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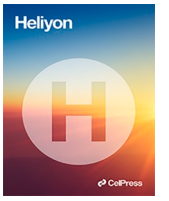

Development and performance of recombinase-aided amplification (RAA) assay for detecting *Schistosoma haematobium* DNA in urine samples

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ABSTRACT

Rapid diagnosis of urogenital schistosomiasis caused by *Schistosoma haematobium* requires an accurate and timely assay, especially for low-intensity *S. haematobium* infection cases and in non-endemic areas. The mitochondrial cytochrome *c* oxidase 1 (cox1) gene fragment of *S. haematobium* was selected as detection target as this short fragment, which can be rapidly sequenced and yet possess good diagnostic resolution. A pair of primers and a fluorescent probe were designed according to the principle of recombinase-aided amplification (RAA), which was subsequently optimized and applied as an *S. haematobium*-specific RAA assay. Its diagnostic performance was validated for sensitivity and specificity in comparison to microscopy-based egg counting after urine filtration. The RAA assay could detect as little as 10 copies/μL of *S. haematobium* recombinant plasmid, and no cross-reactions were observed with *S. mansoni*, *S. japonicum*, *Ancylostoma duodenale*, *Clonorchis sinensis*, *Echinococcus granulosus*, or *Ascaris lumbricoides*. This test can be conducted at 39 °C and the whole RAA reaction can be completed within 20 min. The validation of the RAA assay showed that it had 100 % consistency with urine-egg microscopy, as it does not require an elaborate reading tool, is simple to use, and should be useful for field diagnostics and point-of-care applications.

1. Introduction

Schistosomiasis is a major neglected tropical disease that can lead to severe bladder, ureteral, kidney, and genital pathologies;

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approximately 258 million people are currently infected worldwide, and approximately 779 million people are at risk [1–4]. It is estimated that more than 85 % schistosomiasis cases occur in sub-Saharan Africa, approximately two-thirds of which are caused by *Schistosoma haematobium*, the pathogenic agent of urogenital schistosomiasis [5,6]. This type of schistosomiasis is often misdiagnosed as a urinary tract infection, whereas other schistosomiasis cases, such as *S. japonicum* and *S. mansoni* infections, are often misdiagnosed as gastrointestinal tract infections. *S. haematobium* can cause infections via the bladder vein and pelvic venous plexus, leading to genitourinary tract diseases, such as terminal hematuria, bladder irritation, and urinary tract obstruction, which can lead to renal failure. Thus, the late stages can be life threatening [2]. *S. haematobium* eggs deposited in the bladder tissue can also lead to chronic infection and bladder squamous cell carcinoma [7,8].

Several countries with endemic *S. haematobium* infections have successfully reduced morbidity and, in some cases, interrupted transmission by regularly treating infected or at-risk individuals with schistosomiasis [9]. As the prevalence and intensity of infections have decreased, low-intensity infections have become more common and a high proportion of them are asymptomatic. Molecular diagnostic assays for *S. haematobium* in urine are more sensitive than immunological methods [10,11] and these infections are also more difficult to diagnose using techniques, such as reagent strip testing, to detect microhaematuria or microscopy [12,13] to detect the presence of *S. haematobium* eggs in urine [14–16]. In addition, cases of schistosomiasis in travellers and migrants, especially those with *S. haematobium* infection, still occur in various non-endemic countries. For example, imported schistosomiasis cases were reported in China, with 73.8 % (262/355) of all imported schistosomiasis cases in 1979–2019 being caused by *S. haematobium* [17,18]. Europe has also received imported schistosomiasis in the last decade, mainly from endemic countries in Africa and the Middle East; for example, up to 21.2 % of individuals in cohorts of newly arrived asylum seekers coming to Italy between April 2014 and June 2015 had schistosomiasis [19]. In Corsica (France), an outbreak of locally transmitted urogenital schistosomiasis occurred in 2013, infecting more than 120 people [20]. Owing to the lack of diagnosis and treatment experience among clinicians and specialized diagnostic assays, cases of imported schistosomiasis are prone to be misdiagnosed [21–23].

