Journal of Feline Medicine and Surgery (2010) **12**, 129–137
doi:10.1016/j.jfms.2009.08.006

ELSEVIER

jfms

A dual-strain feline calicivirus vaccine stimulates broader cross-neutralization antibodies than a single-strain vaccine and lessens clinical signs in vaccinated cats when challenged with a homologous feline calicivirus strain associated with virulent systemic disease

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Feline calicivirus (FCV) causes an array of clinical disease in cats. Traditionally this disease has been associated with respiratory disease, limping, or chronic stomatitis. Within the last 10 years, virulent systemic feline calicivirus (VS-FCV) has been recognized which causes additional clinical signs and has a higher fatality rate. A dual-strain FCV vaccine containing a strain of FCV associated with traditional respiratory disease and a VS-FCV strain stimulates serum cross-neutralization antibodies when tested against field strains from Europe and VS-FCV strains from USA. Following challenge with a homologous VS-FCV strain, vaccinated cats had significantly reduced clinical signs.

Date accepted: 18 August 2009

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Feline calicivirus (FCV) has long been recognized as a cause of feline upper respiratory tract disease and oral ulceration. It generally causes chronic infections and intermittent acute respiratory disease and has been associated with chronic stomatitis. Fatalities are rare except in kittens.[1] Two isolates causing lameness were identified in 1983.[2] A more severe clinical syndrome, an acute virulent systemic disease (VSD), was described in 2000 as being caused by a virulent systemic feline calicivirus (VS-FCV) strain called FCV-Ari.[3] Clinical signs reported in the initial VSD outbreak in California and subsequent outbreaks in the UK and US include jaundice, high fever, hair loss, skin ulceration and necrosis, pyoderma, vasculitis, emesis of blood-tinged vomitus, limb and facial subcutaneous edema, and a high incidence of death – up to 67% in some outbreaks.[4–7]

Although there is appreciable evidence of genomic and antigenic variability within FCV viruses, all isolates of FCV are generally considered to belong to a single serotype. This variability appears random with little support for either antigenic or genomic sub-classifications or clusters. In fact, classification of FCV as belonging to different 'strains' is somewhat arbitrary in that isolates are classified as belonging to a different strain only if there is >20% difference between the nucleotide sequences in the capsid region.[8] Even though there is sufficient antigenic overlap among isolates to aggregate all FCV strains within a single serotype, the genomic mutability of this virus is evident as FCV evolves not only within groups of cats, but also within individual cats as well.[8–11] No clear genomic virulence markers have been identified.[12,13]

Viral mutations associated with certain clinical signs such as lameness or VSD appear to arise independently from existing FCVs and with little interrelationship to each other.[14] In VSD cases, the virus is better able to gain access to cellular compartments (examples include skin, liver, kidney, or pancreas) that are normally not associated with traditional FCV infection or disease.[15] It is believed that genetic mutation(s) in the capsid gene may be responsible for this change in cellular tropism.[5] On the other hand, phylogenetic analyses and alignments of the capsid and proteinase–polymerase sequences did not reveal any particular change that correlated with virulence, thus confounding efforts to find a unique VS-FCV clade that could be used to develop a genetic or in vitro diagnostic test.[14]

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The great antigenic variability of FCV raises questions on efficacy of current FCV vaccines against field strains. Using more than one strain in an FCV vaccine to increase cross-reactivity is not a new idea, having been suggested by Povey as early as 1979[16] and more recently by Dawson et al in 1993[17] among others. Recently a dual-strain FCV vaccine was introduced in Europe based on a combination of two traditional FCV isolates. These isolates were not VS-FCV, but were selected to increase cross-protection based on capsid protein sequence and antigenic profile.[18] Cats vaccinated with the dual-isolate FCV vaccine were protected from challenge by two antigenically distant FCV isolates.[19]

To further investigate the potential advantages of a dual-strain FCV vaccine, a vaccine was developed using an FCV strain known to cause VSD signs in cats in addition to the traditional FCV strain already used as a commercially available FCV vaccine (Fel-O-Vax Lv-K IV; Fort Dodge Animal Health). The VS-FCV isolate was obtained from an investigator of a clinical outbreak of VSD.[3] This isolate was purified through three rounds of limiting dilution cloning, and the resulting VS-FCV strain (designated FCV-DD1) was added to an existing FCV vaccine. A multivalent vaccine containing both VS-FCV and traditional FCV was prepared and tested to obtain USDA licensure. A controlled vaccination challenge study was conducted to demonstrate protection against VS-FCV infection. Additionally, to determine if this dual-strain vaccine conferred improved cross-neutralization against both traditional FCV and VS-FCV isolates sera from cats immunized with a dual-strain FCV/VS-FCV vaccine was compared to that of cats immunized with a vaccine containing only a single strain of FCV.

