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HIV-1 Subtyping in Salvador, Bahia, Brazil: A City With African Sociodemographic Characteristics

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Summary: To investigate the prevalence of the HIV-1 subtypes in different populations from Salvador, Bahia, Brazil, blood samples from 72 HIV-1–seropositive injecting drug users (IDUs) and 62 individuals infected sexually were analyzed using the heteroduplex mobility assay (HMA). In the IDU group, 89.5% were classified as subtype B, 3% as subtype F, and 7.5% showed a B/F HMA profile. In the sexual transmission (ST) group, 95% were identified as B subtype, 3.4% showed a B/F profile, and 1.6% a B/C/E HMA profile. All Brazilian samples that showed multiple reactivities in the HMA analysis clustered on sequencing with B North American/European HIV-1 isolates in the phylogenetic analysis, whereas the F subtypes clustered with F Brazilian HIV-1 isolates. Serologic reactivities of IDU's sera were examined using a panel of synthetic V3 loop peptides representative of the different HIV-1 subtypes. No difference in serologic reactivity between F and B subtype plasma could be observed. Predominance of HIV-1 subtype B was identified in both study groups, whereas subtype F was detected only among IDUs in a frequency lower than described for other Brazilian regions. **Key Words:** HIV-1 subtypes—Diversity—Brazil.

HIV-1 isolates are phylogenetically classified in three groups (M, O, and N) (1–3). The prevalent M group (major group) is classified into at least 10 genetically subtypes designed A through J, with distinct distribution in the world. The existence of these HIV-1 groups and subtypes has many important implications for the global evolution of HIV-1 and future immunoprophylaxis programs.

In Brazil, four different clades (B/B′′, F, C, and D) and additional recombinant samples between subtypes B and F were identified in different geographic areas, most obtained in the southeast region (4–10). Moreover, Brazilian subtype B samples show low levels of amino acid sequence conservation in the V3 loop, with 40% of them typically showing the GWGR motif at the crown of the V3 loop, instead of the conserved GPGR observed in the North American/European isolates and the HIV-1 prototypes currently in use for vaccine development (5). In addition, sera from Brazilian HIV-1 patients display a lower specific antibody response against V3 loop peptides of predominant HIV-1 prototypes circulating in developed countries (11). These genetic diversities could have implications for vaccine development, as well as in pathogenesis, disease progression, and virus transmission.

Bahia State has most of the AIDS cases in the Brazilian northeastern region with 3458 cases reported through February 1999 (12). Most were detected in men

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who have sex with men (MSM), followed by injecting drug users (IDUs), however, an increase in the number of cases in heterosexual individuals has been observed in the later years (12). Most AIDS cases are concentrated in Salvador City, which is a seaside city with 2,500,000 habitants, most of African descent, with a high frequency of tourists coming from different continents. Moreover, this city displays several sociodemographic characteristics of large African or Haitian cities. Recent data from a cross sectional study from this region showed a seroprevalence of 44.1% of HIV infection in IDUs, as well as, a high frequency of HTLV-I/II infection (13,14).

To better understand the spectrum of HIV-1 diversity in this region and the potential association with transmission route, we analyzed the prevalence of the HIV-1 subtypes and the spectrum of antigenic diversity in different exposure groups of HIV-1 infection, using the heteroduplex mobility assay (HMA) and a panel of synthetic peptides representative of the different HIV-1 subtypes.

MATERIALS AND METHODS

Patient Population

Blood samples were obtained from 72 HIV-1–seropositive IDUs from Salvador, Bahia, Brazil (Projeto Brasil-Salvador). The study population, demographic data, and diagnosis of HIV infection were described elsewhere (13,14). Another 62 HIV-1–infected individuals, belonging to the sexual transmission (ST) group, were seen at the Federal University of Bahia Hospital and included in this study. Most study subjects were asymptomatic and the serostatus was previously established by commercial immunoassay and confirmed by Western blot (Cambridge Biotech, Worcester, MA, U.S.A.). All individuals donated blood voluntarily after informed consent.

