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Early Diagnosis of *In Utero* and Intrapartum HIV Infection in Infants Prior to 6 Weeks of Age

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Early initiation of antiretroviral therapy reduces HIV-related infant mortality. The early peak of pediatric HIV-related deaths in South Africa occurs at 3 months of age, coinciding with the earliest age at which treatment is initiated following PCR testing at 6 weeks of age. Earlier diagnosis is necessary to reduce infant mortality. The performances of the Amplicor DNA PCR, COBAS AmpliPrep/COBAS TaqMan (CAP/CTM), and Aptima assays for detecting early HIV infection (acquired *in utero* and intrapartum) up to 6 weeks of age were compared. Dried blood spots (DBS) were collected at birth and at 2, 4, and 6 weeks from HIV-exposed infants enrolled in an observational cohort study in Johannesburg, South Africa. HIV status was determined at 6 weeks by DNA PCR on whole blood. Serial DBS samples from all HIV-infected infants and two HIV-uninfected, age-matched controls were tested with the 3 assays. Of 710 infants of known HIV status, 38 (5.4%) had *in utero* ($n = 29$) or intrapartum ($n = 9$) infections. By 14 weeks, when treatment should have been initiated, 13 (45%) *in utero*-infected and 2 (22%) intrapartum-infected infants had died or were lost to follow-up. The CAP/CTM and Aptima assays identified 76.3% of all infants with early HIV infections at birth and by 4 weeks were 96% sensitive. DNA PCR demonstrated lower sensitivities at birth and 4 weeks of 68.4% and 87.5%, respectively. All assays had the lowest sensitivity at 2 weeks of age. CAP/CTM was the only assay with 100% specificity at all ages. Testing at birth versus 6 weeks of age identifies a higher total number of HIV-infected infants, irrespective of the assay.

Human immunodeficiency virus (HIV) infection is a significant cause of pediatric morbidity and mortality in resource-limited settings, where the majority of HIV infection occurs. The CHER study demonstrated that early initiation of highly active antiretroviral therapy (HAART) at a median age of 7 weeks reduces infant mortality and HIV progression by 76% and 75%, respectively (21). However, early initiation of treatment is dependent on an early diagnosis of HIV infection. The identification of HIV-exposed infants for testing and the diagnosis of pediatric infection remain challenges in resource-limited settings. Strategies to implement accurate and timely diagnostic testing are critical to improve infant outcomes.

Prevention of mother-to-child transmission (PMTCT) guidelines in South Africa stipulate that diagnostic testing in infants be performed at 6 weeks of age because this coincides with the 6-week immunization visit and detects virtually all HIV infections acquired during pregnancy (*in utero* [IU]) or during labor and delivery (intrapartum [IP]) (11, 15). Testing is performed with a viral detection assay since HIV antibody detection assays cannot distinguish transplacentally acquired maternal from infant HIV antibodies. Test results are communicated to the infants' caregivers at the 10-week immunization visit. If an infant is HIV infected, additional time is required to prepare the primary caregiver to initiate HAART (11). Initiation of treatment therefore generally occurs at 12 to 14 weeks of age, too late to impact the early peak of HIV-related deaths in South Africa, centered at 8 to 12 weeks of age (1). Diagnostic testing prior to 6 weeks is thus required to initiate HAART earlier, thereby reducing infant mortality.

One of the most widely used assays for early infant diagnosis is the Amplicor HIV DNA PCR assay (Roche Molecular Systems Inc., Branchburg, NJ). Version 1 of this assay was assessed on dried blood spot (DBS) specimens from infants less than 4 days and 10 to 15 days of age (3). The sensitivities in detecting all IU and IP infections were found to be 27.3% and 88.9%, respectively, and the specificities at both ages were 100% (3). This study was subsequently included in a review by Dunn et al. in 1995, together with other studies which used in-house PCR assays on whole blood (5). The overall sensitivities of DNA PCR were found to be 38% at birth, 93% at 14 days, and 96% at 28 days (5). Subsequent studies exclusively assessing the Roche Amplicor assay on whole blood yielded birth sensitivities ranging from 38% to 62% (7, 9, 24) and sensitivities by 1 week, 2 weeks, and 1 month of age of 29%, 67%, and 67 to 92%, respectively (2, 7, 9). The assay was 100% specific using whole blood at birth (excluding cord blood) and at 1 week, 2 weeks, and 1 month of age (2, 7, 24). Antenatal zidovudine (AZT) was provided to selected women in some of these studies (7, 24). The current Roche Amplicor assay, version 1.5, was not assessed on whole blood prior to 6 weeks but has been assessed on DBS samples at 6 weeks of age, yielding a sensitivity and specificity of 100% and 99.6%, respectively (17).

