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Cross-subtype detection of HIV-1 using reverse transcription and recombinase polymerase amplification

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A low complexity diagnostic test that rapidly and reliably detects HIV infection in infants at the point of care could facilitate early treatment, improving outcomes. However, many infant HIV diagnostics can only be performed in laboratory settings. Recombinase polymerase amplification (RPA) is an isothermal amplification technology that can rapidly amplify proviral DNA from multiple subtypes of HIV-1 in under twenty minutes without complex equipment. In this study we added reverse transcription (RT) to RPA to allow detection of both HIV-1 RNA and DNA. We show that this RT-RPA HIV-1 assay has a limit of detection of 10–30 copies of an exact sequence matched DNA or RNA, respectively. In addition, at 100 copies of RNA or DNA, the assay detected 171 of 175 (97.7%) sequence variants that represent all the major subtypes and recombinant forms of HIV-1 Groups M and O. This data suggests that the application of RT-RPA for the combined detection of HIV-1 viral RNA and proviral DNA may prove a highly sensitive tool for rapid and accurate diagnosis of infant HIV.

1. Introduction

Without antiretroviral treatment (ART), it is estimated that over 50% of HIV-infected children die before age two, therefore early diagnosis and immediate treatment of HIV-1 in young infants remains a public health priority (Obimbo et al., 2009; Newell et al., 2004). While HIV can effectively be diagnosed via serologic testing in adults and children older than 18 months (World Health Organization, 2010), the presence of transplacentally acquired maternal antibodies that target HIV-1 make serologic testing unreliable in young infants (Ciaranello et al., 2011). Consequently, diagnosis of HIV-1 infection in young infants currently relies on immunoassays that detect the viral capsid antigen p24 via immunoassays (Palomba et al., 1992; Patton et al., 2008), or most commonly, the amplification and detection of HIV-1 DNA using PCR-based assays with a whole blood specimen or a dried blood spot (Panteleeff et al., 1999; Paterlini et al., 1990). However, these assays are not adequate for use at the point of care and require sophisticated laboratory facilities as well as significant specimen and results tracking logistics (Essajee et al., 2015). Improving outcomes for HIV-infected infants requires a highly sensitive and specific diagnostic that is simple to use, robust, does not require complex laboratory equipment and can be reliably performed at the point of care.

In hospital or clinic-based settings where laboratory equipment is available, PCR-based assays are typically used for HIV diagnosis in infants. Most rely solely on detecting HIV-1 DNA, however, there is evidence that use of RT-PCR to detect HIV-1 RNA improves sensitivity (Lambert et al., 2003; Young et al., 2000; Obaro et al., 2005; Karchava et al., 2006; Swanson et al., 2005). A number of studies have measured HIV-1 RNA levels in infants from birth up to 18 months of age (Obimbo et al., 2009; Ciaranello et al., 2011; Mutasa et al., 2012; Lambert et al., 2003; Young et al., 2000) and suggest that it can take as little as two weeks following infection for HIV-1 RNA to be detected, with an exponential increase thereafter (Butto et al., 2010). The median viral load has been reported from 4.1×10^5 copies/mL at birth (Young et al., 2000); $1.59 - 3.7 \times 10^6$ copies/mL at 6 – 8 weeks (Mutasa et al., 2012; Young et al., 2000; Richardson et al., 2003); $0.6 - 1.6 \times 10^6$ copies/mL at 6 months (Ciaranello et al., 2011; Young et al., 2000) and 6.0×10^5 copies/mL at 18 months (Ciaranello et al., 2011). Elevated levels of HIV-1 viremia in infants may be due to their increased number of CD4+ T cells, the pri-

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mary target cells for HIV-1 replication, as compared to adults or immune systems with slower and weaker responses to viral infection (Richardson et al., 2003). We propose that a highly sensitive and specific assay that detects both HIV-1 RNA and DNA in whole blood will improve sensitivity compared to a diagnostic that detects only DNA.

A reoccurring theme with PCR-based diagnostics for early infant diagnosis (EID) of HIV infection is that they require skilled staff and a dedicated laboratory with complex equipment and reagents (Kiyaga et al., 2013), which are predominately available in urban or resource-rich settings. HIV testing of infants in low resource settings often requires specimen transport to central laboratories for testing. This typically results in large delays in reporting of test results to caregivers, delaying treatment initiation. While some logistical challenges can be addressed to improve turnaround time for infant HIV diagnosis (Finocchario-Kessler et al., 2014; Ghadrshenas et al., 2013; Kiyaga et al., 2013), an alternative solution is to simplify the diagnostics to enable testing at the point of care.

Our group, as well as a number of others, have shown that recombinase polymerase amplification (RPA) can be used for sensitive and specific detection of pathogen DNA or RNA in twenty minutes or less (Boyle et al., 2014; Boyle et al., 2013; Piepenburg et al., 2006; Rohrman and Richards-Kortum, 2012; Euler et al., 2012). RPA is an isothermal amplification method that utilizes a recombinase and a single stranded DNA binding protein to facilitate the insertion and stabilization of oligonucleotide primers into their complement in a double-stranded DNA molecule for subsequent amplification using a strand displacing DNA polymerase (Piepenburg et al., 2006). The use of opposing primers facilitates the exponential amplification of a defined region of DNA in a manner similar to PCR. RPA probes allow detection of amplification in real time via fluorescence, or by end point analysis using an immunochromatographic strip (ICS) (Boyle et al., 2013). In addition, RNA can be detected by RPA if reverse transcription (RT) of RNA into a complementary DNA (cDNA) is accompanied by RPA in an RT-RPA reaction (Rappolee et al., 1989; Euler et al., 2012).

