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Intrahost genetic diversity of Brazilian isolate of feline immunodeficiency virus

Brief Report

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Summary. We isolated *Feline immunodeficiency virus* (FIV) from three adult domestic cats, originating from two open shelters in Brazil. Viruses were isolated from PBMC following co-cultivation with the feline T-lymphoblastoid cell line MYA-1. All amplified env gene products were cloned directly into pGL8MYA. The nucleic acid sequences of seven clones were determined and then compared with those of previously described isolates. The sequences of all of the Brazilian virus clones were distinct and phylogenetic analysis revealed that all belong to subtype B. Three variants isolated from one cat and two variants were isolated from each of the two other cats, indicating that intrahost diversity has the potential to pose problems for the treatment and diagnosis of FIV infection.
Feline immunodeficiency virus (FIV), originally isolated in the United States in 1986 from a cat with chronic opportunistic infections [28], is a member of the Retroviridae family, genus lentivirus. FIV-infected cats can exhibit illnesses including gingivitis, stomatitis, lymphoma, neurological disorders and wasting [13, 28, 33]. The hallmark of FIV infection is a progressive reduction in the number of circulating CD4+ lymphocytes which ultimately results in an impairment of immunity, similar to AIDS caused by human immunodeficiency virus (HIV) [38]. FIV is both a significant pathogen of domestic cats and a widely used model to investigate HIV pathogenesis and approaches to AIDS vaccination [5].

The viral genome consists of three major genes and several smaller regulatory genes. Like other lentiviruses, FIV exhibits extensive genetic variation [18, 32]. The pol and gag genes encode viral enzymes and core proteins respectively, and are relatively highly conserved. The env gene encodes surface and transmembrane glycoproteins and is highly variable [8, 24, 36]. Within the env gene, nine variable regions have been defined, separated by more conserved regions [26]. On the basis of the analysis of envelope glycoprotein variable regions 3-5, FIV has been classified into five subtypes [17, 27, 35], a number that can be expected to increase as further studies reveal additional diversity. Recent studies identified distinct groups of FIV isolates from the United States and New Zealand [40, 10]. Although genetic subtyping, in general, is based on nucleotide sequences from gag [4], [12], the high variability of the lentiviral env gene makes it the preferred region for subtyping [18]. The env region contains determinants important for cell tropism, cytopathogenicity, and infectivity and prominent immunoreactive domains [21, 26, 34].

The rapid evolution of lentiviruses within an infected individual results in the formation of a viral quasispecies, a phenomenon well documented in HIV infection [7, 32]. Similar to all retroviruses, FIV has a relatively high evolutionary rate, attributed largely to substitution errors made during reverse transcription. The existence of multiple variants, or quasispecies, of FIV has been reported as well [19, 35] although information on intrahost sequence variation is sparse and limited [15, 16]. Without
molecular cloning, the existence of minor quasispecies would not have been detected [19].

FIV infection is prevalent worldwide [43]. Preliminary studies carried out suggested that FIV infection is widespread in the domestic cat population of Brazil [29, 1, 37, 22]. Prevalence rates of FIV infection in Brazil have not been well evaluated and regional variations are largely unexplored. Larger surveys of Brazilian isolates are required to determine whether FIV isolates in Brazil have evolved within a single subtype. Preliminary work has suggested that subtype B isolates are present in the domestic cat population of Brazil [1, 22], but a definitive identification of circulating subtypes is essential in order to develop strategies for molecular diagnosis, since the genetic diversity is high [30]. In this study, novel Brazilian strains of FIV were isolated and env gene products were amplified and then cloned directly into pGL8_MYA, a molecular clone of FIV-GL8. The nucleotide sequences of env of seven clones were determined. Phylogenetic analysis was conducted on the nucleotide sequences derived from these clones and other published FIV sequences and the Brazilian sequences were submitted to GeneBank.

