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Prevalence of feline calicivirus and the distribution of serum neutralizing antibody against isolate strains in cats of Hangzhou, China

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ABSTRACT

Background: Feline calicivirus (FCV) is a common pathogen of felids, and FCV vaccination is regularly practiced. The genetic variability and antigenic diversity of FCV hinder the effective control and prevention of infection by vaccination. Improved knowledge of the epidemiological characteristics of FCV should assist in the development of more effective vaccines.
Objectives: This study aims to determine the prevalence of FCV in a population of cats with FCV-suspected clinical signs in Hangzhou and to demonstrate the antigenic and genetic relationships between vaccine status and representative isolated FCV strains.
Methods: Cats (n = 516) from Hangzhou were investigated between 2018 and 2020. The association between risk factors and FCV infection was assessed. Phylogenetic analyses based on a capsid coding sequence were performed to identify the genetic relationships between strains. *In vitro* virus neutralization tests were used to assess antibody levels against isolated FCV strains in client-owned cats.
Results: The FCV-positive rate of the examined cats was 43.0%. Risk factors significantly associated with FCV infection were vaccination status and oral symptoms. Phylogenetic analysis revealed a radial phylogeny with no evidence of temporal or countrywide clusters. There was a significant difference in the distribution of serum antibody titers between vaccinated and unvaccinated cats.
Conclusions: This study revealed a high prevalence and genetic diversity of FCV in Hangzhou. The results indicate that the efficacy of FCV vaccination is unsatisfactory. More comprehensive and refined vaccination protocols are an urgent and unmet need.

Keywords: Feline calicivirus; vaccine; risk factors; phylogeny; cross-neutralization

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Conflict of Interest
The authors declare no conflicts of interest.

Author Contributions
Conceptualization: Yang Y, Shi F; Data curation: Zheng M; Formal analysis: Zheng M, Li Z; Funding acquisition: Yang Y, Shi F; Investigation: Li Z; Methodology: Zheng M, Fu X, Lv Q; Project administration: Shi F; Resources: Yang Y; Software: Zheng M; Supervision: Lv Q; Validation: Fu X; Visualization: Fu X; Writing - original draft: Zheng M, Li Z; Writing - review & editing: Yang Y, Shi F.

INTRODUCTION

Feline calicivirus (FCV) is a common infectious pathogen that causes upper respiratory tract disease in felids. FCV infection is often manifested as fever, stomatitis, gingivitis, and upper respiratory signs (such as rhinitis, sneezing, and conjunctivitis), either alone or in any combination. FCV is a single-stranded positive-sense RNA virus characterized by genetic variability and antigenic diversity [1]. Despite these features, FCV exists as a single serotype [2]. Its genome is approximately 7.7 kb in length and comprises 3 open reading frames (ORFs). ORF2 encodes the capsid protein VP1 and contains both variable and conserved sequences. Thus, comparative analysis of the ORF2 sequence is commonly used in evaluating phylogenetic relationships among FCV isolates [3-5].

Prophylactic vaccination is aimed at protecting cats against FCV infection. The commercially available vaccine in China is based on a single strain, FCV-255. Such vaccination lessens the severity of the clinical signs of a FCV infection rather than blocking the infection [6]. However, controlling and preventing FCV infection through vaccination yields unsatisfactory results. Vaccinated cats might become infected with field strains of the virus [7], and survey results often reveal vaccinated cats infected with FCV [8]. The cross-reactivity of FCV vaccines with FCV isolate strains is controversial, as has been discussed in recent years [9]. Much of the research focus has been on the F9 strain [4,10,11], while the FCV-255 strain has been relatively less mentioned [11-13].

This study aimed to investigate the frequency of FCV infection in cats and evaluate the potential risk factors. On that basis, the genetic relationships between vaccine strains (mainly FCV-255) and a representative panel of FCV isolates were demonstrated. Investigation of the current levels of neutralizing antibodies in cats will contribute to updating advice on vaccination strategies.

MATERIALS AND METHODS

Sample collection

Oropharyngeal, nasal, and conjunctival swab samples were collected from clinically diseased cats (clinical symptoms compatible with FCV infection) attending veterinary practices in Hangzhou from 2018 to 2020. In addition, a questionnaire was completed for each enrolled cat to record relevant demographic data, including the date of visiting, sex, age, clinical signs, and vaccination history. A DirectPrep kit for FCV (Coyote Bioscience, China) was used to confirm FCV presence. Informed consent was obtained from the owners before their cats were sampled.