Efforts have been made to establish a diagnostic method to detect low-intensity and asymptomatic *S. haematobium* infections in endemic areas and to monitor cases of imported *S. haematobium* infections in non-endemic areas. Both polymerase chain reaction (PCR) and real-time fluorescence quantification are sensitive and specific [10,24,25]; however, they are relatively time-consuming and require a laboratory with specific equipment [12]. Loop-mediated isothermal amplification (LAMP) can detect *S. haematobium* DNA with a higher sensitivity than PCR [26–28].

Recombinase-aided amplification (RAA), based on the principle of recombinase-mediated isothermal amplification, can rapidly detect specific nucleic acid fragments, such as *S. japonicum*, *S. mansoni*, SARS-CoV-2, and Orf virus [29–32]. The recombinant enzyme can combine with the primer DNA at room temperature to form an aggregate of enzymes and primers. When primers search for complementary sequences that exactly match the template DNA, the double-stranded structure of the template DNA can be unrolled with the help of a single-stranded DNA-binding protein, resulting in the formation of new complementary DNA strands under the action of DNA polymerase, and the amplification product grows exponentially. It is simple to conduct and allows real-time monitoring at 37–42 °C [29,30,33]. Here, we aimed to establish and assess an RAA assay to detect a *S. haematobium* cytochrome *c* oxidase subunit 1 (cox1) gene fragment in 5 min and validate it using urine samples from *S. haematobium* infection. This assay represents a new tool to detect *S. haematobium* and may play an important role in point-of-care applications and in the diagnosis of imported cases of urogenital schistosomiasis.

2. Methods

2.1. Urine sample collection and processing

Urine samples were collected from 57 pupils from a primary school in Zanzibar (United Republic of Tanzania) maintained by the China-Zanzibar-World Health Organization (WHO) Schistosomiasis Control and Elimination Project [34–36]. All urine samples were collected between 10:00 and 14:00 to determine the optimum egg output [14]. All urine samples had been previously screened for *S. haematobium* infection by urine filtration (10 mL) and examined under a microscope by skilled local technicians [35,37]. They were classified according to the WHO recommendations [38] as no egg load (considered as negative samples), having a light (<50 eggs/10 mL urine) or heavy (≥50 eggs/10 mL urine) egg load. The samples were then lyophilized, stored at −80 °C and transported to our laboratory at the Jiangsu Institute of Parasitic Diseases in Wuxi, China. The Ethics Review Committee of Zanzibar approved sample collection (ZAMREC/002/MAY/014).

Table 1
Primers for *S. haematobium* cox1 gene fragment and internal probe for RAA assay.

Primer/probe	Sequence (5′–3′)
Forward primer	CTATGATTATAGGGATTCCTACAGGTATAAAG
Reverse primer	TAATATATCTAATGAAGAAGCTGATAAAGC
Probe[a]	CTGTGGGTCTCGTGTATGAGATCCTATAGTTTGATGATTGGTTGGTTTTA

[a] The 31st base modifies the fluorescence reporter group, the 35th base modifies the quenching group, and the 33rd base modifies the tetrahydrofuran residue.

2.2. Extraction of nucleic acids

Nucleic acids were extracted from urine samples using an AxyPrep™ Body Fluid Viral DNA/RNA Miniprep Kit (Axygen Scientific, Union City, CA, USA). Thereafter, the DNA concentration was determined using a NanoDrop 2000 system (Thermo Fisher Scientific, Waltham, MA, USA), and the nucleic acid was stored at −80 °C.

According to the principle of recombinase-aided isothermal amplification and with reference to previous research [29,30], primers were designed using an *S. haematobium* cox1 gene fragment as the target sequence (GenBank accession no. MT579449.1) (Table 1). Primers and probes were synthesized by Sangon Biotech (Shanghai, China).

