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Objectives: Rapid human immunodeficiency virus (HIV) antibody tests, routinely used for diagnosis in adults and older children in resource-limited settings (RLS), do not detect early HIV infections prior to seroconversion or when antibody levels are still low. Nucleic acid amplification to detect HIV-1 RNA is the most sensitive method for acute HIV infection diagnosis, but is costly. We therefore investigated HIV- 1 RNA testing of pooled dried blood spots (DBS) to diagnose acute HIV infection.
Design: Laboratory-based investigation.
Methods: DBS were collected from HIV-1 Voluntary Counselling and Testing (HVCT) clients who tested negative on the Advanced Quality™ HIV antibody rapid test. DBS samples from five participants were pooled and tested on the COBAS AmpliPrep/COBAS TaqMan HIV-1 (CAP/CTM) Test v2. Individual DBS were tested when pools tested positive (> 200 RNA copies/ml). Acute infection was confirmed by HIV viral load testing, two fourth-generation HIV serological assays, and Geenius™ HIV 1/2 Assay for antibody band identification.
Results: Of 482 participants who were tested, one (0.2%) had acute HIV infection: Fourth generation serology was low-level positive, the plasma HIV viral load was 15 929 HIV-1 RNA copies/ml, gp160 and gp41 antibody bands were positive and the p31 band was negative, indicating a Fiebig Stage 5 infection.
Conclusions: Pooled DBS HIV-1 RNA testing is efficient compared to individual testing for acute HIV infection diagnosis. Early identification of participants with acute HIV infection facilitates immediate initiation of antiretroviral therapy to improve immune recovery and prevent transmission to others.

Keywords: Dried blood spot testing, HIV, HIV-1 RNA, South Africa

Background

There were an estimated 380 000 new HIV infections in South Africa in 2015,[1] highlighting the urgent need to improve prevention efforts. In the HIV Prevention Trials Network (HPTN) 052 study, antiretroviral therapy (ART) reduced transmission by 96% in serodiscordant couples.[2] At the community level, increased ART coverage is associated with a lower incidence, likely by reducing transmissibility between partners.[3] Epidemiological models support therapy as prevention and early ART in patients with CD_4 counts above 500 cells/µl provides individual benefit.[4–6] Therefore, the latest World Health Organisation (WHO) and South African guidelines recommend initiation of ART as early as feasible. However, many early HIV infections remain undetected. Acute and early HIV infection is defined as the period from exposure until approximately 100 days,[7] when individuals are highly infectious due to high viral loads in blood and genital secretions.[8] International studies have shown that as many as 20-50% of HIV-1 transmission events could be from sexual exposure to an acutely infected individual.[9,10] HIV voluntary counselling and testing (HVCT) relying on rapid antibody tests may miss patients with acute or early HIV infection before antibodies are detectable.[7,11]

Nucleic acid tests (NAT) for HIV-1 RNA and/or DNA are the most sensitive for diagnosing acute HIV infection.[7] In South Africa, due to high costs, NAT to diagnose acute HIV infection is only used in high risk settings.[12,13] As few rapid test-negative individuals are expected to have acute HIV infection, pooled HIV-1 RNA testing on plasma increases testing efficiency. This approach has been established but still requires phlebotomy, which makes it unsuitable for many typical HVCT settings.[14–16]

Many studies have demonstrated that dried blood spots (DBS) are useful for infant HIV diagnosis and monitoring plasma HIV viral loads with an excellent analytical performance.[17–19] However, no data is available for a DBS testing pooling strategy to diagnose acute and early adult HIV infections. In this study, we investigated whether pooled dried blood spot (DBS) testing might be a feasible method to identify acute HIV infection in a moderate risk HVCT setting.

Methods

The study was approved by the Stellenbosch University Health Research Ethics Committee (N15/09/080). To establish the lower limit of detection of the DBS testing approach, we serially diluted HIV positive plasma samples with known HIV RNA loads and artificially reconstituted whole blood by mixing with the cellular fraction of whole blood samples (50%) from HIV-negative donors. Fifty microlitres of each constituted specimen was pipetted onto Whatman 903 sample collection cards (GE Health Sciences, Buckinghamshire, England) and allowed to dry overnight at room temperature. One known positive HIV RNA DBS sample per

dilution was added to four known HIV negative DBS and tested on the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, v2.0 (CAP/CTM) (Roche Molecular Diagnostics, Pleasanton, CA) in 8-10 replicates per dilution. The limit of detection was estimated by probit regression using the R statistical software package, version 3.3.1.[20]

We collected and tested samples from participants who provided informed consent from March 2016 to August 2016. Our study population included participants who self-referred for community-based HIV testing at either a stand-alone centre or at a mobile service in one of five peri-urban communities around Cape Town. Mobile services were typically set up at transport hubs, along major thoroughfares and in high schools. This community-based HIV testing service was part of a routine service delivery. Participants who tested negative on the Advanced Quality™ HIV antibody screening assay (Intec Products Inc., Haicang, Xiamen, China) had five DBS of 50 μl each collected through finger prick on Whatman 903 cards for further testing. The DBS samples were allowed to dry overnight and then kept frozen at –20 °C until testing. We excluded DBS samples that contained visibly less than 50 μl per DBS.

