnormal dogs that will then develop disease if resistance to fluralaner develops.

Wayne Rosenkrantz (*USA*) asked if the treatment was discontinued, or were the puppies also treated after birth, and whether any of the puppies have been followed for long enough to see if they have gone on to develop disease off of therapy because they still have the genetic abnormality as part of their genetic makeup.

Joanna Karas-Tecza replied that the bitches were treated 10 days prior to mating. A second dose was given to the bitches 90 days later, but the puppies were not treated so that the effect of treating the bitch alone could be determined. The puppies are now 11 months old. She agrees that this product may prove to be a problem if breeders use it to allow breeding of bitches that would otherwise have been excluded from a breeding programme due to a genetic problem.

Ralf Mueller commented that because the protocol did not call for the puppies to be treated, it should be possible to determine over the next year or so if these individuals will develop disease, particularly the female puppies.

Question from the audience: *Is it true that if an individual has been found to have developed (juvenile) generalized demodicosis then it should not be bred?*

Ralf Mueller replied this is true and is stated in recent guidelines and supported by European, American and Australasian dermatological societies and colleges, but that many breeders ignore this recommendation

Use of afoxolaner for treatment of generalized demodicosis (W. Rosenkrantz)

Wayne Rosenkrantz presented a study on the use of afoxolaner for generalized demodicosis in the dog. The afoxolaner was used in 102 cases, of which 68 were adult-onset demodicosis. The product was administered at 2.5 mg/kg per os, and initially used every 2 weeks instead of the label interval of every 4 weeks. Most cases were negative for *Demodex* mites after 2 months of treatment, though rarely it took 3 months to achieve negative scrapings. The initial dosing interval was set at 2 weeks, because it was felt that the product would need an increased rate of administration to be effective. However, it has proven to be very efficacious, and the dose is now administered once monthly at the recommended dose for flea and tick control and appears to be as effective. The exception to this is one case that was on immunosuppressive therapy that became mite-positive when the interval was increased to 4 weeks, but remains mite-negative with dosing every 2 weeks.

Both afoxolaner and fluralaner are good products and one does not appear to be superior to the other in terms of efficacy for *Demodex*. The palatability of each is similar. Afoxolaner does not need to be co-administered with food and in the USA afoxolaner can be given to 8-week-old puppies and fluralaner to 6-month-old puppies. In Europe both products are approved for puppies at least 8 weeks old. Both are safe for breeding, pregnant and lactating dogs.

A question can be asked regarding the end point of therapy. It is important to note that dogs will look better long before they are free of mites. However, most dogs

are mite-free by 3 months. As the product is an excellent treatment for flea and tick control, most clients elect to maintain ongoing administration for the other parasites.

Ralf Mueller commented that the experience in Germany (and also supported by Michael Shipstone's results in Australia) is similar. Both products are extremely efficacious. There are only few treatment failures, less than with any other product. The response to treatment also seems to be much faster, with significant improvement seen in as little as 4 weeks. Data being reported confirm this. The question remains whether this class of products actually eliminates demodicosis as a problem, or if it will allow the proliferation of a more genetically predisposed canine population that will manifest clinical disease if resistance to the products develops.

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Otitis controversies

C. Griffin (Chairperson) and J. Aniya (Secretary)

Animal Dermatology Clinic, San Diego, CA, USA

Craig Griffin (*USA*) welcomed participants and discussed the interactive nature of the workshop. The audience would have the opportunity to vote and answer questions through a live poll. Some questions would be asked to gain information about various topics while others were designed to stimulate a conversation and encourage the audience to participate. Prior to the workshop, an email had been sent to participants, asking what topics they thought were controversial and wished to discuss. This workshop combines the topics most frequently asked by the participants as well as selected topics from invited speakers.

Culture and sensitivity (C. Griffin)

Craig Griffin asked: *Which best describes when you culture otitis externa cases?*

1 Most chronic cases (8%)
2 After failure of my initial therapy (23%)
3 When I am going to prescribe systemic therapy (15%)
4 When systemic therapy has failed (2%)
5 When rods are present (31%)
6 Other (21%)

Craig Griffin asked the audience for some other reasons for performing cultures.

Andrew Carter (*Australia*) cultures cases of otitis externa when there is a single population of bacteria after empirical therapy.

Craig Griffin presented a study[1] he performed that questioned the reliability of ear cultures. In this study, he obtained duplicate cultures from the same ear in 15 dogs. An ear loop was used to collect a sample of purulent discharge from the junction between the horizontal and vertical canal. From the purulent material on the ear loop two culture swabs were inoculated and submitted the same day to the same commercial laboratory under different names. Of thirteen cases that grew any bacteria, only one (7.7%) grew the exact same strain of bacteria. In ten cases *Pseudomonas aeruginosa* was cultured in both samples but 70% had different strains and antibiotic sensitivities. In five cases *Corynebacteria* was grown in both samples but 60% of the strains had differing sensitivities. Two samples grew *Staphylococcus pseudintermedius* in both samples and both had different strains. The high occurrence of different culture results and sensitivities raised the question of whether a culture was a cost-effective test.

Another study evaluated the treatment of *Pseudomonas* otitis based on empirical antibiotic selection versus culture and sensitivity.[2] Twenty cases of *Pseudomonas* otitis were cultured and empirical antibiotic treatment was started while awaiting culture results. Of those 20 cases cultured, seven out of 20 cultures grew pure *Pseudomonas* while 13 of the 20 cases had a mixed culture. Seventeen cases completed the study. Eleven cases reported a resistance to the antibiotic chosen empirically. Of those 11 cases, 10 (91%) were successfully treated with topical antimicrobials alone despite the reported resistance on culture. Six cases were treated with antimicrobials that were reported susceptible on culture. Of those six cases, five (83%) were treated successfully. Interestingly, one of those cases still failed to respond to treatment despite a culture showing susceptibility. Results of this study also question the value of a culture and its relevance to treatment if otitis is to be treated with topical therapy alone.

Giovanni Ghibaudo (*Italy*) performs cultures if systemic therapy is to be prescribed.

Craig Griffin asked: *What are your top two keys to treating resistant Pseudomonas?*

1 High-dose systemic antibiotic (2%)
2 Cleaning well (31%)
3 Antiseptic therapy (3%)
4 Rely on synergism (1.5%)
5 Potent topical antibiotic (13.5%)
6 Combination of treatments 2–5 (38%)
7 Other (11%)

Diagnosis of biofilms in otitis (C. Griffin)

Craig Griffin said that the ability to produce biofilms has been shown by some strains of otitis externa bacteria.[3,4] As biofilms can complicate the treatment of otitis, they must first be recognized and diagnosed.

How often do you diagnose a biofilm in otitis cases?

1 Never (27%)
2 Rarely, in under 1% of cases (15%)
3 In 2–5% of cases (15%)
4 In 6–10% of cases (17%)
5 In 11–20% of cases (6%)
6 In 21–30% of cases (19%)
7 Over 30% of cases (2%)

Craig Griffin raised the question of how a biofilm is actually diagnosed. To prove that a biofilm is present in human medicine, tools such as fluorescence *in situ* hybridization or scanning electron microscopy are used.

What are the primary features associated with biofilms?

1 Culture results (2%)
2 Cytology findings (24%)
3 Odour (2%)
4 Presence of otitis media (0%)
5 Poor response to topical therapy (35%)
6 Poor response to systemic antibiotic therapy (9%)
7 Purulent exudate (28%)

What are secondary features associated with biofilms?

1 Culture results (4%)
2 Cytology findings (10%)
3 Odour (18%)
4 Otitis media (11%)
5 Poor response to topical therapy (33%)
6 Poor response to systemic antibiotic therapy (8%)
7 Purulent exudate (15%)

Craig Griffin commented that in human medicine a combination of features is used to clinically diagnose a biofilm. Chronicity of infection and poor response to treatment are considered important to clinicians dealing with wounds. Chronic discharge and presence of an odour after surgical implantation of medical devices are common features of a biofilm. Since biofilms in human tissue are associated with aggregates and most aggregates associated with *Pseudomonas* are over 8–40 μm in size, he uses this feature to diagnose biofilms. A variety of organisms in planktonic form (Figure 1a) were compared to a bacterial aggregate (Figure 1b).

Sue Paterson (*UK*) asked how the ear samples were collected. Rather than obtaining a sample directly from the pus, she believes it is useful to scrape the canal wall where the biofilm may adhere.

Craig Griffin stated his preferred technique for sample collection was to insert a rubber catheter into the canal. He then looks for aggregates on cytology. A critical finding is the ability to focus in and out of the aggregate cluster and observe different organisms at different focal lengths. The audience was asked if they use any other techniques to help diagnose a biofilm.

Jacques Fontaine (*Belgium*) also utilizes the observation that the pus is more adherent on the slide.

Craig Griffin asked: *Which two treatments are used when a biofilm is diagnosed?*

1 More cleaning (40%)
2 Stronger cleansers (13%)
3 Combination of cleansers (26%)
4 Stronger topical antibiotics (6%)
5 Antiseptics (2%)
6 Combination of antiseptics (4%)
7 Other (9%)

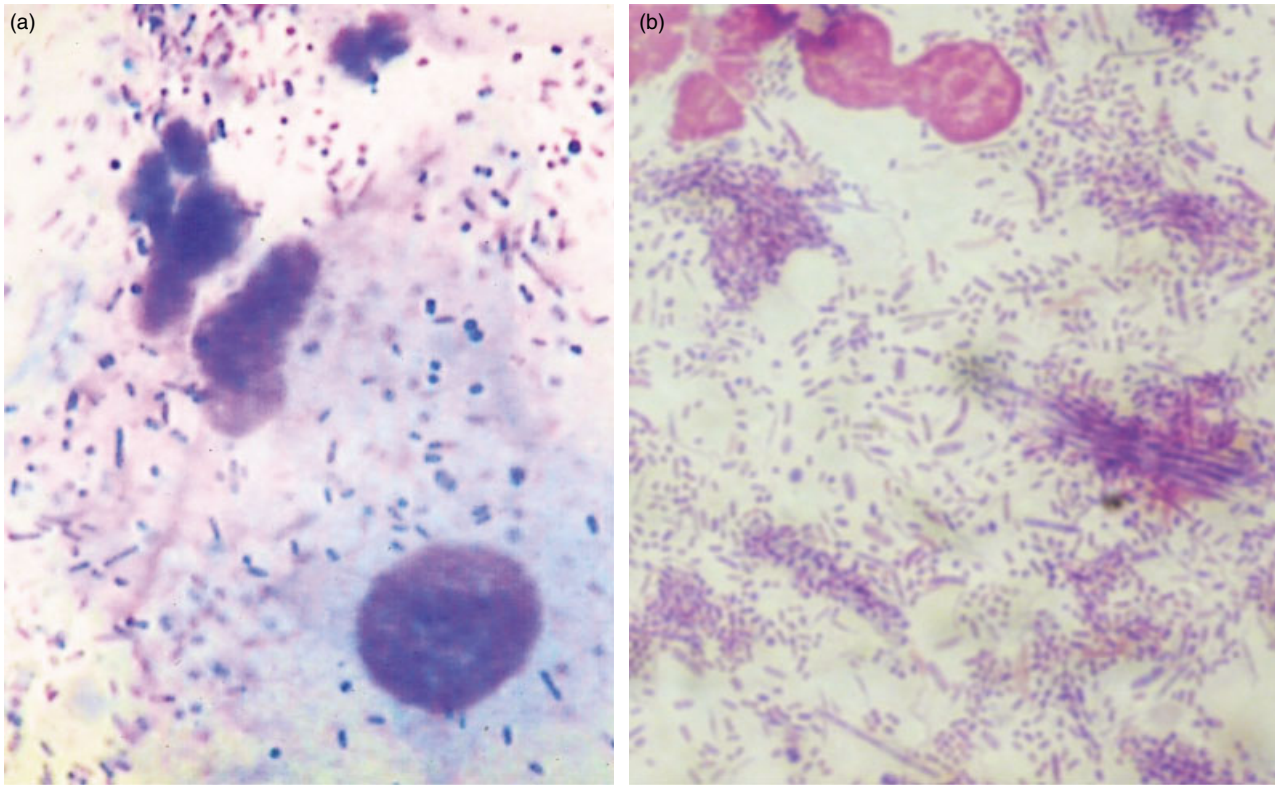

Figure 1. (a) Ear cytology showing planktonic bacteria with a variety of organisms. (b) Ear cytology showing aggregates of bacteria.

Craig Griffin aggressively cleans ears when a biofilm is suspected. Cleaning and utilizing a combination of cleansers is his typical approach to treating these infections. He asked what others were doing to treat biofilms.

Participant stated mucolytic agents.

Craig Griffin agreed and flushes ears with *N*-acetylcysteine whenever he believes a biofilm is present.

Sue Paterson stated that she uses colloidal silver.

Craig Griffin uses silver as well, specifically micronized silver.

Sue Paterson asked whether people were using *N*-acetylcysteine topically, systemically, or both?

Lynette Cole (*USA*) uses *N*-acetylcysteine orally. When used topically for previous cases of biofilm associated with *Pseudomonas*, she didn't believe it improved the success of her treatment.

Craig Griffin cleans the ear thoroughly and then instills a 1–2% solution of *N*-acetylcysteine. He lets it soak for 2 min, and then rinses the *N*-acetylcysteine out with a product like tromethamine (Tris-EDTA) prior to instilling a topical antibiotic.

Jacques Fontaine uses *N*-acetylcysteine topically for otitis externa and occasionally for otitis media. He dissolves a 600 mg tablet in approximately 40–50 mL of water and uses this as a flush for 30 min. Sometimes he follows this with another ear-cleansing product, and also treats with an anti-inflammatory/antibacterial product.

To clean or not to clean ears (P. Bloom)

Paul Bloom (*USA*) introduced himself and the reasoning for his lecture on ear cleaning in dogs. As specialists who train and teach veterinarians to manage otitis in general practice, he wanted to know when and how often specialists clean ears.

He asked: *Do you have puppy owners perform ear cleaning?*

1 Yes (4%)
2 Yes but only in breeds at risk for otitis externa (20%)
3 No (76%)

Do you have owners perform ear cleaning in adult dogs?

1 Yes, since they were a puppy (0%)
2 Only after treatment, as maintenance (8%)
3 During treatment and as maintenance (58%)
4 After bathing (0%)
5 After swimming (0%)
6 I don't routinely recommend this (33%)

How often do you have owners clean ears for routine maintenance?

1 Daily (0%)
2 Twice weekly (10%)
3 Weekly (30%)
4 Monthly (4%)
5 It depends on the case (40%)
6 I don't routinely recommend this (16%)

Do you have groomers or owners routinely pluck hairs from ears when no infection is present?

1 Yes (12%)
2 Yes but only in breeds at risk for otitis externa (6%)
3 Yes but only if they have had an episode of otitis externa (15%)
4 No (67%)

Craig Griffin presented unpublished data from a study in which he collaborated with **Kacie Stetina** and **Stan Marks**. In this study of 314 healthy dogs, owners were asked if they cleaned their dog's ears and, if so, what were their reasons for cleaning. Out of 314 dogs, 146 (47%) owners said they cleaned their dog's ears. Of the 146 owners that cleaned ears, 97 (30.9%) did it for maintenance, 39 (12.4%) did it because the ears would get dirty, fill with debris and/or smell, and 10 (3.2%) cleaned ears because their veterinarians told them to. It was very surprising that such a large percentage (47%) clean their dog's ears. Even more interesting were the reasons for cleaning ears. Only 3.2% of owners did it because their veterinarians told them to. The great majority of owners cleaned ears because they believed they should. Based on this survey, there may be many dogs with allergic ear disease that don't present clinically because owners are cleaning their ears and managing the symptoms on their own.

Paul Bloom asked: *Has ear cleaning resolved or prevented otitis externa?*

1 Yes (47%)
2 No (24%)
3 Not sure (29%)

When do you introduce maintenance ear cleaning in adult dogs that experience an episode of otitis externa?

1 After the dog's first ear infection (20%)
2 Only if there are recurrent episodes of otitis (65%)
3 I don't routinely recommend this (13%)

How often do you instruct owners to clean ears as a preventative or maintenance therapy?

1 Daily (0%)
2 Twice weekly (10%)
3 Weekly (40%)

4 It depends on the case (34%)
5 I don't routinely recommend this (16%)

How do you decide the frequency of ear cleaning during treatment and/or maintenance?

1 It depends on the amount of proliferative changes present (2%)
2 It depends on the amount of discharge present (34%)
3 Combination of 1 and 2 (46%)
4 It depends on the case (reasons other than above) (17%)

How do you decide which ear cleanser to use?

1 By the amount of otic discharge (5%)
2 By the type of otic discharge (79%)
3 Same cleanser is used for all cases (12%)
4 I don't routinely recommend ear cleaning (5%)
5 Whichever company has the best deal (0%)

How do you assess if ear cleaning is effective?

1 By frequent rechecks (73%)
2 By owner feedback (10%)
3 I have no clue if it is effective (6%)
4 I don't recommend ear cleaning (10%)

Topical ototoxicity (S. Paterson)

Sue Paterson stated that while ototoxicity can occur through oral or parenteral administration of drugs, the focus of her presentation was on topical ototoxicity occurring through a ruptured tympanum and absorption through the round window. Interested in finding a way for veterinarians in primary care practice to assess hearing loss, she referred to a study she conducted with **Carly Mason**.[5] Owners completed a questionnaire to assess their dog's responses to common household noises. Owner assessments of hearing were then matched with results from brainstem auditory-evoked response measurements (BAER). The hearing loss was graded using the World Health Organization (WHO) classification system. Utilizing this system, grade 0 ≤ 25 dB (no impairment), grade 1 = 26 – 40 dB (mild impairment), grade 2 = 41 – 60 dB (moderate impairment), grade 3 = 60 – 80 dB (severe impairment) and grade 4 ≥ 81 dB (profound impairment).

