Molecular characterisation of newly identified HIV-1 infections in Curitiba, Brazil: preponderance of clade C among males with recent infections

João Leandro de Paula Ferreira, Mariana Thomaz[1], Rosangela Rodrigues, David Harrad[1], Cristina Mendes Oliveira, Carmem Aparecida de Freitas Oliveira[2], João Paulo Gervasio Batista, Tomoko Sezazake Ito[3], Luis Fernando de Macedo Brigido/[4]/+

Laboratório de Retrovírus [2]Laboratório de Sorologia, Instituto Adolfo Lutz, Av. Dr. Arnaldo 355, 01246-902 São Paulo, SP, Brasil [1]Coordenação Municipal de DST/Aids de Curitiba, PR, Brasil [3]Laboratório Central do Município de Curitiba, Curitiba, PR, Brasil [4]The Research Capacity Program

As in many areas of Brazil, the AIDS epidemic in Curitiba is relatively stable, but surveillance is important to support public policy. The molecular characteristics of HIV may be instrumental for monitoring epidemic trends. We evaluated plasma HIV-1 RNA (n = 37) from 38 cases presenting with positive serology, who were among 820 consenting volunteers visiting the downtown counselling and serology testing centre. Seroprevalence was 4.6% (CI 95% 3.2-6.3) and the estimated HIV incidence, as defined by the BED assay, was 2.86 persons/years (CI 95% 1.04-4.68). An additional set of contemporaneous, anonymous samples from a local laboratory was also analysed (n = 20). Regions of the HIV-1 polymerase (n = 57) and envelope (n = 34) were evaluated for subtyping, determination of mosaic structure, primary drug resistance mutations (pDRM), envelope V3 loop motifs and amino acid signatures related to viral tropism. HIV-1 clade B was observed in 53% of cases; HIV-1C in 30% and BC mosaics in 14%, with one F genome and one CF mosaic. Clade C infection was associated with recent infections among males ($p < 0.03$). Stanford surveillance pDRM was observed in 8.8% of sequences, with 7% showing high level resistance to at least one antiretroviral drug. Tropism for CXCR4 co-receptor was predicted in 18% of envelope sequences, which were exclusively among clade B genomes and cases with serological reactivity to chronic infection.

Key words: epidemiology - antiretroviral resistance - genetic diversity - HIV-1 - tropism - Brazil

Understanding the local HIV-1 epidemic will not only support the regional response, but it may also provide information to nationwide efforts in the fight against AIDS. The variability of HIV-1 has been an obstacle to treatment and to the development of prevention innovations such as vaccines. HIV-1 clusters phylogenetically into distinct clades (subtypes), a fact that has helped to unravel the molecular characteristics of the epidemic. In some regions, differential distribution of HIV-1 clades has been documented (Van Harmelen et al. 1997, Tovanabutra 2001, 2004, Rios et al. 2005, Ryan et al. 2007) and phylogeny may indicate the source(s) of incoming variants (Sarker 2008). Worldwide, clade C is responsible for about half of the epidemic, whereas clade B is the major variant in industrialised nations (Hemelaar et al. 2006). HIV variability may influence the performance of laboratory tools for diagnostic, monitoring and surveillance (Koch et al. 2001). Differential pathogenesis (Baeten et al. 2007, Kaleebu 2007) and transmission potential (Yang et al. 2003, John-Stewart et al. 2005) have been associated to distinct HIV clades, but the actual impact of genetic diversity in HIV disease still remains uncertain (Stebbing & Moyle 2003, Hemelaar et al. 2006, Tebit et al. 2007). However, some characteristics of HIV, such as tropism to either CCR5 or CXCR4 co-receptors, seem to be associated with a distinct pattern of disease progression (Shepherd et al. 2008). Another relevant issue is the identification of primary drug resistance mutations (pDRM) among untreated individuals that may impact future therapeutic options and monitoring of pDRM is recommended by the WHO (http://www.who.int/drugresistance/hivaids/network/en/index.html). Brazil has many studies on HIV-1 subtypes and primary drug resistance from different areas in the country, and most of the recent ones suggest low rates of primary drug resistance, varying from 1.4-8.3% (Brindeiro et al. 2003, Barreto et al. 2006, De Medeiros et al. 2006, Rodrigues et al. 2006, Brigido et al. 2007, Sá-Ferreira et al. 2007, Eyer-Silva et al. 2008). A study performed in Santos has reported the highest drug resistance rates in the country, with 37% among recent infections and 25% among chronic cases (Sucupira et al. 2007). The predominant clade in Brazil is HIV-1 B, except in some areas in the south of the country, where clade C predominates (Bongertz et al. 2000, Brindeiro et al. 2003, Gadelha et al. 2003, Cerqueira et al. 2004, Brigido et al. 2005, 2007, Barreto et al. 2006, Rodrigues et al. 2006, Stefani et al.

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+ Corresponding author: lbrigido@ial.sp.gov.br
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2007). Moreover, different recombinant forms have been described in Brazil, including a BC mosaic, CRF31_BC (Santos et al. 2006). Only one of these studies includes data from Curitiba (Brindeiro et al. 2003). With samples collected in 2001, this study showed 64% of cases with clade B at the polymerase (*pol*) region, 30% with clade C, 4% with clade F and 2% mosaics, with pDRM rates of 2.7% (4 out of 147 sequences).

The use of the BED assay to estimate HIV incidence provides further insights into the understanding of HIV epidemic dynamics (Hall et al. 2008). Along with a relatively well organised public health infrastructure, this epidemiological setting may represent an interesting environment to monitor the potential of clade C expansion in the epidemic and to study the impact of clades B and C on treatment and disease outcome. This study was part of a National Program for Research Capacitation, an effort to identify potential new sites for AIDS related research. The primary objective of this study was to provide information on primary drug resistance and other molecular characteristics of HIV among volunteers from the downtown Curitiba Voluntary Counseling Testing (VCT) site and to correlate these data to the duration of infection (chronic versus recent) as predicted by the BED assay.

SUBJECTS, MATERIALS AND METHODS

Study population - The study was randomly offered to users of the Curitiba downtown VCT from January 2005-February 2006. Socio-behavioural data were obtained by a standard questionnaire from an individual interview with 820 informed and consenting volunteers.

