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First report on the prevalence and genetic relatedness of Feline Foamy Virus (FFV) from Turkish domestic cats

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ABSTRACT

Feline Foamy Virus (FFV) is an important retroviral agent affecting domestic cats in Turkey that has been studied less intensively than *Feline Immunodeficiency Virus* (FIV) and *Feline Leukemia Virus* (FeLV). Accordingly, we aimed to investigate the presence and prevalence of FFV among domestic cats by molecular techniques. PCR was used to amplify the *gag-pol* gene overlap in order to detect the presence of FFV. The gene encoding *bet*, an important accessory gene, was also characterized. Molecular characteristics were analyzed and phylogenetic trees were constructed. We determined the positivity rate as 10% in all samples (20/200) based on the *gag-pol* test. The phylogenetic analysis indicated that the Turkish FFV sequences form a separate cluster among other isolates in the constructed maximum likelihood (ML) tree. bet-based products were obtained for two samples (1%; 2/200) that were also positive for *gag-pol*. These *bet* gene sequences confirm the presence of a separate cluster for the Turkish FFV isolates. The results suggest that FFV is prevalent and widespread in Turkish domestic cats. Additionally, these new FFV sequences represent the first FFV sequences from Turkey to be submitted to GenBank. This study paves the way for studies on the pathogenicity of FFV.

1. Introduction

Feline foamy virus (FFV) taxonomically belongs to the *Spumavirus* genus, subfamily *Spumaretrovirinae* of the family *Retroviridae*. FFV is thought to be non-pathogenic in domestic and wild *felids* (Roy et al., 2003; Winkler et al., 1999). FFV has main molecular motifs (5′*UTR-gag-pro/pol-env*-3′*UTR*) which are present in other feline retroviruses, including *Feline leukaemia virus* (FeLV) and *Feline immunodeficiency virus* (FIV). However, FFV also has unique accessory genes (*bet, tas,etc.*) that differ from those of FeLV and FIV. They express important proteins in the pathogenesis mechanism and restrict the immune response. In particular, *bet* has been shown to interact with the host *APOBECs* (*A3*) and inhibits its intracellular antiviral effects on the replication process (Alke et al., 2000; Löchelt et al., 2005; Lukic et al., 2013). It is not yet known whether these accessory genes are effective in the pathogenesis of FFV *in vivo*. The existence of *bet* may therefore be crucially significant in the elucidation of pathogenesis and clinical investigations of FFV. This potentially allows differentiation between presumptive endogenous variants and facilitates understanding of the relationship between replication-competent FFV and clinical aspects of the disease (Delebecque et al., 2006; Zemba et al., 2000).

The specific clinical symptoms of FFV are still unknown, although it has been shown to have a negative effect on animal and human cells *in vitro*. According to the previous reports, the clinical signs have been mostly assumed about FFV infection are chronic progressive polyarthritis and osteoarthritis (Weissl et al., 2012; Zenger et al., 1993). However, in a number of these studies, FFV has been frequently detected together with other feline retroviruses (FIV, FeLV) (Romen et al., 2006; Weissl et al., 2012; Winkler et al., 1999; Zenger et al., 1993). The lack of specific clinical signs of FFV, and the prominence of FIV and FeLV have led to FFV being overlooked in diagnosis. Accordingly, there are no records of the presence and prevalence of FFV in Turkey. Therefore, we aimed to determine for the first time, the presence and prevalence of FFV and its molecular characteristic status in Turkey. We have also recorded individual features (age, gender, breed, clinical observations and status of vaccination) and a statistical analysis was performed.

2. Materials and methods

2.1. Materials

We collected peripheral blood samples from 200 domestic cats, which were suspected by retroviral infection or were brought by the

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Table 1
Recorded individual features during sampling, and distribution of total cat population (n = 200) according to relevant risk factors.

Gender		Age (month)		Breed		Health Status		Vaccination	
Male	Female	0–12m	12m +	Pure	Mix	Healthy	Diseased	Vaccinated	Unvaccinated
105	95	146	54	15	185	161	39	60	140

Table 2
Information about 20 FFV positive cats in this study. Accession numbers and risk factors for logistic regression analysis. M: Male; F: Female; SV: Status of vaccination to any viral agents; V: Vaccinated; UV: Unvaccinated; N/A: Not Applicable; NC: No Clinical Sign; FIV: Feline Immunodeficiency Virus; exFeLV: Exogenous Feline Leukemia Virus; enFeLV: Endogenous Feline Leukemia Virus.

