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An Assessment of the Timing of Mother-to-Child Transmission of Human Immunodeficiency Virus Type 1 by Means of Polymerase Chain Reaction

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Summary: To approximate the contributions of in utero, intrapartum, and postnatal transmission of human immunodeficiency virus type-1 (HIV-1) and to evaluate polymerase chain reaction (PCR) as a diagnostic tool for pediatric HIV infection, blood was collected at birth (cord blood), and at 3, 6-12, and 13-24 months in 218 children born to HIV-1-seropositive mothers in Kigali, Rwanda. Proviral DNA was detected by a double PCR using two sets of three primers (gag, pol, and env). Pediatric HIV-1 infection was defined according to serological and clinical criteria. The probability of having a positive PCR at a given time was calculated by a nonparametric method. Among children with unequivocal evidence of infection ($n = 47$), it was 30.5% on cord blood and 80.6% at 3 months. Thus, in children born to HIV-1-infected mothers, the estimated rate of transmission in the late postnatal period is 4.9%, and the rate of transmission in the intrapartum plus postnatal periods is 17.6%. Among 117 HIV-1-uninfected children born to HIV-1-infected mothers, six (5%) had a false-positive PCR on cord blood. These results should be taken into account in designing intervention trials aimed at reducing mother-to-child transmission of HIV-1. **Key Words:** Mother-to-child transmission—Timing—Breast-feeding—Africa—Polymerase chain reaction.

Transmission of the human immunodeficiency virus type 1 (HIV-1) from an infected mother to her child occurs in grossly 12 to 35% (1). Mother-to-child transmission of HIV can take place either in utero, during labor and delivery, or postnatally by breast-feeding (2-4). An estimation of the relative contribution of these three modes of transmission has never been reported. However, this is an important issue because the recognition of the mech-

anisms of transmission is precisely what should precede the implementation of rational interventions aimed at reducing the risk of fetus/infant contamination. We describe hereafter an attempt to assess the timing of mother-to-child transmission of HIV-1 by a systematic follow-up of a cohort of 218 children born to HIV-1-infected mothers using the polymerase chain reaction (PCR) in Kigali, Rwanda.

SUBJECTS AND METHODS

Subjects Selection and Follow-up

A prospective cohort study of the perinatal transmission of HIV-1 has been ongoing in Kigali, the capital city of Rwanda, since November 1988. All women delivering at the maternity

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ward of the Centre Hospitalier de Kigali (CHK) were informed by social workers of the objectives, constraints, and advantages of the study. Verbal consent was obtained from each mother participating in the study. Two hundred eighteen children born to HIV-1-seropositive mothers and 218 children born to seronegative mothers were enrolled. Details about enrollment procedures are given elsewhere (5). After delivery, enrolled families were visited every 2 weeks by social workers who collected information on the health status of the children by means of standardized questionnaires. The children and their mothers were systematically examined by a physician every 3 months. At that time, blood was drawn from both mothers and children for laboratory evaluation. The children born to HIV-1-seropositive mothers were breast-fed for a median of 579 days (range, 0-1,302 days) and a mean of 560 days (standard deviation, 332 days).

Definition of Pediatric HIV-1 Infection

Criteria used to define children born to seropositive mothers as infected with HIV-1, uninfected, or with an indeterminate status are drawn from a Working Group consensus on Mother-to-Child Transmission of HIV (6) and have already been used in this cohort (7). The use of this classification (Ghent, 1992) is restricted to children who had reached or could have reached the cut-off age of 15 months at the time of analysis. It takes into account the HIV-1 antibody serostatus at 15 months of age and, when the child died or was lost to follow-up before 15 months of age, the presence or absence of signs and symptoms. A child was considered HIV infected if (a) he or she developed the acquired immunodeficiency syndrome (AIDS) according to the World Health Organization clinical case definition (8) or its revised version (9) regardless of his or her age or (b) died of HIV-related death (6) or (c) was HIV antibody positive at 15 months. A child was considered HIV uninfected if (a) he or she was HIV antibody negative at 15 months or (b) had been lost to follow-up or died of HIV-unrelated cause and was HIV antibody negative at 9 months or older. All other children were considered as having an indeterminate status with regard to HIV infection, especially those who died during the neonatal period.

