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Rapid Screening for Early Detection of Mother-to-Child Transmission of Human Immunodeficiency Virus Type 1

SHARON CASSOL,[1]* ANN BUTCHER,[2] SHARON KINARD,[2] JOANNE SPADORO,[2] TIDIANE SY,[1] NORMAND LAPOINTE,[3] STANLEY READ,[4] PERRY GOMEZ,[5] MICHELINE FAUVEL,[6] CAROL MAJOR,[7] AND MICHAEL O'SHAUGHNESSY[1]

The British Columbia Centre for Excellence in HIV/AIDS, Saint Paul's Hospital, and the Department of Pathology, University of British Columbia, Vancouver, British Columbia,[1] le Centre Maternel et Infantile sur le SIDA, Hôpital Sainte-Justine,[3] and le Laboratoire de Sante Publique du Quebec,[6] Montreal, Quebec, and the Department of Pediatrics, Hospital for Sick Children,[4] and the Ontario Ministry of Health,[7] Toronto, Ontario, Canada; Roche Molecular Systems, Somerville, New Jersey 08876-3771[2]; and Princess Margaret Hospital, Nassau, Bahamas[5]

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The testing of dried blood spots (DBSs) for the presence of human immunodeficiency type 1 (HIV-1) proviral DNA by PCR was first described in 1991. The technology has proven to be particularly valuable for resolving the infection status in HIV-1-indeterminate infants born to HIV-1-seropositive mothers. To broaden the applicability of DBS PCR, we adapted it to a standardized, commercially available microwell plate amplification and detection kit, Amplicor HIV-1, produced by Roche Diagnostic Systems. The microwell assay is rapid and easy to perform and uses equipment that is readily available in routine diagnostic laboratories. The high level of performance of the assay was demonstrated in 1,168 duplicate tests performed on 584 DBSs from 178 uninfected and 100 HIV-1-infected individuals, including 56 children with perinatally acquired HIV-1. Of 12 infants who were followed prospectively from birth, 3 (25%) were infected in utero (PCR positive at birth) and 9 (75%) were infected intrapartum (PCR negative, culture negative at birth). Overall, HIV-1 DNA was identified in 3 of 11 (27.3%) DBSs collected from infected infants during the first 4 days of life, 8 of 9 (88.9%) DBSs collected between 10 and 15 days postpartum, and 166 of 167 (99.4%) DBSs collected after 15 days of age. All 320 DBSs from uninfected children were PCR DNA negative. These findings indicate that use of the Roche microwell DBS PCR assay provides a powerful new approach for large-scale perinatal screening programs and population-based studies of vertical transmission.

Over the past few years, early diagnosis combined with antiviral treatment and prophylaxis of opportunistic infections, especially *Pneumocystis carinii* pneumonia, has significantly reduced morbidity and improved the quality of life for many human immunodeficiency virus type 1 (HIV-1)-infected infants (1, 10, 15, 23, 26, 27). Since maternal antibodies preclude the serological diagnosis of HIV-1 in the newborn period (12, 22), most diagnoses are based on viral culture or PCR (2, 19, 26–28). Both of these technologies are highly specialized, expensive, time-consuming, and inadequate for large-scale perinatal testing, especially in developing countries, where facilities and resources are usually limited. Simple and improved newborn screening assays that could be widely applied in both developing and developed countries would be highly advantageous. These assays are needed to guide therapy, determine prognoses, assess interventions, and monitor the changing dynamics of pediatric HIV-1 infection.

The use of dried blood spots (DBSs) collected on filter paper provide a simple and powerful approach for the testing of neonates. These samples, commonly referred to as Guthrie spots (16), have greatly facilitated genetic and metabolic screening of newborns in more than 20 different countries by simplifying all aspects of sample collection, storage, and transport (16, 25, 29). The first applications of PCR to the detection of HIV-1 in DBS specimens involved the extraction of the HIV-1 proviral DNA; this was followed by PCR amplification and solution hybridization with radiolabeled probes (5, 6). More recently, this technology has been simplified by eliminating the organic extraction step (35, 36). Although both of these methods exhibit high degrees of sensitivity and specificity (5–9, 35, 36), neither approach is suitable for the efficient screening of large numbers of DBS samples.

