Clinical Applications of Isothermal Diagnosis for Human Schistosomiasis

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Definition: About 250 million people affected, 779 million people at risk of infection, and 440 million people with residual morbidity are globally attributable to schistosomiasis. Highly sensitive and specific, simple, and fast to perform diagnostics are required for detecting trace infections, and applications in resource-poor settings and large-scale assessments. Research assessing isothermal diagnoses of S. japonicum, S. haematobium, S. mansoni, mixed infections, and schistosomal hybrids among clinical human specimens was investigated. Loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA) and combined techniques were identified. Both LAMP and RPA reached species-dependent 100% sensitivity, and detection levels within femtogram and nanogram amounts for pure and hybridale breeds. Cross-reactivity among Schistosoma species and co-endemic pathogens was rare, though research on diagnostic markers and primer optimization should continue. Operating with ready-to-use lyophilized reagents, simplified and inexpensive nucleic acid extraction, tolerability to likely inhibitors, and enzyme stability at ambient temperature is advantageous. RPA performed optimal at 35–39 °C within 5–10 min. while LAMP operated at 61–65 °C for up to 120 min.; properties are preferable over assays requiring expensive laboratory equipment. DNA degradation could be prevented by stabilizing substances. A limitation throughout warranting future research is the small sample size reaching a few hundred participants at the maximum. Isothermal diagnostics are highly valuable in detecting trace infections seen subsequent to chemotherapeutic treatment, and among apparently healthy individuals, both constituting likely sources of ongoing pathogen transmission. Its expansion to the vaccine field for assessing parasitological trial endpoints could be considered.

Keywords: schistosomiasis; Schistosoma; isothermal; diagnosis; LAMP; RPA

1. Background

Schistosomiasis as a neglected tropical helminthic disease causes large morbidity and mortality. Among the genus Schistosoma, S. haematobium, S. mansoni, and S. japonicum are the most widespread and clinically relevant species afflicting humans. S. haematobium and S. mansoni are prevalent in Africa and the Middle East; S. mansoni occur also and S. japonicum solely in Latin America and the Caribbean, as well as Asia, respectively [1,2]. The species S. mekongi, S. guineensis, S. intercalatum, and S. malayensis also affect humans, but to a lower extent [1,2]. Of growing concern is cross-species hybridization abated by natural and anthropogenic changes; it leads to a large diversity of novel inter-species and/or inter-lineages through instant acquisition of genetic information [3].

The blood-feeding flukes cause globally more than 250 million people affected, over 779 million people at risk of infection, about 440 million people with residual morbidity, and nearly 500,000 annual deaths [2,4,5]. Infections of vertebrates occur during contact with freshwater infested with skin-penetrating schistosomal cercariae disseminated by species-specific snails; larval cercariae transform into schistosomulae and migrate matured to adult
worms to their oviposition sites within the vasculature for mating and sexual reproduction; see Figure 1 for more details. Intact adult worms can persist in immunocompetent definitive hosts for decades. Purposefully, eggs are shed via fecal or urinary routes to continue the transmission from vertebrates to molluscs for asexual reproduction upon hatching of miracidia into freshwater; see Figure 1 for more details [2,4,5].

Figure 1. Generic Schistosoma life cycle.

