



OPEN Advances in the recombinase polymerase amplification platform for urogenital schistosomiasis diagnosis (ShDral-RPA) towards implementation at the point-of-care

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Accurate diagnosis of schistosomiasis is crucial to achieve disease elimination as a public health problem. Rapid and highly sensitive diagnostic tools that can be used in decentralized environments and/or at the point-of-care are needed. This work optimises and simplifies an existing isothermal molecular diagnostic platform (recombinase polymerase amplification, RPA) for urogenital schistosomiasis, the ShDral-RPA, with a focus on delivering a more accurate diagnosis in endemic settings. The standard ShDral-RPA oligonucleotides were modified, incorporating a phosphorothioate backbone into the reverse primer and inverting the probe fluorophore and quencher, to prevent false positive results due to secondary structure formation. The sensitivity and specificity of the modified assay were evaluated on a donor urine spiked with one *S. haematobium* egg and an array of other schistosomes and human urinary tract pathogens. The stability of RPA reagents was assessed by storing them at ambient temperature ($\pm 27^\circ\text{C}$) in a dark environment for up to 90 days. Sample preparations were explored to develop a simple, rapid and low resource methodology that would complement the ShDral-RPA platform when used in remote settings. The modified ShDral-RPA assay was robust, sensitive and specific to *S. haematobium* group species, detecting down to 10 fg of gDNA and ten synthetic *Dra* I copies. DNA amplification was achieved at 42°C within 20 min and results could easily be visualized using a portable fluorometer or under blue light. RPA reagents remained stable when stored in the absence of light at $\pm 27^\circ\text{C}$ for up to 30 days. A two-step DNA extraction method proved optimal for extracting DNA from single *S. haematobium* eggs in spiked urine. The optimized ShDral-RPA platform shows improved specificity and sensitivity and has now reached several of the target product profile requirements set out by the WHO for the ideal diagnostic test for schistosomiasis.

Keywords *Schistosoma haematobium*, RPA, Optimisation, Schistosomiasis, Urogenital schistosomiasis, Isothermal molecular test

Schistosoma is a diverse genus of parasitic trematodes comprised of 25 species, with six of these being pathogenic to humans and causing schistosomiasis, a neglected tropical disease (NTD). At present, schistosomiasis affects more than 250 million people globally, mainly in Sub-Saharan Africa^{1,2}. *Schistosoma mansoni* and *Schistosoma haematobium* are the most widespread species, causing intestinal and urogenital schistosomiasis, respectively. Urogenital schistosomiasis is often highly endemic in African countries with haematuria, urinary tract infections (UTIs), granuloma formation and chronic inflammation being the most common symptoms³. Furthermore,

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there is a clear correlation between urogenital schistosomiasis and an increased risk of bladder and kidney cancer, infertility and stigmatization, with the last two associated with female genital schistosomiasis (FGS)^{4,5}.

Although highly prevalent, urogenital schistosomiasis is a preventable disease. In 2020, the world health organization (WHO) launched the “Road map for neglected tropical diseases”, in which it established the goal of eliminating schistosomiasis as a public health problem by 2030⁶. Accurate diagnostics play a crucial role in achieving this goal, as it has been defined as “less than 1% prevalence of heavy-intensity infections” in a given area⁷. The WHO-defined target product profile (TPP) for ideal schistosomiasis diagnostics requires improvements in existing tests, with the ambitious goal of developing highly specific and sensitive point-of-care (POC) tests suitable for low infrastructure settings, which could then be used for monitoring, evaluation and surveillance purposes⁸.

Currently, urine filtration followed by the microscopic search for expelled *S. haematobium* eggs is the most routinely used method of diagnosing infections and estimating the disease prevalence in a given region. The estimated prevalence will then, in turn, determine the treatment approach that should be conducted⁸. However, diagnosis by microscopy lacks sensitivity, particularly when attempting to detect low-intensity and/or early stages of infection². Moreover, microscopy is time-consuming and labour-intensive, requiring appropriately trained and skilled microscopists. Recently, an automated mobile phone associated POC tool, the SchistoScope, was developed for rapid microscopy screening of urogenital schistosomiasis⁹. Although the device has shown promising results, it has also shown some flaws with field of vision, issues with image and filter design, and manufacturing. Furthermore, biological aspects of the parasites’ lifecycle (e.g., daily fluctuation in egg release) limit its use in large-scale control programmes. Likewise, artificial intelligence-based image analysis has been developed to detect eggs on a robotised microscope¹⁰. However, this still does not overcome the inherent limitations of egg-based detection, which ultimately require the presence of schistosome eggs in the sample.

Diagnostic nucleic acids amplification tests (NAATs), also known as molecular tests, offer many advantages over traditional parasitological diagnosis due to their high sensitivity and specificity¹¹. Polymerase chain reaction (PCR)-based tests have been widely used in the past decades and have made possible real progress in the diagnostics field. Real-time quantitative PCR (qPCR) is considered more sensitive than PCR, with the additional benefit of providing a quantitative result that can be correlated to the intensity of infection¹². When using qPCR to detect *S. haematobium* DNA in urine samples, Keller et al.¹³ detected twice as many positive cases compared to urine-egg microscopy. Esiere et al.¹⁴ reported a 20-fold higher schistosomiasis prevalence using PCR compared to when egg microscopy was the test of choice. These results reinforce how powerful NAATs can be.

Despite the notable advantages of PCR-based tests, the financial costs and the need for adequate laboratory infrastructure to perform them limit their use in infection surveillance at the POC and in low resource areas¹³. In this context, isothermal DNA amplification methods stand out as promising alternatives for application at the POC, as they provide a rapid and sensitive diagnosis that can be performed with few resources and equipment, and without the need for temperature cycling to amplify DNA¹⁵. Several isothermal amplification methods have been described and two have already been adapted for schistosomiasis diagnosis: loop-mediated isothermal amplification (LAMP)^{16–19} and recombinase polymerase amplification (RPA).

