DISSERTATION

ASSESSMENT OF NOVEL STRATEGIES FOR THE PREVENTION AND TREATMENT OF FELINE UPPER RESPIRATORY TRACT INFECTIONS IN SHELTERS AND FELINE HERPESVIRUS-1 IN LABORATORY SETTINGS

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ABSTRACT

ASSESSMENT OF NOVEL STRATEGIES FOR THE PREVENTION AND TREATMENT OF FELINE UPPER RESPIRATORY TRACT INFECTIONS IN SHELTERS AND FELINE HERPESVIRUS-1 IN LABORATORY SETTINGS

Feline upper respiratory tract infection (URI) and its pathogens are ubiquitous in the feline population. Most URI cases are due to viral infections with feline herpesvirus-1 (FHV-1) and/or feline calicivirus (FCV) with secondary bacterial infections. After acute exposure to FHV-1, most cats develop persistent, latent infections with reactivation particularly during times of stress and immune suppression. Clinical signs including ocular and nasal discharge, sneezing, conjunctivitis, anorexia, lethargy, and pyrexia can vary in severity from mild and transient to severe and life-threatening. Preventive measures such as vaccination, stress reduction, environmental modifications, and infection control have lessened illness, recurrence, and spread, and many successful therapies such as antibiotics for secondary bacterial components, systemic and ocular antivirals for FHV-1, supportive care, and non-specific immune stimulation have helped to reduce the severity of illness and decrease mortality in cats. Despite these advancements in management strategies, many cats and kittens continue to suffer from URI, and those in crowded environments continue to become severely ill and either die or are euthanized. Furthermore, many animal shelters still lack information and resources regarding successful implementation of URI prevention and treatment protocols, and thus URI remains one of the most common medical reasons for euthanasia in shelters. This syndrome results in poor quality of life, and extended lengths of stay in shelters can lead to high financial burdens. Further work

is needed to better understand the pathogenesis of the syndrome as well as improved preventives and treatments. The goals of the work described in this dissertation were to evaluate novel preventive and treatment strategies to decrease the incidence and severity of URI in shelters and with an emphasis on FHV-1 in experimental studies.

This body of work was conducted in both the controlled research environment as well as in the animal shelter environment. Chapter 1 provides an overview of URI with a specific focus on FHV-1 and FCV and Chapter 2 presents the brief research objectives for each of the studies in this body of work. Three of the studies (Chapters 4, 5, and 7) in this body of work evaluated novel immune stimulants and preventive measures for primary FHV-1 infection and recrudescent FHV-1 in purpose-bred, experimentally infected cats in a controlled research setting. Chapter 4 evaluated a plant-based nutraceutical, Carnivora™, with anti-inflammatory and immune modulating components and its effects on recrudescence of clinical signs and viral shedding in young adult cats upon repeat challenge of FHV-1. Our study found that cats that were administered Carnivora™ had significantly less clinical manifestations of FHV-1 disease when compared to the control group. Chapter 5 assessed a new mucosal formulation of a liposomal toll-like receptor immune stimulant (LTC) as both a preventive and treatment for FHV-1 in purpose-bred kittens. This study found that administration of LTC as a preventive 24 hours prior to FHV-1 challenge resulted in some positive clinical effects and decreased shedding of FHV-1 DNA, whereas administration of LTC as a treatment during illness with FHV-1 did not influence clinical course of FHV-1 illness. Chapter 7 explored the use of a pheromone product in these same purpose-bred kittens and its effects on stress reduction, relaxation, and recrudescence of FHV-1 clinical signs. Results indicated that the pheromone product decreased stress, increased relaxation, and decreased some of the clinical signs of FHV-1 recrudescence in the kittens.

Two of the studies (Chapters 3 and 6) evaluated novel immune stimulants and preventives in open-admission shelter environments. Chapter 3 explored whether the addition of an inactivated, broader spectrum FCV vaccine to a standard vaccination protocol at a shelter, would result in decreased incidence, duration, and severity of URI and oral ulceration in cats. The study did not find evidence that the additional vaccine protected cats from developing URI, severe URI, or oral ulceration indicative of calicivirus. Chapter 6 evaluated administration of the LTC discussed in Chapter 5 to cats in an open-admission shelter. Cats were administered the LTC upon admission to determine whether it would result in decreased incidence and severity of URI. The study did not find significant evidence that the LTC protected cats from developing URI or severe URI in the shelter, nor did it significantly impact clinical course of illness. Although neither of the shelter experiments had significant findings regarding the preventive product being tested, results provided additional important information regarding immune compromise and potential for immunomodulatory therapeutics and stimulation in the shelter environment, risk factors contributing to URI onset, timing, prevalence, severity, and outcomes in shelter environments.

The work described in this dissertation has increased our knowledge of FHV-1 preventive and treatment options and mitigating and risk factors that might contribute to URI occurrence, recrudescence, and resolution. We hope that the findings in this work will help to decrease prevalence and severity of URI and improve the outcome for cats and kittens with URI, especially in the shelter environment.

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Through working with and for Dr. Lappin, I became acquainted with worlds of which I had minimal previous knowledge or experience: industry, clinical research, grant writing and funding, purpose-bred animals, and ultimately animal welfare. I learned through observation,

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It also cannot go without saying that it is because of Dr. Lappin and the work we did together, that I quite unexpectedly became a devout feline enthusiast and advocate, a feline veterinarian, and a self-avowed crazy cat lady. Dr. Lappin is also at least indirectly responsible for several of the cats (and one dog) that are now permanent members of my household but that were formerly study failures and apparently my foster failures.

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DEDICATION

This dissertation is dedicated to the animals, some of whom are no longer with us:

the cats: Baloo, Bambi, Christine, Duchess, Edgar, Madame, Oakley, Rajah, Sheer-Khan, Toulouse, Vesper, Waldo, Marnie, Mowgli, Napoleon, O'Malley, Phantom, Skye, Abigail, Bagheera, Lafayette, Nala, Rupert, Sarabi, Simba, Aria, Duke, Fred, Knight, Lady, Pina, Scooby, Sven, Bran (Mikey), Daphne, Kristoff, Merylin, Noria, Olaf, Summer, Velma, Elenacat, Persephone, Kahuna, all the shelter cats, and to snot cat #1:

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and to my dear, departed, beloved amputee dogs, Torpedo and Cotee.

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1.1 Overview of Feline Upper Respiratory Infection (URI)

1.1.1. Etiology and pathogenesis

Feline upper respiratory infection (URI) refers to a multifactorial disease complex commonly caused by one or multiple contagious viral and/or bacterial organisms. It is widespread and an important factor in morbidity, namely in cats in group or crowded settings that can include shelters, multicat households, boarding facilities, and catteries.[1–7] It is usually an acute disease of less than ten days duration but can also be chronic.[2,8] Mortality is more likely to occur in young kittens, geriatric debilitated cats, or immune compromised cats and in crowded and unhygienic conditions.[2,9,10]

It is generally thought that most URI cases, especially in shelter or crowded settings, are due primarily to viral primary infections with secondary bacterial infections.[1,2,8,11] The viral pathogens responsible for most URI infections are feline herpesvirus-1 (FHV-1, Chapter 1.2) and feline calicivirus (FCV, Chapter 1.3).[2,3,9,12] Cellular damage and cytolysis occur in the respiratory and conjunctival mucosa during FHV-1 infection,[13,14] and epithelial cells in oral and respiratory tissues undergo apoptosis and epithelial necrosis during FCV infection.[15–17] The respiratory, oral, and ocular tissues are therefore compromised and damaged by viral infection, also decreasing respiratory immune function, thus increasing susceptibility to secondary bacterial infection. The main bacterial pathogens implicated are gram-negative bacteria that include Chlamydia felis, Bordetella bronchiseptica, and Mycoplasma felis, and some bacteria might be primary pathogens as well.[2,5,7–9,18–21]

1.1.1.1. Viral pathogens (Please also see Chapters 1.2 and 1.3)

Feline herpesvirus-1 is a double-stranded DNA virus that is a member of the Alphaherpesvirinae subfamily consisting of mucosal pathogens characterized by rapid spread between cells, acute cytolysis, and latent infections within the sensory ganglia of the host.[22–26] Infection occurs preferentially through the mucopeithelial cells of the oral and nasal respiratory mucosa as well as conjunctival mucosa and corneal epithelial cells.[13,14,27,28] FHV-1 is primarily transmitted via oronasal and ocular secretions in acutely infected cats.[23,29,30] The incubation period is between 2-6 days and intermittent shedding can occur throughout life, as most cats are latently, persistently infected and become carriers of the virus.[13,14,26,27] Clinical signs can range from subclinical and very mild to severe. These include sneezing, serous to mucopurulent nasal discharge and congestion, as well as pyrexia, ocular disease, conjunctivitis, chemosis, and serous to mucopurulent ocular discharge.[25,30–32]

Feline calicivirus, a member of the Caliciviridae family and Vesivirus genus is a single-stranded nonenveloped RNA virus that, because of its positive-sense RNA genome, lacks proofreading ability and has a high mutation rate and many isolates.[16,33–35] FCV mainly replicates in oral and respiratory tissues and epithelial cells, forming vesicles that rupture, inducing epithelial necrosis.[16,17,36] FCV transmission mainly occurs via oronasal or conjunctival routes through direct contact with an acutely infected cat.[17,37] Incubation period is between 2-10 days, and shedding can persist for months or more.[16,38–40] Persistence in the environment can be up to several weeks.[37,41–43] Clinical signs can include pyrexia, conjunctivitis, and rhinitis; oral ulceration is one of the characteristic lesions, and other clinical signs can include lameness.[9,44–46] The highly virulent systemic strain (VS-FCV) causes severe systemic disease and a systemic inflammatory response.[35,47–49]

1.1.1.2. Bacterial pathogens

Chlamydia felis is an obligate intracellular gram negative bacteria that infects and replicates in epithelial cells of the conjunctiva and is spread mostly through direct or close contact between cats and ocular secretions.[50,51] Incubation period is between 2-5 days and shedding can persist for 60 days after infection.[50–52] Chlamydia felis mostly causes ocular clinical signs including conjunctivitis, chemosis, and hyperemia in feline URI and can also cause upper respiratory signs.[50,51,53]

Mycoplasma felis is a gram-negative bacteria that lacks a cell wall and is found in the conjunctival and respiratory mucous membranes in cats. Although it is a normal commensal organism in the upper respiratory tract, there is growing evidence that Mycoplasma felis has a role as a secondary or even primary pathogen in cats with URI or conjunctivitis.[19,21,54–56] It is thought that Mycoplasma felis might proliferate in cats exposed to unhygienic conditions, overcrowding, concurrent bacterial or viral infections, immunosuppression, or other stressors.[10,57] Clinical signs in the upper respiratory tract can include serous to mucopurulent ocular and nasal discharge, sneezing, conjunctivitis and conjunctival hyperemia.[57]

Bordetella bronchiseptica is an aerobic gram-negative bacterium that can colonize the respiratory tract and cause upper respiratory illness in cats. Transmission is mainly through shedding of organisms through oral and nasal secretions and aerosolization from infected animals and through fomites.[58–60] Bordetella bronchiseptica can persist in the environment for ten days, and shedding from infected cats can occur intermittently for more than a month.[60] Infection is more likely to be associated with crowded and contaminated environments, poor hygiene, group-housing, and contact with dogs.[9,59–61] Incubation period is typically two to six

days.[58,62,63] URI clinical signs that can be attributable to Bordetella bronchiseptica infection include sneezing, serous to mucopurulent ocular or nasal discharge, conjunctivitis, fever, and cough, and uncomplicated cases can resolve within ten days.[58–61,63]

Other secondary bacterial pathogens in cats with URI might include Staphylococcus spp., Streptococcus spp., Pasteurella multocida, and other Mycoplasma spp.[8,64,65]

1.1.1.3. Role of stress in etiology and pathogenesis

Cats that are stressed in a shelter have been reported to be those that are more likely to develop URI as compared to cats with lower stress scores in a shelter.[66] Unpredictability of handlers and environments, altered feeding schedules, altered husbandry activities, cage confinement, impoverished environments, non-stimulating environments, and lack of hiding resources, have been found to cause stress in cats.[67–72] Furthermore, changing the housing status of group housed cats to cages has also been shown to be a trigger for FHV-1 associated disease.[73] Stress also can result from aversive stimuli, such as noise, odors, uncomfortable temperatures, unfamiliar people, animals and environments, as well as unpredictable handling. Even minor changes, such as moving from one cage to another or being placed in a carrier, can be significantly stressful for cats. Stressful effects of aversive stimuli are amplified when events are unpredictable or the animal lacks the opportunity to modulate their effects through behavioral responses.[74]

The mechanism by which stress contributes to URI prevalence or reactivation is unclear. While acute stress can be adaptive, allowing the animal to cope with and avoid or lessen the impact of the stressor, “distress,” can lead to a damaging pathophysiological reaction in the animal, leading to faulty immune responses and disease susceptibility.[75,76] Thus it is this distress,

leading to chronic stress that can be problematic to the health of an animal. Stress can suppress immune system function and potentially reactivate infectious disease.[76,77] If an animal is unable to cope with a stressor or the necessary coping efforts are physiologically demanding, health and immune compromise and pathology can occur due to a continued and sustained over-utilization of the neuroendocrine system.[77] This is a complex interplay between host response to infection, genetic susceptibility, infectious organism, and host response to environment, both perceptually and pathophysiologically. The activation of the stress response system is dependent on individual history, the context in which the stressor occurs, and the expectation the individual has for the outcome of the event.[78,79] The stress response encompasses both immunologic, neurologic, and vascular changes which are associated with or result in the behavioral observed response of stress.[78,80]

1.1.2. Epidemiology

URI can occur in any cat, but URI in the shelter setting poses unique challenges in managing and treating this very frustrating syndrome. Management of URI in shelters is difficult due to the multiple pathogens implicated, the multifactorial etiology, carrier states of the pathogens, multiple cats from different environments, and the inherently stressful situation of a cat entering a new shelter environment.[66,81–83]

There are multiple risk factors for URI in the shelter that household cats do not necessarily experience. For instance, some of the risk factors for owned cats include being less than 1 year of age, intact status, winter season, multicat households, recent antibiotic therapy, and presence of dogs in the household.[9,12,84] In the shelter, risk factors can include those as well as extremely high population density and overcrowding, multiple cats of different ages and

backgrounds, small living enclosures, multiple housing changes, extremely stressful and novel, foreign environment, increased length of stay in the shelter, hygiene and air quality issues, as well as high exposure to multiple pathogens in large quantities.[6,9,66,82,83] And despite shelter intake vaccination protocols, URI remains one of the top diseases of concern to shelters, resulting in high financial burdens, poor quality of life, and extended lengths of stay.[4,6,7,83,85] Although URI is considered ubiquitous within the feline population, there are few studies that define prevalence of URI in the household as opposed to the shelter. Nevertheless, prevalence of URI in household cats has been estimated at approximately 38% in one study.[12]

Mean or median time to develop URI in the shelter has been reported as 8.3 days,[66] between two to eight days for carriers of URI implicated pathogens,[83] or six days in kittens and seven days in adults.[6] Risk of developing URI has been reported to increase with time in the shelter in that over 80% of cats had a risk of URI after two weeks.[6] Prevalence estimates of URI in shelters have ranged between 3% and 58%;[5–7,66,82,83,86] (Table 1.1) however, true prevalence is difficult to calculate due to variation in detecting, defining, and reporting URI in different shelters.

Table 1.1: Examples of studies evaluating URI prevalence in shelters

Reference	Type of facility	Prevalence (%)
Aziz et al 2018	Open admission relocated/transported cats	26%
Wagner et al 2018	9 different shelters	17 (3% to 29%+)
McManus et al 2014	Short-term shelters Long-term sanctuaries Foster care programs TNR programs	44% 57% 44% 48%
Gourkow et al 2013	Open admission SPCA	34%
Tanaka et al 2012	Open-admission municipal	58%
Dinnage et al 2009	Open admission	30%
Bannasch and Foley 2005	Open-admission and adoption guarantee	55%

1.1.3. Clinical signs

The clinical presentation of cats or kittens with URI is mostly similar with a large amount of overlap regardless of the pathogen(s) involved. Clinical signs can range from very mild to very severe and depend on the pathogen(s) involved, pathogenicity of strains and infective dose, number of coinfections, environment, stress, nutrition, individual differences, and individual immune systems.[1,2,12,78,87,88] Sneezing has been reported as the most common clinical sign in URI.[12,89] Other common clinical signs are serous or mucoid nasal discharge, mucopurulent nasal discharge that occurs with inflammation, bacterial infection, and lengthier viral infection, ocular discharge, chemosis, and conjunctivitis, while severe cases also have pyrexia, inappetence, cough, oral or nasal ulceration, and dyspnea.[1,2,12,18,30,88] Some clinical signs might be more common with one pathogen versus another. For instance, limping and oral ulceration are more common with FCV.[16,17,35,44,47] Corneal dendritic ulcers and dermatitis are related to FHV-1.[14,30,90,91] Conjunctivitis without concurrent nasal clinical signs might be indicative of

Chlamydia felis.[2,50,53] Cough can be associated with Bordetella bronchiseptica, although it could also be related to other infections progressing to the lower respiratory tract.[59–61,92]

1.1.4. Diagnosis

The definitive cause of URI is complicated due to the overlapping clinical signs of all pathogens, coinfections are common especially in crowded environments with multiple cats, detection of organisms does not necessarily correlate with cause of illness and clinical signs, clinically healthy animals shed pathogens, vaccination interference, inadequate sample handling procedures and assay detection thresholds, and some pathogens are shed in low and sometimes undetectable numbers or genetic variations.[2–4,12,30,55,93,94] Most cats have resolution of clinical signs without need for diagnosis of the specific pathogens involved, and testing in acute, uncomplicated single cases is not recommended.[3,8,18]

Diagnostic tests might be used if clinical signs are severe and persistent or in outbreak situations in shelters where further diagnostic evaluation would alter treatment and management strategies.[18,95,96] Assays available for viral diagnosis include virus isolation, indirect fluorescent antibody testing, ELISA, and PCR.[17,30,93,94,97–99] Assays available for Chlamydia spp. diagnosis include cell culture from conjunctival swabs, conjunctival smear cytology, serum antibodies, and PCR from conjunctival swabs, scrapes, or biopsies.[3,55,100] Assays available for Mycoplasma spp. diagnosis include culture of oropharyngeal or other tissue swabs or PCR; however, cultures are not as sensitive, and PCRs might amplify the commensal Mycoplasma spp. and dead organisms. Therefore, tests should be interpreted with caution and in conjunction with clinical signs.[56,57,101] Assays available for B. bronchiseptica diagnosis include aerobic bacterial culture from oropharyngeal swabs, PCR, and serology for antibodies against B. bronchiseptica antigens.[58–60]

Each of the assays has multiple limitations, false positives, and false negatives; therefore, each should be interpreted cautiously and in conjunction with clinical signs, clinical course, number of ill animals, vaccinations, and lack of response to empirical treatment. Although a feline URI PCR panel can test for multiple viral and bacterial pathogens in a single sample,[11,102] interpretation of PCR assay results in individual cats is typically not helpful since all viral and bacterial causes of URI in cats can be harbored by healthy cats.[8] The International Society for Companion Animal Infectious Diseases suggests that the use of feline URI PCR panels is best reserved for testing multiple cats in an outbreak situation in an attempt to determine causation.[8] If a shelter has an outbreak with mortality, necropsy and histopathology should be performed.[35,103,104]

1.1.5. Treatment

Supportive care with attention to fluid restoration, food intake, nutrition, and a reduction of stress is important in all management plans, and this might be all that is necessary in uncomplicated cases.[2,18,25,32,105,106] Appetite stimulants such as mirtazapine or cyproheptadine might be considered due to potential blockage of nasal passages thus loss of smell, potential oral pain due to ulceration, and general malaise and inappetence. Nasal and ocular discharge should be cleaned, and nebulization and airway humidification can also be considered.[2,10,105,107,108]

Because many respiratory infections are viral, and considering the increase in antibiotic resistance, antimicrobial treatment should be reserved for cats if the clinical course is not resolving and if the cat might be suspected to have a bacterial infection.[8,18,109] However, although purulent nasal or ocular discharge may increase suspicion of bacterial infection, viral infections can also recruit large numbers of neutrophils, decrease normal secretions, and thus result in

purulent discharge.[30] If supportive care does not resolve clinical signs, and if there is a suspicion of bacterial infection, the antibiotic treatment of choice for upper respiratory bacterial infections is doxycycline; Bordetella bronchiseptica, Chlamydia felis, and Mycoplasma spp. are susceptible to doxycycline.[8,50,59,101,110]

Extra-label antiviral therapies have been used for the treatment of FHV-1. Famciclovir, an orally administered prodrug of penciclovir, is sometimes used for the treatment of severe acute infections, although clinically beneficial doses might vary.[111–114] Ocular anti-viral drugs that have been used in the treatment of human herpesviruses, have also been used for FHV-1. Ophthalmic applications have included idoxuridine, trifluridine, and cidofovir, although idoxuridine and trifluridine necessitate frequent application and are also not well tolerated by cats.[105,113,115,116]

There are currently no safe antiviral medications for FCV infection in cats.[16,17,108]

1.1.5.1. Immune stimulation and protection

Another approach in the treatment of upper respiratory illness has been to upregulate innate immune responses to provide benefits through non-specific stimulation. In one study, prolonged feeding of the probiotic Enterococcus faecium SF68, which has been shown to enhance T-helper lymphocyte numbers in cats, lessened morbidity associated with chronic FHV-1 infection in some cats during stress associated with changing from group housing to individual cage housing.[73]

Although vaccinations, in general, target the adaptive immune system, the innate immune system is necessary for a vaccination to be effective.[117,118] Cell mediated immunity (CMI) can be activated by adjuvants, peptides, lipids, carbohydrates, or nucleic acids within the vaccine, the

vaccine vector, or the modified live pathogen itself, as these are substances introduced into the host, and they are typically recognized as foreign substances by the innate immune system.[117–119] It is therefore thought that the innate cell-mediated immunity provided by a vaccine potentially targets pathogens not included in the vaccine itself. The use of an intranasal vaccine has provided results suggesting immune modulation might be effective in controlling or treating cats with signs of upper respiratory tract disease (URTD).[120–123] Administration of an intranasal vaccination has been shown to induce cross protection against pathogens and decrease some clinical signs of URI, thus imparting non-specific immune responses.[120–122] The intranasal formulation might be effective because it is eliciting local IgA mucosal responses directly in the upper respiratory system.[124–126]

Modulation of cellular activity through toll like receptor (TLR) recognition and signaling, is a target for immunotherapy against viral or microbial infections.[127–129] One immunotherapy platform is based on the triggering of innate immune responses using TLR9 agonists complexed to cationic liposomes; this greatly enhances the activity of the TLR9 agonist.[127,128] In a number of animal challenge studies, parenteral or inhalational administration of liposomal-TLR9 complexes has generated complete or nearly complete protection against highly virulent bacterial and viral pathogens.[129–134] In addition, administration of liposome-TLR9 complexes intraperitoneally (IP) to cats once weekly for four or six weeks resulted in lessening of clinical signs associated with feline URTD and an increase in neutrophils, monocytes, and CD4+ and CD8+ lymphocytes.[135] An intranasal formulation of a liposome-TLR complex (LTC) was developed that includes a TLR9 agonist, a TLR3 agonist, and methylcellulose as a mucosal adhesive agent.[136] In a study of healthy, purpose bred cats, cytokine and cellular immune responses to this LTC were evaluated in vitro and in vivo, and the LTC rapidly activated cat

leukocytes, including upregulation of co-stimulatory molecules and cytokine production.[136] A follow up study was performed to assess the mucosal administration of LTC prior to FHV-1 challenge in purpose-bred kittens in a research facility.[32] The mucosal administration of LTC 24 hours prior to FHV-1 challenge was associated with several positive clinical effects and with decreased shedding of FHV-1 DNA.

The lessening of stress has been shown to decrease signs of upper respiratory infection and shedding of organisms in shelter cats.[81,137] In shelters, stress reduction methods have included gentle stroking and vocalization, petting, grooming, playing, hiding boxes, hiding enrichment; minimal invasive daily cage cleanings.[5,67,68,74,81,137,138]

Feliway (Ceva Santé Animale) is a commercial preparation of feline pheromone fractions, which may be used as another stress reducing modality.[139] The Feliway spray has been shown to reduce other feline diseases sometimes associated with stress, such as urine spraying and feline idiopathic cystitis, and it has been shown to reduce signs of stress during transportation and improvement in appetite in a hospitalized setting.[140–146] It has also been suggested as an enrichment means for cats in the shelter environment.[147] A recent study also showed its efficacy in reducing stress levels when visiting a veterinary clinic.[148]

1.1.6. Vaccination

Although various management strategies have been studied to address feline URI in the shelter, vaccination is imperative.[74,117,149] Cats are vaccinated upon intake as standard of care in shelters, and the standard vaccine protocol is typically the administration of a subcutaneous (SQ) modified live (MLV) feline viral rhinotracheitis, calicivirus, and panleukopenia (FVRCP) vaccine.[117,150] The primary benefit of the MLV FVRCP vaccination is to prevent illness from

parvovirus/panleukopenia in cats entering the shelter.[117,150,151] However, another benefit of vaccination is the potential ability of the vaccine to initiate cell mediated immunity (see section above). Vaccination against FHV-1 and FCV-1 does not confer complete immunity or protection against subsequent infection, but rather helps to potentially lessen severity of illness if exposed.[97,152–154] Furthermore, FCV vaccines do not protect equally against the many field isolates of FCV.

Vaccines for Bordetella bronchiseptica and Chlalmydia felis are available, although they are only recommended for some cats in high-density environments with a history of those infections.[50,59]

1.1.7. Prevention

Although elimination of URI in a shelter environment might be unrealistic, substantially reducing frequency and lessening illness are achievable through the institution of good management strategies aimed at prevention. These strategies can also be used in the home environment.

Since overcrowding, high density environments, and small living enclosures are risk factors for URI, limiting the number of cats and providing larger and more diverse enclosures will reduce URI rates.[18,82,95,155–157] Similarly, extended lengths of stay are associated with increased URI risk, thus decreasing time in shelter should be pursued.[6,66,158,159] Proper, clean, good ventilation with an appropriate number of air exchanges is also necessary to reduce risk of URI, and air quality within the cat's microenvironment enclosure should also be considered.[18,95,96] Cleaning and disinfection should be performed with products effective against the most difficult of the pathogens, calicivirus, to eliminate it from the environment while also

being mindful of necessary contact times and fomite exposure and implementing staff training regarding cleaning and disinfection protocols.[18,96]

As discussed above, stress is an important risk factor for URI in shelters and homes, and this should be mitigated first.[66,76,137,160,161] Many aspects of shelter life or even home life can lead to stress in the cat. Lack of enrichment and barren environments cause stress and distress in cats.[67,70,78,162,163] Witnessing other conspecific distress can also be a stressful event for an animal.[162–165] Food deprivation, inconsistencies in feeding times and cleaning times have all been documented as stressors in cats.[69–71,78,162,163,166,167] As discussed above, stress reduction methods have included gentle stroking, playing, hiding boxes, hiding enrichment; minimal invasive daily cage cleanings, enriched enclosures.[5,67,68,74,81,137,138,156] Other management practices such as spot cleaning, reducing movement of cats to different cages, decreasing noise exposure, providing toys and scratching surfaces, minimizing handling, and socialization are recommended; furthermore, aversive handling for treatment or forceful medication administration could induce undue stress, and thus the stress of treatment must be weighed against the value of a particular treatment.[96,168–170]

1.2. Feline herpesvirus-1

1.2.1. Etiology

Feline herpesvirus-1 (FHV-1), a member of the Alphaherpesvirinae subfamily, is a linear, double-stranded DNA virus contained within a capsid in a glycoprotein-lipid envelope.[23,171] It infects the domestic cat and other members of the Felidae family. Members of the Alphaherpesvirinae subfamily are generally characterized by rapid spread between cells and acute cytolysis within the infected sites.[23,25] Alphaherpesviruses are typically mucosal pathogens

that have the ability for neuronal persistence and latent infections within the sensory ganglia of the host.[22,24] Although it is thought that FHV-1 isolates are mostly similar,[23,24] some isolates have been found to vary genetically and in glycoprotein expression and virulence,[172–174] and some attenuated vaccine strains exist.[24]

1.2.2. Epidemiology

FHV-1 is one of the most common infectious disease viruses in cats and is widespread in the domestic feline population whether a cat is clinically normal or ill.[55,93,175] FHV-1 is also responsible for a high level of morbidity and can contribute to mortality in some environments such as shelters or catteries.[7,11] Reported prevalence in shelter populations has ranged from 2% upon entry[83] to nearly 60% during a stay in the shelter.[7,176,177](Table 1.2) In sick shelter cats, the percentage of cats that are positive for FHV-1 DNA by PCR assay has been over 80% of ill cats, although sample sizes in those studies were very small, and the role of recent MLV vaccination in PCR detection was not detailed.[11,178] However, one study[123] found that kittens that were administered intranasal and subcutaneous MLV vaccines concurrently had significantly lower FHV-GAPDH ratios than unvaccinated kittens after challenge with FHV-1.

FHV-1 is primarily transmitted via direct contact between acutely infected cats in oronasal and ocular secretions and potentially by sneezed macrodroplets that can reach up to two meters.[23,29–31] However, FHV-1 is relatively unstable when aerosolized in regular respiratory secretions.[23,179] FHV-1 is also transmitted indirectly through fomites and contamination, although it is fragile in the external outside environment due to its glycoprotein-lipid envelope, and it is easily inactivated by common disinfectants.[18,23,31] Cats that are acutely infected with active signs of disease are those that are most likely to transmit the virus.[29,31]

Table 1.2: Examples of studies evaluating feline herpesvirus-1 prevalence

Healthy cats	Unhealthy cats	Overall	Reference	Method
Households				
Hx URI 0% No hx URI 1%	11% with URI	5%	Binns et al 2000	OP; VI
Hx conjunctivitis 3% No hx conjunctivitis 3%	Current conjunctivitis: 12% Current, hx conjunctivitis: 9%	7%	Low et al 2007	Conjunctiva; PCR
16%	5% w gingivostomatitis		Dowers et al 2010	Oral biopsy; PCR
	56%		Schulz et al 2015	OP, nasal, conjunctiva; PCR
Carrier suspect: 19-33% Healthy: 32-37%	22-31%		Veir et al 2016	OP; PCR
6% healthy	28% w URI		Fernandez et al 2017	Conjunctiva, OP; PCR
	24% w conjunctivitis		Fernandez et al 2017	Conjunctiva, OP; PCR
	16% w gingivostomatitis		Fernandez et al 2017	Conjunctiva, OP; PCR
Shelters				
4% at admission		52% after 1 week	Pedersen et al 2004	Conjunctiva; PCR
	85%		Veir et al 2004	OP nasal; PCR
		0-41%	Bannasch & Foley 2005	Conjunctiva, OP; PCR, VI
		20%	Zicola et al 2009	OP; PCR
2% at admission		10% on day 10	Gourkow et al 2013	Conjunctiva, OP; PCR
41%	59%		McManus et al 2014	Conjunctiva, OP; PCR
	94% 67%		Litster et al 2015 Litster et al 2015	Conjunctiva, nasal: PCR Conjunctiva, nasal: VI
24% at admission			Wagner et al 2018	Conjunctiva, OP; PCR
Combined household & shelter				
11% 28%	18% 33%		Maggs et al 1999 Maggs et al 1999	Conjunctiva; VI Conjunctiva; IFA
		66% 97%	Maggs et al 1999 Maggs et al 1999	Blood; SN abs Blood; ELISA abs
31% 11% 11%	14% 9% 8%		Burgesser et al 1999 Burgesser et al 1999 Burgesser et al 1999	Conjunctiva; PCR Conjunctiva; VI Conjunctiva; IFA
	27%		Hartmann et al 2010	Conjunctiva; PCR
Catteries				
49%	51%		Helps et al 2005	Conjunctiva, OP; PCR
Trap Neuter Return				
5%	17%		McManus et al 2014	Conjunctiva, OP; PCR

abs: antibodies
FHV: feline herpesvirus
Hx: historical
IFA: Immunofluorescent antibody
OP: oropharyngeal
SN: serum neutralization
URI: upper respiratory infection
VI: virus isolation

1.2.3. Pathogenesis

FHV-1 infection occurs preferentially through the mucoepithelial cells of the oral and nasal respiratory mucosa, and it can also invade the conjunctival mucosa and corneal epithelial cells with a preference for the conjunctiva.[13,14,23,25,28] The primary sites of virus replication in the acute phase are the mucosal cells of the nasal cavity including the nasal septum, turbinates, nasopharynx and tonsils, and replication can also occur in the conjunctivae, mandibular lymph nodes, and upper trachea.[13,23] Replication is rapid, and FHV-1 infected cells can be found in the oropharyngeal and nasal mucosae after 24 hours. Cellular damage and cytolosis occur in those affected cells, and clinical signs typically appear within 2-6 days after infection. The incubation period is between 2-6 days. The virus is typically detected for 7 to 21 days after infection, although viral DNA can be detected for longer periods.[14,23,25,27,180] It is not thought that viremia plays a large role in infection, spread, or reactivation.[1,23,181] Although FHV-1 has been found in the blood of some clinically ill cats and healthy cats, it has not been found in the blood of clinically ill cats in other studies.[181–183] It is thought that viremia may occur for a very brief period during primary infection but is less likely to occur in recrudescent disease.[181]

During active viral replication, there is neutrophilic infiltration, cell lysis, mucosal erosion and ulceration, and epithelial necrosis in the invaded mucosal and epithelial surfaces.[1,30,184,185] Mucosal erosion in the nasal passages results in nasal cartilage and bone exposure, leading to osteolytic damage in the nasal turbinates.[30,186] This damage could be permanent, and subsequent remodeling might contribute to chronic rhinitis. There is also an immune-mediated inflammatory component to FHV-1 infection that could also contribute to chronic inflammatory changes.[23,30,186]

Following acute exposure, most cats are latently, persistently infected and become carriers of the virus. The trigeminal ganglion is a site of latency of FHV-1, although viral DNA has also been found in other neural sites such as the optic nerve and olfactory bulb.[23,24,26,180] FHV-1 has a tropism for conjunctival cells and upper respiratory epithelium, and clinically normal cats have been found to have FHV-1 DNA in their cornea and conjunctiva; it has therefore been suggested that herpesvirus might reside in the cornea in its latent form.[23,24,31]

FHV-1 infection is characterized by intermittent episodes of spontaneous or immune-suppression induced shedding periods and reactivation of the virus. During reactivation, the nasal turbinates are among the first viral replication sites.[23,27,29,187,188] Reactivation and shedding can occur due to other concurrent disease, corticosteroid use, lactation, or after stressful events.[29,137,187,189,190] Stressful events can include housing changes, travelling, crowding, unpredictability, unfamiliarity, impoverished environments, and lack of hiding resources.[66,69,71,73,78,162,187,191,192]

The mechanism by which stress induces reactivation of FHV-1, is unclear. While acute stress can be adaptive, allowing the animal to cope with and avoid or lessen the impact of the stressor, "distress," can lead to a damaging pathophysiological reaction in the animal, leading to faulty immune responses and disease susceptibility[75,76]. Shedding can occur for 3 weeks after a stressful event; after the event, there is a lag phase of 4-11 days (mean 7.2 days), after which FHV-1 shedding occurs for an average of 6.5 days (range 1-13 days).[23,187] After viral shedding, there can be a refractory period during which the virus is less likely to be reactivated.[187,193,194] Some cats may show clinical signs during shedding and reactivation, while other cats are clinically normal during that period.[26,89,187] Risk factors for FHV-1 shedding have included

presence of upper respiratory illness, younger age, large number of cats in environment, and less sanitary conditions.[9,12]

1.2.4. Clinical signs

Clinical signs of FHV-1 infection can range from subclinical and very mild to severe. The uncomplicated typical clinical course of disease can last 10 to 21 days.[30] Kittens exposed to FHV-1 generally develop moderate to severe upper respiratory signs consisting of sneezing, nasal serous to mucopurulent discharge and congestion, as well as pyrexia, lethargy, inappetence, and ocular disease.[23,25,30,30] Ocular clinical signs of FHV-1 infection include conjunctivitis with hyperemia and chemosis, serous ocular discharge that can progress to mucopurulent ocular discharge by day five to seven of infection, and if cytolysis is severe enough in the mucosal surfaces of the conjunctiva, discharge can become serosanguineous.[25,30] In more severe and progressive disease, corneal ulceration and keratitis can occur with dendritic corneal ulceration that is the only pathognomonic clinical sign for FHV-1 infection.[14] Furthermore, severe conjunctivitis and ulceration with corneal ulceration potentially leading to symblepharon and resultant blindness. Chronic corneal tissue damage and associated inflammatory changes can lead to chronic stromal keratitis and blindness. In the neonatal kitten, if infection occurs prior to the opening of eyelids, ophthalmia neonatorum and conjunctivitis neonatorum can occur, sometimes leading to globe rupture and severe and permanent corneal damage.[25,30] Adults with recrudescent disease may have clinical signs as listed above, while other adults with recrudescent disease might have milder signs consisting of conjunctivitis, epiphora, and mild sneezing.[1,25,31]

Severe cases of FHV-1 infection can lead to lower respiratory involvement with secondary bacterial infections, coughing, dyspnea, pneumonia, and occasionally death. Severity

is dependent upon exposure, infective dose, viral isolates, comorbidities, age and immune status, and individual differences.[13,31,32,89,172]

Ulcerative dermatitis is also associated with FHV-1 infection although much less commonly than the other clinical signs.[90,91,195,196] Although rare, when ulcerative dermatitis occurs, it is usually found on the face periorbitally, on the nasal planum, or on the haired facial skin, and very rarely on the extremities or flank.[90,91,196,197] Lesions are usually characterized by ulcerations, erosions, vesicles, and crusts, and histological characteristics include epidermal and dermal ulceration and follicular necrosis, perivascular to diffuse inflammation with eosinophils and neutrophils, necrotic areas, and occasional intranuclear inclusion bodies.[90,91,196,198] It can be mistaken for eosinophilic granuloma complex or vice versa, and it is also possible that both can occur concurrently.[195,198] Many cases of FHV-1 associated ulcerative dermatitis also have concurrent or historical upper respiratory clinical signs.[91,195,196]

1.2.5. Diagnosis

Diagnostic test methods include PCR for amplification of FHV-1 specific DNA, virus isolation in culture, indirect fluorescent antibody (IFA) testing, and antibody detection by serum-neutralization and ELISA (Table 1.2).[30,93,97,98] Samples for evaluation are typically serum (serology) or swabs from the conjunctiva or other ocular tissues, oropharynx, or nasal mucosa for viral isolation or PCR assays.[93,98,100,199] Each of the diagnostic tests for FHV-1 has multiple limitations, false positives, and false negatives.

Clinical signs and the detection of FHV-1 by various molecular methods are not well correlated (Table 1.2).[7,21,98,175] Diagnosis of FHV-1 is complicated by latency and non-clinical infected cats that shed virus, clinically ill cats that do not shed virus, vaccination strains,

components of cell-mediated immunity, co-infection with other pathogens, and detection thresholds.[12,55,93,97,98,100]

Serologic titers are not helpful in diagnosis of illness due to FHV-1 infection, as serology does not distinguish between vaccine or virally-induced antibodies, serum neutralization titers are not high even after primary infection, and there is little correlation between seropositivity, titers, and clinical illness, although cats with FHV-1 antibodies have been found to be resistant to challenge.[30,31,93,97] IFA and viral isolation methods of testing are also unsatisfactory due to their lack of sensitivity and lack of correlation between results and clinical illness.[5,12,30,93] One study isolated FHV-1 virus in 11% of normal cats and only 18% of cats with clinical signs of disease.[93]

PCR can amplify FHV-1 DNA; however, test detection limits, shedding in clinically normal animals, and detection of non-viable virus, inhibit reliable interpretation.[12,23,30,55,93,98,181,200] An important drawback to PCR is that it can also amplify FHV-1 DNA from vaccines and therefore does not differentiate between vaccination or infectious strain.[180,200] In one study,[122] vaccinated kittens had increased FHV-1 DNA copy numbers as compared to unvaccinated kittens on days 0 and 4 of FHV-1 challenge, and in another study,[123] FHV-1 DNA was amplified from only those kittens that were vaccinated before challenge, and not in the unvaccinated group. PCR detection methods are also problematic in that DNA can also be shed intermittently and spontaneously without clinical signs, and so DNA can be detected in clinically normal animals, and therefore, levels of viral shedding do not necessarily correlate with severity of clinical illness.[55,98,187,200,201] And although quantitative PCR might detect more DNA, results do not correlate with clinical disease.[55,122] In two recent studies, neither the quantitative PCR nor titer FHV-1 values differed between the treatment and control groups, even though clinical signs did differ between groups.[89,189] In another recent study, qPCR method did not discriminate between

cats recently recovered from URTD and clinically ill cats with URTD; pharyngeal swabs detected FHV-1 DNA in 37% of healthy cats, 33% of suspect carriers, and only 22% of the clinically ill cats.[175] Positive results from PCR should therefore be interpreted cautiously.

Diagnosis for FHV-related ulcerative dermatitis is typically performed by biopsy and histopathology using IHC for detection; PCR is not recommended due to its overly high sensitivity and detection of positives.[195,198]

Diagnosis of feline herpesvirus as the cause of upper respiratory tract disease is therefore typically based on clinical signs including respiratory and/or ocular disease.[93] Clinical signs can be used to initiate supportive therapy without a positive diagnosis of feline herpesvirus if diagnosing within catteries or shelters. Since coinfections with other pathogens are likely, providing treatment based on suspected pathogens seems reasonable.

1.2.6. Prevention and vaccination

Vaccination against FHV-1, like infectious exposure to FHV-1, does not confer immunity or protection against subsequent infection, but rather helps to potentially lessen severity of illness if exposed.[105,122,153,154,187] Vaccination against FHV-1 is considered a core vaccine component, and it is commonly combined in a vaccine also against FCV and panleukopenia.[108,117,202] There is a modified live (MLV) subcutaneous and intranasal vaccine and an inactivated subcutaneous vaccine against FHV. All are based on the same FHV serotype.[105]

Although vaccinations, in general, target the adaptive immune system, the innate immune system is necessary for a vaccination to be effective.[117,118] Innate CMI can be activated by adjuvants, peptides, lipids, carbohydrates, or nucleic acids within the vaccine, the vaccine vector, or the modified live pathogen itself, as these are substances introduced into the host, and they are

typically recognized as foreign substances by the innate immune system.[117–119] It is thought that cell-mediated immunity plays an important role in protection against FHV-1 illness.[97,152,154,203,204] Non-specific immune responses have been found in cats exposed to FHV-1 challenge; for instance, one study found that cats without detectable antibody to FHV-1, were resistant to challenge with FHV-1, suggesting that CMI might contribute to protection.[97,154] Another study suggested that an early CMI effect was responsible for a decrease in FHV-challenge related clinical signs as early as seven days after vaccination with either the MLV or inactivated vaccine.[154] It is also thought that the intranasal FHV-1 vaccine is better able to modulate non-specific immune responses against pathogens including FHV-1, occurring with more rapidity than vaccination with a subcutaneous vaccine.[120,122,123,204] One study showed that after FHV-1 challenge, a significant reduction in clinical scores was noted in kittens as soon as 4 days after administration of 1 dose of an intranasal vaccine; this occurred prior to the development of specific FHV-1 immune responses. This CMI protection might persist for several months after the IN vaccination.[122,203]

1.2.7. Treatment

Although there are no labeled drugs for treatment of FHV-1, clinical efficacy can sometimes be achieved with various treatment modalities. Supportive care with attention to fluid restoration, food intake, nutrition, and a reduction of stress are important in all management plans, and this might be all that is necessary in uncomplicated cases.[25,32,89,105] Appetite stimulants such as mirtazapine or cyproheptadine might be considered. Broad-spectrum antimicrobials (doxycycline 10mg/kg PO once daily or 5 mg/kg PO twice daily) might be necessary in severe

illness if potential secondary bacterial infection is suspected.[8,23,105] Nasal and ocular discharge should be cleaned, and nebulization and airway humidification can also be considered.

Extra-label antiviral therapies have also been used for the treatment of FHV-1. Famciclovir, an orally administered prodrug of penciclovir, is sometimes used for the treatment of severe acute infections, although clinically beneficial doses might vary.[111–114] Ocular anti-viral drugs that have been used in the treatment of human herpesviruses, have also been used for FHV-1. Ophthalmic applications have included idoxuridine, trifluridine, and cidofovir, although idoxuridine and trifluridine necessitate frequent application and are also not well tolerated by cats.[105,113,115,116]

Adjunctive therapies and preventives have included the administration of the amino acid L-lysine that has been widely used and recommended. Results, however, have been variable with some negative results with use of L-lysine.[106,193,205,206] In vitro studies have shown antiviral effects of feline or human recombinant interferon, but clinical trials have had mostly negative results, although one reported clinical improvement in upper respiratory tract disease in some shelter cats treated with high dose interferon-alpha.[121,207–210]

Another approach in the treatment of FHV-1 illness has been to upregulate innate immune responses to provide benefits through non-specific stimulation. In one study, prolonged feeding of the probiotic Enterococcus faecium SF68, which has been shown to enhance T-helper lymphocyte numbers in cats, lessened morbidity associated with chronic FHV-1 infection in some cats during stress associated with changing from group housing to individual cage housing.[73] The use of an intranasal vaccine has also provided results suggesting immune modulation might be an effective treatment (see “Vaccination” section above).

One reportedly immune enhancing product is Carnivora™, a commercial preparation derived from the extracts of Dionaea muscipula, the Venus fly trap carnivorous plant species. The product contains compounds such as the naphthoquinones hydroplumbagin, plumbagin, and droserone, phenolic acids such as gallic acid, and flavonoids such as quercetin.[211–213] Studies have shown that Carnivora™ and these compounds have immune modulatory, anti-inflammatory, anti-cancer, and antiviral activities in in vitro and some in vivo models.[212–216] Carnivora™ has also been used in some pets and for the treatment of herpesvirus in humans.[213,217,217,218] In Chapter 4, we discuss the use of Carnivora for prevention of FHV-1 recrudescence.