Acute schistosomiasis or Katayama fever seen among individuals without past parasitic exposure presents as debilitating febrile illness coupled with, e.g., headache, myalgia, fatigue, diarrheal, as well as respiratory symptoms, and hepatomegaly following an incubation period of up to 10–12 weeks. Chronic schistosomiasis manifests as host immunoresponses to unreleased retained eggs, that can lead to long-term complications. Host reactivity presents as bleeding and scarring, chronic inflammations and granulomatous-fibrotic formations around eggs trapped in capillaries damaging species-dependent organs inclusive of liver, intestine, spleen, and the urinary bladder [2,3]. Intestinal chronic schistosomiasis may induce diarrhea or constipation including blood admixture with progression to ulcerations, hyperplasia, polyposis, and fibrosis. *S. haematobium* causes urogenital pathologies such as dysuria and hematuria and so called female genital schistosomiasis (FGS) if eggs are deposited in the female genitalia [6]. FGS impairs fertility, e.g., ectopic pregnancy and miscarriage, and susceptibility to viruses, e.g., human immunodeficiency virus [7] and papillomavirus, and progressing to malignancies augmented by calcification, e.g., squamous cell carcinomas and sandy patches [8–12]. Ectopic excess egg deposition or erroneous migrating adult worms within the central nervous system can induce cognitive and physical impairments as seen among infested children in endemic settings. Praziquantel (PZQ) is commonly used [7] to treat schistosomiasis and eliminate adult schistosomes by changing irreversibly the permeability and stability of their tegument, but it requires prevailing host immune defense mechanisms for complete efficacy [13]. Control and prevention measures should be complemented by vaccination given the advances in this field to achieve long-term protection against transmission, infection, and disease recurrence [8].
Conventional schistosomal diagnostics include microscopy as the gold standard to visualize fecal and urinary eggs, and the detection of antibodies, antigen(s) and genetic information. However, they vary in sensitivity and specificity depending on, e.g., disease status, endemcity/co-endemcity levels, and chemotherapeutic treatment [7], in particular post-treatment [2,14–16]. Oviposition is a common marker to indicate active infection. However, egg shedding starting approximately one month post-infection is impacted by day-to-day variations [7], and the acquisition of, e.g., single sexes, infertile females or senile worms, which make this a rather poor indicator [16–18]. Despite, reliable diagnostics are essential for disease monitoring to detect in particular low infection levels promoting pathogen transmission [7], and for evaluating the effectiveness of treatment and control measures [1,17]. Consensus exists that an optimal diagnostic tool must be highly sensitive and specific, simple, and fast to perform and interpret also on different specimen types; favored diagnostics should be capable of detecting acute phase and trace infections supporting early treatment, and cost-effective for use also in resource-limited endemic settings [19].

Nucleic acid amplification is a highly valuable tool for simultaneous detection and species identification at day one post-infection [7] since the introduction of the polymerase chain reaction (PCR) [2,15]. However, PCR-based assays require expensive laboratory and technical equipment besides highly skilled personnel, cold chain for reagent storage, and prolonged reaction times [19,20]. This impacts their large-scale implementation as a genetic high-throughput point-of-care diagnostic measure, especially in resource-constrained settings. Isothermal amplification techniques can overcome aforementioned limitations due to their advancement in speed, simplicity, sensitivity, and specificity [1,21,22]. They detect nucleic acid in an exponential manner without constraints of thermal cycling, and are adaptable to multiplex, quantitative real-time, and reverse transcriptase techniques [23,24]. This is because nucleic acid strands are not heat-denaturated to enable primer binding and initiate amplification reactions since a polymerase of, e.g., Bacillus stearothermophilus (Bst), Bacillus subtilis (Bsu), or Staphylococcus aureus (Sau) with strand-displacement enzymatic activity is used [2,21].

An isothermal amplification technique of great interest is the loop-mediated isothermal amplification (LAMP), first reported by Notomi et al. [25]. LAMP detects few copies of genetic material as seen in low-endemic or newly emerging settings and subsequent to mass drug administration (MDA) [1,17]. Assays exist for various pathogens, e.g., Plasmodium falciparum, Babesia spp., Leishmania spp., Trypanosoma brucei, Ascaris lumbricoides, Ancylostoma spp., Necator spp., Taenia spp., and Toxoplasma gondii [16,19,26–29]. LAMP produces in a one-step reaction large amounts of lengthy double-stranded genetic information with a mutually complementary sequence and an alternate repeated structure at a constant temperature of 60–65 °C during 60 min reaction time on average [30,31]. Dumbbell-shaped DNA with stem-loops at both ends is formed, which activates steps of polymerization and extension [32]. LAMP performs well with simple isothermal equipment, e.g., heat block or water bath [17]. A pair of highly specific internal primers for strand displacement and synthesis, and external primers are required to detect six distinct sequences among the cognate sites [33]. Amplification can be accelerated and targeted regions expanded through additional loop primers [2,25,34]. Amplified products are visualized by agarose gel electrophoresis, turbidity, and colorimetry based on metal ion indicators and dyes causing color changes that are visible to the naked eye or a fluorometer [1,35–38]. Reagents are storable at ambient temperature, and test reactions are robust against inhibitory compounds in specimens, and variations in pH and temperature [9,39,40].

