JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2008, p. 721–726 Vol. 46, No. 2
0095-1137/08/$08.00+0 doi:10.1128/JCM.01539-07

Early Infant Human Immunodeficiency Virus Type 1 Detection Suitable for Resource-Limited Settings with Multiple Circulating Subtypes by Use of Nested Three-Monoplex DNA PCR and Dried Blood Spots▽

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Received 2 August 2007/Returned for modification 7 November 2007/Accepted 26 November 2007

The early detection of human immunodeficiency virus type 1 (HIV-1) infection in infants is complicated by the persistence of maternal antibodies and by diverse HIV-1 subtypes. We developed a nested, three-monoplex HIV-1 DNA PCR (N3M-PCR) assay to detect diverse HIV-1 subtypes in infants born to infected mothers. We optimized the test for use with dried blood spot (DBS) samples for ease of storage and transport from rural China to central laboratories. Six pairs of primers were designed that targeted *env*, *gag*, and *pol* genes, and the test was run in three reactions with an analytical sensitivity of 10 copies DNA per reaction to cover nine HIV-1 subtypes, A, B, C, D, F, G, CRF01-AE, CRF08-BC, and CRF07-BC. The assay performance was evaluated on 347 DBS specimens from 151 exposed infants in four diverse provinces of China in which multiple subtypes were circulating. The results of this test were compared to those of HIV antibody enzyme immunoassay and Western blotting confirmation for the infants at ≥18 months of age or to convincing clinical and epidemiologic data for deceased infants. The sensitivity of the N3M-PCR assay was 30.0% (3/10) for infants at 48 h after birth, 91.7% (11/12) at 1 to 2 months of age, and 93.7% (15/16) at 3 to 6 months of age. The specificity was 100% (94/94) at all three time points. The PCR reproducibility in the three DNA regions was 100% for samples at 48 h after birth, 96.7% at 1 to 2 months, and 100% at 3 to 6 months of age. The HIV-1 DNA N3M-PCR assay on DBSs offers a simple and affordable approach for early infant HIV-1 diagnosis in regions with diverse HIV-1 circulating subtypes.

The recently reported children with HIV early antiretroviral therapy study suggests that infants infected with human immunodeficiency virus type 1 (HIV-1) should be started on antiretroviral therapy in their first 3 months of life (34a). Thus, the accurate diagnosis of HIV-1 infection in infancy becomes the first step toward effective and timely antiretroviral care, now judged to be essential even before previously recommended $CD4^+$ T-cell or viral load thresholds are reached (32). However, the early diagnosis of HIV-1 infection in infancy is not feasible without antigen testing due to the persistence of passively transferred maternal antibodies for up to 18 months after birth. Diverse HIV-1 subtypes may further limit diagnostic options, given that PCR primers may be designed to diagnose, for example, subtypes B and C (10, 28, 30). Conventional virologic tests include virus culture and isolation or the extraction of DNA from peripheral blood mononuclear cells (PBMC); these are costly, are time-consuming and labor-intensive, and do not lend themselves to easy collection, storage, and shipment. Technical difficulties arise with venipuncture in young infants, and the onsite preparation of lymphocyte pellets in rural settings is both impractical and unaffordable, even in rich nations. False-negative results from traditional DNA PCR assays have been reported due to the failure of primers designed for B subtypes to amplify some non-B-subtype viruses (2, 14, 18, 19, 23, 24, 26). An HIV DNA PCR (version 1.5) test is available to diagnose HIV-1 subtype C virus infection at 6 weeks of age or older, but this assay has not been optimized for other subtypes (30).

In China, many disparate HIV-1 subtypes circulate, including A, B, B′ (Thai B), C, D, F, G, circulating recombinant form 01-AE (CRF01-AE), CRF07-BC, and CRF08-BC (15, 20, 33, 35, 38). In both China and other areas where multiple subtypes are circulating, an affordable, rapid, and simple method to detect disparate HIV-1 subtypes among infants born to HIV-infected mothers is needed, especially given the new paradigm for early treatment from the results of the study on children with HIV early antiretroviral therapy (34a). In this study, we designed and evaluated a nested, three-monoplex HIV-1 DNA PCR (N3M-PCR) assay with an optimized DNA extraction procedure using dried blood

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▽ Published ahead of print on 12 December 2007.

