Characterization of a new circulating recombinant form comprising HIV-1 subtypes C and B in southern Brazil

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Objective: To identify a new circulating recombinant form (CRF) of HIV-1 comprising two circulating subtypes in the southern region in Brazil, subtypes B and C.

Methods: A total of 152 HIV-positive patients followed at two hospitals in southern Brazil had their viral *pol* genes isolated by reverse transcriptase–polymerase chain reaction (PCR) from plasma. PCR products were sequenced and phylogenetically analysed using HIV-1 subtype reference sequences. Six full-length subtype C viruses from Brazil previously described as 'pure' strains were included in the analysis. Sequences suggestive of recombination were analysed by bootscanning and phylogenetic analyses of separate fragments. The common ancestry of recombinant strains was evaluated by similarity plot and informative site analyses.

Results: HIV-1 subtypes commonly found in Brazil (B, C and F1) were observed. Sixty-two viruses were initially assigned as subtype C, but 15 viruses clustered in a separate internal clade. *Pol* from two full-length genomes of subtype C viruses grouped together with those samples. Bootscanning analysis showed that all 17 viruses had the same recombinant structure, with a 240 base pair fragment of subtype B in the middle of the reverse transcriptase *pol* region. Subtype B assignment of this fragment was confirmed by phylogenetic analyses using different methods of tree inference and cluster robustness tests. Mosaics were shown to have a common ancestry.

Conclusion: As CRF_BC represents 11% of the HIV-1 viruses circulating in the southern region of the country, which borders several south American countries, the assessment of its spread is of pivotal importance to the HIV/AIDS epidemic in Brazil and Latin America.

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Keywords: Circulating recombinant form, HIV-1, southern Brazil, subtype C, recombinant virus

Introduction

High genetic diversity is one of the major hallmarks of HIV-1. Phylogenetic analysis of numerous strains showed that HIV-1 can be divided into groups, subtypes, sub-subtypes, circulating recombinant forms (CRF) and unique recombinant forms [1]. HIV-1 groups refer to the three very distinct lineages M, N and O. The vast majority of strains found worldwide are classified as group M. This group is the major group responsible for the AIDS

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pandemic. Group O, on the other hand, is endemic to Cameroon and neighbouring countries [2], whereas group N is represented by a limited number of isolates from Cameroon [3]. Within group M nine subtypes have been characterized, A–D, F–H, J and K [1]. Subtypes form roughly equidistant clusters in phylogenetic trees, being separated by 25–35% distance between *env* sequences and by 10–15% between *pol* sequences at the nucleotide level. Within some subtypes further phylogenetic structure can be identified, leading to a classification into sub-subtypes (A1 and A2, F1 and F2) [4,5]. This diversity of HIV-1 is the result of high rates of mutation of its reverse transcriptase [6] and rapid viral turnover in patients [7,8].

The phylogenetic analysis of the HIV-1 full-length genome sequences demonstrated that certain isolates clustered with two or more subtypes depending on the genomic region analysed [9,10]. These mosaic forms appear through HIV-1 recombination during reverse transcription [11,12], and this phenomenon promotes an additional source of HIV-1 variation. Some of the recombinant forms may achieve an epidemic level of dissemination, and currently there are at least 16 published different CRF (http://www.hiv.lanl.gov/content/hiv-db/CRFs/CRFs.html). More recently, CRF18_cpx [13] and CRF19_cpx [14] have been described.

In Brazil, the prevailing subtype is B, but other subtypes, such as F1, C, D, A and B/F co-circulate [15–18]. Analysis of protease (PR) and reverse transcriptase (RT) genomic regions have shown that subtype B was responsible for approximately 65% of infections in 2001, followed by subtype C (approximately 30%) [19]. The same study showed that approximately 15% of samples were recombinants. Recent studies conducted by our group showed a high prevalence of subtype C in the southern region, including the states of Rio Grande do Sul and Paraná (prevalence of 45 and 30%, respectively) [19–21], and more recently six full-length genomes of local subtype C viruses were characterized [22].

A few studies of Brazilian B/F recombinants have been published [23–26], but CRF28_BF and CRF29_BF were described only recently [27]. Given the high and similar frequencies of subtypes B and C in southern Brazil, we decided to investigate the potential generation of a CRF_CB in that part of the country.

Methods

Samples

Samples of 152 HIV-positive patients from two HIV/AIDS reference centres in Rio Grande do Sul, Hospital de Clínicas de Porto Alegre (in the capital of the state) and University Hospital of Rio Grande (in the city of the same name, further south in the state) were included this study. Subjects signed a written consent form for their inclusion in the study, and upon acceptance a single sample of peripheral blood was drawn. The date of HIV diagnosis was extracted from patients' medical records. This study was approved by the local hospitals' internal review boards.

RNA isolation, polymerase chain reaction and sequencing

HIV viral RNA was isolated as previously described [28]. Complementary DNA synthesis and genomic amplification by polymerase chain reaction (PCR) of the HIV-1 *pol* fragment spanning the entire protease gene and approximately two-thirds of the RT gene (285 codons; nucleotides 2201–3353 relative to the HXB2 strain) were conducted in two steps with specific nested primers [28]. A 1152 base pair (bp) fragment was obtained and purified using Microcon PCR cartridges (Millipore Corp., Billerica, Massachusetts, USA). Primers used in the PCR reactions were described elsewhere [28]. Purified products were sequenced in an automated ABI Prism ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, California, USA). All sequencing chromatograms obtained were assembled with PC/Windows computers using the software SeqMan (DNAStar, Madison, Wisconsin, USA) and manually edited. For five strains out of the 15 generated in this study that grouped in a cluster suggestive of recombination (see below), a larger *pol* fragment (nucleotides 2157–5220 relative to the HXB2 strain) was PCR-amplified, sequenced and further analysed.

