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The role of transplacental microtransfusions of maternal lymphocytes in HIV transmission to newborns

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Abstract

Background—Perinatal HIV transmission could occur via microtransfused maternal blood during delivery. If so, detecting maternal cells in umbilical cord blood should correlate with infection risk.

Objective—To develop sensitive assays for maternal DNA in infant's blood stored as dried blood spots (DBS) and examine the correlation between microtransfusion and perinatal HIV infection risk.

Methods—Blood-in-blood serial dilutions were prepared as DBS. Extracted DNA was amplified for unique minor-population sequences using 24 allele-specific polymerase-chain-reaction (AS-PCR) assays. Using newborns born to HIV+ mothers, paired mother-infant samples were similarly examined to identify unique maternal sequences targeted by AS-PCR of DNA extracted from cord blood DBS. Cord-blood PCR-negative infants were categorized as uninfected or perinatally infected by HIV PCR on samples collected 4–8 weeks after birth.

Results—Sequences from added cells were detected at 1:1000 dilutions in 19 of 20 aliquots, and 1:10,000 dilutions in 7 of 20 aliquots; the median limit of detection (probit analysis) was 1 added genomic sequence in 9500 background sequences of amplifiable DNA. Maternal sequences were detected in cord-blood DBS of 50% of infected infants (N=18) and 44% of uninfected infants (N=43). Infection did not correlate with more frequent detection of maternal sequences.

Conclusion—This semi-quantitative assay reliably detected maternal DNA sequences in DBS at levels of less than 1:1000 cells. Maternal sequences were frequently detected but did not correlate infection risk with detection or level of maternal DNA in umbilical cord blood. Therefore we could not demonstrate that microtransfusions at parturition were responsible for perinatal HIV transmission.

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Authors' contributions:

Dr. Biggar conceived of the manuscript, organized the original study, analyzed the data and primarily wrote the manuscript, with the assistance of Dr. Busch. Dr. Broadhead, Taha and Kumwenda contributed to the conduct of the study in Malawi and collecting the samples. Drs. Lee and Wen did the laboratory work, in the laboratory of Dr. Busch.

In settings where maternal antiretroviral treatment is not used, about a quarter of infants born to HIV+ women become HIV-infected before or at birth, as demonstrated by polymerase chain reaction (PCR) tests. Specifically, in Malawi, site of the current study, approximately 6–8% of infants were infected *in utero*, defined as being HIV PCR-positive in umbilical cord blood. Of uninfected infants, 15 to 20% became infected perinatally, defined by being PCR-negative in cord blood but PCR-positive in the first tested blood sample taken 1–2 months after birth^{1, 2}. Thus, the two thirds of the infants who are diagnosed as infected by 1–2 months of age acquired HIV at or very near the time of birth. The route of perinatal transmission seems likely to be across the placenta. There is little support for infection in the birth canal^{1, 3}, and breast feeding in the first weeks of life appears to contribute little to perinatal infection risk⁴.

One plausible mechanism for perinatal transmission is by transfer of HIV-infected maternal cells into the fetal circulation at or near the time of delivery. Several reports describe the detection of fetal DNA in the mother's circulation during pregnancy and this approach has been applied to detect genetic abnormalities in the fetus^{5–6}. Transplacental microtransfusions from the mother to the fetus have also been documented using hybridization⁷ and by PCR testing of DNA extracted from cells obtained from fresh cord blood, where frequency was estimated to be 1 maternal cell per 1000 to 10,000 child cells in mid-trimester fetuses⁸.

However, obtaining this level of sensitivity is challenging when the target DNA is not greatly different from an overwhelming background of quite similar DNA, such as when blood from first degree relatives (i.e., mother and child pairs) is admixed. We report here a semi-quantitative approach using DNA extracted from dried blood spots (DBS), in which levels below 1 added cell per 1000 cells were reliably detected. We further used this technique to examine DBS made from umbilical cord blood to examine whether finding maternal DNA sequences was associated with perinatally-acquired HIV infection in the infant.

