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# Cross-contamination during processing of dried blood spots used for rapid diagnosis of HIV-1 infection of infants is rare and avoidable

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## Summary

Dried blood spot (DBS) samples are a convenient way to collect infant blood for HIV-1 diagnostic testing. Minimizing the risk of false positives is critical for diagnostic tests. A protocol for processing and testing DBS for infant HIV-1 diagnosis was evaluated to identify the rate and source of false positive results. DBS were created on Flinders Technology Associates (FTA) filter paper with 500 copies/punch (high) or 5000 copies/punch (very high) concentrations of HIV-1 DNA. Blank discs of filter paper punched after DBS samples were tested for carry-over of HIV-1 DNA using nested PCR for the *pol* region. No false positives were detected in the 40 series using high concentration DBS. In series with very high concentrations of HIV-1, 8/246 (3%) reactions were falsely positive. When tubes were spun prior to opening, contact with caps minimized, and spaces left between lanes of the gel, repeat second round PCR of five false positives resulted in only one repeat false positive PCR. This study outlines procedures that minimize false positive results for nested PCR of HIV-1 DNA from DBS.

### Keywords

dried blood spots; infant HIV diagnosis; PCR contamination

Over 500,000 children under the age of 15 were infected with HIV-1 in 2006 (UNAIDS, 2007), the majority in the developing world. Recent reports suggest that early diagnosis and treatment improves the outcome for infants infected with HIV-1 (Violari et al., 2008), however, infant diagnosis using “routine” serologic assays is precluded by the presence of maternal HIV-1 antibodies that can persist in the infant until 18 months after birth, and access to PCR



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testing is limited in most developing countries. Multiple non-commercial groups have developed inexpensive PCR of viral nucleic acids to diagnose infant HIV-1 infection during this critical time(1), and new laboratory sites are being developed to conduct this testing. Collection of blood from a heel stick onto filter paper is practical for obtaining diagnostic specimens from infants: only 50-200uL of blood is required, dried blood spots (DBS) can be stored and transported easily, and PCR of DBS has been found to be sensitive and specific for early HIV-1 diagnosis in infants (Beck et al., 2001; Cassol et al., 1996).

DBS samples for metabolic and genetic newborn screening in the United States are primarily collected on 903 filter paper (Whatman Bioscience, Florham Park, NJ) that allows for testing of proteins or nucleic acids. Optimal storage for 903 paper DBS appears to be in a refrigerator or freezer (Mitchell et al., 2008; Youngpairoj et al., 2008). To analyze nucleic acids from samples collected onto 903 filter paper the entire blood-soaked paper is cut into smaller pieces, submitted to DNA extraction and PCR. Flinders Technology Associates (FTA) filter paper (Whatman Bioscience, Florham Park, NJ) is a treated filter paper for specimen collection that is designed to lyse proteins and bind nucleic acids, allowing stable storage of DNA at room temperature for >4 years (Li et al., 2004). When processing samples for HIV-1 diagnosis on FTA paper, one small disc of the sample is excised with a manual hole-punch instrument and washed to remove protein fragments and other potential PCR inhibitors. Then nested PCR is used to amplify HIV-1 to detect infection. The advantages of using this system are its sensitivity for low copy-number HIV-1 infection, achieved through nested PCR (Beck et al., 2001), and its cost efficiency, as a result of less technician time in comparison to the 903 processing.

A critical consideration when performing tests for HIV-1 diagnosis is avoiding false positive or false negative results. The nested PCR is quite specific (1), but during processing of DBS, there are several steps with potential for cross-contamination between specimens. One of these is the instrument used to excise the filter paper discs, which is cleaned between samples by punching blank filter paper to remove any viral nucleic acids adherent to the instrument. A recent study found that when using the Roche Amplicor HIV-1 DNA test, processing of DBS without cleaning the automated hole punch equipment in between patient samples resulted in little to no cross-contamination between HIV-1 positive and negative samples (Driver et al., 2007). It is unknown whether manual punching of specimens in conjunction with the more sensitive method of nested PCR from FTA filter paper requires cleaning of the punch to reduce the risk of contamination between specimens. Thus, a study was conducted to evaluate carry-over of DNA on the hole punch and other potential sources of cross contamination with HIV-1 during processing of DBS and testing using nested PCR. Five steps in the protocol where contamination might have occurred were identified and tested: the punching instrument, the punching surface, tweezers used to transfer discs to PCR tubes, transfer of PCR product between 1$^{st}$ and 2$^{nd}$ round tubes or leakage across wells in the gel used to visualize the product.

