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Prevalence and genetic characterization of feline leukemia virus in portuguese stray cats

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Abstract

Background With its large stray population, Portugal had the highest prevalence of the Feline Leukemia Virus (FeLV) infection in Europe. Progressive infection is still high, especially in southern Europe. In addition to the viral load, the subgroup is a determining factor in the development of the various syndromes associated with this disease, which can be fatal, justifying the morbidity and mortality of this retrovirus. The aim of the study was to determine the prevalence of FeLV, the viral load and to identify the subgroup of circulating virus in stray cats in the Lisbon metropolitan area. This prospective study was conducted at the Veterinary Teaching Hospital of the FMV-ULusófona, from November 2022 to January 2023. Blood samples from 129 stray cats from the Lisbon metropolitan area were included. All samples were tested for p27 antigen detection and proviral DNA by qPCR. For the detection of FeLV subgroups (A, B and C) nested PCR was performed.

Results The results showed that at the time of enrollment, 8.5% ($n=11/129$) of the cats tested positive for FeLV using p27 antigen and qPCR for proviral DNA. Considering the proviral DNA load, 90.9% ($n=10/11$) of the FeLV infections were classified as "likely progressive infection" ($>4.0\times10^5$ copies/mL). In the remaining animal, the FeLV infection was classified as "likely regressive infection" ($\leq 4.0\times10^5$ copies/mL). In this study, the most common FeLV subgroup was a combination of AB (54,5%, $n=6/11$), followed by FeLV-A (45,5%, $n=5/11$). FeLV-C subgroup was not detected.

Conclusions To the best of our knowledge, this is the first study to research FeLV subgroups in Portugal. The prevalence of FeLV in this group was consistent with our expectations. However, the molecular characterization differed significantly from that observed in other countries. These results suggest that stray cats may not be the main epidemiological concern.

Keywords Subgroup classification, FeLV, Viral loads, Phylogenetic diversity, Southern Europe, Virus reservoir, Stray cats

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Background

Since its discovery by William Jarrett in 1960, the Feline Leukemia Virus (FeLV) has been considered to be the most pathogenic, causing a variety of neoplastic and degenerative diseases [1]According to the PAN-European prevalence study published in 2019, Portugal has the highest prevalence of FeLV in Europe (8.8%, compared to a European average of 2.3%). This is thought to be due to the high, and increasing, stray population, combined with outdoor access and intact male cats, as well as low vaccination rates [2].

The prevalence of FeLV in populations of stray cats has been the subject of extensive study. Most of these studies have used point-of-care (PoC) serology tests as was the case in the three studies of Portuguese stray cats conducted over a decade ago [3–5]. European studies on stray cat populations have recently been published, with larger sample sizes and/or molecular characterization of the FeLV [2, 6–9] However, the study of subgroups has rarely been studied along with the quantification of viral load [10, 11]. To the best of our knowledge, this has never been done specifically for stray cats.

The problem of stray cats as a reservoir and a risk factor for FeLV infection is undeniable. Households with several cats, shelters and outdoor access also pose risks, as FeLV is transmitted through social interactions where immunity status and age play an important role too [9, 12]. Like any retrovirus, it is challenging to understand clinically, and its evolution is unpredictable and often frustrating. It has been recognized that this unpredictability is directly related to the FeLV subgroups, infection pressure and viral load in a cat with an immune status and age playing a role for or against [1, 8, 13–17].

Without an effective host immune response, the viral RNA genome is reverse-transcribed and randomly integrated into the host genome following infection. Once the provirus is integrated, cell division produces new cells that also contain viral DNA. The ability of the virus to become part of the host's own DNA is crucial for the virus's persistence throughout life. All cats have non-pathogenic endogenous FeLV (enFeLV) sequences in their genome which seem to have become part of the feline genome prior to the speciation of the Felis genus [18] Although enFeLV sequences do not induce disease in the host, they are highly relevant to FeLV biology, because the recombination of exogenous FeLV (exFeLV) with endogenous sequences (enFeLV) allows the emergence of variants with greater pathogenicity [14].

The propensity of the virus to interact with endogenous elements of the feline genome is related to the existence of different FeLV subgroups. Each subgroup has unique characteristics of infection, replication and viral transmission that significantly affect the outcome of the disease. The different FeLV subgroups are genetically distinct, bind to different cell receptors, but are immunologically similar [2, 18]. Their identification and characterization are carried out using PCR and Sanger sequencing. There are currently seven FeLV subgroups. The three best known, most studied and most common FeLV subgroups are FeLV-A, FeLV-B and FeLV-C [19, 20] Four additional FeLV subgroups have recently been discovered: FeLV-D, FeLV-E, FeLV-T and FeLV Env (TG35-2) [1, 21, 22]. The epidemiological and clinical significance of these last four subgroups is still not fully understood [23–26].

Directly related to the different FeLV subgroups with different levels of pathogenicity and subsequent clinical manifestations, three different syndromes can be induced: immunosuppression, proliferative disease (neoplasms) and degenerative disease (anemias and leucopenia) [1, 18]. FeLV-A is the least pathogenic but most contagious subgroup and is present in all naturally infected cats [18]. FeLV-B results from the recombination of FeLV-A envelope gene (*env*) sequences with enFeLV elements and is associated with the occurrence of lymphomas and leukemias, diseases with high morbidity and mortality rates. However, its transmission between cats has not been demonstrated [21, 22] The FeLV-C subgroup results from mutations in the *env* gene of FeLV-A, associated with non-regenerative anemia [6, 12, 17, 19]. The FeLV-T subgroup is a cytopathic virus, with special tropism for T lymphocytes. It was isolated from a cat with acquired immunodeficiency syndrome (FeLV-FAIDS) caused by mutation of FeLV-A strains [22, 24, 26, 27].

