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Development of a lateral flow recombinase polymerase assay for the diagnosis of *Schistosoma mansoni* infections

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ABSTRACT

Infection with *Schistosoma mansoni* causes intestinal schistosomiasis, a major health problem across Africa. The accurate diagnosis of intestinal schistosomiasis is vital to inform surveillance/control programs. Diagnosis mainly relies on microscopic detection of eggs in faecal samples but many factors affect sensitivity. Molecular diagnostics are sensitive and specific but application is limited as necessary infrastructure, financial resources and skilled personnel are often lacking in endemic settings. Recombinase Polymerase Amplification (RPA) is an isothermal DNA amplification/detection technology that is practical in nearly any setting. Here we developed a RPA lateral flow (LF) assay targeting the 28S rDNA region of *S. mansoni*. The 28S LF-RPA assay's lower limit of detection was 10pg DNA with the lower test parameters permitting sufficient amplification being 6 min and 25°C. Optimal assay parameters were 40–45°C and 10 min with an analytical sensitivity of 10^2 copies of DNA. Additionally the PCRD3 lateral flow detection cassettes proved more robust and sensitive compared to the Milenia HybriDetect strips. This 28S LF-RPA assay produces quick reproducible results that are easy to interpret, require little infrastructure and is a promising PON test for the field molecular diagnosis of intestinal schistosomiasis.

Introduction

Schistosomiasis is a neglected tropical disease (NTD) second only to malaria in infection rates [1] with 230 million people estimated to be infected worldwide [2,3]. It is endemic in Africa, Asia, the Middle East and parts of South America, however the greatest burden of disease is found in sub-Saharan Africa where *S. haematobium* (causing urogenital schistosomiasis) and *S. mansoni* (causing intestinal schistosomiasis) cause 90% of global infections resulting in an estimated mortality rate of around 200,000 per year [4]. Infection occurs when larval stages of the parasite are shed from the specific fresh water snails and enter through the human skin via water contact. These larval forms mature in the liver and then migrate to either the mesenteric veins (*S. mansoni*) or to the perivesicular veins (*S. haematobium*) where adult worms exist as a reproductive pair and produce many thousands of eggs per day [2]. The World Health Organization (WHO) has targeted schistosomiasis for elimination as a public health problem (defined as reducing prevalence of heavy-intensity infection to below 1%) in all endemic countries with a halt in transmission in most of these countries by 2025 [5,6]. This is to be achieved principally through mass drug administration (MDA) of oral praziquantel (PZQ) to kill adult worms [5] but will also require sensitive diagnostics to be able to identify low intensity infections, and also to set effective treatment intervals. The current standard diagnostic test for *S. mansoni* infection involves taking a double smear of a single stool sample for microscopic detection of schistosome eggs [7,8]. This is not only time-consuming, but lacks sensitivity particularly in low-intensity infections [7–15], leading to an under-estimation of disease prevalence. It is probable therefore that many infections are being missed using current diagnostic methods, and elimination goals are unlikely to be achieved unless a more sensitive, quick, easy to perform and cost-effective method of detection is developed.

Molecular diagnostics and molecular technologies are rapidly advancing and offer greater sensitivities and specificities over existing disease diagnostics. For the detection and quantification of *Schistosoma*-specific DNA in clinical samples, a number of molecular techniques and range of molecular targets have been put into practice [16–18]. Polymerase Chain Reaction (PCR) amplification of schistosome DNA within clinical samples has been shown to be highly sensitive in detecting *S. mansoni*, able to detect down to 2.4 eggs per gram of faeces [18]. An added advantage is the flexibility of using different types of sample due to the ability to detect not only eggs but also Cell-Free-Parasite-DNA (CFPD) in clinical samples. However, current molecular diagnostic use

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is hampered by the need for expensive laboratory equipment, highly skilled personnel and cold-chain storage devices, not readily available within most endemic countries [16]. Recombinase Polymerase Amplification (RPA) is a novel isothermal (requiring constant ambient temperatures) DNA amplification technology being developed for the Point-Of-Need (PON) diagnosis of several important pathogens including those causing NTDs [19–22]. The technology is sensitive and specific but also overcomes many of the obstacles faced by existing molecular diagnostics; being rapid, robust, high-throughput, requiring low energy and portable equipment, with results available using field-friendly detection devices, making this a promising technology for molecular PON diagnosis [19,20]. There are an increasing number of reports combining isothermal amplification with simple and rapid lateral flow detection of amplified DNA [20–30], moving nearer to achieving molecular diagnostics that can be used at the PON. A Lateral Flow (LF) RPA assay for the detection of *S. japonicum* DNA targeting the highly repetitive retrotransposon SjR2 region in faecal samples has been shown to have equal sensitivity to both quantitative PCR and real time (RT) RPA, detecting 5fg *S. japonicum* DNA which is reportedly less than that found in one egg [21]. The assay also showed high specificity, showing no cross-reactivity with other helminthic parasites [21]. A previous LF-RPA study targeting the tandem repeat Dra-1 sequence of *S. haematobium* found successful amplification and detection between assay temperatures of 30–45 °C, in 10 min incubation time, and with a sensitivity of 100fg DNA [22]. There is no existing molecular PON diagnostic test for *S. mansoni* and so here we aimed to develop an RPA assay for the sensitive detection of *S. mansoni* DNA, and to test different reaction parameters for the assay's suitability for the endemic PON setting. Two different nuclear DNA targets were evaluated for optimal assay design and performance and the results are discussed in relation to the assay's potential for field PON testing on clinical samples.