Virus isolation

Each swab sample was diluted 1:100 using Dulbecco's Modified Eagle Medium and then centrifuged at 8,000 × *g* for 10 min at 4°C. The supernatant was filtered and inoculated onto a monolayer of Crandell-Reese feline kidney (CRFK) cells at 37°C under 5% CO_2. The cell cultures were incubated for 3-5 days and monitored daily for signs of the typical cytopathic effects (CPEs) of FCV. All samples were passaged at least twice before being considered negative. Positive cell cultures were harvested by performing 3 cycles of freezing and thawing. Supernatants were stored at -80°C for further analysis [14,15].

Sequencing of viral strains

In order to investigate the diversity and relationships among the isolates, total RNAs were extracted from cell culture supernatants of 80 representative FCV-positive samples using RNA-

easy Isolation Reagent (Vazyme Biotech, China) and transcribed into complementary DNA according to the manufacturer's instructions. The ORF2 sequences of 2007 or 2010 base pairs in length were amplified through polymerase chain reaction (PCR; 2×Phanta Max Master Mix, Vazyme Biotech) of each FCV isolate. The primers used were: 5′-TTGAGCATGTGCTCAACCTG-3′ (forward) and 5′-ATTTTGRTTTGTGTATGAGTAAGGG-3′ (reverse). The PCR products were verified and submitted for sequencing. The sequencing results were aligned against the reference sequence utilizing BLAST and Lasergene MegAlign software. Sequence alignments and phylogenetic analyses were performed using MEGA X software.

Viral neutralization test

Blood samples were collected from cats with a known vaccination history. Serum samples were inactivated at 56°C for 30 min and then stored at −20°C until use. Neutralization assays were performed using a constant-virus varying serum method in 96-well plates using 4 wells per serum dilution. Serial 2 fold dilutions of serum were mixed with 200 $TCID_{50}$ of the virus in equal volumes, incubated at 37°C for 1 h, and added to the CRFK cells that had been plated on the 96-well plates. The final serum dilution ranged from 1:10 to 1:5,120. Plates were observed for CPEs after 48 and 72 h. Antibody titers were determined by assessing the highest dilution of serum that resulted in a 50% inhibition of CPEs, as calculated by the Reed-Meunch method.

Statistical analysis

Data from questionnaires were used to evaluate the association between risk factors and FCV infection. Potential risk factors included age (kitten: 0–0.5 yr, juvenile: 0.5–1 yr, adult: 1–7 yr, senior: > 7 yr); sex (male or female); breed (British shorthair, Chinese domestic cats, Ragdoll, American shorthair, Garfield, others); vaccination history (‘proper’ vaccination or not); season (spring: March to May, summer: June to August, autumn: September to November, winter: December to February). Proper vaccination refers to a cat having a regular vaccination (feline rhinotracheitis-calici-panleukopenia vaccine, killed virus). Relationships between FCV infection and clinical signs, including sneeze, nasal discharge, ocular discharge, oral symptoms, conjunctivitis, and cough, were also assessed.

Statistical analysis was performed using SPSS software. To select variables for inclusion in the multivariate analysis, univariate analysis was applied using χ^2 or Fisher's exact tests. Variables with a p value < 0.25 were considered for inclusion in the multivariate analysis. Multivariate analysis was based on using logistic regressions. Model fit was evaluated by applying the Hosmer-Lemeshow goodness-of-fit test with a p value > 0.05 suggesting an adequate model fit. Odds ratio and 95% confidence interval values were calculated. A p value < 0.05 was regarded as statistically significant. The Mann-Whitney U test was used to compare the distribution of neutralization titers between the vaccinated and unvaccinated groups.

RESULTS

Characteristics of study sample

Five hundred-sixteen samples were collected during the survey, and the FCV-positive rate in those samples was 43.0%. Based on univariate analysis, all variables were considered to be associated with FCV infection status (**Table 1**). The results of a multivariate analysis of FCV infection are shown in **Table 2**.

