Phylogenetic analysis and nonsynonymous mutations in a Feline immunodeficiency virus *env* gene genotype A in Colombian cats

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List of Abbreviations

Abreviation	**Term**
AIDS	Feline Acquired Immunodeficiency Syndrome
BIC	Bayesian Information Criterion
bp	base pairs
$CD25^+$ T	T cluster of differentiation 25 (CD25+ regulatory T cells)
$CD4^+$ T	T cluster of differentiation 4 (CD4+ T cells)
$CD8^+$ T	T cluster of differentiation 8 (CD8+ T cells)
cDNA	complementary DNA
ELM	Eukaryotic Linear Motif (database)
env	envelope gene
FASTA	Fast-All: Sequence alignment software that unifies its two predecessor programs: FAST-P (for proteins) and FAST-N (for nucleotides). The "All" indicates that it works with both types of sequences
FIV	Feline immunodeficiency virus
gag	group-specific antigen gene
gp120	glycoprotein 120
HKY+G+I	Hasegawa-Kishino-Yano model with gamma distribution and invariant sites
kb	kilobases
LTR	long terminal repeat
MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum Likelihood

Abreviation	Term
mRNA	messenger RNA
MUSCLE	MUltiple Sequence Comparison by Log-Expectation, and the term "MUSCLE algorithmic" that refers to the computational that uses this famous multiple sequence alignment (MSA) program in bioinformatics
MutPred2	Mutation Predictor version 2
NCBI	National Center for Biotechnology Information
PCR	polymerase chain reaction
pol	polymerase gene
PROSITE	PROtein SITE (sitio proteico). Base de datos de bioinformática, creada en 1988 por Amos Bairoch en el Swiss Institute of Bioinformatics (SIB) y es parte del servidor ExPASy (Expert Protein Analysis System).
QS	Quality Score
RNA	ribonucleic acid
Ser/Thr kinases	serine/threonine kinases, family of enzymes
SRA	Sequence Read Archive
ssRNA(+)	single-stranded positive-sense RNA
SU	surface glycoprotein
Taq	Thermus aquaticus polymerase
TM	transmembrane glycoprotein
V3–V5	Variable regions 3 to 5 of the envelope gene

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Abstract

Feline immunodeficiency virus (FIV) is a retrovirus that infects domestic cats. Genetic variability of the virus, particularly in the V3–V5 region of the envelope gene, may contribute to amino acid changes that affect viral pathogenicity and the development of eight distinct subtypes. The circulating genotype and nonsynonymous mutations in an *env* gene fragment were analyzed in domestic cats from Colombia. Blood samples were collected from 151 felines, and an 859-base pair fragment of the *env* gene was amplified and sequenced. Phylogenetic analysis identified genotype A as the circulating genotype; however, sequences clustered into different clades within this genotype. Twelve nonsynonymous amino acid substitutions were detected, of which H86R, N88K, E41V, and K91D showed a high probability of having a deleterious impact. These findings highlight the genetic diversity within FIV genotype A and underscore the potential impact of specific nonsynonymous mutations on viral pathogenicity.

Keywords: Deleterious; PCR; Sanger sequencing; Amino acids; Retrovirus; Epidemiology.

Study contribution

This study provides novel insights into the molecular epidemiology of FIV in Colombia by characterizing the circulating genotype A and uncovering phylogenetic relationships within the *env* gene fragment. By combining phylogenetic reconstruction and the prediction of nonsynonymous amino acid substitutions with potential deleterious impacts, this research highlights the genetic plasticity of the V3–V5 regions and their role in maintaining viral diversity. These findings contribute to the broader understanding of FIV evolution, regional subtype distribution, and the molecular mechanisms that may influence viral pathogenicity, supporting future strategies for diagnostics, control, and vaccine development.

