Plenary Session Abstracts: Saturday afternoon, 28th August Theme: INFECTIOUS AND PARASITIC DISEASES

State of the Art Address

Mycobacterial diseases affecting the skin or subcutis of cats and dogs

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Mycobacteria are gram-positive bacterial rods that have as their distinguishing feature a cell wall rich in mycolic acids and esters. This waxy layer imparts two important features of the genus: an acid-fast staining characteristic, and the ability to survive and replicate within phagocytic cells. In the history of microbiology, infections caused by mycobacteria feature prominently as two important diseases of antiquity, tuberculosis and leprosy. In veterinary medicine, most attention has been paid to tuberculosis and Johne's diseases (paratuberculosis). The best way to understand mycobacterial syndromes is to develop a conceptual framework based on the host-pathogen relationship. Thus, the mycobacterioses can be divided into: (1) diseases caused by obligate mammalian parasites; (2) localized infections of immunocompetent hosts; and (3) disseminated infections in hosts with defective cell-mediated immunity. Tuberculosis is the prototypic disease caused by mycobacteria that are so adapted that they cannot survive without a mammalian host, i.e. they represent true bacterial parasites. The *Mycobacterium tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*) are primary pathogens capable of producing contagious disease in immunocompetent hosts, although they can also produce devastating disease in immunodeficient hosts. It is important to remember that although the tubercle bacillus can produce florid clinical signs, localized inapparent infections that are constrained and eventually eliminated by a competent immunological response are much more common than cases with symptomatic disease. An atypical form of tuberculosis attributable to *M. microti* is seen with some frequency in cats in the UK as a result of cats being bitten by a vole (field mouse), an environmental reservoir. Feline leprosy attributable to *M. lepraemurium* is probably best considered a mycobacterial disease caused by an obligate mammalian parasite, the murine leprosy bacillus, inoculated subcutaneously following bite injuries from infected rats. Most mycobacterial diseases in cats and dogs fall into the second category of localized infections of immunocompetent hosts. The majority of these infections result from inoculation of environmental mycobacterial species through a breach in the epidermis. Typically, this occurs following a penetrating injury in which there is subsequent contamination of the wound with soil or dirt, although the insult may be as subtle as an insect bite. Rapidly growing mycobacteria like *M. fortuitum* or *M. smegmatis* have a predilection for producing infections in fatty tissues, such as the subcutis of cats and dogs or the mammary glands of cattle. These infections can, with time, become deep-seated and extensive. Treatment typically involves long courses of antimicrobials chosen on the basis of culture and susceptibility data, although some cases require *en bloc* resection of infected tissues and reconstruction of the surgical wound using advancement flaps. Occasionally, *M. avium* and other slow-growing saprophytic species can also give rise to localized cutaneous disease, typically following a cat-scratch injury. Leproid granulomas are localised infections of short-coated dog breeds that probably result from inoculation of a fastidious saprophytic mycobacterial species into the subcutis following fly bites. The majority of these infections resolve spontaneously as a result of an effective host response, although rare cases require systemic or topical therapy. The least common form of mycobacterial disease occurs in patients with immunologic compromise. In these patients, involvement of the skin is the most obvious manifestation of a widespread disseminated process. Surprisingly, these cases may respond favourably if treated in a timely manner with appropriate combinations of antimicrobials, e.g. clarithromycin and rifampicin, and possibly an additional agent.

Supporting Original Study 23

Clinical, pathological, and molecular characterization of feline leprosy syndrome in the western USA

