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Analysis of primary resistance mutations to HIV-1 entry inhibitors in therapy naive subtype C HIV-1 infected mother–infant pairs from Zambia

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Abstract

Background—Small molecular CCR5 inhibitors represent a new class of drugs for treating HIV-1 infection. The evaluation of the primary resistance mutations associated with entry inhibitors during HIV-1 perinatal transmission is required because they may have a profound impact on the clinical management in MTCT.

Objectives—To evaluate the primary resistance mutations to maraviroc and vicriviroc during perinatal transmission and analyze the sensitivity of Env derived from mother–infant pairs to maraviroc.

Study design—Nine MIPs infected by subtype C HIV-1 were recruited to analyze the prevalence and transmission of primary resistance mutations to maraviroc and vicriviroc. Moreover, Env derived from six MIPs were employed to construct provirus clones and to analyze the sensitivity to maraviroc.

Results—Mutations A316T, conferring partial resistance to maraviroc, T307I and R315Q, both conferring partial resistance to vicriviroc are prevalent in mother and infant cohorts, indicating the transmission of primary resistance mutations during HIV-1 perinatal transmission. However, the mutations of acutely infected mothers seem to directly transmit to their corresponding infants, while some mutations at low frequency of chronically infected mothers would be lost during

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Ethical approval: The sample collection and the study were consented by the study subjects, and the study was approved by the University of Nebraska-Lincoln Institutional Review Board and the University of Zambia, School of Medicine Research Ethics Committee. This study was approved by the Institutional Review Board at the Nankai University.

transmission. Moreover, provirus clones derived from acutely infected MIPs are less susceptible to maraviroc than those from chronically infected MIPs.

Conclusions—Our study suggests that the transmission mode of primary resistance mutations and the sensitivity to maraviroc are dependent on infection status of MIPs either acutely or chronically infected. These results may indicate that higher dose of maraviroc could be needed for treatment of acutely infected MIPs compared to chronically infected MIPs.

Keywords

MTCT; HIV-1; Subtype C; Primary resistance; Maraviroc; Vicriviroc

1. Background

Mother-to-child transmission (MTCT) of the human immunodeficiency virus type 1 (HIV-1) remained the major route of infection for children in endemic regions and more than 90% of the infected children acquired HIV-1 from their mothers.[1,2] This is especially significant in sub-Saharan Africa where most of the new perinatal transmission cases have been reported.[3] Moreover the most prevalent HIV-1 subtype which is transmitted perinatally in the setting is subtype C, the much less well characterized HIV-1 subtype.[4]

Antiretroviral drugs (ARVs) for HIV-1-infected pregnant women and their infants are highly effective in reducing MTCT of HIV-1, especially when the WHO guidelines for the prevention of MTCT of 2006 and 2010[5,6] recommend complex antiretroviral prophylaxis to replace single dose nevirapine (sdNVP), a non-nucleoside reverse transcriptase inhibitor (NNRTI).[3,7] However, concerns have been raised about the public health implications of the emergence of resistance to antiretroviral drugs.[8–11]

Small molecular CCR5 inhibitors represent a new class of drugs for treating HIV-1 infection, by binding to a hydrophobic cavity located between the transmembrane domains of CCR5, inducing conformational changes in CCR5 and then inhibiting HIV-1 entry allosterically.[12,13] Thus far, three CCR5 antagonists (maraviroc, vicriviroc and aplaviroc) have been shown to inhibit virus replication in humans.[14] Although vicriviroc and aplaviroc have been tested in clinical trials and were not pursued because of sub-optimal efficacy and liver toxicity respectively,[15] maraviroc was approved by the FDA in 2007 and has been utilized as a salvage therapy for multi-drug resistant patients with R5 tropic virus,[14,16,17] which harbored the potential clinical use to prevent MTCT more efficiently. Drug resistance-associated mutations (DRAMs) have been reported in the V3 region of the *env* against maraviroc[18] and vicriviroc.[19,20] Primary mutations associated with resistance to maraviroc and vicriviroc are also found to be prevalent in adult therapy naive patients.[21,22] However, the prevalence and transmission of primary mutations to HIV-1 entry inhibitors-maraviroc and vicriviroc during MTCT are unclear, and both may have a profound impact on the clinical management of maraviroc.

2. Objective

The study aims to evaluate the presence and transmission of resistance-associated mutations to maraviroc and vicriviroc during MTCT, and to analyze the sensitivity of *env* derived from Mother–Infant Pairs (MIPs) to maraviroc.

