Isolation and partial characterization of Brazilian samples of feline immunodeficiency virus

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Feline immunodeficiency virus (FIV) causes a slow progressive degeneration of the immune system which eventually leads to a disease comparable to acquired immune deficiency syndrome (AIDS) in humans. FIV has extensive sequence variation, a typical feature of lentiviruses. Sequence analysis showed that diversity was not evenly distributed throughout the genome, but was greatest in the envelope gene, env. The virus enters host cells via a sequential interaction, initiated by the envelope glycoprotein (env) binding the primary receptor molecule CD134 and followed by a subsequent interaction with chemokine co-receptor CXCR4. The purpose of this study was to isolate and characterize isolates of FIV from an open shelter in São Paulo, Brazil. The separated PBMC from 11 positive cats were co-cultured with MYA-1 cells. Full-length viral env glycoprotein genes were amplified and determined. Chimeric feline × human CD134 receptors were used to investigate the receptor utilization of 17 clones from Brazilian isolates of FIV. Analyses of the sequence present of molecular clones showed that all clones grouped within subtype B. In contrast to the virulent primary isolate FIV-GL8, expression of the first cysteine-rich domain (CRD1) of feline CD134 in the context of human CD134 was sufficient for optimal receptor function for all Brazilian FIV isolates tested.

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1. Introduction

Feline immunodeficiency virus (FIV) is a widespread pathogen of the domestic cats. Carrying the virus present with a variety of clinical signs, most notably chronic gingivostomatitis and lymphadenopathy (Hosie et al., 1989; Yamamoto et al., 1989). FIV is a member of the lentivirus genus of the Retroviridae family. Lentiviruses like human immunodeficiency virus (HIV) and FIV are notorious for their extensive genetic variation and for their rapid diversification within a single host (Overbaugh and Bangham, 2001). In part, this diversification is due to the virus having a rapid rate of replication and a high error rate of reverse transcription (Mcgrath et al., 2001). FIV is unique amongst the non-primate lentiviruses as in its natural host, the domestic cat, it produces a disease similar to AIDS. Since FIV, like human immunodeficiency virus, has a long and variable disease course, it is often difficult to predict the outcome for an infected cat.

The FIV genome consists of three major genes and several smaller regulatory genes. Like other lentiviruses, FIV exhibits extensive genetic variation (Roberts et al., 1988; Kann et al., 2007). The pol and gag genes encode viral enzymes and core proteins, respectively, these genes are relatively highly conserved. The env gene encodes surface and transmembrane glycoproteins and is highly variable (Olmsted et al., 1989; Talbott et al., 1989; Greene et al., 1993). Within the env gene, nine variable regions have been defined, separated by more conserved regions (Pancino et al., 1993b). On the basis of analyses of env variable regions V3–V5, FIV has been classified into five subtypes (A–E) and recombinant strains (Sodora et al., 1994; Kakinuma et al., 1995; Pecoraro et al., 1996) the number of subtypes may increase as further studies reveal additional diversity. Recent studies identified distinct groups of FIV isolates from the Unites States and New Zealand (Weaver et al., 2004; Hayward et al., 2007). The env region contains determinants important for cell tropism, cytopathogenicity, infectivity and prominent immunoreactive domains (Siebelink et al., 1992; Lombardi et al., 1993; Pancino et al., 1993a).

The prevalence rates of FIV infection in Brazil has not been evaluated extensively and regional variations are largely unexplored (Martins et al., 2008). 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.
be infected by the virus (Reggeti et al., 2008). The specificity of infectivity to support FIV replication. Recently a study has shown that feline susceptibility to opportunistic infections (English et al., 1994; Hosie and Beatty, 2007).

