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## Does endogenous feline leukemia virus occur as a risk factor?: A molecular characterization study from Türkiye

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**ABSTRACT:** Feline leukemia virus (FeLV) is a feline retrovirus that causes various effects on cat health. FeLV, along with other retroviruses, has altered in terms of molecular structure and pathogenetic and clinical status due to integration into the host genome. In this study, we aimed to determine the presence of enFeLV in indoor cats and provide a comparison with potential exFeLV prevalence. Additionally, we aimed to investigate the relationship between positive cases of enFeLV and risk factors including age, gender, and breed. We collected 200 samples from “healthy” or diseased domestic cats in Türkiye for molecular diagnosis and characterization of en- or exFeLV. Amplified products were purified and sequenced using the Sanger method. According to the phylogenetic tree, our sequences constituted two main clusters that were divergent from each other in Group-2 enFeLVs. The “Health status” unit in the overall population comprised 161 healthy and 39 diseased cats according to clinical diagnosis. We found to be positive in terms of enFeLV in 11 (11/161; %6.8) healthy cats whereas, in diseased cats, 17 were found to be enFeLV positive (17/39; 43.6%). “Gender”, “age”, and “breed” were not found to be risk factors for the presence of enFeLV among domestic cats in this study. With regard to the outcomes of the study, we submit that both variants of FeLV should be tested prior to initiating a vaccination program.

**Keywords:** domestic cats; endogenous; exogenous; feline leukemia virus; molecular characterization; Türkiye

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## INTRODUCTION

Feline leukemia virus (FeLV) is the earliest-discovered feline virus and causes significant health problems, especially in the immune and hematopoietic systems. Taxonomically, FeLV belongs to the genus *Gammaretrovirus* in the subfamily *Orthoretrovirinae* of the family *Retroviridae* (Hoover and Mullins 1991; Hartmann 2012). The genome consists of the main gene regions of retroviruses, which are *gag*, *pol*, and *env*, and two *LTR gene regions* that flank structural gene regions on both termini (Berry et al. 1988). Two variants for FeLV have been described, which are designated exogenous and endogenous feline leukemia virus (exFeLV and enFeLV) according to their molecular and pathogenetic dynamics (Roca et al. 2005; Menéndez-Arias 2010; Polani et al. 2010; Stewart et al. 2011; Anai et al. 2012; Krunic et al. 2015; Ledesma-Feliciano et al. 2018; Powers et al. 2018).

ExFeLV is defined as a horizontally transmissible and more pathogenic variant of FeLV that has been associated with clinical signs (Jarrett et al. 1978; Neil et al. 1991; Luring et al. 2001; Dunham and Graham 2008). EnFeLV is defined a replication-defective variant of FeLV and a genetic remnant that has been integrated into the systemic genome of felines. Some studies assumed that the genome fragment of enFeLV completes the puzzle instead of acting as the lost piece for the pathogenetic mechanism of exFeLV. Therefore, molecular dynamics of enFeLV have been considered in the patho-clinical observation of FeLV (Polani et al. 2010; Stewart et al. 2011; Anai et al. 2012; Krunic et al. 2015; Powers et al. 2018).

To our best knowledge, there is a lack of sufficient molecular data on enFeLV in Türkiye (Muz et al. 2021). Therefore, we intend to investigate the molecular status of FeLVs in domestic cats in Türkiye and distinguish exFeLV and enFeLV based on the *LTR gene region* using a diagnostic approach. Interpretation of a potential relationship between individual features and the data is another aim of this study.

## MATERIALS AND METHODS

In the current study, veterinary clinicians collected whole blood samples from 161 healthy-looking domestic cats and 39 clinically diseased domestic cats in several veterinary clinics throughout Türkiye between August 2016 and May 2017. All practices on cats were performed with the permission of their own-

ers under an ethical statement approved by the local ethical committee (Ankara University Local Ethical Committee for Animal Experiments, no: 2015-17-192). Individual features and risk factors (Table 1) for cats were recorded according to declarations by the owners and clinician veterinarians.