Although recombination and mutation events at the origin of the different FeLV subgroups lead to increased pathogenicity, FeLV variants are associated with a decreased capacity for horizontal transmission, which requires FeLV-A as a *helper virus* [22, 24]. At present, phenotypic testing by interference assays is the only method to distinguish between the subgroups - FeLV-A, -B, -C, -E and -T - and may eventually be improved by molecular analysis of the SU gene [22].

In addition to the subgroups, another important factor influencing the course of infection, is the ability of the immune system to cope with FeLV of different virulence, pathogenicity and infectious viral load, resulting in different outcomes: [1, 27] (1) Abortive infection - replication occurs only in oropharyngeal tissue; healthy cats with a robust immune system eliminate the virus shortly after infection, with negative results for PoC capsid protein p27 (PoCp27); Indirect Fluorescent Antibody Assay (IFA) (-); PCR (-); antibodies (+); (2) Regressive infection (transient viremia) - partial immune response following virus integration into organ and/or bone marrow DNA (provirus), with discordant PoCp27 and PCR results, weak viral shedding, and low viral load; (3) Progressive infection

- occurs in cats with an ineffective immune response, characterized by the presence of the virus in all lymphoid tissues, glandular tissues, mucous membranes, etc., with positive results for PoCp27, IFA, and PCR, and viral loads tending to be high [13, 14, 17, 28]. It is estimated that approximately 5% of cats follow a focal (atypical) course of infection, characterized by antigenemia without viremia [14] This is due to the cat's immune system sequestering viral replication in specific tissues, such as the spleen and lymph nodes [1].

The determination of proviral and viral loads, in conjunction with the correlation of these values with those of the FeLV subgroups and their respective tissue tropisms, will facilitate a deeper understanding of the pathogenesis of this virus [11, 13, 28]. To the best of the authors' knowledge, there has never been a study into the prevalence of FeLV subgroups (associated with viral loads) in Portugal.

The aim of this study was to determine the prevalence of FeLV in stray cats in the Lisbon metropolitan area. Additionally, we aimed to identify FeLV in the cat genome and proceed with the genetic characterization and determination of the viral and proviral load present in this population.

Methods

A cross-sectional study was carried out as part of a protocol of the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine - Lusófona University (UL) to support animal protection and care associations in a trap-neuter-return system for stray cat colonies. The stray cats included in the study ($n = 129$) were randomly captured in the Lisbon metropolitan area, between November 2022 and January 2023.

The stray cats were found to weigh a minimum of 1.5 kg, which was assumed to be indicative of an age of over 16 weeks [29]. They were thus classified as kitten (over 16 weeks and less than 1 year), young adult (1–6 years old), and adult (7–10 years old). However, it was not possible to specify the subcategories of mature adult, senior (over 10 years), or super senior (over 15 years) according to the 2021 AAHA/AAFP Feline Life Stage Guidelines [30].

The physical characteristics of the cats were recorded, such as tail features, coat type, ear cropping, and tabby coat patterns of various colors, with brindle and short coats being the most common. The majority of the cats were phenotypically close to Common European breed. Additionally, age, gender, reproductive status, body condition score according to the WSAVA Nutritional Assessment Guidelines [31], external signs of illness, and pre-anesthetic blood tests were conducted. These tests included a hemogram ($n = 61$) and measurement of serum glucose ($n = 91$) and total proteins ($n = 60$), as well as chest radiology if the auscultation or breathing pattern were abnormal during the pre-anesthetic examination.

Parasitic pneumonia is a common cause of respiratory abnormalities in stray cats, so all cats with abnormal respiratory sounds on pre-anesthetic assessment underwent chest radiography. The samples were collected from all sedated cats during the initial phase of the anesthetic protocol as part of the neutering process. Using opportunistic sampling, blood and serum samples were used for FeLV serological screening with the PoCp27. The entire process of sampling and neutering was carried out by the same team, which also completed the database for each of the 129 cats. All the procedures were performed in accordance with animal research standards.

Detection of p27 antigen

Cats were tested for p27 antigen using a point-of-care ELISA test (SNAP® FeLV Antigen Test, IDEXX Laboratories, Inc., Westbrook, ME, USA) on EDTA-anticoagulated whole blood, following the manufacturer's instructions.

All samples with a positive serologic test were subjected to the following molecular analyses: FeLV RNA and DNA qPCR, FeLV genetic characterization including subgroups identification and detection of Mycoplasmas Haemo Group in FeLV (+) samples.

DNA and RNA extraction

Detection and quantification of proviral and viral loads were carried out on DNA and RNA extracted from EDTA whole blood of animals with positive PoC results.

DNA and RNA were extracted using the appropriate extraction kits (Nzytech, Lisbon, Portugal) following the manufacturer's recommendations. The DNA and RNA concentrations were quantified by spectrophotometry (Spex NanoSNAP™ Microvolume Spectrophotometer) and subsequently stored at – 80 °C until use.

Evaluation of the quality of DNA extraction by PCR

DNA samples were subjected to conventional PCR to detect the endogenous GAPDH enzyme (glyceraldehyde 3-phosphate dehydrogenase) using previously described primers (Table 1) [32]. Each reaction contained a final concentration of 500 nM of each primer with 12.5 µL of GoTaq® G2 Hot start Green Master Mix, 2X (Promega, USA), 2 µL of DNA and ultrapure water DNase and RNase free (Promega, USA) in a final volume of 25 µL. The target gene was amplified on a Biometra gradient cycler (Analytik Jena, Germany) under the following conditions: Initial denaturation for 2 min (min) at 95 °C followed by 35 cycles of 94 °C for 1 min, 56 °C for 1 min s, 72 °C for 1 min, and a final extension of 72 °C for 5 min. PCR products were visualized by agarose gel electrophoresis.

