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Distribution and genetic diversity of Feline calicivirus in Moscow metropolitan area

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ABSTRACT

Background: Feline calicivirus (FCV) is widespread throughout the world. An FCV infection is associated with conjunctivitis, rhinitis, and mouth ulcers that can lead to the animal's death. Because vaccination is not always effective, it is necessary to monitor the infection regularly.
Objectives: This study examined the FCV epizootic situation in the Moscow metropolitan area by conducting a molecular phylogenetic analysis of the virus isolates.
Methods: Samples from 6213 animals were examined by a reverse transcription polymerase chain reaction. For phylogenetic analysis, 12 nucleotide sequences obtained from animal samples were selected. Sequencing was performed using the Sanger method. Phylogenetic analysis was conducted using the Maximum Likelihood method.
Results: The FCV genome was detected in 1,596 (25.7%) samples out of 6,213. In 2018, calicivirus was detected in 18.9% of samples, 27.8% in 2019, 21.4% in 2020, and 32.6% in 2021. Phylogenetic analysis of the F ORF2 region and the ORF3 start region led to division into two FCV genogroups. Most of the isolates (8 out of 12) were close to the Chinese strains. On the other hand, there were isolates closely related to European and American strains. The isolates circulating in Moscow were not included in clusters with vaccine strains; their nucleotide similarity varied from 77% to 83%.
Conclusions: This study revealed a high prevalence and genetic diversity of the FCV in Moscow. The epizootic situation remains stably tense because 24 viruses were detected in 25% of animals annually.

Keywords: Feline calicivirus; monitoring; PCR; sequencing; phylogenetic analysis; genogroups

INTRODUCTION

Feline calicivirus (FCV) infections are highly contagious and are associated with conjunctivitis, rhinitis, oral ulcers, lethargy, and fever [1,2]. The clinical signs in infected cats may develop acutely, chronically, or not at all. In the case of a systemic form, infection leads to death in 50–60% of cases [2,3]. Calicivirus is prevalent in domestic and wild feline populations throughout the world. FCV isolation has been reported in the USA [4,5],

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Conflict of Interest
The authors declare no conflicts of interest.

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Australia [6], Brazil [7,8], some European (Great Britain [9-11], Italy [11,12], Germany [9,11,13,14]), and Asian (South Korea [15,16], Japan [17-19], China [20,21]) countries. Data on the distribution of the FCV in Russia are insufficient. Outbreaks of this disease were recorded in Siberia. A study of 1,125 samples from cats in the Tomsk, Omsk, and Novosibirsk regions detected the FCV genome by polymerase chain reaction in 161 animals (14.3%) [22]. The FCV belongs to the family Caliciviridae, genus Vesivirus [1]. The genome is a 7.7 kb positive-polarity single-stranded RNA with three open reading frames (ORFs). ORF1 encodes nonstructural proteins, ORF2 encodes the major VP1 capsid protein, and ORF3 encodes the minor VP2 capsid protein [2]. ORF2 is divided into six regions (A-F) and is commonly used for the phylogenetic differentiation of FCV isolates [2]. Genomic regions A, B, D, and F are largely conserved, while regions C and E are more variable. The F region of ORF2 (7253–7329 bp of the vaccine strain FCV F9 [GenBank M86379]) and the start region of ORF3 (7.465–7.524 bp of the F9 strain) are conserved and therefore convenient for accurate FCV identification [16]. Recombination is a common mechanism of FCV evolution. The majority of reported recombination events in the FCV genome occur at a "hot spot" between the polymerase (ORF1) and capsid (ORF2) regions [21].

Phylogenetic analysis of the FCV usually leads to a star-like phylogenetic tree with little statistical support for clades, which does not allow the identification of reliable virus genotypes either by spatial, temporal, or clinical signs [11]. Coyne et al. [10] examined the prevalence of various FCV strains, but none of the field strains was dominant. They concluded that FCV belongs to one large genogroup. Hou et al. [9] analyzed the sequences of the C and E ORF2 regions of 72 isolates. They identified 46 strains, which confirmed the high level of FCV diversity. On the other hand, some researchers suggested the presence of two different FCV genogroups [7,18,20].

Vaccines from France and the Netherlands based on F9 and F255 reference strains are used widely to prevent calicivirus infections in the cat population. On the other hand, antigenic differences among FCV isolates lead to a decrease in vaccine efficacy [2,21,23]. Thus, data on the genetic structure of local virus strains circulating among animals in a particular territory of the Russian Federation can help in the development of vaccines for animals.