DNA Isolation

Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque gradient centrifugation and 2×10^6 cells were resuspended in lysis buffer containing proteinase K (Sigma Chemical, St. Louis, MO, U.S.A.). After proteinase K digestion, 5 μl of this solution was used directly for polymerase chain reaction (PCR). Nonamplifiable samples were further submitted to genomic DNA extraction using the Isoquick Nucleic Acid Extraction Kit (Microprobe, Garden Grove, MD, U.S.A.) according to the manufacturer's instructions. Samples that were not amplifiable by PCR using this condition were reextracted using the phenol/chloroform method (15).

Nested Polymerase Chain Reaction

PCR amplification was carried out as described previously, with minor modifications (16). Briefly, 1 to 2 μg of genomic DNA was added to a first-round reaction of 50 μl containing 50 mM KCl, 10 mM Tris pH 8.3, 1.5 to 1.75 mM $MgCl_2$, 5 pmol of each primer, and 0.2 mM of each deoxynucleoside triphosphate (dNTP). Standard reaction conditions were 3 cycles of 97°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes, followed by 32 cycles of 95°C for 45 seconds, 55°C for 1 minute, and 72°C for 2 minutes, and a final extension at 72°C for 10 minutes. Second-round reactions (100 μl) were formulated and performed in similar conditions

First-round PCR primers ED3 and ED14 amplified a 2.0 Kb fragment spanning from the first exon of *rev* to the region of the envelope gene coding for the N-terminal half of the transmembrane protein gp41. Subsequently, three sets of primers (ED5/ED12, ED31/ED33, ES7/ES8) were used in the second-round PCR to amplify different regions of the viral envelope, resulting respectively in the expected 1.3 kb, 500 bp, and 700 bp products, respectively. Subtype reference plasmids were amplified using second-round primers and 10 ng of plasmids as templates (17).

Heteroduplex Mobility Assay

HIV-1 subtype was determined by HMA as described elsewhere (16). Incubating in annealing conditions 5 μl (100–250 ng of DNA) of second-round PCR with 5 μl of homologous product from a subtype reference (A–H).

Each unknown sample was amplified with at least two sets of primers, and subtyped on the basis of the higher mobility of the heteroduplexes formed with three reference strains of HIV-1 subtypes A to E and two of F to H (17)

Peptides and Peptide Enzyme-Linked Immunosorbent Assay

The amino acid sequence of the different specific HIV-1 subtypes was based on that in the Los Alamos Database (1). Non-biotinylated HIV-1 peptides HXB2, MN, SF2, RF (subtype B); V3BRcon-CNTRKSIHIGWGRAFYATGE (variant B′′); ELI, MAL, Z6 (subtype D); and the biotinylated peptides bV3FBR-NTRKSIPLGPGRAFY (Brazilian subtype F); bV3C-GKSIRIGPGQTFYAT-OH (subtype C), bV3BRW-NTRKSIHMGWGRAFY, bV3BR5-NTRKSIHLGWGRAFY, bV3Bcons-NTRKSIHIGPGRAFY), were synthesized respectively by Chiron (Clayton, Victoria, Australia) and Neosystem Laboratory (Strasbourg, France).

The HIV-1 peptide enzyme immunoassay was carried out as described previously (18). A pool of HIV-1–negative sera was used to establish the cutoff value for each plate, at 4 times the standard error plus the mean of the negative samples.

DNA Sequencing and Phylogenetic Analysis

PCR products corresponding to the *env* C2-V3 region of gp120 of nine samples were purified using a QIAamp PCR purification kit (Qiagen, Oslo, Norway) according to manufacturer's instructions. Double-stranded PCR fragments were sequenced in both directions using a ABI PRISM fluorescent dye-labeled terminator sequencing kit (ABI-Prism Dye Terminator Cycler Sequencing Ready Reaction Kit, Perkin Elmer Cetus, Norwalk, CT, U.S.A.). The primers ED31 (sense) and ED33 (antisense) were employed for molecular sequencing. DNA sequences were generated using an automated sequencer (ABI Model 370, Perkin Elmer Cetus).