Although the principal hallmark of FIV infection is a decrease in CD4+ cells, the virus has also been shown to infect a variety of cell types including CD4+ and CD8+ lymphocytes, B lymphocytes, cells of neuronal lineage and a monocyte/macrophage lineage (English et al., 1993; Dean et al., 1996; Joshi et al., 2005). Joshi et al. (2005) described feline CD4+ CD25+ T regulatory (Treg) cells with the ability to support FIV replication. Recently a study has shown that feline dendritic cells (DCs) express specific viral receptors for FIV and can be infected by the virus (Reggeti et al., 2008). The specificity of the virus–receptors interaction is the earliest determinant of cell tropism and a decisive factor in the pathogenesis of viral disease (Willett et al., 2006b). FIV is characterized by similar pattern of receptor usage to that of HIV-1; however, CD134 rather than CD4 is the primary receptor, and the subsequent interaction with the coreceptor CXCR4 permits cells entry (Shimojima et al., 2004). CD134 was originally identified as MRC Ox-40, an antigen expressed on activated rat CD4+ T cells which belongs to the tumor necrosis factor receptor/nerve growth factor receptor (TNFR/NGFR) superfamily (Paterson et al., 1987; Mallett et al., 1990). The SU of FIV, gp95, binds to activated cells expressing CD134, a 43-kDa receptor. Studies have indicated that FIV infection of certain cells may result solely through binding the co-receptor molecule CXCR4 providing the expression of the chemokine receptor is sufficiently high (De Parseval and Elder, 2001). Recent studies have revealed differential utilization of feline CD134 by FIV. Primary isolates of FIV can be categorized into at least two groups on the basis of their interactions with CD134. The expression of the first cysteine-rich domain (CRD1) of feline CD134 alone is sufficient to confer nearly optimal receptor function for infection with strains such as PPR, subtype A, and B2542 (subtype B), although pathogenic primary strains of virus, such as GL8, subtype A, GFGammer (subtype C) and NCSU1 (subtype B) require additional determinants within the second cysteine-rich domain, CRD2, of CD134 (Willett et al., 2006a,b).

2. Materials and methods

2.1. Open shelter

Cats from an open shelter in São Paulo, Brazil, with reported cases of FIV infection were tested in this study. Blood samples from 55 domestic, short haired cats were collected by jugular venipuncture and placed in serum separator and EDTA treated glass tubes. Serum and whole blood were stored at −80 °C until diagnostic testing was performed. FIV infection in cats was determined using a polymerase chain reaction (PCR)-based method to amplify the gag gene (Hohdatsu et al., 1998) and the SNAP FIV/FeLV Comb Test, IDEXX™ – Westbrook, EUA to detect antibody.

2.2. Cells and viruses

MYA-1 (Miyazawa et al., 1989) and cell lines derived from the feline large granular lymphoma cell line, MCC cells (Cheney et al., 1990), were cultured in RPMI 1640 medium. HEK-293T cells were maintained in Dulbecco’s modification of Eagle’s medium. All media were supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.11 mg/mL sodium pyruvate, 100 IU/mL penicillin, and 100 µg/mL streptomycin. In addition the medium for MYA-1 cells was supplemented with 100 IU/mL of recombinant human IL-2 (kindly provided by Drs Hattori and Miyazawa) and 50 µM 2-mercaptoethanol. All media and supplements were obtained from Invitrogen Life Technologies Ltda (Paisley, United Kingdom). Cell lines expressing CD134 and chimeric constructs were maintained in G418 (Invitrogen, Paisley, United Kingdom). Brazilian isolates of FIV, the subtype B isolates B2542 (Diehl et al., 1995) and the subtype A isolate GL8 (Hosie and Jarrett, 1990), were tested in parallel.

2.3. Collection of peripheral blood mononuclear cells (PBMC) and sera – virus isolation –

Blood samples were collected from 11 cats in the open shelter that were naturally infected with FIV. The cats were displaying few clinical signs (analogous to the asymptomatic stage of HIV infection). Sera were aliquoted and stored at −80 °C prior to use in neutralization assays. PBMC were fractionated from 5 mL of heparinised whole blood by centrifugation using Ficoll-Paque density separation medium (GE Healthcare, Little Chalfont, United Kingdom). The separated PBMCs were then co-cultivated with MYA-1 cells, which are highly susceptible to infection with FIV (Miyazawa et al., 1989), without mitogenic stimulation. DNA was prepared from cultures identified as FIV-infected using column chromatography (QiAamp DNA maxiprep Kit; Qiagen) as soon as FIV p24 antigen was detected by ELISA; thus, viruses had undergone minimal passage in vitro. Eluted DNAs samples were stored at −20 °C.

