Viral Sequence Analysis of HIV-Positive Women and Their Infected Children: Insight on the Timing of Infection and on the Transmission Network

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Abstract

We used high-resolution phylogenetic methods in the context of mother-to-child transmission to obtain information on the timing of the infection and on the transmission network. A total of 33 *pol* sequences (from maternal peripheral blood, from breast milk, and from plasma of children) belonging to five cases of HIV infant transmission were studied. Using time-scaled phylogeny we were able to estimate that in two cases the transmission occurred after the recommended duration of breastfeeding, supporting a longer, not reported, duration of breastfeeding as a significant factor associated with HIV infant acquisition in this cohort. Among the postnatal infections we were also able to demonstrate that the cell-free virus in breast milk was the most likely population associated with the event of transmission. Our study showed that a coalescent-based model within a Bayesian statistical framework can provide important information that can contribute to optimizing preventive strategies.

ALTHOUGH THE ADMINISTRATION OF antiretroviral drugs for the prevention of HIV mother-to-child transmission is highly efficacious,^{1,2} cases of infant transmissions still occur despite prophylaxis. Understanding the mechanisms of these residual transmissions is critical to optimize future preventive strategies.

Phylogenetic analyses can provide important contributions to this topic by two means: (1) to help establish the exact timing of the transmission to better identify the specific factors associated with HIV infant acquisition, and (2) to determine the maternal viral population more likely associated with the event of transmission (both cell-free and cell-associated viruses either in peripheral blood or in breast milk have been implicated in the transmission).^{3,4}

In the present study we analyzed five cases of mother-to-child transmission occurring in an observational study of maternal antiretroviral therapy (ART) administration for the prevention of mother-to-infant transmission in Malawi.⁵

The mother/child pairs were part of the Safe Milk for African Children (SMAC) study conducted in Malawi within the Drug Resource Enhancement against AIDS and Malnutrition (DREAM) program of the Community of S. Egidio, an Italian faith-based nongovernmental organization. The study was approved by the National Health Sciences Research Committee of Malawi.

In the study HIV-infected pregnant women received a triple drug antiretroviral regimen of either zidovudine or stavudine, plus lamivudine and nevirapine, from week 25 of gestational age until 6 months after delivery (end of the breastfeeding period at the time of the study), or indefinitely if they met the criteria for treatment (baseline CD4⁺ <350/mm³). Infants' infection status was assessed locally at 1, 3, 6, and 12 months using a qualitative HIV-1 DNA polymerase chain reaction (PCR) assay (Amplicor HIV-1 DNA v 1.5 assay, Roche, Branchburg, NJ). In this cohort, including 311 women, there were eight cases of mother-to-child transmission.⁵

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According to the study protocol, collection of blood samples from mothers was scheduled at baseline (before the administration of drugs), at delivery, at 1, 3, and 6 months postpartum, and every 3 months thereafter. Collection of breast milk samples was scheduled at delivery and at 1, 3, and 6 months postpartum. Samples from children were collected at 1, 3, 6, 12, 18, and 24 months.

HIV-RNA was assessed using the kPCR Versant v 1.0 assay (Siemens Diagnostics, Deerfield, IL).

Viral sequences of the *pol* gene were obtained from plasma (HIV-RNA) and breast milk (HIV-RNA) using the Trugene assay (Siemens Diagnostics). The same assay was used to sequence HIV-DNA after extraction from whole blood and whole breast milk (QIAamp DNA Blood Mini Kit, Qiagen, Hilden, Germany). For each mother/child pair between five and eight sequences were obtained from peripheral blood or breast milk at the different time points, for a total of 33 sequences. The sequences were submitted to GenBank (accession numbers KM233418–KM233450).

Three different datasets were built.

The first dataset included the 33 HIV-1 *pol* gene isolates plus 38 subtype-specific reference sequences downloaded from the HIV Los Alamos database (www.hiv.lanl.gov/content/index) and was used to perform the subtype assignment.

