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Detection of Feline Cupripoxvirus and Variation Analysis of Its VP1 Gene in Chengdu from 2020 to 2023

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Abstract. Feline calicivirus (FCV) is an important pathogen causing infectious diseases in the oral cavity and upper respiratory tract of cats. Research on its prevalence and genetic variations contributes to the prevention and treatment of related diseases, as well as the development of new vaccines. In this study, a total of 113 samples of eye, nose, and throat secretions were collected from cats with respiratory and oral inflammation symptoms in Chengdu area from 2020 to 2023. The samples were tested using the RT-PCR method. The VP1 gene region was then cloned, sequenced, and subjected to homology analysis and phylogenetic analysis with domestic and foreign FCV reference strains. The results showed that among the 113 samples, 24 tested positive for FCV, with a positive rate of 21.24%. The nucleotide homology between the 5 FCV VP1 genes obtained in this study and the reference strains ranged from 72.6% to 84.1%, while the amino acid homology ranged from 84.0% to 95.9%. Phylogenetic analysis showed that the strains obtained in this study clustered into two gene groups (GI and GII) in the domestic and foreign FCV strain evolution tree. Strains SMU-M2, SMU-M7, SMU-M13, and SMU-M71 belonged to the GII subtype, while strain SMU-M8 belonged to the (GI subtype. The FCV vaccine strain had a distant genetic relationship with the prevalent FCV strains in Chengdu. The study revealed that FCV is prevalent in the cat population of Chengdu, and there is a low genetic homology and significant sequence differences between the prevalent strains and vaccine strains. This suggests the need to evaluate the actual protective efficiency of existing commercial FCV vaccines and promote the development of new vaccines with better cross-protection against prevalent strains.

Keywords: feline calicivirus; molecular epidemiology; VP1 gene.

1. Introduction

Feline calicivirus (FCV) is an important pathogen that causes oral and upper respiratory infectious diseases in cats [1]. It can lead to symptoms such as conjunctivitis, sneezing, coughing, and difficulty breathing, with oral ulcers being the most common manifestation. In severe cases, it can result in acute systemic illness [2], causing significant impacts on the pet industry. Research has found that this virus can infect not only cats but also other felids of all age groups [3, 4]. In recent years, the virus has been detected in wild felids such as the Siberian tiger, Bengal tiger, East African lion, and cheetah [5]. Infected animals are the main source of transmission, as the virus can be expelled through the nasal and oral secretions, infecting healthy animals [6, 7]. Some cats may continue to carry and shed the virus even after recovery, serving as a source of infection for healthy animals. All these indicate that FCV has strong contagiousness, leading to its worldwide prevalence. Currently, a commercial inactivated vaccine against FCV is widely used for immunization in China. However, due to the high genetic variability of FCV, the vaccine's clinical efficacy is poor, and frequent vaccine failures occur [8].

FCV belongs to the family Caliciviridae and the genus Vesivirus [9, 10]. It is a non-enveloped, positive-sense, single-stranded RNA virus with a genome length of 7683bp, containing three open reading frames (ORFs) [9, 10]. ORF1 encodes the more conserved non-structural proteins, while ORF2 encodes the capsid protein VP1, which comprises six regions (A-E). Regions C and E are

highly variable and prone to mutation, contributing to the decline in vaccine efficacy. FCV can be classified into two subtypes, GI and GII, based on VP1 [11]. Due to the high variability of VP1, cross-reactivity between different FCV strains is related to the variable region E of VP1, making VP1 a target for clinical diagnosis [12]. ORF3 encodes the minor capsid protein VP2, which is involved in the synthesis, maturation, and replication of infectious viral particles [13, 14]. FCV has only one serotype, but due to its high variability, multiple genotypes can emerge, leading to variations in major clinical symptoms. Furthermore, complex and highly pathogenic strains can increase the mortality rate in infected cats [2].

In this study, 113 samples of ocular, nasal, and pharyngeal secretions were collected from cats with respiratory and oral inflammation symptoms in Chengdu. RT-PCR was used for detection, and epidemiological diagnosis was performed to investigate the potential associations between FCV infection and factors such as gender, age, breed, and vaccination status of the cats. Additionally, partial sequences of the VP1 gene were amplified from 24 positive samples, and their homology was analyzed in comparison with vaccine strains and strains circulating in different regions of China. This was done to understand the molecular epidemiological characteristics and genetic variations of FCV in Chengdu and other areas, with the aim of providing theoretical direction for the development of new effective FCV vaccines.