Sample	Accession No. (gag-pol)	Accession No. (bet)	Age (Month)	Gender	Breed	SV	Clinical remarks	FIV	exFeLV	enFeLV
FFV/TR/BTK12/Mia	MH351774	–	8	M	O. Tabby	V	Gingival disease	+	–	+
FFV/TR/BTK13/Webo	MH351775	–	3	M	Mix	UV	N/A	–	–	–
FFV/TR/BTK37/Dizel	MH351776	–	45	F	Mix	V	Gingivitis	+	–	–
FFV/TR/BTK44/1382	MH351777	–	22	M	Persian	UV	Gingivitis	+	–	–
FFV/TR/BTK48/Gofret	MH351778	–	14	M	Mix	UV	Fever, Weakness	–	–	–
FFV/TR/BTK51/Pala	MH351779	–	37	M	Mix	V	Gingivitis, Neurological symptoms, Intermittent fever	+	–	+
FFV/TR/BTK63/Citir	MH351780	MK305123	16	M	O. Tabby	V	Gingival lesions, Leukopenia	+	–	–
FFV/TR/BTK67/Mocha	MH351781	–	25	M	Persian	V	Gingivitis, Anorexia, Low granulocyte-thrombocyte count, Leukopenia, Lymphocytosis, Low RBC count	+	–	+
FFV/TR/BTK79/1887	MH351782	–	3	M	Mix	V	NC	–	–	+
FFV/TR/BTK87/2023	MH351783	–	3	M	Mix	UV	NC	–	–	+
FFV/TR/BTK97/Poki	MH351784	–	4	F	Mix	UV	NC	–	–	+
FFV/TR/BTK109/Hugo	MH351785	–	12	M	Mix	V	NC	–	–	+
FFV/TR/BTK119/Hiro	MH351786	–	2	M	Mix	UV	NC	–	–	+
FFV/TR/BTK126/Zeus	MH351787	–	14	M	Mix	V	NC	–	–	–
FFV/TR/BTK138/3686	MH351788	–	32	F	Siamese	UV	Gingival lesions	+	–	–
FFV/TR/BTK155/KediPgs	MH351789	–	8	F	Tabby	UV	Gingivitis	+	–	+
FFV/TR/BTK163/6518	MH351790	MK305124	12	F	Mix	V	NC	–	–	–
FFV/TR/BTK165/Pegasos	MH351791	–	3	F	Mix	UV	NC	–	–	+
FFV/TR/BTK172/286	MH351792	–	11	M	Mix	UV	NC	–	–	–
FFV/TR/BTK189/3549	MH351793	–	8	M	Mix	V	NC	–	–	–

intent of determination pre-vaccination status in Animal Hospital at Ankara University, Faculty of Veterinary Medicine, and some private veterinary clinics. During the sampling period, the individual features of all cats were recorded according to owners' declarations and clinical observations (Tables 1 and 2). Age, gender, breed, clinical and vaccination statuses were taken into consideration as risk factors in this study. As a risk factor, the statuses of where cats lived were ignored due to all of them were indoors. All examinations on animals were conducted by clinician veterinarians in accordance with the specifications in the approval forms from the owners and the academic ethical committee permission license (Ankara University Local Ethics Committee for Animal Experimentations Permission license no. 2015-17-192).