This study examined the distribution of the causative agent of FCV infections in the Moscow metropolitan area in 2018–2021 and conducted a phylogenetic analysis of the virus isolates.

MATERIALS AND METHODS

From January 2018 to December 2021, 6,213 samples from animals in the Moscow metropolitan area were tested at a commercial laboratory. The ages of the cats ranged from six months to 15 years. To confirm calicivirus infection, nasal, mouth, and eye discharges from 65 animals showing the clinical signs of respiratory diseases were examined by reverse transcription polymerase chain reaction using the commercial test system "Feline Calicivirus" (NanoDiagnostics, Russia) according to the manufacturer's protocol.

Further studies were carried out in the Laboratory of Biochemistry and Molecular Biology of the Research Center "All-Russian Institute of Experimental Veterinary Medicine" (VIEV). For phylogenetic analysis, 12 samples with a low cycle threshold (Ct < 20) were selected (**Table 1**). RNA was extracted from the samples using a commercial reagent kit

Table 1. Characteristics of the study samples for the phylogenetic analysis

Date	FCV isolate	GENBANK ID	Age	Sex, breed	Material
2021-06	FCV-1	ON637643	-	Female, Mestizo	Oral swab
2021-06	FCV-2	ON637644	8 yr	Female, Cornish Rex	Conjunctival swab
2021-06	FCV-9	ON637645	1 yr	Female, Abyssinian	Oral, conjunctival swabs, nasal discharge
2021-07	FCV-10	ON637646	1 yr	Male, Burmese	Oral swab
2021-07	FCV-22	ON637647	1 yr	Male, Scottish Fold	Nasal discharge, conjunctival swab
2021-07	FCV-25	ON637648	-	Female, Mestizo	Conjunctival swab
2021-06	FCV-27	ON637649	11 yr	Male, British Shorthair	Conjunctival swab
2021-11	FCV-32	ON637650	8 mon	Female, Mestizo	Oral swab
2021-11	FCV-34	ON637651	6 mon	Male, Mestizo	Oral, conjunctival swabs
2021-11	FCV-35	ON637652	11 mon	Male, Burmese	Oral swab
2021-10	FCV-41	ON637653	7 mon	Female, Mestizo	Oral, conjunctival swabs, nasal discharge
2021-10	FCV-44	ON637654	5 yr	Female, British Shorthair	Oral, conjunctival swabs

FCV, feline calicivirus.

"RIBO-prep" (InterLabService, Russia). cDNA was obtained by reverse transcription using the Reverta-L RT reagents kit (InterLabService, Russia) according to the manufacturer's instructions. The primers described by Kim et al. [16] were used for DNA sequencing of FCV isolates. The flanking sites were the 324-nucleotide region of the F ORF2 region and the ORF3 start region (7261-7585): ORF2 F 5′ CTGCCTCCTACATGGGAAT 3′, ORF3 R 5′ GTGTATGAGTAAGGGTCRACCC-3′. The 25 μL reaction mixture contained 5 μL cDNA, 2.5 78μl 10X DreamTaq Buffer (Thermo Scientific, USA), 0.5 μL dNTPs mix (Thermo Scientific, USA), 14.6 μL Nuclease-Free water, 0.25 μL DreamTaq DNA Polymerase (Thermo Scientific, USA), and 10 pM each of the forward and reverse primers. The thermal cycle consisted of DNA denaturation (95°C, 5 min), followed by 35 cycles: denaturation (95°C, 30 s), primer annealing (50°C, 30 s), and elongation (72°C, 40 s). The reaction products were analyzed by electrophoresis in 1% agarose gel prepared in a Tris-acetate buffer solution (pH 8.0) with the addition of ethidium bromide (0.5 μg/mL). The gel was examined under ultraviolet light at a wavelength of 254 nm using a Transilluminator instrument (Vilber Lourmat, France).

Extraction of the amplicons from agarose gel was carried out using the method from the commercial kit manufacturer LumiPure (Lumiprobe, Russia). Nucleotide sequences were determined using the BigDye 3.1 kit (Applied Biosystems, USA). The obtained nucleotide sequences of the sequenced fragments were analyzed using the ClustarW alignment method with the published sequences of other FCV strains using the BioEdit 7.0.0 program (https://bioedit.software.informer.com/7.0/). Phylogenetic dendrograms were constructed using the Maximum Likelihood method (MEGA 7.0 software). The topology of the trees was confirmed by 1000 bootstrapping replication steps.