Materials and methods

S. mansoni genomic DNA

S. mansoni (Ugandan Isolate, Lake Victoria) adult worm genomic DNA was obtained from the Schistosomiasis Collection at The Natural History Museum (SCAN) [31], and was quantified using the NanoDrop ND-8000 8-Sample Spectrophotometer.

RPA primer design

The *S. mansoni* 28S and ITS ribosomal DNA regions were targeted for amplification. Their sequences were downloaded from Genbank (NCBI) as these have been used in previous molecular diagnostic assays [16,18] (https://www.ncbi.nlm.nih.gov/genbank/). Accession numbers; JQ289757 (ITS); AY157173.1 (28S). Multiple forward and reverse RPA primers were designed using Primer3 (http://primer3.ut.ee). RPA primers were designed following the TwistDx™ guidelines to be 30-35 bases long with 30–70% GC content for both regions. The primers were designed to produce the smallest possible amplicon to maximize amplification rates. A gap of at least 52bp was maintained between primer pairs to allow for internal probe design for lateral flow detection.

ITS and 28S RPA primer screening

Primers were screened using the TwistAmp™ Basic kit to determine the best primer pairs according to product yield using the TwistAmp™ Basic kit reagents. To prevent cross contamination, preparation of all RPA reactions was carried out in a pre-PCR room under extraction hoods, and all reagents were left under UV light before and after each batch of reactions was run. There were eight possible primer pairs to be tested; six ITS pair combinations covering a 267bp region, and two 28S primer pair combinations covering a 327bp region. All primer screening was done using 1ng of *S. mansoni* genomic DNA.

Reactions were set-up following the TwistDx™ Basic RPA protocol with each reaction containing 29.5μl rehydration buffer, 2.4μl of each forward and reverse primer (10pmol), 12.2μl dH_20, and 1μl of *S. mansoni* genomic DNA for each reaction mix. The RPA pellets were first decanted into 0.2ml PCR tubes for easier handling, the reaction mix was then added, vortexed and spun down. 2.5 μl of magnesium acetate was added to each lid making a total reaction volume of 50μl and a magnetic bead was dispensed into each tube for magnetic mixing. Tubes were centrifuged and immediately placed into the Twirla™, a portable heat block with internal magnetic motor; the motor agitates the mix every 10 s preventing the need for a manual mixing step. Incubations were initially performed at 40 °C for 20 min. Amplification products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany), and run on a 4% gel red agarose gel (Sigma Aldrich, UK). Negative no DNA template controls were incorporated into each set of reactions. Primer pairs that gave strong positive amplification of the correct size amplicon with no non-specific amplification were selected for further development. Additionally the primer pairs that gave the smaller size amplicons were selected to facilitate rapid amplification.

Lateral flow RPA probe design and testing

Internal lateral flow RPA probes were designed for both the 28S and ITS DNA regions following the TwistDx guidelines, with lengths of between 46 and 52 nucleotides, containing either a 6-carboxyfluorescein (FAM) label or a biotin label at the 5′ end. A basic tetrahydrofuran (THF) residue replaced a single nucleotide at least 30bp from the 5′ end and at least 15 nucleotides from the 3′ end. A C3 spacer at the 3′ end prevents extension of any un-hybridised probe. To enable lateral flow detection, reverse primers were modified by attaching either a biotin recognition label or a FAM label to the 5′ end. If the probe was labeled with the FAM then the reverse primer was labeled with biotin and vice versa. The ITS and 28S LF-RPA reactions were performed using the TwistDx nfo kit. Reactions contained 1ng of *S. mansoni* DNA, 29.5μl of rehydration buffer, 2.1μl forward primer (10pmol), 2.1μl labeled reverse primer (10pmol), 0.6μl of the specific internal lateral flow probe (10pmol), and 12.2μl dH_20 for each reaction. These were mixed and added to the RPA nfo pellets. A magnetic bead was added for magnetic mixing and then 2.5 μl of magnesium acetate was added to the lids before the reactions were closed, centrifuged and incubated in the Twirla for 20 min. Negative no DNA template controls were also included with each set of reactions performed.

28S and ITS LF-RPA amplicon detection

S. mansoni DNA amplification was detected using both the Milenia HybriDetect lateral flow dipsticks (Milenia Biotec GmbH, Gießen, Germany) and also the PCRD Nucleic Acid Detector lateral flow assay cassettes (Abingdon Health, York, UK) for comparison. Detection occurs in a typical ‘sandwich’ format, with the target, in this case via the probe, forming a conjugate with recognition anti-FAM antibodies on the sample application area, and is then captured at the test line by anti-biotin antibodies to form a complex with colloidal carbon (PCRD) or colloidal gold (Milenia), producing a coloured signal. A control line is also visualised on the test strips to prevent any false negatives through failure of the lateral flow strips.

To prevent contamination by RPA amplicons post-amplification processing of the ITS and 28S RPA assays for lateral flow detection was carried out in a separate post-PCR area to the reaction set up and also under an extraction hood. For the PCRD strips 5μl of the RPA amplification product was added to 70μl PCRD running buffer. Then 75μl of this mix was pipetted into the sample well on the cassette. Results were read at no later than 10 min. For Milenia HybriDetect strips 5μl of RPA amplification product was added to 100μl HybriDetect buffer. The detection strip was placed vertically into the tube containing the mix with the sample application pad submerged in the solution. Results were