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Molecular Epidemiology of HIV-1 in Brazil: High Prevalence of HIV-1 Subtype B and Identification of an HIV-1 Subtype D Infection in the City of Rio de Janeiro, Brazil

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Summary: HIV-1–positive individuals were recruited from January 1993 to December 1996 from several cohorts receiving follow-up in the city of Rio de Janeiro, Brazil, to evaluate HIV-1 genetic variability and the potential association with modes of transmission. HIV-1 subtyping was carried out using the heteroduplex mobility assay (HMA), and those samples corresponding to the typical Brazilian subtype B variant were further identified based on the *Fok I* restriction fragment length polymorphism (RFLP). DNA sequencing was performed to evaluate one case of subtype D infection. From the 131 HIV-1–positive individuals analyzed, 106 (80.9%) could be identified as infected by subtype B and 20 (15.3%) by subtype F. One of the samples (0.8%) was classified as subtype D. DNA samples from 4 patients (3.0%) did not yield polymerase chain reaction (PCR)-amplified products to be typed. Based on the *Fok I* RFLP, 39 of the 106 subtype B samples (37%) were identified as corresponding to the typical Brazilian subtype B variant containing the GWGR motif at the tip of the V3 loop. No statistically significant association could be detected between HIV-1 subtypes and modes of transmission, exposure categories, or gender. This is the first reported case of HIV-1 subtype D infection in Brazil. **Key Words:** HIV-1 subtypes in Brazil—Heteroduplex mobility assay—HIV-1 subtype D infection.

Phylogenetic analysis of HIV-1 isolates distributed worldwide showed at least 10 HIV-1 subtypes (A–J) included in the M group, some of them corresponding to recombinant genomes (1–4). HIV-1 isolates belonging to B, F, and C subtypes have been found in Brazil (5–8), in addition to B/F recombinant genomes (9,10), with a clear predominance of the B subtype (11). Those studies have also shown that many Brazilian subtype B isolates present a typical amino acid composition (GWGR) at the conserved crown of the gp120 V3 loop, suggesting that subtype B isolates could be split in two main variants in Brazil, one corresponding to the U.S./European consensus B sequence and another, with important differences in the secondary structure and antigenicity of the corresponding proteins (12). Antigenic differences have also been detected in subtype B isolates from Thailand that present a typical amino acid composition PLGPGQAW at the tip of the V3 loop (B′) (13).

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Although the immunologic correlates of HIV-1 vaccines remain unknown, this striking genetic variability of HIV-1 isolates, specially at immunologically important regions of the envelope protein, can be limiting for the development of a broadly protective vaccine.

It has been suggested that HIV-1 subtype could be associated with different modes of transmission (14). Two distinct HIV-1 subtypes were detected in Thailand, where subtype B was currently identified among injecting drug users (IDUs), whereas subtype E was associated to heterosexual transmission (15,16). An association of HIV-1 subtype F infection and IDUs has been recently described in the city of São Paulo, Brazil (17). However, Brazil is a huge country and HIV epidemics may assume different patterns of transmission depending on the geographic region.

In this study, we used a heteroduplex mobility assay (HMA) (18,19) to evaluate the HIV-1 genetic diversity and its potential association with modes of transmission, exposure categories, gender, and distribution over time in the city of Rio de Janeiro, which is the second major city in terms of prevalence of HIV-1 infection in Brazil (after São Paulo) (20).

MATERIALS AND METHODS

Study Group

HIV-1–infected individuals were recruited from January 1993 to December 1996 from several cohorts: 80 patients infected for <2 years observed at the Evandro Chagas Hospital-FIOCRUZ, Rio de Janeiro, an AIDS medical care reference center in the city of Rio de Janeiro; 27 patients from the Ambulatório da Providência, RJ, an outpatient facility assisting basically underprivileged patients, including street/homeless individuals; and 24 injecting drug users (IDUs) recruited both from treatment centers and the streets as part of an ongoing cooperative IDUs multicenter project (21).

