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Feline immunodeficiency virus subtypes A, B and C and intersubtype recombinants in Ontario, Canada

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Knowledge of the geographical distribution of feline immunodeficiency virus (FIV) subtypes is important for understanding different disease courses and for vaccine design. Intersubtype recombination may develop in areas where more than one subtype is prevalent and has the potential to create new transmittable variants with novel pathogenic properties. In this study, 40 FIV-positive DNA samples were classified by sequence analysis of the LTR–*gag* region. Phylogenetic analysis indicated that 32 Canadian FIV isolates clustered with previously identified subtypes A, B and C and that subtype A was most frequent in Ontario. Four strains with inconsistent clade assignment were further analysed by sequencing of the *env*–LTR regions. Comparisons of phylogenetic trees constructed from the two different regions of the genome and analysis of similarities to reference sequences yielded classification of three samples as A/B and one as A/C intersubtype recombinants. Although the A/B recombinant samples were obtained from unrelated cats in geographically disparate regions, a common breakpoint was consistently identified within *gag*. In addition, there was no evidence of co-infection with parental strains of subtypes A and B as indicated by PCR-based limiting dilution assays, although these assays allowed for the identification of two different recombinant viruses co-existing in one sample. Both sequences contained the same breakpoint. These findings suggested that a new circulating recombinant FIV may be enzootic in Ontario.

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INTRODUCTION

Feline immunodeficiency virus (FIV), first identified as a T-lymphotropic virus (Pedersen *et al.*, 1987), is a lentivirus of the family *Retroviridae* related to the human immunodeficiency virus (HIV) (Olmsted *et al.*, 1989a, b; Pedersen *et al.*, 1987; Sparkes *et al.*, 1993). Infected cats typically develop $CD4^+$ T lymphocytopenia, which leads to immunodeficiency, opportunistic infections, increased occurrence of neoplasia or a neurological syndrome. Based on genetic, morphological and clinical similarities, FIV has been a valuable model for understanding aspects of HIV pathogenesis and for developing intervening strategies to control infection and disease (Bendinelli *et al.*, 1995; Johnson *et al.*, 1994; Okada *et al.*, 1994; Pedersen *et al.*, 1987).

FIV is distributed worldwide (Bachmann *et al.*, 1997; Ishida *et al.*, 1989; Nishimura *et al.*, 1998; Pedersen *et al.*, 1989). The prevalence of infection is highly variable ranging from 1 % in cats at low risk in the USA and Canada (Yamamoto *et al.*, 1989) to 44 % in symptomatic cats in Japan (Hohdatsu *et al.*, 1998), depending upon factors such as age, gender and indoor or outdoor housing. Currently, FIV isolates are classified into five different subtypes designated A, B, C, D and E, based on envelope sequence analysis (Nishimura *et al.*, 1998). Subtype A has been reported from California and Northern Europe while subtype B was prevalent in the central and eastern USA and in southern European countries (Sodora *et al.*, 1994). Subtype C has been identified from California and British Columbia (Bachmann *et al.*, 1997; Sodora *et al.*, 1994), while subtypes D and E have been reported from Japan (Hohdatsu *et al.*, 1996, 1998; Nishimura *et al.*, 1998) and Argentina (Pecoraro *et al.*, 1996), respectively. Classification into subtypes may be accomplished by different methods including subtype-specific PCR (Nishimura *et al.*, 1998), heteroduplex mobility assays (Bachmann *et al.*, 1997), restriction fragment polymorphisms (Hohdatsu *et al.*, 1998; Kurosawa *et al.*, 1999) and phylogenetic analysis of sequences (Worobey & Holmes, 1999). Although most studies are based on *env* gene variability, comparison of sequences of the *gag* gene can be useful, since this gene also has marked variability among different FIV isolates (Hohdatsu *et al.*, 1998; Kakinuma *et al.*, 1995; Pistello *et al.*, 1997).

Co-infection with two different lentiviral strains might occur following exposure to a second virus shortly after the initial infection or after the initial infection has been

The GenBank accession numbers of the sequences reported in this article are AY220046–AY220074 and AY369376–AY369384 (LTR–*gag*), and AY221627–AY221629 (*env*–LTR).

established, a condition termed superinfection (Jost *et al.*, 2002). This phenomenon has been experimentally induced with FIV subtypes A and B, both *in vitro* and *in vivo* (Okada *et al.*, 1994; Pistello *et al.*, 1999). In another study, consecutive exposure of cats to two different FIV strains resulted in superinfection in one cat and recombination in another (Kyaw-Tanner *et al.*, 1994). This conclusion was based on sequence analysis of 516 bp of *env*, showing that 106 bp were highly homologous to one parental strain and the remainder to the other parental strain. It was suggested that a high inoculation dose of virus and the route of infection may have favoured superinfection, and that superinfection and recombination should be studied under natural conditions (Kyaw-Tanner *et al.*, 1994). Since genetic diversity among lentiviruses is extensive, it has been debated whether a vaccine should include a single virus strain, a broad spectrum of viral variants or only those that are relevant to a particular geographical area (Gao *et al.*, 1998). Similar to HIV, intersubtype recombination might be an important consideration for development of vaccines effective against multiple FIV subtypes (Siepel *et al.*, 1995).

Neither the prevalence of FIV infection in Canada nor the prevailing subtypes are known. Identification of circulating subtypes is essential to develop strategies for molecular diagnosis, since the genetic diversity is high. In addition, the coexistence of more than one subtype in a particular location raises the possibility of recombination, creating variants with novel features that might interfere with diagnostic tests. Knowing prevalent FIV subtypes is also an important consideration for designing and testing vaccines under field conditions and would aid in establishing potential associations between specific subtypes and the severity of clinical outcomes. Thus, the aims of this study were to identify primary FIV subtypes present in Canada, to detect associated unique clinical conditions and to investigate the occurrence of intersubtype recombination. For this purpose, we sequenced the LTR–*gag* gene region from 40 FIV primary isolates and the *env*–LTR region from selected samples. Recombination was assessed by phylogenetic analysis of nucleotide and amino acid sequences as well as by analysis of similarities to reference sequences.