Introduction

Feline immunodeficiency virus (FIV) is a lentivirus that belongs to the Retroviridae family. It is composed of double single-stranded, positive-sense RNA [ssRNA(+)], its genome is approximately 7 to 11 kb, and it has membrane glycoproteins, making it an enveloped virus. FIV is the etiological agent of Feline Acquired Immunodeficiency Syndrome (feline AIDS), which generates immunosuppression, compromising the health and life of felines by leaving them exposed to secondary diseases caused by opportunistic microorganisms(1) FIV mainly infects $CD4^+$ T and $CD8^+$ T lymphocytes, causing lymphopenia. It also has the ability to infect $CD25^+$ T cells, monocytes, and macrophages(2)

The replication cycle of FIV begins with an incubation period of 8 to 10 days and presents three stages: an initial acute phase in which viral replication occurs in the lymph nodes of the cervical region, epithelial cells of the salivary glands, and $CD4^+$ T, $CD8^+$ T, and B lymphocytes. This stage is characterized by neutropenia, lymphopenia, lymphadenopathy, and a transient febrile episode.(2) After infection is established, the virus invades other defense cell types, including mononuclear cells, and non-lymphoid tissues such as the lung, intestine, and kidney, leading to clinical signs such as acute diarrhea and respiratory and renal conditions.(3) In the final stage of FIV infection, the infected feline enters a chronic phase characterized by infection of bone marrow cells, a progressive decrease in $CD4^+$ T lymphocytes, and consequent impairment of the lymphoid lineage.[(1)]

The FIV genome consists of typical retroviral genes, including *gag*, *pol*, and *env*, flanked by long terminal repeats (LTRs). Among these, the *env* gene encodes the SU and transmembrane (TM) glycoproteins, which are essential for viral attachment and fusion with host cells.[(4)] The *env* gene is also the most variable region of the genome, playing a crucial role in viral tropism, immune evasion, and vaccine design. Based on amino acid variation in the V3–V5 region of the TM,[(5, 6)] eight main subtypes of FIV have been described, denoted as A to F, U, and X-EGY.[(7)] Subtypes A, B, and C are the most common and are distributed throughout the world.[(8)] Molecular surveillance of circulating FIV strains is therefore essential for understanding viral evolution and informing regional diagnostic and control strategies. However, data on the genetic diversity of FIV in South America remain scarce. Prior studies in Brazil and Argentina identified subtype B and novel strains.[(9)]

However, few comprehensive analyses exist for Colombian isolates.[(10, 11)] Although FIV infection has been reported in domestic cats in Colombia, most available data are based on serological testing, and information on the genetic characteristics of circulating viral strains is limited. Seroprevalences ranging from 17 to 18.3 % have been reported for this virus in Colombia.[(12, 13)] Rapid serological techniques have become a diagnostic tool for this viral pathogen; however, these methods can be affected by low antibody levels and have been applied primarily to owned cat populations. The presence of free-roaming and unmanaged feline populations poses a substantial challenge for disease surveillance and control, while simultaneously offering a valuable opportunity to study the epidemiology and genetic diversity of pathogens such as FIV in a natural host setting.

Given the impact of this virus on animal health and on epidemiological surveillance and control, the objective of this study was to determine the circulating genotype and to identify nonsynonymous mutations through the analysis of an *env* gene fragment in a sample of domestic cats from Colombia.

Materials and methods

Ethical statement

This project obtained approval from the Bioethics Committee of Corporación Universitaria Remington [P38 Resolution 06-2022].

Study population and blood collection

Between July 2022 and July 2023, convenience sampling was conducted on 151 domestic cats from four municipalities in Antioquia (**Figure 1**). Blood samples were collected in ethylenediaminetetraacetic acid microtainers, homogenized, and transported to the laboratory under refrigeration (4 °C). The selected felines were owned cats that attended veterinary outreach days organized by the Uniremington Veterinary Clinic. Their owners were informed about the procedure and provided informed consent. Of the sampled felines, 55.63 % were male and 44.37 % were female, with ages ranging from 0 to 13 years. A total of 56.29 % were between 0 and 2 years old, and 11.9 % were over 5 years old. The sampled population consisted of 96 % mixed-breed cats, 2 % Persian cats, and 2 % Angora cats.

