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Epidemiologic and Evolutionary Trends of HIV-1 CRF31_BC-Related Strains in Southern Brazil

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Background: To evaluate the impact of HIV-1 CRF31_BC in the southern Brazilian HIV epidemic.

Methods: Blood plasma from 284 patients was collected from July 2002 to January 2003 at 2 reference HIV/AIDS centers in southern Brazil. Viral protease and reverse transcriptase (RT) genomic regions were amplified by RT polymerase chain reaction, sequenced, and subtyped. Evolutionary analyses were performed to estimate the CRF31_BC most recent common ancestor and its population growth rate with BEAST version 1.3.

Results: CRF31_BC was responsible for 7.4% of infections. The average time of HIV diagnosis and the proportion of patients on antiretroviral treatment were shorter for CRF31_BC and subtype C than for subtype B. CRF31_BC was found as early as in 1990 in the Brazilian epidemic. Evolutionary analysis of CRF31_BC revealed that it appeared immediately after the introduction of subtype C in Brazil and has been growing at a similar rate as subtype C.

Conclusions: CRF31_BC plays an important role in the HIV epidemic of southern Brazil, and its prevalence has increased throughout the years. This circulating recombinant form corresponds to approximately 25% of total HIV isolates in this region in 2004. Understanding the cause of this spread is important for public health strategies in Brazil and in Latin America.

Key Words: Brazil, circulating recombinant form, CRF31_BC, HIV, HIV sequence variability/subtypes, Latin America

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HIV is divided into groups, subtypes, subsubtypes, circulating recombinant forms (CRFs), and unique recombinant forms (URFs).[1] To date, 3 groups (M, N, and O),[2,3] 9 subtypes (A–D, F–H, J, and K),[1] 4 subsubtypes (A1, A2, F1, and F2),[4,5] and more than 30 CRFs have been described worldwide. This diversity is reflective of high rates of virus mutation,[6] rapid viral turnover in vivo,[7,8] and frequent recombination events.[9,10]

Recombination between different subtypes is common within group M, the major group accounting for the HIV/AIDS pandemic, the relevance of which, in global diversity, has been recognized by several authors.[11–14] In 2000, CRFs and other recombinants were responsible for 17.6% of total worldwide infections, although this proportion was higher in some regions, such as southern Asia and Southeast Asia (88.6%) and West Africa (42.6%).[13] In Latin America, CRF12_BF is the most prevalent CRF, and its presence has been detected in Argentina, Peru, Bolivia, and Uruguay.[15] In Cuba, CRF18_cpx and CRF19_cpx were recently characterized, with CRF18_cpx accounting for 7% of infections.[16,17]

In Brazil, CRF28_BF and CRF29_BF were recently identified in the southeastern region.[18] In southern Brazil, our group has recently characterized a new CRF derived from subtypes B and C,[19] named CRF31_BC. In this region, a higher prevalence of subtype C is observed with respect to the rest of country, with a steady increase since its appearance in the Brazilian epidemic.[20–23] Until recently, CRF31_BC was included in the Brazilian subtype C,[19,23] although its epidemiologic relevance is still unknown. In this study, we report the epidemiologic trends of CRF31_BC-related strains in southern Brazil.

METHODS

Samples

Peripheral blood samples were obtained from patients followed at 2 HIV/AIDS reference centers in Rio Grande do Sul state (Hospital de Clínicas de Porto Alegre and University Hospital of Rio Grande), who agreed to participate in this study by signing informed consent forms. All samples were collected between July 2002 and January 2003. Clinical data, such as $CD4^+$ T-cell counts, treatment status, HIV viral load, patient age, and date of diagnosis, were also retrieved from medical records. This study was approved by the internal review boards of both hospitals.