2.3. RAA assay

RAA reactions were performed in a 50-μL reaction volume using RAA Nucleic Acid Amplification Kit (Lot. FEZFB0; Jiangsu Qitian Gene Biotechnology Co., Ltd., China). The reaction mixtures contained 5 μL template DNA, 25 μL reaction buffer, 4.2 μL primers (forward and reverse; 10 μM), 0.6 μL probe (10 μM), 12.7 μL double-distilled water (ddH_2O), and 2.5 μL 280 mM magnesium acetate. The reaction system was mixed and incubated for 4 min in a 0.2-mL PCR tube in a constant-temperature oscillation mixer (RAA B6100; Jiangsu Qitian Gene Biotechnology Co., Ltd., Jiangsu, China). Thereafter, the fluorescence values were assessed every 20 s for the whole 20 min using a real-time fluorometer (RAA F1620, Jiangsu Qitian Gene Biotechnology Co., Ltd., China) at 39 °C.

2.4. RAA assay sensitivity

An amplified and purified *S. haematobium* mitochondrial cox1 gene fragment (approximately 176 bp) was synthesized into a plasmid vector by Sangon Biotech (Shanghai, China), and its initial concentration was determined using a NanoDrop 2000 system (Thermo Fisher Scientific, Waltham, MA, USA). Recombinant plasmid samples with concentrations of 1.0×10^6, 1.0×10^5, 1.0×10^4, 1.0×10^3, 1.0×10^2, and 10 copies/μL were subjected to the RAA assay, involving detection of fluorescence signals. The negative control was ddH_2O.