Table 1 Primers used in this study

Name	Nucleotide Sequence (5'-3')	Amplicon Size (bp)	Target gene	Reference
GAPDH-F	CCTTCATTGACCTCAACTACAT	282	Housekeeping (GAPDH)	33
GAPDH-R	CCAAAGTTGTCATGGATGACC			
H18	ACATATCGTCCTCCTGACCAC	2350	(U3) LTR/*env*	36–39
H20	GAAGGTCGAACTCTGGTCAACT		*pol/env*	
RB59	CAATGTAAAACACGGGGCAC	1071	FeLV-A -*env*	40,41
RB17	TAGTGATATTGGTTCTCTTCG			
RB53	ACAACGGGAGCTAGTG	856	FeLV-B-*env*	40,41
RB17	TAGTGATATTGGTTCTCTTCG			
RB58	AGATCTTGGGCACGTTATTCC	1755	FeLV-C-*env*	40,41
RB47	TTGTGAAATGGCATTGCTGC			

Detection and quantification of FeLV proviral DNA and RNA by qPCR and RT-qPCR

The detection and quantification of FeLV proviral DNA and viral RNA were performed on Rotor-Gene™-Q (Qiagen, USA) following the manufacturer's instructions of FeLVp dtec-qPCR and FeLV dtec-RT-qPCR Kits (Genetic PCR Solutions™, Spain). Standard curves were prepared by dilution of the standard template provided in the kits.

According to new guidelines and recent studies, cats with a PoC positive result suggesting FeLV infection can be classified as "likely" regressive infection with FeLV viral DNA loads qPCR $\leq 4 \times 10^5$ copies/mL and "likely" progressive infection cats with FeLV viral DNA loads qPCR $> 4 \times 10^5$ copies/mL [1, 13, 28, 33]. It is important to note that since there were no quantified antibodies in the samples and it was a one-off test, it was not possible to repeat the test over time, ideally with a minimum interval of six weeks. It should be noted that a single, isolated test cannot accurately determine the course of FeLV in a cat, and any possible abortive or focal stages were not tracked [13–15, 17, 28].

Nested PCR for FeLV subgroups (A, B and C)

FeLV subgroups were characterized using DNA extracted from the EDTA whole blood of the same animals with positive PoC results. A nested PCR was conducted to detect the FeLV subgroups A, B and C. In the first step, a pair of oligonucleotides were used, which recognized the junction sequence between the pol/env genes (start codon) and the sequence in the U3-long terminal repeat (LTR) region (Table 1) [34–37]. Each reaction contained a final concentration of 500 nM of each primer with 12.5 µL of GoTaq® G2 Hot start Green Master Mix, 2X (Promega, USA), 1 µL of DNA and ultrapure water DNase and RNase free (Promega, USA) in a final volume of 25 µL. The target gene was amplified on a Biometra gradient cycler (Analytik Jena, Germany) under the following conditions: Initial denaturation for 2 min at 94 °C followed by 35 cycles of 94 °C for 1 min, 61 °C for 1 min, 72 °C for 2 min, and a final extension of 72 °C for 5 min. PCR products were visualized by agarose gel electrophoresis.

In the second step, one microliter of the first reaction product and specific oligonucleotides for each subgroup were used. RB59 and RB17 were used for FeLV-A, RB53 and RB17 were used for FeLV-B, and RB58 and RB47 were used for FeLV-C (Table 1) [38, 39]. Each reaction contains a final concentration of 500 nM of each primer with 12.5 µL of GoTaq® G2 Hot start Green Master Mix, 2X (Promega, USA) in a final volume of 25 µL. Target sequences were amplified on a Biometra gradient cycler (Analytik Jena, Germany) under the following conditions: Initial denaturation for 2 min at 94 °C followed by 40 cycles of 94 °C for 1 min, 44ºC (FeLV - A) or 50ºC (FeLV- B) or 52 °C (FeLV - C) for 1 min, 72 °C for 2 min, and a final extension of 72 °C for 5 min. PCR products were visualized by agarose gel electrophoresis.

Purification and DNA sequencing

Purification of amplified products was performed using appropriate kit (Nzytech, Portugal), following the manufacturer's instructions and sent to an external laboratory for Sanger sequencing (StabVida, Portugal).

Sequencing and phylogenetic analysis

The resulting sequences were edited and aligned using the *Clustal W* program from the MEGA™ software package, version 11.0.13 for Windows® [40]. The sequences obtained for the FeLV-A and FeLV-B subgroups were compared to highly similar sequences with 95–100% identity from the GenBank database (National Center for Biotechnology Information - NCBI) using the Basic Local Alignment Search Tool (BLAST®) web interface (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree was conducted in the MEGA™ software package, version 11.0.13 for Windows®, using the *Neighbor-Joining (NJ)* method [41] and Kimura 2-parameter method [42]. The significance of the clusters was estimated by analyzing 1,000 bootstrap replicates [43].

Detection of Mycoplasmas Haemo group by qPCR

Additionally, the detection by qPCR for Mycoplasmas Haemo Group (*Mycoplasma haemofelis*, *Candidatus Mycoplasma haemominutum* and *Candidatus Mycoplasma turicensis*) were performed on Rotor-GeneTM-Q (Qiagen, USA) following the manufacturer's instructions of MycHG dtec-qPCR (Genetic PCR Solutions™, Spain), *Mycoplasma haemofelis* and *Candidatus Mycoplasma haemominutum* real-time PCR kits (Genesig®, Spain).