2. Materials and methods

2.1 Materials

2.1.1 Sample collection

Clinical samples were collected from 2020 to 2023 from four animal hospitals and one cat shelter in Chengdu, China. These samples consisted of eye, nose, and throat swabs from cats with respiratory symptoms, oral inflammation, or a history of FCV (Feline Calicivirus). A total of 113 samples were obtained. Clinical information such as the host species, breed, gender, age, symptoms, and immunization status were recorded for each sample. The collected samples were added to virus preservation fluid and stored at -80℃ for future use. The affected cats mainly exhibited clinical symptoms such as sneezing, increased eye and nasal secretions, conjunctivitis, difficulty breathing, coughing, excessive salivation, and oral ulcers, among others.

2.1.2 Main reagents and instruments

Trizol™ Reagent and Prime ScriptTM RT Kit are products of Bao Biological Engineering (Dalian) Co., Ltd.; Quick Taq HS Dye Mix is a product of Oriental Textile (Shanghai) Biotechnology Co., Ltd.; Goldview (EB substitute) is a product of Shaanxi Zhonghui Hecai Biomedical Technology Co., Ltd.; DL2000 DNA Marker and high-purity low electro-osmotic Qianzhi gel are products of Beijing Qingke Xinye Biotechnology Co., Ltd.; general PCR instrument is a product of Thermo Fisher Scientific; Gel imaging system and nucleic acid electrophoresis instrument are products of Bio-Rad Corporation.

2.1.3 Primer information

Based on the GenBank published VP1 gene sequence (Sato et al., 2002), primers were synthesized for FCV detection. The primers used for FCV detection were FCV-F: GAATTGGCTAARATCTTRCATGA, FCV-R: GGRGTTTCAGAGTTDGARGTCA, and the primers used for amplification of the FCV-VP1 gene fragment were FCV-VP1 F: GTTGGTGGTGTGATTGCCGA, FCV-VP1 R: CTCCAGCCAGCAGTTTCTTG. The amplification fragment lengths were 456bp and 720bp, respectively. All of the primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

2.2 Methods

2.2.1 Extraction and detection of sample RNA for FCV

Take 500 μL of processed clinical samples and extract RNA using the TRIzol method. Then, reverse transcribe the RNA into cDNA using the Prime Script™ RT reagent kit. Perform FCV detection using RT-PCR with a target fragment size of 456bp. The reaction system consists of the following steps: 94℃ for 2 minutes, 94℃ for 30 seconds, 53℃ for 30 seconds, 72℃ for 1 minute, and 72℃ for 8 minutes. Repeat this cycle 35 times.

2.2.2 Amplification of FCV-VP1 gene sequence

Using designed primers, FCV-VP1 partial region sequence was amplified by PCR. The primer sequences can be found in Table 2. The PCR reaction system was 25 μL: Quick Taq HS Dye Mix 12.5 μL, FCV-VP1F 1 μL, FCV-VP1R 1 μL, ddH2O 8.5 μL, cDNA 2 μL. The reaction conditions were as follows: 94℃ for 2 min; 94℃ for 30 s; 58℃ for 30 s; 68℃ for 1 min; 72℃ for 8 min. After purification, the PCR product was ligated with the pET28a(+) vector for cloning and transformation. Ten colonies on the plate were selected for PCR identification. The PCR-positive bacteria (10 μL) were sent to Shenggong Biotechnology Engineering (Shanghai) Co., Ltd. for bidirectional sequencing, obtaining the partial region sequence information of the FCV-VP1 gene.

2.2.3 Genetic evolutionary analysis of genes

An analysis and comparison were conducted between the sequencing results of the ORF2 gene (with a total length of 2000bp) of five FCV isolates obtained in this study, the VP1 gene sequence of prevalent strains from different regions in GenBank, and the sequences of published vaccine strains. The MEGA 11.0 software was utilized for multiple sequence alignment (using the ClusterW algorithm) and homology analysis. The neighbor-joining algorithm was employed to construct a clustering tree based on the gene alignment results. The bootstrap method (with 1000 bootstrap replicates) was used to construct a systematic phylogenetic tree based on the alignment of the VP1 gene.

2.2.4 Statistical (Epidemiological) Analysis of FCV Detection Results and Analysis

The sample information of the diseased animals, including gender, age, breed, and immune status, will be collected. Chi-square analysis will be used to compare the detection rate of FCV in terms of breed, age, gender, and immune status. Correlation analysis and two-factor ANOVA analysis will be conducted using IBM SPSS Statistic 28.0. If the corresponding P-value is less than 0.05 or 0.01, the data differences will be considered statistically significant or highly significant.