This report describes the adaptation of DBS PCR to a rapid microwell plate assay (Amplicor HIV-1; Roche Diagnostics Systems, Somerville, N.J.) (4). The use of a standardized, commercially available amplification kit (with an adaptation of the sample preparation protocol) broadens the applicability of DBS technology and renders it suitable for routine screening for HIV-1 in clinical and public health laboratories. The assay will be particularly valuable for determining mother-to-child HIV-1 transmission rates in different populations and assessing the efficacies of therapeutic agents designed to reduce the risk of virus transmission from infected pregnant women to their babies.

MATERIALS AND METHODS

Selection and preparation of blood spot specimens. As in previous studies, low-level-positive controls containing 0 to 128 copies of HIV-1 proviral DNA per blood spot were prepared by serially diluting 8E5 cells into HIV-1-seronegative whole blood (5–7). The 8E5 cell line is stably infected, with each cell containing one integrated copy of HIV-1 proviral DNA defective in the *pol* gene (14). Clinical DBSs were prepared by applying 50-μl aliquots of citrated whole blood to standard newborn screening papers (903; Schleicher & Schuell) by using

* Corresponding author. Mailing address: British Columbia Centre for Excellence in HIV/AIDS, Saint Paul's Hospital, 1081 Burrard Street, Vancouver, British Columbia, Canada V6Z 1Y6. Phone: (604) 631-5281. Fax: (604) 631-5464.

a plugged microliter pipette. Spots were allowed to dry for at least 4 h at room temperature before being stored in plastic bags for periods of up to 36 months at −20°C. Negative controls consisted of DBS samples prepared from an uninfected lymphoid cell line, CEM, diluted in whole blood.

Initially, we analyzed a coded, randomized panel of 116 well-characterized DBS samples (5–7) and compared the results with previous DBS PCR results obtained by organic extraction and radioisotopic detection methods. Infected samples included 54 DBSs from HIV-1-seropositive adults (33 pregnant women with early- to intermediate-stage infection; 21 homosexual men at all stages of infection) and 24 culture-positive children (1 month to 7.5 years of age). Uninfected samples consisted of 23 DBSs from seronegative adults (15 blood donors and 8 HIV-1-seronegative homosexual men) and 15 healthy asymptomatic children of HIV-1-infected mothers who consistently tested negative for HIV-1 by culture, PCR, and p24 antigen capture assay. All 15 children have been followed for >36 months, and all have shown a complete loss of HIV-1 antibodies.

To further assess the performance of the microwell assay in infants and young children, we analyzed a blinded panel of 468 additional blood spots selected from two cohorts of children participating in ongoing collaborative studies of vertical HIV-1 transmission in the Bahamas and Montreal. In those studies, pregnant women are offered HIV-1 testing as part of their antenatal care, and women who are identified as being HIV-1 seropositive are invited to enter the study. The mothers and their infants are followed at regular intervals and are evaluated for general health, growth and development, as well as immunological and virological status. The HIV-1 infection status of each child is determined by extensive laboratory testing (culture, PCR, p24 antigen capture) and long-term clinical follow-up. All projects were approved by the Human Subjects Review Board of the participating center, and informed consent was obtained.

Sample processing and PCR analysis. After optimizing the conditions for DNA recovery, the following protocol was used routinely. Circles of dried blood (0.6 cm in diameter) were excised from the filter with a sterile, flamed punch and were transferred to a 1.5-ml screw-cap tube (Sarstedt). The filters were suspended in 1.0 ml of Specimen Wash Buffer (Amplicor Whole Blood Specimen Preparation Kit; Roche Diagnostics Systems) and were placed on a rotator for 1 h at room temperature. The tubes were then microcentrifuged (1 min at 12,000 × *g*), and the hemoglobin-containing supernatant was removed by aspiration. HIV-1 proviral DNA was recovered from the filter by incubating each circle in 200 μl of a chelex-containing solution for 1 h at 100°C with intermittent vortexing (31).

