Lack of viral selection in human immunodeficiency virus type 1 mother-to-child transmission with primary infection during late pregnancy and/or breastfeeding

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Mother-to-child transmission (MTCT) of human immunodeficiency virus type 1 (HIV-1) as described for women with an established infection is, in most cases, associated with the transmission of few maternal variants. This study analysed virus variability in four cases of maternal primary infection occurring during pregnancy and/or breastfeeding. Estimated time of seroconversion was at 4 months of pregnancy for one woman (early seroconversion) and during the last months of pregnancy and/or breastfeeding for the remaining three (late seroconversion). The C2V3 envelope region was analysed in samples of mother–child pairs by molecular cloning and sequencing. Comparisons of nucleotide and amino acid sequences as well as phylogenetic analysis were performed. The results showed low variability in the virus population of both mother and child. Maximum-likelihood analysis showed that, in the early pregnancy seroconversion case, a minor viral variant with further evolution in the child was transmitted, which could indicate a selection event in MTCT or a stochastic event, whereas in the late seroconversion cases, the mother’s and child’s sequences were intermingled, which is compatible with the transmission of multiple viral variants from the mother’s major population. These results could be explained by the less pronounced selective pressure exerted by the immune system in the early stages of the mother’s infection, which could play a role in MTCT of HIV-1.

INTRODUCTION

Mother-to-child transmission (MTCT) is the overwhelming source of human immunodeficiency virus type 1 (HIV-1) infection in young children. According to the World Health Organization (WHO), during the year 2005, despite effective antiretroviral (ARV) therapy, there were approximately 700 000 new infections among children worldwide, most of whom were from resource-limited countries (UNAIDS, 2007).

HIV-1 MTCT rates without drug access range from 15–25 % in Europe and the USA to 25–40 % in some African and Asian studies (De Cock et al., 2000; Luzuriaga, 2007). Pregnancy, delivery and breastfeeding have been identified as the three different routes of HIV-1 MTCT and contribute to the rate of transmission to different degrees (Scarlatti, 2004). The pathogenic mechanisms and determinants of MTCT of HIV-1 by these three different routes of transmission need to be understood more precisely if they are to contribute to the layout of new approaches to prevention.

Some studies on the viral genotype associated with MTCT of HIV-1 have suggested that the transmitted variant is derived from a maternal minor virus population that has possibly escaped the immune response (Ahmad et al., 1995; Blish et al., 2007; Dickover et al., 2001; Kampingo et al., 1997; Kliks et al., 2000; Verhofstede et al., 2003; Wolinsky et al., 1992; Wu et al., 2006). The minor viral variant detected in the peripheral blood compartment may represent a major variant in local compartments such as the birth canal or the placenta. In addition, selection may occur in the infant through a replication advantage of some transmitted variants, or stochastic effects may play a role.
However, other results have demonstrated children infected by a major or multiple maternal viral variants (Briant et al., 1995; Kampfinga et al., 1997; Pasquier et al., 1998; Scarlatti et al., 1993; Verhofstede et al., 2003; Wade et al., 1998; Zhang et al., 2002). Furthermore, a more heterogeneous virus population, as detected by sequencing, has often been described in mothers who have not transmitted the virus to their child (Ahmad et al., 1995; Sutthent et al., 1998).

The occurrence of seroconversion in women during pregnancy and breastfeeding has been described as a high-risk situation for MTCT (Berkeley et al., 1992; Pizzo & Butler, 1991; van de Perre et al., 1991), despite some evidence against it (Roongpisuthipong et al., 2001).

During primary infection, the virus population is genetically homogeneous in most adults (Derdeyn et al., 2004; Fulcher et al., 2004; Karlsson et al., 1999; McNearney et al., 1992; Pang et al., 1992; Zhang et al., 1993; Zhu et al., 1993), with some individuals showing higher variation (Long et al., 2000; Overbaugh et al., 1996; Poss et al., 1995; Poss & Overbaugh, 1999; Sagar et al., 2004; Zhu et al., 1996), which contrasts with the higher genetic diversity observed in the chronic stages of the disease (Burger et al., 1991; Liu et al., 1997; McNearney et al., 1992; Saag et al., 1988; Wolinsky et al., 1996).

