

MOLECULAR AND BIOLOGICAL DIVERSITY OF HIV-1 IN BRAZIL

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To determine the genomic polymorphism and biological properties present in HIV-1 Brazilian isolates, we analyzed five viral isolates obtained from patients residing in Rio de Janeiro (P1 and P5), São Paulo (P3) and Bahia (P2 and P4) states. For each viral isolate in vitro characteristics such as replication rate, syncytium-inducing capacity and cell death were observed in lymphoblastoid (H9, CEM and peripheral blood mononuclear cells) as well as monocytoid (U937) cells. In addition, the evaluation of the restriction fragment length polymorphism of these isolates was also performed using a panel of endonucleases such as Hind III, Bgl II, Sac I, Pst I, Kpn I and Eco RI. One of the isolates (P1), showed the highest phenotypic and genotypic divergence, when compared to others. The results found suggest a HIV heterogeneity in Brazil similar to that already described in other regions of the world.

Key words: Brazilian HIV – virulence – restriction analysis – viral diversity

In Brazil, acquired immunodeficiency syndrome (AIDS) is a very serious public health problem. As of August 1991, 19,361 cases, most of them in homosexual/bisexual men and intravenous drug users, have been officially reported (Ministério da Saúde, 1991). Since the discovery of human immunodeficiency virus (HIV) as the causative agent of AIDS in 1983 (Barré-Sinoussi et al., 1983), several isolates have been obtained and found to be different with respect to their cell infectivity, replication rate, neutralization pattern and enzyme restriction characteristics (Levy, J. A. et al., 1985; Asjo et al., 1986; Alizon et al., 1986; Prince et al., 1987; Balachandran et al., 1991). Indeed, polymorphic restriction maps have been observed among isolates recovered from the same region (Benn et al., 1985) or even from the same individuals (Saag et al., 1988; Meyerhans et al., 1989).

In 1987 we succeeded in isolating a retrovirus from an AIDS patient suffering of a

neurologic syndrome, which was characterized antigenically as HIV-1 (Galvão-Castro et al., 1987). Since then, further successful viral isolations have been described from patients in states of Rio de Janeiro, São Paulo and Bahia. The states of Rio de Janeiro and São Paulo present an estimated 80% of total AIDS cases.

In this paper, we present results concerning restriction enzyme polymorphism and biological features indicating the diversity of HIV in Brazil.

MATERIALS AND METHODS

Viruses and cell lines – HIV isolation was carried out according to Barré-Sinoussi et al. (1983), with some modifications (Galvão-Castro et al., 1987). Briefly, 2×10^6 patient's peripheral blood mononuclear cells (PBMC) were cocultured with 5×10^6 phytohemagglutinin (PHA)-stimulated healthy donor's PBMC. The cultures were maintained in RPMI-1640 medium containing 20% fetal bovine serum and 50 UI/ml of recombinant interleukin-2 (Innogenetics, Antwerp, Belgium). Every week 2×10^6 PHA-stimulated healthy donor's PBMC were added to the cultures. Cell culture supernatants were collected every two or three days

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and tested for viral antigens by an HIV antigen capture enzyme immunoassay (EIA), (Innogenetics, Antwerp, Belgium). HIV-containing supernatants were kept at -70°C until use. Patient's clinical status and the geographical region where the viruses were isolated are shown in Table I. The cell lines used were H9 (Popovic et al., 1984), CEM (Foley et al., 1965), both derived from human T-lymphocyte lineages. Also, the promonocytic U937 cell line (Sundstrom & Nilsson, 1976) and healthy donor's PHA-stimulated PBMC were infected.