Newer viral detection assays may prove more sensitive for earlier detection of HIV infection in infants. Two such technologies

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have been assessed using DBS samples from pediatric populations, namely, the Roche COBAS AmpliPrep/COBAS TaqMan (CAP/CTM) HIV-1 assay, which detects both HIV-1 RNA and DNA, and the Gen-Probe Aptima HIV-1 screening assay with an automated Tigris analyzer (18, 19). These assays were highly sensitive (99.7% and 100%, respectively) and specific (100% and 97%, respectively) at 6 weeks of age and older (18, 19). The manual version of the Gen-Probe Aptima assay using DBS samples was also sensitive and specific (99.2% and 100%, respectively) for HIV-exposed infants and children (6). Using serum samples, Aptima's specificity was 100% for HIV-exposed, uninfected infants younger than 18 months old (13). However, neither the CAP/CTM nor the Aptima assay has been evaluated for HIV diagnosis in infants before 6 weeks of age.

This study aimed to compare the performances of the Amplicor DNA PCR, CAP/CTM, and Aptima assays using DBS samples to identify IU- and IP-infected infants up to 6 weeks of age.

MATERIALS AND METHODS

A prospective, observational cohort study was conducted at Rahima Moosa Mother and Child Hospital (RMMCH) in Johannesburg, South Africa, from August 2008 to December 2010. HIV-infected women over 18 years of age, admitted to the RMMCH postnatal ward, who were identified during antenatal testing and who planned to attend RMMCH for follow-up PMTCT care were eligible to participate in the study. The Ethics Committee of the University of the Witwatersrand approved the study, and written informed consent was obtained from all mothers prior to enrollment. Mother-infant pairs enrolled in the study continued to receive the routine PMTCT care offered at RMMCH according to national PMTCT guidelines at the time. Initially, single-dose nevirapine (sdNVP) together with maternal AZT from 28 weeks' gestation and infant AZT for 1 week was the recommended PMTCT regimen. If maternal PMTCT care was suboptimal, defined as less than 4 weeks of AZT, the infant received 28 days of AZT. Women with CD4 counts of ≤200 were eligible for HAART. The PMTCT guidelines were updated during 2010 to commence maternal AZT from 14 weeks' gestation and provide HIV-exposed infants with a daily dose of NVP for 6 weeks or for the duration of breast-feeding. Women with CD4 counts of ≤350 were eligible for HAART. At the time of the study, most women at RMMCH elected to exclusively formula feed.

Infants were enrolled at birth in the postnatal ward and seen at 2, 4, 6, and 10 weeks of age. A medical practitioner examined the infant at each visit. The 6- and 10-week visits coincided with routine PMTCT care visits. Infants underwent DNA PCR testing (Amplicor HIV-1 DNA PCR, version 1.5; Roche Molecular Systems Inc., Branchburg, NJ) on peripheral blood at 6 weeks, and test results were provided to the infants' caregivers at the 10-week visit (11). Where the PCR result was negative, the mother was counseled regarding the risk of postnatal transmission of HIV and the infant discharged. Where the PCR result was positive, a confirmatory baseline viral load (VL) test and a CD4 count were performed and the infant was referred for HAART initiation.

For study purposes, DBS samples were obtained from the infants at each visit. At birth and 2 weeks, blood was collected by heel prick onto Schleicher & Schuell 903 (S&S 903 W-041) filter paper cards, and at the 4- and 6-week visits, blood was collected by venesection and spotted onto filter paper cards. Each DBS card was stored at RMMCH at ambient room temperature in a ziplock plastic bag with a desiccant sachet.

