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Single genome amplification of proviral HIV-1 DNA from dried blood spot specimens collected during early infant screening programs in Lusaka, Zambia

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Abstract

The ability to evaluate individual HIV-1 virions from the quasispecies of vertically infected infants was evaluated in a field setting at the Centre for Infectious Disease Research in Zambia. Infant heel-prick blood specimens were spotted onto dried blood spot (DBS) filter paper cards at government health clinics. Nucleic acid was extracted and used as a template for HIV-1 proviral DNA detection by a commercial Amplicor HIV-1 PCR test (Roche, version 1.5). On samples that tested positive by commercial diagnostic assay, amplification of DNA was performed using an in-house assay of the 5' and 3' region of the HIV-1 genome. Additionally, fragments covering 1200 nucleotides within *pol* (full length protease and partial reverse transcriptase) and 1400 nucleotides within *env* (variable 1-variable 5 region) were further analyzed by single genome amplification (SGA). In summary, we have demonstrated an in-house assay for amplifying the 5' and 3' proviral HIV-1 DNA as well as *pol* and *env* proviral DNA fragments from DBS cards collected and analyzed entirely in Zambia. In conclusion, this study shows the feasibility of utilizing DBS cards to amplify the whole proviral HIV-1 genome as well as perform SGA on key HIV-1 genes.

Early infant diagnosis (EID) of HIV infection through rapid detection of proviral DNA within dried blood spots (DBS) is critical in the management of perinatally infected children. DBS reduces costs of prevention of mother-to-child transmission programs by up to 25% when compared to conventional methods (Sherman et al., 2005; Kébé et al., 2011) and facilitates early treatment in a setting where immediate initiation of antiretroviral therapy is associated with significant mortality reduction (Violari et al., 2008).

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The implementation of antiretroviral drugs for the prevention of mother-to-child-transmission has resulted in significant reductions in vertical HIV infections (Torpey et al., 2012). However, infant HIV infections continue to occur: *in utero* (IU), intrapartum (IP), and postnatal (PN) (Mubiana-Mbewe et al., 2009). A study from 2007 to 2010 reported that 3,481 (12.2%) of 28,320 HIV DNA PCR tests from 5 provinces in Zambia were HIV positive (Torpey et al., 2012), though the authors saw an overall decrease in the proportions of positive PCR results over time- likely an indication of better prevention strategies. To address this, the current WHO guidelines emphasize the importance of providing lifelong antiretroviral therapy (ART) to all HIV-infected pregnant women eligible for such treatment (UPdaTE 2012). Review of 2,233 infant DBS samples processed in the on-site laboratory from a recent 3-month period in 2013 (April–June) showed a mean HIV DNA PCR positivity of 4.0% (95% confidence interval: 2.6%–5.4% from 13 district clinics in Lusaka province, LS, MBG unpublished data).

Information obtained from amplified DNA from DBS can be used for more than qualitative diagnostic information. Genomic sequence information obtained from proviral DNA at the time of vertical transmission could be instrumental in furthering HIV pathogenesis, microbicide, and vaccine research by enabling the direct analysis of the virus(es) responsible for productive clinical infection. The transmitted virus is, in fact, the exact virus that a prevention modality must interdict and carries with it the genetic repertoire with which to establish chronic infection (Lehman and Farquhar, 2007).

Very little, however, is known about the actual transmitted virus in vertical infection and the virological determinants that contribute to the high viral loads and poor prognosis in these young children. In this study, a proof-of-concept study of single genome amplification (SGA) methodology was carried out, used previously for drug resistance testing (Palmer et al., 2005) and studies of horizontal transmission (Salazar-Gonzalez et al., 2008; Salazar-Gonzalez et al., 2009) in order to evaluate vertical transmission. Procedures appropriate for application in the field setting were developed in which whole genomes representing unique virions of the quasispecies (Keele et al., 2008) could be prepared for analysis from DBS. Applications for this technology would include determination of transmitted/founder viruses, *pol* mutations/drug resistance, as well as characterization of *env* or other genes facing immunologic pressure over time. Even infectious molecular clones- which could reveal more information about the infectivity of each clone- could be generated from amplified successfully whole genomes (Salazar-Gonzalez et al., 2009).