Nucleotide sequences corresponding to the fragment of 345 pb in the C2V3 env region were aligned using *CLUSTAL W* (19) with minor manual adjustments. The phylogenetic trees using the neighbor-joining method and reliability of the branching orders using bootstrap approach were implemented by using CLUSTAL W. Evolutionary distances

were calculated with Kimura's two-parameter method (20). Various previously determined HIV-1 sequences were included in this analysis for comparative purposes, and the sequence of SIVcpz–gab was used as outgroup. The HIV-1 envelope sequences from Salvador, Bahia described in this study have been deposited in EMBL and the accession numbers are assigned from Y18752 to Y18760.

RESULTS

Study Subjects

Most of the 72 IDUs who participated in the study were male (70.8%), the mean age was 30 years (range, 13–57 years). Most are of African descent and estimated duration of HIV infection ranged from 6 months to 2 years at the sample collection. Data on the percentage of syringe sharing and length of drug consumption among this group was not available.

In the 62 individuals from the ST group, 41 samples were collected during the early HIV-1 infection in Salvador (1988–1990) and 21 samples during the years of 1995 to 1997. Most of these cases (77.4%) occurred among men having sex with men (MSM), the mean age was 35.5 years (range, 24–48 years), and 14 cases (22.6%) reported heterosexual transmission. Individuals from the first group (1988–1990) had been infected for >1 year at the time of blood collection and the $CD4^+$ cell count was not available. Most individuals from the second group (1995–1997) showed a $CD4^+$ count >500 cells/mm^3. In both groups, most HIV-1–infected individuals (83.6%) were not provided any antiretroviral therapy. The racial origin in this group was not available.

Polymerase Chain Reaction and Heteroduplex Mobility Assay Subtyping

About 50% of the samples were directly amplified by nested PCR, using the product of PBMC digestion. The rest of the samples, purified using the commercial DNA extraction IsoQuick kit or the phenol/chloroform procedure, generated PCR products to perform HMA subtyping. However, six specimens from the IDUs and two from HIV-1 ST group, were still PCR-negative and thus not typable by HMA.

Table 1 summarizes the demographic data and the genetic subtypes of HIV-1 characterized in the study population. The genetic HIV-1 subtyping by HMA performed among the IDU group showed that 66 samples (91.6%) could be genetically typed by HMA, 59 (89.5%) were identified as subtype B, 2 (3%) as subtype F and 5 (7.5%) showed a B/F profile (Fig. 1*A*).

In the ST group, 60 HIV-1 samples (96.8%) could be subtyped by HMA. Most (95%) were classified as B subtype, 2 patients (3.4%) showed a B/F, and 1 patient (1.6%) a B/C/E profile (Fig. 1*B*). No F subtype was observed in the HIV-1 ST group in Salvador, Bahia. These results and sociodemographic information of this group are summarized in Table 2.

TABLE 1. *Demographic data and genetic heteroduplex mobility assay subtypes of injecting drug users from Salvador, Bahia, Brazil*

		Subtype		
Variable	Tested (*n*)	B *n* (%)	F *n* (%)	B/F *n* (%)
Total	66	59 (89.5)	2 (3)[a]	5 (7.5)[a]
Gender				
Male	46	42 (91.3)	—	4 (8.7)
Female	20	17 (85)	2 (10)	1 (5)
Age (y)				
<20	11	11 (100)	—	—
20–29	21	18 (85.7)	—	3 (14.3)
30–39	21	17 (80.9)	2 (9.55)	2 (9.55)
>40	10	10 (100)	—	—
Unknown	3	3 (100)	—	—

[a] Samples selected for C2-V3 *env* sequencing.

Phylogenetic Analysis

Five samples from the IDU group (BA95, BA103, BA104, BA118, BA122), one from the ST group (BA75) showing rapidly migrating heteroduplexes with subtypes B and F, one ST sample that had a B/C/E profile (BA23) in the HMA, in addition to the two subtype F samples (BA73 and BA94), were chosen for sequencing of the env C2V3 region. Three samples (BA95, BA118, and BA122) clustered with the MN strain in phylogenetic analysis, whereas two others (BA103 and BA104) clustered with GWGR Brazilian subtype B samples, although none presented the GWGR motif at the crown of the V3 loop. Two samples from the ST group, showing, respectively, B/F (BA75) and B/C/E (BA23) HMA profiles, clustered separately in the subtype B group (Fig. 2). The only two samples identified as subtype F in HMA analysis clustered with typical Brazilian F subtype HIV-1 isolates (Fig. 2).