Where an infant was identified as being HIV infected by the routine 6-week PCR test, stored DBS samples from birth and 2, 4, and 6 weeks were retrieved and retrospectively tested together with samples from two age-matched, HIV-exposed, uninfected controls at the same time points. Laboratory testing was conducted by accredited hospital-based laboratories using 3 assays: HIV DNA PCR, CAP/CTM HIV-1 (version 1; Roche Molecular Systems, Inc., Branchburg, NJ), and the Aptima HIV-1 screening assay with an automated Tigris analyzer (Gen-Probe Inc., San Diego, CA). Samples were retested where discrepant or equivocal results were obtained at a single time point and additional DBS sample was available; however, only the initial test results were included in the analysis. Infants with at least 2 positive DBS test results on different assays at birth were considered to have an IU infection. IP infection was considered to have occurred where the birth results were negative, followed by at least 2 positive test results on different assays by 6 weeks of age. Postpartum (PP) transmission was documented when the birth and 6-week test results were negative, followed by a positive PCR result in the context of breast-feeding. The possibility that infants infected by early PP transmission are counted in the IP-infected group cannot be excluded.

Where an infant was lost to follow-up before undergoing routine 6-week testing at RMMCH, the National Health Laboratory Service's (NHLS) Laboratory Information System (LIS) was searched to determine whether the infant had accessed PCR testing at another facility. If the surname and date of birth matched, the PCR test result from the LIS was used to assign an HIV infection status at 6 weeks of age. At the end of the study, all birth DBS samples of infants with an unknown HIV status were tested using the Aptima assay to identify all remaining IU-infected infants in the cohort. All positive Aptima tests were confirmed with a CAP/CTM and HIV DNA PCR test. Additionally, all stored DBS samples available for enrolled infants known to have died were submitted for testing.

Statistical analyses were performed using Microsoft Excel 2010. Sensitivity [true-positive results/(true-positive + false-negative results)] and specificity [true-negative results/(true-negative + false-positive results)] were calculated with 95% confidence intervals by following the Wilson score method. Positive predictive values [true-positive results/(true-positive + false-positive results)] and negative predictive values [true-negative results/(true-negative + false-negative results)] were calculated to determine the proportion of infants with positive and negative results, respectively, who were correctly diagnosed.

RESULTS

A convenience sample of 848 mother-infant pairs was enrolled. Ten withdrew during the course of the study. Of the remaining 838 HIV-exposed infants, 128 (15.3%) were excluded from all analyses because they were lost to follow-up or died before an HIV status could be established. Of the remaining 710 mother-infant pairs, 557 (78.5%) received optimal maternal PMTCT care, defined as 4 weeks or more of AZT or HAART. sdNVP with various durations of AZT was administered to 653 (92.0%) infants, 56 (7.9%) infants received daily-dose NVP, and a single infant did not receive any PMTCT prophylaxis. Antenatal CD4 counts were available for 638 women (median count of 354.5×10^6/liter; range, 24×10^6/liter to $1{,}221 \times 10^6$/liter). A CD4 count of ≤350×10^6/liter was recorded for 316 (49.5%) women, accounting for 60.6% of vertical transmissions.

The HIV status of 606 (85%) and 85 (12%) of the 710 infants was determined by the 6-week PCR performed at RMMCH and searching the NHLS LIS, respectively. The remaining 19 (3%) infants had their HIV status established from testing stored DBS samples. Of the 710 infants with a known HIV status, 38 (5.4%) were determined to have been infected either IU ($n = 29$) or IP ($n = 9$) (Table 1). IU transmission accounted for 76.3% of infections detected in the first 6 weeks of life. Positive test results were first documented at the 4- or 6-week visit for 8 of the 9 IP-infected infants. For the remaining infant, who was reportedly exclusively formula fed, the first positive test was at 10.7 weeks of age, when the infant was admitted to RMMCH having defaulted after the birth visit. Daily-dose NVP had been administered to 2 (6.9%) IU- and no IP-infected infants. An additional 5 infants from the study cohort were diagnosed as infected by the routine HIV service but are not included in the analysis. Two of these infants who were