The Centre for Infectious Disease Research in Zambia Central Laboratory is a reference facility in Lusaka, Zambia that performs early infant diagnosis testing from DBS cards collected from infants throughout approximately 180 government clinics in 4 of 10 provinces within Zambia (Lusaka, Eastern, Southern, and Western provinces), representing approximately 16,500 tests annually. DBS were prepared by dispensing blood by routine skin puncture procedure (i.e., heel stick) onto filter paper, 5 spots per card (S&S903 filter paper; Schleicher & Schuell, BioScience GmbH, Barcelona, Spain), and dried at room temperature. The DBS were then stored in biospecimen bags with a silica gel desiccant and stored at room temperature (20–30°C) during transport from clinic to the laboratory. The

ethics boards at the University of Zambia and the University of Alabama at Birmingham approved the study.

HIV-1 DNA PCR testing was performed using Roche COBAS® Amplicor HIV-1 DNA test kit (version 1.5, Roche Molecular Systems, Inc., Branchburg, NJ) according to the manufacturer's instructions. Briefly, one 6 mm punch, containing approximately 12.5 uL of dried blood total (O'Broin et al., 1995), from S&S 903 Whatman® paper cards was washed (repeated twice with 700 uL of the kit wash solution) and incubated with 200uL of the kit extraction solution (made up to volume with 2.67 uL of the kit internal control) at 60°C for 30 min and then at 100°C for 30 min (Thermomixer® Compact, Eppendorf). Then, a target 155-bp region of the *gag* gene was amplified using HIV-1-specific complementary primers followed by hybridization of the amplified products to target-specific biotinylated oligonucleotide probes and a streptavidin-peroxidase colorimetric reaction (Package insert: "Roche COBAS® AmpliScreen HIV-1 Test, version 1.5") (Kébé et al., 2011).

Following diagnostic PCR, the genomic DNA (gDNA) extraction from de-identified HIV positive patients was used for down-stream PCR. Genomic DNA from DBS isolated using the stated method- with the final gDNA elution at a concentration of one 6 mm punch (~12.5 uL of blood) in a 200uL elution. Prior to performing the SGA itself, primers and reaction conditions were tested using nested bulk PCR to amplify sub-genomic portions of HIV-1 including its 5' half, 3' half, partial *pol* (all of protease and part of reverse transcriptase), and complete *env*. Round 1 of nested bulk PCR was carried out in a 50 uL reaction using 30 uL of the genomic DNA template, 1 U/μl Platinum® Taq High Fidelity (Invitrogen, Life Technologies, Carlsbad, California, U.S.A.), 1× High Fidelity PCR buffer (2 mM MgSO₄ final concentration), 0.25 mM of each dNTP, and 0.4 μM forward primers and reverse primers, as listed in Table 1. Lengths of the four amplicons and their relative positions within the HIV-1 genome are listed in Table 1. The PCR mixtures were set up in optical 96-well reaction plates (MicroAmp; Applied Biosystems, Life Technologies, Carlsbad, California, U.S.A.) and sealed with Micro-Amp adhesive film and carried out in thermal cycler (PCR system 9700, Applied Biosystem). PCR conditions for both rounds were 94°C for 2 min, followed by 35 cycles of 94°C for 15 sec, 55°C for 30 sec, and 68°C for 6 min, with a final extension period of 7 min at 68°C. Second-round PCR was performed by transferring 2 μl of the first-round product into a final volume of 50 μl of a reaction mixture with the same PCR conditions as for the first-round PCR. Amplicons were sized on 1% agarose gels (Biorad, Hercules, CA, U.S.A.). O'GeneRuler™ DNA ladder (Thermo Scientific, Waltham, Maryland, U.S.A.) was used for the electrophoresis gel marker.