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Feline leprosy syndrome is caused by multiple species of mycobacteria; at least two histomorphologic subtypes have been reported from Australia and Canada, and PCR/DNA sequencing studies suggest that several species of mycobacteria may be implicated. To the authors' knowledge, similar studies from the United States have not been performed. Ten cats with skin lesions characteristic of feline leprosy syndrome and acid-fast confirmation of organisms were included in the study. The cats were evaluated by clinical follow-up, histopathology, and molecular characterization (PCR with DNA sequencing). Eight of 10 cats were from coastal cities of Hawaii, Washington or California; two cats were from the coastal mountain range in California, approximately 30 miles inland. Additional environmental risk factors included access to the outdoors in nine cats, four of which were observed hunting. Skin lesions ranged from mildly alopecic and swollen to nodular and ulcerated. Lesions were evaluated histopathologically, using both H&E and Fites acid-fast stains for necrosis, number and distribution of acid-fast bacteria, and visibility of organisms on H&E examination. PCR and DNA sequencing of a fragment of the 16S rRNA gene was performed in all cases. Lesions from four cats yielded *Mycobacterium visibilis*/IWGMT 90242 species, as previously identified in Canadian but not Australian cases; in each of these, large numbers of acid-fast bacteria were seen diffusely within non-necrotic lesions. These organisms also were visible with H&E examination. Lesions from four cats were associated with *M. lepraemurium:* three had necrosis with few (three cases) to moderate (one case) numbers of acid-

fast bacteria that were not visible upon H&E examination. Organisms tended to cluster in necrotic foci. The remaining lesions from two cats included one each of *Rhodococcus erythropolis* and *M. kansasii*; both of these had *M. lepraemurium*-type histomorphology. Clinically, cats with *M. visibilis*/IWGMT infection tended to be older (mean age 10.3 years, $P = 0.008$) and have larger numbers of lesions (range four to too numerous to count) with recurrence (three cats), unexplained mortality (one cat), or concurrent disease (one cat coinfected with *T. gondii*). Cats with *M. lepraemurium*, or histomorphologically similar *Rhodococcus erythropolis* and *M. kansasii* infections, were younger (mean age 2.2 years) and tended to have few lesions (range 1–5; mean 2.2). These cases responded completely to excision and treatment with miscellaneous broad-spectrum antibiotics. The presence of two clinically and histomorphologically distinct syndromes supports previous reports from Canada and Australia. Clinical differences from Australian cases were identified, specifically the benign course of *M. lepraemurium*-type infections. The significance of the *Rhodococcus erythropolis* and *M. kansasii* is not clear; however, multiple species of mycobacteria have been recently identified by PCR in Canadian cases of feline leprosy syndrome. Geographic differences in specific organisms associated with feline leprosy syndrome may reflect local differences in risk factors, possibly due to differing ecologies of *M. lepraemurium* and *M. visibilis* in Australia, Hawaii and the continental United States.
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Supporting Original Study 24

Rhodococcus spp. as ubiquitous contaminants of paraffin-embedded tissues in PCR analysis for *Mycobacterium* spp. skin infections

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Molecular techniques have greatly enhanced the sensitivity of the laboratory diagnosis of infectious diseases. Highly sensitive polymerase chain reaction (PCR) analysis of tissue DNA is being used to characterize and speciate infectious agents. The use of PCR is increasing in the diagnosis of *Mycobacterium* spp. infections. Frequently, the primers used are not specific for the genus and they may amplify other closely related organisms such as *Rhodococcus* spp. Some of these organisms are ubiquitous and, as for organisms of the genus *Mycobacterium*, they are usually isolated from soil. For this reason, they may be potential contaminants of instruments, reagents or even skin. As part of a study to characterize the causative organisms of feline leprosy, nodular pyogranulomatous lesions from cats with no recognizable organisms in tissue sections, using acid-fast stains or the BCG technique, were analysed by nested-PCR. Briefly, samples from 12 cats were obtained retrospectively from paraffin-embedded tissues, deparaffinized and DNA extracted by standard techniques. Extracted DNA was amplified with previously characterized sets of primers for a fragment of the 16S rRNA gene. Amplified fragments were sequenced in an automated sequencer, analysed and compared by computer analysis with known sequences of related organisms. In addition, 14 feline and canine control tissue samples were analysed. These included mildly inflamed (psychogenic alopecia and allergic skin disease, $n = 5$) and noninflammatory (neoplastic skin masses, $n = 3$) skin lesions, and internal organs (liver, spleen and lung, $n = 6$). The results revealed DNA sequences consistent with *Rhodococcus* spp. (99–100% homology) in nine of 12 (75%) of the acid-fast bacteria-negative nodules. The remaining two samples were negative. None of the samples yielded amplicons with homology to *Mycobacterium* spp. Additionally, nine of 14 (64%) unrelated control tissues showed similar amplification of a bacterium with high homology (98–99%) to *Rhodococcus erythropolis* and other *Rhodococci*; two of 14 amplified a fragment of DNA with homology to *Gordonia* spp. (soil organism), and one sample amplified a fragment of DNA with homology to *Mycobacterium sphagni* (soil organism). The remaining two samples were negative. Parallel controls (PCR master mix inoculated with sterile water and PCR master mix alone) were consistently negative. Based on these results, two main scenarios may be in play: (1) in lesions with histologically undetectable acid-fast organisms, the amount of mycobacterial DNA may be very low, and other dominant contaminant organisms such as *Rhodococcus* spp. may be amplified preferentially; (2) a negative acid-fast result obtained histologically may be accurately predictive for an absence of infection with *Mycobacterium* spp., and only contaminants are amplified. In summary, the significance of *Rhodococcus* spp. as a causative agent of nodular dermal lesions with no recognizable acid-fast organisms, when results are obtained exclusively by PCR analysis, should be questioned. In this event, the aetiology of the lesions should be further investigated using other complementary diagnostic procedures (e.g. DNA capture, *in situ* hybridization, *in situ* PCR).
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Supporting Original Study 25