The use of RPA for a molecular detection of *S. haematobium* was initially described by Rosser et al.²⁰ who developed the lateral flow ShDraI-RPA which targets the genomic region *Dra I* (GenBank accession number: DQ157698.1). The *Dra I* region is a 121-base pair (bp) tandemly arranged repetitive sequence that corresponds to approximately 15% of *S. haematobium* genome²¹. It has similarities with DNA sequences from *Schistosoma bovis*, *Schistosoma magrebowiei*, *Schistosoma mattheei*, *Schistosoma curassoni*, and *Schistosoma intercalatum*, species that are part of the *S. haematobium* group^{21,22}. Indeed Rosser et al.²⁰ reported that the lateral flow test ShDraI-RPA cross-reacted with *S. curassoni*, *S. bovis*, but also *S. mansoni*. The cross-reactivity to the latter could be due to the concentrations used in the tests. Later, Roston et al.²³ developed a real-time fluorescence-based ShDraI-RPA assay targeting the same tandem repeat in the *S. haematobium* genome, however the specificity of the assay was not reassessed by these authors. The ShDraI-RPA assay has been used to detect *S. haematobium* DNA in clinical samples, firstly reported by Archer et al.²⁴, by testing urine samples which were egg-positive by microscopy achieving a high accuracy rate (93.7% sensitivity; 100% specificity). It was also reported an impressive sensitivity of the ShDraI-RPA with a limit of detection (LOD) of 10 synthetic copies of the target DNA region and one *S. haematobium* egg²⁴. Frimpong et al.²⁵ reported cross-reactivity with *S. mansoni* and *Schistosoma japonicum*, but no cross-reactivity was found with a range of other medically important protozoa or bacteria. Later, the ShDraI-RPA was used to detect FGS in cervicovaginal lavage (CVL) and vaginal self-swab samples²⁶. The accuracy of the ShDraI-RPA, when compared to qPCR results, varied according to the sample examined and the DNA extraction method used. As a reaction enhancement strategy to improve ShDraI-RPA performance, Donnelly et al.²⁷ assessed the effect of adding various amounts of betaine on the analytical sensitivity and specificity of the assay. It was found that by adding 2.5 M of betaine, the specificity of the assay was improved while the sensitivity remained the same as observed in studies previously mentioned.

The use of reaction enhancement strategies is a common approach with isothermal molecular methods, aiming to reduce secondary structure formation, improve reaction sensitivity and specificity, and decrease the incubation temperature of the reaction²⁸. Assay optimizations should be considered for assays when moving from centralized laboratory to the POC, where resources are not abundant. Crucially, the pre-analytical stage (i.e., sample collection, storage, preparation, and DNA extraction) and the way fluorometric assay results are visualised should also be adapted to this context²⁹.

With a view to use the ShDraI-RPA test at the POC, this paper focuses on modifications to the assay including simplifications to the initial two-step DNA extraction approach from urine samples, enhancements to the oligonucleotides used in the RPA assay, and improvements in alternative way of visualising results. Additionally, the robustness of the RPA reagents storage at different temperatures was tested to simulate lack of access to cold

chain, and to evaluate their portability and stability in tropical environments. This work significantly advances the ShDraI-RPA platform towards portability and implementation in low resource settings, with results indicating that this newly described platform is a promising approach to support the diagnosis of urogenital schistosomiasis in resource-limited settings.

Results

Analytical specificity and sensitivity of the ShDraI-RPA with modified oligonucleotides

The ShDraI-RPA assay was carried out using the modified ShDraI reverse primer and fluorescent probe, and was incubated at different temperatures (39, 40 and 42 °C), with reactions performed at 42 °C found to be optimal (Supplementary Table S1). The ShDraI-RPA assay, using the modified oligonucleotides and incubated at 42 °C, proved not to be specific to *S. haematobium*. However, the cross-reactivity was limited to members of the *S. haematobium* group: *S. curassoni*, *S. bovis*, *Schistosoma guineensis*, *Schistosoma intercalatum*, *S. magrebowiei* and *S. mattheei*. For *S. mansoni*, reactions demonstrated a weak fluorescence signal, but this did not cross the cut-off for positivity of 500 RFU above the baseline (Fig. 1A) and were therefore deemed negative. No cross-reactivity was observed in reactions containing gDNA from *S. japonicum*, *S. rodhaini*, other medically relevant helminths, or UTI bacteria (Fig. 1B–D).

The incorporation of the modified oligonucleotides in the ShDraI-RPA improved analytical sensitivity, resulting in a lower limit of detection (LOD) of 10 fg of *S. haematobium* gDNA (Fig. 2A). This is a tenfold improvement on the previously reported 100 fg analytical sensitivity of the standard assay²⁷, regardless of whether betaine was added to enhance reaction specificity. When synthetic copies of the *Dra* I target region were tested, assay sensitivity was 10 copies, which confirms the findings from previous studies using the standard ShDraI-RPA design (Fig. 2B)^{24,27}. The ShDraI-RPA also robustly detected *S. haematobium* DNA isolated from a single egg (Fig. 2A) with 100% sensitivity.

Performance of the modified ShDraI-RPA assay with DNA from egg- and gDNA-spiked urine samples

All egg- and gDNA-spiked urine samples had positive RPA results when the gDNA was extracted following the short protocol from the SwiftX DNA kit, regardless of the thermal conditions in which the lysis incubation was held (Table 1). Conversely, false negative qPCR result was observed when samples were incubated for 15 min which could have been due to the high DNA concentration. In urine samples that had not been spiked with