SGA uses limiting dilution to amplify single DNA molecules, in contrast to conventional, bulk, multiple template PCR. Direct sequencing of SGA amplified sequences then allows for accurate characterization of individual viruses within the quasispecies (Keele et al., 2008; Salazar-Gonzalez et al., 2008; Keele et al., 2009; Salazar-Gonzalez et al., 2009; Salazar-Gonzalez et al., 2011). Following diagnostic DNA PCR, proviral DNA was extracted from left over DBS cards as above. Multiple reactions were set up using dilutions of template until fewer than 30% of reactions were positive. In this case, according to Poisson's distribution, greater than 80% of amplified DNAs would have originated from a single-copy template (Salazar-Gonzalez et al., 2009). Nested PCR for SGA were carried out in the same

way as outlined for bulk PCR above, with the same reaction conditions, primers, and thermal cycler conditions. Template DNA, however, was diluted serially until approximately 20% of reactions were positive for the correctly-sized amplification products. All PCR procedures were performed under PCR clean-room conditions.

Infant heel-prick blood were spotted onto S&S 903 Whatman ® paper cards, sealed in individual plastic bags with desiccant, and kept at room temperature. The average transport time for DBS from Zambian government clinics to the Central Laboratory across the 4 provinces was 13.0 days (95% confidence interval 12.7–13.3 days). 6 children were selected randomly for sub-genomic HIV testing from among those testing positive for HIV by commercial DNA PCR.

To amplify the complete HIV-1 genome in two overlapping fragments, in six samples, oligonucleotides were designed to amplify a 5988 base pair (bp) fragment corresponding to the 5' region at HXB2 nucleotide (nt) position 572 to 6500 and a 3441 base pair fragment corresponding to the 3' region at HXB2 nt position 6201 to 9642 (Figure 1, showing 2 representative samples). These two amplicons have an overlap of 300 bp from nt position 6200 to 6501 and in total cover the 3' end of 5' LTR, *gag*, *pol* (*pro*, *rt*, *int*), *vif*, *vpr*, *vpu*, *tat*, *rev*, *env*, *nef*, 3' LTR U3, 3' LTR R region, and the 5' portion of 3' LTR U5. In total, all bases are represented. Additional bulk sequencing of *pol* and *env* using primers as per Table 1 were also performed (data not shown). The in-house assay for *pol* utilized several oligonucleotides designed in a prior in-house HIV drug resistance mutation genotyping study (Wallis et al., 2010).

SGA of partial *pol* (HXB2 nt position 2164 to 3344) and the variable regions 1–5 of *env*, (HXB2 nt position 6583 to 7931) was performed next. Genomic DNA from DBS cards was diluted serially from 1:10 to 1:50, and 12.5 uL aliquots of diluted gDNA were added in replicates of eight PCR wells and subjected to nested PCR amplification with HIV-specific primers that yield the appropriate length fragment. The *pol* gene, including all of protease and part of reverse transcriptase, was amplified as a 1200 bp fragment (Figure 2A shows a representative sample) and was SGA at a dilution of 1:50. The *env* gene (including variable loops 1–5) was amplified as a 1400 bp fragment (Figure 2B shows a representative sample) and was SGA at a dilution of 1:50.

Intrapartum and perinatal transmission are likely to occur across a mucosal surface and may be analogous to mechanisms found during sexual transmission in adults, and accordingly, one or few transmitted viral variants would be expected (Keele et al., 2008). Intrauterine infection may be more akin to mechanisms encountered with acquisition of HIV during a contaminated blood transfusion. These infections would then be likely to transmit multiple viral variants and could explain the poorer prognosis. In horizontal transmission, investigators have used SGA analysis of HIV viruses near the time of transmission to identify the actual sequence of envelope genes responsible for transmitted infection and have described their diversification in the critical period between transmission, peak viremia, and seroconversion (Keele et al., 2008; Salazar-Gonzalez et al., 2008; Salazar-Gonzalez et al., 2009). Essential to this strategy was SGA of HIV-1 plasma viral RNA followed by direct sequencing of uncloned amplicon DNA, which precludes *Taq*

polymerase-induced nucleotide misincorporation and recombination in finished sequences (Palmer et al., 2005; Salazar-Gonzalez et al., 2008). The benefit of SGA over bulk sequencing has been demonstrated in these prior findings- with the former study demonstrating that drug resistance mutations identified by single-genome sequencing were not detected by standard genotype analysis in over 90% of patients studied and the latter demonstrating that identification of the exact transmitted/founder virus during vertical transmission of HIV is not possible to ascertain with traditional bulk PCR methods.