All sequences generated in this study were submitted to the GenBank database and were assigned the accession numbers AY275717–AY275802, AY390076, AY390179–AY390194, DQ190951–DQ191039 and DQ343964–DQ344021.

HIV-1 subtype determination

For HIV-1 genetic subtype determination and to discard sample mix-ups or contaminations, all sequences in FASTA format were aligned with reference sequences representative of all HIV-1 subtypes obtained from the Los Alamos database (http://hiv-web.lanl.gov) in ClustalW [29]. In this analysis, we also included homologous *pol* fragments from six samples of Brazilian subtype C isolates recently described by Sanabani *et al.* [22] (GenBank accession nos. AY727522–AY727527). Aligned sequences were subjected to phylogenetic inference through the neighbour-joining method and Kimura two-parameter model implemented in the MEGA 2.1 package [30]. One thousand bootstrap replicates were used to assess the phylogenetic robustness of the clusters. The sequence of $SIV_{CPZ}GAB$ (GenBank accession no. X52154) was used as an outgroup to root the trees. In addition, maximum parsimony and maximum likelihood analyses were also conducted for

the same dataset in MEGA 2.1 and in PHYML 2.4.4 [31] to corroborate the neighbour-joining findings. In the maximum likelihood analysis, the nucleotide substitution model was estimated using Modeltest 3.7 [32]. In addition to bootstrap analysis, the internal branch length test was used to evaluate the robustness of sequence clusters, implemented in MEGA 2.1.

Recombinant HIV-1 identification

HIV-1 subtype C sequences forming a high bootstrap-supported cluster inside the C clade were further analysed by bootscanning analysis implemented in Simplot 3.5.1 for Windows [10] using representatives of all HIV-1 subtypes. A sliding window of 300 bp, an increment step of 20 bp and the Kimura two-parameter model were used in the analysis. Bootstrap support was calculated based on 100 data replicates. Fragments of sequences assigned to specific HIV-1 subtypes were further confirmed by separate phylogenetic analyses conducted as described above. As two out of the six full-length subtype C genomes recently described in Brazil [22] grouped within the above-mentioned cluster, we re-analysed them by bootscanning in Simplot 3.5.1 with a sliding window of 400 bp and an increment step of 50 bp.

To characterize the recombination breakpoints suggested in the previous analyses more precisely, the putative recombinants were subjected to informative site analysis by generating consensus DNA sequences of CB recombinants, and of local subtype B and C viruses. Consensus sequences were generated with 60% of threshold frequency and were aligned in ClustalW and manually compared for sites that indicated identities between the recombinants and subtypes B or C. Statistical analyses were conducted for nucleotide frequencies around each recombination breakpoint in the datasets used for the generation of subtypes B and C consensus using Fisher's exact test.

In order to assess the common origin of the recombinant isolates, their sequences were subjected to similarity plot analysis using Simplot [10]. Each CB recombinant sequence was compared with consensus sequences of the remaining CB recombinants, and with consensus of local subtype C and B sequences generated in this study.

Results

HIV-1 subtype distribution

Viral RNA samples isolated from plasma of 152 patients were sequenced in the protease and RT genomic regions and subtyped through phylogenetic analysis. Sixty-two samples were initially assigned to subtype C and 70 to subtype B. Another six samples were identified as subtype F1, one as subtype D, and 13 represented mosaic viruses that had the protease gene of one subtype and the RT gene of another. These included the genotypes PR^{D}/RT^{B}, PR^{F1}/RT^{B}, PR^{B}/RT^{C} and PR^{C}/RT^{B}.

Neighbour-joining distance-based phylogenetic analysis has shown that the Brazilian subtype C viruses grouped in a cluster (with a bootstrap of 77%) when compared with subtype C viruses from other countries (Fig. 1). The interior branch test of phylogeny analysis rendered a probability of 93% for that clade, whereas maximum parsimony analysis showed a bootstrap of 65% (data not shown), also corroborating previous observations of monophyly by our group [33]. Samples of subtypes B and F1 characterized in this study clustered into their respective groups with high bootstrap values in all trees (Fig. 1 and data not shown).

HIV-1 CB recombinant identification

Seventeen samples belonging to the Brazilian subtype C clade (15 characterized herein and two described by Sanabani *et al.* [22]) clustered in a group supported by a high bootstrap value (97%) external to the remaining isolates (Fig. 1). This cluster was also supported by maximum parsimony (bootstrap of 90%), by maximum likelihood (bootstrap of 96%) and by the interior branch test of phylogeny (99%) (data not shown). As this tree topology is suggestive of common ancestry, we decided to characterize those samples in further detail. Five representative isolates of the CB recombinant clade had a larger fragment of genomic cDNA amplified, encompassing the whole protease, RT and the 5′ half of integrase. Each of these isolates was subjected to bootscanning analysis. We included isolates 04BR137 and 04BR142 characterized in Sanabani *et al.* [22] that had clustered together with those samples, and the results are depicted in Fig. 2. All isolates had the same pattern of subtype recombination, with an internal *pol* fragment spanning approximately 240 nucleotides assigned to subtype B. All three fragments (both outer subtype C and inner subtype B) were separately subjected to phylogenetic analysis to corroborate the bootscanning results. The internal 240 bp assigned to subtype B from all isolates clustered with a bootstrap value of 64% (Fig. 3b), and an internal branch length test of 91% (data not shown), whereas the remaining fragments grouped with subtype C, particularly with the Brazilian reference isolate C.BR.92.U52953 (Fig. 3a and c). The phylogenetic support has undoubtedly enabled us to confirm the recombinant nature of those isolates. In addition, the bootscanning re-analysis with full-length genome of isolates 04BR137 and 04BR142 (previously described as pure subtype C in Sanabani *et al.* [22]) was performed. To our surprise, this analysis also confirmed the same recombinant breakpoints in the *pol* region (data not shown).