Table 1. Univariate analysis of factors associated with feline calicivirus infection

Variable	Category	Proportion	Frequency (%)	χ^2	*p* value
Overall		222/516	43.0		
Age	Kitten	137/297	46.1	4.474	0.220
	Juvenile	41/117	35.0		
	Adult	39/92	42.3		
	Senior	5/10	50.0		
Sex	Male	138/305	45.2	1.503	0.240
	Female	84/211	39.8		
Season	Spring	45/130	34.6	5.355	0.149
	Summer	68/154	44.2		
	Autumn	67/141	47.5		
	Winter	42/91	46.2		
Vaccination	No	45/87	51.7	15.697	< 0.01
	Not proper	60/128	46.9		
	Proper	30/113	26.5		
Breed	British shorthair	76/173	43.9	9.707	0.084
	Chinese domestic cats	57/110	51.8		
	Ragdoll	17/53	32.1		
	American shorthair	16/45	35.6		
	Garfield	10/34	29.4		
	Others	22/53	41.5		

$p < 0.25$ was considered significant.

Table 2. Multivariate logistic regression analysis of factors associated with feline calicivirus infection

Variable	OR	95% CI	*p* value
Age			
Kitten	1.209	0.288–5.067	0.796
Juvenile	1.200	0.271–5.310	0.810
Adult	1.111	0.245–5.037	0.891
Senior	1.0*	-	-
Sex			
Male	1.0*	-	-
Female	0.690	0.424–1.123	0.135
Season			
Spring	0.556	0.264–1.170	0.122
Summer	0.935	0.464–1.883	0.852
Autumn	1.014	0.495–2.078	0.970
Winter	1.0*	-	-
Vaccination			
No	2.871	1.292–5.564	0.008
Not proper	2.756	1.452–5.229	0.002
Proper	1.0*	-	-
Breed			
British shorthair	0.659	0.285–1.520	0.328
Chinese domestic cats	0.797	0.320–1.986	0.626
Ragdoll	0.318	0.109–0.933	0.037
American shorthair	0.480	0.163–1.412	0.182
Garfield	0.449	0.142–1.420	0.173
Others	1.0*	-	-

$p < 0.05$ was considered significant.
OR, odds ratio; CI, confidence interval.
*Reference category.

There was a significant effect of vaccination on FCV infection, with unvaccinated cats being approximately 2.871 times as likely to be infected with FCV as those that were vaccinated ($p = 0.008$). The presence of FCV was less frequently identified in Ragdoll samples ($p = 0.037$). In the final model, age, sex, and season were not significantly associated with the risk of FCV infection.

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Table 3. Multivariate logistic regression analysis of clinical signs associated with feline calicivirus infection

Variable	OR	95% CI	*p* value
Sneeze	1.262	0.837–1.902	0.267
Nasal discharge	1.117	0.735–1.697	0.605
Ocular discharge	0.661	0.453–0.965	0.032
Oral symptoms	2.796	1.805–4.330	< 0.001
Conjunctivitis	0.587	0.327–1.056	0.075
Cough	1.253	0.645–2.434	0.506

$p < 0.05$ was considered significant.
OR, odds ratio; CI, confidence interval.

Among the clinical signs, oral symptoms were significantly associated with FCV infection (**Table 3**). Cats with oral symptoms were 2.796 times more likely to have FCV than cats without oral symptoms ($p < 0.001$). In addition, FCV was detected in a lower proportion of the cats with ocular discharge than in cats without ocular discharge ($p = 0.032$).

Phylogenetic analysis

A total of 76 ORF2 sequences were obtained from 80 FCV isolates. Isolates whose ORF sequences were not amplified may have resulted from primer mismatches. As presented in **Fig. 1**, phylogenetic analysis displayed a typical radial distribution, with the intermingling of temporally and spatially diverse strains. Thirteen clades were represented by more than one isolate (divergence < 20%, bootstrap values ≥ 80%), indicating possible variants of individual