Another isothermal amplification technique of growing interest is the recombinase polymerase amplification (RPA) [21,41]. It performs similar to LAMP, except it forms in the presence of a recombinase protein derived from, e.g., T4-like bacteriophages or Escherichia coli, and a high molecular crowding agent a recombinase-primer complex; both promote primer invasion into double-stranded DNA at cognate sites [42]. The invasion is stabilized by single-stranded binding proteins. Subsequent polymerization and extension of loop-like
DNA are induced by a chain-replacement polymerase [42]. RPA operates at 22–45 °C, though best at 37–42 °C, that allows simple cycling equipment, e.g., incubator, heatblock, chemical heater, body heat, or ambient temperature, during a reaction time of less than 30 min [10,11]. RPA operates on many specimens, e.g., cultured organisms, body fluids, surgical biopsies, organ tissues, and animal or plant products, and with lyophilized reagent pellets [10,20,43]. Amplicons are visualized similar to LAMP or by oligo chromatographic lateral flow strips, and fluorescence-labeled probes, i.e., dT-fluorophores coupled with corresponding dT-quenchers [44].

Research performed on the isothermal detection of human schistosomiasis within clinical applications was investigated and findings are presented hereby. The progression of isothermal techniques as a high-throughput point-of-care diagnostic measure for single- and multiple-species identification, including schistosomal hybrids, is also addressed. Searches performed in PubMed, Embase and Web of Science, and details of assays identified for S. japonicum, S. haematobium, S. mansoni, and mixed infections are delineated in Figures 2–5, respectively.

**Figure 2.** Articles identified on the isothermal diagnosis of *Schistosoma japonicum* with details on participants, specimens, assay features, and test evaluation in terms of detection limits, sensitivity, specificity, PPV and NPV versus a comparator test by chronological order of publication date. Articles highlighted in light blue delineate schistosomal diagnosis based on loop-mediated isothermal amplification (LAMP) while articles highlighted in blue delineate Schistosoma lateral flow strips, and fluorescence-labeled probes, i.e., dT-fluorophores coupled with corresponding dT-quenchers [44].

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Figure 3. Articles identified on the isothermal diagnosis of Schistosoma haematobium with details on participants, specimens, assay features, and test evaluation in terms of detection limits, sensitivity, specificity, PPV and NPV versus a comparator test by chronological order of publication date. Articles highlighted in light blue delineate Schistosomal diagnosis based on loop-mediated isothermal amplification (LAMP) while articles highlighted in blue delineate Schistosomal diagnosis based on recombinase polymerase amplification (RPA).

For details on participants, specimens, assay features, and test evaluation in terms of detection limits, sensitivity, specificity, PPV and NPV versus a comparator test by chronological order of publication date, please refer to the original article.
We sought databases of PubMed, Embase, and Web of Science for suitable publications on the isothermal diagnosis of schistosomiasis among clinical specimens collected from human subjects by applying the following terms: “schistosomiasis”, “Schistosoma”, “snail fever”, “detection”, and “aspects”.

The last searches were performed on 31 December 2021. Publications included after removing duplicates, screening titles and abstracts, reading full-texts, and complementing through reference searches were not restricted by time period, but by the availability of full-texts available in English. Experimental animal studies, investigations on intermediate mollusk hosts, reviews and mathematical models were excluded unless considered highly relevant. Abbreviations: S. = Schistosoma, KK = Kato-Katz, bp = basepair, min = minutes, EtBr = ethidium bromide, ° = male, ♀ = female, PCR = polymerase chain reaction, PPV = positive predictive value, NPV = negative predictive value, fg = femtogram, Bst = Bacillus steatorrhophilus, NA = not available.

Figure 4. Articles identified on the isothermal diagnosis of Schistosoma mansoni with details on participants, assays, and test evaluation in terms of detection limits, sensitivity, specificity, PPV and NPV versus a comparator test by chronological order of publication date. Articles highlighted in light blue delineate schistosomal diagnosis based on loop-mediated isothermal amplification (LAMP).