HIV serological testing - HIV diagnosis was performed according to Brazilian guidelines. Briefly, enzyme immune assay (EIA) reactive samples were analysed by western blotting (WB) or IFA, with positive samples further confirmed by a subsequent blood collection. EIA non-reactive samples were considered HIV-negative (neg). HIV-1 incidence was estimated by further testing of the first biological sample from HIV-positive subjects using HIV-1 BED Incidence EIA (Calypte, USA) according to manufacturer instructions. Samples with initial reactivity suggesting recent infection pattern (ODn < 1.0) were re-tested in triplicate and all of them yielding an ODn of 0.8 were considered as representing recent infections (inc) [assuming a seroconversion duration of 155 days as the window period (w)]. Incidence estimate (I) and a 95% confidence interval (CI) were calculated as suggested by CDC revised formulas {I = [(365/w) x Ninc/(Nneg + (365/w) x Ninc/2)] x 100}; (95% CI = I +/- 1.96 x I/√Ninc) with adjustments for missing specimens. Cases with reactivity of recent infection, but with evidence of chronic infection (e.g., low TCD4 values), were excluded for incidence estimates.

Molecular analyses - HIV-1 protease (PR), partial reverse transcriptase (RT) and partial envelope (*env*) regions, approximately 700 and 600 kb, respectively, were sequenced by nested PCR followed by Dye Terminator and resolved by an ABI 3100 Genetic Analyzer (Applied Biosystems, USA) as previously described (Stuyver et al. 1997, WHO-UNAIDS 2002, Rodrigues et al. 2005). All sequences obtained were assembled with Sequencher 4.7 software and manually edited to identify mixtures. Automatic assembly (http://www.ial.sp.gov.br/cgi-bin/HIV/submissao) was used for subtyping analysis. HIV-1 clade was screened by BLAST (NCBI) and the REGA HIV-1 Subtyping Tool and further characterised by phylogeny. Multiple alignments were performed using ClustalW (Thompson et al. 1994), with a reference set from the Los Alamos HIV-1 database (www.hiv.lanl.gov) and Neighbour Joining (NJ) and maximum likelihood trees were constructed based in nucleotide substitution models determined by Modeltest v3.7 with Phylogenetic Analysis Using Parsimony (PAUP) v4b10 (Swofford 1999). Sequences showing discordant PR/RT subtyping, outlier behaviours on NJ trees or with ambiguous subtyping at Web resources (NCBI or Rega) were selected for recombination analysis using SIMPLOT 2.5 (Salminen et al. 1995). Statistical support was evaluated using Bootstrap (1000 replicas) and the likelihood ratio test, both performed with PAUP. Mutation profile and predicted ARV susceptibility were analysed by comparison to the Stanford HIV Drug Resistance Database (hivdb.stanford.edu). The analysis was performed both individually and using batch submission to determine primary drug resistance surveillance using the Calibrated Population Resistance tool, with both 2008 Stanford Surveillance Drug Resistance Mutations (SDRM) and IAS-USA major mutation list. Viral tropism was predicted based on visual inspection of amino acid alignments, considering basic amino acids at positions 11 and/or 25 of the V3 loop as predictive of an X4 phenotype (CXCR4-tropic HIV-1) and by submission to the Max Planck Institute site (http://coreceptor.bioinf.mpi-inf.mpg.de/index.php), using both a 2.5% and 20% false positive rate (1-specificity) for prediction of an X4 phenotype. Discordant results or dual tropic predictions were considered as an X4 phenotype.

Statistical analyses - Yates corrected or Fisher test, as appropriate, was used for categorical variables, Chi square was used for comparing proportions and analysis of variance was used for continuous variables, assuming a two tailed test with a level of significance of $p < 0.05$, with Epi Info™ (CDC, USA).

Ethics - The VCT volunteers study was approved by the National Ethical Committee (CONEP #9553), Research Ethical Committee of Instituto Adolfo Lutz and Research Ethical Committee of Curitiba. Written informed consent was obtained at the time of HIV testing visit. The anonymous unlinked component was approved by Instituto Adolfo Lutz Ethical Committee CEPIAL 28/06 and Research Ethical Committee of Curitiba.

RESULTS

HIV infection - HIV-1 seroprevalence was 4.6% (CI95% 3.2-6.3). Out of the 38 confirmed HIV seropositive cases (EIA and WB assays), 32 were available for further HIV testing with the BED assay, with 8/32 (25%) reacting as recent. One case had a TCD4 below 200 cells/mm^3, albeit with a recent infection reaction pattern at BED, was not included as "recent case" for incidence calculation. Estimated HIV incidence was 2.86 persons/

years (CI95% 1.04-4.68). Socio-behavioural data are summarised in Table I.

HIV subtyping - *Pol* and *env* regions of the HIV genome were partially sequenced from HIV infected VCT volunteers and the *pol* region was sequenced from an additional set of anonymous HIV positive samples from the city. Nucleotide sequence data reported in this paper are available in the GenBank database under the accession numbers EU340706 to EU340788. The clade B genome was observed in 52.6% of cases, followed by the clade C in 29.8%. Mosaics BC were observed in 14%, with one CF mosaic (1.8%) and one F genome (1.8%). Overall 15.8% of sequences available had evidence of recombination, more frequently at *pol*, 12.3%, with 2.9% at *env (*p = 0.25) and one case of *pol/env* clade discordance. The additional *pol* sequences were obtained from 20 anonymous HIV positive serology cases from Curitiba, including two cases of suspected recent infection and two young pregnant women. All were ARV naïve and submitted to the same study protocol.

Table II shows demographic and laboratory data by HIV-1 clade. Fig. 1 shows a representative phylogenetic tree of concatenated *pol* and *env* sequences. Most mosaic breakpoint patterns are at RT, but a typical CRF31_BC pattern was not observed. Pattern 6 (BR6PR40) and pattern 7 (BR06PR50 and BR06PR42), the latter with a discordant (clade B) *env* region, have breakpoints at RT at similar regions as the CRF31 pattern, but with a somewhat shorter B segment at RT. This pattern was observed at RT in three samples from another city in south Brazil (Brigido et al. 2007). Pattern 2 was also observed in two sequences (BR06PR52 and BR05PR431). Recombinant patterns are shown in Fig. 2.

