DISSERTATION

# UPPER RESPIRATORY TRACT DISEASE IN CATS: ORGANISMS INVOLVED, MODULATION OF THE IMMUNE RESPONSE, AND ANALYSIS OF A NOVEL TREATMENT

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JULIA K. VEIR ENTITLED UPPER RESPIRATORY TRACT DISEASE IN CATS: ORGANISMS INVOLVED, MODULATION OF THE IMMUNE RESPONSE, AND ANALYSIS OF A NOVEL TREATMENT BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

UPPER RESPIRATORY TRACT DISEASE IN CATS: ORGANISMS INVOLVED, MODULATION OF THE IMMUNE RESPONSE, AND ANALYSIS OF A NOVEL TREATMENT

This work evaluated organisms detected by microbiologic culture and molecular biology techniques in acute upper respiratory disease in shelter cats. Compared to other studies, similar detection rates were found for feline herpesvirus-1 (FHV-1) and *Bordetella bronchiseptica* but in our study population, there was a low incidence of *Chlamydophila felis* and calicivirus. Our results suggest that results from samples collected from the nasal or pharyngeal cavity were similar and detection by nucleic acid amplification techniques were suitable sampling strategies. A quantitative PCR assay was applied to nasal and pharyngeal samples and correlation between disease status and FHV-1 viral load was demonstrated, suggesting the assay may be useful clinically.

A whole-blood proliferation assay was evaluated in order to assess the cellular immune response during an attempt to improve response to FHV-1 vaccination via supplementation with a strain of *Enterococcus faecium* (SF68) in kittens. The assay was shown to be reliable and used little blood, allowing for repeated testing in young animals. An increase in the percentage of CD4+ lymphocytes but not an increase in proliferative response to FHV-1 antigens secondary to supplementation was demonstrated. This may

suggest an improvement in antigen processing abilities of the cats; however, more detailed studies are needed to prove this theory.

In an attempt to ameliorate clinical signs in cats with rhinitis, response to a novel therapy of liposome DNA complexes was reported in three groups of cats: client-owned, shelter-owned, and healthy laboratory-animals. Detection of FHV-1 was low in the diseased animals, suggesting that FHV-1 is not involved in the disease or, alternatively, instigates a pathologic process and then is cleared. Also notable, no bias towards a Th2-type immune response leading to ineffective clearance of virus was detected in diseased cats. Administration of the liposome-complexes produced an innate immune response manifested by fever and malaise in placebo and treatment groups, which may have affected our ability to detect significant differences in the groups, as the placebo appeared to have an effect in cats as well. Reduction in severity of clinical signs was noted in client-owned treatment cats but not more acutely affected shelter-owned cats.

Traditionally defined pathogens were not detected in several cats in our studies; further investigation of all organisms detected in these cats is warranted. *Mycoplasma* species were detected in the majority of these cats, therefore, a real-time PCR assay was developed to allow for quantitation of organismal load in future studies.

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## DEDICATION

This work is dedicated to two great women whom the world has lost while I was writing this dissertation. Each taught me vital lessons and loved me in their own way, though neither had a clue exactly what I was doing in school so long. My grandmother, Helen Hornish, was a strong and stoic matriarch who presided over a family of five children and too numerous to count grandchildren during difficult times. She taught me by example to keep a stiff upper lip and persevere. My childhood neighbor, nanny, designated medic, protector, cheerleader, and Saturday night date, Cleavie Guingerich, always saved a pork chop and a bear hug just for me. She taught me that truly, the best thing in the world is to sit on your front steps and let the sun dry your hair, no matter that the whole neighborhood can see you in curlers. Despite their dichotomous approaches to life, they respected one another's role in my life and contributed equally to my development, giving me two grandmothers to count on during my childhood. You are both greatly missed.

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# INTRODUCTION

Acute upper respiratory tract disease (URTD) is a source of major morbidity and, less frequently, mortality in the domestic cat. It has been reported to be a major financial burden (Foley and Bannasch 2004) and a leading cause of euthanasia in shelters (Pedersen *et al* 2004). The syndrome is characterized by nasal discharge, conjunctivitis, ocular discharge, sinusitis, dyspnea, coughing, inappetence, lethargy, and in kittens or debilitated animals, death. Many pathogens are associated with the syndrome, including feline herpesvirus-1 (FHV-1), feline calicivirus (FCV), *Chlamydophila felis*, and *Bordetella bronchiseptica*. Additionally, several species of *Mycoplasma* have been isolated from cats with URTD (Campbell *et al* 1973, Haesebrouck *et al* 1991, Johnson *et al* 2004, Tan *et al* 1977), however, the role of this group of organisms as primary pathogens in respiratory disease remains contentious, as, similar to the above pathogens, some studies have isolated *Mycoplasma* species from the respiratory tract of healthy cats at a frequency similar to that found in cats with URTD (Foster *et al* 2004b, Haesebrouck *et al* 1991, Tan *et al* 1977, Tan and Miles 1974).

As expected, the relative frequency of organisms associated with URTD in cats varies from study to study. This fact can be attributed to differences in entry criteria (conjunctivitis compared to nasal discharge), geographic distribution, populations sampled (shelter vs. privately owned), detection methods (microbiological culture compared to nucleic acid amplification), and anatomic sampling site. Additionally, most studies to date have examined only a single class of pathogens (i.e. viral or bacterial),

pathogens detected by a single detection method (virus isolation or nucleic acid detection), or a single sampling site (nasal, pharyngeal, or ocular), making identification of co-infections compared to primary infection and comparisons across studies difficult. Therefore, our laboratory examined the prevalence of all proven primary pathogens (FCV, FHV-1, *Chlamydophila*) as well as *Mycoplasma* species in shelter cats with URTD by both microbiologic culture techniques as well as nucleic acid amplification from two anatomic sampling sites (nasal and pharyngeal). The objectives of this study (Chapter 1) were to a) identify pathogens most frequently associated with URTD using both microbiologic and nucleic acid amplification techniques, b) determine if there were cases of URTD not associated with traditional primary pathogens (FCV, FHV-1, *Bordetella bronchiseptica*, *Chlamydophila felis*) in which *Mycoplasma* species were detected, thereby suggesting a primary pathogen role for the group, and c) compare recovery rates for all pathogens studied from the two sampling sites and the two classes of detection assays.

In most prevalence studies of URTD, including work completed in Chapter 1 of this dissertation, FHV-1 emerges as a major pathogen. Feline herpesvirus-1 was first identified as an organism associated with URTD in cats by Crandell et al in 1958 after in vitro culture of nasopharyngeal swabs from both diseased and healthy cats resulted in characteristic cytopathic effects in host feline cells. Primary sites of replication are epithelial cells, as is typical for the α-herpesviruses. These sites in the cat include corneal epithelial cells, conjunctiva, and nasal and pharyngeal epithelium (Gaskell and Povey 1979). The organism is a cause of neonatal death in cats and can be associated with the chronic sequelae of herpetic stromal keratitis (HSK), which is similar to the

disease in man (Stiles 2003). Like herpes simplex virus-1 (HSV-1), the causative agent of HSK, FHV-1 is a classic α-herpesvirus and as such, readily establishes neuronal latency in the trigeminal ganglia with an incidence of up to 80% (Gaskell *et al* 1985, Nasisse *et al* 1992, Ohmura *et al* 1993) and can cause disease secondary to reactivation (Gaskell and Povey 1977). Latency is associated with transcription of short RNA transcripts termed latency associated transcripts (LAT) in infected neurons in experimentally infected animals (Daheshia *et al* 1998) and in naturally infected cats (Townsend *et al* 2004). This makes detection and isolation or removal of carriers difficult. Additionally, the virus is easily spread from cat to cat (Pedersen *et al* 2004) making spread of the virus from stressed, reactivated carriers to healthy animals in isolation or holding areas of shelters a significant problem. In one shelter study, the prevalence of FHV-1 shedding as detected by virus isolation increased from just 4% at admission to over 50% after just one week of housing at the shelter (Pedersen *et al* 2004).

As with all of the primary pathogens, prevalence rates of FHV-1 vary depending on sampling site and method of detection. Studies have reported detection rates of FHV-1 in healthy cats ranging between 0 and 31% and the organism has been detected in up to 69% of cats with nasal discharge as a component of their URTD (Bannasch and Foley 2005, Holst *et al* 2005, Pedersen *et al* 2004, Rampazzo *et al* 2003a, Sykes *et al* 1999). Despite these high rates of detection in affected animals, a recent study has demonstrated that the mere detection of FHV-1 DNA by polymerase chain reaction (PCR) is not significantly correlated with disease (Rampazzo *et al* 2003b) given the high rate of detection in healthy animals. Correspondingly, there was no significant difference in detection of infection with FHV-1 by virus isolation (VI), the immunofluorescent

antibody assay (IFA), or seroprevalence in normal cats, cats with acute URTD or conjunctivitis, or chronically infected cats (Maggs *et al* 1999). Confusing interpretation of test results even further, several studies have demonstrated that many commonly used PCR protocols for FHV-1 are capable of detecting FHV-1 in widely available and utilized commercial vaccines (Lappin *et al* 2006, Maggs and Clarke 2005, Weigler *et al* 1997) both in vivo and in vitro. In a review of FHV-1 diagnosis and pathogenesis, possible reasons for a positive result for FHV-1 by PCR were characterized as: 1) coincidental, 2) consequential (recrudescence secondary to a primary disease), and 3) causal (recrudescence is the primary disease) (Maggs 2005). For these reasons, a positive result by any method of detection cannot be used as the sole means of diagnosis. In lower respiratory tract disease, previous authors have taken the approach that organisms which can be found in normal animals but are also frequently detected at a higher rate in diseased animals can be quantified. Subsequently, a cutoff value at which the presence of the organism is pathologic is determined (Peeters *et al* 2000). In Chapter 2 of this dissertation, we used a similar approach to determine if results from the use of a quantitative assay for detection of FHV-1 DNA from nasal swabs in cats could be used to stratify animals into disease groups, specifically healthy, suspected carriers, and animals with current oculo-nasal disease suggestive of FHV-1.

The exact protective immune response in α-herpesvirus infections are unknown, either in man or cat. However, previous studies initially focused on maximizing antibody responses in the development of vaccines for HSV, perhaps because glycoprotein specific neutralizing antibodies reduced mortality and even protected from infection in some animal models in passive immunity studies (Rajcani and Durmanova 2006) and

antibodies specific for glycoprotein D are able to neutralize the virus in vitro. However, one author suggested a compelling argument against the importance of humoral immunity by pointing out that there is a significantly increased presence of disease in patients lacking cell-mediated immunity (AIDS, immunosuppression secondary to organ transplantation) but not in patients that are agammaglobulinemic (Krause and Straus 1999). In order to fully assess the cell-mediated immune response in future studies in our laboratory, the ability to evaluate the proliferative response to both mitogens and the specific antigen of interest, FHV-1, was needed.

Proliferative assays are commonly used immunologic assays and various methods are used to detect the response to stimuli. However, among the methods of detection, tritium labeled thymidine ([3H]-TdR) is routinely more sensitive and reliable than other methods of detection. Protocols for assessment of proliferative response to mitogens by feline peripheral blood mononuclear cells (PBMC) have been well described (DeBoer and Moriello 1993, Moriello *et al* 2003, Otto *et al* 1993, Sparkes *et al* 1996); however, large volumes of blood are needed for the various separation processes. Additionally, there is evidence in some species that the separation process affects proliferative responses in some species (Confer *et al* 1989). The only direct evaluation of this phenomenon in the cat (Tham *et al* 1982) found a lower response to mitogens in separated mononuclear cells as compared to unadulterated or washed whole blood but did not evaluate antigen specific responses.

Antigen specific responses are less sensitive to extraneous influences such as concurrent disease and age in murine models (Gummert *et al* 1999). Evaluation of proliferative response in cats to specific antigens has focused mainly on infection with

dermatophytes (DeBoer and Moriello 1993, Moriello *et al* 2003, Sparkes *et al* 1996) but some authors have examined the response to incubation with FHV-1 preparations (Otto *et al* 1993, Tham and Studdert 1987). Both studies of response to FHV-1 used derivations of vaccines as antigen preparations. The preparation used by Otto *et al* was simply a diluted commercially available vaccine, however, the authors noted that minimal proliferation was seen and subsequently hypothesized that something in the preparation affected overall viability of the cells. Tham *et al* demonstrated a reliable and sensitive method; however, the antigen used in that case was a vaccine preparation without adjuvants which would be difficult for most investigators to obtain. Therefore, Chapter 3 of this dissertation describes the evaluation of an assay for both concanavalin A and FHV-1 specific responses in whole blood to minimize volume of blood needed, maximize sensitivity to differences among samples, and allow researchers ready access to all necessary components.

Because of the possibility of lifelong infection with periodic recrudescences, shedding, and spread to other cats as well as the confounding nature of diagnostics associated with FHV-1, prevention from infection would be ideal. Vaccination for FHV-1 has been widely administered for decades and has been shown to decrease severity of clinical signs in experimentally infected cats when challenged (Scott and Geissinger 1999). However, vaccination with currently available products does not prevent establishment of latency (Weigler *et al* 1997) and vaccine efficacies do not approach 100% as they do for panleukopenia, another component of multivalent "kittenhood" vaccines. The ideal vaccine would completely prevent infection and subsequent latency and this has been attempted by using both killed (Scott and Geissinger 1999, Tham and

Studdert 1987) and gene deleted or subunit vaccines (Yokoyama *et al* 1996). However, as with all vaccines of these types studied to date, severity of clinical signs on challenge can be reduced but signs were still present but whether the latent state was prevented is unknown.

By definition, probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" beyond nutritional value (Mcfarland *et al* 1995). Probiotics have been commonly used to modulate the course of a variety of infectious diseases in human medicine (Isolauri 2001), most often in the prevention or attenuation of bacterial gastroenteritis. However, in addition to local immunomodulatory mechanisms within the gut and genitourinary system, administration of probiotic organisms has been shown to affect systemic immunity in human studies as well, characterized most frequently by an increase in in vitro phagocytosis of potentially pathogenic bacteria (Schiffrin *et al* 1995) and natural killer cell activity (Schiffrin *et al* 1995). These characteristics have been applied with at least partial success in human studies in an attempt to improve the response to mucosal vaccines (Wold 2001). In murine models, application of three specific strains of probiotics improved the phagocytic response of peripheral white cells, increased the proliferative response of splenocytes in response to both T and B cell mitogens, and increased production of cytokines by splenocytes after stimulation with the T cell mitogen concanavalin A (Gill *et al* 2000).

In contrast, few studies have been performed in veterinary medicine, with the majority of veterinary studies examining the effects of probiotics on large animals to alter the shedding of fecal pathogens (Kim *et al* 2001) or to improve production parameters such as weight gain, feed conversion rate and reduced mortality (Broom *et al* 2006). In

one companion animal study, *Enterococcus faecium* strain SF68 was fed to a group of puppies vaccinated with canine distemper virus (CDV) and compared to a control group that received vaccinations only (Benyacoub *et al* 2003). Puppies supplemented with SF68 had increased serum and fecal total IgA concentrations, increased CDV-specific IgG and IgA serum concentrations, and increased percentage of circulating B lymphocytes compared to control puppies proving an immune enhancing effect induced by this probiotic.

Modulation of the immune response by probiotics is an attractive theory in feline medicine. Probiotics in general are extremely economical in comparison to more expensive immunomodulators such as pharmaceuticals. The strain studied in Chapter 4 of this dissertation, *Enterococcus faecium* SF68, was encapsulated making it durable enough for the food milling process and extremely stable at room temperature. In theory, this would allow the addition of therapeutic levels to daily rations without the need for additional administration by caretakers, a significant concern in cats which may already be averse to oral medications secondary to upper respiratory disease. These qualities, along with the potential to augment the innate immune response to a vaccine that is only moderately effective, led our laboratory to study the effects of the probiotic *Enterococcus faecium* SF68 on the immune response to a routine multivalent feline "kittenhood" vaccine that included FHV-1.

In some cats with URTD, clinical signs continue for prolonged periods of time and may develop into the chronic rhinitis-sinusitis syndrome (CRS). Clinical signs are similar to those in URTD and include nasal discharge, sneezing, anorexia, ocular discharge, and unlike most URTD, can progress to severe turbinate destruction.

Treatment of these animals is frequently unrewarding and frustrating for owners and clinicians. Transient responses to antimicrobials are common, but relapses are frequent. Similar to URTD, the syndrome probably has many etiologies and as such, the role of individual organisms has been hard to define. In the only prospective study of the prevalence of bacteria, viral, and fungal organisms in the literature there was no statistically significant difference in the rate of detection of FHV-1 between the disease and control groups (Johnson *et al* 2005). However, the study was limited in the numbers of animals available to be enrolled in the study and quantitative data regarding viral DNA was not available. Additionally, given the role of FHV-1 in acute disease, the sequelae of turbinate destruction in experimentally induced FHV-1 infection, and the ability of the virus to produce lifelong infection with either chronic clinical signs or intermittent relapses, FHV-1 would seem to be a logical organism to investigate as an etiologic agent in CRS.

In a hallmark study, BenMohamed *et al* (2003) demonstrated that administration of CD4+Th1 peptide epitopes but not with CD4+ Th2 peptide epitopes provided protective immunity in an ocular administration murine model of HSV-1 disease. This study demonstrated the importance of a Th1 type response in protection from disease. In a review of the immunology of HSV, it was pointed out that interferon gamma (IFN- $\gamma$) is important in the primary response to HSV, being released by "$\gamma\delta$ T cells, NK cells, CD4+ T cells and possibly neurons" (Whitley and Miller 2001). Additionally, IFN-$\gamma$ stimulates IgG class switching and downregulation of Th2 T cells, promoting the balance of the immune response to a Th1 type response (Janeway *et al* 2001), which was demonstrated in the study outlined above to be beneficial in HSV immunity.

Administration of cationic lipid DNA complexes (CLDC) in mice was previously shown to be induce release of cytokines which have been shown to have significant anti-viral activity, including IFN-γ, IFN-α, and interleukin 12 (IL-12) as well as partially reversing the Th2 predisposition of the animals in the model (Dow *et al* 1999b). In another study from the same author, administration of CLDC in mice with experimental tumors demonstrated that these substances stimulated release of IFN- γ and IL-12 (Dow *et al* 1999a). Based on these previous findings, we hypothesized in Chapter 5 that cats with CRS may have a Th2 bias that does not allow proper control of viral activity and that administration of CLDC would ameliorate clinical signs.

As previously noted, FHV-1 is not the only organism commonly detected in cats with URTD. *Mycoplasma* species have been isolated in our laboratory from cats with URTD (Veir *et al* 2004) and have been detected at a higher rate in cats with URTD than normal cats by other authors (Bannasch and Foley 2005). However, they are readily detected in the oropharynx and nasal cavity of normal cats as well (Randolph *et al* 1993, Tan *et al* 1977). In cats with chronic upper respiratory tract disease, *Mycoplasma* species were cultured from the nasal cavity in sick cats only, although the control group was small (Johnson *et al* 2005) and a *Mycoplasma* species was grown in pure culture from the nasal cavity in a retrospective study of the disease (Cape 1992). *Mycoplasma* species have also been isolated more frequently from the conjunctiva of cat with conjunctivitis as compared to normal controls and conjunctivitis has been induced experimentally with a *Mycoplasma* species as well, although repeated administration was necessary to produce clinical signs (Haesebrouck *et al* 1991).

The role of *Mycoplasma* species as pathogens in lower airway disease in the feline is hotly debated as well, with many authors reporting isolation of mycoplasmas from lower airway samples (Chandler and Lappin 2002, Crisp *et al* 1987, Foster *et al* 1998, Foster *et al* 2004b, Foster *et al* 2004a), but they are also isolated from normal animals (Padrid *et al* 1991). Anecdotal reports of response to therapy with doxycycline in cats with pure cultures of *Mycoplasma* species from the lower airway tract are suggestive that the organisms can be true pathogens. It should be noted, however, this group of organisms is associated with lower respiratory disease in humans, frequently in association with asthma. As very few centers have the ability to measure airway reactivity in cats, it is difficult to determine how many of these anecdotal reports also involve an underlying subclinical case of feline lower airway disease.

An additional confounding factor in the debate regarding the role of *Mycoplasma* species in respiratory disease in cats is the fastidious nature of the organism. *Mycoplasma* species require rapid transport to a diagnostic facility as they do not survive well outside of the host and special media is required for culture (Johnson *et al* 2004), therefore, the clinician must specifically request culture of the organism in most facilities. Finally, *Mycoplasma* species are slow growing, requiring up to a week of culture.

The advent of nucleic acid based detection methods has greatly improved diagnosis in human disease. Mycoplasmal pneumonia is a frequent cause of community acquired pneumonia in humans. However, because of the slow microbiologic culture characteristics, both serology and culture results are evaluated in diagnosis. In a small comparative study, a real-time PCR and a conventional endpoint PCR were shown to be more sensitive than and as specific as traditional serologic diagnosis (Templeton *et al*

2003) and allows for more targeted therapies earlier in the disease due to decreased turn around time. The improved sensitivity and turn around time has also been applied to patients with mycoplasmal pneumonia in order to decrease morbidity associated with sampling techniques. The results of a real-time PCR applied to serum were as specific as traditional diagnostic methods (serology and clinical signs) although not as sensitive (Daxboeck *et al* 2005). These techniques have been evaluated in the feline species as well. A *Mycoplasma felis*-specific conventional endpoint PCR was recently developed and evaluated for use in clinical specimens from the respiratory tract of cats (Chalker *et al* 2004). The PCR assay was both sensitive and specific, allowing for widespread use in veterinary diagnostic laboratories and decreased turn around time for clinicians awaiting results. Another laboratory applied the results of a conventional endpoint PCR specific for *Mycoplasma* at the genus level to both nasal flush and biopsy specimens in normal cats and compared the results to microbiologic culture (Johnson *et al* 2004). Concordance between the two assays was excellent and the more broadly defined amplicon may allow for a more useful screening assay.

In our investigation of the organisms associated with URTD in cats (Chapter 1), nine cases were identified in which traditional primary pathogens were not detected, either by molecular or microbiological culture techniques, but did have positive samples for *Mycoplasma felis*. We would like to evaluate the pathogenicity and sensitivity to treatment of these samples further, however, a quantitative assay would be preferable to a qualitative assay in this case as response to therapy may be measured in organismal burden. Therefore, in Chapter 6 of this dissertation, the development of a quantitative

real time PCR (q-rtPCR) based assay specific for *Mycoplasma felis* to be used in future studies of the isolates obtained from the cats with URTD is described.

## References


Bannasch MJ, Foley JE (2005), Epidemiologic evaluation of multiple respiratory pathogens in cats in animal shelters, *J Fel Med Surg* 7: 109-119

BenMohamed L, Bertrand G, McNamara CD, Gras-Masse H, Hammer J, Wechsler SL, Nesburn AB (2003), Identification of novel immunodominant CD4(+) Th1-type T-cell peptide epitopes from herpes simplex virus glycoprotein D that confer protective immunity, *J Virol* 77: 9463-9473

Benyacoub J, Czarnecki-Maulden GL, Cavadini C, Sauthier T, Anderson RE, Schiffrin EJ, von der WT (2003), Supplementation of food with *Enterococcus faecium* (SF68) stimulates immune functions in young dogs, *J Nutr* 133: 1158-1162

Broom LJ, Miller HM, Kerr KG, Knapp JS (2006), Effects of zinc oxide and *Enterococcus faecium* SF68 dietary supplementation on the performance, intestinal microbiota and immune status of weaned piglets, *Res Vet Sci* 80: 45-54

Campbell LH, Snyder SB, Reed C, Fox JG (1973), *Mycoplasma felis*-associated conjunctivitis in cats, *J Am Vet Med Assoc* 163: 991-995

Cape L (1992), Feline idiopathic chronic rhinosinusitis: A retrospective study of 30 cases, *JAAHA* 28: 149-155

Chalker VJ, Owen WMA, Paterson CJI, Brownlie J (2004), Development of a polymerase chain reaction for the detection of *Mycoplasma felis* in domestic cats, *Vet Microbiol* 100: 77-82

Chandler JC, Lappin MR (2002), Mycoplasmal respiratory infections in small animals: 17 cases (1988-1999), *JAAHA* 38: 111-119

Confer AW, Wyckoff JH, Oltjen SL, Oltjen JW (1989), Sepracell-Mn for mononuclear cell-separation from cattle - effect on mitogen-induced and antigen-induced lymphocyte blastogenesis, *Vet Immunol Immunopathol* 22: 79-89

Crisp MS, Birchard SJ, Lawrence AE, Fingeroth J (1987), Pulmonary abscess caused by a *Mycoplasma* species in a cat, *J Am Vet Med Assoc* 191: 340-342

Daheshia M, Feldman LT, Rouse BT (1998), Herpes simplex virus latency and the immune response, *Curr Opin Microbiol* 1: 430-5

Daxboeck F, Khanakah G, Bauer C, Stadler M, Hofmann H, Stanek G (2005), Detection of *Mycoplasma pneumoniae* in serum specimens from patients with mycoplasma pneumonia by PCR, *Int J Med Microbiol* 295: 279-285

DeBoer DJ, Moriello KA (1993), Humoral and cellular immune responses to *Microsporum canis* in naturally occurring feline dermatophytosis, *J Med Vet Mycol* 31: 121-132

Dow SW, Fradkin LG, Liggitt DH, Willson AP, Heath TD, Potter TA (1999a), Lipid-DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously, *J Immunol* 163: 1552-1561

Dow SW, Schwarze J, Heath TD, Potter TA, Gelfand EW (1999b), Systemic and local interferon gamma gene delivery to the lungs for treatment of allergen-induced airway hyperresponsiveness in mice, *Hum Gene Ther* 10: 1905-1914

Foley J, Bannasch MJ (2004), Infectious Diseases of Dogs and Cats, in *Shelter Medicine for Veterinarians and Staff*, ed. Miller LS and Zawitowski S, Blackwell Publishing, Ames p 235-284

Foster SF, Barrs VR, Martin P, Malik R (1998), Pneumonia associated with *Mycoplasma* spp in three cats, *Aust Vet J* 76: 460-464

Foster SF, Martin P, Allan GS, Barrs VR, Malik R (2004a), Lower respiratory tract infections in cats: 21 cases (1995-2000), *J Feline Med Surg* 6: 167-180

Foster SF, Martin P, Braddock JA, Malik R (2004b), A retrospective analysis of feline bronchoalveolar lavage cytology and microbiology (1995-2000), *J Feline Med Surg* 6: 189-198

Gaskell RM, Dennis PE, Goddard LE, Cocker FM, Wills JM (1985), Isolation of felid herpesvirus I from the trigeminal ganglia of latently infected cats, *J Gen Virol* 66 ( Pt 2): 391-394

Gaskell RM, Povey RC (1977), Experimental induction of feline viral rhinotracheitis virus re-excretion in Fvr-recovered cats, *Vet Rec* 100: 128-133

Gaskell RM, Povey RC (1979), Feline viral rhinotracheitis - sites of virus-replication and persistence in acutely and persistently infected cats, *Res Vet Sci* 27: 167-174

Gill HS, Rutherfurd KJ, Prasad J, Gopal PK (2000), Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019), *Brit J Nutr* 83: 167-176

Gummert JF, Otto G, Barten MJ, Morris RE (1999), Effect of anesthesia on a whole blood lymphocyte proliferation assay in the rat, *Immunopharmacol Immunotoxicol* 21: 267-276