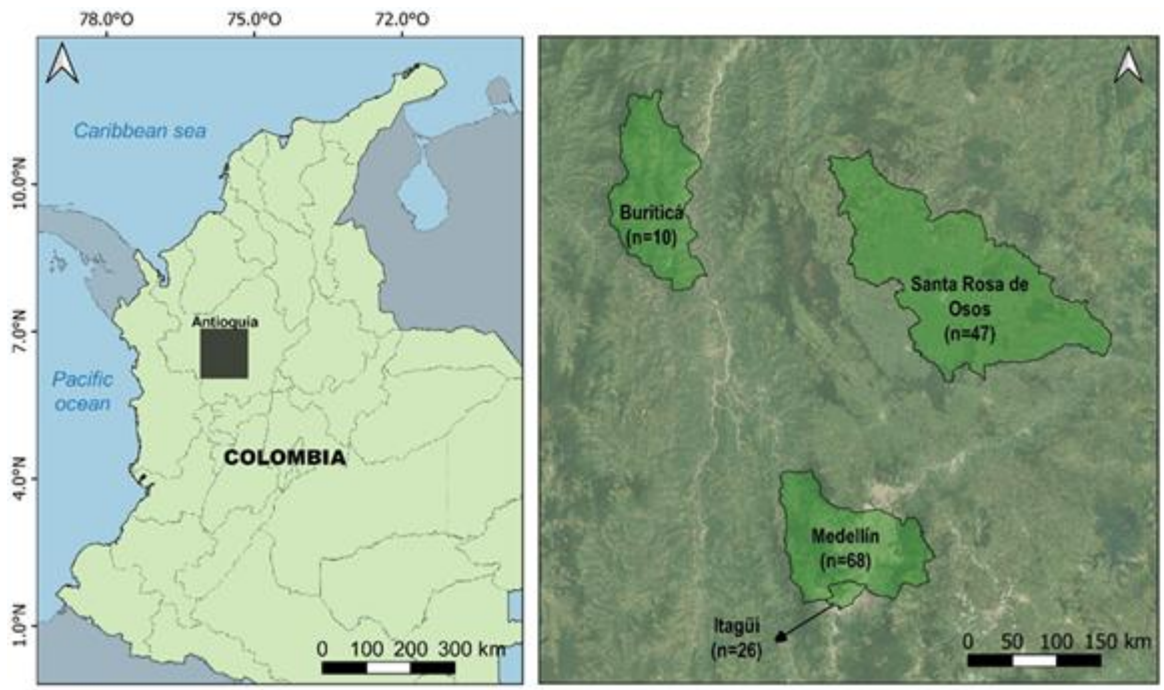


Figure 1. Geographical location of the sampling sites in the department of Antioquia. Analyzed samples from Buriticá (10), Santa Rosa de Osos (47), Medellín (68), and Itagüí (26).

DNA extraction

DNA was extracted using the HigherPurity™ Blood DNA Extraction Kit (Canvax Biotech), following the manufacturer's protocol. DNA was resuspended in 30 µL of elution buffer and stored at -20 °C until further analysis. DNA quality was evaluated based on concentration and purity, using absorbance ratios of 260/280 (acceptable range: 1.8–2) and 260/230 (acceptable range: 2–2.2) measured with a NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific).

PCR amplification of the FIV env *gene*

A nested polymerase chain reaction (PCR) was conducted to amplify an 859 bp fragment of the *env* gene of feline immunodeficiency virus (FIV), following the protocol described by (Arjona et al.).[14] The first-round PCR employed the primers FIV1-*env*FW (5′-

GAGTAGATAC(A/T)TGGTT(G/A)CAAG-3′) and FIV1-*env*RV (5′-CATCCTAATTCTTGCATAGC-3′), while the second-round PCR used FIV2-*env*FW (5′-CAAAATGTGGATGGTGGAA(T/C)C-3′) and FIV2-*env*RW (5′-ACCATTCC(A/T)ATAGCAGT(G/A)GC-3′). The first PCR reaction was performed in a final volume of 30 μL, containing 18.7 μL of nuclease-free water, 2.5 μL of reaction buffer, 0.6 μL of dNTPs (10 mM), 3 μL of $MgCl_2$, 1 μL of each primer (10 μM), 0.2 μL of Horse-Power™ Taq DNA polymerase (Canvax Biotech), and 3 μL (112 ng) of genomic DNA.