To determine whether the DNA was of sufficient quality and quantity for PCR, 50 μl of each DNA sample was initially amplified for 40 cycles with primers to a highly conserved region of the HLA-DQα gene (GH26 and GH27) (30) as described previously (5). One-sixth of the PCR product was resolved by agarose gel electrophoresis and was visualized by ethidium bromide staining. HIV-1-specific amplifications were performed in duplicate in 100-μl reaction mixtures containing 50 μl of DNA and 50 μl of Master Mix consisting of dATP, dCTP, dGTP, dUTP, AmpErase, AmpliTaq, salts, and biotinylated primers (Bio-SK431 and Bio-SK462) (Amplicor HIV-1 Amplification Kit; Roche Diagnostic Systems). Amplifications were performed in a Perkin-Elmer TC9600 thermal cycler by using the following profile: 50°C for 2 min and then 5 cycles at 95°C for 10 s, 55°C for 10 s, and 72°C for 10 s and 30 cycles at 90°C for 10 s, 60°C for 10 s, and 72°C for 10 s (4). Samples were immediately denatured by adding an equal volume of denaturing solution (Amplicor HIV-1; Roche Diagnostic Systems). The denatured amplicons were then analyzed by adding 25 μl of each amplicon to 100 μl of hybridization solution (Amplicor; Roche Diagnostic Systems) contained in individual wells of a microwell plate coated with SK102. After incubation at 37°C for 60 min, the plates were washed and reincubated with 100 μl of an avidin-horseradish peroxidase conjugate per well. Following further washing to remove unbound conjugate, 100 μl of chromogenic substrate (tetramethylbenzidine) was added to each well, and the plates were placed in the dark for 10 min at 25°C. The color reaction was stopped by the addition of 100 μl of stop reagent, and the plate was read on a microwell plate reader (Bio-Tek Instruments) at a wavelength of 450 nm. Optical densities greater than 0.350 were considered positive for HIV-1. When the results of duplicate amplifications were concordant, no further testing was performed. However, when the results were discordant, samples were reanalyzed. If reamplification did not resolve the discordance, the result for the sample was considered indeterminate. Appropriate positive and negative controls were included in all assays.

TABLE 1. Comparison of microwell and radioisotopic methods for detection of HIV-1 proviral DNA in DBS samples

Clinical group and HIV-1 status	No. of DBS samples	PCR (no. of DBSs positive/no. tested)	
		Microwell assay	Radioisotope assay
Adults			
HIV-1-seropositive pregnant women	33	33/33	29/33
HIV-1-seropositive homosexual men	21	20/21	19/21
HIV-1-seronegative homosexual men	8	0/8	0/8
Children born to HIV-1-seropositive mothers			
Culture positive (1 mo to 7.5 yr of age)	24	24/24	24/24
Culture negative (2 days to 17.5 mo of age)	15	0/15	0/15
Control (HIV-1-seronegative blood donors)	15	0/15	0/15

RESULTS

Quality of the DNA. The DNA eluted from the blood spots was intact and suitable for PCR analysis, as shown by the consistent amplification of human HLA-DQα sequences from both control (blood donor, 8E5) and clinical (adult and pediatric) specimens (data not shown). One-sixth of each eluate provided a sufficient amount of DNA to routinely generate a strong HLA-DQα signal when the DNA was amplified for 40 cycles and analyzed on ethidium bromide-stained gels (data not shown). Eluates were further analyzed for the presence of HIV-1 *gag* sequences by using the Roche microwell plate system (Amplicor HIV-1) (4).