Informative site analysis of recombination breakpoints

To characterize the recombination breakpoints of our CRF more precisely, we performed an informative site

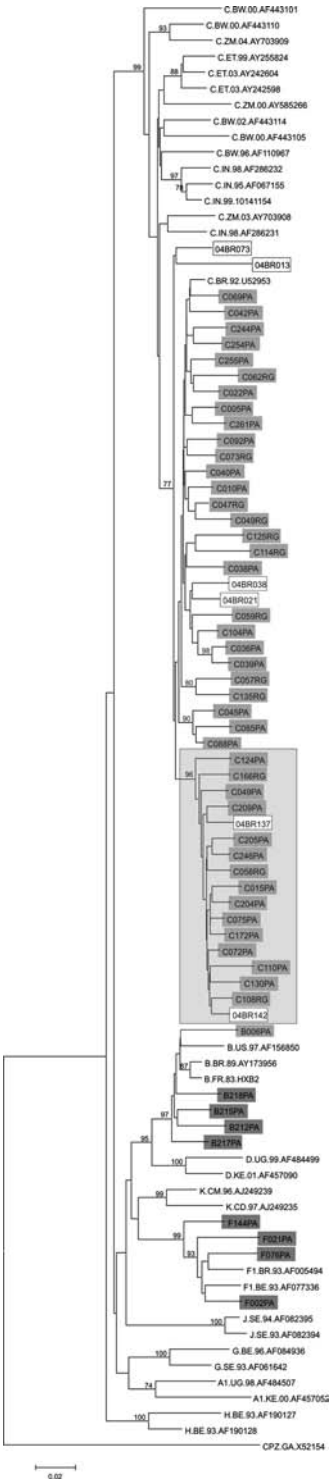

Fig. 1. Phylogenetic analysis of HIV-1 viruses from Rio Grande do Sul, Brazil, characterized in this study. The tree was obtained using neighbour-joining and the Kimura two-parameter model. Bootstrap values supporting HIV-1 subtype clades are depicted close to their branches. Reference subtype sequences were obtained from the Los Alamos HIV Sequence Database and were included in the analysis. The first letter indicates the subtype. BE, Belgium; BR, Brazil; BW, Botswana; CD, Democratic Republic of Congo; CM, Cameroon; ET, Ethiopia; FR, France; IN, India; KE, Kenya; SE, Sweden; UG, Uganda; US, United States; ZA, South Africa. Isolates characterized in this study are shaded in dark grey. The six subtype C viruses recently characterized in Sanabani *et al*. [22] were also included (open boxes). The recombinant clade is indicated in a light grey shaded box.

analysis comparing consensus sequences of the CB recombinants with those of subtypes B and C. That analysis is depicted in Fig. 4. By analysing signature patterns among the three consensus sequences, we were able to narrow the recombination breakpoints down to nucleotides 712–731 and to 925–938 of our fragment (2965–2984 and 3190–3203 of HXB2, respectively). At positions 711, 732 and 924, 100% of the subtype C strains used for the consensus generation presented an adenosine, whereas all subtype B presented a guanine ($P = 0.001082$ for all three cases). At position 939, the frequency of cytosine was 100% in subtype C and 16.7% in subtype B ($P = 0.004079$). In external borders the mosaic consensus showed the same signatures of subtype C, whereas in internal borders it showed the same signatures of subtype B.

Common ancestry of CB recombinant viruses

We wanted to confirm further the epidemically circulating property of the CB recombinant strains. For this, we generated three sets of local sequences, one representing subtype B, another subtype C, and a third one with the CB recombinant strains (with the exception of the one queried). Each recombinant strain was compared against those three consensus sequences through similarity plots. The results of these analyses can be seen in Fig. 5. They revealed that all recombinant strains analysed were closer to their CB consensus throughout the fragment analysed, even when they belonged to subtypes B or C and were compared with subtype B and C strains from the same geographical region (Fig. 5 and data not shown). We used only the initial 950 bp fragment because we only had that information from the 'pure' local subtypes, but on the other hand that allowed us to test all 17 putative recombinant sequences available. All of them showed the same pattern of similarity (data not shown), indicating a common ancestry of all recombinant strains described.

Discussion

Earlier studies of HIV-1 subtype distribution in Brazil have shown the prevalence of approximately 3% of subtype C [15], but we and others have revealed the southern region as an endemic site for subtype C

Fig. 1. (*Continued*)