Statistical analysis

Statistical analysis was conducted using a sample size of 129, with a 95% confidence level., a 5% margin of error, and an estimated prevalence of 8% from existing literature [2] to assess the prevalence of FeLV. Using R (version 4.4.1) and the epiR package (version 2.0.76), an initial sample size of 114 was calculated using the formula by Kelsey et al. (1996) and increased by 12.5% to account for potential losses. A bootstrap simulation with 1000 resamples established a 95% confidence interval for the prevalence. Descriptive and inferential analyses were performed using SPSS (version 30.0.0). Categorical variables were assessed with Fisher's Exact Test, while non-parametric methods, such as the Mann-Whitney and Kruskal-Wallis tests, were used for continuous variables due to non-normality. Trimmed means with confidence intervals were reported for skewed data. Bonferroni-adjusted pairwise comparisons were conducted to identify significant relationships. Statistical significance was considered when $p < 0.05$.

Results

Frequency of feline samples positive for FELV

Of the 129 FeLV SNAP tests performed, 8.5% ($n = 11$) were positive and 91.5% ($n = 118$) were negative. Thus, the absolute prevalence of FeLV in this sample is 8.5% (CI 95%: 4.7-13.2%).

Clinical characterisation of the sample

In the kitten and young adult group ($n = 119$), 10 cats (7.6%) were FeLV (+) and 109 cats (84.5) were FeLV(-). In the adult group ($n = 8$), 1 case (12.5%) was FeLV (+) and 7 cats (87.5%) were FeLV(-) (Table 2). There were thus no statistical differences ($p = 0.526$) between the age groups regarding FeLV status.

Among the FeLV(+) cats, 36.4% ($n = 4$) were males, while 63.6% ($n = 7$) were females, with females being more represented among the FeLV(+) cases. Among the FeLV(-) cases, 53.0% ($n = 62$) were male and 47.0% ($n = 55$) were female, indicating a similar representation of males and females in the FeLV (-) group. However, the differences were not statistically significant ($p = 0.526$).

In both groups, the most frequent BCS was 4/9, accounting for 27.8% ($n = 35$) of the cats, followed by 23.0% ($n = 29$) with a BCS of 5/9, 19.0% ($n = 24$) with a BCS of 3/9, and 15.9% ($n = 20$) with a BCS of 6/9. The mean weight in both groups was 3.62 kg (S.E. = 0.11; 95% CI [3.41, 3.84]). As in the FeLV(-) group, the FeLV (+) cats were overwhelmingly in good condition. In the FeLV (+) group, 3 cats had BCS 4/9 and 3 cats had BCS 6/9. FeLV(+) cats had a mean weight of 4.08 kg (SE = 0.49, 95% CI [2.98–5.18]). In contrast, FeLV(-) cats have a mean weight of 3.56 kg (SE = 0.10, 95% CI [3.36–3.76]).

In the FeLV (+) group, physical examination did not reveal any relevant abnormalities. The most common clinical signs observed in the FeLV(-) group were pulicosis (18%), gingivitis or gingivostomatitis (12%), but in reality, all the cats seemed generally healthy.

The complementary tests performed showed no significant differences in blood tests between FeLV(-) and FeLV(+) cats. Regarding hematological alterations, the most prevalent was non-regenerative mild anemia, occurring in 29.5% ($n = 18/61$) of the cats. In the FeLV(+) group, 2 cats exhibited mild anemia, while mild neutrophilia was also recorded in two cats. Furthermore, one of

Table 2 Signalment, clinical data and results of molecular tests of FeLV-Positive stray cats

Stray Cat (Sc)	Age*	G	RS	W (kg)	BCS**	CM	CI-M	Viremia load/ml	Proviral load/ml	FeLV Subgroup
Sc13	Juvenile	F	Yes	3.25	4	No	No	3.24×10^{7}	3.40×10^{7}	A
Sc17	Adult	M	Yes	5.0	6	No	Yes	3.50×10^{7}	3.35×10^{7}	AB
Sc29	< 1 year	M	Yes	2.5	3	No	Yes	5.15×10^{6}	5.42×10^{6}	AB
Sc30	< 1 year	F	Yes	3.4	3	No	No	2.83×10^{8}	6.06×10^{7}	A
Sc57	Juvenile	F	Yes	7.0	4	No	Yes	1.60×10^{8}	2.56×10^{6}	AB
Sc86	Juvenile	F	Yes	2.3	6	No	Yes	ND	1.06×10^{3}	AB
Sc93	Juvenile	M	Yes	6.5	8	No	No	4.12×10^{8}	5.29×10^{8}	AB
Sc95	< 1 year	M	Yes	5	7	No	Yes	1.22×10^{8}	3.45×10^{8}	AB
Sc117	Juvenile	F	Yes	3.5	4	No	Yes	6.05×10^{5}	6.91×10^{5}	A
Sc122	Juvenile	F	Yes	4.2	3	No	No	2.03×10^{6}	1.53×10^{6}	A
Sc131	Juvenile	F	No	2.2	4	No	No	4.58×10^{5}	1.07×10^{6}	A

G: Gender; RS: Reproductive status; Yes- fertile state; No- infertile state; W: Weight; BCS**: Body condition score**; CI-M: Co-infection haemotropic mycoplasmas; CM: Clinical manifestations, ND: Not detected

* According to the 2021 AAHA/AAFP Feline Life Stage Guidelines [30]. **According to the WSAVA Nutritional Assessment Guidelines [31]

the hemograms from the FeLV-positive group exhibited eosinophilia and monocytosis.

Serum glucose was abnormally high in 67% ($n = 61/91$) of the samples. In the FeLV(+) group the mean glucose level was 207.17 mg/dl (SE = 15.02, 95% CI [168.56–245.77]), whereas in the FeLV(-) group the mean glucose level was 216.34 mg/dl (SE = 9.58 95% CI [197.27–235.41]). These differences were not statistically significant ($p = 0.919$).