Viral infectivity – The infection of the cell types by the viral isolates was carried out in the following way: HIV positive cell culture supernatants, as measured by HIV antigen capture EIA, were diluted serially and used at the highest dilution that presented an optical density of 2.0 or higher with the EIA. One million cells of each cell line were incubated with 1 ml of the appropriate dilution of positive supernatant during 30 min at 37°C . The cells were then washed twice with sterile phosphate-buffered saline (PBS) and maintained in culture medium for at least four weeks. The presence of HIV in the supernatant and cells was monitored by EIA and indirect immunofluorescence assay (IFA) (Sandstrom et al., 1985), respectively. Productive infection of cell cultures was defined as a continuous increase of HIV antigen levels in the supernatants and characteristic fluorescence of the cells. Cytopathic effects (CPE), such as syncytium formation and cell death, were evaluated daily in an inverted microscope. Total cell number and viability was evaluated by trypan blue exclusion in an hemocytometer chamber.

Southern blot hybridization analysis – High-molecular-weight DNA was obtained from PBMC infected with HIV isolates corresponding to samples P1, P2, P4 and P5 as previously described (Morgado et al., 1989). Briefly, HIV-positive cells were recovered after the 10-12th day of culture, washed twice with PBS and the pellet was resuspended in 2 ml of lysis buffer containing 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 200 mM NaCl, 0.5% SDS and 100 $\mu\text{g}/\text{ml}$ proteinase K. The suspensions were then incubated overnight at 37°C . DNA samples were extracted with phenol (saturated with Tris-HCl pH 7.5), for 1 h, phenol/chloroform (1:1) for 30 min, and chloroform, respectively, precipitated with 2.5 vol of ethanol, dried and dissolved in water. Uninfected and

HTLV-III β -infected PBMC and CEM cells were used as negative and positive controls, respectively. In addition, HIV-2rod-infected CEM cell line was used to assess the specificity of the probe for HIV-1. Each DNA sample (10 μg) was then digested with a panel of restriction enzymes, electroforested through 0.8% agarose gels, and blotted onto nylon membranes (Hybond-N, Amersham Co.) overnight according to Southern technique (Southern, 1975). After prehybridization, the membranes were hybridized during 16-20 h at 65°C , in 3 X SSC, 0.1% SDS, 5 mM EDTA, 10% Denhart's solution, 50 $\mu\text{g}/\text{ml}$ sonicated salmon sperm (Sigma Co.) and 10% dextran-sulphate containing 5×10^6 cpm/ml of the 5^{\prime}^{32}P d-CTP nick translated probe.

After hybridization the filters were washed in 3 x SSC, 0.05% SDS for 10 min at room temperature and washed again in 1 x SSC, 0.05% SDS for 30 min at 65°C . The filters were exposed to Kodak X-Omatic AR film using an intensifying screen at -70°C .

DNA probes were prepared from Sac I-Kpn I fragment covering 5'LTR-GAG-POL regions of the HIV-1 proviral genome, cloned on pUC 18 plasmid (pKs.5), kindly provided by Unité d'Oncologie Virale of the Institute Pasteur, Paris. For the Southern blot the total insertion was used as probe, whereas for the construction of the partial restriction endonuclease maps three distinct fragments, Sac I-Kpn I, Hind III and Bgl II-Kpn I, were used as probe as indicated on Fig. 2.

RESULTS

Viral infectivity – The P1 isolate was the most infectious. This isolate successfully infected all cell lines used, and it commenced killing the lymphoblastoid cells by the eighth day post-infection. The cell death was preceded by clear cut syncytium formation (mostly in H9 and PBMC) between the fourth and sixth days. This isolate presented the fastest replication rate, reaching the highest antigenic levels in less than a week. No fluorescence staining could be observed in any cell lines used. Three isolates (P2, P4 and P5), showed a similar growth characteristics in all lymphoblastoid cell lines used (Table II). Culture supernatants were positive by 12-14 days post-infection. H9 and PBMC cells presented the highest HIV antigen levels. All cell types but U-937 presented a large percentage ($> 50\%$) of positivity using

IFA. Only the P2 isolate showed discreet CPE in H9 cells around the 10th day of infection. The P3 isolate was the least infectious exhibiting the lowest culture supernatant levels of HIV antigen (Table II). Fluorescence less than 50% was observed only in H9 cell line and PBMC by the 20th day. No CPE could be observed.