In order to explore the relevance of viral variability in MTCT of HIV-1, we sequenced the virus population from women with HIV-1 primary infection occurring during pregnancy and/or breastfeeding and from their respective children. According to previous findings (Liu et al., 1997; Lukashov et al., 1995; Wolinsky et al., 1996), in these cases there was less selective pressure mediated by the immune system in both mother and child.

**METHODS**

**Patients.** Children under 12 months of age born to HIV-1-infected women were referred from hospitals located in Buenos Aires city and the surrounding area to the National Reference Center for AIDS (CNRS) for HIV paediatric diagnosis.

It was possible to document seroconversion during pregnancy or breastfeeding in four mothers. In these cases, the primary infection was determined by a negative or indeterminate serological sample during pregnancy or breastfeeding, which was later followed by a positive serological test in the mother and subsequent detection of the virus in the newborns (Table 1). The study was approved by the Ethics Committee of the Microbiology Department of the School of Medicine, Buenos Aires University, Argentina, and written informed consent was obtained from the mothers for their participation in the study. The estimated time of seroconversion was at 4 months of pregnancy for one woman (early seroconversion) and the period spanning the last trimester of pregnancy (after 7 months) to breastfeeding (late seroconversion) for the other three. Data on the estimated seroconversion times are summarized in Fig. 1 and clinical data are detailed in Results and Table 1.

**Diagnosis of paediatric and adult HIV-1 infection and viral load studies.** Blood samples from mother–child pairs were collected in tubes containing 3% EDTA at the hospitals where families had been admitted. These samples were tested at the CNRS on the same day. Serum samples were tested by ELISA (HIV Uniform II; Organon Teknika) and a particle agglutination test (Serodia HIV kit; Fujirebio) and confirmed by Western blotting (WB; New Lav Blot-1; Sanofi Diagnostics Pasteur).

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density-gradient separation and thereafter used for virus amplification by PCR as described previously (Albert & Fenyo, 1990). HIV-1 p24 antigen was detected in the plasma by using a kit and following the manufacturer’s instructions (HIVAG 1 Monoclonal Antibody kit; Abbott Laboratories). Viral load in the plasma was analysed using a Quantiplex HIV RNA 3.0 assay (Bayer) according to the manufacturer’s recommendations.

Criteria for HIV paediatric infection proposed by the Centers for Disease Control and Prevention (CDC) were followed (CDC, 1994). Infants were defined as infected when any of the assays for HIV-1 direct detection (DNA detection by PCR, virus isolation or p24 antigen detection) tested positive on two separate occasions or when an AIDS-defining condition was recorded. Clinical status for adults was based on the recommendations of CDC (1992).

**Amplification and cloning of the C2V3 region of HIV-1.** Uncultured PBMCs (2 × 10⁶) were lysed as described by Albert & Fenyo (1990). A nested PCR approach was used to amplify the C2V3 region of the HIV-1 env gene from proviral DNA (nt 6988–7373, numbered as in HIV-1 clone HXB2; GenBank accession no. K03455). Only one sample was processed at a time to avoid cross-contamination.

The sequences of the C2V3 outer primers were JA 167: 5'-TATC-(C/T)TTTGAGCCAATTCCT(C/T)ATACA-3' and JA 170: 5'-GTGAT-GTATT(A/G)CA(A/G)TGAAGAATTCTC-3', and the inner primers were JA 168: 5'-ACATG(C/T)ACATGGGATTIA(A/G)GCCA-3' and JA 169: 5'-AGAAAATTC(C/T)CTCT(C/T)ACATTTAAA-3'. PCR conditions were as described by Leitner et al. (1996).

PCR products were purified using a Qiagen kit and cloned using a TOPO TA kit, according to manufacturer’s recommendations (Invitrogen). Colonies were screened for the insert by PCR and plasmids were purified using a QIAprep kit (Qiagen). Clones were used for ThermoSequenase (Amersham Pharmacia) sequencing with labelled primers for detection in a laser sequencing apparatus (ALF Red; Pharmacia Biotech).

**Phylogenetic and comparative sequence analyses.** Sequence alignment and comparison of nucleotide and amino acid sequences were performed using CLUSTAL_X. For each pair, the most frequent sequence of the mother’s virus population was selected as the major sequence.