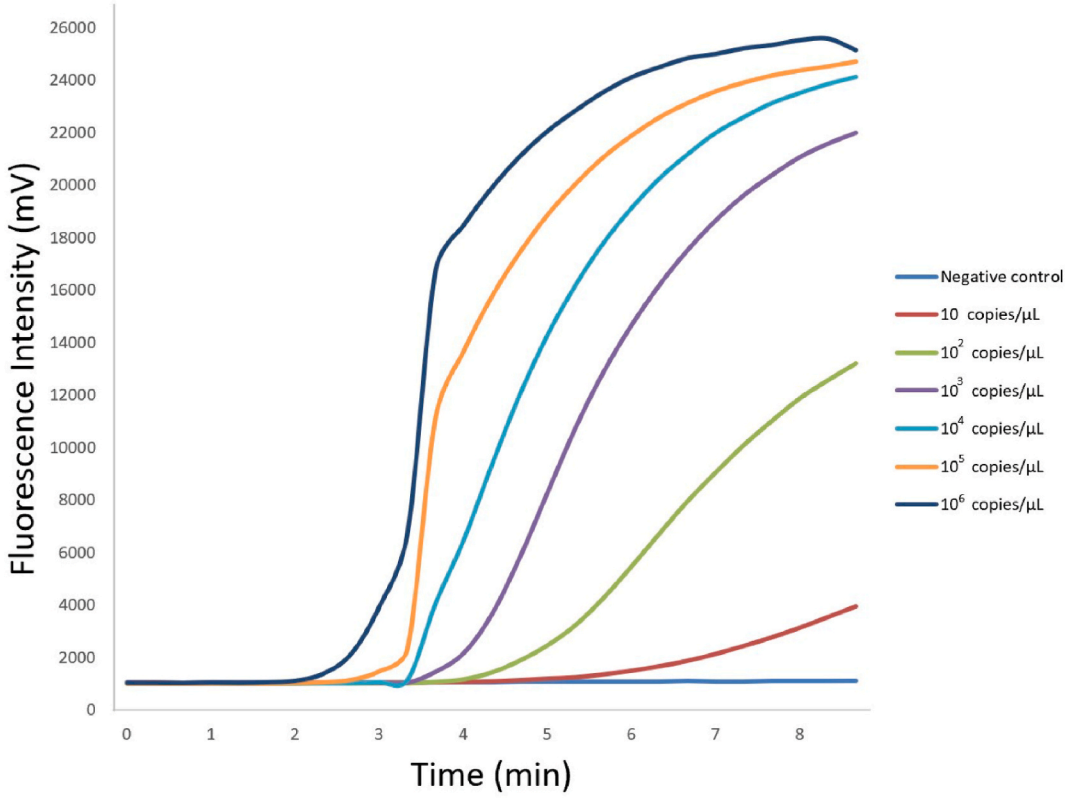

Fig. 1. Sensitivity of the RAA assay in detecting *S. haematobium* cox1 recombinant plasmid at various concentrations
The sensitivity of the RAA assay was demonstrated by its ability to detect and amplify *S. haematobium* cox1 recombinant plasmid at various concentrations. All concentrations of *S. haematobium* cox1 recombinant plasmid were successfully detected and amplified, highlighting the effectiveness of the assay. Notably, the negative control (ddH2O) did not show any amplification, confirming the specificity of the assay.

2.5. RAA assay specificity

The DNA of *S. haematobium, S. mansoni, S. japonicum, Ancylostoma duodenale, Clonorchis sinensi, Echinococcus granulosus*, and *Ascaris lumbricoides* was obtained from the Key Laboratory on Technology for Parasitic Disease Prevention and Control (Wuxi, China). DNA concentrations were determined using the NanoDrop 2000 system (Thermo Fisher Scientific, Waltham, MA, USA), and 2 ng of each DNA was used as a template and then subjected to the RAA assay, involving the detection of fluorescence signals. The negative control was ddH_2O, which was used as a template.

2.6. RAA validation

Among the 57 urine samples, 9 were found positive and 48 were found negative via microscopy-based egg counting. Nine egg-positive urine samples with light or heavy *S. haematobium* egg loads (five with a light load [U1,U82,U89,U171, and U48] and four with a heavy load [U137, U79, U96, and U240]) and 48 egg-negative urine samples were used to validate the RAA assay. Two-hundred microliters of each urine DNA was extracted using an AxyPrep™ Body Fluid Viral DNA/RNA Miniprep Kit (Axygen Scientific, Union City, CA, USA). Lastly, 5 μL extracted DNA from each sample was subjected to the RAA assay, involving detection of fluorescence signals. In addition, 100 copies of the recombinant plasmid were used as a positive control.

3. Results

3.1. RAA assay sensitivity

The *S. haematobium* cox1 recombinant plasmid samples at various concentrations (1.0×10^6, 1.0×10^5, 1.0×10^4, 1.0×10^3, 1.0×10^2, and 10 copies/μL) were amplified via RAA. The fluorescence values of all samples (but not of the negative control) increased within 5 min at the beginning of the detection period. The concentration of 1.0×10^6 copies/μL led to the earliest peak (Fig. 1).

3.2. RAA assay specificity

No fluorescence was observed for *S. japonicum*, *S. mansoni*, *Ancylostoma duodenale*, *Clonorchis sinensis*, *Echinococcus granulosus*, or *Ascaris lumbricoides*, whereas increased fluorescence (starting within 5 min of the detection period) was observed for *S. haematobium* (Fig. 2).