In vivo expression analysis of *Microsporum canis* secreted subtilisin-like serine proteases in feline dermatophytosis

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The cellular and molecular pathophysiology of dermatophytosis is largely unknown in both humans and animals. During the past 5 years, we developed a broad research programme to better understand the host-fungus relationship in *Microsporum canis* ringworm. This allowed us to isolate and characterize several putative fungal virulence factors, especially keratinolytic proteases, belonging to two gene families encoding three subtilisin-like serine proteases (SUBs) and three metalloproteases (MEPs). We demonstrated that all the SUBs and two MEPs were produced in hair of experimentally-infected guinea pigs. Using the same model, we also demonstrated the immunogenicity of at least two proteases, SUB3 and MEP3. However, we recently showed that SUB3 and MEP3 produced as recombinant pro-

teases and used as subunit vaccines failed to protect against a *M. canis* experimental infection in guinea pigs. As a prelude to investigation of the role of SUBs in *M. canis* infection in natural hosts and to their potential use as vaccines in cats, this study was designed to establish their presence in *in vivo* clinical conditions in the cat and human. RT-nested PCRs using specific primers of internal fragments of *SUB1*, *SUB2* and *SUB3* were performed on total RNA extracted from infected hair or scales from naturally infected cats ($n = 4$) and humans ($n = 2$). Two specific primer pairs were also designed for the amplification of an internal fragment of *M. canis* actin mRNA, which was used as a positive control for *M. canis* RNA extraction and RT-nested PCR. Fungal strains from which one or more *SUB* mRNA(s) could not be detected from infected samples were checked by PCR for the presence of the correspondent *SUB* gene(s) in their genomes. Results indicated that *M. canis* actin mRNA was detected in all samples, indicating that both *M. canis* RNA extraction and RT-nested PCR conditions were successful. The three *SUBs* were transcribed in two cats while mRNAs encoding two of them were detected in the other two cats. The *SUB1* gene was found to be transcribed in humans. All *SUB* genes where *in vivo* transcription was not detected were demonstrated to be present in the genomes of the corresponding strains. These results suggest that *M. canis* SUBs are strongly involved in the cat-fungus relationship, whereas SUB1 could play a significant role in *M. canis* dermatophytosis in humans. Indeed, although being a very difficult technique to perform in filamentous fungi, the RT-nested PCR used in this study demonstrated that all *SUB* genes could be transcribed *in vivo* in the cat. It suggests strongly that the corresponding encoded proteases are expressed by the fungus during invasion of feline keratinized structures. Although these data were obtained from a rather small number of samples, they are the first ones dealing with the molecular pathogenesis of dermatophytosis during natural infections. Moreover, they must be included in the frame of our previous and present studies focusing on different aspects of the host-fungus relationship, including both the characterization of *M. canis* virulence factors and the host response to infection.
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Supporting Original Study 26

Cutaneous asymptomatic carriage of *Leishmania* spp.