HIV-1 Peptide Reactivity

Sera from 72 IDUs were tested for reactivity with biotinylated peptides corresponding to the V3 loop sequences of subtype B, subtype F, and subtype C; 45 of these sera were tested against non–biotin-treated HIV-1 peptides from subtype B and subtype D.

The biotinylated V3B peptide (65 of 70 B sera) and the non-biotinylated MN peptide (39 of 43 B sera) were the most frequently recognized. Three sera recognized

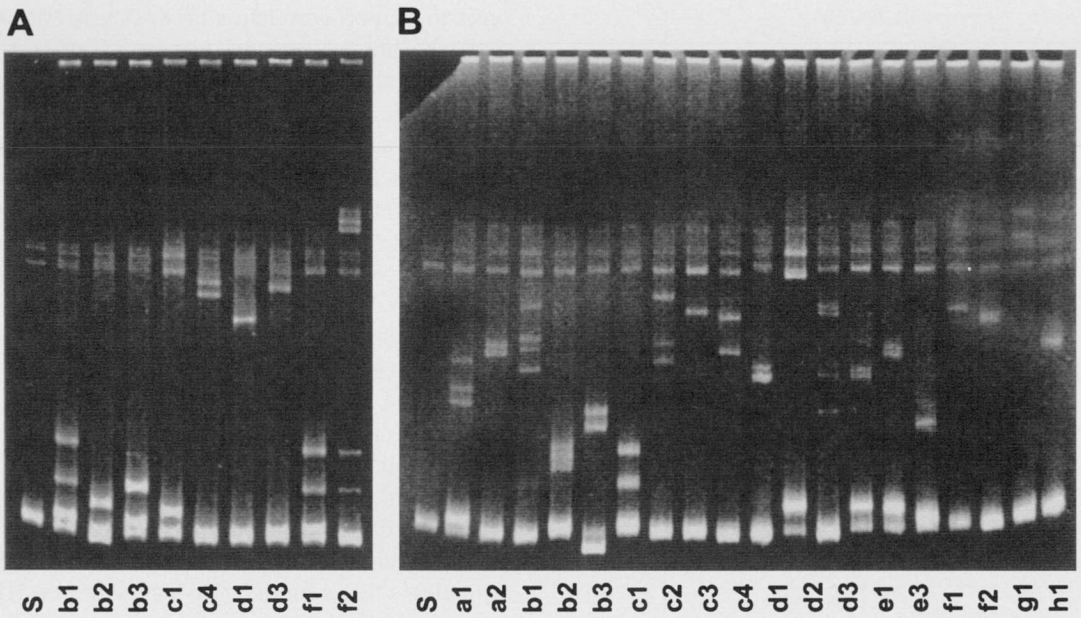

FIG. 1. Molecular characterization of HIV-1 Brazilian subtypes using heteroduplex mobility assay (HMA). (**A**) HIV-1 sample with B/F HMA profile. (**B**) Sample with a B/C/E profile in HMA analysis.

specifically only one B peptide, two reacted with bV3B, and one with BRcon. No IDU serum was negative with all B peptides, however, only two sera were able to react with all B peptides employed in this study.

TABLE 2. *Demographic data and genetic heteroduplex mobility assay subtypes of the sexual transmission group from Salvador, Bahia, Brazil*

Variable	Tested (*n*)	Subtype B *n* (%)	F *n* (%)	B/F *n* (%)	B/C/E *n* (%)
Total	60	57 (95)	—	2 (3.4)	1 (1.6)[a]
Gender					
Male	47	45 (95.8)	—	1 (2.1)[a]	1 (2.1)
Female	13	12 (92.3)	—	1 (7.7)	
Age (y)					
<20	—	—	—	—	—
20–29	11	10 (90.9)	—	1 (9.1)	—
30–39	21	19 (90.5)	—	1 (4.75)	1 (4.75)
>40	11	11 (100)	—	—	—
Unknown	17	17 (100)	—	—	—
$CD4^+$ counts ($cells/mm_3$)[b]					
<200	3	3 (100)	—	—	—
200–500	5	5 (100)	—	—	—
>500	12	9 (75)	—	2 (16.7)	1 (8.3)
Unknown	40	—	—	—	—
Antiretroviral therapy					
Yes	8	7 (87,5)	—	1 (12.5)	—
No	50	48 (96)	—	1 (2.0)	1 (2.0)
Unknown	2	2 (100)	—	—	—

[a] Samples selected for C2-V3 *env* sequencing.
[b] Only available for 1995/1997 sexual transmission subgroup.