3. Study design

3.1. Patient information

Archived nine Mother–Infant Pairs (MIPs) 1084, 1984, 2617, 2669, 2873, 1449, 2660, 834 and 2953 from Zambia were available for this study and described previously.[23,24] The mothers of six MIPs (pairs 1084, 1984, 2617, 2669, 1449 and 2873) were found to be infected at delivery and their infants were determined by PCR to be infected at either 2 months (pairs 2617, 2669, 1449 and 2873) or 4 months (1084 and 1984) after birth. These six MIPs were defined as the chronically transmitted MIPs. For other MIPs (pairs 834, 2660 and 2953), mothers and infants were found to have seroconverted at the same follow-up time point and at 4, 18 and 11 months after birth, respectively. They were defined as acutely infected MIPs. For the chronically infected MIPs, maternal samples collected at delivery and infant samples collected at the first postpartum HIV-1 PCR-positive time point were defined as baseline specimens. For acutely infected MIPs, the baseline specimens were obtained at the time of seroconversion. The baseline HIV-1 serological status of the mother was determined by two rapid assays, Capillus (Cambridge Biotech, Ireland) and Determine (Abbott laboratories, USA). Positive serological results were confirmed by immunofluorescence assay (IFA) as previously described.[25]

3.2. Cloning and sequencing of env derived from patients

To obtain the proviral HIV-1 *env* gene, genomic DNA was extracted from uncultured peripheral blood mononuclear cells (PBMC) for all subjects except for mother 1084. For mother 1084, *env* gene was amplified from placenta tissue since PBMC was not available. Nested PCR was used to amplify a 1100 bp fragment spanning the V1-V5 region of *env* as described previously.[24] Amplified fragments were cloned into the pGEM-T easy vector (Promega) and sequenced in both directions with dideoxy terminators (ABI BigDye Kit). A total of 20–40 clones were sequenced for each sample to obtain a representative measurement for the diversity of the viral population genotypes. A maximum likelihood (ML) tree was constructed for each transmission pair, including the V1-V5 region of *env* gene amplified from nine MIPs and two unrelated subtype C reference sequences from the Los Alamos HIV Sequence Database as outgroup sequences to root the Trees.[26] Subtyping analysis indicated that the clones sequenced of all the MIPs corresponded to HIV-1 subtype C, except for MIP 1449, which were subtype A/C recombination.[23,24] The primary isolates from these MIPs studied here were found to exclusively use CCR5 as a co-receptor, exhibit macrophage-tropism, and do not infect T-cell lines or cause syncytia *in vitro*.[23,24] The analysis of resistance-associated mutations to maraviroc and vicriviroc included: (i) A316T and I323V,[18] and (ii) K305R, S306P, T307, 315Q, F318I, T320R, and G321E,[19,20] respectively.

3.3. Generation of recombinant proviral expression constructs

The patient V1–V5 region of *env* gene was cloned into an Env expression vector pSRH NLA/S/Av (kindly provided by Dr. Eric Hunter, Emory University).[27] All the patient-derived chimeric Env expression constructs were first screened for biological function using the fusion assay.[28] Between 30% and 70% of the selected clones were biologically functional. Finally, four to eight functional envelope constructs derived from patients were subcloned into a proviral expression vector NL4.3ΔEnvEGFP (kindly provided by Dr. Miguel E. Quinones-Mateu, Case Western Reserve University), resulting in the infectious molecular clone plasmids. To eliminate the possibility that the selected clones for the analysis could be outliers, we then calculated the divergence for each selected clone of the MIP as the genetic distance between any sequence and the most recent common ancestor (MRCA) of the total previously analyzed archived virus sequences. It showed that divergence from each selected Env is within the range of the characterized population, and no outlier of divergence was used in our analysis.[29]

3.4. Cells and cell cultures

TZM-bl, COS-1 and 293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), penicillin (50 U/ml) and streptomycin (50 μg/ml).

3.5. Virus stocks

The chimeric viruses bearing patient-derived Env V1-V5 region were produced by transfecting proviral constructs into 293T cells. Culture supernatants were harvest at 48 h post-transfection, and stored at −80 °C. The p24 content of each virus stock was determined using HIV-1 p24 ELISA kit (Perkin-Elmer Life Sciences, Inc.).