TABLE 1. Primers, cycling conditions, and fragment sizes for the N3M-PCR assay

Primer	Sequence	Cycling conditions	No. of cycles and final step (if any)	Fragment size (bp)
Gp40F-1	5′TCTTAGGAGCAGCAGGAAGCACTATGGG	94°C for 5 min, 50°C for 1 min, 72°C for 2 min	1	583
Gp41R-1	3′AACGACAAAGGTGAGTATCCCTGCCTAA	94°C for 30 s, 50°C for 30 s, 70°C for 1 min	30; 10 min at 72°C	
Gp46F-2	5′ACAATTATTGTCTGGTATAGTGCAAC AGCA	94°C for 2 min, 50°C for 50 s, 72°C for 1 min	1	460
Gp47R-2	3′TTAAACCTATCAAGCCTCCTACTATCATTA	94°C for 30 s, 50°C for 30 s, 70°C for 1 min	35; 10 min at 72°C	
Gag f-2	5′ATGGGTGCGAGAGCGTCARTATTA A	94°C for 5 min, 52°C for 1 min, 72°C for 2 min	1	1,242
Gag e-2	3′TCCAACAGCCCTTTTTCCTAGG	94°C for 30 s, 52°C for 30 s, 70°C for 1 min	30; 10 min at 72°C	
306	5′GGGAAA AAATTCGGTTAAGGCC	94°C for 2 min, 50°C for 50 s, 72°C for 1 min	1	671
Cn-gag	3′TAGTTCCTGCTATRTCACTTCC	94°C for 30 s, 52°C for 30 s, 70°C for 1 min	35; 10 min at 72°C	
Pol-1	5′CCCTACAATCCCCAA AGTCAAGG	94°C for 30 s, 50°C for 15 s, 72°C for 45 s	35; 10 min at 72°C	323
Pol-2	3′TACTGCCCCTTCACCTTTCCA			
Pol-3	5′TAAGACAGCAGTACAAATGGCAG	94°C for 30 s, 50°C for 15 s, 72°C for 30 s	30; 10 min at 72°C	175
Pol-4	3′GCTGTCCCTGTAATA AACCCG			
β-actin-1	5′ACACAACTGTGTTCACTAGC	94°C for 30 s, 50°C for 15 s, 72°C for 45 s	35; 10 min at 72°C	300
β-actin-2	3′TGGTCTCCTTAAACCTGTCTTG			

spot (DBS) samples for the early diagnosis of HIV-1 infection among infants vertically exposed to HIV.

MATERIALS AND METHODS

Sample collection. Serial DBS samples (n = 347) were collected from 151 infants born to HIV-1-infected mothers in four geographically diverse regions of China through the HIV-1-infected mothers' vertical transmission to children prospective cohort study, which was conducted by the China Center for Disease Control and Prevention (China CDC) from January 2005 to December 2006. EDTA-anticoagulated blood specimens were collected, from which 70 to 80 μl of whole blood was applied to filter paper (S&S 903; Whatman, New York, NY) and dried. DBS samples were mailed to the National AIDS Reference Laboratory of the China CDC using the national postal service.

DBS DNA extraction. A 6-mm-sized DBS specimen was detached by an ethanol-flamed punch and transferred into a 1.8-ml screw-cap tube. The DBS specimen was washed twice using deionized water and then was suspended with 10% Chelex 100 resin (lot no. 95577; Sigma). After a 2-h incubation at 56°C, the mixture was boiled for 8 min and centrifuged at 12,500 × g for 3 min. The supernatant was collected and stored at −20°C for PCR amplification.