Performance of the Amplicor HIV-1 kit. The sensitivity of the microwell assay was initially assessed in reconstruction experiments by using 8E5 blood spots with known HIV-1 copy numbers. Using these standardized spots, we routinely detected 4 to 16 molecules of HIV-1 DNA in the dried equivalent of 3 μl of whole blood. To evaluate the sensitivity and the specificity of the assay in the clinical setting, we reanalyzed a randomly selected panel of 116 coded DBS specimens and correlated the results with those obtained previously by our radioisotopic DBS method (5). As shown in Table 1, the microwell assay correctly identified 115 of 116 (99.1%) filter paper specimens. Of the 78 positive specimens, 77 (98.7%)

tested positive by the microwell assay. These specimens included 33 DBSs from HIV-1-seropositive pregnant women with asymptomatic infection, 5 DBSs from high-CD4-count (>500 cells per mm^3) HIV-1-seropositive men, 9 DBSs from medium-CD4-count (200 to 500 cells per mm^3) HIV-1-seropositive men, 6 DBSs from low-CD4-count (>200 cells per mm^3) HIV-1-seropositive men, and 24 DBSs from infected, HIV-1 culture-positive children (ages 1 month to 7.5 years). One DBS from an HIV-1-seropositive homosexual man with a CD4 count of 278 cells per mm^3 tested false negative. The microwell results matched those of the radioisotopic method for 110 of the 116 (94.8%) samples tested by both procedures. The lower level of sensitivity of the radioisotopic assay, 92.3% of 78 positive samples, may reflect losses in the DNA template during the organic extraction step. All 38 DBS specimens from HIV-1-seronegative adults and HIV-1 culture-negative children tested PCR negative by the microwell and radioisotopic methods to give a specificity of 100% for both assays.

Diagnosis of infection in infants and young children. Additional testing of pediatric populations was performed on a coded set of 468 DBSs from children in the Bahamas and Montreal: 163 from 56 children with confirmed HIV-1 infection and 305 from 140 uninfected children. Infants were diagnosed with HIV-1 infection when two or more specimens tested culture, PCR, or p24 antigen positive and were diagnosed negative if, at 18 months of age, all cultures, PCR, and serological tests were negative and the child was asymptomatic with normal CD4 counts (6; unpublished data). All results were clearly positive or negative (no grey zone), with >80% of positive samples giving optical density values of >3.500 (range, 2.480 to 4.000), while the majority of negative samples gave optical density values of <0.100 (range, 0.051 to 0.159). Results of duplicate testing were highly concordant, with only 3 of 468 (0.64%) samples yielding indeterminate results (one positive and one negative result). Results for all three samples that gave discordant values were resolved during repeat testing and hence were reclassified accordingly. After reclassification, the microwell assay achieved a specificity of 100%. Overall (including the 24 HIV-1-infected infants from Table 1), HIV-1 DNA was detected in 177 of 187 (94.7%) specimens collected from children who had or who subsequently developed clinical and laboratory evidence of HIV-1 infection. Since newborns were tested at regular intervals, it was possible to determine the sensitivity of the assay at different time points following delivery (Table 2). When stratified according to age, HIV-1 proviral DNA sequences were detected in 11 of 20 (55.0%) specimens collected from infected infants during the first 15 days of life. The assay was least sensitive at the earliest time points, with only 3 of 11 (27.3%) specimens testing PCR positive within the first 4 days of life. Thereafter, the sensitivity of HIV-1 DNA detection increased dramatically, to reach a level of 88.9% in 9 specimens obtained 10 to 15 days postpartum and 97.2% in 36 specimens obtained between 16 days and 3 months of age. The only HIV-1-positive DBS that gave a negative PCR result for a specimen that was obtained after 16 days of age came from a 47-day-old Bahamian infant who tested PCR positive at three other times (19 days and 2.6 and 3.7 months). This sample was found to be PCR positive on repeat testing. Excluding this initial sample that gave a false-negative result, which was probably due to the incomplete recovery of the DNA during elution, the microwell assay correctly identified 100% of 132 specimens from infected infants who were older than 15 days of age at the time of testing. A detailed representative time course showing the onset of HIV-1 DNA positivity in 12 infected infants is presented in Table 3. Overall, 3 of 10 (30%) newborns who subsequently developed laboratory and clinical evidence of HIV-1 infection (Table 3; infants 3, 8, and 10) tested positive for HIV-1 by PCR within the first 96 h of life; 8 of 9 (88.9%) were determined to be HIV-1 positive by PCR by day 15 (Table 3, infants 1, 3, 5, 6, 8, 10, 11, and 12), and by 36 days postpartum, 100% of the 12 infected newborns who had been followed during the neonatal period were found to be HIV-1 positive by PCR (Table 3, infants 1 through 12).