TABLE I

Clinical and epidemiological data of Brazilian HIV-1 isolates

Viral isolate	Clinical stage ^a	Geographical isolation	Year of isolation	Risk factor
P1	IV B	Rio de Janeiro	1987	Polytransfused
P2	IV A	Bahia	1988	Polytransfused
P3	IV D	São Paulo	1988	Homosexual
P4	IV A	Bahia	1988	Homosexual
P5	III	Rio de Janeiro	1988	Homosexual

a: CDC classification system, 1987.

TABLE II

Biological characteristics of Brazilian HIV-1 isolates

Viral isolates	Replication levels ^a				CPE ^b formation
	H9	CEM	U937	PBMC	
P1	++	++	+	++	++
P2	++	++	-	++	+
P3	+	-	-	++	-
P4	++	++	-	++	-
P5	++	++	-	++	-

a: replication levels: (-) = optical density (OD) < cut-off value in HIV antigen capture EIA; (+) = cut-off value < O.D < 1,5; (++) = O.D > 1,5.

b: CPE: cytopathic effect.

In general, PBMC was the most easily infected cell in accordance to Evans et al. (1987). Whereas, the U-937 line was refractory to all but the P1 isolate (Table II).

Restriction enzyme analysis – For the restriction enzyme analysis the DNAs were prepared from infected PHA-stimulated PBMC, since this cell type was susceptible almost equally for all isolates. The analysis of proviral DNA corresponding to samples P1, P2, P4 and P5 showed only one major viral type in each case. No hybridization could be detected for P3 isolate.

Comparisons of proviral DNAs, restricted by Hind III, Bgl II and Sac I endonucleases,

showed a polymorphism among the isolates (Fig. 1). Similar Bgl II patterns could be verified among P2, P4 and P5 isolates, with two restriction sites on the 5'LTR-POL region. P1, however, presented only one site with this enzyme (Fig. 1a). P1 and P2 isolates showed a common 9.0 Kb Sac I restriction fragment; whereas, an internal Sac I site localized 6.0 Kb from the 5'extremity was identified for the P4 and P5 isolates.

Polymorphic Hind III restriction fragments were identified along the GAG-ENV regions of P1 (3.1 Kb), P2 and P5 (6.4 Kb) and P4 (9.7 Kb) (Fig. 1b). The two conserved Hind III fragments of 0.5 Kb and 0.6 Kb, localized at the 5'LTR-GAG region, were observed in all isolates (data not shown).

Uninfected PBMC and CEM cells, used to grow the isolates, did not hybridize with the fragments used as probe. Also, PBMC infected with the HIV-2rod strain did not show any hybridization indicating the specificity of the probes (Fig. 1c).

The most important differences were observed between P1 and all of the other isolates analyzed, with limited conservation of some restriction sites. Based on the biological differences described above, we chose the P5 isolate and the most virulent HIV-1 isolate (P1), to be partially mapped by using restriction fragment length maps for HIV-1 prototype HTLV-IIIβ/LAV (Muesing et al., 1985; Alizon et al., 1985). We could not recover enough DNA containing integrated proviral genome of P3 isolate suggesting differences of integration capacity of this virus isolate.

The partial restriction map showed in Fig. 2, confirms the presence of polymorphic sites between the two Brazilian isolates and also between HTLV-IIIβ/LAV and our isolates. With the exception of the Sac I site, approximately 6,8 Kb from the 5'LTR region, isolate P5 showed high homology level with HTLV-IIIβ/LAV isolate for the region analyzed. The P1 isolate showed polymorphic sites in relation to Pst I, Bgl II, Hind III and Eco RI restriction enzymes.

DISCUSSION

The results of this study suggest that HIV circulating in Brazil are heterogeneous with respect to cytopathogenicity, replication rate and restriction fragment pattern.