Considering stress is thought to play a role in the reactivation of FHV-1, the lessening of stress might also be considered as a preventive or treatment for FHV-1 illness.[23,74,137,187] There have been some reports of stress reducing modalities in shelters resulting in an overall lessening of upper respiratory illness and shedding of upper respiratory organisms.[81,137] In shelters, stress reduction methods have included gentle stroking, petting, grooming, playing, hiding boxes, hiding enrichment; minimal invasive daily cage cleanings, and large, sanitary, uncrowded habitats[5,67,68,74,81,137,138]

Feliway (Ceva Santé Animale) is a commercial preparation of feline pheromone fractions, which may be used as another stress reducing modality.[139] The Feliway spray has been shown to reduce other feline diseases sometimes associated with stress, such as urine spraying and feline idiopathic cystitis, and it has been shown to reduce signs of stress during transporation and improvement in appetite in a hospitalized setting.[140–146] It has also been suggested as an enrichment means for cats in the shelter environment.[147] A recent study also showed its efficacy in reducing stress levels when visiting a veterinary clinic.[148] In this body of work, Chapter 7

evaluated the effects of Feliway on kittens when exposed to the stress associated with changes in housing and being confined in a kennel and resultant effects of FHV-1 recrudescence.

1.3. Feline FCV

1.3.1. Etiology

Feline calicivirus (FCV), a member of the Caliciviridae family and Vesivirus genus is a small, positive-sense single-stranded nonenveloped RNA virus.[33] Because of its positive-sense RNA genome, it lacks proofreading ability and thus has a high mutation rate allowing it to diversify and adapt to new environments and also evade vaccination targets. Multiple strains (having greater than 20% variation in the nucleotide sequence of one of the capsid regions) of the virus have been identified and carry varied pathogenic potential.[35] Interestingly, when outbreaks of the highly virulent systemic FCV have been sequenced, each strain has been distinct, thus some of the mutations seem to evolve independently.[16,219] The E region of the virus's capsid protein p66 is thought to be responsible for much of the genetic and antigenic variability of the virus; the E region, the binding site for virus neutralizing antibodies, has two hypervariable areas, thus allowing escape mutants to evade the host's immune system.[220–222] Many isolates exist; one study found that at least 16 isolates were present in the cats in a well-managed shelter.[223] Another study found 123 strains in the United Kingdom over nine months and 41 strains in two separate communities over 14 months.[34] Furthermore, none of the strains appeared to outcompete the others with a maximum prevalence for any strain of 5% in the country and 14% in the communities.[34]

The viral mutations combined with the cat's genetic immunologic factors very likely contribute to how the strain affects the cat and others in its vicinity.[48,224] Although there are

many isolates with antigenic variation, serum neutralization studies have found cross-reactivity to different isolates and thus a large amount of antigenic overlap.[16,44,221,222,225,226] This has led to the belief that all strains belong to the same serotype, although other studies have suggested that more serotypes exist.[44,227,228]

1.3.2. Pathogenesis

FCV transmission can occur through direct contact with an acutely infected or carrier cat via the oral, nasal, or conjunctival routes, or transmission can occur indirectly through fomites within the environment. Persistence in the environment has been reported from several days to several weeks depending on the conditions.[37,42,43,229] The incubation period is approximately two to 10 days but can be less than 24 hours in the virulent systemic hemorrhagic form.[41]

On a cellular level, when cells are infected with FCV, the cells undergo apoptosis. This occurs through the loss of mitochondrial membrane potential, Bax translocation to the mitochondria, release of cytochrome c from the mitochondria, and then activation of caspase 9 and the executioner caspase 3 which results in apoptosis.[15] Outwardly, vesicles form in tissues and epithelial cells, particularly on the tongue margin. The vesicles become ulcers and rupture and necrosis of the epithelium occurs along with an infiltration of neutrophils surrounding the periphery of the lesions.[16,17,36]

The virus mainly replicates in the oral and respiratory tissues, although the virus has been found in visceral tissues and bodily fluids such as skin, blood, urine, feces, and joint fluid.[16,44] The joint lesions consist of a synovitis and macrophage-like cells in the joints[16,46] Viral shedding mainly occurs through oral, nasal, and ocular secretions.[16,41,224] There is high shedding in some cats and carrier states with up to 75 days post infection, with some cats potentially shedding

virus for up to two years as persistent shedders.[16,17,38,224] Chronic carriers and persistent infections also occur and are thought to be related to viral antigenic changes occurring in the cat, resulting in isolates that allow escape from the host immune response and resultant persistent infections.[221,230] Persistent infection might also be due to reinfection or coinfection considering the large number of isolates and weak antigenic cross-protection.[34,225] Furthermore, vaccinated cats can become carriers of the vaccine strain.[231–234]

1.3.3. Clinical signs

Feline calicivirus is one of the more common pathogens associated with upper respiratory infection in cats.[7,9,17,44,100]. Oral ulceration is one of the characteristic lesions. Pyrexia, conjunctivitis, rhinitis, and other upper respiratory clinical signs are also common. However, respiratory disease is not always present when infection is induced during experimental challenge with FCV or in suspect cases.[44,45] So although FCV is mostly a respiratory and oral pathogen, a variety of clinical signs have been attributed to FCV and its many strains.[35,46,47,49,235] Other clinical signs can include acute lameness ("limping syndrome"), ulcerative dermatitis, and pneumonia.[16,17]

The highly virulent systemic strain (VS-FCV) causes severe systemic disease and a systemic inflammatory response and is associated with hemorrhagic-fever, vascular compromise, multiple organ dysfunction, shock, and a high mortality.[16,35,41,46,47] Some of the characteristic signs are cutaneous edema mostly on the head and limbs and ulcers on the skin, paws, nose, lips, and ears.[41,49,224]

FCV has also been associated with chronic lymphoplasmacytic gingivitis stomatitis (LPGS).[16,236,237]

1.3.4. Epidemiology

FCV is ubiquitous across feline populations worldwide (Table 1.3). A generally high prevalence is likely associated with various factors including the shedding and carrier states mentioned above. Infection and illness are of particular concern in densely populated environments such as catteries and shelters.

Table 1.3: Examples of studies evaluating feline calicivirus prevalence

	Healthy cats	Unhealthy cats	Reference	Method
Households				
	21% healthy	33% with URI	Binns et al 2000	OP swab; VI
	9.2% healthy		Afonso et al 2017	OP swab; VI
	8% healthy	45% suspected FCV ill	Berger et al 2015	OP cytobrush, nasal and conjunctival swabs; VI and PCR
		50% with URI	Schulz et al 2015	OP, conjunctiva, nasal; PCR
		41% with gingivostomatitis	Dowers et al 2010	Oral biopsy; PCR
	16% healthy		Hou et al 2016	OP swab; VI
	15% healthy	48% with URI	Fernandez et al 2017	Conjunctival and OP swab; PCR
		44% with conjunctivitis	Fernandez et al 2017	Conjunctival and OP swab; PCR
		58% with gingivostomatitis	Fernandez et al 2017	Conjunctival and OP swab; PCR
	45% overall		Coyne et al 2006	OP swab; VI
Shelters				
	13-36% overall		Bannasch and Foley 2005	Conjunctival, OP swab; PCR
	51% without URI	67% with URI	McManus et al 2014	Conjunctival, OP swab; PCR
	2.8% at admission		Gourkow et al 2013	Conjunctival, OP swab; PCR
	28% overall		Coyne et al 2007	OP swab; VI and PCR
	14% at admission		Wagner et al 2018	Conjunctival, OP swab; PCR
Catteries				
	29% without URI	47% with URI	Helps et al 2005	Conjunctival, OP swab; PCR
	6% - 75% overall		Coyne et al 2006	OP swab; VI
TNR				
	36% without URI	55% with URI	McManus et al 2014	Conjunctival, OP swab; PCR

FCV: feline FCV
OP: oropharyngeal
TNR: Trap neuter return
URI: upper respiratory infection
VI: virus isolation

1.3.5. Diagnosis

The methods to detect presence of FCV include amplification of nucleic acids via reverse transcriptase PCR assays (RT-PCR), virus isolation in culture, and antibody detection by virus neutralization methods or ELISA.[17,97,99,238–240] Diagnosis of FCV, however, is complicated by subclinical carriers, vaccination strains, co-infection with other pathogens, and detection thresholds; clinical signs and the detection of the virus by various molecular methods are not well correlated (Table 1.3).[17,44,45,99,241–243] Results should therefore be interpreted with caution.

Conventional, nested, RT-PCR, and reverse transcription quantitative PCR (RT-qPCR) assays amplify FCV RNA in tissues, most commonly conjunctival or oropharyngeal swabs or also nasal swabs, blood, tissue scrapings, or other tissues dependent on clinical indications and outcome.[17,99,239,241] These molecular methods are quick and generally sensitive. Namely, more recent RT-qPCR assays can identify unique viral strains, although the high genetic variability of FCV impacts sensitivity and potentially limits strain identification.[17,99,239–241] Also confounding true positive identification of FCV responsible for illness, virus in live vaccinations can be shed, subclinical carriers can test positive, vaccinated cats can become carriers of the vaccine strain, and some cats shed more virus than others.[38,223,224,232,233,243,244]

Virus isolation methods detect the presence of replicating FCV virus in tissues most commonly from conjunctival, oropharyngeal, or nasal swabs.[12,17,99,99,245,246] Virus isolation methods are not as affected as RT-PCR by the high genetic variability of FCV, but potentially low numbers of virions in samples, virus inactivation during transit, or interfering antibodies in the sample, might preclude growth in culture.[17,99,245]

Virus neutralization or ELISA methods can be used to detect FCV antibodies in mainly serum and potentially other bodily fluids. Because antibodies are present in both vaccinated and

previously exposed or infected cats, and because some cats with low antibody titers might be severely ill, serology is not useful for diagnosis of FCV infection.[97,151,152,247,248] Virus neutralization assay titers can be used to predict protection against FCV; however, they are dependent on which strain(s) are used in the test, and considering the high genetic variation of the FCV virus and multiple strains and an unknown influence of CMI, titers likely do not represent an accurate picture of protection for an individual cat.[153,233,249–251] Virus neutralization assays are also used to predict response or cross-reactivity to different virus challenges or novel vaccine strains.[228,230,231,252,253]

Clinical signs can be used to initiate supportive therapy without a positive diagnosis of FCV if diagnosing within catteries or shelters. Since coinfections with other pathogens are likely, providing treatment based on suspected pathogens seems reasonable. If two or more cats have similar severe clinical signs indicative of FCV with oral ulceration and severe systemic disease, VSD is considered more likely.[16] Appropriate disease control, transmission, management, disinfection, and quarantine measures should be followed.[17,42,49,74,254]

1.3.6. Prevention and vaccination

Modified live (MLV) or inactivated vaccines against FCV are considered a core vaccination for cats and are commonly combined in vaccines that also contain FHV-1 and panleukopenia virus.[108,117,202] The MLV form is non-adjuvanted and different products are available for parenteral or intranasal administration. The inactivated forms have both adjuvanted and non-adjuvanted products.[16] Current vaccines typically decrease severity of clinical signs and viral shedding after exposure to field strains, but they do not confer protection against infection.[97,122,153]

FCV vaccines are based on whole viral antigens grown in cell culture and are from strains that have shown to be more immunogenic and cross reactive.[16,225,232,243] Most MLV vaccines are monovalent, based on a single FCV strain, mainly the FCV-F9 strain. Inactivated vaccines are typically based on the FCV-255 strain or newer combinations with the FCV-431 or FCV-G1 strains.[220,231,243,255]

Considering VS has occurred mostly in vaccinated cat populations, vaccination efficacy may be limited; reasons are likely multifactorial.[35,49,242] It has been suggested that some infections could have been obtained during vaccination, as clinical signs can occur if the parenteral MLV vaccine virus reaches the cat's oral or respiratory mucosa, and cats that receive IN vaccines might also develop URI clinical signs.[6,36,44,243,256,257] The FCV-F9 vaccine virus is sometimes shed by vaccinated cats and can be found in the general cat population.[223,224,243,244,258] However, it seems that the levels of virus induced by the vaccine have been tolerated thus far.[34]

The vaccines do not protect equally against the many field isolates of FCV, likely due, in part, to the high antigenic variation and because most of the vaccines contain only one strain of FCV; thus the mutants are likely unaffected by the common vaccines' components of FCV-F9 or FCV-255.[44,220,224,243] It has also been suggested that field isolates may be developing resistance to the commonly used vaccine strains (F9 and 255). This is suggested considering outbreaks of VS have occurred in vaccinated cat populations, and the VS strains are genetically distinct.[35,49,219] Furthermore, one study found that in cats infected w FCV strains F9, 255, G1, and 431, the antisera against the newer strains G1 and 4331 neutralized significantly more of the field isolates that the antisera for the older strains F9 and 255 which lacked neutralization ability.[250] Another study had similar findings, suggesting that more recent vaccine strains G1 and 431 had better neutralizing ability than the older F9 and 255 strains.[253] On the other hand, others found that both

of the vaccine strains for F9 and 255 were successful in neutralizing many field isolates and that antisera against F9 was still broadly cross-reactive against field isolates, in in vitro virus neutralization assays.[231,244]

The needs for broader cross-reactivity and improved immune response have guided development of other vaccination and prevention strategies.[208,252,259–261] Rong et al 2014[260] used a new strain in a vaccine, avirulent strain FCV-21; they found it protected against mortality in cats challenged with a highly virulent VS strain, whereas the typical vaccine with the F9 strain provided poor protection.

Vaccines containing more than one strain of the virus have also been developed for their potential to provide improved protection in the field.[220,252,253] Poulet et al 2005[255] combined two strains FCV-G1 and FCV-431 in a SQ MLV vaccine and compared responses in kittens to responses in kittens that received a monovalent vaccine of each strain; kittens were challenged with different isolates 31 days after vaccination. This dual strain vaccine resulted in fewer clinical signs and less virus shedding when compared to the single strain vaccines. In a later study, Poulet et al 2008[220] compared a SQ inactivated non-adjuvanted vaccine containing strains 431 and G1 to a SQ MLV vaccine containing the F9 strain and to a SQ adjuvanted inactivated vaccine containing strain 255. Kittens were vaccinated twice, four weeks apart and were challenged 1-4 weeks after vaccination. After challenge with FCV 255, the kittens that were vaccinated with the dual strain vaccine had fewer clinical signs compared to the kittens that were vaccinated with the F9 MLV strain, and clinical signs in the dual strain group were comparable to those kittens vaccinated with the adjuvanted inactivated 255 strain. One study[252] compared a SQ killed vaccine containing a VS isolate in a traditional FVRCP/FeLV vaccine to a SQ killed traditional FVRCP/FeLV vaccine. Kittens were vaccinated twice, three weeks apart. The antisera

from kittens that received the dual strain vaccine had higher levels of neutralizing antibodies in assays, and more strains of FCV virus were neutralized when compared to the antisera from cats that received the traditional vaccine alone. Kittens that received the dual strain vaccine were challenged with the homologous VS-FCV isolate two weeks after vaccination, and they were compared to an unvaccinated control group; the vaccinated group did not develop VSD, whereas all kittens in the control group became severely ill with VS signs. Recent in vitro virus neutralization studies have similarly found higher cross-neutralization from antisera against two FCV strains as compared to antisera from one strain.[253]

New vaccine technologies such as DNA vaccines, myxoma recombinant vaccines, and subunit vaccines expressing a portion of the capsid protein have been explored and offer some promise.[262–265] Targeting an immune response against more conserved regions of the capsid variants has also been suggested as vaccination efficacy improvements.[44] Oral administration of an FCV vaccine has also been studied and was found to decrease mortality against a virulent VS strain.[266] However, this route should be explored with caution considering others have found increased clinical signs in cats that were exposed to the vaccine through the oral route.[36,44,243,256] The intranasal route of vaccination was also found to offer some improvement in response to heterologous challenge in a recent study.[261]

Protection against FCV is mainly attributed to antibodies via humoral immunity; therefore, FCV vaccines are designed to mainly work by inducing virus neutralizing antibodies. Although protection against FCV is thought to be primarily mediated via humoral immunity, cell-mediated immunity is uncertain, but local immune responses and innate cell mediated immunity might be important in response to challenge as well.[16,97,243,249,251] In one study, clinical

signs in a kitten declined by 7 days after FCV challenge despite lack of vaccination and antibodies to the antigens.[251]

Many factors related to the virus and vaccination combined with the cat's genetic immunologic factors very likely contribute to how the cat responds to vaccination and challenge.[48,224,242]

1.3.7. Treatment

Supportive care is the mainstay of therapy for FCV infection; fluids and nutritional support are essential. There are currently no safe antiviral medications for FCV infection in cats.[17,108] Antiviral drugs are being explored and there have been some positive results.[259] Feline interferon omega has been studied, and there is a lack of evidence supporting efficacy of this product in cats with FCV infections.[108,208,267] For those cats with severe illness, intensive nursing care might include intravenous fluid administration while correcting electrolyte disturbances. Because many ill cats will not eat due to pyrexia, ulceration in the oral cavity, and/or loss of sense of smell due to respiratory disease, a feeding tube is highly recommended if the cat does not eat for more than three days.[16,17,108] Appetite stimulants such as mirtazapine or cyproheptadine can be considered in less severe cases. Broad-spectrum antimicrobials (doxycycline 10mg/kg PO once daily or 5 mg/kg PO twice daily) might be necessary in severe illness if potential secondary bacterial infection is suspected.[8,17] Non-steroidal anti-inflammatory medications might be used as analgesic and anti-pyretic agents. Nasal and ocular discharge should be cleaned several times per day with physiologic saline.[17,108] Nebulization and airway humidification can also be considered.

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CHAPTER 2: RESEARCH OVERVIEW

Feline upper respiratory infections (URI) are one of the most common infectious disease syndromes in cats and kittens and continues to be a pervasive health and welfare concern for cats in crowded, stressful, or multi-cat environments where morbidity and mortality can be high. URI remains one of the most common medical reasons for euthanasia in animal shelters despite improvements in infection control and increased knowledge regarding stress reduction and the environmental needs of cats. Vaccination protocols and various treatment and management strategies have improved outcomes in some environments, but many cats continue to become severely ill and either die or are euthanized due to URI. The goals of this body of work were to explore alternative preventive and treatment models for URI that had not been previously studied in detail and in the settings described here. Feline herpesvirus-1 (FHV-1) is one of the primary viral pathogens implicated in feline URI, thus three chapters (Chapters 4, 5, 7) evaluated preventives or therapies for primary FHV-1 or FHV-1 recrudescence in purpose-bred, experimentally infected cats in a controlled research setting. Feline calicivirus (FCV) is another viral pathogen implicated in feline URI, thus Chapter 3 investigated the effects of administering an additional dual strain FCV vaccine in a shelter environment. Chapter 6 examined the use of an upper respiratory mucosal immune stimulant in a shelter environment.

FHV-1 is ubiquitous, and immunity against FHV-1 is not complete. Vaccinated or previously infected cats can therefore become ill when re-exposed to FHV-1 or when FHV-1 is reactivated, sometimes in response to stress and immune suppression. Most cats that are infected become latently and persistently infected with intermittent episodes of reactivation and shedding of the virus. Other than vaccination and antiviral therapies, a number of other strategies with

variable outcomes have been employed in an attempt to lessen FHV-1 illness and reactivation in cats. The objectives of Chapter 4 were to determine if oral administration of a plant-based nutraceutical with anti-inflammatory and immune-modulating components would reduce clinical signs of recrudescence and viral shedding in cats with FHV-1 upon repeat challenge. We hypothesized that treatment of cats with the plant-based nutraceutical would be safe and would lessen clinical signs and FHV-1 viral shedding in latently infected, repeat-challenged cats that were administered the nutraceutical as compared to a control group. The objectives of Chapter 5 were to evaluate the efficacy of a new mucosal formulation of a liposomal toll-like receptor immune stimulant as both a preventive for clinical signs of FHV-1 illness and as a treatment for FHV-1 illness in kittens experimentally infected with FHV-1. We hypothesized that administration of the immune stimulant prior to FHV-1 challenge and at the first signs of illness would induce positive clinical outcomes and would result in decreased signs of illness in cats that were administered the immune stimulant as compared to a control group. The objectives of Chapter 7 were to explore the use of a feline pheromone diffuser product and its effects on relaxation and stress reduction in kittens and thus resultant decrease in recrudescent clinical signs of FHV-1 in kittens when exposed to housing change (group to kennel) induced stress. We hypothesized that the pheromones would lessen stress, resulting in decreased recurrence of FHV-1 associated illness in kittens in a room with the pheromone diffuser as compared to kittens in a control group.

Management of URI in shelters is difficult due to the multiple pathogens involved, the multifactorial etiology, carrier states, multiple cats from different environments, and the inherently stressful situation of a cat entering and residing in a new shelter environment. Vaccination upon intake to a shelter is an integral component in preventing or decreasing illness

and lessening transmission of infectious disease; there is also some thought that vaccination might also stimulate local immune responses and innate cell mediated immunity in addition to humoral antibody-induced immunity. The objectives of Chapter 3 were to determine whether the addition of a broader spectrum inactivated vaccine against two FCV isolates to the existing vaccination protocol in a large, open-admission shelter, would result in decreased incidence, severity, and duration of URI and oral ulceration as an indication of FCV associated illness. We hypothesized that the cats that were administered the additional vaccination would have an overall enhanced immune response against FCV and other URI pathogens in the shelter as compared to a control group. The objectives of Chapter 6 were to evaluate whether administration of the Chapter 5 mucosal immune stimulant to cats upon admission to a shelter would result in decreased incidence, severity, and duration of URI. We hypothesized that the cats that were administered the mucosal immune stimulant would have overall less URI and clinical signs as compared to a control group.

CHAPTER 3: CLINICAL EFFECTS INDUCED BY ADMINISTRATION OF A DUAL STRAIN FELINE CALICIVIRUS VACCINE

3.1 Introduction

Feline upper respiratory infection (URI) continues to be a pervasive health and welfare concern in animal shelters.[1–3] Feline URI can result in high morbidity, financial burdens, poor quality of life, extended lengths of stay, and euthanasia.[1,4–6] Multiple pathogens are implicated in feline URI. Feline herpesvirus-1 (FHV-1) and feline calicivirus (FCV) are the most common primary causes, and multiple bacterial pathogens occur as secondary and opportunistic invaders in combination with the viruses.[3,7,8] Management of URI in shelters is difficult due to the multiple pathogens involved, the multifactorial etiology, carrier states, multiple cats from different environments, and the inherently stressful situation of a cat entering and residing in a new shelter environment.[3,9–11]

Vaccination upon intake is an integral component in preventing or decreasing illness and lessening transmission of infectious disease in shelters.[12–15] A modified live (MLV) or inactivated vaccine against FCV is considered a core vaccination for cats and is commonly combined in a vaccination also against FHV-1 and panleukopenia virus.[14,16–19] Vaccination, however, does not prevent infection or viral shedding, and it does not protect against the highly virulent strain of FCV (VS).[20–24] Furthermore, the vaccines do not protect equally against the many field isolates of FCV, likely due, in part, to the high antigenic variation of FCV and because most of the vaccines contain only one strain of FCV, most commonly FCV-F9 or FCV-255.[25–29]

The needs for broader cross-reactivity and improved immune response have guided development of other vaccination and prevention strategies including vaccines containing more

than one strain of the virus.[28,30,31] One study[30] used a dual-strain vaccine containing a VS isolate and a traditional FCV isolate and found that the dual strain vaccine induced cross-neutralization of a wider spectrum of FCV and VS isolates than a single strain vaccine.[30] A broader spectrum inactivated vaccine (hereinafter called "CCVax") was subsequently produced for market and is the only vaccine for cats in the USA that contained two different FCV strains. Because of the hyper-virulence of the VS-FCV isolate in the vaccine, the USDA required it be an inactivated vaccine which at the time of this study also contained an adjuvant.

Although protection against FCV is thought to be primarily mediated via humoral immunity, local immune responses and innate cell mediated immunity are likely important as well.[26,32–35] In one study, clinical signs in a kitten declined by seven days after challenge with FCV despite lack of vaccination and lack of antibodies to FCV.[33] Another study compared subcutaneous (SQ) inactivated adjuvanted and non-adjuvanted FCV vaccines and found that neutralizing antibodies were not predictive of clinical signs or outcome in FCV challenged cats, and decreased FCV excretion was not predictive of cross-neutralization between vaccine and challenge; this suggested that a cell mediated response was likely partially responsible.[28] FHV-1 is the other major viral pathogen implicated in feline URI, and another study showed partial clinical immunity to challenge with FHV-1, as soon as seven days after one SQ inactivated FHV-1 vaccination.[36]

The standard vaccine protocol at the large open admission shelter that collaborated in this study, was the administration of a SQ modified live (MLV) feline viral rhinotracheitis, calicivirus, and panleukopenia (FVRCP) vaccine.[14,15] There was anecdotal evidence of lessened URI in this shelter when the CCVax was used in the past (lead shelter veterinarian, personal communication). The objectives of this study were to determine whether addition of CCVax to

the existing vaccine protocol would result in decreased incidence of URI cases over time, decreased severity and duration of URI in those cats that developed URI despite vaccination, decreased oral ulceration as an indication of FCV associated illness, and increased time to onset of URI as compared to those cats receiving just the FVRCP vaccine upon admission, while also considering the potential effect of multiple other factors on the outcomes. We hypothesized that although the CCVax was inactivated, that it would contribute synergistically with the MLV FVRCP vaccine to enhance the overall immune response against FCV and other URI pathogens in the shelter.

3.2 Methods

3.2.1. Study Design

All cats that were admitted to a north central Colorado open admission shelter between June 8 and September 9, 2015 and that were medically suitable for vaccination were enrolled in this study. Each cat was administered one of two intake vaccination protocols based on day of week. The cats in group 1 were administered a SQ modified live feline viral rhinotracheitis, calicivirus, panleukopenia virus vaccination (Boehringer Ingelheim); the cats in group 2 were administered the SQ FVRCP vaccination and a SQ killed feline calicivirus (FCV) vaccination (Boehringer Ingelheim). Shelter intake staff administered the vaccinations and recorded their administration within the shelter's database system, Chameleon (ChameleonBeach, HLP Inc.). Cats that were still present in the shelter after two weeks were administered booster vaccination(s) identical to those administered on intake. Shelter staff was not masked to vaccination information. The protocol was approved by the shelter and the Clinical Review Board of the sponsor.

3.2.2. Data and categories

Chameleon shelter data from June 8 through October 9, 2015 was downloaded for all study cats. Data included signalment, intake source (owner surrender, stray, transfer, return, foster), intake type (over-the-counter, night dropbox, other); intake reason if owner surrender; declaw status (declawed or not); outcome type (adoption, euthanasia, death, transfer, return); infectious disease test results (FIV, FeLV, parvovirus, DTM culture); the following while each cat was in the shelter: vaccinations, diagnostics, medical conditions, diagnoses, medications, treatments, surgery types, kennel locations, URI scores, URI descriptions, and dates corresponding to each data point.

For this study, age category was defined according to age at intake: kitten if $\leq$ 6 months of age: adolescent if > 6 and $\leq$ 24 months of age; adult if > 24 months and $\leq$ 84 months of age; and senior if > 84 months of age. Intake status was redefined into three categories of owner surrender, transfer and return, and stray. Since there were only three cats that had an intake status of "return," those three cats were included in the transfer category. All but one of the cats that had an intake status of "foster" were kittens or adolescents that entered the shelter previously as neonates or younger kittens and had received vaccinations followed by return to foster care and then readmission to the shelter at an older age; therefore, these foster kittens and adolescents were excluded from the main analyses and were included in separate model analyses and were considered group 1-F and group 2-F.

Cats that had URI within their first three days in the shelter and cats that left the shelter within their first three days in the shelter were removed from the study population since concurrent URI precluded vaccine-induced protective immunity, and results from protective

vaccine-induced humoral immunity could not be expected in that timespan, respectively. Because of varied lengths of stay, expected time to onset of vaccination efficacy, and expected time to first URI occurrence, number of housing changes was calculated as the number of times a cat was moved to a different kennel within the cat's first 17 days within the shelter; the first 17 days were chosen because this was a two-week timeframe after removing the first three days as stated above, and 17 days was the average length of stay for cats in this shelter. Surgery status was recorded as yes or no for this study. "Yes" was recorded for the two following scenarios: when a surgery was performed prior to URI and when a surgery was performed in a cat that did not have URI throughout the cat's days in the shelter.

3.2.3. URI Scoring

Cats were evaluated for general health by shelter intake staff upon admission and were subsequently evaluated for general health by shelter animal care and evaluation staff during daily staff activities. When shelter staff found a cat that had clinical signs such as sneezing, nasal or ocular discharge, or respiratory congestion, staff recorded a notation in Chameleon, requesting an examination by veterinary staff. A veterinary technician subsequently evaluated the cat and assigned a URI score and recorded the URI score and notation in Chameleon (Table 3.1). If a cat was assigned a URI score of 3 or 4, this was considered "severe URI," and a shelter veterinarian was alerted via the Chameleon system to evaluate the cat. Presence or absence of URI, URI scores, and outcome after URI were determined and recorded for each cat in this report.

Table 3.1: Shelter URI scoring system

No URI signs	0
Sneezing cats with or without serous (clear watery) nasal discharge or mild congestion (barely audible)	1
Sneezing cats with mucoid (clear or white) nasal discharge or moderate congestion (easily audible)	2
Sneezing cats with mucopurulent (discolored) or hemorrhagic nasal discharge, severe congestion (open mouth breathing) and/or dehydration.	3
As above with severe respiratory distress, severe lethargy or radiographically diagnosed pneumonia	4

Due to inconsistencies in the recording of the URI score in the appropriate field, data was mined with commercial software by an investigator (EC) to find all cats that had URI while in the shelter. Data was queried on the following terms in appropriate database fields: sneez, ulcer, respiratory, conjunct, bleph, discharge, congest, tongue, oral ulcer, doxy, clav, fmvr, tear/iod, zithr, erythro, URI. Those cats with any of those terms, were then coded as a URI occurrence in those cats. To eliminate inadvertent retrieval of non-URI notations, the queried data was then re-queried on the following terms: no sneez, no discharge, no bleph, urine, ponazuril, pruritus, uria, injur, and the URI codes for those rows of data were removed. Data was also individually mined for entries of non-URI ulcers, non-URI congestion, and clavamox or doxycycline administered for non-URI medical issues.

All data were inspected for accuracy, missing and unassigned values, and dates by one of the investigators (EC) who was masked to vaccination group during the data evaluation. For each row that had missing or unassigned URI score values, a URI score was assigned based on the descriptive entry in the field for that row, using a set of rules (Table 3.2). Dates were entered that indicated first report of URI clinical sign, first date of URI medication, and date transferred to another facility. URI data was included in analyses if the URI occurred while the cat was in the shelter without interruption.

Ulcer type (oral, tongue, lip, nasal) was also assigned based on descriptive entry in a field for that row if ulcer was not recorded in the appropriate categorical field. An ulcer was recorded as present in this study if it was recorded as on the tongue, nose, or oral cavity in combination with other URI clinical signs. Dates were entered that indicated date of first ulcer.

Table 3.2: Rules for URI scores assigned to missing data fields

Rule	Criteria
1.	• If staff assigned URI score of 1, 2, 3, or 4 in descriptive field instead of numeric field, that URI score was transferred to the appropriate numeric field
2.	• If both veterinary technician and veterinarian scored cat differently on two different rows on the same date, used veterinarian's score and row and removed the technician's
3. Assign URI score 0	• No URI signs noted within three days of last score of URI 1 • If a row of data had an entry of URI 0, that row's date was labeled as the resolution date if and only if no other entries three days subsequent to that date, indicated that URI was still observed in the cat • If an entry within three days subsequent to the URI 0 score, indicated a URI score of 1, 2, or 3, the URI 0 score was removed
4. Assign URI score 1	• Sneezing with or without serous nasal discharge or mild congestion, barely audible • Serous or mucoid ocular discharge or mild conjunctivitis ONLY • If nasal upper respiratory congestion noted with no other signs noted • If notations state, "on vet tech check for sneezing or URI" • If oral ulcer present, resolving per notations, without continued URI for nose, eyes
5. Assign URI score 2	• Sneezing with mucoid nasal discharge or moderate congestion, easily audible • Mucopurulent ocular discharge and/or moderate conjunctivitis ONLY • Hypersalivation and colored ocular or nasal discharge • If only oral ulcer noted, prior to other URI signs
6. Assign URI score 3	• Sneezing with mucopurulent, discolored or hemorrhagic nasal discharge, severe congestion, open mouth breathing, and/or dehydration
7. Assign URI score 4	• Severe respiratory distress, severe lethargy, or radiographically diagnosed pneumonia

Resolution date was recorded as the date a URI score of 0 was recorded and no further URI scores were reported. If a URI 0 score was not recorded, resolution date was assigned by the investigator as three days after a cat's last URI 1 date, if the cat had three subsequent,

consecutive dates of no URI clinical signs reported, followed by a subsequent outcome unrelated to the URI. A resolution date was not assigned if the cat was adopted, transferred, or was euthanized or died within three days of the last URI score reported. A resolution date was also not assigned if the cat's URI clinical signs were not reported after another overriding illness such as dermatophytosis took precedence in the medical record; when another overriding illness took precedence, URI notations were rarely recorded in the shelter database.

3.2.4. Statistical evaluation

Descriptive statistics including frequencies, counts, medians, and ranges were calculated. Univariable logistic regression models were used to assess the association of the main independent variable of vaccination group (group 1 or 2) with the outcomes of URI occurrence (yes/no), severe URI occurrence (yes/no), and oral ulceration (yes/no). Other factors potentially associated with the outcomes of URI occurrence and severe URI were also evaluated. These factors included age category (kitten, adolescent, adult, senior), intake status (owner surrender, stray, transfer/return), number of housing changes within 17 days of admission, and the dichotomous variables of declawed (yes/no), FIV/FeLV (positive/negative), sex (male, female), and surgery (yes/no). Housing type (individual kennels and group housing) could not be statistically evaluated because of the much fewer numbers of group housing spots available. Factors that were associated in the univariable analyses with a $P \leq 0.20$ were used to build a multivariable logistic regression model using manual backwards elimination. Vaccination status was retained in the model regardless of P-value. All eliminated variables were re-evaluated for confounding effects within the final model using a change in coefficient estimate approach such that if a change in coefficient estimate were $\geq$ 10%, the variable was considered a confounder

and entered back into the model. Interaction terms between vaccine group and all other independent factors were assessed. Clinically relevant interaction terms were also assessed: age category and FIV/FeLV status, age category and surgery status, age category and intake type, and number of housing changes and surgery status. Odds ratio estimates and 95% confidence intervals were calculated. The Hosmer-Lemeshow test was used to evaluate the overall model fit.

To consider the effect of time to onset of the efficacy of a killed vaccine, the same analyses listed above were performed after excluding those cats that were in the shelter for seven or fewer days and cats that had URI within seven days of admission. Descriptive analyses were performed to evaluate the foster intake category (group 1-F and 2-F).

Due to non-normalcy of variables as assessed by the Shapiro-Wilk test, the Wilcoxon rank-sum test was used to compare the two vaccination groups and median number of days from intake to URI, intake to severe URI, intake to oral ulcer, and number of days from first date of URI to URI resolution. Histograms revealed highly skewed distributions, thus the Kaplan-Meier survival method was used to estimate the median overall time (days) to development of URI and severe URI and the median overall time (days) to resolution of URI. The log-rank test for equality of survivor functions was used to compare differences between curves for the two vaccination groups. The multivariable Cox proportional hazards model was used to assess the association of other variables with time to URI and severe URI. The proportional hazards model assumptions were tested.

All analyses were performed using commercially available statistical software (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP). Significance was defined as $P < 0.05$.

3.3 Results

3.3.1. Study population and cat characteristics

A total of 2,602 cats were admitted to the shelter and assigned to either of the two vaccination protocols; this included 1,390 cats that received only the FVRCP vaccination (group 1) and 1,212 cats that were administered both the FVRCP + FCV vaccinations (group 2) (Figure 3.1). Cats in the shelter for three days or less (n=619) and cats that had URI within three days of admission (n=297) were excluded from the study population. There were 141 kittens and adolescent cats with the foster intake status that were excluded from the main study population. This resulted in a total of 1,545 cats including 811 in group 1 and 734 in group 2 (Figure 3.1).

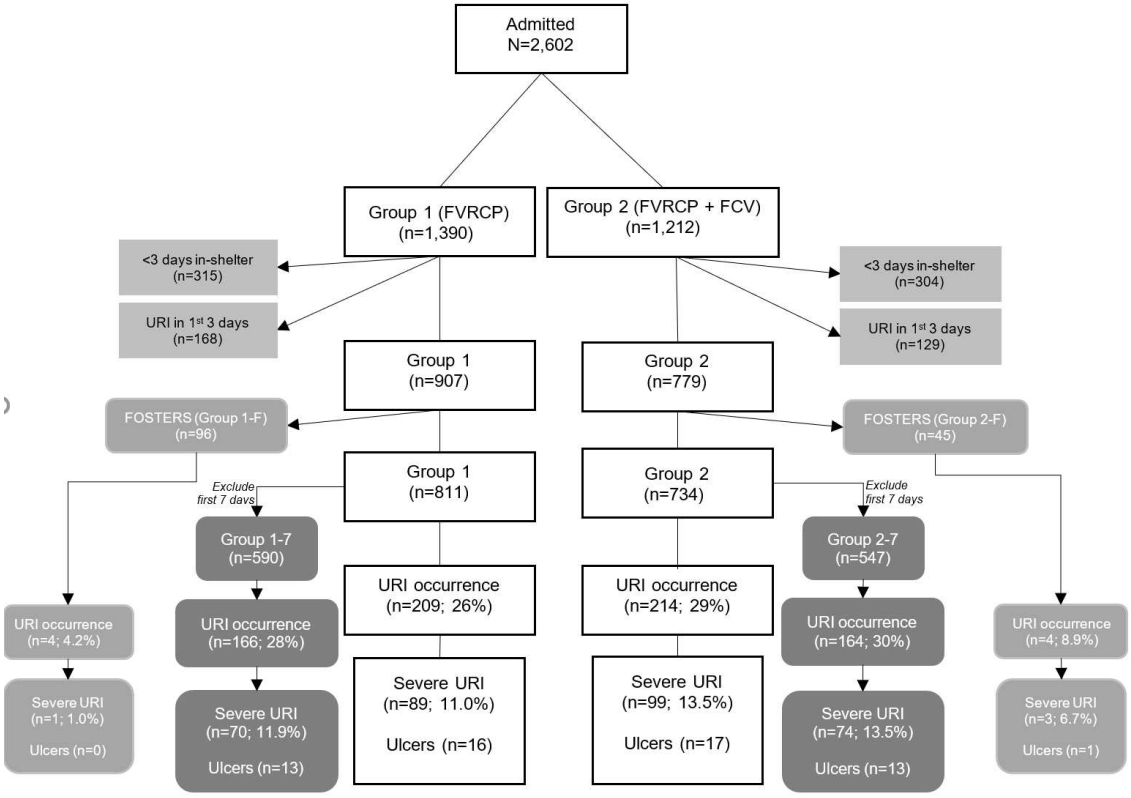

Figure 3.1: Flow diagram of cats entering the study, study groups, and URI and severe URI occurrence. Diagram represents the numbers of cats in the study population, exclusions from the population, numbers in groups 1 and 2, categorizations separated from main analyses (fosters and excluding the first seven days of admission), numbers of cats with URI, severe URI, and oral ulcers in each group and category

All adoptable cats admitted to the shelter were gonadectomized, and neuter status was subsequently automatically updated in the Chameleon system for each cat. Therefore, cats listed as spayed females or neutered males included those cats that were gonadectomized while in the shelter. Sex and age group are listed in Table 3.3.

Table 3.3: Cat sex and age category by vaccination group

	Kitten N=406 (26.2%)		Adolescent N=300 (19.4%)		Adult N=501 (32.4%)		Senior N=338 (21.9%)	
	Group 1 N=208 (25.7%)	Group 2 N=198 (27.0%)	Group 1 N=147 (18.1%)	Group 2 N=153 (20.8%)	Group 1 N=285 (35.1%)	Group 2 N=216 (29.4%)	Group 1 N=171 (21.1%)	Group 2 N=167 (22.8%)
Female spayed N=738 (47.8%)	n=85	n=78	n=78	n=88	n=141	n=100	n=86	n=82
Male neuter N=719 (46.5%)	n=100	n=112	n=61	n=54	n=130	n=101	n=82	n=79
Female intact N=32 (2.1%)	n=6	n=3	n=7	n=5	n=2	n=4	n=2	n=3
Male intact N=26 (1.7%)	n=4	n=5	n=0	n=2	n=7	n=6	n=0	n=2
Unknown N=30 (1.9%)	n=13	n=0	n=1	n=4	n=6	n=6	n=1	n=1

Of the 1,545 cats, 1,309 (84.7%) entered the shelter during regular shelter hours as “over the counter” admissions, while 181 (11.7%) entered the shelter via an overnight “drop box,” and 55 (3.6%) entered the shelter through other means such as transfers from other agencies. Intake types consisted of 876 cats (56.7%) that were surrendered by owners, 622 cats (40.3%) that were surrendered as strays, and 48 cats (3.1%) that were transfers from other facilities or returns from a prior adoption (Table 3.4). Of the 876 cats that were surrendered by owners, the reason for relinquishment was due to moving, traveling, or “no home” for 216 cats (24.7%); too many pets for 121 cats (13.8%); other owner problems for 110 cats (12.6%); house soiling for 83 cats (9.5%); allergies to cat for 82 cats (9.4%); aggression for 48 cats (5.5%); and landlord issues for 37 cats (4.2%) (Figure 3.2).

Table 3.4: Cat intake type and age category by vaccination group

	Kitten N=406 (26.2%)		Adolescent N=300 (19.4%)		Adult N=501 (32.4%)		Senior N=338 (21.9%)	
	Group 1 N=208 (25.7%)	Group 2 N=198 (27.0%)	Group 1 N=147 (18.1%)	Group 2 N=153 (20.8%)	Group 1 N=285 (35.1%)	Group 2 N=216 (29.4%)	Group 1 N=171 (21.1%)	Group 2 N=167 (22.8%)
Owner surrender N=876 (56.7%)	n=68	n=67	n=69	n=56	n=191	n=148	n=142	n=135
Stray N=622 (40.3%)	n=126	n=124	n=74	n=96	n=86	n=63	n=23	n=30
Transfer or return N=48 (3.1%)	n=14	n=7	n=4	n=1	n=8	n=5	n=6	n=2

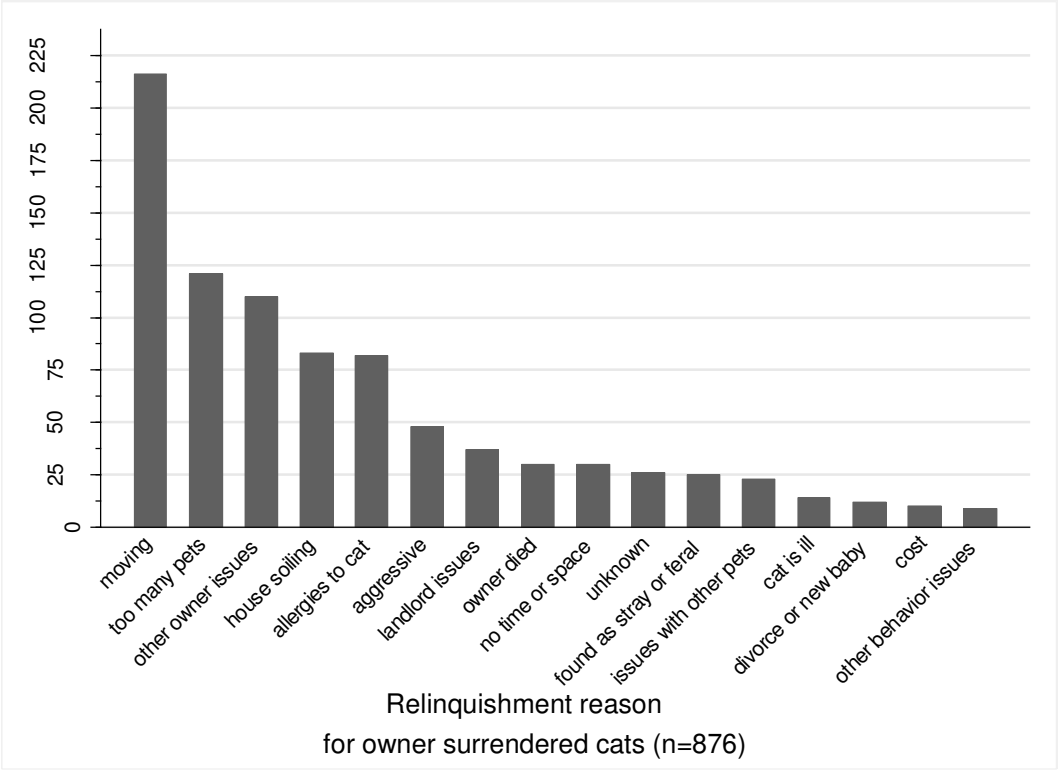

Figure 3.2: Bar graph depicting reason for relinquishment of owner surrendered cats

There were a total of 113 cats (7.3%) that were declawed; this included 63 from group 1 and 50 from group 2. Cats that were FIV or FeLV positive were rare; there were 9 FIV positive and 0 FeLV positive cats in group 1, and there were 12 FIV positive and 3 FeLV positive cats in group 2. Breeds recorded by shelter staff included domestic shorthair (n=1077; group 1 n=554; group 2 n=523), domestic medium hair (n=274; group 1 n=154; group 2 n=120), domestic

longhair (n=1389; group 1 n=71; group 2 n=67), Siamese (n=19; group 1 n=9; group 2 n=10), Persian or Himalayan (n=4; group 1 n=2; group 2 n=2), and others including Balinese, Bengal, Maine Coon, Manx, Ragdoll, and Snowshoe (n=33).

Table 3.5: Breeds of cats by vaccination group

Breed	Group 1	Group 2
DSH	554	523
DMH	154	120
DLH	71	67
Siamese	9	10
Persian or Himalayan	2	2
Other (Balinese, Bengal, Maine Coon, Manx, Ragdoll, Snowshoe)	21	12

3.3.2. Shelter characteristics

Average length of stay for the study population was 17.8 days (median 14 days; range 4 to 110; 95% CI 17.1 - 18.5); average length of stay for group 1 was 18.3 days (median 14 days; range 4 to 110; 95% CI 17.2 - 19.3), while average length of stay for group 2 was 17.3 days (median 13 days; range 4 to 97; 95% CI 16.3 - 18.3). While in the shelter, cats were moved to a different kennel or housing location, on average, 4.8 times (range 1 to 21; 95% CI 4.7 - 4.9); group 1 cats were moved, on average, 5.0 times (range 1 to 18; 95% CI 4.8 - 5.1), and group 2 cats were moved, on average, 4.6 times (range 1 to 21; 95% CI 4.5 - 4.8). Within the first 17 days of admission, group 1 cats were moved to a different kennel, on average, 3.9 times (range 1 to 9; 95% CI 3.8 - 4.0), and group 2 cats were moved to a different kennel, on average, 3.7 times (range 1 to 10; 95% CI 3.7 - 3.9).