The seasonal coefficient (Sc) was calculated using the formula: $Sc = X/Y \times 100$, where X is the number of incident cases in peak months; Y is the total cases of illness [24].

RESULTS

From 2018 to 2021, 6,213 samples from domestic cats in the Moscow metropolitan area were analyzed. In 2018, calicivirus was detected in 18.9% of animals (n = 1,395): 27.8% in 2019 (n = 1,600), 21.4% in 2020 (n = 1,438), and 32.6% in 2021 (n = 1,780). The disease was detected in all age groups of animals from six months to 15 years. Animals infected with FCV were detected throughout the year, regardless of the season. The maximum number of virus-infected animals was detected in winter (28.7%), and the minimum was noted in

Table 2. Detection of FCV cases in the Moscow metropolitan area from 2018 to 2021

Month	2018			2019			2020			2021			Average long-term morbidity rate (for month)
	Total	FCV +	%	Total	FCV +	%	Total	FCV +	%	Total	FCV +	%	
January	129	23	17.8	106	28	26.4	135	43	31.9	151	60	39.7	10%
February	92	19	20.7	97	19	19.6	105	31	29.5	106	44	41.5	7%
March	115	29	25.2	110	27	24.5	122	38	31.1	112	55	49.1	10%
April	87	19	21.8	119	35	29.4	86	15	17.4	131	51	38.9	7%
May	66	6	9.1	174	40	23	103	11	10.7	127	38	29.9	5%
June	112	21	18.8	109	33	30.3	106	25	23.6	126	44	34.9	8%
July	110	13	11.8	144	49	34	124	23	18.5	149	56	37.6	8%
August	113	14	12.4	151	46	30.5	126	18	14.3	192	34	17.7	7%
September	125	23	18.4	132	38	28.8	131	24	18.3	141	31	22	8%
October	160	39	24.4	152	45	29.6	133	25	18.8	196	58	29.6	11%
November	167	33	19,8	171	45	26,3	129	14	10,9	190	58	30,5	9%
December	119	24	20,2	135	40	29,6	138	41	29,7	159	51	32,1	10%
Total	1395	263	18,9	1600	445	27,8	1438	308	21,4	1780	580	32,6	

FCV, feline calicivirus.

autumn (23.7%). In addition, an annual rise in the number of detected cases of calicivirus infection was recorded in March. In May, there was a decrease in positive cases of detection of calicivirus infection (**Table 2**).

The seasonal coefficient from 2018 to 2021, which reflects the number of sick animals each month to the total number of disease cases in a year, was 57.5% (51–60%), indicating weak seasonality. In the months of the seasonal rise (January, March, December, November, and October), 1.35 times more cases of calicivirus were registered than in other months.

For phylogenetic analysis, 12 nucleotide sequences of FCV isolates from the Moscow metropolitan area were analyzed and deposited in GenBank (ON637643-ON637654).

The phylogenetic tree was constructed on the ORF2/ORF3 junction sequences of the FCV virus basis (**Fig. 1**). The sequence distance matrix calculated using the "similarity" algorithm showed that the nucleotide similarity of 12 FCV strains ranged from 75% to 100%. In addition, these viruses shared 73–92% nucleotide identity with the 50 most similar FCV reference sequences by BLAST usage; some of them were also used in other works [6,7,9,10,15,20].

The results of phylogenetic analysis showed that 12 isolates belong to four clades. Cluster 1 was formed by isolates FCV-1, FCV-9, and FCV-2, as well as isolates BJ-112-2021 (MW088951), GX-2019 (MK867378), BJ-DH-2019 (MW088961), and CH-JL4-2015 (KT206207) from China, showing 90–92% similarity. In addition, the FCV-44 isolate stands out as a separate neighboring branch in this clade. FCV-35 belongs to cluster 2, which includes isolates from Germany and Italy; its nucleotide similarity with the German strain FCV/DD/2006/GE (DQ424892) was 89%, and with the Italian strain 1466/2017 (MT062981) was 92%. A separate group II was formed from three and four clusters. Two isolates, FCV 34 and FCV-41, were classified into a branch, along with the Chinese strains SMU-F4-2020 (MW194991), HRB-SS-2014 (KM016908), WZ-1-2015 (KX371573), SH1-2021 (MT239579) and the South Korean isolate 12Q087-5-2012 (KJ572401). Another cluster 4 included five isolates (FCV-10, FCV-22, FCV-25, FCV-32, and FCV-27), Chinese strain GX01 13-2016 (KT970059), and strains from South Korea (14Q315-2014 [MT123328] and 15D022-2015 [MT123329]). None of the isolates were included in the clade with vaccine strains F9 (M86379) and F255 (U07130), and their nucleotide similarity varied from 79% to 83% and from 77% to 81%, respectively.