3.6. Assay of sensitivity to CCR5 antagonists

The drug susceptibility assay was performed using TZM-bl cells as previously described.[20] Briefly, 2×10^4 TZM-bl cells/well were seeded into 96-well plates and incubated at 37 °C overnight. The next day, serial 2-fold dilutions of maraviroc (ranging from 32 nM to 0.125 nM) were added to wells 1 h prior to the addition of 10 ng of p24 of each virus. Plates were incubated for another 48 h and luciferase activity was analyzed by adding 50 μl of SteadyGlo™ luciferase assay buffer according to the manufacturer's instructions (Promega, Madison, WI).

4. Results

4.1. Characteristics of the nine MIPs

As summarized in Table 1, the maternal age at delivery was between 19 and 31 years. The mode of delivery of all MIPs was vaginal except MIP 2617, which was cesarian section. The mothers of chronically infected MIPs were known to be HIV-1 positive at the time of delivery and are likely to have acquired HIV-1 infection heterosexually, but their infants were HIV-1 negative at birth, suggesting the infants were infected either intrapartum or postpartum. For the acutely infected MIPs, mothers and infants were found to have

seroconverted at the same time point after birth, suggesting the infants were infected through breast-feeding (Table 1). All the mothers were asymptomatic without any clinical signs of immunosuppression and the infants were all breast-fed and drug naive. Infants of pairs 2617, 2669, 2873, and 1449 were considered rapid progressors since they died within the first year of life, due to apparent HIV-related complications (Table 1).

4.2. Analysis the primary resistance mutations to maraviroc and vicriviroc in viral quasispecies of mothers of each MIP

Maraviroc and vicriviroc resistance-associated mutations in V3 regions of gp120 are detailed in Fig. 1. For maraviroc, the mutations A316T and I323V have been reported to confer partial resistance for subtype B viruses.[18] For vicriviroc, mutation K305R was reported to confer partial resistance for both subtype C and subtype G viruses.[19,20] However, a number of mutations associated with vicriviroc resistance (S306P, T307I, F318I, T320R, and G321E) were observed only in a subtype C variant.[19] Mutation R315Q was observed in a subtype G variant.[20] As shown in Fig. 1, all samples of 31 Env derived from maternal quasispecies of MIP 2617 harbor mutations T307I and R315Q. The A316T mutation was detected in 69.2% of the samples. A316I and F318Y were the major polymorphisms found in the V3 regions. The resistance mutations in maternal quasispecies of other MIPs are summarized in Table 2. Similarly, mutations A316T, T307I and R315Q were most prevalent in mother quasispecies. Mutation I323V, which also conferred partial resistance to maraviroc, was only found in mother 1449.

4.3. Analysis the transmission of primary resistance mutations from mother quasispecies to their corresponding infants

Maraviroc and vicriviroc mutations related to reduce susceptibility in quasispecies of infants of each MIP are summarized in Table 2. Mutation A316T, T307I and R315Q are still predominant in most infant viral quasispecies, indicating the primary resistance mutations could be transmitted from mothers to infants during perinatal transmission. In the three acutely infected MIPs, the primary resistance mutations seem to be directly transmitted from mothers to infants. However, in the chronically infected MIPs, some of the resistance associated mutations presented in mother quasispecies have been lost during the transmission, such as mutation A316T and R315Q in MIP 1449, mutation K305R and T307I in MIP 2669, and T307I in MIP 2873.

4.4. Sensitivity of proviruses harboring V1-V5 regions derived from patients to maraviroc

To better understand the impact of the primary mutations on viral sensitivity to maraviroc, replication-competent recombinant proviruses harboring NL4-3 backbone and V1–V5 regions derived from representative four chronically and two acutely infected MIPs, were constructed to analyze their susceptibility to maraviroc. Although most of the MIPs except for MIP 2669 harbored one primary mutation site associated to reduce susceptibility to maraviroc, all the proviruses remained fully susceptible to maraviroc as the complete dose response curves were obtained (Fig. 2A–F). And no resistance to maraviroc, characterized by a plateau at less than 90% inhibition[18] was observed. The IC_{50} is variable among all the MIPs (Fig. 3A). Moreover, it should be noticed that mothers and infants of acutely infected

MIPs are less susceptible to maraviroc compared to the mothers and infants of chronically infected MIPs by comparing IC_{50}. ($P = 0.004$ and $P = 0.003$, respectively) (Fig. 3B).