Primer design and selection. Six pairs of primers were designed on the basis of the conserved regions in HIV-1 *env*, *gag*, and *pol* genes (34, 35, 37). Consensus sequences from the three genomic regions were found to be sufficiently complementary to cover the major HIV-1 subtypes circulating in the world, including those in China (A, B, Thai B [B′], C, D, F, G, CRF01-AE, CRF07-BC, and CRF08-BC). Each pair of primers was validated separately by a panel of plasmids containing cloned HIV-1 *env* and *gag* fragments of representative subtypes and CRFs. The plasmids included A1 (PVJ310), AE (PTN238), B2 (PUG280), B1 (PTB132), C (PUG268), D (PK31), F (PBZ162), G (PLBU21-7), H (PUI525), and J (PE1973) and were provided by the NIH AIDS Research and Reference Reagent Program; plasmid stock for CRF07-BC also was used, and it was provided by the National AIDS Reference Laboratory of the China CDC. Primers of the *pol* fragment were described previously (34). The concentration of the plasmid PBH10 solution containing nearly full-length HIV-1 genomes was quantified by UV-visible spectroscopy. The PBH10 solution was diluted 10-fold to cover a test range of 10^1 to 10^9 copies per reaction for determining the relevant analytic sensitivities of the selected primers.

N3M-PCR. The N3M-PCR was performed on a GeneAmp PCR system 9600 (Perkin-Elmer, Waltham, MA). The outer PCR mixture consisted of 5 μl of 10× PCR buffer; 3 μl of 25 mM $MgCl_2$; 1 μl of 10 mM deoxynucleoside triphosphates; 0.5 μl of 5 U/μl *Taq* polymerase enzyme (Takara *Taq*; DaLian, People's Republic of China); 1 μl of each (20 mM) outer primer for the *gag*, *env*, and internal control β-actin genes; and 28.5 μl of sterile deionized water. Five microliters of outer PCR product was added to 45 μl of the inner PCR mixture, which consisted of 5 μl of 10× PCR buffer, 3 μl of 25 mM $MgCl_2$, 1 μl of 10 mM deoxynucleoside triphosphates, 0.5 μl of 5 U/μl *Taq* polymerase enzyme (Takara *Taq*), 1 μl of each 20 mM outer primer for the *gag* and *env* gene, and 33.5 μl of sterile deionized water. The outer and inner PCR master mix of the *pol* region was almost the same as that for the *env* and gag regions, except that 2 μl of 25 mM $MgCl_2$ and 28.5 and 33.5 μl of sterile deionized water were added separately. N3M-PCR products and DNA molecular weight markers were visualized after electrophoresis on an ethidium bromide-stained 1% agarose gel using a UV transilluminator at 300 nm. To test for each extract as an internal control, the human β-actin gene was amplified in a single PCR assay with predesigned primers, β-actin-1 and β-actin-2, under the same conditions as those described above for the outer *pol* fragments (Table 1). As an external control, N3M-PCRs were performed on DBS-positive and -negative control samples provided by the U.S. Centers for Disease Control and Prevention Proficiency Testing Program for DBS-Based Infant Diagnostics. DBS-positive controls were prepared using an 8E5 cell line (which contains a single integrated copy of proviral DNA) diluted in HIV-1-negative human blood samples to reach positive concentrations. DBS-negative controls were prepared using HIV-1-seronegative blood samples.

Determination of infant HIV-1 infection by N3M-PCR. DBS samples collected from the infants were tested by the N3M-PCR assay. If a β-actin fragment was not present, the PCR result for the sample was scored as indeterminate for HIV-1 and the DNA extraction was repeated. If the β-actin gene was present, the results of PCR were interpreted as follows: (i) if more than two regions of the *pol*, *gag*, and *env* genes were positive (i.e., amplified), the samples were identified as HIV-1 positive; (ii) if only one region was positive, the results were reported as indeterminate for HIV-1; (iii) if none of the three viral regions was amplified, the samples were considered HIV-1 negative. All tests detecting HIV-1 DNA-indeterminate and -positive samples were repeated once by N3M-PCR assay. If there was a discordant result between the original and repeated samples from the

FIG. 1. Each pair of primers of the *env* (left) and *gag* (right) fragments was validated separately by a panel of plasmids containing cloned HIV-1 *env* and *gag* fragments from clinical adult samples representing subtypes A, B(B1), C, D, F, G, H, J, CRF01-AE, and CRF07-BC. The duplex bands in lanes G, F, D, and B1 of the ENV panel were caused by plasmids that were not digested using restriction enzymes before the PCR was performed. The first lane in the left panel and last lane in the right panel are the molecular size markers.

same age, the infant had another DBS sample taken for definitive confirmation. Dual concordant results by PCR were classified as HIV infection.