Figure 5. Articles identified on the isothermal diagnosis of mixed Schistosoma infections with details on participants, specimens, assay features, and test evaluation in terms of detection limits, sensitivity, specificity, PPV and NPV versus a comparator test by chronological order of publication date. Articles highlighted in light blue delineate schistosomal diagnosis based on loop-mediated isothermal amplification (LAMP).
We searched databases of PubMed, Embase and Web of Science for suitable publications on the isothermal diagnosis of schistosomiasis among clinical specimens collected from human subjects by applying the following terms: “schistosomiasis”, “Schistosoma”, “snail fever”, “schistosomiasis MeSH Terms”, “isothermal”, “diagnosis/diagnostic”, “detection”, and “assay”. The last searches were performed on 31 December 2021. Publications included after removing duplicates, screening titles and abstracts, reading full-texts, and complementing through reference searches were not restricted by time period, but by the availability of full-texts available in English. Experimental animal studies, investigations on intermediate mollusk hosts, reviews and mathematical models were excluded unless considered highly relevant. Abbreviations: S. = Schistosoma, bp = basepair, min = minutes, EtBr = ethidium bromide, PCR = polymerase chain reaction, PPV = positive predictive value, NPV = negative predictive value, ng = nanogram, Bst = Bacillus stearothermophilus, NA = not available, AM = ante meridien, PM = post meridien.

2. *Schistosoma japonicum* Assays

Searches revealed the application of LAMP on patient sera with microscopy-confirmed *S. japonicum* infection [26]. The assay builds on the non-long terminal highly repetitive retrotransposon SjR2 marker that identifies schistosomal infestation early, and accounts for 14% of the parasite genome [45,46]. LAMP compared to PCR yielded 96.7% versus 60% sensitivity, respectively. Detection limits of 0.08 femtogram and 0.8 picogram were achieved by LAMP and PCR, respectively; LAMP did not cross-react with *S. mansoni* and *Clonorchis sinensis* [42]. This research builds on investigations of experimentally challenged New Zealand rabbits to evaluate LAMP for assessing the chemotherapeutic efficacy of PZQ (150 mg/kg) administered at week-7 and week-8 post-challenge. Rabbit sera were negative for *S. japonicum* at week-12 and week-10 post-treatment by LAMP and PCR, respectively, which indicates better sensitivity of LAMP over PCR. Xu et al. could show that LAMP operates better than conventional diagnostics in amplifying genetic information also within a reaction time of 60 min while using non-sophisticated equipment and easy visualization.

LAMP was in other clinical investigations assessed for detecting trace infections of *S. japonicum* and the chemotherapeutic effectiveness of PZQ [47]. Fecal samples were stratified based on their microscopy-confirmed eggs per gram (EPG) of feces into cohorts of 84.5% (93/110) with <100 EPG, 11.8% (13/110) with 100–400 EPG, and 3.6% (4/110) with >400 EPG [48]. LAMP revealed 95.5% sensitivity and 100% specificity overall, and 95.1% (95% confidence interval (CI): 88.5–100), 97.6% (95%CI: 93.0–100), and 95.1% (95%CI: 88.5–100) sensitivity among cases carrying < 10 EPG, 10–39 EPG, and 40–99 EPG of feces, respectively [47]. The negative conversion rate at three, six, and nine months post-treatment increased from 23.4% (95%CI: 11.3–35.5) to 61.7% (95%: 47.8–75.6), and 83.0% (95%: 72.3–92.7), respectively, while immunoresponses remained at low levels throughout. Notably, 16.7% (10/60; 95%CI: 7.3–26.1) of healthy controls were *S. japonicum* positive by LAMP.

To further examine trace targets of *S. japonicum* among microscopy-confirmed fecal samples, Sun et al. combined the lateral flow dipstick technology with an RPA that utilizes the SjR2 marker and ready-prepared lyophilized reagent pellets [24,49]. The detection limit was five femtogram with no cross-reactivity found for the strains assessed (Figure 2); optimal visibility was seen for cycling conditions of 39 °C for 10–15 min detected 0.9 femtogram schistosomal DNA, no cross-reaction with other *Schistosoma* species, and each 100% sensitivity and specificity. RPA also confirmed *S. japonicum* infections among fecal specimens of highly exposed fishermen with 100% sensitivity and 96.40% (95%CI: 99.32–99.54) specificity, and strong agreement with Kato-Katz by Cohen’s kappa (k = 0.942, 95%CI: 0.89–0.99, p-value < 0.0001).
Applying several primers simultaneously increases chances of primer–primer interactions leading to false positivity [19,38], as seen for the internal transcribed spacer (ITS)/28S-based \textit{S. mansoni} RPA developed by Poulton et al. [20]; this hampers the identification of unique target sequences [14]. To address this, a single set of hairpin primers coupled with a real-time LAMP was evaluated [32]. Primers were designed with a target-unrelated stem-loop domain at the 5′-end, and a target-related binding sequence at the 3′-end. Human sera containing \textit{S. japonicum} antibodies showed 100% (19/19) positivity after seven-time repeated testing, which also indicates LAMP’s robust repeatability strengthened by a relative standard deviation of 3.7%.