In contrast, most sera (70 of 72) were negative with peptides derived from African subtype D strains. Extensive cross-reactivity was observed with the F peptide (100%) and the C peptide (60%). The two subtype F sera recognized the bV3FBR peptide as well most B peptides tested.

DISCUSSION

In this paper, the genetic analysis in the main exposure categories of the HIV-1 epidemic in Bahia in northeastern Brazil, demonstrates the presence of two HIV-1 subtypes circulating in this region, with a clear predominance of subtype B among IDUs and individuals infected by ST. No difference was observed between the two ST groups, as only subtype B was observed in the samples collected both in 1988 to 1990 and 1995 to 1997. Conversely, subtype F was detected only in the IDU group. Similarly, in a previous study conducted with a few samples collected in Salvador, Bahia in the beginning of the 1990s (4), one sample obtained from an IDU was initially described as "A-like," and further characterized as subtype F. The phylogenetic analysis of the B samples from IDU group showed a near relation of their genetic structure, probably due to the extensive needle sharing occurring in this group. However, the two ST samples (BA23 and BA75) clustered together in a separate branch from those including the IDU samples.

Recently, a high prevalence of HIV-1 subtype F was

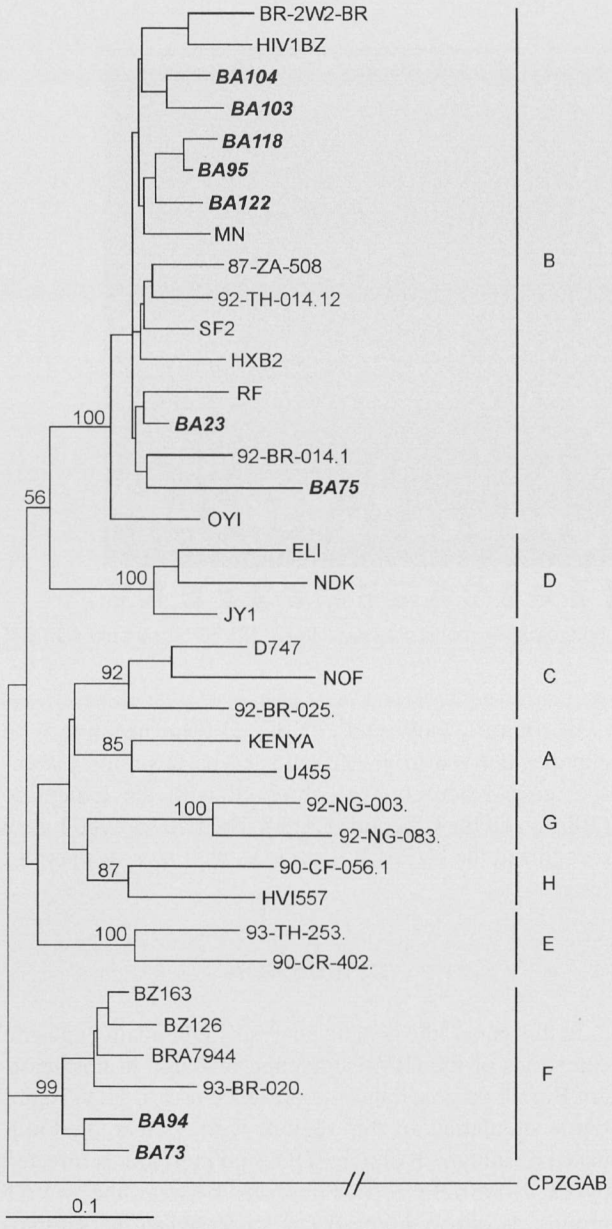

FIG. 2. Phylogenetic tree of HIV-1 C2-V3 *env* sequences from nine Brazilian HIV-1 subtypes. Nucleotide sequences and phylogenetic tree were implemented using CLUSTAL W (19). All Brazilian samples are *bold italicized* and the scale bars represent 10% divergence. The Afro-Brazilian HIV-1 envelope sequences from Salvador, Bahia, have been deposited in EMBL under accession numbers Y18752 through Y18760.

observed in São Paulo (in southeastern Brazil) in IDUs and their female sexual partners, suggesting a higher level of transmissibility of this subtype among this risk group (21). Although in the present study conducted in Bahia, the subtype F was detected only among IDUs, the percentage was very low and no significant statistical basis to support correlation between this HIV-1 subtype and exposure category or gender.