Validation standard. Infants were determined to be definitively HIV-1 infected when (i) both HIV antibody enzyme immunoassay (Organon Teknika, Boxtel, The Netherlands) and Western blotting (Genelab, Singapore) confirmatory tests were positive for the follow-up infant samples at ≥18 months of age or (ii) if they met rigorous clinical and epidemiologic criteria for pediatric AIDS in the case of infants who died prior to the 18-month PCR assessment. All infants who were breastfed after birth ($n = 2$) were excluded from our study; China encourages replacement feeding for all infants of HIV-infected women, since formula is supplied and access to clean water is facilitated by the provincial health authorities. For the infants who were retested at ≥18 months of age, any infant with two negative results by enzyme immunoassay was determined to be HIV negative.

N3M-PCR assay reproducibility. DBS samples ($n = 32$) were randomly selected to be tested in the three genomic regions, and the tests were repeated three times to assess the N3M-PCR assay's reproducibility.

Statistical analyses. All statistical analyses used SPSS/PC+ software, version 13.0 (SPSS Inc., Chicago, IL), and Microsoft Excel (Microsoft Corporation, Redmond, WA). The sensitivity and specificity were calculated from appropriate two-by-two tables.

Ethical considerations. This study was approved by the institutional review boards of the CDC of China and Vanderbilt University. Informed consent was received from all parents of the infants before the samples were collected.

RESULTS

Analytic sensitivity of the N3M-PCR assay. The analytic detection sensitivity of the N3M-PCR assay for each pair of primers based on HIV-1 *env*, *gag*, and *pol* regions reached 10 copies of HIV-1 DNA per reaction. Three sets (Table 1) were able to amplify the targeted regions of HIV-1 subtypes A, B, C, D, F, G, CRF01-AE, CRF08-BC, and CRF07-BC. A panel of plasmids containing cloned full-length HIV-1 or *env* and *gag* fragments was amplified by *env* and *gag* primers (Fig. 1).

HIV-1 infection status among infants determined by the validation standard. We monitored 106 infants from birth to the age of definitive diagnostic assessment (≥18 months of age), and 4 infants died due to clinically and epidemiologically confirmed AIDS. Sixteen infants were diagnosed with HIV-1 infection by our validation standards, 94 infants seroreverted and were considered HIV-1 negative, and 41 infants who were seen from birth but were <6 months of age at the end of the study were classified as indeterminate for HIV-1 infection. Two mother-infant pairs did not receive any treatment due to their having delivered at home; among the other 14 mother-infant pairs, six mothers did not receive a single dose of nevirapine (or any other antiretroviral drug), while eight mothers and all 14 infants received one dose of nevirapine (L. Wang, unpublished data).

Sensitivity of the N3M-PCR assay. To determine the sensitivity of the N3M-PCR assay, the combined results from the three regions were compared to the results of the validation standard. Of the 16 infants, 10 were sampled at 48 h after birth, 12 at 1 to 2 months of age, and all 16 at 3 to 6 months of age. Among the HIV-infected infants sampled at 48 h after birth, 30.0% (3/10) of the HIV-positive samples were detected by N3M-PCR. For HIV-infected infants sampled at 1 to 2 months of age, 91.7% (11/12) of the samples were positive by N3M-PCR. For HIV-infected infants at 3 to 6 months of age, 93.7% (15/16) of the samples were positive by N3M-PCR (Table 2). The sensitivity of the PCR based on a single region was either lower than or equal to that of the combined results of the three viral regions.