METHODS

Viral samples. Reference FIV subtype A, strain Petaluma (clone 34TF10), was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from J. Elder. Subtype C was provided by J. Mullins, University of Washington, Seattle, WA, and subtype D, strain Shizuoka, by T. Hohdatsu, Kitasato University, Japan. Of the 40 unknown samples, four originated from eastern USA, one from British Columbia and 35 from Ontario. All blood samples were positive for FIV antibodies by ELISA (Pet Check ELISA; IDEXX) and yielded products after one or two rounds of PCR. Background information on the samples is provided in Table 1.

DNA isolation. DNA was extracted from 200 µl whole blood (QIAamp DNA Blood Mini kit; Qiagen) following the directions of the manufacturer. The DNA was eluted into 100 µl buffer and stored at −20 °C. First- and second-round amplifications and analysis of amplicons were performed in separate laboratories with dedicated equipment.

LTR–*gag* PCR. Primers were designed to amplify the LTR–*gag* region of proviral DNA. Base pair identification of all sequences including primers and amplicons was in reference to FIV subtype A, strain Petaluma (GenBank accession no. M25381; Talbott *et al.*, 1989). The relative locations of the primers are indicated in Fig. 1. First-round reactions produced amplicons of 1287 bp using primers LTR1-f and Gag1-r (Table 2). The second round of PCR yielded products of 1127 bp using a different forward primer (LTR2-f) and primer Gag1-r. This PCR product included the LTR regions R and U5, the *gag* region encoding the p15 protein and 421 bp of the *gag* gene encoding the p24 protein. Amplifications were performed in 25 µl total volume with 1·25 units *Taq* DNA polymerase (Life Technologies), 1·5 mM $MgCl_2$, 0·2 mM each dNTP, 0·5 µM each primer and 2 µl purified DNA. For second-round reactions, 2 µl of the first-round PCR product was used as a template. Reactions were cycled 35 times with denaturation at 94 °C for 45 s, annealing at 60 °C for 20 s and elongation at 72 °C for 90 s. Second-round conditions were identical except that the annealing temperature was 64 °C for 20 s. PCR products were identified by electrophoresis in 1 % agarose gels and ethidium bromide staining.

***env*–LTR PCR.** A 2 kb fragment of proviral DNA including 1750 bp of the *env* gene and 250 bp of the LTR region was amplified from selected samples, using primers MB-1 (Bachmann *et al.*, 1997) and LTR3-r (Table 2). The relative locations of the primers are indicated in Fig. 1. PCR mixtures and cycling conditions were identical to those described above for the first round of the LTR–*gag* PCR.

PCR-based limiting dilution assay (PLDA). To minimize the likelihood of resampling the same DNA molecule during PCR amplification, the number of copies of provirus from selected samples was estimated. Serial twofold dilutions of template were PCR-amplified using LTR-1 and Gag1-r primers. Five replicates of four template dilutions that yielded a mixture of positive and negative PCR results were then amplified with the same primers. The template copy number was estimated with the software QUALITY, as described previously (Rodrigo *et al.*, 1997).

Cloning experiments and sample preparation for sequencing. Amplicons of the appropriate size were cut out of agarose gels with sterile blades and the DNA was extracted (QIAquick Gel Extraction kit; Qiagen). Purified PCR products for the LTR–*gag* region from all recombinant samples were cloned into a plasmid (TOPO TA Cloning kit; Life Technologies) and sequenced. The concentration of purified DNA samples was determined by fluorimetry (PicoGreen; Roche) and adjusted to 30 ng μl^{-1}. Due to the size of the *env*–LTR fragments, it was not possible to get full sequences with primers MB-1 and LTR3-r; therefore, sequencing primers Env2-f and Env3-f were designed to anneal at nt 7848–7872 and nt 8340–8361 (Table 2), 535 and 1025 bp along the 2 kb fragment, respectively. Results from the four overlapping subfragments were aligned to reassemble contiguous sequences. Sequences were determined by the BigDye Terminator method on an ABI Prism 377 XL DNA Sequencer (DNA Sequencing Facility, Robarts Research Institute, London, ON).

Alignments and phylogenetic analysis. Nucleotide sequence analysis was performed to account for the non-coding function of the LTR. This analysis included 1040 bp (310 bp of LTR and 730 bp of *gag*) from four reference sequences and 40 unknown FIV samples. Sequences were edited using VECTOR NTI (Version 7.0; InforMax, Calgary, AB) and multiple alignments were performed with CLUSTAL_X (Thompson *et al.*, 1997). Gaps were inserted to