According to the proposed classification, 46 of the 218 children born to HIV-1-infected mothers were considered HIV-1 infected at 15 months of age. In addition, one AIDS-free child developed HIV antibodies after 15 months and after a transient period of seroreversion and was considered HIV-1 infected at 24 months (10). Thus the sample of HIV-1-infected children at 24 months consisted of 47 subjects (Group 1). One hundred thirty-nine children were considered HIV-1 uninfected at 24 months (Group 2). For 32 children, the HIV-1 infection status remained indeterminate (Group 3). A random sample of 103 HIV-1-uninfected children born to HIV-1-seronegative mothers was also selected for our study (Group 4).

Serological Methods

Every 3 months, blood was drawn from mothers and children. Serum samples were screened by a commercial enzyme immunoassay (EIA, Vironostika, Organon Teknika, Boxtel, The Netherlands). All positive samples were further confirmed by a commercial Western blot technique (Du Pont de Nemours, Wilmington, DE, U.S.A.) using the Centers for Disease Control criteria for interpretation (11).

Polymerase Chain Reaction (PCR)

DNA from the peripheral blood mononuclear cells (PBMCs) was extracted as described earlier (12). Two rounds of amplification were performed on PBMCs collected at birth (cord blood), 3, 6-12, and 13-24 months of age. The double PCR procedure was performed by means of two sets of three primer pairs (gag 881-882, pol 001-004, env 401-404 and gag 883-990, pol 002-003, env 402-403), 40 cycles in a Perkin-Elmer Cetus DNA Thermal Cycler and Perkin-Elmer Cetus GeneAmp kits. This technique has already been extensively described elsewhere (12). A PCR was considered positive in presence of a detectable signal for at least two of the three primer pairs. All lysates with a negative PCR result on cord blood were amplified for the conserved region human leukocyte antigen (HLA)-DQ by means of the primer Gh26-Gh27 (13). The detection of amplified HLA-DQ alpha nucleic acid sequences provides a measurement of the quality and quantity of each extracted DNA sample. Thus samples with a negative PCR result for HLA-DQ alpha gene were excluded from the analysis. In addition to infants' and children's blood samples, a random sample of 31 HIV-1-seropositive mothers and of 13 HIV-1-seronegative mothers was tested blindly as additional controls.

Statistical Methods

In the group of HIV-1-infected children born to HIV-1-seropositive mothers (Group 1), the probability of having a positive PCR according to the age at the time of testing was calculated and plotted on a curve following the Turnbull method (14). This nonparametric method is most adequate when applied to interval-censored data. The Mann-Whitney test was used to compare the median number of tests performed in each group. Estimations of the transmission rates are presented with their 95% confidence intervals (CI).

RESULTS

Description of the Study Sample

Table 1 gives a summary of the follow-up of the cohort of children born to HIV-1-infected mothers and the number of blood samples available for PCR testing at each time of collection. The follow-up in the comparative cohort of children born to HIV-1-uninfected mothers was grossly equivalent to the follow-up of the exposed cohort (data not shown). Overall, 613 PCR tests were performed in children, 482 in children born to HIV-1-seropositive mothers and 131 in children born to HIV-1-seronegative mothers.

PCR as a Diagnostic Tool

All HIV-1-seropositive mothers tested were PCR positive, and all HIV-1-seronegative mothers tested negative. Only two cord blood samples lacked HLA-DQ alpha gene detectable by PCR and

TABLE 1. Summary of the first two-years of follow-up of the cohort of children born to HIV-1 infected mothers

Time after birth (mo)	Cumulative number of deaths	Cumulative number of children lost to follow-up	Number of children followed-up	Number (%) of samples available for PCR ^a
0	—	—	218	185 (85)
3	11	—	207	151 (73)
12	27	12	179	135 (75)
24	40	21	157	11 (7)

^a Polymerase chain reaction (PCR) on 13–24 months samples was not systematically performed but was restricted to HIV-1-infected children with a previous negative PCR result or with a single positive PCR test at 12 months of age.