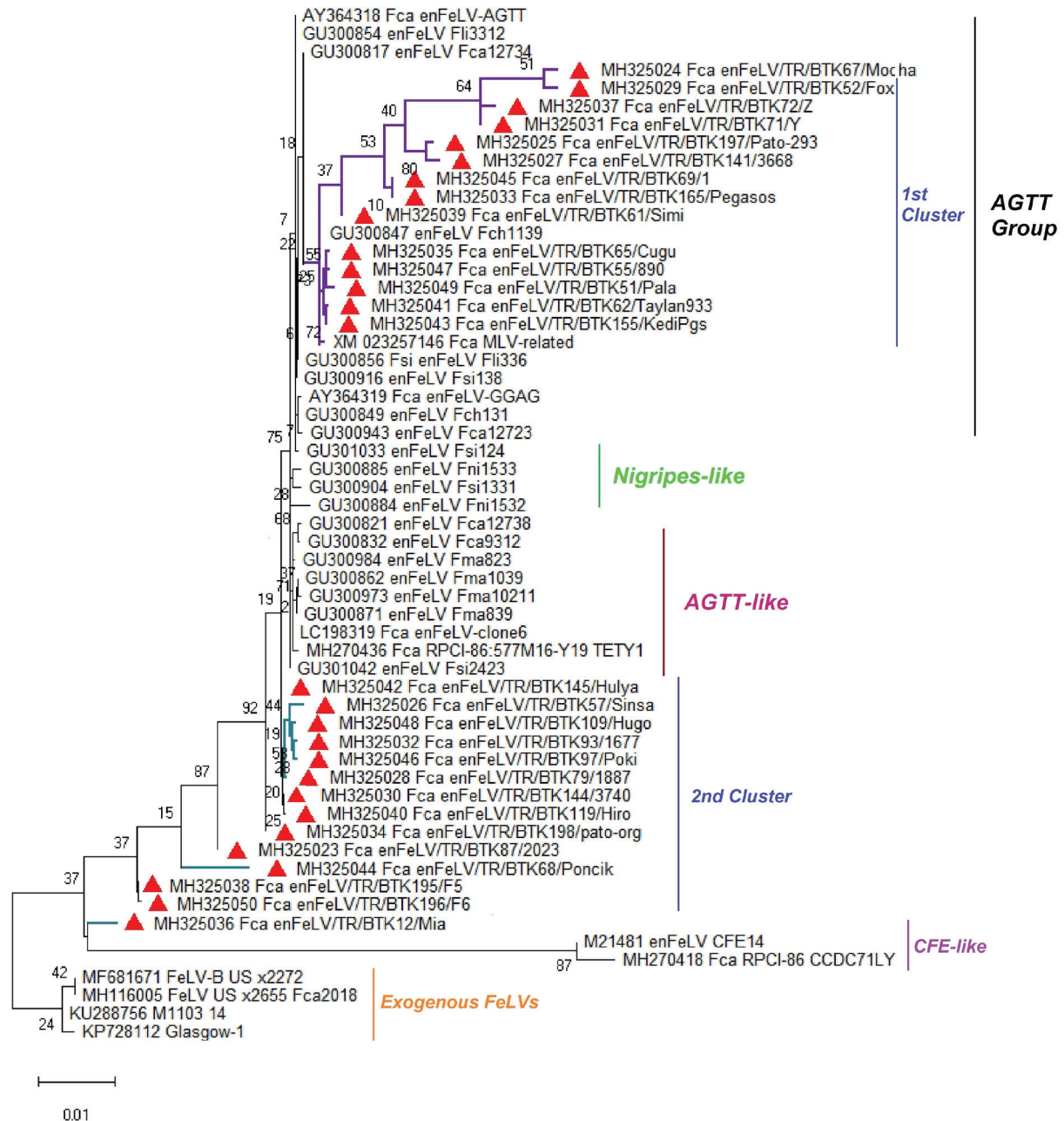
We isolated viral RNA/proviral DNA using a commercial total RNA/DNA isolation kit (*GeneAll® Exgene™ Viral DNA/RNA kit*, Seoul, S. Korea). We conducted a molecular investigation by conventional polymerase chain reaction with the primers designed previously elsewhere (Polani et al. 2010; Roca et al. 2005). Amplified PCR products were sequenced by using the Sanger method for confirmation and phylogenetic evaluation. We cleaned raw data obtained from the sequencing process using commercial histogram tracing software (Finch TV, Geospiza, Inc., Seattle, Washington, USA). After editing, we conduct CLUSTALW multiple alignment with reference strains from GenBank. A phylogenetic tree was constructed according to the Maximum Likelihood (ML) method, Kimura-2 parameters, and 1000 bootstrap replicates in MEGA 10.0 software (Kumar et al. 2020).

Recorded information about cats and the outcomes of the study were also statistically analyzed. Multivariate Logistic regression analysis method implemented in SPSS (SPSS v19.0, Chicago, IL, USA) was used to examine the relationship between risk factor units and enFeLVs. A probability value of less than 0.05 was considered significant ( $P < 0.05$ ).

