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Early HIV-1 diagnosis using in-house real time PCR amplification on dried blood spots for infants in remote and resource limited settings

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Abstract

Background—In resource-limited settings, most perinatally HIV-1-infected infants do not receive timely antiretroviral therapy because early HIV-1 diagnosis is not available or affordable.

Objective—To assess the performance of a low cost in-house real-time PCR assay to detect HIV-1 DNA in infant dried blood spots (DBS).

Methods—1319 DBS collected throughout Thailand from non-breastfed infants born to HIV-1-infected mothers were shipped at room temperature to a central laboratory. In-house real-time DNA-PCR results were compared to Roche Amplicor® HIV-1 DNA test (Version 1.5) results. In addition, we verified the Roche test performance on DBS sampled from 1218 other infants using as reference HIV serology result at 18 months of age.

Results—Real-time DNA-PCR and Roche DNA-PCR results were 100% concordant. Compared to HIV-serology results, the Roche test sensitivity was 98.6% (95% CI: 92.6 to 100.0%) and its specificity at 4 months of age was 99.7% (95% CI: 99.2 to 99.9%).

Conclusions—In-house real-time PCR performed as well as the Roche test in detecting HIV-1 DNA on DBS in Thailand. Combined use of DBS and real-time PCR assays is a reliable and affordable tool to expand access to early HIV-1 diagnosis in remote and resource-limited settings, enabling timely treatment for HIV-1-infected infants.

Keywords

HIV-1 real-time DNA-PCR; Dried Blood Spots; early diagnosis; infant; access to treatment; remote and resource limited-settings

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Introduction

In 2007, there were an estimated 2.1 million children living with HIV, most of whom were infected through perinatal transmission [1]. A WHO survey of 77 countries in the same year, among mostly lower middle income countries found that of the 715,000 infants born to HIV infected women, only 8% (54,900) were tested within the first two months of life [1]. In the absence of antiretroviral treatment, up to 40% of perinatally HIV-infected children in resource limited countries may die before they reach the age of one year[2–4]. Despite the scale up of antiretroviral therapy, children still account for a disproportionately high 16% of all HIV related deaths when they represent only 7% of the HIV infected population [1]. This is partly reflective of their high risk of mortality in the first years of life and limited access to early HIV diagnosis. The World Health Organization (WHO) recommends that all HIV-infected infants, including those in resource limited settings, initiate therapy in the first year of life due to high risk of mortality [5]. However, in these settings, infants born to HIV-1-infected mothers have only access to antibody tests, which are widely available and low cost but only allow for a reliable diagnosis after 18 months of age when maternal antibodies have been cleared from the child's blood. While this enables identification of the HIV-1-infected children who have survived the first 18 months of life for initiation of antiretroviral treatment, it fails to benefit to the infants at greatest risk, i.e. those infants who develop severe immune deficiency early in their life. This highlights the urgent need for widely available and affordable access to early HIV-1 diagnosis in these settings.

Early diagnosis of HIV-1 infection requires the use of assays which can detect HIV-1 virus or its components. Previously, this would require labor intensive virus culture and highly secure laboratory facilities. To date, two techniques are currently used: the ultrasensitive detection of p24 antigen [6–12], that requires minimal laboratory equipment and the polymerase chain reaction (PCR) for the amplification of HIV-1 RNA from plasma [13–18] or DNA from mononuclear cells [13, 15, 17–21], which requires a standard molecular biology facility, allowing for positive diagnosis from as early as one day of age.

Among the PCR techniques currently used, the commercial qualitative Roche Amplicor HIV-1 DNA test has been widely considered as the gold standard test for early diagnosis of HIV-1 infection [19–21], especially since it has been refined to detect various HIV subtypes (version 1.5)[18, 22–24]. In Thailand where CRF01_AE is prevalent, Young et al. found that, using cell pellets, its sensitivity increased from less than 40% at birth to 100% at 2 months of age, while its specificity was 100% regardless of the age of infant [18].