Table 1. Sample descriptions and subtype distribution of 40 FIV primary isolates

Sample	Country	Prov./state	City	Age (years)	Sex*	Remarkable findings	Subtype†
CaONA01	Canada	ON	Mount Hope	10	FS	Anaemia, cutaneous ulceration	A
CaONA02	Canada	ON	Churchill	8	MC	Hyperglobulinaemia, lymphopenia, cardiomyopathy	A
CaONA03	Canada	ON	Guelph	4	M	Proliferative polyarthiritis, lymphopenia	A
CaONA04	Canada	ON	Scarborough	5	FS	Severe stomatitis	A
CaONA05	Canada	ON	Oakville	8	MC	Severe stomatitis, diabetes mellitus	A
CaONA06	Canada	ON	Kincardine	9	MC	Demodecosis, hyperglobulinaemia, lymphopenia	A
CaONA07	Canada	ON	Guelph	10	MC	Stomatitis, diarrhoea	A
CaONA08	Canada	ON	Toronto	6	MC	Anaplastic extranodal lymphoma	A
CaONA09	Canada	ON	Guelph	9	MC	Chronic diarrhoea, stomatitis	A
CaONA10	Canada	ON	Guelph	NA	MC	Pneumonia, cutaneous ulceration, lymphopenia	A
CaONA11	Canada	ON	Guelph	7	MC	Stomatitis, diarrhoea	A
CaONA12	Canada	ON	Toronto	2	MC	Stomatitis, neutropenia	A
CaONA13	Canada	ON	Toronto	4	M	Vomiting, cutaneous ulceration	A
CaONA14	Canada	ON	NA	NA	F	No abnormalities	A
CaONA15	Canada	ON	Toronto	NA	NA	Gingivitis, diarrhoea, multiple cutaneous abscesses	A
CaONA16	Canada	ON	Toronto	2	M	NA	A
CaONA17	Canada	ON	Ottawa	6	MC	Diarrhoea, neutropenia	A
CaONA18	Canada	ON	Ottawa	4	M	Dermatitis	A
CaONA19	Canada	ON	Ottawa	2	M	Otitis	A
CaONA20	Canada	ON	Ottawa	1	M	Non-healing wound	A
CaONA21	Canada	ON	Ottawa	2	M	NA	A
CaONA22	Canada	ON	Ottawa	7	M	Mycotic dermatitis (*Microsporum* spp.)	A
CaONA23	Canada	ON	Ottawa	NA	NA	NA	A
CaONAB01	Canada	ON	Toronto	5	FS	Chronic renal disease	A/B
CaONAB02	Canada	ON	Brampton	5	MC	Multiple cutaneous abscesses	A/B
CaONAB03a	Canada	ON	Kitchener	NA	NA	Anaemia, lymphocytosis	A/B
CaONAB03b	Canada	ON	Kitchener	NA	NA	Anaemia, lymphocytosis	A/B
CaONAC01	Canada	ON	Ottawa	4	M	NA	A/C
CaONB01	Canada	ON	Guelph	7	MC	Uveitis	B
CaONB02	Canada	ON	Guelph	10	F	Encephalopathy, hyperglobulinaemia	B
CaONB03	Canada	ON	Barrie	6	MC	Gingivitis, abscessation, diarrhoea	B
CaONB04	Canada	ON	Toronto	NA	NA	NA	B
CaONB05	Canada	ON	Ottawa	2	M	Abscess	B
CaONB06	Canada	ON	Ottawa	3	M	Entropion, wounds	B
CaONB07	Canada	ON	Ottawa	4	M	NA	B
USctB01	USA	CT	NA	NA	M	Gingivitis, diarrhoea	B
USgaB01	USA	GA	Athens	8	MC	Seizures, lymphopenia	B
USgaB02	USA	GA	Athens	8	MC	Stomatitis, diarrhoea	B
USgaB03	USA	GA	Athens	5	MC	Lymphadenopathy	B
CaBCC01	Canada	BC	Vancouver	8	MC	Stomatitis	C
CaONC01	Canada	ON	Ottawa	3	M	Gingivitis	C

NA, Not available.

*FS, female spayed; MC, male castrated.

†Subtypes were assigned based on phylogenetic analysis of LTR–*gag* sequences.

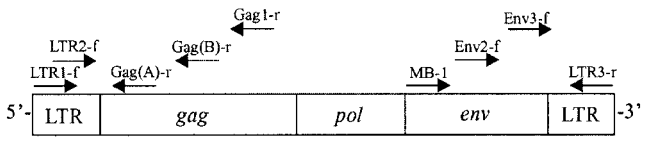

Fig. 1. Relative location of primers for amplification and sequencing of the LTR–*gag* and *env*–LTR genomic regions.

compensate for genetic variation among sequences and penalties for gaps and for gap extensions were assigned to minimize the number of possible insertions without breaking the homology of contiguous sequences. Further adjustments were done manually to maximize similarities. Nucleotide alignments were analysed with MODELTEST (Posada & Crandall, 1998) to determine the correct substitution model. Settings from the best-fit model (selected by hierarchical likelihood ratio tests) were then used to create trees with PAUP (Swofford, 2002), based on the criteria of parsimony, maximum-likelihood and

Table 2. Amplification and sequencing primers for FIV LTR, *gag* and *env* proviral DNA

Primer	Sequence (5′→3′)	Location (nt)*
LTR1-f	TTAACCGCAAAACCACATCC	122–141
LTR2-f	TGAACCCTGTCGTGTATCTGTGTAA	285–309
LTR3-r	CAAGGGAGAACTCGAAAGTCC	9371–9351
Gag1-r	AGATACCATGCTCTACACTGC	1409–1389
Gag(A)-r	CCATCTGAAATTCCCTTCTCC	738–718
Gag(B)-r	TCCTATCCCCATAATCTCTGC	1341–1321
MB-1†	ATACCAAAATGTGGATGGTG	7316–7335
Env2-f	TTGCTGGAAATTGGTCTTGTACATC	7848–7872
Env3-f	TCGCTATGCAAGAATTAGGATG	8340–8361

*All positions are in reference to subtype A Petaluma (GenBank accession no. M25381).

†Previously described in Bachmann *et al.* (1997).

minimum evolution. Bootstrap analyses were performed on 1000 iterations for parsimony and minimum evolution and on 100 iterations for maximum-likelihood. Amino acid sequence analysis was performed using PHYLIP (Felsenstein, 1993) to compare clade assignment of selected sequences based on two different genomic regions. This included 242 and 491 aa sequences deduced from *gag* and *env* sequences, respectively. Alignments were analysed with PROTDIST to create distance matrices based on the Dayhoff PAM model for substitutions between each pair of amino acids. Neighbour-joining trees were then constructed. To compare different measures, additional trees were created based on the criteria of parsimony and maximum-likelihood. For statistical support in consistency of the clades, bootstraps were performed on 1000 iterations for parsimony and minimal evolution and on 100 iterations for maximum-likelihood. Final editing of the trees and graphical representations were obtained using TREEVIEW (Page, 1996).