Methods

Patients and Materials

During a clinical intervention trial in Blantyre, Malawi in 1994, women delivering at Queen Elizabeth Central Hospital were enrolled in a study to determine rates of HIV transmission with and without vaginal cleansing with chlorhexidine, an antiseptic known to neutralize HIV. Details of the design and conduct of that study have been described elsewhere¹. During this study, HIV infection in the mother was determined by testing cord blood for passively-transmitted antibody against HIV. HIV infection in the infant was determined by PCR on DBS made from the umbilical cord blood or heel sticks at various ages. DBS were stored in individual sealed plastic envelopes in a cold room (4 C) until testing in 2007. Laboratory methods for sample collection and HIV testing have been reported in previous publications^{1, 9, 10}. Infants found to be uninfected postnatally were assumed to be HIV-negative at all earlier ages, including at delivery. To confirm that cord blood samples from infants previously found to be HIV-negative were indeed negative, we re-tested cord blood DBS for HIV, using a highly sensitive RT-PCR method to seek sequences specific to HIV¹¹.

For the current study, we examined a subset of maternal-infant pairs from the 1994 intervention study¹. The original study was approved both by the University of Malawi Medical School and the Government of Malawi as well as by the National Institutes of Health (USA). The current study was approved by the National Cancer Institute as exempt from further ethical review because it used only anonymous and un-linkable materials collected as part of previously approved study. Both case and control infants were vaginally-

delivered singleton term infants born to untreated HIV-infected women and all were breast-fed. Infants who were HIV PCR-positive in cord blood samples were considered infected *in utero* and excluded. Infants who were PCR-negative in cord blood samples were all PCR-tested between 4 and 8 weeks after delivery using DNA extracted from DBS obtained by heel stick. Infants who were HIV PCR-positive at 4–8 weeks were considered to have been infected perinatally. By design, the intended ratio of cases to controls was 1:2, but in some mother-child pairs, no PCR target sequences suitable for distinguishing mother and child were identified.

Laboratory Methods

Testing Strategy—The laboratory method required identification of a target sequence that was present in the maternal DNA but not the infant's DNA (i.e., informative allele). We approached this problem using methods recently developed in our laboratory for the purpose of detecting persistent chimeric cells following blood transfusion^{12, 13}. To identify prospective targets, we examined maternal and child sample pairs, seeking a strong PCR target present only in the mother's cells. Once a target was identified, the cord blood spot samples were extracted and sequence-specific PCR testing was performed to detect and quantify maternal cells. All testing of both the added controls and clinical specimens was performed on coded dried blood spot samples so that the status of subjects was unknown to the laboratory.

DNA Preparation for Identification of Informative Alleles—The dried blood spot filter paper was cut along the circumference of each blood spot. Scissors were cleaned with bleach and alcohol after each individual cutting to ensure that the scissors would not cross contaminate the samples. Each dried blood spot, expected to represent about 50 uL of blood, was soaked in 240uL of PBS, vortexed and incubated for overnight at room temperature. After overnight incubation, Corbett Robotics digest buffer (Sigma, St. Louis, MO) was added and incubated at 60C for 2 hours. Both the liquid and the filter paper were carefully transferred to the Corbett extraction plate. DNA extraction using the Universal Liquid Sample Xtractor protocol was followed. The procedure was performed using the automated X-tractor Gene Nucleic Acid Extraction System (Corbett Robotics, QLD, Australia). The DNA was eluted in 150uL of Solution A, to which an additional 150uL Solution B was added¹³.