The second dataset included the 33 HIV-1 *pol* gene isolates previously classified as subtype C, plus 154 HIV-1 *pol* gene subtype C sequences downloaded from the Los Alamos HIV sequence database on the basis of the following inclusion criteria: (1) sequences already published in peer-reviewed journals, (2) no uncertainty about the subtype assignment, and (3) a known sampling date. This dataset was built to estimate the HIV-1 subtype C *pol* gene mean evolutionary rate. The third dataset included only the 33 HIV-1 subtype C *pol* gene isolates from the present study to perform the time-scaled phylogeny. To avoid the influence of convergence evolution at antiretroviral drug resistance mutations, the stripped sequences were analyzed in all datasets. The sequences of all the datasets were aligned using Clustal X and manually edited by Bioedit software v. 7.0 as previously described.⁶ Modeltest 3.7 was used for all the datasets to select the simplest evolutionary model that adequately fitted the sequence data, as already described.⁷

The phylogenetic signal of each dataset was investigated by means of the likelihood mapping analysis of 10,000 random quartets generated using TreePuzzle as previously described.^{8,9} Groups of four randomly chosen sequences (quartets) were evaluated. For each quartet the three possible unrooted trees were reconstructed using the maximum likelihood approach under the selected substitution model. The posterior probabilities of each tree were then plotted on a triangular surface so that fully resolved trees fell into the corners and the unresolved quartets were in the center of the triangle (a star-tree). When using this strategy, if more than 30% of the dots fall into the center of the triangle, the data are considered unreliable for the purposes of phylogenetic inference.

Subtype was determined by uploading sequences individually into the REGA HIV-1 automated Subtyping Tool v2.0 [www.bioafrica.net/rega-genotype/html/subtypinghiv.html] and confirmed by phylogenetic analysis. A maximum likelihood (ML) phylogenetic tree was estimated on the first dataset with the best-fitting nucleotide substitution model

tested by Modeltest⁷ (GTR + I + G) using Phyml 3.0¹⁰ (www.atgc-montpellier.fr/phyml/). The statistical robustness and reliability of the branching order within the phylogenetic tree were confirmed by bootstrap analysis.

The evolutionary rate was estimated on the second dataset by using a Bayesian Markov Chain Monte Carlo (MCMC) approach (Beast v. 1.7.4, <http://beast.bio.ed.ac.uk>)^{11,12} implementing the model selected with ModelTest using both a strict and an uncorrelated log-normal relaxed clock model.

As coalescent priors, four parametric demographic models of population growth (constant size, exponential, logistic growth, and expansion growth) and a Bayesian skyline plot (BSP, a nonparametric piecewise-constant model) were compared. The best fitting models were selected using a Bayes Factor (BF with marginal likelihoods). Chains were conducted for at least 150×10^6 generations and sampled every 15,000 steps. In accordance with Kass and Raftery,¹³ the strength of the evidence against H0 was evaluated as follows: $2 \ln BF < 2$, no evidence; 2–6, weak evidence; 6–10, strong evidence; > 10 , very strong evidence. A negative value indicates evidence in favor of H0. Only values of ≥ 6 were considered significant. Convergence of the MCMC was assessed by calculating the effective sampling size (ESS) for each parameter. Only parameter estimates with ESSs of > 200 were accepted.

The dated tree was estimated on the third dataset by using a Bayesian MCMC approach (Beast v. 1.7.4, <http://beast.bio.ed.ac.uk>)^{11,12} implementing the evolutionary model selected by ModelTest, setting the evolutionary rate to the value previously estimated, and for the clock and the demographic model selecting the best fitting one using a Bayes Factor. Statistical support for specific clades was obtained by calculating the posterior probability of each monophyletic clade. Chains were conducted for 50 million generations and sampled every 5,000 steps. Convergence of the MCMC was assessed by calculating the ESS for each parameter. Only parameter estimates with ESSs of > 200 were accepted.

The tree was summarized in a target tree using the Tree Annotator program included in the Beast package by choosing the tree with the maximum product of posterior probabilities (maximum clade credibility) after a 10% burn-in. Time of the most recent common ancestor (tMRCA) estimates were expressed as mean and 95% high posterior density interval (HPD) months, considering the time of delivery as time zero (the tMRCA estimates were expressed in months before or after delivery). The final trees were edited and displayed using FigTree v. 1.4.