3. \textit{Schistosoma haematobium} Assays

Initial LAMP experiments for \textit{S. haematobium} were conducted on urine fractions, i.e., whole urine, supernatant and sediment, of microscopy-confirmed cases [9] using the species-specific ribosomal intergenic spacer (IGS) marker [50]. LAMP performed best with DNA extracted from sediments using the Rapid-Heat LAMP DNA extraction method, i.e., 100% (18/18) positivity. The Bst 2.0 Warm-Start DNA-Polymerase applied operates faster and with higher stability at room temperature compared to the wild-type Bst, which is advantageous for settings lacking cold chain facilities. Positivity was observed among cases infected with other pathogens and also controls, i.e., 11.1% (1/9) other helminths, 20% (1/5) protozoa/bacteria/viruses, 6.7% (1/15) controls with eosinophilia, and 20.8% (5/24) controls without eosinophilia, though unlikely due to cross-reactivity considering the low detection rates. Finding \textit{S. haematobium} among controls could be explained by low infection levels and day-to-day variability in oviposition. Experiments revealed 100% (95%CI: 81.32–100) sensitivity and 86.67% (95%CI: 75.40–94.05) specificity overall; LAMP’s detection limit was $10^4$ times higher compared to PCR, i.e., 100 femtogram versus one nanogram, and could be optimized to one femtogram or an equivalent of a single cell parasite. LAMP performed similar among 69 urine samples from Egyptian participants, i.e., 100% (95%CI: 88.78–100) sensitivity and 63.16% (95%CI: 48.99–78.19) specificity [12].

Gandasegui et al. evaluated LAMP further among urine sediments of schoolchildren from Angola [51]. Test positivity ranged from 57% (98/172) to 73.8% (127/172) depending on the nucleic acid extraction and visualization methods used (Figure 3). Nevertheless, LAMP revealed tolerability to potential inhibitory substances in biological samples that highlights its practicability for high-throughput large-scale testing [9,51]. Despite SYBR GreenI improved test positivity due to better visibility, approximately 72% positive cases were visible to the naked eye. Interestingly, 78.2% (68/87) to 86.2% (75/87) of microscopy-positive specimens and 48.2% (41/85) to 61.2% (53/85) of microscopy-negative specimens were LAMP-positive. This refers back to LAMP’s capability to detect egg DNA and cell-free circulating DNA of parasitic lysis products [52,53]. Interestingly, 90.3% (56/62) samples positive by microhematuria and microscopy and 57.8% (37/64) samples negative by microhematuria and microscopy were also LAMP-positive. Kabir Patwary et al. found a significant correlation of detecting hematuria and \textit{S. haematobium} DNA (OR: 6.66; 95%CI: 0.98–99.5; $p$-value = 0.4142) among cervicovaginal lavages of Zambian women suffering from FGS; 7.11% (16/225) versus 6.2% (14/225) lavages were positive by RPA and PCR, respectively [6].

A real-time RPA targeting the DraI tandem repeat region [54] of \textit{S. haematobium} was used by Rostron et al. among pediatric urine samples categorized per ova load into very low, low, medium, and high, i.e., 1–10 eggs/10 mL, 11–50 eggs/10mL, 51–400 eggs/10mL, and $>$400 eggs/10mL, respectively [10,55]. DraI accounts for 15% of the parasite genome [45]. Of specimens with medium and high egg loads, 70% (14/20) revealed strong fluorescence subsequent to just eight minutes of cycling; of samples with very low egg loads, 20% (4/20) were positive, 5% (1/20) inconclusive, and 5% (1/20) negative. Despite RPA’s detection limit was one femtogram, which is below the limit of 10 femtogram by PCR and 100 femtogram by lateral flow assays, test inconclusiveness and negativity could have occurred due to sample degradation, false positivity by microscopy, or inhomogeneous egg
distribution in sample aliquots [55]. Rosser and colleagues [10] raised in earlier bench-based experiments of spiked urine the likelihood of cross-amplification with other members of the *S. haematobium* group possessing the DraI region. Future species-specific assays to identify sub-group species [14] or multiple species [20] could address this [14].