Haesebrouck F, Devriese LA, van Rijssen B, Cox E (1991), Incidence and significance of isolation of Mycoplasma felis from conjunctival swabs of cats, *Vet Microbiol* 26: 95-101

Holst BS, Berndtsson LT, Englund L (2005), Isolation of feline herpesvirus-1 and feline calicivirus from healthy cats in Swedish breeding catteries, *J Fel Med Surg* 7: 325-331

Isolauri E (2001), Probiotics in human disease, *Am J Clin Nutr* 73: 1142S-1146S

Janeway CA, Travers P, Walport M, Shlomchik MJ (2001), *Immunobiology: The Immune System in Health and Disease*, Garland Publishing, New York

Johnson LR, Drazenovich NL, Foley JE (2004), A comparison of routine culture with polymerase chain reaction technology for the detection of *Mycoplasma* species in feline nasal samples, *J Vet Diagn Invest* 16: 347-351

Johnson LR, Foley JE, De Cock HE, Clarke HE, Maggs DJ (2005), Assessment of infectious organisms associated with chronic rhinosinusitis in cats, *J Am Vet Med Assoc* 227: 579-585

Kim LM, Morley PS, Traub-Dargatz JL, Salman MD, Gentry-Weeks C (2001), Factors associated with *Salmonella* shedding among equine colic patients at a veterinary teaching hospital, *J Am Vet Med Assoc* 218: 740-748

Krause PR, Straus SE (1999), Herpesvirus vaccines - Development, controversies, and applications, *Infect Dis Clin North Am* 13: 61-+

Lappin MR, Sebring RW, Porter M, Radecki SJ, Veir J (2006), Effects of a single dose of an intranasal feline herpesvirus 1, calicivirus, and panleukopenia vaccine on clinical signs and virus shedding after challenge with virulent feline herpesvirus 1, *J Fel Med Surg* In Press, Corrected Proof

Maggs DJ (2005), Update on pathogenesis, diagnosis, and treatment of feline herpesvirus type 1, *Clin Tech Small Anim Pract* 20: 94-101

Maggs DJ, Clarke HE (2005), Relative sensitivity of polymerase chain reaction assays used for detection of feline herpesvirus type 1 DNA in clinical samples and commercial vaccines, *Am J Vet Res* 66: 1550-1555

Maggs DJ, Lappin MR, Reif JS, Collins JK, Carman J, Dawson DA, Bruns C (1999), Evaluation of serologic and viral detection methods for diagnosing feline herpesvirus-1 infection in cats with acute respiratory tract or chronic ocular disease, *J Am Vet Med Assoc* 214: 502-507

Mcfarland LV, Surawicz CM, Greenberg RN, Elmer GW, Moyer KA, Melcher SA, Bowen KE, Cox JL (1995), Prevention of Beta-Lactam-Associated Diarrhea by *Saccharomyces-Boulardii* Compared with Placebo, *Am J Gastroenterol* 90: 439-448

Moriello KA, DeBoer DJ, Greek J, Kuhl K, Fintelman M (2003), The prevalence of immediate and delayed type hypersensitivity reactions to *Microsporum canis* antigens in cats, *J Fel Med Surg* 5: 161-166

Nasisse MP, Davis BJ, Guy JS, Davidson MG, Sussman W (1992), Isolation of Feline Herpesvirus-1 from the Trigeminal Ganglia of Acutely and Chronically Infected Cats, *J Vet Intern Med* 6: 102-103

Ohmura Y, Ono E, Matsuura T, Kida H, Shimizu Y (1993), Detection of Feline Herpesvirus-1 Transcripts in Trigeminal Ganglia of Latently Infected Cats, *Arch Virol* 129: 341-347

Otto CM, Brown CA, Lindl PA, Dawe DL (1993), Delayed-Hypersensitivity Testing As A Clinical Measure of Cell-Mediated-Immunity in the Cat, *Vet Immunol Immunopathol* 38: 91-102

Padrid PA, Feldman BF, Funk K, Samitz EM, Reil D, Cross CE (1991), Cytologic, microbiologic, and biochemical analysis of bronchoalveolar lavage fluid obtained from 24 healthy cats, *Am J Vet Res* 52: 1300-1307

Pedersen NC, Sato R, Foley JE, Poland AM (2004), Common virus infections in cats, before and after being placed in shelters, with emphasis on feline enteric coronavirus, *J Fel Med Surg* 6: 83-88

Peeters DE, McKiernan BC, Weisiger RM, Schaeffer DJ, Clercx C (2000), Quantitative bacterial cultures and cytological examination of bronchoalveolar lavage specimens in dogs, *J Vet Intern Med* 14: 534-541

Rajcani J, Durmanova V (2006), Developments in herpes simplex virus vaccines: Old problems and new challenges, *Folia Microbiol* 51: 67-85

Rampazzo A, Appino S, Pregel P, Tarducci A, Zini E, Biolatti B (2003b), Prevalence of *Chlamydophila felis* and feline herpesvirus 1 in cats with conjunctivitis in northern Italy, *J Vet Intern Med* 17: 799-807

Randolph JF, Moise NS, Scarlett JM, Shin SJ, Blue JT, Corbett JR (1993), Prevalence of Mycoplasmal and Ureaplasmal Recovery from Traceobronchial Lavages and of Mycoplasmal Recovery from Pharyngeal Swab Specimens in Cats with Or Without Pulmonary-Disease, *Am J Vet Res* 54: 897-900

Schiffrin EJ, Rochat F, Linkamster H, Aeschlimann JM, Donnethughes A (1995), Immunomodulation of Human Blood-Cells Following the Ingestion of Lactic-Acid Bacteria, *J Dairy Sci* 78: 491-497

Scott FW, Geissinger CM (1999), Long-term immunity in cats vaccinated with an inactivated trivalent vaccine, *Am J Vet Res* 60: 652-658

Sparkes AH, Gruffydd-Jones TJ, Stokes CR (1996), Acquired immunity in experimental feline *Microsporum canis* infection, *Res Vet Sci* 61: 165-168

Stiles J (2003), Feline herpesvirus, *Clin Tech Small Anim Pract* 18: 178-185

Sykes JE, Anderson GA, Studdert VP, Browning GF (1999), Prevalence of feline *Chlamydia psittaci* and feline herpesvirus 1 in cats with upper respiratory tract disease, *J Vet Intern Med* 13: 153-162

Tan RJS, Lim EW, Ishak B (1977), Ecology of Mycoplasmas in Clinically Healthy Cats, *Aust Vet J* 53: 515-518

Tan RJS, Miles JAR (1974), Incidence and Significance of Mycoplasmas in Sick Cats, *Res Vet Sci* 16: 27-34

Templeton KE, Scheltinga SA, Graffelman AW, Van Schie JM, Crielaard JW, Sillekens P, Van Den Broek PJ, Goossens H, Beersma MF, Claas EC (2003), Comparison and evaluation of real-time PCR, real-time nucleic acid sequence-based amplification, conventional PCR, and serology for diagnosis of *Mycoplasma pneumoniae*, *J Clin Microbiol* 41: 4366-4371

Tham KM, Studdert MJ (1987), Antibody and Cell-Mediated Immune-Responses to Feline Herpesvirus-1 Following Inactivated Vaccine and Challenge, *Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health* 34: 585-597

Tham KM, Wilks CR, Studdert MJ (1982), Optimal conditions for in vitro blastogenesis of feline peripheral-blood lymphocytes, *Vet Immunol Immunopathol* 3: 485-498

Townsend WM, Stiles J, Guptill-Yoran L, Krohne SG (2004), Development of a reverse transcriptase-polymerase chain reaction assay to detect feline herpesvirus-1 latency-associated transcripts in the trigeminal ganglia and corneas of cats that did not have clinical signs of ocular disease, *Am J Vet Res* 65: 314-319

Veir JK, Ruch-Gallie R, Spindel ME, Lappin MR. Prevalence of FHV-1, *Mycoplasma* spp. and aerobic bacteria in shelter cats with acute upper respiratory tract disease (2004), *J Vet Intern Med* 18: 197

Weigler BJ, Guy JS, Nasisse MP, Hancock SI, Sherry B (1997), Effect of a live attenuated intranasal vaccine on latency and shedding of feline herpesvirus 1 in domestic cats, *Arch Virol* 142: 2389-2400

Whitley RJ, Miller RL (2001), Immunologic approach to herpes simplex virus, *Viral Immunol* 14: 111-118

Wold AE (2001), Immune effects of probiotics, *Scand J Nutr* 45: 76-85

Yokoyama N, Maeda K, Fujita K, Ishiguro S, Sagawa T, Mochizuki M, Tohya Y, Mikami T (1996), Vaccine efficacy of recombinant feline herpesvirus type 1 expressing immunogenic proteins of feline calicivirus in cats, *Arch Virol* 141: 2339-2351

# CHAPTER 1

# PREVALENCE OF SELECTED INFECTIOUS ORGANISMS IN SHELTER CATS WITH UPPER RESPIRATORY TRACT DISEASE

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**Abstract**: In order to describe the isolation rates of potential pathogens and to compare sampling site suitability, nasal and pharyngeal swabs were taken from cats with clinical upper respiratory disease in a humane society. DNA of FHV-1 was amplified from 51 of 52 cats sampled, *Mycoplasma* species were cultured or detected by PCR in samples from 34 of 42 cats sampled for both assays, and *Bordetella bronchiseptica* was isolated from 3 of 59 cats sampled for aerobic culture. A single swab was positive for calicivirus and no swabs were positive for *Chlamydophila felis*. *Mycoplasma, Pasteurella, and Moraxella* species were all isolated from at least one cat in which no primary pathogen was identified. With the exception of *B. bronchiseptica*, which was detected in nasal swabs only, recovery rates for all suspect primary pathogens were comparable between sampling sites.

## Introduction

Upper respiratory tract disease (URTD) continues to be a major problem in shelters and humane societies; many report it to be a leading cause of euthanasia in cats in traditional shelters (Foley and Bannasch 2004) and a major financial burden for no-kill shelters (Pedersen *et al* 2004). Multiple organisms have been isolated from cats with acute upper respiratory tract disease including feline herpesvirus-1 (FHV-1) and feline calicivirus (FCV), as well as many aerobic and anaerobic bacteria (Cape 1992, Helps *et al* 2005). With the exception of *Bordetella bronchiseptica* and *Chlamydophila felis*, most bacterial infections are thought to be secondary to other primary diseases. Although historically implicated in feline lower respiratory tract disease (Foster *et al* 1998, Foster *et al* 2004, Padrid *et al* 1991), *Mycoplasma* species have recently been proposed to be associated with chronic (Johnson *et al* 2005) and acute (Bannasch and Foley 2005) feline upper respiratory tract disease.

Microbial detection techniques utilized in URTD prevalence studies include aerobic culture, anaerobic culture, virus isolation, polymerase chain reaction (PCR) assay for microbial DNA, and reverse transcriptase PCR (RT-PCR) assay for RNA viruses like calicivirus. However, comparison of results amongst previous prevalence studies cannot be made because of lack of standardization in microbial detection techniques and sampling sites. In order to maximize limited diagnostic and treatment resources, shelter personnel must know not only the most common organisms associated with URTD, but also the optimal sampling site and diagnostic assays for use in refractory or sentinel cases. Therefore, the objectives of this study were to determine the prevalence rates of aerobic bacteria, select viruses, and *Mycoplasma* species in clinically ill cats residing in a

humane society by use of standardized methods of microbial isolation applied to samples collected from two separate but commonly referenced sampling sites (nasal and oropharyngeal).

**Materials and Methods**

*Experimental Design*

Samples were collected from cats residing in a combined rural and urban humane society shelter in north central Colorado between January and December, 2003. Sixty-one cats with clinical signs of upper respiratory tract disease (oculonasal discharge, sneezing, and stertorous breathing) with a qualifying clinical score were entered concurrently into the study described here as well as an antibiotic therapy comparison study (Ruch-Gallie *et al*, 2006). Within 24 hours of admission, three swabs from both the nasal cavity and oropharynx were taken from each cat prior to treatment. Nasal swabs were obtained by gently rolling a sterile transurethral culture swab[a] in the anterior aspect of the right nares after removing any excess mucous. Oropharyngeal swabs were obtained using a sterile cotton tipped applicator gently rotated in the oropharynx of each cat. One swab from each sampling site was placed into a commercial transport medium[b] and submitted for culture of *Mycoplasma* species and aerobic bacteria within four hours. A second swab was placed in viral transport medium[c] and submitted for virus isolation within four hours. The final swab was placed in 1 mL sterile phosphate buffered saline solution (PBS), allowed to equilibrate for two to three hours at room temperature according to the extraction kit[d] manufacturer's instructions and then stored at -70°C until assayed for the presence of FHV-1 DNA, *Chlamydophila felis* DNA, *Mycoplasma* species DNA, and calicivirus RNA.

*Aerobic bacteria and Mycoplasma species culture*

Discharge collected on one oropharyngeal and nasal swab from each cat was inoculated onto blood agar, MacConkey, Columbia agar and Friis broth agar plates, then placed in 2 mL of Friis broth and incubated at 37°C with 5% $CO_2$ for 24 hours. One hundred microliters of the resulting liquid culture was again inoculated onto Friis broth agar plates. All plates were incubated at 37°C with room air (blood agar, MacConkey), 5% $CO_2$ (Friis broth) or 10% $CO_2$ (Columbia) for 72 hours immediately after inoculation. Aerobic bacteria were characterized using standard biochemical methods. *Mycoplasma* species cultures were considered positive if colonies had a microscopic "fried egg" appearance on selective agar (Friis broth). Determination of the species of *Mycoplasma* causing infection is difficult and not currently performed at Colorado State University and so all positive results are described as *Mycoplasma* species.

*Virus Isolation*

After low speed centrifugation at 4°C, sample supernatant (~1 mL) was filtered using a sterile 0.45 um filter and inoculated in triplicate onto a monolayer of Crandell Rees Feline Kidney (CRFK) cells. After an adsorption period of 1-2 hours at 37°C in 5% $CO_2$, the sample was decanted and cells were incubated with one mL of Modified Eagle's Medium (MEM) with 3% FBS in the same environment for a maximum of seven days. Cells were checked daily for evidence of cytopathic effect after comparison to a control well of naïve CRFK cells. Samples that did not show evidence of cytopathic effect after seven days were passaged a second time as previously described prior to being declared negative. For positive samples, the virus causing cytopathic effects was assumed to be

either FHV or FCV based on the results of the FHV-1 PCR assay and FCV RT-PCR assay performed on the same sample.

*PCR and RT-PCR assays*

Detection of FHV-1, *Mycoplasma* species, and *Chlamydophila felis* DNA in swabs was performed using previously published assays (Baird *et al* 1999, Burgesser *et al* 1999, Sykes *et al* 1997) after extraction of DNA from one-half the volume of the sample using a commercially available kit[d] according to manufacturer's protocols. RNA was extracted from the remainder of the sample using a commercially available kit[d] and the RNA was assessed using two previously published RT-PCR assays for the amplification of FCV (Radford *et al* 1997; Sykes *et al* 1998).

*Statistical Analyses*

Concordance of assay and sampling site results for FHV-1, FCV, *C. felis*, *B. bronchiseptica*, and *Mycoplasma* species were evaluated using the kappa statistic with indices of specific agreement ($p_{pos}$ and $p_{neg}$) (Feinstein 2002). Definitions of quantitative significance for kappa were as defined by Landis and Koch (1977). Any analysis that required larger than a 2x2 agreement matrix (i.e. comparison of agreement between results for two organisms from both sampling sites) and concordance of assay and sampling site results for the remaining detected organisms were evaluated using a simple proportion of agreement (Feinstein 2002).

**Results**

Overall, 65 cats were enrolled in the study. Due to sample volume limitations, not all assays were run on all samples. The number of cats tested is detailed within the results section for the assay for each organism.

*Comparison of Sampling Sites for Microbial Recovery*

Aerobic bacteria were cultured from the majority of cats regardless of sampling site (nasal: 45/59, 76.3%; pharyngeal: 56/59, 94.9 %, Table 1). However, aerobic bacterial culture results from nasal and pharyngeal swabs were in complete agreement for only seven of 59 cats sampled. Suspected primary pathogens (*C. felis, B. bronchiseptica, Mycoplasma* species, FCV, and FHV-1) were cultured or amplified from a variable number of cats and results differed between sampling sites depending on the organism and assay (Table 2). RNA of FCV was not amplified from any sample using the first RT-PCR assay (Sykes *et al* 1998) we utilized, prompting the assay of the samples in a second assay (Radford *et al* 1997). In the second assay, FCV was detected in a single sample. In addition, *C. felis* was not amplified from any sample assayed. Because of the low prevalence rates of FCV and *C. felis*, analysis of cumulative primary pathogen results between sampling sites were compared only for FHV-1, *Mycoplasma* species and *B. bronchiseptica* (Table 2). For 39 cats, PCR assay results for both FHV-1 and *Mycoplasma* species from both sites were available; 35 of 39 (89.7%) nasal swabs and 37 of 39 (94.9%) pharyngeal swabs were positive for one or both of the organisms. Results were in agreement for the two sampling sites for both organisms via PCR assay in 19 of 39 (48.7%) cats. Culture for FHV-1 (virus isolation), *Mycoplasma* species and *B. bronchiseptica* yielded positive results for at least one of the organisms in 39 of 51

(76.5%) in both nasal and pharyngeal samples. Agreement between the two sampling sites for all three organisms by microbiologic culture was 62.7% (32 of 51 cats, Table 2).

*Comparison of PCR assay and culture for detection of Mycoplasma species*

Results of both PCR assay and culture for *Mycoplasma* species were available for 42 cats. Of the 42 nasal samples, 26 (61.9%) were positive as determined by PCR assay and 21 (50.0%) were positive via bacteriologic culture (kappa = 0.67, $p_{pos}$ = 0.85, $p_{neg}$ = 0.81). Of the 42 pharyngeal samples, 29 (69.0%) were positive by PCR assay and 23 (54.8%) were positive on bacteriologic culture (kappa = 0.41, $p_{pos}$ = 0.77, $p_{neg}$ = 0.63). When both sampling sites were taken into account, there was a 64.3% (27 of 42) agreement rate for detection of *Mycoplasma* species between PCR assay and bacteriologic culture.

*Detection of organisms in which no traditional primary pathogens were detected*

Although *Mycoplasma* species and *Pasteurella* species have been suggested to be primary pathogens in cats with upper respiratory tract disease, these organisms have not been definitively linked to disease to date. Thus, we considered FHV-1, FCV, *C. felis, B. bronchiseptica* primary pathogens because Koch's postulates have been fulfilled. When results of all assays and both sample sites were considered, at least one primary pathogen was cultured or amplified from all but nine cats (Table 3). In these nine cats, *Mycoplasma* species was detected in seven. Six of the *Mycoplasma* species positive cats were co-infected with *Pasteurella* species (*P. multocida* [n = 5], *P. dogmatis* [n = 3]) and four were co-infected with *Moraxella* species.

**Discussion**

A primary goal of this study was to determine whether nasal or pharyngeal samples were optimal for detection of the primary pathogens associated with URTD in shelter cats; detection rates of pathogenic organisms obtained by pharyngeal and nasal swabs were similar between sites for all traditionally defined primary pathogens. With the exception of *Bordetella bronchiseptica*, the kappa statistics for comparison of nasal and pharyngeal sampling sites for all assays and organisms were classified as moderate (FHV-1 PCR, *Mycoplasma* species PCR), substantial (FHV-1 virus isolation, *Mycoplasma* species culture), or perfect (FCV, *C. felis*). In such a poorly distributed population (FCV, *C. felis*, *B. bronchiseptica*), the kappa statistic must be viewed with caution. Nasal swabs performed in the manner used for this study require a great deal more time and discomfort for the cat with minimal improvement in yield, therefore, unless there is a high index of suspicion of FHV-1 and virus isolation is more readily available, swab sampling sites are most likely equivalent. However, to achieve maximal sensitivity for use in prevalence studies, results from both sample sites are needed.

As FHV-1, FCV, and *Mycoplasma* species can be difficult to culture and require minimal transport time before recovery rates fall, nucleic acid amplification assays are being used more frequently. In a previous study, *Mycoplasma* culture and PCR assay results were compared in a small number of samples taken from the nasal cavity of cats of unknown respiratory health status and shown to be similar (Johnson *et al* 2004). In this study, we compared results of culture and PCR assay for FHV-1 and *Mycoplasma* species in a larger number of cats with URTD and determined the techniques to give similar results although the kappa statistics for *Mycoplasma* species were not as high as

previously reported (Johnson *et al* 2004). While PCR assays present several distinct advantages over culture (decreased need for special transport, time for storage, rapid results), the assays detect only the presence of microbial DNA, not viable organisms. However, it is unlikely that DNA of dead organisms persist for any length of time. We conclude that PCR assays for both FHV-1 and *Mycoplasma* species are acceptable alternatives to traditional culture techniques for use in prevalence studies.

Because FHV-1 was so prevalent in the cats of this study, most cats with concurrent infections were FHV-1 positive. FHV-1 can be a primary pathogen and can also damage epithelial tissues allowing for secondary bacterial infection. In addition, currently available assays cannot discriminate between FHV-1 modified live vaccine strains and virulent strains (Maggs 2005). Thus, detection of FHV-1 by culture or PCR assay does not prove illness associated with the virus. In addition, while FHV-1 could be grown or amplified from similar numbers of nasal and pharyngeal samples, maximum sensitivity was not achieved until results of both sites were combined. Because FHV-1 culture and conventional PCR assays are expensive and positive results do not correlate to illness, routine use of these assays in individual shelter cats with acute URTD appears to have little clinical benefit. Results of quantitative PCR assays that document the FHV-1 viral load in a sample may prove to have a better positive predictive value (Veir, 2003), but further studies are needed.

The prevalence of FCV in this study population was lower than in previous studies. Even in normal cats, the rate of detection of calicivirus in the oropharynx via PCR or virus isolation has been reported to be as high as 22% (Binns *et al* 2000) as the virus can be shed continuously despite lack of clinical signs. It is possible to have false-

negative RT-PCR assay results because of RNA degradation, however the samples were handled appropriately and the two RT-PCR assays gave similar results. In addition, while our virus isolation assay does not discriminate between FCV and FHV-1, all samples positive on virus isolation were also positive for FHV-1 via PCR assay. Based on these findings, we believe that calicivirus was not a common cause of disease in this population of cats. It is possible that this is related to breeds of cats seen at this humane society; a previous study showed a correlation with FCV infection and longhair breeds (Coutts *et al* 1994). As for FHV-1, positive FCV culture or RT-PCR assay results do not correlate to FCV-associated disease and the assays are relatively expensive. Based on these facts, routine use of FCV culture or RT-PCR assay in individual shelter cats with acute URTD appears to have minimal clinical benefit.

Both the pharynx and nasal cavity of normal and clinically affected cats are host to a wide variety of organisms (Bannasch and Foley 2005, Johnson *et al* 2005). Thus, detection of different aerobic bacteria from the nasal cavity and pharynx of the cats described here was not unexpected. Results of this study are similar to others and suggest that aerobic culture of nasal discharge or the pharynx of cats with URTD is unlikely to yield useful clinical information and that it may be more cost effective to use logical empirical antibiotic therapy when bacterial URTD is suspected. In a previous study of antibiotic susceptibility testing of aerobic bacterial isolates from cats with URTD, more than 80% of the aerobic bacterial isolates from both the upper and lower airway cultures were susceptible to amoxicillin-clavulanic acid, cephalosporins, chloramphenicol, enrofloxacin, and tetracyclines (Stein and Lappin 2006).

All of the organisms isolated from the cats described here have also been isolated from healthy cats; therefore, determining which are primary pathogens should be based on demonstration of Koch's postulates. While these have been fulfilled for FHV-1, FCV, *B. bronchiseptica*, and *C. felis*, other proposed primary pathogens (*Mycoplasma* species and *Pasteurella* species) have only been implicated by epidemiologic evaluations (Bannasch and Foley 2005, Johnson *et al* 2005). In this study, only nine cats were negative for all the primary pathogens; in seven of these cats *Mycoplasma* species were detected either by PCR assay or bacteriologic culture. In the remaining two cats, *Pasteurella* and *Moraxella* species were the only organisms associated with upper respiratory tract disease detected (Table 3). *Pasteurella multocida* has been suggested to be associated with chronic nasal disease in cats by one author (Johnson *et al* 2005) and is associated with atrophic rhinitis in swine (Eamens *et al* 1988). Similarly, *Moraxella* species are associated with rhinitis in humans (Boyle *et al* 1991), but no evidence for their role as a primary pathogen in acute upper respiratory disease in cats has been published. It is possible that an unknown organism or separate disease process (allergic, foreign body, nasopharyngeal polyp, or anatomical abnormality) may have caused the clinical signs in these nine cats. For example, some authors have suggested that anaerobic bacteria may be associated with disease in cats with chronic rhinitis (Johnson *et al* 2005) and we did not culture for that group of bacteria. However, it is also possible that some *Mycoplasma* species, *Pasteurella* species, or *Moraxella* species can be primary pathogens in cats. Further studies attempting experimental transmission and proof of Koch's postulates with these organisms will be needed to prove or deny the hypothesis.

