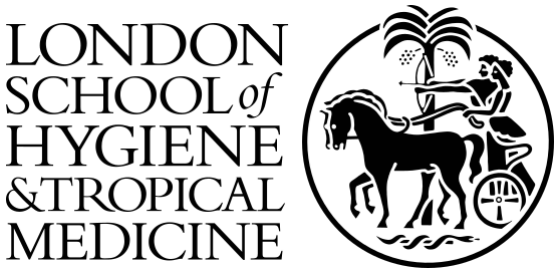

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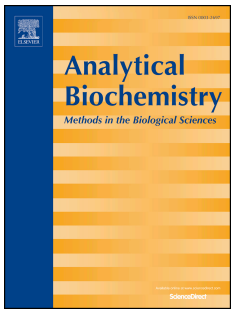

Development of a lateral flow recombinase polymerase assay for the diagnosis of *Schistosoma mansoni* infections

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PII: S0003-2697(18)30070-8

DOI: 10.1016/j.ab.2018.01.031

Reference: YABIO 12922

To appear in: *Analytical Biochemistry*

Received Date: 12 October 2017

Revised Date: 25 January 2018

Accepted Date: 30 January 2018

Please cite this article as: K. Poulton, B. Webster, Development of a lateral flow recombinase polymerase assay for the diagnosis of *Schistosoma mansoni* infections, *Analytical Biochemistry* (2018), doi: 10.1016/j.ab.2018.01.031.

Development of a Lateral Flow Recombinase Polymerase Assay for the diagnosis of *Schistosoma mansoni* infections

Highlights

- A LF-RPA assay has been developed for *S. mansoni*
- Reactions take 6 minutes and can work at 25°C
- The assay can detect 10pg of DNA and 10^2 copies of DNA
- Betaine can rectify false positives but may influence assay sensitivity
- The low resource need and quick time to results may enable PON testing

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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 minutes and 25°C. Optimal assay parameters were 40-45°C and 10 mins 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.

Key Words: Recombinase Polymerase Amplification, molecular diagnostic, schistosomiasis, point-of-need

1. 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 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 minutes 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.

2. Materials and Methods

2.1 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.

2.2 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.

2.3 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. 2.5µl 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

seconds preventing the need for a manual mixing step. Incubations were initially performed at 40°C for 20 minutes. 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.

2.4 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 46-52 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 labelled 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 2.5µl of magnesium acetate was added to the lids before the reactions were closed, centrifuged and incubated in the Twirla for 20 minutes. Negative no DNA template controls were also included with each set of reactions performed.

2.5 28S and ITS LF-RPA amplicon detection

S. mansoni DNA amplification was detected using both the Milenia HybriDetect lateral flow dipsticks (Milenia Biotec GmbH, Gieβen, 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 minutes. 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 read between 5-15 minutes.

2.6 Addition of Betaine

To address problems with false positive results with the ITS LF-RPA assays, the addition of Betaine to the RPA reactions was trialed with an aim to reduce primer noise and mis-priming. 1µl, 5µl and 7.5µl of Betaine (5M) were each substituted for the same amount of dH_20 in the reaction mix in the ITS RPA reactions to maintain reaction volumes at 50µl. This was tested with negative no DNA template control reactions for each primer pair to determine the minimum Betaine concentration that would effectively prevent false positive results from occurring on the detection strips. This was not done for the 28S LF-RPA reactions as no false positives were encountered.

2.7 LF-RPA Reaction parameter testing

Different parameters were tested 10 times to assess accuracy.

2.7.1 Temperature

The LF-RPA assays were tested on template DNA (1ng) at different reaction temperatures; 20, 25, 30, 35, 40, 45 and 50°C for 20 minutes. When using incubation temperature as the RPA assay variable, incubations were performed in a standard PCR machine (GeneAmp PCR System 9700) set at the required temperature. Reactions were manually mixed every 5 minutes to prevent localised depletion of reagents caused by viscosity of the reaction mix

188

189 *2.7.1 Reaction Time*

190 LF-RPA assays were tested on template DNA (1ng) with different reaction times to
191 determine the minimum length of time needed for DNA amplification and detection.
192 Reactions were run at 5, 6, 7, 8, 9, 10 and 15 minutes duration in the Twirla (40°C), and
193 immediately halted by placing tubes on ice before further processing.