## RESULTS

We did not detect any amplified products associated with exFeLV by cDNA or proviral DNA, whereas enFeLV-related sequences were obtained in 28 samples (28/200; 14%), 11 from “healthy” and 17 from clinically diseased domestic cats (Table 1).

Molecular assessments of enFeLVs were provided by Maximum-Likelihood method in the phylogenetic tree. According to the demography of the tree, our sequences constituted two main clusters that were divergent from each other in Group-2 enFeLVs. Following the method used by Polani et al. (2010) for cluster classification with genotyping, we found that one group of sequences was located among the enFeLV-AGTT group, while the other group was closely located near the enFeLV-AGTT-like group. Representative exFeLVs from GenBank diverged from enFeLVs in the phylogenetic tree (Figure 1).



**Figure 1.** Maximum Likelihood (ML) phylogenetic tree. The tree was constructed according to Kimura-2 parameter model and 1000 bootstrap replicates in MEGA X. Our sequences are marked with (▲). Two main clusters occurred, which are annotated as 1<sup>st</sup> and 2<sup>nd</sup> Cluster. Accession numbers are presented in taxon names.

Regarding recorded features and risk factors of cats, two out of five factors - health and vaccination status - were efficient for enFeLV occurrence. The unit of “Health status” in the overall population comprised 161 healthy and 39 diseased cats according to clinical diagnosis. In diseased cats, 17 were found to be enFeLV positive (43.6%;  $P < 0.05$ ). Regarding vaccination status, this factor showed an inverse rela-

tionship with disease occurrence. Interestingly, clinically diseased cats that had been vaccinated against any pathogens had a higher positivity rate of enFeLV compared with non-vaccinated cats (30%;  $P < 0.05$ ). Percentages and statistical rates are shown in Table 1. “Gender”, “age”, and “breed” were not found to be risk factors for the presence of enFeLV among domestic cats in this study.



**Table 1.** enFeLV positivity rates according to risk factor evaluation.

<i>Risk Factors</i>	<i>enFeLV</i>
<b>Gender</b>	
Female(n=95)	12 (%12.6)
Male (n=105)	16 (%15.2)
p-value	>0.05
<b>Age (month)</b>	
0-6 (n=102)	8 (%7.8)
7-12 (n=44)	8 (%18.2)
13-48 (n=50)	10 (%20)
>48 (n=4)	(%50)
p-value	>0.05
<b>Breed</b>	
Pure (n=15)	7 (%46.6)
Mix (n=185)	21 (%11.3)
p-value	>0.05
<b>Clinical status *</b>	
Healthy (n=161)	11 (%6.8)
Diseased (n=39)	17 (%43.6)
p-value	<0.05
<b>Vaccination *</b>	
Vaccinated (n=60)	18 (%30)
Non-vaccinated (n=140)	10 (%7.1)
p-value	<0.05

\*Risk factors that are statistically significant with regard to the presence of enFeLV.

## DISCUSSION

FeLV is one of the most dynamic viral agents in cats based on molecular co-evolution and host interaction. In many recent studies regarding the presence and prevalence of FeLV around the world, it has been emphasized that FeLV strains have not led to clinical diseases to the extent that they did previously (approximately three decades ago) due to the integrative evolutionary status of FeLV. Recently, enFeLV has been found to be far more prevalent than exFeLV in domestic and wild cats. Several reports have therefore focused on enFeLV and its molecular dynamics due to its integrative pathogenetic mechanism to exFeLV (Tandon et al. 2008; Hartmann 2012, Powers et al. 2018). There is not yet sufficient knowledge regarding what triggers effects in the host genome and the type of effect that may be seen. Molecular studies on ex- and enFeLVs are especially important to provide insight to clarify pathogenesis and evolutionary dynamics.

To date, previous studies have substantially focused on the prevalence and clinical status of FeLV in *Türkiye*. There is only a recent study that reports characterization of enFeLVs based on pol gene (Muz