2.2. Methods

Whole blood samples were used for the proviral genome isolation using a commercial viral DNA/RNA isolation kit (Exgene Viral Nucleic Acid Isolation Kit, GeneAll, South Korea). With the aim of the diagnosis and molecular characterization of FFV infection, standard polymerase chain reactions (PCRs) were performed. The oligonucleotides (2510S/3065AS) reported by Winkler et al. (1998), targeted to the nucleocapsid (NC) domain in the overlap region between the *gag-pol* genes, were used for the diagnosis of FFV. In addition, FIV, FeLV (ex/en) and *feline infectious peritonitis* (FIP) were investigated with previously reported PCR primers (Endo et al., 1997; Polani et al., 2010; Simons et al., 2005). An amplifiable primer set of the *bet* gene region, 5′-ATGGCTTCAAAATAC CCGGAAGAAG-3′ and 5′- TTCAGAGTCAGATGACTCAGATGTTG-3′, was designed for a detailed FFV molecular assessment. The PCR cycling conditions were an initial denaturation at 98 °C for 60 s; 30 cycles of 20 s at 98 °C, 30 s at 55 °C and 150 s at 72 °C; and a final elongation at 72 °C for 5 min. After the PCR processes, amplified products were purified using a commercial PCR purification kit (Expin Purification Kit, GeneAll, South Korea). The purified products were Sanger sequenced, and the obtained sequences were confirmed by BLAST (Altschul et al., 1990) in the GenBank (Benson et al., 2012) database to confirm that the relevant gene fragments belong to FFV. The sequences of all FFV strains and some of other FV strains in GenBank were downloaded and then aligned based on the ClustalW method implemented in BioEdit (Hall, 1999). A phylogenetic analysis was performed by selecting "find best model" in MEGA 6.0 (Tamura et al., 2013). Maximum likelihood trees were constructed with 500 bootstrap replicates for all gene regions. Additionally, positive sequences were submitted to GenBank through the BankIt interface to receive an accession number (Table 2). Similarity and identity rates regarding *bet* sequences have been calculated in MatGAT 2.0 (Campanella et al., 2003).

We also statistically evaluated by comparing the results of molecular analyses and recorded individual risk factors of cats. Logistic regression analysis was performed using a computer statistical software (SPSS 22.0) for calculation of cats' relative risks. A probability value of less than 0.05 was considered significant ($p < 0.05$).

3. Results

We detected 20 positive cases by diagnosing oligonucleotides (10%, 20/200), which enabled the amplification of the product in a 456-bp length. FFV's prevalence rate was determined to be 10% among the sampled population (20/200), and the rate of cats with clinical signs