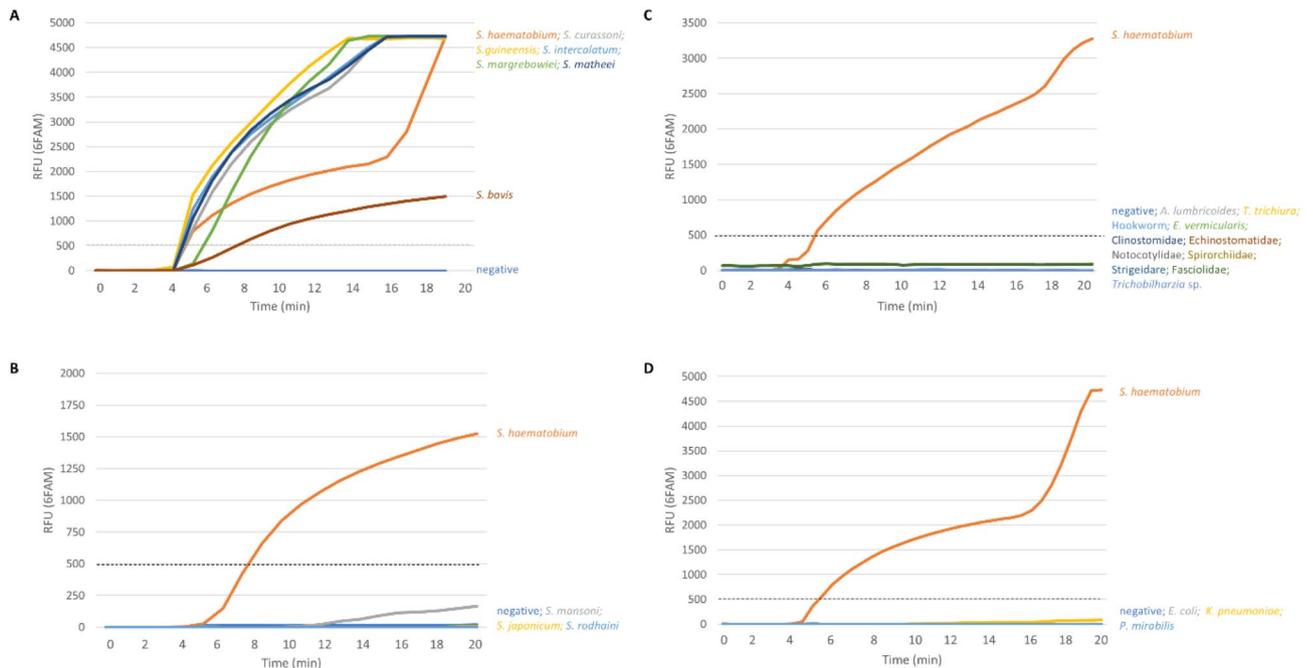


Fig. 1. Graphs showing the fluorescent signal from the modified ShDraI-RPA assay when tested on different *Schistosoma* species and other human pathogens. The graphs show the analytical specificity of the ShDraI-RPA at 42 °C using the modified oligonucleotides and 100 pg of: (A) gDNA of species from the *S. haematobium* group; (B) gDNA of other *Schistosoma* species; (C) gDNA from other helminths and, (D) gDNA from pathogenic bacteria. The assay was specific to the *S. haematobium* group species and did not cross-react with other medically important schistosome species, helminths, nor common urinary tract infection-causing bacteria. Samples were considered positive when the amplification curve crossed the threshold of 500 RFU, which is indicated by the dashed lines. Non uniform fluorescent curves can occur for gDNA samples extracted using the Qiagen DNeasy Blood and Tissue kit. The reason for this is currently unknown. RFU relative fluorescence units.

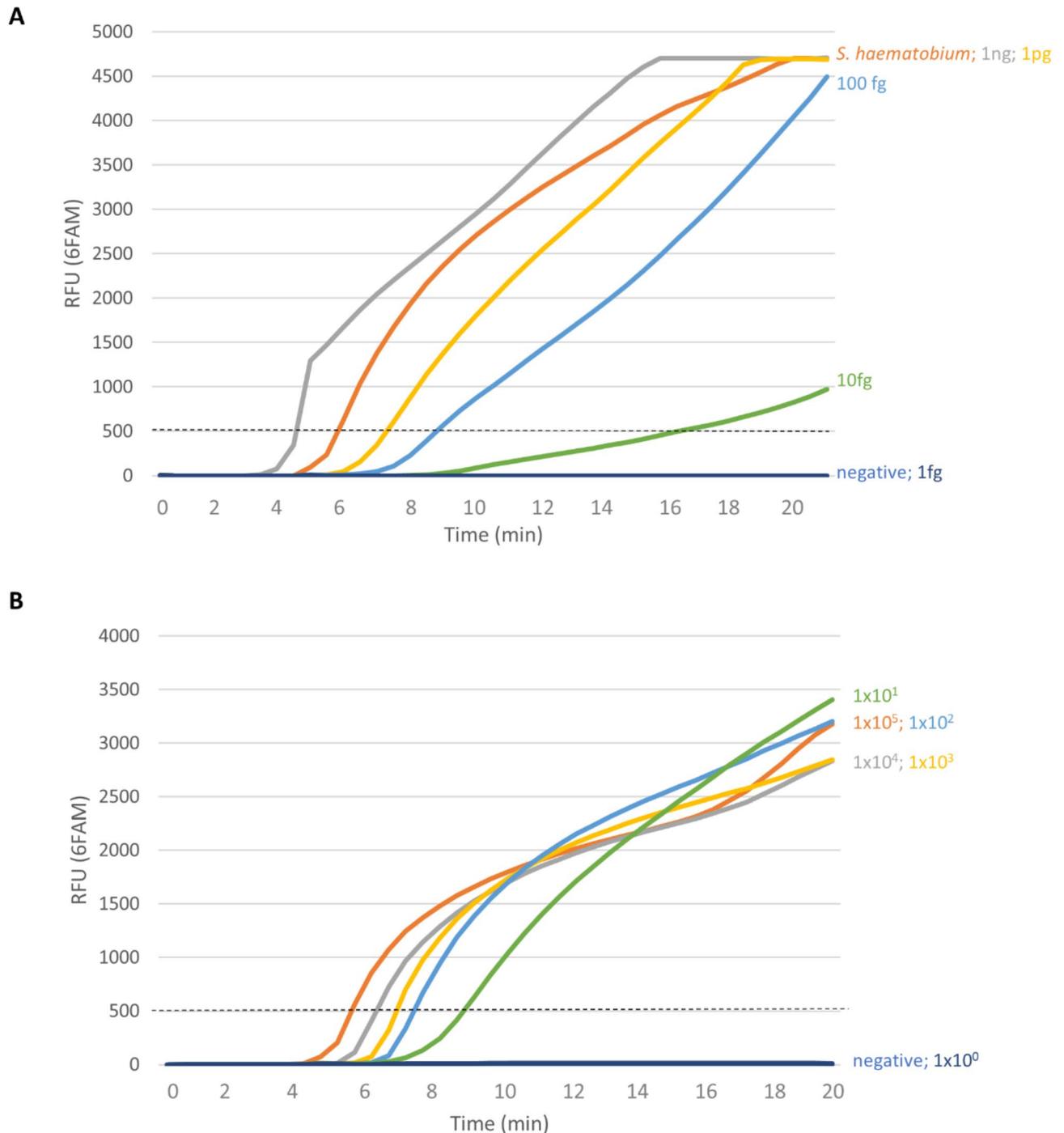


Fig. 2. Graphs showing the fluorescent signal from the modified ShDraI-RPA assay when tested on different amounts of *S. haematobium* DNA. The analytical sensitivity of the modified ShDraI-RPA tested with (A) a single *S. haematobium* egg and dilutions of *S. haematobium* gDNA; (B) synthetic copies of the *Dra I* target. Samples were considered positive when the amplification curve crossed the threshold of 500 RFU, indicated by the dashed lines. The ShDraI-RPA assay robustly detects a single *S. haematobium* egg, 10 fg of gDNA, and 1×10^1 copies of the *Dra I* sequence. RFU relative fluorescence units.

eggs or gDNA, the only extracts in which all three replicates remained negative (i.e. which demonstrated no false positives) by both RPA and qPCR were those incubated at room temperature (regardless of the duration of incubation).