Materials and methods

Field isolates of European origin

Thirty-seven FCV isolates were collected from European cats ranging in age from 26 days to 14 years. The majority of samples were collected from shelters with large feline populations. Most of the cats (27/37) sampled were showing clinical signs consistent with FCV infection while the remaining cats were clinically healthy at the time of sample collection. Previous vaccination history was unknown for the vast majority of cats. Samples were collected by oropharyngeal, nasal, or ocular swabs, which were sent to Langford Veterinary Diagnostics at the University of Bristol where FCV was initially isolated. Positive FCV samples were sent to Fort Dodge Animal Health for further characterization. The samples were purified by one round of limiting dilution cloning.

To assess the virulence of purified virus, virus stocks from the 20 isolates originally obtained from cats with obvious clinical signs were used to challenge specific pathogen-free (SPF) cats. For each isolate, a group of two cats were inoculated intranasally with 1 ml of 10^6–10^7 $TCID_{50}$/ml per cat and monitored daily for 28 days for clinical signs of disease. The 20 FCV isolates were categorized according to the clinical signs observed during a 28-day observation period. A case definition of VSD was modified from previous work.[6] Cats were determined to have experienced VSD if the following were observed: moderate to marked facial, limb or pinna edema not explained by other reasons and associated with one or more of the following signs fever, oral ulcers, lesions on face or limbs, icterus, upper respiratory disease or limping; or moderate to marked pyoderma or alopecia not explained by other causes and associated with one or more of the following fever, oral ulcers, lesions on face or limbs, icterus, upper respiratory disease or limping; or fever followed by sudden death confirmed by necropsy finding consistent with VS-FCV infection; or fever over 105.5°F and one or more of the following signs oral ulcers, sores on face or limbs, icterus, upper respiratory disease or limping. Of the 20 isolates three caused subclinical disease (mild fever), 11 caused signs consistent with traditional respiratory FCV infection, three caused limping in addition to signs consistent with traditional FCV, and three caused signs consistent with VSD (FCV-6147, FCV-6580 and FCV-9292).

VS-FCV isolates of US origin

A total of six VS-FCV isolates from the United States known to cause FCV-associated VSD were obtained from various sources. FCV-Ari was originally isolated from a Northern California cat that died of VS-FCV disease in 1998.[3] FCV-UTCVM-H1 was isolated from a Tennessee cat that died of VS-FCV disease in 1999.[20] FCV-33585, FCV-88287 and FCV-94580 were isolated from kittens that died of VS-FCV disease outbreaks in Massachusetts, Pennsylvania and New York in 2001. FCV-Kaos was isolated from kittens that died of a VS-FCV disease outbreak in Southern California in 2002.[6]

Preparation of antiserum

Two antiserum pools were generated using SPF cats. One serum pool was generated for Fel-O-Vax PCT (Fort Dodge Animal Health, killed FPV, FCV and FHV-1 vaccine) containing a single traditional FCV strain and the other pool was generated for Fel-O-Vax PCT + CaliciVax containing the traditional FCV strain and FCV-DD1 strain. SPF cats were vaccinated with Fel-O-Vax PCT or Fel-O-Vax PCT + CaliciVax twice, 3 weeks apart, by the subcutaneous route. Serum was collected from each vaccinated cat at 7 and 14 or 28 days following the second vaccination. Serum samples collected from 11 (Fel-O-Vax PCT) or 40 (Fel-O-Vax PCT + CaliciVax) vaccinated cats were pooled to generate sufficient antisera for virus neutralization assays. All serum samples and pools were stored at −80°C until testing.