were then excluded from the analysis. Table 2 shows the proportion of children with a positive PCR on at least one occasion during the follow-up, according to their HIV-1 infection status. The median number of blood samples available for PCR was not statistically different in Group 1 and Group 2 (Mann-Whitney test, $p = 0.90$). All children from Group 1 (HIV-1 infected) had at least one positive PCR result. In no instance was a negative PCR result observed after a positive result in the previous trimester. Eleven positive PCR results were detected in the 139 HIV-1-uninfected children born to infected mothers (Group 2). Six of them were obtained from 117 cord blood samples and were followed by repeated negative results in the follow-up, suggesting a contamination by mother's blood when collecting cord blood. Five other positive results were obtained in this group, three at 3 months and two at 9 months. During the follow-up, these positive PCR results were preceded and followed by

negative results. This suggests a laboratory or a labeling error. Clearing of infection is another plausible explanation. By definition, most of the children who were lost to follow-up were classified in Group 3 (undeterminate HIV-1 status). Children from this group had a lower number of blood samples available for testing than children from Groups 1 and 2. Six of the 32 children from this group had at least one positive PCR result. In 103 HIV-1-uninfected children born to HIV-1-seronegative mothers (Group 4), only one child had a positive PCR result. This child was free of any HIV-associated sign or symptom, and his mother remained HIV seronegative during the entire follow-up, but a positive PCR result was obtained on two occasions, on cord blood and at 24 months of age.

The specificity of our PCR technique can be calculated as follows. We have to exclude from this calculation results obtained from cord blood samples of children from Group 2 because maternal blood contamination was suggested in six of 117 cord blood samples (5%; CI, 1–9%). Overall, 342 PCR test results were obtained in HIV-1-uninfected children, 211 (328 – 117) in children from Group 2 and 131 in children from Group 4. Because seven positive results were observed of these 342 tests (five in Group 2 and two in Group 4), an estimate of the specificity of our PCR technique applied to the diagnosis of perinatal HIV-1 infection is 98.0% (CI, 96.5–99.5%).

Assessment of the Timing of Transmission

In the 47 HIV-1-infected children born to HIV-1-infected mothers, 42 had a blood sample tested by

TABLE 2. Proportion of children with a positive polymerase chain reaction on at least one occasion

HIV-1 infection status ^a	Total number of samples available for PCR	Median number of blood samples per child available for PCR (range)	Number of children with at least one positive PCR, n (% = n/N)
Group 1 ($n = 47$)			
Mother HIV ⁺ , child HIV ⁺	112	3 (1–5) ^b	47 (100%)
Group 2 ($n = 139$)			
Mother HIV ⁺ , child HIV ⁻	328	3 (1–4) ^b	11 (8%)
Group 3 ($n = 32$)			
Mother HIV ⁺ , child HIV?	42	2 (1–3)	6 (19%)
Group 4 ($n = 103$)			
Mother HIV ⁻ , child HIV ⁻	131	1 (1–4)	1 (1%)

n , number of children.

^a See Method section for definitions.

^b Mann-Whitney U test (Group 1 versus Group 2), $p = 0.90$.

PCR on cord blood, 35 on blood collected at 3 months, 27 at 6–12 months, and eight at 13–24 months. Figure 1 shows the probability in these children of having a positive PCR according to age at the time of testing. In this group, 30.5% of children (CI, 15.4–51.4%) had a positive PCR on cord blood and 80.6% (CI, 54.4–93.6%) on the blood sample collected at 3 months of age. At 24 months, all HIV-1-infected children had had at least one positive PCR result.

This allows approximation of the timing of transmission. The median estimate of the mother-to-child transmission rate at 24 months in this cohort is $(47 \times 100)/(47 + 139) = 25.3\%$ (CI, 19.0–31.6%). Two extreme estimates of the in utero, intrapartum, and postnatal transmission rates can be calculated from these data. If one considers that all HIV-1-infected children with a negative PCR on cord blood (i.e., 33, or 70% of the 47 HIV-1-infected children) acquired the infection intrapartum or in the postnatal period, the in utero transmission rate is 0.253×0.305 (see Fig. 1) $\times 100\% = 7.7\%$, and intrapartum plus postnatal transmission rate is $0.253 \times (1 -$

$0.305) \times 100\% = 17.6\%$. On the other hand, if one considers that the sensitivity of PCR is optimal only at 3 months of age and that only children with a positive PCR obtained after 3 months of age were indeed infected in the postnatal period, in utero plus intrapartum transmission rate is 0.253×0.806 (see Fig. 1) $\times 100\% = 20.4\%$, and late postnatal transmission rate is $0.253 \times (1 - 0.806) \times 100\% = 4.9\%$.