3. Results

3.1 Detection Results and Analysis

3.1.1 Positive Detection Rate of Sample

Using the RT-PCR method, 113 samples of cat ocular and nasal pharyngeal secretions were tested for FCV. The results showed that out of the 113 samples collected in certain areas of Chengdu from 2020 to 2023, 24 samples tested positive for FCV, accounting for 21.24% (95%CI=13.79%~28.69%) of the total samples.

3.2 Correlation Analysis of FCV Infection Incidence with Cat Gender, Age, Breed, and Immunization Status

In terms of gender, the detection rates of FCV in male cats and female cats were 22.92% and 28.57% respectively, with a slightly higher infection rate in female cats. Statistical analysis results showed that there was no significant correlation between FCV infection rate and cat gender in Chengdu area (P=0.59). In terms of age, the detection rates of FCV in young cats (≤12 months old) and adult cats

(>12 months old) were 25.58% and 25.71% respectively, with a slightly higher infection rate in adult cats. Statistical analysis results showed that there was no correlation between FCV infection rate and cat age in Chengdu area (P=0.82). In terms of breed, the positive detection rates of FCV in Chinese domestic cats, British Shorthairs, American Shorthairs, Silver Tabbies, Golden Tabbies, and Ragdolls were 15.38%, 25%, 50%, 28.57%, 40%, and 16.67% respectively. Among them, American Shorthairs, Golden Tabbies, and British Shorthairs had higher infection rates, but statistical analysis showed no correlation between FCV infection rate and cat breed (P=0.12). In terms of immune status, out of the 113 samples tested, 24 were positive for FCV, among which 8 were immunized, accounting for 33.33% of the total number of positive cases, and 11 were not immunized, accounting for 45.83% of the total number of positive cases. It can be seen that the incidence of FCV is higher in non-immunized cats than in immunized cats, and there is a significant correlation between FCV infection rate and cat immune status (P=0.048).

Table 1. The correlation between the prevalence of FCV and sex,age,breed and immunity

Variables	Number of samples	Number of FCV- positive samples	Number of FCV-negative samples	Positive rate(95%CI)	OR	*P*-value
			Sex (n=76)			
Male	48	11	37	22.91%(10.95%~34.87%)	1.04	0.59
Female	28	8	28	28.57%(11.91%~45.23%)		
			Age (n=78)			
≤6 month	29	7	22	24.14%(8.66%~39.62%)	0.88	0.82
＞6 months	49	13	36	26.53%(14.18%~38.88%)		
			Immunity (n=81)			
Immunized	21	8	17	33.33%(13.14%~53.52%)	2.18	0.048
Non-immunized	60	11	51	45.83%(33.23 %~58.43%)		
			Breed (n=98)			
Chinese native cat	52	8	44	15.39%(5.59%~25.19%)	0.46	0.12
Foreign breed cat	46	13	33	28.26%(15.32%~41.20%)		