Looking for a signature of what allows one virus to be transmitted and another not to be transmitted is important in development of advanced methods of prevention such as microbicides or vaccines. Identifying weaknesses and bottlenecks of transmission from mother to infant remain fundamental to prevention research. Additionally, evaluating signatures in both mother's and infant's viruses will improve the probability of identifying signatures that are present during transmission but are not present when transmission does not occur. Furthermore, cloning amplicons from full-length SGA products allows for the generation of Infectious Molecular Clones (IMCs) (Salazar-Gonzalez et al., 2009). The establishment of IMCs is an important component to describing the transmitted virus as IMC of transmitted/founder viruses allow for phenotypic characterization that can address questions specific to transmission biology, such as the target cells of primary infection, mechanisms of immune escape, and the potential vulnerabilities occurring during the establishment of chronic infection and latency.

The use of DBS simplifies sampling and reduces transport costs, making it a particularly suitable tool in remote and resource-limited settings like Zambia (Lofgren et al., 2009). Packed in zip-lock plastic bags with desiccant, DBS can be stored and shipped at ambient temperature, thus avoiding the need for cold chain and same day transport to the laboratory (Mitchell et al., 2008). However, DBS cards in use for early infant diagnostic purposes can also provide additional information about vertical transmission. The experiments detailed in this protocol outline how to implement the SGA methodology for detailed characterization of the HIV-1 quasispecies from DBS.

In this study, an in-house assay was developed for amplifying HIV-1 DNA from dried blood spots, and performed amplification of the 5' half, 3' half, *pol*, and *env* HIV-1 DNA using oligonucleotides that were optimized for clade C viruses in this geographical region. Patient-specific primers were not required. Furthermore, PCR titration experiments were performed to show proof of principle for the SGA technology. Capacity is in place at the on-site laboratory facilities to run these specimens for sequencing, and Central Laboratory has been running HIV drug resistance mutation sequencing since 2010, with an in-house sequencing assay run on an on-site ABI 3130xl from Applied Biosystems (Life Technologies). Current efforts are in place to utilize these technologies to perform confirmatory experiments using sequencing methods on patient samples. Because this current study does not show sequencing data on the samples that were amplified using this SGA protocol, it is incomplete as presented. Ultimately, the goal of a program that utilizes SGA technology for clinically relevant purposes (e.g., detect minority drug-resistance viral variants from patient samples (Palmer et al., 2005; McKinnon et al., 2011; Geng et al., 2010; Shi et al., 2010) or identify transmitted/founder HIV genomes during MTCT (Permar et al., 2013; Salazar-

Gonzalez et al., 2008; Salazar-Gonzalez et al., 2009) will necessitate the sequencing of these amplicons and can incorporate this into existing HIV drug resistance testing programs (Hamers et al., 2012).

This study shows the feasibility of utilizing DBS cards to amplify the HIV-1 proviral genome as well as perform the initial components of the SGA protocol on key HIV-1 genes. This study is the first of its kind to attempt to characterize the transmitted virus from mother-to-child infection of using proviral HIV from a DBS specimen- although a previous study reported that single molecules of proviral sequences were amplified using genomic DNA isolated from HIV-infected peripheral blood mononuclear cells (Simmonds et al., 1990). Determination of the transmitted/founder virus during vertical HIV transmission will elucidate the genetic repertoire of the exact virus that goes on to establish chronic infection (Lehman and Farquhar, 2007). A secondary innovation of this study is the feasibility of performing this assay from infant DBS specimens from a field laboratory setting in Sub-Saharan Africa. This research is significant because the specimens were collected and analyzed entirely in a field laboratory setting in Zambia. These findings demonstrate as a proof-of-principle that other field sites may be able to adopt this technically rigorous protocol. Current efforts are under way to complete sequence analysis of amplicons generated by SGA near the time of transmission to evaluate for number and complexity of individual viral variants which may shed light on the pathogenesis of vertical transmission.

