Phylogenetic analysis to define feline immunodeficiency virus subtypes in 31 domestic cats in South Africa

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ABSTRACT

Feline immunodeficiency virus (FIV), a lentivirus, is an important pathogen of domestic cats around the world and has many similarities to human immunodeficiency virus (HIV). A characteristic of these lentiviruses is their extensive genetic diversity, which has been an obstacle in the development of successful vaccines. Of the FIV genes, the envelope gene is the most variable and sequence differences in a portion of this gene have been used to define 5 FIV subtypes (A, B, C, D and E). In this study, the proviral DNA sequence of the V3–V5 region of the envelope gene was determined in blood samples from 31 FIV positive cats from 4 different regions of South Africa. Phylogenetic analysis demonstrated the presence of both subtypes A and C, with subtype A predominating. These findings contribute to the understanding of the genetic diversity of FIV.

Key words: domestic cats, feline immunodeficiency virus, subtypes,phylogenetic analysis, South Africa.

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INTRODUCTION

Feline immunodeficiency virus (FIV) was first isolated in 1986 from a group of cats in California that were exhibiting signs of an immunodeficiency syndrome[27]. It has subsequently been classified as a lentivirus in the *Retroviridae* family based on morphology and genome organisation[23,26]. Human immunodeficiency virus (HIV) is also a member of the genus *Lentivirus* and both viruses behave similarly in their respective hosts[2]. Therefore, FIV is becoming an increasingly useful small animal model in understanding HIV infection, particularly in the development of vaccines and therapeutic agents.

In domestic cats, seroepidemiological surveys have revealed a worldwide distribution of FIV with variable prevalence. In some regions of the United States, FIV prevalence is less than 2 % of the healthy cat population whilst in Japan FIV is found in up to 12 % of healthy cats[11,41]. The same surveys recorded prevalences in sick cats of 14 % and 44 %, respectively. South Africa could be considered to have a relatively high FIV seroprevalence with a recent study finding that 22.2 % (101/454) of sick domestic cats were FIV antibody positive[33]. The variation in prevalence is likely to reflect the different systems of cat management throughout the world. Since FIV is spread via saliva during biting, the increased prevalence in certain countries is thought to be associated with cats being allowed to roam more freely between indoors and outdoors, with a consequent increase in territorial fighting[28].

Lentiviruses are characteristically unstable viruses with genetic variants constantly evolving due to the high error rate of the viral polymerase enzyme during DNA synthesis[2]. The complete genome sequence of FIV was first reported in 1989[23,36] and subsequent sequence comparisons have revealed high levels of heterogeneity of the envelope (*env)* gene sequence in particular. Nine distinct variable regions (V1–V9), interspersed by more conserved domains, have been identified within the FIV *env* gene[24]. Based on sequence differences in the variable V3–V5 region of the *env* gene, FIV has been divided into 5 subtypes (designated A, B, C, D, and E)[8,20]. Nucleotide sequence divergence among subtypes is 17.8–38.0 %, whereas intra-subtype differences are 2.5–15.0 %[4,34].

Worldwide, subtypes A and B are the most prevalent subtypes and have a relatively wide geographical distribution in comparison with subtypes C, D and E. Subtypes A and B have been found in the United States, Canada, Europe, Australia and Japan[1,13,21,34]. Subtype C has been identified predominantly in Canada, Vietnam and Taiwan[1,19,39]. Subtypes D and E have a more limited distribution, having been isolated only in Japan and Argentina, respectively[20,25].

The extensive genetic diversity of FIV has been a major obstacle in the development of a successful vaccine[5,40]. In 2002, a dual subtype FIV vaccine became commercially available (Fel-O-Vax FIV®, Fort Dodge Animal Health). This vaccine contains inactivated infected cells and whole viruses from subtypes A and D. In addition to providing protection against homologous subtypes, this vaccine has been shown to be protective against subtype B challenge[16,29,30]. Vaccinated cats developed broad-spectrum humoral and cellular immunity[30].