We failed to detect *C. felis* in any sample. *Chlamydophila felis* is most often associated with conjunctivitis and ocular signs in cats (Bannasch and Foley 2005) and while this was not an exclusionary criterion in our study, very few cats exhibited conjunctivitis. The results of this suggest that *C. felis* was not a common cause of URTD in these cats and that performance of assays to detect *C. felis* on nasal or pharyngeal swabs is unlikely to have significant clinical benefit.

| | Aerobic Culture (n=59) n (% positive) | |
|---|---|---|
| | Nasal | Pharyngeal |
| *Bordetella bronchiseptica* | 3 (5.1%) | 0 (0%) |
| *Mycoplasma spp.* | 28 (47.5%) | 31 (52.5%) |
| *Pasteurella spp.* | 19 (32.2%) | 43 (72.9%) |
| *Moraxella spp.* | 4 (6.8%) | 21 (35.6%) |
| *Coagulase (-) Staphylococcus spp.* | 4 (6.8%) | 6 (10.2%) |
| *Non-hemolytic Streptococcus spp.* | 4 (6.8%) | 7 (11.9%) |
| *Flavobacterium* | 0 (0%) | 10 (17.0%) |
| *Staphylococcus aureus* | 0 (0%) | 2 (3.4%) |
| *Staphylococcus intermedius* | 3 (5.1%) | 0 (0%) |
| *Enterobacter spp.* | 0 (0%) | 1 (1.7%) |
| *Escherich coli* | 0 (0%) | 1 (1.7%) |
| *Streptococcus B-hemolytic* | 7 (11.9%) | 6 (10.2%) |
| *Cornebacterium spp.* | 1 (1.7%) | 1 (1.7%) |
| *Haemophilus spp.* | 0 (0%) | 1 (1.7%) |
| *Actinomyces spp.* | 2 (3.4%) | 1 (1.7%) |
| *Micrococcus spp.* | 1 (1.7%) | 0 (0%) |

**Table 1 Aerobic organisms isolated from nasal or pharyngeal swabs of cats with URTD.** Within 24 hours of admission, samples were collected from consecutive admissions to a combined rural and urban humane society shelter with clinical signs of upper respiratory tract disease. Routine aerobic culture was performed on samples within four hours of collection.

| | Microbiologic Culture (n positive/n total assayed, %) | | | PCR assay (n positive/n total assayed, %) | | |
|---|---|---|---|---|---|---|
| | Nasal | Pharyngeal | Kappa | Nasal | Pharyngeal | Kappa |
| *Bordetella bronchiseptica* | 3/59, 5.1% | 0/59, 0% | 0 | n/a | n/a | n/a |
| *Feline Herpesvirus-1* | 37/54, 68.5% | 27/54, 50.0% | 0.63 | 49/60, 81.7% | 44/60, 73.3% | 0.57 |
| *Mycoplasma spp.* | 28/59, 47.5% | 31/59, 52.5% | 0.62 | 27/50, 54% | 33/51, 64.7% | 0.53 |
| *Total** | 39/51, 76.5% | 39/51, 76.5% | 32/51, 62.7% | 41/45, 91.1% | 42/45, 93.3% | 29/45, 64.4% |

**Table 2 Concordance of results of detection from nasal and pharyngeal swabs of primary pathogens associated with URTD in cats by microbiologic culture or nucleic acid amplification.** Samples were collected as in Table 1. *"Total" represents results from cats in which all assays were performed.

| | | *Mycoplasma spp. PCR assay* | | *Mycoplasma spp. Culture* | | *Aerobic Culture* | |
|---|---|---|---|---|---|---|---|
| | *Case no.* | *Nasal* | *Pharyngeal* | *Nasal* | *Pharyngeal* | *Nasal* | *Pharyngeal* |
| *79004* | *1* | *(+)* | *(+)* | *(+)* | *(+)* | *Pasteurella multocida* | *Pasteurella multocida, Non-hemolytic Streptococcus, Flavobacterium, Haemophilus* |
| *98414* | *2* | *n/a* | *n/a* | *(-)* | *(-)* | *(-)* | *Pasteurella dogmatis* |
| *101005* | *3* | *n/a* | *n/a* | *(+)* | *(+)* | *(-)* | *(-)* |
| *103372* | *4* | *n/a* | *n/a* | *(-)* | *(+)* | *(-)* | *Pasteurella multocida, Moraxella, Simonsiella* |
| *104146* | *5* | *(-)* | *(+)* | *(-)* | *(-)* | *(-)* | *Pasteurella multocida, Flavobacterium* |
| *105577* | *6* | *n/a* | *n/a* | *(-)* | *(-)* | *(-)* | *Moraxella, Simonsiella, Bacillus* |
| *106525* | *7* | *(+)* | *(-)* | *(-)* | *(-)* | *Non-hemolytic Streptococcus* | *Pasteurella multocida, Moraxella, Pasteurella dogmatis* |
| *106548* | *8* | *n/a* | *n/a* | *(-)* | *(+)* | *(-)* | *Pasteurella multocida, Moraxella, Pasteurella dogmatis* |
| *106555* | *9* | *(-)* | *(-)* | *(-)* | *(+)* | *(-)* | *Moraxella, Pasteurella dogmatis* |

**Table 3 Organisms isolated from nine cats with clinical signs of URTD in which no primary pathogens were detected.** Samples were collected as in Table 1. Primary pathogens were defined as: *Bordetella bronchiseptica*, *Chlamydophila felis*, feline calicivirus, and feline herpesvirus-1.

**References**


Baird SC, Carman J, Dinsmore RP, Walker RL, Collins JK (1999), Detection and identification of *Mycoplasma* from bovine mastitis infections using a nested polymerase chain reaction, *J Vet Diagn Invest* 11: 432-435

Bannasch MJ, Foley JE (2005), Epidemiologic evaluation of multiple respiratory pathogens in cats in animal shelters, *J Fel Med Surg* 7: 109-119

Binns SH, Dawson S, Speakman AJ, Cuevas LE, Hart CA, Gaskell CJ, Morgan KL, Gaskell RM (2000), A study of feline upper respiratory tract disease with reference to prevalence and risk factors for infection with feline calicivirus and feline herpesvirus, *J Fel Med Surg* 2: 123-133

Boyle FM, Georghiou PR, Tilse MH, McCormack JG (1991), *Branhamella* (*Moraxella*) *catarrhalis*: pathogenic significance in respiratory infections, *Med J Aust* 154: 592-596

Burgesser KM, Hotaling S, Schiebel A, Ashbaugh SE, Roberts SM, Collins JK (1999), Comparison of PCR, virus isolation, and indirect fluorescent antibody staining in the detection of naturally occurring feline herpesvirus infections, *J Vet Diagn Invest* 11: 122-126

Cape L (1992), Feline idiopathic chronic rhinosinusitis: A retrospective study of 30 cases, *JAAHA* 28: 149-155

Coutts AJ, Dawson S, Willoughby K, Gaskell RM (1994), Isolation of feline respiratory viruses from clinically healthy cats at UK cat shows, *Vet Rec* 135: 555-556

Eamens GJ, Kirkland PD, Turner MJ, Gardner IA, White MP, Hornitzky CL (1988), Identification of toxigenic *Pasteurella multocida* in atrophic rhinitis of pigs by in vitro characterization, *Aust Vet J* 65: 120-123

Feinstein AR (2002), *Principles of Medical Statistics*, CRC Press LLC, Boca Raton, FL

Foley J, Bannasch MJ (2004), Infectious Diseases of Dogs and Cats, in *Shelter Medicine for Veterinarians and Staff*, ed. Miller LS and Zawitowski S, Blackwell Publishing, Ames p 235-284

Foster SF, Barrs VR, Martin P, Malik R (1998), Pneumonia associated with *Mycoplasma* spp in three cats, *Aust Vet J* 76: 460-464

Foster SF, Martin P, Braddock JA, Malik R (2004), A retrospective analysis of feline bronchoalveolar lavage cytology and microbiology (1995-2000), *J Fel Med Surg* 6: 189-198

Helps CR, Lait P, Damhuis A, Bjornehammar U, Bolta D, Brovida C, Chabanne L, Egberink H, Ferrand G, Fontbonne A, Pennisi MG, Gruffydd-Jones T, Gunn-Moore D,

Hartmann K, Lutz H, Malandain E, Mostl K, Stengel C, Harbour DA, Graat EA (2005), Factors associated with upper respiratory tract disease caused by feline herpesvirus, feline calicivirus, *Chlamydophila felis* and *Bordetella bronchiseptica* in cats: experience from 218 European catteries, *Vet Rec* 156: 669-673

Johnson LR, Drazenovich NL, Foley JE (2004), A comparison of routine culture with polymerase chain reaction technology for the detection of *Mycoplasma* species in feline nasal samples, *J Vet Diagn Invest* 16: 347-351

Johnson LR, Foley JE, De Cock HE, Clarke HE, Maggs DJ (2005), Assessment of infectious organisms associated with chronic rhinosinusitis in cats, *J Am Vet Med Assoc* 227: 579-585

Landis JR, Koch GG (1977), The measurement of observer agreement for categorical data, *Biometrics* 33: 159-174

Maggs DJ, Clarke HE (2005), Relative sensitivity of polymerase chain reaction assays used for detection of feline herpesvirus type 1 DNA in clinical samples and commercial vaccines, *Am J Vet Res* 66: 1550-1555

Padrid PA, Feldman BF, Funk K, Samitz EM, Reil D, Cross CE (1991), Cytologic, microbiologic, and biochemical analysis of bronchoalveolar lavage fluid obtained from 24 healthy cats, *Am J Vet Res* 52: 1300-1307

Pedersen NC, Sato R, Foley JE, Poland AM (2004), Common virus infections in cats, before and after being placed in shelters, with emphasis on feline enteric coronavirus, *J Fel Med Surg* 6: 83-88

Radford AD, Bennett M, McArdle F, Dawson S, Turner PC, Glenn MA, Gaskell RM (1997), The use of sequence analysis of feline calicivirus hypervariable region in the epidemiological investigation of FCV related disease and vaccine failures, *Vaccine* 12/13: 1451-1358

Ruch-Gallie R, Veir JK, Spindel M, Lappin MR (2006), Efficacy of amoxicillin and azithromycin for the treatment of cats with upper respiratory infections, In review: *J Fel Med Surg*

Stein JE, Lappin MR (2006), Bacterial culture and antibiotic sensitivity results from the upper and lower airways of cats: 421 cases (1995-2003). In review, *JAAHA*

Sykes JE, Studdert VP, Anderson G, Browning GF (1997), Comparison of Chlamydia psittaci from cats with upper respiratory tract disease by polymerase chain reaction analysis of the ompA gene, *Vet Rec* 140: 310-313

Sykes JE, Studdert VP, Browning GF (1998), Detection and strain differentiation of feline calicivirus in conjunctival swabs by RT-PCR of the hypervariable region of the capsid protein gene, *Arch Virol* 143: 1321-1334

Veir JK, Dow, SW, Lappin SW (2003), Detection of feline herpesvirus-1 DNA from swabs collected from the pharynx or nasal discharges of cats using fluorogenic and conventional PCR, *J Vet Intern Med* 17:425 (abstract)

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ENDNOTES

[a] Ca alginate swabs, Ultrafine Al, product #14-959-78, Fischer Scientific

[b] BBL CultureSwab Plus Amies Medium with Charcoal, Becton, Dickinson and Company; Sparks, MD

[c] Viral transport media: Modified Eagle's Medium with 1% Hepes, 4% bovine growth serum, 0.0025% Amphotericin B + antibiotics

[d] Qiagen, Valencia, CA

CHAPTER 2

# QUANTIFICATION OF FELINE HERPESVIRUS-1 DNA FROM SWABS COLLECTED FROM THE PHARYNX OR NASAL DISCHARGES OF CATS USING REAL TIME AND CONVENTIONAL PCR ASSAYS

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## Introduction

Of the 15,000 cats examined at Colorado State University (CSU) between 1988 and 1999, 1,573 (10.5%) had respiratory signs of disease. Infectious feline upper respiratory tract disease (URTD) is even more common in multi-cat environments like humane shelters and catteries and can result in euthanasia rather than adoption or sale. Feline infectious URTD has multiple causes; feline herpesvirus-1 (FHV-1) and calicivirus are the most common primary viral causes (Binns *et al* 2000, Sykes *et al* 1999). After exposure to FHV-1, cats develop a rapid immune response, however, up to 80% of animals become latently infected (Nasisse *et al* 1992, Ohmura *et al* 1993), typical of the α-herpesviruses. Prior to the widespread clinical use of the polymerase chain reaction (PCR) technology, infection was documented by documenting the organism in cells by use of fluorescent antibody assays and by detection of serum antibodies by serum neutralization (Stiles *et al* 1997b). However, in the past decade, qualitative endpoint FHV-1 PCR assays have been shown to be a sensitive method of documenting the presence of FHV-1 DNA (Burgesser *et al* 1999, Hara *et al* 1996, Reubel *et al* 1993, Stiles *et al* 1997b, Stiles *et al* 1997a, Sykes *et al* 1997, Weigler *et al* 1997). However, because of latency and intermittent shedding or re-activation, results of these PCR assays cannot differentiate between co-incidental shedding in healthy FHV-1 carriers, shedding secondary to immunosuppression from concurrent disease, and the disease entity itself (Maggs *et al* 1999). In addition, FHV-1 strains used in modified live vaccines cannot be distinguished from naturally occurring strains (Maggs and Clarke 2005).

In a recent study of experimentally and naturally-infected cats, results of a quantitative real time PCR (q-rtPCR) assay targeting the FHV-1 glycoprotein B (Vogtlin

*et al* 2002) were compared to those of a FHV-1 endpoint PCR assay using ocular fluids; the q-rtPCR assay had at least equal sensitivity in vitro and greater sensitivity in vivo than endpoint PCR assay for later stages of infection. Additionally, the authors used the quantitative data obtained from the q-rtPCR assay to define "stages" of infection in the experimentally-infected cats and applied the data to naturally-infected cats. Our laboratory has recently used a q-rtPCR to compare viral load in recently vaccinated cats to demonstrate a temporal increase in the presence of viral DNA post-vaccination and viral challenge (Lappin *et al* 2006). However, whether quantitative FHV-1 q-rtPCR assay results can be used to differentiate cats that are FHV-1 carriers versus those that are ill from FHV-1 infection is currently unknown. There are no studies to date evaluating the use of quantitative viral load data of the respiratory tract in an attempt to discriminate between FHV-1 carriers and cats clinically ill from FHV-1 infection. Therefore, the objectives of this pilot study were to amplify FHV-1 DNA from nasal or pharyngeal swabs from cats with or without history of URTD by use of both a q-rtPCR assay and an endpoint PCR assay to determine the optimal sampling site and to determine whether results of the q-rtPCR could discriminate between FHV-1 carriers, suspect carriers of FHV-1, and cats clinically ill from FHV-1 infection.

**Materials and Methods**

*Study groups.* Three groups of cats privately owned by staff or students of Colorado State University Veterinary Teaching Hospital were sampled. Owners were interrogated regarding history of clinical signs associated with URTD (sneezing, nasal discharge, stertorous breathing that resolved without surgical intervention, inappetence, ocular discharge) and the groups were defined and sampled as follows: Group 1: Healthy (no

clinical signs of URTD within the last 2 years): pharyngeal swabs only, Group 2: Suspect carriers: (clinical signs of URTD between 1 and 3 mos prior to sampling but normal at the time of collection): pharyngeal swabs only, and Group 3: Clinically ill (discharge referable to recurrent URTD present at time of sampling): pharyngeal and nasal swabs. All protocols were approved by the Animal Care and Use Committee of Colorado State University.

*Sampling.* Swab samples were obtained using standard cotton tipped wooden applicators (pharynx) or urethral culture swabs[a] (nasal cavity) on awake, non-sedated cats by a single author (JKV). Swabs were immediately placed in 1.0 mL sterile 0.01 M phosphate buffered saline (PBS) and allowed to sit at room temperature for 2-3 hours before being placed into -70°C until processing and analysis according to manufacturer's protocol. DNA was extracted from the PBS using a commercial kit[b] and assayed for FHV-1 using previously published protocols using both endpoint (Weigler *et al* 1997) and q-rtPCR (Vogtlin *et al* 2002). The q-rtPCR was modified for use in nasal and pharyngeal sampling as previously described (Veir *et al* 2006; Chapter 5 of this dissertation). In order to normalize cellular yield due to sampling variability between cats, quantification of feline glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was concurrently assayed using a previously published q-rtPCR (Leutenegger *et al* 1999). Products of the endpoint PCR were separated using agarose gel electrophoresis and considered positive if a single band of the appropriate size (322 bp) was visualized.

Data from the q-rtPCR was analyzed with the instrument software.[c] Samples were considered positive if the fluorescence intensity exceeded ten times the standard deviation of the baseline fluorescence (threshold cycle [Ct]). All reactions were run in

duplicate. A control sample consisting of DNA pooled from swabs from 10 normal cats spiked with plasmid derived FHV-1 DNA was run on each plate to ensure repeatable thermocycler conditions.

Standard curves for GAPDH and FHV-1 were generated as previously described (Veir *et al* 2006; Chapter 5 of this dissertation). Briefly, a standard curve for GAPDH-cell equivalent was generated using RNA isolated from a feline lung epithelial cell line (Dow unpublished data) that was digested in the same manner as the test samples (cell equivalent). The standard curve for FHV-1 was generated using a 10-fold dilution series using plasmid generated DNA (pDNA). The FHV-1 pDNA was produced using a commercially available vector[d] after purification[e] of product obtained from a conventional PCR reaction using the primers used in the real time assay. Viral load was then defined as the calculated FHV copy number divided by the calculated GAPDH-cell equivalent copy number of each sample.

*Analysis.* Concordance rates of detection of FHV-1 DNA between the two methods (all sites grouped together) and the two sampling sites (pharyngeal compared to nasal, Clinically ill group only) were analyzed using the kappa statistic as defined by Feinstein (2002) and evaluated for significance as defined by Landis and Koch (1977). Quantitative data was compared between disease groups using the student's unpaired t test. The level of significance was set at $p < 0.05$ for between disease group comparisons.

**Results**

Twenty one cats were enrolled in the study between November 2002 and January 2004. Six were classified as Healthy (mean age 6.4 years), six were classified as Suspect

carriers (mean age 5.3 years), and nine were classified as Clinically ill (mean age 4.9 years). Breeds represented were domestic shorthair (n = 20) and domestic longhair (n = 1). There was no statistical difference in mean age among groups (p = 0.86).

Other than transient sneezing associated with nasal swab sample collection, no hemorrhage or other adverse effects from either sampling technique were observed. The two assays showed similar detection rates (Table 1) in all disease groups, with disagreement in the Suspect carrier group only. There was a low rate of detection of FHV-1 in all groups; therefore, indices of specific agreement were calculated to determine the effects of the skewed distribution of challenge. In the only disease group with a kappa between the assays of less than one, the specific proportionate agreement for negative results was higher than that for positive results, as can be expected in a group with such low rates of positive results (Table 1). In the single disease group in which sampling sites were compared (Clinically ill), correlation between results for the assays was substantial as defined by parameters set forth by Koch and Landis (1977) (kappa=0.73, Table 2).

After normalization to starting sample size with GAPDH, there was a significant difference between the copy number of FHV-1 per cell equivalent in pharyngeal samples between the Healthy (mean = 0.27 ± 0.03) and Clinically ill groups (mean = 20.64 ± 13.28, p = 0.027) but no difference between the Suspect carrier group (mean 0.70 ± 0.13) and any other disease group (Healthy: p = 0.098, Clinically ill: p = 0.066, Figure 1).

**Discussion**

All pharyngeal samples except one taken from the pharynx of a healthy cat were adequate for amplification (with no evidence of PCR inhibitors) as evidenced by GAPDH

values of at least sixteen cell equivalents, the lower level of detection of the assay. The sample with inadequate DNA for amplification was taken from a fractious cat which may have decreased sample size as opposed to the presence of inhibitors of PCR. In support of this, a Ct value was obtained, but it was not within the linear range of the standard curve, making quantification inaccurate. All nasal samples had adequate DNA for amplification of feline GAPDH, indicating that inhibitors of PCR should not confound sampling from this area.

Sensitivity of the q-rtPCR assay was at least as sensitive as the endpoint PCR assay across all groups, with one additional positive in the suspect carrier group. Without a third assay such as virus isolation to be used as a gold standard, it is impossible to say whether this is a false positive or increased sensitivity of the q-rtPCR assay. However, the detection limit for the q-rtPCR assay has been shown to be lower than the endpoint assay in vitro (Vogtlin *et al* 2002), lending support that this is truly a difference in sensitivity, not specificity. Regardless, there was a high correlation between the two assays indicating q-rtPCR is a valid assay for detection of FHV-1 from the pharynx and the nasal cavity of cats.

Sampling from the nasal cavity of cats with URTD yielded a larger number of positives (3/9, 33%) compared to the pharyngeal cavity (2/9, 22%), However, again, concordance between the two sites was high (kappa = 0.73, both assays) indicating that for cats with nasal signs, the nasal cavity is a valid sampling site that carries less risk for the sampler when using urethral culture swabs than pharyngeal swabs.

In this population, the q-rtPCR assay was able to discriminate between the Healthy and Clinically ill group. However, the assay was not able to discriminate

between those animals that had recently recovered from an episode of URTD and those with current clinical signs, negating the usefulness of the assay in some cases. However, given the population size, outliers and large standard deviations as were present in the Clinically ill group, will affect significance. Studies of larger populations are needed to determine if quantification of the targets used in this assay will be able to discriminate between these two disease states. As previously demonstrated, stress from other diseases can also induce shedding. Addition of a fourth group with animals suffering from a concurrent systemic illness such as neoplasia or endocrine disorders but not showing clinical signs of URTD as well as a group of cats recently given a modified live vaccine should be included in future studies as well.

Finally, as stated above, a large proportion of FHV-1 infected cats are in the latent state of disease and therefore samples from these animals may be negative when assayed for target genes generally expressed during active infection. Other authors (Townsend *et al* 2004) have investigated the use of assays targeting latency associated genes in order to improve detection. Studying these targets in a quantitative manner may help further discriminate those cats in which FHV-1 is inducing disease as compared to co-incidental shedding or latency.

Results of this study suggest that detection of FHV-1 in nasal or pharyngeal swabs by q-rtPCR is a valid method of diagnosis and quantitation can discriminate between healthy and clinically ill animals but not between animals recently recovered from a suspicious episode and any other disease group. Studies with larger populations, the addition of animals suffering from concurrent systemic illness associated with immunosuppression but not URTD signs, and targets associated with latency are needed

prior to determining if quantification of FHV-1 can be a useful clinical diagnostic tool in the diagnosis and ultimately, guide the treatment decision tree of upper respiratory tract infection in cats.

| URTD Disease Group | Endpoint PCR: detection rate | q-rtPCR: detection rate | Concordance (kappa) | $p_{pos}$ | $p_{neg}$ |
|---|---|---|---|---|---|
| **Healthy (n=6, pharyngeal only)** | 2 (33.3%) | 2 (33.3%) | 1 | 1 | 1 |
| **Suspect carriers (n=6, pharyngeal only)** | 1 (16.7%) | 2 (33.3%) | 0.57 | 0.67 | 0.89 |
| **Clinically ill (n=9, pharyngeal)** | 2 (22.2%) | 2 (22.2%) | 1 | 1 | 1 |
| **Clinically ill (n=9, nasal)** | 3 (33.3%) | 3 (33.3%) | 1 | 1 | 1 |

**Table 1 Comparison of two methods of detection by PCR of FHV-1 DNA in respiratory swabs from privately owned adult cats** Samples were obtained using standard cotton tipped applicators (pharyngeal) or urethral culture swabs (nasal) from privately owned cats. Endpoint and q-rtPCR targeting FHV-1 DNA were performed on each sample in duplicate. Mean ages at time of sampling were statistically similar between disease groups. Disease group definitions are defined in text. Indices of specific agreement ($p_{pos}$ and $p_{neg}$) were calculated according to Feinstein (2002).

| Assay | Pharyngeal: detection rate | Nasal: detection rate | Concordance (kappa) | $p_{pos}$ | $p_{neg}$ |
|---|---|---|---|---|---|
| **Endpoint PCR** | 2 (22.2%) | 3 (33.3%) | 0.73 | 0.80 | 0.92 |
| **q-rtPCR** | 2 (22.2%) | 3 (33.3%) | 0.73 | 0.80 | 0.92 |

**Table 2 Comparison of sampling sites for detection of FHV-1 DNA in respiratory swabs from privately owned adult cats** Two samples of the respiratory tract from each of nine cats (Clinically ill group, Table 1) were obtained using standard cotton tipped applicators (pharyngeal) or urethral culture swabs (nasal). All cats were currently showing clinical signs consistent with URTD at the time of sampling. Indices of specific agreement ($p_{pos}$ and $p_{neg}$) were calculated according to Feinstein (2002).

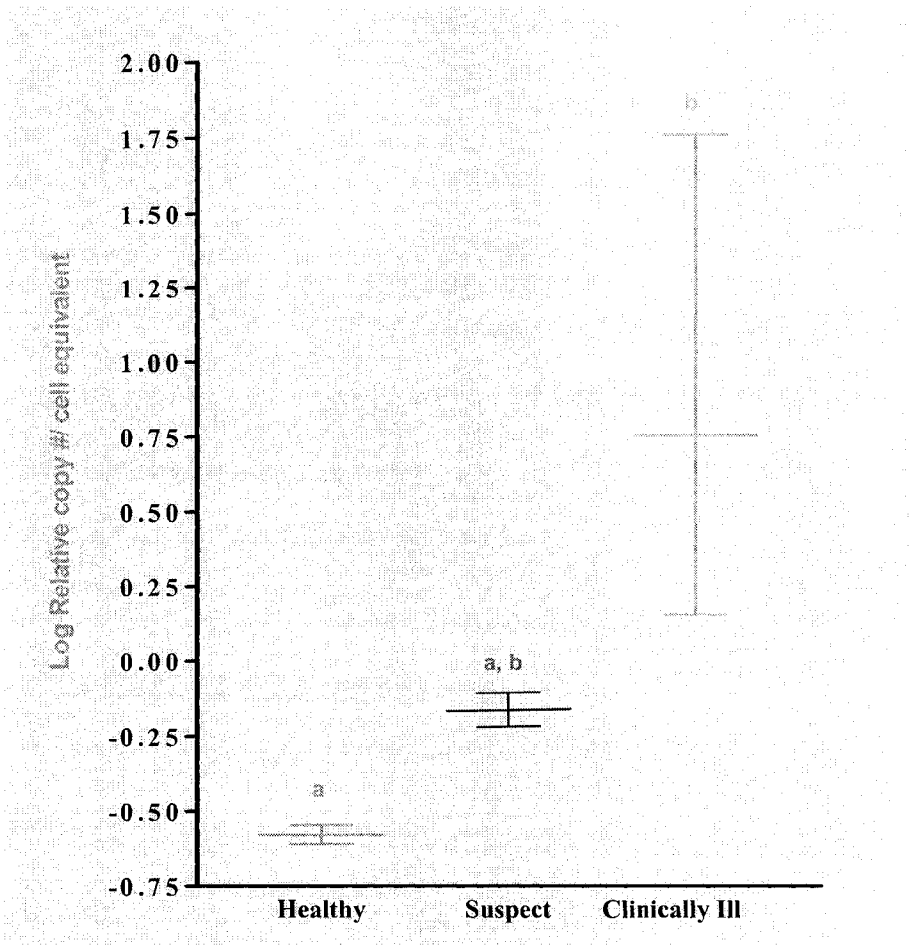


**Figure 1 Comparison of copy number of FHV-1 per cell equivalent in pharyngeal swabs from three groups of privately owned cats** Samples were obtained using standard cotton tipped applicators from privately owned cats. Endpoint and q-rtPCR targeting FHV-1 DNA were performed on each sample in duplicate. Mean copy number FHV-1/cell equivalent with interquartile ranges for each group are graphed. Mean ages at time of sampling were statistically similar between disease groups. Disease group definitions are defined in text. Groups labeled with different letters are statistically different ($p<0.05$).

**References**


Binns SH, Dawson S, Speakman AJ, Cuevas LE, Hart CA, Gaskell CJ, Morgan KL, Gaskell RM (2000), A study of feline upper respiratory tract disease with reference to prevalence and risk factors for infection with feline calicivirus and feline herpesvirus, *J Fel Med Surg* 2: 123-133

Burgesser KM, Hotaling S, Schiebel A, Ashbaugh SE, Roberts SM, Collins JK (1999), Comparison of PCR, virus isolation, and indirect fluorescent antibody staining in the detection of naturally occurring feline herpesvirus infections, *J Vet Diagn Invest* 11: 122-126

Feinstein AR (2002), *Principles of Medical Statistics*, CRC Press LLC, Boca Raton, FL

Hara M, Fukuyama M, Suzuki Y, Kisikawa S, Ikeda T, Kiuchi A, Tabuchi K (1996), Detection of feline herpesvirus 1 DNA by the nested polymerase chain reaction, *Vet Microbiol* 48: 345-52

Landis JR, Koch GG (1977), The measurement of observer agreement for categorical data, *Biometrics* 33: 159-174

Lappin MR, Sebring RW, Porter M, Radecki SJ, Veir J (2006), Effects of a single dose of an intranasal feline herpesvirus 1, calicivirus, and panleukopenia vaccine on clinical signs and virus shedding after challenge with virulent feline herpesvirus 1, *J Fel Med Surg* 8: 158-163.