Total protein was normal in 51.7% ($n = 31/60$) and slightly elevated in 48.3% ($n = 29/60$) of the samples. Hypoproteinemia was not detected in any cat.

Interestingly, in both the FeLV(+) and FeLV(-) groups, exactly 27% of the cats required a chest x-ray for respiratory findings, with the patterns found being bronchial and interstitial/alveolar.

The 11 FeLV(+) cats were tested for co-infection with hemotropic mycoplasmas. Five (45.5%) FeLV(+) cases were negative and 6 (54.5%) were positive for mycoplasma. The qPCR for hemotropic mycoplasmas, showed that 3 cats were co-infected with *M. haemofelis* and *M. haemominutum (canditatus*), 1 was co-infected with *M. haemominutum* and in 2 cats the species could not be determined.

Proviral load results

All cats that serologically tested positive for Ag p27 were also FeLV(+) by qPCR blood testing. Only one case ($n = 1$) out of 11 positive cases was "likely regressive" with a proviral load of 1.06×10^3 copy/mL. Of the ten likely progressive samples, 90% of the cats ($n = 9$) had a proviral load above 10^6 and only one had a viral load of 10^5. The mean level of proviral DNA quantified by qPCR was approximately 4.45×10^7 copies/mL, with a standard error (SE) of 3.06×10^7 copies/mL, indicating variability in the mean measurement. The standard deviation (SD) of 1.02×10^8 copies/mL also highlights the high variability in proviral DNA levels between observations.

Viremia (viral load) results

No viremia was detected by RT-qPCR in the "likely regressive" case, although the ELISA p27 antigen test was positive in this cat. The blood viral loads of the "likely progressive" cats showed a high mean level of viral RNA quantified by RT-qPCR of approximately 9.57×10^7 copies/mL, with an SD of 1.4×10^8 copies/mL, indicating, as with the proviral load, variability in the mean measurements.

Subgroup results (Nested PCR for FeLV A/B/C *env* gene)

Subgroup results by nested PCR showed that of the eleven FeLV-positive samples, 5 were amplified for subgroup A and 6 were amplified for subgroups A and B with a combination of AB and no amplification for subgroup C and/or ABC. Nested PCR also allowed the identification of FeLV subgroups, with 45.5% (95% CI 27.3%-81.8%) for subgroup A and 54.5% (95% CI 18.2%-72.7%) for subgroup AB. Confidence intervals were calculated using bootstrap resampling.

To understand which of the two subgroups is most viremic, an analysis of FeLV RT-qPCR results across FeLV subgroups (A, AB) was performed and shows notable differences in viral load mean and variability. For subgroup A ($n = 5$), the mean RT-qPCR result is 6.3×10^7 copies/mL with a SE of 5.5×10^7 copies/mL, indicating moderate levels with substantial variability and positive skewness. Subgroup AB ($n = 6$) has the highest mean of 1.2×10^8 copies/mL, with a larger SE of 6.3×10^7 copies/mL. These descriptive findings suggest different distributions of viral load results across FeLV subgroups; however, inferential analysis using Mann-Whitney did not suggest a statistically significant difference between subgroups ($p = 0.429$).

FeLV phylogenetic analysis

The sequences of the stray cat samples, based on the *env*/FeLV-A *env*/FeLV-B genes were compared with sequences obtained from GenBank. The BLAST analysis for the FeLV-A subgroup showed that the Portuguese stray cat samples shared a significant identity with the Brazilian variant G21A-SC (OP205371) and with several domestic cat's variants from Italy: 308/2019 A (OR227257), 124/2020 A (OR227270), 1127/2019 A (OR227264), and 324/2021 (OR227275) (Figs. 1 and 2). The BLAST analysis of the FeLV-B subgroup showed that the Portuguese stray cat samples are closely related to the variants found in the USA (see Figs. 1 and 2). However, the specific FeLV-B subgroup sequences (PTSc57 B PT and PTSc95 B PT) were found to be phylogenetically distant from the remaining sequences of the Portuguese FeLV-B group. This suggests there has been a divergence of the virus in the Portuguese variants (Figs. 1 and Additional file 1- Estimates of evolutionary divergence between sequences).

Discussion

Regarding stray cats, there is currently a lack of comprehensive data for Portugal, as well as for the majority of other European countries, particularly in Southern Europe. However, in recent years there has been an increased interest in the study of the prevalence and molecular characterization of FeLV in the stray population worldwide [6, 12, 44]. In this stray cat population, the prevalence of FeLV-positive cats was found to be 8.5% ($n = 11/129$). To date, only three serological screening studies have been carried out in the stray cat population in Portugal. The first study, conducted between November 2003 and July 2005 [4], demonstrated a prevalence of 7.1% ($n = 14/189$), while the remaining two studies, both