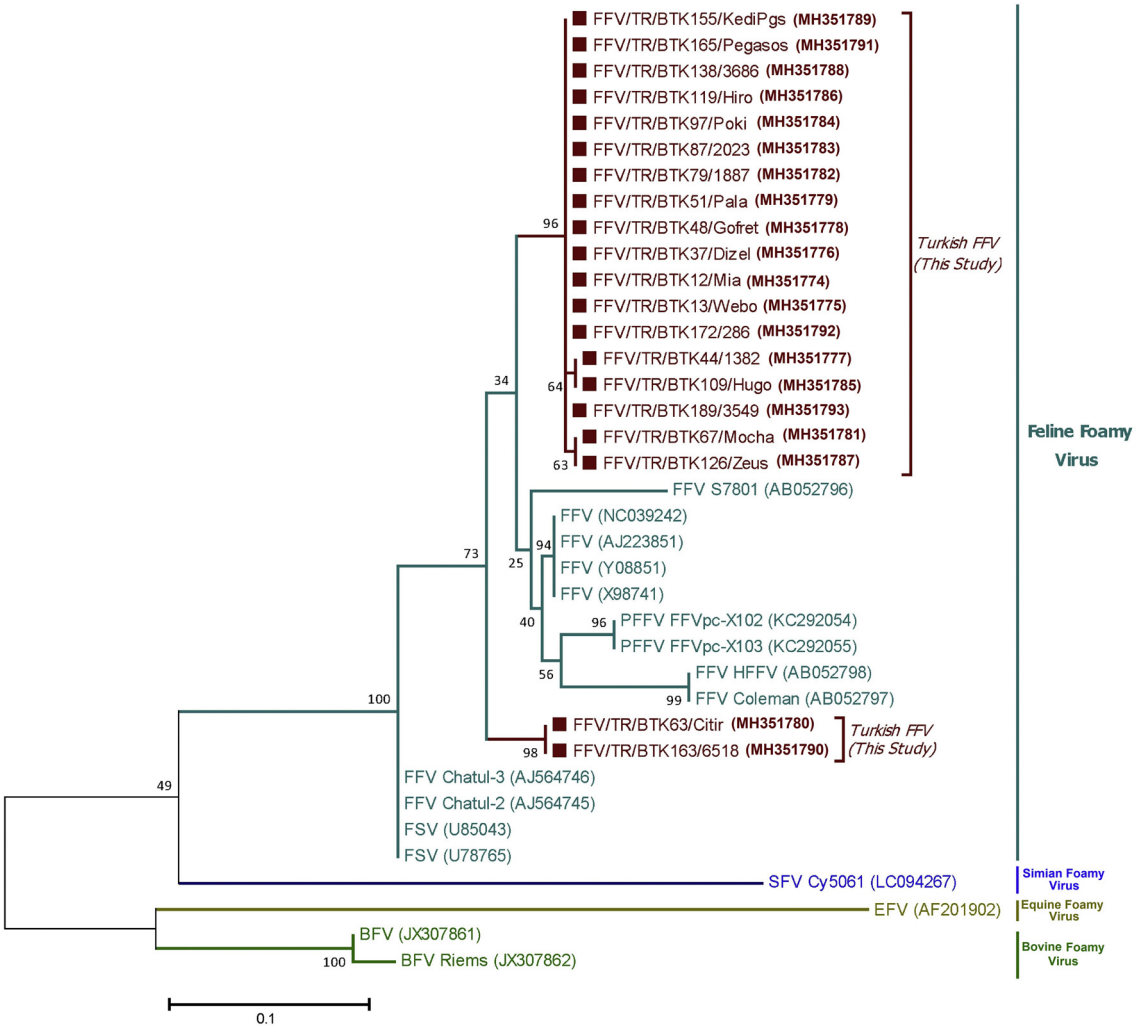

Fig. 1. Phylogenetic tree in terms of FFV partial *gag* gene region. Our sequences were marked with "▪" and their accession numbers were presented in tree. Bovine, Equine, and Simian Foamy virus sequences were determined as outgroup. The molecular evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model with 1000 bootstrap replicates. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 13.7224)).

was observed to be 5.5% (11/200) (Table 2). The BLAST result has shown a high rate of similarity (98%–99%) to the reference sequences from GenBank. In BLAST, the closest reference sequence was the FUV strain (Y08851), defined for all our sequences by Winkler et al. (1999).

In the maximum likelihood tree, the sequences in this study constituted a separate cluster, but were localized as a sister branch near the reference FFVs (Fig. 1). Only two positive reactions were detected by both primers, which recognized complete *bet* gene regions that were 1350 bp in length. According to the *bet* gene phylogenetic tree, our *bet* sequences were similar the partial *gag-pol* tree located on a separate branch (Fig. 2). In a similarity matrix table, our sequences have shown homology rates between 85.8% and 87.7% with reference FFV sequences, except HFFV (AB052798). Additionally, we found low homology rates, between 43.1% and 46.5%, among other FVs (Fig. 3).

4. Discussion

FFV is a viral agent widely spread amongst domestic and wild cats (Romen et al., 2006). It is considered apathogenic or pathogenic only under certain circumstances, therefore, the influence on the cat's health is still unclear. Unlike other feline retroviruses, Turkish veterinarian clinicians are generally unaware of the existence of FFV and there have been no reports in the literature of occurrences in Turkey. This study is the first report on the presence and epidemiology of FFV in Turkey. A remarkable positivity rate (20/200; 10%) in all population was observed in domestic cats. In all cats (n = 200), the effects of all risk factors except the "breed" factor on FFV, were found to be non-significant. The breed of FFV-infected cats is statistically significant ($p < 0.05$) and the rate of FFV infection was detected to be 70% (14/20). Mixed-breed cats were 4.3-fold more prone to FFV infection than purebred cats according to the statistical analysis. The lower prevalence rate in purebred animals may be a result of the limited numbers of animals sampled but it might also be a consequence of purebred animals often being kept as single animals in comparison to mixed-breed animals who are often housed in groups. Concerning only FFV-positive cats (n = 20), the positivity rates were detected to be higher in males than females, and the rate of 70% was statistically significant ($p < 0.05$). Interestingly, an epidemiological study by Winkler et al. (1999) found that females had high positivity rates in comparison to males in terms of FFV, which led to comments on the possible transmission of FFV between female cats living in the same habitat. Contrary to the aforementioned study, we observed that males with FFV infection are more prevalent than females. Therefore, it led to the hypothesis of a vertical transmission route by sexual intercourse. Although coinfection with FIV and FeLV was found to be statistically non-significant in this study, it might be considered as relevant in terms of molecular and