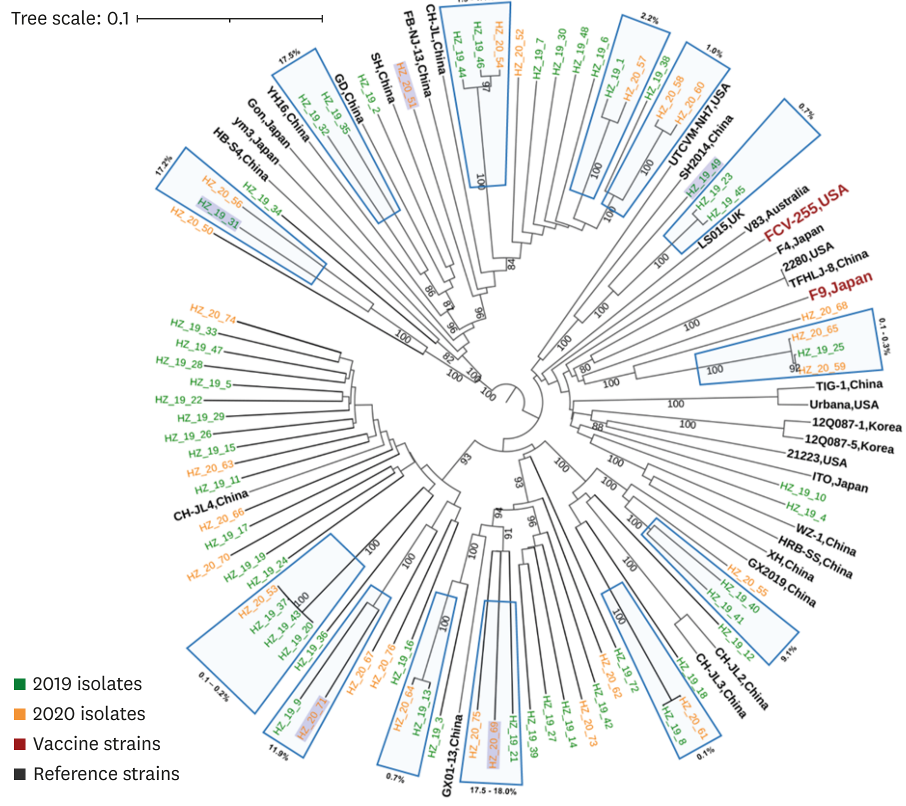

Fig. 1. Unrooted neighbor-joining tree of 107 feline feline calicivirus open reading frame 2 sequences from 76 study isolates and 31 reference strains. Only bootstrap values ≥ 80% are indicated. The evolutionary distances were computed using the Tamura-Nei method. Isolates with a gray label background were subsequently used in the viral neutralization tests. Clades represented by more than a single sequence (divergence < 20%, bootstrap values ≥ 80%) are boxed. The intra-clade diversity is indicated next to the box.

strains. Of the 13 clades, 9 contained isolates from different years, suggesting the possibility of local circulation of these strains. The phylogenetic tree could be further divided into 2 major groups. One group contained strains isolated from several countries, whereas the other group only included strains isolated from China and Japan. Notably, no isolated strain was assigned to the FCV-255 branch.

Viral neutralization assays

A total of 80 serum samples from vaccinated cats (experimental group) and 40 serum samples from unvaccinated cats (control group) were collected and underwent neutralization assays. The virus neutralization results for 5 isolated strains are shown in **Fig. 2**.

Of the 80 serum samples in the experimental group, 75 (94%) showed neutralization of at least one isolate strain at titers ranging from 1:10 to 1:5120. In vaccinated cats, the proportions of serum samples with antibody titers above the level of the suggested protective neutralizing titer (≥ 1:32) [16] against HZ_19_31, HZ_19_49, HZ_20_51, HZ_20_71, HZ_20_69 were 40%, 51%, 84%, 79%, and 73%, respectively. The results showed distinct variation in individual cat responses, with some cats' serum neutralizing some isolates particularly well but neutralizing others less well. As shown in **Table 4**, there were significant differences in the distribution of neutralizing titers between vaccinated and unvaccinated cats ($p < 0.001$). Median neutralizing titer values for unvaccinated cats were all below 10, while median rates among vaccinated cats ranged from 20 to 320.

DISCUSSION

FCV is a common and important pathogen of felids. Widespread vaccination has reduced the morbidity and lethality associated with FCV infection; nevertheless, vaccination does not offer complete protection. FCV is still a significant causative agent of feline viral diseases. This study was conducted to assess the prevalence of FCV in a population of cats exhibiting clinical signs. The results showed an FCV-positive rate of 43.0%, which was higher than that detected in 16 Chinese cities (14.2%) [17], but lower than that reported in Beijing (46.3%) [18] and Switzerland (45.0%) [19]. The high risk of FCV infection may be due to the carrier state of the virus as FCV is generally shed more or less continuously by infected cats [20]. Geographical location, sample population, and sample size probably account for the observed variation.