Among the 1,545 cats, final outcomes included 1,189 adoptions (77.0%), 170 euthanasias or deaths (11.0%), and the remaining 186 (12.0%) cats had outcomes of foster, return to owner, release, transfer, or still in shelter at the study's conclusion. Outcomes for each group are listed in Table 3.6.

Table 3.6: Shelter outcomes by vaccination group

Outcome	Group 1	Group 2
Adoption	n=625 77.1%	n=564 76.8%
Euthanasia / Death	n=82 10.1%	n=88 11.9%
Foster	n=63 7.8%	n=43 5.9%
Return to owner	n=17 2.1%	n=18 2.5%
Spay, neuter, release	n=8 1.0%	n=14 1.9%
Transfer	n=6 0.7%	n=5 0.7%
Still in shelter	n=10 1.2%	n=2 0.3%

3.3.3. Outcome: URI occurrence

Of the 1,545 cats, 423 (27.4%) had at least one occurrence of URI; there was no difference when comparing URI occurrence in univariate analyses for group 1 (n=209; 26%) as compared to group 2 (n=214; 29%, P=0.14) (Table 3.7a). Univariable logistic regression modeling identified five variables associated with URI occurrence (P<0.20) including vaccine

administered, intake type, FIV/FeLV status, surgery status, and number of housing/kennel changes (Table 3.7a, b).

Table 3.7a: Summary of univariable analysis of URI presence/absence and categorical variables

Predictor (Risk factor)	Level	URI Yes (n)	URI No (n)	TOTAL (n)	URI Yes (%)	P-value	Odds Ratio	95% Confidence Interval
[a] **Vaccine (Main)**	CCvax + FVRCP (Group 2)	214	520	734	29%	0.14	1.19	0.95 - 1.48
	FVRCP only (Group 1)	209	602	811	26%	reference	reference	reference
Age group	Senior (>7yr)	101	237	338	30%	0.20	1.24	0.90 - 1.71
	Adult (>2yr-7yr)	135	366	501	27%	0.65	1.07	0.80 - 1.44
	Adolescent (6mo-2yr)	83	217	300	28%	0.54	1.11	0.80 - 1.56
	Kitten (<6 mos)	104	302	406	26%	reference	reference	reference
[a] Sex	Male	218	527	745	29%	0.15	1.18	0.94 - 1.48
	Female	200	570	770	26%	reference	reference	reference
[a]Intake type	Owner surrender	219	657	876	25%	**0.007***	0.73	0.58 - 0.91
	Transfer/Return	9	38	47	19%	0.09	0.52	0.25 - 1.09
	Stray	195	427	622	31%	reference	reference	reference
Declaw status	Has claws	388	1044	1432	27%	0.37	0.83	0.55 - 1.25
	Declawed	35	78	113	31%	reference	reference	reference
[a] FIV/FeLV status	FIV/FeLV negative	420	1101	1521	28%	0.11	2.67	0.79 - 9.00
	FIV/FeLV positive	3	21	24	13%	reference	reference	reference
[a] Surgery (if before URI)	Did not have surgery	263	543	806	33%	**0.0001***	1.75	1.39 - 2.20
	Had surgery (before URI)	160	579	739	22%	reference	reference	reference

[a] Included in multivariable analysis
*: statistically significant at $P < 0.05$
n: number
URI: Upper respiratory infection

Table 3.7b: Summary of univariable analysis of URI presence/absence and continuous variables

Predictor (Risk factor)	URI	Number	mean	SD	Range	P-value	Odds Ratio	95% Confidence Interval
[a] number of housing changes	Yes	423	4.3	1.5	1 - 10	**0.0001***	1.38	1.28 - 1.48
	No	1122	3.7	1.4	1 - 10			

[a] Included in multivariable analysis
*: statistically significant at $P < 0.05$
URI: Upper respiratory infection

The final multivariable logistic regression model for risk of URI included factors for vaccination, intake category, number of kennel changes, and the interaction between age and surgery (Table 3.8). Controlling for other variables in the model, the odds of URI were, on average, 1.37 times higher (95% CI 1.07-1.76, P=0.01) in group 2 as compared to group 1. As the number of housing changes increased by 1, the odds of URI increased, on average, by 1.75 (95% CI 1.58 to 1.94; P<0.0001). The odds of having URI were, on average, 1.54 times lower (95% CI 1.16 to 2.04; P=0.002) and 3.70 times lower (95% CI 1.64 to 8.33; P=0.002) in cats that had an intake status of owner surrender and transfer/return, respectively, as compared to cats that had an intake status of stray. A significant interaction was found between age and surgery status (Figure 3.3). As age increased in cats that had surgery, the probability of URI increased (OR=1.21; CI 1.13-1.28; P<0.001 for every one-year age increase). Conversely, as age increased in cats that didn't have surgery, the probability of URI decreased.

There were no significant confounding variables. The final multivariable model showed acceptable model-fit (Hosmer-Lemeshow P=0.68). The model's sensitivity was low at 28% with a positive predictive value of 63%, but the specificity was high at 94% and a 78% negative predictive value.

Table 3.8: Summary of multivariable analysis of URI presence/absence

Predictor (Risk factor)	Level	Coefficient	OR	95% CI	P-value
Vaccine (Main)	CCvax+FVRCP (Group 2)	0.32	1.37	1.07 - 1.76	**0.012***
	FVRCP (Group1)	reference	reference	reference	reference
Number of housing changes	1 - 10	0.56	1.75	1.58 - 1.94	**<0.001***
Intake group	owner surrender	-0.43	0.65	0.49 - 0.86	**0.002***
	transfer/return	-1.3	0.27	0.12 - 0.61	**0.002***
	stray	reference	reference	reference	reference
Interaction age x surgery	adolescent x surgery	1.67	5.30	2.44 - 11.51	**<0.001***
	adult x surgery	2.74	15.56	7.65 - 31.66	**<0.001***
	senior x surgery	2.42	11.29	5.31 - 24.01	**<0.001***
	kitten x surgery	reference	reference	reference	reference

*significant P-value at P<0.05
OR: Odds ratio
CI: Confidence interval

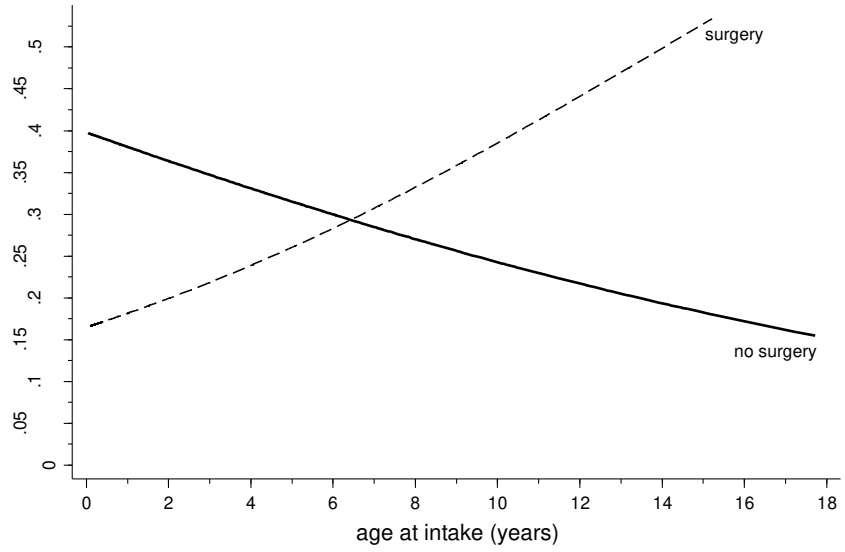

Figure 3.3: Interaction plot depicting the interaction between surgery and age of cats and probability of having an occurrence of URI. Dashed line: cats that had surgery; Solid line: cats that did not have surgery.

3.3.4. Outcome: Severe URI occurrence

Of the 423 cats that had URI, 188 cats had severe URI (44.4%); this included 89 cats out of the total 907 cats (11.0%) from group 1 and 99 cats out of the total 779 cats (13.5%) from group 2; this difference was not significant (P=0.13). The final multivariable logistic regression model for risk of severe URI included factors for vaccination, number of kennel changes, intake group, and the interaction between age and surgery (Table 3.9). Controlling for other variables in the model, the odds of severe URI were 1.4 times higher (95% CI 1.02 to 1.96; P=0.04) in group 2 as compared to group 1. The final multivariable model showed acceptable model-fit (Hosmer-Lemeshow P=0.79). The model's sensitivity was extremely low at 2.7% with a positive predictive value of 31%, but the specificity was high at 99% with an 88% negative predictive value.

Table 3.9: Summary of multivariable analysis of severe URI

Predictor (Risk factor)	Level	Coefficient	OR	95% CI	P-value
Vaccine (Main)	CCvax+FVRCP (Group 2)	0.35	1.41	1.02 - 1.96	**0.04***
	FVRCP (Group1)	reference	reference	reference	reference
Number of housing changes	1 - 10	0.47	1.60	1.42 - 1.81	**<0.001***
Intake group	owner surrender	-0.41	0.66	0.46 - 0.95	**0.03***
	transfer/return	-1.86	0.16	0.04 - 0.67	**0.01***
	stray	reference	reference	reference	reference
Interaction age x surgery	adolescent x surgery	1.83	6.22	2.19 - 17.66	**0.001***
	adult x surgery	2.73	15.26	6.05 - 38.48	**<0.001***
	senior x surgery	2.05	7.80	2.89 - 21.04	**<0.001***
	kitten x surgery	reference	reference	reference	reference

* significant P-value at < 0.05

3.3.5. Shelter outcomes for cats with severe and non-severe URI

Of the 423 cats that had URI, there were 235 cats (55.6%) that had URI that did not progress to severe URI; this included 120 cats from group 1 and 115 cats from group 2. Of the 235 cats that had non-severe URI, 222 (94.5%) had an outcome of adoption, 11 (4.7%) had an outcome of foster, return to owner, spay neuter return, or transfer, and 2 (0.9%) were still in the shelter at the study's conclusion. None of these 235 cats were euthanized or died. Of the 188 cats that had severe URI, 133 (70.7%) had an outcome of adoption, 36 (19.1%) had an outcome of euthanasia or died in shelter, 17 (9.0%) had an outcome of foster, return to owner, or transfer, and 2 (1.1%) were still in the shelter at the study's conclusion.

3.3.6. Outcome: Oral ulceration

There were 16 cats (2.0% of 907 cats) from group 1 and 17 cats (2.3% of 734 cats) from group 2 that had oral ulceration (Figure 3.1); this difference was not significant (P=0.64). The multivariable logistic regression model for risk of ulcers did not include vaccination group (P=0.5) but did include housing changes (OR 1.35; 95% CI 1.10 to 1.67; P = 0.005) and age (OR 1.11; 95% CI 1.02 to 1.20; P=0.01). Of the 16 cats from group 1 that had ulcers, nine were ultimately adopted, two were sent to a foster home, one was still in the shelter at the conclusion of the study, and four were euthanized. Of the 17 cats from group 2 that had oral ulceration, ten cats were ultimately adopted, three were sent to a foster home, one was transferred to another shelter, and three were euthanized.

3.3.7. Excluding first 7 days: URI occurrence, severe URI, and oral ulceration

After excluding those cats that were in the shelter for seven or fewer days and cats that had URI within seven days of admission (n=1137), a total of 330 cats were identified with at least one occurrence of URI clinical signs (Figure 3.1). Of the 590 cats in group 1-7, 166 cats (28.1%) had at least one occurrence of URI, and of the 547 cats in group 2-7, 164 cats (30.0%) had at least one occurrence. This difference was not significant (P = 0.5). The final multivariable logistic regression model for risk of URI excluding the first seven days, included factors for vaccination (NS), number of kennel changes, the interaction between age and surgery, and the interaction between intake group and surgery (Table 3.10). The final multivariable model showed acceptable model-fit (Hosmer-Lemeshow P=0.50). The model's sensitivity was low at 26% with a positive predictive value of 65%, but the specificity was high at 94% and a 75% negative predictive value.

Table 3.10: Summary of multivariable analysis of URI presence/absence excluding first 7 days

Predictor (Risk factor)	Level	Coefficient	OR	95% CI	P-value
Vaccine (Main)	Calicivax+FVRCP (Group 2)	0.2	1.22	0.92 - 1.62	0.17
	FVRCP (Group1)	reference	reference	reference	reference
Number of housing changes	1 - 10	0.45	1.57	1.40 - 1.76	**<0.001***
Interaction age x surgery	adolescent x surgery	1.41	4.08	1.67 - 9.97	**0.002***
	adult x surgery	2.25	9.50	4.10 - 21.99	**<0.001***
	senior x surgery	1.72	5.59	2.22 - 14.08	**<0.001***
	kitten x surgery	reference	reference	reference	reference
Interaction intake group x surgery	owner surrender x surgery	1.12	3.08	1.66 - 5.72	**<0.001***
	transfer/return x surgery	n/a	n/a	n/a	n/a**
	stray x surgery	reference	reference	reference	reference

* significant P-value at < 0.05

** There were 14 cats in the transfer/return category that had surgery, and 0 of those 14 cats had URI; therefore, those 14 observations could not be used in the analysis.

After excluding those cats that were in the shelter for seven or fewer days and cats that had URI within seven days of admission, a total of 144 cats were identified with severe URI; this included 70 cats (11.9%) from group 1 and 74 cats (13.5%) from group 2 (Figure 3.1). This difference was not significant (P = 0.4). The final multivariable logistic regression model for risk of severe URI excluding the first seven days included factors for vaccination (NS, P = 0.12), number of housing changes (P < 0.001), and the interaction between age and surgery. The final multivariable model showed acceptable model-fit (Hosmer-Lemeshow P=0.16).

Excluding those cats that were in the shelter for 7 or fewer days and cats that had URI within 7 days of admission, 13 cats (2.2%) from group 1 and 13 cats (2.4%) from group 2 had oral ulceration; this difference was not significant (P=0.87). Due to the limited number of affected cats, no further analyses were performed for this outcome.

3.3.8. Fosters

Of the 141 foster kittens and adolescents, only eight kittens had a URI occurrence; this included four kittens (4.2%) from group 1-F and four kittens (8.9%) from group 2-F (Figure 3.1). Only four kittens had severe URI; this included one kitten (1.0%) from group 1 and three kittens (6.7%) from group 2, and there was only one kitten that had oral ulceration; that kitten was from group 2 (Figure 3.1). Due to the limited number of affected kittens, no further analyses were performed for the foster category.

3.3.9. Time Outcome: Intake to URI

Of the 423 cats that had URI, 385 (91.0%) had their first occurrence of URI within 17 days of admission; this included 194 cats from group 1 (92.8%) and 191 cats from group 2

(89.3%). The median number of days to URI was ten days for both group 1 cats (range 4 to 87) and group 2 cats (range 4 to 55). When using the Wilcoxon rank sum method to compare median number of days to URI for those cats that had URI, the difference in number of days to URI between vaccination groups was not significant (P=0.8). When using the Kaplan Meier survival method that also accounted for cats remaining in the shelter without URI, the cats in group 2 had a higher probability of developing URI sooner over time as compared to cats in group 1 (P = 0.047) (Figure 3.4; Table 3.11). Number of housing changes, intake category, and the interaction between surgery and age were also significantly associated with time to URI (P < 0.001, P < 0.001, P = 0.004, respectively). When including those variables in the model, vaccination group was also significantly associated with the outcome (P =0.001). The assumptions of the proportional hazards model were met.

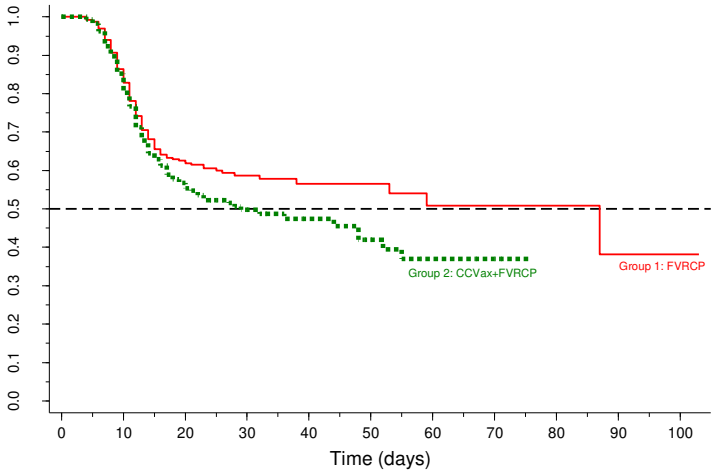

Figure 3.4: Kaplan-Meier probability curve depicting time (days) from intake to probability of not developing URI comparing group 1 (n=811 cats; 209 cats developed URI) and group 2 (n=734 cats; 214 cats developed URI).

Table 3.11: Time to URI table depicting days at-risk for URI, day interval, censored cats, and probabilities of having and not having URI

	Day interval		Number of cats at risk	new URI case	censored[a]	Probability of having URI	Probability of not having URI
Group 1 (n=811)	0	5	811	6	51	0.01	0.99
	5	7	754	17	80	0.03	0.97
	7	10	657	66	120	0.14	0.86
	10	14	471	77	96	0.30	0.70
	14	21	298	32	111	0.39	0.61
	21	28	155	5	61	0.41	0.59
	28	35	89	2	25	0.43	0.57
	35	50	62	1	35	0.44	0.56
	50	70	26	2	14	0.50	0.50
	70	90	10	1	7	0.58	0.42
	90	100	2	0	1	0.58	0.42
	100	.	1	0	1	0.58	0.42
Group 2 (n=734)	0	5	734	5	46	0.01	0.99
	5	7	683	20	62	0.04	0.96
	7	10	601	64	127	0.15	0.85
	10	14	410	75	103	0.33	0.67
	14	21	232	35	96	0.46	0.54
	21	28	101	6	33	0.50	0.50
	28	35	62	3	21	0.53	0.48
	35	50	38	4	13	0.59	0.41
	50	70	21	2	12	0.64	0.36
	70	90	7	0	7	0.64	0.36

[a]censored cats include those that left the shelter prior to development of URI

3.3.10. Time Outcome: Intake to severe URI

The median number of days to severe URI was 13 days (range 4 to 53) for group 1 cats and 12 days (range 4 to 31) for group 2 cats. When using the Wilcoxon rank sum method to compare median number of days to severe URI for those cats that had severe URI, the difference in number of days to severe URI between vaccination groups was not significant (P=0.70). When using the Kaplan Meier survival method that also accounted for cats remaining in the shelter without severe URI, the cats in group 2 had a higher probability of developing severe URI sooner over time as compared to cats in group 1 (P = 0.048) (Figure 3.5). Number of housing changes, surgery, and intake group were also significantly associated with time to severe URI (P < 0.001 for all), and vaccination group was also significantly associated with time to severe URI (P = 0.007).

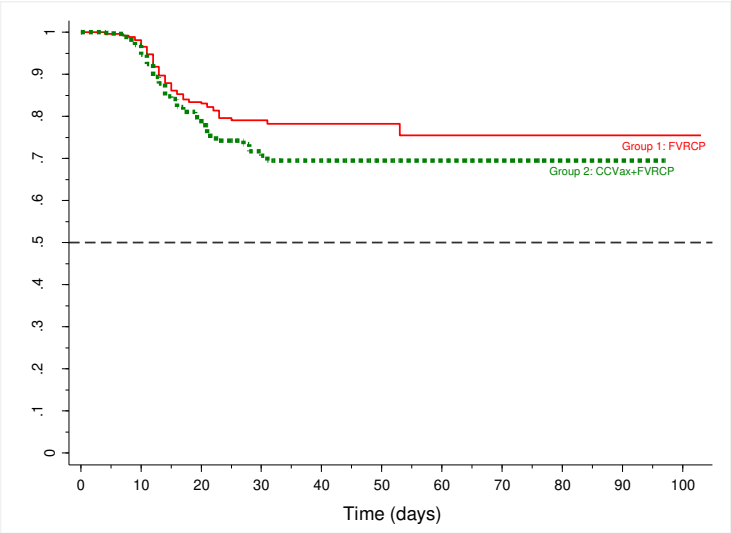

Figure 3.5: Kaplan-Meier probability curve depicting time (days) from intake to probability of not developing severe URI comparing group 1 (n=811 cats; 89 cats developed severe URI) and group 2 (n=734 cats; 99 cats developed severe URI).

3.3.11. Time outcome: URI to resolution of illness

Of the 209 cats from group 1 with URI, 83 cats (39.7%) had a resolution date recorded, 99 cats (47.4%) were adopted prior to a recorded resolution date, 15 (7.2%) were euthanized or died, and 12 (5.7%) were transferred out of the shelter. Of the 214 cats from group 2 with URI, 55 cats (25.7%) had a resolution date recorded, 123 cats (57.5%) were adopted prior to a recorded resolution date, 21 (9.8%) were euthanized or died, and 15 (7.0%) were transferred out of the shelter. The median number of days from URI occurrence to resolution of URI was nine days in group 1 (range 2 to 39) and nine days in group 2 (range 3 to 59). When using the Wilcoxon rank sum method to compare median number of days to resolution for those cats that had a resolution date, the difference in number of days to resolution between vaccination groups was not significant (P=0.9). Because nearly 60% of the cats left the shelter prior to a resolution date, the Kaplan Meier survival method was not used to evaluate this variable further.

3.3.12. Time outcome: Intake to ulceration

The median number of days from intake to an oral ulcer was 21 days (range 8 to 27) in group 1 (n=16) and 20 days (range 13 to 28) in group 2 (n=17). This difference was not significant (P = 0.6).

3.4 Discussion

Approximately a quarter of the feline population in a large open-admission shelter had URI clinical signs; this proportion was comparable to that found in other recent studies of URI in shelter cat populations.[1,3,9,11] Nearly half of the cats with URI had severe URI clinical signs, and a very small proportion had oral ulceration identified; this distinction in prevalence between mild versus severe clinical URI signs has not previously been reported for a feline shelter population. However, this study did not support our original hypotheses that the cats that received the CCVax vaccine in addition to the standard FVRCP vaccine would have enhanced protection from URI and severe URI clinical signs. After exclusion of the first seven days in the shelter, there was no difference between the two vaccine groups and occurrence of URI and severe URI. However, after exclusion of just the first three days in the shelter and after controlling for other variables, cats that received the CCVax (group 2) had significantly greater odds of having URI and severe URI as compared to those that did not receive the CCVax (group 1). These were unexpected findings and did not support our hypotheses.

3.4.1. Limited efficacy: Humoral immunity, inactivated vaccines, and pathogen exposure

There are several possible reasons why the CCVax might not have provided enhanced protection against URI, severe URI, and oral ulceration in our study. First, the CCVax was an

inactivated vaccine that was administered along with the standard MLV FVRCP vaccination upon admission and 2 to 3 weeks later as a booster revaccination. Unfortunately, however, killed vaccines usually require at least two initial doses, 3 to 4 weeks apart to induce protective immunity that occurs typically within 7-10 days after the second dose.[14,16,37] In one study, kittens were administered an MLV or an inactivated FVRCP vaccine at two week intervals for three total vaccinations, and antibody titers were measured; kittens that received the inactivated vaccine were slower to show an FCV antibody response, and only two out of the six kittens receiving the inactivated vaccine were seropositive after receiving two doses.[38] Therefore, it seems that in the study reported here, the inactivated vaccine was unlikely to provide humoral protection until day 21 of the cat's stay in the shelter. Considering the average length of stay for cats in this shelter was 17-18 days, and the median length of stay was 13-14 days, humoral immunity likely activated after the cat had already left the shelter. Furthermore, the median time to URI for the cats in this shelter was 10 days. It therefore seems very likely that the cats in this study were exposed to upper respiratory pathogens much earlier than onset of humoral immunity to the pathogens. The practicality and requisite efficiency of shelter intake procedures and flow through a shelter precluded a quarantine area or quarantine period for incoming cats; therefore, cats were exposed to a variety of pathogens upon admission.[4,6,39]

Previous studies have found that the average time to develop URI in a shelter while adhering to intake vaccination protocols was approximately eight days.[1,11] Our study had similar findings. Therefore, if the following events occurred: pathogen exposure early in the cat's stay in the shelter, incubation periods of the pathogens within the timeframe of 2-10 days, URI within the first 1-2 weeks within the shelter, and humoral immunity by inactivated vaccine requiring two vaccinations at least two weeks apart to provide protection, it was a reasonable finding that

the CCVax vaccine would not have been efficacious in this environment. Humoral immunity might have been upregulated if the cats entering the shelter had previously received the CCVax bivalent vaccine. However, that scenario was highly unlikely, as the CCVax vaccine was not part of the "core" vaccination series recommended in regular clinics or shelters.[14,16]

It might be suggested that lack of efficacy could have been due, in part, to low prevalence or incidence of FCV in this shelter setting. However, as discussed above, even if cats were largely exposed to FCV in this shelter, they would likely have been exposed prior to onset of humoral immunity, and thus the vaccine would likely not have been able to induce protective humoral immunity against FCV since the cat would have already been exposed. Nevertheless, if there was a very low incidence of FCV, if the cats had an unusually long length of stay, and if cats were exposed to FCV rarely after humoral immunity could confer protection, any potential benefits might have been lost due to the small numbers of FCV in this population. However, the vaccine's potential ability to confer additional protective mechanisms against URI and severe URI and not merely humoral immunity against FCV, was also part of the impetus for this study.

3.4.2. Evidence to try CCVax: Dual strain calicivirus vaccines

This dual strain FCV vaccine was chosen due to efficacy in previous studies, anecdotal reports, and because VS FCV had occurred primarily in vaccinated cats.[30,40] Dual strain FCV vaccines have performed well in research settings.[28,30,31,41] One study combined two strains, FCV-G1 and FCV-431, in a SQ MLV vaccine and compared responses to kittens that received a monovalent vaccine of each strain; kittens were challenged with different isolates 31 days after vaccination, and the dual strain vaccine resulted in fewer clinical signs and less virus shedding when compared to the single strain vaccines.[41] In a later study, a SQ inactivated non-adjuvanted

vaccine containing the same 2 previous strains, FCV-431 and FCV-G1 was compared to a SQ MLV vaccine containing the FCV-F9 strain and to a SQ adjuvanted inactivated vaccine containing strain FCV-255.[28] In that study, kittens were vaccinated twice, four weeks apart and were challenged 1-4 weeks after the vaccination series. After challenge with FCV-255, the kittens that were vaccinated with the dual strain vaccine had fewer clinical signs compared to the kittens that were vaccinated with the FCV-F9 MLV strain, and clinical signs in the dual strain group were comparable to those kittens vaccinated with the adjuvanted inactivated FCV-255 strain.[28] Another study compared a SQ inactivated vaccine containing a VS isolate in a traditional FVRCP/FeLV vaccine to a SQ inactivated traditional FVRCP/FeLV vaccine.[30] In that study, kittens were vaccinated twice, three weeks apart. The antisera from kittens that received the dual strain vaccine had higher levels of neutralizing antibodies in assays, and more strains of FCV virus were neutralized when compared to the antisera from cats that received the traditional vaccine alone. Kittens that received the dual strain vaccine were challenged with the homologous VS-FCV isolate two weeks after vaccination, and they were compared to an unvaccinated control group; the vaccinated group did not develop VS, whereas all kittens in the control group became severely ill with VS clinical signs. The study did not compare challenge to the traditionally vaccinated group.[30] Recent in vitro virus neutralization studies have similarly found higher cross-neutralization from antisera against two FCV strains as compared to antisera from one strain.[31] The challenge experiments in each of the aforementioned studies, however, were performed after the complete vaccination series had a sufficient time to activate humoral immunity. This differs markedly from the shelter scenario in which cats were likely exposed to infectious pathogens upon entry/admission to the shelter or very shortly thereafter, and some likely entered the shelter as carriers of pathogens.[3,9,39] Due to vaccination and exposure timing differences, the previous

research environment experiments regarding dual strain FCV vaccines should likely not be extrapolated to the shelter environment in future studies. This suggestion was confirmed by the findings of our study.

As the CCVax vaccine had been shown to have good cross neutralization abilities for FCV,[30] it might have been possible that cats that had previously received an FVRCP vaccine prior to shelter entry, would have had an anamnestic response to the CCVax vaccine when administered. Prior vaccination history, however, was unknown for the cats in this shelter. And if FCV incidence was low in this study, potential benefits would have been lost due to the small numbers of FCV in the environment.

3.4.3. Evidence to try CCVax: Innate cell mediated immunity

If humoral immunity were unlikely to occur, the other reason this study design was implemented was because of the possible protection conferred by innate cell mediated immunity (CMI). Although vaccinations, in general, target the adaptive immune system, the innate immune system also is necessary for a vaccination to be effective.[14,42] Innate CMI can be activated by adjuvants, peptides, lipids, carbohydrates, or nucleic acids within the vaccine, the vaccine vector, or a modified live pathogen, as these are substances introduced into the host, and they are typically recognized as foreign substances by the innate immune system.[14,42,43] The inactivated CCVax likely had components able to trigger an innate CMI response.

The innate CMI response might also target the other pathogens responsible for URI. In one study, cats without detectable antibody to FHV-1 (or FPV) were still resistant to challenge with those pathogens, suggesting that innate CMI might contribute to protection.[32] In another study, kittens were either not vaccinated or vaccinated with a SQ MLV or inactivated version of

an FVRCP vaccination, and seven days later they were challenged with an FHV-1 strain.[36] Total clinical scores in both vaccination groups were similar post-challenge and significantly less than total clinical scores in the unvaccinated group, and on days 15-21, the group that received the inactivated vaccine had lower total respiratory scores as compared to the group that received the MLV vaccine. The study suggested that an early CMI effect was responsible for the lowered clinical signs as early as seven days after vaccination with either the MLV or inactivated vaccine.[36] However, in our study reported here, innate CMI that might have been conferred by the CCVax did not appear to improve responses for those cats vaccinated with the CCVax. We conducted analyses after removing the first three and seven days of data to allow time for early CMI; however, there was no difference between groups after removing the first seven days of data, further suggesting that early CMI was not preferentially activated for those cats that received the CCVax.

3.4.4. Limited efficacy: Resistance

It has been suggested that field isolates of FCV may be developing resistance to the commonly used vaccine strains (FCV-F9 and FCV-255). This is suggested considering outbreaks of VS have occurred in vaccinated cat populations, and the VS strains were genetically distinct.[22–24,44–46] Furthermore, another study found that in cats infected with FCV, the antisera against the newer strains FCV-G1 and FCV-433 neutralized significantly more of the field isolates than the antisera for the older strains FCV-F9 and FCV-255 that lacked neutralization ability.[47] Another study had similar findings, suggesting that more recent vaccine strains FCV-G1 and FCV-431 had better neutralizing ability than the older FCV-F9 and FCV-255 strains.[31] On the other hand, Porter et al 2008[48] found that both of the vaccine strains for FCV-F9 and

FCV-255 were successful in neutralizing field isolates. And Afonso et al 2017[49] also found that antisera against FCV-F9 was still broadly cross-reactive against field isolates in in vitro virus neutralization assays. The cats in the study reported here that received the CCVax had a similar occurrence of reported oral ulceration as the cats that did not receive the CCVax; it therefore might be possible that vaccine resistance played some part in the lack of efficacy of the CCVax. However, this is also presupposing that humoral immunity would have defined the vaccine response.

3.4.5. CCVax higher risk

In our main multivariable models that excluded only the first three days after admission, cats in group 2 had an increased risk of URI, increased risk of severe URI, and higher probability of developing URI and severe URI sooner when compared to cats in group 1. The significance of this risk factor of CCVax, however, was lost when the first seven days were removed from the data. Nevertheless, this unexpected finding of the potentially short-term negative effects of the CCVax were worth exploring.

The FCV strains used in the CCVax were evaluated in another study[30], and those kittens that received the vaccine were not reported to have clinical illness from vaccine administration. In that study, the kittens were 8 weeks old and housed in a barrier control research facility, administered one vaccine, and exposed only to FCV.[30] In contrast, the cats in the study reported here were of various backgrounds and entering the unfamiliar shelter environment, administered two vaccines concurrently, and were likely exposed to a variety of pathogens upon intake and throughout their stay. The findings in the previous study were therefore not directly applicable to the study reported here.

Some of the most common adverse events reported for vaccines in companion animals are transient pyrexia, lethargy, and inappetence within a few days after vaccination.[50–54] As discussed above, this could occur due to a highly upregulated innate immune response to the vaccine components and resultant cytokine and chemokine production, and this could occur after receiving an adjuvanted product.[43,50] Although adjuvants typically improve the stability of or enhance presentation of the antigen in the vaccine to the host, or they act as immunomodulators by altering cytokine upregulation and downregulation, the immune activation and upregulation of cytokine responses could theoretically increase some of the adverse effects of vaccination responses such as pyrexia or lethargy.[55] This excessive upregulation could be related to the interaction of the adjuvant and antigen.[55]

There has also been some evidence of vaccination inducing a transient immunosuppression or shift of innate CMI and humoral responses.[50,56–58] One study found a significant decline of CMI function post vaccination in dogs; they found a decline in an in vitro response of lymphocytes and also found that peripheral neutrophils and Th1 immune reactions decreased after vaccination.[58] In general, this would not cause disease. However, in the shelter environment, such a response might then lead to increased illness considering the amount of stress endured in the new shelter environment as well as likely exposure to many pathogens, all contributing to lowered immune responsiveness and tendency to develop disease.[5,59] There was one report of several kittens from a cattery developing fatal salmonellosis and panleukopenia after vaccination and potentially related the mortalities to immunosuppression induced by the vaccine.[56] Furthermore, another study found cats had delayed aberrant reactions 7 to 21 days after vaccination with an FVRCP-Chlamydia psitacci vaccine.[54] Some of the reactions included upper respiratory inflammation, inappetence, and lethargy.[52,54] These findings are in contrast to

the innate CMI response expected as reported in other studies.[32,36] However, our study supports the possibility that immunosuppression or a shift in immune responses occurred.

The administration of the CCVax with the FVRCP vaccine had not been evaluated previously. It is possible that the administration of the two vaccines concurrently in our setting, led to a higher overall impact to the cats' immune system and a subsequent partial immunological compromise instead of enhancement. Since some MLV vaccines have been implicated in clinical signs consistent with the virus for which the cat is being vaccinated, it seems possible that the combination used in this study could also lead to clinical signs.[45,60] If this occurred in response to the CCVax components combined with the MLV FVRCP components to enhance the response, this could have led to increased URI in that group. In a study of canines receiving a polyvalent vaccine, significant lymphocyte suppression was found within five days of the vaccinations.[57] The study suggested that the combination of and interaction between one of the viral strains in one vaccine and an attenuated strain of another virus resulted in the significant decrease in lymphocyte response, potentially related to lytic action or suppression of proliferation. Another study found that in cats, the greatest risk for adverse vaccine reactions including lethargy and pyrexia, was the number of concurrently administered vaccines, and the risk increased with each additional vaccine, and risk of lethargy post-vaccination was significantly associated with the multivalent FVRCP vaccine.[53] Starr (1993) had similar results in that the administration of multiple vaccines to cats resulted in an increased risk for adverse reactions such as lethargy and pyrexia.[54] On the other hand, another study found that combining the FVRCP and rabies vaccines in cats did not significantly impact their serological response and concluded that the two could be administered together.[61] It cannot be ruled out, however, that the

combination of the MLV FVRCP and CCVax with the inactivated VS component might have interacted in an unexpected manner in this particular environment.

Another hypothetical possibility is a combination of increased innate CMI response combined with elimination of the CCVax antigens. If the MLV vaccine upregulated CMI and antibody responses rapidly, this might have led to removal of the antigens introduced by the CCVax while also concomitantly directing the immune response toward the CCVax antigens thus away from other pathogens encountered in the shelter. There is no data to support such a theory, but it is one worth mentioning. Another unexpected finding was that cats in group 1 were more likely to remain in the shelter for a longer period of time and not develop URI or severe URI, whereas those cats in group 2 had a higher probability as compared to group 1 cats of developing URI and severe URI as length of time in the shelter increased. The longer lengths of stay for the cats in both groups included the 2-3 week booster vaccination period; therefore, humoral immunity from the CCVax would be expected to be upregulated after that time. But on the contrary, there were more cats from group 1 that remained in the shelter for longer periods of time without URI. Our results, therefore, did not support the presence of increased protective immunity for the cats that were still in the shelter weeks after their booster revaccinations. Instead, the group 2 CCVax cats continued to become sick with URI with a higher probability as time progressed (Figure 3.4, 3.5). Our findings suggested that the CCVax combination might have encouraged increased pathogen invasion and infection. On the other hand, if FCV prevalence in the shelter was low, and if the main benefit of the CCVax was humoral protection, then even those cats that remained in the shelter long enough for the booster vaccination benefits, differences would be lost due to the small numbers of FCV in the environment.

Differences in outcomes comparing vaccine groups were lost when analyses did not use cats that became ill or left the shelter within the first seven days of admission. These findings lend some further support to the above theories involving some component of early CMI related to the CCVax adversely contributing to the increased illness in the group 2 cats. Loss of significant differences between vaccine groups could be because cats with a more robust immune system or cats less susceptible to the stress of the shelter, were able to avoid the initial seemingly adverse effects associated with the combination of the CCVax and FVRCP vaccine. A complex interaction of factors related to the virus and vaccine combined with the environment, cat's genetics, experiences, nutrition, and immunologic factors contribute to how each cat responds to vaccination and pathogen exposure.[10,14,25,46,62] However, there was still no additive protective effect with the addition of the CCVax when evaluating the groups excluding the first 7 days. Potential reasons are discussed above.

3.4.6. Oral ulceration

Oral ulceration was a rare occurrence in this study, and it occurred with similar frequency in both vaccination groups. However, all cats with URI or inappetence were not necessarily evaluated nor evaluated adequately for oral ulceration; therefore, oral ulceration could have been under reported. Nevertheless, because the risk of oral ulceration was similar between vaccination groups, it seems likely that the pathogens affecting the cats and responsible for clinical URI illness were not solely FCV, but rather a combination of viral and bacterial pathogens as would be expected in a shelter setting.[9,63–65] It also may suggest that the prevalence of FCV was low. There was also no evidence of VSD in this shelter during this time period.

3.4.7. Prevalence of URI and severe URI

The overall prevalence (27%) of URI in the shelter in our study was similar to that found in other feline shelter studies, although both ours and the other studies varied in geographical location, timeframe evaluated, and type of facility.[1,3,9,11] The median number of days for cats to develop URI in our study was ten days; this was also similar to findings in other studies. Other studies have found mean or median time to develop URI as 8.3 days,[11] 14 days,[9] between two to eight days for carriers of URI implicated pathogens,[9] and six days in kittens and seven days in adults.[1] Any cats that developed URI within the first three days of admission, however, were removed from analyses in our study; therefore, this artificially increased the median length of time to URI and decreased the probability of developing URI (Table 3.11; Figure 3.4). Whereas another study[1] found that the probability of developing URI was between 26-32% by day 7 in the shelter and between 80-86% by day 14 in the shelter, our study found that probability of developing URI was 31% by day 14 and 42% by day 21, but 91% of the cats in our study that had URI, had their first occurrence within their first 17 days in the shelter. It is also possible that some of the differences are because many variables differ between shelters such as definitions and protocols for reporting of URI; prevention, management, and treatment of URI; and changes in implementation of shelter practices since the previous studies were performed.[13,15]

Most previous reports of URI in shelters have addressed URI by pathogens involved or a general URI diagnosis including both mild and severe forms of URI together in rates.[1,3,7,9,66] To the authors' knowledge, ours is the first report distinguishing severe URI from occurrence of any URI. Almost half of the cats that had any URI occurrence, had a severe URI occurrence as well. This was an important finding, as it indicated the gravity of having URI in a shelter if nearly half of the cats will have progression of disease with a potentially poor outcome. Of the cats in the

shelter that had severe URI, almost 20% of those cats were euthanized or died in the shelter; this was twice the number of overall deaths or euthanasias of cats in this shelter. This indicated that URI continues to be one of the more common medical reasons for euthanasia of cats in shelters.[1,66] Therefore effective control of URI in the shelter to prevent progression to severe disease is an important objective of shelter population management.

Interestingly, of the cats that had only non-severe URI, none of those cats were euthanized or died, and their adoption rate (94.5%) was much higher than the overall shelter cat adoption rate (77%). It is possible that those cats had mild clinical signs from the vaccines or, more likely, the vaccines helped to prevent serious clinical disease in those cats.

3.4.8. Other shelter and risk factors

The number of housing changes was a significant risk factor in all analyses; as the number of housing changes increased, the risk of URI and severe URI increased. Although thought to be a factor responsible for increased stress or URI in shelter cats, kennel or housing changes in the shelter environment have only been evaluated in one other study.[3] Similar to our findings, that study found that cats that were moved to different kennels more than two times within the first week of the shelter stay, had a significantly higher risk of URI.[3] Other studies evaluated FHV-1 and housing changes in purpose-bred cats in research facilities.[67–70] Increased FHV-1 viral shedding occurred after cats were exposed to a variety of housing changes.[67,68] Since cats experience stress with moves or changes in their environments and unfamiliar places, unusual or novel sights, sounds, smells, and inconsistencies, it has been inferred that cats may become stressed with shelter kennel or housing changes.[70–75] Our results supported that theory. In our study, cats were moved to a different kennel or housing location an average of five times

throughout their stay and four times within the first 17 days of admission. The maximum number of kennel changes for a cat was 21 moves. Although the large number of housing changes could have included moves to a nearby kennel, various group housing rooms, or to different kennels before, during, and after surgical procedures, it intuitively seems that moving a cat to a different environment so many times might result in stress and consequently illness. Although it might be considered that the number of kennel or housing changes might increase the risk of URI because of the increased exposure to more pathogens, this is less likely because of the large amount of movement throughout the shelter so that all pathogens are likely distributed throughout the shelter fairly similarly.

Cats that are declawed might engage in more inappropriate behaviors that might be a manifestation of stress related to chronic pain or discomfort.[76,77] Our study therefore evaluated a cat's declaw status (declawed or not) as another measure of stress that could influence and act as a risk factor for URI occurrence. Although the prevalence of declawed cats in the U.S. is unknown, geographical prevalence rates have been reported at approximately 20% in the early to mid 2000's.[78,79] Only 7.3% of the cats in our study were listed as declawed; this is lower than other reported prevalence rates. This could be due to lack of identification or recording a cat's declaw status in the shelter, geographical area, or a decrease in the number of declaw procedures being performed due to onychectomy being increasingly considered an inappropriate procedure.[80–82] Nevertheless, declaw status was not associated with URI occurrence in our study.

In most of this report's models, stray cats were more likely than owner surrendered, transfer, and returned cats to develop URI and severe URI. This has been found in other studies as well.[1,9,83] This could be due, in part, to the stress experienced when placed in the completely foreign indoor environment and kennel, although that is in contrast to the findings of one study

that suggested that owner surrendered cats experienced more stress than stray cats during the first three days of shelter admission.[84] The increased URI in stray cats in our study could also be due, in part, to likely being unvaccinated in combination with existing latent infections due to their outdoor and territorial lifestyle.[83,85] It is also possible that stray cats might be more susceptible to illness due to poor nutrition; however, BCS and general appearance (unkempt versus groomed) were not evaluated in this study so this theory cannot be confirmed. Another consideration is that the category of stray was not necessarily defined as a feral, unsocialized, outdoor, unowned cat. Stray cats also included those that previously belonged to a household but wandered away or were abandoned by the previous household, and some stray cats might be brought to the shelter by an individual that reported the animal as a stray instead of owner surrender to avoid stigmatization for surrendering an animal. However, because our study as well as other recent studies have found that stray cats are at significantly increased risk of URI in the shelter, it seems that most cats categorized as strays possess certain features rendering them more susceptible to illness induced by shelter life.[1,9,83]

Interestingly, when we excluded the first seven days in the shelter, there was a significant interaction between intake group and surgery. Strays that did not have surgery were the most likely to have URI, and strays that had surgery were the least likely to have URI when compared to owner surrendered cats. This could be because the legal requisite in this shelter was to enact a "stray hold" for five days on a cat to allow an owner to reclaim the cat. Those cats that were unclaimed after day 5 and became ill shortly thereafter (after day 7) would likely not undergo gonadectomy or other surgery until healthy again, whereas those cats that were owner surrendered likely underwent surgery sooner because they did not have to wait for the stray hold. On the other hand, if a stray cat were unclaimed and healthy on or after day 7, those cats were

likely very adoptable and would subsequently be scheduled for surgery very soon after their stray hold had expired. In fact, a disproportionate number of stray cats underwent surgery (62.7%) as compared to strays that did not have surgery (37.3%) and owner-surrendered cats that had surgery (41.2%); this could have artificially resulted in statistical significance and a type 1 error. Other surgical procedures could have also been responsible for the higher occurrence of URI in owner surrendered cats that had surgery.

Although we would expect that kittens, in general, would be more likely to have URI in the shelter[39,66] we instead found that there was an interaction between surgery and age. Of those cats that did not have surgery, kittens were more likely to have URI. Since URI is common in kittens in crowded environments, it is possible that gonadectomy surgery was delayed until after the kittens' health had returned; URI would have therefore occurred prior to surgery. Kittens that had surgery were significantly less likely to have URI. This is likely because those kittens in the shelter that had surgery were healthy adoptable kittens having gonadectomy surgery and were immediately adopted after their surgeries. In general, performing prepubertal gonadectomy is considered a quicker, simpler, and safe procedure with few complications as compared to performing gonadectomy in older cats.[86–88] If surgical time and complications are reduced during prepubescent gonadectomy, those kittens likely recover more uneventfully and without as much stress subsequently. This decrease in stress could therefore have led to a decrease in URI in those kittens that underwent surgery as compared to adults that underwent surgery. As age increased in cats that had surgery, the likelihood of URI increased. Those adult or senior cats in the shelter that had surgery were either having gonadectomy surgery or another surgical procedure such as a dental procedure, cystotomy, wound management, or other. Other studies have also found an

increase in risk of URI with increasing age.[1] If the additional stress of surgery is combined with increasing age, it seems reasonable that risk of URI would increase.