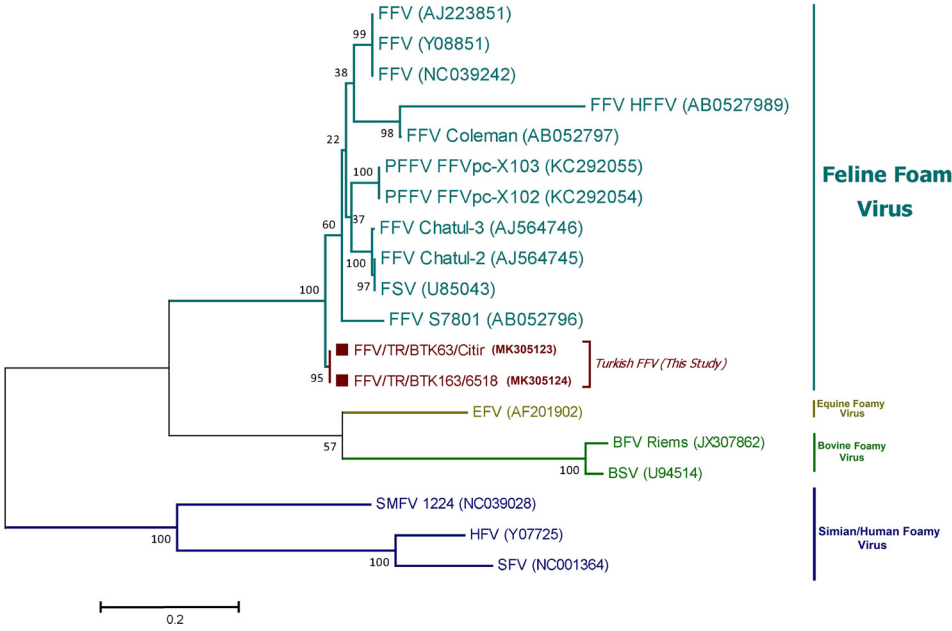

Fig. 2. *bet* gene phylogenetic tree. Our sequences were marked with "▪" and their accession numbers were presented in tree. Bovine, Equine, and Simian Foamy virus sequences were determined as outgroup. Maximum-Likelihood statistical method, Hasegawa-Kishino-Yano model and 500 bootstrapping were chosen for phylogenetic analysis.

genetic interactions ($p > 0.05$).

Interestingly, some papers on FFV pathogenesis and clinical perspectives have indicated a strong association with chronic progressive polyarthritis (100%) (Pedersen et al., 1980; Winkler et al., 1999). Moreover, *in vitro* studies have identified a negative effect of FFV on some tissues (kidney and lung), the immune system and mesenchymal stem cells of cats (Arzi et al., 2015; German et al., 2008). In this context, we investigated a possible association between the cats' clinical status and the presence of FFV. Nine out of 20 FFV-positive cats had at least one clinical symptom, mainly oral lesions or gingivitis (40%; 8/20). However, we have also detected proviral FIV DNA in whole blood samples belonging to the same cats, which might have caused the oral lesions and gingivitis. It is therefore not possible to state that FFV alone induced the described clinical signs, because the occurrence of gingivitis is a shared clinical sign between both FIV and FFV in the same eight out of 20 cats. However, FFV may trigger the course of feline retrovirus infections with clinical manifestations as prevalence rates of 40% (8/20) and 50% (10/20) for FIV and enFeLV were detected respectively in FFV-positive cats. This finding is in accordance with a previous report which emphasized the FFV could be a cofactor of FIV (German et al., 2008). In this study, we did not sample any cats with chronic progressive polyarthritis. Additionally, 11 of 20 FFV-positive cats had no clinical symptoms (55%). The lack of clinical symptoms in FFV-infected cats, despite new data on FFV pathogenesis and clinical association, has still provided further information confirming similar conclusions in other reports (German et al., 2008; Winkler et al., 1999).

In Turkey, the molecular status and epidemiological information related to feline retroviral infections are limited, and only two retroviruses, namely FIV and FeLV, have been previously investigated (Oğuzoğlu et al., 2010, 2013). Globally, the molecular phylogeny of FFV has also been ignored in many studies; thus, available molecular data on FFV based on different gene regions are extremely limited in the literature and GenBank database. Substantially different prevalence rates of FFV have been observed in the few available serological studies found in the literature; for instance, Winkler et al. (1999) detected 221 FFV-positive cats in 389 cases (56.8%), whereas in another study (Romen et al., 2006), positivity rates of FFV were determined to be 36%, 25% and 19% against *gag, env* and *bet,* respectively. Comparing the results of our molecular study with the report by Romen et al. (2006), we have found 9% against both FFV's *gag* and *bet*, suggesting that the detected prevalence rate is extremely low. The reasons for the low prevalence rate might be that our study does not involve any stray