Since the early 1990's, the use of microsamples spotted on filter paper or dried blood spots (DBS) has been proposed to make state of the art PCR techniques for the early HIV-1 diagnosis of infants accessible to rural and remote settings [23, 25–35]. Only 50 microliters of blood are required per spot and the DBS can be safely and easily stored and shipped to a central laboratory using standard mail at room temperature.

For more than 10 years, early HIV-1 diagnosis using DBS and the Roche Amplicor HIV-1 DNA test (Version 1.5) has been widely used in studies on prevention of mother to child transmission of HIV (PMTCT) in several research settings, in particular in the United States [20], Thailand [31, 32], and South Africa [23]. Although the results of these studies have translated into interventions for prevention of MTCT in the clinics, the early diagnosis technique utilized in the studies has not been widely transferred to these settings, partly due to its cost [36] and the limitations of shipping liquid blood samples to molecular biology facilities. Indeed, there are safety, temperature and time constraints for shipping liquid blood samples. Samples have to be appropriately packaged to prevent exposure to blood in case of leak or breakage; exposure to high ambient temperature during transportation may damage

cells with the release of substances inhibiting PCR reaction; timely shipment at a cool temperature (2–10°C) is often not feasible on long distance in low resource settings. The shipment of DBS samples bears none of these limitations.

By the end of the 1990's, a new technique, the real-time PCR technology was widely used in research laboratories for the detection of HIV-1 DNA. Assays based on real-time PCR display very high sensitivity and specificity, provide results more rapidly than classical PCR and avoid cross over PCR contamination, thus do not require separated amplification and detection rooms. Moreover, equipment costs have dramatically reduced with increasing competition and improvement in detection system: standard RT machines, priced USD 90,000 in 1995, are now priced from $20,000. With this increasing affordability, several groups developed real-time PCR assays to detect different gene regions of HIV-1, with the aim of expanding access to early diagnosis to resource limited settings[37–42].

In this paper, we describe the performance of an in-house real time PCR method versus the Roche Amplicor® HIV-1 DNA test (Version 1.5), for the detection of HIV-1 DNA on DBS collected from infants born to HIV-1-infected mothers in Thailand. Also, as the Roche test had never been validated on DBS in this specific setting, we assessed its ability to detect HIV-1 CRF01_AE, the most prevalent strain in Thailand, using a HIV serology result at 18 months of age as the reference.

Materials and Methods

Origin of samples

a) Comparison of in-house Real- Time PCR test versus commercial of the Roche Amplicor® HIV-1 DNA test (Version 1.5)—We used all DBS from non breastfed infants born to HIV-1 infected mothers collected (1) between July 2002 and November 2002 in the context of a perinatal HIV prevention trial study (PHPT-2, ClinicalTrials.gov NCT00398684) [32] and (2) between September 2004 and September 2006 in the context of the pilot phase of a program to provide free access to early HIV-1 diagnosis in 31 public hospitals throughout Thailand, [43]

b) Validation of the Roche test—We used all DBS from non breastfed infants born to HIV-1 infected mothers collected at their four month visit between January 1998 and July 2000 in the context of another perinatal HIV prevention trial study (PHPT-1, ClinicalTrials.gov NCT00386230) [31]. These DBS were shipped at room temperature to the New England Newborn Screening Program (Boston, Massachusetts) laboratory for HIV-1 DNA PCR testing. For this analysis, we choose the 4 month samples as it has been shown that the Roche test can detect virtually all perinatal HIV-1 infections by this age in other epidemiological contexts[23, 29] and on liquid blood [18, 23].

The study was reviewed and approved by the Ethical Committee of the Faculty of Associated Medical Sciences, Chiang Mai University.