Sue Paterson asked: *How much hearing loss needs to occur for an owner to detect it?*

1 Complete hearing loss in one ear (8%)
2 Grade 4 hearing loss in both ears (27%)
3 Grade 3 hearing loss in both ears (59%)
4 Grade 2 hearing loss in both ears (6%)

Based on her study, owners could not tell if a dog was deaf in one ear nor could owners detect whether grade 1 bilateral hearing loss was present. Dogs had to have quite profound hearing loss between 41 and 60 dB (grade 2) before owners could perceive it.

Sue Paterson asked: *Is there a way to clinically detect unilateral hearing loss?*

1 Yes (22.5%)
2 No (52.5%)
3 Sometimes (25%)

Sue Paterson asked: *Which of these ceruminolytic agents do you think is safe to use based on our current knowledge of these particular products?*

1 Carbamide peroxide (0%)
2 Sodium docusate (11%)
3 Squalene (69%)
4 Triethanolamine (20%)

Sue Paterson presented a study that investigated the effects of squalene, dioctyl sodium succinate, carbamide peroxide and triethanolamine on the middle ear.[6] In this study, each product was instilled into the middle ear of dogs and guinea pigs via myringotomy. Animals were then sacrificed and the untreated ear was used for comparison in each case. Only the ear flushed with squalene failed to show neurological or morphological damage. These results suggest squalene to be a safe product. When a patient is under anaesthesia she instills squalene into the middle ear, lets it soak for 10–15 min, then flushes it out. She uses a 22% squalene product.

Rod Rosychuk (*USA*) also uses squalene routinely in the middle ears of dogs and cats. He often injects it through a catheter as deep as he can get into the middle ear and agrees that it disperses the debris nicely.

Sue Paterson commented that during treatment of a dog with purulent otitis or *Pseudomonas* otitis, the flushing agents she would normally use include tromethamine (Tris-EDTA), chlorhexidine and lactic acid.

She asked: *What is the highest level of chlorhexidine believed to be safe for use in the middle ear of dogs?*

1 It is not safe at any percentage (33%)
2 0.15% (54%)
3 0.5% (8%)
4 1.0% (4%)

Sue Paterson reviewed a series of studies in cats suggesting marked ototoxicity for vestibular and cochlear function at 2% chlorhexidine.[7–9] Fewer ototoxic reactions occurred at 0.05% chlorhexidine but vestibular damage was reported. Damage to the mucocillary clearance mechanism in the tympanic bulla was also described. She reviewed another study examining the safety of chlorhexidine at low concentrations in dogs.[10] In that study, 0.2%

chlorhexidine was instilled into canine ears after experimental myringotomy and no significant abnormalities were detected. Her experience is that low concentrations of chlorhexidine (0.15%) and tromethamine (Tris-EDTA) are very safe. She routinely flushes middle ears with proprietary ear cleaners containing those ingredients. Lactic acid appears to be ototoxic. She has seen several cases where it had been used in dogs and cats with damaged tympanic membranes and it caused deafness.

Dilated pars flaccida (R. Rosychuk)

Rod Rosychuk began his lecture with a case report on Jackson, a 7-year old male castrated yellow Labrador. This dog had a 4 year history of seasonal (spring, summer, fall) allergic otitis externa that responded well to topical therapy. His left ear had always been more significantly affected and seemed more bothersome over the past 4 months. Diagnosed with a secondary *Malassezia* infection, he showed a partial response to Mometamax® (Merck, NJ, USA) and TrizUltra Plus Keto (DermaPet®; Potomac, MD, USA) flushes. Two weeks previously a mass had been visualized in the left ear. Examination revealed a mass (Figure 2).

Looking at Figure 2, what is the most likely cause of this mass-like structure?

1. Ceruminous gland cyst (20%)
2. Neoplasia 'of some sort' (6%)
3. Inflammatory/fibrous aural polyp (18%)
4. Dilated fluid/debris filled pars flaccida associated with an otitis media (42%)
5. 'Bulging' pars tensa (2%)
6. Associated with a fluid-/debris-filled otitis media (12%)

CT images were shown (Figure 3). The scan revealed evidence of a chronic otitis media with proliferative changes and a fluid- or tissue-filled density in the middle ear.

What would you do next to work up/treat this problem?

1. Use biopsy forceps to obtain sample for histopathology (2%)
2. Use grabbing forceps to attempt to 'pull out' the mass (2%)
3. Needle aspirate and cytology (38%)
4. Probe with catheter or ear loop to determine attachment site and consistency (58%)

Rod Rosychuk played a video of Jackson's ear being examined under anaesthesia. The mass was probed and fixed dorsally whereby a catheter could be passed beneath it. A needle was inserted into the dilatation and the material within it was flushed out. Removal of the material minimized the prominence of the structure and helped to identify it as a dilated pars flaccida. The material from within the middle ear was histologically identified as a cholesterol granuloma, which is a very common change associated with chronic otitis media in dogs (Figure 4). Figure 5 shows the canal after flushing of the middle ear. Jackson was ultimately diagnosed with a dilated, fluid-/debris-filled pars flaccida with concurrent active otitis media. Many patients with a dilated pars flaccida are incorrectly diagnosed as having cysts. The 'cyst' is a part of the tympanum that has developed significant pathologic changes. Figure 6 shows the pathologic changes that occur as a result of chronic inflammation. The respiratory-like epithelium on the medial aspect of the pars flaccida becomes more squamous-like. Communication between

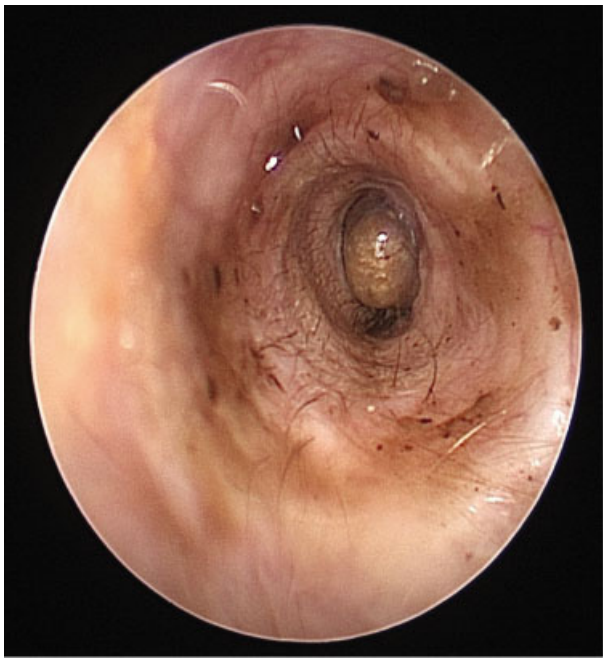

Figure 2. Structure visualized deep in the horizontal canal.

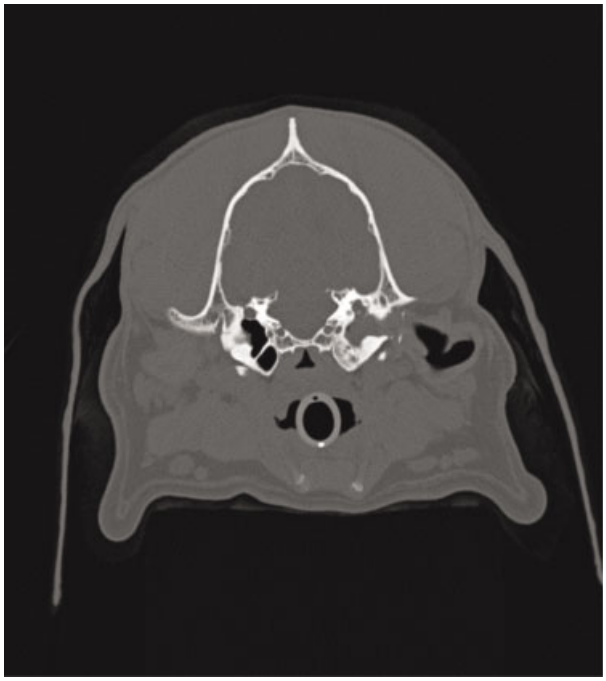

Figure 3. CT revealed a mass occluding the horizontal canal. Proliferative changes suggested a chronic otitis media with a fluid- or tissue-filled density in the middle ear.

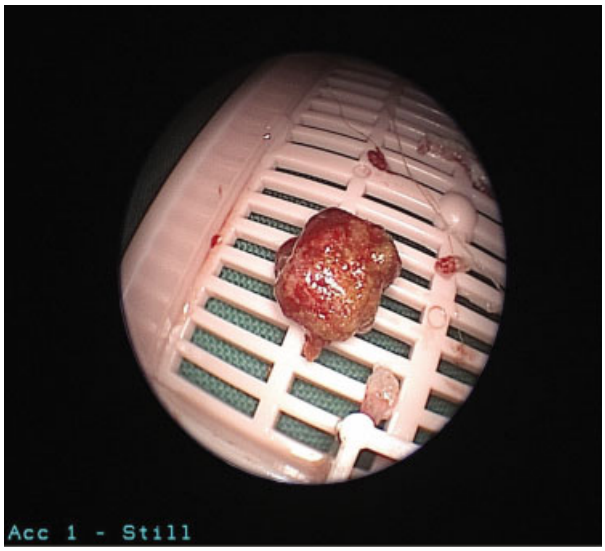

Figure 4. Material removed behind the dilated pars flaccida and within the middle ear revealed a cholesterol granuloma. Such formations are a common change with otitis media.

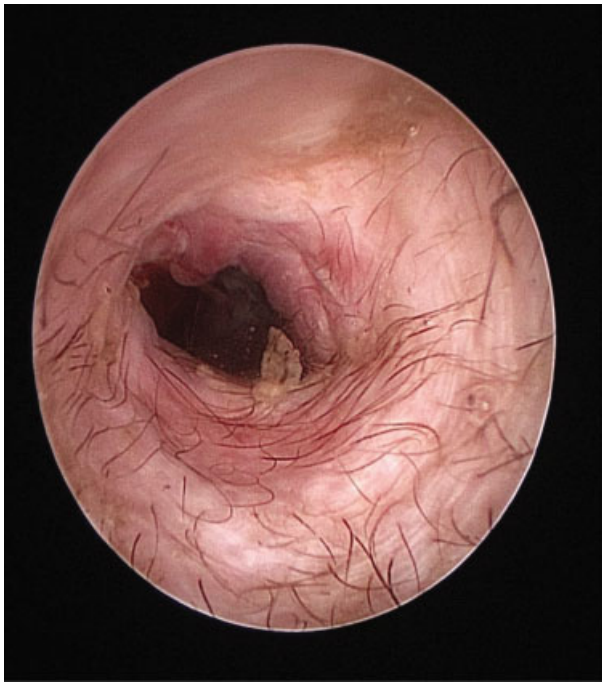

Figure 5. Ear canal post flush. The pars flaccida is seen on the dorsal canal wall and the middle ear is now free of debris and discharge.

the middle ear and pars flaccida can even be lost due to fibrosis (Figure 7).

Craig Griffin concluded the workshop and thanked the audience for their participation. The interactive nature of the workshop showed how specialists around the world approach and treat various aspects of otitis from ear cleaning to biofilms. Through **Sue Paterson's** owner-based hearing questionnaire it was established that dogs had to have quite profound hearing loss before owners could perceive a hearing deficit. Squalene and low concentrations of chlorhexidine (0.15%) and tromethamine (Tris-EDTA) were deemed safe ceruminolytic agents to instill into middle ears. In chronic cases of otitis media, **Rod Rosychuk** discussed how patients can develop such severe pathologic changes to their pars flaccida that they may be mistakenly diagnosed as cysts.

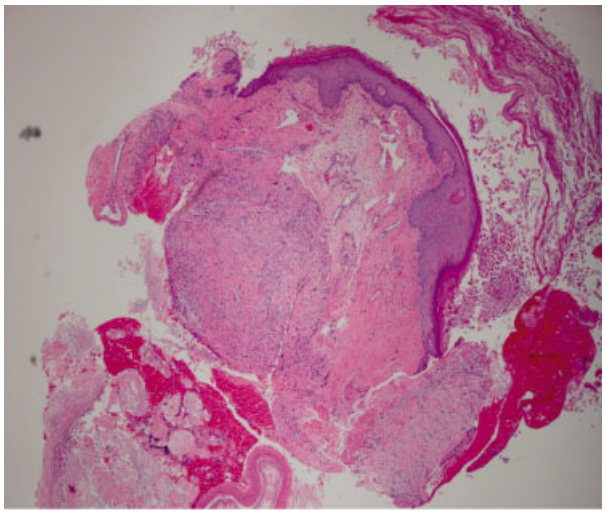

Figure 6. Histopathology of a chronically dilated pars flaccida. The pars flaccida thickens as a result of chronic inflammation and the respiratory epithelium on the medial aspect becomes more squamous.

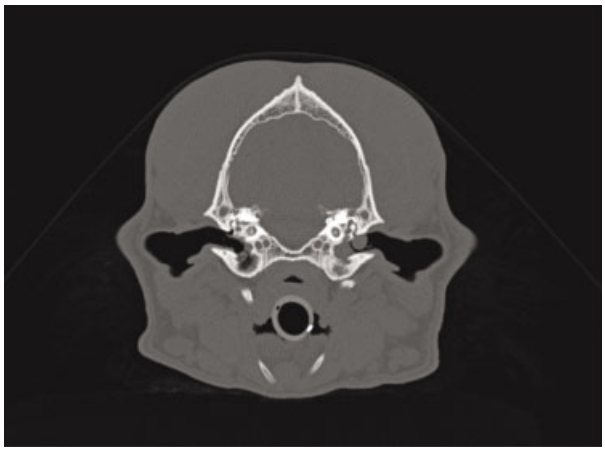

Figure 7. CT scan showing a chronically dilated pars flaccida that became fibrosed in such a way as to become isolated, with loss of communication with the middle ear.

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Immune-modulating drugs in cats: new perspectives and old favourites

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Amanda Burrows (*Australia*) welcomed the audience to the workshop and introduced the four speakers (Petra Bizikova, Samantha Lockwood, Christian Ortalda and Patrizia Pandolfi).

Feline pemphigus foliaceus: retrospective case study and systematic literature review (P. Bizikova)

Petra Bizikova (*USA*) began her presentation by stating that pemphigus foliaceus (PF) is the most common autoimmune skin disease in cats. Her goal was to summarize the clinical features and treatment outcomes from a systematic literature review (165 cats) and a retrospective evaluation of medical records of cases of feline PF from the dermatology service at North Carolina State University (NCSU) (19 cats) for a total of 184 cats evaluated. All cats exhibited a superficial pustular and/or crusting dermatitis with acantholysis confirmed histologically. The mean age of onset of affected cats was 7 years with a male/female ratio of one. Lesion distribution was reported for 166 cats. Lesions were symmetrical in 92% of affected cats and involved either a single region (28%) or multiple (two or more) regions (72%). The head was affected in 81% of cats with lesions affecting the pinnae, nasal planum and eyelids in 77, 35 and 14% of cats respectively. Claw folds and footpads were affected in 54 and 22% of cats respectively. Paronychia was the only presenting clinical sign in 10% of cats. The peri-areolar region was affected in 8% of cats. Approximately 50% of the cats demonstrated pruritus. Lethargy and fever were reported in 37 and 26% of affected cats respectively. Treatment outcome was known for 158 cats. Complete remission was reported for 146 cats (92%) with a mean time to remission of 39 days. Immunosuppressive drug treatment at the time of complete remission was reported for 97 cats and included either oral glucocorticoid monotherapy (55%) or combination drug therapy (38%). High-dose pulse glucocorticoid therapy did not appear to offer any advantage in treating cats with PF. Complete withdrawal of drug therapy was achieved for only 17% of cats. In summary, cats with PF exhibit symmetrical skin lesions most frequently affecting the head and feet. Disease remission can be achieved for a majority of cats in a relatively short time-frame; however, complete drug withdrawal is uncommon.

Kim Boyanowski (*USA*) asked why oral triamcinolone was not used in the high-dose pulse glucocorticoid therapy protocol at NCSU.

Petra Bizikova responded that there was controversy about the potency of triamcinolone compared to other glucocorticoids and that she had wanted to ensure that a consistent dosage for standard pulse therapy (i.e. prednisolone at 10 mg/kg) was being utilized when she began using the NCSU protocol. Subsequent to this, however, she has gained confidence that triamcinolone could be used for pulse glucocorticoid therapy.

Natalie Van Hoe (*Belgium*) asked about the success rate when using ciclosporin as monotherapy for the management of feline PF.

Petra Bizikova responded that there was only a single case with ciclosporin monotherapy in her study and the cat achieved complete remission. She indicated that she does not routinely use ciclosporin for monotherapy of feline PF. In a practice setting, she would recommend commencing treatment with oral corticosteroids for rapid effect administered concurrently with ciclosporin, and then tapering the dose of corticosteroids when the cat achieved remission.

Christoph Klinger (*Germany*) asked if there was any information regarding the use of other immunodulatory drugs, such as pentoxifylline, for the management of feline PF.

Petra Bizikova responded that there was no such information that she was aware of in the veterinary literature.

Amanda Burrows asked if anyone from the audience had experience using pentoxifylline for the management of feline PF and there were no responses.

Samantha Lockwood (*USA*) asked if cats receiving combination drug therapy in the study commenced treatment with both drugs or initially just received oral glucocorticoids.

Petra Bizikova responded that many cats received combination drug therapy from the onset of treatment. In contrast to dogs, cats respond rapidly to glucocorticoid monotherapy and in the majority of cases cats achieve remission within a month. She cited a published case series evaluating glucocorticoid monotherapy for the treatment of feline PF.[1]

Amanda Burrows indicated that the author of the aforementioned study, Debbie Simpson, was an invited guest and in the audience.