The foster kitten and adolescent group in our study had a very low occurrence of URI compared to the occurrence of URI in the rest of the study. The foster group were vaccinated in the shelter and then returned to home environments that usually also included their siblings. The low occurrence of URI in this group of fosters was very likely related to the individual attention and care they received in a home environment, decreased stress in that environment, and less exposure to the load of bacterial and viral pathogens in a shelter environment.[7,10,39]

3.4.9. Limitations

There were several limitations to this study. First, we did not identify the bacterial or viral pathogens that might have been contributing to URI clinical signs in this study, nor did we evaluate cellular responses. Although levels of viral shedding do not necessarily correlate with severity of clinical illness, measurement of these variables might have added some explanatory data to our study.[19,89,90] The methods to detect presence of FCV include amplification of nucleic acid via PCR, virus isolation in culture, and antibody detection by virus neutralization methods or ELISA.[19,32,91–93] Diagnosis of FCV associated illness , however, is complicated by subclinical carriers, vaccination strains, components of cell-mediated immunity, co-infection with other pathogens, and detection thresholds; clinical signs and the detection of the virus by various molecular methods are not well correlated (Table 1.3).[19,27,46,49,90,92,94] The same diagnostic methods and issues similarly occur for the molecular diagnosis of FHV-1 viral shedding. Molecular detection of bacterial pathogens might also have assisted to account for differences in URI occurrence and severity within vaccination groups and relationship with other factors.

Identification and quantification of cytokines and leukocyte or other cellular components to assess the upregulation or downregulation of immune function components prior to and subsequent to vaccination and onset of URI might have also added important information to our study. Sampling methods for the above procedures were not approved in the shelter. Our results were therefore based solely on clinical signs; therefore, interpretations must be made with caution, as it is unknown whether cats in group 2 had a higher or altered viral or bacterial load or a shift in leukocyte or cytokine responses as compared to group 1.

Another limitation was that URI reporting and scoring were dependent on whether a shelter staff member or volunteer, while performing another shelter duty, noticed and recognized that a cat had a URI clinical sign. Therefore, it is possible that URI could have been under reported and over reported dependent on vigilance and timing. Similarly, oral ulceration was not evaluated or noticed in all cats that had URI or severe URI; this would have resulted in under reporting of oral ulceration. Problematic under reporting of illness in the appropriate database fields was noticed after the study's conclusion and was remedied by the investigators thorough searching through all database fields for key words. Because this was a very large shelter with hundreds of staff and volunteers throughout the time period of the study, data errors and inconsistencies were expected. However, also because of the large sample size, this also allowed evaluation and summarization of data for a very large number of cats; large study numbers decreased chances of a type II error. The large study sample size also reinforced confidence that the failure to detect a difference in favor of the CCVax was a true negative finding.

The evaluation of several other variables might have helped to explain some of the differences in URI and severe URI. Type of surgery was not identified in our analyses; although gonadectomy was very likely the most common surgery performed, dental procedures were also

performed with frequency but not evaluated here. Dental as compared to gonadectomy surgery might have been associated with a higher occurrence of URI, severe URI, and potentially oral ulceration. Other surgeries such as wound repair, amputation, cystotomy, fracture repair, enucleation, or other surgical procedures were all combined together under the category of surgery, although each type of surgery could have very different recovery times and resultant stress and therefore risk of URI.

Dental disease was also not evaluated in our study, although many cats in this shelter had dental release/waiver forms in their records indicating that the cat, upon adoption, had dental disease which might require attention by a veterinarian. Similarly, chronic gingivitis and stomatitis were not evaluated in our analyses, and this might have contributed more explanatory data since FCV has been associated with chronic lymphoplasmacytic gingivitis stomatitis (LPGS).[34,95,96]

Treatment regime instituted for the cats was also not evaluated. Some cats might have received antibiotics at different timepoints, and some might have received oral medications in combination with ocular and/or nasal treatments. In severe cases, some might have received fluids, appetite stimulants, face washing, antiviral medications, and supplements such as probiotics or lysine. All of these treatments and durations could have affected outcome.

Other limitations included those similar to other shelter studies in that we were unaware of the cats' physical, environmental, behavioral, health, or vaccination histories. Any of these variables could have affected outcomes. Furthermore, data was collected from a single shelter during one summer; this limits generalizability of our findings. However, this was a very large open-admission shelter in a metropolitan area that likely has cats from varied backgrounds similar to many other shelters and households throughout the US. At the time of this study, the

shelter also had a variety of different kennel sizes, single and double compartments, different locations with varied amounts of staff and public traffic, older stainless steel kennels and newly constructed kennels, and group rooms of varied sizes with many permutations of numbers of cats within the group rooms. Cats were therefore exposed to a variety of cats, staff, volunteers, public, and environmental surroundings that could have affected individual responses and infection rates. Cats were also often transported to the shelter's clinic area to be evaluated by the veterinarian thus adding additional unreported levels of stress and potential exposure to additional pathogens. Similarly, air exchange rates, cleaning protocols, and fomite transmission were factors likely to affect any shelter study, and these were also not evaluated. Although food was generally standardized within the shelter, some cats received special diets, all canned, all dry, or mixed foods. Neither food composition nor consumption were evaluated in our study.

Despite the limitations of conducting a study in a large municipal open-admission shelter, this also allowed evaluation of a large number of shelter cats thus making extrapolation of results tenable. The shelter had one of the largest shelter cat populations in the country with an annual intake number of approximately 10,000 cats per year, and their overall live release rate for cats was above the national average.[97] We feel our results provide useful information and insights that might be utilized by other shelters and their management of URI and cat vaccination protocols.

3.5. Conclusions

This study did not find evidence that the CCVax protected cats from developing URI, severe URI, or oral ulceration indicative of calicivirus. Furthermore, negative effects were found in cats that received the CCVax in our study. Although a potential protective benefit against

VSD might be found if cats received the full CCVax vaccination series prior to entering a shelter and being exposed to VSD, that scenario is highly improbable; thus, the potential risks and incurred extra expenses of this additional vaccine in a shelter setting, far outweigh any likelihood of benefits. Although numerous factors could be responsible for both no differences between the groups as well as increased illness in the CCVax group, our study, overall, does not support the use of this additional vaccine in this shelter setting.

Some of our other findings might guide improved URI prevention and management in shelters. Since nearly half of the cats that had URI eventually developed severe URI, further attention might be directed toward improved URI detection and identification protocols and evaluation of management methods after detection; evaluation of this shelter's treatment data and future studies could help to guide treatment decisions. The numerous housing changes experienced by an individual cat in the shelter should also be considered, as housing changes was the one factor that was a significant risk factor for URI, severe URI, and time to URI in all models. Repeated alteration of a cat's environment and surroundings induces stress and further pathogen exposure; therefore, minimizing housing changes might reduce stress and exposure to more organisms, and thus occurrence of URI and severe URI. Since the foster kittens and adolescents had the lowest occurrence of URI, foster homes for ill cats might also help to reduce URI spread and hasten recovery for ill cats.

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CHAPTER 4: PILOT STUDY TO EVALUATE THE EFFECTS OF AN ORAL SUPPLEMENT, CARNIVORA™, ON CLINICAL SIGNS OF FELINE HERPESVIRUS-1 IN CATS

4.1. Introduction

Feline herpesvirus-1 (FHV-1) infection is a common and highly contagious feline upper respiratory pathogen. FHV-1 infection can be subclinical or can result in severe clinical disease including fever, sneezing, nasal discharge, conjunctivitis, keratitis, cough, dyspnea, and occasionally death.[1–5] While FHV-1 infected cats can be clinically normal for periods of time, the infection can be reactivated by crowding, other concurrent diseases, and other forms of stress.[2,6,7] Immunity against FHV-1 is not complete; therefore, vaccinated or previously infected cats can become ill when re-exposed to FHV-1.[6,8–11] In addition, during periods of reactivation, FHV-1 can be shed again in ocular or respiratory secretions, potentially resulting in the infection of other cats.[6,12]

Currently, oral administration of famciclovir or topical administration of cidofovir (ocular cases) are considered by many veterinarians to be the optimal treatments for cats with clinical signs of FHV-1 associated disease that are severe enough to warrant more than supportive treatment.[13–17] A number of strategies with variable outcomes have been employed in an attempt to lessen FHV-1 reactivation in cats.[9] Lessening stress, administration of lysine, and feeding an immune enhancing probiotic have been recently studied or reviewed.[18–21] Feeding of the immune-enhancing probiotic, administration of alpha 2b interferon, and use of an intranasal vaccine as a potential immune therapy have provided information suggesting that immune modulation could be effective for the treatment or control of FHV-1.[19,22]

Carnivora™ is a commercial preparation derived from the extracts of Dionaea muscipula, the Venus fly trap carnivorous plant species (Figure 4.1). The product contains compounds including naphthoquinones such as hydroplumbagin, plumbagin, and droserone, phenolic acids such as gallic acid, and flavonoids such as quercetin.[23–26] Studies have reported that Carnivora™ and these compounds might have immune modulatory, anti-inflammatory, anti-neoplastic, antimicrobial, and antiviral activities in in vitro and some in vivo models.[23,27–29] Carnivora™ has also been used in some pets and might have some antiviral activity against herpes simplex type 2 of humans.[26,27,30] The hypotheses of this study were that treatment of cats with Carnivora™ would be safe and would lessen clinical signs of FHV-1 as well as viral shedding in cats that underwent repeat exposure inoculation to FHV-1.

Jennifer Koches, USFWS; https://www.fws.gov

Figure 4.1: Dionaea muscipula

4.2. Materials and methods

4.2.1. Treatment groups

A total of 16, two-year-old cats were used with Institutional Animal Care and Use Committee (IACUC) approval. One year before the study described here, each of the eight intact female and eight neutered male cats had been in a FHV-1, calicivirus, and panleukopenia vaccine study and were first infected with FHV-1 via aerosolization.[31] In that study, FHV-1 infection was

confirmed in all cats and each developed clinical signs consisting of sneezing, ocular and/or nasal discharge, and/or conjunctivitis.

For use in this study, cats were randomized into a treatment group (n = 8) or control group (n = 8) and were individually kenneled in two separate rooms. The cats were provided dry food and water ad libitum and received daily group socialization. The treatment group was administered Carnivora™ orally as either capsules (1-2 capsules in morning, afternoon, and/or evening) or drops (5, 8, or 10 drops in morning, afternoon, and/or evening) following the manufacturers' instructions (Table 4.1). The control group of cats was administered saline and empty capsules in a similar volume, concentration, and manner to simulate the same degree of stress induced by medicating the treatment group of cats. Body weights were measured weekly and doses for individual cats adjusted based on whether the body weight was above or below 4 kg.

Table 4.1: Carnivora™ dosing protocol

Cats < 4 kg	**Cats ≥ 4 kg**
Week 1 (Monday through Saturday)	Weeks 1 and 2 (Monday through Saturday)
Morning. 1 capsule with food or water	Morning. 2 capsules with food or water
Afternoon. 5 drops by syringe directly into mouth	Afternoon. 5 drops by syringe directly into mouth
Evening. 1 capsule with food or water	Evening. 2 capsules with food or water
Week 1 (Sunday)	Weeks 1 and 2 (Sunday)
Morning. 1 capsule without food	Morning. 1 capsule without food
Evening. 1 capsule without food	Evening. 1 capsule without food
Weeks 2, 3, 5, 6, 7, 9, 10, 11 (Monday through Saturday)*	Weeks 3, 5, 6, 7, 9, 10, 11 (Monday through Saturday)*
Morning. 2 capsules with food or water	Morning. 2 capsules with food or water
Afternoon. 8 drops by syringe directly into mouth	Afternoon. 10 drops by syringe directly into mouth
Evening. 2 capsules with food or water	Evening. 2 capsules with food or water
Weeks 2, 3, 5, 6, 7, 9, 10, 11 (Sunday)	Weeks 3, 5, 6, 7, 9, 10, 11 (Sunday)
Morning. 1 capsule without food	Morning. 1 capsule without food
Evening. 1 capsule without food	Evening. 1 capsule without food
Weeks 4, 8, 12 (Monday through Saturday)	Weeks 4, 8, 12 (Monday through Saturday)
Morning. 1 capsule with food or water	Morning. 1 capsule with food or water
Evening. 1 capsule with food or water	Afternoon. 2 capsules
Weeks 4, 8, 12 (Sunday)	Evening. 1 capsule with food or water
No treatment	Weeks 4, 8, 12 (Sunday)
	No treatment

4.2.2. Experimental design

Two trained, masked observers assessed the cats for 30 minutes at approximately the same time in the mornings and recorded observations using a standardized score sheet consisting of seven variables including body temperature (Figure 4.2, Table 4.2). Body temperatures were evaluated by subcutaneous microchip probe in 15 cats and axillary temperature in one cat due to two malfunctioning microchips.[32] Clinical scores were determined from Days -14 to 0 and Days 42 to 84, and temperatures were recorded from Days -11 to 0 and Days 42 to 84 (Figure 4.2). For Days 0 to 42, the cats were observed daily for attitude and the presence of sneezing and ocular or nasal discharges, but a clinical score was not determined. Total number of clinical scores > 0 in the seven clinical score categories (Table 4.2), were compared between treatment and control groups within each of the three treatment periods: pre-treatment equilibration period (14 days), pre-inoculation treatment period (15 days), and post-inoculation treatment period (28 days).

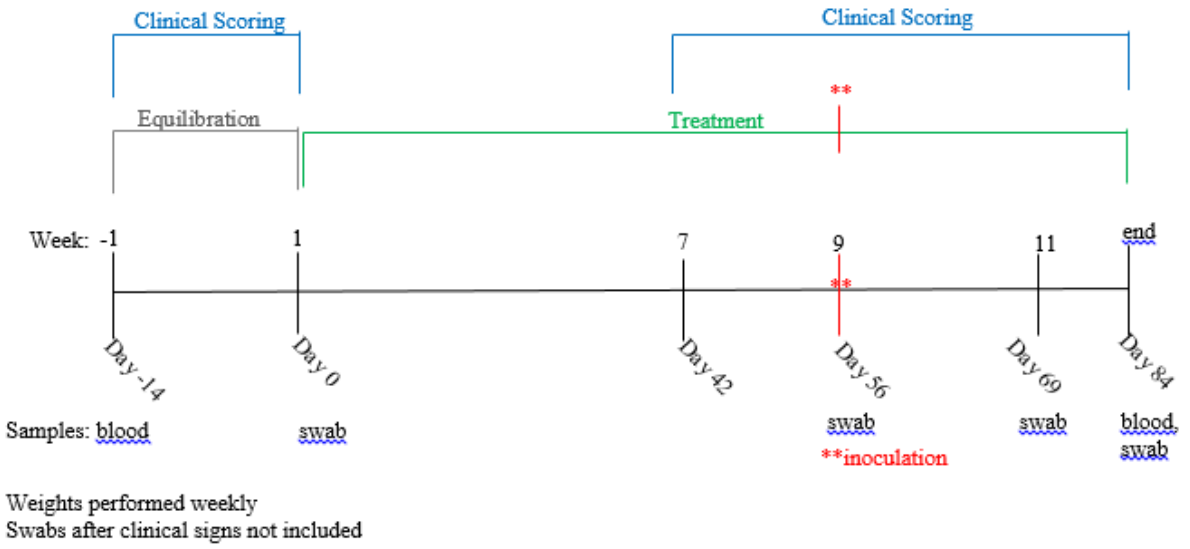

Figure 4.2: Treatment, monitoring, and sampling schedule

Table 4.2. Clinical Scoring Chart

Conjunctivitis	0 = None 1 = Mild conjunctival hyperemia 2 = Moderate to severe conjunctival hyperemia 3 = Moderate to severe conjunctival hyperemia and chemosis
Blepharospasm	0 = None 1 = Eye < 25% closed 2 = Eye 25 – 50% closed 3 = Eye 50 to 75% closed 4 = Eye completely closed
Ocular discharge	0 = None 1 = Minor serous discharge 2 = Moderate mucoid discharge 3 = Marked mucopurulent discharge
Sneezing	0 = None 1 = Observed
Nasal discharge	0 = None 1 = Minor serous discharge 2 = Moderate mucoid discharge 3 = Marked mucopurulent discharge
Nasal congestion	0 = None 1 = Minor congestion (barely audible) 2 = Moderate congestion (easily audible) 3 = Marked congestion with open mouth breathing
Body temperature (microchip)	0 = ≤ 103 °F 1 = ≥ 103 °F

Body weights were measured weekly for all cats as a surrogate marker of appetite. To assess primary, stress-associated, or FHV-1 associated weight loss, the percent of increase or decrease in each cat's body weight between Day 0 and Day 84, between Day 0 and Day 56, and between Day 56 and Day 84, respectively, was assessed. The percent change in the different periods was compared between the treatment and placebo groups.

Blood was collected on Day -14 and Day 84 (Figure 4.2). Mucosal cells were collected from the caudal pharynx of each cat on Day 0, Day 56, Day 69, and Day 84 by gently rolling a swab against the mucosa in the region (oropharyngeal swabs) for performance of FHV-1 PCR assays. In addition, oropharyngeal swabs were also collected from individual cats on the first day after challenge that clinical signs were noted and then again seven and 14 days later.

On Day 56, all cats were inoculated with a USDA challenge strain of FHV-1 via nostril and oropharynx, as previously described.[31]

4.2.3. Clinical and laboratory evaluations

On the day the samples were collected, total DNA and RNA was extracted, as previously described, from blood in EDTA. Samples were assayed for FHV-1 DNA using a previously described conventional FHV-1 PCR assay (cPCR).[33] Sera and oropharyngeal swabs were stored at -80°C until assayed in batches. Serum biochemical values were measured at a commercial laboratory (Veterinary Diagnostic Laboratory, Colorado State University, Fort Collins, Colorado). Total DNA and RNA was extracted from the oropharyngeal swabs, and the FHV-1 cPCR assay was performed as well as quantitative PCR assays for FHV-1 DNA (qPCR) and GAPDH.[17] Results of the FHV-1 qPCR assay were presented as the ratio of FHV-1 DNA/GAPDH DNA. Serum antibodies against FHV-1 were measured using a previously reported ELISA; the results were reported as absorbance values.[34] The pre and post-inoculation absorbance values were converted to a percent change value by use of the following formula: FHV-1 absorbance value post FHV-1 challenge/FHV-1 absorbance value pre FHV-1 challenge X 100.

4.2.4. Statistical evaluation

The Fisher's exact test was used to compare total number of clinical scores > 0 (Table 4.2) in the treatment group as compared to the control group within each of the three treatment periods: pre-treatment equilibration period (14 days), pre-inoculation treatment period (15 days), and post-inoculation treatment period (28 days). The Shapiro-Wilk test was used to evaluate

body weights, blood chemistry values, and FHV-1 titer changes for normality. Due to non-normalcy, the Wilcoxon rank-sum test was used to compare body weight changes between the treatment and control groups; FHV-1 absorbance value changes between the treatment and control groups at start and end of study; and the treatment group's blood chemistry values at the start and end of study. Results of the FHV-1 PCR assays performed on DNA extracted from blood and the oropharyngeal swabs were reported descriptively. Commercially available software was used for all comparisons (StatCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP). Significance was defined as $P < 0.05$.

4.3. Results

4.3.1. Serum biochemistry

There were no clinically significant differences when comparing serum biochemical values from the Carnivora™ treated cats before and after treatment.

4.3.2. FHV-1 associated clinical parameters

4.3.2.1. Equilibration period

There were a maximum of 760 scores potentially collected during the 14-day equilibration period. One cat in the treatment group had a clinical score of 1 due to mild serous ocular discharge for five days, and one cat in the control group had a clinical score of 1 due to a sneeze on one day. All other cats had clinical scores of 0 every day. No cats in either room had temperatures above 103.0°F. The differences in cumulative total clinical scores between the treatment group (5 of 760 observations) and the control group (1 of 759 observations) were not statistically significantly different ($p = 0.2$) (Table 4.3).

Table 4.3: Total clinical scores and evidence of FHV-1 recrudescence across sampling periods

	Equilibration (Day -14 to 0)				Pre-inoculation/ Treatment Weeks 7-8 (Day 42 to 55)				Post-inoculation/ Treatment Weeks 9-12 (Day 56 to 84)				
Name	Wt (kg)	CS	PCR		Wt (kg)	CS	PCR		Wt (kg)	CS	PCR	Wt (kg)	FHV titer % change (shaded=decrease; bold=increase)
CONTROL GROUP													
Bran	5.4	1			5.3	6			5.3	14		5.5	-1.97
Daphne	3.6				2.9				2.7		+	2.4	5.54
Kristoff	4.2				3.9				3.9			4.0	-18.13
Merylin	3.4				3.1		+		2.9	2	+	3.2	-3.48
Noria	2.5				2.3				2.2	1		2.0	-6.80
Olaf	5.2				4.7				4.5			4.4	98.96
Summer	3.1				3.1	1			2.8	8		2.2	-24.41
Velma	2.4		++		2.4	6			2.2	12	++	2.6	7.92
TREATMENT GROUP													
Aria	3.1	5			2.7				2.4	4		2.2	6.68
Duke	5.6				5.3				5.3			5.1	16.38
Fred	4.2				4.0				3.8			3.9	2.15
Knight	5.8				5.8				5.8			5.5	-9.22
Lady	3.5				2.9				2.8			2.4	-2.67
Pina	3.7				3.3				3.2			3.0	-8.68
Scooby	6.9				6.4		+		6.3			5.9	-25.41
Sven	5.0				5.0	1			4.9	2		4.8	3.31
unknown						2							

CS: total clinical score throughout period
Wt: weight (kg)
+: positive via cPCR or qPCR
++: positive via cPCR and qPCR

4.3.2.2. Treatment period prior to FHV-1 challenge

There were a maximum of 840 scores potentially collected during the 15-day treatment period prior to FHV-1 inoculation. The total clinical scores for the treatment group (3 of 840 observations) compared to the control group (13 of 840 observations) were significantly different ($p = 0.02$). In the treatment group, there were a total of three occurrences of a clinical score of 1 due to sneezing from at least one cat; the other two sneeze occurrences were heard but the specific cat was not identified. In the control group, there were a total of 13 occurrences of a clinical score of 1; this included 12 occurrences in which the body temperature was above 103.0°F for three different cats and one episode of sneezing by one of these cats. The percentages of treatment cats with fever (0 of 8 cats; 0%) or any clinical sign (1 of 8 cats;

12.5%), were lower than the percentages of control cats with fever (3 of 8 cats; 37.5%) or any clinical sign (3 of 8 cats; 37.5%), but these results were not statistically significantly different (Table 3).

4.3.2.3. Observation period after FHV-1 challenge

There were a maximum of 1,568 scores potentially collected during the 28-day period after FHV-1 inoculation. The total clinical scores for the treatment group (5 of 1,568 observations) compared to the control group (37 of 1,568 observations) were significantly different ($p < 0.0001$). In the treatment group, there were a total of five occurrences of a clinical score of 1; this included three sneezes from two cats and mild serous ocular discharge on two days from the same cat who had ocular discharge during the equilibration period. In the control group, there were a total of 37 occurrences of a clinical score of 1; this included 28 occurrences in which the body temperature was above 103.0°F in four different cats, and nine sneeze occurrences from those four cats and one other cat. Overall, two of the treatment group cats (25%) accounted for all of the > 0 scores after FHV-1 inoculation; one of these cats also accounted for all > 0 scores during the equilibration period. Overall, five of the control group cats (63%) accounted for all of the > 0 scores after FHV-1 inoculation; four of these cats had no >0 scores during the equilibration period. The percentages of treatment cats with fever (0 of 8 cats; 0%) or any clinical sign (3 of 8 cats; 37.5%); were lower than the percentages of control cats with fever (4 of 8 cats; 50%) or any clinical sign (5 of 8 cats; 62.5%), but these results were not significantly different.

4.3.3. Body weights

Body weights on Day 0 were not significantly different (p=0.09) when comparing the treatment group (median 4.6 kg; range 2.9-6.8) and the control group (median 3.3 kg; range, 2.4-5.5). All (94%; n=15/16) but one of the cats experienced weight loss between Day 0 and Day 56 (Table 3). The amount of weight loss between Day 0 and Day 56, did not significantly differ (p=0.8) when comparing the treatment group (median -8%; range -20% to 1%) and the control group (median -10%; range -22% to -3%). Weight changes potentially related to FHV-1 infection between Day 56 and Day 84, also did not significantly differ (p=0.4) when comparing the treatment group (median -6%; range -14% to 3%) and the control group (median 0%; range -21% to 18%). Overall weight changes between Day 0 and Day 84, also did not significantly differ (p=0.4) when comparing the treatment group (median -12%; range -31% to -4%) and the control group (median -8%; range -30% to 7%).

4.3.4. Assays

None of the cats were positive for FHV-1 DNA in blood by cPCR assay. From oropharyngeal swabs, one control cat was positive for FHV-1 via cPCR, both during the equilibration period and after FHV-1 re-inoculation; the cat was also positive for FHV-1 DNA via qPCR assay during the equilibration period and post-inoculation. Prior to inoculation, after eight weeks of treatment, one cat from the control group and one cat from the treatment group were positive for FHV-1 via qPCR assay. After FHV-1 inoculation, three cats from the control group (37.5%) were positive for FHV-1 via qPCR assay but none of the cats from the treatment group (0%) were positive for FHV-1 via qPCR assay (Table 4.3); the difference was not

significant. There were not enough positive samples to statistically compare magnitude of FHV-1 DNA shedding between groups.

Four cats in the treatment group and three cats in the control group had increased FHV-1 antibody absorbance values in the final sample when compared to the pre-treatment sample. However, the percent changes between the groups were not statistically different ($p = 0.9$) (Table 4.3).

4.4. Discussion

Cats that received Carnivora™ had fewer clinical signs of FHV-1 when compared to those cats that received the control. Carnivora™ was also well tolerated, as neither vomiting nor diarrhea was reported by the research facility. In addition, there were no significant differences in serum biochemical panel findings in the Carnivora™ treated cats over the study. These results confirm unpublished observations that Carnivora™ is safe to use in cats at the doses and intervals described.

4.4.1. Carnivora compounds

Compounds found in extracts of Dionaea muscipula, have been shown to have anti-inflammatory, immunomodulatory, antimicrobial, and antiviral properties.[27,29,35–37] Among the multiple compounds listed in the Carnivora formulation, the naphthoquinone compounds and flavonoids are those that might have contributed to the positive effects seen in this study.[25,26,29,35,38–40]

4.4.1.1. Plumbagin

Dionaea muscipula contains naphthoquinone compounds that are derivatives of naphthalene, an organic compound with the formula C10H8.[29] Likely the most important of the naphthoquinone compounds in D. muscipula is plumbagin, a 5-hydroxy-2-methyl-1,4-naphthoquinone.[27,29] Plumbagin has cytotoxic, apoptotic properties. Potential mechanisms of action include formation of reactive oxygen species, disruption of cellular microtubules through tubulin binding, modulation and inhibition of NF-KB activation, and induction of p21, a cyclin-dependent kinase inhibitor.[36,41–43] Plumbagin has also been shown to have antibacterial and antiviral properties.[29,44–46] Plumbagin or its derivatives also have reported efficacy against human herpes simplex virus type 2.[27,47]

4.4.1.2. Quercetin

D. muscipula also contains flavonoids, 15-Carbon compounds with two benzene rings connected by a herocyclic pyrene ring.[48] The most studied of the flavonoids in D. muscipula is quercetin, a member of the flavonol subgroup and characterized by a 3-hydroxy-w-phenylchromen-4-one backbone (Figure 4.3).[37,48,49] Quercetin has been reported to have anti-inflammatory, immunomodulatory, antioxidant, antitumor, antibacterial, and antiviral properties including actions against herpes simplex virus.[29,46,49–51] The antiviral effects have received attention for years, dating back to the 1950's when it was shown to have a protective effect in mice against viral infection.[52,53] Antiherpetic activities of the flavonols including queretin have also been reported.[27,30,39,40,54] A different plant that also has quercetin as its major active component induced secretion of type I IFN and decreased replication of herpes simplex virus-1 (HSV-1) in vitro and in vivo.[48,55] It has been suggested that quercetin exerts antiviral effects on HSV-1 by

blocking virus binding and penetrating cells and that quercetin can also suppress NF-kB activation.[56] Another study reported a synergistic effect against HSV-1 and HSV-2 when combining quercetin with acyclovir.[40] The suggested mechanism of action was the elevation of intracellular levels of cAMP and inhibition of cAMP phosphodiesterase, suggesting a relationship between increased levels of cAMP and antiviral activity.[40,57] Some of the other possible mechanisms of action for quercetin include blocking the arachidonic acid pathway, suppression of prostaglandin synthesis, inhibition of the NF-kB pathway thus inhibiting cytokine nitric oxide synthase expression, and inhibition of macrophage proliferation and activation.[29,37,50]

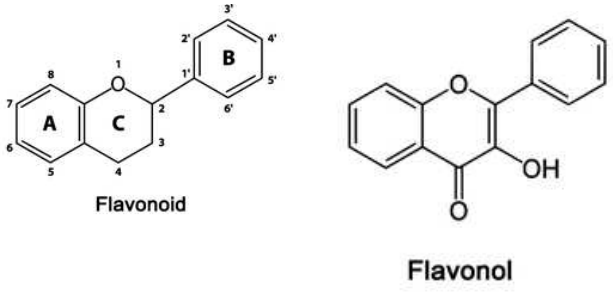

Figure 4.3: Flavonoid molecular structure

4.4.2 Clinical signs decreased in treatment group

The potential positive effects associated with Carnivora™ were demonstrated in this study in the cats after repeat exposure to FHV-1, as cumulative clinical scores were lower in Carnivora™ treated cats when compared to controls. As Carnivora™ has been used as a treatment for herpesvirus in other species, this study provided evidence of efficacy against FHV-1 in cats as well. The decreased FHV-1 clinical signs including pyrexia in the treatment group as compared to the control group could be due to Carnivora's potential antiviral, immunomodulatory, and/or anti-inflammatory properties.[27,40,50,53] Overall, the percentage of cats developing clinical signs of activated FHV-1 after challenge was numerically greater in the control group (62.5%) than in the

treatment group (25%). The failure to achieve statistical significance may reflect the small sample size.

In contrast, the clinical scores did not vary between the groups during the equilibration period. In fact, the only treatment-group cat exhibiting clinical signs of FHV-1 during the equilibration period, was one of the only two cats in the treatment-group that exhibited clinical signs post-inoculation. On the other hand, only one cat in the control group exhibited clinical signs on one day on one occasion during the equilibration period, yet this cat had a total of 20 occurrences of clinical signs after the treatment periods started. And four other control cats without any clinical signs during the equilibration period, had clinical signs post-inoculation. This provides evidence that Carnivora™ helped to prevent recrudescence of FHV-1 related clinical signs.

In this study, the clinical signs after FHV-1 challenge were mild, as expected, as the cats were previously infected and should have had partial immunity. However, the results of the study document that FHV-1 immunity is not complete nor sterilizing as multiple control cats became ill and shed FHV-1 again. Fever was common in the control cats but never detected in the treatment cats. While FHV-1 viremia was not detected in these cats, the PCR assay on blood was not performed on the days that fevers were present. Thus, we cannot confirm a correlation between fever and viremia. It is more likely that local inflammation in the upper respiratory tract was adequate to induce a febrile response. Because of the potential anti-inflammatory effects of plumbagin and quercetin and lack of fever in the treatment group cats, this seems a likely explanation.[29,36,37,50]

Plumbagin has also been shown to prevent weight loss in mice models.[58] Thus, we evaluated body weight in the two groups of cats over time both as a surrogate marker of appetite and to assess whether Carnivora™ could induce any positive effect on body weight during

stressful periods. In our model, body weight changes did not differ between the treatment and control groups.

4.4.3. Clinical signs overall increases

Clinical signs such as elevated temperature and sneezing occurred during the pre-inoculation phase of the study during Weeks 7-8 of treatment or placebo administration. Furthermore, weight loss occurred in 94% of the cats (n=15/16) throughout the study, including the pre-inoculation phase of the study. Although signs of FHV-1 recrudescence were less in those cats receiving Carnivora™ compared to those receiving placebo, an increase was present in both groups, including similar magnitude of weight loss. This recrudescence of signs and weight loss are likely attributed to stress accompanying the multiple daily administrations of either the treatment or placebo.[7,59–67] Although other stressors such as cage housing or estrous related behaviors in the females could have contributed to increased stress levels, those factors were present during the first two weeks of clinical scoring during the equilibration period when weights were higher and when few clinical signs occurred in any of the cats. It therefore seems more likely that stress due to restraint and multiple daily treatment administration events resulted in increased daily stress in all of the cats. Restraint and forceful handling have been associated with stress in cats.[60,61,65–68] Stressors can lead to recrudescence of FHV-1 and other immune compromising sequelae.[59,63,69,70] Although Carnivora™ did not contribute to weight loss in the treatment group to a greater extent than the control group, the results also failed to show a positive effect of Carnivora™ on lessening stress-associated weight loss or potential FHV-1 associated weight loss. It is therefore recommended that a less invasive, less stressful method or different administration regime for Carnivora™ be implemented for cats.

4.4.4. Assays

After FHV-1 challenge, three of the control cats but none of the treatment cats had repeat FHV-1 shedding; although the numerical difference in shedding between groups suggested a treatment response induced by Carnivora™, this result was not statistically significant using the small sample size in this study. FHV-1 antibody absorbance values increased in four treatment cats and three control cats, and there was no difference in the magnitude of the antibody changes between groups. It could therefore not be documented whether Carnivora™ administration potentiated FHV-1 humoral immunity. In a different study of an immune-enhancing probiotic, FHV-1 antibody titers were also not enhanced. It was proposed in that study that the cats were healthy and immune competent and thus, already had titers close to maximal, potentially masking a treatment effect.[19] That hypothesis may also be true for this study. In this study, neither titer values nor shedding corresponded to clinical signs; clinical signs and the detection of FHV-1 by various molecular methods have lacked correlation in other studies as well.[71–73]

4.5. Conclusions

Overall, we conclude that this pilot study documented that Carnivora™ might have immune modulating effects that might influence the course of FHV-1 infections in cats. The results should be confirmed in larger field studies, and future studies should evaluate the mechanisms of action and also establish bioavailability and pharmacokinetic parameters, as bioavailability can be low for some of the metabolites in rodent models.[74–76] Further feline safety and efficacy studies with varied dosing regimens should also be considered.

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CHAPTER 5: EFFECT OF IMMUNE MODULATION THROUGH A LIPOSOME-TOLL-LIKE-RECEPTOR (TLR) INTRANASAL AND MUCOSAL IMMUNE STIMULANT (LTC) ON THE CLINICAL COURSE OF FHV-1[1]

5.1. Introduction

Feline herpesvirus-1 (FHV-1) is one of the most common causes of ocular and upper respiratory infections (URI) in cats and can be a major cause of morbidity and sometimes mortality, especially in young kittens.[1–4] Routes of infection and viral replication predominantly occur within the conjunctival, nasal, and oral mucous membranes and respiratory epithelium, leading to upper respiratory clinical signs.[5–7] Incubation period is typically between two to six days, and clinical signs in uncomplicated cases usually resolve after 10 to 21 days.[6,8–11] However, viral latency within the trigeminal ganglia can lead to recrudescence, and viral damage might lead to chronic changes including permanent nasal turbinate osteolysis and resorption, secondary bacterial infections and continued inflammation throughout the cat's life.[5–7,12]

Prior administration of FHV-1 vaccines may lessen illness if exposed, but vaccination against FHV-1 provides incomplete immunity.[13–16] Clinical signs of FHV-1 infection can be reactivated with repeat exposure, after induction of stress, or administration of immune suppressive drugs.[17–19]

Upregulation of innate immune responses might provide benefits through non-specific immune stimulation. Feeding of an immune-enhancing probiotic or a Dionaea muscipula extract, administration of alpha 2b interferon, and use of an intranasal vaccine have provided results suggesting immune modulation might be effective in controlling or treating feline

[1] Part of this research is in print: Contreras ET, Olea-Popelka F, Wheat W, Dow S, Hawley J, Lappin MR. Evaluation of liposome toll-like receptor ligand complexes for non-specific mucosal immunoprotection from feline herpesvirus-1 infection. J Vet Int Med. 2019;33(2):831-837.

URTD.[13,15,17,20–22] One study showed that after FHV-1 challenge, a significant reduction in clinical scores was noted in kittens as soon as four days after administration of one dose of an intranasal vaccine; this occurred prior to the development of specific FHV-1 immune responses.[13] Administration of an intranasal FHV-1 vaccine was shown to induce cross protection against Bordetella bronchiseptica, a primary bacterial pathogen in cats that was not contained within the vaccine.[20] These findings suggested that intranasal administration of these two vaccines were inducing non-specific immune responses that were imparting a positive effect against the primary pathogen. This supports continued work evaluating stimulation of innate immunity for protection of infections in cats.

Viruses and bacteria express evolutionarily conserved and specific molecular structures with ligands that mammalian cellular toll-like receptors (TLRs) recognize.[23–25] The mammalian cellular TLRs then activate intracellular signaling pathways, leading to dendritic and natural killer cell activation, release of type 1 interferons, and pro-inflammatory cytokines and chemokines, thus a cellular response is mounted against the invading pathogen.[24–28]

Modulation of cellular antiviral activity through TLR recognition and signaling, is therefore a clear target for immunotherapy against viral or microbial infections. Nucleic acid therapeutics utilize cationic liposomes to deliver plasmid DNA intracellularly, thereby activating the TLRs and stimulating the immune response.[23,25,29–33] Cationic lipid DNA complexes (CLDC) have been shown to induce strong innate immune responses and to augment non-specific protection against viral and bacterial diseases in multiple species.[30–32,34–37] More specifically, one widely studied immunotherapy platform is based on the triggering of innate immune responses using TLR9 agonists complexed to cationic liposomes; this greatly enhances the activity of the TLR9 agonist.[29,30] In a number of animal challenge studies, parenteral or inhalational

administration of liposomal-TLR9 complexes has generated complete or nearly complete protection against highly virulent bacterial and viral pathogens.[31,32,34–36,38] In addition, administration of liposome-TLR9 complexes intraperitoneally to cats once weekly for four to six weeks resulted in lessening of clinical signs associated with feline URTD and an increase in neutrophils, monocytes, and CD4+ and CD8+ lymphocytes.[37]

Recently, a new intranasal formulation of a liposome-TLR complex (LTC) was developed that includes a TLR9 agonist, a TLR3 agonist, and methylcellulose as a mucosal adhesive agent.[39] In a study of healthy, purpose bred cats, cytokine and cellular immune responses to this LTC were evaluated in vitro and in vivo. Quantitative PCR assays, ELISA assays, and flow cytometry were used to evaluate nasal lavage specimens and pharyngeal swabs.[39] In that study, the in vitro experiment showed that the LTC rapidly activated cat leukocytes, including upregulation of co-stimulatory molecules and cytokine production. The in vivo experiment showed that topical administration of the LTC triggered rapid recruitment of monocytes to the nasal and oropharyngeal mucosa in the healthy cats.[39]

Based on the results from the in vivo and in vitro experiments in healthy cats, there were two objectives of this pilot study. The first objective was to assess whether mucosal administration of LTC prior to FHV-1 challenge could decrease clinical signs and severity, hasten resolution of clinical signs, and decrease FHV-1 DNA shedding in the kittens. The second objective was to assess whether mucosal administration of LTC at the first signs of clinical illness, followed by a 2nd dose in 24 hours could decrease clinical signs and severity, hasten resolution of clinical signs, and decrease FHV-1 DNA shedding in the kittens. The primary hypotheses were that administration of LTC prior to FHV-1 challenge and at the first signs of illness would induce positive clinical outcomes to infection.

5.2. Materials and Methods

5.2.1. Animals

Twelve female and 13 male, 14-week old, purpose-bred non-vaccinated domestic shorthair kittens were included in this 28-day pilot study. Prior to the start of the study, all kittens were serologically negative for FHV-1, and pharyngeal swab samples obtained from each kitten were negative for DNA of FHV-1 by PCR assay (Center for Companion Animal Studies, Colorado State University, Fort Collins, CO). Six of the females were randomly selected and gonadectomized 26 days prior to the start of the study, and all of the males were gonadectomized 19 days prior to the start of the study.

5.2.2. LTC formulation

Liposomal toll-like receptor (TLR) complexes (LTC) were prepared using cationic liposomes [cholesterol and 1,2-dioleoyl-3-trimethylammonium propane (DOTAP; Avanti, Alabaster, AL)] admixed with two TLR agonists: polyinosinic-polycytidylic acid (poly I:C, InVivoGen, San Diego, CA) as a TLR3 and RIG-I and MDA5 agonist and non-coding commercial plasmid PCR2.1 DNA (pDNA; Life Sciences) as a TLR9 agonist rich in CpG. Carboxymethycellulose (Sigma-Aldrich, St. Louis, MO) was then added to the LTC to increase adhesion to mucosal cell surfaces. The complexes were prepared in 1mM Tris-buffered 5% dextrose in water (D5W); pH = 7.4.[39] The placebo solution was prepared using only the 1mM Tris-buffered D5W. The LTC used in this study was tested and shown to be safe in experiments in healthy young cats.[39]

5.2.3. Study design

The 25 kittens were divided into three separate rooms in three separate groups, the Preventive Group, Group A (n = 7), Treatment Group, Group B (n = 6), and Control Group, Group C (n = 12). Due to future study plans,[18] all six spayed females were assigned to Group C, while the six intact females were randomized to either Group A or Group B, and the remaining seven neutered males were randomized into either Group A (n=4) or Group B (n=3).[40,41] Five days prior to the start of the study, the kittens were moved into their respective rooms.

The room sizes were 104.6 square feet for Group A (n=7), 66.3 square feet for Group B (n=6), and 140.3 square feet for Group C (n=12). The room for Group C had attached ante-rooms, separated from the study room by plastic mesh climbing surfaces. Protective barrier control outerwear for observers was stored and donned in the ante-room. The room for Group A did not have an attached ante-room, thus barrier control outerwear for observers was stored and donned in the research facility hallway. The kittens were housed and cared for in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the contract research facility.

Twenty four hours prior to FHV-1 inoculation, the kittens in Group A (n=7) were administered 1 ml LTC, delivered by both the intranasal route (0.2 ml in each nare) and oral route (0.6 ml administered to the caudal oropharynx) using a 1 ml syringe. On Day 0 of the study, within a 75-minute time span, all 25 kittens were sedated and inoculated with a United States Department of Agriculture (USDA) challenge strain of FHV-1 at a $10^{5.3}$ tissue culture infective dose $(TCID)_{50}$ divided equally between the nares and oropharynx.[15,16] The kittens in Group B (n=6) were individually administered 1ml LTC delivered by both the intranasal route (0.2 ml in each nare) and oral route (0.6 ml administered to the caudal oropharynx) using a 1 ml

syringe on the first day of clinical illness as determined by the investigators; a second dose was individually administered 24 hours later.

Table 5.1: Daily clinical scoring rubric applied by trained, masked observers to each kitten each day

Clinical sign	Score
Conjunctivitis	0=None 1=Mild 2=Moderate 3=Severe
Blepharospasm	0=None 1=Eye<25% closed 2=Eye 25-50% closed 3=Eye 50-75% closed 4=Eye completely closed
Ocular discharge	0=None 1=Mild serous (clear) discharge 2=Moderate mucoid (white) discharge 3=Severe mucopurulent (moist yellow-green) discharge
Body temperature (microchip)	0: ≤ 102.5 1: > 102.5
Cough	0=None 1=Observed
Sneezing (yes/no)	0=None 1=Observed
Nasal discharge	0=None 1=Mild serous (clear) discharge 2=Moderate mucoid (white) discharge 3=Severe mucopurulent (moist yellow-green) discharge or hemorrhagic (bloody/red) discharge
Nasal congestion (if score varies during observation period, record highest score observed)	0=None (no congestion present; able to breathe through both nares without difficulty) 1=Mild / Minor congestion (barely audible; audible on close listening, subtle snoring sounds on inhalation ANY time during the observation period 2=Moderate congestion (easily audible; consistently audible throughout observation period; audible snoring sounds on inhalation or expiration that are likely to originate from the nasal cavity) 3=Severe congestion (audible across the room, with or without open mouth breathing; minimal nasal air flow noted from one or both nares after local debris is cleared away)

5.2.4. Clinical monitoring

Two trained observers (masked as to assignment of animals to the study groups) assessed the kittens for 30 minutes at approximately the same time every morning beginning on Day 0 prior to FHV-1 inoculation and continuing through Day 28 post-inoculation. Observers used a clinical score sheet adapted from other FHV-1 vaccination or treatment studies (Table 1).[16,17] Body temperatures were estimated by microchip.[42] Elevated body temperature was defined as >102.5°F (39.2°C), and pyrexia was therefore classified as presence or absence per kitten per day. Body weights were measured weekly. Overall health was monitored daily by one of the study investigators. The protocol included a rescue clause for those kittens that developed moderate to severe signs of FHV-1 and a loss of appetite for 48 hours; daily appetite during severe illness days was determined by interest in and willingness to consume a canned food formulated for critical care cases (Hill's Prescription Diet a/d, KS); it was offered in equal small quantities to the kittens in each room. Supportive care and treatment that could be administered included subcutaneous fluids, buprenorphine for discomfort, topical cidofovir, or oral famciclovir as needed and determined by the investigators.

5.2.5. Laboratory evaluations

Mucosal cells were collected on sterile swabs from the caudal pharynx from each kitten under manual restraint without sedation. For Groups A and C, swabs were collected on Days 7, 14, and 21; for Group B, swabs were collected on each kitten's first day of clinical signs and 7, 14, and 21 days subsequently. Swabs were collected from all kittens in all groups on Day 28. Swabs were stored at -80°C until assayed in batches. Total DNA was extracted from the oropharyngeal swabs and evaluated for DNA of FHV-1 and DNA of the glyceraldehyde 3-

phosphate dehydrogenase (GAPDH) housekeeping gene by quantitative PCR (qPCR) as previously described.[43] Results of the FHV-1 qPCR assay were presented as the ratio of FHV-1 DNA/GAPDH DNA to attempt to standardize specimens and to ensure sample adequacy by presence of GAPDH in the sample. FHV-1/GAPDH ratios could only be compared between Group B and Group C on Day 28, since swab collection dates were not the same for other study days.

5.2.6. Statistical analyses

Soon after the FHV-1 challenge and onset of clinic signs, it was determined that sneezing and nasal congestion were being underestimated in Group C because of the larger number of kittens (n=12) in a larger room; the scorers were therefore unable to reliably capture sneezing and nasal congestion occurrences in the larger study group in the timeframe allotted. Sneezing and nasal congestion were therefore excluded from all subsequent data analyses.