TABLE 2. Performance of microwell DBS PCR assay in infants and young children

DBS PCR result	No. with the following HIV-1 status:		Age
	+	−	
+	11	0	Birth–15 days
−	9	68	
+	35	0	16 days–3 mo
−	1[a]	72	
+	14	0	4–6 mo
−	0	35	
+	18	0	7–9 mo
−	0	30	
+	14	0	10–12 mo
−	0	22	
+	7	0	13–15 mo
−	0	21	
+	54	0	>15 mo
−	0	57	

[a] This specimen tested PCR positive on repeat testing.

DISCUSSION

The results of the double-blind study reported here demonstrate that the Amplicor HIV-1 kit can be used to identify HIV-1 proviral sequences in perinatal DBS specimens with a high degree of sensitivity and specificity. The ability to rapidly screen large numbers of blood spots represents a major advance over time-consuming radiometric DBS PCR methods (25, 36). The microwell assay is easy to perform, involves few manipulations, uses minimal amounts of whole blood (approximately 3 μl per PCR test), eliminates the need for gel analysis and Southern blotting, and takes advantage of automated equipment (plate washers, enzyme-linked immunosorbent assay readers) that is readily available in many clinical and public health laboratories.

In the present study, with one exception at day 47, the rates of successful HIV-1 DNA detection in infected infants during early infancy were 100% after day 15, 88.9% between days 10 and 15, and 27.3% in the interval between birth and 4 days of life. Comparative testing indicated that the sensitivity of the microwell assay exceeded that of the radioisotopic method (Table 1) and was equivalent to the levels of HIV-1 detectability reported in several published studies. In one DBS-based study, Yourno et al. (35) reported the detection of HIV-1 DNA in 100% of five infected infants who ranged in age from 8 weeks to 7 years, while in a second study, Comeau et al. (9) were able to identify HIV-1 proviral sequences in 55% of 42 DBSs from neonates. The report of a third study, involving

TABLE 3. HIV-1 detection in infected newborns over time

Infant no.	Age	Result after amplification reaction:	
		1	2
1	24 h	–	–
	3 days	–	–
	15 days	+	+
	30 days	+	+
	6 mo	+	+
2	24 h	–	–
	2 mo	+	+
	4 mo	+	+
	6 mo	+	+
	9 mo	+	+
	12 mo	+	+
3	24 h	+	+
	13 days	+	+
4	3 days	–	–
	30 days	+	+
	4 mo	+	+
	6 mo	+	+
	9 mo	+	+
	12 mo	+	+
5	3 days	–	–
	15 days	+	+
	30 days	+	+
	6 mo	+	+
6	24 h	–	–
	14 days	+	+
	30 days	+	+
7	24 h	–	–
	15 days	–	–
	36 days	+	+
8	2 days	+	+
	11 days	+	+
	30 days	+	+
9	4 days	–	–
	30 days	+	+
10	4 days	+	+
	15 days	ND[a]	ND
11	10 days	+	+
	6 mo	+	+
	9 mo	+	+
	21 mo	+	+
12	15 days	+	+
	5 mo	+	+
	7 mo	+	+
	8 mo	+	+

[a] ND, not done.

viral culture and conventional PCR performed on mononuclear cell lysates, indicated that only 5 of 50 (10%) infected infants were PCR positive for HIV-1 at birth, whereas 16 of 50 (32%) were identified as HIV-1 positive by PCR or culture at 4 to 9 weeks of age (18).