Sequences were available for five out of the eight cases of transmission in the cohort. Characteristics of mother/child pairs included in this study are reported in Table 1. Maternal baseline HIV-RNA levels ranged from 8,664 to 153,688 copies/ml and CD4⁺ cell count varied from 68 to 723/mm³. During treatment both plasma and breast milk viral load were undetectable in most cases with the exception of one mother who had high HIV-RNA levels in both compartments 1 month after delivery. One child first tested positive at month 1 (no birth samples were collected in the study so it is impossible to discriminate between *in utero*, intrapartum, or early breastfeeding transmission), whereas four first tested positive after month 1 (all postnatal transmissions). No mother had drug resistance-associated mutations either in plasma or breast milk. In children nevirapine-associated mutations were detected during breastfeeding due to exposure to suboptimal

TABLE 1. PATIENT CHARACTERISTICS

Patient ^a	Maternal CD4 ⁺ count	Plasma HIV-RNA (copies/ml)	Breast milk HIV-RNA (copies/ml)	HIV-DNA infant test	Available sequences ^b
A¹					
Enrollment	68	153,688			
Month 1	113	44	101	—	BM RNA; BM DNA
Month 3	133	43	<37	—	
Month 6	224	<37	41	—	M RNA; M DNA
Month 12	265	422	—	+	C RNA
Month 18	155	14,840	—	+	C RNA
B^{2c}					
Enrollment	723	56,709			M RNA; M DNA
Month 1	NA	12,452	8,753	—	BM RNA; BM DNA
Month 3	NA	NA	NA	—	
Month 6	880	668	<37	+	M RNA; C RNA
Month 8	NA	7,791	—	+	M RNA
Month 12	NA	NA	—	+	C RNA
C^{2c}					
Enrollment	649	19,700			M DNA
Month 1	578	67	<37	+	M RNA; C RNA
Month 3	789	<37	<37	+	
Month 6	539	<37	<37	+	C RNA
Month 8	NA	973	—	+	M RNA
Month 12	600	NA	—	+	C RNA
D²					
Enrollment	462	132,000			
Month 1	NA	<37	<37	—	M RNA; BM RNA ^d ; BM DNA ^d
Month 3	514	<37	90	+	
Month 6	1,016	<37	<37	+	M DNA; C RNA
E¹					
Enrollment	124	8,664			M RNA; M DNA
Month 1	253	<37	293	—	M RNA ^d ; BM RNA; BM DNA ^d
Month 3	NA	<37	<37	—	
Month 6	261	<37	<37	—	
Month 12	331	<37	—	+	M DNA; C RNA
Month 24	NA	<37	—	+	C RNA

^aTreatment regimen: ¹stavudine/lamivudine/nevirapine; ²zidovudine/lamivudine/nevirapine.

^bM RNA: HIV-RNA sequence obtained from maternal plasma; M DNA: HIV-DNA sequence obtained from maternal whole blood; BM RNA: HIV-RNA sequence obtained from breast milk; BM DNA: HIV-DNA sequence obtained from breast milk; C RNA: HIV-RNA sequence obtained from child plasma.

^cTreatment interrupted at month 6.

^dSequences obtained from samples collected at the time of delivery.

doses of this drug through breast milk (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/aid).

The phylogenetic noise of each data set was investigated by means of likelihood mapping. The percentage of dots falling in the central area of the triangles was 2.9%, 16.4%, and 6.2% for the first, second, and third dataset, respectively (Supplementary Fig. S1): as none of the datasets showed more than 30% of noise, all of them contained a sufficient phylogenetic signal.

Rega subtyping analysis and phylogenetic analysis gave the same results in terms of subtype assignment. All the 33 HIV-1 *pol* gene sequences were classified as subtype C (data not shown).

The mean evolutionary rate of HIV-1 subtype C *pol* gene sequences was estimated on the second dataset by using a Bayesian MCMC approach implementing the best fitting nucleotide substitution model selected with ModelTest (GTR+I+G). BF analysis showed that the relaxed clock

fitted the data significantly better than the strict clock (2 ln BF between the strict and relaxed clock was 137,744 in favor of the second). Under the relaxed clock, BF analysis showed that the BSP was better than the other models (2 ln BF > 400). The estimated mean value of the HIV-1 subtype C *pol* gene evolutionary rate was 1.601×10^{-3} substitutions/site/year (95% HPD: 1.2806×10^{-3} – 1.9548×10^{-3}).

Figure 1 shows the Bayesian time-scaled phylogenetic tree of the third dataset reconstructed assuming the GTR+I+G model of nucleotide substitution, under the relaxed clock and BSP demographic model. The tree shows five statistically supported clades (with a posterior probability ≥ 0.99), including the sequences from the five mother/child pairs.