Frequency of urinary tract infections in feline patients with dermatologic disease receiving long-term glucocorticoids and/or ciclosporin (S. Lockwood)

Samantha Lockwood presented a summary of her study investigating the frequency of urinary tract infections (UTIs) in cats receiving long-term glucocorticoid and/or ciclosporin therapy for dermatologic disease compared to normal cats. Dogs receiving long-term glucocorticoids or ciclosporin have been demonstrated to have an increased frequency of UTIs. No studies had previously investigated the frequency of UTIs in cats receiving long-term glucocorticoids and/or ciclosporin. Thirty-three cats being treated for dermatologic disease with oral glucocorticoids and/or ciclosporin for longer than 3 months or having received at least two injections of long-acting glucocorticoid treatment within the previous 6 months were included. The majority of cats were being treated for allergic skin disease. Thirty-four normal cats were used as a control group. In all cats, blood samples were collected and a complete blood count (CBC) and serum biochemical (SBC) profile performed. A urine sample was collected by cystocentesis and a urinalysis and urine culture performed. In the study group, all urine samples were negative for bacterial growth. In the control group, 1/34 cats had bacteria isolated on urine culture. In this study, there was no evidence to suggest that receiving long-term glucocorticoids and/or ciclosporin was a risk factor for UTIs in cats.

Petra Bizikova asked if the cats received different types of glucocorticoid drug therapy.

Samantha Lockwood responded that the cats received oral prednisolone, methylprednisolone or triamcinolone, or injections of dexamethasone sodium succinate or methylprednisolone acetate (Depo-Medrol®).

Petra Bizikova commented that the type of glucocorticoid should be recorded along with the mean dose of glucocorticoid medication administered.

Meng Siak (*Australia*) asked if there were selection criteria applied with regard to the length of time that glucocorticoid and/or ciclosporin therapy had been administered.

Samantha Lockwood responded that the minimum period of drug administration was 3 months.

Christina Gentry (*USA*) asked if the cats received antibiotic therapy prior to or during the study.

Samantha Lockwood responded that antibiotics had to be withdrawn for at least 1 month before inclusion in the study and that no cat received antibiotic therapy during the study.

The use of oclacitinib for allergic pruritus in cats: an overview (C. Ortalda)

Christian Ortalda (*Italy*) presented an overview of the use of oclacitinib for the management of pruritic cats. In a previous open study, oclacitinib decreased pruritus and skin lesions in five of 12 allergic cats at a dosage of 0.4–0.6 mg/kg once daily.[2] In this preliminary double-blind randomized controlled study 11 cats with feline atopic dermatitis received oclacitinib at a dosage of 0.7–1.3 mg/kg twice daily and seven control cats received oral methylprednisolone at a dosage of 0.5–1 mg/kg twice daily. SCORing Feline Allergic Dermatitis (SCORFAD) lesion and pruritus visual analogue scale (PVAS) scores decreased markedly and quality of life (QoL) scores improved moderately in both groups. For all parameters, the improvement was more significant in the control cats receiving methylprednisolone than those cats receiving oclacitinib. Ninety percent of cats receiving methylprednisolone improved their lesional scores by greater than 50% compared to 60% of cats receiving oclacitinib. While 60–70% of cats in the oclacitinib and methylprednisolone groups demonstrated a greater than 50% reduction in pruritus scores, respectively, only 25% of cats receiving oclacitinib acquired a low pruritus score (<2) compared with approximately 60% of cats receiving methylprednisolone. No adverse events were recorded and no significant abnormalities were observed in CBC and SBC profiles. The conclusion was that oclacitinib demonstrates a rapid onset of action and good efficacy for decreasing pruritus and clinical signs of feline allergic dermatitis for many cats. Oclacitinib may be an option for the treatment of feline allergic skin disease.

Head and neck feline dermatitis: response to oclacitinib treatment (M. Beccati and P. Pandolfi)

Patrizia Pandolfi (*Italy*) presented a non-randomized, open clinical study evaluating the management of pruritus and clinical signs in 15 cats referred for an allergic or idiopathic head and neck pruritus that had failed to respond to alternate antipruritic therapies. Oclacitinib was administered at a dose of 0.5–0.8 mg/kg twice daily for 14 days reducing to 0.5–0.8 mg/kg once daily for 14 days. Clinical lesions were evaluated using the SCORFAD system and the intensity of pruritus was measured using the PVAS scale. Ten cats demonstrated a reduction in both pruritus and lesion scores and two cats failed to demonstrate any improvement. Three cats were withdrawn from the study due to difficulty in administering the tablets. The conclusion was that oclacitinib may be useful for the management of pruritus and clinical lesions for cats with an allergic/idiopathic head and neck pruritus.

Isabel Bisse (*Germany*) asked if any behavioural side effects were noted with the administration of oclacitinib. She had observed one cat that displayed aggression and another cat that experienced lethargy with the use of this drug.

Amanda Burrows asked what doses of oclacitinib were being used.

Isabel Bisse replied that oclacitinib had been dosed at 0.5 mg/kg twice daily for the cat that displayed aggression and 0.5 mg/kg once daily for the cat that developed lethargy.

Patrizia Pandolfi responded that she had not observed behavioural side effects with the use of oclacitinib in cats.

Amanda Burrows commented that anecdotal reports of aggression being displayed by dogs receiving oclacitinib had been shared on the Derm Listserv and discussed in Wayne Rosenkrantz's WCVD8 workshop (see Workshop 7.2 in this volume). She had observed one case of reversible aggression displayed by a dog receiving oclacitinib.

Petra Bizikova commented that there was at least one research group evaluating the potential influence of oclacitinib on brain signalling and that there was potential for the drug to change endorphin levels or similar factors that could affect behaviour.

Astrid Thelen (*Germany*) asked how long corticosteroids were withdrawn for prior to the administration of the oclacitinib in the study cats.

Patrizia Pandolfi responded that all corticosteroids had been withdrawn for 1 month.

Petra Bizikova commented that, from the data presented, it was evident that all the flea-allergic cats had responded to oclacitinib; she asked if the cats received flea control during the study.

Patrizia Pandolfi responded that the cats that were receiving flea control at the onset of the study remained on the same endectocide but no new flea control was introduced.

Amanda Burrows asked the audience if they had utilized oclacitinib for the management of pruritic skin disease in cats and about half the audience responded in the affirmative. She suggested that because the drug was a relatively new treatment option and unregistered for cats, it perhaps was being used selectively for cats that had failed conventional and registered treatments. She asked the audience if anyone had observed other adverse events with the use of oclacitinib in cats apart from hyperactivity and aggression.

Christian Ortalda commented that he had one cat (ragdoll breed) in his first study that presented with nystagmus after receiving 6 months of oclacitinib therapy. The owner refused to withdraw the drug as it was the only treatment effective for control of pruritus.

Amanda Burrows commented that she had observed cats that developed mild gastrointestinal adverse events but which were not significant enough for owners to withdraw the drug.

Samantha Lockwood asked if the audience had noted any difference in the level of pruritus exhibited by cats when oclacitinib was reduced from twice a day to once a day dosing.

Christian Ortalda commented that in his first study the only cat in which he reduced oclacitinib to once a day dosing did not flare.[2]

Amanda Burrows asked if he would use oclacitinib as the first option for the management of feline allergic pruritus in his practice.

Christian Ortalda replied that he would still use oral corticosteroid therapy as the first option at present; following completion of his current study he will have further data regarding the use of oclacitinib in cats.

Amanda Burrows asked if the twice-a-day dosing posed a burden to owners.

Christian Ortalda replied that the oclacitinib caplet was easier to administer than ciclosporin capsules and that in general owners did not have a problem with twice-a-day dosing.

Natalie Barnard (*UK*) asked how the cats' QoL was assessed.

Christian Ortalda replied that they used a validated questionnaire of 14–15 questions assessing both the cat's and owner's QoL.[2]

Amanda Burrows commented that she was interested in the measurement of QoL and how owning an atopic dog or cat impacts owner QoL indices. In atopic children there has been evidence that QoL indices could improve for the patient but the burden and costs of care could result in a concurrent reduced QoL score for parents and families.[3]

Amanda Burrows asked the audience for any further comments or discussion and thanked the presenters and attendees for their participation.

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7.7

Topical therapy for atopic dermatitis. A practical option? Are expectations met?

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Valerie Fadok (*USA*) opened the workshop by describing the importance of topical therapy and shared her data from an open-label study on the effect of a sodium hypochlorite shampoo for the management of canine pyoderma. The study was stimulated by the use of diluted bleach baths in human patients with atopic dermatitis (AD) to improve clinical signs and decrease staphylococcal colonization. A product containing sodium hypochlorite with salicylic acid (Command™ Therapeutic Shampoo for Animals; VetriMAX, College Station, Texas, USA) was assessed for the treatment of canine pyoderma in atopic dogs. The study focused on the efficacy of the shampoo in methicillin-resistant staphylococcal pyoderma. Other therapies needed to control signs of AD were allowed and owners were asked to bathe their dogs three times per week. Dogs were re-evaluated after 2 and 4 weeks. No antibiotics or other shampoos were allowed during the study. At each visit dogs were photographed and cytology was done to assess bacterial load. Both the owner and the investigator made assessments. There was no control group. A client questionnaire with a scoring system of 0–3 for redness, crusting, odour, percentage of the body affected and severity of itch was used. Nineteen patients were enrolled and 17 completed the study. Out of this population of 19 dogs, 17 dogs were infected with methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) and two were infected with methicillin-sensitive staphylococci. Two patients dropped out; one each in the methicillin-resistant and -sensitive groups; of these, one dog dropped out because it was doing so well that the owner did not feel the need to come back for a recheck and the other one because of worsening pyoderma. After 2 weeks of bathing, a significant decrease in number of bacteria was found on cytology and by 4 weeks most dogs had no bacteria. In terms of clinical assessment, there was a statistically reduced clinical score at 2 and 4 weeks compared to baseline but no difference between weeks 2 and 4. The shampoo was well tolerated by the dogs and owners. The formulation was 5 ppm sodium hypochlorite without considering the dilution factor of the additional water used during bathing. Comments were made that mechanical removal of bacteria during bathing may also be a method to disrupt biofilm formation.

Dunbar Gram (*USA*) shared the results of an online survey of 822 dermatology clients at the University of Florida focused on bathing frequencies and preferences. Surveys were returned by 253 clients. Seventy-five percent of clients reported bathing their dogs at least once a month and 50% reported bathing their dogs every 1–2 weeks. The majority (81%) of the respondents never bathe their cat. About half of the respondents were willing to pay $20–50 to have their dog bathed by someone else. About two-thirds of the pet owners had an interest in having the bathing done at a veterinary hospital, highlighting the importance for veterinarians to offer a bathing service. Routine interaction with pet owners as they come in to have their pets bathed could help with following a schedule for topical therapy. More than half of these pet owners were interested in a package deal which included other services such as nail trims and ear cleaning or a discount for the pre-purchase of multiple visits.

Ralf Mueller (*Germany*) commented that some practitioners in Australia simply make a facility available for bathing on the weekend. Pet owners bathe their dogs themselves but prefer to pay a small fee to do it at another facility so that there is no mess at home. Comments were made about contact time and how some people recommend 5 min, others 10–15 min. Currently there is no study to support one recommendation over another for length of contact time.

Topical antimicrobial therapy has also been applied to treatment of canine demodicosis. A study was reviewed in which over 50 dogs were enrolled with half randomized into a group that was treated with benzoyl peroxide shampoo and ivermectin, and the other half were treated with benzoyl peroxide shampoo, ivermectin and oral antibiotics.[1] There was no difference in the time to negative skin scrapes between the two groups.

Ralf Mueller reviewed several studies involving topical antibacterial therapy. The first study evaluated a chlorhexidine shampoo (Douxo®; Ceva Animal Health, Lenexa, KA, USA) versus placebo in the treatment of canine pyoderma.[2] Dogs were shampooed weekly; cytology and corneocyte bacterial counts were assessed before and after treatment with the placebo or chlorhexidine. There was a significant decrease in bacteria in both groups. The chlorhexidine shampoo decreased counts more but the difference at the end of the study between the placebo and chlorhexidine group was not statistically different. This suggests that mechanical removal of material with bathing is important regardless of the shampoo used.

The second study investigated a variety of antibacterial shampoos (four chlorhexidine shampoos in varying concentrations from varying manufacturers, benzoyl peroxide shampoo and a placebo control).[3] Forty dogs were bathed with two shampoos each: the left side with one, the right side with the other. Hair was sampled after 4 weeks of twice-weekly shampooing. When hairs were incubated on agar with *Staphylococcus* cultures, very large inhibition zones occurred around some hairs. This seemed to indicate that bacterial growth was inhibited in hair from some dogs after topical therapy. This suggests some residual antibacterial effect on hair shafts after bathing but this effect varies dramatically between shampoos. For example, there were four chlorhexidine shampoos, two of which had very large inhibition zones and two of which had very small inhibition zones. To make it more confusing, success was not necessarily related to the concentration of chlorhexidine in the shampoos. The formulation as well as the ingredients may be important. Chlorhexidine is a challenging ingredient to keep in solution as it tends to deposit on the bottom on the bottle.

Ralf Mueller reported on an unpublished study that used a titanium shower head with holes that produce foam-like water. It was theorized that this type of treatment might cause water to push oxygen into the tissue. This additional oxygen could potentially increase healing, decrease pruritus and have antibacterial activity. The recommended shampoo with the device contained basic cleansing ingredients. Twenty dogs were enrolled in the trial and 10 of them also had pyoderma. For relief of pruritus, about 10% did very well and another 20–30% had mixed results.

The antipruritic effect of shampoo with dogs in a whirlpool was compared to dogs bathed with the same shampoo in a traditional bathtub environment.[4] This single-blind crossover study found that the effect of the whirlpool with the shampoo was clearly better than bathing in the tub alone. The shampoo without the whirlpool was better than the whirlpool with water alone. However, the whirlpool with water alone also had a positive effect on the dogs. This indicates that hydration alone and the removal of skin debris and allergens may lead to clinical improvement. The degree to which it helps varies from dog to dog and water alone could be helpful.

Ralf Mueller reviewed another study looking at an antipruritic shampoo compared to a vehicle control.[5] There was significant improvement of pruritus in both groups, but there was no statistically significant difference between the placebo and shampoo group. Thus, a placebo effect should be considered when clients report that a shampoo product was effective. One important consideration is the dramatic difference in individual animal response to shampoo products; there is not one shampoo that serves all dogs.

Stephany Peng (*Thailand*) asked how often bathing is recommended for antipruritic effect.

Ralf Mueller replied that this depends on the owner and the severity of the dog's pruritus. The frequency can be three times per week on a regular basis. For clients with time and who are often willing to do it, this can be a positive bonding experience. Most owners bathe twice weekly or weekly due to time constraints. Many dogs will acclimate to the regular bathing over time even if they are not initially amenable to the process.

The use of topical corticosteroids was reviewed next. Cortavance® (Hydrocortisone Aceponate Topical Spray; Virbac, Carros, France) is considered the topical steroid of choice because it does not cause systemic effects.

Kerstin Bergvall (*Sweden*) was mentioned as someone who started using this product for otitis treatment and prophylaxis. She has also used Cortavance® for the treatment of *Malassezia*-infected ears. This may sound counterintuitive as steroids are immunosuppressive, so one might expect that infection could worsen. However, if inflammation decreases then ceruminous gland secretion decreases and *Malassezia* numbers normalize. Cortavance® should only be used in ears with intact tympanic membranes because of unknown effects of this drug and product vehicle in the middle ear. Many patients relapse when therapy is discontinued, so Cortavance® can be applied prophylactically. This seems to work very well in patients with chronic atopic otitis and remission often occurs with only once or twice weekly treatment in both ears.

Johan Van Leuven (*Belgium*) asked how much Cortavance® should be applied to the ear canal.

Ralf Mueller stated that 0.1–0.5 mL is typically used.

Vanessa Schmidt (*UK*) addressed the concept of proactive therapy. AD is a multivariable/multifactorial disease that requires a multimodal treatment plan. This involves preventive care, allergen avoidance and immunotherapy, avoiding flare factors, ectoparasite control and use of anti-inflammatory therapy. The aim of anti-inflammatory drugs is to reduce skin inflammation and avoid the peak and trough cycles of inflammation since AD is a naturally waxing and waning disease. The overall goal is to restore the normal skin environment and prevent microbial overgrowth and ongoing infections. Once the patient is stabilized, it is best to reduce therapy to the lowest dose and frequency possible for long-term remission. This approach has been used in the human field for a long time and is known as 'proactive intermittent therapy'. Many owners stop using treatments once the skin starts looking normal and the dog is stable. Regardless of what the skin looks like, owners are encouraged to continue with intermittent proactive therapy on a regular basis to prevent or decrease the severity of flares.

Vanessa Schmidt reviewed a double-blind placebo-controlled study to evaluate the long-term maintenance use of hydrocortisone aceponate topical spray. The dogs recruited in this study were allocated to placebo or hydrocortisone aceponate groups.[6] Dogs were allocated after they had been treated and were regarded as being stable or having a very low Canine Atopic Dermatitis Extent and

Severity Index (CADESI) score. All dogs had a clinical diagnosis of AD with a compatible history, an appropriate clinical picture and exclusion of other conditions. All the dogs in the study had positive intradermal skin tests. Dogs were randomized into two placebo and two hydrocortisone aceponate groups. Once they were stable they were treated with placebo or hydrocortisone aceponate twice a week. Owners filled in questionnaires to provide feedback during trial. To improve compliance, regular phone calls were also made to the owners and the bottle was brought back to the practice to be weighed at the end of the trial. The outcome of the study was to look for recurrence of clinical symptoms measured as the median time until relapse. The time to relapse was significantly longer in the hydrocortisone aceponate group (median 115 days) compared to the placebo group (median 33 days). At day 50, more than 85% of the dogs in the placebo group had relapsed. There were four dogs lost to follow up and four dogs withdrawn. Dogs were withdrawn because they needed medications that were not allowed on the study, to treat other diseases. There were no adverse effects attributed to treatment. This study supports the use of proactive anti-inflammatory treatment in atopic canine patients when the skin appears normal and the patient is stable to prevent the frequency of flares and reduce the severity of flares when they occur. Vanessa Schmidt currently uses hydrocortisone aceponate on localized pruritic areas in addition to systemic anti-inflammatory drugs.