All patients were serologically positive for HIV-1 as determined by enzyme-linked immunosorbent assay (ELISA) commercial kits, immunofluorescence (Biomanguinhos-FIOCRUZ, Rio de Janeiro, Brazil), Western blot (Cambridge Biotech, Worcester, MA, U.S.A.), or a combination of these. Clinical and epidemiologic data were assessed by physician evaluation and the patients were scored according to the U.S. Centers for Disease Control and Prevention (CDC) clinical classification (22). $CD4^+$ T-cell determinations were done by flow cytometry.

DNA Preparation and Polymerase Chain Reaction Amplification of HIV-1 *env* Sequences

First, 5 ml of ethylene diamine tetraacetic acid (EDTA)-anticoagulated blood was obtained from each patient. After centrifugation, the plasma was collected and the pellet (2–3 ml) resuspended in 0.5% Saponin/0.4% NaCl (vol/vol), thoroughly vortexed, centrifuged (400 × g, 5 minutes, room temperature) and washed twice with phosphate-buffered saline (PBS) by centrifugation in the same conditions. Genomic DNA was extracted as previously described (6).

DNA samples (~1 μg) were amplified by polymerase chain reaction (PCR) by a nested protocol in a Perkin Elmer 480 Thermal Cycle (Perkin Elmer-Cetus, Norwalk, CT, U.S.A.) and consisted of three cycles at 97°C (1 minute), 55°C (1 minute), and 72°C (2 minutes), followed by 32 cycles at 95°C (45 seconds), 55°C (1 minute), and 72°C (2 minutes), with a 10-minute extension at 72°C in the last cycle. PCR reactions were performed in a volume of 100 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 mM of each deoxynucleoside triphosphate (dNTP), 20 pmol of each primer, 1.25 mM $MgCl_2$ (first round) or 1.8 mM $MgCl_2$ (second round), and 2.5 U Taq polymerase (Pharmacia, Fine Chemicals, Uppsala, Sweden). First-round primers ED3 (5′TTAGGCATCTCCTATGGCAGGAAGAAGCGG) and ED14 (5′TCTTGCCTGGAGCTGTTTGATGCCCCAGAC), corresponding, respectively, to bases 5959-5985 and 7960-7931 of the HIV-1 HXB2 genome (GenBank acession no. K03455), were used to amplify a 2.0-kb fragment of the HIV-1 *env* gene (18,19). For the second-round PCR, different sets of inner primers were used as follows: ED5 5′ATGGGATCAAAGCCTAAAGCCATGTG (6556–6581) and ED12 5′AGTGCTTCCTGCTGCTCCCAAGAACCCAAG (7822–7792) for the V1-V5 region (1.2 kb); ED31 5′CCTCAGCCATTACACAGGCCTGTCCAAAG (6816–6844) and ED33 5′TTACAGTAGAAAAATTCCCCTC (7359–7380) for the C2-C3 region (600 bp); or ES7 5′tgtaaaacgacggccagtCTGTTAAATGGCAGTCTAGC (7001–7020) and ES8 5′caggaaacagctatgaccCACTTCTCCAATTGTCCCTCA (7667–7647) for the V3-V5 *env* region (700 bp).

DNA samples that did not give amplified products using this protocol were directly amplified using the ED5/ED12 set of primers in the first round and ED31/ED33 or ES7/ES8 sets in the nested PCR.

Heteroduplex Mobility Assay

HIV-1 subtyping was determined by heteroduplex mobility assay (HMA) as described elsewhere (18). Briefly, 5 μl of ED31/ED33 or ED5/ED12 PCR-amplified products was mixed with 5 μl of PCR-amplified plasmids (94°C, 2 minutes) containing *env* fragments of the HIV-1 subtypes A to H reference samples included in the Heteroduplex Mobility Analysis HIV-1 *env* Subtyping Kit (23) provided by the NIH AIDS Research and Reference Reagent Program. Each unknown sample was tested respectively against three reference plasmids of HIV-1 subtypes A to E and and two of F to H.