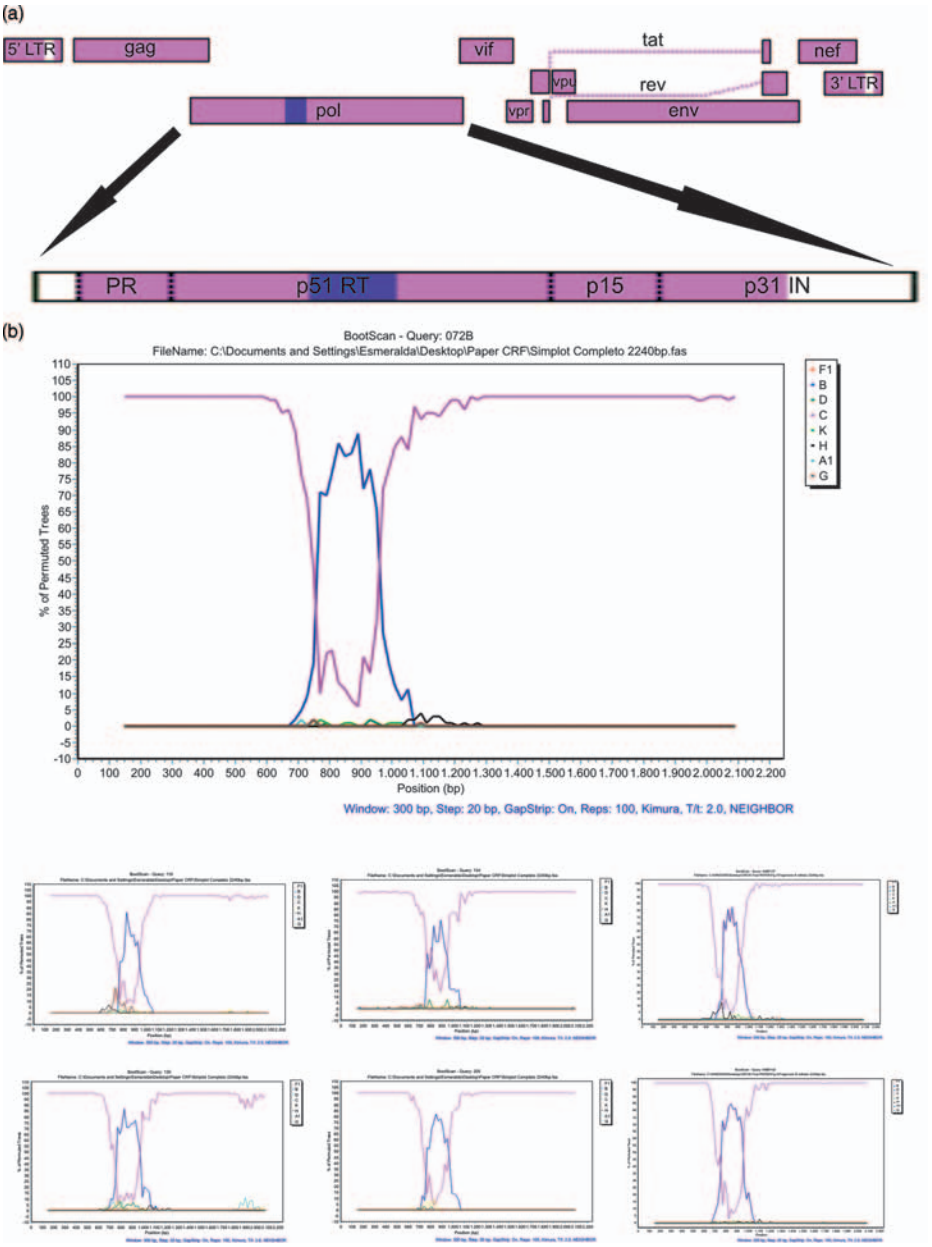

Fig. 2. (a) Schematic structure of the CB recombinant virus genome analysed. (b) Bootscanning analysis of the genomic region depicted in (a) for seven representative recombinant viruses. Horizontal axes represent nucleotide position in this region, whereas vertical axes depict bootscanning values (%) that support the grouping of the isolate with each HIV-1 subtype.

[19,20,33–35], notably in the state of Rio Grande do Sul. The molecular profile of HIV-1 subtypes observed in this study corroborates those previous observations. We found 62 samples belonging to subtype C, which represented 41% of the total.

Phylogenetic analyses have shown that Brazilian HIV subtype C viruses clustered in a well-defined clade (Fig. 1), in agreement with previous studies [22,33]. Altogether, these data show that the introduction of subtype C viruses in Brazil was probably a single event. The maximum parsimony and interior branch test of phylogeny with the neighbour-joining distance method tree and Kimura two-parameter model confirmed such an hypothesis (data not shown).

We have observed a subcluster within the Brazilian subtype C clade in this study comprising 17 samples with high bootstrap value, including two samples, 04BR137 and 04BR142, which had previously been described as pure subtype C strains [22]. A more detailed analysis revealed that all samples were CB recombinants and