Table 4. Comparation of neutralizing antibody titers against 5 feline calicivirus isolate strains between vaccinated and unvaccinated cats

Strain	Vaccination	Average rank	P_{50}	Z	p value
HZ_19_31	No	43.55	< 10	−4.199	< 0.001
	Yes	68.97	20		
HZ_19_49	No	40.55	< 10	−4.791	< 0.001
	Yes	70.47	40		
HZ_20_51	No	31.5	< 10	−6.598	< 0.001
	Yes	75	320		
HZ_20_71	No	31.48	< 10	−6.566	< 0.001
	Yes	75.01	80		
HZ_20_69	No	34.35	< 10	−6.009	< 0.001
	Yes	73.58	160		

$p < 0.05$ was considered significant.
P_{50}, median.

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A

Vaccinated cats | Unvaccinated cats

Frequency (%)

< 10 | 10 | 20 | 40 | 80 | 160 | 320 | > 320
n = 35 | n = 4 | n = 9 | n = 8 | n = 8 | n = 7 | n = 1 | n = 8

< 10 | 10 | 20 | 40 | 80 | 160 | 320 | > 320
n = 34 | n = 0 | n = 1 | n = 3 | n = 1 | n = 1 | n = 0 | n = 0

B

< 10 | 10 | 20 | 40 | 80 | 160 | 320 | > 320
n = 29 | n = 6 | n = 4 | n = 7 | n = 12 | n = 4 | n = 5 | n = 13

< 10 | 10 | 20 | 40 | 80 | 160 | 320 | > 320
n = 33 | n = 2 | n = 1 | n = 1 | n = 1 | n = 1 | n = 0 | n = 1

C

< 10 | 10 | 20 | 40 | 80 | 160 | 320 | > 320
n = 9 | n = 1 | n = 3 | n = 10 | n = 5 | n = 11 | n = 2 | n = 39

< 10 | 10 | 20 | 40 | 80 | 160 | 320 | > 320
n = 31 | n = 1 | n = 0 | n = 2 | n = 1 | n = 1 | n = 0 | n = 4

D

< 10 | 10 | 20 | 40 | 80 | 160 | 320 | > 320
n = 9 | n = 5 | n = 3 | n = 16 | n = 9 | n = 10 | n = 14 | n = 14

< 10 | 10 | 20 | 40 | 80 | 160 | 320 | > 320
n = 25 | n = 8 | n = 1 | n = 2 | n = 2 | n = 2 | n = 0 | n = 0

E

< 10 | 10 | 20 | 40 | 80 | 160 | 320 | > 320
n = 15 | n = 4 | n = 3 | n = 8 | n = 7 | n = 12 | n = 13 | n = 18

< 10 | 10 | 20 | 40 | 80 | 160 | 320 | > 320
n = 31 | n = 3 | n = 0 | n = 2 | n = 0 | n = 1 | n = 2 | n = 1

Fig. 2. Distribution of neutralizing antibody titers against 5 FCV isolate strains in vaccinated and unvaccinated cats. (A-E) represent antibody titers against FCV isolate strains HZ_19_31, HZ_19_49, HZ_20_51, HZ_20_71, and HZ_20_69, respectively.
FCV, feline calicivirus.

The correlations between FCV infection and specific factors were analyzed. Our results demonstrated that vaccination has a significant protective effect against FCV infection, similar to results obtained in previous studies [21]. We also observed that cats with oral symptoms have a higher prevalence of FCV infection than those without it. Similarly, oral symptoms such as chronic gingivostomatitis and oral ulcers are reported to affect FCV prevalence [4,13,22]. However, it has been reported that the severity of oral lesions in cats is not related to FCV load [23]. The FCV-positive percentage was higher for males than females, but the difference was not statistically significant. A higher rate in males is likely attributable to the more aggressive nature of male cats; male cats and intact cats have been shown to have more aggressive tendencies, leading to a greater risk of bite wounds [24]. In this study, multivariate analysis showed that FCV infection was not associated with age, although other researchers considered younger cats were more likely to be infected with FCV [13]. The latter result could be because younger cats may have less access to vaccination. The potential for interference in the successful vaccination by maternally-derived antibodies (MDA) may also account for a higher FCV prevalence in kittens. Inactivated vaccines against FCV have been shown to be less effective in the presence of high MDA than low MDA levels [25]. Thus, our results suggest that vaccination protocols have a more crucial role than age in FCV infection status.