Leutenegger CM, Mislin CN, Sigrist B, Ehrengruber MU, Hofmann-Lehmann R, Lutz H (1999), Quantitative real-time PCR for the measurement of feline cytokine mRNA, *Vet Immunol Immunopathol* 71: 291-305

Maggs DJ, Clarke HE (2005), Relative sensitivity of polymerase chain reaction assays used for detection of feline herpesvirus type 1 DNA in clinical samples and commercial vaccines, *Am J Vet Res* 66: 1550-1555

Maggs DJ, Lappin MR, Nasisse MP (1999), Detection of feline herpesvirus-specific antibodies and DNA in aqueous humor from cats with or without uveitis, *Am J Vet Res* 60: 932-936

Nasisse MP, Davis BJ, Guy JS, Davidson MG, Sussman W (1992), Isolation of feline herpesvirus-1 from the trigeminal ganglia of acutely and chronically infected cats, *J Vet Intern Med* 6: 102-103

Ohmura Y, Ono E, Matsuura T, Kida H, Shimizu Y (1993), Detection of feline herpesvirus-1 transcripts in trigeminal ganglia of latently infected cats, *Arch Virol* 129: 341-347

Reubel GH, Ramos RA, Hickman MA, Rimstad E, Hoffmann DE, Pedersen NC (1993), Detection of active and latent feline herpesvirus-1 infections using the polymerase chain reaction, *Arch Virol* 132: 409-420

Stiles J, McDermott M, Bigsby D, Willis M, Martin C, Roberts W, Greene C (1997a), Use of nested polymerase chain reaction to identify feline herpesvirus in ocular tissue from clinically normal cats and cats with corneal sequestra or conjunctivitis, *Am J Vet Res* 58: 338-342

Stiles J, McDermott M, Willis M, Roberts W, Greene C (1997b), Comparison of nested polymerase chain reaction, virus isolation, and fluorescent antibody testing for identifying feline herpesvirus in cats with conjunctivitis, *Am J Vet Res* 58: 804-7

Sykes JE, Anderson GA, Studdert VP, Browning GF (1999), Prevalence of feline Chlamydia psittaci and feline herpesvirus 1 in cats with upper respiratory tract disease, *J Vet Intern Med* 13: 153-162

Sykes JE, Browning GF, Anderson G, Studdert VP, Smith HV (1997), Differential sensitivity of culture and the polymerase chain reaction for detection of feline herpesvirus 1 in vaccinated and unvaccinated cats, *Arch Virol* 142: 65-74

Townsend WM, Stiles J, Guptill-Yoran L, Krohne SG (2004), Development of a reverse transcriptase-polymerase chain reaction assay to detect feline herpesvirus-1 latency-associated transcripts in the trigeminal ganglia and corneas of cats that did not have clinical signs of ocular disease, *Am J Vet Res* 65: 314-319

Veir JK, Lappin MR, Dow SW (2006), Evaluation of a novel immunotherapy for treatment of chronic rhinitis in cats, *J Fel Med Surg* Jul 17; [Epub ahead of print].

Vogtlin A, Fraefel C, Albini S, Leutenegger CM, Schraner E, Spiess B, Lutz H, Ackermann M (2002), Quantification of feline herpesvirus 1 DNA in ocular fluid samples of clinically diseased cats by real-time TaqMan PCR, *J Clin Microbiol* 40: 519-523

Weigler BJ, Babineau CA, Sherry B, Nasisse MP (1997), High sensitivity polymerase chain reaction assay for active and latent feline herpesvirus-1 infections in domestic cats, *Vet Rec* 140: 335-338

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ENDNOTES

[a] Ultrafine aluminum applicator swab, Fisher Scientific, Pittsburgh, PA

[b] QIAamp DNA minikit, Qiagen, Inc, Valencia, CA

[c] ABI Prism 7000 SDS Software Version 1.0 (build 81 rev 3): Applied Biosystems, Foster City, CA

[d] TA Cloning Vector: Invitrogen Corporation, Carlsbad, CA

[e] QIAquick PCR Purification kit, Qiagen, Inc, Valencia, CA

# CHAPTER 3

# DEVELOPMENT OF A WHOLE BLOOD PROLIFERATION ASSAY TO ASSESS CELL-MEDIATED IMMUNE RESPONSE TO FHV-1 IN CATS

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Lymphocyte proliferation assays using incorporation of tritium labeled thymidine ([3H]-TdR) are frequently used as an in vitro measure of cell-mediated immunity in both humans and veterinary species, including cats (Brown 1977, Hill *et al* 2005, Horohov *et al* 1985, Kim *et al* 2000, Otto *et al* 1993, Weiss and Oostromram 1990). These assays can be used clinically to assess response to vaccination and the need to revaccinate, detect hypersensitivity reactions, and predict response to infection. These assays are both sensitive and repeatable but have traditionally required large volumes of blood, typically between 4 and 8 mL minimum per test sample, due to requirements of lymphocyte purification techniques typically used. The purification techniques, including low speed differential density centrifugation and defibrination, are both labor intensive and extremely user dependent. Incomplete or inadequate layering can result in a dramatic loss of certain lymphocyte populations (Confer *et al* 1989) and disturb normal cell-cell interactions (Barten *et al* 2001). Finally, the separation techniques themselves as well as certain sedation protocols required for collecting such a large volume of blood may influence lymphocyte population dynamics and activation state (Gummert *et al* 1999). An alternative method is to use whole blood in the assays. Whole blood proliferation assays utilize less blood (typically 100 microliters) allowing more frequent sampling, the use of less or no sedation that may affect proliferative capacity, and smaller animals. Additionally, potential bias towards certain T cell populations is avoided as there is no separation step required (Barten *et al* 2001).

Feline herpesvirus-1 (FHV-1) is a viral infection of cats that can cause chronic morbidity and is a leading cause of both euthanasia (Foley and Bannasch 2004) and financial expense for animal shelters (Pedersen *et al* 2004). FHV-1 infection is common

in cats and extremely contagious between cats. Fever, sneezing, nasal discharge, inflamed eyes (conjunctivitis), ulcers of the cornea (keratitis), cough, difficult breathing (dyspnea), and death can occur following FHV-1 infection (Hickman *et al* 1994). In a recent prevalence study at a humane society in north central Colorado, FHV-1 DNA was amplified from oropharyngeal or nasal swabs collected from 49 of 60 cats (81.7%) showing clinical signs of upper respiratory tract disease (Veir *et al* 2004, Chapter 1). Protection against FHV-1 is mediated by both humoral and cell-mediated immunity (Tham and Studdert 1987). While serological tests for FHV-1 antibodies have been studied extensively, reports of technique for assessment of cell-mediated immune responses are less common. The purpose of this study was to develop and evaluate a whole blood [3H]-TdR proliferation assay using both a non-specific T cell mitogen (concanavalin A) and an inactivated FHV-1 preparation for potential use in FHV-1 vaccine and treatment studies.

All use of animals was approved by the Colorado State University Animal Care and Use Committee. Feline herpesvirus-1 antigen was prepared from an isolate obtained from a swab of the nasal cavity of a cat in northern Colorado. After initial isolation, virus was propagated on a monolayer of Crandall Reese feline kidney (CRFK) cells to a final concentration of $10^{7.85}$ pfu/mL. Viral stock was exposed to UV light in a sterile environment for varying lengths of time (10, 20, and 30 minutes). An aliquot of stock was then inoculated onto CRFK cells and confirmed to be inactivated by demonstration of no evidence of characteristic CPE. Five hundred microliters of whole heparinized blood was obtained from the jugular vein of each of four two year old domestic short hair cats (2 female intact, 2 male castrated) previously vaccinated with a killed FVRCP

vaccine[a]. Assays were performed in 96-well plates using 10 uL whole heparinized blood added to 100 uL complete tumor media with various concentrations of concanavalin A (ConA) (0, 5, 10, 20 μg/mL) and dilutions of FHV-1 ($1x10^{0}$, $1x10^{-1}$, $1x10^{-2}$) per well. Cells were incubated with media only (control), concanavalin A and media (ConA), or FHV-1 and media. After 102 hours of incubation in a humidified environment at $37^{0}$C with 5% $CO_2$, wells were aseptically pulsed with 0.5 uCi tritiated thymidine for 18 hours before being harvested onto filter mats and counted.[b] All samples were assayed in triplicate and stimulation indexes (SI) were calculated by dividing the average of the stimulated cell count by the control count for each cat and mitogen or antigen concentration.

All UV exposure time groups eliminated viable organisms in preparations as demonstrated by incubation with confluent CrFK cells for 48 hours at 37°C in a 5% $CO_2$ atmosphere. Additionally, incubation with the non-UV inactivated did not allow any proliferative response. Therefore, all further comparisons considered only the shortest UV exposure time of 10 minutes for FHV-1 antigen preparation. Results of the effects of different concentrations of ConA and FHV-1 antigen preparation are summarized in Figures 1 and 2 and Table 1.

While 5 ug/mL ConA yielded a higher mean counts per minute (CPM) and mean stimulation index (SI), the ranges and standard deviations obtained for both CPM and SI using 10 ug/mL were smaller, therefore, 10 ug/mL was chosen as the preferable ConA concentration. When the results for the FHV-1 antigen preparation were analyzed for optimal concentration, an unexpected result was found. The undiluted viral preparation ($10^{7.85}$ pfu/mL) seemed to have an inhibitory effect as both CPM and SI were

dramatically lower than the $10^{-1}$ and $10^{-2}$ dilution (Figure 2). The finding potentially could be explained by cytopathic effect secondary to infection with viable virus. However, as previously stated, no differences were seen in titration experiments using the FHV-1 preparation after exposure to UV light at 10, 20, and 30 minutes, therefore, no virus should have remained viable. Additionally, viability staining with trypan blue of control wells (no [3H]-TdR added) revealed greater than 80% viability in all preparations, including the wells to which the undiluted antigen preparation had been added. This finding is similar to a previous study in which the most concentrated preparation of an FHV-1 antigen stock inactivated by chemical means yielded one of the lowest stimulation indices of those tested as well (Tham and Studdert 1987). In that study, the authors hypothesized presence of FHV-1 antibodies inhibited exposure of antigen and subsequent T cell proliferation. However, this seems unlikely because antibodies in the whole blood cell preparation should have also inhibited the lower concentrations of virus, perhaps even to a greater degree and a more likely explanation may be the presence of cytotoxic additives in the viral preparation. Of the remaining two concentrations, the $10^{-1}$ dilution yielded a higher CPM and SI, however, a smaller standard deviation and range were obtained using the higher dilution ($10^{-2}$, Figure 2).

Concanavalin A stimulation produced reliable results at 10 ug/mL using a very small volume of whole blood. This assay can be used to assess in vitro T cell-mediated immunity in cats with a very small volume of blood for very little cost as long as a radioactive safety and usage program is in place in the performing laboratory. In the only other published report of a FHV-1 whole blood proliferation assay, the authors used inactivated FHV-1 vaccine without adjuvant to determine cell-mediated immune

response to vaccine and infection (Tham and Studdert 1987). However, this preparation may contain cytotoxic preservatives and additives. In contrast, crude viral stocks are available to any veterinary diagnostic laboratory performing viral isolation and do not have additives that affect cell growth. With the simple inactivation procedure presented in this study, laboratories can easily produce their own viral stock, quantify it with standard laboratory methods, and inactivate with simple ultraviolet light exposure. This inexpensive, easy to prepare, and readily available reagent can then be used to assess cell-mediated immunity to a relevant viral antigen for both research and clinical purposes. However, prior to the use of the assay to predict need for vaccination (along with already available commercial assays for antibody dependent immunity), challenge studies correlating clinical signs to SI levels need to be performed. This assay was used in Chapters 4 and 5 of this dissertation to evaluate proliferative ability secondary to stimulation with the specific antigen (FHV-1).

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ENDNOTES
[a] Fel-o-vax®, Fort Dodge Animal Health, Overland Park, KS
[b]Wallac-Microbeta; Perkin Elmer, Boston, MA

| *Treatment* | *Concentration* | *Mean CPM (± SD)* | *Mean SI (± SD)* |
|---|---|---|---|
| *Concanavalin A* | ***5 μg/Ml*** | ***11595** (6216)* | ***42.09** (26.31)* |
| | ***10 μg/mL*** | ***6427** (2601)* | ***22.04** (3.090)* |
| | ***20 μg/mL*** | ***6328** (1301)* | ***26.31** (36.92)* |
| *FHV-1 Antigen Prep* | ***7.079x10$^5$ pfu/mL*** | ***5382** (1028)* | ***1.035** (0.9510)* |
| | ***7.079x10$^6$ pfu/mL*** | ***11956** (6159)* | ***43.78** (29.21)* |
| | ***7.079x10$^7$ pfu/mL*** | ***238** (133)* | ***20.05** (5.578)* |

**Table 1. Mean counts per minute (CPM) and stimulation indices (SI) of 10 uL whole heparinized blood obtained from four mature FHV-1 vaccinated domestic shorthair cats.** Blood was incubated at 37oC with 5% CO2 for 102 hours in complete tumor media with respective treatments prior to being pulsed with 1 uCi [3H]-TdR and incubated in the same environment for an additional 18 hours. All samples were run in triplicate and stimulation indices were calculated by dividing the average of the stimulated cell count by the control count for each cat and mitogen or antigen concentration.

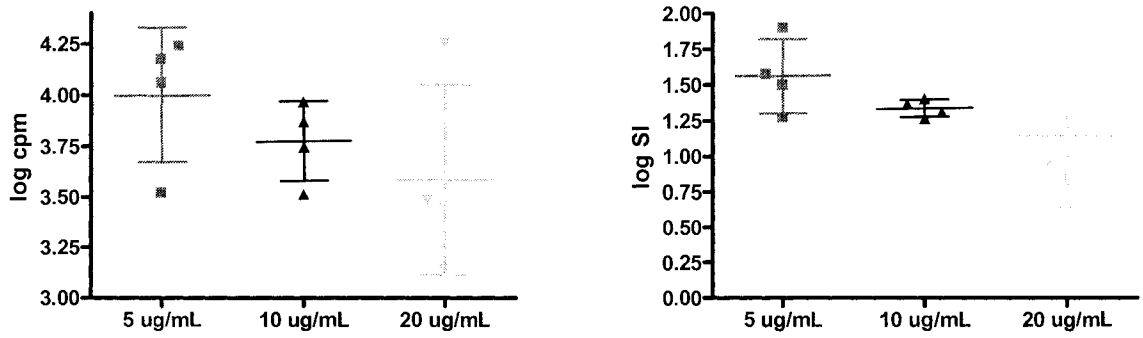


**Figure 1. Mean (with interquartile range) counts per minute (CPM, left panel) and stimulation indices (SI, right panel) of 10 uL whole heparinized blood obtained from four mature FHV-1 vaccinated domestic shorthair cats stimulated with varying concentrations of concanavalin A in vitro.** Conditions were as described for Table 1.

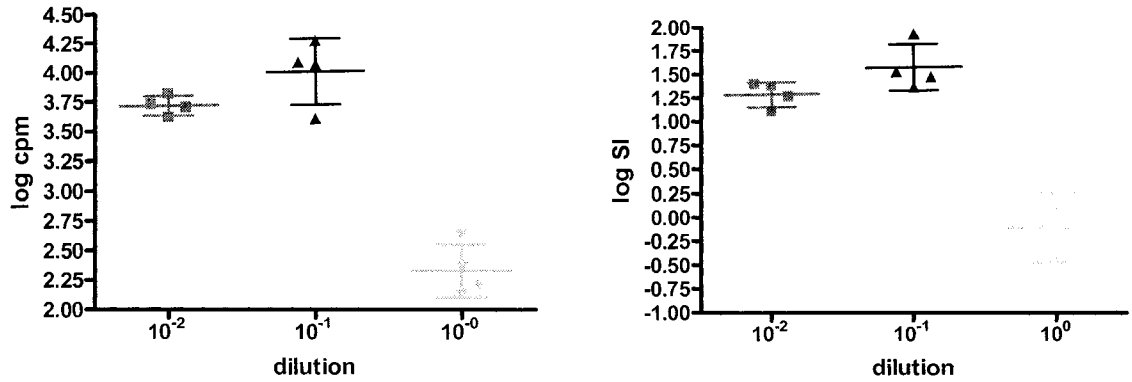


**Figure 2. Mean (with interquartile range) counts per minute (CPM, left panel) and stimulation indices (SI, right panel) of 10 uL whole heparinized blood obtained from four mature FHV-1 vaccinated domestic shorthair cats stimulated with varying concentrations of an FHV-1 viral stock culture (original stock: 1x107.85 pfu/mL) inactivated by exposure to UV light for 10 minutes.** Conditions were as described for Table 1.

**References**


Barten MJ, Gummert JF, van Gelder T, Shorthouse R, Morris RE (2001), Flow cytometric quantitation of calcium-dependent and -independent mitogen-stimulation of T cell functions in whole blood: inhibition by immunosuppressive drugs in vitro, *J Immunol Meth* 253: 95-112

Brown J (1977), Micromethod for study of in vitro lymphocyte-transformation to specific antigens, *J Immunol Meth* 18: 17-32

Confer AW, Wyckoff JH, Oltjen SL, Oltjen JW (1989), Sepracell-Mn for mononuclear cell-separation from cattle - Effect on mitogen-induced and antigen-induced lymphocyte blastogenesis, *Vet Immunol Immunopathol* 22: 79-89

Foley J, Bannasch MJ (2004), Infectious Diseases of Dogs and Cats, in *Shelter Medicine for Veterinarians and Staff*, ed. Miller LS and Zawitowski S, Blackwell Publishing, Ames p 235-284

Gummert JF, Otto G, Barten MJ, Morris RE (1999), Effect of anesthesia on a whole blood lymphocyte proliferation assay in the rat, *Immunopharmacol Immunotoxicol* 21: 267-276

Hickman MA, Reubel GH, Hoffman DE, Morris JG, Rogers QR, Pedersen NC (1994), An epizootic of feline herpesvirus, type 1 in a large specific pathogen-free cat colony and attempts to eradicate the infection by identification and culling of carriers, *Lab Anim* 28: 320-329

Hill AS, Rogers QR, O'Neill SL, Christopher MM (2005), Effects of dietary antioxidant supplementation before and after oral acetaminophen challenge in cats, *Am J Vet Res* 66: 196-204

Horohov DW, Moore RN, Rouse BT (1985), Herpes simplex virus-specific lymphoproliferation: an analysis of the involvement of lymphocyte subsets, *Immunobiol* 170: 460-73

Kim HW, Chew BP, Wong TS, Park JS, Weng BC, Byrne KM, Hayek MG, Reinhart GA (2000), Modulation of humoral and cell-mediated immune responses by dietary lutein in cats, *Vet Immunol Immunopathol* 73: 331-341

Otto CM, Brown CA, Lindl PA, Dawe DL (1993), Delayed-hypersensitivity testing as a clinical measure of cell-mediated-immunity in the cat, *Vet Immunol Immunopathol* 38: 91-102

Pedersen NC, Sato R, Foley JE, Poland AM (2004), Common virus infections in cats, before and after being placed in shelters, with emphasis on feline enteric coronavirus, *J Fel Med Surg* 6: 83-88

Tham KM, Studdert MJ (1987), Antibody and cell-mediated immune-responses to feline herpesvirus-1 following inactivated vaccine and challenge, *Journal of Veterinary Medicine Series B-Zentralblatt fur Veterinarmedizin Reihe B-Infectious Diseases and Veterinary Public Health* 34: 585-597

Veir JK, Ruch-Gallie R, Spindel ME, Lappin MR (2004). Prevalence of FHV-1, *Mycoplasma* spp., and aerobic bacteria in shelter cats with acute upper respiratory tract disease. *J Vet Intern Med* 18[3]: 197 (abstract)

Weiss RC, Oostromram T (1990), Effect of recombinant human interferon-alpha in vitro and in vivo on mitogen-induced lymphocyte blastogenesis in cats, *Vet Immunol Immunopathol* 24: 147-157

# CHAPTER 4

## THE EFFECT OF AN *ENTEROCOCCUS FAECIUM* (SF68) ENHANCED DIET ON THE IMMUNE RESPONSES TO A FELINE HERPESVIRUS 1, FELINE CALICIVIRUS, AND PANLEUKOPENIA VACCINE IN CATS

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Abstract: To evaluate the effect of supplementation with *Enteroccocus faecium* (SF68) on non-specific and antigen-specific humoral and cell-mediated immune responses 18 purpose bred kittens were divided into two groups and were given palatability enhancer with (Treatment) or without (Placebo) SF68 daily for 20 weeks starting at 7 weeks of age. Feline viral rhinotracheitis (FHV-1), calicivirus (FCV), and panleukopenia virus (FPV) vaccine was administered at 9 and 12 weeks of age. Survival of SF68 after ingestion, presence of *Clostridium* spp. products, fecal cultures, CBC, biochemical profiles, CD4+, CD8+ and B cell populations, CD44 and MHC Class II expression, Concanavalin-A and FHV-1-specific proliferation, total serum, salivary and fecal IgG and IgA, FHV-1-specific serum and salivary IgG and IgA, and FCV- and FPV-specific serum IgG were evaluated at 7, 9, 15, 21, and 27 weeks of age. No significant differences were seen in developmental parameters. SF68 was demonstrated in feces of seven of nine treatment cats during the study. The percentage of CD4+ lymphocytes was significantly higher in the treatment group at 27 weeks of age. Supplementation of kittens with SF68 does not negatively affect measured developmental parameters. The results suggest that supplementation modulates systemic immune responses but longer supplementation may be needed to reach statistical significance and more detailed studies of the cell populations affected are needed to determine what these effects are.

## Introduction

Probiotics have been defined as live microorganisms that when administered in adequate amounts confer a health effect on the host (Schrezenmeir and de Vrese 2001). It is theorized that probiotics may impart their beneficial health effects either by increasing the resistance to colonization of mucosal surfaces by pathogenic bacteria (colonization resistance) (Sanders 2003) or by exerting an indirect effect on gut associated lymphoid tissue (GALT) resulting in the production of immunomodulating substances (Isolauri *et al* 2001, Macpherson and Uhr 2004).

Probiotics have been commonly used to modulate the course of a variety of infectious diseases in human medicine (Isolauri 2001). In contrast, few studies have been performed in veterinary medicine, with the majority of veterinary studies being in large animals where probiotics were used to attempt to alter the shedding of fecal pathogens (Kim *et al* 2001) or to improve production parameters such as weight gain, feed conversion rate and reduced mortality. In one small animal study, *Enterococcus faecium* strain SF68 (NCIMB10415) was fed to a group of puppies vaccinated with canine distemper virus (CDV) and compared to a control group that received vaccinations only (Benyacoub *et al* 2003). Puppies supplemented with SF68 had increased serum and fecal total IgA concentrations, increased CDV-specific IgG and IgA serum concentrations, and increased percentage of circulating B lymphocytes compared to control puppies proving an immune enhancing effect induced by this probiotic.

Feline panleukopenia (FPV) is a virus resulting in viremia followed by severe gastrointestinal disease; appropriately vaccinated kittens have sterilizing immunity (Richards *et al* 2001). However, viral upper respiratory tract infections continue to be a

major problem in feline medicine (Sykes *et al* 1999). At Colorado State University, approximately 10% of the feline admissions are for the evaluation of respiratory disease. Feline rhinotracheitis (FHV-1) and feline calicivirus (FCV) are the two viral pathogens implicated in the syndrome. While FCV vaccines induce > 95% relative efficacy in vaccinates when compared to unvaccinated controls after being inoculated with a pathogenic challenge strain, FHV-1 vaccines only induce approximately 60% relative efficacy (Lappin *et al* 2002). Thus, FHV-1 continues to be a major problem in humane shelters and client-owned cats despite widespread vaccination (Sykes *et al* 1999). Previous attempts at improving efficacy of vaccination have included intranasal administration, which leads to greater side effects (Scott and Geissinger 1999), and manipulation of virulent strains, which leads to decreased disease severity but does not decrease the prevalence of the carrier state (Slater and York 1976). The carrier state can lead to recrudescence or reinfection of the host as well as transmission to house mates. Multiple therapies for chronic FHV-1 infections have been tried, including interferon alpha, trephination, antiviral drugs, rhinotomy, glucocorticoids, topical decongestants, and antibiotics directed at secondary bacterial infection (Van Pelt and Lappin 1994). However, none of these have been able to clear the chronic viral infection; therefore recurrences of viral shedding and clinical illness are common. Both cell-mediated and IgA mucosal immune responses are considered important in prevention and control of α-herpesvirus infections (Lappin *et al* 2002, Slater and York 1976). Improved FHV-1 vaccines or responses to vaccinations are needed to lessen morbidity induced by this pathogen.

In this study, we hypothesized that feeding *E. faecium* SF68 to kittens would enhance non-specific immune responses, FHV-1, FCV, and FPV-specific humoral immune responses, and FHV-1-specific cell-mediated immune responses of kittens.

**Materials and Methods**

*Feline study population.* Twenty, six-week old SPF kittens were purchased from a commercial vendor.[a] The kittens were shown to be seronegative for feline leukemia virus antigen and feline immunodeficiency virus antibodies by ELISA.[b]

*Experimental design.* All procedures were approved by the Colorado State University Animal Care and Use Committee. After a 10 day equilibration period, the kittens were randomized into two groups of ten kittens each and the treatment study started at 7 weeks of age. Between 0.25 and 0.28 g (~ $5x10^9$ colony forming units [CFU] based on dilution count assays) of LBC ME5 PET *E. faecium* SF68 (NCIMB 10415)[c] were added into individual 50 mL conical bottom polypropylene centrifuge tubes, capped, and stored at 4°C for the duration of the study. Similar preparations were used for aliquots of the palatability enhancer[d] using 150 mg per tube. Aliquots were monitored for water absorption and were to be discarded if there appeared to be any clumping of either the probiotic or palatability enhancer. Just before administration, one aliquot of palatability enhancer was transferred to one of the stored *E. faecium* SF68 tubes (treatment group) or an empty tube (placebo group) and diluted using room temperature tap water to a total volume of 10 mL. Contents were vortexed for at least three minutes and aspirated into a 12 cc syringe. Immediately after vortexing the suspension, appropriate kittens were orally administered 1 ml of either the *E. faecium* SF68 (total daily dose $5x10^8$ CFU per day) or the palatability enhancer alone (placebo kittens) daily until they were 27 weeks of age. Both groups were fed dry

kitten food ad libitum[e] and 'group' housed in 2 separate rooms to avoid cross-contamination with the probiotic. At 9 and 12 weeks of age, all kittens were vaccinated subcutaneously with a modified live combination vaccine[f] for feline herpesvirus-1, calicivirus, and panleukopenia virus as recommended by the American Association of Feline Practitioners (Richards *et al* 2001).

*Sample collection and clinical monitoring.* The attitudes and behavior of the kittens were monitored daily throughout the study. Body weight was measured weekly. Blood, saliva, and feces were collected from all cats prior to starting probiotic or palatability enhancer supplementation at 7 weeks of age and at 9, 15, 21 and 27 weeks of age. In addition, feces were collected from kittens in the treatment group at 28 weeks of age. For each group of kittens, 5 fecal samples per day were randomly selected from the shared litter box and scored using a standardized graphic scoring card[g] and the daily group means determined. Fecal extracts from samples taken at 9 and 27 weeks of ages were analyzed for total IgA and total IgG measurement according to a previously published protocol (Benyacoub *et al* 2003). All samples were stored at -80°C until assayed in batches.