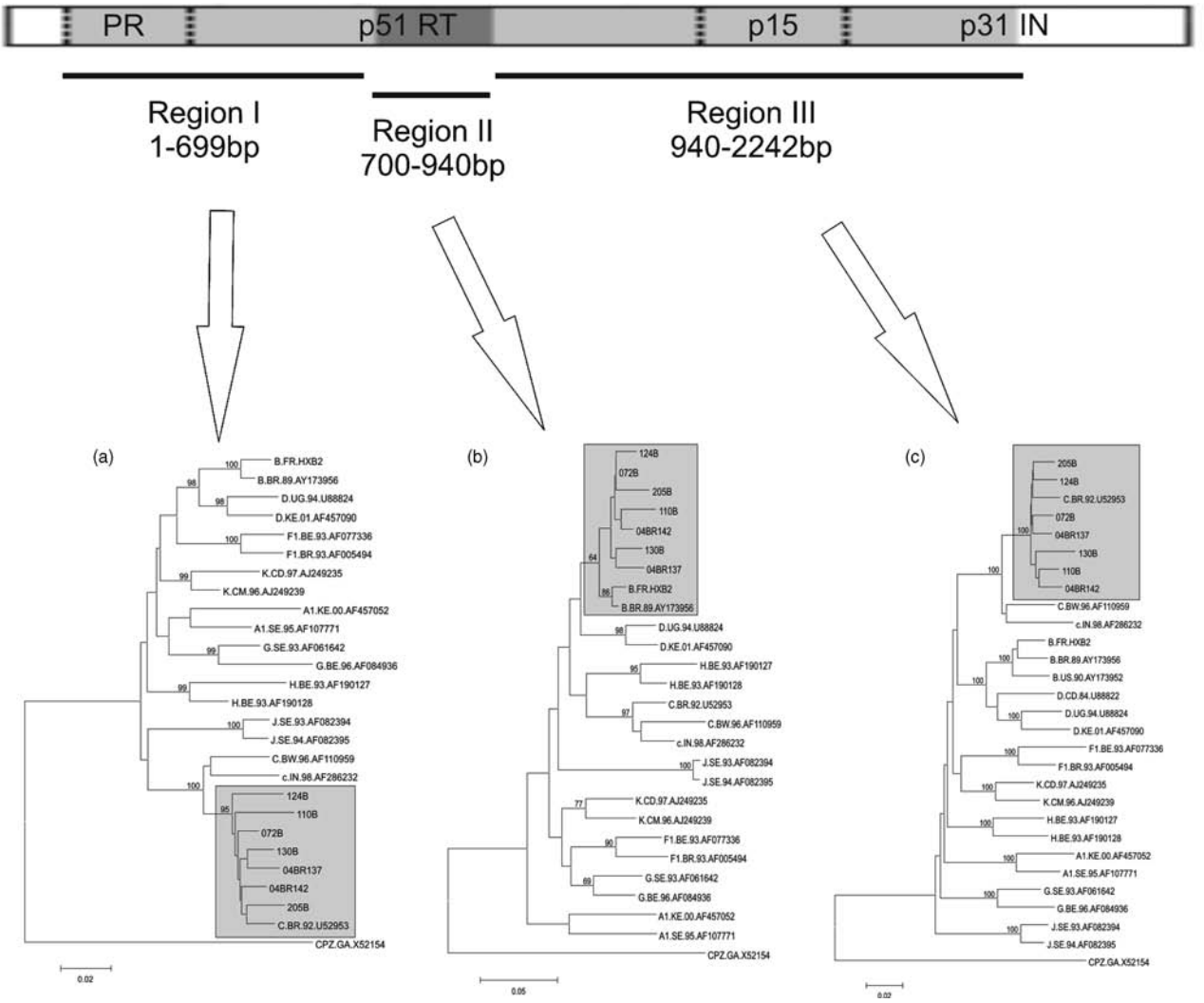

Fig. 3. Phylogenetic analysis of the three *pol* gene fragments assigned to different subtypes according to the bootscanning analyses (Fig. 2). The grey shaded boxes indicate clades (HIV-1 subtypes) within which the recombinant strains clustered with high bootstrap value.

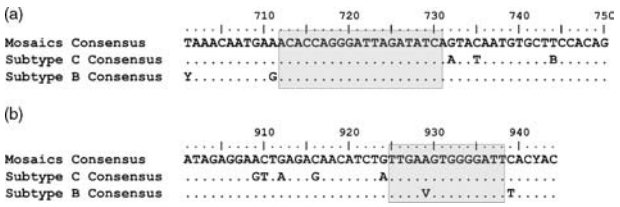

Fig. 4. Informative site analyses of the CB strain recombination breakpoints. Consensus sequences for local subtypes B and C and for the CB recombinant form were generated around the putative left (a) and right (b) breakpoints pointed out in the bootscanning analysis (Fig. 3). Shaded boxes indicate the narrowed regions where the breakpoints most likely happened, which are constrained by informative sites. Genetic code ambiguities are defined as follows: Y–C or T; B–C, G or T; V–A, C or G.

shared the same recombination breakpoint, suggesting that mosaic viruses shared a single ancestor. A 2242 bp fragment within the *pol* gene was confirmed by bootscanning analysis and further phylogenetic inference.

The probability of generating a CRF of HIV requires and is directly related to the co-circulation of distinct subtypes in a population. The high co-prevalence of subtypes B and C, which together made up 87% of the HIV viruses in the state of Rio Grande do Sul, is thus likely to generate CB recombinants. The presence of CB recombinants is also common in countries where these subtypes predominate, such as India [36,37], China [38–40], and in south American countries where subtype C has recently been introduced [41–43]. An informative site analysis that compared the CB recombinants with local