Time periods were defined as Days 1-14, 15-28, and 1-28. Observation occurrences were defined as the number of times a clinical sign (Table 5.1) was observed as present within a time period and group. For each 14-day time period, Group A (n = 7) had a total of 98 possible observation occurrences, Group B (n=6) had a total of 84 possible observation occurrences, and Group C (n=12) had 168 possible observation occurrences. For the 28-day time period, Group A had a total of 196 possible observation occurrences, Group B had a total of 168 possible observation occurrences, and Group C had 336 possible observation occurrences. Total scores were calculated for each kitten each day by adding the individual clinical score parameters recorded for that day, excluding sneezing and nasal congestion as previously detailed (Table 5.1). Total ocular scores were calculated as the sum of conjunctivitis, blepharospasm, and ocular

discharge. Total respiratory scores were calculated as the sum of nasal discharge and cough. Total clinical scores were calculated as the sum of total ocular, total respiratory, and pyrexia. Presence versus absence of severe ocular disease and severe respiratory disease were recorded on each day that the kitten's total ocular or total respiratory score was > 2. Presence of severe total clinical disease was recorded on each day that the kitten's total clinical score was > 3. Day of illness resolution was defined as the study day in which total clinical score was less than 2 and remained as such.

Descriptive statistics were calculated. Clinical scores were expressed as frequencies (presence or absence) of observations. Total ocular, respiratory, and clinical scores, FHV-1/GAPDH ratios, change in body weights, and day of illness resolution were expressed as median, mean, and range. The Shapiro-Wilk test was used to evaluate outcome variables for normality. Due to non-normalcy of variables and clinical outcome ordinal data, the Wilcoxon rank sum test was used to compare median clinical scores between Group A and Group C and between Group B and Group C for each of the three time periods; body weight changes; and FHV-1 DNA and FHV/GAPDH ratios on Days 7, 14, 21, and 28. The number of days to illness resolution was evaluated using Kaplan Meier curves and compared between groups using the log-rank test for equality of survivor functions. The proportion of observations of the dichotomous (presence or absence) clinical parameters of pyrexia, severe ocular, severe respiratory, and severe total clinical scores were compared between Group A and Group C and between Group B and Group C by the 2-tailed Fisher exact test. Mixed model regression analyses were used to control for lack of independence among observations due to repeated measurements on the same kitten through time; ranked data was used for continuous variables, and conjunctivitis and nasal discharge were converted to dichotomous variables of presence or

absence. Odds ratios and 95% confidence intervals (CI) were calculated for some parameters. Commercially available software (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX, StataCorp LP) was used for all comparisons. Significance was defined as $P < 0.05$.

5.3. Results

5.3.1. Preventive Experiment: Group A versus Group C

5.3.1.1. Overall clinical findings

All 19 kittens exhibited clinical signs of FHV-1 infection after inoculation and throughout the study. Pyrexia, ocular discharge, and nasal discharge occurred in all 19 kittens on at least one day. All kittens had a minimum of 12 days of observation with a total clinical score > 0 and a minimum of 3 days with a total clinical score > 1. Subcutaneous fluids at 15 ml/kg were needed for 1 Group A kitten on Day 9 and 1 Group C kitten on Day 10. Both kittens resumed drinking and eating on subsequent days, and all 17 other kittens recovered uneventfully with no rescue treatment needed.

Body weight changes on Days 7, 14, 21, and 28 did not statistically significantly differ between groups; on Day 28, body weight increased more for Group A as compared to Group C, but this difference was not statistically significant ($P = 0.08$).

Coughing was rare in this study and was only observed on day 6 from one Group A kitten and three Group C kittens and on day 10 from one Group C kitten. Pyrexia occurred at 54 out of 168 (32.1%) Group C observation points and at 21 out of 98 (21.4%) Group A observation points during Days 1-14; this difference was not significant ($P = 0.07$) (Figure 5.1).

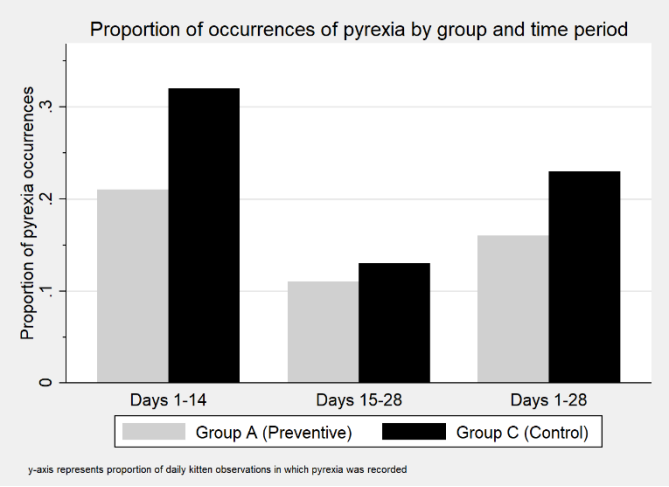

Figure 5.1: Proportion of pyrexia occurrences, Group A and Group C throughout Days 1-14, 15-28, and overall Days 1-28. Bars on Days 1-14 and Days 15-28 represent Group A (n=98 observations from 7 kittens over 14 days; grey bar) and Group C (n=168 observations from 12 kittens over 14 days; solid black bar). Bars on Days 1-28 represent Group A (n=196 observations from 7 kittens over 28 days; grey bar) and Group C (n=336 observations from 12 kittens over 28 days; solid black bar).

Total clinical scores (conjunctivitis, blepharospasm, ocular discharge, nasal discharge, cough, pyrexia) did not statistically differ between groups. Severe total clinical disease occurred primarily on Days 1-14, and there were not enough occurrences on Days 15-28 to compare between groups. Severe total clinical disease on Days 1-14 occurred more frequently in Group C as compared to Group A (P = .03) (Figure 5.2). After controlling for lack of independence, there was not a statistically significant difference between groups when comparing severity of total clinical disease.

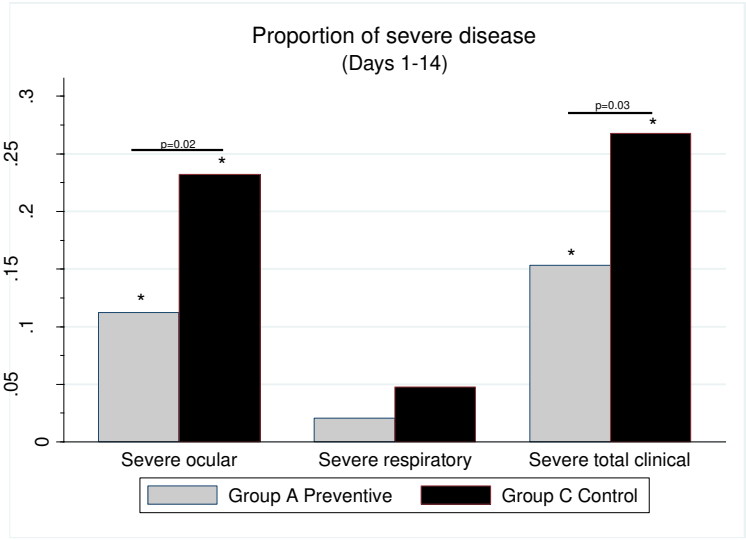

Figure 5.2: Proportion of severe disease occurrences, Group A and Group C on Days 1-14. * P-values are from the Fisher 2-tailed exact test prior to adjusting for lack of independence among kittens through time; n=14 observations per kitten in each group. Group A: n=98 observations (7 kittens for 14 days). Group C: n=168 observations (12 kittens for 14 days).

5.3.1.2. Ocular clinical findings

Conjunctivitis and blepharospasm occurred on at least one day in all 7 Group A kittens and 11 of 12 Group C kittens (overall = 95% of kittens). Conjunctivitis scores were higher and more frequent in Group C as compared to Group A on Days 1- 14 (P = .01), Days 15-28 (P < .001), and Days 1-28 (P < .001) (Table 5.2). After controlling for lack of independence due to repeated measurements on the same kitten through time, the odds of having conjunctivitis were greater among kittens in Group C as compared with kittens in Group A on Days 15-28 (OR, 4.4; 95% CI, 1.4 to 14; P = .01) and Days 1-28 (OR 3.1; 95% CI, 1.2 to 7.6; P = .02) (Figure 5.3). On Days 1-14, kittens in Group C had greater odds (OR, 2.4; 95% CI, 1.1 to 6.4) of having conjunctivitis compared with kittens in Group C, but this difference was not significant (P = .07).

Table 5.2: Percentage of conjunctivitis and nasal discharge observation occurrences by 14-day time period and severity score in Group A and Group C. Unadjusted P-values represent results from Wilcoxon rank sum test. Adjusted P-values represent logistic regression analyses on presence or absence of clinical sign and adjusted for lack of independence due to repeated measurements on the same kitten through time. Group A: n=98 observations (7 kittens for 14 days). Group C: n=168 observations (12 kittens for 14 days). Score=1 is mild severity; score=2 is moderate severity; score=3 is severe clinical sign (Table 5.1)

		Group A Preventive	Group C Control	Unadjusted p-value	Adjusted p-value
		Days 1-14			
	score=1	12%	20%		
	score=2 or 3	4%	10%		
	total:	16%	30%	P=0.01	P=0.07
Conjunctivitis					
		Days 15-28			
	score=1	10%	28%		
	score=2 or 3	0%	0.6%		
	total:	10%	29%	P<0.001	P=0.01
		Days 1-14			
	score=1	42%	35%		
	score=2 or 3	15%	14%		
	total:	57%	49%	P=0.26	P=0.30
Nasal discharge					
		Days 15-28			
	score=1	47%	42%		
	score=2 or 3	21%	2%		
	total:	68%	45%	P<0.001	P=0.06

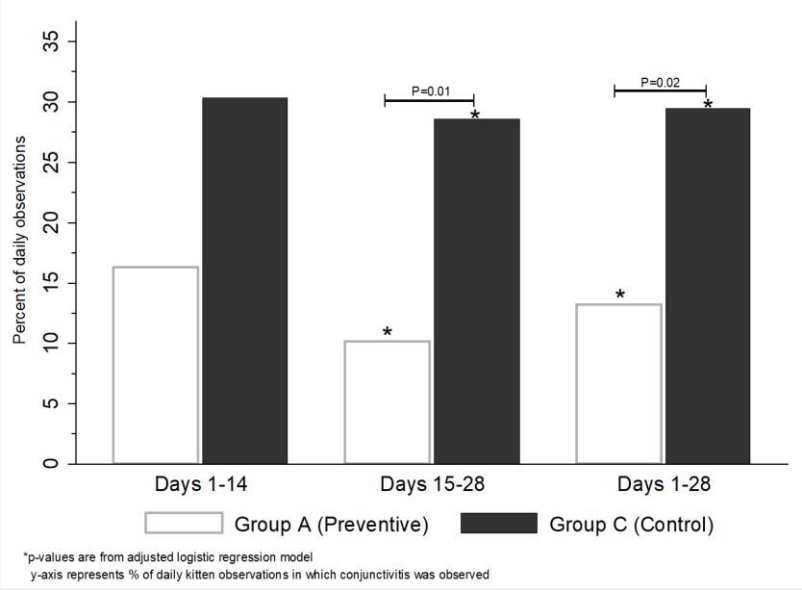

Figure 5.3: Percentages of observations with conjunctivitis after FHV-1 inoculation of kittens in Group A and Group C. P-values are from logistic regression models adjusted for lack of independence due to repeated measures on the same kitten through time.

There were no statistically significant differences between groups for ocular discharge or blepharospasm. Total ocular scores were higher and more frequent in Group C as compared to Group A on Days 15-28 (P = .04). After controlling for lack of independence, total ocular scores did not significantly differ between groups. Severe ocular disease occurred primarily on Days 1-14, and there were not enough occurrences on Days 15-28 to compare between groups. Severe ocular disease on Days 1-14 occurred more frequently in Group C as compared to Group A (P = .02) (Figure 2: Severity). After controlling for lack of independence, the odds of having severe ocular disease was greater for kittens in Group C as compared to kittens in Group A, but this difference was not statistically significant (OR, 2.5; 95% CI, 0.9 to 6.9; P = .08).

5.3.1.3. Nasal and respiratory clinical findings

Nasal discharge scores were higher and more frequent in Group A as compared to Group C on Days 15-28 (P = .04). After controlling for lack of independence, presence of nasal

discharge was not statistically different between groups (Table 5.2). Total respiratory scores were higher and more frequent in Group A as compared to Group C on Days 15-28 ($P < .001$) and Days 1-28 ($P < .001$). After controlling for lack of independence, the odds of having higher total respiratory scores were greater for kittens in Group A as compared with kittens in Group C on Days 15-28 (OR, 3.9; 95% CI, 0.0002 to 9.0; $P = .03$) and Days 1-28 ($P = .06$). All cats in both groups had total respiratory scores of ≤ 2 by Day 28. Severe respiratory disease occurred primarily on Days 1-14, and there were not enough occurrences on Days 15-28 to compare between groups. There were no statistically significant differences between groups for severe respiratory disease (Figure 5.2).

5.3.1.4. Resolution of illness

Four Group A kittens had a total clinical score of < 2 (resolution of illness) by Day 28, and three Group A kittens that were clinically well had a total clinical score of 2 (did not meet criteria for defined resolution of illness) by Day 28. In contrast, all 12 Group C kittens had a total clinical score of < 2 (resolution of illness) by Day 28. When the cats with resolution of clinical disease (total clinical score of < 2 by Day 28) were compared, median number of days to illness resolution was 17.5 days (range 10 to 23) for Group A kittens and 23 days (range 20 to 28) for Group C kittens; this difference was not significant groups ($P = 0.18$).

5.3.1.5. FHV-1/GAPDH ratios

Detectable FHV-1/GAPDH ratios > 0 were detected on at least three out of the four dates of sample collection for sis of the seven kittens in the LTC group and all 12 kittens in the control group. The largest amount of FHV-1 DNA was recovered on Day 7 for all kittens. The amount

of FHV-1 DNA recovered was significantly higher in the control group on Day 21 (P = 0.04), Day 28 (P = 0.0018), and when results from Days 21 and 28 were combined (P = 0.0002). Of the 76 swabs obtained from the kittens in Group A (n=28 swabs from 7 kittens on 4 dates) and Group C (n = 48 swabs from 12 kittens on 4 dates), a total of 71 swabs (26 from Group A and 45 from Group C) contained quantifiable GAPDH and were thus used in analyses. FHV/GAPDH ratios did not differ between Groups A and C on Days 7 and 14 (Figure 5.4). On Day 21, Group C had significantly higher FHV-1/GAPDH ratios as compared to Group A (P = .04). On Day 28, Group C had higher FHV-1/GAPDH ratios as compared to Group A; this difference was not significant (P = .06). When the ratios from Days 21 and 28 were combined, Group C had significantly higher ratios as compared to Group A (P = .01) (Figure 5.4).

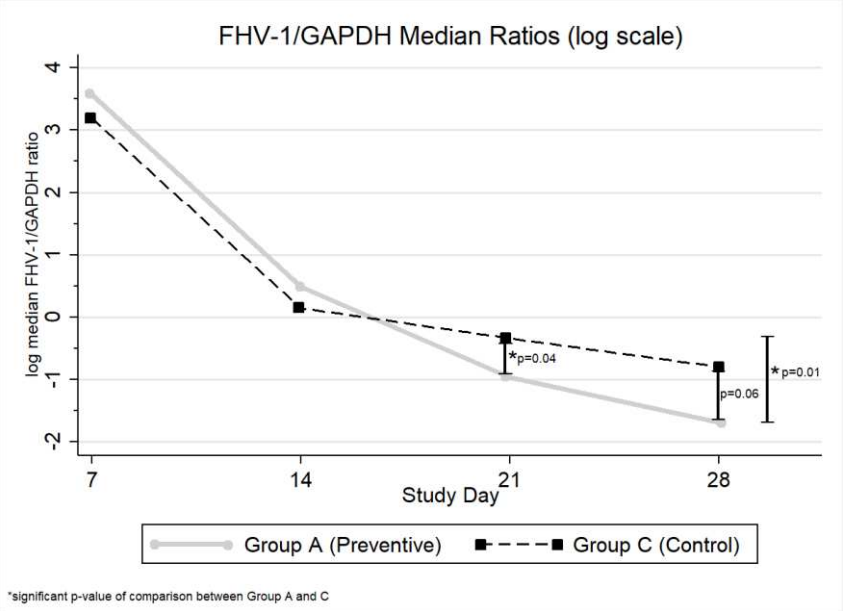

Figure 5.4: FHV-1/GAPDH ratios (medians in logarithmic scale) as detected by qPCR on Days 7, 14, 21, 28 in Group A and Group C. Median values in logarithmic scale are depicted for Group A (n=7, solid grey line with circles) and Group C (n=12, dotted black line with squares). P values are based on Wilcoxon rank sum test to compare median values for Group A and Group C. *Statistical significance: P < .05.

5.3.2. Treatment experiment: Group B versus Group C

5.3.2.1. Clinical findings

As noted for Group C, all six kittens in Group B exhibited clinical signs of FHV-1 infection after inoculation and throughout the study. Pyrexia, ocular discharge, and nasal discharge occurred in all 18 kittens on at least one day. All kittens had a minimum of 12 days of observation with a total clinical score > 0 and a minimum of three days with a total clinical score > 1. Subcutaneous fluids at 15 ml/kg were needed for one Group B kitten on both Days 8 and 9 and one Group C kitten on Day 10. Both kittens resumed drinking and eating on subsequent days, and all 16 other kittens recovered uneventfully with no rescue treatment needed. Body weight changes on Days 7, 14, 21, and 28 did not differ between groups.

Pyrexia occurred more frequently in Group C kittens (76 out of 336 observation occurrences; 22.7%) as compared to Group B kittens (24 out of 168 observation occurrences; 14.3%) on Days 1-28 (P=0.03) (Figure 5.5). On Days 1-14, pyrexia occurred in 32.1% (54 out of 168) of Group C observation occurrences and in 21.4% (18 out of 84) of Group B observation occurrences on Days 1-14 (P=0.08) (Figure 5.5). After controlling for lack of independence due to repeated measurements on the same kitten through time, proportion of pyrexia occurrences was not statistically different between groups on Days 1-14 (p=0.25) nor on Days 1-28 (p=0.41).

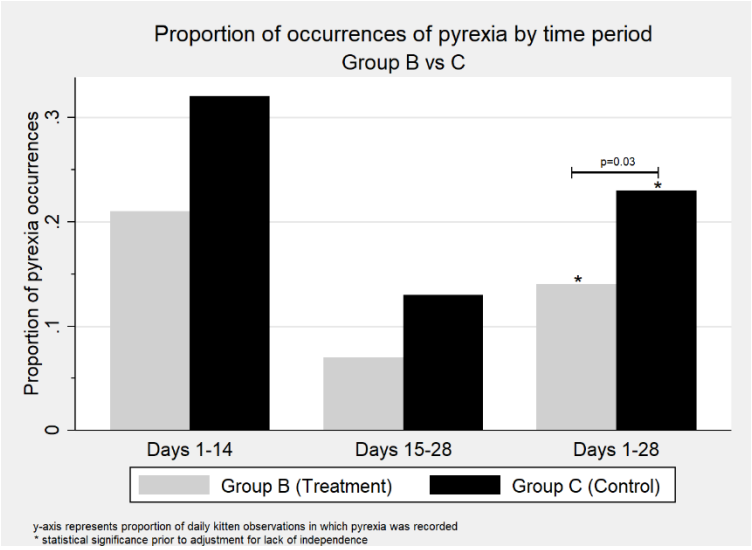

Figure 5.5: Proportion of pyrexia occurrences in Group B and Group C throughout Days 1-14, 15-28, and overall Days 1-28. Bars on Days 1-14 and Days 15-28 represent Group B (n=84 observations from 6 kittens over 14 days; grey bar) and Group C (n=168 observations from 12 kittens over 14 days; solid black bar). Bars on Days 1-28 represent Group B (n=168 observations from 6 kittens over 28 days; grey bar) and Group C (n=336 observations from 12 kittens over 28 days; solid black bar).

Conjunctivitis occurred in 94% (17 of 18 kittens), and blepharospasm occurred in 89% (16 of 18 kittens) of the kittens on at least one day; one kitten from Group C had no recorded occurrences of conjunctivitis or blepharospasm, and one kitten from Group B had no recorded occurrences of blepharospasm. Coughing was observed in Group B from one kitten on Days 4, 6, 7, 10, and 12, from a 2nd kitten on Days 10 and 13, and a 3rd kitten on Day 12 (8 total occurrences from 3 kittens) (Table 5.3). Coughing was observed in Group C from 3 kittens on day 6 and from a 4th kitten on day 10 (4 total occurrences from 4 kittens). All kittens in both Groups B and C had resolution of illness by day 28. The median number of days to illness resolution was 23 days (range 20 to 28) for Group C kittens and 23 days (range 14 to 27) for Group B kittens; this difference was not significant (P = 0.92). There were no differences in clinical scores between Group B and Group C (Table 5.3).

Table 5.3: Means, medians, and ranges of the cumulative scores in Group B vs Group C on Days 1-14, 15-28, and 1-28 inclusive. Means and medians represent each kitten's daily total scores, summed across the time period (Days 1-14, 15-28) and per both time periods combined (Days 1-28) for each kitten. Range represents the lowest (minimum) and highest (maximum) among the group's kittens' daily total scores. There were no statistically significant differences between groups. Group B: n=84 observations per 14-day period. Group C: n=168 observations per 14-day period.

	Days 1-14 mean, median (range)	Days 15-28 mean, median (range)	Days 1-28 mean, median (range)
Total ocular scores			
Group B Treatment (n=6)	18, 17 (8-36)	8, 7.5 (4-13)	26, 22 (16-46)
Group C Control (n=12)	18.1, 15.5 (6-35)	7.2, 6 (1-14)	25.3, 21 (7-46)
P-value	0.85	0.51	0.76
Total respiratory scores			
Group B	10.5, 7.5 (4-20)	6.3, 5.5 (3-12)	16.8, 15 (8-29)
Group C	9.8, 8.5 (4-16)	6.6, 7 (0-12)	16.3, 15.5 (6-27)
P-value	0.96	0.67	0.67
Total clinical scores			
Group B	31.5, 25.5 (19-57)	15.3, 16 (7-25)	46.8, 39.5 (33-82)
Group C	32.3, 29 (19-50)	15.6, 16.5 (7-26)	47.9, 47 (26-73)
P-value	0.61	0.85	0.71

5.3.2.2. FHV-1/GAPDH Ratios

All swabs from the kittens in Groups B and C contained GAPDH on Day 28. FHV/GAPDH ratios did not differ between Groups B and C on Day 28 (P = 0.21) (Figure 5.6).

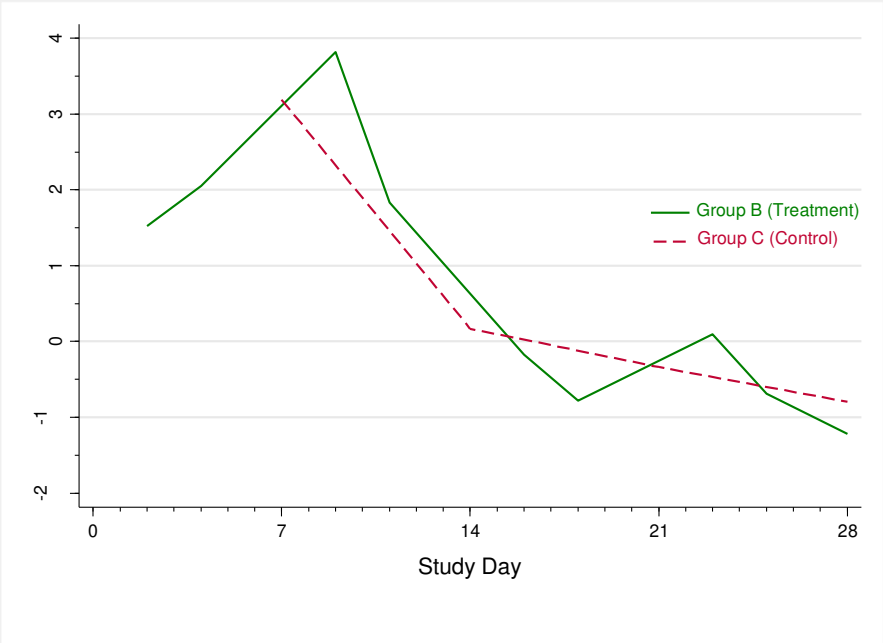

Figure 5.6: Logarithmic scale of median FHV-1/GAPDH ratios as detected by qPCR in Group B and Group C.

5.4. Discussion

All kittens in this study experienced acute clinical signs of FHV-1 infection, and all had measurable shedding of FHV-1 DNA documenting induction of FHV-1 infection. Findings of this study documenting an LTC preventive effect on the course of FHV-1 infection include significantly less conjunctivitis in the Group A (Preventive group) kittens and decreased shedding of FHV-1 DNA on some post-inoculation days in Group A when compared to Group C (Control group). Among other findings, including pyrexia, body weight, total ocular scores, severe total clinical disease scores, and severe ocular disease, the clinical scores also were consistent with a positive preventive effect, though statistical significance was lost after

adjusting for repeated kitten observations over time. The loss of statistical significance after this adjustment can likely be attributed to small sample sizes.[44] On the other hand, LTC did not appear to provide a treatment effect in the Group B (Treatment group) kittens. Group A kittens also had increased occurrences of moderate to severe nasal discharge on Days 15-28 when compared to Group C kittens. On the other hand, Group B kittens had statistically similar clinical signs, duration, severity, and viral shedding when compared to Group C. Pyrexia was the only variable which differed between Group B as compared to Group C kittens; Group B kittens had less occurrences of pyrexia overall, but statistical significance was lost after adjusting for repeated kitten observations over time, again likely attributable to small sample sizes.[44] Additional studies are indicated to determine whether these findings would show statistical significance with greater animal numbers.

5.4.1. LTC as preventive: Group A versus Group C

The effect of the LTC as a preventive administered 24 hours prior to FHV-1 inoculation was likely from upregulated innate antiviral immune responses, most apparent in the first 2 weeks after inoculation. When normal cats were administered LTC in a companion study to this work, most of the immunological effects were noted within hours to days after administration.[39] This LTC formulation utilizes multiple mechanisms to stimulate the innate immune system, targeting antiviral pathways. The cationic liposome (DOTAP) allows the delivery of the LTC nucleic acids to the endosomal compartment of dendritic cells and antigen-presenting cells.[29,30,34,45] The non-coding plasmid DNA molecules delivered, have cytidine-phosphate-guanosine (CpG) sequences recognized as pathogen associated molecular patterns (PAMPs) by intracellular endosomal toll like receptors (TLRs), namely TLR9, which recognizes viral nucleic

acids. This triggers activation of dendritic cells, macrophages, and B cells, resulting in Th1 activation.[30,46,47] The polyI:C is a synthetic analog of double-stranded RNA and so mimics viral infection. TLR3 recognizes double-stranded RNA and thus the polyI:C; this triggers the signaling cascade and production of inflammatory cytokines and type I interferons.[30,46,48–50] Both TLR9 and TLR3 pathways are important in innate defense against viral infection.[49] The TLR3 pathway in particular is important in innate immune defense against viral infections.[49] It has also been shown that deficiency of TLR3 in humans can be associated with susceptibility to herpes simplex virus 1 (HSV-1) encephalitis (HSE).[48] It might therefore be conversely plausible, that an increase in TLR3 agonists would have an inhibitory effect on FHV-1.

Research in healthy cats in a companion study to this work, demonstrated that oral and intranasal administration of the LTC immunotherapeutic significantly activated immune responses locally in the mucosa of cats, as reflected by recruitment of activated monocytes into the nose and oropharynx.[39] In addition, production of several key antiviral and antibacterial cytokines was triggered by LTC treatment, including IFN-alpha, IFN-gamma, and IL-12.[39]

5.4.2. LTC preventive and nasal disease

The kittens in Group A had increased total respiratory scores (nasal discharge and cough) as compared to Group C. As cough was only observed on one occasion in Group A, the difference is due to the increased nasal discharge in Group A, primarily attributed to nasal discharge scores of 2 or 3 reported in 21% of the LTC observations compared to only 2% of the control group observations on Days 15-28 (Table 5.2). Of further note is that four out of seven kittens (57%) in Group A had increased nasal discharge scores on Days 15-28 as compared to those kittens' nasal discharge scores on Days 1-14 (individual kitten results not shown). In

contrast, only one out of the six kittens (17%) in Group B and four out of 12 kittens (33%) in Group C had nasal discharge scores which increased from Days 1-14 to Days 15-28. These were unexpected findings, as LTC was tested on healthy cats, no adverse clinical signs such as nasal discharge were reported by the research facility,[39] and when tested on healthy dogs and healthy cattle (Dow, unpublished data), no adverse clinical signs such as nasal discharge were recognized. Furthermore, the kittens in Group B, which received two dosages of LTC, had nasal discharge scores similar to those in Group C (Table 5.2). It is therefore unlikely that there was a causal relationship between LTC administration and increased nasal discharge.

As our sample size was small, outliers and individual differences in immunological responses to FHV-1 infection might account for differences between groups.[8,11,44,51] Indeed, during Days 15-28, two kittens in Group A had summed nasal discharge scores that were twice as high as nearly all other kittens in all three Groups; however, during Days 1-14, these same two kittens had nasal discharge scores which were similar to the other kittens in the three Groups (Individual kitten results not shown). It therefore seems unlikely that they were outliers during Days 15-28 if not outliers during Days 1-14. Furthermore, over half of the kittens in Group A had increased nasal discharge scores during Days 15-28, in contrast to the few kittens from Groups B or C with increased nasal discharge scores during Days 15-28. Individual differences are unlikely to account for the differences between the groups.

Another possible explanation for increased respiratory scores in Group A cats versus Group C cats is overstimulation of immune and inflammatory responses in the nasal cavity.[30,46] Although TLRs activate the protective antiviral immune response and recruitment of cytokines, chemokines, dendritic cells, neutrophils, and monocytes responsible for the removal of organismal load, some TLRs contribute to sterile inflammatory disease or excessive

inflammation as well.[23,24,46]. It is possible that the degree of inflammation induced by a pathogen could be magnified in some animals,[5,12,52] and there could have been overstimulation of immune and inflammatory responses against FHV-1, as FHV-1 damage has been implicated, in part, in chronic rhinitis in cats.[12,52,53] This might have occurred primarily in the nasal cavity because of direct inoculation of LTC and FHV-1 into the nasal cavity. The nasal turbinates are likely more vulnerable to excess inflammation and damage, as acute viral replication occurs in the mucosa of the nasal septum as well as turbinates, and osteolytic changes in the turbinate bones can occur due to damage.[5,12,54]

5.4.3. Viral shedding

Detection of higher total respiratory scores in the Group A LTC group compared to the control group on Days 15-28 was also unexpected as that was also the time that significant decreases in FHV-1 DNA shedding in the LTC group was occurring. We therefore do not feel that the increase in nasal signs on Days 15-28 is due to increased viral replication and damage, as no other measures indicated increased viral load either. There were no increases in ocular disease, pyrexia, or cough detected concurrently, and most of the kittens were systemically healthy by Days 15-28.

Cats with primary FHV-1 infection typically shed plentiful virus for measurement by various assay methods, in particular quantitative PCR methods.[9,11,13,43,55–58]. As such, this study detected large amounts of FHV-1 and GAPDH DNA for quantitative assessment, and significantly less viral shedding was found on days 21 and 28 in Group A as compared to Group C. This is likely due to the immune stimulatory properties of LTC, which might have cleared the virus more rapidly. As Group A kittens also had less severe clinical signs, and duration of viral

excretion in primary infection is typically related to the severity of clinical signs [11], this lends further support to this explanation. However, other FHV-1 experimental treatment studies have found decreases in viral shedding at earlier timepoints from inoculation. Fontenelle [43] found that cats treated with ocular cidofovir, stopped viral excretion between 7 and 9 days after inoculation, whereas their placebo cats stopped shedding between days 12 and 18. Thomasy[59] found that cats treated with famciclovir had a significantly lower viral DNA load on day 4, but load was again similar to controls until after approximately day 13. However, FHV-1 viral RNA load was significantly less in the cats which received famciclovir as compared to controls by day 21.[59] It is likely that a significant difference between groups in our study was not found until day 21 because of the very large infective dose of FHV-1 administered to the kittens, as a higher infective dose is related to longer duration of viral excretion.[11]. The decreased viral excretion in the Group A kittens, however, lends further support to the protective effects of LTC administered 24 hours prior to inoculation.

5.4.4. LTC as treatment: Group B versus Group C

Although this study partially supported the hypotheses that LTC would be beneficial in lessening a number of FHV-1 infection-related variables related to FHV-1 infection when administered prior to viral challenge, the second objective of the study was not achieved. LTC administered to Group B kittens after the first signs of clinical illness, affected neither clinical signs nor viral shedding, and outcome variables for Group B were very similar as compared to those for Group C. Pyrexia was numerically lower in Group B as compared to Group C, but those differences were lost after adjusting for repeated measures. The failure to reject the null hypotheses for Group B kittens is likely due to systemic illness and FHV-1 viremia already

present in the Group B kittens during LTC administration, due to the large inoculation dose used in experimental kittens.[60] It is suspected that the local nasal and oropharyngeal mucosal delivery was an inadequate means of counteracting the viremia. Goodyear [31] found similar results in a bacterial infection model, as they compared administration of intranasal cationic liposomal DNA complexes (CLDC) 24 hours prior to, during, six hours after, or 24 hours after infection of mice with Burkholderia spp. Those mice that received the intranasal mucosal CLDC immunotherapy 24 hours prior to infection with Burkholderia spp., were completely protected from the lethal infection. Those mice which were administered the CLDC during infection were partially protected, and those mice which were administered CLDC 6 or 24 hours after infection were not protected.[31] Logue[35] found that the location of the subcutaneous injection site of CLDC administration during or after infection, influenced efficacy of CLDC in improving mouse survival in a viral model. Future studies should consider a more natural model of illness with a lower infective dose in order to test the treatment LTC, and systemic administration of LTC should be considered in future studies to determine if a treatment effect is possible with LTC.

Furthermore, the second dose administered to the Group B kittens in this study, likely conferred no additional benefits to the kittens, as in vivo and in vitro studies showed that effects of mucosal administration lasted for at least 72 hours.[39]

And although Group B received 2 dosages of LTC intranasally and oropharyngeally, Group B kittens did not experience increased nasal discharge nor coughing. This is likely because the excessive inflammatory response as described for Group A kittens, did not occur in Group B kittens due to the lack of efficacy of the LTC immune stimulating properties post-infection.

5.4.5. Limitations and future directions

One of the biggest limitations of this study was unequal group sizes and differences in the size of the evaluation rooms. These inequalities confounded the ability of the clinical observers to record sneezing and nasal congestion variables accurately. Sneezing is one of the most objective and quantifiable respiratory clinical signs of FHV-1 infection and should be included in the analyses in future studies.[18] This disparity in group sizes and rooms also resulted in potential confounding factors due to the lack of an ante-room, and the lack of an ante-room mesh divider which provided access to climbing surfaces in Groups B and C, whereas no vertical climbing surfaces were available in the Group A room. Future studies will ensure more equal distributions between groups.

Another potential limitation was to have all three intact females assigned to the LTC group. Although all kittens were sexually immature at the time of the study, the effects of early gonad removal on immune function are unknown, especially in prepubescent animals. Future studies should ensure more equal distributions between groups.

In future studies, a natural model of infection may show different magnitude and timing of potential treatment effects. Studies to determine whether these findings are replicated in cats with FHV-1 infection induced by contact with infected cats and in cats inoculated with the LTC orally or parenterally should be performed. Future studies might instead utilize a more natural means of FHV-1 inoculation with a lower infective dose. This could be done through cat-to-cat contact instead of direct nasal inoculation. Not only would this mimic natural disease to a greater extent, but it would also prevent direct inoculation into the nares of cats, thus avoiding the nasal mucosal sensitivity and propensity to turbinate damage. Second, LTC also might be delivered via a systemic route instead of a nasal mucosal route, as the nasal cavity in the cat might have more

of a predilection toward excess inflammation and damage. In ongoing studies with the LTC described here, a different plasmid is being used that might improve immune stimulating effects. Study rooms should also be equivalent in every manner, including ante-room availability and precautions against inadvertent contamination.

Since PCR assay results do not prove the presence of live virus, quantitative FHV-1 culture might also provide additional information and should be considered for use in future studies. Culturing of other bacterial pathogens from the nasal and oropharyngeal mucosa at the end of the study might also be useful in case of inadvertent exposure to outside organisms in the research setting.

5.5. Conclusions

A single mucosal administration of LTC 24 hours prior to FHV-1 challenge in kittens was associated with several positive clinical effects and with decreased shedding of FHV-1 DNA. Mucosal administration of LTC during illness did not appear to influence clinical course of FHV-1 illness using the current formulation and delivery model. The results of this pilot study support additional larger studies with LTC in client-owned cat populations or in shelter settings where there is a high risk of exposure to FHV-1 and other pathogens and where transmission is by more natural routes and doses of the agents.

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CHAPTER 6: STIMULATION OF MUCOSAL INNATE IMMUNITY THROUGH A LIPOSOME-TOLL-LIKE-RECEPTOR COMPLEX (LTC) FOR THE PREVENTION OR LESSENING OF UPPER RESPIRATORY INFECTION CLINICAL SIGNS IN SHELTER CATS

6.1. Introduction

Feline upper respiratory infection (URI) remains one of the most common medical reasons for euthanasia in shelters and also results in high financial burdens, poor quality of life, and extended lengths of stay in shelters.[1–4] Management of URI in shelters is difficult due to the multiple pathogens implicated, the multifactorial etiology, carrier states of the pathogens, multiple cats from different environments, and the inherently stressful situation of a cat entering a new shelter environment.[4–7] Cats with URTD in shelters are typically infected with feline herpesvirus-1 (FHV-1) and/or feline calicivirus (FCV) and/or with secondary bacterial infections from Chlamydia felis, Bordetella bronchiseptica, and/or Mycoplasma spp.[2,8–11] Other secondary bacterial pathogens can include Staphylococcus spp., Streptococcus spp., and Pasteurella multocida or others.[12,13]

Treatment of URI in the shelter can be partially accomplished with antimicrobials for bacterial pathogens, oral famciclovir for FHV-1, and sometimes extensive supportive care based on the severity and duration of clinical disease.[13–16] Vaccination with a FHV-1, FCV and feline panleukopenia virus (FVRCP) modified life vaccine (MLV) parenterally upon intake is an integral component in preventing or decreasing illness and lessening transmission of infectious disease in shelters.[17–21] Vaccination, however, provides incomplete immunity and does not prevent infection, disease, or viral shedding.[22–25]

Upregulation of innate immune responses might provide benefits through non-specific immune stimulation in shelter cats facing exposure to URI and clinical illness. Feeding of an

immune-enhancing probiotic or a Dionaea muscipula extract, administration of alpha 2b interferon, and use of an intranasal vaccine have provided results suggesting immune modulation might be effective in controlling or treating feline URTD.[26–30] Administration of an intranasal vaccination has been shown to induce cross protection against pathogens and decrease some clinical signs of URI, thus imparting non-specific immune responses.[26,27,31]

Modulation of cellular activity through toll like receptor (TLR) recognition and signaling is a target for immunotherapy against viral or microbial infections. Viruses and bacteria express evolutionarily conserved and specific molecular structures with ligands that TLRs recognize.[32–34] Nucleic acid therapeutics utilize cationic liposomes to deliver plasmid DNA intracellularly, thereby activating the TLRs and stimulating the immune response.[33–37] One immunotherapy platform is based on the triggering of innate immune responses using TLR9 agonists complexed to cationic liposomes; this greatly enhances the activity of the TLR9 agonist.[35,37] In a number of animal challenge studies, parenteral or inhalational administration of liposomal-TLR9 complexes has generated complete or nearly complete protection against highly virulent bacterial and viral pathogens.[36,38–42] In addition, administration of liposome-TLR9 complexes intraperitoneally (IP) to cats once weekly for four or six weeks resulted in lessening of clinical signs associated with feline URTD and an increase in neutrophils, monocytes, and CD4+ and CD8+ lymphocytes.[43]

An intranasal formulation of a liposome-TLR complex (LTC) was developed that includes a TLR9 agonist, a TLR3 agonist, and methylcellulose as a mucosal adhesive agent.[44] In a study of healthy, purpose bred cats, cytokine and cellular immune responses to this LTC were evaluated in vitro and in vivo, and the LTC rapidly activated cat leukocytes, including upregulation of co-stimulatory molecules and cytokine production.[44] A follow up study was performed to assess the mucosal administration of LTC prior to FHV-1 challenge in purpose-

bred kittens in a research facility.[45] The mucosal administration of LTC 24 hours prior to FHV-1 challenge was associated with several positive clinical effects and with decreased shedding of FHV-1 DNA.

Based on the results from these studies, the objectives of this study were to determine whether mucosal administration of LTC to cats upon intake to an open-admission shelter would result in decreased incidence of URI cases over time, decreased clinical signs and severity of URI in those cats that developed URI, increased time to onset of URI as compared to those cats receiving placebo, while also considering the potential effect of multiple other factors on the outcomes. We hypothesized that shelter cats administered the LTC upon admission would have overall less URI and clinical disease as compared to shelter cats receiving the placebo.

6.2 Materials and methods

6.2.1. Study solution

Liposomal toll-like receptor (TLR) complexes (LTC) were prepared using cationic liposomes [cholesterol and 1,2-dioleoyl-3-trimethylammonium propane (DOTAP; Avanti, Alabaster, AL)] admixed with two TLR agonists: polyinosinic-polycytidylic acid (poly I:C, InVivoGen, San Diego, CA) as a TLR3 and RIG-I and MDA5 agonist and non-coding commercial plasmid PCR2.1 DNA (pDNA; Life Sciences) as a TLR9 agonist rich in CpG. Carboxymethycellulose (Sigma-Aldrich, St. Louis, MO) was then added to the LTC to increase adhesion to mucosal cell surfaces. The complexes were prepared in 1mM Tris-buffered 5% dextrose in water (D5W); pH = 7.4.[44] The Placebo (P) solution was prepared using only the 1mM Tris-buffered D5W. The LTC used in this study was tested and shown to be safe and potentially efficacious in two previous studies.[44,45]

6.2.2. Shelter and cats

This study was approved by the Institutional Animal Care and Use Committee at Colorado State University and by the open admission north central Colorado shelter at which the study was conducted. All cats that were admitted to the shelter between July 7, 2016 and September 5, 2016 (60 days) and deemed behaviorally and medically suitable for intake procedures and vaccination (modified live parenteral FVRCP subcutaneous vaccine) per shelter protocol, and remained in the shelter after intake, were eligible for enrollment in this study. Cats had to be greater than six weeks of age or deemed age-appropriate for intake vaccination and in-shelter housing. Exclusion criteria included cats or kittens that were not tractable or behaviorally appropriate for intranasal and oropharyngeal administration of a study solution upon intake. Per shelter protocol, cats that entered the shelter as intractable feral cats or cats that entered the shelter with microchips or collars were not administered vaccinations or other procedures until a 5- or 7-day waiting period, respectively, had elapsed; these cats were therefore also excluded from entry into the study. If a cat was subsequently cleared for intake vaccination administration after a waiting period or if a cat that was previously intractable or considered a feral cat later became tractable, the cat then became eligible to participate in the study while the cat was processed through intake. All cats that entered the shelter were housed in individual holding kennels in an intake room; after intake procedures were performed, they were relocated within the next several days into another area of the shelter.

6.2.3. Study groups

This was a blinded, placebo-controlled clinical trial. Cats were assigned to one of two study groups, LTC or Placebo (P) on alternating days, and the schedule was determined by an

investigator (EC) that did not have daily contact with the shelter cats. The assigned study group corresponded to the solution that the cats received. Only one solution was assigned per intake day.

Because all cats did not receive the solution upon the first day of entry into the shelter, the LTC and P groups were further divided according to when each cat received the solution. Those cats that received the solution on their first day of shelter entry were assigned to the LTC0 and P0 groups; cats that received the solution on the next one or two days after their arrival into the shelter were assigned to the LTC12 and P12 groups; cats that received the solution 3 or more days after their arrival into the shelter were assigned to the LTC3+ and P3+ groups; cats that did not receive any solution were assigned to the "N" group.

6.2.4. Solution administration

An investigator (EC) filled individual 1.0ml syringes with either the LTC or P solution. The syringes were masked in an opaque label affixed to the syringe, color-coded in orange or silver, and labeled with either an "O" or "S" respectively and date of preparation (Figure 6.1a, 6.1b); the letters corresponded to the LTC or P solutions, respectively. A small nasal applicator was attached to the tip of the syringe, and plungers were marked with a small indentation and pen mark at three 0.2 ml increments to indicate amount of solution to be dispensed to each nare and oropharynx. Syringes were kept refrigerated until used, and newly prepared syringes were delivered to the shelter approximately every three days.

Figure 6.1a (left): “O” syringes with LTC solution
Figure 6.1b (right): “S” syringes with Placebo solution

Each cat received either the LTC or P solution during shelter intake performed by a trained and blinded veterinary student. This first day of solution administration was recorded as study day (SD) 0 (SD0) for that cat. To minimize stress associated with intake procedures, the cat was gently wrapped in a towel sprayed with Feliway® (Ceva Sante Animale, Libourne, France) during solution administration; the student was assisted by a shelter staff member. To administer the solution, the student tilted the cat’s head upward at an angle and dispensed 0.2 ml of the solution toward the medial septum of each nare; the student then slowly dispensed the remaining 0.6 ml of solution into the cat’s oropharynx and mid-portion of the tongue. Cats that were still present in the shelter after seven days were administered another dose of the same solution that they received on their first day. The same solution administration procedures were used on day 7.

6.2.5. Clinical monitoring

Cats were evaluated for general health by the veterinary students and shelter intake staff upon admission. Medical concerns were reported to the shelter veterinarian. Body weights were measured during intake and weekly thereafter, and students were instructed to report weight

losses to the shelter veterinarian. All cats that were admitted to the shelter during the study period were assigned daily URI nasal and ocular scores and oral scores (Table 6.1) upon intake and each morning by the trained blinded veterinary student. Daily scoring continued through October 5, 2016, which was 30 days after the last intake date for the study. Oral scores were assigned on SD0 and SD7 during solution administration and during daily scoring if an oral ulcer was suspected due to inappetence, drooling, or other indicative clinical signs. The scoring system was based on a scoring system used in a previous URI shelter study (Chapter 3). The total daily score was the sum of the nasal (0-4), ocular (0-3), and ulcer scores (0-1).