It is conceivable that in many newborns the virus exists in an extrachromosomal form (RNA or cDNA) and becomes integrated only following delivery and immune activation of cells (20, 33). This concept is consistent with the pronounced increase in detectable proviral DNA that we observed between days 4 and 15 and suggests that, following delivery, there is a burst of viral replication similar to that observed in acute-phase seroconverters (11, 37). Studies to quantitate and further define the nature (RNA, cDNA, proviral DNA) of the viral burden in the newborn period are in progress. As described previously, the high degree of specificity and the avoidance of false-positive results is presumably due to the use of AmpErase containing uracil *N*-glycosylase, which provides an effective safeguard against carryover contamination (4, 21).

It has been suggested that the split HIV-1 detection patterns observed at birth may be related to the timing of transmission. Infants who test PCR (or culture) positive during the first 48 h of life may have acquired their infections in utero, while those infants who test negative at birth and who are positive after 7 days may have become infected during labor and delivery, through exposure to contaminated maternal blood or vaginal secretions (15, 26). As a direct corollary, it has also been suggested that infants infected in utero would be more likely to show rapid disease progression (3, 9). To date, however, the total number of infants tested in the newborn period has been too small to discriminate between these possibilities. The availability of a DBS-based screening assay would allow for the systematic and organized collection of large numbers of perinatal specimens and would provide a potentially powerful tool for addressing these unresolved issues. The ability to collect large numbers of samples in an unbiased manner is also important for monitoring the changing dynamics of the HIV-AIDS pandemic in children, determining precise vertical transmission rates in different populations over time, and assessing the effects of therapeutic and preventive intervention strategies. Some of the biases and logistical problems of perinatal sample collection could be overcome by incorporating DBS sampling into sentinel surveillance programs (17, 32). Infants who tested positive for the presence of maternal HIV-1 antibodies could then be retested by PCR at birth and at 15 days and 30 days of age to determine their true infection status.

In summary, the DBS microwell assay described in this report, in combination with adequate controls and a quality assurance program, shows significant promise of becoming a routine perinatal screening assay. Early diagnosis and then careful monitoring are critical for early and more efficacious treatment and improved clinical management of HIV-1-infected infants. At present most of the world's HIV-1-infected children are not diagnosed until after the development of clinical symptoms. If recent therapeutic gains are to be translated into improved quality of life, these infants will need to be diagnosed earlier, before the onset of disease (15). It is of note that even simple measures such as basic care and support (especially nutrition) can have a significant positive impact on the duration and quality of life for a child infected with HIV-1 (24). If DBS PCR is to be applied internationally for diagnostic purposes and to provide a background for surveillance monitoring and evaluating preventive strategies, the assay(s) will have to recognize most, if not all, of the global variants of HIV-1. In a preliminary screen of 36 DBSs, obtained internationally, performed in collaboration with B. Weniger and C.-Y. Ou (Centers for Disease Control and Prevention, Atlanta) (34), Maria Wawer (Columbia University), and Nelson Sewankambo (Makerere Medical School, Kampala, Uganda), our microwell plate assay correctly identified 23 of 26 (88.5%) positive specimens collected from HIV-1-infected individuals in five Asian and one African country by using a single primer pair (Bio-SK462 and Bio-SK431) (unpublished data). In the

future, it should be possible to adapt this technology to heel-prick specimens (13) and to the detection of other viral pathogens that are of clinical relevance to neonates, including cytomegalovirus and hepatitis C virus, and to further increase the throughput of the microwell plate assay by interfacing it with PCR-based robotic workstations such as the Catalyst model 800 Molecular Biology Lab Station from the Applied Biosystems Division of Perkin-Elmer.

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