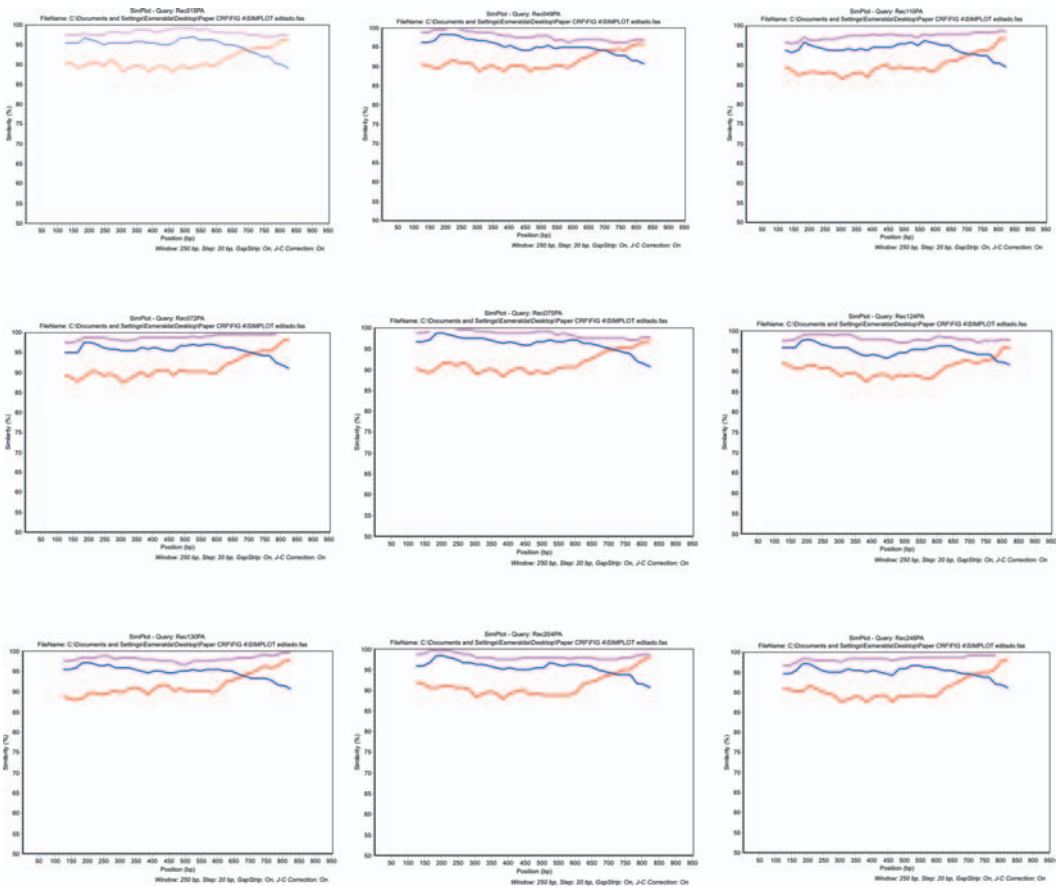

Fig. 5. Similarity plot analyses of nine representative putative CRF_CB strains. Vertical axes represent the similarity percentage at the nucleotide level with each sequence group (local subtype B, local subtype C or CB recombinants), whereas horizontal axes depict nucleotide positions along the alignment. Although only nine isolates are represented here (for space reasons), all 17 recombinants tested in the study showed identical plots. — Local subtype B; — local subtype C; — CB recombinants.

subtype B and C viruses has also shown that the former shared signatures at the DNA level with the latter, suggesting a local origin (Fig. 4).

The subtype B genomic fragment incorporated into the recombinant form described here comprises codons 138–217 of RT, and therefore harbours several positions associated with resistance to nucleoside (codons 151, 184, 210 and 215) and non-nucleoside (codons 181, 188 and 190) RT inhibitors. None of the 15 mosaic isolates found here harboured drug resistance-associated mutations at those positions, and they were all drug naive at the time of sample collection (data not shown). The nature of the selective advantage in incorporating such an RT region into the CRF remains to be determined. One explanation could be that such a region is more prone to develop drug resistance in subtype B than in C, but there is no current evidence for this bias. Previous work from our group has failed to show any differences in genetic barrier for those two subtypes in developing RT drug resistance [44].

Many unique recombinant forms have been described in Brazil, mostly BF mosaics [19,20,23,25,26,45]. Previous studies by our group also identified unique CB recombinant forms in Brazil [19–21], but all have failed to detect CRF. More recently, two CRF_BF viruses were isolated in southeastern Brazil [27]. In the present study, we gathered two full-length genomes previously sequenced in another study [22] and five partial genomes of different samples with the same mosaic CB structure, which formally characterizes the required evidence for describing a new CRF [1]. Our bootscanning re-analyses of the isolates 04BR137 and 04BR142 showed that these are mosaics (data not shown) and not pure subtype C as previously characterized [22]. One possible explanation for the discrepancy in the results obtained by us and in Sanabani *et al.* [22] is the size of the sliding window used in the bootscanning analyses. We used here a sliding window of 400 bp and an increment step of 50 bp, whereas Sanabani *et al.* [22] used a sliding window of 500 bp and the same increment step. A small region of approximately 200 bp could not be noticed with this size window, but only a peak for subtype B bootstrap.

Although both analyses also differed in the substitution model used (Kimura two-parameter versus F84), this difference did not influence the results (data not shown).

Our samples presented the same breakpoint structure, a common origin and nucleotide signatures that indicated a local origin from subtype B and C isolates. To the best of our knowledge, this is the first concrete evidence of the existence of a Brazilian HIV-1 CRF comprising subtypes B and C.

Despite the fact that subtype C is responsible for more than 56% of HIV-1 infections worldwide [46], until now only three out of 29 CRF comprising subtype C had been described, CRF07_BC [47], CRF08_BC [48] and CRF10_CD [49]. Our study may provide a new exemplar of CRF comprising subtype C in its structure.

It is noteworthy that among all samples from Rio Grande do Sul analysed in this study, 24% of the initially assigned subtype C samples now represent a CRF_BC. An independent study recently published [33] found mosaic samples with this same breakpoint structure in 43% of samples previously described as subtype C, corresponding approximately to 25% of the total viruses circulating in newly infected individuals. Interestingly, as we have data on the time of diagnosis for our patients, we can trace this CRF back to at least 1990, which means that it has been circulating in southern Brazil for over 15 years. We are currently estimating the more precise time of generation of this CRF in the region using coalescence techniques. Additional studies are necessary to obtain a complete understanding of the role of this CRF in the Brazilian and ultimately in the Latin American HIV/AIDS epidemic.