For the second round, 3 μL of the first-round product was used as template, with all other reagent concentrations and volumes kept identical. PCR amplification was performed using a SimpliAmp™ Thermal Cycler (Applied Biosystems™). The thermal profile for the first-round PCR consisted of an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 35 seconds, annealing at 53 °C for 35 seconds, and extension at 72 °C for a minute and 20 seconds, with a final extension at 72 °C for 7 minutes. The second-round PCR followed a similar profile, with 25 cycles and an annealing temperature of 51 °C. Negative controls lacking template DNA and positive controls containing genomic DNA from a feline previously confirmed FIV-positive by serology and molecular testing (TOM_Colombia) were included in each PCR run. Second-round amplification products were visualized on a 2 % agarose gel using a gel documentation system (Labnet International, Inc.).

Sequencing and phylogenetic analysis

Six PCR-positive *env* gene products were purified and sequenced by a commercial provider (Macrogen Inc., Korea). The obtained sequences were aligned with 18 previously reported FIV *env* sequences available in GenBank to determine phylogenetic relationships. Sequence alignment was conducted using the MUSCLE algorithm implemented in MEGA v12.0.7. The best-fit nucleotide substitution model, determined according to the Bayesian Information Criterion (BIC), was the Hasegawa-Kishino-Yano model with gamma distribution and invariant sites (HKY+G+I). Phylogenetic reconstruction was carried out using the Maximum Likelihood (ML) method with 1 000 bootstrap replicates.

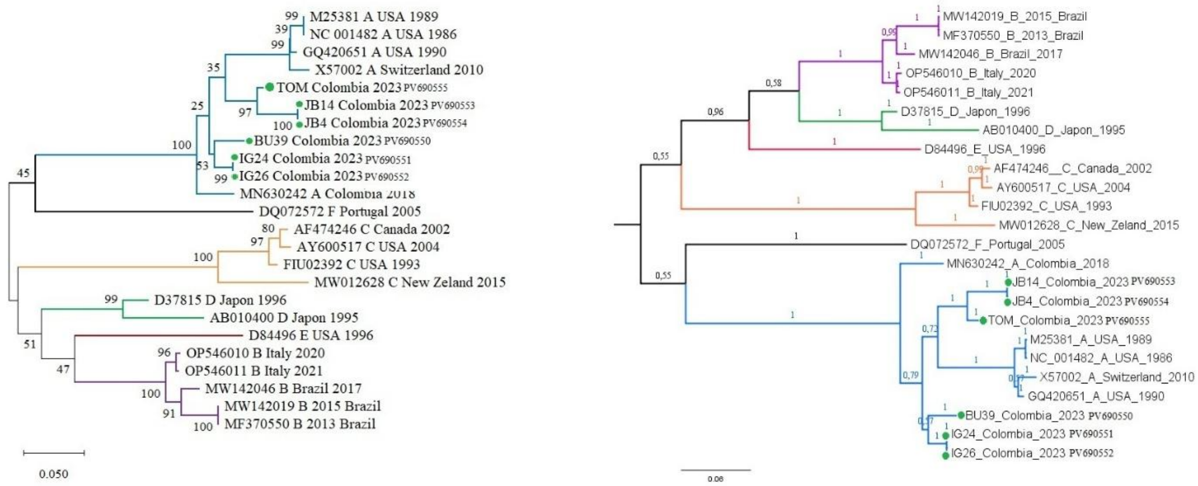
Only bootstrap values ≥ 75 % were considered statistically significant. The resulting phylogenetic tree was visualized using MEGA v12.0.7. The dataset was also analyzed using a Bayesian inference approach implemented in MrBayes v3.2.2. The substitution model applied was the same as that used for ML analysis. Each run consisted of four Markov chains (one cold and three heated), with trees sampled every 500 generations. For the *env* gene, a total of 1 000 000 generations were run until stationarity was achieved, as confirmed using Tracer v1.6. Convergence was indicated by an average standard deviation of split frequencies of 0.003769. A burn-in of 25 % of the sampled trees was applied prior to generating the consensus tree.