194

195 *2.8 Analytical Sensitivity and specificity*

196 *2.8.1 Sensitivity*

197 Serial dilutions of the *S. mansoni* DNA were made by diluting the 1ng/µl stock DNA
198 with water to give dilutions of 100pg /µl, 10pg/µl, 1pg/µl, and 100fg/µl of DNA.
199 ITS and 28S LF-RPA reactions were tested on these DNA dilutions to determine the analytical
200 sensitivity of these assays. All reactions were run in the Twirla device with magnetic mixing
201 at 40°C for 20 minutes.

202

203 *2.8.2 Specificity*

204 The LF-RPA assays were tested on DNA from other important schistosome species found co-
205 endemically with *S. mansoni.* The ITS and 28S LF-RPA assays were tested using 1ng of *S.*
206 *haematobium* and *S. bovis* DNA from the Schistosomiasis Collection at the NHM (SCAN) to
207 determine cross reactivity of the LF-RPA assays. Other pathogens samples were not
208 available for testing so the primers were Blasted against the NCBI Genbank database
209 (https://blast.ncbi.nlm.nih.gov/Blast) to evaluate which other organisms they might also be
210 compatible with.

211

212 *2.9 Analytical limit of detection*

213 As the 28S DNA region and primer probe combination proved the most robust with no
214 issues with false positive reactions a DNA standard was created for the 28S DNA target to
215 determine the analytical limit of detection of the 28S LF-RPA assay. A linearized molecular
216 28S DNA standard was synthesized (GeneArt, Invitrogen, Darmstadt Germany) and diluted
217 to give a measure of numbers of molecules per µl. The 28S LF-RPA assay was tested on 10^8,
218 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 copies of the molecular DNA standard. All reactions were run

using the standard 28S LF-RPA protocol and the reactions were performed in the Twirla device with magnetic mixing at 40°C for 20 minutes. Amplicon detection was performed using the PCRD reaction strips as described previously. The limit of detection was tested multiple times to assess robustness of the assay.

3. Results

3.1 ITS and 28S RPA primers and probes

Sequences for all ITS and 28S primers and probes are shown in Table 1, including reverse primer modifications for LF-RPA testing.

Name	Sequence (5'-3')
ITS 59 F	ACCAGAGACAAGATCAAGTGATTAACGTAG
ITS 65 F	GACAAGATCAAGTGATTAACGTAGCATACG
ITS 70 F	GATCAAGTGATTAACGTAGCATACGATAGG
ITS 192 R	[Btn]CTAGTCTGGTCTAGATGACTTGATTGAGATG
ITS 312 R	[Btn]CTTTTCATCTATCACGACGCACATTAAGTC
ITS probe	[6FAM]TCCCGAGCGTGTATAATGTCATTAAGCCACGA[THF]TCGAGCACAACCCACCGCA[SpcC3]
28S 335 F	GTTTGCTATCGGACTCGTGTAAGTATTTAG
28S 353 F	GTAAGTATTTAGCCTTGGATGGAGTTTACC
28S 647 R	[6FAM]AGAAACTAACAAGGATTCCCCTAGTAACTG
28S probe	[Btn]TAAACACCACATTGCCTTACGATCAAATAAC[THF]CGCAGGCTTTCGGTGTTGGGCT[SpcC3]

Table 1. ITS and 28S primer and probe sequences designed for the LF-RPA assays. Modification codes: Btn = Biotin label, 6FAM = FAM label, THF = basic tetrahydrofuran residue, SpcC3= C3Spacer.

3.2 Primer pair screening

All ITS and 28S primer combinations successfully amplified 1ng of *S. mansoni* DNA. Results visualised on a agarose gel confirmed the correct DNA amplicon lengths expected from each primer pair (Figure 1). Three ITS primer pairs (1. 70F + 192R (122bp*), 2. 65F + 192R

(127bp*), 3. 59F + 192R (133bp*) and one 28S primer pair (353F + 647R (294bp*)), were selected for further optimisation based on the smaller amplicon lengths produced and strong band signal intensity (Figure 1). A shorter amplicon is more desirable as it will equate to a faster amplification rate and generates fewer primer 'artefacts', providing greater sensitivity [32].