Pairwise evolutionary distances (using DNADIST in the PHYLIP package; Felsenstein, 1993) were estimated using the Kimura two-parameter method with transition/transversion = 2/1. Pairwise distances were estimated in order to study the genetic variability within a sequence set and between sets. For intrapatient variability, pairwise distances were calculated between each pair of sequences within the set. For interpatient variability, between epidemiologically linked mother–child sets and epidemiologically unlinked mothers, each sequence from one set was compared with each sequence from the other set. The selection pressure was estimated by calculating the ratio of non-synonymous/synonymous (dN/dS) substitutions using the Datamonkey website (http://www.datamonkey.org/) (Pond & Frost, 2005a, b; Pond et al., 2005) in infant and mother sequences.

**RESULTS**

**Table 1.** Details of the samples and clinical events. The numbers of children and adults are shown. Time of seroconversion is in years and months.
evaluated by performing 1000 bootstrap replicates. Maximum-likelihood (ML) trees were constructed with PAUP 4.0 (http://paup.csit.fsu.edu/) using a nucleotide substitution model chosen by MODELEST software (Posada & Crandall, 1998) and visualized in TreeView (http://taxonomy.zoology.gla.ac.uk/rod/TreeView.html). In order to determine whether monophyly of the children’s sequences had been rejected, the Shimodaira–Hasegawa test from PAUP was used, with a significance value of $P<0.05$.

Table 1. Clinical data and genetic distances of four mother–child cases of MTCT during primary infection

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age</th>
<th>Viral load*</th>
<th>Clinical status†</th>
<th>No.‡</th>
<th>Genetic distances (%)§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Intrapatient</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>M1</td>
<td>25 years</td>
<td>5.56</td>
<td>A2</td>
<td>10</td>
<td>0.9</td>
</tr>
<tr>
<td>C1</td>
<td>0.8 months</td>
<td>4.77</td>
<td>A1</td>
<td>11</td>
<td>1.0</td>
</tr>
<tr>
<td>M2</td>
<td>38 years</td>
<td>4.62</td>
<td>A1</td>
<td>12</td>
<td>0.4</td>
</tr>
<tr>
<td>C2</td>
<td>1 month</td>
<td>5.9</td>
<td>A1</td>
<td>10</td>
<td>0.4</td>
</tr>
<tr>
<td>M3</td>
<td>21 years</td>
<td>ND</td>
<td>A2</td>
<td>5</td>
<td>3.0</td>
</tr>
<tr>
<td>C3</td>
<td>3 months</td>
<td>ND</td>
<td>C3</td>
<td>8</td>
<td>1.2</td>
</tr>
<tr>
<td>M4</td>
<td>22 years</td>
<td>3.7</td>
<td>A1</td>
<td>10</td>
<td>1.1</td>
</tr>
<tr>
<td>C4</td>
<td>5 months</td>
<td>5.59</td>
<td>A1</td>
<td>10</td>
<td>2.1</td>
</tr>
</tbody>
</table>

*Viral load: $\log_{10}$ RNA copies ml$^{-1}$.
†Based on the recommendations of CDC (1992).
‡No. of clones per sample.
§Genetic distances were obtained using DNADIST of the PHYLIP package (Felsenstein, 1993).
ND, Not determined.

Fig. 1. Timelines showing the main time points for results of HIV tests, estimated times of infection and time of sampling for sequencing for each mother–child pair of the four cases of primary infection related to MTCT.
To characterize the HIV-1 subtype in the C2V3 env region, the sequences were aligned with reference sequences from the HIV sequence database alignment from Los Alamos National Laboratory and from Argentinean sequences that included some from the same geographical region.

The analysis of recombinant forms and bootscanning were performed using SimPlot version 2.5 (Salminen et al., 1995). The following GenBank C2V3 env reference sequences from the Los Alamos National Laboratory HIV sequence database were included: M17451, U08974, M66533, U63632, M26727, L22948, U53953, AF005494, L22082, L22085, U88826, U27426, AF005496, AY037275, AY037277, AY037278, AY037266, AY037283, AY037281, AF385936 (CRF12-BF), AY037280, AY037267, AY037282 and AY037276.

RESULTS

Patient characteristics

An overview of the clinical findings is summarized in Fig. 1.