Functional analysis of mutations using MutPred2

To evaluate the potential functional impact of the nonsynonymous mutations identified in the partial *env* gene sequences of FIV, the bioinformatics tool MutPred2 (http://mutpred.mutdb.org/) was used. The sequences were first aligned with the reference sequence NC_001484 (genotype A, US isolate) using MEGA v12.0.7, and amino acid substitutions were identified relative to this reference. The nonsynonymous mutations were individually submitted to MutPred2 along with the corresponding protein sequence in FASTA format. MutPred2 uses machine learning models trained on sequence, structural, and functional features to predict the pathogenic potential of amino acid variants. Each mutation was assessed for its overall probability of pathogenicity, with scores ≥ 0.5 suggesting functional effects and scores ≥ 0.8 indicating a high likelihood of deleterious impact.

Results

A total of eight FIV-positive felines were identified through PCR amplification of the *env* gene. The overall molecular prevalence in the region was 5.29 % (8/151); prevalence by municipality was as follows: Medellín 7.35 % (5/68), Itagüí 7.69 % (2/26), Buriticá 10 % (1/10), and Santa Rosa de Osos 0 % (0/47). The PCR products were sequenced using the Sanger method; however, only six met the required quality standards for downstream analysis Quality Score (QS) > 40. The sequences were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under the following accession numbers: PV690550, PV690551, PV690552, PV690553, PV690554, and PV690555. Phylogenetic analysis grouped all analyzed sequences within subtype A (**Figure 2**). However, the sequences did not form a single cluster, indicating substantial genetic variability in the *env* gene among isolates from different localities.



A B

Figure 2. A. Phylogenetic tree of the FIV *env* gene constructed using the Maximum Likelihood method. B. Phylogenetic tree of the FIV *env* gene constructed using the Bayesian method. The evolutionary history of the *env* gene was inferred using the HKY+G+I model. The tree is drawn to scale, with a scale bar. Six partial sequences obtained in the present study from the Department of Antioquia, Colombia, are indicated with a green dot (●). Clades are color-coded on the branches as follows: blue (A), black (F), orange (C), green (D), red (E), and purple (B). Evolutionary analyses were performed in MEGA v12.0.7 and MrBayes v3.2.2.

The final sequence dataset consisted of 812 nucleotide sites, of which 706 were conserved, 112 were variable, and 61 were parsimony-informative. Nucleotide analysis revealed a three-nucleotide deletion at positions 583 (7929 pb) to 585 (7931 pb) in all sequences, and an additional nine-nucleotide deletion from positions 579 (7925 pb) to 587 (7933 pb) in five sequences. These deletions resulted in the loss of amino acids (highlighted in gray) in all sequences analyzed in this study, as shown in **Figure 3**.