TABLE 1 Description of study cohort and total number of samples from HIV-uninfected and *in utero*- and intrapartum-infected infants tested with each diagnostic assay

Time of visit	Study cohort			No. of samples tested with each diagnostic assay[b]		
	Median age (wk)	Total no. of infants	No. (%) HIV infected[a]	HIV DNA PCR	CAP/CTM	Aptima
Birth	0.1	710	38 (5.4)	107	107	144[c]
2 wk	2.1	566	25 (4.4)	86	86	86
4 wk	4.6	556	25 (4.5)	88	88	88
6 wk	6.6	595	26 (4.4)[d]	85	84[e]	85
Total		710	38 (5.4)	366	365	403

[a] *In utero* and intrapartum infections. HIV infection status was determined retrospectively where the routine 6-week PCR test identified an infant as HIV infected.
[b] Samples were obtained from infants later determined to be HIV infected, together with two age-matched uninfected controls, as well as from infants who died; birth samples were also obtained from infants of an unknown status.
[c] The screen of birth samples from infants with an unknown HIV status using the Aptima assay accounts for the additional Aptima tests.
[d] Samples were not prepared for 2 HIV-infected infants who attended the 6-week visit.
[e] One 6-week sample produced an invalid CAP/CTM result and could not be retested.

reportedly breast-fed demonstrated PP transmission, as their 6-week PCR tests were negative but repeat PCR tests at 9.4 and 15.6 months of age were positive. Three other infants tested negative at birth, defaulted at 2 weeks of age, and had their first positive test at 6 months or older. Since it was unclear whether HIV transmission had occurred IP or PP, they were excluded from the cohort of IP-infected infants. Two had reportedly been breast-fed, and 1 had reportedly received mixed feeding.

Only 26 (68%) of the 38 IU- and IP-infected infants presented for routine DNA PCR testing at 6 weeks as part of the study (Table 1). Of these 26 infants, 18 were IU infected. Of the 12 infants who did not attend the 6-week visit, 11 (92%) were IU infected. By 14 weeks of age, 23 (61%) of the 38 IU- and IP-infected infants were in care to commence treatment. Of these 23 infants, 16 were IU infected. Of the 29 IU- and 9 IP-infected infants identified, 13 (45%) IU- and 2 (22%) IP-infected infants had died or were lost to follow-up before HAART could be commenced.

The sensitivities of the HIV DNA PCR, CAP/CTM, and Aptima assays in identifying IU- and IP-infected infants are depicted in Table 2. Equivocal test results were obtained only with the HIV DNA PCR assay and were omitted from this analysis. At birth, the CAP/CTM and Aptima assays identified 76.3% of all infants with early infections detectable by 6 weeks of age. The DNA PCR assay demonstrated a lower sensitivity (68.4%), missing 3 IU-infected infants. At 2 weeks, all the assays showed reduced sensitivity. Four HIV-infected infants had positive tests at birth that became negative at 2 weeks of age and became positive again at 4 weeks of age. All 4 infants had received sdNVP at birth. Two infants tested negative at 2 weeks on a single assay (*viz.*, one each on the Aptima and CAP/CTM assays), and 2 infants tested negative on 2 assays (CAP/CTM and Aptima assays in one instance and DNA PCR and CAP/CTM assays in the second). By 4 weeks, the CAP/CTM and Aptima assays demonstrated 96.0% sensitivity, missing 1 IP-infected infant who was only identifiable by any assay from 6 weeks of age. This infant could have undergone an early PP transmission but was reportedly exclusively formula fed. Furthermore, the infant received sdNVP prophylaxis, so the delay in diagnosis could not be attributed to viral suppression as a result of daily-dose NVP. At 6 weeks, all the assays demonstrated 100% sensitivity; however, the diagnosis of 2 HIV-infected infants would have been delayed with the DNA PCR assay due to equivocal results that would have required retesting.

CAP/CTM was the only assay to demonstrate 100% specificity for identifying HIV-uninfected infants of all ages (Table 2). The Aptima assay yielded 3 false-positive results that were all negative upon repeat testing. DNA PCR demonstrated the poorest specificity at all ages except birth, with 5 of the 7 false-positive results occurring on a single day. All false-positive and equivocal DNA PCR results that were retested yielded true-negative results.