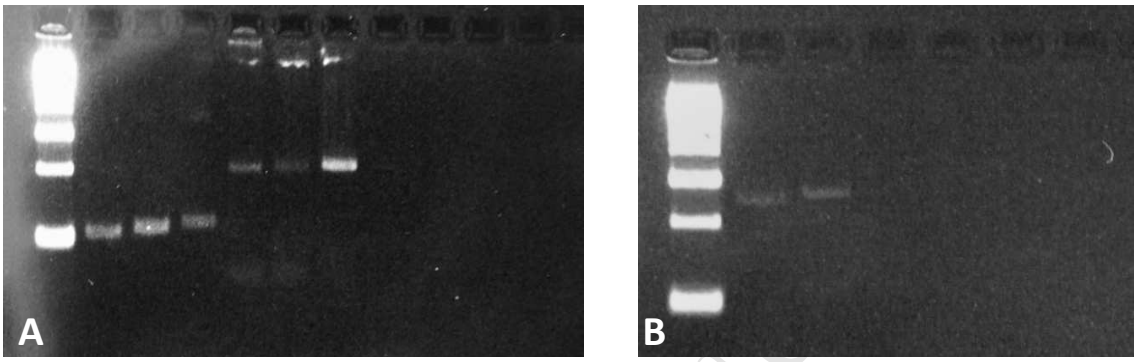

Figure 1. A) amplicons produced by the 6 ITS primer pair combinations. 1. 70F + 192R (122bp*), 2. 65F + 192R (127bp*), 3. 59F + 192R (133bp*), 4. 70F + 312R (242bp), 5. 65F + 312R (247bp), 6. 59F + 312R (253bp) B) amplicons produced by the 2 28S primer pair combinations. 1. 353F + 647R (294bp*), 2. 335F + 647R (312bp). The ladder in lane 1 is Hyperladder IV. *These primer pairs were chosen for further optimization and LF RPA development.

3.3 Lateral Flow (LF) RPA development

Due to the high cost of the internal LF probes only a single ITS and 28S probe were designed and tested.

3.3.1 ITS LF-RPA assays

The ITS LF-RPA reactions were hampered by false positive results with all the primer probe combinations tested. It was suspected that false positives might be caused by a possible overlap of reverse primer with the probe due to them only being separated by just one base. We substituted the biotinylated ITS 312R primer in place of the ITS 192R primer in the reactions to increase reverse primer-probe gap to 122 bases, however this was unsuccessful in preventing false positives occurring (Figure 2a). The ITS LF-RPA amplification products were purified and run on agarose gel to visualise any non-specific DNA products that might be causing the anomaly. It was evident that additional non-specific bands slightly smaller

than the target DNA bands were present in all lanes including the negative control, suggesting possible primer-dimer or primer-probe dimer products (Figure 2b). To try and reduce the possible primer-probe artefacts the addition of Betaine to the reactions was trialled. It was found that with the addition of 7.5µl of Betaine one of the primer pairs, ITS 70F/ITS 312R, with an amplicon size of 242 bases did not produce false positives (Figure 3). This primer pair was used in all further ITS LF-RPA parameter-testing assays.

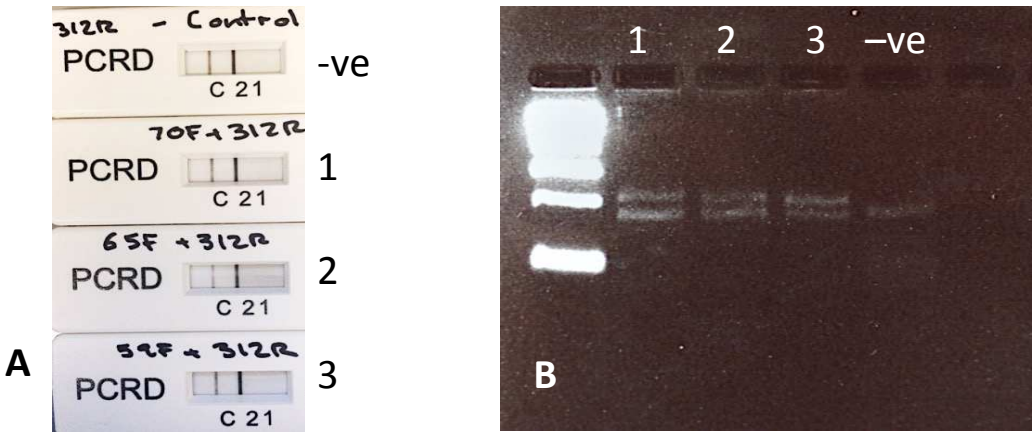

Figure 2. A) Lateral flow results of the different ITS LF-RPA primer combinations plus a negative control that shows a false positive result. **B)** Gel images of the ITS LF-RPA reaction amplicons. A non-specific amplicon (the smaller band) can be seen in all the reactions and the negative control. The ladder in lane 1 is Hyperladder IV.