DBS collection

Blood was collected on EDTA tubes then spotted onto a #903 filter paper (Schleicher & Schuell BioScience GmbH, Dassel, Germany), to fill at least two preprinted circles (approximately 50 ul of blood per spot). After drying for at least 4 hours in an air conditioned room or overnight at room temperature, DBS were individually inserted in a gas-impermeable bag (Bitran bag, VWR, USA) with a desiccant, and then stored at room temperature or −20°C until shipment to our laboratory in Chiang Mai, Thailand.

DNA extraction

Genomic DNA was isolated from DBS by extraction with the polyvalent cationic resin, chelex 100 ((Biorad, Marnes-la-Coquette, France). Each spot was punched for 3 pieces with a ¼ inch hole puncher into a 2 ml screw-cap tube. After washing for 1 hour with a Triton X-100 Buffer (1X PBS, 0.5% Triton X-100), 230 ul of 10% chelex solution was added and incubated at 100°C for 30 minutes. After centrifugation, 200 microliters of DNA-containing chelex supernatant were then transferred into a new tube to be used directly for PCR amplification.

To ensure quality of testing, one blank filter paper spot was randomly placed every 15 – 20 samples in the sample set. As the cleaning of punchers with HCl can lead to rusting and may subsequently cause specimen-to-specimen contamination, the puncher was cleaned between specimens by punching a blank filter papers six times. This cleaning process was previously tested and validated at the New England Newborn Screening Program, Boston, Massachusetts (Dr Comeau, Personal Communication)

Amplification and detection of proviral HIV DNA using the the Roche Amplicor® HIV-1 DNA test (Version 1.5)

Fifty microliters of DNA-containing chelex supernatant were used for HIV-1 DNA amplification and detection using the commercial DNA PCR assay (Roche Amplicor HIV-1 DNA PCR test, version 1.5, NJ, USA) according to the manufacturer‘s instructions.

Amplification and detection of proviral HIV-1 DNA using in-house real-time PCR assay

Amplification and detection of cellular HIV-1 DNA using in-house real-time PCR assay was performed as previously described by the Agence Nationale de Recherches sur le SIDA et les hépatites virales (ANRS) working group on HIV quantitation [37]. The test is based on a 5’ nuclease assay in the long terminal repeat (LTR) gene using the ABI PRISM 7000 (Applied Biosystems, USA). Briefly, 20 uL of DNA-containing chelex supernatant was amplified in duplicate with sense primer NEC 152 5’-GCCTCAATAAAGCTTGCCTTGA-3’ and reverse primer NEC 131 5’-TTTTAGAGATCGTCACCGCGG-3’ (Bioservice Unit, Bangkok, Thailand) in the presence of a labeled LTR probe 6FAM-CTGGTAACTAGAGATCCCT-MGB (Applied Biosystems, UK) and Taqman Universal PCR master mix (Applied Biosystems, Foster City, CA, USA). The probe was designed to be able to detect the two main subtypes circulating in Thailand, CRF01_AE and B, using sequences available in the Los Alamos sequence database.

All tests were performed independently from each other and blinded to clinical data.

Serology

HIV serology diagnosis was performed on site using either ELISA or GPA test kits approved by the Ministry of Public Health of Thailand and according to WHO guidelines (1997–2001)[44]. Children with negative serology result at any age were considered HIV uninfected.

Statistical analysis

To evaluate the performance of the in-house real-time PCR test, the Roche DNA PCR test was used as the reference. We calculated the concordance rate of positivity and negativity restricting the analysis to the first sample drawn in each infant and the 95% confidence intervals using exact binomial distribution.