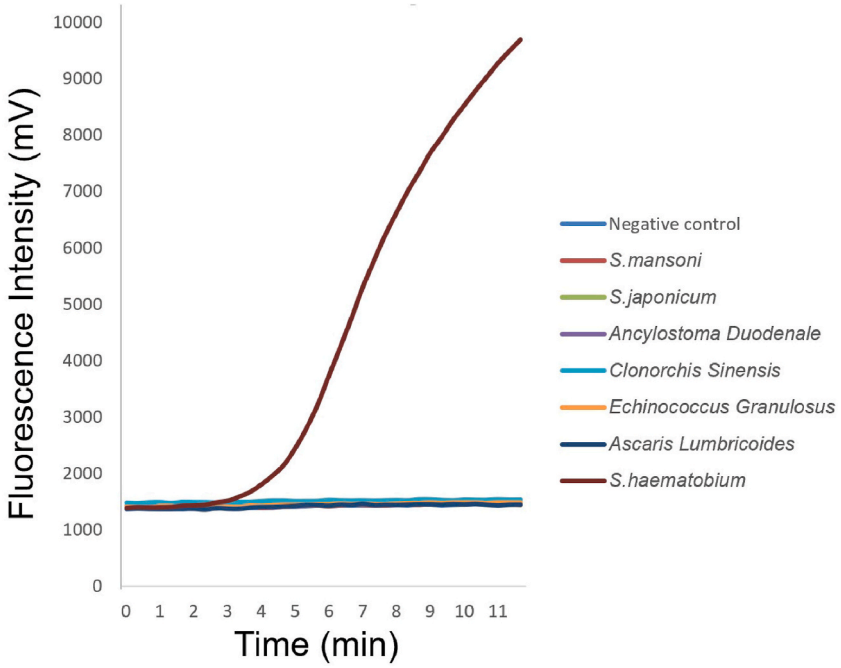

Fig. 2. Specificity of the RAA assay in detecting various parasitic species
This figure illustrates the specificity of the RAA assay in detecting different parasite species. Notably, no fluorescence was observed in *Schistosoma japonicum*, *Schistosoma mansoni*, *Ancylostoma duodenale*, *Clonorchis sinensis*, *Echinococcus granulosus*, or *Ascaris lumbricoides*. In contrast, a noticeable increase in fluorescence that began within 5 min of the detection period was observed in *S. haematobium*. This highlights the ability of the assay to accurately differentiate and detect *S. haematobium* from other parasitic species.

3.3. RAA assay performance

Nine urine samples with light or heavy *S. haematobium* egg loads (five with light loads [U1,U82,U89,U171, and U48] and four with heavy loads [U137, U79, U96, and U240]) and 48 egg-negative urine samples were amplified using RAA assay (Fig. 3, Supplementary Table 1). The fluorescence signal of all the nine of the samples (U1, U48, U79, U82, U89, U96, U137, U171, and U240) increased rapidly within 5 min of the beginning of the detection period (Fig. 3). No fluorescence was observed in any of the negative samples. Thus, among the nine egg-positive urine samples, none led to a false-negative result based on RAA (Fig. 3, Table 2), while none led to a false-positive result among the 48 egg-negative urine samples. The consistency between the RAA assay and the microscopy-based egg counting was 100 % (kappa value = 1). The positive and negative predictive values were 100 %, and nine egg-positive urine samples (from patients with *S. haematobium* infection) and 48 egg-negative urine samples were examined using microscopy-based egg counts in Zanzibar and RAA assay in China.

4. Discussion

Sensitive and specific diagnostic methods are critical for the elimination of schistosomiasis as they enable the timely and accurate identification and treatment of cases. In addition, better diagnostics can enable accurate surveillance of cases (including imported cases) in low- and non-endemic areas [39,40]. Molecular detection of *S. haematobium* DNA in urine is considered a highly sensitive indicator of infection [12,13] and RAA assay is a highly sensitive method that does not require costly tools or a sophisticated laboratory and can deliver results within 5–15 min.

The results of the RAA assay of the recombinant plasmids harboring the *S. haematobium* mitochondrial cox1 gene fragment showed that the higher the concentration, the earlier the fluorescent signal appeared; conversely, the lower the concentration, the later the fluorescent signal appeared (Fig. 1). This shows that the RAA assay has the potential to be used as a semi-quantitative assay for the detection *S. haematobium* in urine. In the present study, the RAA assay could detect 10 copies/μL of recombinant plasmids harboring the *S. haematobium* mitochondrial cox1 gene fragment, which was the lowest concentration assessed (Fig. 1). In theory, a single copy could be detected using the RAA assay if the assay is optimized to increase sensitivity (focusing on the primer and probe combination,