2.4. Cloning and pseudotype virus production

The FIV envelope glycoprotein gene expression constructs GL8 and B2542, have been described previously (Shimojima et al., 2004; Willett et al., 2006b; Samman et al., 2010). Full length env genes from all Brazilian isolates, replication-competent viruses, were amplified by using the polymerase chain reaction (PCR) using a high fidelity enzyme mix (Expand High Fidelity PCR system; Roche Diagnostics Ltda., Burgess Hill, United Kingdom) and degenerate primers based on the Leviano clone (FJ347697 in GenBank). The primers LevianoSal 5′-GGGACACATGGCAGAAGGAGGTTACCTCATAA-3′, and LevianoNot 5′-GGCGGCGGCCCATCTTCTTCTTCTCAGATAT-3′.
at 0.5 µM and 0.2 µL of DNA extract were combined in reaction mixture comprising 4 µL buffer, 0.2 mM 2′-deoxynucleoside 5′-triphosphates (dNTPs) (Invitrogen, Paisley, United Kingdom), 4% (v/v) DMSO (Sigma, Poole, United Kingdom), 1 U Phusion™ Polymerase and nuclease-free water (Promega UK Ltd., Southampton, United Kingdom) to 20 µL. Amplifications were performed using a “touch down” PCR protocol consisting of an initial denaturation at 98 °C for 3 min, followed by 14 cycles of 98 °C for 10 s, 69 °C for 30 s (decreasing by 0.5 °C/cycle), 72 °C for 2 min, then a further 21 cycles of 98 °C for 10 s, 62 °C for 30 s, 72 °C for 2 min, followed by 72 °C for 10 min and a final hold at 4 °C. Primers were designed to incorporate Sal-I and Not-I restriction enzymes (sites are underlined), the env genes were then cloned into a low-copy number eukaryotic expression vector, VR1012 (VICAL Inc., San Diego, CA, USA). Twelve clones from each Brazilian isolate, same amplified DNA, were generated. These clones were named C1–C12. HIV(FIV) pseudotypes were prepared by transfecting 3.3 × 10⁵ HEK-293T cells with 5 µg of the different VR1012/env construct together with 5 µg of pNL-Luc-E.R+ (Connor et al., 1995), an env-deleted HIV provirus incorporating a luciferase reporter gene. The transfection was carried out using SuperFect® reagent – Qiagen – following the manufacturer’s instructions. Culture supernatants were harvested at 72 h post-transfection, filtered at 0.45 µm and stored at −80 °C.

2.5. HIV pseudotype assays

To assess whether CD134 acts as a functional viral receptor and to define the region of CD134 that confers functional FIV receptor activity, we used MCC cells (Cheney et al., 1990), that are refractory to infection with primary strains of FIV. MCC cells had been stably transduced with the feline CD134, human CD134, and a feline × human CD134 chimera, F(F/H)H (Fig. 1) (Willett et al., 2006b). The target cell lines were seeded at 1 × 10⁴ cells per well in a CulturPlate-96 assay plate (Perkin-Elmer, Life and Analytical Sciences, Beaconsfield, United Kingdom) and cultured overnight. The cells were then infected with 50 µL of the each HIV(FIV) luciferase pseudotype and cultured for 72 h until luciferase activity was quantified through the addition of 50 µL of Steadylite HTS luciferase substrate (Perkin-Elmer) and measurements were taken through single photon counting on a MicroBeta Luminometer (Perkin-Elmer).

2.6. PCR product purification and DNA sequencing

PCR products were purified using the Qiaquick gel extraction kit, Qiagen. DNA was eluted in nuclease-free water and used immediately or stored at −20 °C. Sequencing of the FIV env genes was carried out using the Genetic Analyzer AB3130X (Applied Biosystems) and the Big Dye Terminator v1.1 Kit (Applied Biosystems). The reaction mixture consisted of 100 µg/µL purified DNA, 3.2 pmol of each of the primers, 4 µL sequencing buffer and 2 µL sequencing enzyme in 20 µL reactions. Cycling conditions were as per the manufacturer’s protocol. Raw chromatographic data were analysed using ‘Contig Express’ sequence analysis software within the Vector NTI suite of programs (Invitrogen Ltda., Paisley, United Kingdom). Chromatogram traces which appeared to contain multiple peaks were discarded.

2.7. Phylogenetic analysis

Nucleotide sequence analysis was performed on full-length env genes from 16 clones from Brazilian isolates included in the study, as well as selected representative sequences from different subtypes. The generated consensus sequence comprised sequences of three Brazilian isolates and reference sequences. Multiple alignments were performed using the ClustalX (version 2.0) (Larkin et al., 2007) and BioEdit (version 7.0.9.0) applications, followed by manual adjustment to maximize similarities. Amino acid alignments were used as an exact guide for re-positioning of improper gapping, particularly where sequences differed in length. Nucleotide Maximum Likelihood (ML) tree was generated with the GTR+I+G substitution model and 1000 bootstrap replicates with PAUP*4.0b10 (Swoford, 1993, 2000).

2.8. Nucleotide sequence accession numbers

The FIV sequences included in the study were as follows (GenBank accession number, name of isolates, country and subtype of FIV env sequences): L00608.1, Dixon, United States, A; L00607.1, Dixon, United States, A; M25381.1, Petaluma, United States, A; X69496.1, UK8, England, A; M36968.1, PPR, United States, A; D37814.1, Sendai 2, Japan, B; D37817.1, Aomori 2, Japan, B; FJ374696, Leviano C1, Brazil, B; FJ374697, Leviano C7, Brazil, B; FJ374695, Leviano C8, Brazil, B; AF474246.1, Canada, C; D37815.1, Fukuoka, Japan, D; D37811.1, Shizuoka, Japan, D. All sequences reported in this study have been deposited in GenBank.