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References

1. Robertson DL, Anderson JP, Bradac JA, Carr JK, Foley B, Funkhouser RK, *et al*. **HIV-1 nomenclature proposal.** *Science* 2000; **288**:55–56.
2. Gürtler LG, Hauser PH, Eberle J, Brunn AV, Knapp S, Zekeng L, *et al*. **A new subtype of human immunodeficiency virus type 1 (MVP-5180) from Cameroon.** *J Virol* 1994; **68**:1581–1585.
3. Simon F, Mauclère P, Roques P, Loussert-Ajaka I, Müller-Trutwin MC, Saragosti S, *et al*. **Identification of a new human immunodeficiency virus type 1 distinct from group M and group O.** *Nat Med* 1998; **4**:1032–1037.
4. Triques K, Bourgeois A, Saragosti S, Vidal N, Mpoudi-Ngole E, Nzilambi N, *et al*. **High diversity of HIV-1 subtype F strains in Central Africa.** *Virology* 1999; **259**:99–109.
5. Gao F, Vidal N, Li Y, Trask SA, Chen Y, Kostrikis LG, *et al*. **Evidence of two distinct subsubtypes within the HIV-1 subtype A radiation.** *AIDS Res Hum Retroviruses* 2001; **17**:675–688.
6. Preston BD, Poiesz BJ, Loeb LA. **Fidelity oh HIV-1 reverse transcriptase.** *Science* 1988; **242**:1168–1171.
7. Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M. **Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection.** *Nature* 1995; **373**:123–126.
8. Wei X, Ghosh SK, Taylor ME, Johnson VA, Emini EA, Deutsch P, *et al*. **Viral dynamics in human immunodeficiency virus type 1 infection.** *Nature* 1995; **373**:117–122.
9. Robertson DL, Hahn BH, Sharp PM. **Recombination in AIDS viruses.** *J Mol Evol* 1995; **40**:249–259.
10. Salminen MO, Carr JK, Burke DS, McCutchan FE. **Identification of breakpoints in intergenotypic recombinants of HIV type 1 by bootscanning.** *AIDS Res Hum Retroviruses* 1995; **11**:1423–1425.
11. Goodrich DW, Duesberg PH. **Retroviral recombination during reverse transcription.** *Proc Natl Acad Sci U S A* 1990; **87**:2052–2056.
12. Hu WS, Temin HM. **Retroviral recombination and reverse transcription.** *Science* 1990; **250**:1227–1233.
13. Thomson MM, Casado G, Posada D, Sierra M, Najera R. **Identification of a novel HIV-1 complex circulating recombinant form (CRF18_cpx) of Central African origin in Cuba.** *AIDS* 2005; **22**:1155–1163.
14. Casado G, Thomson MM, Sierra M, Najera R. **Identification of a novel HIV-1 circulating ADG intersubtype recombinant form (CRF19_cpx) in Cuba.** *J Acquir Immune Defic Syndr* 2005; **40**:532–537.
15. Bongertz V, Bou-Habib DC, Brigido LF, Caseiro M, Chequer PJN, Couto-Fernandez JC, *et al*. **HIV-1 diversity in Brazil: genetic, biologic, and immunologic characterization of HIV-1 strains in three potential HIV vaccine evaluation sites.** *J Acquir Immune Defic Syndr* 2000; **23**:184–193.
16. Caride E, Brindeiro R, Hertogs K, Brendan L, Pascale D, Elizabeth M, *et al*. **Drug-resistant reverse transcriptase genotyping and phenotyping of B and non-B subtypes (F and A) of human immunodeficiency virus type I found in Brazilian patients failing HAART.** *Virology* 2000; **275**:107–115.
17. Couto-Fernandez JC, Morgado MG, Bongertz V, Tanuri A, Andrade T, Brites C, *et al*. **HIV-1 subtyping in Salvador, Bahia, Brazil: a city with African sociodemographic characteristics.** *J Acquir Immune Defic Syndr* 1999; **22**:288–293.
18. Sabino EC, Shpaer EG, Morgado MG, Korber BT, Diaz RS, Bongertz V, *et al*. **Identification of human immunodeficiency virus type 1 envelope genes recombinant between subtypes B and F in two epidemiologically linked individuals from Brazil.** *J Virol* 1994; **68**:6340–6346.
19. Brindeiro RM, Diaz RS, Sabino EC, Morgado MG, Pires IL, Brigido L, *et al*. **Brazilian Network for HIV Drug Resistance Surveillance (HIV-BResNet): a survey of chronically infected individuals.** *AIDS* 2003; **17**:1063–1069.
20. Soares EA, Santos RP, Pellegrini JA, Sprinz E, Tanuri A, Soares MA. **Epidemiologic and molecular characterization of human immunodeficiency virus type 1 in southern Brazil.** *J Acquir Immune Defic Syndr* 2003; **34**:520–526.