Vanessa Schmidt also reviewed a study that evaluated the use of Cortavance® to treat 'hot spots' (acute moist dermatitis). Other conditions where she uses the product include sterile interdigital nodules; localized lesions around the face, muzzle, axillae or lip folds; skin fold dermatitis; and perianal pruritus not associated with the anal sac involvement, especially in atopic patients. She also reviewed the use of hydrocortisone aceponate in the ears. When looking at otitis relapse, they found that in the placebo group (weekly cleaning only) the median time to relapse was 90 days with 50% chance to remain free of relapse. When using the hydrocortisone aceponate on two consecutive days (in addition to weekly cleaning), the median time to relapse was 180 days with a 95% chance to remain free of relapse.

Johan Van Leuven asked, what was in the placebo?

Vanessa Schmidt confirmed that the placebo contained the base without any active ingredients. She suggested that the base may have had some antibacterial properties.

Genevieve Marignac (*France*) commented that she does not use Cortavance® before cleaning the ear but recommends cleaning the ear and waiting 30 min before using any other drug.

Vanessa Schmidt commented that she cleans the ear on a different day from when the Cortavance® is applied.

Genevieve Marignac commented that removal of biofilms with topical therapy is important but it is also important to maintain skin lipids, which may help the efficacy of some topical drugs. She reviewed a study involving lipids and the presence or absence of biofilms on the skin; changing the skin lipid composition can make it more difficult for a biofilm to develop. Atopy disrupts the stratum corneum, which may lead to a larger biofilm; small biofilms with a large diversity of microbes are optimal for normal skin.

Shampoos and conditioners may effect skin biofilms differently: shampoo may help remove biofilms and conditioners may make heavy monomorphous biofilms more difficult to adhere.

Genevieve Marignac also reviewed a study in which solubilized climbazole was used as a shampoo with efficacy against *Malassezia* infection.[7]

Sergi Segarra (*Spain*) discussed the combination of sphingolipids and glycosaminoglycans (GAGs) for the topical treatment of AD. It is still not clear whether these abnormalities in AD are primary or secondary due to inflammation. Two groups of compounds, GAGs and polysaccharides like chondroitin sulfate and dermatan sulfate, are some of the core components in the body that form proteoglycans. They play a role in maintaining skin hydration with viscoelasticity and have been suggested as being useful in therapies for wound healing and also to maintain the skin homeostasis. Given that in AD there is a strong inflammatory component, the goal of their work is to decrease the inflammation using a topical treatment and help to restore the abnormal skin barrier with a topical treatment. A study was presented where treatments involved a combination of topical lipids and GAGs to see if this could be effective in the management of canine AD.

Using cultures of human dermal fibroblasts, skin hydration and the potential for increased skin elasticity and for the proliferation and migration of fibroblasts was assessed using this combination. A significant increase in skin hydration, skin fibroblast proliferation and migration of fibroblasts was seen.

Defects in the skin barrier of dogs with AD was the focus of another study.[8] The objective was to assess the changes induced by three different sphingolipid extracts that were produced by Bioiberica (Barcelona, Spain): sphingolipid extracts 1, 2 and 3. Using a well-developed *in vitro* model for canine skin, the morphology by histopathology and also quantification of lipids was analysed after exposure to the three different sphingolipid extracts. Data showed that sphingolipid extract 1 stimulates the production of ceramides. Sphingolipid extract 1 contributes to the formation of a well-organized stratum corneum and represents a potential therapy for improving the skin barrier function in AD. The main lipid in the sphingolipid extract 1 is a sphingomyelin, which also has anti-inflammatory activity through inhibition of prostaglandin E_2 production.

The sphingolipid and GAGs were combined into a single product and were assessed in a randomized double-blind placebo-controlled study using beagles that were experimentally sensitized to house dust mites. The control group had six dogs that received no treatment. The

treatment group received the combination of sphingolipids and GAGs applied to different parts of the body twice weekly. CADESI scores, transepidermal water loss, pruritus, pruritus visual analogue scale (PVAS) scores and other clinical signs (time samples of biting, licking and scratching) were evaluated. Blood samples were collected and tape stripping was done to look at lipid profiles. A significantly lower CADESI was achieved at 1 week with the topical treatment compared to the control group. A significant increase in CADESI was observed in the control group from the beginning of the treatment to the end. A significantly lower global pruritus score was achieved after 8 weeks in the treatment group and the pruritus increased significantly only in the control group. There was a significant increase in skin polyunsaturated fatty acids with treatment after 8 weeks. There were no significant differences in any other parameters and the product appeared safe with no reported side effects.

Topical formulations containing sphingolipids and GAG extracts may be useful for the management of canine AD either alone or in combination with other therapies. Future studies should include a larger number of patients with naturally occurring disease and formulations with sphingolipids and GAGs as single active ingredients.

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Ectoparasitic agents

P. Bourdeau[1] (Chairperson) and A.G. Cannon[2] (Secretary)

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Patrick Bourdeau (*France*) opened the workshop with a summary of presenters and topics and showed a graph of the variation in effectiveness of topical products applied by professionals versus owners (Figure 1).

Regulatory aspects of ectoparasiticides (L. Halos)

Lenaig Halos (*France*) explained there are national, international and regional guidelines that define the technical requirements to demonstrate safety and efficacy necessary for drug registration by the European Medicines Agency (EMA). The EMA guidelines for 2016 are available.[a] The guidelines are to ensure the accuracy and integrity of data.

There are two types of study: laboratory studies (for dose confirmation) and field studies (to confirm the laboratory studies). For registration, two laboratory studies and one field study need to be performed and they must follow standard protocols. All studies must have controls: (1) a negative control (untreated) group, (2) a positive control (for field studies) group treated with a registered product and (3) a treated animals group treated with the product in question at the minimum clinical dose.

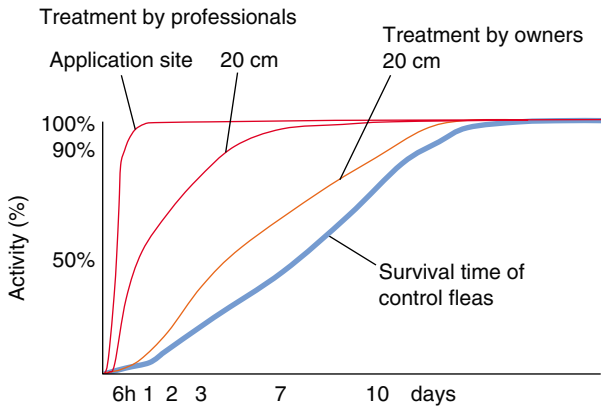

Figure 1. Variation in effectiveness of topical flea products applied by owners compared with trained professionals. Spot-on treatment; survival of fleas on treated hairs 5 weeks post-application. Treatment was applied at application site and 20 cm away by professionals; and at 20 cm by owners. Blue line shows survival of control fleas kept with non-treated hairs. Data from Bourdeau et al. 2003.[1]

Parasite counts are performed using well-described protocols. The mean of live parasite counts allow a calculation of efficacy using the following formula:

$$\text{Efficacy}\% = 100(m_C - m_T/m_C)$$

where m_C is the number of live parasites in the control group and m_T is the number of live parasites in the treatment group. Note that Europe uses arithmetic means while the USA uses geometric means; therefore, the same product may have different efficacy claims in different countries.

Efficacy criteria for registration are well established. For fleas, products must achieve >95% efficacy at 24 h (24 h is the new period for EMA in 2016; previously it was 48 h); for ticks they must achieve >90% efficacy at 48 h post-treatment or challenge. Parasites are re-applied weekly for products with a duration of 30 days and bi-weekly for longer-lasting products. The number of parasites required are 100 fleas and 50 ticks. Adequate infestation levels must be demonstrated by having 25–50% of ticks and 50% of fleas remaining in the untreated groups. It can be difficult to achieve adequate tick infestations in cats; therefore, efficacy results may not be available for control of ticks in cats. For registration there must be a statistical difference between untreated and treated groups.

There are several definitions of efficacy. *Immediate efficacy* is against pre-existing infestation (also known as curative efficacy). *Persistent efficacy* is against new infestations. For topical ectoparasiticides the term *preventative efficacy* may be used, while systemic products may use the term *persistent killing effect*. Other important definitions are length of efficacy: 4 weeks is considered *short lasting persistent efficacy*, while greater than 4 weeks is considered *long lasting persistent efficacy*. *Repellence efficacy* is defined as a 95% reduction within 24 h post-infestation in the number ticks (dead or alive) or number of fleas that have taken a blood meal (anti-feeding effects).

The current definition of *speed of kill* is the earliest time at which complete efficacy is achieved. *Onset of action* is the first time significant mortality is demonstrated.

Topical ectoparasiticides (M. Varloud)

Marie Varloud (*France*) discussed current aspects of topical ectoparasiticides. An increasing variety of products are available for ectoparasite control in companion animals. Unlike oral systemic products, most of the topical products work by contact and, therefore, share a common rationale of treating the skin to protect against external parasites. With the expanding numbers of topical products, users should review the characteristics of topicals (e.g. sprays and spot-ons) and be familiar with their administration, distribution, persistency and efficacy.

While sprays have a quick onset of action and may reduce the number of products needed by multi-pet owners or professionals, they can lack persistency of effect and convenience of administration, and the risk of inaccurate dosing is a concern. We can assume that the theoretical distribution of the active ingredient after a spray administration is immediate. This rapid onset of action was substantiated by efficacy data against fleas including knock-down 1 h post-treatment[2] and anti-feeding.[3]

Spot-on pipettes are designed for easy administration directly to the skin; however, run-off can happen when large volumes are applied in single spots and owners may apply to hairs instead of directly to the skin. Active ingredients from spot-on solutions must distribute from the administration area and cover the body surface to reach effective concentrations. Although the exact mechanisms are unclear, their onset of efficacy is generally 6 h against fleas,[4] 24 h against mosquitoes[5] and 24 h against ticks.[6] Their duration of efficacy and pharmacokinetic data[7–9] provide evidence of their distribution and persistency. The influence of parameters such as species, hair length, skin lipid layer and removal of skin lipids by shampoo prior to a topical treatment are hypothetical but not documented to our knowledge. The performance of spot-on solutions has been investigated under different challenges including outdoor housing,[10] weekly water immersion,[11] and immediate,[12] weekly,[13] fortnightly[14] or monthly[11] shampooing. Only weekly cleansing shampoos were shown to have a moderate effect in decreasing insecticidal or acaricidal efficacy. However, the nature of the shampoo can influence the effect on the parasiticide performances.[10] Although topical ectoparasiticides are often considered less suitable than oral products when shampoos are required, this hypothesis was not assessed until recently and several factors must be considered, including owner adherence to treatment protocols.[15] Investigations comparing the performance of oral and topical ectoparasiticides have found good efficacy with both treatment methods.[16]

In summary, topical ectoparasiticides such as sprays and spot-ons represent a well-established category of products providing reliable and unique weapons against parasites of companion animals. However, only some of the contact-based topicals provide repellence. Links to some humorous and informational videos were provided.[b]

Revival of collars and protective bands for delivery of ectoparasiticides (R. Armstrong)

Robert Armstrong (*USA*) discussed the evolution of technologies that has resulted in a revival of collars and protective bands. These new collars are made by extrusion and injection moulding and are registered medicinal products produced under good manufacturing practices with an associated guarantee of quality. These products work by cutaneous diffusion of active ingredients from a solid matrix. Some collars may diffuse not only cutaneously but also in the air surrounding the pet. Collars have the potential to deliver high levels of a parasiticide over extended periods of time. One limitation is the potential loss of collars. Future opportunities in collars include 3D printing, inclusion of novel and potentially systemic active ingredients, improved application and release systems, better safety and improved resistance to environmental conditions.

Systemic ectoparasiticides (P. Bourdeau)

Patrick Bourdeau reviewed the systemic ectoparasiticides and their roles in parasite prevention in food animals, animal welfare and disease prevention. Systemic products have been available for a long time. Newer products have improved efficacy and safety.

Selamectin (Revolution™, Stronghold™) is a macrocyclic lactone that is applied topically, absorbed rapidly through the skin and distributed via the blood and has activity against a variety of internal and external parasites. The exact mode of action of macrocyclic lactones is not fully elucidated, but it is believed that they bind to glutamate-gated chloride channels in the parasite's nervous system, increasing their permeability. This inhibits nerve activity and causes paralysis of the parasite. Systemic therapies may have variable results; for example, selamectin varies in its bioavailability based on the sex of a dog (Figure 2). The other macrocyclic lactones have no activity against fleas and variable activities against other ectoparasites.

Neonicotinoids include nitroquanidines, neonicotinyls, chloronicotines and chloronicotinyls. Neonicotinoids are modelled after natural nicotine and act as agonists on the postsynaptic acetylcholine receptors in insects. Nitenpyram (Capstar™) is administered orally in pill form to kill fleas in both dogs and cats. It is absorbed rapidly, with maximal blood concentrations reached within 1.2 h in dogs and 0.6 h in cats. Fleas begin to die within 20–30 m of administration, with 100% flea mortality within 3–4 h. The compound is rapidly eliminated, with >90% excreted in the urine within 24–48 h, primarily as unchanged nitenpyram.

Spinosyns are a novel family of insecticides derived from the fermentation of the actinomycete, *Saccharopolyspora spinosa*. The two most abundant products derived from the fermentation process are spinosyns A and D, and these are the major active components of spinosad

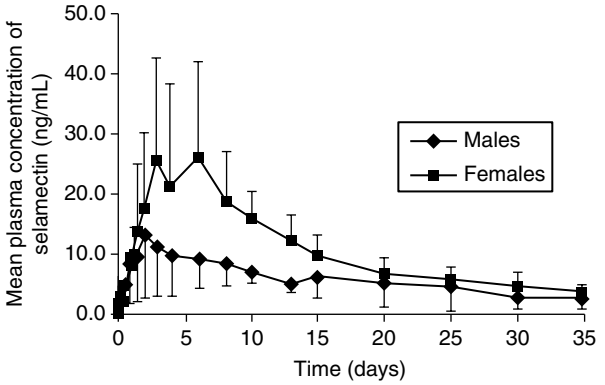

Figure 2. Variation in pharmacokinetics of spot-on (systemic) medications on male and female dogs. Five male and five female beagles were tested with 6 mg/kg selamectin (Stronghold™). Plasmatic dosage was tested with HPLC + fluorescence. Clearance was signficantly higher in males. Data from Dupuy et al. 2004.[17]

(Comfortis™). Spinosad is used to control a wide variety of insects, including flies and fleas. Spinosyns have a novel mode of action, primarily targeting binding sites on nicotinic acetylcholine receptors distinct from other insecticides including neonicotinoids. Spinosyns also affect gamma-aminobutyric acid (GABA) receptor function, which may contribute further to their insecticidal activity. These actions result in excitation of the insect nervous system, leading to involuntary muscle contractions, prostration with tremors and, finally, paralysis. Spinosad has activity against fleas and lice and is formulated as a chewable tablet for dogs and cats. Spinosyns also have an acaricidal activity. Spinosad has a strong and rapid activity against ticks but this activity is shorter than the 4 weeks expected of the ixodicidal products recently launched for dogs. Spinetoram is a second molecule in the spinosyns group that is used topically and does not have systemic diffusion.

Isoxazolines are a new class of compounds that have potent insecticidal and acaricidal activities. Isoxazolines have a novel mode of action and specifically block arthropod ligand-gated chloride channels. They are eliminated through the biliary system. Afoxolaner, fluralaner and sarolaner (NexGard™, Bravecto™ and Simparica™) are currently approved for use in veterinary medicine. The compounds are readily absorbed after oral administration and provide 4–12 weeks of insecticide and acaricide activity. These products have very high initial concentrations and an extended time above the effective dose.

Insect growth regulators (C. LeSueur)

Christophe LeSueur (*France*) introduced the use of insect growth regulators (IGRs) by noting that fleas have been treated with highly effective products but still remain a problem. Focusing on treating animals ignores the overall flea population problem. Fortunately, fleas have a vulnerable life cycle. Veterinarians are often involved in explaining the life cycle of the flea to clients on a daily basis, informing pet owners that 95% of fleas are in the environment. Resistance to ectoparasiticides is still rare, so reasons why fleas are still a problem include: (1) not all pets in the household are treated, (2) neighbours' pets are not treated, (3) wildlife is not treated, (4) products are applied incorrectly and (5) owner compliance is lacking. Attempts can be made to improve owner compliance through education. Other strategies include overcoming lack of compliance by using long-acting products and breaking the flea life cycle with IGRs.

There are two major classes of IGR: (1) insect development inhibitors (e.g. chitin inhibitors such as lufenuron) and (2) juvenile hormone analogues (e.g. pyriproxifen and S-methoprene). Juvenile hormone analogues (JHAs) affect fertility and development of eggs and larvae through modification of the moulting process. The juvenile hormone is produced by corpora allata. The moulting hormone is ecdysone. For the juvenile moult, both ecdysone and JHA are produced. For nymphal and adult moults only ecdysone is produced. Consequently, when JHAs are applied, the treated larvae never reach the adult stage and die.

Two approaches are: (1) to use IGRs in combination with insecticides or (2) keep the products separate. IGRs can be applied in the environment or on the pet. IGRs are available for environmental treatment as sprays and foggers. Sprays are multi-dose and have larger particles and may penetrate carpets and fabrics more effectively than foggers. Delivering IGRs to the pet can be accomplished through spot-ons, collars, sprays and oral products. IGRs are very safe for both mammals and the environment. IGRs only affect insects, and fleas are highly sensitive. There are many benefits to using IGRs including increased speed of efficacy, preventing treatment gaps and the safety of these products.

Experimental models for ectoparasiticide studies (C. Becske)

Csilla Becske (*Belgium*) discussed advantages and limitations of experimental models used in ectoparasiticide studies. Experimental models include both laboratory *in vivo* and *in vitro* studies. *In vitro* studies are useful for comparisons of potency of different compounds and for initial dose selection. The *in vitro* studies can model disease transmission and determine how long it takes for disease transmission. *In vitro* studies are quick and inexpensive and help to reduce the use of animals in studies. The disadvantage is the lack of the biological component of the animal. *In vivo* models are very important for product registration.