3. Results

Five of 55 cats (9.09%) tested positive for both feline leukemia virus (FeLV) and FIV. The seroprevalence of FIV in the open shelter tested in this study was 50.90% (28/55), based on FIV ELISA and PCR results. Five of these animals (5/55, 9.09%) tested positive for both feline leukemia virus (FeLV) and FIV, and 23 (23/55, 41.82%) were infected with FIV but were not co-infected with FeLV (Fig. 1).

We prepared 11 isolates of FIV from samples collected from Brazilian cats. Sequence analyses demonstrated that all clones...
belonged to subtype B. Unexpectedly, little sequence variation was observed in circulating viruses isolated from the infected cats but, as described previously (Teixeira et al., 2010), the sequences of clones obtained from one cat (Didi) shown were heterogeneous. The divergence between clone 2 and clone 10 from this cat was just 0.05%. Nevertheless, this divergence was the biggest in our study. It was notable that the majority of sequences amplified was identical to another species within the individual cat, indicating the dominance of individual variants within each of the quasispecies (Fig. 3). Analysis of isolates from patients demonstrated that intra-patient isolates were more closely related than inter-patient isolates and that intra-patient isolates evolved over time (data not shown).

The receptor usage of each of the variant env clones was assessed in vitro. All Brazilian isolates of FIV used CD134 as the primary receptor and we tested the interaction of each strain with the receptor. In contrast to the virulent primary isolate FIV-GL8, expression of the first cysteine-rich domain (CRD1) of feline CD134 in the context of human CD134 was sufficient for optimal receptor function for all Brazilian FIV isolates tested-ratios obtained by dividing the luciferase activity of each pseudotype on fCD134-expressing cells by that on fεCRD1-expressing cells. As described previously (Willett et al., 2006b), F(FH)H did not support infection with the FIV-GL8 pseudotype (Fig. 4).

For purposes of comparison, we examined the receptor utilization from another subtype B strain of FIV, in order to discern whether these isolates of FIV interacted with CD134 in a similar way. All subtype B strains tested in this study required the expression of feline CD134 CRD1 alone for infection.

4. Discussion

In our study, the prevalence of FIV was higher than that published previously for cats in Brazil (Reche et al., 1997; Souza et al., 2002; Caxito et al., 2006; Teixeira et al., 2007; Lara et al., 2008); the frequency of FIV was up to 50% in the shelter cats. This result is higher than highest seroprevalence found in sick cats in a population sampled in Japan (Ishida et al., 1989) and should be interpreted with caution as the samples came from just one shelter and the cats had been feral before being placed in the shelter or were free-roaming strays, which a consequent lack of confinement and ownership. It is known that the prevalence of FIV infection is greater in such cats. Hence the cats assessed in the study appear to be of greater risk of infection with FIV compared to other cats and represent an important reservoir for this retrovirus.

To characterize Brazilian FIV isolates, our study focused on the env gene since this gene encodes the surface unit (SU) glycoprotein, which plays a key role in viral entry and is a target of both humoral and cellular responses (Willett and Hosie, 2008). In most studies, 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, analysing therefore the most abundant proviral genomes rather than the full repertoire of virus sequences in circulation. For this reason, we cloned all Brazilian variants detected. Our analyses demonstrated that all clones grouped within subtype B (Fig. 3). It is important to remember that since neither the prevalence of FIV infection in Brazil, nor all prevailing subtypes are known, the determination of prevalence of infection in cats in all Brazilian regions will be important to establish. The majority of studies have been concentrated in one area, namely the Brazilian south-east (Martins et al., 2008; Teixeira et al., 2010).

The existence of multiple variants (or quasispecies) of FIV has been reported previously (Huisman et al., 2008; Teixeira et al., 2010) as well as in this study; FIV demonstrates a relatively high evolutionary rate, attributed largely to substitution errors made during reverse transcription. However, there is also evidence that viruses evolve under selection pressure to both evade the host immune response and to achieve higher levels of replication fitness. Consequently, lentiviruses such as FIV and HIV undergo continual evolution in the host. FIV evolution during the chronic phase of infection is potentiated by the high levels of virus replication and the inherent error-prone nature of reverse transcription. At any given time, as shown here, the virus population within a FIV infected cat is heterogeneous and dynamic. Longer term monitoring will be necessary to establish whether this sequence diversity leads to a more significant and effective population size that could impact upon viral evolution and viral fitness. It is unclear to what extent the genetic variation of FIV that was observed in these isolates was influenced by the properties of the infecting strain, the level of replication, or the immune response to the virus.

