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Evaluation of Dried Whole Blood Spots Obtained by Heel or Finger Stick as an Alternative to Venous Blood for Diagnosis of Human Immunodeficiency Virus Type 1 Infection in Vertically Exposed Infants in the Routine Diagnostic Laboratory▽

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The diagnostic accuracy of the Roche Amplicor human immunodeficiency virus type 1 DNA PCR assay (version 1.5) on DNA extracted from pediatric heel prick dried blood spots using Roche MagNA Pure nucleic acid purification technology was evaluated. The methodologies transfer successfully from the labor-intensive research laboratory to the high-throughput automated routine laboratory.

Mortality rates for human immunodeficiency virus type 1 (HIV-1) vertically exposed infants remain unacceptably high on the African continent (12). In order to initiate medical care as soon as possible, access to an early, accurate diagnosis of HIV infection must be made available as a matter of urgency to all infants born to seropositive mothers.

The diagnostic gold standard laboratory test for HIV-1 infection is the HIV-1 qualitative DNA PCR, with sensitivities and specificities in the high 90th percentile in Southern Africa (17, 18). Current South African Department of Health guidelines recommend an initial HIV DNA PCR at 6 weeks of age for all exposed babies (11). A major stumbling block to the implementation of these guidelines is the widespread lack of the expertise needed to venesect infants. This deficit is delaying efforts to fast-track early pediatric HIV-1 diagnosis in South Africa and other developing countries.

Screening technology using dried whole blood spots (DBS), used for over 50 years to screen for metabolic disorders in neonates (8, 9), has been successfully extended to PCR-based detection of human immunodeficiency virus (1, 3, 6, 13, 16). Two studies have been undertaken in Africa to examine the utility of heel prick DBS for HIV DNA PCR (6, 13). Although both have shown excellent results, the laborious methodologies used in the studies are more appropriate to the research laboratory than the routine diagnostic laboratory.

In this report, we evaluate the validity of the use of pediatric heel stick DBS to diagnose HIV-1 infection in the busy routine laboratory setting, using Roche's automated technology to extract and amplify HIV-1 DNA from DBS.

Heel stick DBS were collected at the same time as venous blood samples from 2 groups of children for routine virological assay for HIV. The larger group consisted of vertically exposed 6-week-old babies followed up at the Prevention of Mother to Child Transmission Clinic at the Coronation Women and Children's Hospital. The other group was composed of older HIV-infected children (up to 6 years) attending the Harriet Shezi Children's Clinic at the Chris Hani Baragwanath Hospital, Johannesburg, South Africa. Collection of heel stick DBS was approved by the University of the Witwatersrand Human Ethics Committee (M04-10-05), and informed consent was obtained. At least two circles of a Whatman (formerly Schleicher & Schuell, S&S) grade 903 "Guthrie" card were completely filled with capillary blood and air-dried for at least 3 h before being stored at room temperature in individual ziplock plastic bags with a desiccant sachet. DBS that contained insufficient blood or appeared "layered," crusty, clotted, or wetted were discarded (10).

The laboratory test used to determine the HIV infection status of the 6-week-old infants was a PCR assay targeting HIV DNA in liquid blood (Amplicor HIV-1 DNA assay [version 1.5]; Roche Molecular Systems, Inc., Branchburg, NJ). The viral load of the older children was monitored by HIV RNA PCR (Roche COBAS Amplicor HIV-1 monitor assay [version 1.5]) or by an NASBA (nucleic acid sequence-based amplification) EasyQ HIV-1 RNA assay (Nuclisens; bioMérieux, Boxtel, The Netherlands).

One whole blood spot (approximately 50 μl) was completely excised from the Whatman 903 card and proviral DNA extracted from the DBS using a MagNA Pure LC DNA isolation kit III (Roche Molecular Systems, Branchburg, NJ) protocol with some modifications for DBS. After initially using scissors to remove the DBS from the card and cut it into quarters, we found that using an ordinary paper punch cleaned by punching twice through a clean card was a much quicker, less tiring option for the technologist, with no greater risk of cross contamination (unpublished data).

Using forceps disinfected with a 2% solution of Virkon and dried on paper, the excised pieces of a DBS were transferred to a labeled 2-ml Sarstedt tube. Total hands-on time for

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DBS excision from 30 cards was 45 min. A working lysis buffer (LB) was prepared from the bacterial lysis buffer supplied with the DNA isolation kit III by mixing 260 μl with 40 μl prepared proteinase K solution, also from the DNA kit III, per test. One microliter of supplied internal control per 300 μl was added to the working LB solution to identify processed samples containing substances that could interfere with PCR amplification. To each tube containing a DBS, 300 μl working LB was added and the tube was incubated at 65°C for 10 min, vortexed gently, and incubated at 95°C for a further 10 min. Following centrifugation at 1,500 × *g* for 1 min, 150 μl of lysate was transferred to a MagNA Pure sample cartridge, with care taken to avoid contact with the filter paper. Sample cartridges containing 30 tests plus 2 controls were placed in the MagNA Pure LC instrument, and the standard DNA I high performance protocol was followed, with the exception that no further proteinase K was added for extraction purposes. Automated DNA isolation and purification time for 30 samples and 2 controls was 85 min.