FIV infected cats were identified using the polymerase chain reaction (PCR) to amplify the gag gene [11] and using the SNAP FIV/FeLV Comb Test, IDEXX™ – Westbrook, EUA. One cat, Leviano, originated from an open shelter in Minas Gerais, Brazil [37], and two other cats, Didi and Dengosa, originated from another open shelter in São Paulo, Brazil. All three cats were chronically infected. Virus were isolated by cultivation of the cats’ PBMC [14]. PBMCs were fractionated from 5 ml of heparinised whole blood by centrifugation over a Ficoll-Paque solution with a density of 1.077 g/ml. The separated PBMCs were then co-cultivated with MYA-1 cells in the absence of mitogenic stimulation [23]. DNAs were prepared from positive viral isolations by column chromatography (QIAamp DNA maxiprep Kit; Qiagen) as soon as a positive ELISA for FIV p24 was recorded; thus, viruses had undergone minimal passage in vitro. Full-length viral envelope glycoprotein (env) genes were amplified from these replication-competent viruses using a high-fidelity (proofreading) PCR (High Fidelity PCR system; Roche) using primers corresponding to the 5’ L-SU cleavage site (TAGACCGTAAGATTTTTTAAAGGTATTC) and NdeI site 3’ of the Rev responsive element (CCCTTTGAGGAGAAGATGTGTCATATGAATCCATT) incorporating MluI and NdeI restriction sites, respectively. Due to the inherent instability of the full-length env genes from primary isolates of FIV such as GL8, standard high-copy-number PCR
product cloning vectors could not be used; thus, all amplified env gene products were digested with MluI/Ndel and were cloned directly into pGL8\textsubscript{MYA}, a molecular clone of FIV-GL8 in the low-copy-number plasmid pBR328 and in which an MluI site had been introduced at the L-SU junction.

The nucleic acid sequence of seven independent env clones from each cat was determined using IRD800-labeled oligonucleotides on an automated sequencer, LI-COR Biosciences, Lincoln, Nebr. [15]. DNA sequence alignments and analyses were conducted using BioEdit version 5.0.6 software [9]. The first analysis included 2375 bp of the envelope glycoprotein sequences analyses, from the V2 region to the end of the env open reading frame (orf), 791 amino acids (Fig. 1). The env gene V3-V5 regions encode neutralizing epitopes, such that mutations in these regions induce resistance to viral neutralizing antibodies. Analyses have reported that a major neutralizing epitope is present in the V3 region [21, 31]. In addition, V3-V5 region has been identified as having an important role in cell tropism, with mutations in V3 affecting potential sites for N-linked glycosylation which influence cell tropism [15] and therefore there are many V3-V5 sequences in GenBank, including those from other FIV sequences reported previously from Brazil. In addition, a second analysis was conducted with 473 bp of sequence encoding a region of 157 amino acids comprising the V3-V4 region (Fig. 2).
Fig. 1. Phylogenetic tree of 2375 bp sequences from the region of FIV \textit{env}. The subtype of the obtained sequences was determined by phylogenetic analyses, using a rooted Neighbour-joining tree with Kimura 2-parameter genetic distances and bootstrap analysis with 1000 iterations to evaluate clade consistency.
Fig. 2. Phylogenetic tree of 473 bp sequences from the V3-V4 region of FIV env. The subtype of the obtained sequences was determined by phylogenetic analyses, using an unrooted Neighbour-joining tree with Kimura 2-parameter genetic distances and bootstrap analysis with 1000 iterations to evaluate clade consistency.
Nucleotide sequence data from Leviano’s clones reported in this paper have been deposited in the GenBank database under accession numbers FJ374695, Leviano C8, FJ374696, Leviano C1 and FJ374697, Leviano C7. Others FIV sequences included in the first phylogenetic tree are as follows (the GenBank accession numbers, names of isolates, country and subtype for the FIV env sequences are listed): X60725.1, Dutch 113, Netherlands, A; X69494.1, Scotland, A; X57001.1, SwissZ2, Swiss, A; L00608.1, DIXON, United States, A; M73964.1, Dutch 19K1, Netherlands, A; M59418.1, TM2, Japan, B; M36968.1, PPR, United States, A; X69496.1, UK8, England, A; D37813.1, Sendai 1, Japan, A; D37814.1, Sendai 2, Japan, B; D37815.1, Fukuoka, Japan, D; D37816.1, Aomori 1, Japan, B; D37817.1, Aomori 2, Japan, B; D37811.1, Shizuoka, Japan, D; AF474246.1, Canada, C; NC_001482.1, refseq fiv, United States, A and M25381.1, Petaluma, United States, A. The NC_001413.1, Bovine immunodeficiency virus was used as an outlier.