Analysis of recombinants. Identification of specific patterns of recombination was by direct examination of multiple alignments and sequence analysis using SIMPLOT. This software was developed to demonstrate recombination among HIV sequences and is similar in design to the Recombination Inference Program (RIP) (Lole *et al.*, 1999; Papa *et al.*, 2002; Siepel *et al.*, 1995). Briefly, the similarities of a testing sequence compared with two reference sequences and a third sequence included as an outgroup are analysed. In a window of user-specified size (200 bp in this study), similarity values are calculated and repeated for the entire region with 20 bp overlaps. Similarity values are then plotted to indicate the percentage of similarity along the entire sequence. To predict the location of breakpoints, the software analyses the alignments by maximization of χ^2 (Smith, 1992).

Subtype-specific PCR. To identify co-infection with a recombinant virus plus a subtype A or B virus, subtype-specific PCR assays were designed. A common forward primer (LTR1-f) annealed to a conserved region in the LTR and was combined with reverse primers homologous to subtype A or B. The reverse primers, Gag(A)-r and Gag(B)-r, had two or more mismatches at the 3′ end relative to the other subtype (Table 2). Each was designed to anneal upstream or downstream of the consistent A/B breakpoint identified in *gag* at the junction between the *p15* and *p24* coding regions (Fig. 2). Gag(A)-r annealed on the 5′ side of the breakpoint, while primer Gag(B)-r annealed to the 3′ side, but neither matched the sequence of the A/B recombinants in this region. Therefore, these primers identified

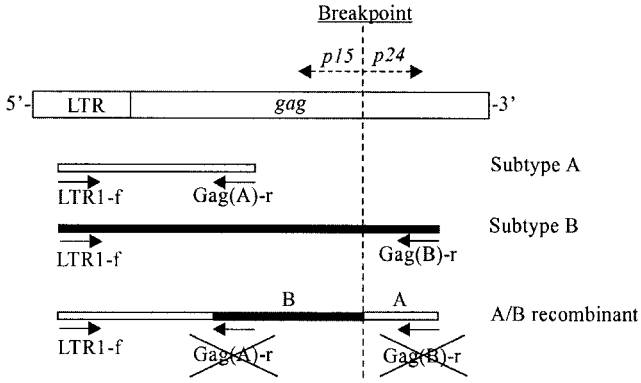

Fig. 2. PCR primers specific for amplification of FIV subtypes A and B. The A/B recombinant site is located at the junction of the *p15* and *p24* coding regions within the *gag* gene. The forward primer (LTR1-f) anneals to all LTR regions. Primers Gag(A)-r and Gag(B)-r located at either side of the breakpoint should not anneal to recombinant target DNA since these regions correspond to the opposite subtype on these particular samples.

subtypes A and B in the presence of A/B recombinant sequences. PCR reagents and cycling conditions were as described above for the first round of the LTR–*gag* PCR.

RESULTS

Provirus amplification

Primers designed to encompass the LTR–*gag* region consistently amplified proviral DNA of subtypes A, B, C and D. Reference strains Petaluma (subtype A), FIV-C (subtype C) and Shizuoka (subtype D), as well as 38 of the 40 primary isolates, were efficiently amplified after a single round of PCR, while two of the 40 samples required a second round of PCR for amplicon detection. Subtype E samples were not available, but LTR1-f and Gag1-r primers were 100 % identical to published sequences of this particular subtype (Pecoraro *et al.*, 1996). Recently, partial sequences for a presumptive novel FIV subtype termed ‘F’ were deposited in GenBank (accession nos AY139105–AY139112). Primer Gag1-r is homologous to these new sequences; however, LTR sequences were not available. The PCR assay designed for the *env*–LTR region consistently amplified subtypes A, B and C in a similar manner.

Phylogenetic analysis

To classify unknown isolates within their respective subtypes, a parsimony tree was constructed based on 1040 bp from the LTR–*gag* region, including reference sequences from subtypes A, B and C (Fig. 3a). To assess the consistency of clade assignment (Hall, 2001), additional trees were created based on the criteria of maximum-likelihood (Fig. 3b) and minimum evolution (not shown). Trees from the three different methods showed that similar structure