Mother–child pair 1 (M1–C1). The woman attended the Hospital Service during her 5th month of pregnancy with an indeterminate result in her WB. Treatment with zidovudine (ZDV) and lamivudine (3TC) was started in her 7th month of pregnancy, while still showing indeterminate results. Intercourse with an HIV-infected person during the first trimester presumptively places infection at her 4th month of pregnancy. At 2 weeks post-partum, the woman tested positive on serological testing, but had not experienced symptoms of primary infection. She tested negative for hepatitis B and C viruses. Her child was born via vaginal delivery and was fed on formula milk. The baby had been treated with ZDV from shortly after his birth and, following diagnosis of HIV infection by PCR and virus isolation, he had been treated with different combinations of triple therapies up to the present time. The child, now 8, has so far maintained a good immunological status.

Mother–child pair 2 (M2–C2). The HIV antibody test at 5 months of pregnancy was negative. The following test at 1 month post-partum proved positive and was performed because the mother’s sexual partner had presented symptoms of opportunistic infection and had been diagnosed with HIV infection. The baby was breastfed until she was 18 days old, when HIV infection was diagnosed in both mother and child and treatment with ARV started. The child has remained healthy so far up to the age of 6.

Mother–child pair 3 (M3–C3). Maternal primary infection was detected during breastfeeding, when the 3-month-old baby was referred to hospital for severe dehydration. WB results for both mother and child were indeterminate, whereas PCR results for HIV and virus isolation were positive. The child developed Pneumocystis jiroveci pneumonia and cytomegalovirus chorioretinitis and died when he was 5 months old. No ARV treatment had been provided.

Mother–child pair 4 (M4–C4). The mother was HIV-negative in her 6th month of pregnancy and was diagnosed as having HIV infection 5 months after delivery, when the child became symptomatic and was referred to hospital. The child was born by vaginal delivery and was bottle-fed. Treatment with combined ARV therapy for both mother and child started at the time of diagnosis.

Global analysis of C2V3 env HIV-1 sequences

A total of 76 C2V3 env sequences, each from independent PCRs, was obtained and sequenced from DNA from each mother and child sample (Table 1). Analysed samples were always the first ones with positive diagnosis (by WB for mothers and by PCR for children) for all mother–child pairs, except for M3–C3, where samples with indeterminate WB results were studied.

Neighbour-joining analysis of all 76 C2V3 env sequences revealed clear epidemiological linkage between each mother and her infant, with no evidence of cross-subject contamination. The phylogenetic analysis showed that sequences of M2–C2 clustered with the reference sequences of subtype B, whilst those of M1–C1, M3–C3 and M4–C4 clustered with the subtype B/F Argentinean recombinant and subtype F reference sequences (Fig. 2). Both SimPlot and bootscanning analysis confirmed these results (data not shown). These results are according to Carr et al. (2001), where the C2V3 region of the B/F Argentinean sequences are mostly subtype F; this is also true in children born to HIV-positive women (Gomez Carrillo et al., 2002).

The coding potential of the env reading frame was maintained in all analysed sequences. The two cysteines residues flanking the V3 loop at positions 296 and 330 (numbered according to clone HXB2) involved in disulfide-bridge formation were conserved in all analysed clones.

The N-linked glycosylation site (NXT or NXS) proximal to the first cysteine of the V3 loop (position 299) was present in all mother–child pair sequences (Fig. 3), except in one C1 clone (ch1c). The other N-linked glycosylation sites showed some minor variations among different patient samples (Fig. 3).

The loop tip showed a GPGPQ motif in all sequences from M3–C3 and M4–C4 (Fig. 3c, d), a GPGQ motif in M1–C1 (Fig. 3a) and a GQGR motif in M2–C2 (Fig. 3b). All sequenced viruses had neutral or negatively charged amino acids at positions 11 and 25 of the V3 loop and might therefore be considered to be non-syncytium-inducing (Milich et al., 1993) or ascribed to the R5 genotype.