Laboratory *in vivo* studies using dogs and cats are required for product registration. The *in vivo* studies are used to select dose and justify the minimal effective dose. These studies are very controlled and all conditions are specified. Specific species and strains of insects are used which may not be the same as those in the natural environment. The species sensitivities of laboratory strains to parasiticides are known and the insects are pathogen-free. Environmental conditions can be kept within a narrow range to ensure that conditions are exactly the same for treated and untreated animals. In the field, environmental conditions may vary widely. Individual animals may also have variations and differences in grooming, immunologic status and other factors that affect product efficacy. In the laboratory, cats are often kept in e-collars to provide higher levels of infestation. Owner compliance issues are excluded in laboratory studies. Laboratory studies are the best model to establish speed of kill as the exact time of infestation is known. Technical aspects of conducting laboratory studies can be difficult: some ectoparasites are difficult to breed, it may be difficult to establish an infestation (e.g. *Demodex*), it is difficult to evaluate flea allergic dermatitis in a laboratory setting and owner compliance cannot be modelled. However, laboratory studies are best for evaluation of resistance and repellence.

Field studies are needed to model real life. In the field, variations in ectoparasite species and strains, breeds and genetic makeup of the host animals, environmental conditions, infestation levels, individual host susceptibility, products and frequency of bathing, grooming, sun exposure, owner compliance and many other variations may impact product efficacy.

For other ectoparasites, such as mites, the same limitations apply. However, the availability of laboratory-raised

mites is very limited and researchers often need to use naturally infected animals.

Researchers can look for ways to optimize laboratory studies. Importantly, attempts can be made to increase the variability of the biological aspects of hosts. This can be accomplished by using different breeds, genders, ages, coat lengths and housing arrangements. To increase the parasite variability, studies can use multiple strains obtained from different geographical regions.

The floor was then opened for questions.

Monika Linek (*Germany*) asked for comments on knock-down times because this was important in the past.

Lenaig Halos replied that knock-down time is primarily a factor with pyrethroids and relates to immediate paralytic effects. However, it is not a factor for registration of products. Knock-down time is determined by counting the number of fleas around the animals at a specified time.

Luc Beco (*Belgium*) asked whether there is a cumulative effect of new oral products at the end of the time of efficacy, and which product is considered the best.

Robert Armstrong responded that most products will have residual activity against some parasites, but not all. The effective part is the free, unbound drug; total plasma concentrations may be higher than required to kill parasites. Pharmacokinetics studies are performed and, if there is bioaccumulation, there can be regulatory approval issues.

Patrick Bourdeau asked if the companies can comment on speed of kill.

Lenaig Halos replied that speed of kill needs to be determined at the end of the efficacy time as is now determined in the new EMA regulations.

Veronika Balazs (*Chile*) asked about geographic differences that may exist in product efficacy. She has seen fleas on dogs 2 months after a 3-month product was given.

Robert Armstrong replied that studies are done in multiple countries and many geographic areas. He would be concerned about seeing fleas earlier than 3 months and recommends contacting the company to report failures.

Lenaig Halos commented that in the field he does not expect to reach the same levels as the controlled studies and, in cases where fleas are seen after 2 months in products with a 3-month label claim, it may not be a product failure but just real life.

Veronika Balazs remarked that the 3-month or 1-month efficacy time-frames likely represent averages and she suspects that there will be some variations.

Patrick Bourdeau agreed there will be variations in real-world use. In certain seasons some fleas will leave the dog and you may have very aggressive flea populations; in these cases it may be better to shorten the interval of treatment in adaptation to the conditions. In a recent paper, Michael Dryden reported using light traps for a study in Tampa, Florida, and found that 40% of fleas caught in the traps were already fed.[18] These fleas had left a dog or another host after feeding. This is not normal in most areas.

Jare Pipp (*Slovenia*) asked if generics are the same as brand-name products.

Patrick Bourdeau replied that generics must go through the same equivalency testing so they should be the same. However, this could depend on where they are manufactured and every compound is different.

Robert Armstrong commented that generics contain active and inactive ingredients. Some of the inactive ingredients can affect the delivery of the active ingredient. Generics that are manufactured by a reputable company should be fine, but others manufactured by companies without a known reputation may not.

Cathy Curtis (*England*) commented that following the launch of a product, resistance is talked about frequently. She asked how often products are checked. For example, she is aware that Bayer has conducted tests of imidacloprid for resistance by developing a test for the viability of flea eggs. The studies have found negligible resistance. She suspects the same is true for other companies.

Lenaig Halos reported that Bayer performs resistance tests most frequently and proposed that the companies work together to monitor resistance. Perhaps they can build a global laboratory to test for resistance.

Patrick Bourdeau commented that resistance has a precise definition. An apparent lack of efficacy cannot be called resistance until proven otherwise. It is also important to demonstrate that it is not a tolerance. This is a problem for all molecules. While researchers can look for resistance genes, merely finding these genes does not mean that there is clinical resistance.

Notes

[a]www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2016/07/WC500210927.pdf

[b]Links to some humorous and informational videos: www.youtube.com/watch?v=vgCEn_fBj2E and www.youtube.com/watch?v=Z8GzJCznb7g

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Why biopsy bald patients?

M. Linek[1] (Chairperson) and E. Kuznetsova[2] (Secretary)

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Monika Linek (*Germany*) opened the workshop by welcoming all the participants and commenting she had learned that in human medicine the term 'bald patient' is exclusively used for male-pattern baldness, and therefore perhaps not suitable to be used in reference to animals. She briefly described the schedule of the workshop and introduced the speakers.

Monika Linek continued with a poll showing clinical pictures of different alopecic patients, questioning who would biopsy each particular patient (assuming that all of the routine clinical work-up, including the necessary blood tests, have been performed and were normal). There was never an unanimous vote, but in some of the more straightforward cases (e.g. colour dilution alopecia, alopecia X, localized post-ischaemic alopecia in a poodle) some participants, mainly pathologists, raised their hands explaining that they would biopsy the patient but primarily for research purposes rather than diagnosis.

Why does a patient get alopecic? (M. Welle)

Monika Welle (*Switzerland*)**)** stated that the regulation of the embryonic hair follicle development and the hair cycle during postnatal life are highly conserved processes that involve a series of interactive signals. Knowledge about these signals is still poor in our companion animals. It is, however, known from rodents that hair growth is dependent on complex interactions between follicular stem cells and signals from their microenvironment, extrafollicular factors such as the adipose tissue, systemic factors such as the genetic background or the hormonal influence, and numerous environmental factors. Non-inflammatory alopecia is largely attributed to genetic variants (also known as dysplasias), hair cycle dysregulation or ischaemia. Only a few causative variants responsible for the different forms of follicular dysplasia have been described in our domestic animals but it has been shown in humans that the identified genetic variants influence, for example, the transcriptional regulation or the cell–cell adhesion and communication. In all disorders associated with hair cycle arrest the number of kenogen follicles is increased 3–4-fold, whereas the number of anagen follicles decreases dramatically. The increased number of kenogen follicles indicates that the promotion of a new anagen phase is insufficient because the stem cell compartment or the cellular crosstalk stimulating the new anagen is impaired. Over time, these missing signals may lead to an increased number of atrophic follicles. Since hair follicles have only a limited way to respond to missing signals, the definitive pathogenesis will often remain unclear after histopathological assessment and a clinical work-up is required.

From Monika Welle's lecture slides, Monika Linek summarized the following as 'take-home' messages.

1. Definite diagnosis is based on histopathology. In some cases histological diagnosis is impossible.
2. Follicular dysplasias are histologically very diverse and not all of them are well defined.
3. The causative genetic variants are unknown in most cases.
4. It is important to take biopsies and EDTA blood to increase knowledge and understanding.
5. The hair follicle has a limited way to respond.
6. Hair follicle physiology is complex, differs between species and breeds and is not yet fully understood.
7. The microenvironmental and macroenvironmental factors that influence the hair cycle are ill defined.
8. Histology is helpful to exclude other possible causes and to suggest a pathogenesis in some cases.
9. The definitive pathogenesis will remain unclear in many cases.
10. A detailed clinical history is necessary.

The dermatologist view: why would you biopsy a bald dog with most likely hair cycle arrest? What do you expect the pathologist will tell you? (L. Frank)

Linda Frank (*USA*) explained that her reason for collecting biopsies from a bald dog with apparent non-inflammatory alopecia would be to rule out inflammatory causes that she might have missed. Additional reasons for obtaining a biopsy include an unusual breed, an unusual presentation, a lack of response to what should be an appropriate therapy or a breed in which both inflammatory and non-inflammatory diseases can occur. An example is the Samoyed, which can develop both sebaceous adenitis and alopecia X.

What tests do you do prior to biopsy to rule out endocrinopathies first and, if ruled out, do you still take biopsies?

Linda Frank explained that if she suspects a non-inflammatory alopecia, she usually performs a minimum database consisting of a complete blood count, chemistry panel and urinalysis. If these are within normal limits and

an endocrine disease is a valid differential diagnosis (based on age, signalment and distribution of alopecia), then she performs thyroxine/thyroid stimulating hormone (T_4/TSH) and low-dose dexamethasone suppression (LDDS) and/or adrenocorticotrophic hormone (ACTH) stimulation tests to further screen the dog for endocrine disease. If these are normal, she considers collecting biopsies only if the above conditions apply, namely to check whether she is missing an inflammatory diagnosis. She does not biopsy to help differentiate non-inflammatory causes of alopecia.

David Shearer (*UK*) thanked Monika Welle for her fantastic presentation, which included a discussion of many different influences on the hair cycle. He mentioned that to him it raised more questions than providing answers. He added that he sees follicular changes and often wonders whether they are post-inflammatory changes that are mimicking an endocrine problem or producing cycling problems. He asked whether Monika Welle supports the hypothesis that inflammation in the skin can lead to changes in the hair cycle.

Monika Welle agreed that inflammation can affect the hair cycle and commented that there are a number of papers in mice that clearly show the interaction between mast cell degranulation (regardless of cause) and hair cycle arrest or slowing of the hair cycle. She thinks that in many atopic dogs we see some sort of hair cycle arrest. When owners report that their dogs are hypotrichotic, she now suspects that some develop a mild form of hair cycle arrest due to the degranulation of mast cells and neurogenic influences, rather than just hair loss from scratching or licking.

David Shearer said that he receives biopsies from many dogs presented with alopecia in which the clinician suspects a non-inflammatory alopecia. However, he sees inflammation and some degree of what he suspects is a follicular change. The inflammation is not necessarily dramatic. He has been wondering for years whether what looks like a bilateral symmetrical alopecia in Labrador retrievers, for instance, is actually a post-inflammatory change and it is all part of a recurrent dermatitis issue rather than a primary or cycle abnormality. Collecting biopsies from these dogs to rule out inflammation is very important, because while clinically some of them do not exhibit pruritus, histologically some dermatitis is present.

Monika Welle absolutely agreed with David Shearer. She commented that 'we tend to break these dogs down into endocrinopathies and alopecia X, but we have no clue what they have.' Monika Welle added that she quite frequently sees biopsies from pinchers, Prager Rattlers and Jack Russell terriers with hypotrichosis on the ventrum, but that she thinks that we actually have no idea what is causing it.

Sonya Bettenay (*Germany*) commented that Monika Welle had mentioned that endocrinopathies and alopecia X both have mostly kenogen hair follicles, and that the distinguishing difference for alopecia X is that there are more telogen hair follicles, Since Linda Frank said that she would only biopsy a suspected alopecia X in an unusual breed, Sonya Bettenay's question comes back to 'do we ever need then to biopsy Pomeranians?'

Monika Welle replied that Pomeranians could also have an underlying cause for alopecia, other than alopecia X.

Linda Frank agreed with Monika Welle and added that she would always perform screening for endocrine diseases. She added that in the 10-year-old Pomeranian that was shown by Monika Linek (in the poll at the beginning) she would not necessarily biopsy. She has seen alopecia X developing for the first time at 10 years of age, but she has also seen Cushing's disease in Pomeranians. That is why she would work them up first, to rule out an endocrine cause. She would not necessarily biopsy a Pomeranian, at least not unless there is something unusual such as losing hair on the head or feet, or other atypical findings.

Emily Walder (*USA*) replied that in real life, when pathologists are not working at a university or at a tertiary referral practice but in large laboratories or even small facilities, on some occasions they might get biopsies from clinicians who did not complete an appropriate work-up. In those cases the responsibility falls on the pathologist to say: 'well, this is alopecia X' or 'go back and do an ACTH stimulation test, because it does not quite look like alopecia X.'

Linda Frank agreed that clinicians have to perform the work-up, because there might be a case of alopecia X with Cushing's disease and it is difficult to tell the difference.

Emily Walder agreed with this but mentioned that if the pathologist is the first one to suggest that, then he or she will have the responsibility to give other possible differentials for the case.

Julie Yager (*Canada*) also agreed with Emily Walder and said that pathologists do have a responsibility, because if they even mention a diagnosis it *becomes* a diagnosis. They must, therefore, be careful because clinicians might not even do the follow-up because the pathologist mentioned the word hypothyroidism or alopecia X.

Monika Linek asked Monika Welle about what would help her as a pathologist when clinicians biopsy, for example, a dog with pattern alopecia? Would it help to find the ethiopathogenesis?

Monika Welle replied that it would not help the clinician. This is really research and might help in the long run to obtain further knowledge. Currently they are collecting RNA from these dogs to perform next-generation sequencing, looking for differences between the different alopecic disorders: which signalling events might be affected and by what. She thinks it would be very helpful to get more biopsies from alopecic areas and haired areas of the same alopecic dog in order to be able to do these experiments and to examine what signalling events are

disturbed. This might allow a more complete picture to be obtained.

David Shearer then asked if any pathologist in the audience had any comments about how to evaluate hair follicles stages or hair follicle morphology in biopsies of a Labradoodle or other crossbred 'doodle'. He said he often thinks there is no clear understanding of these breeds, compared to, for example, a Labrador retriever, German shepherd dog or other breed from which there is knowledge of normal histology based on many biopsies.

Emily Walder answered that it should be always kept in mind that dogs with a long anagen cycle, like the poodles and their crosses, are not going to look like a short-coated dog that has half telogen/half anagen on a normal biopsy. If more than a few telogen follicles are observed in a poodle or Labradoodle, it indicates pathology.

David Shearer replied that in the UK they have many poodle crosses other than Labradoodles, and that they do not all look clinically the same. They can resemble one parent or the other. He asked what other pathologists consider to be 'normal' in these crosses between anagen-dominant and telogen-dominant coats. He said that he has seen dogs claimed to be Labradoodles that have a coat more like a poodle, and others that look more like a Labrador. He does not think that all Labradoodles have an anagen-dominant coat but asked whether others agreed.

Emily Walder replied that most Labradoodles in the USA are poodle-like.

Due to technical problems Ross Bond's presentation could not be started. Silvia Rüfenacht's lecture was presented next.

Trichogram of the bald patient: when is it diagnostic? (S. Rüfenacht)

Silvia Rüfenacht (*Switzerland*) stated that in cats, the trichogram clearly distinguishes between traumatic (self-induced) alopecia and a follicular disorder. In dogs, together with a very typical clinical presentation and a specific breed, the trichogram can be diagnostic in colour dilution alopecia (in her hands, with high sensitivity but low specificity). Excessive trichilemmal keratinization, which can be assessed in the trichogram, can be seen in various hair cycle disorders and thus is not specific for a certain disease. There is no correlation between the different follicular stages (anagen, telogen, exogen) seen in a trichogram and the histopathology of the same patient. Whereas kenogen is the most prominent stage of an alopecic dog on histopathology, it is not visible in the trichogram, and in most anagen stages epilated hairs have a telogen or exogen root. In typical clinical cases of sebaceous adenitis, follicular casts are seen macroscopically. However, since follicular casts can occur in many skin disorders, they are not diagnostic for sebaceous adenitis. In summary, although the trichogram is a helpful additional diagnostic tool, it is not pathognomonic in any of the non-inflammatory alopecias in animals.

A trichogram of a horse was shown with mostly 'exogen hair roots': these are brush-like telogen roots that lack any material around the hair and, therefore, are different from telogen roots, which must be epilated with some force and contain some material around the hair root. However, on histopathology in this case many anagen hair follicles were seen.

One must keep in mind that in a trichogram one can see many exogen and telogen stages of hair roots although hair might already be growing back. In a trichogram one can see only the latest anagen stages, when the hair shaft reaches the surface of the orificium; one cannot see the early stages. Additionally, if kenogen is the most prominent stage of an alopecic dog in histopathology, it is not visible in the trichogram. Thus hair cycle arrest cannot be diagnosed by trichogram.

In human medicine, in alopecia areata, the trichogram might be diagnostic if one observes an exclamation-mark type of hair with a normal hair bulb, thinning of the hair shaft in the middle and a thicker tip. There were some similarities in trichograms from Eringer cows with alopecia areata, but they were not as diagnostic as they are in humans.

Julie Yager had a question for other pathologists regarding a picture in the presentation of a dystrophic trichogram hair in sebaceous adenitis. Julie Yager has seen a subpopulation of sebaceous adenitis cases in which examination of the hair shafts showed them to be dysplastic. She wondered if other pathologists see this. She would like to know what it means; why some of them have dysplastic hair shafts and some do not.

Monika Welle replied that she does not know what it means but suggested that it is possible that the inflammation is influencing signalling events involved in the stem cell activation or the differentiation processes within the hair follicle. There could be a different outcome depending on the type and severity of inflammation. However, she said she is not aware of any study investigating the effects of inflammation on hair follicle or hair shaft development or differentiation.

Ariane Neuber (*UK*) commented that a trichogram does have some value, because it is ruling out *Demodex*, and probably helps in checking for dermatophytes as well. It is one of the basic tests that should always be done in an alopecic dog.

Linda Frank commented that she was interested that Silvia Rüfenacht said that she could not diagnose telogen defluxion with a trichogram. Linda Frank suggested that it might be possible, because she never sees all telogen hairs in any alopecic condition except in telogen defluxion. In this condition, hair also epilates very easily compared to the little tug needed with other conditions.