The protocol used for processing infant DBS uses five punches of a blank filter paper disc to clean the hole punch instrument. The risk of DNA carry-over was evaluated by testing blank filter paper discs punched after sampling a DBS containing HIV-1: the first two and sixth blank discs out of a series of six blanks. Two sets of DBS were created in the laboratory: one with a concentration of 500 HIV-1 DNA copies/3.175mm punch, which is at the high end of viral DNA loads in infected infants' samples, and a second set with 5000 HIV-1 DNA copies/punch, a concentration greater than the highest ever measured in infant samples in our laboratory (unpublished data). HIV-1 uninfected whole blood collected in an anticoagulant EDTA tube was spiked with 8E5 cells (NIH AIDS Reagent Program, lot# 9020006), spotted onto FTA Mini cards (Whatman Bioscience #30), and allowed to dry overnight at room temperature. The discs were then prepared for PCR according to the lab protocol as previously described (Beck et al., 2001). Nested PCR was used to amplify a 520bp region of HIV-1 encoding *pol* (HXB2: outer - 2011-2701bp, inner - 2068-2588bp) from the DBS, first blank (#1), second blank (#2),

and sixth blank (#6) discs. Amplicons were detected on a 1% agarose gel stained with ethidium-bromide. Between experimental series, the hole punch instruments (Lab quality: Model number 10495010, Whatman, UK; Craft supply: McGill, Marengo, IL) were cleaned with a 10% bleach solution or ethanol, rinsed with hot water, and allowed to dry overnight. Statistical analysis was performed using STATA, version 10.0 (STAT Corp, College Station, TX). Counts and rates of contamination in the DBS were generated. The binomial distribution was used to calculate two-sided 95% exact confidence intervals (CI) around rates of contamination.

A total of 122 experimental series were performed: 40 with a DBS containing 500 copies/3mm punch ("high" concentration) and 82 with a DBS containing 5000 HIV-1 copies/punch ("very high" concentration). Testing of 120 blank discs from 40 series with the high concentration DBS produced no false positives. Across all experimental series with the very high concentration of HIV-1, 8 false positive reactions were detected. These included 5 of 82 (6%, 95% CI 2-14%) Blank #1 discs (punched immediately following the sample DBS); 1 of 82 (1%, 0-6%) Blank #2, which followed one of the Blank #1 false positives; and 2 of 82 Blank #6 (2%, 0-9%) that were from different series from the aforementioned falsely positive Blank #1 or #2 discs (Table 1).

Use of bleach to clean the craft store puncher left the punching surface of the instruments with rough, corroded edges that tore the paper rather than cutting cleanly. In the series using the corroded craft store instruments rates of false positives tended to be high: 1/5 (20%) for Blank #1 and 1/5 (20%) for Blank #6 (Table 1). In the first four series, all filter paper discs were punched onto the same tissue and transferred with the same forceps. The rate of contamination tended to be high: 2/4 (50%) for Blank #1 and 1/4 (25%) for Blank #2 samples (Table 1). It was noted that small flecks of blood-soaked FTA card fell from the HIV-1-spiked DBS onto the tissue; subsequently the tissue was changed between samples. When blank discs were transferred using forceps that had just picked up a highly positive (5000 HIV-1 DNA copies/punch) disc 39/39 DNA PCR were negative, suggesting no transfer of DNA occurred via forceps.

Two steps in the nested PCR protocol could contribute to false positive PCR: (1) transfer of PCR product from one tube to another due to aerosolization when opening tubes or contamination on the technician's gloves (either from one first-round tube to another, or from a first round tube to an unrelated second-round tube), (2) mis-loading or leakage between lanes of the gel. When the same second round product was run on a new gel, the false-positive results persisted. To test whether these were due to transfer of DNA between first round PCR tubes, we repeated the second round PCR for five of the weakly false positive samples. On repeat testing 4/5 false positives were negative, suggesting the false positive was due to carryover from a $1^{st}$ round tube into a $2^{nd}$-round PCR. The single repeatable false positive suggests contamination of the $1^{st}$-round PCR amplification – possibly when opening tubes after the first round of PCR versus during processing of the DBS specimen.