et al. 2021). LTR is more considerable to differentiate exFeLV and enFeLV, therefore, unverified pol gene data have restricted comparison with our sequences. Although prevalence rates have been determined to be ranging between 0-20%, exact outcomes have not been explored due to enFeLV having been overlooked in previous studies in *Türkiye* (Yilmaz et al. 2001; Yüksek et al. 2005; Erol and Pasa 2013; Oğuzoğlu et al. 2013). Comparison of output in this study with previous results from *Türkiye* was not possible. We performed phylogenetic tree demography according to classification by *Polani et al.* (2010). Our sequences were gathered in two main clusters; one cluster referred to as “1st Cluster” in the phylogenetic tree was located in the enFeLV-AGTT group, while the others irregularly constituted a group designated “2nd Cluster” (Figure 1). Six cats (enFeLV/TR/BTK79/1887, enFeLV/TR/BTK87/2023, enFeLV/TR/BTK109/Hugo, enFeLV/TR/BTK97/Poki, enFeLV/TR/BTK119/Hiro, enFeLV/TR/BTK145/Hulya) infected with enFeLV had no clinical signs and participated in the AGTT-like group, excluding one in the phylogenetic tree (Figure 1). Accordingly, the authors of a recent study of multiple infections in cats reported that non-progressive co-infection of a chronic viral infection and enFeLV was detected in two-thirds of cats in a colony (Powers et al. 2018). In our assessment, the sequences that belonged to “2nd Cluster” might likely be recombinant strains of FeLV-B.

We detected env-LTR products associated with enFeLV in 16 male and 12 female cats. There were no significant differences between male and female cats regarding the risk factor “Gender” ( $P > 0.05$ ). “Age”, “Breed”, and “Gender” factors have been discussed in some previous studies; however, there is no specific knowledge as to whether these factors are efficient in predicting the prevalence and frequency of enFeLV in *Türkiye* (Yilmaz et al. 2001; Yüksek et al. 2005; Erol and Pasa 2013; Oğuzoğlu et al. 2013). Our study presents preliminary data that can be compared for novel extensive studies. In statistical analyses, 17 out of 39 clinically diseased cats were found to be enFeLV positive (43.6%), and this finding was significant ( $P < 0.05$ ). Accordingly, previously reports have highlighted that enFeLV might induce a predisposition to exFeLV and other chronic viral infections (Liu 2016; Powers et al. 2018). *Tandon et al.* (2008) reported a decrease in copy number of enFeLV during experimental exFeLV infection, as detected by quantitative PCR (qPCR). The authors performed qPCR that targeted the U3 region of LTR to distinguish endogenous

and exogenous variants. In our study, we similarly targeted differences in the U3 region and our results support the findings from *Tandon et al.* (2008).

Globally, many studies have explored the prevalence or frequency of FeLV; however, there is no sufficient knowledge to specifically reveal the epidemiological status of enFeLV (Yilmaz et al. 2001; Yuksek et al. 2005; Anai et al. 2012; Erol and Pasa 2013; Oğuzoğlu et al. 2013; Krunić et al. 2015; Powers et al. 2018). As previously emphasized, the reason for this might be that both FeLV and enFeLV are in high homology and convertible to each other (Chiu et al. 2021; Erbeck et al. 2021). Epidemiological data on enFeLV would likely be accessible after determining the exact genomic mechanism between exogenous and endogenous variants. Env-pol-gag gene regions mostly were used to be targeted for diagnosis and characterization of en- and exFeLV until the last decade. In recent years, owing to increased molecular analysis, LTR is defined as the region that reveals dissimilarities among strains, particularly between en- and exFeLVs (Chiu et al. 2020; Polani et al. 2010; Stewart et al. 2011; Tandon et al. 2008). We, thus, chose the U3 domain of LTR to strictly detect and discriminate FeLV types. In this study, we found to be 14% positivity rate among the sampled cats. Our study might possess a limitation regarding the characterization of larger gene regions belonging to FeLV. However, if it is considered gene fragments might have been embedded in different lengths, further next-generation sequencing, and viral metadata analysis would provide more accurate evaluations in terms of viral integration sites of enFeLVs into the host genome.

Vaccines might be another important risk factor for our study, likewise, that were claimed before (Hofmann-Lehmann et al. 2007; Miyazawa et al. 2010). Knowledge of vaccines performed has not been applicable enable for each domestic cat; thus,

we have not specifically discussed the relationship between vaccines and enFeLVs. However, a study in 2010 from Japan implied that some commercial vaccines for cat health have included endogenous retrovirus, which may trigger some progressive diseases (Miyazawa et al. 2010). Coinciding with this prediction, we have detected a remarkable positivity rate (14/28, 50%) of enFeLV in vaccinated cats. The cell cultures originating from felines might act in transmission of enFeLV in the vaccination production process. To address this risk, cell cultures used to produce vaccines should be checked for endogenous retroviral elements, especially enFeLVs. Additionally, cats that are suspected of having retroviral infection should be tested before beginning a vaccination program in clinics.

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## DATA AVAILABILITY

Sequence data that support the findings of this study have been deposited in “GenBank” with the primary accession codes ranging from “MH325023” to “MH325050”.

## CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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