Complementary DNA Synthesis, Nested Polymerase Chain Reaction, and Sequencing

Viral RNA was extracted from plasma, followed by complementary DNA (cDNA) synthesis using random primers.[24] A nested polymerase chain reaction (PCR) assay was performed with specific primers, as previously reported,[24] to amplify a *pol* fragment of the viral genome spanning across regions coding for the entire protease (PR; 99 codons) and half of the reverse transcriptase (RT; 285 codons), resulting in a fragment of 1152 base pairs (bp) that was subsequently purified with Microcon PCR cartridges (Millipore Corporation, Billerica, MA). Two separate PCR assays, for amplifying PR and RT fragments, were carried out when the entire 1152-bp fragment failed to be amplified. All PCR products were sequenced with an automated ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Chromatograms were manually edited with SeqMan software (DNAStar, Madison, WI). Sequences were deposited in the GenBank, with accession numbers AY275717–807, AY390079–81, AY390178–90, DQ190951–1039, DQ343964–4016, and DQ659454–87, and were previously characterized by Santos et al,[19] Soares et al,[21,22] and Rodrigues et al.[23]

HIV-1 Subtype Determination

To determine the genetic subtype and to discard sample mix-ups or contaminations, all sequences were aligned with reference sequences of all representative HIV-1 subtypes, available at the Los Alamos database (http://hiv-web.lanl.gov), using ClustalW.[25] Phylogenetic analyses were carried out by neighbor-joining, with the Kimura 2-parameter correction, using MEGA 3.1[26] and 1000 bootstrap replicates. The sequence of SIV_{CPZ} GA (GenBank accession number X52154) was used as an outgroup.

Viral isolates of different subtypes provided by discordant PR and RT sequence data were considered to be mosaics and submitted to bootscanning analysis with Simplot 3.5.1 software.[10] Isolates grouping within the CRF31_BC clade were also submitted to bootscanning for confirming their recombinant origin.

Epidemiologic Profiling and Statistical Analyses

Clinical and laboratory data, such as age, CD4 T-cell counts, HIV-1 RNA viral load, time of treatment, and time since HIV diagnosis, were compared between subtype B–, subtype C–, and CRF31_BC-related strains with the Student *t* statistical test. Proportions of gender, Centers for Disease Control and Prevention (CDC) clinical stage, CDC immune stage, transmission route, and treatment status were evaluated by the 1-tailed Fisher exact test. A multivariate analysis to assess the independence of the associations found previously was further conducted. Because the outcomes were categoric and not ordered, multinomial logistic regression was applied. Two models were constructed. Model *A* compared subtype C and CRF31_BC separately with subtype B (base outcome for comparison). Model *B* compared factors associated with subtypes B and C, taking as a base category the CRF31_BC group. All variables were included in the models. A significance level of $P < 0.05$ was considered in all statistical tests.

For the purpose of following HIV subtype and CRF prevalence across the epidemic (Fig. 1), we have also included data by Rodrigues et al.[23]

Evolutionary Analysis of the Epidemic

We estimated the age of the most recent common ancestor (MRCA) of CRF31_BC-related sequences and its growth rate in the viral population. Inference of coalescence was estimated by a Bayesian skyline plot using BEAST version 1.3[27] under the HKY85 + G4 model of evolution,[28] with each codon position allowed to evolve independently. The Markov chain Monte Carlo algorithm available in BEAST was set to run for 10 million generations. After a burning period of 5×50^5 generations, samples were collected at every 1000 steps to build the posterior distribution of parameters. Chains were run twice to check convergence of parametric estimates. Priors for the operators were set at default values and were later tweaked according to the diagnosis automatically performed by the program. The population growth rate was estimated using the exponential growth model. We tested whether the *r* parameter was significantly different from 0 (constant population size) by checking if the 95% confidence interval (CI) for the parameter contained such value. For the sake of comparison, all evolutionary analyses were also carried out for Brazilian subtype B and C sequences isolated in the same geographic setting. For all evolutionary inferences, additional subtype B, subtype C, and CRF31_BC sequences from Porto Alegre from samples collected in 2004 and published by Rodrigues et al[23] were also included. Only sequences for which the PR and RT were both available were used in the coalescence analyses.