V3 loop and viral tropism - *Env* sequences allowed the evaluation of the V3 loop signature and prediction of viral tropism (Fig. 3). All clade C sequences and the BC mosaic showed a GPGQ motif. Among clade B samples, GPGR predominates and 26% (6/23) bear either the motif GWGR or GPGQ. The former has been observed in Brazilian B sequences, but the latter is typical for some non-B clades, such as HIV-1 C. The proportion of GPGQ among our clade B dataset is significantly higher than that of the Los Alamos B clade reference set ($p < 0.04$). Using a composite of the Max Planck geno2pheno bioinformatics tool and sequence inspections for basic amino acids at key positions, all genomes with clade C at *env* had a predicted R5 tropism, whereas 26% of clade B had a predicted X4 tropism. Table III shows envelope molecular information along with available clinical information obtained from each case that entered a follow up at the municipal unit after HIV diagnosis in the study.

HIV genotype and BED reactivity - Mosaic genomes showed mostly chronic infection reactivity as shown by the BED assay, with one out of eight samples (13%) reacting as a recent infection, which was similar to the proportion of clade B infected cases (18%, 3 out of 17) (Table II). Overall, 63% of clade C infected cases had a recent reactivity pattern as shown by BED. Among males, 71% (5/7) of clade C infection sera showed a recent reactivity pattern as shown by BED, with three out of 16 clade B HIV infected males reacting as a recent infection ($p < 0.03$). Among cases with serological reactivity of recent infection, all had a predicted R5 tropism; among those with reactivity of chronic infection, 14% had a predicted X4 tropism (Table III).

Genotypic resistance - SDRM-2008, as defined by the HIV Drug Resistance Database, was observed in 8.8% (CI 95% 3-18) of sequences, being 7% to non-nucleoside reverse transcriptase inhibitors (NNRTI) and 1.8% to nucleoside RT inhibitors (NRTI) drug classes. Using the IAS list, which includes some additional mutations, the proportion of genomes with DRMs is 10.5%. The most prevalent antiretroviral (ARV) primary resistance mutation was K103N, observed in three cases. Accessory

TABLE I

Socio-behavioral characteristics of VCT volunteers[a] from Curitiba. Data was obtained during consenting individual interview at recruitment.

Median age (25th-75th)	31 (23-37)
Males	63.4%
Monthly family income over US$ 450	29%
Over eight years do study	81%
Unemployed	10.3%
MSM	29%
IDU (ever)	1.8%
Wiliness to participate in further HIV related research	99%
Seropositive at study evaluation	4.6%
Total	820

a: 38 cases (4.6%) were found seropositive at entry and were further evaluated for serological reactivity and HIV-1 molecular characteristics (Tables II-IV). IDU: ever had used intravenous recreational drug; MSM: men referring sex with men.

TABLE II

Study population characteristics by HIV clade. Characteristics of HIV infected volunteers at entering the study by viral clades at *pol* and *env*.

	Clade B	Clade C	Cb mosaics
Male gender (%)	83	71	75
Median age[a]	29 (25-35)	37 (25-39)	38 (27-43)
MSM (%)	65	43	67
Viral load[b]	4.06 (0.98)	4.22 (1.25)	3.80 (1.69)
TCD4[b]	570 (423)	563 (280)	486 (198)
Recent infection (%)	18	63	13
N	30	17	8

a: median age (25th, 75th percentiles); *b*: viral load (log_{10}) and TCD4 (cells mm^3) at first determination during clinical follow up (mean and standard deviation) obtained after initiating follow up at municipal clinic; MSM: males referring sex with another man; N: absolute number of individuals in each subtype category (2 cases, 1 F and 1 CF mosaic, are not included in the table).

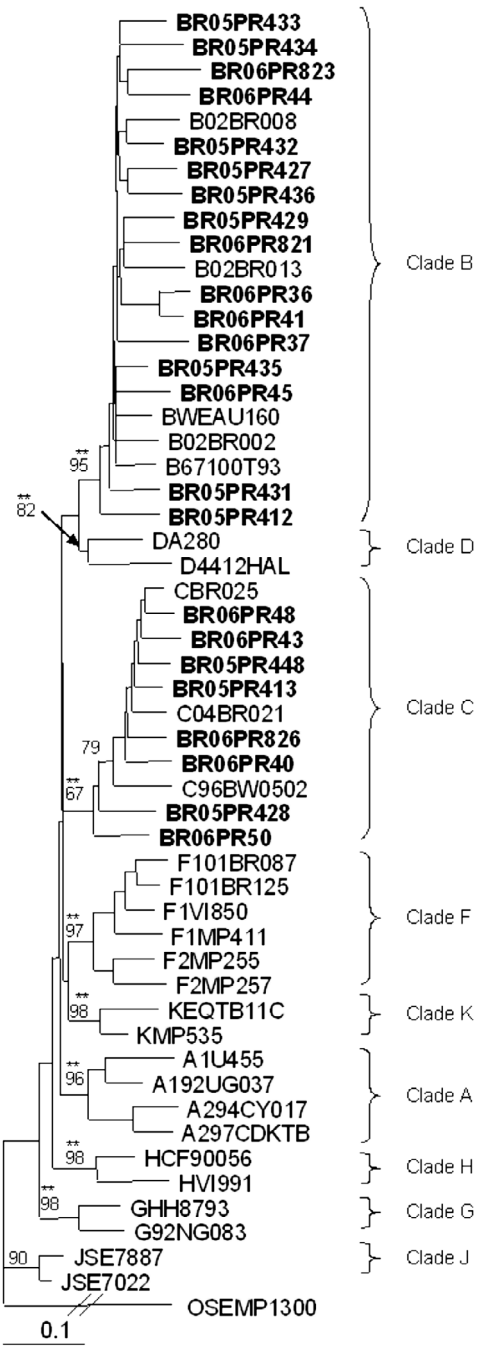

Fig. 1: depicts Neighbor Joining phylogenetic tree of representative concatenated HIV *pol* and *env* partial genes from HIV infected volunteers generated at PAUP 4.0 software using evolutionary model selected at Modeltest (TVM+I+G). Reference HIV-1 subtype were obtained from Los Alamos HIV Sequence database. Tree was rooted with a group O sequence. Bootstrap values above 70% in key branches are depicted. Additional statistical support was evaluated using the likelihood ratio test (PAUP) marked as * for $p < 0.005$ and ** for $p < 0.001$. Most mosaic patterns included in the tree fall outside HIV clade B (BR05PR412) or HIV clade C (BR06PR50, BR05PR428) clusters but some mosaics (BR05PR431 , BR06PR40) fall within clade B or C cluster, respectively, possibly due to the small discordant segment on the genome available for analysis. The ID sequences are named as BR for Brazil, followed by 05 or 06 for year of sample collection, PR for Paraná, state where Curitiba is located, followed by the Laboratory ID number.