In general, the frequency of subtype F in Bahia seems to be lower than described for other cities in Brazil (5, 6,21). No subtype D or C infection was identified in the subjects included in our study group as previously detected in Brazil (7,8). However, more recent data showed the presence of subtype C in 8 of 177 samples from other states in northeastern Brazil, including Salvador, Bahia (22).

Comparing our present results with those obtained in the AIDS epidemic in Africa, we have not observed the presence of multiple HIV-1 subtypes circulating in this African related community in Northeast Brazil, suggesting an original introduction into this area of strains derived from North American/European HIV-1 isolates. However, the permanent contact of Brazilian travelers with African communities represents an important epidemiologic factor for the introduction of new HIV-1 subtypes in Brazil.

A recently described study using HMA analysis described subtype F viruses in the Philippines from overseas contract workers, which played an important role in the introduction of this subtype in this area (23). Salvador, Bahia is a seaside city with several ports. Although the prevalence of non-B subtype is lower in comparison with other regions, such a route for the introduction of new HIV-1 variants should be considered.

The reactivity of sera from HIV-1–infected IDUs were highest with bV3B and MN peptides. All sera were reactive with the peptide derived from a Brazilian HIV-1 F isolate, possibly due to the highly conserved GPGR sequence situated at the top of the V3 loop, common to both F and B peptides, described as being the immunodominant part of the V3 loop.

The reliability of V3 loop serotyping is highly dependent on the viral diversity within the analyzed population. In contradistinction to results reported in Thailand (24), where the enzyme immunoassay with synthetic peptides is highly effective in HIV-1 subtyping, in Bahia, Brazil, the seroreactivity assay used in this study did not allow distinction between the subtype B and subtype F HIV-1 infections. However, this high serologic cross-reactivity observed among the different peptides indicates the occurrence of common epitopes among and within the HIV-1 subtypes prevalent in Brazil and, taken together with data concerning cross-neutralizing antibodies among these subtypes (25), could represent promising data for vaccine development.

In conclusion, the genetic variability of HIV-1 could have some implications on transmission, disease progression, and emphasis on the need of continued epidemio-

logic and molecular surveys in Brazil, mainly in areas that were not previously investigated.

Several epidemiologic studies reported the occurrence of different HIV-1 subtypes and recombinant viruses composed of genetic material derived from two genotypes (9,10). Although in our study we have not found combinations between the nine strains sequenced within the C2-V3 region of the envelope, further complementary studies analyzing other genomic regions will be necessary to assess this issue.