Specificity of the N3M-PCR assay. All 94 HIV-1-negative infants were tested at the same three time points by PCR, and all were confirmed to be HIV-1 negative by the validation standard. When all three regions were used, the specificity of the PCR was 100% (94/94) for the infants at 48 h after birth,

TABLE 2. Sensitivity and specificity of the N3M-PCR assay on *env*, *gag*, and *pol* regions and on the three regions combined

HIV-1 gene region(s)	Result for infants aged:					
	48 h		1–2 mo		3–6 mo	
	Sensitivity[a] ($n = 10$)	Specificity[b] ($n = 94$)	Sensitivity ($n = 12$)	Specificity ($n = 94$)	Sensitivity ($n = 16$)	Specificity ($n = 94$)
env	40.0	100	91.7	98.9	87.5	100
gag	30.0	100	75.0	100	75.0	100
pol	50.0	100	66.7	100	87.5	98.9
Three regions combined[c]	30.0	100	91.7	100	93.7	100

[a] Sensitivity is derived from the number of HIV-1-positive infants determined by the N3M-PCR assay divided by the number of HIV-1-positive infants determined by the validation standard.

[b] Specificity is derived from the number of HIV-1-negative infants determined by the N3M-PCR assay divided by the number of HIV-1-positive infants determined by the validation standard.

[c] Combined results from all three regions were interpreted as follows: (i) if more than two regions of the *pol*, *gag*, and *env* genes were positive, the samples were determined to be HIV-1 positive; (ii) if only one region was positive, the results were reported as indeterminate for HIV-1; moreover, HIV-1 DNA samples determined to be indeterminate or positive were retested by the N3M-PCR assay; (iii) if none of the three regions was amplified, the samples were considered HIV-1 negative.

TABLE 3. Reproducibility of the N3M-PCR assay for detecting HIV-1 infection among infants in different age groups

HIV-1 gene region(s)	Results for infants aged[a]:											
	48 h (n = 7)				1–2 mo (n = 10)				3–6 mo (n = 15)			
	1st test	2nd test	3rd test	Reproducibility (%)	1st test	2nd test	3rd test	Reproducibility (%)	1st test	2nd test	3rd test	Reproducibility (%)
env	7	7	7	100	10	10	10	100	14	14	15	95.4
gag	7	6	7	95.2	10	9	10	96.7	15	15	13	95.6
pol	7	7	7	100	10	9	9	93.3	15	12	13	88.9
Three regions combined[b]	7	7	7	100	10	9	10	96.7	15	15	15	100

[a] Values in the 1st test, 2nd test, and 3rd test columns indicate the numbers of samples with identical results. The reproducibility values are derived from the average number of samples with identical results for three repeated testings divided by the number of samples used for reproducibility testing.

[b] Combined results from all three regions were interpreted as follows: (i) if more than two regions of the *pol*, *gag*, and *env* genes were positive, the samples were determined to be HIV-1 positive; (ii) if only one region was positive, the results were reported as indeterminate for HIV-1; moreover, HIV-1 DNA samples determined to be indeterminate or positive were retested by the N3M-PCR assay; (iii) if none of the three regions was amplified, the samples were considered HIV-1 negative.

at 1 to 2 months, and at 3 to 6 months. This also was true when only one primer set was tested, either *env* or *pol*, except that at 1 to 2 months the test with the *env* primer set gave one false-positive result (98.9%), and at 3 to 6 months the *pol* primer set gave one false-positive result; both of these results were very weak positives (Table 2).

Reproducibility. The reproducibility of the N3M-PCR assay was 100% at 48 h after birth, 96.7% at 1 to 2 months, and 100% at 3 to 6 months for detecting HIV-1 infection based on the three regions combined (Table 3).

DISCUSSION

This study is the first to assess the performance of a novel HIV-1 N3M-PCR assay for diagnosing HIV-1 infection in infants from DBS. It performed very well in China, where diverse HIV-1 subtypes are circulating (15, 20, 33, 35, 38). Such tools are vital to improve the relevance and affordability of fledging infant diagnostic programs emerging in rural China and elsewhere.

It is understandable that our assay sensitivity was only 30.0% for infants at 48 h after birth. All antigen-based tests underestimate the infection rates of children <6 weeks of age. The sensitivities of the test when all three genomic regions were amplified were 91.7% for 1- to 2-month-old infants and 93.7% for 3- to 6-month-old infants, which are higher than the sensitivities obtained when only one or two genomic regions are amplified. The specificity of the N3M-PCR assay based on the three regions combined was 100% at all three time points and was slightly higher when one or two primer sets were used. The reproducibility of the PCR was excellent (>95%). The persistence of passively maternal antibodies for up to 18 months after birth has been the challenge for the early diagnosis of HIV-1 infections in infancy (28). The performance of this assay is comparable to those of other virological assays, including other HIV-1 DNA nested PCRs (3, 5, 27, 30).