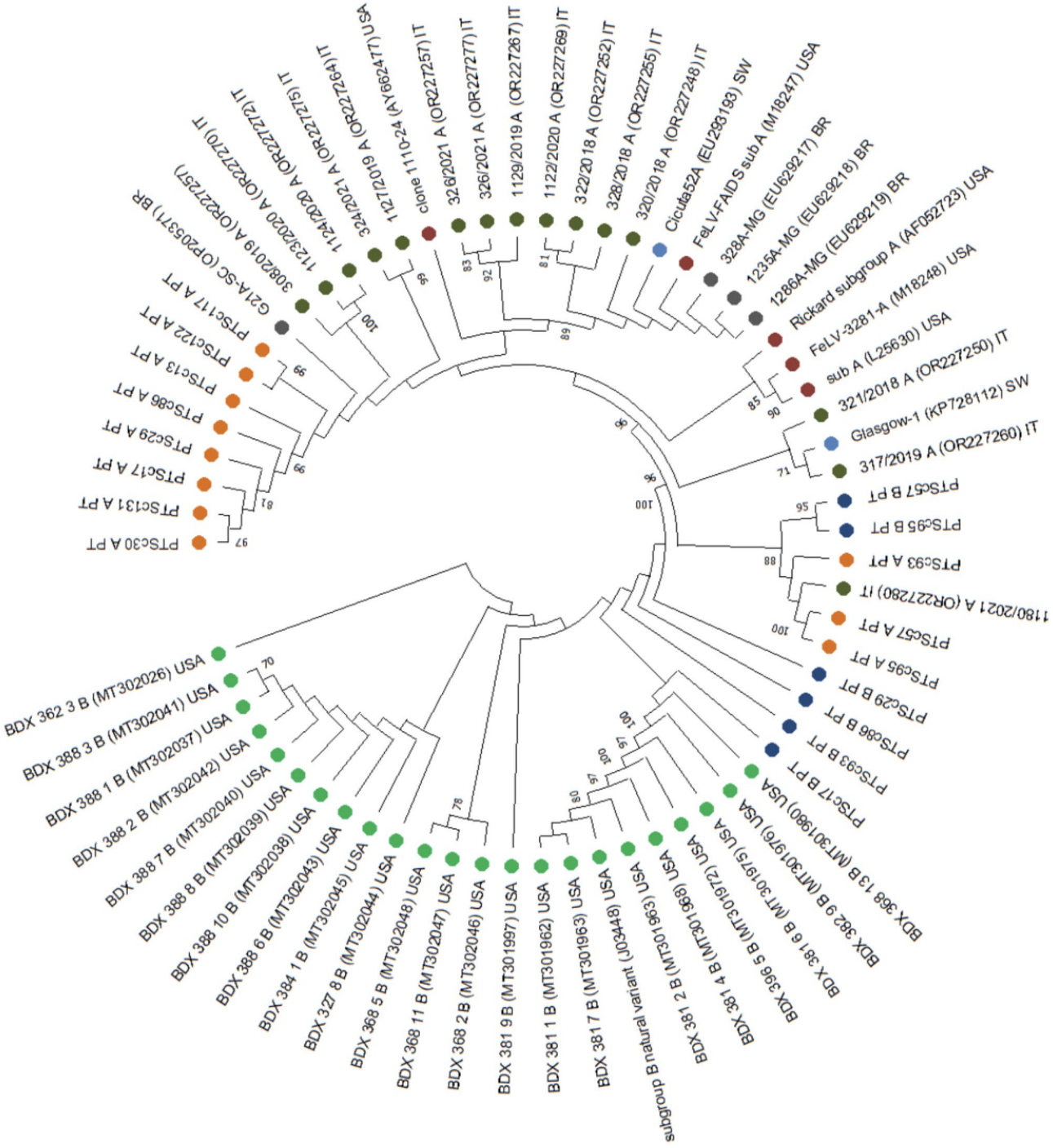

Fig. 1 Phylogenetic tree based on *Neighbor-Joining* method and Kimura 2-parameter model [42]. The bootstrap consensus tree derived from 1000 replicates represents the evolutionary history of the analyzed taxa. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Bootstrap values are given at the nodes of the tree and only values 70% ae shown. Were included sequences from viral subgroups. Colors represent the countries where the FeLV subgroups A and B were obtained. BR-Brazil; IT-Italy; PT-Portugal; SW-Switzerland

published over a decade ago, indicated a prevalence of 5.7% ($n = 5/88$) [3] and 26% ($n = 13/50$) [5] although this latter study took place between 1997 and 2007 and essentially tested feral and wild cats.

All prevalence rates are higher than, for instance, the 4% from a Swiss (Western Europe) stray cat study [6] which took place around the same time period as the work we are presenting. Portugal continues to have