21. Soares EA, Martinez AM, Souza TM, Santos AF, Hora VD, Silveira J, *et al*. **HIV-1 subtype C dissemination in southern Brazil.** *AIDS* 2005; **19 (Suppl. 4)**:S81–S86.
22. Sanabani S, Neto WK, Filho DJ, Diaz RS, Munerato P, Janini LM, *et al*. **Full-length genome analysis of human immunodeficiency virus type 1 subtype C in Brazil.** *AIDS Res Hum Retroviruses* 2006; **22**:171–176.
23. Ramos A, Tanuri A, Schechter M, Rayfield MA, Hu DJ, Cabral MC, *et al*. **Dual and recombinant infections: an integral part of the HIV-1 epidemic in Brazil.** *Emerg Infect Dis* 1999; **5**:65–74.
24. Vicente AC, Otsuki K, Silva NB, Castilho MC, Barros FS, Pieniazek D, *et al*. **The HIV epidemic in the Amazon Basin is driven by prototypic and recombinant HIV-1 subtypes B and F.** *J Acquir Immune Defic Syndr* 2000; **23**:327–331.
25. Thomson MM, Sierra M, Tanuri A, May S, Casado G, Manjon N, *et al*. **Analysis of near full-length genome sequences of HIV type 1 BF intersubtype recombinant viruses from Brazil reveals their independent origins and their lack of relationship to CRF12_BF.** *AIDS Res Hum Retroviruses* 2004; **20**:1126–1133.
26. Sa ilho##Dh#D, Sanabani S, Diaz RS, Munerato P, Brunstein A, Fusuma E, *et al*. **Analysis of full-length human immunodeficiency virus type 1 genome reveals a variable spectrum of subtypes B and f recombinants in Sao Paulo, Brazil.** *AIDS Res Hum Retroviruses* 2005; **21**:145–151.
27. Sa Filho DJ, Sucupira MC, Casiero MM, Sabino EC, Diaz RS, Janini LM. **Identification of two HIV type 1 circulating recombinant forms in Brazil.** *AIDS Res Hum Retroviruses* 2006; **22**:1–13.
28. Stuyver L, Wyseur A, Rombout A, Louwagie J, Scarcez T, Verhofstede C, *et al*. **Line probe assay for rapid detection of drug-selected mutations in the human immunodeficiency virus type 1 reverse transcriptase gene.** *Antimicrob Agents Chemother* 1997; **41**:284–291.
29. Thompson JD, Higgins DG, Gibson TJ. **CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice.** *Nucleic Acids Res* 1994; **22**:4673–4680.
30. Kumar S, Tamura JK, Jakobsen IB, Nei M. **MEGA2: molecular evolutionary genetics analysis software.** *Bioinformatics* 2001; **17**:1244–1245.
31. Guindon S, Gascuel O. **A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood.** *Syst Biol* 2003; **52**:696–704.
32. Posada D, Crandall KA. **Modeltest: testing the model of DNA substitution.** *Bioinformatics* 1998; **14**:817–818.
33. Soares MA, De Oliveira T, Brindeiro RM, Diaz RS, Sabino EC, Brigido L, *et al*. **A specific subtype C of human immunodeficiency virus type 1 circulates in Brazil.** *AIDS* 2003; **17**:11–21.
34. Martinez AM, Barbosa EF, Ferreira PC, Cardoso FA, Silveira J, Sassi G, *et al*. **Molecular epidemiology of HIV-1 in Rio Grande, RS, Brazil.** *Rev Soc Bras Med Trop* 2002; **35**:471–476.
35. Rodrigues R, Scherer LC, Oliveira CM, Franco HM, Sperhacke RD, Paula erreira##Jria, *et al*. **Low prevalence of primary antiretroviral resistance mutations and predominance of HIV-1 clade C at polymerase gene in newly diagnosed individuals from south Brazil.** *Virus Res* 2006; **116**:201–207.
36. Siddappa NB, Dash PK, Mahadevan A, Desai A, Jayasuryan N, Ravi V, *et al*. **Identification of unique B/C recombinant strains of HIV-1 in the southern state of Karnataka, India.** *AIDS* 2005; **19**:1426–1429.
37. Tripathy SP, Kulkarni SS, Jadhav SD, Kalpana DA, Abhay JJ, Swarali NK, *et al*. **Subtype B and subtype C HIV type 1 recombinants in the northeastern state of Manipur, India.** *AIDS Res Hum Retroviruses* 2005; **21**:152–157.
38. Rodenburg CM, Li Y, Trask SA, Chen Y, Decker J, Robertson DL, *et al*. **Near full-length clones and reference sequences for subtype C isolates of HIV type 1 from three different continents.** *AIDS Res Hum Retroviruses* 2001; **17**:161–168.
39. Yang R, Xia X, Kusagawa S, Zhang C, Bem K, Tayebe Y. **Ongoing generation of multiple forms of HIV-1 intersubtype recombinants in the Yunnan Province of China.** *AIDS* 2002; **16**:1401–1407.
40. Yu XF, Liu W, Chen J, Kong W, Liu B, Zhu Q, *et al*. **Maintaining low HIV type 1 *env* genetic diversity among injection drug users infected with a B/C recombinant and CRF01_AE HIV type 1 in southern China.** *AIDS Res Hum Retroviruses* 2002; **18**:167–170.
41. Carrion G, Eyzaguirre L, Montano SM, Laguna-Torres V, Serra M, Aguayo N, *et al*. **Documentation of subtype C HIV type 1 strains in Argentina, Paraguay, and Uruguay.** *AIDS Res Hum Retroviruses* 2004; **20**:1022–1025.
42. Gomez-Carrillo M, Quarleri JF, Rubio AE, Carobene MG, Dilernia D, Carr JK, *et al*. **Drug resistance testing provides evidence of the globalization of HIV type 1: a new circulating recombinant form.** *AIDS Res Hum Retrovirus* 2004; **20**:885–888.
43. Aulicino PC, Kopka J, Mangano AM, Rocco C, Iacono M, Bologna R, *et al*. **Circulation of novel HIV type 1 A, B/C, and F subtypes in Argentina.** *AIDS Res Hum Retroviruses* 2005; **21**:158–164.
44. Dumans AT, Soares MA, Machado ES, Hue S, Brindeiro RM, Pillay D, *et al*. **Synonymous genetic polymorphisms within Brazilian human immunodeficiency virus type 1 subtypes may influence mutational routes to drug resistance.** *J Infect Dis* 2004; **189**:1232–1238.
45. Gadelha SR, Shindo N, Cruz JN, Morgado MG, Galvao-Castro B. **Molecular epidemiology of human immunodeficiency virus-1 in the state of Ceara, Northeast, Brazil.** *Mem Inst Oswaldo Cruz* 2003; **98**:461–463.
46. Osmanov S, Pattou C, Walker N, Schwardlander B, Esparza J, WHO–UNAIDS Network for HIV Isolation and Characterization. **Estimated global distribution and regional spread of HIV-1 genetic subtypes in the year 2000.** *J Acquir Immune Defic Syndr* 2002; **29**:184–190.
47. Su L, Graf M, Zhang Y, Von Briesen H, Xing H, Köstler J, *et al*. **Characterization of a virtually full-length human immunodeficiency virus type 1 genome of a prevalent intersubtype (C/B′) recombinant strain in China.** *J Virol* 2000; **74**:11367–11376.
48. Piyasirisilp S, McCutchan FE, Carr JK, Sanders-Buell E, Liu B, Chen J, *et al*. **A recent outbreak of human immunodeficiency virus type 1 infection in southern China was initiated by two highly homogeneous, geographically separated strains, circulating recombinant form AE and a novel BC recombinant.** *J Virol* 2000; **74**:11286–11295.
49. Koulinska IN, Ndung'u T, Mwakagile D, Msamanga G, Kagoma C, Fawzi W, *et al*. **A new human immunodeficiency virus type 1 circulating recombinant form from Tanzania.** *AIDS Res Hum Retroviruses* 2001; **17**:423–431.