DISCUSSION

Several mechanisms of mother-to-child transmission of HIV-1 have been suggested and probably coexist (2). In utero transmission as early as in the first or second trimester of gestation has been suggested by viral isolation from fetal tissues (15) and by identification of the CD4 molecules on the cells of the stroma and of the placental villi (16). Recently, nucleic acid sequences specific for HIV-1 have been detected in fetal DNA obtained in the third trimester of pregnancy (17). Intrapartum transmission of HIV-1 has been suggested by a retrospective study of twin pairs born to HIV-1-

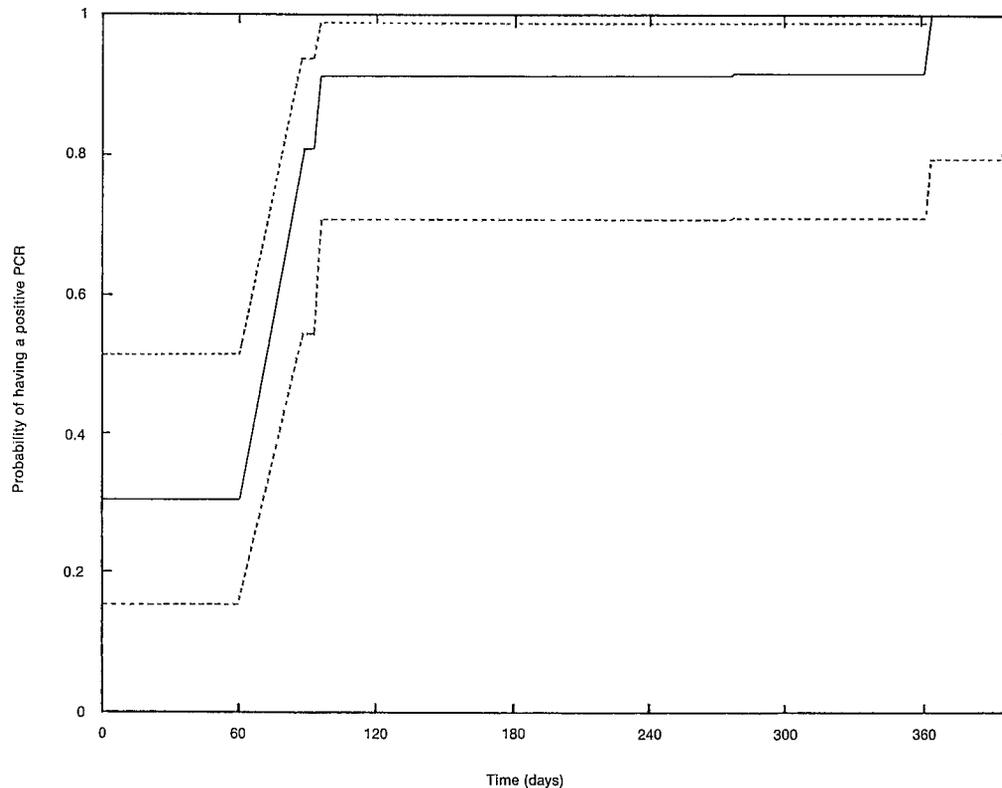


FIG. 1. Turnbull probability curve (with 95% confidence intervals) of having a positive polymerase chain reaction (PCR) according to age at the time of testing in 47 HIV-1-infected children born to HIV-1-infected mothers in Kigali (Rwanda), 1988–1991. Dotted lines represent 95% confidence intervals.

infected mothers (18). In this international study of 66 twin pairs, the first-born twin had a significantly higher likelihood of becoming infected than the second-born twin. It is possible that, during delivery, the first-born twin is exposed to a larger amount of contaminated cervico-vaginal secretion than is the second one. Finally, postnatal transmission of HIV-1 has been demonstrated in mothers seroconverting during the lactation period (12). In addition, David Dunn and colleagues performed a meta-analysis of existing data on mother-to-child transmission rates estimated from cohort studies and evaluated the risk of transmission in the postpartum period from mothers already infected before delivery at 14% (CI, 7–22%) (4).