Restriction Fragment Length Polymorphism Determination

After the second-round PCR with ED31/ED33 primers, 10 μl of HIV-1 amplified DNA was digested with 6 U of *Fok I* restriction enzyme, in a final volume of 25 μl of 1× buffer (Amersham Life Science, Cleveland, IL, U.S.A.), for 2 hours at 37°C. DNA samples were eletrophoresed through 2% agarose gels for 1 hour at 50 V in 1 × Tris-borate-EDTA (TBE) buffer, and the restriction fragments were evaluated under ultraviolet (UV) illumination. The molecular weight of the restriction fragments was determined based on the migration of the phiX-HaeIII digested molecular weight marker (Sigma Chemical, St. Louis, MO, U.S.A.).

Direct DNA Sequencing of Polymerase Chain Reaction Products

A sample of 5 μl (50–150 ng of DNA) of the ED31/ED33 PCR reaction was submitted to enzymatic treatment with 1 μl of shrimp alkaline phosphatase (10 U/μl) and 1 μl of exonuclease I (2 U/μl) hy-

drolytic enzymes according to the manufacturer's protocol and directly sequenced using the dideoxy chain termination method kit (Sequenase Version 2.0 DNA Polymerase, Amersham Life Science). The PCR products were sequenced in both senses with ED31, ED33, and ES7 oligonucleotides as sequencing primers. Alignment of multiple nucleotide and predicted amino acid sequences was performed with the University of Wiscosin Genetic Computer Group (GCG) package.

Phylogenetic tree analysis was done using the Neighbor Joining method (24), as implemented in the program Phylo_Win (25). The distances were estimated using the Tajima-Nei method (26) with pairwise gap deletion. The resulting tree was bootstrapped 100 times.

Statistical Analysis

Fisher's exact test was employed to evaluate possible correlations between HIV-1 subtypes and sociodemographic, behavioral, and other laboratory variables ($CD4^+$ count ranges). A p level of .05 was defined as statistically significant.

RESULTS

Study Group

We defined the subtypes of HIV-1 infection among 131 individuals (77 men and 54 women), who were distributed by the exposure categories as follows: 28 homosexual and 15 bisexual men, 55 heterosexuals (6 men), 24 IDUs (3 women), 1 woman who had received blood transfusions, and eight patients without available information. From this sample of 131 persons, 90 (69%) were clinically classified as group II, 15 (11%) as group III, and 17 (13%) as group IV according to the CDC clinical classification. Clinical data were not available for 9 patients (7%).

HIV-1 *env* Subtyping by Heteroduplex Mobility Assay and Restriction Fragment Length Polymorphism with *Fok I* Restriction Enzyme

From the 131 HIV-1–positive individuals analyzed, 106 (80.9%) could be typed as infected by B subtype and 20 (15.3%) by subtype F. One of the samples (0.8%) was classified as subtype D and will be detailed in the next section. DNA samples from 4 patients (3.0%), 3 infected by heterosexual intercourse and 1 intravenous drug user, did not yield PCR amplified products to be typed. These results are presented in Table 1.

Further digestion of the 600 bp ED31/ED33 PCR amplified DNA products, previously typed by HMA as B subtype, with *Fok I* restriction enzyme, which recognizes DNA sequence coding to the GW motif, generates two patterns of two (400 bp–200 bp) or three (400 bp–120 bp–80 bp) restriction fragments, detected by agarose gel electrophoresis and confirmed by DNA sequencing (data not shown). Using this approach, 39 (37%) of the 106 subtype B samples from the city of Rio de Janeiro were identified as corresponding to the typical GWGR subtype B samples found predominantly in Brazil.