*Fecal assays.* On each sample date, feces from each kitten were plated in eight serial 10-fold dilutions onto KF Streptococcus Agar and incubated for 48 hours at 37°C aerobically. Ten colonies of each morphology type were picked off using sterile loops and placed in 1.2 mL brain heart infusion (BHI)[h] and stored at -80°C pending analysis. Randomly amplified polymorphic DNA (RAPD)-PCR was performed on bacterial isolates from each sample to determine if viable *E. faecium* SF68 was in the stools of treated cats and to assess whether the probiotic was accidentally transmitted from the treated kittens to the control kittens. The thermocycler parameters were as follows: 30 cycles of one minute

of denaturation at 95°C, one minute of annealing 40°C, four minutes extension at 72°C. The 25.5 uL reaction mixture included 2.45 uL 10x magnesium-free buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 3.22 mM MgCl2, 0.4 uL (1 Unit), JumpStart Taq DNA polymerase[i], 1.9 uL dNTP mix (2.5 mM), 1 uL primer (100 uM), 15.47 uL PCR water, and 1 uL bacterial culture. The sequence of the primer used was GGT TGG GTG AGA ATT GCA CG. Five to ten uL of the PCR product was run on a two percent agarose gel and patterns of banding were compared to a positive SF68 control. Negative controls from non-SF68 exposed cats were run on each gel. Commercially available ELISAs[j] were used to determine whether *Clostridium perfringens* enterotoxins or *C. difficile* toxins A/B were present in the feces of all kittens. Routine aerobic fecal cultures for *Salmonella* spp. and *Campylobacter* spp. were performed by the Colorado State University Diagnostic Laboratory.

*Immunologic Assays.* Complete blood counts, serum biochemical panels, and urinalyses were performed at the Clinical Pathology Laboratory at Colorado State University. Antigen specific humoral immune responses were estimated by measuring serum FHV-1-specific IgG, FHV-1-specific IgA, FCV-specific IgG, and feline panleukopenia-specific IgG in sera as well as FHV-1 specific IgG and IgA levels in saliva using adaptations of previously published ELISA assays (Ditmer *et al* 1998, Lappin *et al* 2002). For FHV-1 specific IgG and IgA, results were calculated by both the mean absorbance for the triplicate test wells for each sample and by calculation of percentage ELISA units (test sample mean absorbance minus the negative control sample mean absorbance/positive control sample mean absorbance minus the negative control sample mean absorbance multiplied by 100). For FCV and FPV, mean absorbances were used. Total IgG and IgA concentrations in

sera, fecal extracts, and saliva were estimated by use of commercially available ELISA assays or radial immunodiffusion assay[k] and expressed as amount of immunoglobulin per volume unit serum after comparison to the supplied standards.

Cellular immune responses were assessed via flow cytometry and whole blood proliferation assays. Flow cytometry was performed within 12 hours of blood collection using 500 μL of anticoagulated (EDTA) blood incubated at room temperature in red cell lysis buffer.[l] Cells were washed two times with phosphate buffered saline (PBS) and the resultant cell pellets were resuspended in flow cytometry buffer containing PBS, 0.1% sodium azide and 2% fetal bovine serum to attain a concentration of $1x10^6$ cells/100 μL if possible. Samples with insufficient volume for at least 500 μL of the above suspension were counted and cell concentration recorded. One hundred μL of each cell suspension was added to individual wells in a round-bottom 96 well plate for immunostaining. Non-specific binding was blocked by addition of 10% normal cat serum.[m] Immunostaining was done at 4°C in the dark in flow cytometry buffer. Lymphocytes were stained for expression of CD4 and CD8[n] and expression of CD44.[o] For analysis of B cells, lysed whole blood was immunostained with cross-reactive antibodies to B220[p] and CD21[q] and MHC class II.[r] Cells for analysis were gated on live lymphocyte populations based on forward and side-scatter characteristics. Data were collected on a Cyan MLE cytometer[p] and analyzed using Summit software.[s]

Proliferation assays were performed in triplicate using 10 μL whole heparinized blood preconditioned by incubating in 100 μL complete tumor media[t] at 37°C with 5% $CO_2$ for 30 minutes before addition of the mitogen or antigen. Cells were stimulated with nothing (unstimulated), concanavalin A[u] (10 μg/mL: Con A), or a FHV-1 antigen

preparation (1 μL/well, prepared prior to the start of the study and stored aliquotted at -80°C) for 96 hours at 37°C with 5% $CO_2$. Cells were pulsed with 1 uCi tritiated thymidine per well and harvested 18 hours later onto fiberglass filter mats.[v] Mats were read using a MicroBeta[s] liquid scintillation counter. The mean stimulation index (mean maximum count per stimulated sample divided by mean maximum count per unstimulated sample) was calculated for all samples.

*Statistical evaluation.* On each sample date, group mean values for all measured parameters were calculated. Differences between the probiotic-treated group and placebo group were analyzed using a mixed ANOVA model appropriate for a repeated measures experiment. Time was included in the model as a continuous variable. Percentages of cat samples positive for *C. perfringens* enterotoxin or *C. difficile* toxins A or B and percentages of gated cells positive for cell surface markers were calculated for each group of cats over the duration of the study and compared by a two tailed t test.[w] Statistical significance was considered to be $p < 0.05$.

**Results**

*Clinical monitoring.* The stools of all kittens were normal at the beginning of the supplementation period (7 weeks of age). One kitten in each group was removed from the study for reasons unrelated to the study (femoral fracture, *Isospora felis* infection prior to supplementation) and were therefore removed from the final data analysis. Body weight and fecal scores were not statistically different between the two groups over time or at any individual time points (Figure 1).

*Fecal Assays.* Feces from seven of nine treatment cats were positive for SF68 on at least one time point during the study. However, SF68 DNA was not amplified from feces of

any treated cat one week after stopping supplementation (week 28). Neither *Salmonella* spp. nor *Campylobacter* spp. were grown from feces. All samples from placebo cats were negative for SF68 by RAPD-PCR. Numbers of positive samples for *C. difficile* toxins A/B or *C. perfringens* enterotoxin (Table 1) were not significantly different between the groups over the course of the study.

*Immunologic Assays.* Complete blood counts and biochemical profiles were within normal limits for the age group for all cats at all time points. There was no statistical difference between the groups over time or at any individual time points among the assays analyzed. No FHV-1 specific IgG was detected in saliva. FCV-specific-IgG levels in serum were similar between groups (Figure 4). At 15 weeks of age, the treatment group serum mean FPV-specific IgG levels were numerically greater than those of the placebo group, but the differences were not statistically significantly different (Figure 5). Concentrations of total IgG and IgA in serum were similar between groups (data not shown). Total IgG was not detected in saliva and total IgA concentrations in saliva were similar between groups (data not shown). Total IgA concentrations in fecal extracts were similar between groups (Figure 6).

Proliferation assays using either 10 μg/mL concanavalin A or 1 μL FHV-1 antigen preparation as the stimulants did not produce significantly different mean maximum counts between groups at any time points. There were no statistical differences between the groups for any cell surface markers at the first four time points (Figure 7). At 27 weeks of age, the treatment group had a significantly higher percentage of gated lymphocytes positive for CD4 (mean 13.87%) than the placebo group (mean 10.61%, $p = 0.022$). As could be expected, the calculated number of circulating CD4+

lymphocytes based on the above percentages were also greater in the treatment group than in the placebo group as there was no difference between groups in the total number of circulating lymphocytes as determined by complete blood counts at the same time points. No other comparisons were significantly different.

**Discussion**

Complete blood cell counts, biochemistry parameters, and body weights were similar between groups of cats over the course of the study. Fecal scores were similar between groups as well suggesting that use of SF68 at the dosage described here will induce no noticeable clinical abnormalities. *Salmonella* spp. and *Campylobacter* spp. shedding was not induced by SF68 supplementation. Several fecal samples in both groups of kittens were positive for *C. difficile* or *C. perfringens* toxins; however, there was no significant difference in number of positive samples between groups and positive results did not correlate to the presence of diarrhea. While we documented SF68 in the feces of the majority of treated cats, we failed to detect the organism one week after stopping supplementation. This indicates that the organism persisted in the cats only transiently. We conclude that administration of SF68 using the dosage described here has no deleterious effects and is safe for administration in the time period studied.

After vaccinations, each of the kittens developed FHV-1, FCV, and FPV-specific serum antibody responses that are similar to other studies suggesting they were immunocompetent and that the modified live vaccine used was viable (Lappin *et al* 2002). The increase in the percentage of gated lymphocytes positive for CD4 in the treatment group at 27 weeks of age suggested that feeding of the probiotic influenced cell-mediated immune responses of these kittens. However, because these lymphocytes

were not further characterized via antigen specificity or cytokine expression, it unknown at this time how this increase would impact the overall immune response.

The failure to achieve statistical significance for some parameters could be explained several ways. First, it is possible that SF68 did not influence humoral immune responses using the protocol designed here and that a different protocol or probiotic is needed to induce greater measurable effects. Additionally, while we documented SF68 in the feces of the majority of treated cats, we failed to detect the organism one week after stopping supplementation. This may indicate that the organism did not persist in the cats at a level higher than our detection limit which could be required for maximal immune stimulating effects. However, detection of SF68 in feces is likely to correlate most closely to the presence of the organism in the large intestine and may not reflect presence or absence of the organism in the small intestine. Thus, it is possible that SF68 was present in the small intestine of the treatment group cats but was just not detectable by our assays when used on feces.

In a similar study that supplemented puppies with SF68, several parameters for which we saw numerical trends in our kittens achieved statistical significance (Benyacoub *et al* 2003). For example, canine distemper virus-specific IgG and IgA antibodies in serum were statistically greater in SF68 supplemented puppies, but only after the puppies had been supplemented for 31 and 44 weeks. It is believed that in these puppies, SF68 induced immune enhancement resulted in persistence of high antibody levels in supplemented puppies as the antibody levels in the non-supplemented puppies began to wane. In the study described here, we stopped collections in the kittens after 20 weeks of supplementation but FPV, FCV, and FHV-1 humoral responses may persist for

months to years after vaccination (Scott and Geissinger 1999). Based on these results, a longer collection period is indicated in future feline studies of this type. Lastly, there was a large degree of inter- and intra-individual variation for results of several assays. Because of the relatively small numbers of cats in the groups, this variation may have led to masking of significant results. With this degree of variability, it is difficult to achieve statistical power with the small group sizes used in this study.

The increase in the percentage of CD4+ lymphocytes in the treatment group compared to the placebo group without a concurrent increase in CD8+ counts at 27 weeks of age demonstrates systemic immune modulating effects by the probiotic. Because we did not show a significant increase in lymphocyte stimulation by FHV-1 or an increase in the expression of the memory cell marker CD44 on the CD4+ lymphocytes in the treatment group, the increase in CD4+ T lymphocytes may have been non-specific as the cells appear to be unprimed. In man, there is evidence that CD4+ T lymphocytes dominate the response to herpes simplex virus-1 (HSV1) infection over CD8+ T lymphocytes (Niemialtowski *et al* 1994). In mice, a subset of CD4+ T lymphocytes are believed to be responsible for decreased mucosal replication and latency when challenged with HSV-1 (Morrison and Knipe 1997). In addition, a subset of CD4+ antigen specific T lymphocytes have been shown to be necessary for induction of protective immunity from HSV-2 infection in mice (Zhao *et al* 2003). As the CD4+ T lymphocytes of kittens in this study were not additionally characterized via cytokine production profiles or additional cell surface marker characterization; it cannot be determined whether a Th1 or Th2 response predominated. In future feline studies, challenge inoculation with virulent

FHV-1 could be used to better determine whether SF68 supplementation exerts protective effects.

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ENDNOTES

[a] Liberty Laboratories, Liberty, NY

[b] Snap Combo, IDEXX Laboratories, Portland, ME

[c] Cerbios-Pharma SA, Switzerland

[d] Typical pet food coating (main component liver digest) was used

[e] Typical kitten growth formula meeting all AAFCO requirements and was based on chicken and rice as main ingredients.

Macronutrient composition:

| ingredient | unit | value |
|---|---|---|
| D.E. | cal/kg | 4200 |
| moisture | g/100g | 22 |
| protein | g/100g | 35 |
| carbohydrates | g/100g | 29 |
| fat | g/100g | 18 |
| crude fiber | g/100g | 1.3 |
| ash | g/100g | 5.8 |

[f] Pfizer Animal Health, Exton, PA

[g] Nestlé Purina PetCare, St. Louis, MO

[h] Becton-Dickinson, Franklin Lakes, NJ

[i] Sigma D-4184, Sigma-Aldrich, Inc., St. Louis, MO

[j] *C. perfringens* (ELISA, Kit No. 92-000-22) and *C. difficile* (ELISA, Kit No. 94-0150-KT), Techlabs, Blacksburg, VA

[k] Bethyl Laboratories, INC, Montgomery, TX

[l] Red blood cell lysis buffer: Ammonium Chloride (Buffered)

0.155 M $NH_4Cl$ (Ammonium Chloride)

0.010 M $KHCO_3$ (Potassium Bicarbonate)

$5X10^{-4}$ % Phenol Red (0.5%)

[m] Jackson ImmunoResearch Laboratories, Inc., West Grove, PA

[n] vpg34; CD4-fitc, vpg9; CD8-rpe; Serotec, Raleigh, NC (Oxford, UK)

[o] IM7; CD44-pe/cy5 Pharmingen, Franklin Lakes, NJ

[p] ra3-b62; B220-biotinylated eBioscience, San Diego, CA

[q] b-ly4; CD21-apc; BD-Biosciences, Franklin Lakes, NJ

[r] MHC class II-fitc; clone CAG5-3D1, Serotec, Raleigh, NC (Oxford, UK)

[s] Dako-Cytomation, Fort Collins, CO

[t] Complete tumor media: modified Eagle's medium supplemented with essential and non-essential amino acids + 10% FBS

[u] Sigma-Aldrich, St. Louis, MO

[v] Wallac-Microbeta Perkin –Elmer, Boston, MA

[w] GRAPHPAD Prism, GRAPHPAD Software, Inc., San Diego, CA

**References**


Benyacoub J, Czarnecki-Maulden GL, Cavadini C, Sauthier T, Anderson RE, Schiffrin EJ, von der WT (2003), Supplementation of food with *Enterococcus faecium* (SF68) stimulates immune functions in young dogs, *J Nutr* 133: 1158-1162

Ditmer DA, Lappin MR, Carman J, Collins JK (1998), Enzyme-linked immunosorbent assay for detection of feline herpesvirus 1 IgG in the serum, aqueous humor, and cerebrospinal fluid, *J Vet Diagn Invest* 10: 315-319

Isolauri E (2001), Probiotics in human disease, *Am J Clin Nutr* 73: 1142S-1146S

Isolauri E, Sutas Y, Kankaanpaa P, Arvilommi H, Salminen S (2001), Probiotics: effects on immunity, *Am J Clin Nutr* 73: 444S

Kim LM, Morley PS, Traub-Dargatz JL, Salman MD, Gentry-Weeks C (2001), Factors associated with *Salmonella* shedding among equine colic patients at a veterinary teaching hospital, *J Am Vet Med Assoc* 218: 740-748

Lappin MR, Andrews J, Simpson D, Jensen WA (2002), Use of serologic tests to predict resistance to feline herpesvirus 1, feline calicivirus, and feline parvovirus infection in cats, *J Am Vet Med Assoc* 220: 38-42

Macpherson AJ, Uhr T (2004), Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria, *Science* 303: 1662-1665

Morrison LA, Knipe DM (1997), Contributions of antibody and T cell subsets to protection elicited by immunization with a replication-defective mutant of herpes simplex virus type 1, *Virology* 239: 315-326

Niemialtowski MG, Godfrey VL, Rouse BT (1994), Quantitative studies on CD4+ and CD8+ cytotoxic T lymphocyte responses against herpes simplex virus type 1 in normal and beta 2-m deficient mice, *Immunobiology* 190: 183-94

Richards J, Rodan I, Elston T, Flemmin D, Ford R, Henry S, Hustead D, Lappin MR, Paul M, Rosen D, Scherk M, Scott F, Welborn L (2001), Feline Vaccine Selection and Administration, *Compend Cont Ed Pract Vet* 23: 71-80

Sanders ME (2003), Probiotics: considerations for human health, *Nutr Rev* 61: 91-99

Schrezenmeir J, de Vrese M (2001), Probiotics, prebiotics, and synbiotics--approaching a definition, *Amer J Clin Nutr* 73: 361S

Scott FW, Geissinger CM (1999), Long-term immunity in cats vaccinated with an inactivated trivalent vaccine, *Amer J Vet Res* 60: 652-658

Slater E, York C (1976), Comparative studies on parenteral and intranasal inoculation of an attenuated feline herpes virus, *Dev Biol Stand* 33: 410-6

Sykes JE, Anderson GA, Studdert VP, Browning GF (1999), Prevalence of feline Chlamydia psittaci and feline herpesvirus 1 in cats with upper respiratory tract disease, *J Vet Intern Med* 13: 153-162

Van Pelt DR, Lappin MR (1994), Pathogenesis and treatment of feline rhinitis. [Review] [48 refs], *Vet Clin N Am - Sm An Pract* 24: 807-823

Zhao X, Deak E, Soderberg K, Linehan M, Spezzano D, Zhu J, Knipe DM, Iwasaki A (2003), Vaginal submucosal dendritic cells, but not Langerhans cells, induce protective Th1 responses to herpes simplex virus-2, *J Exp Med* 197: 153-162

## Figures

| | | *C. difficile* | | *C. perfringens* | |
|---|---|---|---|---|---|
| Age (wks) | Weeks on trial | Placebo n=9 | Treatment | Placebo n=9 | Treatment |
| 7 | 0 | **0** | **11.1** | **0** | **0** |
| 9 | 2 | **0** | **0** | **0** | **0** |
| 15 | 8 | **33.3** | **33.3** | **88.9** | **55.6** |
| 21 | 14 | **0** | **11.1** | **0** | **11.1** |
| 27 | 20 | **11.1** | **0** | **22.2** | **11.1** |
| Mean | | 8.8 | 11.1 | 22.2 | 15.6 |
| P value | | 0.69 | | 0.75 | |

**Table 1. Percent of cats positive for *C. difficile* toxins A/B and *C. perfringens* enterotoxin during and after 20 weeks of daily feeding a diet containing 5 x $10^8$ CFU/kitten/day *E. faecium* SF68.** See figure 1 for the complete legend.

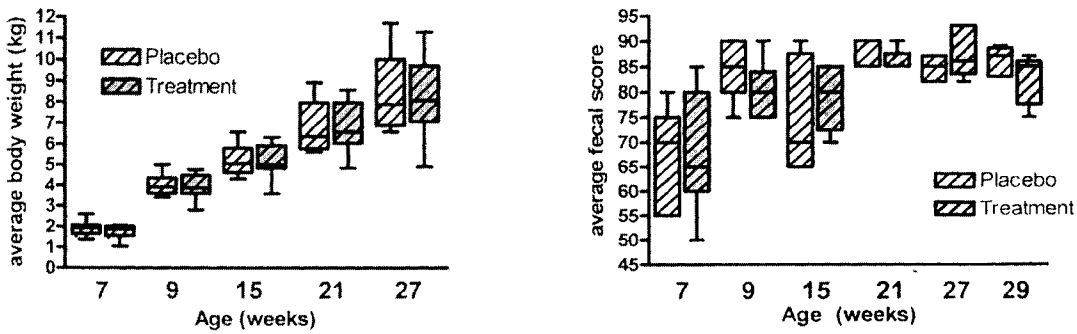


**Figure 1. Body weights (left panel) and fecal scores (right panel) over time of kittens supplemented with 150 mg chicken digest PO (Placebo, n=9) or 150 mg chicken digest mixed with 5 x $10^8$ cfu/day *Enterococcus faecium* strain SF68 (Treatment, n=9) daily starting at 7 weeks of age until 27 weeks of age.** Kittens were vaccinated subcutaneously with a commercially available, modified live FHV-1 vaccine[f] at 9 and 12 weeks of age. Box and whiskers represent the minimum, maximum, median and 25th and 75th percentiles. P>0.05 at all time points.

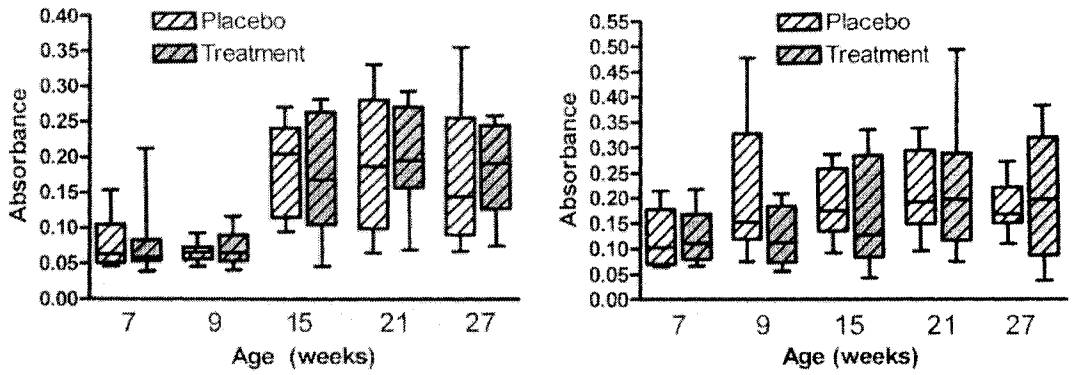


**Figure 2. FHV-1 specific IgA results in serum (left panel) and saliva (right panel) from kittens with (Treatment) or without (Placebo) SF68 supplementation.** See figure 1 for the complete legend. P>0.05 for all time points.

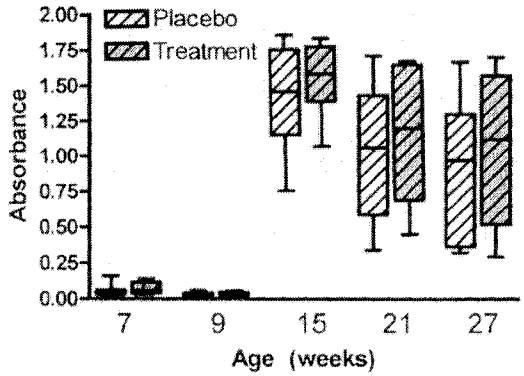


**Figure 3. FHV-1 specific IgG results in serum from kittens with (Treatment) or without (Placebo) SF68 supplementation.** See figure 1 for the complete legend. P>0.05 for all time points.

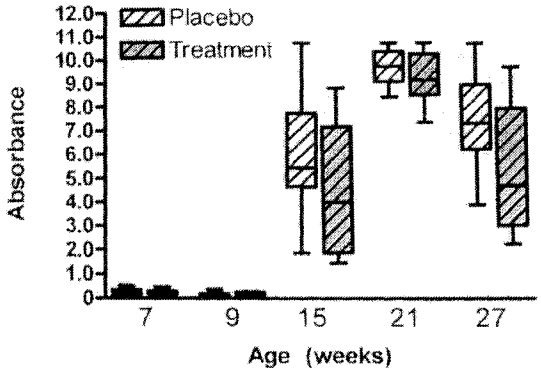


**Figure 4. FCV specific IgG results from kittens with (Treatment) or without (Placebo) SF68 supplementation.** See figure 1 for the complete legend. P>0.05 for all time points.

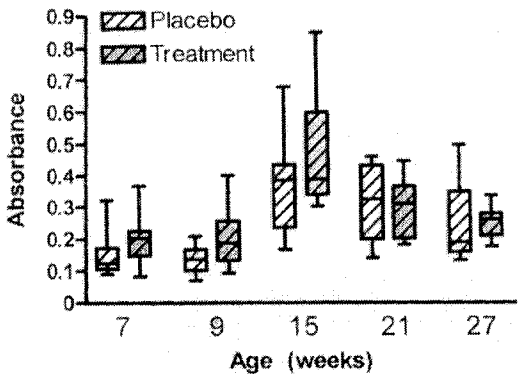


**Figure 5. FPV specific IgG results from kittens with (Treatment) or without (Placebo) SF68 supplementation.** See figure 1 for the complete legend. P>0.05 for all time points.

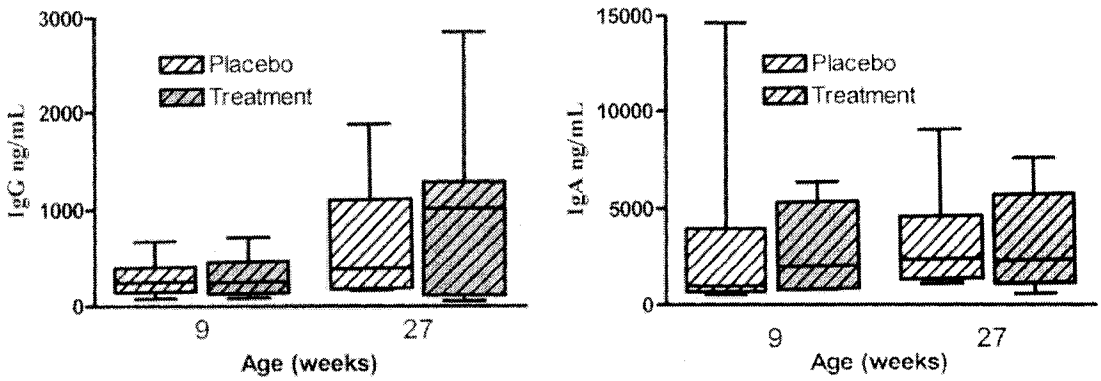


**Figure 6. Total IgG (left panel) and IgA (right panel) in fecal extracts from kittens with (Treatment) or without (Placebo) SF68 supplementation.** See figure 1 for the complete legend. P>0.05 for all time points.

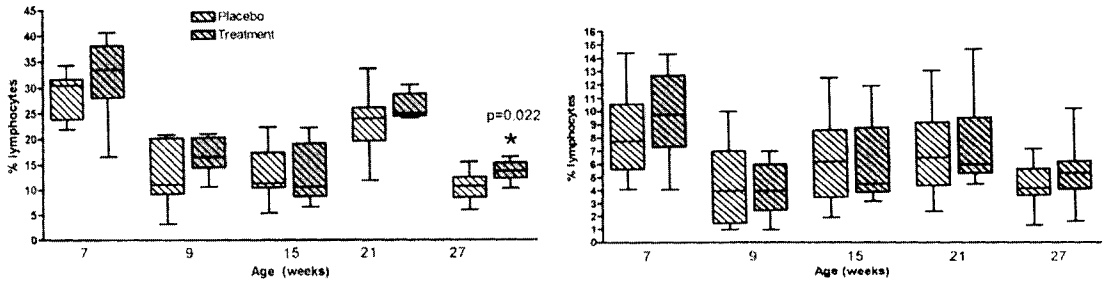


**Figure 7. Percent of gated lymphocytes positive for CD4 (left panel) and CD8 (right panel) in peripheral blood by flow cytometry in kittens with (Treatment) or without (Placebo) SF68 supplementation.** See figure 1 for the complete legend. * denotes time points at which treatment group was significantly (P<0.05) higher than placebo group.

CHAPTER 5

# EVALUATION OF A NOVEL IMMUNOTHERAPY FOR TREATMENT OF CHRONIC RHINITIS IN CATS

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***Summary***

The pathogenesis of chronic rhinitis in cats is poorly understood and consistently effective therapies are not currently available. Therefore, randomized clinical trials were conducted to evaluate a novel immunotherapy for treatment of chronic rhinitis in adult (n=12) and young cats (n=28). In addition, cytokine profiles in cats with chronic rhinitis were compared to those of healthy cats. Cats were treated with a potent stimulator of innate immunity (liposome-IL-2 DNA complexes) and the effects of treatment on clinical signs and immune function were assessed. In adult cats with chronic rhinitis, immunotherapy led to significant improvement in frequency of sneezing but not in other clinical signs when compared to the placebo group, whereas immunotherapy failed to improve clinical signs in younger cats. Analysis of cytokine expression in cats with rhinitis did not reveal evidence of a Th2 cytokine bias in cats with rhinitis. We conclude that chronic rhinitis in cats is not a Th2-biased disease and that immunotherapy may lead to clinical improvement in adult cats with the disease.