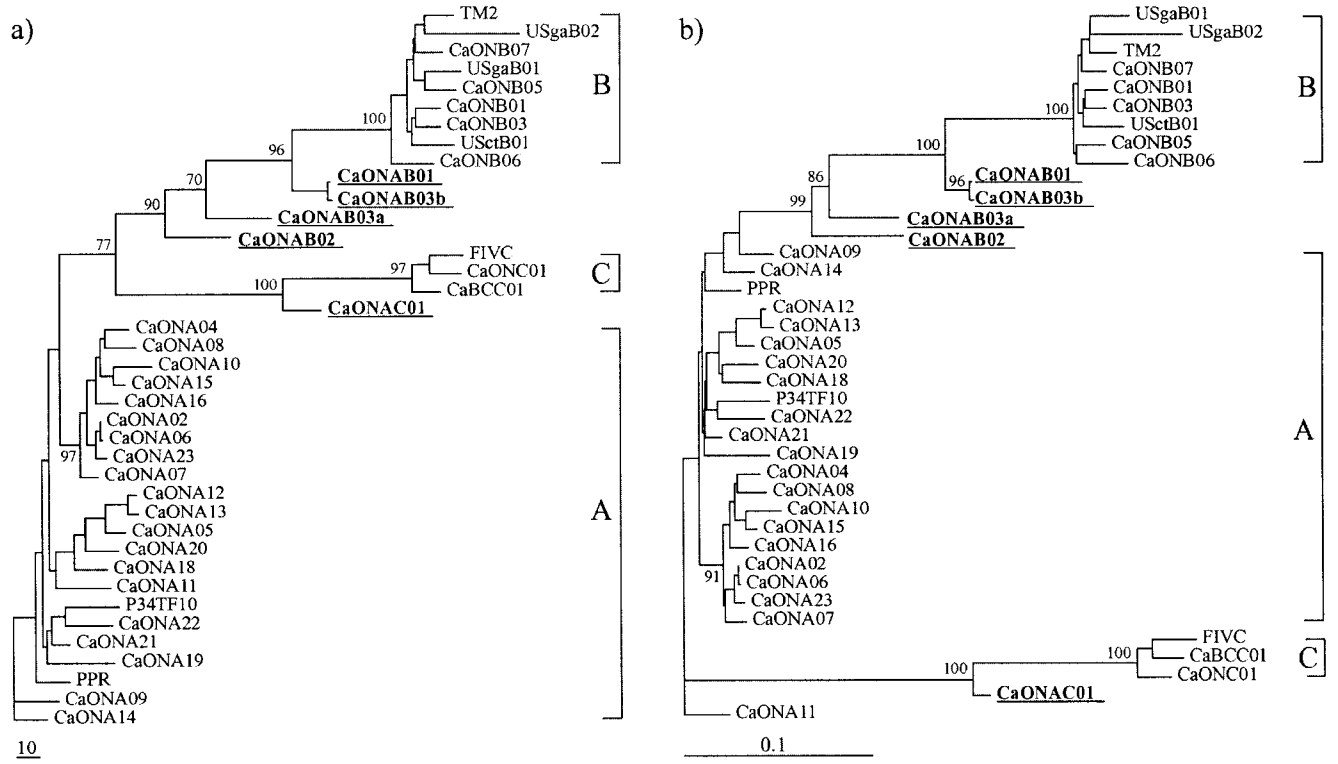

Fig. 3. Parsimony and maximum-likelihood analyses of FIV LTR–*gag* nucleotide sequences. (a) Parsimony tree of 1040 bp between nt 330 and 1370. Sequences located between subgroups A/B or A/C (underlined) were characterized as intersubtype recombinants. Bootstrap values (1000 iterations) greater than 70 are in reference to the node located to the right. CaONA09 and CaONA14 were used as outgroups. The scale bar represents the number of nucleotide changes along the branch. Only horizontal branches are informative. (b) Maximum-likelihood tree for the same sequences indicating consistency of clade assignment. Bootstrap values were based on 100 iterations. CaONA11 was used as an outgroup. Reference sequences: Petaluma, clone 34TF10 (GenBank accession no. M25381; Talbott *et al.*, 1989); PPR (M36968; Phillips *et al.*, 1990); TM2 (M59418; Kiyomasu *et al.*, 1991); subtype C (AF474246) (unpublished).

and group assignment of samples was well supported by bootstrapping (Fig. 3a, b). All four US samples were classified as subtype B. Of the 36 Canadian samples, 23 (64 %) were identified as subgroup A, seven (19 %) as subgroup B and two (6 %) as subgroup C. Four (11 %) samples (CaONAB01, CaONAB02, CaONAB03 and CaONAC01) showed inconsistent subgroup assignment. Three of these sequences (CaONAB01, CaONAB02 and CaONAB03) appeared related to subtype B while the remaining sequence (CaONAC01) was most similar to subtype C. This suggested that these four sequences were either highly divergent or potentially derived from more than one subtype. To investigate these possibilities further, the relationship with prototype FIV subtypes was examined through minimum evolution trees based on translated Gag and Env regions. Additional parsimony and maximum-likelihood trees were also created to compare different methods (not shown). Trees from the three different methods showed similar structure and the group assignment was well supported by bootstrapping (Fig. 4). Three samples (CaONAB01, CaONAB02 and CaONAB03) clustered with subtype B and the remaining sample (CaONAC01) was a subtype C within the Gag region (Fig. 4a). In contrast, three sequences (CaONAB02, CaONAB03 and CaONAC01) clustered with subtype A in the Env region (Fig. 4b) while the other sample (CaONAB01) remained within subtype B. Inconsistent clade assignment in LTR–*gag* and discrepancies between Gag and Env indicated that these samples were A/B (CaONAB01, CaONAB02 and CaONAB03) and A/C (CaONAC01) intersubtype recombinants. To investigate these possibilities, an additional 2 kb of proviral DNA encompassing the *env*–LTR junction was amplified and sequenced.

Intersubtype recombination

The phylogenetic analysis thus suggested that three (CaONAB01, CaONAB02 and CaONAB03) and one (CaONAC01) FIV-positive samples were A/B and A/C intersubtype recombinant viruses, respectively. Visual inspection of multiple alignments of nucleotide sequences identified regions highly similar to their subtypes of origin. Sequences were then further interrogated with SIMPLOT to characterize patterns of recombination throughout the