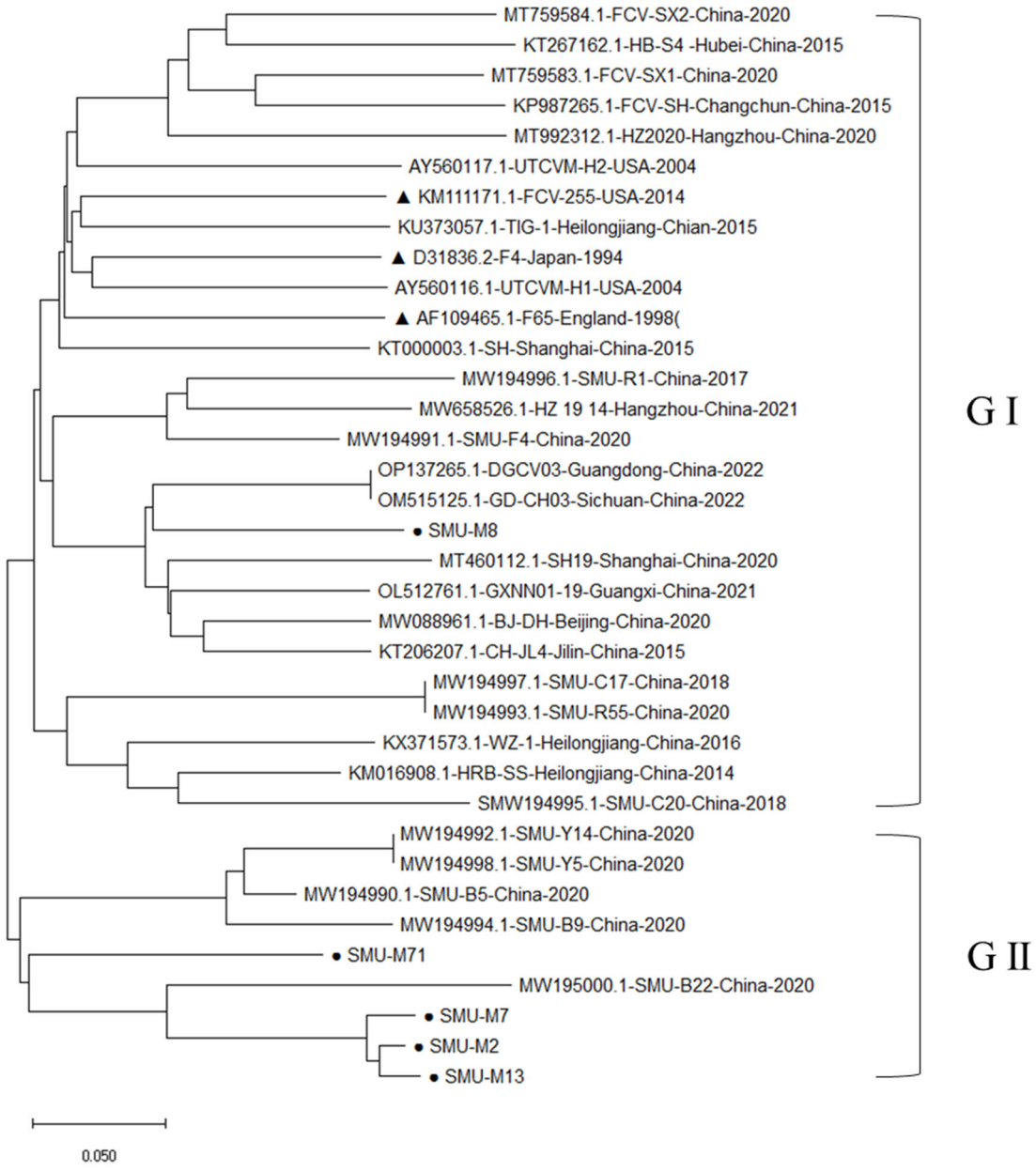
3.3 Homology Analysis of FCV-VP1 Gene

To further investigate the molecular characteristics and genetic variations of FCV-VP1 gene in Chengdu area, we used designed primers to amplify the VP1 partial region sequence of 24 positive samples. Eventually, we obtained 5 strains of FCV-VP1 sequences, named as SMU-M2, SMU-M7, SMU-M8, SMU-M13, SMU-M71, with sequence lengths of 719bp, 717bp, 724bp, 743bp, and 737bp, respectively. The results showed that the nucleotide sequence homology among the 5 strains in this study ranged from 76.2% to 97.3%, while the amino acid sequence homology ranged from 84.0% to 95.9%. The nucleotide homology between these 5 strains and the VP1 gene reference sequences of 31 epidemic strains from five regions in China (North China, East China, Central China, Northeast China, and Southwest China) in GenBank, as well as the sequences of 3 vaccine strains, ranged from 72.6% to 84.1% at the nucleotide level and 80.7% to 94.2% at the amino acid level.

3.4 Genetic Evolution Analysis of FCV-VP1 Gene

The genetic sequences of 5 FCV isolates were amplified and used to construct genetic evolution trees and clustering trees with the genetic sequences of prevalent strains and vaccine strains from five major regions in China (see Figure 1). The results showed that these FCV isolates can be classified into two major groups, Group I and Group II. The SMU-M2, SMU-M7, SMU-M13, and SMU-M71 strains belong to the GII subtype, while the SMU-M8 strain belongs to the GI subtype. Among them, the SMU-M2, SMU-M7, and SMU-M13 strains are clustered together, while the other two strains (SMU-M8 and SMU-M71) form separate branches within the GI and GII types, respectively. Sequence analysis of the 5 FCV isolates revealed that they are genetically distant from the vaccine

strains and do not belong to the same branch. These results indicate that the 5 isolates obtained in this study exhibit significant genetic diversity compared to the existing virus strains from different regions in China. The genetic evolution relationship among the 5 strains is complex, and all of them are genetically distant from the FCV vaccine strains. This may be one of the reasons for the failure of immune response to commercial inactivated FCV vaccines currently used in clinical practice.