Table 6.1: Daily clinical scoring rubric

Nasal score	Description
0	No outwardly apparent URI signs
1	1 or more of: • sneezing, • Serous (clear, watery) nasal discharge, (can DRY/CRUST and appear yellow/brown) • mild/minor congestion (barely audible)
2	+/- sneezing and 1 or more of: • Moderate / Mucoid (white) nasal discharge, (can DRY/CRUST and appear yellow/brown) • moderate congestion (easily and consistently audible; snoring sounds on inhale or exhale – from nasal cavity
3	+/- sneezing and 1 or more of: • Severe / Mucopurulent (moist yellow-green) or hemorrhagic (bloody/red) nasal discharge • Severe congestion (audible across room, with or without open mouth breathing; minimal nasal air flow noted from 1 or both nostrils after local debris cleared away)
4	Severe respiratory distress indicative of pneumonia, with severe lethargy and critical illness

Ocular score	Description (can be in 1 or both eyes. Score WORST eye)
0	No outwardly apparent ocular signs
1	1 or more of: • Mild conjunctivitis (inflammation including nictitans/3rd eyelid and/or redness/blood vessels: hyperemia) • Mild Blepharospasm (squinting) Eye < 25% closed • Mild to moderate serous (clear, watery) ocular discharge, (can DRY/CRUST and appear yellow/brown). Do NOT confuse this with NORMAL morning ocular crusts
2	1 or more of: • Moderate conjunctivitis (inflammation and/or hyperemia) • Moderate blepharospasm. Eye 25-50% closed • Mucoid (white) ocular discharge. (can DRY/CRUST and appear yellow/brown)
3	1 or more of: • Severe conjunctivitis (inflammation and/or hyperemia) • Marked blepharospasm. Eye >50% closed • Mucopurulent (moist yellow-green) or hemorrhagic ocular discharge

Oral lesion
specify yes/no or n/a. Specify tongue, palate, buccal, gingiva. If anyone has an ORAL lesion, please ALERT shelter veterinarian

Both during and after study completion, all data were inspected for accuracy, missing values, and dates by another student and one of the investigators (EC) that was masked to solution group during the data evaluation. URI was recorded as present if the cat had a score of 1

or higher for nasal or ocular URI for two consecutive days or two days with one day in between of a score of 0, or any day that a cat had a score of 3. Presence or absence of severe URI was also recorded and defined as a total URI score of greater than 2. Presence or absence of URI and severe URI per cat, total URI scores, days to URI and severe URI, and days to resolution were determined and recorded for each cat in this report. Number of days to resolution was defined as the first of 2 consecutive days of a score of 0. A resolution date was not assigned if the cat was adopted, transferred, or was euthanized or died prior to resolution.

6.2.6. Detection of FHV-1

If a cat received a total daily clinical score of $\geq$ 3 on either the nasal or ocular scores, or $\geq$ 2 on both the nasal and ocular scores, or if the cat had oral ulceration, the student collected caudal pharyngeal mucosal cells on a sterile swab from the cat and stored the swab in a sterile tube in a freezer until assays were performed in batches. Total DNA was extracted from the samples and evaluated for DNA of FHV-1 and DNA of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) house-keeping gene by quantitative PCR (qPCR) as previously described.[46] Results were expressed as the ratio of FHV-1 DNA to GAPDH DNA. If a cat had a swab taken more than once, the highest of the values was used for analyses.

6.2.7. Covariates

Shelter data was recorded in the shelter's PetPoint Data Management System (Pethealth Inc., Rolling Meadows, IL). Data from July 7 - October 4, 2016 for each cat in the study was downloaded from PetPoint and transferred onto spreadsheets by a veterinary student and rechecked by another veterinary student. Data included signalment, intake source (owner

surrender, stray, return, born in care, safe keeping, and seized due to bite quarantine), intake date, outcome type (adoption, euthanized, died, foster, return to owner, transfer), and outcome date. If no outcome date was recorded, the cat was recorded as being still in the shelter. Length of stay was calculated, and if the cat was still in the shelter upon study completion, the date of October 4, 2016 was used to calculate length of stay.

Due to the very small number of cats that entered the shelter from any source other than stray or owner surrender, intake source was redefined into three categories of owner surrender, stray, and other. Age category was defined by the shelter and was assigned according to the cat's suspected or reported age at intake. Age categories defined as: kitten if < 4 months of age; adolescent if $\geq$ 4 and $\leq$ 12 months; adult if > 12 months and < 84 months; senior if $\geq$ 84 months of age. All adoptable cats admitted to the shelter were gonadectomized, and sex and neuter statuses were not reliably recorded upon intake; therefore, cats were recategorized into the categories of female, male, and unknown.

Kennel locations were recorded separately by the veterinary student during daily clinical scoring. Number of kennel changes throughout the cat's stay in the shelter were calculated. Because of varied lengths of stay and expected time to first URI occurrence, number of housing changes was calculated as the number of times a cat was moved to a different kennel within the cat's first 10 days within the shelter. The first 10 days were chosen based on previous studies that found the mean number of days to develop URI in a shelter ranged between six and 10 days (Chapter 3).[2,7] For those cats that had URI prior to the first 10 days, the number of kennel changes recorded prior to URI onset was recorded.

6.2.8. Statistical evaluation

Descriptive statistics including frequencies, counts, medians, and ranges were calculated. Unless stated otherwise, cats that had URI within their first two days after shelter entry were removed from analyses in comparisons between the LTC0, P0, and N0 groups, and cats that had URI on SD0-SD2 were removed from analyses in comparisons between the LTC12 and P12 groups. Univariable logistic regression models were used to assess the association of the main independent variable of solution group, LTC0 or P0, with the outcome of URI occurrence (yes/no); solution group of N0 was also included as one of the solution groups in analyses. Other factors potentially associated with the outcome of URI occurrence were also evaluated. These factors included age category (kitten, adolescent, adult, senior), intake status (owner surrender, stray, other), number of housing changes within 10 days of admission, weight change between SD0 and SD7, SD0 and SD14, and SD0 and SD21, and the dichotomous variable of sex (male, female). Housing type (individual kennels and group housing) could not be statistically evaluated because of the much fewer numbers of group housing spots available. Interactions between solution and other variables were also assessed. Factors that were associated in the univariable analyses with a $P \leq 0.20$ were used to build a multivariable logistic regression model using manual backwards elimination; if the main independent variable of solution group had a $P > 0.25$, the multivariable model was not built. Odds ratio estimates and 95% confidence intervals were calculated. The same univariable logistic regression analyses were also used to assess the association of the main independent variable of solution group, LTC12 and P12 and other independent variables, with the outcome of URI occurrence (yes/no). Due to the small sample numbers, the 2-tailed Fisher exact test was also used to compare the proportion of cats that had

URI in the LTC12 and P12 groups. The 2-tailed Fisher exact test was used to compare the proportions of cats that had severe URI in the LTC0 and P0 groups.

Due to non-normalcy of variables as assessed by the Shapiro-Wilk test, the Wilcoxon rank-sum test was used to compare the median number of days from intake to URI in the LTC0 group as compared to the P0 solution group and in the N0 group as compared to the two main solution groups. The Kruskal Wallis test was used to compare median number of days from intake to URI in the LTC0, P0, and N0 groups and to compare FHV/GAPDH ratios. The Wilcoxon rank-sum test was also used to compare the median number of days from first date of URI to URI resolution in the LTC0 as compared to the P0 solution groups. To further assess differences in time to development of URI between the LTC0 and P0 groups on specific days after receiving the solution, the 2-tailed Fisher exact test was used to compare the proportion of cats with URI on SD7 in both groups and the proportion of cats with URI on SD10 in both groups.

Kaplan Meier analysis was performed to estimate the median overall time (days) to development of URI and median overall time to resolution in the LTC0 and P0 solution groups. The log-rank test for event-time analyses was used to evaluate the association between categorical variables (solution group, age group, intake status, and sex) and time to development of URI (dependent variable). Cox proportional hazards regression was used to evaluate associations between time to development of URI and number of housing changes within 10 days of admission, weight change between SD0 and SD7, and weight change between SD0 and SD14. Factors with a $P \leq 0.20$ were included in a final multivariable Cox proportional hazards model to control for potential confounding. The log-rank test was also used to evaluate the association between solution group and time to resolution of URI. Kaplan Meier analysis was

also performed to estimate the median overall time to development of URI in the LTC12 and P12 solution groups, and the differences were assessed by the log-rank test.

To assess the efficacy of the LTC solution as a treatment in the LTC3+ group and P3+ group, the 2-tailed Fisher exact test was used to compare the proportion of cats that developed severe URI and to compare the proportion of cats that had a resolution date. To assess the efficacy of the LTC solution as a treatment in the LTC0 group and P0 group, only cats that had URI on SD0-SD2 were included in analyses and designated the LTC-ill and P-ill groups; the 2-tailed Fisher exact test was used to compare the proportion of cats that developed severe URI and to compare the proportion of cats that had a resolution date. Kaplan Meier analysis was performed to estimate the median overall time (days) to resolution in the solution groups, and the differences were assessed by the log-rank test for event-time analyses.

All analyses were performed using commercially available statistical software (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP). Significance was defined as $P < 0.05$.

6.3 Results

6.3.1. Study population and overall cat characteristics

During the 60-day study period, there were 261 cats admitted to the shelter; 250 of the cats stayed at the shelter for more than one day and were eligible for inclusion in the study (Figure 6.2). Of the 250 cats, 199 (79.6%) had an intake status of stray, 35 (14.0%) had an intake status of owner surrender, and 16 (6.4%) were classified as other that included return from previous adoption, born in care, safe keeping, and seized due to bite quarantine. There were 132 kittens (52.8%), 29 adolescents (11.6%), 79 adults (31.6%), and 10 senior cats (4.0%). There

were 118 females (47.2%), 109 males (43.6%), and 23 cats' (9.2%) gender status was unknown and not identified. The 23 unknown gender cats included 19 cats that had intake status of stray and were subsequently euthanized (n=14), sent to foster (n=1), or transferred (n=4), and four cats that had an intake status of seized due to bite quarantine; of the four cats, one was subsequently transferred, and three were still in the shelter at the end of the study.

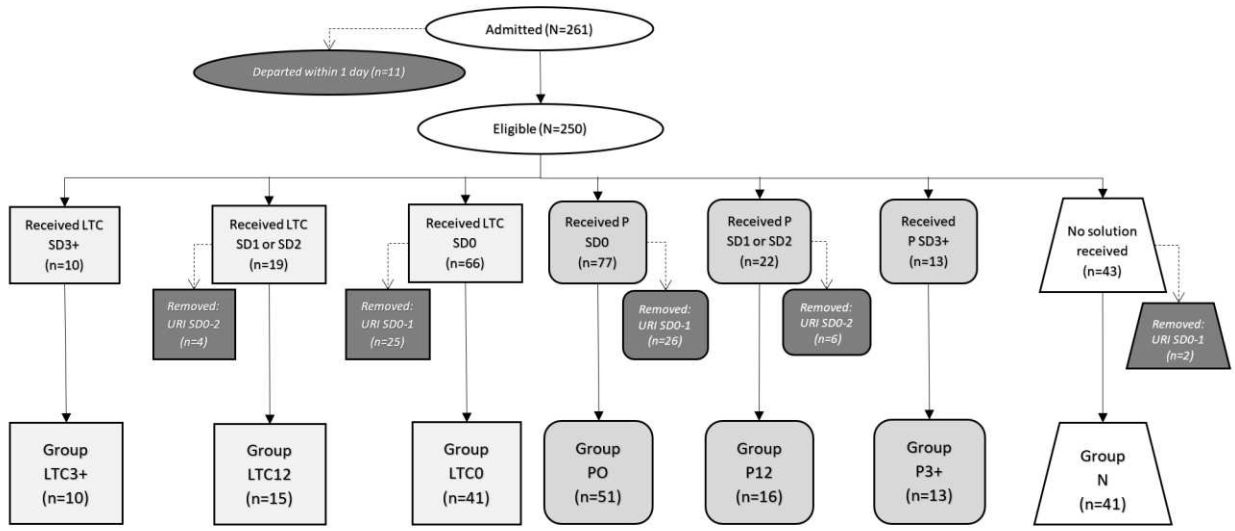


Figure 6.2: Flow diagram representing numbers of cats entering the shelter, the study, and solution groups

Of the 250 cats admitted, 121 (48.4%) had an outcome of adoption, 38 (15.2%) were transferred, 30 (12.0%) were euthanized or died, 22 (8.8%) went to foster, three (1.2%) were returned to their owner, and 36 (14.4%) were still in the shelter at the end of the study. The overall median length of stay was 16 days with a range of 1-89 days. Cats had an overall median of three (range 1 to 8) different kennels or locations throughout their time in the shelter. Within the first ten days of shelter admission, 183 cats (73.2%) stayed in the same kennel without

changing locations. Cats that were amenable to handling were weighed every seven days (Figure 6.3).

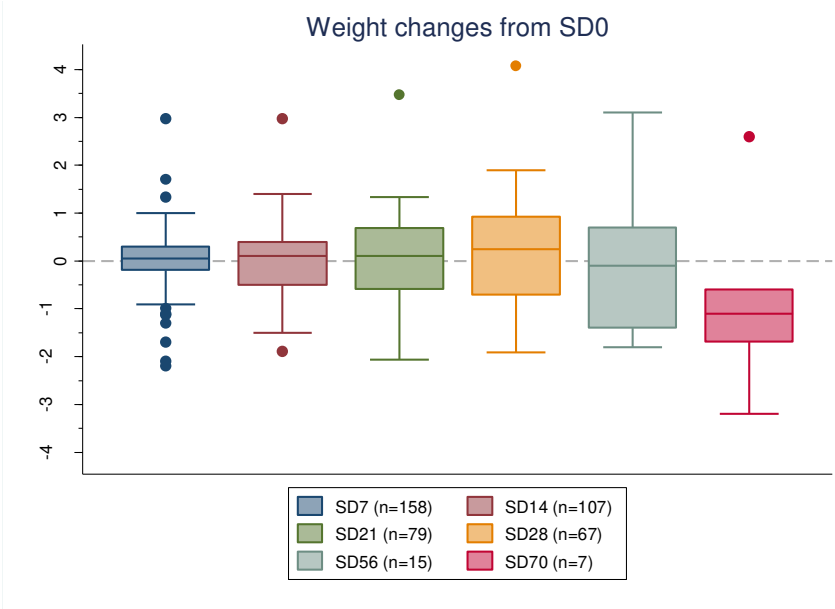


Figure 6.3: Box plot of cats' weight (kilograms) changes from SD0 to SD7, SD14, SD21, SD28, SD56, and SD70

There were 143 cats that were administered either LTC (n=66) or P (n=77) solution on SD0, 41 cats that were administered either LTC (n=19) or P (n=22) solution on SD1 or SD2, 23 cats that were administered either LTC (n=10) or P (n=13) solution on SD3 or higher, and 43 cats that were not administered any solution (Figure 6.2).

6.3.2. Overall upper respiratory illness

Of the 250 study cats, there were 163 cats (65.2%) that had URI and 64 (25.6%) that had severe URI. It took an average of 7.4 days (median 4 days; range 1-47 days) from intake to URI, and it took an average of 15.4 days (median 14 days; range 1 to 51 days) from intake to severe URI. Of the 163 cats that had URI, 120 cats (73%) had their first URI occurrence within the first

ten days of entering the shelter, and 131 (80.4%) of those cats stayed in the same kennel and had no kennel changes during those first ten days. Of the cats with URI, 79 (48.5%) had a resolution date. The average number of days from first URI to resolution was 8.7 days (median 6 days; range 2 to 48 days).

There were 51 oropharyngeal swabs obtained, and 5 of the swabs were from cats that had swabs taken more than once; for all 5 cats that had swabs taken more than once, at least 1 of the swabs had no FHV-1 or GAPDH DNA recovered (Table 6.2).

Table 6.2: FHV/GAPDH ratios by solution group

FHV/GAPDH	LTC0[a]	LTC12[b]	LTC3+	P0[a]	P12[b]	P3+	N[a]	All[a,b]	All
N	9	0	2	5	3	4	7	30	51
Median	197	n/a	4.74	0.23	0	49.2	0.29	0.53	0.16
Minimum	0	n/a	0	0	0	0	0.09	0	0
Maximum	3321	n/a	9.47	199	0	115	1562	3321	3321
Mean (SD)	544 (1073)	n/a	4.74 (6.70)	44.5 (87)	0 0	53.3 (50.7)	373 (654)	265 (679)	163 (533)

[a] Not including those cats from groups LTC0, P0, N that were sick with URI on SD0 or SD1 (first day of intake or day after for cats in N group)
[b] Not including those cats from groups LTC12 and P12 that were sick with URI on SD0-SD2

6.3.3. LTC0 and P0 groups

After removing cats that had URI on SD0 or SD1, there were 41 cats in the LTC0 group, 51 cats in the P0 group, and 41 cats in the N group (Figure 6.2). Characteristics of the groups are summarized in Table 6.3.

Table 6.3: Characteristics of the LTC0, P0, and N groups

	LTC0 (n=41)	P0 (n=51)	N (n=41)	Total (n=133)
Age group				
Kitten	N=22 (53.7%)	N=22 (43.1%)	N=12 (29.3%)	N=56 (42.1%)
Adolescent	N=6 (14.6%)	N=11 (21.6%)	N=2 (4.9%)	N=19 (14.3%)
Adult	N=10 (24.4%)	N=16 (31.4%)	N=27 (65.9%)	N=53 (39.8%)
Senior	N=3 (7.3%)	N=2 (3.9%)	N=0 (0%)	N=5 (3.8%)
Sex category				
Male	N=20 (48.8%)	N=27 (52.9%)	N=6 (14.6%)	N=53 (39.8%)
Female	N=21 (51.2%)	N=24 (47.1%)	N=13 (31.7%)	N=58 (43.6%)
Unknown	N=0 (0%)	N=0 (0%)	N=22 (53.7%)	N=22 (16.5%)
Intake category				
Owner surrender	N=12 (29.3%)	N=6 (11.8%)	N=1 (2.4%)	N=19 (14.3%)
Stray	N=26 (63.4%)	N=44 (86.3%)	N=37 (90.2%)	N=107 (80.5%)
Other	N=3 (7.3%)	N=1 (2.0%)	N=3 (7.3%)	N=7 (5.3%)
Outcome				
Adoption	N=22 (53.7%)	N=30 (58.8%)	N=0 (0%)	N=52 (39.1%)
Euthanized/Died	N=2 (4.9%)	N=2 (3.9%)	N=20 (48.8%)	N=24 (18.0%)
Foster/Transfer/RTO	N=8 (19.5%)	N=12 (23.5%)	N=11 (26.8%)	N=31 (23.3%)
Still in shelter	N=9 (22.0%)	N=7 (13.7%)	N=10 (24.4%)	N=26 (19.5%)
URI (yes)	N=23 (56.1%)	N=31 (60.8%)	N=19 (46.3%)	N=73 (54.9%)
Severe URI (yes)	N=11 (26.8%)	N=7 (13.7%)	N=9 (22.0%)	N=27 (20.3%)
Had URI resolution (yes)	N=13 of 23 (56.5%)	N=17 of 31 (54.8%)	N=4 of 19 (21.1%)	N=34 of 73 (46.6%)
Median (days) length of stay (min-max)	15 (1-89)	17 (2-77)	16 (6-66)	16 (1-89)

There were no differences in URI occurrence in univariate analyses comparing the LTC0 group (n=23; 56.1%), the P0 group (n=31; 60.8%), and the N group (n=19; 46.3%; P=0.38), and there were no differences in URI occurrence in the LTC0 group compared to only the P0 group (P=0.65) (Table 6.4a) or the N group compared to both of the solution groups (P=0.19). Univariable logistic regression modeling with the LTC0 and P0 groups only identified two variables, weight change from SD0 to SD7 and housing changes that were associated with URI occurrence with P<0.20; no significant variables were identified (Table 6.4a, 6.4b). There were no significant interactions between solution group and other independent variables; only one interaction had a P-value < 0.20 (Figure 6.4). There were a total of 18 cats that had severe URI. Of the 23 cats in the LTC0 group that had URI, 11 (47.8%) had severe URI, and of the 31 cats in the P0 group that had URI, 7 (22.6%) had severe URI; this difference was not significant (P = 0.19) (Table 6.3). There was only one cat that had an oral ulcer; that cat was in the LTC0 group.

Table 6.4a: Summary of univariable analyses of URI presence/absence and categorical variables in LTC0 and P0 groups

Predictor (Risk factor)	Level	URI Yes (n)	URI No (n)	TOTAL (n)	URI Yes (%)	P-value	Odds Ratio	95% Confidence Interval
Solution (Main)	Placebo (SD0)	31	20	51	29%	0.65	1.21	0.53 - 2.79
	LTC (SD0)	23	18	41	56%	reference	reference	reference
Age group	Senior (>7yr)	4	1	5	80%	0.34	3.04	0.31 - 29.46
	Adult (>1yr-7yr)	16	10	26	62%	0.70	1.22	0.45 - 3.27
	Adolescent (4mo-1yr)	9	8	17	53%	0.79	0.86	0.28 - 2.63
	Kitten (<4 mos)	25	19	44	57%	reference	reference	reference
Sex	Female	29	16	45	64%	0.27	1.60	0.69 - 3.68
	Male	25	22	47	53%	reference	reference	reference
	Other	1	3	4	25%	0.24	0.25	0.02 - 2.52
Intake type	Owner surrender	13	5	18	72%	0.25	1.95	0.63 - 6.07
	Stray	40	30	70	57%	reference	reference	reference

Table 6.4b: Summary of univariable analyses of URI presence/absence and continuous variables in LTC0 and P0 groups

Predictor (Risk factor)	URI	Number	mean	SD	Range	P-value	Odds Ratio	95% Confidence Interval
number of housing changes (n=92)	Yes	54	1.4	0.74	1 - 4	0.11	0.66	0.40 - 1.10
	No	38	1.7	0.93	1 - 4			
weight change between SD0 - SD7 (n=77)	Yes	53	-0.09	0.52	-2.2 - 0.6	0.16	0.52	0.20 - 1.36
	No	24	0.1	0.66	-1.7 - 1.7			
weight change between SD0 - SD14 (n=56)	Yes	42	-0.15	0.72	-1.9 - 0.9	0.31	0.62	0.24 - 1.61
	No	14	0.1	0.63	-1.0 - 0.9			
weight change between SD0 - SD21 (n=39)	Yes	30	-0.2	0.95	-2.1 - 1.3	0.32	0.64	0.26 - 1.59
	No	9	0.14	0.71	-0.9 - 1.3			

There were nine cats (42.9%) from the LTC0 group, five cats (23.8%) from the P0 group, and seven cats (33.3%) from the N group that had oropharyngeal swabs evaluated for presence of FHV-1 DNA (Table 6.2). The differences in FHV/GAPDH values for the LTC0 group as

compared to the P0 group were not significant (P=0.12), and the differences in FHV/GAPDH values among the three groups (LTC0, P0, N) were not significant (P = 0.31).

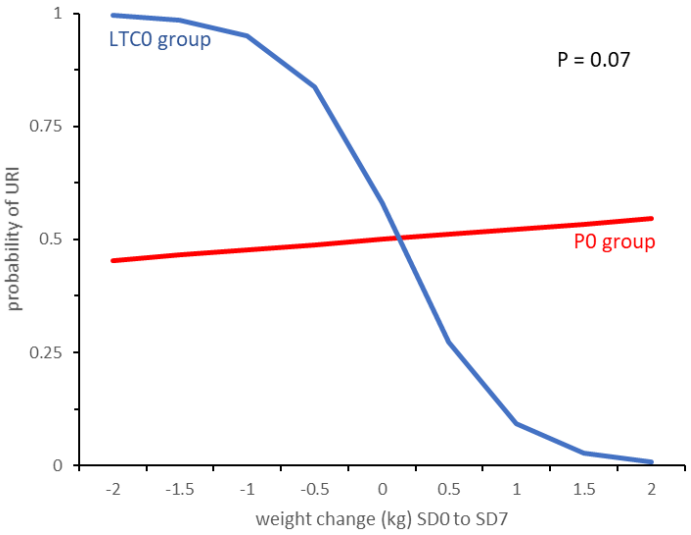


Figure 6.4: Interaction plot depicting the interaction between weight change from SD0 to SD7 and solution group (LTC0 and P0) and probability of URI occurrence

There was not a significant difference (P = 0.65) in median number of days to URI when comparing the LTC0 group (median 8 days; range 2 - 40 days) and the P0 group (median 6 days, range 2 - 47 days). Median number of days to URI in the N group (median 13 days; range 2 - 25 days) was longer than median number of days to URI in both solution groups combined, but this difference was not significant (P = 0.05). The proportion of cats in the LTC0 group that had URI on SD7 (19.5%) and SD10 (34.1%) did not significantly differ from the proportion of cats in the P0 group that had URI on SD7 (33.3%; P = 0.16) and SD10 (43.1%; P = 0.40). When using the Kaplan Meier survival method that also accounted for cats that remained in the shelter without URI, time to event (time to URI) curves did not significantly differ between the LTC0 and P0 groups (P = 0.86) (Figure 6.5; Table 6.5). Univariable analyses of the time to event data identified two variables, age category (P = 0.04) and housing changes (P = 0.01) that were significantly associated with time to URI. Results of the Cox proportional hazard analysis that

included both variables in a final multivariable model are listed in Table 6.6. Risk of URI decreased by 62% if the cat was an adult as compared to a kitten (P = 0.009), and as number of housing changes increased by one, risk of URI decreased by 43.9% (P = 0.006) (Table 6.6).

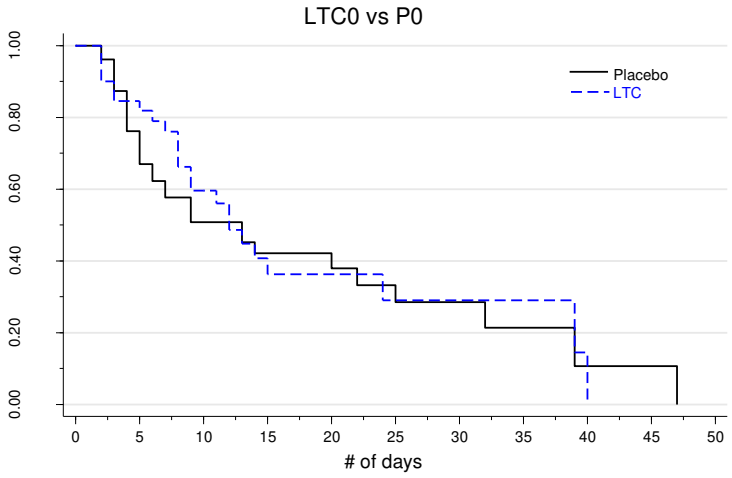


Figure 6.5: Kaplan-Meier probability curve depicting time (days) from SD0 to probability of not developing URI comparing group LTC0 (n=41 cats; 23 developed URI) and P0 (n=51 cats; 31 developed URI).

Table 6.5: LTC0 and P0 days at-risk for URI, day interval, censored cats, and probabilities of having and not having URI

	Day interval		Number of cats at risk	new URI case	censored[a]	Probability of having URI	Probability of not having URI
Placebo (n=51)	0	5	51	11	7	0.23	0.77
	5	10	33	11	0	0.49	0.51
	10	15	22	3	5	0.57	0.43
	15	20	14	0	4	0.57	0.43
	20	30	10	3	3	0.72	0.28
	30	40	4	2	1	0.88	0.12
	40	50	1	1	0	1.00	0.00
LTC (n=41)	0	5	41	6	4	0.15	0.85
	5	10	31	8	5	0.39	0.61
	10	15	18	5	4	0.58	0.42
	15	20	9	1	3	0.64	0.36
	20	30	5	1	0	0.71	0.29
	30	40	4	1	2	0.81	0.19
	40	50	1	1	0	1.00	0.00

[a]censored: cats that left the shelter (adoption, euthanasia/death, transfer, etc) prior to development of URI

Table 6.6: Multivariable Cox proportional hazard model of time to URI in LTC0 and P0 groups

Predictor (Risk factor)	Number	Hazard ratio	95% Confidence Interval	P-value
Age category				
Kitten (<4 mos)	44	reference	reference	reference
Adolescent (4mo-1yr)	17	0.64	0.29 - 1.40	0.27
Adult (>1yr-7yr)	26	0.38	0.18 - 0.78	**0.009**
Senior (>7yr)	5	1.8	0.62 - 5.22	0.28
Number of housing changes	92	0.56	0.37 - 0.85	**0.006**

Of the 23 LTC0 cats that had URI, 13 (43.5%) had a resolution date, and of the 31 P0 cats that had URI, 17 (45.2%) had a resolution date. There was not a significant difference (P = 0.34) in the number of days from first URI to resolution in the LTC0 group (median 7 days; range 3-48 days) as compared to the number of days in the P0 group (median 4 days; range 3-24 days). When using the Kaplan Meier survival method to consider those cats that did not have a resolution date, the time to event (days to resolution) curves did not significantly differ between the LTC0 and P0 groups (P = 0.51) (Table 6.7).

Table 6.7: Probability of resolution or no resolution of URI, day interval, and censored cats in the LTC0 and P0 groups

	Day interval		Starting number of cats	Number of cats that had resolution event	censored[a]	Probability of having resolution	Probability of not having resolution
Placebo (n=31)	0	5	31	9	2	0.30	0.70
	5	10	20	5	5	0.50	0.50
	10	15	10	1	3	0.56	0.44
	15	20	6	1	1	0.64	0.36
	20	30	4	1	1	0.74	0.26
	30	40	2	0	2	0.74	0.26
LTC (n=24)	0	5	23	5	3	0.23	0.77
	5	10	15	4	2	0.45	0.55
	10	15	9	1	1	0.52	0.48
	15	20	7	1	1	0.59	0.41
	30	40	5	1	2	0.69	0.31
	40	50	2	1	1	0.90	0.10

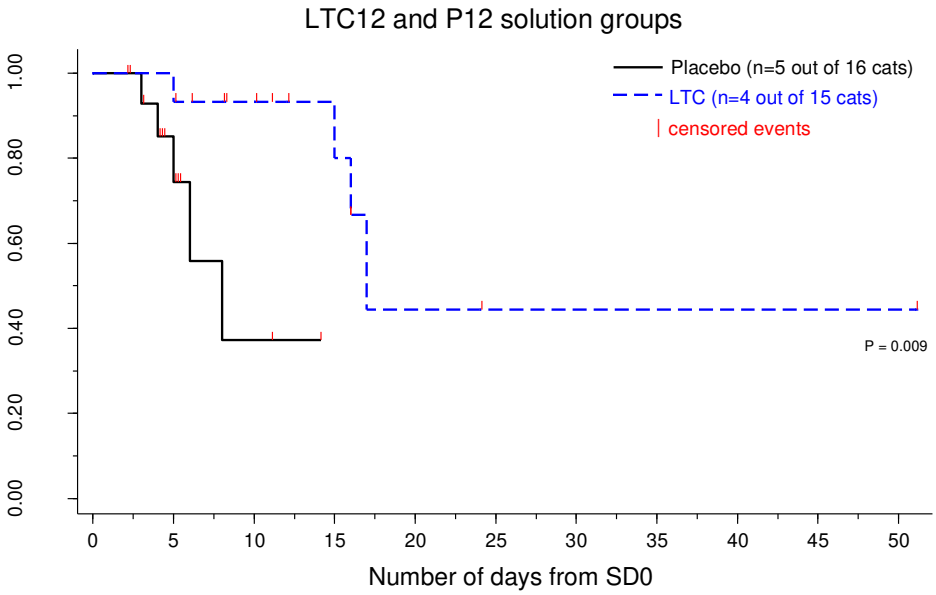
[a]censored: cats that left the shelter (adoption, euthanasia/death, transfer, etc.) before resolution

6.3.4. LTC12 and P12 Groups

There were 19 cats that received LTC on SD1 or SD2 and 22 cats that received P on SD1 or SD2. After removing cats that had URI on SD0, SD1, or SD2, there were 15 cats in the LTC12 group and 16 cats in the P12 group (Figure 6.2). Characteristics of the groups are summarized in Table 6.8. There were no differences in URI occurrence in univariate analyses comparing the LTC12 group (n=4; 26.7%) and the P12 group (n=5; 31.3%; P=0.78). The 2-tailed Fisher exact test also found no significant difference in proportions of cats with URI (P = 0.57) in the LTC12 group as compared to the P12 group. No other independent variables were significantly associated with URI occurrence. Median number of days to URI in the LTC12 group (median 15.5 days; range 5 - 17 days) was longer than median number of days to URI in the P12 solution group (median 5 days; range 3-8 days), but this difference was not significant (P = 0.07). When using the Kaplan Meier survival method that also accounted for cats remaining in the shelter without URI, the cats in the P12 group had a higher probability of developing URI sooner over time as compared to the cats in group LTC12 (P = 0.009) (Figure 6.6). FHV/GAPDH results are listed in Table 6.2.

Table 6.8: Characteristics of the LTC12 and P12 groups

	LTC12 (n=15)	P12 (n=16)
Age group		
Kitten	N=10 (66.7%)	N=14 (87.5%)
Adolescent	N=1 (6.7%)	N=0 (0%)
Adult	N=4 (26.7%)	N=2 (12.5%)
Senior	N=0 (0%)	N=0 (0%)
Sex category		
Male	N=3 (20%)	N=6 (37.5%)
Female	N=12 (80%)	N=10 (62.5%)
Intake category		
Owner surrender	N=0 (0%)	N=4 (25%)
Stray	N=12 (80%)	N=11 (68.8%)
Other	N=3 (20%)	N=1 (6.3%)
Median (days) length of stay (min-max)	12 (5-51)	5 (2-37)
Outcome		
Adoption	N=9 (60%)	N=11 (68.8%)
Euthanized/Died	N=0 (0%)	N=0 (0%)
Foster/Transfer/RTO	N=5 (33.3%)	N=4 (25%)
Still in shelter	N=1 (6.7%)	N=0 (0%)
URI (yes)	N=4 (26.7%)	N=5 (31.3%)
Severe URI (yes)	N=0 (0%)	N=1 (6.3%)
Had URI resolution (yes)	N=1 of 4 (25%)	N=4 of 5 (80%)



censored events: cats that left the shelter (adopted or transferred) prior to development of URI

Figure 6.6: Kaplan-Meier curve depicting time (days) from SD0 to probability of not developing URI comparing the LTC12 group (n=15 cats; 4 cats developed URI) and the P12 group (n=16 cats; 5 cats developed URI)

6.3.5. LTC as treatment

There were 10 cats in the LTC3 group and 13 cats in the P3 group (Figure 6.2). One cat from the LTC3 group (10%) and five cats from the P3 group (38.5%) developed severe URI; this difference was not significant (P = 0.18). There were eight cats in the LTC3 group that had URI, and there were 11 cats in the P3 group that had URI. Of those cats that had URI, three of the LTC3 cats (37.5%) had resolution of URI, and seven of the P3 cats (63.6%) had resolution of URI; this difference was not significant (P = 0.37). FHV/GAPDH data is listed in Table 2.

There were 29 cats in the LTC-ill group, and there were 28 cats in the P-ill group. Thirteen cats from the LTC-ill group (44.8%) developed severe URI, and 15 cats from the P-ill group (53.6%) developed severe URI; this difference was not significant (P = 0.6). Sixteen cats from the LTC-ill group (55.2%) had resolution of URI, and 15 cats from the P-ill group (53.6%) had resolution of URI; this difference was not significant (P = 1.0). When using the Kaplan Meier survival method that also accounted for cats that did not have a resolution date, the time to event (days to resolution) curves did not significantly differ between the LTC-ill and P-ill groups (P = 0.68).

6.4 Discussion

This study did not support our hypothesis that mucosal administration of LTC to cats upon admission to a shelter would lead to decreased upper respiratory illness. Although previous studies[44,45] supported the use of the mucosal LTC formulation in preventing some clinical signs of FHV-1 illness, those studies were in controlled environments and evaluated only one pathogen, whereas this study evaluated efficacy in an environment with likely numerous pathogens and variations. In this study population, the time to develop URI was only associated

with age category and housing changes; kittens and senior cats and cats that had fewer kennel changes were at increased risk. These unexpected results were likely partly due to the peculiarities of the shelter's protocols and demographics.

6.4.1. LTC in the shelter environment

Previous studies evaluating efficacy of cationic liposome-DNA complexes have shown protection in small mammals against mostly single viruses or one bacterial genus in research settings.[38–42] Similarly, the LTC formulation used in our study was also shown to decrease clinical signs in purpose-bred kittens infected with one virus, FHV-1 (Chapter 5).[45] In contrast, the natural illness model in a shelter setting in which cats were likely infected with multiple viral and bacterial pathogens, was used for the study described here. Multiple pathogens are responsible for URI in the shelter setting, and it is thought that most cats have co-infections consisting of FHV-1, FCV, Chlamydia felis, Bordetella bronchiseptica, and Mycoplasma felis.[2–4,8,47,48] The likely multiple co-infections thus high infectious load might have contributed to the failure of the LTC formulation to elicit a positive clinical benefit. Therefore, although the LTC formulation might have decreased clinical signs in kittens infected with FHV-1 in other studies, LTC had not been used in cats with unknown histories and backgrounds and infected with multiple pathogens.

The mucosally administered LTC solution might require a robust and intact immune system without interference from overwhelming infectious or immune-compromising stress burdens. It is possible that an individual must be healthy in order for the LTC immune enhancing properties to work, as this formulation showed positive results when administered to healthy cats.[44] Intranasal CLDC formulations administered to mice 24 hours prior to bacterial challenge

resulted in protection, but when administered intranasally either during infection or 6 or 24 hours post-infection, protection was lost.[41,42] In our study of purpose-bred FHV-1 infected kittens (Chapter 5)[45], LTC administered mucosally prior to FHV-1 infection appeared to confer some protection, but LTC administered after the kittens were infected and displaying clinical signs, did not appear to confer a benefit. Similarly, in this study, LTC administered to those cats that were already ill did not decrease clinical signs, severity, or length of illness. We found some differences in clinical disease in cats that received LTC solution on the first or second day after admission as compared to cats that received P solution on the first or second day after admission. It is possible that these cats receiving the solutions on the first or second days had an extra day or two to adjust to their new environment and were consequently less impacted by stress when the solution was administered, thus their immune systems might have allowed for the LTC solution to confer its protective effects. However, the numbers of cats that had URI in the LTC12 group (n=4) and P12 group (n=5) were very small, thus these results should be interpreted with caution. The significant results could be due to individual differences or type 1 error.[49,50] Future studies might consider administration of the formulation to cats as they are being handed from the surrenderer or animal control officer to the intake staff. Although this would still be a very stressful time point for the cat, it might be early enough in the stress response to allow the LTC formulation to impact the mucosal immune system and confer some benefit.

The failure to reject our null hypotheses could be due to the inability of the LTC in its current formulation to overcome the overwhelming infectious and other burdens encountered in this type of stressful and contaminated shelter environment. Previous work with CLDC formulations administered prior to bacterial or viral challenges in mouse models, have shown positive results when the CLDC was administered prophylactically intraperitoneally,[39] intravenously,[39]

subcutaneously (SQ)[40], and intranasally (IN)[41,42] prior to infectious challenge. However, when the CLDC formulation was administered at the time of infectious challenge or after challenge, minimal to no protective benefits were found.[39–41] One of those studies,[40] however, found that when the CLDC was administered SQ to mice 12 hours after viral infection, some protection was provided dependent on location of the SQ injection in that administration had to be performed dorsal to the cervical spine.[40] Another of these studies[41] found that when IN treatment was provided during infection, partial protection from lethal outcomes were achieved, but protection from chronic infection was still lacking.[41] One study showed that administration of liposome-TLR9 complexes intraperitoneally to cats once weekly for 4 or 6 weeks resulted in lessening of some clinical signs associated with feline URTD in 12 client-owned cats and 28 shelter cats with unresolved rhinitis.[43] However, that study also failed to find differences in other URTD clinical signs and some cytokine parameters.[43] Nevertheless, future studies with this LTC formulation or another might be considered in client owned cats prior to boarding, for instance. The LTC that was use in this study is currently being reformulated with a new plasmid that may have improved efficacy to be trialed in additional studies.

6.4.2. Shelter prevalence of URI

Over half of the cats in the shelter in this study had clinical signs of URI either at admission or during their stay in the shelter whether they received the LTC solution, placebo, or no solution. Other recent studies have reported average URI percentages of approximately 17%,[5] 35%,[4] 55%,[8] and 58%.[7] This high rate of URI in the shelter of this study is likely due to various factors including those related to intake procedures. Due to logistical issues, cats often entered the shelter and were placed in an intake kennel to await intake and vaccination that sometimes

occurred the next day or later. If the cat was overly resistant to handling or if the cat had a microchip or collar, the cat was not vaccinated and remained either in the intake area or an adjacent room or hallway, potentially for several days or more. Due to staffing and logistical issues, some stray cats that were not able to be handled remained unvaccinated in the shelter up to 14 days or more until euthanasia occurred. It is rather recommended that cats should be vaccinated upon entering the shelter; otherwise risk of exposure to and/or transmission of disease is high.[19,20,51] These issues likely contributed to high infectious burdens in this shelter. Other shelter management issues potentially contributing to high infectious burden and URI rates included a stressful environment consisting of aversive noises and scents including dogs and barking; small, unkempt kennels; unpredictability of times of feedings and cleanings; potential breaks in barrier precautions; inadequate disinfection protocols; fomite transmission by untrained staff or volunteers; and high population numbers.[52–64] Not only did these factors likely contribute to the high URI incidence, but these factors could have also interfered with the ability of the LTC formulation to confer benefits to outweigh the multiple competing adverse variables that might have contributed to lowered immune responsiveness.

The probability of having URI more than doubled between days 5 and 10 in our study, and of all the cats in the shelter, mean number of days to URI was 7.4 days, and median number of days to URI was 4 days. This time to onset of URI was similar to findings in previous studies.[2,4,7] Mean or median time to develop URI in the shelter has been reported as 8.3 days,[7] between 2 to 8 days for carriers of URI implicated pathogens,[4] or 6 days in kittens and 7 days in adults.[2] In our study, excluding those that already had URI upon entry, there was over a 50% risk of developing URI by the second week in the shelter. Similarly, another study found that the probability of developing URI was between 80-86% by day 14 in the shelter.[2]

6.4.3. Shelter variables and risk factors for URI

The number of housing changes in our study seemed to have a protective effect in the LTC0 and P0 groups. This was an unexpected finding. In another study (Chapter 3), the number of housing changes was a significant risk factor in all analyses; as the number of housing changes increased, the risk of URI and severe URI increased (Chapter 3). Another study found that cats that were moved to different kennels more than two times within the first week of the shelter stay, had a significantly higher risk of URI.[5] Others have evaluated FHV-1 and housing changes in purpose-bred cats in research facilities. One found that increased FHV-1 viral shedding occurred after cats were rehoused from a group setting into an individual cage setting.[65] Another found that some of the cats had increased FHV-1 viral shedding after a variety of housing changes.[66] One of the chapters in this body of work (Chapter 7)[67] evaluated stress, behavior, the use of a pheromone, and clinical signs in kittens with FHV-1 and utilized the housing change of group to kennel housing as an inducer of acute stress. Since cats are stressed with moves or changes in their environments and unfamiliar places, unusual or novel sights, sounds, smells, and inconsistencies, it has been inferred that cats may become stressed with shelter kennel or housing changes.[55,56,68–73] And because stress can reactivate FHV-1 and contribute to lowered immune responsiveness, housing changes would be expected to be a risk factor for increased incidence of URI.

The unexpected contrary finding in this study that housing changes conferred a protective effect on the cats could be due to factors unique to this shelter. The LTC0 and P0 cats in our study had between one and four housing changes within their first ten days in the shelter. Those cats that stayed in one kennel for a longer period of time were those that remained in the intake and holding areas. Those areas contained the unvaccinated cats and were especially noisy and

chaotic because dog intake occurred in the adjacent area. Considering that nearly 75% of the cats that had URI, had their first URI occurrence within the first ten days, and over 80% of the cats with URI stayed in one kennel without moving by the time their URI occurred, it is likely that staying in those initial intake and holding area kennels increased risk of URI for all cats due to transmission, infectious load, and increased stress.[54,62,63] It therefore follows that it was protective if a cat moved to either the surgery or clinic area for gonadectomy or to the adoption floor for adoption, thus increasing the number of kennel locations but decreasing the time spent in the higher risk and infectious environment.

Another disparate characteristic of this shelter was that over 80% of the cats had an intake status of stray. This was unexpected considering the shelter was in a primarily residential community in the third largest county in the state (www.weldgov.com). In contrast, owner surrender is the most common intake source in other studies.[2,4] In Colorado, stray cats account for less than half of the intakes (https://shelteranimalscount.org). One explanation is that patrons of the shelter in this study might claim that the animal that they are relinquishing to the shelter is a stray animal and to avoid the stigma and repercussions of surrendering an owned animal, especially if they intend to adopt another animal (shelter staff personnel, personal communication). Nevertheless, stray cats have been found to be at increased risk of URI in other studies, and this could have further contributed to the high URI rate in this shelter if the cats were truly stray cats.[2,4,49]

Kittens and senior cats were at increased risk for URI in this study. Previous studies have also found that kittens were more likely to have URI in the shelter and that there is an increased risk of URI with increasing age.[2,8,74] Alternative methods to house or care for these at-risk groups might be considered in shelter settings.