HIV DNA PCR was performed in the routine molecular diagnostic laboratory in Johannesburg, South Africa, by technologists blinded to the HIV infection status of the infants. Fifty-microliter aliquots containing DNA extracted from the DBS were subjected to qualitative HIV DNA PCR using the Roche Amplicor HIV-1 DNA assay (version 1.5). The normal quality control program used for the assay on whole blood was included. In line with the laboratory's routine testing algorithm, all positive and equivocal results were repeated. The cutoff optical density (OD) readings for negativity and positivity were 0.2 and 1.5, respectively, at a 450-nm wavelength. OD readings between 0.2 and 1.5 were considered equivocal or inconclusive. The threshold OD values of $\geq$0.2 and $<$0.8 recommended in the kit insert have been modified and validated by the routine molecular laboratory to take into account our specific population and local conditions. This policy widens the "safety net" to ensure fewer false positive results are reported and increases the number of equivocal results when a repeat specimen is requested for testing. A repeat specimen is also requested from patients with initial positive results on specimens that retest negative.

A total of 206 heel stick DBS were collected, of which 161 were from approximately 6-week-old infants (median age, 47 days; range, 34 to 81 days) and 45 from older children (median age, 18 months; range, 3 to 72 months). On the basis of clinical assessment and an HIV DNA PCR test on venous blood, 12 of the 6-week-old babies were diagnosed HIV seropositive, giving a prevalence rate of 7.45% for this group. To more accurately assess the sensitivity of the testing procedures on heel prick DBS, 45 older HIV-infected children were included in the study, bringing the overall prevalence to 27.7%. One hundred forty-nine children were diagnosed as HIV-1 uninfected.

With one persistently false negative result, the sensitivity of the testing procedures (calculated as the number of true positive results divided by the sum of true positive and false negative results, expressed as a percentage), was 98.28%. However, a second heel prick DBS collected subsequently from the same child tested positive, so the possibility of a specimen mix-up cannot be excluded. Although there was only one false-negative result in this study, subsequent work has shown a low but persistent incidence of false negatives following MagNA Pure extraction of DNA from DBS compared with results from manual extraction. This has been observed by others extracting from EDTA-anticoagulated plasma and whole blood (14) and is currently under investigation by this laboratory. With two false-positive results on first testing, the diagnostic specificity (calculated as the number of true negative results divided by the sum of true negative and false positive results, expressed as a percentage), was 98.68%. However, both DBS retested negative, giving an effective specificity of 100%. Because the prevalence was artificially manipulated, likelihood ratios rather than the positive and negative predictive values were used to examine the validity of the testing procedures for diagnostic purposes. A positive likelihood ratio (sensitivity/1 − specificity) greater than 10 strongly suggests that a positive test result indicates disease, whereas a negative likelihood ratio (1 − sensitivity/specificity) of less than 0.1 virtually rules out the chance that the patient has the disease (information found at the Oxford Centre for Evidence-Based Medicine website, http://www.cebm.net/likelihood_ratios.asp).

Our positive and negative likelihood ratio values of 74.2 and 0.0175, respectively, strongly support the validity of the Roche Amplicor HIV-1 DNA assay (version 1.5) for diagnosis of HIV infection in children by detection of DNA isolated from heel stick DBS with the Roche MagNA Pure technology.

These results are in agreement with the findings of Nyambi et al. (13) in Kenya, which showed a sensitivity from 95% to 100% and a specificity of 100% for heel stick DBS. However, an in-house nested PCR was used to detect HIV-1 proviral DNA, and the manual DNA extraction method was complex and lengthy, rendering both these protocols unsuitable for use in the busy routine laboratory. In Rwanda, where HIV infection is predominately by subtype A virus, Fischer et al. (6) demonstrated the efficiency of the Roche Amplicor assay (version 1.5) on heel stick DBS for the diagnosis of HIV infection in vertically exposed children. However, their DNA isolation method, using Chelex-100 resin, is too labor-intensive to be practical for high volumes in the routine laboratory, in contrast to the automated MagNA Pure protocol adapted for DBS that was utilized in this study. One constraint to the use of automated technology to analyze DBS is the labor- and time-consuming process of excising the blood spots. The introduction of an automated punch, currently being validated, should appreciably improve the efficiency of DBS processing.

In South Africa, the Amplicor assay has proved 100% sensitive and 99.6% specific on venous DBS, applied to Whatman No. 1 filter paper, from 288 infants perinatally exposed to HIV subtype C virus (16). These results highlight the success of version 1.5 of the Roche Amplicor DNA-1 qualitative assay in its ability to detect non-subtype B virus (4, 18), responsible for the majority of HIV infections in Africa and other developing countries which bear the brunt of HIV infection worldwide (7).

Because of the persistence of maternal anti-HIV antibodies in the infant circulatory system, the HIV antibody enzyme-linked immunosorbent assay can only be used at 12 to 18 months of age (5), by which time more than 80% of vertically exposed children in Johannesburg, South Africa, have been lost to follow-up (15). The established reference test for the diagnosis of pediatric HIV-1 infection is HIV DNA PCR on liquid blood. However, the automated technology used to per-

form molecular virological assays for the diagnosis of HIV infection is expensive and technically complex. This being the case, these assays are performed in a few centralized laboratories in urban centers in most developing countries.

DBS on filter paper, in use by developed countries for over 50 years (8, 9), are the ideal vehicle for blood collection in countries where the expertise needed to venesect infants is in short supply. The skills required for proper collection of DBS from infants by heel stick are quickly learned by unskilled workers following appropriate training. Other advantages of DBS collection by heel stick over liquid blood collection include reduced biohazard status once dry, no need for further processing in the field, and HIV-1 DNA stability on filter paper, which eliminates the need for refrigeration (2, 8). Importantly, they can be easily transported via the postal system to a reference laboratory for molecular virological testing. In this study, we show that heel stick DBS provide an accurate, reliable means for HIV detection using Roche's automated technology. Data on the efficiency of MagNA Pure's DNA extraction from DBS will be available in the coming months. Once this issue and that of manual DBS processing have been addressed, DBS can effectively substitute for liquid blood in a busy centralized laboratory.

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