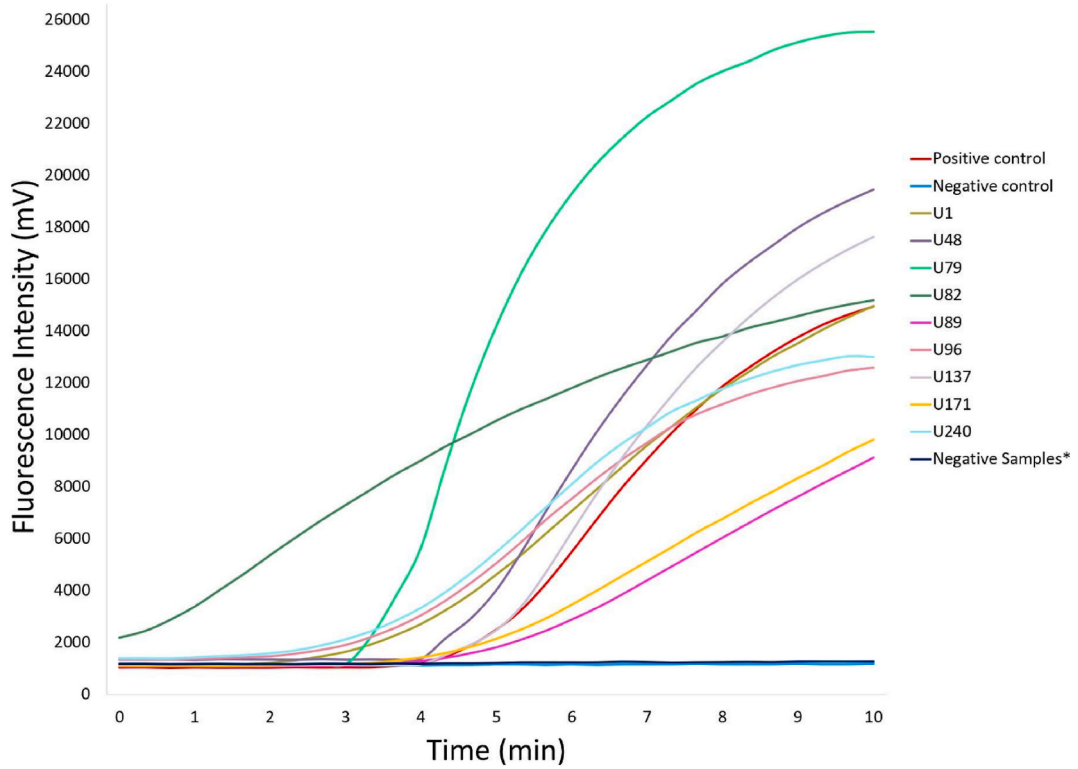

Fig. 3. Evaluation of the RAA assay's performance using field-collected urine samples
The performance of the RAA assay was assessed using field-collected urine samples. A total of 57 urine samples were included in the study, 9 of them tested positive and 48 tested negative based on microscopy-based egg counting. Specifically, among the 57 samples:Nine urine samples were positive for *S. haematobium* eggs with varying egg loads (five with a light load [U1, U82, U89, and U171, and U48] and four with a heavy load [U137, U79, U96, and U240]). All 48 egg-negative urine samples were used as negative controls. This validation process helped confirm the accuracy and reliability of the RAA assay in diagnosing *S. haematobium* infections in a field setting.

Table 2
Diagnostic performance of the RAA assay.

Reference test (microscopy-based egg counting*)					
		+	–	Total	
RAA assay	+	9	0	9	PPV: 100 %
	–	0	48	48	NPV: 100 %
	Total	9 Sensitivity: 100 % (9/9)	48 Specificity: 100 % (48/48)	57	

PPV, positive predictive value; NPV, negative predictive value. Fifty seven egg-positive urine samples (from patients with *S. haematobium* infection) were examined using microscopy-based egg counting in Zanzibar and RAA assays in China. *Test conducted in duplicate; positive: ≥1 egg was observed; negative: 0 eggs were observed in either reading.

target gene region, and concentration) in future research.

No cross-reactivity was observed with the other two major schistosome species or with other common parasitic helminths (Fig. 2), indicating that targeting the *S. haematobium* cox1 gene fragment led to high specificity for detecting *S. haematobium* infection using the RAA assay. The mitochondrial *S. haematobium* cox1 gene fragment was selected as the specific target to design the primers because it is conserved (despite the natural intra-species genetic variation observed across Africa) and can be used for species identification [41–43].