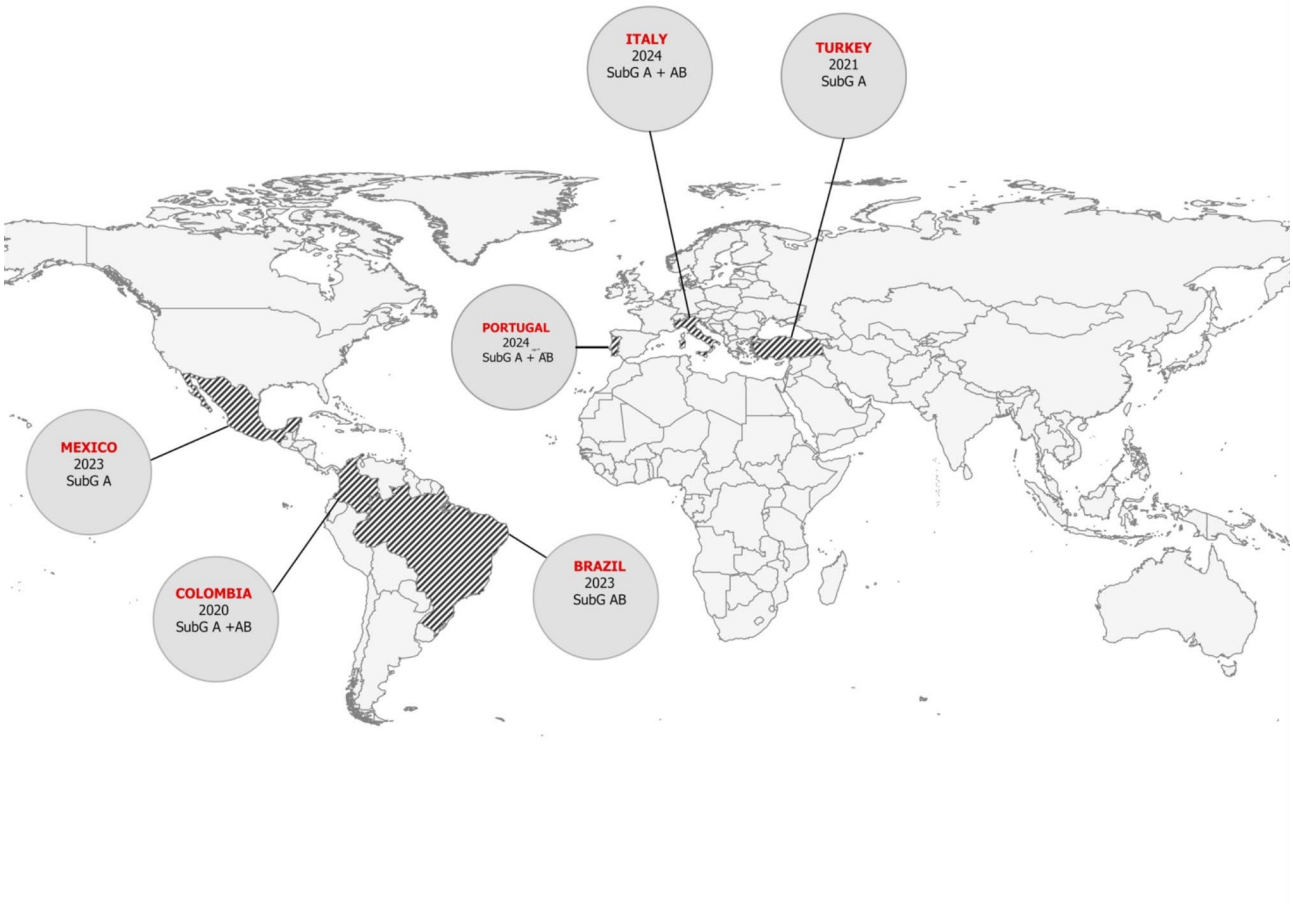

Fig. 2 Worldwide distribution of the different FeLV subgroups based on various studies published to date

higher stray cat prevalence rates, but these are lower than those found in cats receiving veterinary care, as found in the 2019 pan-European study, in a study published in 2023 in 4 countries, including Portugal, and in another study from the same geographical area as this one, presented at WSAVA 2023. They reported a prevalence of 8.8% [2], 20.4% [8] and 12.3% [45]respectively. In contrast to some European countries where the prevalence of FeLV appears to be stagnating [2, 46] or decreasing, in Portugal it continues to increase both in the stray cat population and the overall cat population [45]. Stray cats do not appear to be the most affected demographic population. However, they are a significant reservoir of FeLV, especially in Portugal. From an epidemiological perspective, the highest risk may be associated with shelters and the absence of comprehensive vaccination protocols. Surprisingly, in 2024 a seroprevalence study was published in a single shelter in a different geographical area, in northern Portugal, which found a seroprevalence of 2.1% ($n = 7/326$). These results must be seen in the context of the specific conditions and management of this shelter [47].

Previous studies have shown that the prevalence of FeLV and feline immunodeficiency virus (FIV) infection varies according to various characteristics such as: age, gender, and healthy/sick [13, 48, 49]. Young adult age is a factor with a higher risk of FeLV infection [2, 14]. This is confirmed by our data with 10 of the 11 FeLV(+) cats where in the kitten and young adult age group. Conversely, approximately two-thirds of the FeLV(+) cats were intact females, which is an unexpected finding in comparison to the literature where intact males are more likely to be FeLV(+) [50] The Y chromosome concentrates a large number of enFeLV. There is a lack of consensus regarding whether enFeLV confers protection against exogenous infection or increases the pathogenicity of exFeLV [11]. On the other hand, five of the seven female FeLV(+) cats are subgroup AB, exhibiting an identical proviral load as the male cats. This is in contrast to previous findings which indicated that female cats were more likely to be associated with progressive stages and subgroups B [11].

Interestingly, none of the FeLV(+) cats showed any outward signs of disease and were in good physical condition, which can be attributed to the fact that they were

closely monitored and remotely fed and cared for by their careers. No clinical, laboratory or radiographic alterations were found that would indicate the FeLV(+) cats were in a better or worse clinical condition compared to the FeLV(-) cats. While FeLV infection is commonly associated with various hematological disorders, the absence of significant hematological alterations in FeLV(+) cats has also been reported, with cytopenias being the most frequently reported hematological disorders linked to FeLV infection [51–53].

A prevalence of 50% was observed for Mycoplasma co-infection by qPCR in FeLV(+) cats, a figure that is higher than the 43.43% recorded by qPCR in Portugal in a sample of 320 cats [54] Co-infection with more than one hemoplasma species was observed in 50% of FeLV(+) cats with *Mycoplasma* spp. (+) infection. Although only one cat had mild anemia, the association between a FeLV (+) condition and its co-infection with *Mycoplasma spp*. also appears to occur in this population of stray cats, as has been previously reported in the literature [14, 17].

All but one of the cats that tested positive for p27 serologic PoC had positive DNA qPCRs with significantly high loads. This is consistent with the concept that the lower the proviral load, the greater the discordance with p27 serologic results, even by immunofluorescence [13]. The majority of cats in this study, had a high ("likely" progressive) proviral load. The proviral load in these cats was greater than 4×10^5 copies/mL. This finding contrasts with that of a recent Swiss study [6], which reported a lower prevalence of 32.4% with a viral load of $> 10^6$ copies/PCR. The fact that the samples were saliva and that RNA rather than DNA was quantified may have an impact on these differences, and the results cannot be accurately compared. The presence of high viral loads, coupled with a notable prevalence of subgroup AB, represents a significant risk for other cats. These cats act as a more potent reservoir for FeLV, leading to the emergence of diseases associated with the progressive state and subgroup B, such as lymphoma and leukemia [1, 13, 14, 17]. A study conducted by Power and colleagues in the United States revealed a positive correlation between elevated viral loads and the prevalence of subgroup B [11].