Silvia Rüfenacht agreed, and remarked that given the uncertainties of those cases she likes to have a very good clinical picture and history too.

Ross Bond (*UK*) commented that he thinks trichography can be quite useful when an owner is distraught that the

pet does not possess a normal hair coat. Plucking the hairs and showing the owners the malformed and misshaped hairs helps them to understand their pet's condition. He added that his consult room has a video screen connected to the microscope, so owners can see the cytology or trichography.

Silvia Rüfenacht agreed with Ross Bond and added that it also might be helpful in a case in which an owner thinks that a cat is not pruritic to show them that the hairs are damaged.

Where can dermoscopy replace skin biopsies as a diagnostic tool, or are they another helpful extra? (F. Scarampella)

Fabia Scarampella (*Italy*) commented that only a few studies on the application of dermoscopy are available in veterinary dermatology. One can assume that, as in humans, this technique may have the potential for obviating unnecessary biopsies. When a biopsy is still needed, it could be helpful in choosing an ideal biopsy site. Dermoscopy allows one to observe *in vivo* hair shaft abnormalities and measure and assess the number of hairs in one follicular unit. Therefore, it can provide additional information beyond that obtained by clinical examination in some visually striking diseases such as feline self-induced alopecia, canine black hair follicle dysplasia, colour dilution alopecia and pattern alopecia. Dermoscopy may also distinguish whether hair follicle openings contain hair residues or hyperkeratotic plugs. Hence, it may provide some suggestive features in disorders of hair cycling such as those occurring in dogs affected by alopecia X or recurrent flank alopecia.

Transverse sectional biopsies (R. Bond)

Ross Bond stated that the normal practice of vertical sectioning of skin biopsy specimens from alopecic dogs contrasts sharply with the approach in human dermatopathology, wherein combined transverse (the 'Headington technique') and vertical sectioning is considered optimal.[1,2] Whereas transverse sectioning is effectively dismissed by the authors of one textbook of hair loss in animals,[3] others have reported significant benefits. For example, Credille and others concluded that transverse sectioning was 'critical' for accurate assessment of canine hair follicle morphology. The author's group has reported that transverse sectioning enhanced the assessment of sebaceous glands in both health and disease, as well as canine hair follicle abnormalities (especially anagen/telogen ratios and numbers of hair shafts per unit). Histomorphometry of transverse sections provided compelling statistical confirmation of the subjective impression of increased telogenization in transverse sections from alopecic areas in curly coated retrievers with follicular dysplasia. This technique deserves more frequent utilization in veterinary dermatological practice.

David Shearer asked Ross Bond if he was performing sectioning all the way through the biopsies. He asked how practical it would be for pathologists to train technicians to make fewer sections, but sections that give pathologists more valuable information. He asked how could we apply these great research results in practice.

Ross Bond answered that in their transverse sectional studies[2] they had multiple different levels so they could see the common infundibulum, more inferior portion or isthmus level, but when it comes to anagen/telogen ratio they absolutely needed the isthmus level. He commented that if there is a very thin piece of skin, particularly in alopecic dogs, they would probably cut it only once transversally, making two halves in a paraffin block. They would then start step sectioning. He added that the questions become 'how many slides do we need?' and 'how much is it going to cost?'.

They also found that in biopsies from the trunk, and particularly the dorsal trunk where the skin is thicker and is technically easier to cut transversally, they might cut twice (to obtain three pieces) or three times (to obtain four) with a microtome blade or scalpel blade. In their study, in only one of these 31 dogs did they fail to capture the isthmus level. It was normally straightforward to visualize this level.

David Shearer asked Ross Bond whether he and his colleagues could develop guidelines for preparation of the samples that could be used in a commercial environment in the future.

Ross Bond replied that it is not difficult. In his university referral setting, he or his team can follow a biopsy to the pathology laboratory and talk to the technicians about cutting it in the aforementioned way. In some cases they just keep the biopsies for a day in formalin and then proceed to cut them together with the technicians. He added that it is also possible to do this on archived tissue as they did in their sebaceous adenitis study. It is possible to melt down the block to obtain transverse sections.

Julie Yager added two quick points. She thanked Ross Bond for the presentation, said that it was great and that she totally agrees: probably including transverse sections should be the route as a standard practice. On a historical note, as veterinarians we should be very pleased to know that the technique was pioneered in the 1950s in Australia by Margaret Hardy, the most wonderful hair follicle biologist working in Australia, because of funding from the wool industry. She also commented that she thinks that in human laboratories they just take two transverse levels and they are so used to it that it seems not a great effort.

Ross Bond explained that if the skin layers are appreciated (epidermis, dermis and fat) then it would be possible to cut it exactly at the desired level.

Monika Welle stated that vertical sections are optimal for appreciating kenogen follicles. However, she thinks that both vertical and transverse cuts are needed. There are reports from human medicine that show that it is possible to use either technique as long as they are cut very well. It is not easy to obtain a good longitudinal section, but

with a good section she thinks that you can appreciate the cycle stages equally. What could be missed in cross-sections are dysplastic or dystrophic changes within the hair follicles. She has never been able to see this aspect as well in the transverse sections, so she thinks it is better to use one half for a longitudinal section and one half for a transverse one.

Ross Bond absolutely agreed, but mentioned that sometimes in the dystrophic hair follicle, where it is possible to see very malformed hair in transverse section, it is perhaps one that could be invisible as a bit of hair in a vertical section. In transverse section they see it sideways. He agreed with Monika Welle's point and said that in a curly coated retriever to be sure to see the disarranged keratinocytes in the outer root sheath, a vertical section is needed. He commented that in human medicine, vertical sections are often better for some of the infiltrative lymphocytic cytotoxic processes, although in his study they had a case of mural folliculitis that was discovered transversally, but not vertically.

Monika Welle commented that it is necessary to get the right level; for example, for alopecia areata it is necessary to cut numerous step sections to avoid missing the diagnostic level.

Ross Bond replied that he has a spreadsheet with all the block numbers for all the alopecia areata cases where he wanted to do transverse sections. The problem in alopecia areata is the small number of bulbs in which lymphocytic infiltration can be seen. To see more bulbs the cut should be done transversally through the inferior portion rather than vertically, so there should be more hair follicles seen, therefore, with a better chance of detecting lymphocytic infiltration.

Monika Welle replied that this might be a question of the cost/benefit of obtaining multiple sections.

Ross Bond commented that in a case of demodicosis or lymphoma it does not matter how many levels are looked at in a biopsy. In pemphigus foliaceus, however, the level might matter, because there are cases of pemphigus foliaceus where pyoderma is on one level and acantholytic 'intragranular level' pustules on another.

Linda Frank asked if Ross Bond had any advice for alopecia X? She said that he had mentioned that in a thick biopsy it is possible to get three transverse sections. She had tried doing it in their alopecia X study but, despite their best efforts, the tissue was so thin that they could only obtain two slices.

Ross Bond said that, undoubtedly, the thinner the skin becomes the more difficult it is to obtain multiple transverse sections. For the very thin ones, one can actually hold the biopsy between the ends of ordinary forceps and cut it.

Derick Whitley (*USA*) asked Ross Bond if he has a rough idea as to how many slides he needs to evaluate a case. He also asked whether he felt that there is an increased chance of destroying a biopsy sample by using this method if someone doesn't have a properly trained technician.

Ross Bond answered that his only experience is with an excellent histotechnologist, or by doing it themselves. He said usually you can cut just two or three times (more often two) and then just cut one level of that, exposed on the paraffin block. Most of the time this has provided the levels that were needed. And, of course, if they don't obtain the level they needed, they just return and cut again until they get there.

Inflammatory alopecia: can histopathology diagnose or rule out infection? (D. Shearer)

David Shearer began by stating that it is rarely, if ever, possible to prove a negative histopathology. In this case, you cannot completely rule an infectious aetiology for a folliculitis by histopathology alone. If an organism is present, but cannot be visualized in H&E-stained sections, it cannot be ruled out. The presence of folliculitis and the types of inflammatory cells present provide us with some clues as to whether or not an organism could be present and responsible for the folliculitis. One can perform special staining (e.g. Gram, PAS, Giemsa) and, with some organisms, immunohistochemistry (e.g. feline herpes virus). In general terms the presence of a neutrophilic folliculitis makes him consider bacterial infection. Apart from an allergic or parasitic aetiology, the presence of an eosinophilic folliculitis makes him consider a viral or fungal infection. If there is a mononuclear folliculitis then he looks for *Demodex* mites and dermatophytes.

Monika Welle commented that Michael Goldschmidt once told her that the presence of eosinophils within furunculosis points towards either dermatophytes or bacteria.

David Shearer replied that he had an eosinophilic folliculitis case in the presentation that was actually caused by a dermatophyte.

Monika Welle agreed, but mentioned that dermatophytosis does not have eosinophilic lumen folliculitis, it is a mural folliculitis with furunculosis.

David Shearer commented that without PAS they did not see the dermatophytes. In fact, in the case, which was in the presentation, they had a culture which was positive. They returned to their sections and did PAS staining and they then found the hyphae. So eosinophil-rich lesions can be caused by fungi.

Monika Welle summarized that dermatophytes tend to have more eosinophils than bacteria.

Emily Walder commented that it is not that simple because with methicillin-resistant *Staphylococcus pseudintermedius* and *Staphylococcus aureus* (MRSP/MRSA) it is also possible to have eosinophils as a primary granulocyte in the inflammatory infiltrate.

David Shearer agreed that bacteria must be considered too.

Monika Linek asked about the use of dermoscopy for choosing a more appropriate place from which to collect a biopsy. She asked if we could take a smaller biopsy in hairless dogs and do transversal sections. Could smaller biopsies chosen by dermoscopy and then transversally sectioned make the biopsy more diagnostic with less trauma?

Ross Bond replied that he has never seen a pathologist asking for a smaller biopsy and that he wonders if it makes any difference.

Fabia Scarampella also replied that she thinks that it is too early, and that we must work to define the clues of the dermoscopy and the correlation between the dermoscopic findings and the histopathological features.

Monika Welle commented that biopsies are not always perfectly oriented on the slide. Even in a larger biopsy one may have only two or three compound hair follicles that are more or less perfect. She added that she would prefer to cut every sample in half: one half to be cut transversally, and another half to be cut vertically, which means that the size of each specimen should be large enough to obtain two halves from each sample.

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Performing and interpreting allergy tests: their value and pitfalls

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Richard Halliwell (*UK*) welcomed participants to the workshop and thanked Veterinary Allergy Reference Laboratory (VARL) for their sponsorship. He asked that presenters declare any conflict of interest, and acknowledged that he acted as a consultant for Virbac and Avacta Animal Health.

Comparison of serology performed in serum and plasma (J. Bexley)

Jennifer Bexley (*UK*) reported results of a study undertaken comparing EDTA plasma and serum for analysis of *Dermatophagoides farinae* (DF-specific) IgE and IgG. Matched samples taken before and after intralymphatic immunotherapy from the University of Zurich were assayed employing a monoclonal anti-IgE and a polyclonal anti-IgG antibody. Results of dilution curves for a high-responder dog were almost identical for plasma and serum (mean $r = 0.999 \pm 0.001$ and $r = 0.996 \pm 0.004$ for IgE and IgG respectively in a high-responder dog with similar results from all four dogs including one low responder). She concluded that EDTA plasma and serum could be used interchangeably for the measurement of allergen-specific IgE and IgG.

Ken Lee (*USA*) remarked that Greer Laboratories had performed similar studies on three separate occasions with at least 10 different animals using EDTA, heparin and sodium citrate as well as clotted blood.[1–3] No difference was seen with not only *D. farinae* but also with 50 different allergens. Either serum or plasma can be used, and haemolysis and lipaemia do not affect assay results.

Peter Hill (*Australia*) queried if there was any reason to suggest that this might be a problem from a theoretical basis.

Richard Halliwell responded that he was unaware of any reason. He commented that serum has always been used and some laboratories will not accept anything else, including haemolysed serum.

Characterization of *mac*ELISA responses in dogs: a 10-year history (K. Lee)

Ken Lee started by explaining what the *mac*ELISA is. Because of the potential cross-reactivity between horseradish peroxidase and some pollens he used alkaline phosphatase-coupled antibody. The cut-off level for this assay was 150 ELISA absorbance unit (EAU), which might be adjusted with different allergens. The correlation with different laboratories running the same assay against all of the allergens is 0.99%, and the assay detects as little as 10 pg of allergen-specific IgE. The assay is sensitive over a 200-fold concentration range. Serum samples were obtained from 74,044 presumed atopic dogs from eight different regions across North America and evaluated against a panel of 48 regionally specific allergens of which 36 are common to all regions. Sixty percent of the dogs were younger than 5 years old. More than 75% were shown to be IgE-reactive to at least one allergen, approximately 40% to five or more allergens and 25% reacted to 10 or more allergens. Labrador retrievers were the most commonly tested breed and accounted for 16.7% of positive responders. The breed with the highest percentage of reactors was the American Staffordshire bull terrier, which represented 4.2% of the positive test population. The same kind of response was seen among the various breeds and within the various American Kennel Club groupings. The highest overall rate of sensitization (about 54%) was directed toward mites, followed by different grasses (range 15.0–23.5%), weeds (9.5–23.8%) and trees (3.6–22.8%) which suggested possible cross-reactivity because most of the reactive samples were reactive to multiple allergens and very few were reactive to a given group of allergens. Individual responses to single grass allergen were extremely rare and the magnitude of responses to all grass allergens in an individual sample was of the same order. The magnitude of responses to weeds and trees was somewhat less compared to grasses and most weed and tree responses were evident in samples that were reactive to grasses. Although multiple sensitizations might be possible, these results are indicative of cross-reactions among allergen extracts.

Malassezia had the highest reactive level. Approximately half of the dogs tested reacted to *Tyrophagus putrescentiae* storage mites as well as *D. farinae* with some 25% reacting to *Blomia tropicalis* and *Dermatophagoides pteronyssinus*. Almost half of the dogs were non-reactive to *D. farinae*, *D. pteronyssinus* and *T. putrescentiae*, and roughly 20% were positive to all three. *T. putrescentiae* reactivity alone accounted for about 6% of the population of dogs. Reactivity to the five mites was seen

in 74,000 samples with roughly the same reactivity levels for each, which suggests a substantial degree of cross-reactivity among mites. Sensitization to all individual mites in an individual dog is unlikely. Thus, the observation of multiple mite reactions in most of the sera indicates cross-reaction among the epitopes. There were very few flea reactivities in Greer Laboratories' *mac*ELISA test because it uses whole body flea for the allergen while the major flea allergens are present in saliva.

Richard Halliwell questioned whether Greer would test 1000 normal dogs at various ages and breeds and look at the incidence of positive reactions in normal dogs.

Ken Lee responded that he would commit Greer to that as he would not be doing it himself! He stated they would be more than happy to run the samples.

IgE-binding proteins: can they affect ELISA-based measurements (B. Hammerberg)

Conflict of interest statement: Bruce Hammerberg is working with Avacta Animal Health as well as providing some materials to ALK-Abello.

Bruce Hammerberg (*USA*) discussed the current state of knowledge concerning naturally occurring proteins that bind specifically or non-specifically to circulating IgE and thus may interfere with serological assays.[4] This includes naturally occurring IgG anti-IgE autoantibodies, circulating soluble high-affinity receptor for IgE (Fc epsilon RI), soluble low-affinity receptor for IgE (sCD23) and galectin-3. These are serum proteins, most often auto-anti-IgE, an IgE class of antibodies that the host makes against its own IgE. These form complexes with IgE that may render circulating IgE undetectable by certain ELISA systems through masking of target IgE epitopes.

Conversely, opportunities exist in that serum proteins which bind to the circulating IgE may reveal pathogenic potential or explain lack of clinical disease. He questions why IgE is detectable to specific allergens when disease is not evident and postulates the answer may lie in looking at these complexes. Naturally occurring IgG anti-IgE antibodies may inhibit allergen-induced basophil activation. IgG auto-antibodies were isolated and examined to see how they influence the ability of basophils that are armed with IgE to function. A question may be whether they actually stimulate the release of IgE or stimulate the release of histamine from basophils that are pre-armed with IgE. The answer to this is that they do the latter. Another question is whether they inhibit the cross-linking of bound IgE on the basophil to an allergen (in this case house dust mite allergen). It was found that some of the components of antibodies they isolated could indeed inhibit the actual cross-linking of the IgE on the surface of basophils by allergen. This is important when looking at the IgG component of auto-anti-IgE and deciding how to examine potential correlations with manifestation of disease or lack of manifestation of disease. This could be important moving forward in terms of making our assay for IgE more correlated to the actual clinical status of the animal being tested.

Other components circulating in serum include the alpha chain that is the part of the high-affinity receptor that binds IgE. This can be found in serum and is becoming an intense area of research in the human field. It could also be affecting dendritic cell function because dendritic cells also have the alpha chain in association with the gamma chain portion that is actually the signalling part. In addition to the alpha chain portion of the high-affinity receptor, we have sCD23 or the low-affinity receptor for IgE. Another potential player is galectin-3, and it is really not known how it might influence IgE functionality in disease pathogenesis. The free alpha chain of the high-affinity receptor in blood basically has the capability of interacting with IgE that is already on B cells. Many questions are yet to be answered: does this have an effect on B-cell function? Does it have an effect on the ability of the B cell to maintain itself and produce IgE or does it become apoptotic? Similarly, if you have IgE bound with the alpha chain, it is not going to be able to bind to the high-affinity receptors expressed on basophils or mast cells. The alpha chain in circulation might be binding to IgE that is already bound to multivalent antigens. Typically, that would sit on dendritic cells and act as a way of enhancing further IgE responses to that particular allergen. That also can be blocked by circulating alpha chains. There are dendritic cells in circulation as well as dendritic cells in tissue.

Peter Hill inquired if there were any data as to how much IgE might be bound up in the circulation just due to antigen and immune complexes.

Bruce Hammerberg responded that he had not found such data in the literature.