Our genetic evolution analysis results are broadly in agreement with those in previous studies and indicate a radial phylogeny that contains many strains [10,21]. Looking at the diversity within the observed clades, 6 groups of the isolated strains were < 1% divergence from each other, suggesting those isolates may not replicate for an extended period in cats. The presence of 2 distinct groups with high bootstrap values has been reported previously [5]. Strains isolated in China and Japan formed a single subgroup, which may be due to their geographical proximity [26]. However, an association between geographical distribution and clustering in the phylogenetic tree was not observed, which is in accordance with the findings in previous studies [15,27,28]. The observation that inactivated viruses does not shed following vaccination is generally corroborated by the absence of an FCV-255-like strain in the phylogeny tree.

Despite the high variability, FCV strains are generally considered to have only one serotype. Previous studies have confirmed the cross-reactivity of vaccine strains [10,11]. Nevertheless, there were several ‘properly’ vaccinated cats that were FCV positive (30/113). The seroprevalence of FCV in vaccinated cats observed in virus neutralization assays indicated an insufficient vaccination efficacy, enabling us to suggest that the balance between cell- and antibody-mediated immunity in FCV protection is not unequivocal, and cellular immunity may contribute to FCV protection. Negative neutralizing titers against FCV should not necessarily be regarded as indicative of lack of protection [29]. However, it is believed that antibody levels reflect protective capacity to a certain extent; thus, *in vitro* virus neutralization assays remain an accepted method of assessing cross-reactivity [11-13]. The presence of high antibody titers suggests that anti-sera against FCV-255 remains cross-reactive against recently isolated strains. Cross-neutralization tests have been used to evaluate the potency of vaccine strains against field strains [10,13,30,31]. Our results align with previous studies in which virus neutralization assays and enzyme-linked immunosorbent assay (ELISA) have demonstrated good concordance. Therefore, the use of ELISA to detect antibody levels in previously vaccinated cats might help in the development of concise booster vaccination regimens. Differing neutralizing antibody titer levels may be related to the characteristics of the cats in the study, such as age, vaccination time, and blood collection time. The cats in this study varied remarkably in their immune responses against the same viral strain, which is consistent with previous observations [12]. The potency of the protection offered

by immunization with current commercial vaccines has been questioned [17]. One possible explanation for this observation is that multiple factors can affect vaccination efficacy. For example, cats with MDA could potentially fail to develop protective antibodies by the end of their initial vaccination series [16,32].

Given the widespread presence of FCV, providing sufficient FCV-related disease protection is a major concern. Vaccination is considered a generally safe and effective approach to reducing clinical disease in cats; however, challenges remain. The quality of vaccine-induced immunity is affected by the environment, the pathogen, the characteristics of the vaccine, and the animal's immune competence. Factors that influence an individual cat's ability to respond to vaccination include immunodeficiencies, interference from MDA, inadequate nutrition, concurrent diseases, and chronic stress. In short, in order to achieve greater vaccination efficacy, cat vaccination should be patient-specific and based on an individualized risk-benefit assessment [33]. Hence, the importance of regular and annual evaluations of vaccination status.

There are 2 main ways to produce anti-sera for testing: infection and vaccination. Differences in viral replication and antigen presentation are likely to be affected in unknown way, so that produce anti-sera by vaccination seems be better. Previous studies indicate a higher response than that from routine vaccination can be obtained by vaccinating with ten doses of a commercial vaccine [4]. Clearly, vaccination can reveal the cross-reactivity of a vaccine strains to a field strain; however, this approach fails to uncover vaccination efficacy exactly. High antibody titers and long-lasting antibody levels may not be achieved for all individuals. Contrastingly, the approach used in this study more closely mirrors that in current practice. Regrettably, this study did not include the FCV-255 strain as a homologous control in the neutralization assays. Without antibody units as a measure, comparisons among the 5 strains may be biased. To overcome this problem, unvaccinated cats were used as the control group, which, in this study, may have compensated for the homologous control deficiency. Although conclusions about vaccination efficacy based only on *in vitro* results may be inconclusive, such results may help optimize vaccination protocols.

Overall, the data presented in this study suggest that cat populations in Hangzhou are inadequately protected by vaccination against FCV. Thus, more comprehensive and refined vaccination protocols should be considered. Additional studies should be undertaken to learn more about the exact efficacy of FCV vaccination. The results of this study are crucial in elucidating the epidemiology and prevention of FCV; moreover, they provide theoretical and experimental foundations for further assessment and screening of vaccine strains.

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