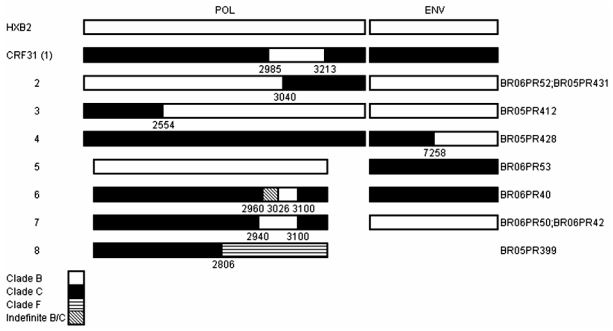

Fig. 2: depicts breakpoint pattern of mosaic structure along with reference genomes (HXB2, CRF31). Breakpoints estimate position as numbered according to HXB2; pattern 1(CRF-31) to 4 (2243 to 3569 *pol*); pattern 5-8 (2277 to 3218 *pol*) and all *env* (pattern 1 to 7) spanning nucleotides 7002-7597 of HXB2 envelope. Pattern 6 breakpoint (2960-3026) could not be clearly delimitated and the area of transition is marked as indefinite B/C region. It is similar to pattern 7, observed in two samples, with a discordant *env* genome. These RT breakpoints are similar to that observed in samples from another city in the south Brazil (Brígido 2007).

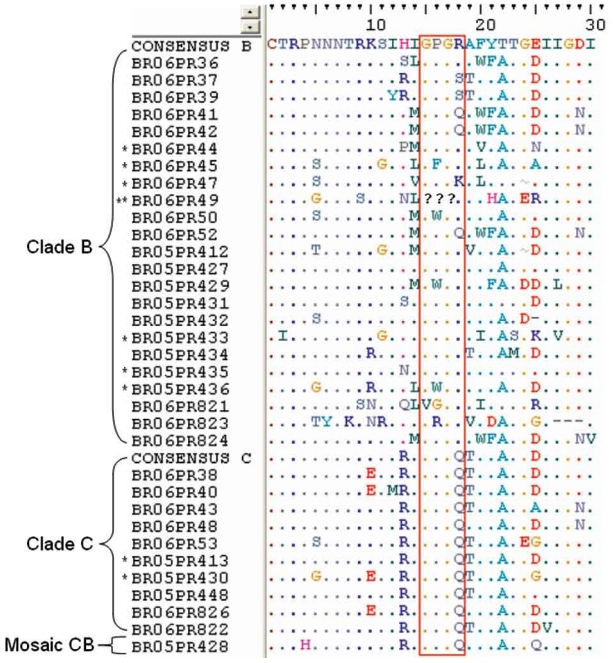

Fig. 3: depicts amino acids alignment of V3 region (7110 -7203, position related to HXB2) from study population along with HIV-1 clades B and C consensus (NCBI). Amino acids at positions 11 and 25 were used to build the prediction of viral tropism (R5 or X4). V3 loop crown amino acids have been highlighted (box). *: sequences with non synonymous mixtures; the figure depicts either GWGR or GPGR whenever they are one of the variants; **: the sequence BR06PR49 showed more than two possible variants (none GPGR or GWGR) at manual edition; A or G at position one, I,T or S at position two, R or G at position three and R at position four of the V3 crown.

mutations and polymorphisms not included in the surveillance system were also observed and, overall, 14% of cases showed some degree of loss of susceptibility to at least one ARV, with 7% showing high level resistance to

TABLE III

Socio-demographic and laboratory data of HIV infected volunteers

ID	BED	Sex	Age	Sex orient	Clade *env*	*env* V3	Tropism	VL Baseline	CD4 Baseline	ARV	VL Last	CD4 Last
3606	R	M	19	MSM	B	GPGR	R5	2,63	532			
3706	L	M	25	MSM	B	GPGS	R5		263	Yes		
3806	L	F	25	Hetero	C	GPGQ	R5					
3906	L	M	33	MSM	B	GPGS	R5	4,95	386	Yes	4,1	138
4006	L	F	43	Hetero	C	GPGQ	R5	5,57	683		4,99	737
4106	L	M	32	MSM	B	GPGQ	R5					
4206	L	F	22	Hetero	B	GPGQ	R5					
4306	R	M	25	MSM	C	GPGQ	R5	3,95	801			
4406	L	M	26	Hetero	B	GPGR	R5					
4506	L	F	26	Hetero	B	GFGR	X4			Yes		
4606	L	M	22	MSM	ND	ND	ND					
4706	L	M	24	Hetero	B	GPGR[b]	R5					
4806	R	M	39	Hetero	C	GPGQ	R5					
4906	L	M	26	MSM	B	[c]	X4					
5006	L	M	42	Hetero	B	GWGR	R5					
5106	R	M	42	Bi	ND	ND	ND					
5206	L	M	26	MSM	B	GPGQ	R5					
5306	L	M	38	Hetero	C	GPGQ	R5	4,65	630			
41205	L	M	52	Bi	B	GPGR	R5					
41305	R	M	53	Hetero	C	GPGQ	R5	4,88	349			417
42705	L	M	29	Bi	B	GPGR	R5					
42805	R	M	37	MSM	CB	GPGQ	R5	5,17	300	Yes	< 400	355
42905	R	M	36	Hetero	B	GWGR	R5	3,77	927		3,13	1170
43005	L	M	39	Hetero	C	GPGQ	R5	5,43	170	Yes	< 400	214
43105	L	M	27	MSM	B	GPGR	R5	4,15	332			268
43205	L	M	70	Hetero	B	GPGR	R5	4,1	1211		4,89	885
43305	R/L[a]	M	44	Hetero	B	GPGR	X4	5,20	10	Yes	< 400	72
43405	L	M	29	MSM	B	GPGR	R5	3,74	1147		3,84	1009
43505	L	M	24	MSM	B	GPGR	X4	5,18	299	Yes	5,42	242
43605	L	M	20	Bi	B	GWGR	R5	4,18	355		4,42	556
43705	L	M	30	Hetero	ND	ND	ND					
44805	R	M	29	MSM	C	GPGQ	R5	5,5	472		5,4	444
82106	N.D	M	28	Hetero	B	VGGR	X4					
82206	N.D	M	28	MSM	C	GPGQ	R5					
82306	N.D	M	41	Hetero	B	GRGR	X4					
82406	N.D	M	27	MSM	B	GPGR	R5					
82606	N.D	M	47	Hetero	C	GPGQ	R5					