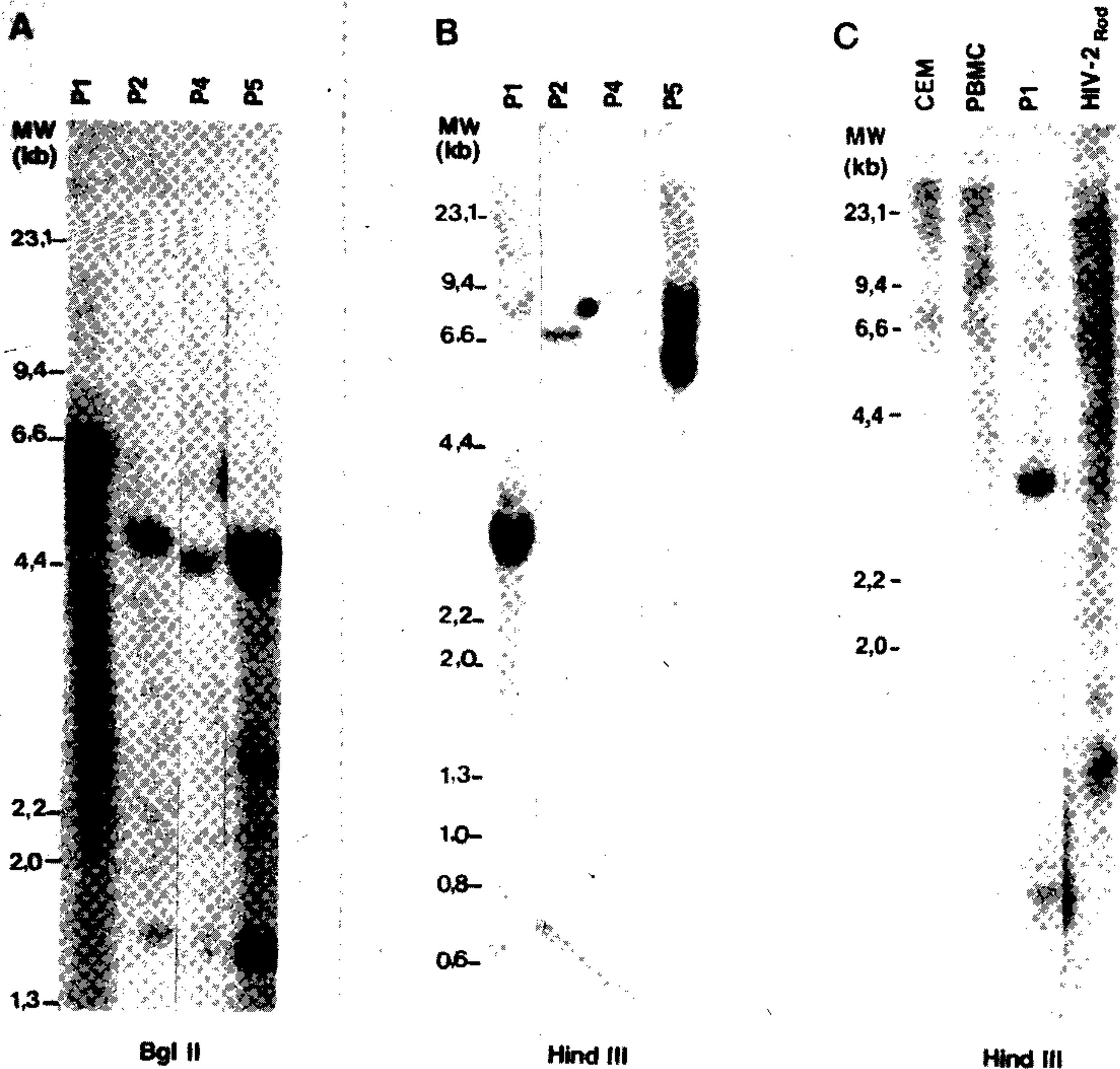


Fig. 1: representative restriction endonuclease fragment patterns of HIV-1 proviral DNA from PBMC infected with Brazilian isolates digested with Bgl II (A) and Hind III (B). The specificity of the probe was analyzed using uninfected CEM cell line and PBMC and HIV-2rod infected CEM (C).