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
			Similarity																	
1. FFV (AJ223851)			82.0	95.0	95.0	96.1	96.0	96.2	96.3	96.0	100.0	100.0	43.7	42.9	43.8	43.8	40.8	42.7	96.3	96.3
2. FFV HFFV (AB0527989)		75.1		85.8	80.4	80.8	80.8	80.9	80.9	80.8	82.0	82.0	43.0	43.2	42.7	52.6	40.6	48.7	81.1	81.1
3. FFV Coleman (AB052797)		86.5	78.8		93.7	93.9	93.9	94.1	94.2	93.9	95.0	95.0	43.8	44.0	44.1	41.9	41.1	42.7	94.3	94.3
4. FFV S7801 (AB052796)		86.5	73.9	85.2		94.8	94.8	94.7	94.8	94.8	95.0	95.0	44.5	43.2	44.7	42.7	40.6	42.4	95.2	95.2
5. FFV Chatul-3 (AJ564746)		87.6	73.8	85.4	86.3		99.7	96.0	96.1	99.7	96.1	96.1	44.4	42.9	44.1	42.8	40.7	42.0	96.2	96.2
6. FFV Chatul-2 (AJ564745)		87.5	73.8	85.4	86.3	91.2		95.9	96.0	100.0	96.0	96.0	44.9	43.6	44.4	43.0	40.8	41.7	96.2	96.2
7. PFFV FFVpc-X103 (KC292055)		87.8	74.0	85.7	86.3	87.6	87.5		99.9	95.9	96.2	96.2	44.5	44.2	43.8	42.0	40.9	43.6	95.7	95.7
8. PFFV FFVpc-X102 (KC292054)		87.8	74.0	85.7	86.3	87.6	87.5	91.5		96.0	96.3	96.3	44.5	44.2	43.7	42.0	41.1	43.6	95.8	95.8
9. FSV (U85043)		87.5	73.8	85.4	86.3	91.2	91.5	87.5	87.5		96.0	96.0	44.9	43.6	44.4	43.0	40.8	41.7	96.2	96.2
10. FFV (NC039242)	Identity	91.5	75.1	86.5	86.5	87.6	87.5	87.8	87.8	87.5		100.0	43.7	42.9	43.8	43.8	40.8	42.7	96.3	96.3
11. FFV (Y08851)		91.5	75.1	86.5	86.5	87.6	87.5	87.8	87.8	87.5	91.5		43.7	42.9	43.8	43.8	40.8	42.7	96.3	96.3
12. BFV Riems (JX307862)		44.2	43.8	44.7	44.9	45.5	46.3	45.0	45.0	46.3	44.2	44.2		96.8	49.9	44.2	42.4	43.8	45.0	45.0
13. BSV (U94514)		43.6	44.0	44.2	43.9	44.1	45.3	45.2	45.1	45.3	43.6	43.6	91.7		49.4	43.6	43.3	43.6	44.4	44.4
14. EFV (AF201902)		43.2	42.3	43.2	44.8	44.5	44.9	43.7	43.6	44.9	43.2	43.2	49.0	48.3		44.9	40.9	44.5	44.5	44.5
15. HFV (Y07725)		46.7	55.7	44.0	44.4	44.9	45.1	44.1	44.1	45.1	46.7	46.7	46.7	46.0	47.7		43.6	86.9	43.5	43.5
16. SMFV 1224 (NC039028)		43.2	42.3	43.7	43.1	43.3	43.4	43.8	43.9	43.4	43.2	43.2	44.5	44.8	42.8	43.4		43.2	40.5	40.5
17. SFV (NC001364)		44.3	51.5	45.3	44.4	43.0	42.7	44.9	44.9	42.7	44.3	44.3	46.3	46.3	47.2	84.8	43.2		42.4	42.4
▪18. FFV/TR/BTK63/Citir		87.8	74.2	85.8	86.7	87.7	87.7	87.3	87.3	87.7	87.8	87.8	44.6	44.8	44.3	46.5	43.1	44.0		100.0
▪19. FFV/TR/BTK163/6518		87.8	74.2	85.8	86.7	87.7	87.7	87.3	87.3	87.7	87.8	87.8	44.6	44.8	44.3	46.5	43.1	44.0	91.5	

Fig. 3. FFV *bet* gene based on similarity and identity matrix table. FV *bet* genes were aligned and rates were calculated using the MatGAT 2.0. Turkish FFV sequences were marked by "▪" at no.18 and 19.

cats and also each cat in the study lives in a separate house. In addition to this, we have not performed serological assays which are stated to be much more sensitive in comparison to molecular methods (Romen et al., 2006; Ledesma-Feliciano et al., 2018). Contrary to these reports, Winkler et al. (1998) claimed no significant difference in terms of emerged results between serological and molecular tests. Any commercial serological ELISA kit is not available, thus, we could not compare with serological data. Hence, virus isolation is a requirement to observe serological status for Turkish cats. This study shows preliminary data about FFV in Turkey and we assume it is valuable for the next studies. As a further investigation, we aim to isolate Turkish FFV and to reveal more accurate information by comparison of molecular and serological results.