In the primary analysis, we determined the sensitivity, specificity, positive predictive value and negative predictive value of the Roche Amplicor® HIV-1 DNA test (Version 1.5) at the 4-month visit by using the HIV serology results at the 18-month visit as reference. Sensitivity and specificity were calculated as the percentage of children with a positive and negative Roche DNA-PCR test among all children with a positive and negative HIV serology test at 18 months, respectively. The positive predictive value (PPV) and the negative predictive value (NPV) of the Roche DNA-PCR test at 4 months were defined as the percentage of HIV-1-infected and uninfected children correctly diagnosed, respectively. The PPV and NPV were calculated for a mother-to-child-transmission rate of 3.9%, the overall rate reported by the national program in Thailand between 2001 and 2003 [45] as follows: [sensitivity*prevalence] / [sensitivity*prevalence + (1-specificity)*(1-prevalence)] and [specificity*(1-prevalence)] / [specificity*(1-prevalence) + (1-sensitivity)*prevalence], respectively [46]

In a secondary analysis, we also included the children with a 4-month visit DNA PCR result but no available serology result at the 18-month visit, assigning these cases a presumptive HIV status blindly to Roche DNA PCR results. Children were classified as HIV-infected if a plasma viral load was above 10,000 copies/ml or two above 1,000 copies/mL [5], or if clinical symptoms, anthropometric, or hematological data were presumptive of HIV infection [5, 47]; otherwise they were classified HIV-uninfected.

Results

Comparison between the Roche Amplicor® HIV-1 DNA test (Version 1.5) and in-house HIV-1 real-time DNA PCR

One thousand three hundred and nineteen DBS collected from 287 infants born in PHPT-2 and 645 infants enrolled in the pilot program were tested for HIV-1 DNA using the real-time PCR and Roche test. At the time of testing, the median age was 4.1 months (10^{th} – 90^{th} percentiles: 1.7 to 7.2 months). The detection of HIV-1 by the two techniques was concordant in all 1319 DBS analyzed (Table 1). In the subset restricted to the first sample drawn in each of the 932 infants (median age: 3.5 months, 10^{th}–90^{th} percentiles: 1.5–6.7 months) the concordance rate was 100% (95% CI: 94.6 to 100%) for the 66 positive samples and 100% (95% CI: 99.6 to 100%) for the 866 negative samples. The earliest positive tests were obtained at birth.

Assessment of the specificity and sensitivity of the Roche Amplicor® HIV-1 DNA test, (Version 1.5) on DBS as compared to HIV serology at 18 months for the diagnosis of HIV-1 infection in infants

Among the 1409 children born in the PHPT- 1 trial, 1240 children had a blood sample available at the 4-month and 18-month visits, tested by Roche DNA PCR on DBS and HIV-serology, respectively (Table 2).

For the specificity and sensitivity analyses, 3 cases were excluded: one HIV-1-infected infant with a negative serology result suspected to be related to severe immune deficiency (Table 3, Case 1), and two infants with positive serology tests at 18 months but all negative virology tests until 6 months of age, suggesting late post-natal infections (Cases 2 and 3).

Sensitivity of the Roche test was 98.6% (95% CI: 92.6 to 100.0%) and specificity was 99.7% (95% CI: 99.2 to 99.9%). Using the reported rate of HIV-1 transmission in Thailand (3.9%)[45], the estimated positive predictive value would be 94.0% and the negative predictive value of 99.9%.

There were four discordant results. Three children tested Roche DNA PCR positive at 4 months of age but had a negative HIV serology at 18 months, and one child tested DNA PCR negative at 4 months of age with a positive HIV serology at 18 months. Table 3 summarizes the results of our investigations of these cases. Three discordant results were suspected to be due to human error: sample mislabeling (Case 4) and laboratory error (Cases 5 and 6). In the fourth case (Case 7), three sequential HIV-1 DNA PCR positive results were followed by two negative DNA PCR results and a negative serology diagnosis. If we excluded the three discordant cases due to human error, as they do not reflect an intrinsic lack of performance of the test, the sensitivity and specificity would be100% and 99.91 %, respectively, with a positive predictive value of 97.9% and negative predictive value of 100%.