Sensitivity Testing—To determine sensitivity, we used 10-fold serial dilutions of blood-in-blood admixtures made into DBS in 1998 and stored in the same freezers as the test samples. Four undiluted test samples were initially tested to find appropriate target sequences. The dilution series were then tested blindly to determine the lowest dilution at which the target sequence could be detected. In all results, the starting material was a unique dried blood spot (i.e., not pooled DNA from a common extract). Initial trials using a quantitative DQ alpha assay¹² indicated that each spot extract yielded an average of 14,983 amplifiable sequences (standard deviation: 5153) of amplifiable DNA. Individual dried blood spots from each dilution level were extracted and tested 4 (two series) or 6 (two series) times to assess replication of the results. Results for each set of samples is presented as the number of replicates detected as positive for the added DNA alleles divided by the number of replicates tested at each dilution. Sensitivity was assessed by probit analysis, pooling replicate results from all series for robustness.

DNA Typing of Maternal and Cord Blood Samples—Plates for PCR typing were prepared and frozen at –80C. The 24×16 typing plate format consisted of 12 columns of Insertion/Deletion PCR assays and 12 columns of HLA-DR PCR assays. The 12 Insertion/Deletion PCR assays were specific for insertion/deletion polymorphisms and the PCR

conditions were described elsewhere^{12, 13}. The twelve HLA-DR PCR assays targeted DR1, DR3, DR4, DR7, DR8, DR9, DR10, DR11, DR12, DR13, DR15, and DR16 and the PCR conditions were also described in the same publications^{12, 13}. Five microliters of DNA were added to 10uL of PCR mix. The Roche LightCycler 486 (Roche Diagnostics, Basel, Switzerland) was used to perform the DNA typing using PCR amplification.

DNA Preparation for Microchimerism Detection—The dried blood spot filter papers were cut as described above and soaked in 400uL PBS overnight at room temperature. For extraction, a modified protocol using Qiagen DNA mini kit reagents (Valencia, CA) was performed by adding 400uL of Qiagen buffer AL and 20uL protease. The filter and solution was vortexed and protein digestion occurred at 60C for 30 minutes. Four hundred microliters of 200 proof ethanol was added and vortexed. Both the liquid and filter were carefully transferred to the Qiagen DNA column. Wash procedures, per manufacturer's protocol, were then followed. The DNA was eluted in 200uL of nucleic acid free water, and dried at 100C before the addition of 200uL Solution A and B¹³. The preparation was vortexed to reconstitute the DNA.

Amplification for Microchimerism—Maternal and infant blood samples were typed and compared to identify informative strong-signal alleles present in maternal blood but not in offspring blood. After identification of informative maternal alleles, the paired cord blood DBS was extracted and amplified for the informative allele. For each subject, we amplified 6 replicates per informative allele. Twenty-five microliters of DNA was added to 50uL of PCR mix. Amplification conditions were as described previously^{12, 13}.

Statistical Analysis—The correlation between mother-infant HIV transmission and detection of microchimerism was assessed by exact odds ratios¹⁴ without further adjustments, because controls were closely matched to cases, as described above. Analysis of CD4 count and maternal viral level could not be done because the information was not available.

Results

In the serial dilution blood-in-blood sensitivity studies (Table 1), added cell populations in the archived test samples were almost always detected below 1:1000 cell dilutions (19 (95%) of 20 samples), often at 1:10,000 cell dilutions (7 (35%) of 20 samples) and sometimes at 1:100,000 dilutions (3 (15%) of 20 samples). Dilutions made to 1:1,000,000 and un-added controls were uniformly negative. The effective median detection (probit analysis) was 1 maternal genomic sequence in 9,500 infant sequences, indicated that we were likely to detect a sample as positive for microtransfusion if 1–2 maternal sequences were present in a blood volume containing 15,000 infant alleles, the average number of DNA copies extracted from a single DBS, based on DQ alpha testing.

In the tested samples from the study population, 68 mother-child pairs had adequate samples in both mother and child, including 22 case pairs in which perinatal infection occurred and 46 control pairs in which no infection occurred. No suitable target maternal alleles were identified in 4 case pairs (18%) and 3 control pairs (7%), leaving 18 case pairs and 43 control pairs for amplification. Results (Table 2) were scored by the number of six replicated samples of umbilical cord blood that tested positive for informative maternal DNA target alleles. Of all umbilical cords tests, 46% had maternal DNA detected in at least one replicate and 13% had all replicates positive.