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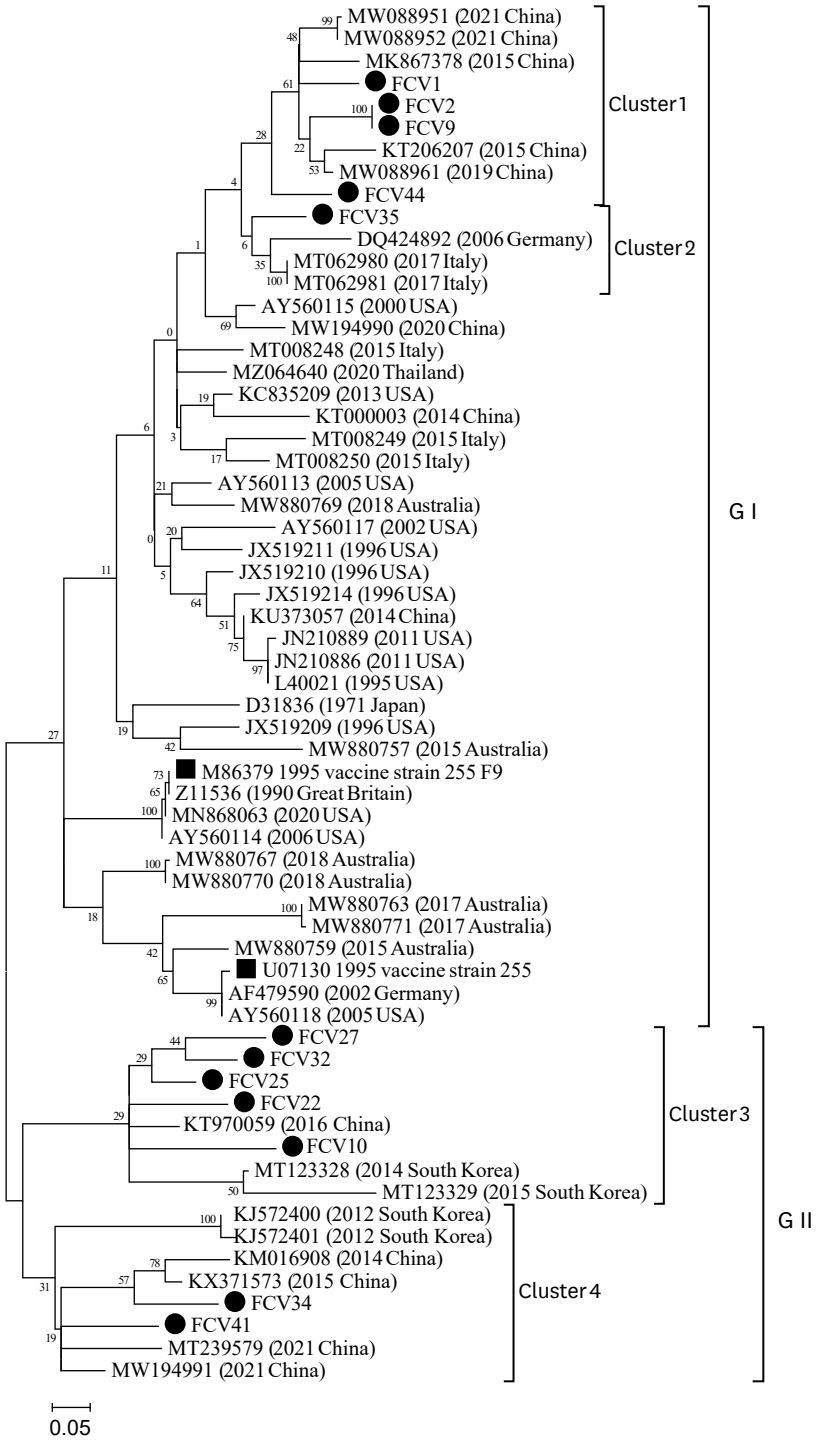

Fig. 1. Phylogenetic tree based on the alignment of nucleotide sequences of the ORF2/ORF3 junction of the FCV isolates from Moscow metropolitan area and 50 strains from GenBank. The multiple sequence alignment was performed using the ClustalW method. A maximum likelihood tree was constructed using MEGA 7.0 software using a nucleotide substitution model (General time reversible model). The resulting tree was midpoint rooted. The scale bar indicates the substitution rates. The topology of the trees was confirmed by 1000 bootstrap replications. Node supports are shown at each node. The FCV isolates sequenced in this study are designated by a circle (●), the vaccine strains are designated by a square (■).
FCV, feline calicivirus.