5. Discussion

To the best of our knowledge, this is the first study that evaluates the prevalence and transmission of the primary resistance mutations to CCR5 antagonists-maraviroc and vicriviroc during perinatal transmission. We found a high prevalence of mutations A316T, R315Q and R307I, conferring partial resistance to maraviroc[18] and vicriviroc[20] respectively, in the viral quasispecies of Zambia pregnant women infected by subtype C HIV-1. The mutation R315Q seems to be a specific natural polymorphism for the subtype C HIV-1, in agreement with the recent reports.[21,22] ART was available to the public sector in 2002 when the Zambian Ministry of Health initiated an ART program at the country's two largest hospitals, and scaled up in 2004. However, entry inhibitors are not available in the ART therapy in Zambia.[22] Therefore, the resistance associated mutations in our ARV-naive HIV-1 infected mothers are related to naturally occurring mutations to maraviroc and vicriviroc rather than selected and transmitted from patients with administration of entry inhibitors.

Primary mutations were also observed in infant quasispecies, indicating they could be transmitted during perinatal transmission. In our acutely infected MIPs, the mutations present in mothers were found to be directly transmitted to their infants. However, in the chronically infected MIPs, the mode of transmission is complex and variable. Some mutations at low frequency in maternal quasispecies have been lost during perinatal transmission. The differences may be due to a selection event in our chronically infected MIPs while lack of viral selection occurred in our acutely infected mothers. The results are supported by our previously phylogenetic analyses, a genetic bottleneck was observed in our chronically infected MIPs but not in acutely infected MIPs.[23,26] This lack of bottleneck selection in acutely infected MIPs is unlikely to be due to the high viral load of the mothers due to acute infection as the viral burden was found to be similar in the mothers for both acute and chronic transmission.

We also analyzed the sensitivity of chronically infected MIPs and acutely infected MIPs to maraviroc as the prevalence and transmission of primary mutations may compromise the use of its treatment strategy. All the proviruses containing V1–V5 regions derived from chronically and acutely infected MIPs showed fully susceptible to maraviroc. And there seems to be no difference of IC_{50} between the proviruses containing A316T or I323V mutation and the proviruses which did not harboring the mutation. The A316T and I323V have been reported to confer partial resistance for subtype B viruses. And complete resistance to this entry inhibitor occurs when both mutations (A316T/I323V) are present.[18] However, in our study, the subtype C viruses harboring A316T or I323V mutation did not show partial resistance to maraviroc as subtype B did. The results indicated that the genetic background of HIV-1 subtypes may influence the mutations associated resistance to maraviroc. This is consistent with the study which showed the three-amino-acid QAI (315–317) deletion in the V3 region conferred the resistance phenotype for a subtype G strain.[18]

It should be noted that mothers and infants of acutely infected MIPs were less susceptible to maraviroc than chronically infected MIPs. One possible explanation is that viral quasispecies of chronically infected mothers have been selected for effective replication in the host and hence more sensitivity to maraviroc. However, proviruses of acutely infected mothers did not have enough time to adapt. This supports the previous study which showed maraviroc resistance-associated mutations may compromise viral replication *in vitro*.[18] However, our numbers of chronically and acutely infected mothers are not sufficient and a much larger number of subtype C infected mothers will need to be analyzed to determine if such a pattern persists. These results nevertheless indicate that higher doses of maraviroc would be needed for treatment of acutely infected as compared to chronically infected individuals.

In conclusion, although the relatively small sample size, our results indicate that the natural accumulation of polymorphisms that can generate resistance to maraviroc and vicriviroc could be transmitted during perinatal transmission and the transmission mode of chronically infected MIPs is more complex than acutely infected MIPs. Moreover, provirus clones derived from acutely infected MIPs are less susceptible to maraviroc than chronically infected MIPs. Further studies of larger sample size from other endemic regions are needed. Nevertheless, these findings may help define the clinical management of maraviroc for the treatment of Zambian patients infected with HIV-1 subtype C since the high prevalence and transmission of these primary mutations during MTCT may compromise the efficiency of maraviroc therapy.