Virus neutralization assays

Virus neutralization assays for FCV including European FCV isolates and US VS-FCV strains were performed against serum pools or samples using 96-well plates. Briefly, serial twofold dilutions of heat-inactivated sera were mixed with equal volumes of viral suspensions (50−400 $TCID_{50}$). The serum-virus mixture was incubated at 37°C with 5% CO_2 for 1 h and then inoculated into cell suspensions of Crandell Rees feline kidney (CRFK) cells in 96-well plates. The plates were incubated in a humidified incubator with 5% CO_2 at 37°C for 3−4 days and viral growth was determined by microscopic examination for cytopathic effect characteristic of FCV infection. The FCV-specific titers were calculated as the serum dilution causing 50% inhibition of virus replication.[21] Antibody titers for feline herpesvirus type 1 (FHV-1) were determined using the same method as for FCV. Antibody titers for feline panleukopenia virus (FPV) were determined using the same method except that 50−400 $TCID_{50}$ of FPV was used in the serum-virus mixture and endpoints were read by detection of virus infection in CRFK cells by immunofluorescence using FPV-specific antiserum conjugated to a fluorochrome.

Feline leukemia virus (FeLV) and FCP serological assays

Serum antibodies to FeLV proteins were measured using a modified enzyme-linked immunosorbent assay (ELISA).[22] Serum antibodies to *Chlamydophila felis* (FCP) were measured using the microfluorescence test. Briefly, FCP infected cells, which were fixed on plates, were reacted with varying dilutions of serum starting from 1:20. The plates were stained using indirect immunofluorescence using goat anti-cat antibody conjugated to a fluorochrome and titers were calculated as the highest serum dilution that specifically binds to the *Chlamydophila* species infected cells. The antibody titers were calculated as the reciprocal of the highest serum dilution which specifically binds to the *Chlamydia* species infected cells.

VS-FCV challenge

A challenge study was conducted using a double blind, randomized, parallel group design. There were three groups of 8-week-old, American shorthair male and female kittens in the study with 20 kittens in both the principal group and the active control group, and nine kittens in the challenge control group. All study kittens were seronegative to FCV, FPV, FHV-1, FCP, and FeLV, as demonstrated by a lack of virus neutralizing antibody titers to FHV-1, FPV and FCV-255 (<2) and a lack of binding antibody titers to FeLV (<200) and FCP (<20), at the start of the study. Study animals where gang housed in an environmentally controlled facility.

The challenge control group kittens were administered no vaccine. Those in the active control group were administered a killed FPV, FCV, FHV-1, FCP, FeLV vaccine (Fel-O-Vax Lv-K IV; Fort Dodge Animal Health), which contained a traditional FCV isolate only without a VS-FCV isolate. Kittens in the principal group were administered a killed FPV, FCV, FHV-1, FCP, FeLV vaccine with FCV-DD1 added (Fel-O-Vax Lv-K IV + CaliciVax; Fort Dodge Animal Health), thus containing both traditional FCV and VS-FCV isolates. A single dose of vaccine was administered subcutaneously twice to each kitten 3 weeks apart. Serum samples were taken to determine antibody titers on the day of the first vaccination (0DPV1), the day of the second vaccination (0DPV2), 7 days post second vaccination (7DPV2), and 14 days after the second vaccination (14DPV2). Two weeks after the second vaccination, kittens in the principal group, and challenge control group were challenged with an isolate of VS-FCV homologous to the VS-FCV isolate in the dual-strain FCV vaccine. Each cat was challenged with $10^{5.1}$ $TCID_{50}$ of FCV-DD1 oro-nasally to stimulate the natural route of exposure. Post challenge all kittens were observed for clinical signs daily for 14 days. The signs monitored were rectal temperature, facial and limb edema, ulceration of skin, pyoderma, crusting and focal hair loss, dyspnea, nasal discharge, ocular discharge, limping, weight, and death. A case definition was used to determine if cats experienced VSD. The VSD case definition was described previously in this paper.

Results

Cross-neutralization

Antisera from cats vaccinated with Fel-O-Vax PCT (single-strain antisera) neutralized 10 of 43 (23%) FCV isolates at a serum dilution of 1:2 or greater, while antisera from cats vaccinated with Fel-O-Vax PCT + CalicicVax (dual-strain antisera) neutralized 30/43 (70%) FCV isolates tested, as shown in Table 1. Single-strain antisera neutralized only 1/9 (11%) of VS-FCV isolates. The VS-FCV isolate neutralized by single-strain antisera was from the US. None of the VS-FCV isolates from Europe were neutralized by the single-strain antisera. In contrast, the dual-strain antisera neutralized 6/9 (67%) of VS-FCV isolates including 2/3 (67%) from Europe and 4/6 (67%) from the US. The mean titer for the isolates demonstrating cross-neutralization from the single-strain FCV vaccine group was 1:6, while the mean cross-neutralization titer for the dual-strain FCV/VS-FCV vaccine group was 1:55.