Comparison of the C2V3 env sequences in mother–child pairs

The degree of variability of the nucleotide distances of the C2V3 env region sequences from the four mother–child
pairs is shown in Table 1. Pairwise sequence analysis showed that nucleotide sequences were highly conserved between each mother and her infant (mean values of 2.0, 0.4, 1.8 and 1.2 for M1–C1, M2–C2, M3–C3 and M4–C4, respectively), with genetic differences ranging from 0 to 9.1%. The intrapatient variability was also low and mean values were 0.9, 0.4, 3.0 and 1.1 for M1, M2, M3 and M4, respectively, and 1.0, 0.4, 1.2 and 2.1 for C1, C2, C3 and C4, respectively.

The multiple analyses of amino acid sequences derived from the HIV-1 C2V3 region of the mother–child pairs are shown in Fig. 3. For M2–C2, M3–C3 and M4–C4, the major viral sequence from each pair was identical to the consensus sequence deduced from the most frequent amino acids observed when the sequence alignment from the corresponding mother was carried out. In these mother–child pairs, the major viral sequence from each infant was identical to the major sequence from the mother, whilst in the M1–C1 pair, the major viral sequence from the baby was not present in the mother.

**Phylogenetic tree analysis of mother–child pairs**

Fig. 4 shows the ML trees for the four mother–child pairs and the outgroup (reference sequence U52953, subtype C). Using MODELTEST to select the best-fit model, the tree for M1–C1 was performed with the F81 model (Felsenstein, 1981), whilst the trees for M2–C2, M3–C3 and M4–C4 (late seroconversions) were performed with the HKY model (Hasegawa et al., 1985). In the M1–C1 pair (early seroconversion), the tree topology suggested the transmission of one maternal variant of unique origin, with subsequent independent evolution in the child.

The trees for M2–C2, M3–C3 and M4–C4 showed a homogeneous virus population where mother and child sequences were intermingled. These results are compatible with the transmission of multiple and major maternal variants, which suggests the absence of viral selection during MTCT. In the M2–C2 pair, there was little variation in viral sequences and therefore it was difficult to determine whether multiple variants from the mother...

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**Fig. 2.** Phylogenetic tree from the C2V3 region of HIV-1, from clones of the four mother–child pairs and reference sequences of HIV-1 subtypes and B/F recombinants. The tree was constructed using the neighbour-joining method and Kimura’s two-parameter model (transversion/transition = 2/1). Bootstrap values (1000 replicates) higher than 50 are indicated on the tree. The bar indicates branch lengths corresponding to 2% sequence divergence.
had been transmitted or not. This tree might be consistent with a single variant emerging in the child. Next, we wondered whether we could reject monophyly of the children’s sequences from M2–C2, M3–C3 and M4–C4, because these phylogenetic relationships could only be due to noisy, indecisive data. To determine this, we first looked for the best tree where the children’s sequences were constrained to be in the same group. Then, using the Shimodaira–Hasegawa test, we determined whether the ML tree was significantly better than the constrained tree. According to this test, we were able to determine that, in ML trees corresponding to M2–C2, M3–C3 and M4–C4, there was statistically significant evidence to reject the hypothesis of monophyly of the children’s taxa (M2–C2: $P < 0.001$; M3–C3: $P = 0.042$; M4–C4: $P = 0.041$).

### DISCUSSION

To date, studies performed on the genetic analysis of HIV-1 in MTCT have been based on mothers with established infections. We analysed viral variation in four cases of MTCT with primary infection during pregnancy and/or breastfeeding in order to study transmission before the appearance of the maternal HIV-1-specific immune response. As would be expected in early infection, all maternal sequences presented a homogeneous viral population, in agreement with the results of Zhang et al. (1993) who demonstrated low viral diversity in the HIV-1 env gene during primary infection in adults.
Our results showed that the presence or absence of the N-glycosylation site next to the cysteine at position 296, and the motif at the tip of the V3 loop, did not play a significant role in MTCT.