RESULTS

Genetic Diversity of HIV-1 in Southern Brazil

In the present study, we analyzed 284 patients, 181 (63.7%) from the Hospital de Clínicas de Porto Alegre and

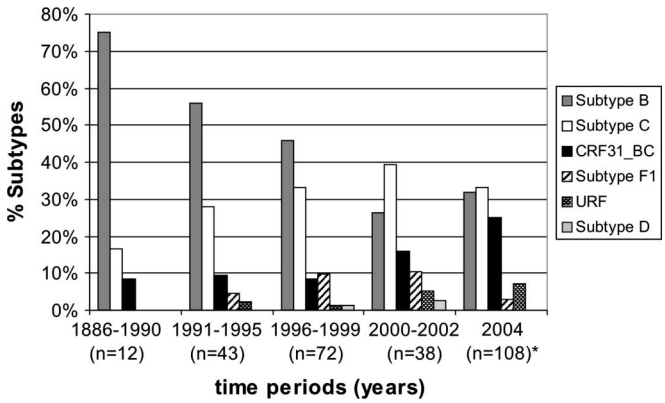

FIGURE 1. HIV-1 subtype distribution according to HIV diagnosis periods (1986–2002) in Porto Alegre, Brazil. The asterisk denotes samples isolated by Rodrigues et al[23] that were included in the analysis.

103 (36.3%) from the University Hospital of Rio Grande. The PR and RT regions were sequenced for 162 patients (57%), whereas only the PR or the RT region was amplified for 72 (25.4%) and 50 patients (17.6%), respectively.

Phylogenetic analyses showed that subtype B was the most prevalent subtype in southern Brazil (45.4%), followed by subtype C (34.9%)– and CRF31_BC-related strains (7.4%). Subtype F1, mosaics, and subtype D represented 6.3%, 4.2%, and 1.8%, respectively. Mosaics were represented by diverse URFs harboring genomic fragments of HIV subtypes commonly found in the area (Fig. 2). When comparing the 2 municipalities, significant differences were found with respect to subtype distribution. Prevalence of subtype C in the city of Rio Grande (41.8%) was higher than in the capital (Porto Alegre, with 30.9%; $P = 0.019$); conversely, prevalence of CRF31_BC in Rio Grande (3.8%) was lower than in the capital (9.4%; $P = 0.045$). The distribution of the remaining subtypes and mosaics did not differ significantly between cities.

Epidemiology Data of Patients Infected With Different HIV-1 Subtypes

Demographic and clinical data were analyzed for patients infected with subtype B–, subtype C–, and CRF31_BC-related strains (Table 1). Differences between patients infected with different subtypes were observed; in those infected with subtype B, the proportion of infected men (60.7%, male-to-female ratio = 1.46) was significantly higher than in those infected with subtype C (51%, male-to-female ratio = 1.04) or with CRF31_BC (38.1%, male-to-female ratio = 0.62; $P = 0.041$ and $P = 0.031$, respectively). Mean time of HIV infection since diagnosis for subtype B was longer than for subtype C and for CRF31_BC ($P < 0.001$ and $P = 0.039$, respectively). In relation to CDC clinical stage, 41% of patients with subtype B were in stage A, whereas 56% with subtype C were in this stage ($P = 0.010$). Conversely, 38% with subtype B were in stage C versus only 21% with subtype C ($P < 0.01$). With respect to transmission, 41% with subtype B were heterosexual versus 59% with subtype C and 63% with CRF31_BC ($P < 0.01$ and $P < 0.044$, respectively), and at the time of sample collection, 65% of patients with subtype B were receiving HIV treatment, as were 44% with subtype C ($P < 0.01$) and 38% with CRF31_BC ($P = 0.013$). The remaining parameters did not differ between subtypes and CRF31_BC.

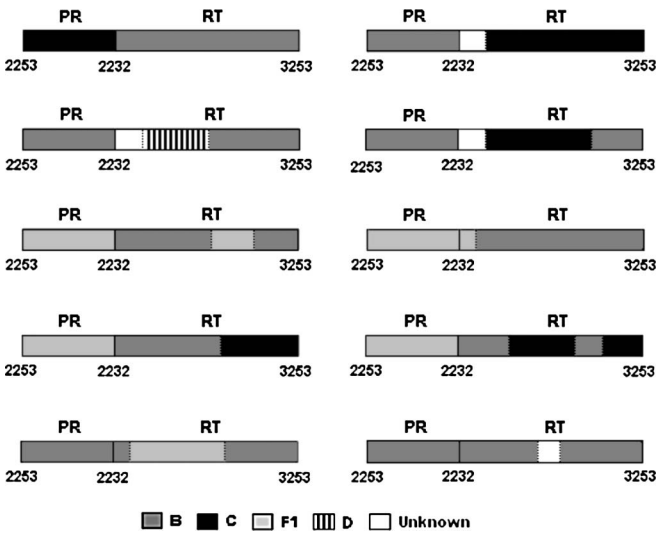

FIGURE 2. URFs comprising different HIV-1 subtypes found in the study.