The spiked urine samples extracted using the long protocol demonstrated a reduced sensitivity and reproducibility in the ShDraI-RPA when compared to the short protocol, with false negative results obtained in urine samples spiked with one egg or with 1 ng of *S. haematobium* gDNA. One negative urine sample (which had not been spiked) gave a false positive result in one of the RPA repeats performed (Table 1).

DNA extraction kit	Protocol	Lysis step		Urine Samples														
				Urine spiked with a single <i>Schistosoma haematobium</i> egg									Urine spiked with 1 ng of <i>Schistosoma haematobium</i> gDNA			Non-spiked urine		
		Temperature (°C)	Time (min)	Sample 1			Sample 2			Sample 3			Sample 1			Sample 1		
				NanoDrop (ng/μl)	RPA result	qPCR result (\bar{x} Ct)	NanoDrop (ng/μl)	RPA result	qPCR result (\bar{x} Ct)	NanoDrop (ng/μl)	RPA result	qPCR result (\bar{x} Ct)	NanoDrop (ng/μl)	RPA result	qPCR result (\bar{x} Ct)	NanoDrop (ng/μl)	RPA result	qPCR result (\bar{x} Ct)
SwiftX DNA (Xpedite)	1: Short	95	5	313.35	32.44	291.72	25.73	267.27	34.13	374.63	25.63	288.58						
				253.79	26.75	242.52	27.8	232.55	26.01	277.17	27.85	324.74						
		RT	15	366.86	26.03	296.22	36.06	369.33	31.62	293.62	26.44	212	34.29					
				449.57		254.97	35.2	379.29	25.75	291.6	28.32	263.76						
	2: Long	95	5	66.7	25.6	21.81	26.44	177.17	23.86	46.28	34.8	34.99						
				17.81	26.84	58.76	25.28	29.43	26.43	32.03	39.2							
		RT	15	59.62	23.7	209.73	23.53	79.3	26.32	45.71	35.48	8.35						
				47.54	25.71	41.36	28.81	46.68	28.47	24.22	36.13							
DNeasy Blood & Tissue (Qiagen)	Standard	56	60	6.98	25.71	3.08	23.62	6.37	24.50	5.86	23.50	3.12						

Table 1. Table of ShDraI-RPA and qPCR results with gDNA from different extraction methods, and three different sample types. ShDraI-RPA and qPCR results, and nanodrop quantification of the spiked and non-spiked urine extracts obtained using the SwiftX DNA (Xpedite) and DNeasy blood & tissue (Qiagen) kits. Both assays were performed in triplicate for each sample used. Green cells represent three positive results among the three replicates performed; yellow cells represent two positive results among the three replicates performed; orange cells represent one positive result among the three replicates performed and, red cells represent three negative results among the three replicates performed. *RT* room temperature, \bar{x} *CT* average of the threshold cycles of three qPCR replicates.

The urine samples processed using the short protocol of the SwiftX DNA kit, with the lysis incubation step being performed at room temperature for both 5 and 15 min, presented 100% accuracy with no false positive nor false negative ShDraI-RPA results. However, a false negative qPCR result was observed when the short protocol was performed at room temperature incubation for 15 min, whilst a 5-minute incubation was 100% accurate with both ShDraI-RPA and qPCR, indicating that the latter may be the optimal method for the molecular detection of *S. haematobium* in urine samples (Table 1).

The DNeasy blood & tissue kit (Qiagen) was used according to the standard protocol from the manufacturer and gave accurate results in all replicates with no false positive or false negative results recorded.

Six microliters of urine sample spiked with a single *S. haematobium* egg were also directly added to the RPA reaction tube without any prior DNA extraction step. All the replicates tested provided a positive result, indicating that the RPA assay tolerates the presence of typical PCR inhibitors present in urine samples, such as urea (Fig. 3).

Blue-light exposure

The fluorescence from all the RPA assays performed in this study was visualised in real-time via the T16-ISO Software, and at the endpoint, by exposing the reaction tubes to blue-light. The results observed by both approaches gave concordant results and representative images are displayed in (Fig. 4).

The reaction tubes containing samples in which gDNA amplification occurred glowed when under the blue-light exposure, whilst negative samples remained dark. The tubes containing samples that presented a slight fluorescence that did not cross the 500 RFU threshold or those that had a background fluorescence observed at the fluorometer, appeared turbid under the blue-light exposure. However, this could clearly be differentiated from the glow observed in positive samples (Fig. 4A,B).

Stability and longevity of the ShDraI-RPA

The ShDraI-RPA performed well and consistently when all the reagents were stored in the absence of light at ± 27 °C for up to 30 days. One replicate on both days one and two provided a false positive result in the negative control (i.e. nuclease-free water) which is likely to be a result of contamination during the assay set-up. At the 60-day time point, two out of three replicates gave a negative result, which may relate to the degradation of the reagents (Table 2).

Discussion

The present study aimed to advance the performance of the real-time fluorescence-based ShDraI-RPA platform by modifying essential components of the reaction such as the reverse primer and the fluorophore. The reverse primer was modified by incorporating a phosphorothioate backbone to its sequence. Several benefits and applications of phosphorothioate-modified (PM) oligonucleotides have been reported since its first use³⁰. PM primers have been used in PCR³⁰, LAMP³¹, CRISPR-Cas12a diagnostics (Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR- associated protein 12a)³², NPSA (Nested Phosphorothioated Hybrid Primer-mediated Isothermal Amplification)³³, and RPA^{34,35} assays. They have been shown to provide better results in terms of specificity, stability against degradation by nucleases, reduction in the incubation temperature and/or reaction time, and prevention of primer-dimer formation, as well as providing more accurate discrimination