TABLE 1. *HIV-1 subtypes distributed by the exposure categories of the infected individuals in the city of Rio de Janeiro, Brazil*

Categories	HIV-1 subtypes				Total
	B (%)	F (%)	D (%)	NA (%)	
Homosexual/ Bisexual	35	8	0	0	43
Heterosexual	46	6	0	3	55
IDUs	18	5	0	1	24
ND/other	7[a]	1	1	0	9
Total	106 (80.9)	20 (15.3)	1 (0.8)	4 (3.0)	131

[a] One HIV-1–positive patient included in this group was infected by blood transfusion.

NA, not amplified by polymerase chain reaction; ND, not determined; IDU, injection drug user.

Detection of Subtype D Infection in Rio de Janeiro, Brazil

Heteroduplex mobility assay of ED31/ED33 DNA PCR-amplified sample obtained from one of the patients included in this study (96BRRJ100), showed the fastest heteroduplex mobility with the three plasmids containing *env* genes of subtype D isolates from Uganda included in the HMA kit (Fig. 1*A*). Slightly less rapidly migrating heteroduplexes were also verified with the E1 and E2 plasmids containing the *env* gene of E subtype isolates from Thailand. No heteroduplex reaction was detected against the E3 plasmid corresponding to an E subtype isolate from the Central African Republic. Rapidly migrating heteroduplexes were detected only with subtype D isolates when HMA was performed with the 1.2-kb PCR-amplified products, corresponding to the V1-V5 region of the gp120. No reaction was detected among the three respective D and E subtype reference samples. A new blood sample of this patient was collected 4 months later and similar results were obtained (data not shown).

Phylogenetic tree analysis of *env* sequence data (Fig. 1*B*) showed that the C2-V3 sequence obtained from this sample clustered with other subtype D sequences with a bootstrap value of 90%. Comparing with other subtype D sequences deposited in the GenBank, the highest identity, varying from 92.0% to 89.9%, was observed with a set of subtype D samples from South Africa (27). However, the patient analyzed in this study has never been in any African country and denies any behavior associated with risk with people from that continent.