***Introduction***

Chronic rhinitis in cats is a syndrome associated with inflammation of the nasal cavity; it is frequently associated with secondary infection of the frontal sinuses, and it typically lasts greater than one month (Cape 1992). Clinical signs in cats include nasal discharge, sneezing, ocular discharge, anorexia, and stertorous breathing. Possible causes for chronic rhinitis include neoplasia, foreign bodies, viral infections (feline herpesvirus-1 [FHV-1], calicivirus), mechanical obstruction (dental disease, polyps, or nasopharyngeal stenosis), bacterial infections (*Bordetella bronchiseptica, Chlamydophila felis* and others), allergic diseases, or fungal diseases (*Cryptococcus neoformans* or less commonly, *Aspergillus* spp. or *Penicillium* spp.). It is relatively easy to remove foreign body, neoplasia, fungal infection, dental disease, polyps, and nasopharyngeal stenosis from the differential list with a diagnostic workup. Once these diseases are excluded, many chronic rhinitis cases have been attributed to the effects of chronic viral infections or bacterial infections (Helps *et al* 2005, Johnson *et al* 2005b); however it is virtually impossible to prove a cause and effect relationship, as many potentially pathogenic organisms are also detected in clinically normal cats (Van Pelt and Lappin 1994). In addition, multiple causes of chronic rhinitis may coexist in some cats.

Once foreign bodies, dental disease, neoplasia, polyps, fungal disease, and nasopharyngeal stenosis are ruled out or considered unlikely, multiple treatments for chronic rhinitis can be attempted. These include use of recombinant human interferon (IFN)-$\alpha$, antiviral drugs, systemic or inhaled corticosteroids, topical decongestants, antibiotics, rhinotomy, or nasal sinus trephination (Van Pelt and Lappin 1994). However,

none of these treatments have been shown to be consistently effective and recurrences are common.

New treatments are needed for cats with chronic rhinitis. Because many cases are thought to have viral or bacterial involvement, enhancement of innate immune responses may be of benefit. Modulation of the innate immune responses through the systemic administration of liposomal DNA complexes, either containing non-coding plasmid DNA or plasmid DNA encoding the gene for interleukin-2 (IL-2), has been demonstrated to have substantial activity in rodent tumor models (Dow *et al* 1999a). In addition, complexes of cationic liposomes and plasmid DNA (CLDC) are extremely potent inducers of Th1 cytokine responses, triggering the release of several cytokines with potent antiviral activity, including IFN-γ, IFN-α, and IL-12. Moreover, systemic administration of these complexes has also been shown to partially reverse the Th2 mediated immunological abnormalities present in a mouse model of asthma (Dow *et al* 1999b). More recently, we have also found that CLDC can be used as vaccine adjuvants in dogs with refractory atopy (Mueller *et al* 2005). Studies in mice have used treatment with recombinant interferon-α to successfully reduce the severity of clinical signs in experimentally induced herpes virus infection (Lenzo *et al* 2003). Moreover, *scid-hu* mice infected with γ-herpesvirus have been treated effectively with recombinant human IL-2, illustrating that several different Th1 cytokines may elicit potent anti-viral activity (Baiocchi *et al* 2001).

The results of these prior studies suggested that liposome-DNA complexes might be useful as an immunotherapeutic in feline chronic rhinitis, particularly in cases for which inflammation is related to viral or bacterial infections. We hypothesized that

chronic rhinitis in cats could be mediated at least in part by an ineffective or subverted immune response triggered initially by chronic viral or bacterial infection and that stimulation of Th1 immune responses may aid in the treatment of affected cats. The purpose of this study was to administer CLDC to different groups of cats to determine the immunological and clinical effects.

***Materials and Methods***

**Animals and study inclusion criteria**. All protocols and procedures were approved by the Animal Care and Use Committee of Colorado State University. In total, 4 groups of healthy or clinically ill cats were studied in a series of 4 experiments.

*Group 1 cats.* The 6 cats in this group were laboratory-reared and apparently healthy when evaluated at 6 to 8 months of age. There was no history of upper respiratory disease, a complete blood cell count, serum biochemistry profile, and urinalysis were normal, and the cats were negative for FeLV p27 antigen and FIV antibodies in serum.[a]

*Group 2 cats.* The 8 domestic shorthair, laboratory-reared cats (5 male neutered, 3 female spayed) in this group were evaluated at 1-2 years of age (median age 17 months, range 15-25 months) and were apparently healthy on physical examination. There was no history of upper respiratory disease, a complete blood cell count, serum biochemistry profile, and urinalysis were normal, and the cats were negative for FeLV p27 antigen and FIV antibodies in serum.

*Group 3 cats.* The 12 older cats in this group (median age 102 months, range 12-168 months; 5 male neutered, 7 female spayed) were client-owned and had clinical signs of chronic rhinitis, the cause of which was most consistent with chronic viral disease.

Cats were owned by clients of either a university teaching hospital (n = 5) or private secondary referral center (n = 7). To be entered into the study, the cats had to have: 1) a non-progressive nasal discharge of > 1 year duration that was incompletely cleared after administration of oral antibiotics for 4-6 weeks; 2) minimal to no dental disease on oral examination; 3) lack of other systemic signs besides anorexia and lethargy; 4) lack of evidence of facial deformity; and 5) discontinuation of antibiotic therapy or immunomodulating therapy for at least 3 weeks. All 12 cats were determined to be free of concurrent systemic disease by means of a routine complete blood count, serum biochemistry profile, urinalysis, and results of FeLV p27 antigen and FIV antibody testing. For some cats, other diagnostic tests were performed to further exclude other common causes of chronic rhinitis. These included *Cryptococcus neoformans* antigen test on serum for cats with travel to endemic areas (n = 2; both negative), thoracic radiographs (n = 9), nasal radiographs (n = 8), computed tomography of the nasal cavity and sinuses (n = 3), or rhinoscopy with biopsy, histopathology, and bacterial culture (n = 8).

*Group 4 cats.* The 28 cats in this group (estimated median age 17 months, range 6 – 96 months; 13 male neutered, 15 female spayed) came from a local animal shelter and had clinical signs of rhinitis, the cause of which was most consistent with viral disease. To be entered into the study, Group 4 cats had to have: 1) persistence of clinical signs precluding adoption despite treatment with two different antibiotics (amoxicillin-clavulanic acid and azithromycin) over 21 days; 2) minimal to no dental disease on oral examination; 3) lack of systemic signs besides anorexia and lethargy; 4) lack of evidence of facial deformity; and 5) discontinuation of antibiotic therapy or immunomodulating

therapy for at least four days. All 28 cats were determined to be free of concurrent systemic disease by means of a routine complete blood count, serum biochemistry profile, urinalysis, and results of FeLV p27 antigen and FIV antibody testing.

**Preparation of cationic liposome-DNA complexes**. The feline IL-2 cDNA was cloned by RT-PCR from normal cat peripheral blood mononuclear cells (PBMC), based on published sequence data. The cDNA was cloned into a eukaryotic expression vector (pMB75.6) that utilized the CMV immediate-early promoter-enhancer region, a synthetic intron (pGL3) immediately upstream of the start site, an SV40 early poly(A) site, and the kanamycin resistance gene, as described previously (Fairman *et al* 1999). Expression was confirmed by assaying the ability of supernatants from transiently transfected feline CrFK cells to support proliferation of normal feline PBMC *in vitro* (data not shown). Plasmid DNA encoding feline IL-2 was prepared by alkaline lysis followed by chromatographic purification, as described previously (Dow *et al* 1999a). The endotoxin content of the DNA was less than 0.1 EU/ug DNA, as determined by the LAL kinetic assay.[b] Cationic liposomes comprised of DOTIM (octadecenoyloxy [ethyl-2-heptadecenyl-3-hydroxyethyl] imidazolinium chloride)[c] and cholesterol[d] were prepared as described previously by Templeton et al (2003). Briefly, equimolar amounts of DOTIM and cholesterol were evaporated to dryness, then rehydrated in 5% dextrose in water (D5W) at $50^0$C, then extruded sequentially through filters of decreasing diameter (1000 nm, 450 nm, and 200 nm) to form extruded liposomes. Immediately prior to treatment of cats, CLDC were prepared by addition of 100 uL of liposomes to 1.5 mL D5W. One hundred fifty ug feline IL-2 plasmid DNA was then added to the liposomes and mixed by gentle pipetting of the solution. Additional D5W was then added to bring

the solution to a final volume of three milliliters prior to injection. The CLDC solution was administered to non-anesthetized cats by intraperitoneal injection.

**Treatment protocols.**

*Group 1 cats.* These 6 cats were randomly assigned by coin flip to 2 treatment groups in an unblinded randomized trial. Group 1 cats were administered either CLDC (treatment group [n = 3]) or 0.9% NaCl solution (placebo group [n = 3]) by intraperitoneal injection (IP) once weekly for six weeks. This route of administration was selected because pilot studies in mice indicated that this route gave the highest degree of systemic immune activation with minimal toxicity (data not shown).

*Group 2 cats.* These 8 cats were randomly assigned by coin flip and assessed in a double blinded trial. Group 2 cats were administered either CLDC (treatment group [n = 4]) or cationic liposomes (placebo group [n = 4]) by IP injection, once weekly for four weeks. To determine humoral responses to a novel antigen, the cats were administered 100 ug of keyhole limpet hemocyanin (KLH)[c] dissolved in sterile phosphate buffered saline solution (PBS) intradermally (ID) at week 0 and week 2.

*Group 3 cats.* These 12 cats were randomly assigned by coin flip and assessed in a double blinded trial. Group 3 cats were administered either CLDC (treatment group [n = 7]) or cationic liposomes (placebo group [n = 5]) by IP injection once weekly for four weeks. To determine humoral responses to a novel antigen, the cats were administered 100 ug of KLH dissolved in PBS ID at week 0 and week 2.

*Group 4 cats.* These 28 cats were randomly assigned by coin flip and assessed in a double blinded trial. Group 4 cats were administered either CLDC (treatment group [n = 13]) or cationic liposomes (placebo group [n = 15]) by IP injection once weekly for

four weeks. To determine humoral responses to a novel antigen, the cats were administered 100 ug of KLH dissolved in PBS, ID at week 0 and week 2.

**Clinical monitoring and sample collections.**

*Group 1 cats.* Body temperature was assessed prior to treatment and at 2-4 hour intervals after injection for a 12 hour period and then again at 24-hours after injection. A complete blood cell count was performed on blood collected on days -14, 0, 1, 7, and 21. Percentage of the total lymphocyte population that was CD4+ and CD8+ was determined by flow cytometry on samples collected on days 0, 21, and 42.

*Group 2 cats.* Body temperature was assessed prior to treatment and at 4 hour intervals after injection for a 24-hour period. A complete blood cell count was performed on blood collected on days 0, 1, 7, and 28. Antibodies to KLH were determined on serum collected on days 0 and 28. Cytokine profiles were determined on samples collected prior to treatment as well as 8 hours after the first treatment and on day 28.

*Group 3 cats.* Each of these 12 cats was monitored closely for clinical abnormalities in the hospital for 12-24 hours following the first injection of CLDC or liposomes. The cats were then monitored by their owners at home for the duration of the study. Owners were blinded as to treatment groups and were asked to assess any changes in clinical signs relative to the cat's clinical condition at the start of the study. Clinical responses were measured in 25% increments of improvement or decline from baseline over the preceding week in specific clinical signs, based on standardized scoring criteria as follows: serous discharge alone occurring on at least one day but less than four days: one point; serous discharge occurring anymore than three days of the preceding seven days: two points; mucopurulent discharge present either one or two days: two points were

assigned for the period; three to five days of mucopurulent discharge: four points, and the same discharge for five to seven days: five points. Any day on which sneezing was observed was also recorded and contributed one point per day observed. Other clinical signs anticipated but not analyzed due to infrequency of occurrence were severity of conjunctivitis, amount of coughing, and increase or decrease in appetite. Complete blood counts and serum biochemical profiles values were also determined on blood samples collected on days 0 and 28. Percentage of the total lymphocyte population that was CD4+ and CD8+ was determined by flow cytometry on samples collected on days 0, 1, 7, and 28. Antibodies to KLH were determined on serum collected on days 0 and 28. Cytokine profiles were determined on samples collected prior to treatment as well as 8 hours after the first treatment and on day 28. Samples for concanavalin A stimulated whole blood proliferation were collected on days 0, 14, and 28.

*Group 4 cats.* Each of these 28 cats was hospitalized for the duration of the study. On day 1 of the study and then weekly, a nasal swab was collected from each cat, placed in sterile PBS (0.01 M; ph = 7.2), incubated at room temperature for 2 hours, and then stored at -70°C until assayed for FHV-1 by quantitative PCR assay. Clinical findings were scored as for Group 3 cats and the person determining the score was blinded as to the treatment groups. Complete blood counts and serum biochemical profiles values were also determined on blood samples collected on days 0 and 28. Percentage of the total lymphocyte population that was CD4+ and CD8+ was determined by flow cytometry on samples collected on days 0, 1, 7, and 28. Antibodies to KLH were determined on serum collected on days 0 and 28. Cytokine profiles were determined on samples collected prior to treatment as well as 8 hours after the first treatment and on day

28. Samples for concanavalin A stimulated whole blood proliferation were collected on days 0, 14, and 28.

**Quantitation of FHV-1 copy number by quantitative PCR**. Quantitation of FHV-1 copy number in nasal swabs taken weekly from Group 4 cats was performed by quantitative PCR, using a previously published assay (Vogtlin *et al* 2002) adapted for nasal swabs. DNA was extracted from samples using a commercially available kit[e] according to manufacturer's protocols with 50 ug salmon sperm DNA[f] added per mL of manufacturer's lysis buffer. The 25 uL PCR reaction contained 12.5 uL TaqMan Mastermix,[g] 0.5 uL (400 nM) of each primer, 0.2 uL (80 nM) of probe (FAM/TAMRA), 1.3 uL of sterile water, and 10 uL of extracted template DNA. All reactions were carried out on an ABI 7000[g] using the following conditions: two minutes at 50°C, 10 minutes at 95°C, and then 40 cycles consisting of a 15 second 95°C denaturation step followed by a one minute 60°C annealing step. Parallel reactions for feline GAPDH using a previously published protocol (Leutenegger *et al* 1999) were performed on the same plate to account for variations in DNA quantity in each sample secondary to different yields of cells during swab sampling. GAPDH reaction conditions were the same as for FHV-1 with the exception that only 5 uL of template DNA was added with a corresponding increase in the volume of sterile water (6.3 uL). Data was analyzed with the instrument software.[h] Signals were considered positive if the fluorescence intensity exceeded ten times the standard deviation of the baseline fluorescence (threshold cycle [Ct]). All reactions were run in duplicate. A control sample consisting of DNA pooled from swabs from 10 normal cats spiked with plasmid derived FHV-1 DNA was run on each plate to ensure repeatable thermocycler conditions.

A standard curve for GAPDH-cell equivalent was generated using DNA isolated from a feline lung epithelial cell line (Dow unpublished data) that was digested in the same manner as the test samples. The standard curve for FHV-1 was generated using a 10-fold dilution series using plasmid generated DNA. The FHV-1 pDNA was produced using a commercially available vector[i] after purification[e] of product obtained from a conventional PCR reaction using the primers used in the real time assay. Viral load was then defined as the calculated FHV copy number divided by the calculated GAPDH-cell equivalent copy number of each sample.

**KLH antibody ELISA.** Sera for use in this assay was stored at -70°C until assayed. An indirect ELISA was developed to measure antibodies to KLH. Sera collected from 11 cats prior to inoculation and on day 28 (after receiving two inoculations of KLH) were used for ELISA titrations. Multiple antigen concentrations, microELISA plates, buffers, blocks, and secondary antibody concentrations were assessed while optimizing the ELISAs. In the optimized ELISA, 100 µl of a 1:1600 dilution of 1 mg/mL KLH solution in 0.01M phosphate buffered saline solution (PBSS; pH 7.2) was added to a microELISA plate[j] and incubated at 4°C for a minimum of 16 hours. Plates were washed three times with 200 µl PBS solution containing 0.05% Tween 20[c] (PBS-TW solution). The sera from the study cats were serially diluted 1:50 to 1:51200 in PBS-TW solution in a final volume per well of 100 µl. The plate was then incubated at 37°C for 30 minutes. Plates were then washed 3 times with 200 µl of PBS-TW solution. Horseradish peroxidase conjugated goat anti-cat IgG (heavy chain specific)[k] was diluted 1:2000 in PBS-TW solution and 100 µl was pipetted into the appropriate wells. After a 30 minute incubation at 37°C, the plates were washed three times (as before) and 100 µl of substrate[l] was

added to all wells. The enzyme reaction was stopped after an incubation period of ten minutes at room temperature (approximately 20°C) by pipetting 100 µl of 0.18M $H_2SO_4$ into each well. The optical density of each well (compared to a substrate control blank row) was read at 450 nm with an automated microELISA reader.[m] Mean values for this calculation were used in statistical comparisons. Positive (sera from previously inoculated cats) and negative (sera from naïve cats) controls were included on each plate.

**Flow cytometric evaluation of peripheral blood mononuclear cells.** All samples for PBMC collection were frozen and stored in liquid nitrogen until analysis. The PBMC were separated from heparinized whole blood by density gradient centrifugation using lymphocyte separation medium.[n] Prior to immunostaining and flow cytometric analysis, PBMC samples were rapidly thawed and washed in complete medium. Cells were placed in round-bottom 96 well plates for immunostaining and non-specific binding was first blocked by addition of 10% normal cat serum. Immunostaining was done at 4°C in FACS buffer containing PBS, 0.1% sodium azide and 2% fetal bovine serum. Cat lymphocytes were immunostained with FITC-conjugated antibodies to feline CD4[o] and phycoerythrin-conjugated antibodies to feline CD8[p] and with a cross-reactive PE/cy5 labeled antibody to CD44.[q] For analysis of monocytes, PBMC were immunostained with a cross-reactive antibody to CD11b[r] and a cross-reactive antibody to MHC class II.[s] Analysis gates were set on the live lymphocyte population based on forward and side-scatter characteristics. Data were collected using either a FACSCalibur cytometer[t] or a Cyan MLE cytometer,[u] and data were analyzed using either CellQuest[t] or Summit software.[u]

**Real-time PCR analysis for cytokines.** The level of mRNA transcript expression for the cytokines IL-4, IL-6, IL-10 and IL-12 and IFN-γand IFN-α and tumor necrosis factor (TNF)-α was assessed with quantitative real-time PCR using previously published assays (Kipar *et al* 2001, Leutenegger *et al* 1999). Samples of PBMC were resuspended in complete tissue culture medium (CTM: modified Eagle's medium supplemented with essential and non-essential amino acids + 10% FBS) at 37°C for ten minutes immediately after a rapid thaw and then washed two times with PBS. Total RNA was extracted using a commercially available kit[e] according to manufacturer's directions. Residual genomic DNA was removed using an on-column DNase 1 treatment. The final product was eluted into 30 uL of DEPC treated water. The cDNA was synthesized the same day using 10 uL of the above RNA product, random primers,[f] dNTP mix (2.5 mM each dNTP),[f] MMLV reverse transcriptase,[f] and an RNase inhibitor.[f] Final product was brought up to volume (60 uL) in DEPC treated water.

TaqMan primer probe pairs were prepared based on previously published sequences (Leutenegger *et al* 1999, Kipar *et al* 2001). The probes were labeled at the 5' and 3'ends with a reporter fluorescent dye, FAM, and a quencher, TAMRA, respectively. Amplifications were carried out in duplicate in 25 uL reaction mixtures containing (final concentration) 12.5 uL Mastermix,[v] 0.5 uL (400 nM) of each primer, 0.2 uL (80 nM) of probe, 6.3 uL PCR grade water, and 5 uL template cDNA. Thermocycler conditions and background were set using the same parameters as for FHV-1. Relative quantification was carried out using the comparative $C_T$ method (Applied Biosystems 2001) with feline GAPDH as the endogenous reference gene and each cat's pre-treatment PBMC sample as the control sample for across time comparisons. Analysis was done using either

unstimulated PBMC (Controls) or following stimulation for six hours with phorbol myristate acetate[c] (PMA: 10 ng/mL) plus ionomycin[c] (500 ng/mL) (week 0 samples only). Samples from Group 3 and Group 4 cats obtained at times zero, eight hours, two weeks, and four weeks were analyzed for cytokine mRNA expression.

**Whole blood proliferation assays.** Assays were performed in 96-well plates using 10 uL whole heparinized blood diluted in 100 uL complete tumor media (modified Eagle's medium supplemented with essential and non-essential amino acids + 10% FBS) per well. Cells were incubated with media only (control) or 10 ug/mL concanavalin A and media (ConA). After 96 hours of incubation at 37°C with 5% $CO_2$, wells were pulsed with 1 uCi tritiated thymidine for 24 hours before being harvested onto filter mats[w] and counted.[b] All samples were run in triplicate and stimulation indexes (SI) were calculated by dividing the average of the stimulated cell count by the control count for each cat and time point.

**Statistical analysis.** Statistical differences in clinical scores were assessed using the sign rank test. KLH antibody titers were log transformed in order to normalize data and then compared using a paired t-test. One way ANOVA with Tukey's multiple comparison test was used to identify significant changes between more than two groups of variables. The level of significance was set at $p < 0.05$ for all comparisons.

***Results***

**CLDC treatment effects on innate and adaptive immune responses in healthy cats.** Intraperitoneal administration of CLDC rapidly induced a febrile response in Group 1 cats that was significantly different than Group 1 cats inoculated with saline. Fever was detectable by 6 hours post-injection, peaked by 10 hours, and declined thereafter (Figure

1). Fever was detected in some CLDC-inoculated, Group 2 cats (3 episodes) and some liposome-inoculated Group 2 cats (2 episodes) but statistically significant differences between the groups were not noted.

CLDC-inoculated, Group 1 cats had a significant increase in neutrophil numbers within 24 hours of injection of CLDC when compared to saline-inoculated Group 1 cats (Figure 2A). The neutrophil numbers continued to increase until day 7 and plateaued by day 21. CLDC-inoculated, Group 1 cats also had a significant increase in monocyte numbers when compared to saline-inoculated Group 1 cats, but differences were not noted until day 7 (Figure 2B). Compared to baseline values, a significant increase in neutrophil counts was detected in healthy adult Group 2 cats administered CLDC and Group 2 cats administered liposomes alone by day 14 (data not shown). However, differences between CLDC-inoculated and liposome-inoculated Group 2 cats were not detected. Changes in monocyte counts were not detected in Group 2 cats regardless of treatment assignment.

Group 1 cats administered CLDC weekly had a significantly higher percentage of CD4+ lymphocytes (Figure 3A) and significantly a higher percentage of CD8+ lymphocytes (Figure 3B) than Group 1 cats administered saline by 6 weeks of treatment. KLH antibody responses were similar in Group 2 cats regardless of treatment.

**Clinical effects of CLDC administration to cats with rhinitis.** In Group 3 cats, fever was detected in 4 of 7 cats (57%) administered CLDC and 2 of 5 cats (40%) administered liposomes. Vomiting was detected in 0 of 7 Group 3 cats administered CLDC and 1 of 5 cats (20%) administered liposomes. In Group 4 cats, fever was detected in 1 of 13 cats (8%) administered CLDC and 1 of 15 cats (7%) administered liposomes. Vomiting was

detected in 3 of 13 (23%) Group 4 cats administered CLDC and 2 of 15 cats (13%) administered liposomes. None of these differences were statistically significant. Differences between complete blood cell counts, serum biochemical panel, or urinalysis between the baseline and day 28 samples or between treatment groups at any time points in Group 3 and Group 4 cats were not detected regardless if CLDC or liposomes were administered.

For Group 3, 5 of 7 (71%) of owners of cats administered CLDC reported at least a 25% improvement in the rate of sneezing, whereas none of the 5 cats administered liposomes alone were reported by the owners to have improved (Figure 4); this difference was significantly different ($p < 0.05$). However, there were no differences between CLDC-inoculated or liposome-inoculated Group 3 cats in regards to nasal discharge (Figure 4) or weekly clinical score (data not shown). There were also no differences in nasal discharge (Figure 5A), sneezing, (Figure 5B) or weekly clinical score (data not shown) between CLDC-inoculated or liposome-inoculated Group 4 cats. Differences in FHV-1 viral load (data not shown) between CLDC-inoculated or liposome-inoculated Group 4 cats were not detected. Similar numbers of Group 4 cats were positive for FHV-1 DNA at the beginning of the study (CLDC cats = 2 of 13; liposome cats = 1 of 15) and the end of the study (CLDC cats = 0 of 13; liposome cats = 0 of 15), regardless of whether the cats were administered CLDC or liposomes.

**Th1 and Th2 cytokine profiles in cats with and without rhinitis.** Results for IL4, IL6, IL10, IL12, IFNγ, and TNFα cytokine expression from PMBC samples from cats in Groups 2-4 collected prior to treatment were compared before and after activation with PMA plus ionomycin at time zero. Results from unstimulated PBMC did not vary

between the 3 groups of cats for any of the 6 cytokines evaluated (Figure 6A). However, after stimulation, both Group 3 and Group 4 cats had significantly higher levels of mRNA transcript expression of TNF-α than Group 2 cats (Figure 6B).

**Effect of repeated CLDC administration on cytokine mRNA expression in cats with and without rhinitis.** Results for IL4, IL6, IL10, IL12, IFNγ, and TNFα cytokine expression from unstimulated PMBC samples from cats in Groups 2-4 collected prior to treatment were compared to those in samples collected 8 hours after the first treatment and on day 28 of the treatment period. Significant differences in the expression of IL-4, IL-6, IL-10, IL-12, or IFN-γ transcripts were not noted in CLDC-treated cats compared to liposome-only treated cats in either Group 3 or Group 4, when compared to pre-treatment levels (data not shown). Expression of TNF-α mRNA decreased between week zero and week four in CLDC-inoculated Group 3 cats ($p = 0.025$) but not in CLDC-inoculated Group 4 cats ($p = 0.42$). In cats treated with liposomes only, there were no significant differences in TNF-α mRNA levels in either Group 3 ($p = 0.39$) or Group 4 cats ($p = 0.38$) when compared to pre-treatment levels.

**Effects of CLDC treatment on adaptive immune responses in cats with chronic rhinitis**. Whole blood proliferative responses to concanavalin A were significantly increased ($p = 0.05$) from baseline in CLDC-inoculated Group 4 cats but not liposome-inoculated Group 4 cats or CLDC-inoculated or liposome-inoculated Group 3 cats (data not shown).

KLH antibody levels from day 28 were significantly greater than day 0 in Group 4 cats inoculated with liposomes only ($p=0.0004$) or CLDC ($p<0.0001$). In addition, there were no detectable differences between treatment groups within Group 2 or 3.