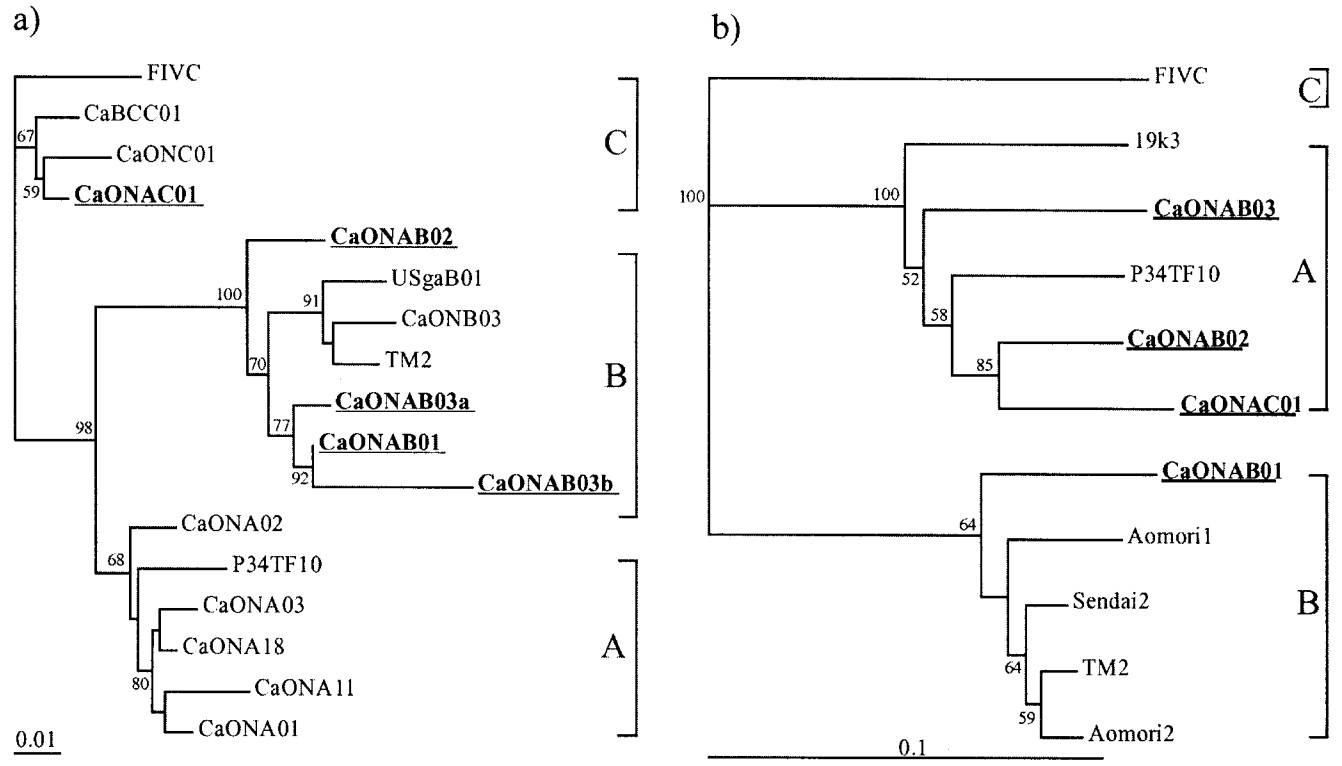

Fig. 4. Minimum evolution trees of FIV Gag and Env amino acid sequences. Recombinant strains grouped within different clusters depending upon the region analysed. (a) Minimum evolution tree of 242 aa within Gag. All A/B recombinant isolates clustered with subtype B sequences, while the A/C recombinant was classified as subtype C. (b) Minimum evolution tree of 491 aa within Env. Isolates CaONAC01, CaONAB02 and CaONAB03 clustered within subgroup A, while CaONAB01 was consistently classified as subtype B. Bootstrap values greater than 50 are indicated. Reference sequences: Dutch 19K3 (GenBank accession no. M73965; Siebelink *et al.*, 1992); Sendai 2 (D37814); Aomori 1 (D37816); Aomori 2 (D37817; Kakinuma *et al.*, 1995). Accession numbers and the sources of the remaining sequences are as in Fig. 3.

entire LTR–*gag* and *env*–LTR regions (Fig. 5). Plots of similarities demonstrated an identical 'B–A' breakpoint located between nt 1029 and 1060 in the LTR–*gag* sequences from the three A/B recombinants. This 31 bp fragment was identical in the three samples and corresponded to the junction between the *p15* and *p24* coding regions. Samples CaONAB02 and CaONAB03 showed a second recombination site at nt 690 and 750, respectively. Sample CaONAC01 showed only one A/C recombination site located at nt 680, between 300 bp of subtype A and 700 bp of subtype C fragments. This breakpoint was located at the beginning of the *gag* gene, explaining why this sample was classified as subtype C by amino acid sequence analysis. To investigate recombination further, an additional 2 kb of proviral DNA encompassing the *env*–LTR junction was amplified and sequenced. Analysis with SIMPLOT identified recombination sites only in CaONAB01 at nt 8860 and 9160 (Fig. 5). This 300 bp fragment was homologous to subtype A while the remaining sequence (85 %) was homologous to subtype B. Parsimony trees were created from each DNA subfragment delimited by the breakpoints (Fig. 5). This analysis provided further support to the subtype assignment within each recombinant, as indicated by bootstrap values ranging from 95 to 100, based on 1000 replicates. To preclude the possibility of chimera generation during nested PCR amplifications and to verify the presence of recombinant sequences, first-round PCR products were cloned and sequenced. Results indicated 100 % identity with sequences derived from second-round PCR products.

Intrinsic genetic diversity

Since a common breakpoint was identified in all A/B recombinants, we further characterized the genetic variability within these samples by PLDA. The number of proviral DNA copies was estimated and the concentration of the template was adjusted to prevent reamplification of the same molecule during PCR. This procedure increases the likelihood of amplifying variant sequences from the same sample (Rodrigo *et al.*, 1997). Five replicates from each A/B recombinant were then amplified and sequenced. Overall identities of the replicates from each sample were 98 % or higher, except for CaONAB03 where one of the five replicates differed by 10 %. These strains were identified as CaONAB03a and CaONAB03b and included in the phylogenetic analysis (Fig. 2a, b). Analysis of both sequences with