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REFERENCES

1. Myers G, Korber B, Foley B, et al. *Human retrovirus and AIDS 1997: a compilation and analysis of nucleic acid and amino acid sequences.* Los Alamos, NM: Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, 1997.
2. Van den Haesevelde M, Decourt J-L, et al. Genomic cloning and complete sequence analysis of a highly divergent African human immunodeficiency virus isolate. *J Virol* 1994;68:1586–96.
3. Simon F, Mauclère P, Roques P, et al. Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. *Nat Med* 1998;4:1032–7.
4. Potts KE, Kalish ML, Lott T, et al. Genetic heterogeneity of the V3 region of the HIV-1 envelope glycoprotein in Brazil. *AIDS* 1993; 7:1191–7.
5. Morgado MG, Sabino EC, Sphaer EG, et al. V3 region polymorphisms in HIV-1 from Brazil: prevalence of subtype B strains divergent from North American/European prototypes and detection of subtype F. *AIDS Res Hum Retroviruses* 1994;10:569–76.
6. Couto-Fernandez JC, Janssens W, Heyndrickx L, et al. Genetic and antigenic variability of HIV type 1 in Brazil. *AIDS Res Hum Retroviruses* 1994;10:1157–63.
7. WHO Network for HIV isolation and characterization: HIV-1 variation in WHO-sponsored vaccine evaluation sites: genetic screening, sequence analysis and preliminary biological characterization of representative viral strains. *AIDS Res Hum Retroviruses* 1994;10:1927–43.
8. Morgado MG, Guimar es ML, Gripp CBG, et al. High prevalence of B subtype and recent introduction of HIV-1 subtype D infection in Rio de Janeiro city, Brazil: no evidence of association between HIV-1 subtypes and exposure categories. *J Acquir Immune Defic Syndr Hum Retrovirol* 1998;18:488–94.
9. Sabino EC, Sphaer EG, Morgado MG, et al. Identification of HIV-1 envelope genes recombinant between subtypes B and F in two epidemiologically-linked individuals from Brazil. *J Virol* 1994;68:6340–6.
10. Gao F, Robertson DL, Carruthers D, et al. A comprehensive panel of near-full-length clones and reference sequences for non-B isolates of human immunodeficiency virus type 1. *J Virol* 1998;72: 5680–98.
11. Bongertz V, Jansson M, Flodby P, et al. Analysis of antibody specificity against the third variable region of the envelope gp 120 of HIV-1 in plasma from HIV-1 positive individuals resident in Brazil. *Braz J Med Res* 1994;27:1225–36.
12. Brazilian Ministry of Health. *AIDS epidemiological bulletin* [in Portuguese]. PNDST/AIDS, year XII, no, 01. Brasilia: Ministério da Saúde, 1999.
13. Andrade TM, Dourado I, Galvão-Castro B. Association among HTLV-I, HTLV-II and HIV in injecting drug users in Salvador, Brazil. *J Acquir Immune Defic Syndr Hum Retrovirol* 1998;18: 186–87.
14. Dourado I, Andrade T, Carpenter CL, et al. Risk factor for human T cell lymphotropic virus type I among injecting drug users in Northeast Brazil: possibly greater efficiency of male to female transmission. *Mem Inst Oswaldo Cruz* 1999;94:13–18.
15. Maniatis T, Fritsch EF, Sambrook J. *Molecular cloning: a laboratory manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
16. Delwart EL, Sphaer EG, Louwagie J, et al. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 env genes. *Science* 1993;262:1257–61.
17. Delwart EL, Herring B, Learn Jr, GH, et al. *Heteroduplex mobility analysis HIV-1 env subtyping kit,* protocol version 3. Rockville, MD: NIH AIDS Research and Reference Program, 1995.
18. Bongertz V, Costa CI, Guimarães ML, et al. HIV specific humoral immune response in Rio de Janeiro, Brazil. *Mem Inst Oswaldo Cruz* 1998;93:391–8.
19. Thompson J, Higgins D, Gibson T. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight choice. *Nucleic Acid Res* 1994;22:4673–80.
20. Kimura M. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotides sequences. *J Mol Evol* 1980;16:111–20.
21. Sabino EC, Diaz RS, Brigido LF, et al. Distribution of HIV-1 subtypes seen in an AIDS clinic in São Paulo City, Brazil. *AIDS* 1996;10:1579–84.
22. Shindo N, Dube I, Souza IOM, et al. Prevalence of HIV-1 subtype C in northeast and central east states in Brazil [abstract 343]. I Bienal de Pesquisa, Fundação Oswaldo Cruz, 1998.
23. Santiago ML, Santiago EG, Hafalla JCR, et al. Molecular epidemiology of HIV-1 infection in the Philippines, 1985 to 1997: transmission of subtypes B and E and potential emergence of subtypes C and F. *J Acquir Immune Defic Syndr Hum Retrovirol* 1998;18: 260–9.
24. Pau CP, Lee-Thomas S, Auwanit W, et al. Highly specific V3 peptide enzyme immunoassay for serotyping HIV-1 specimens from Thailand. *AIDS* 1993;7:337–40.
25. Bongertz V, Costa CI, Guimar es ML, et al. Neutralization susceptibility of B subtype variant B primary HIV-1 isolates. *Scand J Immunol* 1998;47:603–8.