Identifying the type and diversity of FIV strains is essential, not only to minimise the chance of vaccine breakdown in regions where vaccination is to be implemented but it will also assist in the development of molecular based diagnostic assays. Currently, vaccinated and infected cats cannot be distinguished because diagnostic tests rely on the detection of FIV antibodies[18]. Therefore, new assays based on viral nucleic acid or antigen detection will become increasingly important as they directly detect components of the virus. However, the accuracy of some of these diagnostic tests may be affected by variability in the target sequence, and therefore an understanding of subtype variation is required.

The prevailing FIV subtype(s) present in the South African domestic cat population is unknown. Preliminary data from a worldwide survey were based on only 2 naturally infected South African cats whose FIV clustered with subtype A isolates[1]. This was based on an indirect measure of variability, a heteroduplex mobility assay on PCR fragments encompassing the V3–V4 region of the *env* gene. In this

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study, we present sequence diversity data of the V3–V5 region of the FIV *env* gene from domestic cats in South Africa.

MATERIALS AND METHODS

Viral samples

Thirty-three blood samples from South Africa were sent to the Veterinary Virology Laboratory, University of Queensland, Australia. All samples were collected from FIV positive cats, based on antibody detection by the ELISA method (FeLV antigen/FIV antibody Snap Combo Plus, IDEXX Laboratories). The samples originated from cats presented to the Onderstepoort Veterinary Academic Hospital in Pretoria, private veterinary practices in Durban and Cape Town, and a cat rescue centre in Johannesburg. The cats were bled from either the jugular or cephalic veins and blood was drawn into heparin tubes. The samples required gamma irradiation (an Australian Quarantine and Inspection Service requirement) before handling in Australia. Before the samples were received, 2 Australian samples collected during a similar survey underwent a 'trial' irradiation to ensure that the PCR was not adversely affected by the irradiation procedure. The 2 samples were successfully amplified following the 50 k Gray irradiation.

DNA extraction

Genomic DNA was extracted from 200 $\mu\ell$ of whole blood using the Wizard® SV Genomic DNA Purification System (Promega), according to the manufacturer's instructions. The genomic DNA was eluted in a total volume of 250 $\mu\ell$ nuclease-free water and stored in aliquots at –20 °C until use.

The genomic DNA of 4 samples was extracted using the BioRobot® EZ1 Workstation and EZ1 DNA blood cards (Qiagen) following manufacturer's protocols. This system utilises magnetic beads to bind DNA.

Polymerase chain reaction (PCR)

The V3–V5 region of the *env* gene was amplified in 2 overlapping fragments using the primer pairs su3-su4 and su5-su6. Primers su3-su4 amplify the region between nucleotides 7314 and 7806 of the published Petaluma sequence (GenBank accession number M25381) and the primer pair su5-su6 amplify from nucleotides 7660 to 7942[23]. Primer sequences are as follows:

su3 5′ ATWCCAAAATGTGGATGGTGG 3′
su4 5′ AATAAGGTCATCTACCTTCAT 3′
su5 5′ AATCCTGTAGATTGTACCATG 3′
su6 5′ TCCTGCYACTGGRTTATACCA 3′

PCR amplification was performed in a reaction mixture (12.5 $\mu\ell$) containing 2 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 0.8 μM of each primer and 1 unit Expand High Fidelity Enzyme mix (Roche®). Reaction conditions were an initial denaturation at 94 °C for 1 minute, followed by 40 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute and extension at 72 °C for 1 minute. This was followed by a final extension at 72 °C for 10 minutes.

For some products, 2 rounds of PCR were performed. The band of the appropriate size was excised from an agarose gel and gel purified followed by sodium acetate and isopropanol precipitation and used to reseed a PCR with conditions as described above, with the exception of 1.5 mM $MgCl_2$ and an annealing temperature of 60 °C in a 30 cycle PCR program.

Sequencing

PCR amplified fragments were purified using the MinElute™ PCR Purification Kit (Qiagen) and directly sequenced using the Big Dye Terminator Cycle Sequencing Kit (v3.1) (Applied Biosystems) using both forward and reverse PCR primers or using 1 primer at least twice. Full length (V3–V5) sequences were deduced from alignment of the overlapping fragments. Owing to poor sample quality, 6 samples (Ca10, Jo6, Pr4, Pr5, Pr6, and Pr8) could be sequenced for the shorter su5-su6 fragment only and 1 sample (Ca6) could be sequenced for the su3-su4 fragment only. Two samples from Johannesburg were excluded from the analysis as they could not be amplified.