As is known, for other feline retroviruses, FIV and FeLV prevalence rates in stray cats are higher than housed cats (Levy et al., 2008; Little, 2011). Likewise, FFV is likely to be more prevalent among stray cats in Turkey. Further studies are required to confirm this assumption. A more recent molecular study by Weissl et al. (2012), demonstrated the existence of FFV in cats with degenerative joint disease (DJD) of varying severity, with FFV nucleic acid detected in 13% (8/58) of the total population. This is an important result as it is also based on molecular data and gave a similar result to our study.

We have molecularly characterized two different gene regions, but a comprehensive comparison was not possible due to a lack of reference sequences in GenBank. In a phylogenetic tree of the partial *gag-pol* overlapping region, sequences have split into two distinct clusters for all evaluated reference sequences (Fig. 1). The two mentioned sequences have also created a divergent branch in the *bet* phylogenetic tree (Fig. 2). Additionally, homology rates for the *bet* gene have shown a remarkable divergence to the reference sequence rather than to the *gag-pol* partial gene. Thus, we propose that the *bet* gene should be considered important for molecular studies of FFV. An accessory gene, *vif,* has also been shown to play an important role in FIV pathogenesis as well as for genotyping (Yoshikawa et al., 2017). According to Yoshikawa et al. (2017), attenuation of *vif* has only been encountered in the B subtype of FIV rather than other subtypes. Likewise, *bet* has also been shown to have a similar role in impeding APOBEC antiretroviral mechanism (Löchelt et al., 2005; Ledesma-Feliciano et al., 2018). This group reported that the *bet* gene of FFV was inactivated in APOBEC-expressed CRFK cell line *in vitro*. Likewise, potential attenuation or similar actions (genetic diversifications or mutations) in *bet* might have led to negative PCR results in approximately 18 FFV sequences *in vivo*. This led us to conclude that we could not have amplified the relevant gene regions by PCR. Only two *bet* sequences were produced which were also positive by *gag-pol* overlapped region. These two sequences might belong to a putative different genotype, which accordingly might have led to the monophyletic divergent branch in the gag-pol overlapped tree. This potential diversity in the genetic composition of FFV might trigger different pathogenesis and clinical observation of infection in cats. In other words, the *bet* gene might be a key-factor for molecular status determination of FFV. Further analyses need to be performed to understand *bet* gene influence on host cells during the pathogenesis of FFV.

This is the first report showing the presence and prevalence of FFV in Turkey. In the statistical analysis, we observed FFV infection among Turkish cats at significant levels which has not been described in previous studies. A comprehensive comparison study also needs to be performed on all significant viral agents to enable the most accurate evaluation of potential co-infections or -factors. We recommend, based on our data that FFV, in addition to FIV and FeLV, should also be considered in suspected clinical cases during clinical examinations and laboratory diagnoses. FFV has been developed as a potential candidate for gene therapy and a vaccine vector against cancer, autoimmune disease and other infectious diseases due to its non-pathogenic status (Ledesma-Feliciano et al., 2018; Schwantes et al., 2003). Despite these beneficial features, there is a need to consider the current biosafety, efficacy and applicability status of FFV, because it may lead to unpredictable infection in the long term in both cats and other mammalian hosts in terms of differences in genetic composition. Further multidisciplinary (pathological, virological, epidemiological and clinical) studies of FFV could clarify the effects on cats and public health. Additional molecular data for FFV are required to understand potential associations in terms of its clinical, pathological and geographical statuses.

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Author contributions

BTK and TÇO have equally contributed to all sections of this study.

Compliance with ethical standards

All examinations in cats were conducted by specialist veterinarians under the specifications in the approval form from owners' and academic ethical committee permission license (Ankara University Local Ethics Committee for Animal Experimentations Permission license no. 2015-17-192). This study does not contain any studies with human participants or experimental animals performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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