Forty series were performed with an "optimized" protocol. Significant modification to the protocol included: use of a sharp, non-corroded instrument, punching discs onto a clean tissue that was discarded after each specimen, spinning PCR tubes before opening to clear amplicon from the top of the tube, and use of a tissue to open the tubes between nested rounds of PCR to capture any aerosol and minimize contamination of technician's gloves. In series performed with optimized procedures to reduce contamination, and using the "very high" HIV-1 DNA DBS only two HIV-1 PCR were false positives, including 1/40 (2.5%) Blank #1 and 1/40 (2.5%) Blank #6. Both false positives were re-tested with a new second round PCR and were negative, suggesting that contamination occurred during transfer of first round product to second round.

This study used blood spiked with HIV-1 DNA to simulate infant DBS specimens to evaluate cross-contamination during specimen processing and HIV-1 testing using a sensitive nested PCR. In experiments using concentrations of HIV-1 DNA at the higher end of those in infected infants no false positive results were detected, suggesting that even with no cleaning of instruments used to excise and transfer the filter paper that cross-contamination is unlikely. In experiments using samples with a very high concentration of HIV- DNA, two risky steps led to falsely positive tests: 1) excising a DBS disc using a low quality hole punches, and 2) transfer of PCR product from first to second round nested PCR. Most false positive results were related to cross contamination at several steps while transferring first round to second round, since repeat gels of the original product remained positive, but new second round amplification was negative for 4/5 samples.

The range of HIV-1 DNA concentrations in infected newborns' samples analyzed in our laboratory (n= 82) is <1 copies/punch to 4880 copies/punch, with a median of 53.5 HIV-1 DNA copies/punch. In this study simulating diagnostic testing of infants, cross-contamination was only evident after processing DBS with very high concentrations of HIV-1 DNA (5000 copies/punch). However, using this set of extreme DBS allowed identification of risky steps in our protocol. Using optimal techniques, application of this nested PCR assay to over 2900 specimens collected every two weeks for 2 months from over 800 at risk infants resulted in only 1 false positive result (Dross et al., in preparation). To reduce any potential for cross-contamination we recommend the use of high-quality lab-supply instruments, cleaning these instruments with simple soap and water at the end of each day of use, and using a clean disposable surface to catch each new punched-out disc. In addition, when using nested PCR we recommend centrifugation of tubes following the first round PCR to clear amplicon from the lid prior to opening tubes, and careful manipulation of PCR tubes to avoid contamination of gloves or creating an aerosol with PCR product.

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## Table 1

**Simulations to assess carryover contamination of HIV-1 at multiple steps of testing dried whole blood spots (DBS) by nested PCR for infant diagnosis**

Rates of false positive results when processing blank filter paper following DBS spiked with an average or a high concentration of HIV-1 DNA are shown by potential risk factors for contamination. The sampling procedure was to first punch a disc from a DBS containing HIV-1 followed by six punches of clean filter paper (blank discs 1-6) using the same instrument without any cleaning between punches.

| Variations in Processing DBS | # of series tested | Sequence of Blank Disc punched following HIV-1(+) Disc | | |
|---|---|---|---|---|
| | | #1 | #2 | #6 |
| **DBS containing "high" (500 copies/punch) concentration of HIV-1 DNA** | | | | |
| Use of non-corroded hole punchers, and discs transferred on clean surface | 40 | 0 (0%) | 0 (0%) | 0 (0%) |
| **DBS containing "very high" (5000 copies/punch) concentration of HIV-1 DNA** | | | | |
| Optimized protocol[b] | 40 | 1[c] (2.5%) | 0 (0%) | 1[c] (2.5%) |
| Discs punched onto shared surface | 4 | 2 (50%) | 1 (25%) | 0 (0%) |
| Use of corroded hole punchers | 5 | 1 (20%) | 0 (0%) | 1[c](20%) |
| No "precautions"[a] when transferring 1st to 2nd round PCR product | 33 | 1[c] (3%) | 0 (0%) | 0 (0%) |

[a] "Precautions" refers to centrifugation of tubes post PCR prior to opening, and using a tissue to open the tubes, especially important to minimize 1st round PCR product at top of tubes that can contaminate gloves or splash into adjacent tubes upon opening lids to transfer amplicon to 2nd round PCR

[b] Optimized protocol refers to using sharp punch instrument, individual clean disposable surface to collect disc punched from DBS, and precautions described above

[c] Repeat gels of second-round PCR revealed persistent false (+), however when the original first-round PCR product was re-amplified in new 2nd round PCR, these yielded negative results, suggesting the original false positive result was due to inadvertent transfer (cross contamination) of PCR product between tubes between 1st- and 2nd-round PCR