Clade A included six sequences, two from the child and four from the mother. The sequence obtained from maternal plasma (HIV-RNA) represented the outgroup of the clade. Inside it, the statistically supported cluster A1 included two sequences from breast milk (one for HIV-DNA and the other for HIV-RNA) and two child sequences. Cluster A1 was

FIG. 1. (a) Bayesian phylogenetic tree of the 33 HIV-1 subtype C *pol* gene sequences from mother/child pairs (third dataset). Clade and cluster are indicated with *letters* and *numbers*. The *asterisk* (*) along the branch represents significant statistical support for the clade subtending that branch (posterior probability >99%). Time of the most recent common ancestor (tMRCA) estimates were expressed as mean and 95% high posterior density interval (HPD) months, considering the time of delivery as time zero (tMRCA was expressed in months before or after delivery). The tips of the five clades are shown with different symbols in the tree depending on the viral population: *filled circle*, maternal HIV-DNA of the peripheral blood; *empty circle*, maternal HIV-RNA of the peripheral blood; *empty rhombus*, HIV-RNA from breast milk; *filled rhombus*, HIV-DNA from breast milk; *rectangle*, HIV-RNA from children. (b) The five clades (from A to E) are highlighted. tMRCA estimates were expressed as mean and 95% HPD months, considering the time of delivery as time zero (tMRCA were expressed in months before or after delivery). The *asterisk* (*) along the branch represents significant statistical support for the clade subtending that branch (posterior probability >99%). The tips of the five clades are shown with different symbols in the tree depending on the viral population: *filled circle*, maternal HIV-DNA of the peripheral blood; *empty circle*, maternal HIV-RNA of the peripheral blood; *empty rhombus*, HIV-RNA from breast milk; *filled rhombus*, HIV-DNA from breast milk; *rectangle*, HIV-RNA from children.

dated 12 months before childbirth, with 95% HPD between 24 months and 1 month before delivery, including the time of the beginning of lactogenesis. The two child sequences clustered together significantly and dated to month 8 (8 months after delivery with 95% HPD: month 1, month 12). The child most probably was infected through breast milk HIV-RNA as this sequence was at the outgroup of the cluster. The estimated time of infection fell within the time frame of the last HIV PCR-negative (6 months) and first PCR-positive test (12 months). The mother of this child reported that she had breastfed up to 15 months.

Clade B included eight sequences, two from the child, two from breast milk (one for HIV-DNA and the other for HIV-RNA), and four sequences from maternal peripheral blood (three for HIV-RNA and one for HIV-DNA). Inside clade B a group of two sequences (B1) and a cluster (B2), both statistically supported, can be identified. Group B1 included two maternal sequences from peripheral blood (HIV-RNA and HIV-DNA). Cluster B2 included two maternal sequences from peripheral blood (HIV-RNA) and a cluster (B3) including a sequence from breast milk HIV-RNA and two child sequences. Cluster B3 dated to 5 months before delivery

(95% HPD: between 15 months and 1 month before delivery). Also in this case, the timing corresponds to the passage of the virus into the breast milk compartment. The two child sequences clustered together significantly and dated to month 4 (95% HPD: 1 month before delivery, 6 months after delivery); the child most probably was infected by breast milk HIV-RNA as this sequence was the outgroup of the cluster. The estimated time of infection fell within the time frame of the last HIV PCR-negative (month 1) and first PCR-positive test (month 6). No samples were collected at month 3 for this child. Detectable viral load levels were present both in plasma and in breast milk of this mother 1 month after delivery.

Clade C was composed of six sequences (three from the child and three from the mother). Inside clade C, there was evidence of a statistically supported cluster (C1). The three sequences from the child clustered together significantly and dated to month 0 (95% HPD: between 5 months before delivery and 2 months after delivery). The child most probably was infected from maternal HIV-RNA of the peripheral blood as this sequence was in the outgroup of the child sequences. The timing of the infection was compatible with an infection at the moment of delivery. This mother was diagnosed with a vaginal infection during pregnancy (an association between inflammatory genital tract conditions and an increase in shedding of genital HIV-RNA has been noted elsewhere¹⁴). No breast milk sequences were available for this mother.