DISCUSSION

The Amplicor HIV DNA PCR, CAP/CTM, and Aptima assays were assessed in infants born to a population of women predominantly infected by subtype C, with 50% having antenatal CD4 counts over 350×10^6/liter and 79% having received at least 4 weeks of antenatal PMTCT prophylaxis. Over 90% of infants in this cohort received sdNVP at birth. In this setting, 29 (76%) of the 38 HIV-infected infants diagnosed at 6 weeks of age were identifiable at birth.

The CAP/CTM and Aptima assays were more sensitive and specific than DNA PCR for identifying IU- and IP-infected and HIV-uninfected infants at any age up to 6 weeks using DBS samples. The World Health Organization (WHO) recommends testing of all HIV-exposed infants at 4 to 6 weeks of age and requires that viral detection assays have a minimum sensitivity of 95% and a specificity of 98% (23). At 4 weeks of age, the CAP/CTM and Aptima assays were 96% sensitive and 100% specific, fulfilling WHO requirements, yet failed to identify one HIV-infected infant. The sensitivity of the DNA PCR assay complied with the WHO standards only at 6 weeks of age. There were a number of false-positive DNA PCR results at all ages besides birth, yielding specificities below the WHO-recommended threshold of 98% (23). This is likely a result of contamination, which can be a consequence of the manual nature of the DNA PCR assay. However, these results are not necessarily representative, since higher specificities have previously been reported (17). The Aptima test also demonstrated false-positive results, most likely due to low-level contamination which may have occurred during the initial sample preparation, which is performed manually. CAP/CTM was the only assay with a specificity of 100% at all ages and would be the best confirmatory assay for infants under 6 weeks of age.

At birth, the CAP/CTM and Aptima assays identified 76.3% of all HIV-infected infants detectable at 6 weeks. This is higher than previous descriptions of 27.3% to 62% using earlier viral detection

TABLE 2 Performance of the HIV DNA PCR, CAP/CTM, and Aptima assays in identifying *in utero*- and intrapartum-infected and HIV-uninfected infants in the first 6 weeks of life[a]

Infection status and time of visit	HIV DNA PCR				CAP/CTM				Aptima			
	Total no. of infants tested[b]	No. with true-positive result	% Sensitivity (95% CI)	PPV (%)	Total no. of infants tested	No. with true-positive result	% Sensitivity (95% CI)	PPV (%)	Total no. of infants tested	No. with true-positive result	% Sensitivity (95% CI)	PPV (%)
Infected												
Birth	38	26	68.4 (52.5–80.9)	100	38	29	76.3 (60.8–87.0)	100	38	29	76.3 (60.8–87.0)	93.5
2 wk	25[1]	16	64.0 (44.5–79.8)	84.2	25[3]	15	60.0 (40.7–76.6)	100	25[2]	16	64.0 (44.5–79.8)	100
4 wk	24	21	87.5 (69.0–95.7)	91.3	25	24[c]	96.0 (80.5–99.3)	100	25	24[c]	96.0 (80.5–99.3)	100
6 wk	22	22	100 (85.1–100)	91.7	24	24	100 (86.2–100)	100	24	24	100 (86.2–100)	96.0
Uninfected												
	Total no. of infants tested[d]	No. with true-negative result	% Specificity (95% CI)	NPV (%)	Total no. of infants tested	No. with true-negative result	% Specificity (95% CI)	NPV (%)	Total no. of infants tested	No. with true-negative result	% Specificity (95% CI)	NPV (%)
Birth	67	67	100 (94.6–100)	84.8	69	69	100 (94.7–100)	88.5	106	104	98.1 (93.4–99.5)	92.0
2 wk	60	57	95.0 (86.3–98.3)	86.4	61	61	100 (94.1–100)	85.9	61	61	100 (94.1–100)	87.1
4 wk	62	60	96.8 (89.0–99.1)	95.2	63	63	100 (94.3–100)	98.4	63	63	100 (94.3–100)	98.4
6 wk	61	59	96.7 (88.8–99.1)	100	60	60	100 (94.0–100)	100	61	60	98.4 (91.3–99.7)	100