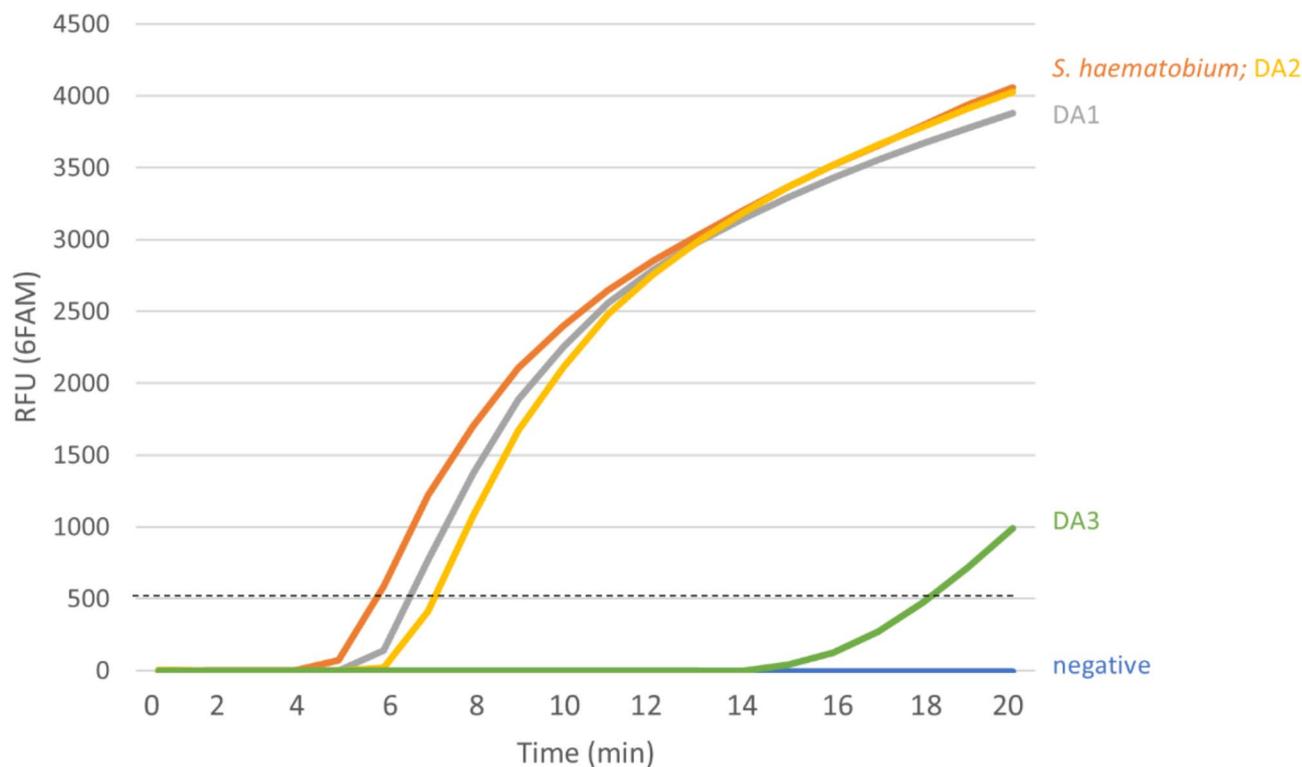


Fig. 3. Graph showing the fluorescent signal from the modified ShDraI-RPA assay when tested on *S. haematobium* eggs without a preceding DNA extraction step. Six μL of urine spiked with a single *S. haematobium* egg was directly added to the RPA reaction, without any prior sample preparation. Samples were considered positive when the amplification curve crossed the threshold of 500 RFU, which is indicated by the dashed line. RFU relative fluorescence units; *S. haematobium* = gDNA control; DA direct addition replicates 1,2 and 3.

between matched and mismatched regions of the target^{28,36}. To further enhance the efficiency of the assay, the positions of the fluorophore (6FAM) and the quencher (BHQ1) were inverted within the fluorescent probe sequence, as previously proposed by Frimpong et al.²⁵. This modification aims to keep the 6FAM away from the nearest guanine, since this base is known to act as a quencher for FAM due to its electron-donating properties³⁷. With this alteration, the assay sensitivity increased, detecting down to 10 fg of *S. haematobium* gDNA.

A broader evaluation of the assay specificity was performed in this study compared to previous ones. The ShDraI-RPA using the modified oligonucleotides and incubated at 42 °C was specific to the *S. haematobium* species group with no cross-reactivity against *S. mansoni*, *S. japonicum*, *S. rodhaini*, nor to other helminths and bacteria that were tested. The *S. haematobium* species group is composed of *S. haematobium*, *S. intercalatum*, *S. guineensis*, *S. margrebowiei*, *Schistosoma leiperi*, *S. mattheei*, *S. bovis* and *S. curassoni*. Three of these (*S. haematobium*, *S. intercalatum* and *S. guineensis*) are of medical importance as causative agents of human schistosomiasis²², whereas the remaining species have veterinary importance. The occurrence of hybrids between human and veterinary schistosomes have also been reported³⁸. Since the ShDraI-RPA is not specific to *S. haematobium*, this approach should not be used for studies that require species-specific identification of the parasite. Nevertheless, in a human diagnostic context, detecting infections caused by medically relevant schistosome species with high sensitivity is an asset. Currently, praziquantel is the drug of choice recommended by the WHO to treat schistosomiasis, regardless of the of the causative species³⁹. New molecular targets should be explored in the future to enable the development of a species-specific assay, that would be used in cases where detecting the exact species is important. Alternatively, a genus-specific assay that can detect all schistosome species, including the epidemiologically important *S. mansoni* and *S. japonicum*, would be extremely useful for worldwide public health and disease control measures.

In this study, naive urine samples were spiked with single *S. haematobium* eggs to evaluate the efficacy of the ShDraI-RPA assay using newly modified oligonucleotides on clinical samples. Naive urine samples were also spiked with 1 ng of *S. haematobium* DNA to simulate the detection of cell-free DNA (cfDNA). Spiked samples had their DNA isolated using two types of DNA extraction methods, a pure column-based method (Qiagen DNeasy Blood & Tissue) and a crude bead-based one (Xpedite SwiftX DNA). Archer et al.²⁶ reported that the sensitivity and specificity of the ShDraI-RPA differed depending on the DNA extraction approach used in CVL samples, with the highest sensitivity and specificity values observed in gDNA samples that were extracted with the discontinued SpeedXtract Nucleic Acid Kit (Qiagen, Germany), now superseded by the SwiftX DNA kit which uses similar technology. Donnelly et al.²⁷ evaluated the performance of the ShDraI-RPA on DNA extracts obtained using the SwiftX DNA and SpeedXtract Nucleic Acid Kits, and no difference was observed in terms of