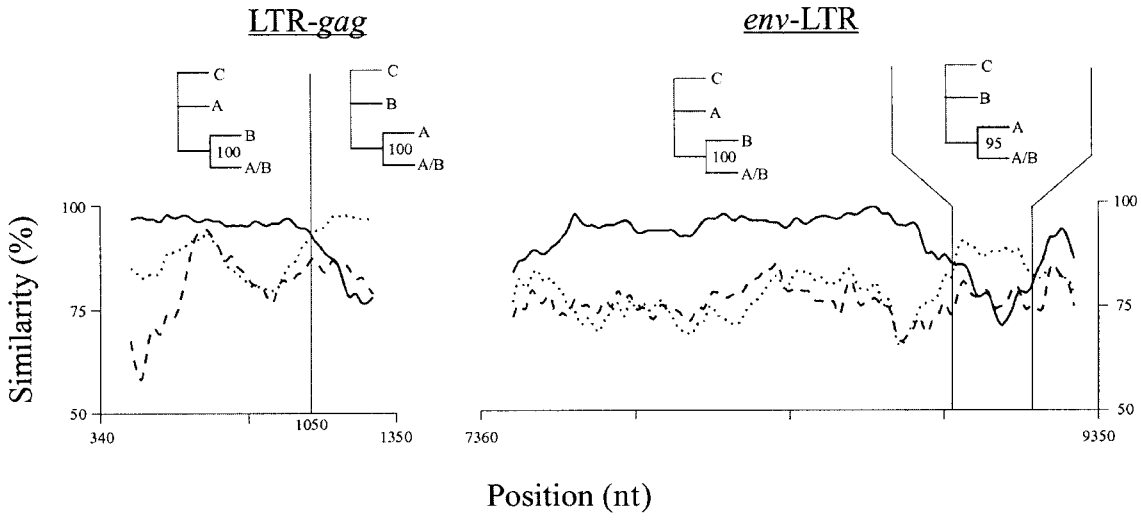

Fig. 5. Similarity analysis of A/B recombinant CaONAB01 and reference sequences with SIMPLOT. Fragments include LTR–*gag* (nt 340–1350) and *env*–LTR (nt 7360–9350). Each curve represents the percentage similarity of the test sequence to the reference sequence: subtype A (P34TF10, dotted line); subtype B (TM2, solid line). Subtype C (dashed line) was included as outgroup. LTR–*gag* similarity plots of 1·1 kb identified a recombination breakpoint consisting of a highly conserved region between nt 1037 and 1061. This breakpoint was present in all A/B recombinants. Recombination was also identified within 2 kb of the *env*–LTR region. Unrooted trees (on top) created from the subfragments of nucleotide sequences limited by the breakpoints confirmed subtype assignment. Bootstrap values based on 1000 replicates are indicated. Reference sequences are as in Fig. 3.

SIMPLOT confirmed the presence of the breakpoint described above. PLDA was also performed on this particular DNA sample using MB-1 and LTR3-r primers to amplify the *env*–LTR junction, but all sequences were 99 % similar. Thus, while two different sequences were identified in the LTR–*gag* region, only one was recovered from *env*–LTR. This might represent an additional recombination event within *gag*.

Co-infection

For recombination to occur within an infected cat, genomic RNA from two different viruses has to be present simultaneously within the same cell. Cats infected with recombinant viruses might concurrently have been infected with their parental subtypes. To distinguish parental subtypes A and/or B from A/B recombinants, DNA from these samples was subjected to subtype-specific PCR. Isolates CaONAB01 and CaONAB03 yielded neither subtype A- nor subtype B-specific products, indicating absence of co-infection; however, subtype-specific PCR amplification of CaONAB02 yielded a product with both sets of primers. Close visual inspection of the sequence from CaONAB02 revealed high similarity relative to subtype A and B sequences in the primer-annealing region. To characterize this sample further, PLDA was performed. Multiple alignments with at least five replicate sequences indicated identities of 99 %. We therefore concluded that in this case the subtype-specific PCR had insufficient discriminatory ability and that the cat was infected with a single recombinant virus.

FIV subtypes and clinical outcomes

Relevant clinical information was available for the majority of FIV-infected cats (Table 1). Clinical conditions ranged from no abnormalities to stomatitis, abscessation, diarrhoea, lymphoma and neuropathy. Associations between clinical conditions and FIV subtype could not be identified.

DISCUSSION

Canadian FIV strains have not been well characterized. FIV subtypes B and C were identified previously in Canada (Bachmann *et al.*, 1997; Sodora *et al.*, 1994) and subtype B was the predominant FIV subtype in eastern and central USA (Sodora *et al.*, 1994). In this study, classification of 40 FIV proviral DNA samples based on sequencing analyses encompassing the viral LTR, *gag* and *env* identified A as the most common subtype in Ontario. Subtype B was present in a minority of Ontario samples and all of the US samples, while subtype C was found in two samples from western Canada and Ontario. In addition, FIV A/B or A/C intersubtype recombination was discovered in four samples. Geographical proximity to the USA suggested that FIV B would be most common in Ontario. Instead, subtype A was predominant, which might reflect the introduction of FIV directly to Canada commensurate with immigrants and their cats from northern Europe, rather than movement of FIV-infected cats from the USA to Canada. Most samples originated from cats with clinical illness. Unique gene

sequences emerge with disease progression of HIV infection (Liu *et al.*, 2002) and may potentially occur in FIV infection and thus might have influenced the sequences obtained.

Recovery of recombinant virus sequences from four (11 %) of 36 Canadian samples is an intriguing finding that may be due to the intrinsic propensity of retroviruses for recombination or perhaps due to transmission of recombinant strains among cats. Sequence and phylogenetic analysis are considered effective tools to identify recombination (Worobey & Holmes, 1999). DNA phylogenetic analysis from LTR–*gag* was performed because 30 % of the sequences corresponded to the LTR, which are non-coding regions, thus providing the advantage of analysing longer sequences. However, this analysis showed that four samples did not group within any particular subtype. Since nucleotide changes have different impacts on the amino acid sequence depending on the relative location of the substitutions, parsimony trees were created with translated Gag and Env sequences. These analyses not only have more biological relevance but they also allowed for comparison between two distant genomic regions of the virus, thus providing further consistency of clade assignment. Phylogenetic trees based on Gag classified the A/C recombinant as subtype C, while all A/B samples were subtype B. In contrast, analysis of Env sequences indicated that the A/C and two of the A/B recombinants were subtypes A, while the remaining sample was consistently classified as subtype B. If subtyping had been based solely on data from Gag or Env amino acid sequences, these viruses would have been erroneously classified as subtypes A, B or C, without recognition of intersubtype recombination in the *gag* gene. These findings illustrate the advantage of contrasting nucleotide with amino acid sequence analyses. They also show the limitations of phylogenetic assignment based on subgenomic DNA fragments, suggesting that full genome sequence analysis should be the preferred method for accurate characterization of FIV isolates. Thus, additional recombination sites could have been identified through whole-genome sequencing of the four recombinant proviruses.