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Parasites establish dynamic relationships with their hosts that allow their persistence. *Leishmania* spp. are unicellular parasites that have two hosts: a haematophageous insect, the sand fly, and a vertebrate. Both in animals and humans, absence of clinical lesions does not imply absence of infection. This phenomenon has major implications in epidemiology, preventive medicine, and pharmacology. Parasitic load determination in mammalian tissues or organs is currently performed by culture-based methods that are time consuming and require samples harbouring cultivable parasites. Recently, we developed a real-time PCR assay to quantify the parasitic burden of several *Leishmania* spp. based on detection of kinetoplastic DNA (kDNA). The efficacy of this real-time PCR assay was first evaluated in monitoring the fate of *Leishmania major* in C57Bl/6 mice following intradermal inoculation of the right ear pinna with low doses of infective metacyclics at 24 h, 96 h, and weekly for 7 weeks. *Leishmania* were established in the center of the inoculated pinna, to a lesser extent in the pinna border, and in the draining lymph node. The parasite was detected, but not quantified, in distant cutaneous sites (left ear pinna, thorax skin, tail base) and in the femoral bone marrow. Throughout the whole experiment, the parasite was detected in the glabrous tail base skin. We also investigated whether the parasites detected by our method were still viable or if we were also detecting DNA from dead parasites. Technical and biological constraints have been overcome by developing an *in vitro* model to monitor the persistence of *Leishmania* kDNA in cultured mouse macrophages after host leucocytes were exposed to *L*-leucine-methyl ester, a leishmanicidal molecule. The detection of kDNA by real-time PCR was correlated with the viability of the parasites in macrophages. These results show that laboratory mice (experimental host) or *in vitro*-grown mouse macrophages infected with *Leishmania* parasites can be considered useful systems in two areas. First, in pharmacology, preventive medicine and epidemiology, such a system would allow pharmacological evaluation of *Leishmania*-targeted drugs or vaccines to a broader extent than merely monitoring the reduction of the lesion or the prevention of lesion onset. A similar system could be developed in other animal species, particularly the dog, in order to detect asymptomatic carriage at the borders or in quarantines, or to investigate the real prevalence of *Leishmania* spp. The growing importance of *Leishmania infantum*-related disorders in humans makes such a method very interesting. A second use for this experimental system is the investigation of the prompt and renewed interactions that *Leishmania* parasites establish within their mammalian hosts. Establishment of a system to quantify the parasitic burden of *Leishmania* spp. is the perspective of this preliminary research. Recently published data show that the apparent lack of symptoms hides complex mechanisms involving very different factors such as keratinocytes, epidermal and dermal leucocytes, the extracellular matrix, and sand fly saliva.
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Supporting Original Study 27

Adherence of *Staphylococcus intermedius* to canine corneocytes involves a protein–protein interaction that is sensitive to trypsin but resistant to cold

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Adherence is a prerequisite for canine cutaneous bacterial infection that is potentially affected by a large number of variables. The aim of this study was to explore the nature of this interaction by investigating the effects of