Among 18 perinatally-infected infants, 9 (50%) had maternal DNA detected in at least one umbilical cord blood replicate, but the percentage with maternal DNA was similar in 43

uninfected infants, with maternal sequences amplified at least once in 19 cord bloods (44%) (exact odds ratio: 1.26; 95% confidence interval: 0.36–4.39). Similarly, 2 (11%) of those infected had all replicates positive (high levels of maternal DNA), compared to 6 (14%) of those uninfected (exact odds ratio: 0.77; 0.07–4.97).

Discussion

The frequency of finding maternal DNA in newborn blood has not been adequately studied, possibly because there are few applications for such knowledge. However, emerging interest is being generated by concern over graft-vs-host disease associated with placenta-derived hematopoietic stem cell transplantations^{15–16}. These studies have documented very low concentrations of maternal leukocytes in 20–50% of cord blood samples, the frequency of detection depending on the sensitivity of the assays used. The PCR methods we present detected 95% of test samples with a concentration of 1 target cell per 1000 background cells. At this concentration, the test samples would have had approximately 15 target sequences in 15,000 amplifiable sequences, the typical extract yield in study samples. Using probit analysis for all dilutions, the effective median detection level was 1 copy in 9500 genomes. Thus, we had a 50% chance of detecting a sample as positive if one or two sequences were present in the dried blood spot extract. When samples had very low levels of target sequences, the probability that the tested extract contained even a single target sequence governed the likelihood that the samples would be detected as positive.

We applied this assay to examine the frequency and magnitude of mother-to-fetus leukocyte transfer, as measured by the presence of maternal DNA in cord blood, in the context of perinatal HIV transmission. Despite finding that maternal DNA was commonly detected in cord blood, we did not find that detection correlated with HIV infection risk either qualitatively (any versus none) or semi-quantitatively (replicate level). Indeed, perinatal HIV infection appeared to be equally common when no microtransfused maternal cells were detected and when all replicates were positive. Even if free virus in maternal plasma were responsible for HIV infection, the volume of plasma microtransfused would be expected to be approximately proportional to the number of cells found.

It seems likely that HIV transmission occurring at delivery results from transplacental HIV transfer. In previous studies, we¹ and others³ were unable to demonstrate HIV transmission during passage through the birth canal. Since all infants in this study were breast-fed, some post-natal infection might have occurred by breast feeding during the first month. Infection via breast feeding no doubt can occur in the first weeks of life^{17–20} but appears to be uncommon. In South Africa, total infection rates at 6 weeks of age were similar in breast-fed only and exclusively bottle-fed infants⁴, indicating that infection via breast milk was not a major contributor to infection risk.

The current study was intended to determine if microtransfusion during labor might explain the high risk of HIV transmission during the first weeks of life. Our study failed to support this hypothesis despite finding a high frequency of detectable maternal DNA in cord blood. However, Kwiek et al.²¹, working independently in Malawi, recently reported a correlation between maternal DNA in cord blood and perinatal HIV transmission risk. Their report was based on data from 9 infected and 69 uninfected infants, but despite small numbers, they observed significant associations between levels of detected maternal DNA in umbilical cord blood and intrapartum HIV transmission ($p=0.02$), as well as a surprising possible association with *in utero* transmission (13 infected infants, $p=0.06$).

Several reservations need to be considered in evaluating our results. Other factors could have affected our ability to find an association. Mothers who transmitted HIV infection to

their infants were likely to have higher HIV levels than those who did not transmit infection^{22, 23} but maternal HIV levels were not available in the current study. Likewise, although all infants were vaginally-delivered, other delivery factors such as labor duration could have affected the results slightly. Furthermore, mothers who share more HLA concordance with their children are more likely to transmit HIV to their newborns^{24–25}. Testing for maternal DNA depleted our samples on these subjects, precluding further genetic analyses to determine relative histocompatibility. However, we doubt that these small influences would have affected the outcome of this study.