Multiple alignment of the amino acid sequences of

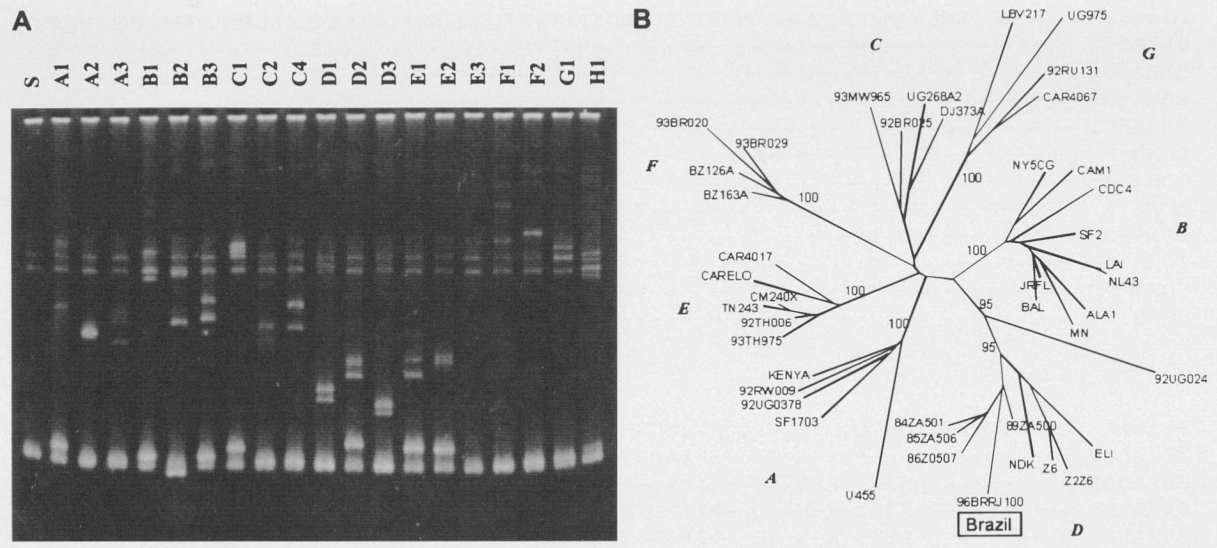

FIG. 1. Molecular characterization of an HIV-1 subtype D sample from Rio de Janeiro, Brazil. (**A**) Heteroduplex mobility assay of PCR amplified product of an HIV *env* subtype D sequence (96BRRJ100) spanning from the C2-C3 region of the gp120 annealed against three reference samples from A to E, and two reference samples from F to G HIV-1 subtypes. (**B**) Phylogenetic tree of HIV-1 C2-V3 *env* sequences.

sample 96BRRJ100 (Brazil) with other D subtype sequences, in addition to the HIV-1 subtype B MN strain is shown in Figure 2. The non–subtype B–conserved GPGQ motif in the tip of the V3 loop was detected in the Brazilian D subtype samples analyzed in this study. The presence of the positively charged amino acids at the positions 11 or 25, currently associated with the simian immunodeficiency viruses (28), was not observed in these Brazilian D subtype samples, suggesting a non–syncytium-inducing biologic profile.

No Segregation of HIV-1 Subtypes by Exposure Categories nor Over Time

To evaluate whether HIV-1 subtype could be segregated by exposure categories in Rio de Janeiro, we distributed the more prevalent B subtype– and F subtype–infected individuals by this parameter. As shown in Table 1, similar proportions of both HIV-1 subtypes were detected among them. Although a higher frequency of HIV-1 subtype F samples was determined in the IDU group, no statistically significant association (Fisher's exact test) was detected when compared with that of heterosexual ($p = .296$) or homosexual/bisexual ($p = .296$) risk groups. Moreover, no association with HIV-1 subtype distribution could be verified when patients were distributed by gender ($p = .807$) or by sexual and parenteral routes of transmission ($p = .534$). Similarly, HIV-1 infection by the typical GWGR Brazilian HIV-1 subtype B samples was not statistically associated to exposure categories, gender, and routes of transmission.

Based on our previous study (6) in which only one F subtype was identified among 25 DNA samples obtained from HIV-1–positive individuals from Rio de Janeiro, recruited for the study from 1990 to 1992, we hypothesized that subtype F frequency could be increasing over time. Indeed, 20 new subtype F samples were identified among the 131 HIV-1–infected individuals in this study. Considering the heterogeneity of the patient populations studied at that time and now, we decided to assess the question of temporal trends in HIV subtype distributions using the $CD4^+$ counts per cubic millimeter of the patients included in the present study, infected with B or F HIV-1 subtypes, as surrogates for duration of infection. However, the frequencies of subtype B and subtype F infections were similar in the >400 cells/mm^3 (83% B; 17% F) and <400 cells/mm^3 (84% B; 16% F) groups.

DISCUSSION

In this paper, we describe the identification of subtype D infection, in addition to the distribution of subtypes B and F by sociodemographic and behavioral variables in a molecular epidemiologic study of HIV-1 diversity in Rio de Janeiro, Brazil.

Based on our study group, which is representative of the main exposure categories of HIV-1 epidemics in Rio de Janeiro, a clear predominance of B subtype (80.9%), followed by subtype F (15.3%) was verified, similar to