VS-FCV challenge

Equivalent serological responses to FPV, FCV, FHV-1, FCP and FeLV were observed for the principal group and the active control group (data not shown) indicating the addition of the VS-FCV antigen, FCV-DD1, resulted in a product that was not serologically inferior to the vaccine without FCV-DD1.

Table 1. Serum neutralizing antibody titers to various FCV isolates.

Virus isolate	Origin of FCV isolate	Induce VSD	Antiserum to Fel-O-Vax PCT	Antiserum to Fel-O-Vax PCT + Calicivax
FCV-Ari	Northern California, US	Yes	<2	>256
FCV-UTCVM-H1	Tennessee, US	Yes	<2	6
FCV-33585	New England, US	Yes	<2	<2
FCV-88287	New England, US	Yes	<2	20
FCV-94580	New England, US	Yes	9	6
FCV-Kaos	Southern California, US	Yes	<2	<2
FCV-6123	West Sussex, UK		<2	<2
FCV-6125	West Sussex, UK		<2	<2
FCV-6147	West Sussex, UK	Yes	<2	<2
FCV-6148	West Sussex, UK		<2	6
FCV-6150	West Sussex, UK		<2	20
FCV-6197	Madrid, Spain		<2	2
FCV-6198	Madrid, Spain		3	3
FCV-6199	Madrid, Spain		<2	<2
FCV-6205	Madrid, Spain		<2	69
FCV-6207	Madrid, Spain		<2	3
FCV-6209	Madrid, Spain		2	3
FCV-6306	Norfolk, UK		9	4
FCV-6307	Norfolk, UK		3	2
FCV-6404	Meaux, France		<2	7
FCV-6405	Meaux, France		13	208
FCV-6406	Meaux, France		7	17
FCV-6479	Parma, Italy		2	<2
FCV-6507	West Sussex, UK		<2	69
FCV-6510	West Sussex, UK		<2	<2
FCV-6511	West Sussex, UK		<2	5
FCV-6512	West Sussex, UK		<2	3
FCV-6513	West Sussex, UK		<2	6
FCV-6514	West Sussex, UK		<2	23
FCV-6515	West Sussex, UK		<2	3
FCV-6580	Normandy, France	Yes	<2	45
FCV-6582	Normandy, France		5	>256
FCV-6583	Normandy, France		<2	>256
FCV-6738	Noirot, France		<2	<2
FCV-9047	Cornwall, UK		<2	11
FCV-9065	Perpignan, France		<2	<2
FCV-9066	Perpignan, France		<2	<2
FCV-9067	Perpignan, France		<2	<2
FCV-9068	Perpignan, France		<2	>256
FCV-9069	Perpignan, France		<2	<2
FCV-9292	Cornwall, UK	Yes	<2	39
FCV-9561	Sur Loire, France		<2	26
FCV-9694	Norfolk, UK		6	39

FCV-DD1 serum neutralizing antibody (SN Ab) titers were evident in the principal group for 18/20 kittens 3 weeks after the initial vaccination. By 7DPV2 all kittens in this group had seroconverted and FCV-DD1 SN Ab titers were >256 for 18/20 kittens. By 14DPV2 all kittens in the group had FCV-DD1 SN Ab titers >256. As expected, FCV-DD1 SN Ab titers of kittens in both the active control group and the challenge control group remained negative (<2) during that time, which confirms the immunity of the vaccinates was induced by vaccination, not due to environmental FCV.

The clinical signs observed post challenge are described in Tables 2 and 3. All nine of the unvaccinated controls exhibited VSD with signs that included high fever ($T \geq 105.5°F$), facial, limb, or pinna edema, pyoderma, and alopecia. Ulcers, either oral or dermal, were noted on multiple days in 8/9

Table 2. Challenge control group; signs observed post challenge.