Sequence and phylogenetic analysis

Multiple sequence alignments were created with ClustalW[37] aligning the sequences in this study with up to 38 previously reported sequences outlined in the caption of Fig. 1. Amino acid sequences were compared to evaluate the conservation of amino acid sites such as cysteine residues and *N* linked glycosylation sites. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1[15]. Genetic distances between pairs of sequences were determined using the Kimura 2-parameter model[14]. An unrooted neighbour joining tree[32] was constructed to determine the phylogenetic relationships. In each instance, bootstrap analysis[7] was performed with 1000 repetitions to evaluate clade consistency. Information on the direction of evolutionary change could not be deduced as there is no outgroup sequence. Phylogenetic trees were generated using full length sequences and sequences obtained from the individual fragments (5′ su3-su4 and 3′ su5-su6).

RESULTS

Sequence analysis

Up to 682 nucleotides, representing the V3–V5 region of the *env* gene were sequenced from 31 blood samples of FIV infected cats from 4 regions of South Africa (GenBank accession numbers DQ873700-DQ873733). Sequences of this whole region (in 2 overlapping fragments) were achieved for 24 samples while only the 3′ su5-su6 region was able to be sequenced for 6 samples and for 1 sample only the 5′ su3-su4 region was successfully amplified and sequenced. Comparisons of the predicted amino acid sequences with representatives from each of the subtypes confirmed that the targeted region was sequenced. In concordance with previous reports, cysteine residues and *N*-linked glycosylation sites were relatively well conserved, which reflects their functional importance to the envelope glycoprotein (data not shown)[12,20,25].

Phylogenetic analysis

Analysis of pairwise genetic distances is indicative of the presence of 2 subtypes within South Africa with pairwise genetic distances of up to 38.1 % observed with the su5-su6 sequences, whereas the su3-su4 sequences showed a maximum pairwise genetic distance of 14.8 %[4,12].

In order to classify the sequences from this study into the appropriate subtypes, neighbour joining phylogenetic trees were constructed with sequences representative of each of the subtypes. Phylogenetic analyses showed that the majority of South African sequences clustered within subtype A (Fig. 1). In Fig. 1 (a) samples were excluded from the phylogenetic analysis if only 1 fragment was amplified or if inconsistencies in subtype assignment between the 2 fragments were observed. For these sequences, separate phylogenetic analyses of the su3-su4 and su5-su6 fragments were performed. The su3-su4 fragments (25 samples) clustered within subtype A. The su5-su6 fragments clustered either within subtype A or subtype C (Fig. 1 b).

For the majority of samples, phylogenetic grouping of sequences of the individual fragments was consistent with respect to subtype classification when analysed separately. However, for 3 samples (Pr9, Ca11, Ca12), an inconsistency with subtype assignment was noted when the different fragments were subjected to phylogenetic analysis. The 5′ fragments, amplified with the primers su3-su4, clustered within subtype A while the 3′ fragments, amplified with the primers su5-su6, grouped with other subtype C viruses confirming the presence of 2 sub-

types, A and C, in domestic cats in South Africa.

In this study, isolates within South Africa did not cluster by geographical region, with sequences from each region distributed throughout the tree. The South African A and C isolates did not cluster separately from the other A or C isolates from other regions of the world. However, bootstrap values are generally quite low within the subtypes, and therefore a definitive association between subtype and geographical location cannot be made.

Subtype relationship to clinical disease

The cats in this study showed clinical signs that are often associated with FIV infection and these are summarised in Table 1. Only the 4 feral cats sampled from Johannesburg were considered to be free of clinical disease. A wide variety of clinical signs was reported, the most common being anorexia and weight loss. Other common presenting signs were pyrexia, lethargy, inflammation of the mouth, skin disease, haematological disorders, neoplasia, hepatic and respiratory disease. No obvious relationship of subtype to clinical disease was apparent.