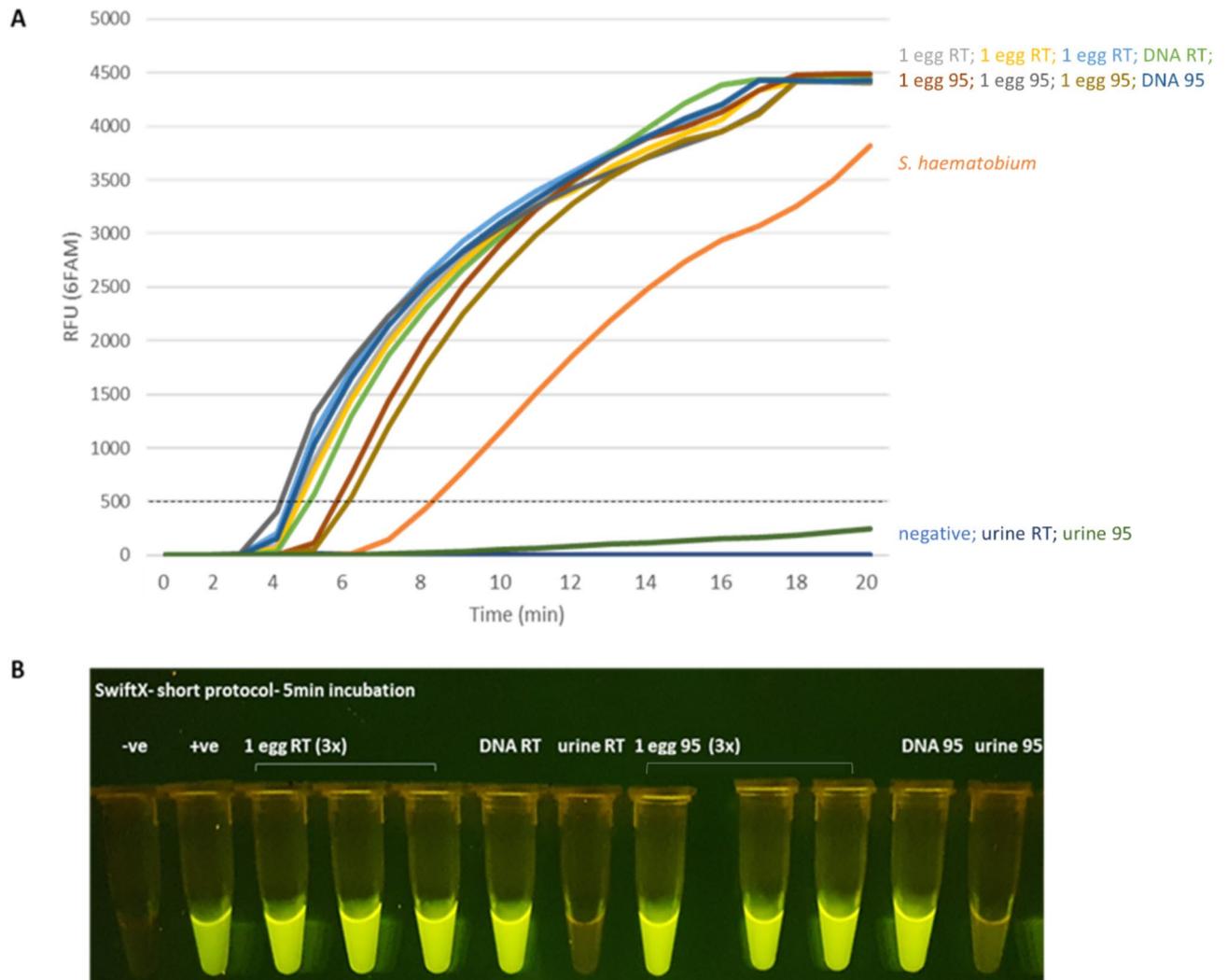


Fig. 4. (A) Graph showing the fluorescent signal from the modified ShDraI-RPA assay when tested on gDNA extracted from urine samples containing a single *S. haematobium* egg and with 1 ng of *S. haematobium* gDNA following the short protocol from SwiftX DNA (Xpedite diagnostics- i.e. protocol one; incubation step at room temperature and 95 °C for 5 min). Samples were considered positive when the amplification curve crossed the threshold of 500 RFU. Dashed lines indicate the 500 RFU cut-off. RFU relative fluorescence units, RT room temperature; 95 = 95 °C. (B) Reaction tubes exposed to a source of blue light after ShDraI-RPA reactions. Exposing to blue light proved to be a robust and simple alternative method of visualising the results of ShDraI-RPA reactions. +ve- *Schistosoma haematobium* gDNA control, -ve- negative control.

reproducibility among the replicates or compatibility with the RPA components. Likewise, in the present work, the SwiftX DNA kit showed promising RPA results with all variations of the protocol evaluated (i.e. short and long protocols with different time and temperature for lysis incubation). However, false-negative results were observed in urine samples spiked with 1 ng of gDNA when the long protocol was followed, indicating that this protocol may not be appropriate for the identification of cfDNA that may be present in urine samples of infected individuals⁴⁰. False-positive results have occasionally also been observed and reported by others^{26,27}. Here, all the samples were also analysed by qPCR (used as reference test), with false-negative results being observed in only one of the egg-spiked samples and the gDNA-spiked samples. Additionally, two false-positive results were observed when non-spiked urine samples were analysed by qPCR, with one of these also being RPA-positive. The short protocol, modified for the lysis incubation at room temperature for five minutes, was the only one that provided samples that had 100% ShDraI-RPA and qPCR specificity, indicating that this may be the best SwiftX DNA protocol to follow for this assay. This is a promising result considering potential POC use, as the short protocol with the five-minute incubation at room temperature is faster and simpler. Additionally, this is a rapid protocol that takes approximately 15 min to process eight samples, simple to perform as it is a two-step approach, with an estimated cost of 3.20 USD²⁷. However, to confirm these findings, further studies should be conducted using a larger clinical sample set, ideally from endemic areas. The data also highlights the importance of robust methods to mitigate against cross-sample contamination.

Days	Replicate 1		Replicate 2		Replicate 3	
	PCT	NCT	PCT	NCT	PCT	NCT
0	Green	Red	Green	Red	Green	Red
1	Green	Green	Green	Red	Green	Red
2	Green	Red	Green	Green	Green	Red
3	Green	Red	Green	Red	Green	Red
7	Green	Red	Green	Red	Green	Red
14	Green	Red	Green	Red	Green	Red
21	Green	Red	Green	Red	Green	Red
30	Green	Red	Green	Red	Green	Red
60	Red	Red	Red	Red	Green	Red
90	Red	Red	Red	Red	Red	Red

Table 2. Data showing the stability of the ShDraI-RPA assay reagents stored $\pm 27^\circ\text{C}$ for up to 30 days. False positive results were observed in one replicate on days 1 and 2 after the start of the test. False negative results were observed after 60 days after the start of the test. PCT positive control (100 pg of *S. haematobium* gDNA); NCT negative control (nuclease-free water); green cells- positive result; red cells- negative result.

Interestingly, despite the SwiftX DNA kit being designed to isolate the gDNA through cell-capture followed by a cell-lysis step, it was possible to recover and detect *S. haematobium* DNA from gDNA spiked urine samples, designed to mimic cfDNA. The potential detection of *S. haematobium* cfDNA in urine samples should be further investigated using clinical samples from endemic areas.