***Discussion***

There are several significant findings from the experiments performed on the healthy cats in Group 1 and Group 2. Detection of fever, increased neutrophil count, monocytosis, increased percentage of CD4+ lymphocytes, and increased percentage of CD8+ lymphocytes in Group 1 healthy cats after the administration of CLDC documents that CLDC are activators of innate immunity in cats, similar to what was previously shown in mice (Dow *et al* 1999a). These effects may vary by the age of the cat as evidenced by detection of persistent monocytosis in the CLDC-inoculated young Group 1 cats but not the CLDC-inoculated older Group 2 cats. Previous studies in mice had shown that injections of cationic liposomes alone were inert in vivo (Dow *et al* 1999a). Data from this study suggests the liposome component of CLDC is not inert when administered to cats. For example, both liposome-inoculated and CLDC-inoculated Group 2 cats developed fever and increase in neutrophil count, as did CLDC-inoculated Group 1 cats. However, cats in the Group 1 placebo group received saline in contrast to liposomes and did not develop fever or change in neutrophil count. Though the CLDC used in these studies encoded the feline IL-2 gene, the immune stimulatory effects observed were most likely the result of the CLDC themselves, rather than due to DNA specifically coding for IL-2. In support of this, we have previously observed equivalent degrees of immune activation in mice administered IL-2 encoding CLDC or CLDC prepared with non-coding DNA (data not shown) by IP injection.

In general, the IP inoculations were well tolerated by the cats. Fever, lethargy, and vomiting following administration of liposomes or CLDC to cats were generally transient and mild; we attributed the findings to the rapid induction of innate immunity.

Serum biochemical profiles remained normal over time and so there appeared to be no adverse effects on liver or kidney function. The results should be viewed cautiously as the duration of this study was relatively short; it is possible that longer treatment might have elicited changes. However, we have treated several cats repeatedly with CLDC over a 2-4 year period without induction of notable side effects (unpublished data).

The biggest limitations to the experiments performed in the Group 3 and 4 cats with rhinitis are sample size and inability to perform a complete diagnostic workup in all cats which did not allow us to further stratify the cases by cause (i.e. bacterial vs. viral). However, based on the entry criteria utilized, we believe the majority of otherwise treatable diseases such as nasopharyngeal polyp, dental disease, neoplasia, and fungal disease were unlikely. Because all cats had previously been treated with antibiotics considered appropriate for bacterial rhinitis, we believe that chronic viral disease or another undetermined cause of chronic inflammatory disease was the cause of illness in these cats. In future prospective studies of this type, it would be optimal to apply a standardized workup and pre-study therapeutic trials to all cats.

Administration of CLDC but not liposomes to Group 3 cats with chronic rhinitis resulted in decreased sneezing which suggests that the CLDC protocol used here may be a useful adjunctive therapy for these patients. As virological data was not available for this group of cats, it cannot be determined whether induction of anti-viral activity could have accounted for the lessening of sneezing. Based on results of the experiments in healthy Group 1 and Group 2 cats, we believe activation of innate immunity is most likely responsible for the clinical improvement observed, though the exact mechanism of this effect could not be ascertained in this group of study animals. However, CLDC

administration may also improve T cell-mediated immunity as evidenced by increased percentages of CD4+ and CD8+ lymphocytes in healthy Group 1 kittens and by increased concanavalin A stimulated whole blood proliferative responses in CLDC treated Group 4 cats.

In Group 4 cats, improvement in clinical parameters was noted over time regardless of administration of CLDC or liposomes alone. It is possible that the clinical abnormalities were naturally resolving as these were young cats with a relatively short duration of clinical illness compared to Group 3 cats. However, since we showed evidence of enhanced innate immunity in healthy Group 2 cats after administration of liposomes alone, the liposomes may have had a treatment effect in Group 4 cats. Group 4 cats developed detectable KLH antibody responses regardless of treatment with CLDC or liposomes whereas KLH antibody responses were not detected in Group 2 or 3 cats. The failure of healthy Group 2 cats to develop detectable KLH responses suggests that CLDC or liposome associated immune stimulation alone was not potent enough to enhance responses to this non-adjuvanted antigen in normal cats. The detection of KLH antibodies in Group 4 cats with rhinitis, regardless of treatment with CLDC or liposomes suggests the cats had non-specific immune enhancement induced by their disease process. The failure to detect the same KLH responses in Group 3 cats with chronic rhinitis may relate to their age and immune function. Alternately, because Group 3 cats had very chronic illnesses, any non-specific immune enhancement associated with acute rhinitis may have waned.

Because complete virological assessments were not performed, we cannot make definitive statements about the role viruses played in the disease process. In Group 4

cats, FHV-1 DNA was detected in some cats but because healthy cats can also be positive, this finding does not prove disease causation. Similar viral loads and percentage positive results were detected before and after CLDC or liposome administration to Group 4 cats, suggesting that these protocols did not influence the infection rate or shedding rates of FHV-1. The FHV-1 infection rate of our Group 4 cats with persistent disease was much lower than a group of cats with acute, treatment responsive disease (52 of 61 cats) evaluated at the same shelter during the same time period (Veir *et al* 2004). This finding may indicate that FHV-1 was not associated with the syndrome in our population of shelter cats with resistant disease or that the superficial swabs lead to falsely negative results. However, it is also possible that FHV-1 had caused the initial insult but was not necessary for the persistence of clinical signs or that FHV-1 copy numbers within the sensitivity limits of the PCR assay used are only present during acute disease. We believe the latter hypothesis is most likely because similar methods were used in our study of cats with acute, treatment responsive disease.

Cytokine profiles showed no bias towards a Th2 type response in cats with chronic rhinitis in this study. Since the completion of this study, this has been supported by other publications in which a bias towards a Th1 type response was demonstrated in nasal biopsies from cats with chronic rhinitis (Johnson *et al* 2005a). The decrease in TNF-α mRNA expression over time in older Group 3 cats with chronic rhinitis following CLDC treatment may actually be a beneficial effect, as excessive concentrations of TNF-α can lead to chronic weight loss and other signs associated with chronic rhinitis. Overproduction of TNF-α can also lead to an inappropriate inflammatory response to pathogens, which could be a contributing factor to disease in cats with chronic rhinitis.

The failure to observe changes in cytokine mRNA expression by PBMC in response to treatment with CLDC may be attributable to the effects of sample collection timing as activation of innate immunity by CLDC in mice is typically transient (Dow *et al* 1999a). In addition, CLDC are more likely to traffic to lymph nodes than to the bloodstream following IP injection (Zaks et al, 2006), so that assessment of PBMC for cytokine changes may fail to sample the relevant cell populations.

In summary, we have shown that relatively low doses of CLDC, which are a potent activator of innate immunity, are capable of eliciting activation of both innate and adaptive immune responses in cats. Adult, client-owned cats with chronic rhinitis of greater than one year appear to be more likely to experience clinical benefit from treatment with CLDC compared to younger shelter cats. However, ages in the humane society group were estimated and so this age comparison must be interpreted cautiously. The potency of CLDC and their favorable safety profile in both healthy and sick cats suggest that further evaluation as an immunotherapeutic in chronic infectious or inflammatory diseases of cats is warranted.

***References***

Applied Biosystems (2001), User Bulletin #2: ABI PRISM 7700 Sequence Detection System. Applied Biosystems.

Baiocchi RA, Ward JS, Carrodeguas L, Eisenbeis CF, Peng R, Roychowdhury S, Vourganti S, Sekula T, O'Brien M, Moeschberger M, Caligiuri MA (2001), GM-CSF and IL-2 induce specific cellular immunity and provide protection against Epstein-Barr virus lymphoproliferative disorder, *J Clin Invest* 108: 887-894.

Cape L (1992), Feline idiopathic chronic rhinosinusitis: A retrospective study of 30 cases, *JAAHA* 28: 149-155.

Dow SW, Fradkin LG, Liggitt DH, Willson AP, Heath TD, Potter TA (1999a), Lipid-DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously, *J Immunol* 163: 1552-1561.

Dow SW, Schwarze J, Heath TD, Potter TA, Gelfand EW (1999b), Systemic and local interferon gamma gene delivery to the lungs for treatment of allergen-induced airway hyperresponsiveness in mice, *Hum Gene Ther* 10: 1905-1914.

Fairman J, Roche L, Pieslak I, Lay M, Corson S, Fox E, Luong C, Koe G, Lemos B, Grove R, Fradkin L, Vernachio J (1999), Quantitative RT-PCR to evaluate in vivo expression of multiple transgenes using a common intron, *Biotech* 27: 566-4.

Helps CR, Lait P, Damhuis A, Bjornehammar U, Bolta D, Brovida C, Chabanne L, Egberink H, Ferrand G, Fontbonne A, Pennisi MG, Gruffydd-Jones T, Gunn-Moore D, Hartmann K, Lutz H, Malandain E, Mostl K, Stengel C, Harbour DA, Graat EA (2005), Factors associated with upper respiratory tract disease caused by feline herpesvirus, feline calicivirus, Chlamydophila felis and Bordetella bronchiseptica in cats: experience from 218 European catteries, *Vet Rec* 156: 669-673.

Johnson LR, De Cock HE, Sykes JE, Kass PH, Maggs DJ, Leutenegger CM (2005a), Cytokine gene transcription in feline nasal tissue with histologic evidence of inflammation, *Am J Vet Res* 66: 996-1001.

Johnson LR, Foley JE, De Cock HE, Clarke HE, Maggs DJ (2005b), Assessment of infectious organisms associated with chronic rhinosinusitis in cats, *J Am Vet Med Assoc* 227: 579-585.

Kipar A, Leutenegger CM, Hetzel U, Akens MK, Mislin CN, Reinacher M, Lutz H (2001), Cytokine mRNA levels in isolated feline monocytes, *Vet Immunol Immunopathol* 78: 305-315.

Lenzo JC, Mansfield JP, Sivamoorthy S, Cull VS, James CM (2003), Cytokine expression in murine cytomegalovirus-induced myocarditis: modulation with interferon-alpha therapy, *Cell Immunol* 223: 77-86.

Leutenegger CM, Mislin CN, Sigrist B, Ehrengruber MU, Hofmann-Lehmann R, Lutz H (1999), Quantitative real-time PCR for the measurement of feline cytokine mRNA, *Vet Immunol Immunopathol* 71: 291-305.

Mueller RS, Veir J, Fieseler KV, Dow SW (2005), Use of immunostimulatory liposome-nucleic acid complexes in allergen-specific immunotherapy of dogs with refractory atopic dermatitis - a pilot study, *Vet Dermatol* 16: 61-68.

Templeton KE, Scheltinga SA, Graffelman AW, Van Schie JM, Crielaard JW, Sillekens P, Van Den Broek PJ, Goossens H, Beersma MF, Claas EC (2003), Comparison and evaluation of real-time PCR, real-time nucleic acid sequence-based amplification, conventional PCR, and serology for diagnosis of Mycoplasma pneumoniae, *J Clin Microbiol* 41: 4366-4371.

Van Pelt DR, Lappin MR (1994), Pathogenesis and treatment of feline rhinitis, *Vet Clin N Am - Sm An Pract* 24: 807-823.

Veir JK, Ruch-Gallie R, Spindel ME, Lappin MR (2004). Prevalence of FHV-1, *Mycoplasma* spp., and aerobic bacteria in shelter cats with acute upper respiratory tract disease. *J Vet Intern Med* 18[3]: 197.

Vogtlin A, Fraefel C, Albini S, Leutenegger CM, Schraner E, Spiess B, Lutz H, Ackermann M (2002), Quantification of feline herpesvirus 1 DNA in ocular fluid samples of clinically diseased cats by real-time TaqMan PCR, *J Clin Microbiol* 40: 519-523.

Zaks K, Jordan M, Guth A, Sellins K, Kedl R, Izzo A, Bosio C, Dow S (2006), Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes, *J Immunol* 176: 7335-7345.

*Figures*

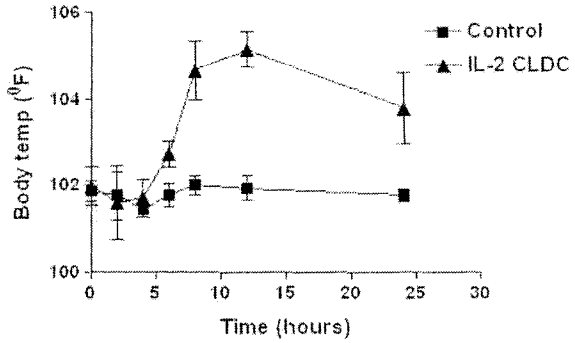


**Figure 1. Effects of injection of IL-2 CLDC on body temperature in healthy cats.** Six healthy, 6-8-month old purpose-bred cats were randomized to two treatment groups of 3 cats each (Group 1). One subgroup of 3 cats was injected intraperitoneally once weekly with liposome-DNA complexes (CLDC) containing 150 ug plasmid DNA encoding the feline IL-2 cDNA (IL-2 CLDC group). A second subgroup of 3 cats was injected intraperitoneally with saline only (Control). Body temperature was assessed prior to treatment and at 2-4 hour intervals after injection for a 24-hour period. The mean body temperature (± SD) for each group was plotted. Body temperature increased significantly ($p< 0.05$) in the IL-2 CLDC group beginning 6 hours after injection and remained significantly elevated for a 24-hour period.

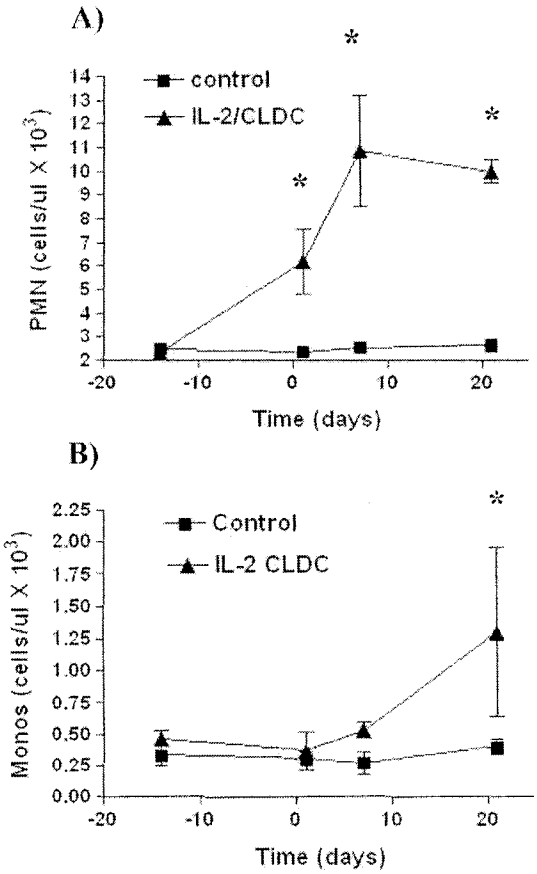


**Figure 2. Effects of IL-2 CLDC treatment on neutrophil and monocyte counts.** Six healthy, 6-8-month old purpose-bred cats were randomized to two treatment groups of 3 cats each (Group 1). One subgroup of 3 cats was injected intraperitoneally once weekly with 150 ug IL-2 CLDC (IL-2 CLDC). A second subgroup of 3 cats was injected intraperitoneally with saline only (Control). Neutrophil (reference range 2-12.0 x $10^3$/uL) and monocyte (reference range 0.0 -0.8 x $10^3$/uL) counts were determined for each group of animals and the mean (± SD) was plotted. (A) Neutrophil ("PMN") counts were significantly increased ($p < 0.05$) in cats treated with IL-2 CLDC, beginning within 24 hours of the first injection. (B) Monocyte ("Monos") counts were significantly increased ($p < 0.05$) by the first week after treatment.

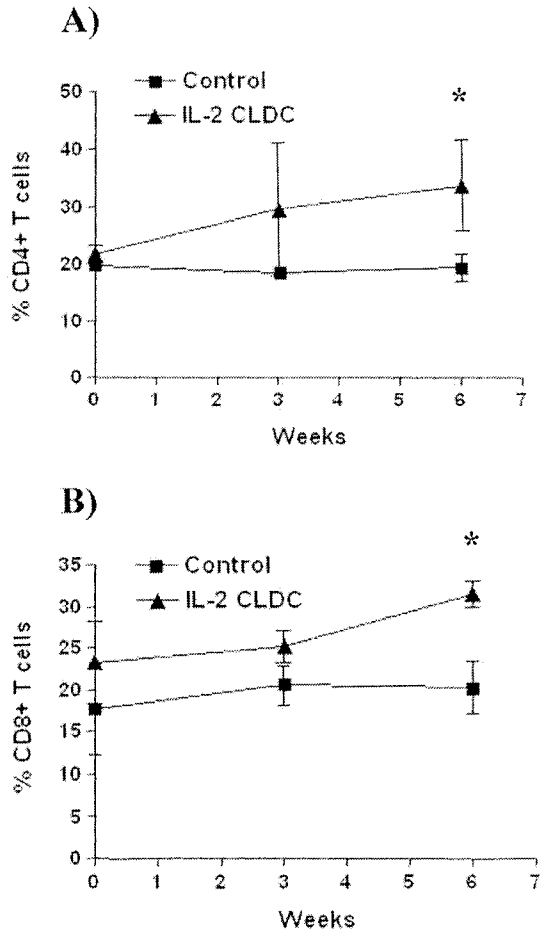


**Figure 3. Effects of IL-2 CLDC treatment on CD4+ and CD8+ lymphocytes in cats.** Six healthy, 6-8-month old purpose-bred cats were randomized to two treatment groups of 3 cats each (Group 1). One subgroup of 3 cats was injected intraperitoneally once weekly with 150 ug IL-2 CLDC (IL-2 CLDC). A second subgroup of 3 cats was injected intraperitoneally with saline only (Control). Peripheral blood mononuclear cells were collected prior to treatment and again at 3 weeks and 6 weeks of treatment. (A) The percentage of CD4+ lymphocytes in each cat was assessed by flow cytometry, as described in Methods, and the mean (± SD) percentage of CD4+ lymphocytes for each group was plotted. (B) The mean (± SD) percentage of CD8+ Lymphocytes was determined and plotted as for CD4+ lymphocytes. * denotes values that are significantly ($p < 0.05$) different between control and IL-2 CLDC treated cats.

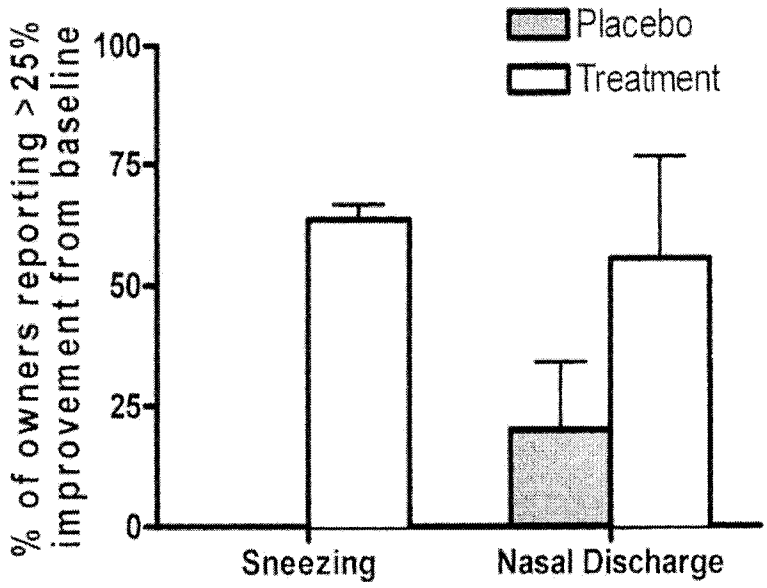


**Figure 4. Effect of IL-2 CLDC treatment on clinical signs in adult client-owned cats with chronic rhinitis.** Client-owned adult cats (Group 3) were randomized to 4 weeks of treatment with intraperitoneal injection once weekly with 150 ug IL-2 CLDC (Treatment, n=5), or were randomized to treatment with liposomes only (Placebo, n=7). The mean (+ SD) of the owners' evaluations of changes in clinical scores at each week, compared to baseline values, were tabulated and plotted. Compared to pre-treatment signs, cats randomized to the IL-2 CLDC treatment group had a significant improvement in sneezing ($p = 0.016$) but not in the frequency of nasal discharge ($p = 0.063$).

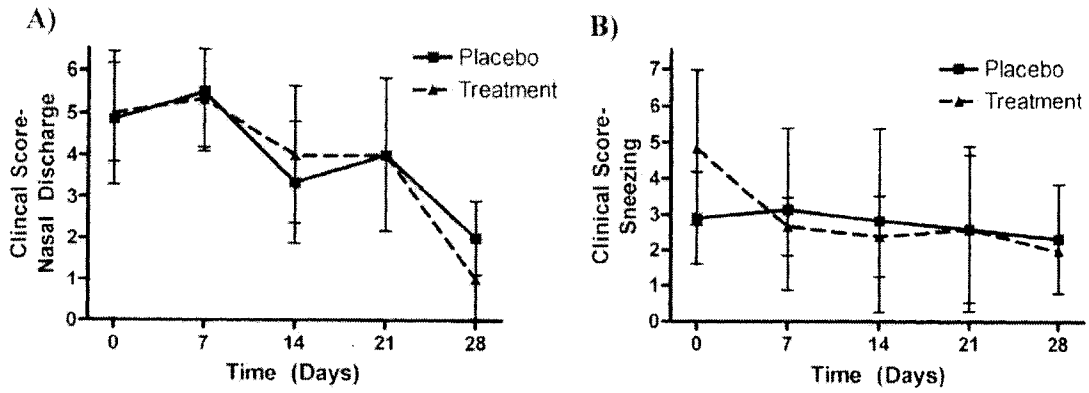


**Figure 5. Effect of IL-2 CLDC treatment on clinical scores in Shelter-owned cats with chronic rhinitis.** Shelter cats were randomized to 4 weeks of treatment with intraperitoneal injection once weekly with 150 ug IL-2 CLDC or with liposomes only, as described in Methods (Group 4). The clinical scores were assessed by assistants blinded to the treatment groups. The mean (± SD) scores for nasal discharge (A) and sneezing (B) were plotted. Differences in placebo (n=13) and treatment (n=15) groups were not statistically different at any time point (p>0.05).

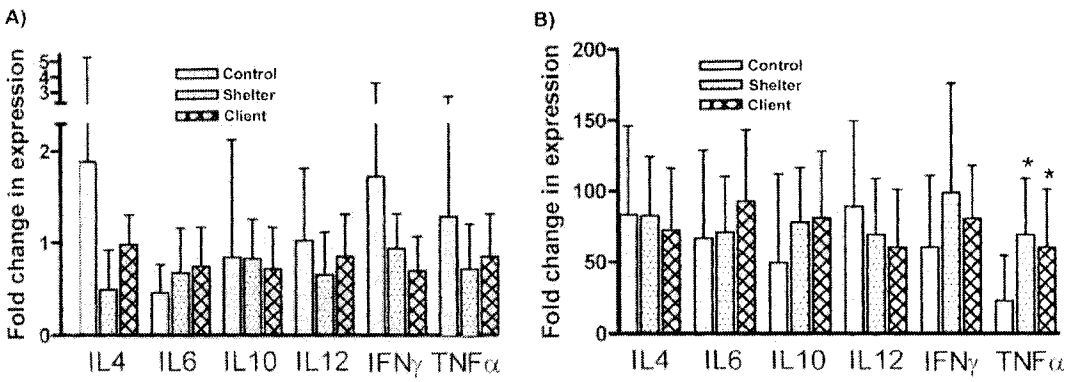


**Figure 6. Cytokine mRNA expression in PBMC from normal cats and cats with rhinitis.** PBMC were isolated by density centrifugation from blood of Group 2 cats (Control n = 8), Group 3 cats (Client n =9), and Group 4 cats (Shelter n = 28), prior to treatment based on sample availability. Total RNA was isolated from cells immediately after isolation (A) or following stimulation for six hours with phorbol myristate acetate (PMA: 10 ng/mL) plus ionomycin (500 ng/mL) (B) and cytokine mRNA expression was quantitated as described in Methods. Mean cytokine expression levels (± SD) were expressed relative to a sample of pooled cDNA and plotted. * denotes values that are statistically different ($p < 0.05$) from the Control group.

ENDNOTES
[a]IDEXX, Westbrook, ME
[b]Biowhittaker, Walkersville, MD
[c]Sigma-Aldrich Chemical Co, St Louis, MO
[d]Avanti Polar Lipids, Alabaster, AL
[e]Qiagen, Valencia, CA
[f]Invitrogen Corporation, Carlsbad, CA
[g]Applied Biosystems, Foster City, CA
[h]ABI Prism 7000 SDS Software Version 1.0 (build 81 rev 3): Applied Biosystems, Foster City, CA
[i]TA Cloning Vector: Invitrogen Corporation, Carlsbad, CA
[j]Immulon-1 96-well microtitre plates, Thermo LabSystems, Franklin, MA
[k]Kirkegaard and Perry Laboratories Inc, Gaithersburg, MD
[l]TMB microwell peroxidase system, Kirkegaard and Perry Laboratories Inc, Gaithersburg, MD
[m]Multiskan Ascent plate reader, ThermoElectron Corporation, Beverly, MA
[n]ICN Biomedical, Aurora, OH
[o]vpg34; CD4-fitc: Serotec, Raleigh, NC
[p]vpg9; CD8-rpe; Serotec, Raleigh, NC
[q]IM7; CD44-rpe/cy5 Pharmingen, Franklin Lakes, NJ
[r]5C6, CD11b-rpe Serotec
[s]CAG5-3D1; MHC Class II: Serotec, Raleigh, NC
[t]BD Biosciences, San Jose, CA
[u]DAKO-Cytomation, Fort Collins, CO
[v]Applied Biosystems, Foster City, CA
[w]Wallac-Microbeta; Perkin–Elmer, Boston, MA

CHAPTER 6

# DEVELOPMENT OF A REAL TIME POLYMERASE CHAIN REACTION FOR THE DETECTION OF *MYCOPLASMA FELIS* IN DOMESTIC CATS

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## Introduction

Mycoplasmas are the smallest self replicating bacteria known to man (Dussurget and Roulland-Dussoix 1994). These bacteria are common cell culture contaminants; some Mycoplasma species have the potential to induce clinical disease in humans and animals (Stakenborg *et al* 2005). While pathogenic *Mycoplasma* species causing anemia (Tasker *et al* 2003, Willi *et al* 2005) and arthritis (Bonilla *et al* 1997, Ernst and Goggin 1999, Moise *et al* 1983) are well documented, the role of *Mycoplasma* species in respiratory disease in cats is unknown. *Mycoplasma* species have been isolated from the respiratory tracts of both healthy and clinically ill cats (Bannasch and Foley 2005, Chandler and Lappin 2002, Crisp *et al* 1987, Foster *et al* 1998, Foster *et al* 2004, Johnson *et al* 2005, Padrid *et al* 1991, Randolph *et al* 1993, Tan *et al* 1977, Tan and Miles 1974). Respiratory disease has been experimentally induced by inoculation with a *Mycoplasma* species in at least one SPF kitten (Tan 1974). However, the numbers of organism in the inoculum seemed to be greater than what would be encountered naturally and the kitten was most likely immunosuppressed secondary to its age at inoculation and sampling via cardiac puncture. Due to the difficulty in speciating mycoplasmas, veterinary studies typically identify them only to the genus level. However, in those studies that do identify the species of mycoplasmas involved, *Mycoplasma felis* is frequently encountered.