The same DBS testing algorithm was followed throughout (Figure 1). DBS from five sequential participants constituted a minipool. The five punched-out DBS were placed in a 1.5 ml Eppendorf tube together with 1.4 ml of pre-extraction reagent (SPEX) buffer and then placed on a shaker at 56-57 °C for 30 minutes. Thereafter the sample was centrifuged at 13 000 g for 2 min and 1.1 ml of supernatant was removed and placed in a clean 1.5 ml Eppendorf tube to be used for further testing.

The pooled sample was tested for HIV-1 RNA on the CAP/CTM system. As plasma volumes in DBS were variable and our purpose was diagnosis, raw assay values rather than extrapolated plasma RNA loads were reported. Pools testing positive were deconvoluted by testing the five constituent samples separately. For this, individual DBS samples were placed in 1.5 ml Eppendorf tubes and 1.1 ml of SPEX buffer was added. Pre-extraction and testing were the same as for the sample pools. At the study outset, we found a case with detectable pool and detectable

individual viral load < 200 copies/ml, who was recalled for a confirmatory sample that tested RNA and antibody negative. We also found 4 detectable pools < 200 copies/ml without detectable individual samples. Therefore, considering the impact of a false provisional positive diagnosis using an approach not yet validated for diagnosis, we subsequently regarded > 200 copies/ml as a threshold for deconvolution of pools and confirmatory testing of individual DBS positive samples.

To confirm acute HIV infection and perform Fiebig staging, an individual testing positive on DBS was asked to visit a health care site for phlebotomy to collect a confirmatory sample as soon as possible. The HIV viral load in the confirmatory sample was determined by the CAP/CTM. In addition, it was tested on two sensitive fourth generation HIV serology platforms: The Abbott Architect i200SR immunoassay analyser (Abbott Laboratories, Abbott Park, Illinois, USA) and the Mini-Vidas HIV Duo immunoassay (Biomerieux Vitek Inc., Missouri, USA) which is designed to specifically and sensitively detect the p24 antigen in the absence of HIV antibody. In participants positive for antibodies, the HIV antigen reactivity pattern was confirmed with the Geenius™ HIV 1/2 Assay (BioRad, Marnes-la-Coquette, France).

Results
The pooled DBS HIV-1 RNA testing lower limit of detection (95% hit rate) was an individual sample plasma HIV-1 RNA load of 2 629 (95% confidence interval: 1 258 – 7 236) copies/ml.

Two samples were excluded from the study for the following reasons: one mistaken enrolment of a known antibody positive individual with chronic HIV infection; and, one individual without a second DBS, not allowing deconvolution. Of the 482 samples included, one showed acute HIV infection (0.2%). For this patient, the HIV viral load of the minipool was 29 649 copies/ml and the deconvoluted individual DBS HIV viral load 15 929 copies/ml. There was a delay of 14 days from the original DBS sample to testing a confirmatory sample. At this stage, the laboratory serology was already a low positive for HIV antibody and the plasma HIV-1 RNA load was 58 092 copies/ml. The Geenius™ assay showed gp160 and gp41 bands, but p31 was negative. This patient was classified with acute HIV infection, Fiebig Stage 5, but declined immediate ART.

Discussion
Diagnosing and treating HIV infection as early as possible has individual as well as community benefit. It reduces the risk of HIV-associated disease and limits transmission.[4,5,21,22] Patients treated during acute infection are also ideal candidates for curative interventions as early therapy prevents immune damage and limits seeding of reservoirs.[23,24]