long-term storage, cold temperatures and trypsin on staphylococcal adherence to canine corneocytes. Sheets of corneocytes were collected using double-sided tape from the ventral abdomen of healthy dogs at 5, 3 and 2 months before use and stored at –80°C, 4°C and room temperature (approximately 20°C) in dry and dark conditions; corneocytes were also collected on the day of each experiment. *Staphylococcus intermedius* from a case of bacterial pyoderma was prepared in phosphate-buffered saline (PBS) and applied in triplicate to the canine corneocyte-covered tapes using PBS as the negative control. After incubation, rinsing and staining with crystal violet, quantification of the adherent bacteria was carried out blindly by computerised image analysis of 15 fields. All the experiments were processed in two duplicate, identical experiments. There were no significant differences in the adherence of *S. intermedius* to fresh corneocytes and to those that had been stored at –80°C ($P = 0.3703$) or 4°C ($P = 0.1129$) at any time point and by subanalysis ($P > 0.05$ in each case). By contrast, the adherence to corneocytes stored at room temperature showed significant variation ($P = 0.0079$), with the values for fresh samples being higher than those stored for 5 months; however, subanalysis did not reveal significant differences between individual time points ($P > 0.05$). These results indicate that the mechanisms of adherence are not affected by even prolonged storage at cold temperatures. Such storage greatly facilitates the performance and comparison of adherence studies. To determine if adherence involved interaction of proteins on the surface of the organism with those on corneocytes, trypsin, a potent broad-spectrum proteinase, was added to the incubation buffers. Preliminary experiments showed that trypsin concentrations of 1, 10^{-2}, 10^{-4} and 10^{-6}% did not affect the viability of *S. intermedius* in incubations lasting up to 4 h. Subsequent adherence assays were therefore conducted using a 4-h incubation period and the above concentrations. Adherence of *S. intermedius* to canine corneocytes was completely abolished following incubation of the organism with trypsin at concentrations ranging from 1 to 10^{-2}%, but adherence still occurred at the lower concentrations, demonstrating a dose–response phenomenon. These results suggest that a protein receptor on the surface of the organism was digested, thus eliminating the capacity for adherence. Paradoxically, pretreatment of the corneocytes with trypsin resulted in a significant enhancement in adherence of the organism ($P < 0.0035$). This may result from exposure of surface receptors after digestion of surface proteins. Alternatively, trypsin may damage the corneocyte envelope and expose cytoplasmic keratin fibres and matrix which express greater affinity for the bacterium. These studies provide evidence that adherence of *S. intermedius* to canine corneocytes involves a specific protein–protein interaction. Elucidation of the precise proteins involved could provide molecules that might be amenable to therapeutic targeting.

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Supporting Original Study 28

Histopathological and clinical characterization of dermatopathy associated with toxic shock-like syndrome in dogs

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Toxic shock syndrome (TSS) in humans is a systemic disease caused by the release of bacterial toxins with superantigen activity. Most cases in humans are related to production of toxic shock syndrome toxin-1 (TSST-1) by strains of *Staphylococcus aureus*. Clinically, they manifest with high fever, hypotension, diarrhoea, myalgia, skin rash and oedema, often progressing to desquamation of the skin. External or internal infections may be the origin. In dogs, the most common bacterial organism isolated from cases of bacterial pyoderma is *Staphylococcus intermedius*. This bacterium has also been shown to produce exotoxins, including TSST-1. Retrospectively, 11 dogs with clinical presentations and histopathological lesions closely resembling TSS in humans were evaluated. The animals ranged in age from 1.5 to 13 years, with a mean of 4.65 years. No sex predisposition was found. The breeds represented in the study were varied, including small breeds (pug, Boston terrier) and large breeds (golden retriever, Labrador retriever). The most common clinical presentation was multicentric to generalized cutaneous erythema ($n = 10$) and oedema ($n = 7$), in some cases with vesicles ($n = 4$) and pustules ($n = 3$) evolving to ulcers ($n = 3$). The extremities, ears and ventrum were frequently involved. Systemic signs were invariably present. Mild anaemia ($n = 8$), hypoalbuminaemia ($n = 8$), depression ($n = 8$) and neutrophilia ($n = 7$) were the most consistent abnormalities. Fever ($n = 5$) and thombocytopenia ($n = 5$) were also observed. Complete clinical history was not obtained in two cases that were compatible with this condition on histopathological exam. In general, there was a good response to systemic treatment with cephalexin ($n = 4$); in cases for which early antibiotic therapy was not instituted, the outcome was usually fatal ($n = 5$). In the only case cultured, pustular lesions yielded a mixed population of coagulase-positive *Staphylococcus* spp. and *Pseudomonas* spp. Histologically, the most characteristic pattern was superficial dermatitis with epidermal neutrophilic exocytosis and necrotic (apoptotic) keratinocytes, often with neutrophilic satellitosis. The lesions evolved to panepidermal necrosis with ulceration in some dogs, a phenomenon unrelated to the final outcome. The pathogenesis of the skin lesions in human cases of TSS seems to be related to the nonspecific stimulation of lymphocytes by the bacterial superantigen and the release of high levels of cytokines, specifically TNFα. Until further characterization of the process is obtained, biopsies with features of neutrophilic exocytosis and apoptosis should warrant consideration of TSS, and immediate antibiotic treatment (cephalexin) should be instituted to prevent potential fatal progression of the disease.

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