Historically, detection of mycoplasmas has been achieved through microbiologic culture. However, *Mycoplasma* species are difficult to culture in vitro. Even if a diagnostic laboratory equipped to culture the organism is available to the general practitioner, the need for specialized transport media, the poor survival of the organisms during transport, and the slow growth of the organisms make diagnosis of *Mycoplasma*

species infection cumbersome at best. The development of assays based on detection of the DNA of *Mycoplasma* species has eased specialized sample submission requirements and decreased delays in treatment because the results can be returned in much shorter time than for culture. Recently, a conventional endpoint PCR assay for the detection of *M. felis* has been developed for use in veterinary medicine (Chalker *et al* 2004).

Because *Mycoplasma* species can be detected in normal animals as well as those with clinical illness, culture results and conventional PCR assay results that are reported only as positive have minimal positive predictive value. In an attempt to differentiate normal commensal levels of bacteria from pathologic levels, previous authors have defined "cutoff values" of bacterial counts from quantitative culture of airway samples obtained from dogs (Peeters *et al* 2000). Using this same theory, the quantitative results from a rapid, reproducible, real time PCR (rtPCR) based assay for *M. felis* might differentiate a clinically ill cat from a carrier. Therefore, the purpose of this study was to develop a q-rtPCR based assay for the detection of *M. felis* in respiratory samples collected from cats. If successful, the assay could be used in future studies to determine if there is level of *M. felis* DNA that correlates to the presence of illness and could also be used to monitor response to administration of drugs with an anti-*Mycoplasma* spectrum.

**Materials and Methods**

*Bacterial Strain Sources and Detection*

*Mycoplasma* species isolated from four cats enrolled in a separate study (Veir *et al* 2004) were used for PCR amplification and sequencing. Briefly, oropharyngeal swabs from cats showing clinical signs of upper respiratory disease were inoculated on to Friis broth agar plates, then placed in 2 mL of Friis broth and incubated at 37°C with 5% $CO_2$

for 24 hours. The resulting culture was again inoculated onto Friis broth agar plates. All plates were incubated at 37°C with 5% $CO_2$ for 72 hours immediately after inoculation. *Mycoplasma* cultures were considered positive if colonies had a microscopic "fried egg" appearance on selective agar (Friis broth). All microbiologically positive *Mycoplasma* species isolates were confirmed by a previously published endpoint PCR (Baird *et al* 1999). *Mycoplasma* isolates from cats negative by nucleic acid detection methods and virus isolation for FHV-1 and feline calicivirus were selected for use in this study in order to increase the likelihood of detection of pathogenic strains. As a positive control, *M. felis* strain 23391 was obtained from the American Type Culture Collection (ATCC).[a]

*Amplification of Isolate DNA and Sequencing*

DNA from 200 uL of each bacterial culture and the ATCC strain was extracted according to the manufacturer's suggested protocol using a commercially available kit.[b] 16s rDNA was amplified from all isolates using a previously published nested PCR protocol (Baird *et al* 1999). Amplification products were visualized with ethidium bromide staining and ultraviolet illumination. Bands were excised from the agarose gel and purified using a commercially available kit.[c] Sequencing was performed using a commercial genetic sequencing service[d] using an ABI3100 Genetic Analyzer[l] and the ABI V3.1 Big Dye ready reaction kit.[l] Results for all samples were compared to available 16s rDNA sequences[e] using the BLAST algorithm and all samples used in this study had >95% sequence homology with *M. felis*.

*Design and Selection of Primer Pairs*

A consensus sequence of the four 16s rDNA PCR amplification products was generated using Clustal W[f] and default parameters.[g] Primer pairs were designed using

commercially available software[h] with the following limits: primer $T_m$ 58-60°C, primer GC content 30-80%, primer length 9-40 base pairs, amplicon size 50-150 base pairs, and no GC clamp. Secondary structure was evaluated using the mFold program.[i] Four primer pairs were selected and tested at varying concentrations (Table 1) using DNA extracted from the ATCC strain and SYBR® Green[j] as the detection chemistry. The 25 µL reaction contained the following: 12.5 µL SYBR® Green master mix,[k] 5 µL template DNA, and a primer/water mix. Primer volumes and concentrations were varied according to a trial primer matrix (Table 1) and water was added to bring total volume of the mixture added to the reaction to 7.5 µL. All reactions were carried out on an ABI 7000[l] or Bio-Rad iCycler[j] using the following conditions: 2 minutes at 50°C, 10 minutes at 95°C, and then 40 cycles consisting of a 15 second 95°C denaturation step followed by a one minute 60°C annealing step. Amplification was followed by a melt curve consisting of 80 cycles with a hold time of 10 seconds and a step of 0.5°C. As no pair gave acceptable results, a second analysis of the consensus sequence using the same primer design software was performed after changing the GC clamp setting to 1. In order to determine the linear range of the reaction, a log dilution series of the ATCC isolate was performed ($1 \times 10^0$ to $1 \times 10^{-6}$ dilutions) and regression analysis was performed on the resulting standard curve using the instrument software.[j] Intra- and interassay variability were determined by running five replicates of the entire dilution series in a single run and the same dilution series in duplicate for three consecutive days, respectively. Finally, DNA isolated from the four field isolates were subjected to the q-rtPCR assay on a single plate along with appropriate positive (ATCC *M. felis* strain 23391) and negative (no template control) samples in duplicate. In order to determine

specificity for *M. felis* compared to other *Mycoplasma* species, previously extracted DNA from *M. gateae*, *M. californicum* and *M. bovis* was applied in duplicate to the assay. All products that were defined as positive (mean fluorescence ten times the standard deviation of background) were separated via agarose gel electrophoresis and purified, sequenced, and compared to published sequences as above.

**Results**

*Assay Characteristics*

Characteristics of the primer pair selected and resulting amplicon are shown in Table 2. The amplified fragment contains 108 base pairs. Moderate secondary structure was detected with the mFold software program; however, no other suitable pairs within the consensus sequence existed given the constraints of the primer design. Initial concentration of the type strain stock was 16.56 µg/mL resulting in a dilution series ranging from $8.28 \times 10^{-2}$ to $8.28 \times 10^{4}$ pg DNA applied to the reaction. The lowest reliable level of detection was $8.28 \times 10^{1}$ pg DNA/reaction and the assay was linear over a range of four dilutions with an $r^2$ value of 0.968 and reaction efficiency of 87.2% (Figure 1). Intraassay variability was within acceptable limits; maximal coefficient of variation of Ct for any single dilution over 5 replicates was 8.0% (Figure 1). The maximal coefficient of variation of mean calculated starting quantity for any single dilution over 3 days was 7.3% (Figure 2) and correlation coefficient ($r^2$) ranged from 0.9581 to 0.9995. The melting curve showed that the *T*m values for the type strain ranged between 81.0 and 83.5°C. Agarose gel electrophoresis demonstrated a single product between 105 and 110 bp correlating with the predicted size of 108 bp. Sequence

analysis of the product showed >99% homology with the previously demonstrated type strain sequence.

*Sensitivity and Specificity of the Assay using Field Isolates and other Mycoplasma species.*

The assay was able to demonstrate *M. felis* DNA in all four field isolates. Initial starting concentrations were not able to be determined spectrophotometrically as they were out of the working range of the instrument. Calculated starting quantity ranged from 22 to 38 pg DNA applied to the reaction. Melting curves showed a single peak in each of the four field isolates with a range of Tm values between 78.5°C and 83.0°C. No reaction containing water as template (no template control) was positive based on maximal fluorescence or demonstrated a peak on melt curve analysis. Additionally, all three non-*M. felis* species were negative in all reactions applied. Sequencing of the products from the four field isolates showed >93% homology with the type strain.

**Discussion**

The SYBR® Green based assay demonstrated a linear range of four log dilutions and good sensitivity. Melt curve analysis demonstrated that a single product was responsible for fluorescence in the assay and that the assay is reproducible. Correlation of detection rates between a previously published endpoint PCR (Baird *et al* 1999) and culture methods in field isolates demonstrates that this assay is useful in field applications as well. Finally, the assay can accurately differentiate between *M. felis* and the selected strains tested in this study. The variability in *T*m values is not a concern based on the data from this study, as no products were formed that were not consistent with *M. felis*. However, limited numbers of negative controls were applied and further studies should

include additional *Mycoplasma* species previously isolated from the airways of cats (Ureaplasmas, *Mycoplasma laidlawii*, *M. arginii*).

This study used DNA obtained from pharyngeal swabs because of availability. However, quantitation is impossible because sample collection cannot be standardized between cats. For this assay to be more clinically useful than conventional PCR assays, a method for standardizing between samples must be employed. With most quantitative PCR assays developed to date, normalization between samples is achieved by comparing the specific gene of interest with a second, so called housekeeping gene. However, SYBR® Green technology does not allow multiplex assays. If the assay described in this chapter is used in future studies, a second reaction targeting a housekeeping gene will be applied to all samples. However, this will double costs and decreases throughput capabilities. Ideally, fluorescence resonance energy transfer systems would be applied to allow multiplexing, thereby allowing rapid and more economical normalization and those experiments will be performed in future experiments. Additionally, further application of this assay to a more easily standardized sample such as bronchoalveolar lavage may aid in delineating the role of *M. felis* in lower respiratory disease in cats. Although this assay demonstrated the presence of *M. felis* in ill cats, sample size precluded the stratification of ill vs. healthy cats in an attempt to define a cutoff value for pathogenic organismal load which is planned in future studies.

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ENDNOTES

[a] American Type Culture Collection, Manassas, VA

[b] DNA Minikit, Qiagen, Incorporated, Valencia, CA

[c] QiaQuick PCR Purification Kit, Qiagen, Incorporated, Valencia, CA

[d] Macromolecular Resources, Fort Collins, CO

[e] NCBI: National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi

[f] European Bioinformatics Institute: http://www.ebi.ac.uk/clustalw/index.html#

[g] DNA Gap Open Penalty = 15.0

DNA Gap Extension Penalty = 6.66

DNA Matrix = Identity
DNA ENDGAP = -1
DNA GAPDIST = 4
[h] Primer Express, version 1.0, Applied Biosystems, Foster City, CA
[i] mFold DNA program: http://www.bioinfo.rpi.edu/applications/mfold/old/dna/
[j] Bio-Rad, INC, Hercules, CA
[k] iQ SYBR® Green Supermix, Bio-Rad, Hercules, CA
[l] Applied Biosystems, Foster City, CA

| Final Primer Concentration | Volume in Reaction | Final Primer Concentration | Volume in Reaction | Final Primer Concentration | Volume in Reaction | Final Primer Concentration | Volume in Reaction |
|---|---|---|---|---|---|---|---|
| F(50nM) | 1.25 uL | F(50nM) | 1.25 uL | F(50nM) | 1.25 uL | F(50nM) | 1.25 uL |
| R(50nM) | 1.25 uL | R(100nM) | 0.50 uL | R(300nM) | 1.50 uL | R(600nM) | 3.00 uL |
| F(100nM) | 0.50 uL | F(100nM) | 0.50 uL | F(100nM) | 0.50 uL | F(100nM) | 0.50 uL |
| R(50nM) | 1.25 uL | R(100nM) | 0.50 uL | R(300nM) | 1.50 uL | R(600nM) | 3.00 uL |
| F(300nM) | 1.50 uL | F(300nM) | 1.50 uL | F(300nM) | 1.50 uL | F(300nM) | 1.50 uL |
| R(50nM) | 1.25 uL | R(100nM) | 0.50 uL | R(300nM) | 1.50 uL | R(600nM) | 3.00 uL |
| F(600nM) | 3.00 uL | F(600nM) | 3.00 uL | F(600nM) | 3.00 uL | F(600nM) | 3.00 uL |
| R(50nM) | 1.25 uL | R(100nM) | 0.50 uL | R(300nM) | 1.50 uL | R(600nM) | 3.00 uL |

**Table 1. Matrix of primer concentrations used to develop quantitative real time PCR for *Mycoplasma felis* 16s rDNA** A matrix of primer concentrations were evaluated for use in a real time quantitative SYBR® Green based PCR assay for *Mycoplasma felis*. Primers and amplicon length are listed in Table 2. Reactions were evaluated for presence of primer-dimers, a single, uniform melt curve peak, and finally, lowest $C_t$ value. All 50 nM of final concentration primers use 1 µM of primer stock, remaining concentrations use 5 µM stock. Total volume of primer/water mixture added to reaction was 7.5 µL. PCR grade water was added to bring the mixture to final volume. F=concentration of forward primer, R=concentration of reverse primer. Highlighted combination was chosen as the final selection. Adapted from Nolan, T (2004).

| | Start Position | Length | Tm | Sequence |
|---|---|---|---|---|
| **Forward** | 8 | 22 | 59 | GGAGCTGGTAATACCCGAAGTC |
| **Reverse** | 115 | 21 | 59 | CCCACGTTCTCGTAGGGATAC |
| **Amplicon** | | 108 | 82 | |

**Table 2. Primer pair and amplicon used in quantitative real time PCR for *Mycoplasma felis* 16s rDNA** Using a consensus sequence derived from four field isolates, primers were designed using Primer Express software[l] to detect 16s rDNA of *Mycoplasma felis* using SYBR® Green[k] as the detection chemistry. Criteria for selection were as in Table 1.Parameters used in the software and thermocycler protocol is listed in the text.

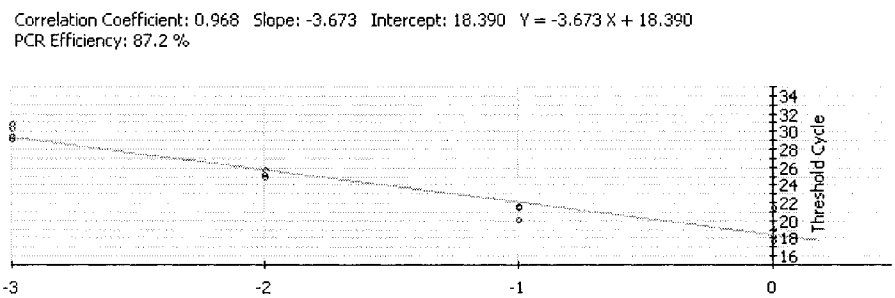


**Figure 1. Dilution series for quantitative real time PCR for *Mycoplasma felis* 16s rDNA** Genomic DNA was isolated from Mycoplasma felis strain 23391 and was serially diluted 10 fold. Stock solution (dilution log $10^0$) concentration was determined by spectrophotometry. Five replicates of each point of the dilution series were run on a single assay. Starting quantity was calculated using the best fit line derived by the instrument software.[j] Individual points represent the mean of each run.

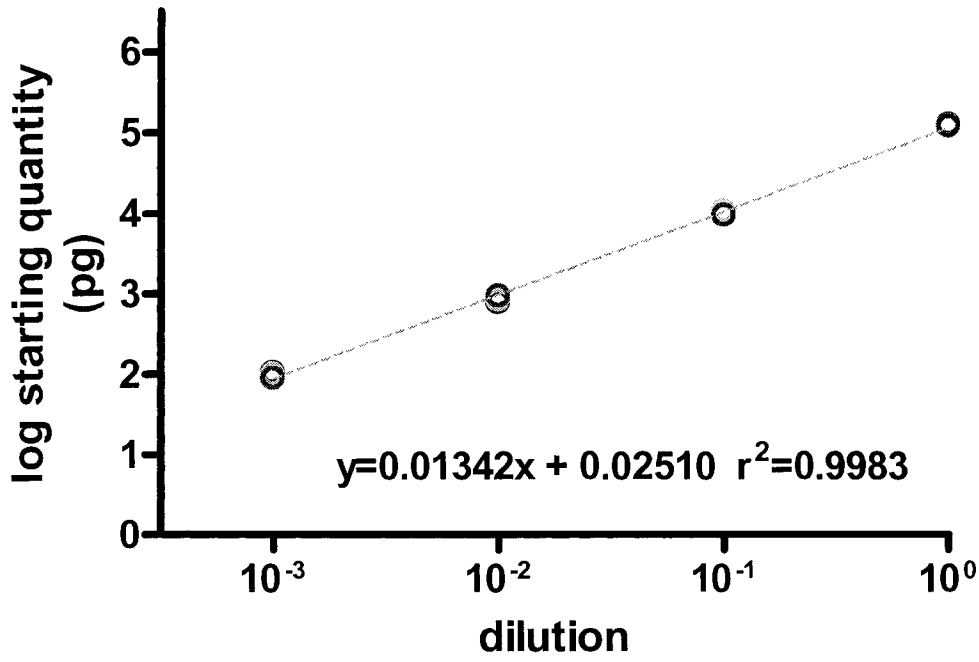


**Figure 2. Dilution series for quantitative real time PCR for *Mycoplasma felis* 16s rDNA** Genomic DNA was isolated from *Mycoplasma felis* strain 23391 and was serially diluted 10 fold. Stock solution (dilution log $10^0$) concentration was determined by spectrophotometry. Dilution series were run in duplicate on three consecutive days. Starting quantity was calculated using the best fit line derived by the instrument software[j] on each day. Individual points represent the mean of each run.

**References**


Baird SC, Carman J, Dinsmore RP, Walker RL, Collins JK (1999), Detection and identification of *Mycoplasma* from bovine mastitis infections using a nested polymerase chain reaction, *J Vet Diagn Invest* 11: 432-435

Bannasch MJ, Foley JE (2005), Epidemiologic evaluation of multiple respiratory pathogens in cats in animal shelters, *J Fel Med Surg* 7: 109-119

Bonilla HF, Chenoweth CE, Tully JG, Blythe LK, Robertson JA, Ognenovski VM, Kauffman CA (1997), *Mycoplasma felis* septic arthritis in a patient with hypogammaglobulinemia, *Clin Infect Dis* 24: 222-225

Chalker VJ, Owen WMA, Paterson CJI, Brownlie J (2004), Development of a polymerase chain reaction for the detection of *Mycoplasma felis* in domestic cats, *Vet Microbiol* 100: 77-82

Chandler JC, Lappin MR (2002), Mycoplasmal respiratory infections in small animals: 17 Cases (1988-1999), *JAAHA* 38: 111-119

Crisp MS, Birchard SJ, Lawrence AE, Fingeroth J (1987), Pulmonary abscess caused by a *Mycoplasma sp.* in a cat, *J Am Vet Med Assoc* 191: 340-342

Dussurget O, Roulland-Dussoix D (1994), Rapid, sensitive PCR-based detection of mycoplasmas in simulated samples of animal sera, *Appl Envir Microbiol* 60: 953-959

Ernst S, Goggin JM (1999), What is your diagnosis? *Mycoplasma* arthritis in a cat, *J Am Vet Med Assoc* 215: 19-20

Foster SF, Barrs VR, Martin P, Malik R (1998), Pneumonia associated with *Mycoplasma spp.* in three cats, *Aust Vet J* 76: 460-464

Foster SF, Martin P, Allan GS, Barrs VR, Malik R (2004), Lower respiratory tract infections in cats: 21 cases (1995-2000), *J Feline Med Surg* 6: 167-180

Johnson LR, Foley JE, De Cock HE, Clarke HE, Maggs DJ (2005), Assessment of infectious organisms associated with chronic rhinosinusitis in cats, *J Am Vet Med Assoc* 227: 579-585

Klein D (2002), Quantification using real-time PCR technology: applications and limitations, *Trends Mol Med* 8: 257-260

Moise NS, Crissman JW, Fairbrother JF, Baldwin C (1983), *Mycoplasma gateae* arthritis and tenosynovitis in cats: case report and experimental reproduction of the disease, *Am J Vet Res* 44: 16-21

Nolan T (2004), Getting Started - The Basics of Setting Up a qPCR Assay, in *A-Z of Quantitative PCR*, ed. Bustin SA, International University Line, La Jolla p 529-543

Padrid PA, Feldman BF, Funk K, Samitz EM, Reil D, Cross CE (1991), Cytologic, microbiologic, and biochemical analysis of bronchoalveolar lavage fluid obtained from 24 healthy cats, *Am J Vet Res* 52: 1300-1307

Peeters DE, McKiernan BC, Weisiger RM, Schaeffer DJ, Clercx C (2000), Quantitative bacterial cultures and cytological examination of bronchoalveolar lavage specimens in dogs, *J Vet Intern Med* 14: 534-541

Randolph JF, Moise NS, Scarlett JM, Shin SJ, Blue JT, Bookbinder PR (1993), Prevalence of mycoplasmal and ureaplasmal recovery from tracheobronchial lavages and prevalence of mycoplasmal recovery from pharyngeal swab specimens in dogs with or without pulmonary disease, *Am J Vet Res* 54: 387-391

Stakenborg T, Vicca J, Butaye P, Maes D, De Baere T, Verhelst R, Peeters J, de Kruif A, Haesebrouck F, Vaneechoutte M (2005), Evaluation of amplified rDNA restriction analysis (ARDRA) for the identification of *Mycoplasma* species, *BMC Infect Dis* 5: 46 (abstract)

Tan RJ (1974), Susceptibility of kittens to *Mycoplasma felis* infection, *J Exp Med* 44: 235-240

Tan RJS, Lim EW, Ishak B (1977), Ecology of mycoplasmas in clinically healthy cats, *Aust Vet J* 53: 515-518

Tan RJS, Miles JAR (1974), Incidence and significance of mycoplasmas in sick cats, *Res Vet Sci* 16: 27-34

Tasker S, Helps CR, Day MJ, Gruffydd-Jones TJ, Harbour DA (2003), Use of real-time PCR to detect and quantify *Mycoplasma haemofelis* and "*Candidatus Mycoplasma haemominutum*" DNA, *J Clin Microbiol* 41: 439-441

Veir JK, Ruch-Gallie R, Spindel ME, Lappin MR (2004). Prevalence of FHV-1, *Mycoplasma* spp., and aerobic bacteria in shelter cats with acute upper respiratory tract disease. *J Vet Intern Med* 18[3]: 197 (abstract)

Willi B, Boretti FS, Cattori V, Tasker S, Meli ML, Reusch C, Lutz H, Hofmann-Lehmann R (2005), Identification, molecular characterization, and experimental transmission of a new hemoplasma isolate from a cat with hemolytic anemia in Switzerland, *J Clin Microbiol* 43: 2581-2585

# CONCLUSION

Completion of the work described in this dissertation answers some questions generated by previous studies of URTD in cats. However, there are still many unanswered questions for future study.

*Chapter 1*

One of our first goals was to evaluate the organisms associated with URTD in cats, comparing several methods of detection and sampling sites. The results of this study differed somewhat from previously published studies in that *Chlamydophila felis* was not detected in any samples and FCV was present in only a single sample. However, as our study focused on nasal discharge as a key criterion for entry this may have discriminated against cats infected with these organisms as ocular and oral signs are the disease syndromes most commonly caused by these organisms. We confirmed the presence of other organisms previously implicated as primary pathogens including FHV-1, *Bordetella bronchiseptica*, and *Mycoplasma* species. The study demonstrated that sampling from either the nasal or pharyngeal cavity by swab technique is equivalent at detection of most organisms traditionally associated with URTD. The exception to this may be *Bordetella bronchiseptica*, which was detected only in nasal swabs. Finally, the data generated from this study suggested that molecular biology based techniques for detection of both FHV-1 and *Mycoplasma* species are an acceptable alternative to microbiologic culture techniques. Comparison of detection methods for other organisms, such as FCV, *Bordetella bronchiseptica*, and *Chlamydophila* species, would need to be carried out in a population that was first surveyed and determined to have an established prevalence of these organisms, perhaps in a retrospective manner using stored nucleic

acid from samples previously cultured for the organisms. Additionally, this study identified several cases in which traditionally defined primary pathogens were not identified, but in which *Mycoplasma, Pasteurella, and Moraxella* species were detected. Studies investigating the pathogenicity of theses strains should be conducted.

*Chapter 2*

In continuing to evaluate optimal sampling protocols in URTD, Chapter 2 describes the evaluation of the use of a previously developed q-rt-PCR assay for FHV-1 and demonstrated that the assay could discriminate between healthy cats and cats with significant upper respiratory disease by quantitating the amount of viral DNA present in nasal swabs. However, the assay failed to discriminate between a group of cats that had recently shown clinical signs of URTD but had recovered and either healthy cats or those currently clinical signs. Therefore, results must be correlated with clinical history in order to be useful in the clinical setting. Further studies using larger sample populations as well as the addition of two more disease groups consisting of animals affected with concurrent immunosuppressive systemic disease (i.e. neoplasia, diabetes mellitus) and a group of recently vaccinated cats would better evaluate the clinical utility of this assay.

*Chapter 3*

Evaluation of an antigen specific proliferative response using a small volume of blood due to the age of the animals was necessary in order to assess the cell-mediated immune response to FHV-1 vaccination for the study conducted in Chapter 4. And so Chapter 3 describes the evaluation of an assay to fulfill this need using easily acquired reagents. The use of tritium-labeled thymidine does restrict the use of this assay to specialized laboratories. Conversion of this assay from a radioactive isotope based assay

to one based on the Alamar Blue™ technology would allow further utility of this assay. Along with previous developed ELISAs for FHV-1 antibodies, the completion of Chapter 3 gave an assay that allows in vitro investigation of both arms of the immune system.

*Chapter 4*

Concurrent oral administration of a proprietary probiotic and administration of a multivalent URTD vaccine demonstrated that moderately term administration does not adversely affect the developmental parameters of kittens. Unfortunately, only numerically greater means were detected in many of the measures of humoral and cell-mediated immune responses. This may be secondary to insufficient sampling and treatment time and the lack of viral challenge in order to evaluate clinical signs, the most significant outcome. Assessing the effect of supplementation during or after challenge with FHV-1 on clinical signs may be a more valuable test of efficacy. Additionally, because of probiotics' effect on local gut health, this supplement may be more useful to ameliorate the clinical signs of gastrointestinal infections in contrast to the application to a respiratory disease.

*Chapter 5*

In an attempt to improve therapeutic options for URTD, the use of a novel immunotherapy for use in cats in which upper respiratory signs were more prolonged is described in Chapter 5. In the course of this study, we identified a surprisingly low detection rate of FHV-1 as compared to animals with presumptive acute disease from the same shelter. This pathogen has traditionally been associated with this syndrome, but based on our data, either the organism is an instigating factor but then plays no further role, is below the level of detection of our assay, or does not factor in the syndrome at all.

Additionally, we discovered that liposomes used as the placebo treatment in our study may have immunostimulatory properties independent of incorporation of additional substances as there was evidence of innate immune stimulation in even our placebo group animals. Our study produced data that suggests that, contrary to our initial hypothesis, there is no systemic bias toward the Th2 type cytokines in cats with this syndrome. This supports previously published findings that there is no local predilection for Th1 in the nasal cavity of cats with chronic rhinitis. The use of the liposome preparation to stimulate innate immunity may be more useful in primary viral infections in contrast to the more chronic process which was examined in Chapter 5. Additionally, if there truly is a local Th1 type bias in chronic rhinitis, daily local administration of an antiinflammatory Th2 type cytokine such as IL-10 in a vehicle that would allow expression until the next dose may improve the clinical signs.

*Chapter 6*

Finally, the development of a q-rtPCR for *Mycoplasma felis* will allow a more detailed evaluation of the role of this organism in URTD. The assay describe in this chapter is sensitive and as tested so far, specific. However, further samples should be evaluated, including *Ureaplasmas* and samples collected from the lower respiratory tracts of cats as well as the equine species, as this organism has been implicated in disease in that species as well. Development of an assay based on a fluorescent probe as opposed to detection of double stranded DNA will increase specificity and allow for the possibility of multiplex assays, perhaps in conjunction with other respiratory pathogens such as FHV-1 and FCV and housekeeping genes for normalization. However, the increased cost of this technology should be weighed against the possible benefits.