In the secondary analysis (see Methods), when we used the clinical, anthropometric and hematological criteria to assign a presumptive infectious status to the 137 children with a 4-month DNA PCR result but no further HIV serology results, 18 were classified HIV-infected and 119 HIV-uninfected. All but one of the 4-month Roche DNA PCR results were concordant with the assumed HIV-1 status; the discordant case was DNA PCR negative at the 4-month visit (as well as at birth, 6 weeks and 6-month visits) but was presumed HIV-1-infected because of failure to thrive, although with no infectious events. When including all 1374 children with a DNA PCR result at the 4-month visit, using the presumptive classification when serology was not available, the sensitivity of the Roche DNA PCR was 97.8% (95% CI: 92.3 to 99.7%) and specificity was 99.8% (95% CI: 99.3 to 100%). Using the rate of HIV-1 transmission in Thailand (3.9%), the estimated positive predictive value would be 94.4% and the negative predictive value 99.9%.

Cost of the tests

We calculated that the cost per reportable test, including cost of filter papers, reagents, equipment maintenance and human resources, was US$20 for the in-house assay and US$42 for the Roche Amplicor® HIV-1 DNA test (Version 1.5).

Discussion

This is the first report describing the use of an in-house real-time HIV-1 DNA PCR test on DBS for the early diagnosis of HIV-1 infection in a very large number of non breastfed infants born to HIV-1-infected mothers. The results using this test were identical to those obtained using the Roche DNA PCR on DBS.

Ideally, the specificity and sensitivity of the PCR based HIV-1 diagnosis assays should be determined using a direct comparison with the HIV serology at 18 months. This was possible for the validation of the Roche Amplicor® HIV-1 DNA test (Version 1.5), in the context of the PHPT-1 study, a clinical trial where children were not breast-fed and followed until 18 months of age or further until a serology confirmation test could be obtained. Although the Roche Amplicor® HIV-1 DNA test (Version 1.5) has been widely validated and is the reference test for HIV-1 diagnosis in infants born to HIV-1 infected mothers, our concern was a theoretical lack of sensitivity due to the fact that we were dealing with subtype CRF01_AE. This concern was fully addressed as all positive serology tests at 18 months were also detected by the Roche Amplicor® HIV-1 DNA test (Version 1.5).

This strategy could not be pursued for the validation of the in-house assay as the samples were either from children enrolled in the PHPT-2 study who were followed up to 12 months only, i.e. before a reliable serology test could be done or from children followed in the context of routine clinical care. Therefore we validated our new technique against the Roche Amplicor® HIV-1 DNA test (Version 1.5), which has been validated in Thailand.

In our clinical studies, it was required that venous blood samples were collected in an EDTA tube for both safety laboratory tests and DBS preparation, therefore heel sticks were not used. In other settings, obtaining whole blood via heel stick would be easier. Several studies have shown that diagnosis of HIV infection using heel stick DBS is as reliable as venous blood draw [30, 48, 49]. Our approach, using a LTR specific probe to test DBS samples with an in-house real time PCR assay, which is simpler and less prone to contamination than conventional PCR assays, is generalizable to other settings. Our probe was adapted to the B and CRF01_AE subtypes circulating in Thailand which can be easily modified to fit other settings. The LTR region is one of the most conserved regions of HIV-1, and this is the reason why most of the real time tests more recently developed [39, 50] are also using a LTR probe. For example, the ARNS generic real-time RT-PCR assay used in France, Africa and Cambodia was shown to be very reliable [50]

It was important to verify that the use of DBS, which contain only 50 microliters of blood, did not compromise the performance of the Roche DNA PCR assay. The sensitivity and specificity of the Roche assay on DBS at around 4 months of age, compared to HIV serology at around 18 months, were 98.6% and 99.7%, respectively. These values were not significantly modified when including the cases with no available serology results. These results are consistent with those reported by Young et al. [18] (sensitivity 100%; specificity 100%), using a larger quantity of blood (100 microliters of whole blood) from 317 infants at 6 months of age in Thailand, and by Sherman et al. [23] with 98.8% sensitivity and 99.4% specificity of HIV-1 DNA PCR on DBS collected from 627 infants at 6-weeks of age in South Africa. Our results indicate a very high sensitivity of Roche HIV-1 DNA PCR on DBS when compared to the serology results.