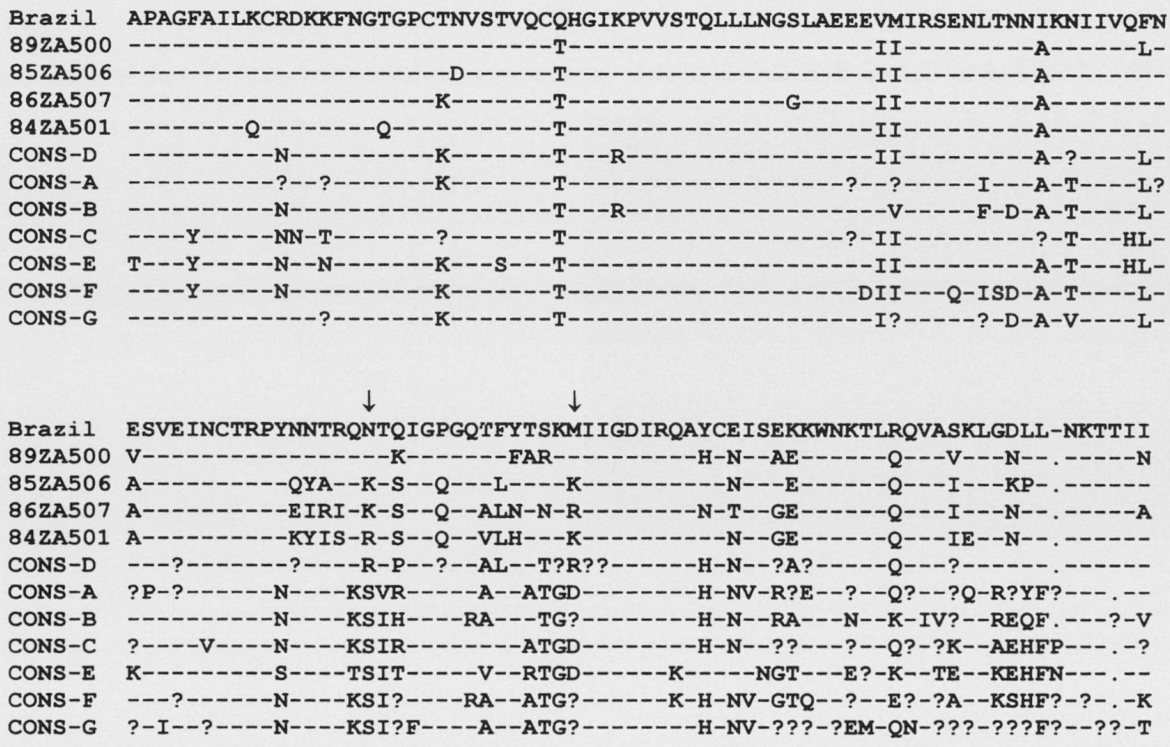

FIG. 2. Alignment of the C2-V3 gp120 amino acid sequence of the Brazilian HIV-1 subtype D with other subtype D samples from the African continent (GenBank accession numbers L47608, L48061, L48062, L48070) and HIV-1 subtypes D, A, B, C, E and F consensus sequences. Identical amino acids are indicated by dots. Arrows indicate positions 11 and 25 of the gp120 V3 loop (bold) which usually contains basic amino acids in the SI HIV-1 biologic pattern. Brazilian subtype D sequence 96BRRJ100 (Brazil) has been assigned GenBank accession number AF000238.

that described for the whole country (11). Among the subtype B samples identified in this study, 37% correspond to those presenting the typical GWGR sequence in the tip of the V3 loop. Although the use of the Fok I restriction enzyme could underestimate the frequency of this subtype B variant as a result of the limitation to the GGA codon usage for the glycine in the GW motif, all Brazilian GWGR subtype B samples sequenced so far have presented the GGA codon for glycine at that position (5, 6, 11, and a subset of samples included in this paper). Moreover, the percentage of GWGR samples typed by Fok I RFLP was similar to those previously described in Brazil based on the sequencing of the gp120 V3 loop.

No subtype C infection was identified in the present study, as previously demonstrated in Southern Brazil (8) and, more recently, in IDUs from Santos, the main port of the Southeast region, with one of the highest prevalence of HIV-1 infection among IDUs in Brazil (29). Such geographic differences in HIV-1 subtype distribution possibly result from distinct entries of the AIDS virus in Brazil.