DISCUSSION

This preliminary study demonstrates the presence of FIV subtypes A and C in the domestic cat population of South Africa, with subtype A the most prevalent. The presence of subtype A had previously been suggested based on heteroduplex mobility analysis utilising the V3–V4 region of the *env* gene of the virus from only 2 cats from South Africa[1].

Sequences were generated for 2 overlapping fragments encompassing the V3–V5 region of the *env* gene. For most samples, (21/24), the individual fragments grouped consistently within subtype A when phylogenetic analyses was performed. However, 3 isolates (Ca11, Ca12, Pr9) showed an inconsistency with subtype assignment. It was observed that the 5′ region of these sequences (amplified by su3-su4) most closely resembled subtype A while the remaining downstream sequence appeared most closely related to subtype C. Dual infection and/or intersubtype recombination, either within an individual or as a PCR artefact, may explain some of these findings. However, to definitively confirm the presence of recombinants, analysis of full length genomes is necessary.

The genetic diversity of lentiviruses, including FIV, is a result of errors that occur during replication, in addition to recombination[1,2]. Superinfection and recombination in FIV infected cats have been demonstrated under experimental conditions[1,17,22] and as more sequence data become available from around the world, there is increasing evidence to support their occurrence during natural FIV infection, particularly in areas where different subtypes are known to exist[31]. Dual infection and recombination are, therefore, theoretical possibilities in South Africa and may explain the subtype discrepancies observed with the 3 discordant isolates.

Although many disease states are associated with FIV, the association between subtype and severity of disease is still uncertain. Potential correlations between FIV subtype and disease have been reported[1,3,21]. Cats infected with subtypes A or C are generally more likely to be symptomatic than cats infected with subtype B[1,20]. Ikeda *et al.*[10] also found that subtype A infected cats developed AIDS-related clinical signs earlier than cats with subtype B infection[10]. In the present study, cats harboured subtype A or C viruses and except for the 4 feral cats from Johannesburg, all cats demonstrated some sign of disease. The sample population could be considered to be biased because, apart from the feral cats, all the sampled cats were visiting a veterinary clinic. Nonetheless, there appeared to be no difference in clinical status between the cats infected with subtype C or subtype A in this study. In humans, several studies investigating the relationship between HIV subtype and pathogenicity have been performed with varying conclusions[9,38]. It is possible that any observed differences could be due to confounding factors such as environmental factors and, therefore, more studies are required to determine whether any significant correlation exists between subtype and pathogenicity.

FIV isolates have been observed to cluster according to geographical location. Two types of geographical clustering have been noted. Firstly, subtypes may group by geographical location within a country. An example of this is seen among FIV infected cats in Japan where subtype B is found predominantly in eastern regions and subtype D is found mainly in western regions[20]. Secondly, the presence of geographically localised subclusters within a subtype has been observed. For example, Austrian and Portugese isolates form distinct clusters within subtype B[4,35]. In the phylogenetic trees constructed from sequences in this study, no clustering of isolates from similar geographical regions was observed, nor did the South African isolates form a unique cluster within subtypes A or C. However, it is premature to infer that this is not occurring due to the low bootstrap values observed within subtype A in particular.

The substantial and increasing genetic diversity of FIV has made the development of a successful vaccine difficult. However, the Fel-O-Vax FIV® dual subtype vaccine has been shown experimentally to provide good protection against homologous and heterologous subtype

Figure 1: **Unrooted neighbour joining phylogenetic trees inferred from the alignment of previously described FIV nucleotide sequences and the sequences determined in this study using Kimura's 2-parameter model. Bootstrap values, as a percentage of 1000 bootstrap replicates, are shown on branches where values are greater than 50%. Branch lengths are drawn to scale. The subtypes A, B, C, D and E are indicated. The South African isolates from this study are designated Ca for Cape Town samples, Du for Durban samples, Jo for Johannesburg samples and Pr for Pretoria samples and are shown in bold. The isolate name, country of origin and GenBank accession numbers for the published sequences included in this study are**:

Subtype A: Petaluma (USA) M25381, Dixon (USA) L00608, PPR (USA) M36968, USCAtt01A (USA) U02411, USCAlemy01A (USA) U02404, DEBAb91 (Germany) AF531043, UK2 (United Kingdom) X69494, UK8 (United Kingdom) X69496, UT113 (Netherlands) X60725, DutchK1 (Netherlands) M73964, SwissZ2 (Switzerland) X57001, Wo (France) L06135, Sendai1 (Japan) D37813.