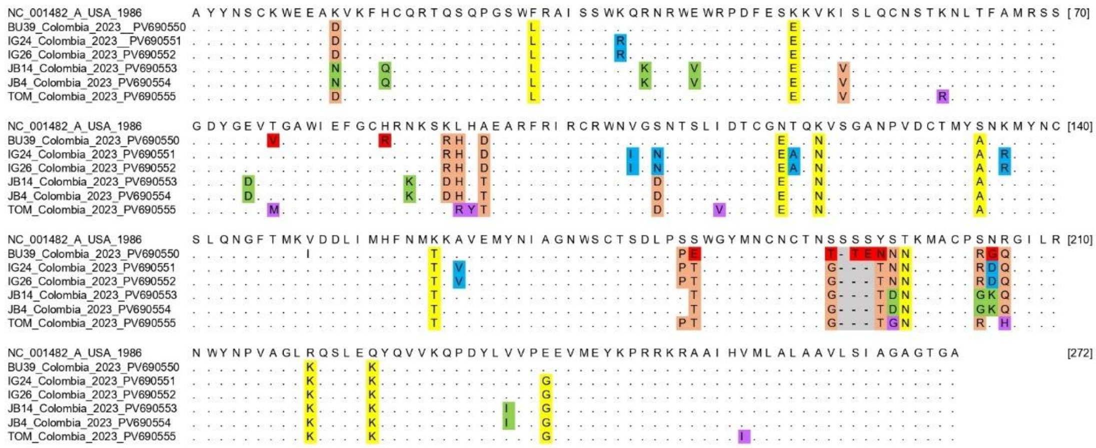


Figure 3. Partial amino acid alignment (position 272) corresponding to a partial region of the FIV *env* gene. The reference sequence used was NC_001484, genotype A, from a US isolate. In the alignment, the F28L substitution observed in all sequences is highlighted in yellow. Unique substitutions found in two sequences from Medellín are shown in green and purple. Unique changes in two isolates from Itagüí are highlighted in blue, while red indicates unique substitutions found in the isolate from Buriticá.

A total of 12 nonsynonymous amino acid substitutions identified in the GP120 region of the FIV *env* gene were evaluated using MutPred2. The resulting scores ranged from 0.506 to 0.857, with the highest values observed for mutations H86R (0.857) in the Buriticá isolate PV690550, N88K (0.777) and E41V (0.773) in the Medellín isolates PV690553 and PV690554, and K91D (0.743) in the Medellín isolates PV690553, PV690554, and PV690555, suggesting a high probability of functional impact (deleterious). **Figure 4** visually summarizes the predicted pathogenicity based on MutPred2 scores, while **Table 1** summarizes the functional predictions of the mutations.

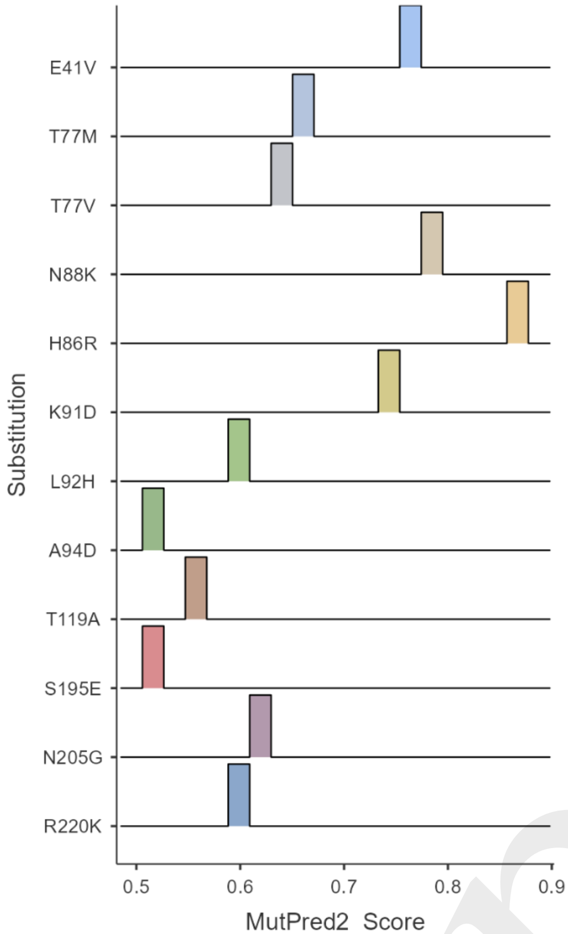


Figure 4. Predicted functional impact of nonsynonymous mutations in the FIV GP120 protein based on MutPred2 scores. The bar chart represents the MutPred2 score for each amino acid substitution, indicating the likelihood that each mutation affects protein function. A MutPred2 score ≥ 0.5 suggests a probable functional effect, and scores ≥ 0.8 indicate a high likelihood of deleterious impact.

Several mutations were predicted to interfere with key structural and regulatory elements of the protein. For example, H86R and N88K overlapped with conserved PROSITE and ELM motifs and were associated with disruptions in metal and DNA binding, loss of catalytic sites, and alterations in glycosylation at position N88. Additionally, E41V and

S195E were linked to changes in transmembrane structure and disordered regions, which may affect protein folding or membrane integration. Mutations such as T77V and T77M, although affecting the same residue, exhibited different predicted mechanisms, highlighting the potential role of amino acid context in structural rearrangement and functional outcomes (**Table 1**).