HIV-1 clade at envelope, V3 loop crown motif and predicted tropism as R5 for CCR5 tropic or X4 for CXCR4 or dual tropic viruses. TCD4 (CD4) (cells mm^3) and Viral Load (VL) (log_{10}) and information on whether treatment was introduced after collection was obtained at the first and last determinations during the subsequent clinical follow up. Some patients were either not followed at a Municipal Health Care Unit or had not initiated follow up to the last observation about one year after interview. *a*: patient 43305 albeit having a BED assay reactivity of recent infection was considered chronic case due to low TCD4: *b*: mixture with GPGK; *c:* more than one possible amino acid, mixture, at position 1 (A or G), 2 (I,T or S), 3 (R or G) and R at position 4 of V3 loop crown. L and R: long term infection reactivity (chronic) and recent infection reactivity, respectively, according to BED assay performed with the first available serum sample; Bi: bisexual; Hetero: heterosexual; MSM: men who have sex with men.

at least one ARV drug. Table IV depicts the HIV clade at the *pol* gene, the observed ARV resistance-related mutations and the predicted susceptibility profile.

DISCUSSION

This paper describes the molecular characteristics of HIV-1 genomic RNA isolated from newly identified infected subjects at a VCT serological screening, along with an additional set of contemporaneous HIV-1 positive serum samples from the same city. Moreover, retesting HIV reactive samples with the BED assay allowed the identification of recently infected cases.

The prevalence of pDRM was similar to most of the studies in the country (Brindeiro et al. 2003, Barreto et al. 2006, Rodrigues et al. 2006, Brigido et al. 2007, Eyer-Silva et al. 2008), but some studies showed higher rates (Pedroso et al. 2007, Sucupira et al. 2007). These studies varied in patient population selection, type of genetic

TABLE IV

Pol clades[a], drug resistance mutations[a] and predicted ARV susceptibility

ID	Clade *pol*	NRTI	NNRTI	PI	Drug susceptibility[b]
3606	B			L10V, A71V	
3706	B				
3806	C				
3906	B				
4006	CB[c]				
4106	B	T69S			
4206	CB[c]				
4306	C	V118I			
4406	B		V108I		PLLR (DLV; EFV; NVP)
4506	B			L10V, A71T	
4606	B	L210F		A71T	
4706	B				
4806	C				
4906	B				
5006	CB[c]				
5106	B	K70R, M184V, K219Q			HLR (3TC; FTC), LLR (AZT; D4T), PLLR (ABC)
5206	BC[c]		A71V		
5306	B[d]				
6304	C				
6404	C				
21102	B				
21202	B				
35505	B				
36005	C				
36605	B				
37105	B				
38105	C				
38205	C			L33I	
39905	CF[c]				
40005	B				
40105	B	A62V			
40305	C				
40705	B				
40905	F			L10V	
41105	C				
41205	CB[c]		K103N		HLR (DLV; EFV; NVP), PLLR (ETR)
41305	C				
41705	C				
42305	B		K103N		HLR (DLV; EFV; NVP), PLLR (ETR)
42405	C				
42705	B				
42805	C[d]				
42905	B				
43005	C				
43105	BC[c]		A71IV		
43205	B			A71T	
43305	B	L210M			
43405	B		E138K		PLLR (EFV), LLR (DLV; ETR; NVP)
43505	B				
43605	B				
43705	B				
44805	C			L10I	
82106	B		K103N	A71V	HLR (DLV; EFV; NVP), PLLR (ETR)
82206	C				
82306	B			F227Y	PLLR (NVP)
82406	B			A71V	
82606	C		V179D	T74S	PLLR (NFV), PLLR (DLV; EFV; ETR; NVP)

a: HIV-1 clade at pol region and drug resistance mutations (DRM) according to Stanford HIV Resistance Database by sample analyzed and listed according to antiretroviral class as analogous nucleosid(t)es (NRTI), non analogous nucleosides (NNRTI) or *Proteae* inhibitor (PI). Major and minor (accessory) DRMs are listed; *b*: predicted susceptibility is described by antiretroviral; *c*: mosaic genomes; *d*: note that in two cases, 42805 and 5306, although only one clade was assigned at *pol*, these samples show a discordant or mosaic genome at envelope; HLR: high level resistance; LLR: low level resistance; PLLR: potencial low level resistance.

material analysed and the list of mutations considered for surveillance purposes. The study with higher levels of resistance was based on proviral DNA (Sucupira et al. 2007), whereas most of the other studies were based on HIV-1 virion RNA. Few studies have compared plasma and cell mutation profiles, but cell/plasma discordance is not uncommon (Bon et al. 2007) and one recent study suggested an astonishingly high degree of heterogeneity among peripheral blood mononuclear cells (PBMCs) in regards to resistance (Quan et al. 2008). On the other hand, at least one study reporting a low primary resistance was also performed using the cell provirus (Eyer-Silva et al. 2008). The comparability of these studies must take this into consideration, but PBMCs may have the advantage of reflecting archival genomes, and, as it is less costly, it may be a useful tool to monitor resistance in a more limited setting.

Another factor that may impact these estimates is the use of samples obtained from VCTs without adequate questioning about ARV exposure. Although no studies have evaluated, how many people go to these testing sites to check if ARV treatment has provided a "cure", this possibility may have an important impact on pDRM estimates.

The list used to define a pDRM is also an issue in comparing different studies. In our study, we used the SDRM list that was organised to provide a simple, unambiguous and stable measure of transmitted HIV-1 drug resistance (Shafer et al. 2007). Moreover, the SDRM list provides an estimate of transmitted drug resistance in accordance with WHO guidelines.