For the second phylogenetic tree the GenBank accession numbers, names, country and subtype for the FIV env sequences included were: M25381.1, Petaluma, United States, A; L00608.1, DIXON, United States, A; X6075, FIV-UT 113, Netherlands, A; M59418.1, TM2, Japan, B; M36968.1, PPR, United States, A; X69496.1, UK8, England, A; D37813.1, Sendai 1, Japan, A; D37814.1, Sendai 2, Japan, B; D37815.1, Fukuoka, Japan, D; D37816, Aomori 1, Japan, B; D37817.1, Aomori 2, Japan, B; D37812, Yokohama, Japan, B; D37815.1, Fukuoka, Japan, D; D37811.1, Shizuoka, Japan, D; AY139094.1, TX125, United States (Texas), F; AY139096.1, TX200, United States (Texas), F; AY139097.1, TXMK, United States (Texas), F; EF153977.1, TKP88, New Zealand, U; EF153979.1, TKP22, New Zealand, U; EU375619, RJ35, Brazil, B; EU375617, RJ24, Brazil, B; EU375616, RJ23, Brazil, B; EU375614, RJ21, Brazil, B; EU375597.1, strain RJ04, Brazil, B; EU375604.1, strain RJ11, Brazil, B; EU375606.1, strain RJ13, Brazil, B; EU375608.1, strain RJ15, Brazil, B; EU375611.1, strain RJ18, Brazil, B; EU375612.1, strain RJ19, Brazil, B;
Analyses of the viral sequences present of molecular clones showed that all clones grouped within subtype B and that those clones varied from each other even the clones from the same cat (Fig. 1 and table 1). Neither the prevalence of FIV infection in Brazil nor all prevailing subtypes are known. Identification of circulating subtypes from different areas of Brazil is essential to develop strategies for molecular diagnosis, since the genetic diversity is high [18]. A recent publication declared that Brazilian FIV strains and their epidemic spread have not been deeply characterized [22]. In this study the analyses classified the clones as subtype B in accordance with other reports from Minas Gerais and also the Brazilian state of Rio de Janeiro [1, 22]. The isolation and characterization of FIV env genes from Minas Gerais and São Paulo states is the first of its kind. It is important to state that Minas Gerais, São Paulo and Rio de Janeiro are neighbouring states and Brazil is a huge country. To verify the difference between the clones from Brazilian isolates, HIV pseudotype assays were performed using the methods described previously [41]. In addition, the viral variants were compared for receptor usage. All clones were infected MCC cells expressing the native feline CD134 (FFF) and the FFHH chimaeric version of CD134 but the luciferase activity from all clones showed different values (data not shown).

We observed variability amongst the clones isolated from three infected cats and, in agreement with a recent study [16], our results confirmed that biological isolates contain heterogeneous viral quasispecies [16]. These results are consistent with the emergence of a quasispecies and the diversity of the viruses examined confirmed previous observations [19]. Quasispecies arise as the virus-encoded reverse transcriptase (RT) enzyme, an RNA-dependent DNA polymerase that is present in mature virions of all members of the Retroviridae, lacks a proofreading mechanism that corrects for erroneously incorporated nucleotides, resulting in extensive sequence variation, a typical feature of lentiviruses that includes base substitution, addition, and deletion [32]. Previously, intrahost sequence variation has been assessed based on sequence analyses of PCR amplicons obtained using DNA isolated from the PBMC of infected cats as a template, reflecting the most abundant proviral genomes rather than the complete repertoire of virus sequences in circulation [16]. In this study, FIV env was analyzed since this gene encodes the protein that is a target for the humoral response and is
known for its capacity to escape from recognition by antibodies by the accumulation of mutations in the variable regions especially. Sequence variation was most extensive in the V3-V6 regions, where none of the analyzed sequences were identical.

Knowing the prevalence and variability of FIV is of importance for designing and testing vaccines under field conditions. The sequence of the env gene is particularly important since the envelope glycoprotein is the major target for virus neutralization [21, 31] and it may be possible to establish potential associations between specific subtypes and the severity of clinical outcomes [30].

In this study we observed that FIV displays genetic variation amongst the variants identified within three biological isolates (Figures 1 and 2). Further studies will be required to identify the importance of the variability amongst Brazilian FIV isolates identified in this study. Preliminary data have shown variable cell tropism amongst the clones in their ability to infect MCC cell expressing CD134. Since the in vivo cell tropism of FIV expands as disease progresses [3, 6] and a single potential site for N-linked glycosylation in the envelope glycoprotein of FIV modulates the virus-receptor interaction [42], even minor variation in the env gene could have great significance. Sequence variations and conformational changes within the Env protein are responsible for determining the receptor usage of FIV [20]. Chemokine receptor tropism has been linked to specific sequence variations in the Env of primate lentiviruses [2]. For FIV, a mutation in V3 is sufficient to convert a non-CRFK tropic virus into a CRFK-tropic virus [39]. Other domains of SU distinct from V3 have also been identified as important determinants and/or co-determinants of cell tropism in HIV-1 [25]. The results of this work would provide further information for studies involving structure, diagnosis, vaccine development and phylogenetic analysis of FIV.

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References