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Abbreviations

HIV-1	human immunodeficiency virus type 1
MTCT	mother-to-child transmission
MIP	mother–infant pair
NNRTI	non-nucleoside reverse transcriptase inhibitor
DRAM	drug resistance-associated mutation
IFA	immunofluorescence assay
PBMC	peripheral blood mononuclear cells
DMEM	Dulbecco's Modified Eagle's Medium
FBS	fetal bovine serum

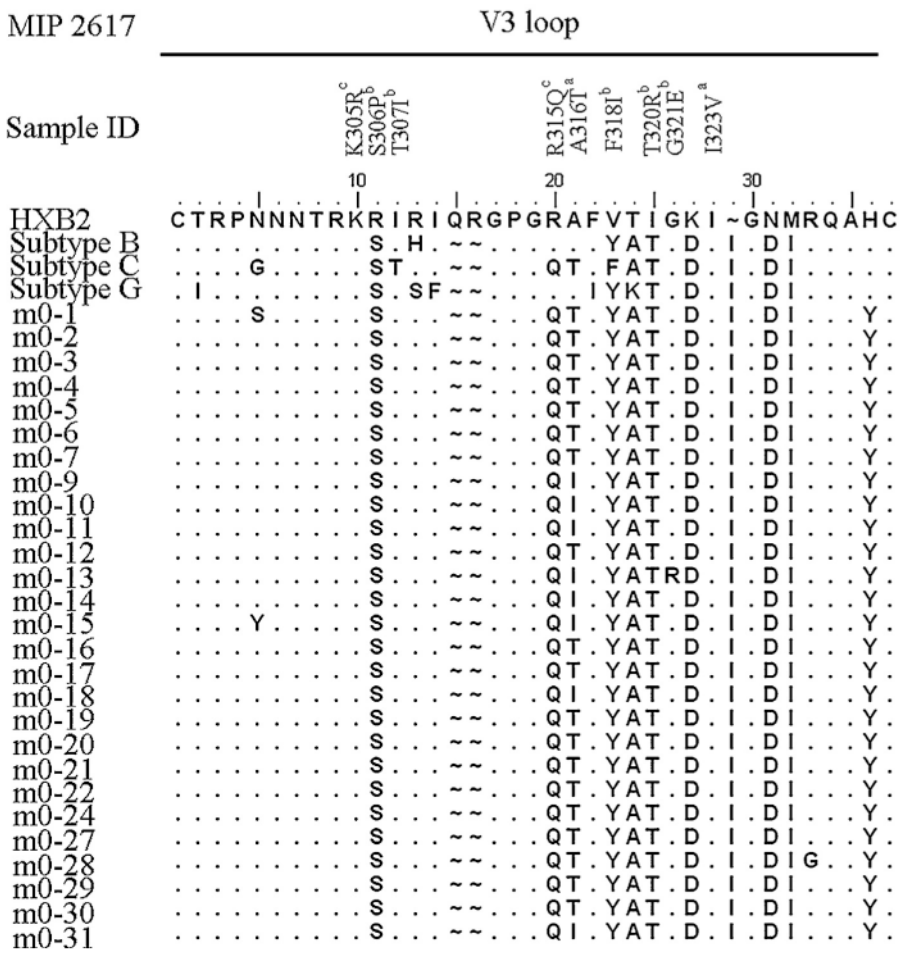

Fig. 1.

Analyze the primary resistance mutations to maraviroc and vicriviroc in the V3 loop of maternal variants of MIP 2617. This alignment includes V3 sequences derived from HXB2, subtype B, subtype C and G HIV-1 sequences reported previously, and 27 maternal variants sequences from MIP 2617. Resistance mutations to maraviroc and vicriviroc are shown above and numbering of amino acid position is based upon the HXB2 envelope sequence. (a) The subtype B HIV-1 isolate was used as reference sequence of amino acid position 316 and 323. (b) The subtype C HIV-1 isolate was used as reference sequence of amino acid position 306, 307, 318, 320, and 321. (c) The subtype G HIV-1 isolate was used as reference sequence of amino acid position 305 and 315. Maternal samples were collected at the delivery, indicated as m0. The number after the dash indicates the clone number. Amino acid identity (·), insertions/deletions (~) and substitutions are indicated.