Of the four discordant results between HIV-1 DNA-PCR and serology, three were suspected to be due to human error (Table 3), emphasizing the importance of blood sample identification and labeling to ensure reliable diagnosis and the need for confirmation testing when important clinical decisions have to be taken on the basis of such results. Additionally, there was an HIV-1-infected child for whom HIV-serology was negative at 18 and 24 months of age and the absence of detectable antibodies was suspected to be related to severe immune deficiency (Table 3). This further reinforces the benefit of HIV-1 DNA-PCR for the diagnosis of HIV-1.

In summary, for more than 15 years, commercial tests such as the Roche DNA PCR test have been used in research settings for the diagnosis of HIV-1 infection in infants born to HIV-1-infected mothers. However, few countries have implemented this technique in their national antiretroviral programs. Even fewer countries are using DBS to facilitate remote access to early diagnosis of HIV-1 infection in infants [1, 29, 36, 51]. In 2005, fewer than 2% of infants had access to early PCR testing worldwide [52]. For lower-middle income countries, such as Thailand, who rely predominantly upon domestic funding for the scale up of antiretroviral therapy, the cost of HIV related services remains a significant issue. The use of low cost in-house real-time HIV-1 DNA PCR test that can be used on DBS will facilitate access to early diagnosis of HIV-1 infection for all infants born to HIV-1 infected mothers, including those in rural and remote settings by transportation of samples by standard mail. Today, this is probably the easiest, most affordable and reliable method ensuring that infected infants have timely access to the life saving antiretroviral therapy.

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Table 1

Comparison of HIV DNA PCR results of all DBS tested with in-house Real Time DNA PCR test versus Roche test

Roche DNA	Real Time PCR results		
PCR results	Positive	Negative	Total
Positive	99	0	99
Negative	0	1220	1220
Total	99	1220	1319

Table 2

Comparison of Roche HIV DNA PCR versus HIV serology results

HIV Serology	Roche DNA PCR results		
	Positive	Negative	Total
Positive	72	3	75
(%)	(96%)	(4%)	(100%)
Negative	4	1161	1165
(%)	(0.3%)	(99.7%)	(100%)
Total	76	1164	1240

Table 3

History of HIV testing for children with discordant DNA PCR and HIV serology results

Children	HIV DNA PCR results					HIV serology	Conclusions
	Birth	6-week visit	4-month visit	6-month visit	12-month visit	18-month visit	
1*	Neg	Pos/Retest Pos	Pos	Pos		Neg	HIV-infected child
2	Neg	Neg	Neg	Neg		Pos	Late HIV infection
3	Neg	Neg	Neg	Neg		Pos	Late HIV infection
4**	Neg	Neg/Retest Neg	Pos/Retest Pos	Neg/Retest Neg		Neg	Possible sample mislabeling
5	Neg	Neg	Pos/Retest Neg	Neg	Neg	Neg	Possible laboratory error
6	NA	Pos/Retest Pos	Neg/Retest Pos	Pos		Pos	Possible laboratory error
7***	Pos/Pos	Neg/Retest Pos	Pos	Neg	Neg	Neg	HIV uninfected child

Neg; negative result, Pos: positive result

* Child with severe immunodeficiency, 1% and 2% CD4 at 12 and 25 months, and plasma HIV RNA: 631,000 copies/mL at 1.5 month and 389,000 copies/mL at 10 months of age. HIV serology was found negative at 15, 18, and 24 months of age.

** At 6-week visit, two different DBS collected at 31 days and 48 days were tested and found HIV DNA PCR negative.

*** Two additional samples drawn at 14 and 15 months were tested and found negative for HIV DNA. NA: No sample available.

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