The extensive genetic diversity of lentiviruses derives from their high mutation, recombination and turnover rates (Blackard *et al.*, 2002; Thomson *et al.*, 2002). The high recombinogenic capacity of retroviruses is due to their peculiar mode of replication involving alternating jumps of the reverse transcriptase from one RNA template to the other, combined with its limited proofreading activity (Peliska & Benkovic, 1992; Robertson *et al.*, 1995; Thomson *et al.*, 2002). Recombination as studied *in vitro* occurs at a very high rate. Using single-replication vector systems, recombination occurred at a frequency of between 2 and 3 % per genome per cycle (Zhang & Temin, 1993). Factors that predispose a viral genomic site to recombination are not clearly understood but strand transfers in retroviruses appear to occur non-randomly, involving specific sites ('hot spots') at high frequency (Zhuang *et al.*, 2002). In addition, it seems that once recombination occurs, chimeric molecules become more susceptible to undergoing additional events (Zhuang *et al.*, 2002). A recombination-prone site may exist in FIV since all A/B recombinant samples, including the two different strains co-infecting one cat (CaONAB03a and CaONAB03b), had a common breakpoint between the *p24* and *p15* coding regions. However, it is unlikely that all four A/B recombinants had the same breakpoint as a consequence of independent recombination events around a 'hot spot'. Instead, this might indicate transmission of a related field strain among cats. Similarly, in an HIV study in Argentina, 13 out of 15 samples harbouring a BF intersubtype recombinant shared a common breakpoint at the 5′ end of *env* (gp120). Sequencing of the genomic region including the breakpoint enabled samples to be readily identified (Gomez Carrillo *et al.*, 2002). HIV-1 isolates are classified as 'circulating recombinant forms' if they show inconsistent clade assignment when different genome regions are compared and if they are identified from at least three individuals with no direct linkage (Blackard *et al.*, 2002; Lole *et al.*, 1999; Robertson *et al.*, 2000). The A/B recombinant samples described here were classified differently depending on the analysed genomic region; they originated from three different cities in Ontario (Table 1) and all of them shared a common recombination site in *gag*. Although full sequencing would be desirable, these findings suggest transmission of a circulating recombinant form of FIV among cats in Ontario.

Intersubtype recombination results from coincident infection of a cell by more than one subtype and subsequent exchange of gene regions producing replication-competent heterozygous virions (Goff, 2001). Superinfection with different FIV strains has been reported and concurrent infection with subtypes A and B has been experimentally induced both *in vivo* and *in vitro* (Okada *et al.*, 1994; Pistello *et al.*, 1999). In another study, recombination occurred in one out of three cats inoculated with two different FIV strains (Kyaw-Tanner *et al.*, 1994). Since both FIV subtypes A and B were present in Ontario, theoretically opportunities for dual infection of cats and therefore recombination existed. Using subtype A- or B-specific PCR assays, we investigated the possibility of co-infection in recombinant samples. Results suggested that only recombinant sequences were present. We then further investigated the possibility of co-infection by PLDA. While sequence identities among at least five replicates from each sample were of 99 %, one replicate from one sample was 10 % different. This particular sequence was 99·8 % similar to a recombinant identified in a different cat. Parental strains of subtypes A and/or B were not identified in either cat after limiting dilutions. Thus, one cat was infected with at least two different A/B recombinants, one of which was almost identical to the virus in another cat from a different geographical region and neither cat had parental A or B strains. These findings further support transmission of an A/B recombinant strain of FIV among cats in Ontario.

Recombination greatly accelerates the evolutionary rate of retroviruses by creating new variants much faster than mutations will, thereby rapidly increasing genetic diversity. Large-scale recombination allows selection of traits that increase virus fitness and permits the elimination of disadvantageous gene regions (Worobey & Holmes, 1999). The sudden emergence of new variants may further serve as a mechanism to evade the selective pressure imposed by the immune system, leading to better adaptations between the virus and the host or perhaps to more severe clinical outcomes. The evolution of recombinant viruses with enhanced pathogenicity and immune evasion was recently described in an HIV-infected individual with an exceptionally rapid disease course (Liu *et al.*, 2002). In another report, superinfection and subsequent recombination were necessary to create replicative-competent viruses, suggesting that superinfection may favour virus synergism (Blackard *et al.*, 2002). Whether recombination had an impact on the clinical outcomes of the cats in this study was unknown, since the available clinical data resulted from one assessment and the duration of infection was unknown in most cases. Similar difficulties would be expected under field conditions, since the disease affects predominantly feral, sexually intact cats that are relatively inaccessible for study.

At least 13 different HIV-1 circulating recombinant forms have been characterized (Thomson *et al.*, 2002). In particular geographical areas, some of these variants are currently more prevalent than the native subtypes, which might be an indication of increased virus fitness after recombination. For example, the recombinant virus CRF02_AG is one of two predominant HIV-1 variants in Africa (Vergne *et al.*, 2003) and in the year 2000 was found to be the second most important cause of the global pandemic (Osmanov *et al.*, 2002). Little is known about FIV recombination under natural conditions. Here, we have shown evidence that three of the four main FIV subtypes are represented in Canada and provide the first indication that a recombinant form of FIV circulates among cats in Ontario. These findings have implications for molecular diagnosis and vaccine development.

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