Our present results disagree with those described previously concerning the degree of CPE (Asjo et al., 1986). We observed a lack of this phenomenon with the majority of our isolates, which were obtained from AIDS patients. However, we should take into account that this lack of viral pathogenicity could be due to the fact that we maintained our isolates in several H9 replicative cycles, which could select subpopulations of virus with altered virulence (Meyerhans et al., 1989). According to the replication/virulence classification system of Fenyo et al. (1988), our P1 isolate had the characteristics of the described "rapid/high" viruses. P2, P3, P4 and P5, on the other hand,

exhibited replication rates and virulence compatible with a "slow/low" classification.

We also observed that the promonocytoid cell line U937 was refractory to infection by most of our isolates. We succeeded only in infecting this cell line with the viral isolate from the patient with neurological symptoms. This fact could be explained by easier infectability of phagocytic cells by HIV-related neurotropic strains (Levy et al., 1985; Meltzer et al., 1990).

In studying only five isolates no conclusion can be drawn regarding the correlation

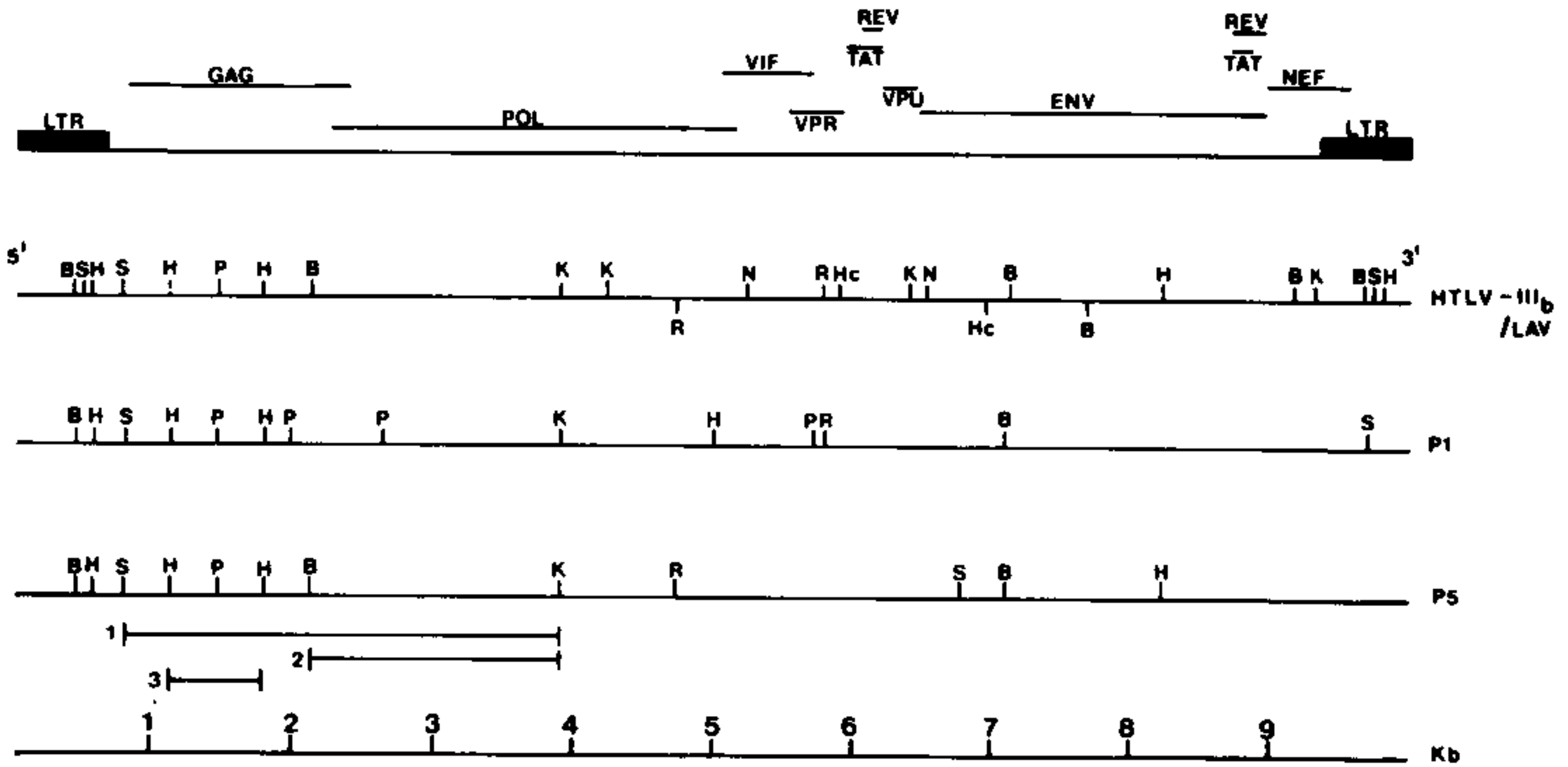


Fig. 2: comparison of the partial restriction endonuclease maps of proviral DNA from PBMC infected with Brazilian isolates with published restriction maps of HTLV-III_B/LAV isolates (Muesing et al., 1985; Wain-Robson et al., 1985). The probes used are indicated above the scale. B, Bgl II; S, Sac I; H, Hind III; P, Pst I; K, Kpn I; R, Eco RI; Hc, Hinc II, N, Nde. The last two enzymes were not used to map our isolates.

between clinical status or geographical distribution and biological or molecular characteristics. However, a limited biological and molecular polymorphism was observed between the isolates obtained from the P5 patient (a homosexual man from Rio de Janeiro) and isolates P2 and P4 (respectively obtained from a blood transfused woman and a homosexual man both from Salvador city, 1,700 km from Rio de Janeiro). However, an extensive polymorphism was observed among P1 isolate (obtained from a transfused man residing in Rio de Janeiro) and the other isolates, each of them corresponding to different clinical forms (Table I). This suggests no correlation between geographic distribution and risk group or clinical status, as already described (Benn et al., 1985).