The prevalence of HIV infection has continued to rise globally. The variation among HIV strains will increase as additional mosaic strains are discovered. Conventional PCR assays were developed and optimized for use with certain subtypes, but they potentially could result in false-negative findings when used for other virus subtypes (2, 14, 18, 19, 23, 24, 26). This N3M-PCR assay incorporates three sets of primers carefully selected from the conservative regions of *env*, *gag*, and *pol* from multiple subtypes of HIV-1 based on data from the Los Alamos National Laboratory database (http://www.hiv.lanl.gov). Consensus sequences from the three sets of primers were found to be sufficiently divergent and representative of the subtypes of HIV-1 prevalent in China and various other areas of the world. Hence, such an assay is likely to perform similarly among populations infected with different subtypes and can be used in China and worldwide.

In 2004, the U.S. Public Health Service recommended that diagnostic testing should be performed before the infant is aged 48 h, 1 to 2 months, and 3 to 6 months. Additional testing at 14 days might allow for the early detection of infection. HIV DNA PCR is the preferred virologic method for diagnosing HIV infection during infancy (http://aidsinfo.nih.gov/ContentFiles/PediatricGuidelines.pdf). We followed these guidelines in our selection of sampling times for our study, permitting comparisons between our research and other research in this area.

The implementation of HIV DNA PCR diagnosis faces challenges due to the uneven economic development in China. In the remote poor rural areas, vertically exposed infants still were diagnosed at 15 months of age using HIV-1 antibody testing because of the lack of available laboratories for early diagnosis. In contrast, in the more urban and/or prosperous eastern provinces of China, HIV DNA PCR diagnosis has been widely available. This disparity is what motivated us to find a reliable technique covering all of the circulating subtypes and using DBSs suitable for collection, storage, and shipment even from the most remote rural areas.

The use of DBS samples to detect HIV-1 infection by PCR was first demonstrated in 1991 (9). Multiple replicate testing of samples and specialized filter paper types have been used to improve the accuracy of the PCR testing for HIV DNA (4). DBS samples collected from infant diagnosis programs have been used to determine maternal HIV seroprevalence rates, estimate maternal-infant HIV transmission rates, measure viral loads, and perform drug resistance genotyping (4, 13, 22). DBS samples carry less of a biohazard risk than liquid samples and require minimal storage facilities, since the samples are stable at room temperature for prolonged periods and are easy to ship, facilitating centralized laboratory testing in developed regions. Centralized testing creates economies of scale and is

an especially attractive approach in any country with a functioning postal system.

We doubt that antiretroviral treatment affected our results. Two mother-infant pairs were not treated at all due to home delivery. Fourteen infants and eight mothers received a single dose of nevirapine, while six mothers did not received nevirapine (L. Wang, unpublished). During the life cycle of HIV-1 after its entry into human target cells, the virus is integrated into the host cell chromosome by a reverse transcription step with the synthesis of complementary HIV-1 proviral DNA and viral integrase. The integrated proviral HIV-1 DNA genome induces productive replication through the transcription of the full-length viral RNA genome, enabling the manufacture and subsequent transmission of HIV-1 virions (31). Even though highly active antiretroviral therapy can inhibit HIV-1 replication to the extent that the plasma viremia level falls below the detectable threshold of HIV-1 RNA copies (<50 copies/ml) (16, 25), HIV-1 proviral DNA persists in PBMC and in lymph nodes (7, 12). Therefore, receiving antiretroviral treatment lowers the maternal viral load and would be expected to lower the infant's plasma viral load, but it would not be expected to affect the detection of proviral DNA in infants infected in utero or intrapartum.