The diagnosis of HIV-1 infection in infants and children by means of such a sensitive technique as PCR applied on serial blood samples systematically collected at various ages in a single cohort provided a unique opportunity to determine the timing of transmission of HIV-1 from mother to child. Our data provide evidence that our double PCR technique is extremely sensitive. When applied blindly, it discriminated between HIV infected and uninfected mothers with a 100% concordance with antibody detection. Our PCR technique was also 100% sensitive to diagnose HIV infection in our infected children. The specificity was 98.0% (CI, 96.5–99.5%). Our study showed also that cord blood is probably not suitable for early diagnosis of HIV-1 infection in newborns because a probable contamination of the newborn blood sample by maternal blood and the consequent false-positive PCR result was observed in six of 117 cord blood samples from newborns further shown to be uninfected. In contrast to other recent studies, venous blood samples collected in the first days of life were not available in our study. Testing of these samples by PCR could have allowed a better description of the different transmission groups. It can indeed reasonably be expected that a limited number of children with a positive PCR on cord blood had in fact been infected peripartum or early in life but had a positive test on cord blood due to a contamination by maternal blood.

Our methodology of evaluating the timing of transmission suffered a major constraint. It has been already demonstrated that PCR or viral isolation performed before the first month of life is positive in only one third to one half of the children further shown to be infected (19–22). Various explanations have been proposed for this phenome-

non. It is possible that fetuses infected in utero express only minute viral replication at birth. The number of viral copies could then fall beyond the detectable level of standard PCR and viral isolation. Alternatively, this low rate of positivity of PCR and virus isolation at birth could simply reflect transmission of HIV-1 during labor or delivery or both. These uncertainties as to the interpretation of PCR results at birth and in the first month of life have major consequences for the analysis of our data.

Our method of estimation of the relative importance of each mechanism of transmission seems in close agreement with the definition endorsed by the Pediatric Virology Committee of the AIDS Clinical Trial Group (23). If we consider that all infected children who had a negative PCR at birth were indeed infected either during delivery or postnatally via breast-feeding, the estimate of in utero transmission rate would be 7.7%, and the maximum combined intrapartum and postnatal transmission rate, 17.6%. This rate of in utero transmission is probably overestimated. Indeed, it can be anticipated that in a subset of infected children with a positive PCR on cord blood, a contamination of cord blood sample by maternal blood has occurred. If only children with a positive PCR obtained after 3 months of age were indeed infected in the postnatal period, the maximum estimate of the combined in utero and intrapartum transmission rate would be 20.4% and the minimal estimate of the postnatal transmission rate, 4.9%. It should be kept in mind that early postnatal transmission is also likely to lead to a positive PCR at 3 months of age. Thus this figure of 4.9% of postnatal transmission really represents late postnatal transmission. It is worth noting that our estimation of minimal and maximal postnatal transmission rate (4.9 to 17.6%) fits in the range of estimations obtained by meta-analysis (7 to 22%) (4).

In cohort studies performed in populations of infants in whom breast-feeding is uncommon, such as in most industrialized countries, at least 95% of the HIV-1-infected children have a positive PCR by 3 months of age (22, and C. Peckham, personal communication). This contrasts sharply with the 80.6% (CI, 54.4–93.6%) positivity rate of PCR at 3 months observed in our cohort of breast-fed children. This observation supports the hypothesis that postnatal transmission of HIV-1 by breast-feeding is at least partly responsible for the excess of mother-to-child transmission rate observed in populations in which breast-feeding is the rule as compared with industrialized countries in which breast-feeding is dis-

couraged in HIV-1-infected mothers. Nevertheless, in the developing world as well as in pockets of poverty in industrialized countries, the potential benefits of breast-feeding still outweigh the risk of HIV transmission, and breast-feeding should continue to be recommended and promoted (24,25). In this context, however, known HIV-1-infected mothers who can afford and apply safely artificial feeding may be counselled individually against breast-feeding.

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