It is noteworthy the predominance of mutations to NNRTI observed in this study, a finding reported by other groups in South America (Petroni et al. 2006) and elsewhere (Grant et al. 2002). Trends in primary drug resistance to NNRTI should be carefully monitored. The NNRTI class has a low resistance barrier (single mutations may cause a significant decrease in drug susceptibility) and NNRTI based highly active treatment combinations are a popular initial regimens in the country as well in the WHO treatment program. The presence of K103N, the most common DRM observed, may be partially explained by the low impact on the viral fitness of this mutation, a fact that favours its persistence (Cong et al. 2007). Another mutation associated with NNRTI, at codon P236, probably represents a natural polymorphism, unrelated to primary resistance secondary to transmission, as Delavirdine is rarely used in this area and Efavirenz and Nevirapine do not select for this mutation.

HIV-1 diversity in this city is distinct from that observed in the major metropolitan areas (São Paulo and Rio de Janeiro), where clade B predominates and, apart from clade F, other subtypes are rarely identified (Bongertz et al. 2000, Brigido et al. 2005, Barreto et al. 2006). The same is true in other regions of the country (Gadelha et al. 2003, Cerqueira et al. 2004, Stefani et al. 2007) and elsewhere in South America (Hemellar et al. 2004, Montano et al. 2005), with the exception of Argentina, where BF recombinants predominate. The local molecular epidemiology scenario is more comparable to other cities in south Brazil, where clade C predominates (Martinez et al. 2002, Soares et al. 2005, Rodrigues et al. 2006). Here, HIV-1 C is present in 30% of cases, and together with BC mosaics, it represents a significant proportion of HIV infections. A much higher prevalence of clade C was recently observed in Itajaí, about 250 kilometres from Curitiba (Brigido et al. 2007). The molecular epidemiology described here also contrasts with the mostly clade B epidemic in the nearby (about 400 km) São Paulo metropolitan area, where there is an intense cultural and economic interaction with Curitiba. These findings highlight the potential for compartmentalisation of HIV dynamics into sub-epidemics, even in related settings. Comprehensive studies are needed for a better understanding of the molecular epidemiology dynamics in the region, an effort that may provide pivotal information on the co-evolution of clades B and C.

When compared to a previous study at this VCT (Brindeiro et al. 2003), the proportion of mosaics significantly increased, from 2-15.8% ($p < 0.02$). Though both studies sequenced a similar region of *pol* from a population attending the same VCT, the studies were independent, which limits this tentative evaluation of molecular epidemiology trends.

The possibility of dual infections also cannot be ruled out. In a previous study, protease and transcriptase regions were amplified separately, favouring the eventual amplification of distinct species in cases of dual infection. Here, the first round generated a single amplicon, thus decreasing, but not eliminating this possibility. Our study also provides, for the first time, information on the *env* region. GPGR predominates among B sequences, and GWGR, a motif found in the Brazilian envelope, was observed in 13% of sequences. The relatively high proportion of GPGQ motifs among the clade B envelopes may be related to the co-circulation of clade B and clade C infections. Many BC mosaics were identified in this study, but the Brazilian CRF31_BC breakpoint pattern was not found. The small sample size does not allow us to rule out its presence in the area. Another aspect that deserves further evaluation is the correlation of recent infection, as defined by the BED assay, with the infection of males with clade C, compared to long term, chronic infections ($p < 0.026$). This methodology has been used to estimate HIV incidence in Brazil and abroad (Teixeira et al. 2004, Oliveira et al. 2005, Hall et al. 2008). However, our small data set and the recent documentation of discrepancies of the use of the assay with non-B isolates (hyperlink http://www.epidem.org/Publications/BED%20statement.pdf) limits the strength of these observations. Follow-up samples from the volunteers, collected after the maturation of the immune response, were analysed and they may provide support for the ascribed recent serological reactivity observed with these samples. The concurrent circulation of two important HIV-1 clades, good adherence and collaboration of VTS users in this study, and good local public health services make this unit an important asset to HIV-related research.

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REFERENCES

Baeten JM, Chohan B, Lavreys L, Chohan V, McClelland RS, Certain L, Mandaliya K, Jaoko W, Overbaugh J 2007. HIV-1 subtype D infection is associated with faster disease progression than subtype A in spite of similar plasma HIV-1 loads. *J Infect Dis 195*: 1177-1180.

Barreto CC, Nishyia A, Araújo LV, Ferreira JE, Busch MP, Sabino EC 2006. Trends in antiretroviral drug resistance and clade distributions among HIV-1-infected blood donors in São Paulo, Brazil. *J Acquir Immune Defic Syndr 41*: 338-341.

Bon I, Gibellini D, Borderi M, Alessandrini F, Vitone F, Schiavone P, Maria Carla Re 2007. Genotypic resistance in plasma and peripheral blood lymphocytes in a group of naive HIV-1 patients. *J Clin Virol 38*: 313-320.

Bongertz V, Bou-Habib DC, Brigido LF, Caseiro M, Chequer PJ, Couto-Fernandez JC, Ferreira PC, Galvão-Castro B, Greco D, Guimarães ML, Linhares de Carvalho MI, Morgado MG, Oliveira CA, Osmanov S, Ramos CA, Rossini M, Sabino E, Tanuri A, Ueda M 2000. 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 23*: 184-193.

Brigido LF, Franco HM, Custodio RM, Oliveira CA, P Ferreira JL, Eira M, Bergel F, Araújo F, Carvalheiro JR, Rodrigues R 2005. Molecular characteristics of HIV type 1 circulating in São Paulo, Brazil. *AIDS Res Hum Retroviruses 21*: 673-682.

Brigido LF, Nunes CC, Oliveira CM, Knoll R, Ferreira JLP, Oliveira CAF, Alves MA, Dias CF, Rodrigues R, Research Capacity Program 2007. HIV-1 Subtype C and Cb *Pol* Recombinants prevail at the cities with the highest Aids prevalence rate of Brazil. *AIDS Res Hum Retroviruses 23*: 1579-1585.

Brindeiro RM, Diaz RS, Sabino EC, Morgado MG, Pires IL, Brigido L, Dantas MC, Barreira D, Teixeira PR, Tanuri A 2003. Brazilian Network for HIV Drug Resistance Surveillance (HIV-BResNet): a survey of chronically infected individuals. *AIDS 17*: 1063-1069.