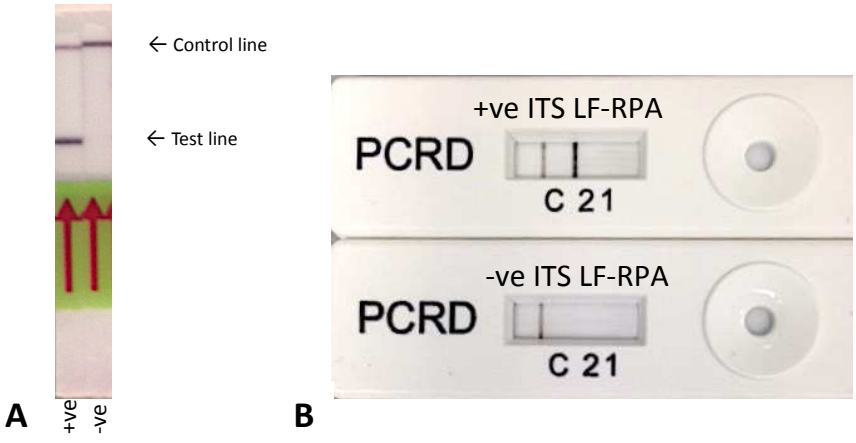

Figure 3. Positive and negative ITS LF-RPA reactions with the addition of 7.5µl of Betaine to the reactions. No false positives were obtained; **A)** using Milenia HybriDetect strips and **B)** PCRD Nucleic Acid Detector strips.

3.3.2 28S LF-RPA assays

The 28S primer / probe combinations did not produce false positive results thus the 28S primer pair that gave the shortest amplicon of 294 bases (28S 353F/28S 647R) was used in all 28S LF-RPA parameter testing assays (Figure 4).

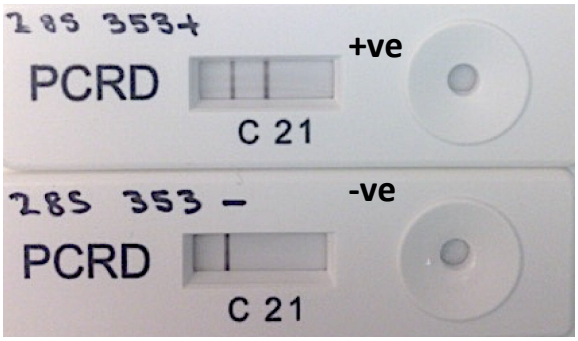

Figure 4. Positive and negative 28S LF-RPA reactions using the primer pair 353F and 647R.

3.4 Performance of the Milenia vs. PCRD Nucleic Acid Detection Strips

Both Milenia HybriDetect lateral flow strips and PCRD Nucleic Acid Detection strips were used for detection of ITS LF-RPA amplified products, to compare their performance (Figure 5). We found that PCRD cassettes were more sensitive than the Milenia HybriDetect strips, able to detect ITS target DNA with one minute less RPA reaction incubation time than the Milenia HybriDetect strips (Figure 5). PCRD test lines consistently appeared more prominently than on the Milenia HybriDetect strips, which varied in intensity and were not uniform in clarity. Test lines also developed at a faster rate on PCRD strips. Due to their better performance, it was decided to use only PCRD cassettes for the remainder of the assays.

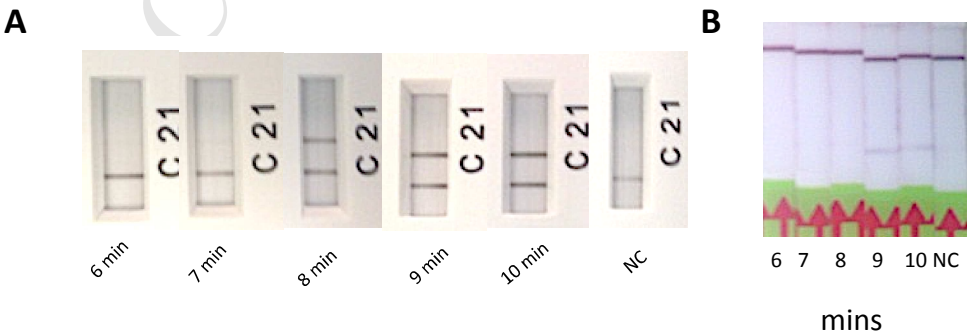

Figure 5. PCRD cassettes (A) gave more consistent results and were more sensitive able to detect DNA amplification after 8 mins of reaction time, whereas the limit of reaction time for the Milenia HybriDetect strips (B) was 9 minutes. NC = negative control

3.5 LF-RPA ITS and 28S temperature and time parameter testing

3.5.1 LF-RPA reaction temperature testing

A positive signal was detectable at incubation temperatures between 30-45°C and 25-45°C for the ITS and 28S assays respectively, with the strongest signals occurring at 40°C and 45°C, and no signal detected at 50°C (Supplementary File Figures 6+7).