[a] Superscripts denote numbers of HIV-infected infants with positive tests at birth but negative 2-week results. CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value.
[b] Excluding 3 equivocal tests ($n = 1$ at 4 weeks and $n = 2$ at 6 weeks).
[c] Same IP-infected infant missed by CAP/CTM and Aptima assays at 4 weeks.
[d] Excluding 4 equivocal tests ($n = 2$ at birth, $n = 1$ at 2 weeks, and $n = 1$ at 4 weeks).

assays in populations receiving little, if any, PMTCT prophylaxis (3, 5, 7, 9, 24). Possible explanations include a reduction in IP transmission due to enhanced PMTCT prophylaxis, resulting in a proportionate increase of IU-infected infants in the first 6 weeks of life, and/or improved sensitivity of newer viral detection assays. Version 1.5 of the DNA PCR assay using DBS samples achieved a better sensitivity at birth than earlier versions of the assay (3) but is not as sensitive as CAP/CTM or Aptima at this age.

The benefits of a birth diagnosis need to be considered in the context of the high rate of loss to follow-up in routine settings where infants do not return for 6-week testing or test results (16). At RMMCH, the current 6-week testing program identified 26 HIV-infected infants, whereas testing at birth would have identified 29 HIV-infected infants without the need for an additional health care visit or risking the demise of infants prior to testing. Of the 12 (32%) HIV-infected infants who did not return for testing at 6 weeks, 11 (92%) infants could have been identified through birth testing. Whether IU-infected infants are at higher risk of rapid disease progression than IP-infected infants remains controversial (8, 12), but if true, then early identification and HAART initiation in this group of infants would be especially important in reducing infant mortality. The disadvantage of birth testing is the need for a second test for early identification of IP-infected infants, substantially increasing the costs of early infant diagnosis programs. Considering the 2010 WHO infant feeding policy of exclusive breast-feeding (22), which requires additional testing 6 weeks after weaning, IP-infected infants would be identified later in life but delayed testing will put them at increased risk of morbidity and mortality.

A limitation of this study is that the findings are skewed in favor of identifying IU- compared with IP-infected infants. *All* IU-infected infants in the cohort were identified, since birth DBS samples were available for testing. In contrast, the LIS lookup could identify only IP-infected infants who accessed diagnostic testing after defaulting from the study, and the true number of IP-infected infants is therefore unknown. However, this mimics the reality in routine settings, where IP-infected infants who default by 6 weeks do not access an early diagnosis, whereas IU-infected infants who are in the health care system at the time of birth represent missed opportunities for early identification. This assumes that the majority of births occur in health care facilities, as is the case in South Africa (20).

The lowest sensitivity in all 3 assays occurred at 2 weeks of age. We speculate that this is a consequence of the sdNVP prophylaxis administered to the infants at birth. Daily NVP, as recommended by current South African PMTCT guidelines (10), was administered to 2 IU-infected infants but not to any of the IP-infected infants in this cohort. Delayed diagnosis as a result of this regimen was not demonstrated, but the study was not designed to assess this possibility. However, the possibility that daily NVP may suppress viral replication and delay the diagnosis of IP infections beyond 6 weeks of age cannot be excluded and requires further investigation. The 2-week results may be improved with the newer version of the quantitative CAP/CTM assay (Roche CAP/CTM, version 2), which has improved sensitivity and viral load quantification compared to version 1 (4, 14). This remains to be assessed in future studies.

In conclusion, the CAP/CTM and Aptima technologies demonstrated superior performance to the HIV DNA PCR assay at all ages, detecting 96% of all early HIV infections by 4 weeks and 76%

at birth. Infant diagnosis at 4 weeks is an option but will lead to earlier treatment only if turnaround times improve to guarantee the availability of test results by 6 weeks. Testing at 4 weeks, however, may not be viable in the context of daily-dose NVP that could potentially delay the diagnosis of IP infections; this requires further study. Alternatively, birth testing can be conducted to identify IU-infected infants who are in the health care system at the time of birth, with IP infections being detected by a later test. Birth testing would ensure that more HIV-infected infants access testing and would enable treatment to be initiated early enough to improve outcomes and reduce infant mortality.

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