Cerqueira DM, Amorim RM, Silva RR, Camara GN, Brígido MM, Martins CR 2004. Antiretroviral resistance and genetic diversity of human immunodeficiency virus type 1 isolates from the Federal District, Central Brazil. *Mem Inst Oswaldo Cruz 99*: 877-882.

Cong M, Heneine W, J Gerardo García-Lerma 2007. The fitness cost of mutations associated with human immunodeficiency virus type 1 drug resistance is modulated by mutational interactions. *J Virol 6:* 3037-3041.

De Medeiros LB, Lacerda HR, Cavalcanti AM, de Albuquerque MF 2006. Primary resistance of human immunodeficiency virus type 1 in a reference center in Recife, Pernambuco, Brazil. *Mem Inst Oswaldo Cruz 101*: 845-849.

Eyer-Silva WA, Couto-Fernandez JC, Silva-de-Jesus C, Morgado MG 2008. Prevalence of HIV type 1 drug resistance mutations in treatment-naïve and experienced patients from resource-limited settings with universal access to antiretroviral therapy: a survey in two small Brazilian cities. *Mem Inst Oswaldo Cruz 103*: 143-149.

Gadelha SR, Shindo N, Cruz JN, Morgado MG, Galvão-Castro B 2003. Molecular epidemiology of human immunodeficiency virus-1 in the state of Ceara, Northeast Brazil. *Mem Inst Oswaldo Cruz 98*: 461-463.

Grant RM, Hecht FM, Warmerdam M, Liu L, Liegler T, Petropoulos CJ, Hellmann NS, Chesney M, Busch MP, Kahn JO 2002. Time trends in primary HIV-1 drug resistance among recently infected persons. *JAMA 288*: 181-188.

Hall HI, Song R, Rhodes P, Prejean J, An Q, Lee LM, Karon J, Brookmeyer R, Kaplan EH, McKenna MT, Janssen RS, Merriweather A, Mergenthaler H, Donnelly JA, Noga H, White S, Crippen D, Merritt M, Benbow N, Fields DK, Ramirez S, O'Connor M, Van Dyne M, Singh S, Cross H, Smith L, Bennani Y, Padgett PJ, Harris T, Obiri G, Brady KA, McCormick K, Shavor TJ, Jablonski CL, Chan S, Diallo N, Exarchos A, DeCausey B, Bodnar U, Glynn MK, Green T, Hernandez A, Kline R, Lin LS, Linley L, Walker F, Wheeler W, HIV Incidence Surveillance Group 2008. Estimation of HIV incidence in the United States. *JAMA 6*; *300*: 520-529.

Hemelaar J, Gouws E, Ghys PD, Osmanov S 2006. Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. *AIDS 20*: W13-23.

John-Stewart GC, Nduati RW, Rousseau CM, Mbori-Ngacha DA, Richardson BA, Rainwater S, Panteleeff DD, Overbaugh J 2005. Subtype C is associated with increased vaginal shedding of HIV-1. *J Infect Dis 192*: 492-496.

Kaleebu P, Nankya IL, Yirrell DL, Shafer LA, Kyosiimire-Lugemwa J, Lule DB, Morgan D, Beddows S, Weber J, Whitworth JA 2007. Relation between chemokine receptor use, disease stage and HIV-1 subtypes A and D: results from a rural Ugandan cohort. *J Acquir Immune Defic Syndr 1: 45*: 28-33.

Koch WH, Sullivan PS, Roberts C, Francis K, Downing R, Mastro TD, Nkengasong J, Hu D, Masciotra S, Schable C, Lal RB 2001. Evaluation of United States-licensed human immunodeficiency virus immunoassays for detection of group M viral variants. *J Clin Microbiol 39*: 1017-1020.

Martinez AMB, Barbosa EF, Ferreira PCP, Cardoso FA, Silveira J, Sassi G, da Silva CM, Mendonça-Signorini V, Antunes CM 2002. Molecular epidemiology of HIV-1 in Rio Grande, RS, Brazil. *Rev Soc Bras Med Trop 35*: 471-476.

Montano SM, Sanchez JL, Laguna-Torres A, Cuchi P, Avila MM, Weissenbacher M, Serra M, Viñoles J, Russi JC, Aguayo N, Galeano AH, Gianella A, Andrade R, Arredondo A, Ramirez E, Acosta ME, Alava A, Montoya O, Guevara A, Manrique H, Sanchez JL, Lama JR, de la Hoz F, Sanchez GI, Ayala C, Pacheco ME, Carrion G, Chauca G, Perez JJ, Negrete M, Russell KL, Bautista CT, Olson JG, Watts DM, Birx DL, Carr JK, the South American HIV Molecular Surveillance Working Group 2005. Prevalences, genotypes and risk factors for HIV transmission in South America. *J Acquir Immune Defic Syndr 40*: 57-64.

Oliveira CAF, Ueda M, Yamashiro R, Rodrigues R, Sheppard HW, de Macedo Brigido LF 2005. Rate and incidence estimates of recent human immunodeficiency virus type 1 infections among pregnant women in São Paulo, Brazil, from 1991 to 2002. *J Clin Microbiol 43*: 1439-1442.

Pedroso C, Queiroz ATL, Alcântara L, Drexler JF, Diaz RS, Wey N, Brites C 2007. High prevalence primary antiretroviral resistance among HIV-1-infected adults and children in Bahia, a northeast state of Brazil. *J Acquir Immune Defic Syndr 45*: 251-252.

Petroni A, Deluchi G, Pryluka D, Rotryng F, Bortolozzi R, Lopardo G, Bouzas MB, Zapiola I, Garone D, Rodríguez C, Chiocconi E, Lázaro ME, Murano F, Maranzana A, Oliva SM, Aparicio M, Beltrán M, Benetucci JA 2006. Update on primary HIV-1 resistance in Argentina: emergence of mutations conferring high-level resistance to nonnucleoside reverse transcriptase inhibitors in drug-naive patients. *J Acquir Immune Defic Syndr 42*: 506-510.

Quan Y, Brenner, BG, Dascal A, Wainberg MA 2008. Highly diversified multiply drug-resistant HIV-1 quasispecies in PBMCs. *Retrovirology 5*:43.

Rios M, Fernandez J, Jaramillo P, Paredes V, Sanchez JL, Laguna-Torres VA, Carr JK, Ramirez E 2005. Molecular epidemiology of HIV type 1 in Chile: differential geographic and transmission route distribution of B and F subtypes. *AIDS Res Hum Retroviruses 21*: 835-840.