Table 1. Functional predictions for nonsynonymous mutations in the FIV GP120 protein using MutPred2

Substitution	**MutPred2 score**	**Functional motifs affected**	**Significant molecular mechanisms ($P \leq 0.05$)**
E41V	0.773	ELME000155	Disordered and ordered interface, transmembrane, gain pyrrolidone acid (Q36)
T77M	0.661	None	Ordered interface, loss solvent accessibility, loss allosteric site (W80), metal binding, transmembrane, gain catalytic site (D72), gain sulfation (Y73)
T77V	0.642	None	Altered metal binding, ordered interface, loss solvent accessibility, gain allosteric site (W80), transmembrane, loss catalytic site (D72), gain sulfation (Y73)
N88K	0.777	ELME000008, ELME000070, ELME000102, ELME000271,	DNA binding, gain helix, gain allosteric site (F83), disordered interface, loss catalytic site (C85),

		ELME000278, PS00001, PS00004, PS00008	transmembrane, loss glycosylation (N88)
H86R	0.857	ELME000102, ELME000108, PS00008	Altered metal and DNA binding, gain allosteric site (F83), transmembrane, disordered interface, loss catalytic site (C85), gain disulfide linkage, gain glycosylation (N88)
K91D	0.743	ELME000070, PS00001	DNA binding, loss helix, loss allosteric site (R87), disordered interface, transmembrane, gain glycosylation (N88)
L92H	0.597	ELME000070	Altered metal and DNA binding, gain intrinsic disorder, loss allosteric site (R87), disordered interface, transmembrane, stability, loss glycosylation (N88)
A94D	0.506	None	DNA binding, transmembrane, disordered interface, gain allosteric site (R99)
T119A	0.556	ELME000052, ELME000053, ELME000202, PS00005	Loss catalytic site (C116), metal binding, ordered interface, loss solvent accessibility, transmembrane, loss allosteric site (T115), loss O-linked glycosylation at S123, loss disulfide linkage
S195E	0.506	ELME000063, ELME000064, PS00006	Transmembrane, loss B-factor, loss GPI-anchor amidation (N191)

N205G	0.628	ELME000085, ELME000239, PS00005	Transmembrane, disordered interface, loss solvent accessibility, metal binding, loss GPI-anchor amidation (N205)
R220K	0.598	ELME000062, PS00008	DNA binding, transmembrane

Discussion

In this study, we analyzed partial sequences of the *env* gene of the feline immunodeficiency virus (FIV) obtained from domestic cats in Colombia, with the aim of contributing to the molecular characterization of circulating viral strains in the region. Although all analyzed sequences corresponded to subtype A, as previously reported for the country,[15] our findings revealed notable sequence diversity among the analyzed samples. This is consistent with reports from other geographic regions, including Australia,[16] the United Kingdom,[17] China,[18] and Belgium and the Netherlands,[19] and suggests ongoing viral evolution and the potential circulation of multiple variants.

Genetic variability is a hallmark of FIV and is largely driven by mutations and recombination events in the envelope gene, which encodes the surface glycoproteins (SU). Variations in these glycoproteins, particularly within the V3–V5 regions, play a critical role in determining viral tropism, facilitating immune evasion, and driving the emergence of distinct genotypes worldwide.[20] The V3–V5 regions of the envelope gene are among the most variable segments of the viral genome, and mutations and insertions within these hypervariable loops contribute significantly to the genetic diversity of viral genotypes.[8] This genetic plasticity underlies the classification of FIV into multiple subtypes (A–E) and recombinant forms documented in domestic cat populations.[5]

It is attributable to the lack of proofreading activity by reverse transcriptase, strong immune selection on neutralizing epitopes, and frequent recombination, all of which drive rapid viral adaptation and genotype diversification. Although the V3–V5 regions encode key neutralizing epitopes, they exhibit limited amino acid variation *in vivo*, suggesting that these regions can remain genetically stable for at least 1–2 years post-infection, despite their importance in immune escape and vaccine resistance. This stability occurs because only mutations that preserve essential functions —such as receptor binding and viral entry— while allowing partial immune evasion, become fixed within the viral population. Consequently, beneficial mutations persist and are vertically or horizontally transmitted, while deleterious changes are purged by selective pressure.[(21)]