●.Strains in this study; ▲.Feline calicivirus vaccine strains

Fig. 1 Phylogenetic tree based on FCV-VP1 sequence constructed using the neighbor-joining (NJ) method

4. Discussion

FCV was first isolated by Fastier in 1957[7]. Subsequently, FCV strains have been isolated in Europe, Asia, Africa, and the Americas, and it is now globally distributed [15, 16]. Moreover, FCV prevalence varies among different countries. Due to the fast mutation rate of FCV as an RNA virus [2], different strains are prevalent in different regions within China, resulting in varying prevalence rates. In this study, representative regions, including North China [17, 18], Northeast China [19, 20], East China

[21, 22], Northwest China [23], and Southwest China [24, 25], were selected to compare and analyze the prevalence of FCV in Chengdu region.

In this test, out of 113 samples, 24 were positive, with a positivity rate of 21.24%. The positivity rates in the northwest, north, northeast, east, and southwest regions were 46.30%, 46.30%, 13.75%, 13.42%, 27.20%, and 30.29%, respectively. Compared with the five domestic regions, the positivity rate was lower. Among them, the highest positivity rate was in the east region represented by Anhui (58.67%), which may be due to the higher economic level and more cat owners in the east region, resulting in a higher probability of cats being infected with FCV. Statistical analysis of cat data with different ages, genders, breeds, and immune conditions in Chengdu region found that the FCV infection rate had no correlation with the cat's gender, age, or breed, but there were significant differences among cats with different immune conditions. This result is consistent with the results of investigations in the other five regions. In terms of immune conditions, the majority of positive cases were cats in the unimmunized state, and there were also some cases of cats that had been immunized. This result is consistent with the study by Wang Jie, indicating that vaccine immunization can only provide certain protection against FCV, but the cross-immunization effect against different strains is not ideal[2].

The nucleotide homology between the VP1 gene reference sequences of these 5 isolated strains and 31 epidemic strains from five regions in China (North China, East China, Central China, Northeast China, Southwest China) and foreign countries in GenBank ranges from 72.6% to 84.1%, while the amino acid homology ranges from 80.7% to 94.2%. From the homology analysis table of the FCV ORF2 fragment, it can be observed that the nucleotide sequence homology within the 5 gene fragment groups obtained in this study ranges from 76.2% to 97.3%, indicating a relatively low within-group homology. The homology with FCV strains from different regions in China is also relatively low, with higher homology observed with the Sichuan strain (GD-CH03) and Guangxi strain (GXNN01-19), reaching 84.1%. This suggests that FCV is spreading across different regions in China and that the epidemic FCV strains also exhibit some variations. The homology with the three vaccine strains (KM111171, D31836, AF109465) is relatively low, ranging from 75.0% to 79.8%, which may be one of the reasons why some cats in Chengdu area still get infected with FCV after vaccination.

Systematic phylogenetic analysis showed that the 5 FCV strains in this study and the FCV reference strains in GenBank are divided into two genotypes on the evolutionary tree. The FCV strains prevalent in Chengdu are distributed in both subtypes GI and GII, among which the strains SMU-M2, SMU-M7, SMU-M13, and SMU-M71 are located in subtype GII, and the strain SMU-M8 is located in subtype GI. This indicates that subtype GI is the predominant subtype in Chengdu, which is different from the reports in the Central China region that state that subtype GII is the predominant subtype in China[26]. Among them, the sequences of M2, M7, and M13 have the closest genetic relationship with the strain SMU-B22 (GenBank No. MW195000.1) isolated in China in 2020 and are located on the same branch. The strain SMU-M8 has a more distant genetic relationship with the other 4 strains and is relatively closer to the strain DGCV03 (GenBank No. OP137265.1) isolated in Guangdong in 2022 and the strain GD-CH03 (GenBank No. OM515125.1) isolated in Sichuan. The 5 strains discovered in this study have a distant genetic distance from strains in other regions and are only closely related to the local strain SMU-B22 in Chengdu. This indicates that there is considerable variation in the genotypes of FCV strains and significant differences in the strains distributed in different regions. There is also diversity among the strains prevalent in the same region, and different regions have their own distinct characteristics of prevalence. It is worth noting that the 5 FCV strains in this study have a distant genetic relationship with the vaccine strains FCV-255 (KM111171), F65 (AF109465), and F4 (D31836). This suggests that the FCV strains currently prevalent in Chengdu may have undergone certain immunogenic drift, which may lead to a decrease in the protective efficacy of existing commercial vaccines against prevalent strains. Future research can focus on the immunogenic changes in the current prevalent strains of FCV and evaluate the protective efficacy of existing vaccines against prevalent strains, providing insights for the development of related vaccines.

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