To validate the RAA assay, 57 lyophilized urine samples collected from a primary school were subjected to the RAA assay, including 9 egg-positive urine samples (five with a light infection and four with a heavy infection) and 48 egg-negative urine samples. The RAA assay correctly categorized all 9 (100 %) egg-positive samples and all 48 egg-negative samples (Table 2), indicating that the RAA assay can detect *S. haematobium* DNA in urine when patients have mild infections. For the nine egg-positive urine samples (five samples with a light load [U1,U82,U89,U171, and U48] and all four with a heavy load [U137, U79, U96, and U240]), fluorescence values were rapidly observed within 5 min, and the signals were strong (Fig. 3) and observed at the very beginning of the detection period.

Regarding the fluorescence values of all urine samples, the onset of fluorescence values varied and were not related to the egg load. Because the fluorescence values varied despite the samples having a low infection load, the difference may have been due to the original sample characteristics or sample degradation. As all egg-positive urine samples were collected from patients with *S. haematobium* infection in Zanzibar, lyophilized, transported to China, and then subjected to the RAA assay, differences among samples in the processes of sample collection, lyophilization, and transportation (which involve factors that can lead to DNA degradation) could be the reason for the difference in fluorescence values. However, although the urine samples were lyophilized and reconstituted in normal physiological saline prior to DNA extraction, the strong fluorescence values detected in the RAA assay (Fig. 3) showed that these processes had little effect on the detection of the *S. haematobium* cox1 fragment in egg-positive urine samples. The AxyPrep™ Body Fluid Viral DNA/RNA Miniprep Kit was used for DNA extraction because it is simple and does not require complex equipment. It can extract low quantities of nucleic acids at a low cost ($2.45/sample). The source of DNA in the RAA assay still needs to be determined; however, it has been reported that cell-free parasite DNA or *S. haematobium* eggs could be the source when DNA is detected using molecular methods [44].

The RAA assay requires a short reaction time (~20 min), whereas the LAMP assay requires approximately 35–40 min to amplify DNA [45](Supplementary Table 2). Moreover, unlike the reference test (microscopy-based egg counting), the RAA assay is not time-consuming, which implies that the RAA assay has huge potential to rapidly detect low-intensity *S. haematobium* infections. RAA and recombinase polymerase amplification (RPA), which rely on the principle of isothermal nucleic acid amplification, have recently been developed as molecular diagnostic methods. The difference between these two methods is the source of the recombinase, with RPA using UvsX from the T4 phage and RAA using recombinases from an extensive range of sources, such as bacteria and fungi [46]. In addition, the reaction sample (urine) was lyophilized into a single unit, which simplified the sample pretreatment process, facilitated transportation, and allowed long-term preservation at room temperature. The limitation of our study is that the shortage of urine samples and the RAA cross-reaction test did not involve other pathogens that could cause urinary tract infection due to the shortage of samples.

5. Conclusion

The RAA assay presented here is a rapid and sensitive approach for the diagnosis of *S. haematobium* infection and can be used to assess schistosomiasis control measures and diagnose *S. haematobium* infection in low-endemic and non-endemic areas with imported cases of schistosomiasis.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All aspects of the study were performed in accordance with the national ethics regulations and approved by the Ethics Review Committee of Zanzibar (ZAMREC/002/MAY/014).

Consent for publication

Not applicable.

Data availability statement

Data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Song Zhao: Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Qiaoqiao Zhang:** Writing – original draft, Validation, Project administration, Data curation. **Xinyao Wang:** Validation. **Wei Li:** Validation, Resources. **Saleh Juma:** Validation, Resources, Data curation. **Robert Berquist:** Writing – review & editing. **Jianfeng Zhang:** Validation, Resources. **Kun Yang:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Kun Yang reports financial support was provided by National Natural Science Foundation of China. Kun Yang reports financial support was provided by Jiangsu Provincial Department of Science and Technology.

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No applicable.

Abbreviations

RAA	Recombinase-aided amplification
cox1	cytochrome *c* oxidase subunit I
RPA	Recombinase polymerase amplification
LAMP	Loop-mediated isothermal amplification
PCR	Polymerase chain reaction

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23031.

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