The sequence changes identified in the *env* gene are of particular interest, as most substitutions surpassed the functional threshold (score ≥ 0.5). These substitutions are likely to produce structural or functional modifications in the envelope protein, consistent with observations in feline and human lentiviruses.[(22)] Overall, these predicted alterations in protein function suggest that specific mutations in the FIV envelope protein could influence viral behavior, including potential changes in infectivity, immune evasion, or pathogenicity. Our data show that the GP120 protein is sensitive to point mutations such as H86R, N88K, E41V, and K91D, which may be deleterious by affecting different molecular mechanisms of the protein. The functional predictions generated by MutPred2 suggest that nonsynonymous mutations in the FIV *env* gene may significantly influence the biological properties of the virus.

Although originally trained on eukaryotic proteins, MutPred2 remains applicable to viral proteins, as it evaluates the structural and functional consequences of point mutations using generalizable biophysical and evolutionary features. For example, HIV and FIV utilize N-linked glycosylation signals (classified as PROSITE motifs) to mask epitopes and evade host antibodies.[23] Additionally, viruses exploit ELM motifs, such as phosphorylation sites, to recruit host kinases and modulate viral protein function.[24] Some flavivirus RNA polymerases have retained Ser/Thr kinase sites phosphorylated through ELM, which are related to the viral life cycle. This plasticity allows gain or loss of the motif, enabling different interactions with host receptors.[25]

Within the limitations of this study, it is not possible to determine whether the sequenced viral variants represent dominant quasispecies, as deep sequencing techniques were not employed to obtain a detailed profile of viral diversity or the frequency of each variant. Furthermore, the statistical approach used cannot ascertain whether the identified sequences are more abundant than others. Moreover, viral samples obtained as cDNA (complementary DNA) do not allow evaluation of viral replication capacity; therefore, it cannot be determined whether the detected products are replication-competent. Additionally, as sampling was conducted at a single time point, infection dynamics cannot be assessed. Future research should adopt a multidisciplinary approach combining deep sequencing, quantitative analysis, and longitudinal sampling designs to accurately characterize the dominant quasispecies in FIV infections.

High-scoring variants such as H86R and N88K were associated with multiple deleterious mechanisms, including disruption of catalytic and glycosylation sites — features critical for viral infectivity and immune evasion—. The presence of these mutations in highly conserved motifs underscores their potential relevance for viral fitness. Furthermore, the variability in predicted effects between substitutions at the same residue (e.g., T77V vs. T77M) points to the importance of specific physicochemical properties of amino acids in shaping protein function. These findings align with previous studies in other retroviruses, where envelope mutations modulate receptor binding, tropism, and immune recognition. Functional validation of these variants will be essential to confirm their role in FIV pathogenesis and to identify potential targets for therapeutic or diagnostic development. These results highlight the importance of continued molecular surveillance in domestic cat populations, particularly in understudied regions such as Colombia, to better understand FIV epidemiology and inform diagnostic and preventive strategies.

Conclusions

This study enhances the molecular characterization of FIV in Colombia by analyzing partial *env* gene sequences from domestic cats. All samples belonged to subtype A yet showed notable intragroup diversity. Functional predictions indicate that nonsynonymous mutations such as H86R and N88K may impact GP120 structure and disrupt conserved motifs, including glycosylation and phosphorylation sites, with potential effects on viral fitness and host interaction. These results highlight the importance of site-specific mutations in viral evolution and support the use of predictive tools such as MutPred2. Ongoing surveillance and experimental validation are crucial for advancing FIV diagnostics, treatment, and prevention, particularly in understudied regions.

Data availability

All relevant data are within the paper and its supporting information files. Raw sequences are publicly available at NCBI (https://www.ncbi.nlm.nih.gov/sra) through the accession numbers: PV690550, PV690551, PV690552, PV690553, PV690554, and PV690555.

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Conflicts of interest

The authors have no conflict of interest to declare in regard to this publication.

Author contributions

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