In the analysed sequences, no positively charged amino acids were recorded at positions 11 and 25 of the env V3 loop. As viral variants with positively charged amino acids at these positions are associated with lymphotrophic strains (Milich et al., 1993) or CXCR4 usage, the viral variants detected in these four mothers were possibly using CCR5 as a co-receptor. In two pairs, M1–C1 and M2–C2, we analysed co-receptor usage taking advantage of the recombinant viruses obtained by homologous recombination of the PCR-amplified V1V3 env sequences of the patient’s virus with an HIV plasmid deleted from the complementary region (Trouplin et al., 2001). The recombinant viruses of both mother–child pairs were only

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**Fig. 4.** Phylogenetic trees from the C2V3 region of HIV-1 from clones obtained from the four mother–child pairs and a reference sequence (U52953). The trees were constructed by ML using modeltest to select the best-fit model. Trees for M2–C2, M3–C3 and M4–C4 (late seroconversions) were performed with the HKY model, whilst the tree for M1–C1 was performed with the F81 model. Bootstrap values (1000 replicates) were obtained from neighbour-joining trees. Values greater than 50 are indicated on the trees. The bar shows the number of nucleotide substitutions per site.
able to replicate in U87 cells transfected with CD4 and CCR5, thus confirming the genetically predicted phenotype.

In M1–C1, in spite of the absence of a correct diagnosis of an early in utero transmission, due to lack of samples from C1 in his first 48 h of life, it is highly probable that MTCT had occurred transplacentally early in pregnancy, considering the high viraemia during primary infection and the fact that ZDV treatment had been started in the mother from her 7th month of pregnancy, which had probably lowered the viral load. In the other three cases described, the earliest possible transmission could be placed at the 7th month of pregnancy with a possibility of subsequent transmission by breastfeeding in M2–C2 and M3–C3.

Our results are compatible with the notion that multiple viral variants of the mother’s major population had been transmitted in the cases of M2–C2, M3–C3 and M4–C4. Despite the difficulty of establishing the M2–C2 transmission, in these mother–child pairs the most frequent sequence in the child was identical to the most frequent one detected in the mother. Moreover, as shown in the amino acid alignment (Fig. 3), the remaining sequences in the child had a pattern similar to those in the maternal sequences, which were intermingled with the child’s in the phylogenetic tree. In these pairs, all of them late maternal seroconversions that had taken place during pregnancy or while breastfeeding, a variant selection event during transmission to the child is not suggested, as the major sequences derived from the mother were found in the child. Also, in the analysis performed using the Shimodaira–Hasegawa test in these three cases, there was statistically significant evidence to reject the hypothesis of a single variant transfer. In M1–C1, the phylogenetic tree suggested a single origin and an independent evolution of the child’s viral sequences. The most plausible explanation for the longer genetic distances observed between the child and his mother’s viral sequences could be the transmission of a minor variant. These sequences clustered in a subtree but into different clades. Therefore, this lack of close relationship between the mother's and child's viral sequences might be due to (i) the transmission of a virus that was able to escape the maternal immune response, as shown by Wu et al. (2006), (ii) selection due to viral treatment, as ZDV variants can normally be selected weeks after treatment (Richman, 2006) or (iii) a stochastic event.

In M1 – unlike M2, M3 and M4 – an earlier maternal seroconversion may have occurred around months 4–5 of pregnancy and the mother would have carried specific circulating antibodies at least during the last trimester. Therefore, this case could be compared with those of MTCT where mothers have already been infected before pregnancy.

Natural selection is assumed to operate mainly at the amino acid sequence level because most of the important biological functions in organisms are performed mainly by proteins. The rate of synonymous substitutions (dS) may be more or less similar to the mutation rate, whereas the rate of non-synonymous substitutions (dN) may vary according to the type and strength of natural selection. It is reportedly known that if the dN/dS substitutions ratio is higher than 1, positive selection is inferred. The results obtained in this analysis showed no site-specific evidence for positive selection in the mother and child sequences analysed. Considering the M1–C1 case, these results could suggest a stochastic event to explain the single origin and an independent evolution of the child’s viral sequences.

In conclusion, these results – the first analyses performed on maternal seroconversion and MTCT of HIV-1 – support two dissimilar transmission patterns. In the early pregnancy seroconversion, a single, minor viral variant was transmitted to the child, suggesting viral selection or a stochastic event in MTCT, whereas in the late seroconversion cases (during the last trimester of pregnancy and/or breastfeeding), mother and child sequences were intermingled in the phylogenetic tree, which is compatible with the transmission of multiple viral variants from the mother’s major population. These results could be explained by the less pronounced selective pressure exerted by the immune system in the early stages of maternal infection, which could play a role in MTCT of HIV-1.

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