Cat ID		03 ARO1	03 ARO2	03 ARP2	03 ARP4	03 ARP5	03 ARR2	03 ARV4	03 IPU2	03 IPV3
0 DCP										
1 DCP	Skin									
	Resp/ocular									
	General									
	Fever			■						
2 DCP	Skin			■						
	Resp/ocular									
	General			■	■					
	Fever			■	■	■	■		■	
3 DCP	Skin			■				■		
	Resp/ocular									
	General			■	■				■	
	Fever		■	■	■	■	■	■	■	■
4 DCP	Skin									
	Resp/ocular		■							
	General	■	■	■		■	■	■	■	
	Fever	■	■	■	■	■	■	■	■	■
5 DCP	Skin	■	■	■			■	■		■
	Resp/ocular		■					■		
	General	■	■	■	■	■	■	■	■	■
	Fever	■	■	■	■	■	■	■	■	■
6 DCP	Skin	■	■	■	■	■	■	■	■	■
	Resp/ocular									■
	General		■	■	■	■		■	■	■
	Fever	■				■				■
7 DCP	Skin	■	■	■	■	■	■	■	■	■
	Resp/ocular		■	■				■		
	General		■	■		■		■	■	
	Fever	■	■		■	■	■	■		
8 DCP	Skin	■	■	■	■	■	■	■	■	
	Resp/ocular							■		■
	General							■	■	
	Fever				■	■	■			
9 DCP	Skin	■	■	■	■	■		DEAD	■	
	Resp/ocular		■	■	■					
	General	■	■	■					■	
	Fever	■			■	■	■			
10 DCP	Skin	■	■	■	■	■	■		■	
	Resp/ocular		■	■						
	General	■	■	■	■				■	
	Fever			■	■					
11 DCP	Skin	■	■	■		■	■		■	
	Resp/ocular		■							
	General		■							
	Fever			■	■					
12 DCP	Skin	■	■	■		■	■			
	Resp/ocular		■							
	General	■	■	■						
	Fever			■						
13 DCP	Skin	■	■			■	■			
	Resp/ocular		■							
	General	■	■							
	Fever									
14 DCP	Skin	■	■			■	■			
	Resp/ocular		■							
	General	■	■							
	Fever									

■ Indicates cat observed with one or more signs listed. *Skin observations*: alopecia, edema, pyoderma, ulcer. *Respiratory/ocular observations*: dyspnea, nasal discharge, ocular discharge. *General observations*: anorexia, dehydration, depression/lethargy, hyperesthesia, limping, salivating. *Fever*: temperature >103° F and >2° F above base line or >105.5° F.

Table 3. Vaccinates; signs observed post challenge.

Cat ID		03 ARO5	03 ARO6	03 ARQ2	03 ARQ3	03 ARQ5	03 ARR3	03 ARS1	03 ARV1	03 ARV5	03 ARW2	03 IPO4	03 IPQ3	03 IPQ4	03 IPS3	03 IPU5	03 IPU6	03 IPV1	03 IPV4	03 IPV6	03 IPW1
0 DCP																					
1 DCP	Skin																				
	Resp/ocular																				
	General																				
	Fever																			■	
2 DCP	Skin																				
	Resp/ocular																				
	General								■			■			■				■		
	Fever								■			■	■		■						■
3 DCP	Skin																				
	Resp/ocular																				
	General																				
	Fever																				
4 DCP	Skin																				
	Resp/ocular																				
	General		■																		
	Fever																				
5 DCP	Skin																				
	Resp/ocular																				
	General																				
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6 DCP	Skin																				
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9 DCP	Skin												
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	Fever												
10 DCP	Skin									■			
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	General												
	Fever												
14 DCP	Skin												
	Resp/ocular												
	General												
	Fever												

■ Indicates cat observed with one or more signs listed. *Skin observations*: alopecia, edema, pyoderma, ulcer. *Respiratory/ocular observations*: dyspnea, nasal discharge, ocular discharge. *General observations*: anorexia, dehydration, depression/lethargy, hyperesthesia, limping, salivating. *Fever*: temperature >103° F and >2° F above base line or >105.5° F.

controls, and one control kitten found dead on day 9 post challenge. Upon necropsy this cat was found to have hemorrhagic, edematous and congested lungs. As this FCV challenge resulted in disease in the challenge control group the in vitro passages previously described did not significantly alter the challenge virus' pathogenicity. Non-vaccinates were considered to have experienced VSD disease for a mean of 10.5 days (95% CI 8.9,12.32). In contrast, all 20 vaccinates were protected from developing VSD. The vaccinates had minimal clinical signs that lasted only 1 day. All vaccinates returned to normal within 24 h without medical intervention. Based on the presence of VSD in all non-vaccinated cats and no observation of VSD in the dual strain vaccinated cats the effectiveness, or the prevented fraction, of the vaccine was 100% (95%CI 83.2%, 100%).