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DISCUSSION

Feline calicivirus is widespread in the cat population worldwide [1,2]. In the present study, FCV was detected in 1596 out of 6213 cases (25.7%). Calicivirus in cats was diagnosed regardless of season, sex, age, and breed, as well as the results published by other researchers [9,10]. In Russia, in 2018–2019, FCV was detected in 21.3% of cases (n = 1,340), which correlates with the present data [25].

Hou J. et al. reported the frequent virus detection in European countries. In France, UK, Portugal, and Germany, the FCV was detected in 26% (n = 102), 16% (n = 83), 23% (n = 80), and 23% (n = 102) of cases, respectively [9]. In addition, a high prevalence of the virus was also noted in China, 23% (n = 162) [21].

Cases of FCV infections were noted throughout the year, but with weak seasonality (the seasonal coefficient for 2018–2021 was 57.5%). Glotova et al. [26] also recorded the FCV throughout the year, but the increase in incidence occurred in the spring. In the present work, the maximum number of virus-infected animals was detected in winter (28.7%), which can be connected with a decrease in the body's natural resistance. The annual rise of infected animals in March may be because of the start of the breeding season, as well as an increased manifestation of aggression in male cats.

According to the data, the FCV epizootic situation in the Moscow metropolitan area remains stably tense because the infection has been detected in 25% of animals annually. Glotova et al. [22] reported a similar situation in Siberia in 2018. This can be associated with the application of vaccines, developed on a strain basis isolated in the 1960s, and poor awareness of pet owners about this infection. In such situations, the pathogen spreading risk increases.

Phylogenetic analysis showed that different FCV strains from different clusters simultaneously circulate in Moscow, which correlates with the results of studies by Hou et al. [9] and Coyne et al. [10] in Europe. Most of the isolates (FCV-1, FCV-2, FCV-9, FCV-34, FCV-41, FCV-10, FCV-22, FCV-25, FCV-32, and FCV-27) are nucleotide similar to strains from China, probably because of geographical proximity. On the other hand, there are also close isolates to European and American strains.

Phylogenetic analysis of the FCV isolates usually results in a star-like phylogenetic tree with little statistical support for subspecies clusters. Therefore, FCV belongs to one diverse genotype [9-11]. On the other hand, Sato et al. [19] and Sun et al. [20] proposed a classification dividing FCV isolates into two different genogroups based on the phylogenetic analysis of ORF2. The first genogroup included global isolates, while the second included only isolates from South Korea and China. In the present study, phylogenetic analysis of the RF2/ORF3 junction regions showed the division of FCV isolates into two genogroups. The genogroup presents the global strains and five isolates from these studies, genogroup II - isolates of the East Asian group, and seven of the present study that correlates with previous studies by Sato et al. [19] and Sun et al. [20]. As the phylogenetic analysis was connected with other genomic regions, it will be necessary to conduct a comparative analysis of the nucleotide sequences of the entire viral genome to confirm the classification into two separate genogroups.

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The genetic variability of FCV leads to the "obsolescence" of vaccines based on the F9 and F255 strains [21-23]. Seal reported a connection between the phylogenetic relationships and serum neutralization by analyzing 32 nucleotide sequences of the ORF2 region (regions B, C, D, E, and F) of FCV isolates. A lower nucleotide similarity of the isolates with vaccine strains indicated a lower titer of neutralizing antibodies [27]. Ohe et al. [18] reported low nucleotide similarity between 15 Japanese virus isolates and vaccine strains F9 and FCV-255 (70.6–82.9%). A low titer of neutralizing antibodies was also noted when testing the efficacy of vaccines. Wensman et al. [23] reported a decrease in the efficacy of vaccines based on F9 strains. The antisera obtained from four vaccinated cats (two vaccinated with F9 and two with F255) neutralized 47.4 and 64.1% (strain 255) and 20.5 and 11.5% (strain F9), respectively, from 72 field isolates. Therefore, the nucleotide mismatch in the conservative F region of the Moscow isolates and vaccine strains (F9 and F255) used in the present country can lead to ineffective vaccination. The obtained information may be useful for studying the molecular epizootology of viruses and developing diagnostic test systems, new vaccines, and programs for the epizootic control of FCV.

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