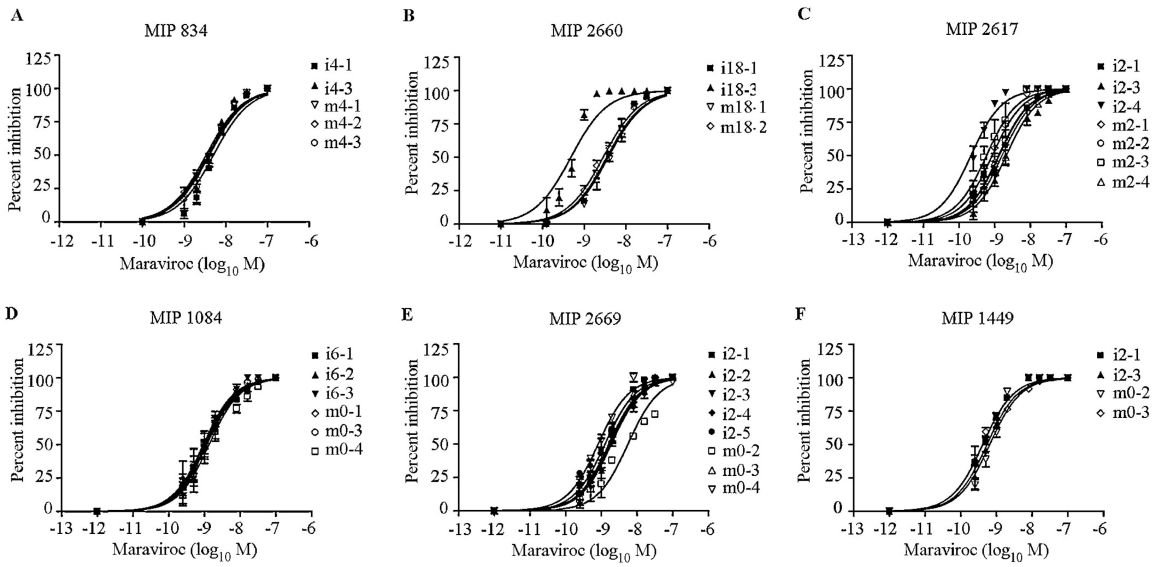

Fig. 2.

Susceptibility of provirus clones harboring NL4.3 backbone and V1-V5 regions derived from MIP 834 (A), MIP 2660 (B), MIP 1084 (C), MIP 2617 (D), MIP 2669 (E), MIP 1449 (F) to maraviroc. Maraviroc was added to TZM-bl cells in concentrations ranging from 0.125 to 32 nM, incubated for 1 h. And then TZM-bl cells were infected with a standardized input of viruses from each MIP. Data points represent percent replication in the presence of drug relative to no drug. Maternal samples were collected at the delivery, indicated as m0. Infant samples were collected at the time of the first HIV PCR-positive result after birth, which for most infants was at 2 months after birth (i2), for some was at 4 months (i4) and for some was at 6 months (i6). The number after the dash indicates the clone number. The bars represent the SD. of the mean from three independent experiments.

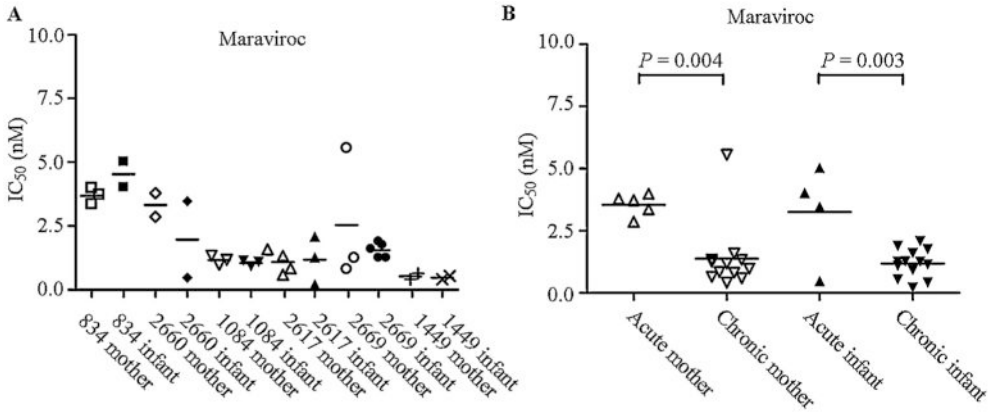

Fig. 3.

Analyze the 50% inhibitory concentrations of maraviroc (IC_{50} values). (A) IC_{50} values were computed from the curves and plotted for maternal and infant provirus of each MIP. (B) Comparable analysis the average IC_{50} of all the maternal and infant proviruses between acutely infected MIPs and chronically infected MIPs.

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Table 1

Summary of clinical information for all the mothers and infants derived from each MIP recruited in this study.