The case of subtype D infection described in our study seems to be a relatively recent infection, because this individual was diagnosed as HIV-1–seropositive in September 1996, and he was a blood donor until the beginning of 1995. However, the $CD4^+$ counts were very low (96 cells/mm^3) at this first visit, but had risen to 290 cells/mm^3 on the second visit 4 months later, the improvement due to antiretroviral therapy. Previous evidence of subtype D infection in Brazil was presented in a dually infected individual, based on the RFLP of the protease gene (30). In the present study, the subtype D infection was defined based on the HMA result and confirmed by nucleotide sequence and phylogenetic analysis of the envelope gene.

Based on a study conducted in the city of São Paulo (17), it was recently suggested that subtype F could be related to injecting drug use in Brazil. However, no distinct pattern could be disclosed in our study conducted in Rio de Janeiro, with no statistically significant association found between HIV-1 subtypes and exposure categories, gender, or mode of transmission. Differences in the dynamics of the infection in our country could explain these findings. Indeed, a serious HIV-1 epidemic is taking place among IDUs in São Paulo, corresponding to 20% to 25% of the new AIDS cases registered there in the last few years, whereas <6% of the identified AIDS

cases in Rio de Janeiro belong to this exposure category (20). Moreover, in a previous study, we found 13.5% average nucleotide diversity in the C2-V3 region of 26 Brazilian subtype B samples obtained from 1990 to 1992 from asymptomatic HIV-1–positive individuals (6). Such diversity is related to the fact that HIV-1 has been circulating in Brazil since the beginning of the 1980s (20). This long-lasting AIDS epidemic may favor the dispersion of the HIV-1 subtypes among the exposure categories. This contrasts with countries with relatively recent epidemics, such as Thailand (15), where the AIDS epidemic was described only after 1988, showing a clear association of E and B HIV-1 subtypes with heterosexual and blood (i.e., IDU) transmission, respectively. More recent data have shown important changes in this pattern over time, with an increase in the proportion of new infections due to subtype E among IDUs (31).

''Old'' epidemics, with relatively high levels of seroprevalence among different population subgroups, were correlated with complex patterns of HIV transmission through social networks, making it difficult to establish a linear correlation between HIV-1 subtypes and epidemiologic and sociodemographic parameters such as exposure categories, age cohorts, or gender. If recent findings of both biologic (14) and epidemiologic (16) researches, showing different transmission rates through distinct transmission routes among diverse HIV-1 subtypes, prove to be true, they will reinforce the premises that Rio de Janeiro's epidemic is a rather old and interactive one in which the putative differential transmission rates have been overcome by a diffuse and efficient mixing pattern of HIV-1 spread.

Considering the $CD4^+$ counts as a surrogate marker to estimate time of infection, and trends of HIV-1 subtype distribution along the time, no increase in the frequency of F subtype was verified in the present sample. However, the implications of HIV-1 subtypes for pathogenesis and epidemiologic parameters are not clearly understood (32). Indeed, the non–subtype B AIDS epidemic in Africa is quite different from that in North America and Europe (33). The high genetic variability of HIV-1 subtypes described in that continent, as well as important differences in the genetic background, nutritional conditions, other sexually transmitted diseases, and endemic parasitic infections (34) may account for the observed differences. The identification of distinct HIV-1 subtypes circulating in Brazil, independent of the exposure categories, gender, or mode of transmission, may represent an important field for further studies of the implications of the HIV-1 subtypes for the natural history of HIV-1 infection in a more similar background.

APPENDIX

The Hospital Evandro Chagas AIDS Clinical Research Group includes B. Grinsztejn, V. C. Rolla, M. C. G. Gallhardo, M. R. C. Guimaraes, S. Cavalcante, and F. Sutmoller.

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