3.5.2 LF-RPA reaction time testing

A positive signal could be detected with a limit of eight and six minute RPA reaction incubation time for the ITS and 28S assays respectively (Supplementary File Figures 8+9).

3.6 Sensitivity testing

The ITS LF-RPA assay was tested on serial dilutions of *S. mansoni* genomic DNA. Unfortunately at lower concentrations inconsistent results were obtained from reactions on the same concentration of DNA and so the limit of detection could not be evaluated further. For the 28S LF-RPA assay consistent results were obtained from replicate reactions and the 28S LF-RPA assay limit of sensitivity was 10pg of DNA (Supplementary File Figure 10).

3.7 Analytical limit of Detection of the 28S LF-RPA assay

The analytical lower limit of detection for the 28S LF-RPA assay was 10^2 copies of DNA (Supplementary File Figure 11).

3.8 Specificity with other important schistosome species

The ITS LF-RPA assay proved to be not specific to *S. mansoni* and was also able to amplify *S. haematobium* and *S. bovis* DNA (Supplementary File Figure 12A) whereas the 28S LF-RPA assay proved to be more specific to *S. mansoni* but was still able to amplify *S. haematobium* and *S. bovis* DNA but at a lower sensitivity and with faint detection lines observed on the PCRD strips (Supplementary File Figure 12B).

4. Discussion

A diagnostic test that is both sensitive and specific is of paramount importance to successfully monitor schistosomiasis transmission, particularly in low endemic areas. Inaccuracies in prevalence data due to lack of sensitivity of diagnostics could lead to a premature reduction/withdrawal of MDA, leading to a rapid resurgence of transmission and disease. Alternatively over-prescription of the drugs caused by a lack of diagnostic specificity causes wastage of medicine, insufficient supply of PZQ, systematic non-compliance and potentially could lead to the development of PZQ resistance. Ultimately this will prevent WHO elimination targets from being reached [5].

Molecular diagnostics are the gold standard for several pathogens due to their greater specificity and sensitivity. However PCR based methods although sensitive, are expensive, difficult to use in low resource settings and experience slow turnaround times [16-18]. RPA is an alternative DNA-based method and overcomes several of the obstacles encountered by traditional DNA-based diagnostics [33-35]. RPA also demonstrates performance characteristics particularly suiting it to PON use. RPA reactions can operate at temperatures as low as 25 °C, albeit slower, so DNA amplification can be achieved using ambient temperature, body heat, room temperature or low power/ battery powered incubators, making the assay highly feasible in low resource settings where a reliable mains power supply may not be available [36]. This together with the LF detection system is simple to use and requires little in the way of training with results easily interpreted by untrained personnel. It is quick to turnaround with results available in less than 30 minutes after incubation initiation [36,37], which is particularly advantageous over existing isothermal systems such as Loop Mediated Isothermal Amplification (LAMP) that needs higher temperatures of 64°C for 90 mins [38]. Here, using the existing TwistDx platform, RPA has also been combined with the LF-RPA DNA amplification and detection system to detect *S. mansoni* DNA, which could be further optimised for the PON molecular diagnosis of human *S. mansoni* infections.

Two DNA targets, the ribosomal ITS and 28S regions, were tested for their RPA performance with different primer and probe combinations. All primer pairs produced promising results with correct amplicon size amplification, however the ITS LF-RPA assays consistently produced false positive results probably due to some primer/probe secondary structure or hairpins possibly causing non-specific amplification creating false positive

results. These non-specific products could be visualised by gel electrophoresis of the ITS LF-RPA reactions (Figure 2B). False positives were controlled with the addition of Betaine, which is often used in nucleic acid amplification to prevent secondary structures such as hairpins from forming, particularly where there is a high GC content [39]. With the addition of Betaine the ITS LF-RPA assay worked at a low temperature of 30°C with an eight-minute reaction time. The 28S LF-RPA assay proved more robust with no false positive reactions and the assay could be performed at lower temperatures (25°C) and with a shorter reaction time of six minutes.