Assays to determine receptor utilization demonstrated that, similar to other isolates of FIV (Willett et al., 2006b), the Brazilian isolates in this study used CD134 as the primary receptor in conjunction with the chemokine co-receptor CXCR4 for infection (Shimojima et al., 2004). Furthermore, for the Brazilian strains, the CRD1 of CD134 (amino acids 1–64) alone was sufficient to confer nearly optimal receptor function (Figs. 1 and 4). Preliminary works have suggested that subtype B isolates are present in the domestic cat population of Brazil (Caxito et al., 2006; Martins et al., 2008; Teixeira et al., 2010) and an another study (Willett et al.,

Fig. 4. Utilization of CD134 chimeras by FIV strains. MCC cells stably transduced with a retroviral vector (pDONAI) bearing each of the chimeras, FFF and F(FH)H, were infected with HIV (FIV) luciferase pseudotypes bearing the GL8 and Brazilian isolate Env. Luciferase activities were assayed at 72 h postinfection and are expressed as ratios derived by dividing the mean (n = 3) counts per minute (CPM) on fCD134-expressing cells by the CPM on fCRD1-expressing cells.

2006b) has indicated a minimal requirement in the CRD1 of feline CD134 for the B2542 isolate, a subtype B virus that may be more host-adapted. Subtype B isolates appear to require a less stringent interaction with the primary receptor for infection to proceed compared to the pathogenic primary strains of virus, such as GL8; this subtype A isolate dominates in early infection and requires additional determinants on the primary receptor, included within the second cysteine-rich domain, CRD2, in order to form a functional receptor, requiring a “complex” interaction (Willett et al., 2006a,b). It is important to remember that in the FIV infection the provirus levels are higher in CD4+ T cells during the acute phase of infection, while B cells contain the majority of provirus during the chronic phase (English et al., 1993; Dean et al., 1996). With disease progression it is possible that FIV may either lose its dependence on binding to CD134 or interact more efficiently with CXCR4 (Willett and Hosie, 2008). Whether there is a link between the nature of the env-CD134 interaction and the broadening of viral cell tropism with disease progression is a topic of ongoing research. An analysis of variants in follow-up samples from the FIV-infected cats tested in this study would assist in this endeavour.

Although it had been suspected that the majority of FIV isolates circulating within the southeastern region of Brazil belong to subtype B, this work presents the first evidence of genetic diversity, based on full-length FIV env gene, in the state of São Paulo. The results of the current study, based on sequence from 16 naturally occurring isolates of FIV from São Paulo state, confirm that subtype B predominates (Fig. 3). The isolation, phenotype and envelope gene diversity of a considerable number of samples of FIV from Brazil is the first of its kind. Now experimental infection in animal model systems using virus stocks can facilitate the study of determinants within the viral genome that may contribute to disease pathogenesis and the development of immunity. It is important to stress that Brazilian FIV strains have not been well characterized previously. Our results support a model of FIV pathogenesis whereby strains of FIV that are transmitted preferentially (“early” viruses) bind CD134 via a complex, high affinity interaction whereas, with disease progression, variants arise (“late” viruses) that have a simple, reduced affinity interaction through a direct interaction with CXCR4 (Willett and Hosie, 2008). All of the Brazilian FIV strains, examined here, are thought to represent “late” strains of FIV. Previous studies have suggested that CD134-independent strains of FIV (for example FIV-PET) are readily neutralized by antibody (Osborne et al., 1994) and thus may be suppressed in peripheral blood, while being tolerated in privileged tissue compartments where they may disseminate more efficiently. It has been suggested that subtype B isolates may be more ancient ad host adapted and as a consequence may be less virulent (Bachmann et al., 1997). However, was shown recently that long-lived hematopoietic stem cells (HSCs) can be infected by HIV and that infection is accomplished primarily by CXCR4-tropic HIVs (Carter et al., 2011). The conversion of HIV env from R5-tropic to X4-tropic has been associated with more rapid disease progression, manifested as reduced CD4+ T cell counts and a poor clinical prognosis (Connor et al., 1997; Daar et al., 2007). Future studies will be designed to test whether strains of Brazilian FIV present in either the early or asymptomatic phases of infection have a different cell tropism and receptor usage compared to strains isolated from animals displaying clinical signs.

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