Patient ID	Subject	Age (year)[a]	Mode of delivery	Birth weight (g)	Breast feeding	Time point analyzed[b]	Clinical information
834	Mother	26	Vaginal	N/A	N/A	4	Seroconvert at 4 month after delivery; sexually transmitted infection; malaria at 6 and 18 months.
	Infant, male	N/A	N/A	3400	Yes	4	Papullae non-specific rash on lower limb at 4 months.
2660	Mother	21	Vaginal	N/A	N/A	18	Seroconvert at 18 month after delivery; fever and headache at 2 and 18 months; sexually transmitted infection: chancroid at 2 months; vaginal discharge at 18 months.
	Infant, male	N/A	N/A	2760	Yes	18	Existing pulmonary tuberculosis; accepted anti-tuberculosis treatment from 30 months; malnourished-looking child.
2953	Mother	24	Vaginal	N/A	N/A	11	Seroconvert at 11 month after delivery; abdominal pains at 11 months.
	Infant, female	N/A	N/A	3250	Yes	18[c]	Anti-malaria treatment at 5 and 11 months after delivery; chicken pox at 18 months; cough at 24months.
1084	Mother	28	Vaginal	N/A	N/A	M0	Found to be HIV-1 positive at the time of delivery; clinically asymptomatic.
	Infant, male	N/A	N/A	3400	Yes	6	After followed for more than 4 years, remained clinically asymptomatic.
1984	Mother	26	Vaginal	N/A	N/A	M0	Found to be HIV-1 positive at the time of delivery; clinically asymptomatic.
	Infant, male	N/A	N/A	3200	Yes	4	After followed for more than 4 years, remained clinically asymptomatic.
2617	Mother	20	Cesarian section	N/A	N/A	M0	Found to be HIV-1 positive at the time of delivery; clinically asymptomatic.
	Infant, female	N/A	N/A	2560	Yes	2	Died within the first year of life.
2669	Mother	19	Vaginal	N/A	N/A	M0	Found to be HIV-1 positive at the time of delivery; clinically asymptomatic.
	Infant, male	N/A	N/A	3260	Yes	2	Died within the first year of life.
2873	Mother	31	Vaginal	N/A	N/A	M0	Found to be HIV-1 positive at the time of delivery; clinically asymptomatic.
	Infant, female	N/A	N/A	3080	Yes	2	Died within the first year of life.
1449	Mother	30	Vaginal	N/A	N/A	M0	Found to be HIV-1 positive at the time of delivery; clinically asymptomatic.
	Infant, male	N/A	N/A	2540	Yes	2	Died within the first year of life.

[a] Age of the mother in years at the time of delivery.

[b] Times are expressed in months after infant birth. MO: maternal samples were collected at the delivery.

[c] No specimen was available for nucleic acid extraction at the 11-month time point.

Table 2

Analyze the prevalence of primary mutations to maraviroc and vicriviroc in maternal and infant quasispecies of each MIP.

Patient ID	Subject	No. of sequences	Mutation CCR5 inhibitors					
			Maraviroc			Vicriviroc		
			A316T	I323V	K305R	T307I	R315Q	G321E
834	Mother	32	100%	0%	0%	100%	100%	0%
	Infant	31	96.8%	0%	0%	100%	100%	0%
2660	Mother	27	100%	0%	0%	96.3%	100%	0%
	Infant	29	100%	0%	0%	100%	100%	0%
2953	Mother	32	100%	0%	14.8%	0%	100%	52%
	Infant	29	100%	0%	21%	0%	96.6%	55%
1084	Mother	31	87.1%	0%	0%	0%	100%	0%
	Infant	25	4%	0%	0%	0%	100%	0%
1984	Mother	29	0%	0%	0%	100%	96.6%	0%
	Infant	25	0%	0%	0%	96%	100%	0%
2617	Mother	26	69.2%	0%	0%	100%	100%	0%
	Infant	27	96.3%	0%	0%	100%	100%	0%
2669	Mother	32	0%	0%	28.1%	25%	100%	0%
	Infant	30	0%	0%	0%	0%	100%	0%
2873	Mother	30	100%	0%	0%	6.7%	100%	0%
	Infant	28	100%	0%	0%	0%	100%	0%
1449	Mother	37	56.7%	100%	0%	100%	5.4%	0%
	Infant	20	0%	100%	100%	100%	0%	0%