The only interaction that approached significance in this study was the interaction between solution group (LTC0 and P0) and weight change from day 0 to day 7. As the LTC0 group gained weight in the first seven days, they were less likely to have URI, whereas the P0 group's risk of having URI remained more constant regardless of weight gain (Figure 6.3). The cats that gained weight were likely those that were overall healthier or less susceptible to the other variables implicated in URI occurrence in the shelter. However, weight gain alone was not associated with a decrease in URI; rather, the interaction between weight gain and LTC administration showed decreased risk of URI. It therefore seems plausible that LTC administered to those cats with immune systems that were overall more robust as evidenced by weight gain, then conferred a protective advantage to those cats and helped to upregulate a stronger immune response to the pathogens. This finding might lend further support to administration of LTC to healthy individuals to detect a beneficial effect. Unfortunately, this finding might also lead to questioning of its use in the shelter setting, since many cats will likely encounter and perceive the new shelter environment as extremely stressful rendering the cats more susceptible to illness due to subsequent immune compromise.[5,54,59,69,75]

6.4.4. Assays

Results from PCR analyses of the oropharyngeal swabs should be interpreted with caution. There were very few swabs taken from each group, and swabs were only analyzed for presence of one virus, FHV-1. Differences were not significant. Nevertheless, false negatives occur in FHV-1 assays.[76,77] One study[76] isolated FHV-1 virus in 11% of normal cats and only 18% of cats with clinical signs of disease, while another study[77] detected FHV-1 DNA in 37% of healthy cats and only 22% of clinically ill cats. Testing for FCV might have also provided more

information, although levels of viral shedding do not necessarily correlate with severity of clinical illness.[21,78,79] Since PCR assay results do not prove the presence of live virus, quantitative culture might also provide additional information and should be considered for use in future studies. Culturing and molecular detection of other bacterial pathogens from the nasal and oropharyngeal mucosa might also be useful for future studies and might have assisted to account for differences in URI occurrence and severity within groups and relationship with other factors. Identification and quantification of cytokines and leukocyte or other cellular components to assess the upregulation or downregulation of immune function components prior to and subsequent to solution administration and onset of URI might have also added important information to our study.

6.4.5. Other limitations

In addition to the disparities listed above, there were several other limitations to this study. The evaluation of several other variables might have helped to explain some of the differences in URI. Surgery was not evaluated in our analyses and might have contributed to URI occurrence. Dental disease was also not evaluated in our study nor were chronic gingivitis and stomatitis evaluated in our analyses, and this might have contributed more explanatory data since FCV has been associated with chronic lymphoplasmacytic gingivitis stomatitis (LPGS).[80–82] Treatment regime instituted for the cats was also not evaluated. Some cats might have received antibiotics at different timepoints, and some might have received oral medications in combination with ocular and/or nasal treatments. In severe cases, some might have received fluids, appetite stimulants, face washing, antiviral medications, and supplements such as probiotics or lysine. All of these treatments and durations could have affected outcome. Other

limitations included those similar to other shelter studies in that we were unaware of the cats' physical, environmental, behavioral, health, or vaccination histories. Any of these variables could have affected outcomes. Furthermore, data was collected from a single shelter during one summer; this limits generalizability of our findings. Similarly, air exchange rates, cleaning protocols, and fomite transmission were factors likely to affect any shelter study, and these were also not evaluated. Although we evaluated weight changes, food composition such as canned versus dry versus brand were not evaluated, and these differences might have affected general health and outcomes.

6.5. Conclusions

This study did not find evidence that the LTC mucosally administered solution protected cats from developing URI nor severe URI, nor did it hasten resolution of illness in this shelter setting. The only interaction that approached significance in this study was the interaction between solution group (LTC0 and P0) and weight change from day 0 to day 7 in that the potentially healthier cats might have been better able to benefit from the LTC. Although many uncontrolled variables in such a setting could be partly responsible for the lack of efficacy, this shelter environment was not unlike many other shelter environments. We therefore do not have evidence to support the use of the LTC in the current formulation in the manner that it was delivered in the shelter setting. Delayed intake vaccinations, inadequate barrier precaution procedures, environmental stressors, and slow movement of cats in this shelter likely highly impacted the development of and increased transmission of URI.

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CHAPTER 7: EFFECT OF A PHEROMONE[2] ON STRESS-ASSOCIATED REACTIVATION OF FELINE HERPESVIRUS-1[3]

7.1. Introduction

Feline herpesvirus-1 (FHV-1) is a common infectious disease of cats. Infection can be subclinical, or it may result in clinical signs of disease including pyrexia, conjunctivitis, keratitis, sneezing, cough, dyspnea, inappetence, lethargy, and occasionally, pneumonia and death.[1–4] Morbidity and mortality in crowded or stressful environments, such as shelters, can be high.[5–8]

After acute exposure, most cats develop persistent infection, with the trigeminal ganglia serving as the main site of viral latency.[2,9–11] Reactivation of FHV-1 then can occur, resulting in clinical signs and increases in FHV-1 shedding.[10,12–14] Stressful events may precipitate FHV-1 reactivation in some instances.[2,8,10,12] In a shelter study, cats with the highest stress scores during the first week in the shelter were more likely to develop upper respiratory infection (URI).[15] Unfamiliar handlers and environments, altered feeding schedules or husbandry activities, kennel confinement, impoverished or non-stimulating environments, aversive stimuli such as noise, odors, uncomfortable temperatures, and lack of hiding resources all can cause stress in cats.[10,16–24] Furthermore, changing housing from group-housing to kennels also has been shown to trigger FHV-1 associated disease.[10,25,26] Stressful events are believed to result in FHV-1 reactivation or shedding within the first three weeks with an approximate lag phase of 4 to 11 days after the stress.[2,10,15]

[2] Feliway®; Ceva Santé Animale, Libourne, France

[3] Part of this research is in print: Contreras ET, Hodgkins E, Tynes V, Beck A, Olea-Popelka F, Lappin MR. Effect of a pheromone on stress-associated reactivation of feline herpesvirus-1 in experimentally inoculated kittens. J Vet Intern Med. 2018;32(1):406-417.

Several strategies with variable outcomes have been employed in an attempt to mitigate FHV-1 reactivation in cats.[25–29] Lessening stress is a strategy that may decrease signs of URI and viral shedding in shelter cats.[8,30–32] In shelters, stress reduction methods have included gentle stroking and speaking to the cat, grooming, playing, and use of hiding enrichment and minimally invasive daily kennel cleaning.[19,22,30,31,33–37]

Feline facial pheromone fractions contained in a commercial preparation (Feliway®; Ceva Sante Animale, Libourne, France) have been assessed in a variety of studies as another potential stress reducing modality.[38,39] Use of this product has been evaluated in the management of feline behaviors sometimes associated with stress, such as urine spraying, as well as stress-associated diseases such as feline idiopathic cystitis. Use of the product also has been shown to decrease signs of stress during transportation or when visiting a veterinary clinic and to improve appetite in hospitalized patients.[38–42]

Our study was designed to determine whether experimentally-induced FHV-1-infected kittens housed in equivalent rooms containing either a pheromone diffuser or a placebo diffuser and subjected to housing-change induced stress, would differ in behavioral scores, clinical scores, FHV-1 shedding, or serum cortisol concentrations.

7.2. Materials and methods

7.2.1. Cats

Six neutered male and six spayed female, 5-month-old, mixed breed kittens bred for use in research projects were used in this 12-week pilot study. Eight weeks before the study, each of the 12 kittens was infected with FHV-1 by intranasal instillation for a study (Chapter 5)[43] in which the 12 kittens were the group-housed control group. Using these control kittens from a

prior study for our study eliminated the need for experimental inoculation of additional kittens, an objective of both the investigators and the sponsor. In that previous study, FHV-1 infection was confirmed in all kittens by quantitative polymerase chain reaction (qPCR), and each developed clinical signs consisting of fever, sneezing, ocular discharge, nasal discharge, nasal congestion, conjunctivitis, blepharospasm, or some combination of these. At the time of the study, none were undergoing treatment for URI, and occasional sneezing, serous nasal discharge, or serous ocular discharge were the only potential manifestations of FHV-1 present intermittently in the kittens. Both the prior study and our study were approved by the Institutional Animal Care and Use Committee.

7.2.2. Housing

Kittens were randomized into a pheromone group (diffuser containing feline facial pheromone F3 fraction) or a placebo group (placebo diffuser); each group consisted of three males and three females. The kittens were housed in two separate but similarly sized rooms (pheromone group = 8’6” width × 10’ 3” length × 9’ height; placebo group = 9’7” width × 9’ length × 9’ height) within the research facility; each room had a separate air exchanger and two similarly-sized litter boxes. During kennel-housing, each room contained three sets of top and bottom individual wire kennels (36” × 25.5” × 22”); kittens spent one 2-week kennel period in a top kennel and the other 2-week kennel period in a bottom kennel. During kennel housing, kittens were in visual contact with the other kittens in kennels, other than the one kitten in a respective top or bottom kennel. Some of the kittens also had physical contact with the other kittens through the bars of the kennels. All kittens were provided dry food and water ad libitum and were provided a table with two levels that were approximately 36” height × 15” width × 36”

length during group-housing and a similarly-sized kennel perch during kennel housing. Kittens were provided two white ping-pong balls during group housing and one white ping-pong ball per kennel during kennel housing. Between two and four cardboard boxes of various shapes and sizes also were provided during group housing; boxes were identical between rooms and were exchanged for new, different boxes every week. The litter boxes, table, and enrichment devices were movable and not always in the same position within the rooms. While observers were interacting with the kittens during group housing, novel objects such as paper balls, writing implements, and the observers' outer garments were used as toys and enrichment devices by the kittens; the novel objects were removed when the observers departed after scoring. Enrichment was kept to a minimum because the study was designed to evaluate the effect of the pheromone on stress.

7.2.3. Clinical scoring

A clinical score sheet adapted from other FHV-1 vaccination and treatment studies including the previous study of which these kittens were a part, was used in our study (Table 7.1).[29,29,44] A total clinical score was calculated for each kitten each day by adding the individual clinical score variables recorded for that day. Body temperatures were estimated by microchip.[45] Increased body temperature was defined as >102.5°F. Heart rates were measured daily when auscultation was not obscured by purring. Body weights were measured weekly.

The protocol included a rescue clause for kittens that developed moderate to severe signs of FHV-1 infection and a loss of appetite for 48 hours. Supportive care and treatment that could be administered included subcutaneous administration of fluids, buprenorphine for discomfort, topical cidofovir, or oral famciclovir as needed and determined by the investigators.

Table 7.1: Daily clinical scoring rubric

Clinical sign	Score
Conjunctivitis*	0=None 1=Mild 2=Moderate 3=Severe
Blepharospasm*	0=None 1=Eye<25% closed 2=Eye 25-50% closed 3=Eye 50-75% closed 4=Eye completely closed
Ocular discharge*	0=None 1=Mild serous (clear) discharge 2=Moderate mucoid (white) discharge 3=Severe mucopurulent (moist yellow-green) discharge
Body temperature (microchip)	0: ≤ 102.5 1: > 102.5
Cough	0=None 1=Observed
Sneezing (yes/no)	0=None 1=Observed
Nasal discharge*	0=None 1=Mild serous (clear) discharge 2=Moderate mucoid (white) discharge 3=Severe mucopurulent (moist yellow-green) discharge or hemorrhagic (bloody/red) discharge
Nasal congestion* (if score varies during observation period, record highest score observed)	0=None (no congestion present; able to breathe through both nares without difficulty) 1=Mild / Minor congestion (barely audible; audible on close listening, subtle snoring sounds on inhalation ANY time during the observation period 2=Moderate congestion (easily audible; consistently audible throughout observation period; audible snoring sounds on inhalation or expiration that are likely to originate from the nasal cavity) 3=Severe congestion (audible across the room, with or without open mouth breathing; minimal nasal air flow noted from one or both nares after local debris is cleared away)
*Due to statistically low occurrence of scores >1, binomial analyses were performed using 0 or 1 to indicate presence or absence of clinical sign.	

7.2.4. Behavioral scoring

Several different behavioral assessment scales used in previous shelter and other studies were reviewed as tools to assess stress and behavior in the kittens.[16,18,22,46–48] Based on observations of the kittens during the study in which they were inoculated with FHV-1, and because these purpose-bred research kittens already were habituated to the research facility,

housing, each other, and human interactions, a modified scale was designed (Table 7.2a,b,c). The behavioral observation metrics also were designed to accommodate ease and efficiency and to avoid distracting from FHV-1 clinical scoring. The rubric contained lists of typical feline postures, vocalizations, and actions that represented either normal, relaxed calm, or stress-related behaviors that could be observed and objectively scored (Table 7.2a,b,c). Because of the overall engaging personalities and temperaments of the kittens in the study, the rubric was further adapted before and during the equilibration period. The final rubric that was applied when the diffusers were introduced into the rooms contained 28 individual behaviors, recorded at five different specified time points during the 45-minute scoring period per room each morning (Table 7.2a,b,c). Snapshot observations were performed for 15 seconds per kitten at the following four time points: upon entry into room (SS1 time point), during clinical scoring handling (SS2 time point), immediately after clinical scoring handling (SS3 time point), and at the 45-minute mark (SS4 time point). Behaviors also were recorded throughout the 20 to 30 minutes between SS3 and SS4 (Long time point) (Table 7.2c).

Table 7.2a: Daily behavioral scoring rubric, time points SS1 and SS2

BEHAVIOR (italics indicate only scored in EITHER group- OR kennel-housing)	SCORE Y = yes or 1; N = no or 0
Snapshot "SS1" Time point: Upon entry into room	
(Group-housed only) Greeting: Greets me at door	Y / N
Vocalization: Meow	Y / N
Vocalization: Hiss/growl	Y / N
(Kennel-housed only) Pawing through kennel	Y / N
(Kennel-housed only) Pacing (repetitive walking back and forth)	Y / N
Snapshot "SS2" Time point: During clinical scoring	
Reaction to clinical scoring period, temp wand/handling	L: allows, leans in, purr, soft body posture, ears forward F: freeze, crouch, immobile, dilated pupils, ears not forward, tense, stiff R: mildly resistant, fidgets, some squirming, some displacement grooming V: very resistant, "obsessively" attempts escape, scratches; doesn't allow, won't stay still
Reaction to petting DURING CLINICAL SCORING PERIOD (scorers will pet a few times after temperature wand/clinical scoring in order to assess)	L: leans in, "enjoys" R: moves away, flinches, not interested, "done with you"
Vocalization: Meow	Y / N
Vocalization: Purring	Y / N
Kneading	Y / N
Vocalization: Hiss/growl	Y / N

Table 7.2b: Daily behavioral scoring rubric, time points SS3 and SS4

BEHAVIOR (italics indicate only scored in EITHER group- OR kennel-housing)	SCORE Y = yes or 1; N = no or 0
Snapshots "SS3" and "SS4" Time points: approximately 15 seconds per each cat SS3: After clinical scoring, in same order as when performed clinical scoring SS4: At 45-minute mark	
Is cat "up" with 4 paws on floor? Standing, walking, running, pacing (Okay to have "Up" AND "Not standing" categories if performed >1 in 15 seconds)	Y: up, walking, climbing, standing N: sleeping, lying down, sitting
IF NOT "up", cat is: (Okay to have >1 category if cat is positioned in >1 way during 15 seconds)	ZZ: sleeping; S: sitting; LE: lying on side, legs extended; LA: Lying down, abdomen exposed; LVU: Lying down ventrally, head up and alert; LVD: Lying down ventrally, head down
Active / Passive: Is cat "Active?" doing something, acting, reacting, watching, ready to pounce, or is cat instead absorbing, relaxing, sleeping	Y: Active N: Passive
Urinating/Defecating	Y / N
Eating/drinking	Y / N
Vocalization: Meow	Y / N
Interacting/playing with objects or other cat(s) or human	Y / N
Vocalization: Hiss	Y / N
(Group-housed only) Climbing on objects or on object	Y / N
(Group-housed only) Climbing on person; or currently on person	Y / N
Grooming self	Y / N
(Group-housed only) Grooming another cat	Y / N
(Group-housed only) Licking person	Y / N
(Kennel-housed only) Pawing through kennel	Y / N
(Kennel-housed only) Pacing (repetitive walking back and forth)	Y / N

Table 7.2c: Daily behavioral scoring rubric, Long time point

BEHAVIOR (italics indicate only scored in EITHER group- OR kennel-housing)	SCORE Y = yes or 1; N = no or 0
"Long" time point: While sitting down in room, approximately 20-30 minutes, during time between SS3 and SS4	
Housing disarray: Litterbox overturned with litter, feces on floor during group-housing. During kennel-housing: kennel disarray - kibble, water, urine, litter scattered throughout kennel (litterboxes were permanently affixed to kennels so they could not easily be overturned by kitten)	Y / N (scored during Long time-point because more time allotted; however, this housing disarray score represents the condition of room or kennel upon scorers' entry into room)
Diarrhea present?	Y / N
Urinated/Defecated (did you SEE the kitten urinate/defecate)	Y / N
Eating/drinking observed? (did you SEE the kitten eat/drink)	Y / N
(Group-housed only) Vocalization: Purring	Y / N (N also includes unknown - if not close enough to hear)
Vocalization: Meow (0, 1 during group-housing) (0, 1, 2 during kennel-housing)	0: no meows 1: some occasional meows during period 2: excessive meowing during period (during kennel-housing)
Vocalization: Hiss/growl	Y / N
Any fighting/spats/aggression toward other cats or humans - present?	Y / N
Hiding behavior	Y / N
Interacting/playing with objects or other cat(s) (or human during group-housing)	Y / N
Kneading	Y / N
(Group-housed only) Climbing on objects or on object	Y / N
(Group-housed only) Climbing on person; or currently on person	Y / N
Groomed self	Y / N
(Group-housed only) Groomed another cat	Y / N
(Group-housed only) Licked person	Y / N
(Kennel-housed only) Pawing through kennel	Y / N
(Kennel-housed only) Pacing (repetitive walking back and forth)	Y / N

7.2.5. Experimental design

Kittens were housed in their respective group rooms on weeks 1, 2 (period E), 3, 4 (period G0), 7, 8 (period G1), and 11, 12 (period G2) (Figure 7.1). Kittens were housed in kennels in their respective rooms on weeks 5, 6 (period K1) and 9, 10 (period K2). Two trained scorers, blinded regarding treatment allocation, applied the standardized clinical and behavioral scoring system at approximately the same time and order every morning, for 45 minutes per room, throughout the study (Figure 7.1).

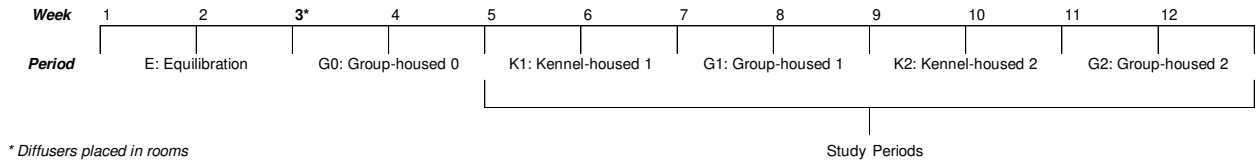

Figure 7.1: Study timeline by week and corresponding group- or kennel-housing period number

7.2.6. Assays

At the beginning of the study and after each of the six periods (E, G0, K1, G1, K2, G2), kittens were sedated, proparacaine was applied to their corneas, and blood, caudal pharynx mucosal cells, and conjunctival swabs were collected. Sera, oropharyngeal, and conjunctival swabs were stored at -80°C until assayed in batches. Total DNA was extracted from the oropharyngeal and conjunctival samples and evaluated for DNA of FHV-1 and DNA of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene by quantitative PCR (qPCR) as previously described.[49] Results of the GAPDH assay were used as a positive control for sample adequacy because this house-keeping gene is present in all feline cells. Results of the FHV-1 qPCR assay were expressed as the ratio of FHV-1 DNA/GAPDH DNA to standardize specimens. Serum cortisol concentrations were measured at a commercial laboratory (Endocrinology Laboratory, Michigan State University, Lansing, Michigan).

7.2.7. Statistical evaluation

After randomization but before starting the study, the total clinical scores associated with FHV-1 that developed after primary infection in the previous study were compared between the

pheromone group and the placebo group using the Wilcoxon rank sum test, and the groups were found to not have different median scores (P = .9).

Because there were no manipulations of the kittens in G0 other than adding the diffusers, this was not considered a stress period for the final comparisons between groups. Only the results from periods K1, G1, K2, and G2 in which the kittens' routines were disrupted by housing changes potentially associated with stress, were evaluated individually and in combinations. Descriptive statistics were calculated, and categorical data were expressed as frequencies, whereas continuous data were expressed as means, medians and ranges. The Shapiro-Wilk test was used to assess normalcy of data. Because of non-normalcy of all variables, the Wilcoxon rank sum test was used to compare group median results for total clinical score, total stress score, FHV-1/GAPDH ratios, heart rate, and weekly body weight changes in the pheromone group as compared to the placebo group. The Wilcoxon rank sum test also was used to compare median heart rates in kenneled periods as compared to group-housed periods, median serum cortisol concentrations at the beginning of the study as compared to the end of the study, and median serum cortisol concentrations in kittens that shed FHV-1 during any study period as compared to serum cortisol concentrations in those kittens that did not shed FHV-1 during any study period. Individual clinical and behavioral variables were categorized into dichotomous variables of presence or absence of these variables each day. The proportions of observations of dichotomous clinical or behavioral variables were compared between the pheromone group and placebo group by use of the 2-tailed Fisher exact test. The 2-tailed Fisher exact test also was used to compare dichotomous variables at the beginning of the study with dichotomous variables at the end of the study. To control for lack of independence among observations because of repeated

measurements on the same kitten over time, mixed model regression analyses were used, and odds ratios (OR) and 95% confidence intervals (CI) were calculated.

Commercially available software (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX, StataCorp LP) was used for all comparisons. Significance was defined as $P < .05$.

7.3. Results

7.3.1. Clinical findings

At the end of the equilibration period, no significant differences were found in total clinical scores between groups ($P = .58$). All kittens had normal appetites, gained weight consistently, and had soft, groomed hair coats each day throughout the study. None of the cats required medical intervention for FHV-1 infection. Weight changes did not differ significantly between groups during the study periods ($P = 0.83$), and pyrexia occurrences did not differ between groups during the study periods ($P=0.07$). For one of the 12 kittens, the temperature sensing microchip malfunctioned, and body temperature in this kitten was measured in the axillary space. Total clinical scores did not differ significantly between groups (Table 7.3). Median, mean, range, and group comparison results for the total clinical score listed by groups and study period are presented in Table 7.3.

Table 7.3: Medians, means, and ranges for the total clinical scores by group and study period

Study Period	G0*	K1*	G1*	K2*	G2*	K1,G1,K2,G2
Group	median, mean (range)	median, mean (range)	median, mean (range)	median, mean (range)	median, mean (range)	median, mean
Placebo	13, 13.8 (5 - 31)	14.5, 15.3 (2 - 29)	12.5, 16.8 (8 - 36)	14, 15.5 (3 - 33)	13.5, 15.3 (4 - 34)	58, 63
Pheromone	9.5, 12.8 (3 - 31)	15, 15 (2 - 28)	12, 12.3 (3 -28)	16, 16 (5 - 29)	11, 13.2 (3 - 31)	55.5, 56.5
P value	.58	.92	.06	.66	.32	.23

G#, Group-housed period number; K#, Kennel-housed period number.
* n=84 observations: 14 observations per 6 kittens per 2-week study period per group.
G1, G2: the two, 2-week group-housing periods after placement of the diffusers in G0; K1, K2: the two, 2-week kenneled periods. The means and ranges are shown to demonstrate variation between the 6 kittens per group; the group medians are compared by Wilcoxon rank sum test.
Statistical significance: $P < .05$.

Heart rates did not differ significantly between groups (Table 3). Heart rates, however, were significantly higher during kennel-housed periods when compared to group-housed periods in the pheromone group ($P < .001$), placebo group ($P < .001$), and both groups combined ($P < .001$); significant differences were retained after adjustment for lack of independence, but no clinical sequelae were noted.

Table 7.4: Heart rate ranges in placebo compared to pheromone groups and kennel-housed compared to group-housed during the treatment periods

	Kennel-housed		*Group-housed*	
GROUP	**Placebo**	**Pheromone**	**Placebo**	**Pheromone**
PERIOD	***K1***		***G1***	
HR mean	214	213	198	196
HR median	216	216	200	195
HR minimum	160	150	160	160
HR maximum	258	300	260	288
PERIOD	***K2***		***G2***	
HR mean	221	214	192	196
HR median	220	213	190	192
HR minimum	168	150	160	150
HR maximum	270	280	230	250

Mild ocular discharge (score = 1) was recorded at many observation points for several kittens before and during the study periods and potentially associated with stress (Table 7.5), but it was never associated with conjunctivitis (Tables 7.6a, 7.6b). Coughing was rarely heard during the study (Tables 7.6a, 7.6b).

Table 7.5: Frequencies of clinical signs in the placebo and pheromone groups in each period

	Period	**E1**	**G0**	**K1**	**G1**	**K2**	**G2**
Clinical sign							
Ocular discharge	**Placebo group**	29%	33%	29%	33%	32%	45%
	Pheromone group	14%	17%	24%	17%	26%	29%
	P-value	0.11	0.01	n/a			
Nasal discharge	**Placebo group**	14%	17%	23%	18%	21%	26%
	Pheromone group	***38%****	44%	56%	36%	51%	49%
	P-value	***0.01****	<0.001	n/a			
Nasal congestion	**Placebo group**	***12%****	4%	29%	27%	40%	5%
	Pheromone group	0%	1%	17%	14%	24%	5%
	P-value	***0.02****	0.31	n/a			
Sneeze	**Placebo group**	55%	32%	21%	27%	11%	29%
	Pheromone group	40%	25%	5%	17%	7%	10%
	P-value	0.19	0.31	<0.001* (Placebo increased)			

P-values from results of logistic regression (ocular discharge, nasal discharge, sneeze) and Wilcoxon rank sum (nasal congestion)

*significant P-value at P<0.05

%: represents the number of occurrences of the clinical sign in each kitten in each period out of the total number of possible occurrences of the clinical sign in each kitten in each period

n/a: not applicable due to discrepancies and potential introduced selection bias

Table 7.6a: Frequency of clinical sign (score = 1 or 2) for each kitten in the placebo group in each 14-day period

PLACEBO ROOM									
Name	Period	ocular discharge	conjunctivitis	blepharospasm	nasal discharge	nasal congestion	sneeze	cough	pyrexia
Baloo JBI1	G0	0%	7%	7%	0%	0%	21%	0%	7%
	K1	0%	0%	0%	7%	14%	29%	0%	0%
	G1	0%	0%	0%	0%	21%	14%	0%	21%
	K2	0%	0%	0%	0%	29%	7%	0%	0%
	G2	0%	7%	0%	0%	0%	29%	0%	0%
Bambi JBI5									
	G0	50%	0%	7%	0%	0%	50%	0%	0%
	K1	57%	0%	0%	7%	57%	36%	0%	0%
	G1	57%	0%	0%	7%	7%	21%	0%	0%
	K2	50%	0%	0%	7%	86%	21%	0%	0%
	G2	93%	0%	0%	29%	0%	36%	0%	0%
Christine JBI4									
	G0	36%	0%	0%	0%	0%	43%	0%	14%
	K1	7%	0%	0%	0%	14%	14%	0%	7%
	G1	36%	0%	0%	0%	14%	14%	0%	21%
	K2	14%	0%	0%	0%	36%	7%	0%	29%
	G2	64%	0%	0%	0%	0%	7%	0%	21%
Madame JBH4									
	G0	100%	0%	7%	93%	0%	21%	0%	0%
	K1	100%	0%	0%	100%	0%	0%	0%	0%
	G1	86%	0%	0%	100%	14%	50%	0%	0%
	K2	100%	0%	0%	100%	21%	14%	0%	0%
	G2	100%	0%	0%	100%	0%	43%	0%	0%
Rajah JBM1									
	G0	14%	0%	7%	7%	14%	50%	0%	0%
	K1	7%	0%	0%	21%	86%	36%	0%	0%
	G1	14%	0%	0%	0%	71%	43%	0%	0%
	K2	29%	0%	0%	21%	64%	0%	0%	0%
	G2	14%	0%	0%	29%	14%	43%	0%	0%
Waldo JBG2									
	G0	0%	0%	0%	0%	7%	7%	0%	21%
	K1	0%	0%	0%	0%	0%	14%	0%	0%
	G1	7%	0%	0%	0%	36%	21%	0%	7%
	K2	0%	0%	0%	0%	7%	14%	0%	0%
	G2	0%	0%	0%	0%	14%	14%	0%	0%

Table 7.6b: Frequency of clinical sign (score = 1 or 2) for each kitten in the pheromone group in each 14-day period

PHEROMONE ROOM									
Name	Period	ocular discharge	conjunctivitis	blepharospasm	nasal discharge	nasal congestion	sneeze	cough	pyrexia
Duchess JBH2	G0	86%	0%	0%	93%	7%	29%	0%	7%
	K1	93%	0%	0%	100%	0%	7%	0%	0%
	G1	79%	0%	0%	100%	0%	21%	0%	0%
	K2	79%	0%	0%	100%	7%	21%	0%	0%
	G2	100%	0%	0%	100%	0%	21%	0%	0%
Edgar JBG1									
	G0	0%	0%	0%	64%	0%	50%	0%	0%
	K1	21%	0%	0%	71%	0%	14%	0%	7%
	G1	14%	0%	0%	43%	0%	14%	0%	7%
	K2	7%	0%	0%	100%	0%	0%	0%	0%
	G2	50%	0%	0%	64%	14%	7%	0%	0%
Oakley JBF4									
	G0	0%	0%	0%	14%	0%	7%	0%	0%
	K1	14%	0%	0%	0%	0%	0%	0%	0%
	G1	0%	0%	0%	7%	0%	14%	0%	0%
	K2	7%	0%	0%	14%	7%	7%	0%	0%
	G2	7%	0%	0%	21%	0%	0%	0%	0%
Sheer-Khan JBF2									
	G0	7%	0%	0%	36%	0%	7%	0%	14%
	K1	14%	0%	0%	71%	36%	0%	0%	0%
	G1	7%	0%	0%	50%	43%	7%	0%	7%
	K2	0%	0%	0%	57%	57%	7%	0%	0%
	G2	7%	0%	0%	50%	7%	14%	7%	7%
Toulouse JBJ1									
	G0	7%	0%	0%	14%	0%	36%	0%	0%
	K1	0%	0%	0%	50%	21%	7%	0%	7%
	G1	0%	0%	0%	0%	0%	21%	0%	0%
	K2	29%	0%	0%	29%	0%	7%	0%	0%
	G2	0%	0%	0%	50%	0%	14%	0%	0%
Vesper JBI3									
	G0	0%	0%	7%	43%	0%	21%	0%	0%
	K1	0%	0%	0%	43%	43%	0%	0%	7%
	G1	0%	0%	7%	14%	43%	21%	0%	7%
	K2	36%	21%	7%	7%	71%	0%	0%	0%
	G2	7%	0%	0%	7%	7%	0%	0%	0%

Mild nasal discharge (score = 1) was recorded frequently (Table 7.5; Figures 7.2a, 7.2b), but moderate mucoid discharge (score = 2) associated with nasal congestion was not detected during the study (Tables 7.6a, 7.6b). Mild nasal congestion (score = 1) was reported commonly (Table 7.5; Figures 7.2a, 7.2b), but moderate congestion was rarely reported (Tables 7.6a, 7.6b). Furthermore, at the end of the equilibration period (Table 7.5), nasal discharge was already significantly higher (P=0.01) in the pheromone group as compared to the placebo group, while nasal congestion was significantly higher (P=0.02) in the placebo group as compared to the pheromone group, thus potentially introducing unintended selection bias. Therefore, coughing, ocular discharge, nasal discharge, and nasal congestion were not evaluated further.

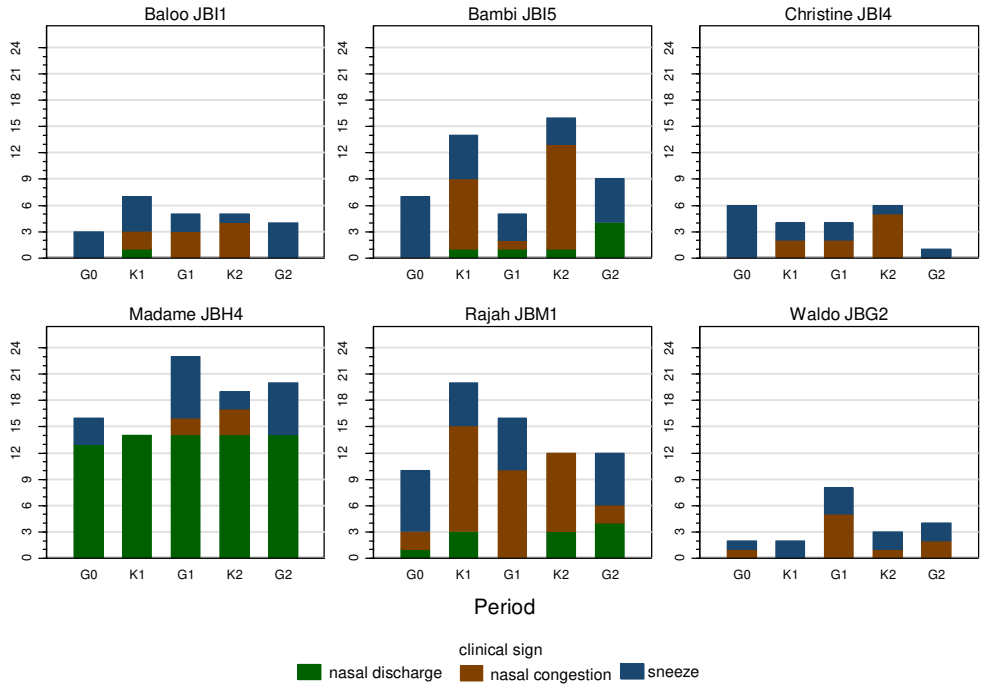

Figure 7.2a: Bar graphs depicting number of days each kitten (n=6) in the placebo group had nasal discharge, nasal congestion, and sneeze recorded during each 14-day period

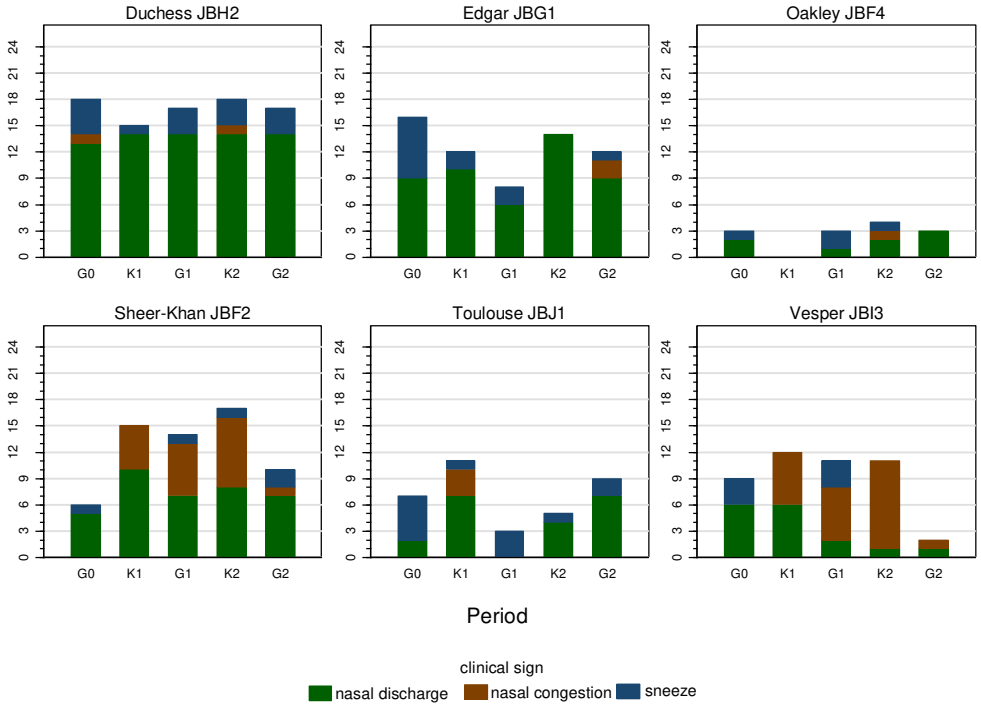

Figure 7.2b: Bar graphs depicting number of days each kitten (n=6) in the pheromone group had nasal discharge, nasal congestion, and sneeze recorded during each 14-day period

Sneezing was the most common finding that likely was associated with FHV-1 infection. After the diffusers were placed during G0 but before the induction of stress, no differences were observed (P = .31) between the placebo group (32%; n=27/84 observations) and the pheromone group (25%; n=21/84 observations) in occurrence of sneezing (Figure 7.3, Tables 7.5, 7.6a, 7.6b).

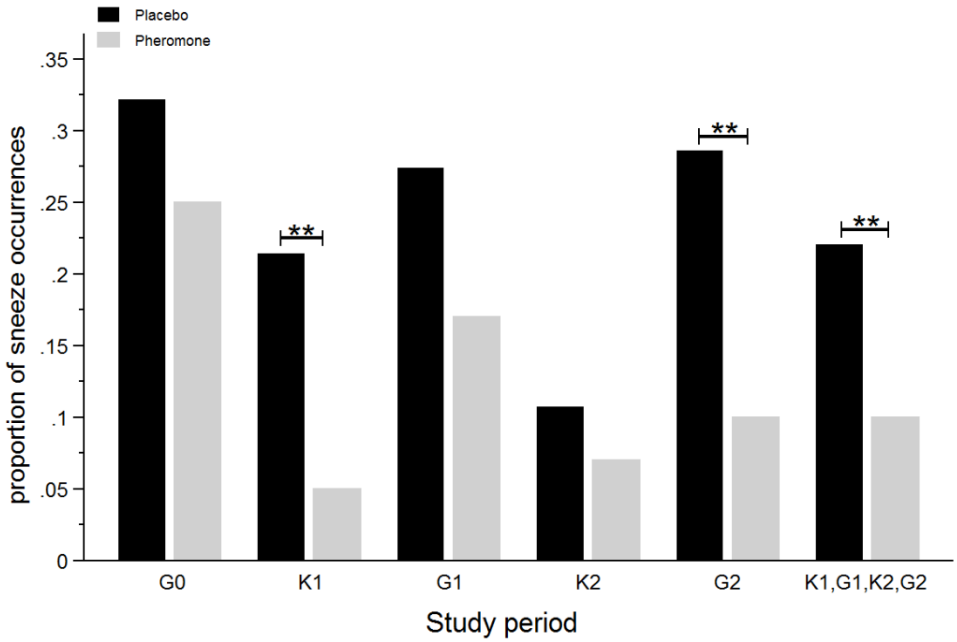

Figure 7.3: Bar graphs depicting proportion of sneezing occurrences (presence) by group and study periods including combined study periods of K1,G1,K2,G2

K#, kennel-housed period number; G#, group-housed period number.
*Statistical significance of P < .05 for group comparisons using multivariate logistic regression, adjusted for lack of independence.

After adjusting for lack of independence, sneezing (presence) occurred less frequently in kittens in the pheromone group when compared to the placebo group during period K1 (P = .006), period G2 (P = .005), and when the study periods potentially associated with stress were combined (P < .001, Figure 7.3). In the combined study periods, kittens in the placebo group were 2.7 (95% confidence interval [CI], 1.7 – 4.6) times more likely to have a sneezing occurrence than kittens in the pheromone group (Figure 7.3). Kittens in the pheromone group also were 3.3 (95% CI, 1.3 to 8.0; P = .009) times more likely to have a sneezing occurrence at the beginning of the study (G0) when compared to the end of the study (G2; Figure 7.3). In contrast, no significant differences were identified in sneezing occurrence among these study periods for the placebo group (P = .61).

7.3.2. Behavioral findings

The frequency of occurrences of all behaviors evaluated, separated by study period and time point, are listed in Tables 7.7a and 7.7b. Based on review of the literature and agreement by the clinical scorers at the end of the study, four behaviors were believed to be objective indicators of stress in this cohort of kittens: kennel pacing, kennel pawing, kennel disarray, and excessive vocalization. Kennel pacing and kennel pawing were evaluated in the SS1, SS3, SS4, and Long time points (Table 7.2a,b,c), but there were too few occurrences of kennel pacing in SS4 to be evaluated. Kennel disarray and excessive vocalization were evaluated in the Long time point (Table 7.2c).

Table 7.7a: Frequency of behaviors displayed by time point (SS1, SS2, SS3) and study period in the placebo and pheromone groups

Frequency (%) represents the number of occurrences per time point per study period per group. Each behavior was evaluated for 14 days per study period in 6 cats in each group = 84 possible occurrences per each behavior during each time point.
SS: Snapshot; TP: time point; Rxn CS: Reaction during clinical scoring; Rxn petting: Reaction during petting during clinical scoring; L: lets/allows; R: resists; V: very resistant; ZZ: sleeping; S: sitting; LE: lying on side, legs extended; LA: lying down, abdomen exposed; LVU: lying down ventrally, head up and alert; LVD: lying down ventrally, head down; Ur/Def: urinating/defecating; U/F: urine/feces; Scr post: scratching post behavior
Red: items used in analyses; * significant unadjusted P value at $P < 0.05$

	PERIOD	*Equilibration*		*G0*		*K1*		*G1*		*K2*		*G2*	
	GROUP	Placebo	Pheromone	Placebo	Pheromone	Placebo	Pheromone	Placebo	Pheromone	Placebo	Pheromone	Placebo	Pheromone
TP	BEHAVIOR												
SS1	Greeting	100%	100%	99%	100%	n/a	n/a	100%	100%	n/a	n/a	99%	100%
	Vocalize	13%	8%	2%	10%	71%	81%	15%	15%	75%	71%	15%	20%
	Pawing - cage	n/a	n/a	n/a	n/a	21%	11%	n/a	n/a	11%	7%	n/a	n/a
	Pacing - cage	n/a	n/a	n/a	n/a	52%	45%	n/a	n/a	33%	36%	n/a	n/a
SS2	Rxn CS: L	43%	38%	57%	68%	56%	67%	74%	86%	61%	60%	79%	81%
	Rxn CS: R	54%	58%	40%	32%	40%	32%	25%	13%	38%	40%	21%	19%
	Rxn CS: V	4%	4%	2%	0%	4%	1%	0%	1%	1%	0%	0%	0%
	Rxn petting: L	79%	81%	87%	86%	88%	94%	92%	98%	100%	100%	100%	98%
	Rxn petting: R	21%	19%	13%	14%	12%	6%	7%	2%	0%	0%	0%	2%
	Rxn petting: V	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	Vocalize	7%	18%	4%	21%	4%	4%	5%	20%	1%	0%	5%	40%
	Purr	95%	73%	87%	69%	100%	95%	86%	79%	100%	99%	75%	80%
	Kneading	8%	3%	7%	10%	40%	20%	0%	1%	42%	40%	4%	6%
SS3	Up (4 paws)	87%	85%	61%	56%	39%	36%	56%	50%	23%	25%	48%	49%
	Not up ZZ	0%	0%	0%	0%	0%	0%	0%	0%	0%	2%	0%	2%
	Not up S	33%	40%	46%	42%	61%	69%	43%	38%	62%	60%	35%	40%
	Not up LE	10%	8%	5%	10%	12%	10%	13%	10%	10%	7%	13%	17%
	Not up LA	0%	4%	5%	1%	1%	6%	6%	5%	1%	2%	7%	2%
	Not up LVU	17%	14%	17%	29%	18%	19%	14%	19%	18%	25%	23%	14%
	Not up LVD	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	Active	85%	86%	86%	86%	86%	96%	94%	89%	98%	96%	99%	96%
	Ur/Def	4%	0%	1%	1%	1%	1%	0%	0%	1%	0%	0%	1%
	Eat/Drink	15%	8%	21%	15%	18%	17%	13%	7%	20%	5%	15%	15%
	Vocalize	0%	2%	1%	1%	27%	42%	1%	0%	8%	27%	1%	4%
	Play	62%	54%	50%	36%	5%	14%	49%	30%	10%	8%	35%	29%
	Hiss	0%	2%	0%	1%	0%	0%	0%	0%	0%	0%	0%	0%
	Climb objects	6%	12%	13%	29%	n/a	n/a	17%	30%	n/a	n/a	17%	14%
	Climb person	42%	33%	42%	40%	n/a	n/a	35%	24%	n/a	n/a	40%	33%
	Groom self	2%	6%	7%	10%	6%	12%	6%	7%	8%	10%	6%	10%
	Groom cat	0%	0%	1%	1%	n/a	n/a	1%	2%	n/a	n/a	4%	1%
	Groom person	4%	2%	4%	0%	n/a	n/a	2%	0%	n/a	n/a	0%	1%
	Pawing - cage	n/a	n/a	n/a	n/a	6%	10%	n/a	n/a	1%	1%	n/a	n/a
	* Pacing - cage	n/a	n/a	n/a	n/a	7%	1%	n/a	n/a	2%	0%	n/a	n/a

Table 7.7b: Frequency of behaviors displayed by time point (Long, SS4) and study period in the placebo and pheromone groups

Frequency (%) represents the number of occurrences per time point per study period per group. Each behavior was evaluated for 14 days per study period in 6 cats in each group = 84 possible occurrences per each behavior during each time point.
SS: Snapshot; TP: time point; Rxn CS: Reaction during clinical scoring; Rxn petting: Reaction during petting during clinical scoring; L: lets/allows; R: resists; V: very resistant; ZZ: sleeping; S: sitting; LE: lying on side, legs extended; LA: lying down, abdomen exposed; LVU: lying down ventrally, head up and alert; LVD: lying down ventrally, head down; Ur/Def: urinating/defecating; U/F: urine/feces; Scr post: scratching post behavior
Red: items used in analyses; * significant unadjusted P value at $P < 0.05$

Long	* Kennel disarray	n/a	n/a	n/a	n/a	44%	36%	n/a	n/a	48%	25%	n/a	n/a
	U/F on floor	7%	36%	7%	21%	2%	0%	29%	64%	5%	6%	21%	7%
	U/F on bowls	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	Diarrhea	0%	0%	0%	0%	4%	2%	0%	0%	2%	0%	0%	0%
	Ur/Def	6%	2%	7%	5%	17%	2%	10%	5%	13%	1%	10%	4%
	Eat/Drink	90%	75%	88%	89%	89%	81%	88%	80%	88%	51%	94%	74%
	Purr	78%	64%	73%	60%	n/a	n/a	52%	61%	n/a	n/a	51%	75%
	Excessive vocal	0%	0%	0%	0%	57%	43%	0%	0%	37%	29%	0%	0%
	Hiss	5%	5%	2%	2%	0%	0%	5%	2%	0%	0%	2%	0%
	Fight	0%	2%	0%	1%	n/a	n/a	5%	0%	n/a	n/a	0%	0%
	Hide	1%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	Play	100%	100%	100%	100%	71%	70%	100%	100%	89%	63%	100%	98%
	Kneading	5%	7%	4%	6%	7%	10%	1%	2%	0%	1%	0%	8%
	Scr post	4%	4%	8%	2%	2%	7%	8%	4%	5%	4%	4%	6%
	Climb object	68%	60%	92%	98%	n/a	n/a	96%	96%	n/a	n/a	82%	85%
	Climb person	100%	98%	99%	100%	n/a	n/a	99%	100%	n/a	n/a	100%	95%
	Groom self	63%	67%	82%	93%	92%	93%	90%	95%	93%	88%	88%	92%
	Groom cat	7%	12%	11%	18%	n/a	n/a	19%	25%	n/a	n/a	32%	23%
	Groom person	31%	24%	50%	40%	n/a	n/a	44%	35%	n/a	n/a	38%	37%
	Pawing - cage	n/a	n/a	n/a	n/a	35%	30%	n/a	n/a	14%	5%	n/a	n/a
	Pacing - cage	n/a	n/a	n/a	n/a	25%	17%	n/a	n/a	6%	2%	n/a	n/a
SS4	Up (4 paws)	73%	60%	48%	60%	18%	26%	49%	50%	25%	21%	46%	46%
	* Not up ZZ	0%	0%	0%	2%	0%	10%	0%	4%	0%	13%	1%	20%
	Not up S	40%	46%	42%	40%	51%	39%	38%	42%	49%	43%	43%	38%
	Not up LE	11%	13%	6%	14%	13%	13%	17%	12%	10%	15%	11%	14%
	Not up LA	2%	1%	2%	1%	7%	7%	6%	5%	6%	8%	10%	6%
	Not up LVU	30%	20%	32%	27%	27%	32%	18%	14%	29%	31%	18%	24%
	Not up LVD	0%	0%	0%	0%	1%	2%	0%	1%	0%	2%	0%	0%
	Active	87%	81%	74%	83%	82%	70%	93%	86%	96%	75%	96%	81%
	Ur/Def	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1%	0%
	Eat/Drink	7%	14%	10%	8%	5%	5%	8%	6%	11%	8%	7%	8%
	Vocalize	2%	5%	0%	2%	23%	17%	2%	2%	8%	8%	0%	0%
	Play	69%	55%	36%	30%	15%	21%	52%	33%	21%	17%	32%	30%
	Hiss	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	Climb object	6%	11%	17%	30%	n/a	n/a	24%	31%	n/a	n/a	19%	29%
	Climb person	35%	33%	36%	30%	n/a	n/a	35%	23%	n/a	n/a	38%	23%
	Groom self	10%	12%	12%	11%	24%	15%	10%	8%	19%	17%	13%	6%
	Groom cat	0%	0%	0%	0%	n/a	n/a	1%	0%	n/a	n/a	6%	0%
	Groom person	4%	0%	0%	1%	n/a	n/a	0%	1%	n/a	n/a	1%	2%
	Pawing - cage	n/a	n/a	n/a	n/a	5%	7%	n/a	n/a	0%	0%	n/a	n/a
	Pacing - cage	n/a	n/a	n/a	n/a	0%	2%	n/a	n/a	0%	0%	n/a	n/a

Differences were detected between the two groups during the kenneled periods (Table 7.8). More frequent kennel pacing in placebo group kittens was borderline significant during the SS3 time point (P = .05). A total stress score for each kitten was calculated by adding the 4 stress-related behavior scores within the K1 and K2 study periods; the total stress score then was compared between groups. The pheromone group had significantly lower total stress scores as compared to the placebo group in univariate analyses (Table 7.9). However, after controlling for lack of independence between kitten observations over repeated measures, the total kitten stress scores were not significantly different between groups.