In the multivariate analysis, using multinomial logistic regression for comparison of outcome category CRF31_BC and the 2 subtypes (Model *A*), the only significant association found was between treatment and subtype B, where those who received treatment had a higher probability of belonging to that group when compared with CRF31_BC (Table 2). The other factors did not show any significant association when adjusted. In the model that used subtype B as the outcome category (Model *B*), it was observed that the mean time of HIV diagnosis reduced the probability of infection by subtype C when compared with subtype B. Also, those who were treated had less probability of belonging to subtype C or CRF31_BC compared with subtype B.

Dynamics of HIV-1 Diversity During the Epidemic

CRF31_BC has recently been characterized in southern Brazil,[19] showing that this CRF accounts for 10% of the infected population. To verify the behavior of this viral variant throughout the HIV/AIDS epidemic, all Porto Alegre isolates were divided in 5 periods according to time of HIV diagnosis and viral subtype (see Fig. 1). CRF31_BC was found to be present as early as 1990, with a stable prevalence until 2000, when it increased to approximately 9% by 2002 and, subsequently, to 25% by 2004. To our knowledge, this is the oldest CRF31_BC strain in Brazil.

Evolutionary Demography

The age of the CRF31_BC MRCA sequence was estimated as 16.9 years before 2004 (95% CI: 5.7 to 36.6). A similar estimate was obtained for Brazilian subtype C, for which MRCA age was 17.2 years before 2004 (95% CI: 5.5 to 37.6). The coalescence of Brazilian subtype B sequences was inferred to have occurred 98.1 years before 2004 (95% CI: 46.4 to 164.3). Estimates of growth rate (r) were 0.7 (95% CI: 0.3 to 1.0) for subtype C and 0.5 (95% CI: 0.2 to 1.0) for CRF31_BC. Subtype B showed an r value of 0.4 (95% CI: 0.2 to 0.7).

DISCUSSION

Previous studies showed a high coprevalence of B and C subtypes in the southern region of Brazil,[20–23] a finding that was corroborated in this study. CRF31_BC, initially considered a divergent clade within the Brazilian subtype C,[19] was responsible for 7.4% of HIV infections in this area, and it actually represents 21.2% of infections previously attributed to subtype C. This observation is in agreement with another recently published study of our group showing that approximately one fourth of "subtype C" viruses were, in fact, CRF31_BC.[19] In Porto Alegre, CRF31_BC prevalence was

TABLE 1. Epidemiologic Features of Patients in the Southern Region of Brazil, 2002 to 2003