The primer pair used for the 28S LF-RPA assay (28S 353F/28S 647R), generated an amplicon of 295bp, longer than that recommended for RPA (<200bp) and had a slightly higher than recommended GC content (49.8% compared to 43.5% ITS). Longer amplicons generate a larger proportion of DNA artefacts such as hairpins and primer-dimers, due to the relatively faster replication of the shorter 'noise' fragments, and thus reduces sensitivity. In theory then the ITS assays (127 bp) would be predicted to outperform the 28S assays; however the 28S assays showed greater performance and reliability. These results highlight the importance of multiple primer and probe screening to develop optimal and robust RPA assays.

The sensitivity of the ITS LF-RPA assays could not be assessed due to inconsistent results with low DNA concentrations probably due to inhibition caused by the Betaine in the reactions. The 28S LF-RPA had a limit of detection of 10pg of genomic DNA and 10^2 DNA copies, which was not as sensitive as previous studies where LF-RPA assays were able to detect down to 100fg of DNA for *S. haematobium* [22], and 5fg for *S. japonicum* [21]. Additionally, Pontes *et al* (2002) [18] were able to detect down to 1fg of pure *S. mansoni* DNA using PCR. However, all of these studies targeted highly repetitive elements within the genome, which have a higher copy number than the regions tested here, and thus could explain why the targeted regions showed lower sensitivity. Sandoval *et al* (2006) [40] found that a 28S PCR was able to detect down to 1pg of DNA, but that the sensitivity of the genus-specific 28S region varied significantly depending on which primer pair was used. Sandoval *et al* (2006) [40] also reported the ITS region to be much less sensitive at detecting schistosome DNA than the 28S region when using PCR. However, an LF-RPA on the ITS1 region of *Fasciola hepatica* was able to detect down to 1pg/µl in stool samples [41].

Designing and testing alternative 28S and ITS primer and probe combinations and/or other DNA regions could therefore improve sensitivity, and should be investigated further [42,43].

LF-RPA amplification detection was trialled both using the PCRD nucleic acid detectors and the Milenia HybriDetect lateral flow dipsticks. The PCRD cassettes were more user friendly and the PCRD test bands were more uniform, easier to read, and developed more rapidly than the Milenia HybriDetect strips. According to the manufacturers, PCRD cassettes have a detection limit of 0.001µg/ml DNA. In this study PCRD did demonstrate a higher sensitivity: able to detect amplification products after shorter RPA assay incubation times than the Milenia HybriDetect strips. This could make PCRD a preferable detection tool in low intensity infections.

The assays were shown to not be specific to *S. mansoni* but had some cross reactivity to S. *haematobium* and *S. bovis.* This offers both advantages in terms of being able to detect multiple schistosome species but also disadvantages in terms of specificity to *S. mansoni.* The cross-reactivity of the assay was not tested on negative control clinical samples or other pathogens that can also be found within stool samples and this will need to be done to evaluate clinical specificity. However, the compatibility of the primers to other organisms was checked using the NCBI blast tool with compatibility only shown with other non-parasitic organisms. The assay's utility will depend on what the diagnostic requirements are for the assays and if specificity is a priority. Other more species-specific DNA targets can be trialled in future work to improve specificity.

This preliminary study offers a basis on which to conduct further RPA research and development with an aim to develop species-specific RPA assays to enable rapid field identification of different and multiple species involved in schistosomiasis infections. With further optimisation and validation the LF-RPA assays may prove to be a feasible diagnostic test for *S. mansoni* infection bringing PCR level sensitivity to the PON settings. Further development is needed to investigate different target DNA regions and primer/probe combinations to improve sensitivity and specificity. Field evaluation is also required to investigate the reliability of the targeted amplification of free schistosome DNA excreted in different types of clinical samples e.g. saliva, urine and plasma, as a by-product of the infection, together with the DNA that can be obtained from the excreted *S. mansoni* eggs in faecal samples [44].

In conclusion, the 28S LF-RPA assay developed here has high potential as a PON

diagnostic test for *S. mansoni* infections. It requires little in the way of equipment and technical support and results are quick to obtain and easy to interpret enhancing its suitability as a PON diagnostic test for individual patient management and disease mapping/surveillance as part of schistosomiasis control programmes [45,46].

Funding

This work received funding from the Royal Society Small Grant Scheme and also the London School of Hygiene and Tropical Medicine for masters research funds.

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