Recently, it was suggested that the restriction pattern of Bgl II was quite distinct between asymptomatic individuals and AIDS patients (Balachandran et al., 1991). Even considering our small sample size, both patterns could be verified within our isolates, which were obtained from symptomatic patients. Three of them (P2, P4 and P5), presented the Bgl II site approximately 2.0 Kb from the 5'LTR region, as described for asymptomatic patients (Balachandran et al., 1991). Moreover, this site seems to be very conserved, as it is verified on the majority of HIV-1 isolates already described in the USA, Europe and Africa (Muesing et al., 1985; Benn

et al., 1985; Alizon et al., 1986). In the P1 sample, isolated from a patient with neurological disorders, this site was absent. This isolate also showed polymorphism in Hind III and Pst I sites. Based on the comparative analysis of the respective Pst I site positions with the nucleotide sequences published for the HTLV-III_B/LAV isolate (Muesing et al., 1985; Alizon et al., 1986), these differences could correspond to point mutations. Moreover, the presence of 3-4 polymorphic Pst I sites was also verified in some American (Saag et al., 1988) and Zairian (LAV_{ELI} and LAV_{MAL}) HIV isolates (Alizon et al., 1986), although not exactly on the same positions as those verified for P1 isolate.

Indeed, these African isolates also showed extensive polymorphism in the region corresponding to the HIV-1 principal neutralizing determinant (PND) on the gp 120 antigen (La Rosa et al., 1990). In this region, which is very important to antibody recognition, even the HTLV-III_B and LAV-1_{BRU} isolates, widely used as reference strains, showed rare PND sequences when compared to the consensus sequence described by La Rosa et al. (1990).

In conclusion, although we did not analyze a large number of samples and did not perform the genomic sequences in our isolates, we could observe a clear heterogeneity in HIV-1 Brazilian samples. Further molecular clon-

ing and sequencing of envelope of HIV circulating in Brazil will be necessary to assess the viability of future immunoprophylaxis programmes, based on the external glycoproteins fragments which display the PND. In addition, this information could be useful for a future subtyping of HIV, due to the marked genomic differences of the viruses identified.

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