Table 7.8: Frequency of potential stress-related behaviors during time points within kennel periods

Time point	Behavior	Group	K1 *	K2 *	unadjusted P value [a]	adjusted P value [b]
SS1	Kennel Pacing	Placebo	52%	33%	.74	n/a
		Pheromone	45%	36%		
	Kennel Pawing	Placebo	21%	11%	.13	.19
		Pheromone	11%	7%		
SS3	Kennel Pacing	Placebo	7%	2%	.04**	.05
		Pheromone	1%	0%		
	Kennel Pawing	Placebo	6%	1%	.60	n/a
		Pheromone	10%	1%		
LONG	Kennel disarray	Placebo	44%	48%	.005**	.44
		Pheromone	36%	25%		
	Kennel Pacing	Placebo	25%	6%	.14	.12
		Pheromone	17%	2%		
	Kennel Pawing	Placebo	35%	14%	.14	.12
		Pheromone	30%	5%		
	Kennel Meow excessive	Placebo	57%	37%	.046	.57
		Pheromone	43%	29%		
SS4	Kennel Pacing	Placebo	0%	0%	n/a	n/a
		Pheromone	2%	0%		
	Kennel Pawing	Placebo	5%	0%	.75	n/a
		Pheromone	7%	0%		

SS#, snapshot time point number; K#, kennel-housed period number.

* n=84 observations: 14 observations per 6 kittens per 2-week study period per group; n/a = not applicable due to too few observations.

[a] Fisher 2-tailed exact test comparing groups.

[b] Multivariate logistic regression, comparing groups, adjusted for lack of independence due to repeated kitten observations.
**Statistical significance: $P < .05$.

Table 7.9: Median and range for the total stress score by group and kennel study period

Study Period	K1*	K2*	K1 and K2 combined
Group	Median (range)	Median (range)	Median (range)
Placebo	36 (19-52)	19 (6-45)	55 (26-97)
Pheromone	25 (19-46)	16 (4-27)	42 (23-73)
P value[a]	.04**	.04**	.004**

K#, kennel-housed period number.
*n=84 observations: 14 observations per 6 kittens per 2-week study period per group. Median represents the 6 kittens' total stress scores in each group, per kennel period and per both kennel periods combined. Range represents the lowest (minimum) and highest (maximum) among the 6 kittens' total stress scores in each group.
[a]Unadjusted P values from Wilcoxon rank sum test are displayed for the comparison between groups.
**Statistical significance: P < .05.

Sleep was used as a correlate for calm/relaxed behavior, as it was observed and recorded at the SS4 time point, after the room gradually quieted after the previous 45 minutes of activity. At that last time point, if the kitten had a relaxed body posture and closed eyes, sleep was recorded.[18,22,23,50] During the equilibration period, none of the kittens in either group had any occurrences of sleeping (Table 7.10).

During study period G0, there were only two occurrences of sleeping in the pheromone group and no occurrences of sleeping in the placebo group. In univariate analyses, the pheromone group had significantly more sleeping events during the K1, K2, and G2 study periods (Table 7.10), when K1 and K2 were combined ($P < 0.001$) and when all four of the study periods (K1, G1, K2, and G2) potentially associated with stress were combined ($P < 0.001$). After controlling for lack of independence between kitten observations over repeated measures, the pheromone group had significantly more sleeping occurrences during the final G2 study period ($P = .006$) and when all four of the study periods potentially associated with stress were

combined (P = .002). In the combined study periods, kittens in the pheromone group were 3.9 (95% CI, 1.4 – 6.4) times more likely to sleep than kittens in the placebo group (Table 7.10).

Table 7.10: Frequency of observations of kittens sleeping during SS4 time point, by group and study period

Study Period Group	Equilibration	G0*	K1*	G1*	K2*	G2*
Placebo	0%	0%	0%	0%	0%	1%
Pheromone	0%	2%	10%	4%	13%	20%
P value[a]	n/a	.5	.007**	.25	.0007**	.0001**

G#, Group-housed period number; K#, Kennel-housed period number; SS4, snapshot-4 time point.
Bold G0: diffusers placed at the start of G0.
*n=84 observations: 14 observations per 6 kittens per 2-week study period per group; n/a=not applicable due to too few observations.
[a] Unadjusted P values from Fisher 2-tailed exact test are displayed for the comparison between groups.
**Statistical significance achieved at $P < .05$.

Other potential behavioral indicators of stress occurred infrequently in this kitten cohort. Isolated hissing events occurred between kittens during group play with novel objects on four occasions in both groups during period E, twice in both groups during period G0, and twice in the pheromone group and on 6 occasions in the placebo group during G1 and G2 combined. Similarly, fighting was observed during group play with novel objects, twice during period E and once during period G0 in the pheromone group, and it occurred on four occasions during period G1 in the placebo group. During the kenneled periods when fecal character could be ascribed to an individual kitten, diarrhea was reported on one occasion in three placebo group kittens and two pheromone group kittens and on four occasions in one placebo group kitten. When group-housed, all kittens greeted scorers at the door daily. Hiding was not observed among the kittens during the study.

7.3.3. Cortisol

All serum cortisol concentrations were within the normal range reported by the laboratory. The results for the pheromone group and the placebo group did not differ over the course of the study. However, the median cortisol results were higher at the end of the last study period (G2) when compared with the start of the first study period (K1) in the pheromone group (P = .02), placebo group (P = .05), and the two groups combined (P = .001).

7.3.4. FHV-1/GAPDH ratios

GAPDH DNA, as an indicator of viable feline cells (GAPDH-positive), was amplified from almost all swabs collected from the kittens, thus adequate sample collections were obtained. However, FHV-1 DNA was only amplified from two kittens in the placebo group and four kittens in the pheromone group after starting the first study period (K1) potentially associated with stress. Because of the low number of samples with detectable FHV-1 DNA, comparisons of numbers of positive or negative samples between groups or comparison of the FHV-1/GAPDH ratios as a measure of viral shedding magnitude between groups were considered inaccurate and thus results are not presented. Serum cortisol concentrations at the end of the study, after period G2, did not differ significantly when comparing kittens that did shed FHV-1 (n=6) to kittens that did not shed FHV-1 (n=6) (P = .09).

7.4. Discussion

After controlling for lack of independence between kitten observations over repeated measures, kittens in the room with the pheromone diffuser had increased sleeping and less sneezing when compared to kittens in the room with the placebo diffuser; these findings support

a treatment effect. Although significant differences between groups were detected in other behavioral indicators of stress, significance was lost when controlling for lack of independence. Collection of objective data to assess behavior in cats can be difficult, especially as our study necessitated development of our own behavioral metric due to the unique environment and histories of the kittens; detection of behavioral changes was further complicated because individual cats respond to stress differently.[18,20,46] In addition, reactivation of FHV-1-associated illness in response to stress may occur in varying degrees depending on the individual cat or kitten, and when reactivation does occur, time until shedding or recognition of clinical signs also varies.[2,10,15,18] In fact, even when a standardized dose of FHV-1 is used to inoculate kittens born to FHV-1 naïve queens, variations occur in the clinical signs of disease.[49,51] We believe the most important limitation to this pilot study was the inclusion of only six cats per group; this may have lessened the chances of detecting significant differences between groups.[52]

7.4.1. Inoculation model

This stress model to attempt to reactivate FHV-1 was used in another 12-cat study that evaluated a probiotic with presumed immunomodulating activity.[25] Similar to the results described in the current study, evidence for reactivation of FHV-1 varied among cats, and a treatment effect was documented.[25] In the previous study, conjunctivitis was common, and sneezing was rare, whereas in the study described here, conjunctivitis was rare, but sneezing was common. These differences between studies likely arose, in part, from the use of two different FHV-1 strains and inoculation methods. In the previous study, the field strain of FHV-1 was administered into the conjunctiva fornix, whereas the different FHV-1 strain used in the study described here was administered by nasal inoculation. If a similar study were to be performed

again in the future, splitting the FHV-1 inoculum between the nose and eyes may better mimic a natural infection, potentially resulting in clearer reactivation of disease. Alternately, exposing the naïve cats to a cat with active clinical illness due to FHV-1 so that infection is acquired more naturally might be an effective strategy for mimicking what occurs in nature.

7.4.2. Overall clinical signs

In this study, the clinical signs in both groups were mild, and the kittens were overall clinically healthy. This was a similar finding in another recent study (Chapter 4)[27] in which recrudescence only resulted in mild clinical signs of FHV-1. Because the kittens in both groups in this study were not overtly clinically ill, the blinded scorers sometimes designated some clinical attributes of the kittens with a score of "1" (Table 7.1), even though there were not necessarily other indicators of recrudescence for the kittens.

7.4.3. Objective clinical signs

The objective clinical sign of sneezing (yes/no) was reliably scored and measured by two scorers present at the same time during the observation periods. All kittens still had intermittent sneezing from the primary FHV-1 infection at the time they entered our study, and sneezing still occurred in 32.1% of the observations for the placebo group and in 25.0% of the observations for the pheromone group during G0 when the diffusers were first introduced (Figure 7.3). However, over time in the four study periods that may have been associated with stress (K1,G1,K2,G2), the pheromone group had decreased proportions of observation points with sneezing, whereas the placebo group did not (Figure 7.3). These findings could indicate reactivation or maintenance of

FHV-1 associated sneezing in the placebo group, presumably because of greater vulnerability to stress exposure.

Sneezing was easily detected within the 45-minute scoring period and was a consistent measure. This was in contrast to a previous study (Chapter 5)[43] in which sneezing could not be reliably evaluated in these same kittens due to the severe primary FHV-1 illness and the larger number of severely ill kittens to evaluate (n=12) in a shorter evaluation time period (30 minutes). Pyrexia was also objectively evaluated in the current study, but pyrexia was infrequently experienced by the kittens in this study (Tables 7.6a, 7.6b). Several of the other clinical signs were removed from analyses due to inconsistencies that potentially rendered inaccurate representations of clinical illness as well as due to potential selection bias. Nevertheless, these warrant further discussion below.

7.4.4. Discrepancies in ocular and nasal clinical signs

Ocular discharge scores increased in both groups throughout the study, but the placebo group had higher scores. Individual differences and genetic predisposition could have contributed to these results. The kittens in this study that had more frequent ocular discharge were Duchess from the pheromone group and Madame, Bambi, and Christine from the placebo group (Tables 7.6a, 7.6b). When comparing the severity and frequency of ocular clinical signs in this study to the ocular clinical signs of the same kittens in a previous study (Chapter 5)[43] both Madame and Duchess had more severe ocular clinical signs in the previous study as well (unpublished data from Chapter 5). Since Madame and Duchess were siblings, it is possible that genetic predisposition contributed to the serous discharge and crusts encountered daily in these two kittens. In contrast, in the current study, the siblings Bambi and Christine in the placebo

group had frequent ocular discharge; however, they did not have an increase in ocular clinical signs in the previous study (unpublished data from Chapter 5). Vesper in the pheromone group was another sibling of Bambi and Christine. In this study, Vesper had increased ocular discharge during the last kennel period, and she also had increased ocular clinical signs in the previous study. Because these kittens with increased ocular clinical signs were siblings, their individual predispositions toward ocular discharge and crusts might be considered as a contributing factor.

It was rare for any kittens in this study to have conjunctivitis or blepharospasm even though ocular discharge was frequently recorded (Tables 7.6a, 7.6b). Although conjunctivitis is a frequent clinical sign of FHV-1 infection, it is possible that epiphora could occur without apparent conjunctivitis or blepaharospasm; however, because both ocular accumulated crusted debris and serous ocular discharge were both considered a clinical sign (score = 1), the association with FHV-1 recrudescence could not be established.

Kittens in the pheromone group had more overall nasal discharge occurrences but fewer occurrences of sneezing and nasal congestion, while kittens in the placebo group had more overall nasal congestion and sneezing occurrences but fewer occurrences of nasal discharge (Table 7.5; Figures 7.2a, 7.2b). These discrepancies were unexpected findings that did not correspond to expected clinical respiratory signs.

One explanation for the discrepancies in nasal discharge, congestion and sneezing occurrences could be due to individual outliers. The two kittens with the most occurrences of nasal discharge in this study and the previous study (unpublished data from Chapter 5) were the siblings, Madame and Duchess (Figures 7.2a, 7.2b). Considering the increased occurrences of ocular discharge in Madame and Duchess as well, it seems possible that genetic predisposition to

FHV-1 induced damage and discharge or conformational differences could have contributed to their overrepresented oculonasal discharge.

Another possible explanation for the discrepancies could be that the kittens might have experienced osteolytic changes in the turbinate bones after prior infection and viral damage from FHV-1 in the previous study.[2,10,12,53] The FHV-1 virus has a predilection for areas of skeletal growth, including the turbinates, and damage could be permanent.[2,10,12,53] The turbinates are also one of the earliest sites of viral replication during reactivation of FHV-1.[12] This could account for these kittens having continued nasal discharge or congestion, unrelated to sneezing as a sign of clinical illness.

7.4.5. Clinical scoring system

The other likely possibility for the discrepancies in ocular and nasal clinical signs and illness could be due to the potentially problematic application and interpretation of the clinical scoring system (Table 7.1). Versions of this clinical scoring system had been successfully used in previous studies.[44,54,55] The clinical scoring system was originally adapted from a scoring method applied in USDA APHIS testing protocols (Supplemental Assay Method [SAM] for Scoring Feline Rhinotracheitis Virus in Cats following challenge and supplemental assay method for scoring feline calicivirus in cats following challenge, Center for Veterinary Biologics and National Veterinary Services Laboratories, Ames, Iowa, SAM 310, 311) that has since been withdrawn due to the need for more specific clinical outcomes.[56] The adapted system used in this study had not been tested for validity or reliability.[57] Although the moderate to severe clinical signs listed in Table 7.1 were likely accurate representations of FHV-1 illness, the mild clinical signs might not validly represent FHV-1 illness. For example, mild ocular crusts in the medial

canthi and mild nasal crusts adhered to the nasal philthrum were frequently observed and assigned a score of 1 for ocular or nasal discharge, respectively (veterinary students, personal communication). These crusts could have resulted from morning accumulation unrelated to FHV-1 recrudescence and rather related to individual conformation and normal variation. Or the crusts could have been due to ocular or nasal irritation. As the kittens' litter material consisted of wood shavings that were consistently dispersed throughout the rooms, it is not unreasonable to suspect that these shavings could have contributed to mucosal surface irritation. As another example, nasal congestion was frequently observed and assigned a score of 1 if the kitten's respiratory patterns were audible, even though that kitten might not have had concurrent sneezing or nasal discharge. It seems unlikely that this audible breathing pattern was due to FHV-1 recrudescence if there were no other indicators of inflammatory debris occluding the nasal passageways. It seems more likely that the reported congestion could have been due to individual variation and facial and nasopharyngeal conformation.[58]

As this clinical scoring system is likely to be used in future studies, its validity and reliability should be further evaluated, namely for the mild clinical scores.[57] Evaluating severity of clinical illness and range of response to treatment requires consistency in scoring and scores that accurately represent illness. Because there is no gold standard for assigning a severity rating to clinical illness in feline URI, interobserver agreement trials might be considered.[59,60] After clinical validation of the scale is performed, masked observers should then be trained and rigorously tested individually to ensure intra-and -inter-observer reliability in assessments. Retraining and retesting should also be considered during a long-term study. These implementations would decrease the possibility of assigning clinical scores to non-clinical signs.

7.4.6. Behavioral observations: Socialization

In this study, we designed our own behavioral metric. A limitation of our study was the use of kittens that were socialized, affectionate, attention-seeking of people, and habituated to the research environment, each other, and the observers. All kittens accepted and often sought gentle handling and human contact, petting, and play with each other and the observers. Therefore, the typical indicators of stress and fear such as hiding, freezing, stiffness, crouching, hissing (unrelated to guarding of novel objects), dilated pupils, and holding ears back could not be evaluated in our study because those behaviors were not displayed by these particular kittens.[18,20,21,47,48,50]

7.4.7. Pheromones

Pheromones are chemical signaling molecules, semiochemicals, that transmit specific information and that are intended to exert a specific influence on members of only that species.[42,61] Five different feline facial pheromones, F1 to F5, that are emitted through the sebaceous secretions in cats' cheeks have been identified.[42,61] Species-specific receptors for the pheromones are in the vomeronasal organ above the hard palate near the internasal septum.[61] Although they are present within the nasal cavity, activation is not affected by the respiratory air flow through the nose, and rather the molecules are thought to be sucked into the vomeronasal organ due to vasoconstriction of the epithelial wall thus creating a pumping action into the lumen of the vomeronasal organ.[42,61] Primer pheromones induce their effects by activating the neuroendocrine limbic system that modulates fears and emotions.[61] Synthetic analogues of these pheromones have been created.

Our study used diffusers containing synthetic analogues of the feline facial pheromone F3 fraction, a fraction thought to instill comfort and familiarity related to the objects or environment over which the pheromone is deposited. The F3 fraction is one of the more studied fractions. A cat deposits the F3 pheromone fraction by rubbing its face on preferred locations in its territory. It is thought this has an appeasing effect and helps the cat to indicate which objects in the environment are safe and known.[42] The F3 facial pheromone fraction has been synthesized to aid in behavioral medication of the domestic cat. The components of the F3 fraction are oleic acid, azelaic acid, pimelic acid, and palmitic acid.[42] Use of this pheromone has been shown to decrease undesirable urine marking behaviors, feline idiopathic cystitis occurrence, and stress associated with veterinary visits, hospitalization, and during transport, and it has been shown to improve general attitude and appetite in cats.[39–41,62–65]

Our study provided further evidence that the F3 pheromone fraction contributed to calm and relaxed behavior and appeared to be related to fewer kennel stress behaviors. And since the kittens in the pheromone group were more relaxed and exhibited less stress-related behaviors, those kittens also experienced less sneezing, a clinical sign of FHV-1. Our findings were in contrast to a recent study[66] performed in an animal shelter; that study found that shelter cats in the room with a pheromone diffuser did not differ with respect to incidence of URI as compared to shelter cats in a room with a placebo diffuser. However, many confounding variables were present in that study that render comparisons between that study and ours, inconsequential. For instance, shelter cats experience a much more pronounced and different level of stress as compared to the kittens in our study; furthermore, interpretation and extrapolation of that studies' findings should be interpreted cautiously. Confounding variables such as ventilation and air exchange variations, secondary bacterial infections, sizes of rooms and placements of diffusers

allowing for equivalent dispersals, markedly different histories and underlying illnesses in cats, and low incidence of URI in that study, can result in highly variable and insensitive results. In contrast, our study provided a controlled environment in which many factors were held constant while altering the one variable of pheromone versus placebo diffuser. This allowed more direct assessment of the pheromones' relationship to the behavior of the cats and their subsequent clinical signs.

7.4.8. Behavioral metric issues

Although data was collected for 28 behaviors recorded during multiple time points, only kennel pacing, kennel pawing, kennel disarray and excessive vocalization (Tables 7.7, 7.8) could be evaluated as stress indicators for statistical comparisons between groups. The other behaviors could not be evaluated for multiple reasons. Examples are provided below.

Behaviors that were recorded during the long time-point did not allow for differentiation of time-to-behavior, length of time performing behavior, nor repetition of behavior. Therefore, nearly all kittens received a score of "1" or "present" for most of the same behaviors every day, even though the behaviors might have indicated stress in some kittens. For instance, some kittens were excessively grooming during the kennel-stress period; however, the scoring rubric did not allow for capturing that information. There were also atypical occurrences during some time points. The SS1 time point during the kennel stress periods was comprised of a high proportion of stress behaviors in both groups, because at that time-point, observers were entering the room; therefore, the kittens were eager to interact with the observers and be released from their kennels. Observers were also overwhelmed by the chaotic pacing, jumping, and excessive meowing from the kittens, thus making it difficult to reliably record all of their behaviors concurrently during

SS1 when in the kennel periods. Therefore, during the kennel-stress periods, stress behaviors between groups were not significantly different at the SS1 time-point.

Other recorded behavioral observations were not meaningful in terms of a stress model. For example, the active versus passive score was recorded as "active" for a kitten if the kitten was eating, playing, grooming, sniffing, defecating, actively watching a cobweb, actively listening to the noises outside of the door, or performing any other normal, alert behavior, while "passive" was recorded if the kitten was in a relaxed state, being pet, or nearing sleep. So although "passive" indicated a calm state, the active score did not indicate a conversely stressed state. Furthermore, if a kitten were calmly and comfortably sleeping, the kitten would also receive a positive score for sleeping as well as passive, which then resulted in counting the same behavior twice. As another example, climbing object was defined as being on top of a cardboard box, on top of an observer clipboard, or on the table in the room. This did not indicate stress or calm behavior, but rather sitting location preference, a spot on which to play and from which to launch, or exploration of box or observer's scoring implements.

The small sample size also likely contributed to the failure to find differences between groups in some parameters during some time points.[52] During the long time-point in the study during the kennel stress periods, although statistical significance wasn't achieved, numerical differences for kennel pacing and pawing occurred. Total stress scores also were numerically higher in the placebo group, but statistical differences were lost when analyses were adjusted for individual kitten variations.

7.4.9. Behavior versus temperament

Presence of other behaviors were dependent on the temperament of the individual kitten and individual predilections and did not accurately indicate stress or relaxation.[46–48,67–69] For instance, during clinical assessment, those kittens that were more resistant to remaining still for the clinical exam, did not hiss nor scratch nor exhibit tense postures, but they rather varied in temperament, exhibited through restlessness and desire for increased play activity as opposed to sit-in-lap activity. As another example, some of the kittens rarely purred during group housing, yet they purred when they were removed from kennels for clinical scoring, as they became very excited and were soothed due to observer handling, contact, and release from confinement. When these kittens were returned to their kennels, they clearly exhibited stress and frustration behaviors, resisting placement back into the kennel. Therefore, recording no purr during group housing versus presence of purr during kennel-housing, as a measure of stress or relaxation, would be highly inaccurate.

Due to the small sample size, differences among groups were not observed across many measures. This could also be due in part to individual differences in stress responses among the kittens (Figure 7.4). Individuals experience wide variations in stress responses, even when exposed to the same stress.[17,69–74] Individual differences could be the result of genetic factors as well as previous confinement or other experiences.[73,75] As seen in Figure 7.4, total stress scores appeared similar among siblings in our study. Animals differ not only in their outward behavioral response to the stress, but also in their neurophysiologic and immune response.[71] Thus individual variations in our kittens' responses could have also resulted in individual variations in reactivation of FHV-1 and outward clinical signs, because coping mechanisms and coping efficiencies differ between individuals.

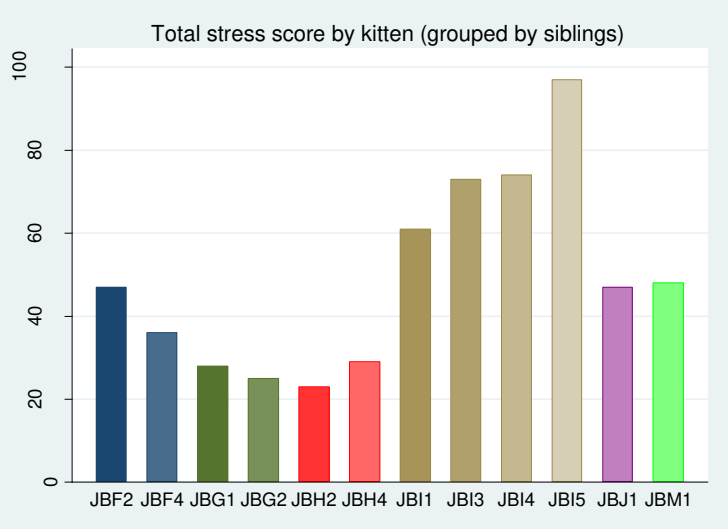

Figure 7.4: Bar graph depicting total stress score by individual kitten, grouped in colors according to siblings

7.4.10. Limited observation time

As the behavioral metric for this study was also designed with speed and ease as primary considerations in addition to attention to the clinical scoring metric, it might not have adequately evaluated overall behavior of the kittens. We created a behavioral scoring protocol that might not have captured sufficient data to assess overall stress or relaxation, as we only used one 45-minute time period and did not observe the kittens for the other 23 hours of the day. Therefore, although the kennel-stress period clearly resulted in acute stress in the kittens, reflected in the analyzed behaviors as well as increased body temperature and significantly increased heart rates during kenneled observation periods, we do not know the kittens' behaviors for the other 23 hours of the day. Future studies should consider improved behavioral and stress scoring metrics that include more frequent time-points.[16,18,22,46,76] Ideally, video recording of the kittens or observation through a one-way mirror should be considered, including multiple observational time-points throughout the day.

Considering that all kittens retained well-groomed fur, all ate and gained weight appropriately, and no relevant diarrhea nor vomiting was observed, kittens might not have experienced prolonged stress while the observers were not in the room. Rather, the kittens might have only experienced acute stress during scoring periods. Stress amount, type, frequency, and duration necessary to reactivate FHV-1 are unclear.

Upper respiratory tract disease is a frequent finding in shelters after a week in the shelter, and risk of URTD can increase to over 80% by two weeks in the shelter.[15,77] Although shelters expose cats and kittens to a multitude of other stress and infectious disease related variables, it would be thought that kittens in our study would have exhibited increased stress related behaviors and subsequently greater increases in illness after being confined for two weeks; however, that did not occur to a large extent.

7.4.11. Counteracting stress

Another consideration is that the amount and quality of contact time provided to the kittens by the observers during the group-housed period counteracted chronic stress responses that might have otherwise been manifested during the kennel-housed periods. Other studies might consider further evaluating the amount of contact time and socialization necessary to counteract a prolonged stress response. One study[73] evaluated the addition of human social interactions with owned housecats for 20 minutes or three sessions of 20 minutes and found that the cats that received the three sessions of 20 minute social interactions had lower stress scores over time.[73]

7.4.12. Unaccounted stress

On the other hand, our kittens were also exposed to unaccounted stressors. During the equilibration period, the facility did not provide environmental enrichment to the kittens other than two ping pong balls. Lack of enrichment and barren environments can cause boredom, frustration, stress and distress in cats.[17,20,36,78–82] Furthermore, blood and assay sampling days, performed at the beginning of the equilibration period and every two weeks thereafter, subjected the kittens to restraint, pain, and handling stress, during which many of the kittens hissed, scratched, and screamed. These procedures induce stress responses in cats and other animals.[16,78–80,83–85] The kittens also witnessed the other kittens in the room being restrained, heard them screaming outside the room, and witnessed them being placed back in the room, immobile due to sedation. Witnessing other conspecific distress can also be a stressful event for the animal, and this has been studied extensively in rodents.[78,79,84,85] None of these stress responses or behaviors in the kittens in this study, were recorded.

There were also other unaccounted stressors such as food deprivation in that sometimes all of the food was consumed in the group rooms, and some of the individual kittens quickly consumed all of their food in their kennels or spilled it outside of their kennels.[16] There was also inconsistency in the facility in terms of when feeding occurred and when rooms were cleaned, which are documented stressors in laboratory and shelter cats and other captive animals.[21,24,78,79] The effects that these might have had on the kittens, were not evaluated.

Furthermore, the pheromone group likely experienced additional daily stress because observers were instructed to perform observations and scoring in the same room order each day. The pheromone group was therefore always scored after the placebo group. The kittens in both groups could hear the observers in the hallway prior to the initiation of scoring. And the

observers likewise heard the kittens meowing and seeking human contact and attention while on the other side of the closed door. Yet the pheromone group always had to wait for 45-60 minutes prior to the observers entering the room and providing attention and interaction with the kittens. While group housed, vocalization ceased when the observers entered the room and the kittens were reunited with the scorers. Future studies might consider alternating room order, although that would lead to variation in kitten expectations. It is therefore suggested that rooms be better sound-proofed or that absolutely no noise occurs outside of the rooms prior to entry; however, due to the facility procedures and variation in schedule, such a scenario would not have been feasible.

7.4.13. Calm, relaxed state

In the kittens of our study, the best indicator of a relaxed state was sleeping at the end of the 45-minute observation period (SS4). We believe this behavior differed from feigning sleep, which has been used as an indicator of stress in cats, particularly shelter and kenneled cats and has been described as a defensive sleeping posture in captive felids in zoos, for example.[18,22,23,68] In contrast, the kittens recorded as sleeping in our study did not have a tense, immobile, or defensive posture, but rather a relaxed body posture with closed eyes, and the kittens responded positively if awakened (Figure 7.5).

Figure 7.5: Photo of relaxed kittens sleeping in pheromone room during SS4 time point

The sleeping behaviors in this study's kittens were only witnessed and recorded during the quietest time, the SS4 time-point. The only other occurrence of sleeping occurred during the SS3 time-point in the G3 period when one of the kittens in the pheromone group fell asleep after the clinical scoring period, as that kitten was the first kitten to be scored, and therefore at least 15 minutes had already passed before he was recorded as sleeping. The SS4 time point consisted of behaviors that the kittens were performing after the kittens were already accustomed to the observers having been in the room for 45 minutes and after the room gradually quieted subsequent to other activities. At the end of the 45-minute observation time when the observers rose to depart the room, the sleeping kittens awakened, occasionally stretched, and actively sought engagement again from the observers. Sleeping observations did not occur during the equilibration period, during times of room activity such as clinical scoring times, when observers first entered the room, or if facility noise was audible outside of the room. Sleeping also was observed with gradually increasing frequency in the room with the pheromone diffuser after the diffusers were in place (Table 7.10), and only one kitten in the placebo group had a sleeping

occurrence, and that occurred during the last study period. These findings supported our assessment of this behavior as an indicator of a relaxed state and not a fear or stress state.

7.4.14. Stress and FHV-1

The mechanisms by which stress induces reactivation of FHV-1 are unclear. Although acute stress can be adaptive, allowing the animal to cope with and avoid or lessen the impact of the stressor, persistent distress can lead to a damaging pathophysiological reaction in the animal, leading to faulty immune responses and disease susceptibility.[86,87] The persistent distress might be one of the factors that contributes to reactivation of FHV-1. In studies within shelter or other environments, stress has been found to be associated with clinical illness, while measures to decrease stress such as provision of hiding spots, vertical surfaces, olfactory stimulants, and human social contact have been found to be associated with improved health.[15,30,35,87–89]

Activation of the stress response system is dependent on individual history, the context in which the stressor occurs, and the expectation the individual has for the outcome of the event as discussed above.[17,71,75,90] Measurement of a stress or distress response can be challenging, but despite these limitations in the study question, our study provided evidence to support the association between stress, behavior, pheromones, and FHV-1.

7.4.15. Assays

GAPDH was amplified from nearly all of the oropharyngeal and conjunctival swabs suggesting that sample collections were adequate. However, FHV-1 DNA was rarely amplified. Because FHV-1 is not eliminated after inoculation, it is likely that many of the FHV-1 PCR assay results were falsely negative. Several studies have shown that FHV-1 PCR assay results

can be negative even in the presence of disease, because numbers of infectious viral particles are suppressed by the immune responses.[1,91,92] Furthermore, even during optimal sample handling in one study, FHV-1 was found in 11% of normal cats and 18% of cats with clinical signs of disease.[13]

Test detection limits, shedding in clinically normal animals, and detection of non-viable or vaccine virus, further inhibit reliable interpretation.[1,2,13,93–95] In Chapter 4,[27] neither the PCR nor titer FHV-1 values differed between the treatment and control groups, even though clinical signs differed between groups. We had similar findings in the current study. In another recent study,[96] qPCR method did not discriminate between animals recently recovered from URTD and clinically ill animals with URTD. Furthermore, pharyngeal swabs detected FHV-1 DNA in 37% of healthy cats, 33% of suspect carriers, and only 22% of the clinically ill cats.[96] Thus FHV-1 qPCR methods are also prone to false negatives.

The serum cortisol ratios in this study should be interpreted with caution. All values at all time-points were within normal range, and the variations between the kittens and between the time-points do not show an association or pattern. Cortisol can vary with diurnal rhythm and other metabolic processes, and serum cortisol is merely a measure of short-term and instantaneous cortisol that can vary within minutes; serum cortisol levels measured at one time-point and measured during stress-inducing restraint and procedures, can be unreliable.[16,83,97–100] We also did not obtain baseline equilibrium period cortisol measures due to laboratory issues. Furthermore, glucocorticoid secretion gradually attenuates during sustained stressors, and acute increases in corticosteroids could be adaptive instead of maladaptive.[16,24,83,101] Although a significant difference between samples collected before attempting to induce housing change stress and the end of study observed in both groups of kittens may suggest that stress occurred

over time, it could also be related to aging of the kittens over the course of the study or normal variation since all were within the reference range. Furthermore, cortisol levels did not correlate with clinical illness nor behavioral measures nor did levels differ between rooms. Serum cortisol was therefore not an accurate means of assessing stress in this model.[16,20,101] Future studies should consider non-invasive measures that evaluate long term cortisol in order to evaluate the chronic physiologic response to stress.[97,99,102–105]

7.5. Conclusions

We believe our data support reactivation or maintenance of sneezing associated with FHV-1 in the kittens in the placebo group when compared to the kittens in the pheromone group. This difference may result from a different response to stress associated with the study design between treatment groups. The evidence for decreased stress and increased relaxation evidenced by increased sleeping at the end of the observation period in the kittens in the pheromone group compared to the placebo group supports the hypothesis that exposure to the pheromone lessened stress and sneezing associated with FHV-1.

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CHAPTER 8: CONCLUDING REMARKS AND FUTURE DIRECTIONS

8.1. Significance of work

Feline upper respiratory infection (URI) and its pathogens have been studied for decades, and vaccines to reduce morbidity and mortality have been available since the 1970's.[1–4] Many therapies such as antibiotics for the bacterial component and antivirals for feline herpesvirus-1 (FHV-1), have helped to reduce the severity of illness and decrease mortality in cats.[5,6] Information regarding stress reduction, environmental needs of cats, and infection control have lessened illness, recurrence, and infection spread.[7–10] Despite these advances, many cats and kittens continue to suffer from URI, and many in crowded environments continue to become severely ill and either die or are euthanized.[11,12] Furthermore, many shelters are still burdened with lack of information and resources regarding risk factors for URI and how to successfully manage URI. Novel preventives, treatments, and understandings are still needed. The goals of this body of work were to explore alternative preventive and treatment models that had not been previously studied in detail and in specific settings. Our tests were performed in both the controlled research environment (Chapters 4, 5, and 7) as well as in the field shelter environments (Chapters 3 and 6).

Three chapters evaluated preventives or therapies for FHV-1 in purpose-bred, experimentally infected cats in a controlled research setting. In Chapter 4, a plant-based nutraceutical with anti-inflammatory and immune modulating components was evaluated. In Chapter 5, a new formulation of a liposomal toll-like receptor immune stimulant was assessed as both a preventive and treatment for FHV-1 in purpose-bred kittens. To determine efficacy in stress reduction and subsequent decrease in FHV-1 clinical signs, in Chapter 7, a pheromone

product was evaluated in those same purpose-bred kittens. All three of these chapters had positive findings that lend support to further exploration of immune stimulation and stress reduction to decrease severity of clinical signs of FHV-1.

Two chapters tested the addition of either a second vaccine with different targets (Chapter 3) or an intranasal immune stimulant (Chapter 6) on URI onset, prevalence, severity, and resolution at two different open admission shelters. In contrast to the FHV-1 studies in a controlled research setting, the findings in the shelter-based studies did not support those interventions in those settings. Both of these chapters, however, provided additional important information regarding the complexities of immune modulation and responses in a shelter environment with innumerable interacting variables as well as risk factors contributing to URI onset, timing, prevalence, severity, and resolution in shelter populations. The findings in the shelter studies as compared to the FHV-1 studies suggest that the real-life multifactorial nature of URI might not be as susceptible to the effects attained in a controlled setting with only one viral organism resulting in clinical illness. URI in the shelter setting is a multifactorial issue that might need to be addressed in a multifactorial multimodal manner.

8.2. Future directions

This body of work has contributed to the further understanding of URI in shelters and FHV-1 in general. Through this understanding, additional questions and research needs have been generated. Although many advances have been made in understanding URI in shelter or crowded cat populations, and some shelter veterinarians have stated that URI is not a major problem in some shelters anymore (lead shelter veterinarians, personal communication), we found that severe URI is still prevalent and a high risk factor for euthanasia even in one of the

largest, premiere shelters in the United States. It would be beneficial to continue to conduct large epidemiologic studies regarding prevalence, geographic region, and risk factors for URI in different types of shelters, catteries, and individual homes as well as trends through time. This could help inform which changes and improvements are influencing outcome in different scenarios.

To evaluate illness and treatment responses, this body of work measured clinical signs (chapters 3,4,5,6,7), behavioral observations (Chapter 7), FHV-1 antibodies in the blood (Chapter 4), and FHV-1 DNA in the blood (Chapters 4, 7), conjunctiva (Chapter 7), and oropharyngeal mucosa (Chapters 5, 6, 7). The role of innate non-specific immunity against FHV-1 or FCV induced illness might warrant further investigation. Innate immune activation has been studied in healthy cats. Natural killer cells and their surface markers have been evaluated in healthy cats in blood, lymph nodes, and the spleen.[13] A companion study[14] to Chapter 5 evaluated cellular responses to the LTC immune stimulant; inflammatory leukocytes and cytokines associated with innate immune responses were measured in healthy feline blood, nasal, and oropharyngeal samples.[14] Other studies have suggested roles of innate CMI through vaccination or other immune stimulants,[15–20] but to this author's knowledge, there are no studies that have evaluated cellular responses in vivo during feline URI illness. Determining innate CMI responses in blood or preferably in mucosal tissues in cats during URI illness, might help in the targeting of development or use of other novel preventives or treatments. Similarly, Chapter 3 found that adding an inactivated vaccine to an intake protocol, did not confer benefit but rather might have led to increased illness in those cats receiving the additional vaccination. Differences in cytokine upregulation and leukocyte composition after vaccination (24 hours, 72 hours, 7

days) thus relegating the cat more or less susceptible to infection or recrudescence might warrant further investigation.

Studying innate immune responses in cats that are clinically ill and infected with URI pathogens would necessitate procedures such as venipuncture during illness, and this is not recommended. On the other hand, oropharyngeal swabbing is a relatively innocuous procedure and can be performed with minimal restraint and a fear free approach; therefore, non-invasive methodologies to identify NK cells, leukocytes, or cytokines associated with innate immune responses might be further explored.[14,21]

Future laboratory studies might also instead utilize a more natural means of pathogen inoculation with a lower infective dose. This could be done through cat-to-cat contact instead of direct nasal inoculation. Not only would this mimic natural disease to a greater extent, but it would also prevent direct inoculation into the nares of cats, potentially avoiding the nasal mucosal sensitivity and propensity to turbinate damage.

Neither of the studies performed in shelters (Chapters 3,6) in this body of work found significantly positive effects of the preventives being trialed. And although many plausible explanations might be given for the findings, the multitude of interacting and confounding variables in shelters are the reality. Although healthy and potentially non-stressed cats in shelters might respond favorably to an immune stimulant as they might have in Chapter 6, most cats will likely not be in prime physical, mental, and emotional health when entering the shelter. Thus potentially different management and prevention strategies might be needed. Several of our findings unrelated to the preventives or treatments being trialed, might guide future work to assist more shelters. For instance, in Chapter 3, we found that as number of moves to different locations and changing kennels increased, the risk for URI increased; housing changes was the

one factor that was a significant risk factor for URI, severe URI, and time to URI in all models. Other considerations might include: how many moves is "too many" and how that might differ per cat. It is presumed that this effect was due, in part, to the stress associated with changing locations and entering new surroundings. The risk might also be associated with type of kennel or size or type of room that a cat is entering or leaving, whether each of the new kennels had hiding areas, vertical surfaces, or other modifications to decrease stress.[22–25] For instance, if a cat were leaving a small kennel to enter a large, environmentally enriching area, it is presumed that the cat would then experience less stress and then less URI. This was discussed as a possibility for the findings in Chapter 6.

Since nearly half of the cats that had URI in Chapter 3 eventually developed severe URI, further attention might be directed toward improved URI detection and identification protocols and evaluation of management methods after detection; evaluation of this shelter's treatment data and future studies could help to guide treatment decisions. Also in Chapter 3, the kittens and adolescents that were fostered had the lowest occurrence of URI, thus foster homes for ill cats might also help to reduce URI spread and hasten recovery for ill cats. Another finding in Chapter 3 was that stray cats were more likely to have URI. Although this might be related to vaccination status and poor nutrition and medical care as a stray, it might also be related to the stress encountered when entering the foreign environment and confinement amidst threatening or unfamiliar scents, sights, and sounds. These details regarding associations should be further explored, since studies have had conflicting findings regarding stray cats and risk for URI.[26–28] Very few studies have evaluated the many intertwining factors that could have a role in the development of URI and severe URI in the shelter; some of these other factors might include type of surgical procedure performed, if any, prior to URI, oral health, air flow and exchange

rates in different areas, food intake and type, visitor (potential adopters, volunteers, and staff members) interactions, noise, and staff members' cleaning routines on different days in different areas.

Finally, a key area that warrants additional research, is chronic stress experienced by cats in crowded shelters, catteries, or homes that do not meet the emotional and physical needs of the cats.[23,27,29–32] Stress has been shown to reactivate FHV-1 in cats, and chronic stress detrimentally impacts the immune system in many species.[29–31,33–37] In another study (unpublished data, bacterial URI study), shelter cats with suspected bacterial URI were transferred to the university for an antimicrobial treatment clinical trial. The university setting consisted of large rooms with many vertical and horizontal structures, toys, and human companionship for two, 30-minute periods each day. Some of the cats did not receive the antimicrobial for the first several days, and some of those cats recovered from URI without intervention. It was thought that this recovery was due to the calmer, more cat-friendly, comforting environment that reduced their stress levels thus hastening their recovery. Similarly, in Chapter 7, the kittens experienced very acute stress and displayed behaviors indicative of frustration-induced stress while in confinement in the kennels and unable to interact with the study personnel; however, we suspect that they did not experience prolonged or chronic stress as anticipated, considering their groomed fur, good appetites, and mild clinical signs. One theory is that the lack of prolonged or chronic stress might have been due, in part, to the amount and quality of contact time provided to the kittens during group housing (veterinary behaviorist, personal communication). One study[32] evaluated stress responses to social interactions with humans in the home environment and found that three sessions of 20 minute social interaction resulted in lower stress scores over time. Future studies might consider further evaluation of the amount of contact time and socialization necessary to

counteract a prolonged stress response in a shelter setting and the amount of social time necessary to concomitantly potentially improve resistance to clinical illness.

Because of this body of work and particularly findings in Chapter 7, we have started to evaluate non-invasive measures of chronic stress in cats. The new studies have involved measurement of claw and fur cortisol in cats as an indicator of chronic stress. The information collected from these studies will be important to help clarify the role of chronic stress in illnesses such as URI.

Our knowledge regarding infection control against and vaccinations for feline URI has improved dramatically over the past few decades, and this has resulted in vast improvements in feline welfare in the home, shelter, cattery, and other environments. The future therefore consists of attaining improvements in the understanding of the feline experience and emotional state. Considering the apparent association between immune and emotional health and welfare, further insight into chronic stress, identification, and prevention strategies might be the most useful and effective means of preventing or treating URI in the home, shelter, research, or other environments.

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