Clade D was composed of five sequences (one from the child and four from the mother). Inside clade D, two statistically supported groups of two sequences each (D1 and D2) can be evidenced. The sequence from the child and the HIV-DNA sequence isolated from the maternal peripheral blood clustered significantly in group D1 and dated 3 months before delivery (95% HPD: between 4 months before delivery and 5 months after delivery). The child most probably was infected through maternal HIV-DNA of the peripheral blood. The child was HIV-negative at month 1 and first tested positive at month 3.

Clade E included eight sequences, two from the child and six from the mother. Inside clade E, a statistically supported cluster of six sequences (E1) can be evidenced. Inside cluster E1, the two child sequences formed a statistically supported group and dated to month 9 (95% HPD: between 2 months and 12 months after delivery). This child tested negative at month 6 and first tested positive at month 12; the mother had breastfed until month 9. These data support the result of the phylogenetic analysis. For this mother/child pair, due to the low posterior probability, it was not possible to discriminate and establish the source of infection for the child.

The application of high-resolution phylogenetic methods and the development of coalescence as tools to study the evolutionary dynamics of pathogens have been significant developments in recent years.^{9,15} A Bayesian statistical inference framework can allow the reconstruction of the temporal history of an epidemic on the basis of isolates randomly sampled at known times.^{11,12,16,17} Phylogenetic analysis can also make it possible to determine the most probable source of HIV infection.

In this study we analyzed the viral sequences of five mothers who transmitted HIV infection and of their children. Using time-scaled phylogeny we were able to provide insight

on the timing of HIV infant acquisition and, in four out of five cases, on the viral population more likely associated with the event of transmission.

As for the time of infection, in two cases of transmission the time corresponded to a period after the recommended duration of breastfeeding, supporting a longer, not reported, duration of breastfeeding as a significant factor associated with transmission in this cohort. In one other case the time corresponded to the presence of high levels of breast milk HIV-RNA, while in the last case of postnatal transmission the transmission event was estimated at a time before delivery, although the 95% HPD included a possible transmission during the first months of breastfeeding.

One recent study¹⁸ used for the first time the Bayesian approach to determine the timing of mother-to-infant transmission of HIV and showed that estimates using a time-scaled phylogeny were reliable, as also supported by our findings. In that study the estimates were consistent with the reference diagnostic procedures based on timing of HIV-DNA positivity in eight out of nine cases, similar to our results. The authors did not exclude a possible misclassification by the standard diagnostic assays for the last case.

In our study the genetic approach has also been used to help determine the viral population associated with the transmission event, which has important implications for breastfeeding transmission. In fact, it has been demonstrated that ART is highly effective in reducing breast milk HIV RNA but that it has no impact on HIV-DNA, the levels of which remain stable despite therapy.¹⁹ The different contribution of the two populations is still debated. Some⁴ have reported that HIV-DNA may be more important in early breastfeeding events (within the first 6 weeks) and that HIV-RNA may be more relevant in late transmissions (up to 6 months). The definition of the different contribution is critical in terms of future strategies. If only cell-free virus is associated with transmission, efforts to optimize treatment adherence to efficacious regimens can represent the critical step. On the other hand, if the persistence of cell-associated virus in breast milk is responsible for the transmission, infant infections can continue to occur despite effective regimens, and further strategies have to be put in place.

Our study, although including a very limited number of cases, supports the hypothesis that breastfeeding transmission is mainly associated with cell-free virus in breast milk and also suggests that not all of the postnatal transmissions are correlated with the virus passage from breast milk. In fact, in two out of the three cases of postnatal transmission the analysis showed that the HIV-RNA in breast milk was most closely correlated with the child sequences, while in the last case the HIV-DNA obtained from peripheral blood was associated, suggesting transmission through exchange of maternal blood probably due to breast cracks or mastitis in this mother who did not have significant levels of HIV-RNA in her breast milk.

The findings of an apparently more significant role for HIV-RNA (which can be suppressed by antiretroviral therapy) in breastfeeding transmission is reassuring in light of the new World Health Organization guidelines²⁰ recommending a long duration of breastfeeding (up to 12 months) due to the increased morbidity and mortality associated with no breastfeeding or with breastfeeding of limited duration.

In conclusion, our study shows that Bayesian methods can reliably estimate the timing of transmission events and can be used to provide information on the viral population more likely associated with the event of transmission, contributing to a greater understanding of the mechanisms of HIV residual mother-to-child transmissions in the context of antiretroviral therapy administration.

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