According to the 2012 Human Science Research Council South African National HIV Prevalence, Incidence and Behaviour Survey, the overall HIV incidence in 2 – 49-year-old individuals was 1.07%. However, as the epidemic is generalised, it is difficult to define risk groups, apart from a higher incidence among young black female survey participants.[25] Moreover, as symptoms of acute HIV infection are nonspecific, it is challenging to identify those cases. One approach is to perform two parallel rapid tests and, when discordant, test individuals for acute infection.[26] Another is to use epidemiologic and clinical scoring algorithms to identify which patients with negative rapid tests are more likely to have acute HIV, warranting point-of-care (PoC) HIV NAT testing.[7] However, such individual PoC NAT testing remains costly.
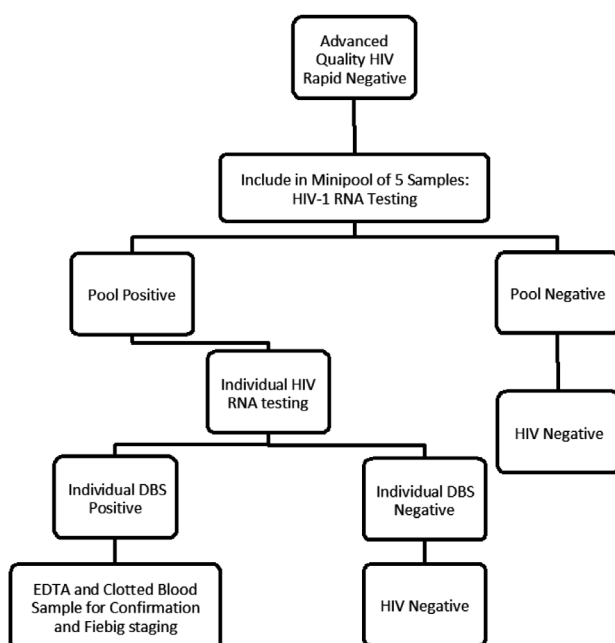

Figure 1: Algorithm for pooled DBS HIV RNA testing.

When the frequency of samples with acute HIV infection is low, pooled testing would increase efficiency. In settings without access to phlebotomy, pooled DBS testing offers a practical alternative to pooled plasma testing. However, in contrast to plasma, the number of samples that can be pooled with DBS testing is limited due to PCR inhibition by haemoglobin or other impurities.

There is currently no ideal acute HIV diagnostic testing strategy available in resource limited settings. Pooled HIV-1 RNA testing is a cost-effective way to utilise HIV-1 RNA testing in trying to diagnose acute HIV. Furthermore, a pooled DBS HIV RNA strategy foregoes the need for phlebotomy, which will make it easier to collect specimens in resource limited settings. Fourth generation HIV laboratory based tests are more sensitive than third generation rapid tests, but have other problems such as increased turnaround time (resulting in possible delays in treatment and loss to follow up) and particular automated assays may be prone to sample contamination.[28] Most fourth generation HIV serology platforms are also not validated for testing with DBS, but some studies have shown promise.[31] Given that the DBS technique is currently the only means of sample transport over long distances and without the need for cold storage, it will be important for manufacturers of laboratory-based tests to validate their platforms for use with DBS.[29] There is also a need for scalable molecular PoC tests and as nucleic acid testing technology becomes more compact and affordable, this may be possible in the foreseeable future.[29]

Our study had several limitations. As participants were from community-based HIV testing sites, where clinical information was not collected, we could not identify individual infection risks. Deconvolution of positive pools through individual re-testing causes delay of a few days, which would likely result in Fiebig stage progression by time of follow-up. We were also operating in a setting with a high treatment coverage, which likely lowered incidence and contributed to finding only one true case of acute infection. In such a setting the use of nucleic acid testing, even when testing pooled samples, is costly (a single HIV viral load test is approximately US$ 25). We also found that low positive pools (< 200 copies/ml) were not predictive of true infections. The Advanced Quality™ HIV antibody screening assay sensitivity is 98.8% (93.2 – 100.0%).[27] The most common causes for false negativites are early HIV infection and advanced HIV infection. In our study, we did not find any chronic HIV infections that were missed with initial rapid HIV testing. This is in contrast to the previously published study by Bassett *et al*[30] that found 2% of patients with chronic HIV infection. This might be explained by the difference in the size of the patient cohorts, the geographical differences in HIV prevalence and the fact that we did not include discordant HIV results in our study.

As far as we know, this is the first report of using pooled DBS testing to diagnose acute HIV infection in adults. Our investigation showed that pooled DBS testing could be used to identify acute HIV infection in rapid test negative individuals at a lower cost than individual testing. DBS collection was also feasible at sites that perform finger prick for HIV rapid testing. Due to the high disease burden, we found it challenging to prioritise diagnosing acute HIV infection, both at clinical and laboratory level. The study team has been involved in cases, outside this study, where treatment of acute HIV infection was delayed, curbing the benefit that very early therapy could achieve. Considering the accumulating evidence of the benefit of immediate therapy, identifying HIV infection could be prioritised as a medical emergency. Increased health care worker awareness, with improved affordable and feasible diagnostic modalities, is needed to initiate immediate ART. Further, larger scale research projects evaluating the use of pooled DBS and similar strategies are urgently needed to inform future best practice.

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