The principle advantages of using RPA as a potential diagnostic in low resource settings (LRS) include rapid reaction time and the fact that amplification occurs over a broad range of temperatures (25–42 °C) allowing ambient temperature or body heat to be used to incubate RPA reactions (Boyle et al., 2014; Piepenburg et al., 2006; Lillis et al., 2014; Crannell et al., 2014a). Most pertinently for the detection of pathogens with high genetic diversity, such as HIV-1, RPA assays can accommodate some target sequence variation (Boyle et al., 2013; Euler et al., 2012). In this study we improved upon our previously described HIV-1 RPA assay (Boyle et al., 2013) by altering the primer and probe sequences and adding an RT step to allow for detection of both HIV-1 DNA and RNA. We assessed the performance of this new RT-RPA HIV-1 assay on a large panel of 175 highly diverse HIV-1 sequence variants including subtypes in groups M and O. Ultimately we aim to integrate this assay with a simple sample preparation method to create a high performance, yet easy to use, rapid HIV-1 diagnostic test that can reliably detect HIV-1 infection in infants at the point of care.

2. Materials and methods

2.1. RPA and RT-RPA amplification and detection

RPA reactions were supplied by TwistDx Ltd. (Cambridge, United Kingdom) in the TwistAmp Exo (probe cleavage via exonuclease III) and TwistAmp Nfo (probe cleavage via endonuclease IV) format for real time or end point detection of RPA amplicons respectively. Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, USA) and oligonucleotide probes from Biosearch Technologies (Novato, USA). Our original HIV *pol* RPA primers and probe sequences were previously described (Boyle et al., 2013). The Twist Alpha HIV-1 assay used novel primers and probe designed by our team at TwistDx. All TwistAmp reaction mixtures were prepared in a final volume of 50 μL per the TwistAmp protocols as previously described (Boyle et al., 2013). Reaction incubation and detection via real time fluorescence measurement was performed in a Twista™ real time reactor at 39 °C for 20 min, with a brief mixing step after 4–5 min carried out by inverting reactions 3 times. Positivity was scored as a double or greater level of fluorescence from the baseline value read at 4 min. For RT-RPA, a range of commercially available reverse transcriptases were screened for compatibility with RT-RPA by adding 2–5 units to each reaction. The best performing reverse transcriptase, OmniScript RT (Qiagen Valencia, USA) was subsequently selected to be used in all subsequent RT-RPA reactions. The final reaction volume and incubation conditions were otherwise unchanged for RT-RPA assays. The TwistAmp Nfo assays were prepared and incubated in an identical manner, except that the probe was labeled at the 5′ end with FAM and the reverse primer was labeled with biotin at the 5′ end to allow for immunochromatographic strip detection of hapten labeled RPA products. Immunochromatographic strips to detect Nfo-based RPA reactions were purchased from Milenia Biotech (Gießen, Germany) and Ustar Biotechnologies (Hangzhou, China). After incubation, RPA reaction tubes were immediately placed on ice and 5 μL of 0.5 M EDTA added to each tube to terminate amplification. Prior to immunochromatographic strip detection, 2 μL of each stopped reaction was diluted by mixing in 98 μL PBS/0.1% Tween buffer (flow buffer) and 10 μL of this solution applied to the capture pad of the strip. The strip was then placed in 100 μL of flow buffer and left to develop for 5 min. Positive results were scored with the development of a stripe on the test line and at the control line while negative results displayed only the control line. Tests that did not develop any lines were repeated.

2.2. Preparation of DNA and RNA

Exact sequence match HIV-1 proviral target DNA was extracted from the ACH-2 cell line that contains a single full-length integrated copy of subtype B HIV-1 *Bru* (subtype B; GenBank accession number K02013.1) using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, USA) (Clouse et al., 1989). The DNA was prepared and quantified as previously described (Boyle et al., 2013). To test for amplification of diverse HIV DNA sequences, plasmid clones of sequence variants of the HIV-1 *pol* gene derived from 56HIV-1 strains of the International Reference Panel from the NIH AIDS Research and Reference Reagent Program and from an existing library of subtype A, C and D primary isolates derived by short-term co-culture from the Overbaugh laboratory were prepared as described previously (Boyle et al., 2013). Plasmid DNAs were purified using QIAprep Spin Miniprep Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions, and concentrations of the purified plasmids were first quantified by spectrophotometry. Plasmid dilutions were then further quantified by real-time PCR to create samples with specific HIV-1 copy numbers as described in (Rousseau et al., 2003). To examine RNA amplification, a panel of 104 culture supernatants of HIV-1 strains that span common subtypes, circulating recombinant forms (CRFs) and unique recombinant forms (URFs) within HIV-1 group M and also group O was obtained from the External Quality Assurance Program Oversight Laboratory (EQAPOL; Duke University, USA) (Sanchez et al., 2014). The genome sequence of each isolate is publically available in GenBank (Benson et al., 2013) and in addition the viral load of each culture supernate was quantified via the COBAS AmpliPrep/COBAS TaqMan 48HIV-1 Test Version 2.0 (Roche Diagnostics, Pleasanton, CA, USA) (Sanchez et al., 2014).