Naive urine samples spiked with *S. haematobium* single eggs (without any prior extraction or other sample processing) were directly added to an ShDraI-RPA reaction, with a positive result observed in all three replicates. The direct addition of schistosomes eggs has been previously proved to be possible^{27,35} but this is the first time that it was tested with eggs resuspended in urine, suggesting that RPA may tolerate common inhibitors in clinical samples that must be removed prior to using PCR⁴¹. Again, although very promising, this finding should be further evaluated and confirmed by repeating the test in a larger number of clinical samples from endemic areas.

A significant consideration when developing and validating new diagnostic tests is their accessibility and affordability to those most in need of them⁴². Schistosomiasis is a neglected tropical disease, and endemic areas usually lack significant financial and technical resources. In this context, we assessed an alternative method to visualise the RPA result, independent of the fluorometer, which consists of exposing the reaction tubes to a source of blue light after a 20-minute incubation at 42°C , so that positive samples would glow. Blue-light results obtained in this work corresponded to results from the Axxin fluorometer, with the additional benefit of simpler result interpretation. The stability and longevity evaluation of the ShDraI-RPA using the modified oligonucleotides indicated that the assay components may be stable for up to one month at 27°C , facilitating their transportation and storage in the absence of a cold-chain, which is of significant benefit when considering outreach from a regional centre to more remote disease-endemic settings. The tests were conducted in triplicate and false-positive results on negative controls were observed at the first two days of the test. Since this did not occur on the following days and in the other replicates, we consider this to be unlikely to be due to degradation of the RPA components and instead may represent contamination during assay set-up.

In conclusion, the optimized ShDraI-RPA assay looks to be a promising and efficient alternative for a fast and portable schistosomiasis diagnostic, able to support the detection of schistosomiasis infection, especially in low-resource settings.

Methods

ShDraI-RPA modifications and optimisation

Modified oligonucleotides

RPA primers and probe targeting the *S. haematobium* Dra I tandem repeat region (GenBank accession number: DQ157698.1) described by Rostron et al.²³ were modified with the aim of increasing assay specificity, whilst maintaining high levels of sensitivity. Modifications included the addition of a phosphorothioate backbone (denoted as “s” in the primer sequence) before the last 3’ base of the reverse primer, and inversion of the position of the fluorophore 6-carboxyfluorescein (6FAM) and the black-hole quencher 1 (BHQ1) within the fluorescent probe (Table 3).

RPA reaction and visualisation of the results

ShDraI-RPA reactions were performed using the TwistAmp Exo Kit (TwistDx, Cambridge, UK) following the manufacturer instructions and as described by Rostron et al.²³. All reactions were performed in triplicate, and

Oligonucleotide	Modified		Sequence (5'-3')
	Yes	No	
Forward primer		x	ATC TCA CCT ATC AGA CGA AAC AAA GAA AAT
Reverse primer	x		AAT ATG AAA CAA TTT TCA CAA CGA TAC GAsC
Fluorescent probe	x		AAT TGT TGG TGG AAG TGC CTG TTT CGC AA (BHQ1) (THF) (6FAM) CTC CGG AAT GGT TG (C3)

Table 3. Sequences of the primers and the fluorescent probe for the modified ShDra1-RPA assay. Modifications were made to the original ShDra1-RPA assay reverse primer and probe, however, the forward primer remained unchanged. *s* phosphorothioate backbone, *BHQ1* black hole quencher 1, *THF* tetrahydrofuran, *6FAM* 6-carboxyfluorescein, *C3* spacer C3.

positive (100 pg of *S. haematobium* DNA) and negative (nuclease-free water) controls were included with each RPA run of 16 reactions. RPA reaction tubes were incubated at 42 °C for 20 min in the portable fluorometer Axxin T16-ISO (Axxin Diagnostics, Australia) where the fluorescence was measured, and results were observed in real-time via T16-ISO Software (V2.1.0.3- Axxin Diagnostics, Australia). Samples were considered positive for *S. haematobium* when an increase of ≥ 500 relative units of fluorescence (RFU) above the baseline was observed²⁷. Additionally, to verify whether the assay could provide a qualitative binary diagnosis without the need for a fluorometer, the end-point fluorescence was observed by exposing the reaction tubes to a source of blue light after the amplification. The blue light has a wavelength of approximately 450 to 495 nm and the absorbance of 6FAM is 493 nm. This means that in positive samples (i.e. which contain amplified DNA), where the fluorophore, 6FAM, has been released from the quencher, is able to absorb the blue light and produce a fluorescence that is visible to the naked eye.

Source of biological samples

Genomic DNA (gDNA) isolated from *S. haematobium* and other *Schistosoma* species (*S. mansoni*, *S. curassoni*, *S. bovis*, *S. rodhaini*, *S. matthei*, *S. japonicum*, *S. margrebowiei* and *S. guineensis*) were obtained from the Schistosomiasis Collection at The Natural History Museum- UK (SCAN)⁴³ repository. The Medical Malacology Collection at René Rachou Institute, Fiocruz Minas- Brazil (Fiocruz-CMM) provided samples from trematodes belonging to the families Clinostomidae, Echinostomatidae, Fasciolidae, Notocotylidae, Spirorchidae and Strigeidae, and SCAN provided trematodes belonging to the genus *Tricobilharzia*. Genomic DNA from helminths of medical importance (i.e. *Ascaris lumbricoides*, *Enterobius vermicularis*, *Trichuris trichiura*, and hookworms) were provided by the Helminthology and Medical Malacology Laboratory from the René Rachou Institute, Fiocruz Minas- Brazil. To test for Gram-negative bacteria which commonly cause UTIs, gDNA previously obtained by Rosser et al.²⁰ from *Escherichia coli* and *Klebsiella pneumoniae* were used. In addition, Charing Cross Hospital (London- UK) supplied a reference colony of *Proteus mirabilis* (ATCC strain 35659; <https://www.atcc.org/products/35659>) from their microbiology laboratory. Single *S. haematobium* ova were provided frozen by the Snail Schistosome Resource (SSR, Natural History Museum, UK) via the NIAID Schistosomiasis Resource Centre (SRC, Biomedical Research Institute, USA). All DNA samples were stored at -20 °C before use.

Analytical specificity testing

Genomic DNA was isolated from all organisms listed above using the DNeasy Blood & Tissue (Qiagen GmbH, Germany) kit, according to the manufacturer's instructions. All gDNA isolates were quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and normalised to a working concentration of 100 pg/ μ L using nuclease-free water. All samples were analysed using the modified ShDra1-RPA oligonucleotides using various incubation temperature (39, 40 and 42 °C) to determine the optimal temperature for the assay specificity.