Reports of spontaneous HIV-1 clearance or transient HIV-1 infections have been published since 1988 (6, 11, 21). An infant with a transient infection is defined as an infant with one or more positive cultures or PCR assays for HIV-1 followed by a subsequent inability to detect the virus in specimens on multiple occasions, an infant who has undergone seroconversion, or both (14). Hypotheses formed to explain HIV-1 clearance include infections due to virus with less pathogenic attenuated strains, virus with replicative defects, very effective local mucosal immunity and/or highly effective natural immunity, and even the presence of a hidden HIV-1 infection that eludes antigen-based detection and that does not elicit a measurable antibody response (17, 29). The weight of the evidence suggests that transient HIV-1 infection in children born to infected mothers has occurred but is very rare (1, 14). These alleged transient infections have occurred with and without the use of antiretroviral therapy (6, 14).

With the carefully selected primers and optimized DNA purification procedure, this N3M-PCR assay detects 10 copies of HIV-1 DNA. The HIV-1 DNA copy number varies greatly in infected humans but trends higher in perinatally infected infants than in adults. Younger children at ≤2 years of age have significantly higher cell-associated HIV-1 DNA loads (mean, 75.4 ± 104.3 copies/10^3 PBMC) than children >5 years old (mean, 13 ± 17.8/10^3 PBMC), regardless of disease status (1). Assuming that healthy children at ≤2 years of age have 5 to 6 million PBMC/ml of blood (8) and each 6-mm filter paper punch holds 5 μl of blood, the N3M-PCR assay incorporating both DBS sample collection methods and the optimized DNA purification procedures has the volume and sensitivity to diagnose nearly all infected infants more than 6 weeks of age.

The limitations of this study include the relatively small number of infants with HIV infection used to assess this N3M-PCR assay, such that we could not assess the sensitivity and specificity of the assay for each subtype. HIV-1 subtypes of our 16 infected infants included subtypes B′, CRF01-AE, CRF07-BC, and CRF08-BC, which represent the predominant subtypes in China (15, 20, 33, 35, 36, 38). Hence, further validation with larger sample sizes and various subtypes in different settings needed before its implementation in a non-Chinese setting.

The strengths of our study include the fact that our selected primers were able to amplify fragments of 175, 671, and 460 bp from the *pol*, *gag*, and *env* regions, respectively, confirming that DNA fragments of different sizes can be amplified from DBSs. These fragments could be used for other applications, e.g., sequencing. We also optimized the DNA extraction procedure to use only two reagents (deionized water and Chelex 100 resin) that adequately released DNA within lymphocyte pellets. We suppose that the presence of the PCR inhibitors could be due to hemoglobin remaining in the pellets. We found no indeterminate samples due to the presence of PCR inhibitors. Chelex 100 resin may have eliminated potential PCR inhibitors, since it is a cation-chelating resin; positively charged ions are captured by the resin, whereas the DNA, negatively charged, remains free in the solution (13).

Since the DNA extraction method using DBS samples developed here is rapid and easy to perform, its link to the N3M-PCR assay offers the promise of an improved diagnostic approach for HIV-1 infection in infants. The HIV-1 N3M-PCR assay circumvents the drawback associated with subtype-specific DNA PCR. The use of three sets of primers carefully selected from three genomic regions (*env*, *gag*, and *pol*) in major subtypes allowed similarly effective detection of infection with several HIV-1 subtypes. We have implemented a nested protocol with gel electrophoresis detection, a technique that merits further investigation for its potential role for user-friendly use in routine diagnosis services. Our approach may offer a simpler, more affordable, and more transportable approach to the early detection of diverse HIV-1 subtypes in infants in China and worldwide.

ACKNOWLEDGMENTS

This study was supported, in part, by the National AIDS Reference Laboratory, National Center for AIDS & STD Control and Prevention, China Center for Disease Control and Prevention, and the Yunnan, Xinjiang, Henan, Guangxi Provincial (or Autonomous Regional) Center for Disease Control and Prevention, U.S. National Institutes of Health (grants R03 AI067349, R03 AI073134, D43 TW001035-09, and P30 AI 54999), and the Vanderbilt University School of Medicine Institute for Global Health.

We acknowledge the technicians from the National AIDS Reference Laboratory, Center for Women and Children's Health, in Yunnan, Henan, Xinjiang, and Guangxi Provinces and the Autonomous Region who assisted with data collection and DBS sample collection. We thank J. E. Tillman from Vanderbilt University for manuscript assistance.

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