The reason behind the variations observed in the distribution of FeLV strains and prevalent viral subgroups across different countries in Europe remains uncertain. It is plausible that the observed differences in FeLV subgroup distribution may be attributed to inherent genetic variations between the respective countries [18, 24, 25]. This is crucial greater understanding of the prevalence, pathogenesis and disease progression [11]. As the subgroups are related to the various disease syndromes, their knowledge will help us to draw up a more realistic and well-founded prognosis in a highly unpredictable and distressing disease for the guardians of stray cats. The findings revealed the presence of 45% (5/11) subgroup A and 55% (6/11) subgroup AB. To date, there has been limited research conducted on the subgroups in European stray cat colonies or hotspots. In a Turkish study [12] of stray cats, only subgroup A was found in the samples tested; however, it is not clear whether the AB subgroup was searched for. In Italian cats presented to veterinary facilities, subgroup B was detected in around 30% of those samples in which sequencing was performed [10]. Subgroup A has been identified as the most prevalent subgroup across the American Continent. In Mexico, the prevalence of Subgroup A is 100%. In Brazil, Subgroup AB is 100% prevalent. Colombia exhibits a 93% prevalence of Subgroup A, with a 0.7% prevalence of Subgroup B. Occasional records of subgroups B and AB have been documented in de domestic cat population (Fig. 2) [7, 9, 37, 55].

The main limitation of this study, besides the sample size, is that the molecular characterization was only performed on samples that tested positive in the serologic screening tests. This may have caused us to overlook regressive states with very low viral loads. Moreover, it would have been crucial to test all 129 cats for antibodies to more accurately identify the four courses of FeLV. Since it was a one-off test, it was not possible to repeat the test over time, ideally with a minimum interval of six weeks according to the guidelines [14, 15, 17]. From an epidemiological standpoint, it would have been highly informative to assess the status of both retroviruses. This is particularly important regarding stray cats that are co-infected with FeLV and FIV. Further studies are needed, along with more in-depth phylogenetic analyses, which will facilitate the confirmation of the strains of the virus in circulation and enable comparisons to be made with strains worldwide.

The data collected in this study is an update on the prevalence of FeLV in stray cats in Portugal after more than a decade. It also includes the genetic characterization of the prevalent FeLV virus, which was done in a pioneering way by linking subgroups and both viral loads in a stray cat population. To the best of our knowledge, few studies worldwide have employed this approach concurrently. Our study of the FeLV population in Portugal will proceed with an expanded sample size and a more comprehensive genetic analysis. This will facilitate the characterization of the genomic retroviral profile of the stray population and Portuguese cats in general.

Conclusion

This study suggests that stray cats may not represent the primary epidemiological challenge in southern Europe regarding FeLV prevalence. Instead, factors such as shelter conditions, vaccination rates, and the lack of supervised adoption programs may contribute more

significantly to the high FeLV prevalence in Portugal. While stray cats pose a risk to other outdoor cats, overcrowded shelters and the absence of vaccination protocols appear to play a critical role in the virus's spread. Given the current lack of comprehensive data on stray cats in Portugal and much of southern Europe, further research focusing on owned and sheltered cats is needed to draw more definitive conclusions. Moreover, implementing vaccination for all outdoor-access cats, including strays, through trap-neuter-return programs could help reduce FeLV prevalence in Portugal.

Abbreviations

AAFP	American Association of Feline Practitioners
AAHA	American Animal Hospital Association
BCS	Body condition score
BLAST	Basic Local Alignment Search Tool
CI	Confidence Interval
CI-M	Co-infection haemotropic mycoplasmas
CM	Clinical manifestations
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
enFeLV	Endogenous Feline Leukemia Virus
env	Envelope gene
exFeLV	Exogenous Feline Leukemia Virus
FeLV	Feline Leukemia Virus
FeLV-FAIDS	Feline Leukemia Virus - Feline Acquired Immunodeficiency Syndrome
FIV	Feline immunodeficiency virus
G	Gender
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IFA	Indirect Fluorescent Antibody Assay
MEGA	Molecular Evolutionary Genetics Analysis
NCBI	National Center for Biotechnology Information
ND	Not detected
NJ	Neighbor-Joining
PCR	Polymerase chain reaction
PoC	Point-of-care
PoCp27	Point-of-care capsid protein p27
qPCR	Real-time quantitative polymerase chain reaction
RS	Reproductive status
S.E.	Standard Error
W	Weight
WSAVA	World Small Animal Veterinary Association

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04691-2.

Supplementary Material 1: Estimates of Evolutionary Divergence between Sequences. The number of base substitutions per site between sequences are shown. Analyses were conducted using the Kimura 2-parameter model [40, 42].

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Author contributions

P.M.A and A.B designed the study. P.M.A, A.B and M.B did the data curation and analysis, P.M.A did the formal analysis, P.M.A and A.B designed the methodology. Tables and figures were prepared by P.M.A, A.B and M.B. P.M.A wrote the original manuscript. P.M.A, A.B, J.O and C.V reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding authors on request.

Declarations

Ethics approval and consent to participate

The work described in this manuscript involved the use of non-experimental unowned animals. Established internationally recognized high standards ('best practice') of veterinary clinical care for the individual patient was always followed. Also, following the guidelines for experimental procedures and approved by the Ethics and Animal Welfare Commission (CEBEA) of the Faculty of Veterinary Medicine of Lusófona University (Protocol number 120/2022).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Authors' information

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