Peter Hill asked whether those immune complexes could assume configurations that would prevent them from doing any type of cell activation.

Bruce Hammerberg stated that he thinks this needs to be looked at to ascertain its actual potential effect. Does it really have a practical application? The intention is to try to bring the diagnostic ELISAs more closely in line with the clinical presentation to make them more diagnostically powerful.

Richard Halliwell commented that he is fascinated with the alpha chain being present free in circulation and asked if anyone had an idea as to how much alpha chain there is free in circulation.

Bruce Hammerberg stated that there are a couple of articles from Harvard which have quantitated that in humans.[5,6] He was unsure as to what level that would be relative to its IgE concentration, which would be an important point. Is it really there in a saturating level or just a trivial amount? It is free in their measures so it is not all bound to IgE. This implies that it is in excess of the IgE concentration. This needs to be investigated further.

Experiences with intradermal testing and serology in allergic cats (C. Prost)

Christine Prost (*USA*) presented a review of allergy testing in cats. Allergic cats present with one or more cutaneous reaction patterns or with respiratory disease, such as asthma. Pruritus is the most consistent clinical feature but is not specific and the clinical diagnosis is based on history, physical findings and the exclusion of other pruritic dermatoses. Intradermal testing (IDT) or serology cannot be used for diagnosis but only for identifying causative allergens prior to exclusion or allergen-specific immunotherapy, which has a success rate of approximately 70% in cats. IDT was for a long time the only test available in the cat and it is still considered to be the gold standard yielding biologically relevant results, but it lacks standardization. IDT is considered to be difficult to perform and difficult to interpret in the cat. This is mostly due to stress that induces a rise in serum cortisol which interferes with the reactivity of the test and can cause some false negative reactions. However, several studies have demonstrated that cats can respond in exactly the same way as dogs if the tests are performed properly.

Cats should always be sedated for IDT in order to minimize stress. A clinical diagnosis consultation should be undertaken first to determine the need for IDT so that when the cat returns for testing it can immediately being sedated rather than going to the waiting room. At this consultation the ears should be examined to rule out *Otodectes cynotis* infestation which could yield positive reactions to house dust mites due to cross-reactivity. Results must be interpreted in light of the patient's history and clinical signs. Steroids should be stopped 2 months before skin testing is performed and antihistamines 2 weeks before. The skin of the cat is thinner than that of a dog so dexterity is needed. The tests are read 15 min following the last injection, evaluating erythema, swelling and firmness. There is no consensus as to what constitutes a positive reaction other than a subjective comparison between the positive and negative controls. The offending allergens are usually *D. farinae* and pollens in about 40% of cases depending on the way the cat lives; that is, whether it is an outdoor or an indoor cat. Ten percent fluorescein saline solution can be injected intravenously after the last injection to facilitate the assessment of the skin test response.

Recently there was a pilot study published looking at the prick test versus IDT and this preliminary study showed that the histamine wheals made by the prick test were equal in size to the ones with IDT. Further studies are continuing to validate the use of a prick test for allergens.

Serology for allergen-specific IgE has several advantages over IDT as sedation is unnecessary, and IDT is less traumatic for the patient and much less time consuming. There is also a lesser risk of drugs interfering with the test result. However, it measures only circulating allergen-specific IgE so it does not take into account allergic pathways, and it has shown positive reactions in non-allergic cats and specific pathogen-free cats. Several assays, mostly based on solid-phase ELISA, have been tested for serum IgE and there is good standardization. However, a study on an experimental model of feline asthma using a liquid-phase assay showed there were some unreliable results whereas using the Fc epsilon R1-based ELISA the results showed better specificity but lower sensitivity than IDT. This Fc epsilon R1-based ELISA has been used now for several studies. However, assays should not be used for the diagnosis of non-flea/non-food hypersensitivity in cats because there are cats with high positive specific IgE that have no clinical signs and are healthy and, conversely, there are cats with clinical diagnosis of environmental allergies with no detectable IgE.

When IDT is undertaken for flea hypersensitivity interpretation is required at 15 min and also at 24 and 48 h. Flea control status will influence the IgE production and a cat with inadequate flea control will have a stronger positive reaction than a cat with adequate flea control. The environment also plays a role as does the way the cat is living. The Fc epsilon R1 alpha-based ELISA using flea saliva has been considered, so far, as a reliable test for the diagnosis of flea-bite hypersensitivity in cats, with a positive predictive value of 82%. However, these assays may indicate that there is an allergic sensitivity in an individual cat but cannot prove that signs in that individual are caused by fleas. Only a positive response to rigorous flea control can prove flea-bite hypersensitivity.

For food hypersensitivity, neither IDT nor serology testing should be used to make a diagnosis. An elimination diet is, so far, the only way to diagnose an adverse food reaction. There have been interesting results with patch testing for foods but research is very preliminary and will require further evaluation.

Richard Halliwell remarked that one of the dilemmas in the cat, and in the dog, is the specificity of the test in that positive reactions may be seen in normal animals. He asked for clarification of the speaker's comment regarding positive *in vitro* test serology in specific pathogen-free cats and who published this data.

Christine Prost answered that she did not remember.

Richard Halliwell commented that **Sophie Gilbert** found differently when she undertook work in Edinburgh.

Christine Prost answered that **Sophie Gilbert's** work was published later.

Richard Halliwell added that he would find it difficult to believe the validity of an assay if it is detecting house dust mite with specific IgE in essentially gnotobiotic cats. He stated he would think that the assay must be wrong.

Christine Prost responded that it might be but questioned how that could be proven because not much is known about IgE in the cat.

Jennifer Bexley remarked that Avacta looked at dust mite-specific IgE in the cat and found some reactivity in minimally diseased cats. In this case the cats had been fed dried food and she wondered if it could be storage mites in the food that caused cross-reactivity with dust mites.

Christine Prost replied that this possibility of cross-reaction was a good point.

Richard Halliwell asked if anyone in the audience had good experience with allergy testing in cats. He asked who was undertaking IDT in cats and if it was working.

Rose Miller (*USA*) agreed that IDT is not always as good as it is in dogs with impressive positive reactions but she has found it helpful. She feels that cats do not respond at the same percentage as her canine patients, but that it is still worthwhile and she recommends IDT.

Ashley Bourgeois (*USA*) commented that some clinics have been using fluorescein stain testing and have been very happy with the results. There were no adverse reactions she knew of but obviously that is a concern.

Richard Halliwell enquired if anyone was using serology in cats with good results.

Debra Simpson (*New Zealand*) responded that they do seem to correlate very well, about the same as in dogs, although often not quite as clearly.

Christine Prost asked the audience how many were using IDT in asthmatic cats.

Ashley Bourgeois responded that in her experience asthmatic cats have had higher positive reactions than atopic cats. However, they use a liquid-based ELISA which is interesting in view of the comment about results not being as clear. She also feels that asthmatic cats do extremely well on immunotherapy and wondered if there was a correlation with that.

Richard Halliwell commented regarding **Christine Prost's** publication in a French journal on immunotherapy in asthmatic cats. He asked if anyone else was using immunotherapy in asthmatic cats.

Ashley Bourgeois asked what the conclusions were.

Christine Prost responded that it was a study of about 20 cats and her conclusion was that clinically immunotherapy was very helpful. She had more than 70% good results. She commented on the sparing of steroids as most of the cats had steroid therapy which could be reduced or withdrawn. She remarked that the radiographic appearance was often unchanged despite a good clinical response.

Claire Doyle (*UK*) asked if the speaker worried about injection-site sarcomas with immunotherapy in cats.

Christine Prost responded that she had no experience of this but personally she was using phosphate-buffered saline and not aluminium hydroxide.

Claire Doyle commented that she thought it was very hard to get aqueous aluminium in the UK so she is concerned.

Christine Prost responded that she had not heard that was a big issue.

Richard Halliwell asked if anybody had seen injection-site sarcomas when giving immunotherapy to cats.

Christine Prost added that she asks the owner to change the injection area and not always give the injection in the same place.

Debra Simpson suggested trying sublingual immunotherapy if there was concern about injection-site sarcomas.

Richard Halliwell commented that cats probably do not enjoy sublingual immunotherapy.

Comparison of IDT and serology at three different dermatology centres in Australia (P. Hill)

Peter Hill explained that in studies comparing results of IDT and serology, IDT is normally regarded as the gold standard by which the accuracy of IgE serology is judged. However, this concept is flawed because there is no evidence that IDT can be considered a gold standard. In this study, the two testing methodologies were compared to each other at three different dermatology referral centres. The same blood test (Heska IgE ALLERCEPT assay) was used at each of the three centres. This is an ELISA assay with very strict quality controls and objective readouts whereas the skin test is a much more dynamic individualistic procedure meaning there can be variation in how the test is performed and how it is interpreted. Hence, as the IgE assay was constant throughout, any differences in agreement had to be attributable to the IDT results.

The speaker explained (using a slide to illustrate) that the data are restricted to those allergens that were included in both the blood test and the skin test at each centre. Within each of these three sites the data show the agreement between the IDT and the Heska test. This gives some indication as to what the most commonly reacting allergens were and also the degree of agreement. The results from the two tests agreed between 72 and 88% of the time, depending on the centre. However, the number of discordant results also varied widely between the three centres. There are some interesting differences between the three test sites and these differences cannot be explained by anything to do with the blood test. They can only be explained by differences in the results of the IDT. In site 1 most of the disagreement came about because there were a significant number of positive blood tests to grasses that were negative on the skin test. Comparing that to site 2 the results were quite different. There was a lot of overall agreement between the two tests but, in this particular centre, that was not so much because of a lack of discordance but because of a high degree of double-negative tests (negative in both the blood test and the skin test). The outlier among these three sites was site 3 and in this site there were very few antigens in which the blood test and the skin test agreed with each other. The three dust mites were the exception

but for all the pollens there were very few examples in which the skin and blood test agreed. There were very few double-negative tests and in >10% of the cases the results did not agree. There were many cases in which the skin test was positive and the Heska test was negative. For many allergens, in 20–30% of the tests the IDT was positive and serology negative but, as in the other sites, there were a significant number of positive blood tests and negative skin tests.

In summary, the constant in these three test centres was the Heska test, and the results of the Heska test were likely to be – whether correct or incorrect – consistent. Clearly, IDT is producing some diverse results in how many allergens are reported as positive, and how they agree with the Heska test. In summary, these data emphasize that IDT is a subjective procedure. It is open to individual variation, everyone has probably been taught to do it slightly differently and there is probably some drift over time with how people modify the way they do their tests and interpret them. The outcome is a diverse set of results (which could possibly be used to formulate immunotherapy) that would be completely different if an animal was seen at one centre compared to at another. This is obviously of concern.

Christine Prost asked how many dogs were included in this study at each site.

Peter Hill responded that the number of dogs differed between each site, but was typically somewhere in the region of 30–50.

Porfirio Trapala (*Mexico*) asked if the speaker liked undertaking immunotherapy in dogs.

Peter Hill replied that he was not sure he liked it but he certainly did perform immunotherapy in dogs.

Claudia Pellicoro (*Italy*) asked what the speaker does on a day-to-day basis. That is, does he choose his allergens based on the IDT and also the blood test or, if the IDT matches the clinical signs and history, does he choose only the positive reactions he sees on this test?

Peter Hill responded that the conclusion from this study is that if you have the opportunity to do both a skin test and a blood test in every patient then you will maximize your likelihood of finding all the positive reactions. Whether this makes any difference in the selection of allergens for immunotherapy and the subsequent success rate is not possible to say.

Antoine Adam (*Switzerland*) commented that on site 2 there are so many negative reactions to trees and grasses and asked how this was explained compared to site 1 and site 3. Is this a regional difference?

Peter Hill was not sure that this was a regional difference. He felt it just reflected the case selection in the three different sites. This is another variable. There has to be a decision made as to whether or not an animal has an allergy test undertaken and it may be that at site 2 some of the dogs were being tested before food trials and at the other sites they were being tested after food trials; that is, some of the cases are pre-filtered. These kinds of factors come into it but otherwise he did not know the reason for the high rate of double negatives.

Testing for allergic reactions to grasses: IDT versus scratch tests using pulverized extracts (P. Hill)

Peter Hill explained that intradermal allergy tests and IgE serology can be negative in dogs suspected of having allergic skin disease. Once food allergy has been ruled out, clinicians are often left with a diagnosis of 'atopic-like dermatitis'. However, in some of these dogs the owners are convinced that the pet is allergic to grass. This dilemma led to experimentation with a technique developed by Ken Mason which involved testing for contact reactions to grass by making a grass extract and then applying it to a superficial scratch on the skin. If there is a suspicion of a grass contact allergy the owner is asked to bring in some samples of low-lying plants and grasses from their environment. The speaker explained that it was a crude and archaic technique (even more so than skin testing for immunotherapy) as the samples were just mashed up by pestle and mortar. In the past, this extract was applied to the skin surface without success. However, actually scratching the epidermis very gently with a fine needle aids penetration of the allergen into the underlying dermis. The various extracts are painted onto the test site and immediate or delayed reactions are awaited. Typically contact dermatitis has been considered to be a delayed-type hypersensitivity and, therefore, you might expect not to see any results at these test sites until 2–4 days after application. A series of cases was described showing positive reactions in the face of negative IDTs and/or IgE serology. A positive reaction appears as a linear urticarial wheal along the scratch line, indicating an IgE-mediated reaction. This method demonstrated that a number of dogs had developed allergic reactions to grass stems or foliage but not to pollens (hence the negative IDT result). In summary, this test should be considered in cases that have a contact distribution but defy diagnosis by conventional investigations. **Ken Lee** has taken this a step further by looking at the different allergens that are present in a commercial allergen extract (which is typically derived from pollen) and the antigens that are present in the stem of the grass. By using western blotting it has been possible to show that these dogs do not have IgE against the pollen allergen but they do against protein present in the grass stem. It is quite convincing that this is a valid and legitimate additional test that can uncover genuine allergic reactions when a skin test or blood test is negative.

Richard Halliwell enquired how these animals were treated.

Peter Hill replied that they were usually treated symptomatically with environmental exclusion, topical glucocorticoids or systemic symptomatic treatment.

Christine Prost wondered if the fact that German shorthaired pointers are hunting dogs was pertinent.

Peter Hill responded that none of these were working dogs so lifestyle differences were minimal between this breed and others.

Rares Capitan (*Romania*) commented that this grass study was really interesting for him and thanked the speaker. He asked for more technical information as he felt he would like to replicate this technique.

Peter Hill explained that two very gentle scratches were used with no blood seen.

Rares Capitan asked how the plant was prepared to put on the skin. Was it mixed with anything?

Peter Hill responded that saline was used. He would normally take a small quantity of the plant, mix it with about 5 mL of saline, mash it up, then draw it up into a little pipette and apply it directly immediately.

Rares Capitan asked how long it was necessary to wait before interpreting the test.

Peter Hill stated it was more or less the same as with an IDT. The immediate reactions come up within 15–20 min. He asks the owner to look for the delayed reactions and finds that they very rarely discover any further reaction at the test site.

Duncan Graham (*New Zealand*) asked if attempts were made to identify the grasses more specifically.

Peter Hill clarified that in terms of identification of species, the owner was relied upon to bring the sample and he also relied on their own identification of the species. Some of the owners know what species of grass they have in their lawn and others do not. He tends not to get a complete example of the plant with roots, flowers, etc. so accurate identification is not possible. He then asked what **Richard Halliwell** thought and whether he ever got into this type of testing.

Richard Halliwell responded that he only undertook regular patch testing and with some he got good results and with some he did not. He has not known what to do with dogs when the owner is certain they are reacting to grasses. Sometimes he has patch-tested them with some good positive results which correlated with the results of restriction therapy but he felt that this technique opened up a whole new avenue of investigation.

Peter Hill then went on to ask the North American members of the audience whether they were undertaking this type of testing in any of their clinics. He explained that this was discussed in Australia last year at their Annual Congress and further data on this type of testing was presented. Danny Scott had commented that he had never made that diagnosis, he had never seen such cases and that he did not think it existed in the USA.

Richard Halliwell commented that he had certainly seen it in North America.

Peter Hill was unsure whether it alters the clinical approach dramatically; as in the case of an allergic dog in which no response was seen to a food trial, the skin test was negative, the blood test was negative and it was diagnosed as 'atopic-like dermatitis', it will be treated symptomatically. These dogs fall into that category and they would be treated symptomatically.

Richard Halliwell stated that in these cases you could apply some restrictions which is very important.

Peter Hill said they had definitely seen cases where the owner had reported back saying that, yes, restriction appeared to resolve the problem.

Debra Simpson commented that they often undertake contact avoidance trials so that the dog is kept off all grass for a week. This is performed in stages to ascertain which grass it is. She described a buffalo grass cage at one of the specialist centres that a dog is put into for a day for observation.

Richard Halliwell suggested the next thing to do was to determine whether those dogs were suffering from classical allergic contact dermatitis or this immediate-contact reaction.

Debra Simpson replied that she thought some were a combination of both because when they are restricted from the grass the ventral parts improve, but the skin around the eyes and the top of the paws is still itchy. They would then be skin-tested and started on immunotherapy.

Richard Halliwell closed the workshop by thanking the presenters and participants.

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Flavour of the month: foods as diagnosis and/or treatment

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Phil Roudebush (*USA*) welcomed the audience and opened the workshop with general comments and an overview of the topic.

Brief clinical review

The discussion started with two sample cases showing the interaction between food and allergies in animals. The first case was a golden retriever that was part of a clinical trial in Japan. The dog was diagnosed with atopic dermatitis and found to be mostly reactive to house dust mite allergens. He had been on hyposensitization injections for 2 years and continued to have problems. The dog had periocular oedema, conjunctivitis and lesions around the muzzle. There was a concern whether the food was a component of the dog's disease and a remarkable improvement was observed 2 weeks after the dog was placed on a commercial veterinary therapeutic food containing a hydrolysed protein source. The second case was an extremely pruritic cat with lesions on the medial forelimbs, neck and around the face, all due to self-trauma. Similar to the dog, the cat was considered to have both environmental and food components to its allergic disease.