Analytical sensitivity testing

Genomic DNA isolated from adult worms of *S. haematobium* was used to obtain tenfold serial dilutions from 1 ng/ μ L to 1 fg/ μ L. Synthetic copies of the *Dra I* target region were obtained as described by Donnelly et al.²⁷ and dilutions ranging from 1×10^5 to 1×10^0 copies/ μ L were prepared. Genomic DNA from single eggs was isolated using the SwiftX DNA extraction kit (Xpedite Diagnostics GmbH, Germany) following the manufacturer's instruction (protocol 1 from the handbook). All DNA (genomic and synthetic) samples were analysed using the modified ShDra1-RPA, as described above, to determine assay sensitivity. The direct addition of single *S. haematobium* eggs (i.e. without their gDNA extracted) to the RPA reactions was also tested.

Spiked urine samples

Aliquots (100 μ L) of urine from a donor (from a non-endemic country) were spiked with: (i) a single egg of *S. haematobium* and, (ii) 1 ng of *S. haematobium* gDNA. Also, non-spiked aliquots were processed as negative controls.

All of the spiked and non-spiked aliquots were processed and used as follows: (i) direct addition to the RPA reaction; (ii) DNA extraction using DNeasy Blood & Tissue kit (Qiagen), following the manufacturer's protocol; (iii) DNA extraction using a rapid, low resource method for POC use, SwiftX DNA kit (Xpedite), according to the manufacturer's protocols one and two, testing variations on the time and temperature of lysis incubation.

Briefly, the SwiftX DNA kit protocol one (hereon, the short protocol) consists of the addition of 100 μ L of the lysis buffer DL and 30 μ L of Beads A to the sample (100 μ L). The mixture was homogenised for 10 s by vortexing

and incubated at 95 °C (or alternatively, at room temperature- RT) for five or 15 min. The tubes were placed in a magnetic rack for one minute and the supernatant (containing the gDNA) was transferred to a fresh 1.5 mL microcentrifuge tube and stored at -20 °C until use. Thus, four variations of the short protocol were tested: (i) lysis incubation at 95 °C for 5 min; (ii) lysis incubation at 95 °C for 15 min; (iii) lysis incubation at RT for five min; (iv) lysis incubation at RT for 15 min.

The SwiftX DNA kit protocol two (hereon, the long protocol) consists of the addition of 100 µL of the buffer EN and 30 µL of Beads A to the sample (100 µL). The mixture was homogenised for 10 s by vortexing and incubated at RT for 3 min. The tubes were then placed in a magnetic stand for 1 min and the supernatant was discarded. 100 µL of the lysis buffer DN was added to each tube that was subsequently homogenised by vortexing. The mixture was incubated at 95 °C (or alternatively, at RT) for five or 15 min and then placed into a magnetic stand for 1 min. The supernatant (containing the gDNA) was transferred to a fresh 1.5 mL microcentrifuge tube and stored at -20 °C until use. Again, four variations of the long protocol were tested: (i) lysis incubation at 95 °C for 5 min; (ii) lysis incubation at 95 °C for 15 min; (iii) lysis incubation at RT for 5 min; (iv) lysis incubation at RT for 15 min.

In total, eight protocols were tested for the SwiftX DNA kit. The concentration of gDNA in all the extracts was measured using a Nanodrop™ spectrophotometer.

qPCR analysis

DNA isolated from all egg- and gDNA-spiked urine samples extracted using the different methods were analysed by qPCR. This was performed as a confirmatory test for the isolation of *S. haematobium* DNA from each sample type by the different methods tested^{27,44,45}. The quantitative nature of this assay also allowed comparison of the DNA yield of the different sample preparation methodologies described above. *Schistosoma* genus-specific oligonucleotides targeting the internal transcribed spacer region 2 (*ITS2*) were used in a 20 µL reaction containing 0.3 µM of each primer (Forward- Ssp48F: 5'-GGTCTAGATGACTTGATYGAGATGCT- 3'; Reverse- Ssp124R: 5'-TCCCAGCGYGTATAATGTCATTA-3'), 0.1 µM of the probe (Ssp78T: 5'- FAM-TGGGTTGTG/ZEN/CTCGAGTCGTGGC-IowaBlack-FQ -3'), 1× Luna Universal Probe qPCR MasterMix (New England Biolabs), and 1 µL of template DNA. The StepOne Plus thermocycler (Applied Biosystems) was used with the following cycling conditions: 2 min initial denaturation at 95 °C, and 50 cycles of 95 °C denaturation for 15 s followed by combined 60 °C annealing/extension for 60 s. Samples were considered positive when the cycle threshold (Ct) value was < 38. This step was particularly important for assessing the different sample preparation methodologies.

Assay stability and longevity

To determine the stability of the ShDraI-RPA reagents (i.e. primers, probe, and components from the TwistAmp exo kit- RPA pellet, RPA rehydration buffer and magnesium acetate MgAc) at ambient temperature in schistosomiasis-endemic settings, all reagents were stored at ± 27 °C in a dark environment without the addition of any stabilizers. The reagents were then used to perform ShDraI-RPA reactions on days: 0, 1, 2, 3, 7, 14, 21, 30, 60 and 90. The ShDraI-RPA reactions were set up as described above and positive (100 pg of *S. haematobium* DNA) and negative (nuclease-free water) controls were included in all tests. Results were observed in real time via the Axxin T16- ISO portable fluorometer and software, and post-amplification exposure of the tubes to a blue light.

Data availability

All data generated and analysed during this study are included in this publication (and its Supplementary Information file).

Received: 31 December 2024; Accepted: 25 March 2025

Published online: 19 April 2025

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Acknowledgements

The authors would like to thank the Wellcome Trust funded Schistosome and Snail Resource (SSR) Wellcome Trust Biomedical Resource Grant 221368/Z/20/Z (2021–2026), the Collection of Medical Malacology (Fiocruz-CMM- Brazil), the Helminthology and Medical Malacology Laboratory (Fiocruz, Brazil), Schistosomiasis Collection at the Natural History Museum (SCAN- UK) and the Charing Cross Hospital (London, UK) for the provision the samples used in this study; Andy Wende and Ludovic Ebert from Xpedite Diagnostics for advice and support during the study. This work received financial support from the Coalition for Operational Research on Neglected Tropical Diseases (COR-NTD), which is funded at The Task Force for Global Health primarily by the Bill & Melinda Gates Foundation (OPP1190754), by UK aid from the British government, and by the United

States Agency for International Development through its Neglected Tropical Diseases Program. Under the grant conditions of the Foundation, a Creative Commons Attribution 4.0 Generic License has already been assigned to the Author Accepted Manuscript version that might arise from this submission. Support was also provided via the Departmental Investment Fund of the Natural History Museum.

Author contributions

SGM, OD and BLW conceived the experiment(s); SGM, OD, EBL conducted the experiment(s); SGM, OD, JA, EBL and BLW analysed the results. All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-95887-x>.

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