Subtype B: TM2 (Japan) M59418, Yokohama (Japan) D37812, Sendai2 (Japan) D37814, Aomori2 (Japan) D37817, TAU01 (Japan) AB010405, Italy M2 (Italy) X69501, ATVIa33 (Austria) AF531045, LP9 (Argentina) D84497, FP4 (Portugal) AJ304983, TLP3 (Portugal) AJ304987.

Subtype C: FIVC (Canada) AF474246, CABCpady02C (Canada) U02392, CABCpbar07 (Canada) U02397, AIC01 (Japan) AB010396, VND-3 (Vietnam) AB083509, TI-4 (Taiwan) ABO16028, MU-3 (Taiwan) AB016668.

Subtype D: Shizuoka (Japan) D37811, Fukuoka (Japan) D37815, OKA01 (Japan) AB010400, KUM02 (Japan) AB010399, MY8 (Japan) D67063.

Subtype E: LP-3 (Argentina) D84496, LP-20 (Argentina) D84498, LP-24 (Argentina) D84500.

(**a**) Tree constructed from 'full length' V3–V5 *env* nucleotide sequence alignment.

(**b**) Tree constructed from su5-su6 fragment of *env* nucleotide sequence alignment.

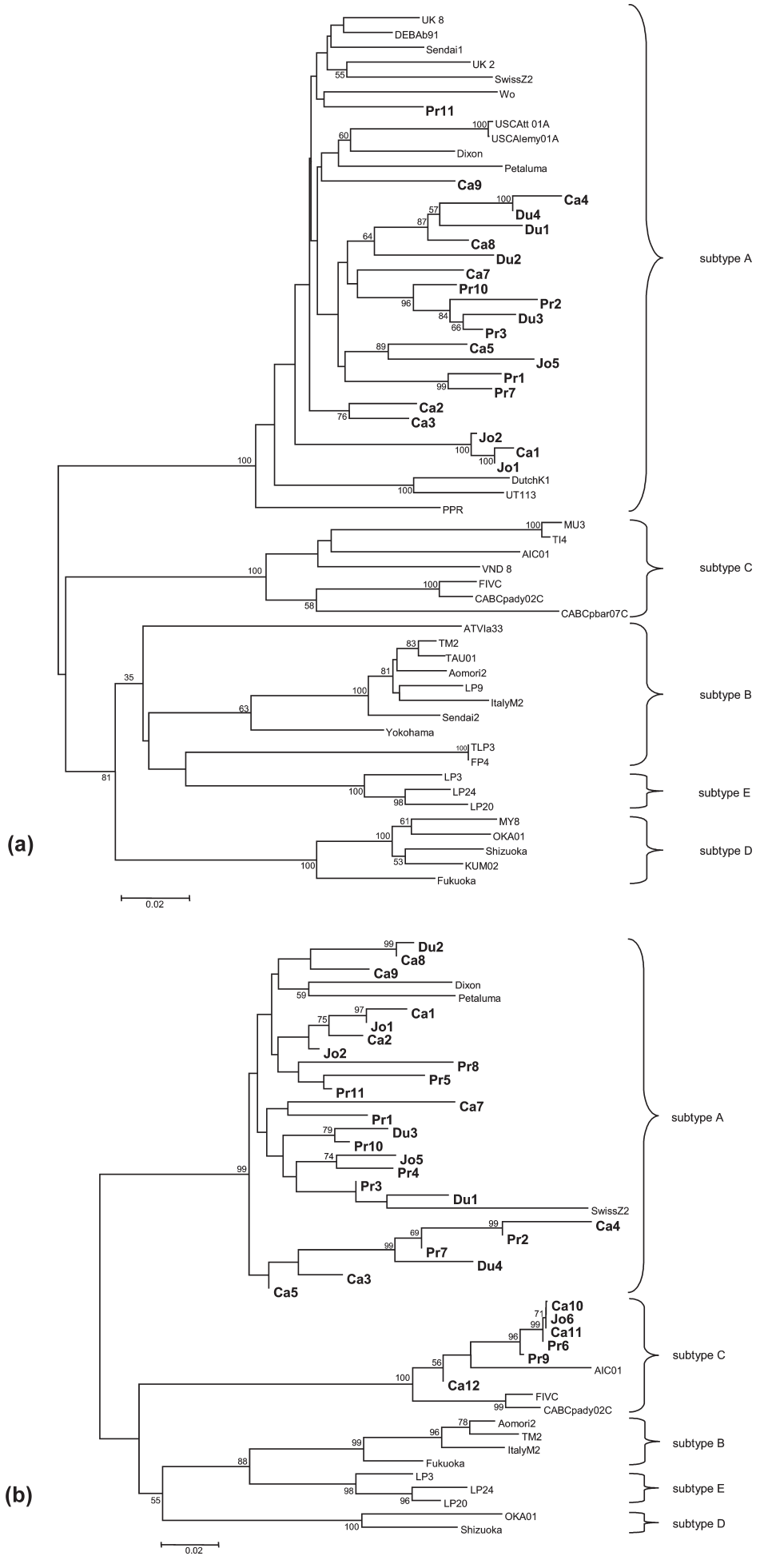
(a)
UK 8
DEBAb91
Sendai1
UK 2
SwissZ2
Wo
Pr11
USCAtt 01A
USCAlemy01A
Dixon
Petaluma
Ca9
Ca4
Du4
Du1
Ca8
Du2
Ca7
Pr10
Pr2
Du3
Pr3
Ca5
Jo5
Pr1
Pr7
Ca2
Ca3
Jo2
Ca1
Jo1
DutchK1
UT113
PPR
MU3
TI4
AIC01
VND 8
FIVC
CABCpady02C
CABCpbar07C
ATVIa33
TM2
TAU01
Aomori2
LP9
ItalyM2
Sendai2
Yokohama
TLP3
FP4
LP3
LP24
LP20
MY8
OKA01
Shizuoka
KUM02
Fukuoka
0.02
subtype A
subtype C
subtype B
subtype E
subtype D
(b)
Du2
Ca8
Ca9
Dixon
Petaluma
Ca1
Jo1
Ca2
Jo2
Pr8
Pr5
Pr11
Ca7
Pr1
Du3
Pr10
Jo5
Pr4
Pr3
Du1
SwissZ2
Ca4
Pr2
Pr7
Du4
Ca3
Ca5
Ca10
Jo6
Ca11
Pr6
Pr9
AIC01
Ca12
FIVC
CABCpady02C
Aomori2
TM2
ItalyM2
Fukuoka
LP3
LP24
LP20
OKA01
Shizuoka
0.02
subtype A
subtype C
subtype B
subtype E
subtype D