Phil Roudebush commented that studies' nomenclature is an area of controversy, whether there is truly food allergy or there are atopic animals in which food triggers clinical signs. For the workshop, he chose to use the terms atopic dermatitis and food allergy as separate diseases.

Current data on adverse reactions to food associated with allergic dermatitis were presented, giving a snapshot from different parts of the world.[1,2] These publications suggest that adverse reactions to food can occur alone, but often occur as part of the complex, multifaceted appearance of allergic and infectious skin disease in both dogs and cats.

The audience was asked if they see patients with food allergy occurring alone or if they think patients present food allergy as a manifestation of a more complex disease such as atopic dermatitis or infectious disease. Three attendees replied that they have seen patients with food-allergic reactions occurring alone. All other attendees see adverse food reactions occurring in conjunction with other conditions. The audience was asked if they have seen cases of food allergy happening in conjunction with flea allergies. Two attendees responded 'yes', while many others responded 'no'. The audience was asked how often they saw the following conditions in patients with suspected or confirmed adverse food reactions (recurrent pyoderma, chronic otitis externa, conjunctivitis, chronic diarrhoea/soft stools). An attendee from Thailand commented that he commonly sees recurrent pyoderma in his food-allergic patients.

Deborah Simpson (*New Zealand*) commented that she sees otitis externa frequently in association with food allergy and that she had a few patients whose owners report the ears flaring up within hours after a food challenge. She felt that in 50% of her canine and feline patients with chronic otitis externa the signs were associated with food allergy. In one case, the owner of a food-allergic cat dropped a piece of beef on the floor; the cat ate it and within 2 hours its ears were bright red and pruritic.

Mark Allington (*UK*) commented that of all of the conditions mentioned previously, chronic diarrhoea was probably seen less frequently.

Eleni Lazou Gialama (*Greece*) stated that chronic diarrhoea occurs more often than people think in food-allergic pets. The animals are usually first treated for various gastrointestinal disorders before a diet change is attempted.

Ekaterina Kuznetsova (*Russia*) commented that she sees very few cases of confirmed adverse food reactions and that she believes there is a difference in how food allergy is diagnosed by specialists in different countries. She also believes that many patients showing mild signs of skin disease or otitis externa are not noticed by owners as allergic dogs, while the patients with chronic diarrhoea are taken to veterinary internists and not seen by dermatologists.

Estelle Louarduzzi (*New Zealand*) commented that she personally thinks it is easy to diagnose food allergy if the patient is pruritic; however, the condition may not be recognized if pruritus is a minor clinical sign.

Food allergens

The audience was asked to mention four food ingredients that are allergens in dogs and three food ingredients that are allergens in cats, either in their experiences or based on the literature. Responses from the audience included

chicken egg, beef, chicken, dairy, soy, lamb, rice, corn and pork in dogs and fish, chicken, beef, pork, duck and wheat in cats.

Food ingredients causing adverse reactions in dogs and cats have been published in the veterinary literature for the last 45 years, including studies from North America, Europe, Australia, New Zealand and Japan. Two recent reviews summarized the data.[3,4] The top four ingredients causing adverse reactions in dogs (n = 340) are: beef (31%), chicken (18%), dairy (17%) and wheat (12%), and the top three ingredients causing adverse reactions in cats (n = 60) are: beef (28%), dairy (27%) and fish (25%).

Corn is commonly implicated in allergic pets but has only been confirmed in 3–4% of dog and 7% of cat adverse reactions. The small number of cats with adverse reactions to corn had only gastrointestinal signs (inflammatory bowel disease). There are no reports of cats with exclusively cutaneous adverse reactions that reacted to corn. Soy is also commonly implicated but only confirmed in 4–5% of dog adverse reactions. Rice was only confirmed in 1.5–2% of dog adverse reactions. Artificial colours and preservatives are commonly implicated but rarely confirmed in adverse reactions in pet animals. This may be because artificial colours and preservatives are rarely included in the challenge phases of food trials.

The actual food allergens (not just ingredients) that have been confirmed in dogs are:

- bovine serum albumin (beef allergy),
- bovine IgG (cow's milk, beef),
- chicken serum albumin (chicken),
- Gly proteins, 50 and 75 kDa (soy),
- muscle phosphoglucomutase (beef, lamb),
- nut protein 22 kDa (walnut),
- ovine IgG (lamb).

To date, there have been no actual food allergens identified in cats.

Rob Hilton (*Australia*) said he does not understand why cats rechallenged with a commercial hydrolysed diet relapse, considering that the hydrolysed food would probably not contain traces of the top three ingredients reported to cause adverse reactions.

Phil Roudebush replied that we talk about ingredients frequently, while we really need to be talking about specific food allergens. There are hundreds if not thousands of potential haptens that could be allergens.

Ekaterina Kuznetsova commented that many studies were done a long time ago and some data might not reflect the current reality. **Phil Roudebush** agreed that there are no studies over time, although current studies are not tremendously different than the ones 20 or more years ago.

Lawan Larsuprom (*Thailand*) commented that most dogs eat chicken in her country and beef is probably not a common allergen.

Food allergen cross-reactivity[5]

Common cross-reactivity in human beings with adverse food reactions was summarized by **Phil Roudebush**. We currently do not know whether these apply to dogs and cats. Allergy to cow's milk is often associated with allergy to milk from other animals (90%); allergy to chicken eggs is often associated with allergy to eggs from other bird species; allergy to one species of fish is often associated with allergy to other fish (50%); allergy to chicken meat is often associated with allergy to other birds. Cross-reactivity is common among all invertebrates [e.g. mites, insects, molluscs (squid), crustaceans (shrimp, lobster, crab)] because of the pan-allergen tropomyosin.

Uncommon cross-reactivity in human beings with adverse food reactions was summarized. Adverse reactions to soy do not predict adverse reactions to other legumes, and human beings with adverse reactions to soy or peanut proteins can safely consume soybean oil and peanut oil, respectively (vegetable oils are highly refined with no protein content).

Cross-reactivity in dogs with adverse food reactions have been reported; there is no information on allergen or ingredient cross-reactivity in cats. Dogs with adverse reactions to soy are not likely to have adverse reactions to wheat. Dogs with adverse reactions to beef are likely to have adverse reactions to lamb (and other mammals?), possibly because of phosphoglucomutase being a common allergen. Dogs with adverse reactions to beef and lamb may or may not have reactions to cow's milk (there are conflicting data between *in vitro* and clinical results). Reactivity to fish oil is possible; processes used for fish oil extraction are not as highly refined and some fish oil sources have detectable levels of protein. It is not known if the proteins in fish oil contain allergens.

Ursula Thomas (*USA*) reported she is concerned about cross-reactivity in patients suspected to have chicken allergy and the diet is changed to duck protein. She is also concerned about potential cross-reactivity between environmental allergens such as tree pollens and some food sources.

Phil Roudebush commented that people allergic to chicken are more likely to react to other poultry like duck, but there are no actual studies in animals.

Ekaterina Kuznetsova commented that people can have cross-reactivity between tomato and cedar pollen and she wondered if that type of cross-reactivity occurs in dogs.

Mite contamination of food

In vitro cross-reactivity between dust and storage mites appears to occur in dogs. What about clinical cross-reactivity or cross-sensitization?

Investigators found no evidence of house dust mite contamination in newly purchased or stored commercial dry dog food in the north central USA.[6] House dust and storage mites and their allergens can contaminate dry dog food stored in household conditions. However, there is little evidence that this is a clinically significant source of

exposure for allergic dogs in temperate climates.[7] Storage mite contamination may be more common in subtropical and tropical climates.[8]

Investigators looked into *in vitro* reactivity between two house dust mite allergens and three storage mites allergens, and found about 50% cross-reactivity.[9] Phil Roudebush commented that this raises the question of whether individuals become sensitized to storage mites and then cross-react with house dust mites, or is it the other way? He thinks that from a clinical standpoint it might be related to tropomyosin, which is a highly allergenic protein for some individuals and is highly conserved across invertebrate species. He expects that some cross-reactivity does occur.

Phil Roudebush added that taking food out of the original bag adversely affects the palatability of commercial dry pet foods. His recommendations for food storage of commercial dry pet foods to minimize house dust or storage mite contamination and maintain food palatability are to: (1) store food in a cool (10–27 °C), clean, dry location; (2) store commercial pet food in the original packaging; and (3) ask pet owners to purchase food that will be consumed within 3 months.

Phil Roudebush asked the audience what they hear or what they (and their clients) are concerned about with regards to mite contamination.

Lotta Pankala (*Finland*) commented this is a common concern among her patients' owners. When she performs skin tests, quite often dogs show positive reactions to house dust mites and storage mites, then owners try to find where the mites come from.

Phil Roudebush added the question, *Is this serological cross-reactivity or is it true cross-sensitization?*

Ariane Neuber (*UK*) wondered if there are low numbers of storage mites in food, might that possibly induce oral tolerance?

Ursula Thomas commented that she has a similar experience as the colleague in Finland about the cross-reactivity in allergy tests and she talks to her clients about how they store the pet food. However, when she talks to general practitioners, she notes that even though they manage allergic patients and recommend vacuuming the house, food storage is not addressed.

Phil Roudebush added that in pet-food manufacturing plants there are large silos containing the ingredients that go into the food. It is known that many of these ingredients may be contaminated with storage mites. These storage mites (dead or alive) might be going into a pet food recipe before the food is cooked. A proposed study might look at individual ingredients for storage mites, or storage mite allergens, before food processing and as soon as the dry or canned food comes off the production line. This is a question unanswered in both human foods and pet food.

Commercial versus homemade pet foods

Phil Roudebush commented that over 60 commercial pet foods are available to aid in management of dogs and cats with skin and hair disorders. Fewer than 20 of these commercial products have had clinical studies to document their efficacy. In published clinical studies, their efficacy has ranged from 65 to 85% but entry criteria and outcomes have varied greatly, which makes comparison of products difficult.

The rationales for pet owners choosing homemade pet foods were summarized:[10]

- appeal of natural, organic, fresh or wild-grown ingredients,
- appeal of vegetarian and vegan foods,
- concerns about additives, preservatives and contaminants,
- inability to understand pet food labels (list of indecipherable list of chemicals),
- table foods have become a bad habit or effort to maintain adequate food intake in a finicky pet,
- feed a pet according to human nutritional guidelines (e.g. low-fat, low-cholesterol, gluten-free),
- lower feeding costs,
- veterinary therapeutic food is unavailable or unacceptable,
- dietary elimination trials or long-term feeding to avoid food allergens,
- wish to support a sick or terminally ill animal,
- desire to personally cook for the pet.

Common nutrient problems in homemade foods have been reported:[11]

- excessive protein levels,
- deficiencies in calories, calcium, vitamins and micro-minerals,
- inverse calcium/phosphorus ratio,
- feline homemade foods: unpalatable fat source, deficient in taurine and arachidonic acid.

In one study many years ago, only 10% of homemade foods recommended by veterinary dermatologists for dietary elimination trials were complete and balanced, while many homemade foods recommended for long-term maintenance feeding (65% feline foods; 50% canine foods) were also not nutritionally adequate.[12]

Assessing homemade food recipes ('quick check' guidelines)[10]

1. Do five food groups appear in the recipe?
   - Protein source, preferably of animal origin
   - Carbohydrate/fibre source
   - Fat source
   - Source of minerals, especially calcium
   - Multivitamin and trace mineral source
2. Is the carbohydrate source present in a higher or equal quantity than the protein source?

- 1:1 to 2:1 for cat foods; 2:1 to 3:1 for dog foods

3 What is the type and quantity of the primary protein source?
- Skeletal muscle protein from different animal species has very similar amino acid profiles.
- The final food should contain 25–30% cooked meat for dogs and 35–50% cooked meat for cats.
- Providing liver once a week corrects most amino acid deficiencies.
- Eggs are the best protein substitute for meat; soybeans or other legumes are the best vegetarian option.

4 Is the primary protein source lean or fatty?
- Fat sources are needed for appropriate energy density and essential fatty acids.

5 Is a source of calcium and other macrominerals provided?
- Calcium carbonate if protein ≥ carbohydrate
- Calcium-phosphorus (bone meal, dicalcium phosphate) if protein < carbohydrate

6 Is a source of vitamins and other micronutrients provided?
- For an adult animal, supplement iodized salt and half to one human multivitamin daily.
- For cats, add taurine, carnitine and arachidonic acid supplements or sources.

For any homemade food, it is important to be aware of 'diet drift' by the pet owner and to always give specific diet recommendations or recipes including how to make the diet and how much to feed.

Phil Roudebush asked the audience the following questions: *Do you prefer using commercial versus homemade pet foods for patients with skin disease?*

What are your favourite homemade food options?

Have you and your clients had success (or not) using homemade foods?

Sonja Zabel (*USA*) commented she has many clients who want home-cooked diets for their pets, but that it is difficult to make sure that the protein is being acquired from a source without contamination.

Deborah Simpson commented that in New Zealand, venison and goat are common protein sources used for diets.

Lawan Larsuprom (*Thailand*) asked if there would be nutritional problems with recommending only an animal protein and potato diet for 2 months.

Phil Roudebush responded that nutritional problems (e.g. water-soluble vitamin deficiency) with incomplete or unbalanced foods could occur within 3 weeks, especially in growing animals.

Jakaphan Wonnawong (*Thailand*) asked if the concept of a single-source protein and a single-source carbohydrate should be considered when recommending a home-cooked diet for an allergic dog or cat.

Phil Roudebush reminded the audience that all carbohydrate sources contain proteins as well; even starch that goes into pet food or is purchased in a store contains small amounts (0.5%) protein. There is no readily available carbohydrate source that is protein-free.

Rob Hilton agreed with the colleague from Thailand about the variability of food allergy in different countries. He stated that in the post-oclacitinib (Apoquel®; Zoetis) era, the method of diagnosing food allergy has completely changed. He reported that it is easier to have owners perform an elimination food trial while receiving Apoquel®, because the dogs don't have to be so uncomfortable for 7–8 weeks.

Raw (BARF) versus cooked/processed pet foods

BARF is an abbreviation for bones and raw food, biologically appropriate raw food or biologically active raw food. This is a popular topic for pet owners; an Internet search in May 2016 showed 5.04 million entries for raw pet food diets and 1.45 million entries for raw pet food recipes.

Nutritional concerns with raw pet foods have been reported. One study found excessive levels of vitamin D, vitamin E, fat and protein; inadequate amounts of potassium, manganese and iron; variable levels of zinc; and inappropriate ratios of calcium to phosphorus.[11]

Phil Roudebush stated that to his knowledge no feeding trials have been completed with raw food diets (e.g. no nutritional adequacy statements confirming feeding trials appear on raw food products in the USA).

Food safety concerns with raw pet foods have been reported from three different geographical areas. A study conducted by the Centers for Disease Control in the USA investigated *Listeria*, *Salmonella* and toxigenic *Escherichia coli* in various pet foods.[13] In phase 1, 480 commercial foods were cultured; one was positive for *Salmonella* and one was positive for *Listeria*. In phase 2, 576 raw/jerky foods were cultured; 15 were positive for *Salmonella* and 66 were positive for *Listeria*.

Phil Roudebush commented that in the USA *Listeria* has been one of the leading bacteria associated with food recalls. *Salmonella* contamination is most commonly associated with chicken. A study from Sweden evaluated 39 samples of raw dog foods. *E. coli* was found in all 39 cultured samples and cephalosporin-resistant *E. coli* in nine products.[14] Risk factors for carriage of antimicrobial-resistant *Salmonella* spp. and *E. coli* in pet foods were reported in Ontario, Canada.[15] Dogs were cultured for *E. coli* and *Salmonella* (138 dogs in 84 households). Dogs positive for these organisms were more likely to be fed a homemade or raw diet, have homemade food or raw food added to a commercial diet or be fed raw chicken in the past week.

Phil Roudebush asked the audience: What experience do you have with raw food diets? Do you recommend them? What problems, if any, have you seen in patients eating raw foods?

Ursula Thomas commented that she has many clients asking about raw food diets and that she generally does not recommend them for food safety reasons. She was also curious to know about how cooking could change the allergenicity of proteins. For example, would it make a difference whether a patient eats raw versus cooked beef?

Phil Roudebush mentioned a poster/abstract presented at the conference showing possible allergenic changes in cooked proteins. Future studies should look at how specific food allergens change with cooking (e.g. bovine IgG or bovine serum albumin levels in raw beef versus cooked beef).

Mark Allington (*UK*) commented that *Campylobacter* bacteria have been a problem associated with raw foods in people in the UK. He asked if there was any information on similar problems in the veterinary literature. A literature search by **Clarissa Souza** (USA) after the workshop found that raw meat and internal organs, possibly contaminated by pathogens such as *Campylobacter* spp. might pose a risk of transmission of zoonoses to the pet owners.[16]

Katja Kyrolainen (*Finland*) commented she also has many clients that are interested in raw food diets. She expressed concerns about the findings of multiresistant bacteria in meat for human consumption and the impact it can have on dermatological patients.

Deborah Simpson said that raw food diets are very popular and many clients ask about them. Some clients believe their pets have improved just from changing from a commercial food to a raw food diet. She encourages pet owners to not feed raw foods for safety reasons, especially in patients that are being given immunosuppressive medications.

Clinical tips

A diet diary is essential when performing dietary elimination food trials (see reference 5 as an example).

Recommend disposable pet food bowls if using raw food diets (or homemade food) since *Salmonella* often persists in stainless steel or plastic pet food bowls. In one study, stainless steel and plastic dog bowls were contaminated with pathogenic *Salmonella*, then different ways of cleaning were tried (warm water rinse, rinse and scrub, scrub and soap, soap and bleach, dish washer, scrub with bleach): 42% retained *Salmonella*.[17]

Board-certified veterinary nutritionists can help with homemade food assessment or recipes. Examples include www.petdiets.com (Dr Rebecca Remillard) and www.balanceIT.com (Drs Sean Delaney and Sally Perella).

Phil Roudebush closed the workshop, thanking everyone for participating and wishing them a safe trip home.

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