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Highlights

- Developed an in-house assay for amplifying HIV-1 DNA from dried blood spot cards
- Performed bulk amplification of the 5' half, 3' half, *pol*, and *env* HIV-1 DNA
- Performed single genome amplification on *pol* and *env* HIV-1 DNA
- Specimens were collected and analyzed in a field laboratory setting in Zambia.

Figure 1.

Lane 1 is O'GeneRuler™ DNA ladder (Thermo Scientific) with standard DNA sizes indicated. Lane 2 shows positive 1200 bp control DNA. Lanes 3 and 4 (labeled 1 and 2) demonstrate two example samples of HIV-1 proviral DNA bulk PCR amplification of 5988 bp 5' half (1A) and 3411 bp 3' half (1B). PCR products were run on a 1% agarose gel and visualized with ethidium bromide and a UV source.

A. Pol serial dilution PCR

B. Env (V1-V5) serial dilution PCR

Figure 2.

HIV-1 proviral DNA single genome amplification (SGA) by serial dilution PCR on (A) *pol* and (B) *env* (variable domains 1- variable domains 5) genes. Lane 1 (M) of each gel is O'GeneRuler™ DNA ladder (Thermo Scientific) with standard DNA sizes indicated. 8 lanes each were for 1:10, 1:20, 1:30, and 1:50 dilutions of PCR products, which were run on a 1% agarose gel and visualized with ethidium bromide and a UV source.

Table 1

Oligonucleotides used for bulk and single genome PCR amplification

	HIV-1 HXB2 genome position	Oligonucleotide sequences	HXB2 amplified region	Expected band size
5' half (1st Round)	1 → 29	V15'F[*] : 5'-TGGAAGGGTTAATTACTCCAAGAAAAGG-3'		
	6990 ← 7015	REV15 : 5'-CTACCATTTAACACAGTAGTTGAGTTGA-3'		
5' half (2nd Round)	538 → 571	WGAF1 : 5'-CCTTGAGTGCTCTAAGTAGTGTCGCCGCTCTGT-3'	5' Long Terminal Repeat R repeat end	5988
	6501 ← 6526	REV14 : 5'-ACCATGTCAATTTTCCACATGTTAAA-3'	Env gp120 start	
3' half (1st Round)	6151 → 6184	V13'F[*] : 5'-TAGGAAAATTGGTAAGACAAAAGAAAATAGACTGG-3'		
	9689 ← 9719	V13'R[*] : 5'-TGCTAGAGATTTTCCACACTACCAAAAATGG-3'		
3' half (2nd Round)	6201 → 6222	FOR13 : 5'-GGGAAAAGAGCAGAGACAGTGG-3'	Env start	3441
	9611 ← 9642	1.R3.B3R[*] : 5'-ACTACTTAGAGCACTCAAGGCAAGCTTTATTG-3'	3' Long Terminal Repeat R repeat end	
POL (1st Round)	2044 → 2066	CWF[^] : 5'-GAAGGACACCAAAATGAAAAGAYTG-3'		
	3604 ← 3628	LSR1 : 5'-ACTGTTTTACATCATTAATGTTGGG-3'		
POL (2nd Round)	2136 → 2163	LSF1 : 5'-TCAGAGCAGACCCAGAGCCAAAGCCCA-3'		1200
	3345 ← 3370	REV7 : 5'-ATCCCTGGGTAAATCTGACTTGCCCA-3'	Gag p6 start p51 Reverse Transcriptase (middle)	
Env (V1-V5) (1st Round)	6201 → 6222	FOR13 : 5'-GGGAAAAGAGCAGAGACAGTGG-3'		
	8365 ← 8341	REV18 : 5'-GGTGAGTATCCCTGCCTAACTCTAT-3'		
Env (V1-V5) (2nd Round)	6556 → 6582	FOR14 : 5'-TATGGGATCAAAAGCCTAAAGCCATGTG-3'	Env gp120 start	1400
	7956 ← 7932	REV17 : 5'-CCTGGAGCTGCTTAATGCCCCAGAC-3'	Rev Responsive Element (RRE) end	

* Referenced in (Salazar-Gonzalez et al 2009)

[^] Referenced in (Wallis et al 2010)