Table 1: **Summary of the South African FIV strains reported in this study.**

Sample	Province	City	Age (years)	Sex	Breed	Clinical findings	Subtype
Pr1	Gauteng	Pretoria	9	FS	DSH	Stomatitis, anorexia, weight loss, abdominal mass	A
Pr2	Gauteng	Pretoria	1-2	M	DSH	Dog attack wounds	A
Pr3	Gauteng	Pretoria	10	M	DSH	Anorexia, panleukopenia, azotaemia	A
Pr4	Gauteng	Pretoria	10	MC	DSH	Epiphora	A*
Pr5	Gauteng	Prctoria	13	FS	DSH	Unwell 6 months ago, anorexia	A*
Pr6	Gauteng	Pretoria	7	MC	DSH	Stomatitis, anorexia	C*
Pr7	Gauteng	Pretoria	13	MC	DSH	Stomatitis, anorexia	A
Pr8	Gauteng	Pretoria	4	MC	DSH	Anorexia, weight loss, leukopenia	A*
Pr9	Gauteng	Pretoria	9	MC	DSH	Anorexia, otitis, abdominal mass	A/C†
Pr10	Gauteng	Pretoria	11	MC	Persian	Anorexia, weight loss, chronic sneezing, haemangiosarcoma ventral tongue	A
Pr11	Gauteng	Pretoria	9	MC	DSH	Anorexia, leukopenia, pyrexia	A
Jo1	Gauteng	Johannesburg	Unknown	Unknown	DSH	NAD	A
Jo2	Gauteng	Johannesburg	Unknown	Unknown	DSH	NAD	A
Jo5	Gauteng	Johannesburg	Unknown	Unknown	DSH	NAD	A
Jo6	Gauteng	Johannesburg	Unknown	Unknown	DSH	NAD	C*
Du1	KZN	Durban	8	M	DSH	Non-responsive dermatitis/pyoderma	A
Du2	KZN	Durban	7	FS	DSH	Non-responsive dermatitis, *Demodex cati*	A
Du3	KZN	Durban	2	F	DSH	Pyrexia, vomiting	A
Du4	KZN	Durban	10+	F	DSH	Lethargy	A
Ca1	W-Cape	Cape Town	8	MC	DSH	Recurrent cholangiohepatitis	A
Ca2	W-Cape	Cape Town	>7	M	DSH	Weight loss, ataxia/weakness	A
Ca3	W-Cape	Cape Town	5	MC	Siamese	Delayed wound healing	A
Ca4	W-Cape	Cape Town	7	MC	DSH	Chronic recurrent stomatitis	A
Ca5	W-Cape	Cape Town	4	M	DSH	Weight loss, pyrexia	A
Ca6	W-Cape	Cape Town	>8	MC	DSH	Weight loss, lethargy, alopecia	A^
Ca7	W-Cape	Cape Town	1	MC	Siamese	Pyrexia, lethargy, inappetence, anaemia	A
Ca8	W-Cape	Cape Town	>8	M	Persian	Weight loss, chronic gingivitis/stomatitis	A
Ca9	W-Cape	Cape Town	10	FS	DSH	Weight loss, gingivitis, lethargy	A
Ca10	W-Cape	Cape Town	8	MC	DSH	Weight loss, lethargy	C*
Ca11	W-Cape	Cape Town	>10	F	DSH	Recurrent pyrexia, anorexia, lethargy, anaemia	A/C†
Ca12	W-Cape	Cape Town	2	MC	DSH	Pyrexia, stomatitis, recurrent abscesses	A/C†

FS, female spayed; MC, male castrated.
DSH, domestic shorthair cat.
NAD, nothing abnormal detected.
KZN, KwaZulu-Natal.
W-Cape, Western Cape.
^, Sequence from su3-su4 fragment only.
*Sequence from su5-su6 fragment only.
†, Subtype assignment discrepancy between the 2 fragments.

challenge[16,29,30]. Its efficacy in the field had been uncertain but recent studies designed to mimic natural conditions have supported its efficacy against more diverse FIV[16,29]. It was found to afford good protection against a subtype B (Aomori2) infection when vaccinated, unvaccinated and infected cats were mingled. Additionally, it was found to be efficacious against a virulent in vivo derived subtype B challenge[29]. However, others have reported that the vaccine afforded no protection against a virulent subtype A challenge[6].

It is difficult to speculate on the potential efficacy of this FIV vaccine in South Africa. The majority of evidence suggests that it would be beneficial in reducing infection with subtype A viruses (which predominate in South Africa) but its efficacy against subtype C infection is uncertain. The ability of the vaccine to protect against heterologous subtype challenge is suggestive of the development of broad spectrum immunity. This broad spectrum immunity may be the consequence of utilising 2 subtypes (subtypes A and D) within the vaccine[30]. It has been speculated that using 2 subtypes may direct the immune system to common epitopes which could enhance vaccine immunity across the subtypes[42]. Given that the vaccine provides protection against diverse subtype B infection, it is possible that protection will be achieved against other subtypes and more diverse viral strains. However, this cannot be confirmed until vaccine trials are performed with more diverse challenges.

In conclusion, this study has found FIV subtypes A and C in the South African domestic cat population, with subtype A predominant. Of interest is the finding of a high degree of FIV genetic diversity among infected cats in South Africa and the detection of diverse sequences within some individual cats. Further research is required to fully elucidate these findings. This knowledge assists in our understanding of FIV diversity which may have implications in preventative and diagnostic strategies.

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