Sample quality also needs to be considered. The sensitivity panel and test dried blood spot samples were both prepared years ago (1998 and 1994, respectively) and stored similarly, so results in the sensitivity panel were likely to apply to tested samples. We appeared to have adequate samples, given the high frequency of detection we found. We could not determine how much blood might have entered the newborn because transfused maternal cells might have been introduced into the cord blood just prior to sampling, and thus, maternal cell concentration in cord blood could have been higher than in the systemic circulation of the infant. Finally, contamination of the cord blood sample by maternal blood could have occurred during collection. The collection procedures, described elsewhere¹, were intended to be simple and straight forward since samples were obtained by midwives in an active obstetrical service in an African hospital. In a previous report², using samples from this study, we assessed the frequency of HIV contamination by PCR-testing 48 cord blood samples from infants who had been born to HIV-infected women but were not infected with HIV. None was positive (upper 95% confidence interval: 6%). However, PCR testing for HIV may not rule out a very low level of contamination by maternal cells since the majority of circulation lymphocytes would not be HIV-infected. Only a few hundred nucleated maternal cells contaminating the initial cord blood collection before the blood was spotted on Guthrie cards for storage would have resulted in maternal DNA levels at a level (1 in 10,000) we might have detected as positive. Although the high level of sensitivity we achieved in the current study makes us concerned about detection of maternal cells contaminating the cord blood during initial specimen collection, we would still expect to have observed correlations with perinatal HIV infection rates if microtransfusion had been a major contributor to perinatal infection.

Based on the current study, the route by which infants become HIV-infected remains an enigma. Given the contrast between our findings and those of Kwiek et al.²¹, further work will be required. The approach and techniques described in this paper for detecting low level chimerism should have many applications in other settings.

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Table 1

Detection of donor-specific DNA sequences in 10-fold serial dilutions of whole blood prepared using 4 control blood samples (A added into B, B added into A, C added into D and D added into C). These dilution series were processed into dried blood spots and stored in parallel with clinical specimens from infected mothers and umbilical cord blood from exposed infants (see methods). The target informative sequence (present in the added DNA but not the diluent DNA) sought in each set is given in brackets. Numbers indicate the number of replicates found positive (+) for the informative target sequence / number tested at that concentration.

Dilution (Target sequence)	A in B (DR8)	B in A (SO7B)	C in D (DR1)	D in C (DR7)	All tests
	+/total	+/total	+/total	+/total	+/total %+
1:10	4 / 4	4 / 4	6 / 6	6 / 6	20 / 20 (100%)
1:100	4 / 4	4 / 4	6 / 6	6 / 6	20 / 20 (100%)
1:1,000	4 / 4	3 / 4	6 / 6	6 / 6	19 / 20 (95%)
1:10,000	2 / 4	3 / 4	1 / 6	1 / 6	7 / 20 (35%)
1:100,000	1 / 4	2 / 4	0 / 6	0 / 6	3 / 20 (15%)
1:1,000,000	0 / 4	0 / 4	0 / 6	0 / 6	0 / 20 (0%)

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Table 2

Detection of maternal DNA in the umbilical cord blood of infants who were HIV-infected and -uninfected perinatally. (+ / total) is the number of replicates positive for the informative maternal sequence divided by the total number of replicates examined.

Maternal DNA detection frequency (+ / total)	Number (%) of infants found positive at this frequency		Perinatal HIV infection		Total
	Yes	No	Yes	No	
0 / 6	9 (50%)	24 (56%)	33 (54%)		
1 / 6	1	3	4		
2 / 6	0	3	3		
3 / 6	5	0	5		
4 / 6	0	3	3		
5 / 6	1	4	5		
6 / 6	2 (11%)	6 (14%)	8 (13%)		
Total	18	43	61		