	Total	Subtype B	Subtype C	CRF31_BC
Mean age (y) (SD)	37.6 (10.6)	38.5 (10.6)	36.6 (11.3)	33.9 (7.6)
Gender (%)				
Male	144 (54.1)	71 (60.7)	49 (51)	8 (38.1)
Female	122 (45.9)	46 (39.3)	47 (49)	13 (61.9)
Mean time of HIV diagnosis (y) (SD)	4.92 (3.62)	5.76 (3.80)	4.10 (3.24)	4.12 (3.12)
CDC clinical stage (%)				
A	122 (47.3)	46 (40.7)	51 (56)	11 (55)
B	56 (21.7)	24 (21.3)	21 (23.1)	3 (15)
C	80 (31)	43 (38)	19 (20.9)	6 (30)
CDC immunologic stage (%)				
1	58 (21.7)	25 (21.4)	23 (24.2)	4 (19)
2	141 (52.8)	61 (52.1)	53 (55.8)	11 (52.4)
3	68 (25.5)	31 (26.5)	19 (20)	6 (28.6)
Mean CD4 cell count $\times 10^6$/L (SD)	348.9 (221.6)	348.3 (235.9)	370.9 (213.4)	343.8 (234.9)
Median CD4 cell count $\times 10^6$/L	320	320	347	297
Treated	304	327	349	218
Naive	342	306	363	344
Mean HIV RNA $\times 10^3$/L (SD)	72,977 (489,698)	120,661 (729,903)	34,516 (95,305)	8,515 (12,196)
Median HIV RNA $\log_{10} \times 10^3$/L	3.52	3.41	3.61	3.64
Treated	2.29	2.58	2.18	1.97
Naive	3.99	4.09	3.89	3.76
Undetectable* viral load (%)	60 (37.5)	28 (33.3)	19 (42.2)	3 (37.5)
HIV transmission routes (%)				
Men who have sex with men	52 (24.1)	35 (5)	13 (16.1)	1 (5.3)
Heterosexual	110 (51.2)	40 (41.2)	48 (59.2)	12 (63.1)
Intravenous drug users + sexual	7 (3.2)	1 (1)	3 (3.7)	2 (10.5)
Intravenous drug users	26 (12.1)	9 (9.3)	12 (14.8)	3 (15.8)
Transfusion	2 (1)	2 (2.1)	0	0
Blood derivatives	2 (1)	2 (2.1)	0	0
Unknown	16 (7.4)	9 (9.3)	5 (6.2)	1 (5.3)
Treatment status† (%)				
Treated	160 (56.3)	84 (65.2)	44 (44.4)	8 (38.1)
Naive	72 (25.3)	20 (15.5)	38 (38.4)	9 (42.8)
Interrupted treatment	36 (12.7)	13 (10)	14 (14.1)	4 (19.1)
Not available	16 (5.7)	12 (9.3)	3 (3.1)	0

*Defined as HIV RNA level less than 80 copies/mL of plasma.
†At the time of sample collection.

higher than 7.4% (9.4%), whereas in the city of Rio Grande, it was only 3.9%, indicating that this CRF probably originated in or near the state capital. The emergence of a CRF derived from subtypes B and C is not surprising. Multiple mosaic forms of these subtypes were observed in recent surveys, although no CRF has been characterized.[22,23,29]

The recent introduction of subtype C in southern Brazil[22,29,30] explains some significant differences between this subtype and the older subtype B of the Brazilian AIDS epidemic. Although we do not know the time of HIV infection of our patients, analysis of other parameters, such as CDC stage or being under antiretroviral treatment, reinforced the hypothesis that the subtype B epidemic is older than the subtype C or CRF31_BC epidemic in Brazil. In fact, we confirmed that the time elapsed from diagnosis to collection in patients with subtype B was longer than in patients with subtype C and CRF31_BC (see Table 1, 2). Being more recent in the Brazilian epidemic, subtype C and, consequently, CRF31_BC, are expected to be less subjected to antiretroviral therapy, a factor also depicted in the univariate and multivariate analyses (see Table 1, 2).

When analyzing the dynamics of CRF31_BC throughout the HIV/AIDS epidemic in Porto Alegre, we found that this CRF has been present since 1990, together with the oldest C subtype isolate in Brazil.[21] The prevalence of CRF31_BC increased over time and reached 15.4% between 2000 and 2002. This analysis, however, has been limited to the state capital, because HIV diagnosis has been available for a longer time there than in Rio Grande. Additionally, another study of more recent samples from Porto Alegre published before CRF31_BC characterization[23] showed that this form was responsible for some 25% of total infections.