Rodrigues R, Custodio RM, Bueno SM, Eira M, Ferreira JL, Jamal L, Duarte AJ, Brígido LF 2005. Prevalence of ARV resistance mutations and impact of genotyping test in HIV patients with advance disease in São Paulo, Brazil. *J Clin Virol 32*: 336-337.

Rodrigues R, Scherer LC, Oliveira CM, Franco HM, Sperhacke RD, Ferreira JL, Castro SM, Stella IM, Brigido LF 2006. 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 Research 16*: 201-207.

Ryan CE, Janet G, Crowe SM, Reeder JC, Oelrichs RB 2007. The heterosexual HIV type 1 epidemic in Papua, New Guinea is dominated by subtype C. *AIDS Res Hum Retroviruses 23*: 941-944.

Sá-Ferreira JA, Brindeiro PA, Chequer-Fernandez S, Tanuri A, Morgado MG 2007. Human immunodeficiency virus-1 subtypes and antiretroviral drug resistance profiles among drug-naïve Brazilian blood donors. *Transfusion 47*: 97-102.

Salminen MO, Carr JK, Burke DS, McCutchan FE 1995. Identification of breakpoints in intergenotypic recombinants of HIV type 1 by bootscanning. *AIDS Res Hum Retroviruses 11*: 1423-1425.

Santos AF, Sousa TM, Soares EA, Sanabani S, Martinez AM, Sprinz E, Silveira J, Sabino EC, Tanuri A, Soares MA 2006. Characterization of a new circulating recombinant form comprising HIV-1 subtypes C and B in Southern Brazil. *AIDS 20*: 2011-2019.

Sarker MS, Rahmana M, Yirrell D, Campbell E, Rahmane AS, Islamd LN, Azima T 2008. Molecular evidence for polyphyletic origin of human immunodeficiency virus type 1 subtype C in Bangladesh. *Virus Research 135*: 89-94.

Shafer RW, Rhee SY, Pillay D, Miller V, Sandstrom P, Schapiro JM, Kuritzkes DR, Bennett D 2007. HIV-1 protease and reverse transcriptase mutations for drug resistance surveillance. *AIDS 21*: 215-223.

Shepherd JC, Jacobson LP, Qiao W, Jamieson BD, Phair JP, Piazza P, Quinn TC, Margolick JB 2008. Emergence and persistence of CXCR4-Tropic HIV-1 in a population of men from the Multicenter AIDS Cohort Study. *J Infect Dis 198*: 1104-1112.

Soares EA, Martinez AM, Souza TM, Santos AF, Da Hora V, Silveira J, Bastos FI, Tanuri A, Soares MA 2005. HIV-1 subtype C dissemination in Southern Brazil. *AIDS 19* S: S81-86.

Stebbing J, Moyle G 2003. The clades of HIV: their origins and clinical significance. *AIDS Rev 5*: 205-213.

Stefani MM, Pereira GA, Lins JA, Alcantara KC, Silveira AA, Viegas AA, Maya NC, Mussi AH 2007. Molecular screening shows extensive HIV-1 genetic diversity in Central West Brazil. *J Clin Virol 39*: 205-209.

Stuyver L, Wyseur A, Rombout A, Louwagie J, Scarcez T, Verhofstede C, Rimland D, Schinazi RF, Rossau R 1997. Line probe assay for rapid detection of drug-selected mutations in the human immunodeficiency virus type 1 reverse transcriptase gene. *Antimicrob Agents Chemother 41*: 284-291.

Sucupira MCA, Caseiro MM, Alves K, Tescarollo G, Janini LM, Sabino EC, Castelo A, Shafer K, Diaz RS 2007. High levels of primary antiretroviral resistance genotypic mutations and B/F recombinants in Santos, Brazil. *Aids Patient Care STDS 21*: 116-128.

Swofford D 1999. PAUP 4.0: Phylogenetic analysis with parsimony (and other methods), version 4.0b2a. Sunderland, Mass: Sinauer Associates Inc.

Tebit DM , Nankya I, Arts EJ, Gao Y 2007. HIV diversity, recombination and disease progression: how does fitness "fit" into the puzzle? *AIDS Reviews 9*: 75-87.

Teixeira SL, Bastos FI, Telles PR, Hacker MA, Brigido LF, de F Oliveira CA, Bongertz V, Morgado MG 2004. HIV-1 infection among injection and ex-injection drug users from Rio de Janeiro, Brazil: prevalence, estimated incidence and genetic diversity. *J Clin Virol 31*: 221-226.

Thompson J, Higgins D, Gibson T 1994. Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight choice. *Nucleic Acid Res 22*: 4673-4680.

Tovanabutra S, Beyrer C, Sakkhachornphop S, Razak MH, Ramos GI, Yongchak T, Rungruengthanakit K, Saokhieo P, Tejafong K, Kim B, de Souza M, Robb MI, Birx DI, Jittiwutikarn J, Suriyanon V, Celentano DD, Mccutcha F 2004. The changing molecular epidemiology of HIV type 1 among Northern Thai drug users, 1999 to 2002. *Aids Res Hum Retroviruses 20*: 465-475.

Tovanabutra S, Polonis V, De Souza M, Trichavaroj R, Chanbancherd P, Kim B, Sanders-Buell E, Nitayaphan S, Brown A, Robb MR, Birx DL, McCutchan FE, Carr JK 2001. First CRF01_AE/B recombinant of HIV-1 is found in Thailand. *AIDS 15*: 1063-1065.

Van-Harmelen J, Wood R, Lambrick M, Rybicki EP, Williamson AL, Williamson C 1997. An association between HIV-1 subtypes and mode of transmission in Cape Town, south Africa. *AIDS 11*: 81-87.

WHO-UNAIDS 2002. *Guidelines for standard hiv isolation and characterization procedures*, 2nd ed., Health and Development Ed, Geneva, 140 pp.

Yang C, Li M, Newman RD, Shi YP, Ayisi J, van Eijk AM, Otieno J, Misore AO, Steketee RW, Nahlen BL, Lal RB 2003. Genetic diversity of HIV-1 in Western Kenya: subtype-specific differences in mother-to-child transmission. *AIDS 17*: 1667-1674.