Discussion

These studies investigate an area that has been identified as key for future FCV vaccine development, broadening the cross-reactivity of vaccine immunity to field viruses.[8] While the first clinical report of VS-FCV appeared in 2000, it is likely these events have occurred in cats previously but they were not adequately observed to justify publication. Since 2000 many VS-FCV isolates have been described. They appear to have arisen independently based on genetic analysis as these isolates vary from each other and from traditional FCV.[14] The VS-FCV challenge study demonstrated this dual-strain FCV vaccine can provide vaccine mediated protection against a FCV challenge that causes significant VSD disease in non-vaccinates. While this demonstration of challenge protection is valuable information, the direct clinical application of this information cannot be precisely known as this laboratory challenge may or may not reflect 'typical' challenges experienced by cats and field VS-FCV isolates have different characteristics.

Protection from FCV disease is generally considered to be mediated mainly by humoral immunity (virus neutralizing antibodies). Evidence indicates it is reasonable to estimate clinical protection from disease based on SN antibody titers.[9,17,23–25] Cross-neutralization is classically used to predict efficacy of FCV vaccines against heterologous strains.[17] The higher mean SN titer for the dual-strain FCV/VS-FCV vaccine group indicates there was generally higher levels of neutralizing antibody in this antisera as compared to the antisera from the single-strain vaccine group. The cross-neutralization finding clearly demonstrates that dual-strain FCV/VS-FCV vaccine containing a traditional FCV strain and a VS-FCV strain is able to induce production of antibodies capable of neutralizing a wider spectrum of both traditional FCV isolates and VS-FCV isolates as compared to a single-strain FCV vaccine. This ability to cross-neutralize a greater number of FCV isolates may equate to improved vaccine efficacy in naturally exposed cats.

The low percentage of isolates neutralized by the single-strain vaccine is surprising given previous experience with this single-strain vaccine. In an unpublished study,[26] antiserum from an equivalent single-strain vaccine neutralized all but one isolate from a similar sized pool of FCV isolates. These isolates were collected by sampling a sequential set of cats presented to private practices in the UK, thus the vast majority of these cats were healthy at the time of sample collection. A similar pattern of high percentage of neutralization was seen in a recently published report from the UK,[23] in which antisera made from a similar isolate neutralized 75% of a pool of FCV isolates. In this study, veterinary practice staff swabbed 20 cats sequentially presented to their facility to collect the isolates. These cats appear to be clinically similar to the cats in the unpublished study in that they were predominantly healthy. The findings of these two studies, where antisera from a similar single-strain vaccine neutralized a high percentage of general cat population isolates, are in contrast to a study conducted in Japan[24] and to our study. In the Japanese study, sick cats were sampled and antisera from a similar single antigen preparation neutralized only about 30% of these isolates, which is similar to the results of our study (23%). Therefore, we conclude the pattern of low neutralization seen in this study with our single antigen vaccine may be related to sampling sick cats and collecting isolates with a higher pathogenicity. Clearly, neutralization patterns should be evaluated in terms of the disease status of the cats sampled for FCV.

The VS-FCV challenge study demonstrated the dual-strain vaccine protected cats from signs of VSD when challenged with a homologous VS-FCV strain. Certainly more *in vivo* studies are needed to evaluate the competence of immune system performance in cats after immunization with a dual-strain FCV/VS-FCV vaccine and to determine the result of challenge to various FCV strains, but improved cross-neutralization is suggestive of better cross-protection against a wider spectrum of both VS-FCV isolates and traditional FCV isolates than a single-strain FCV vaccines.

Acknowledgements

The authors wish to thank Drs Niels Pedersen and Kate Hurley at the University of California for providing FCV-Ari and FCV-Kaos strains, Dr Melissa Kennedy at the University of Tennessee for providing FCV-UTCVM-H1 strain, and Dr Ed Dubovi at the Cornell University for providing FCV-33585, FCV-88287 and FCV-94580 strains. We also thank Dr Andrew Little for collecting swab samples and Dr Tim Gruffydd-Jones at the University of Bristol for initial FCV isolation. All the animal experiments described in this paper were conducted in compliance with

the Animal Welfare Act Regulation (9CFR Parts 1, 2 and 3), and approved by the Institutional Animal Care and Use Committee of Fort Dodge Animal Health. Fort Dodge Animal Health, Overland Park, Kansas 66210, provided funding for this study. We thank Tad B Coles DVM, of Overland Park, Kansas for technical assistance with writing and editing this manuscript.

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