TABLE 2. Multinomial Logistic Regression Analysis for HIV-1 Subtypes and Sociodemographic, Clinical, and Laboratory Characteristics (n = 218)

	Model *A**		Model *B*†	
Variable	Subtype B OR (95% CI)	Subtype C OR (95% CI)	Subtype C OR (95% CI)	CRF31_BC OR (95% CI)
Age	1.03 (0.97 to 1.01)	1.04 (0.98 to 1.11)	1.00 (0.98 to 1.04)	0.97 (0.91 to 1.03)
Male	2.15 (0.70 to 6.62)	1.34 (0.43 to 4.13)	0.62 (0.33 to 1.17)	0.47 (0.15 to 1.43)
Time of HIV diagnosis	1.00 (0.99 to 1.018)	0.99 (0.98 to 1.01)	0.99 (0.98 to 0.99)‡	0.99 (0.98 to 1.01)
CDC clinical stage				
A	1	1	1	1
B	0.71 (0.13 to 3.85)	0.94 (0.17 to 5.07)	1.31 (0.55 to 3.13)	1.40 (0.26 to 7.55)
C	0.61 (0.14 to 2.70)	0.43 (0.94 to 1.96)	0.71 (0.31 to 1.61)	1.65 (0.37 to 7.37)
CDC immunologic stage				
1	1	1	1	1
2	1.08 (0.14 to 8.60)	0.75 (0.97 to 5.77)	0.69 (0.21 to 2.26)	0.92 (0.12 to 7.31)
3	0.51 (0.27 to 9.62)	0.22 (0.12 to 4.03)	0.43 (0.75 to 2.49)	1.95 (0.10 to 36.6)
Treatment status				
Naive	1	1	1	1
Treated	7.72 (1.61 to 37.07)‡	2.94 (0.62 to 13.91)	0.38 (0.16 to 0.92)‡	0.13 (0.27 to 0.62)‡
Interrupted treatment	0.99 (0.19 to 5.17)	0.91 (0.18 to 4.45)	0.91 (0.31 to 2.68)	1.00 (0.19 to 5.18)
Median CD4 cell count	1.000 (0.99 to 1.01)	0.99 (0.99 to 1.01)	0.99 (0.99 to 1.00)	0.99 (0.99 to 1.00)
Median HIV RNA level	1.000 (0.99 to 1.01)	0.99 (0.99 to 1.01)	0.999 (0.99 to 1.00)	0.99 (0.99 to 1.00)

*Model *A*: outcome category CRF31_BC (n = 216).
†Model *B*: outcome category subtype B (n = 216).
‡$P < 0.05$.
OR indicates odds ratio.

Evolutionary analysis showed that the origin of the CRF31_BC epidemic dates back to approximately 1990, a finding that is corroborated by the earliest records of subtype C in Brazil.[21] Moreover, Santos et al[19] identified this CRF in 1 patient diagnosed in 1990. Altogether, these data strongly indicate that the recombination event between subtypes B and C that generated CRF31_BC occurred immediately after the introduction of subtype C in Brazil. Because we rely on the dates of HIV diagnosis and not infection, we cannot guarantee that the earliest subjects carrying CRF31_BC-related strains have not been superinfected with that strain later in the epidemic. However, we found 5 subjects infected with this variant who were diagnosed before 1996, which strengthens the idea that CRF31_BC was already circulating in southern Brazil for a long time. The age of the Brazilian subtype B MRCA sequences was not coincident with a previous estimate of the coalescence time of Brazilian sequences around 1950 through 1960,[30] because, in our study, this date was shifted to the early years of the 20th century. Our 95% CI for this estimate was wide, however, and included the dates calculated by Salemi et al.[30]

The population growth rate inferred for CRF31_BC remained between those for subtypes C and B. These values were close to the estimates of Salemi et al[30] and reaffirmed that the number of HIV-1 subtype C infections in Brazil grows faster than the number of HIV-1 subtype B infections. In addition, the constant population size could be excluded for both subtypes and CRF31_BC, because CIs for r estimates did not include 0. It seems that the epidemic potential of CRF31_BC is intermediate when compared with those of Brazilian subtypes B and C. Nevertheless, these estimates are still preliminary, and more samples are needed for a rigorous statistical evaluation.

This is the first study showing the epidemiologic impact of a CRF derived from subtype C in Brazil, which accounted for 1 in every 4 new infections in the past 2 years. We expect these data to be useful for clarifying the complex dynamics of HIV-1 subtypes in southern Brazil. Although the cause of the escalating subtype C and CRF31_BC epidemics is still unknown, their expansion is a matter of concern and may drive the HIV molecular diversity in the rest of the country as well in neighboring countries around the south of Brazil in the near future.

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