RPA was further applied among pediatric urine samples grouped per ova load and found overall 94% (158/168) positivity, 93.7% (95%CI: 88.7–96.9) sensitivity and 100% (95%CI: 69.1–100) specificity [11]. Interestingly, RPA correctly identified 64 of 70 specimens with ultra-low and 113 of 122 specimens with ultra-low and low *S. haematobium* egg loads, indicating 91.4% (95%CI: 82.2–96.8) sensitivity and 92.6% (95%CI: 86.5–96.6) specificity; also 93.8% (15/16) specimens containing ≤ 1 egg/10mL urine were positive. This highlights RPA’s capability of detecting trace infection levels.

Frimpong et al. validated a real-time RPA among urine samples of Ghanaian participants [56]. They detected 98.4% (95%CI: 91.6–100) sensitivity and 100% (95%CI: 94.9–100) specificity compared to real-time PCR versus 89.9% (95%CI: 80.2–95.8) sensitivity and 98.5% (95%CI: 91.8–100) specificity compared to microscopy. Degradation of *S. haematobium* DNA in the urinary milieu could have impacted RPA’s sensitivity. Nevertheless, RPA’s lower detection limit was less than one femtogram. Cross-reactivity with *S. mansoni* and *S. japonicum* likely occurred due to sample contamination or DraI being a rather sensitive than specific marker.

### 4. *Schistosoma mansoni* Assays

An Sm1–7 sequence-based LAMP was assessed initially among fecal samples of Kenyan schoolchildren; Sm1–7 as a species-specific repeat tandem sequence is present in all life cycle stages [27], and comprises 12% of the *S. mansoni* genome [45]. Specimens were categorized based on their microscopy-confirmed egg loads [48], i.e., <100 EPG, 100–399 EPG, and >400 EPG, resembling low, moderate, and high infection intensities, respectively. LAMP amplified *S. mansoni* DNA across all categories while its detection limit was 32 femtogram; its overall positivity was 44.6% (171/383) compared to 46% (176/383) by Kato-Katz, which indicates strong test agreement (k = 0.9). Sensitivity scored 97% and specificity 100%, and no co-endemic helminths, i.e., *Ascaris lumbricoides*, hookworm and *Trichuris trichiura*, were detected (Figure 4).

The species-specific repeat tandem mitochondrial *S. mansoni* minisatellite marker was used in further LAMP experiments [57], i.e., SmMIT-LAMP, among fecal samples of residents from Brazil [28]. SmMIT-LAMP detected parasitic DNA among 8% (13/162) specimens with low eggs counts, i.e., 12–180 EPG confirmed by double Kato-Katz, and 7.4% (12/162) smear-positive and 22.8% (37/162) smear-negative samples; 24.5% (12/49) children aged ≤ 12 years were *S. mansoni* infected that were missed by microscopy earlier. SmMIT-LAMP demonstrated 92.86% (95%CI: 66.13–99.82) sensitivity and 80.11% (95%CI: 73.64–85.59) specificity overall.

Building on murine experiments [58], Fernández-Soto et al. applied SmMIT-LAMP on urine specimens of infected subjects from sub-Saharan Africa, and urine samples of healthy donors spiked with DNA of *S. mansoni*, and *S. mansoni* coupled with *S. haematobium, S. intercalatum, S. bovis, S. japonicum, Fasciola hepatica, Amphimerus spp.*, or *Strongyloides venezuelensis* [52]. The detection limit of *S. mansoni* among spiked samples was 100 and 0.01 femtogram following 60 and 120 min cycling, respectively; DNA of other helminths was not amplified. Solely 120 min cycling duration revealed positivity among naturally infected subjects, i.e., 71.4% (5/7) of microscopy-confirmed *S. mansoni* cases, and 42.9% (3/7) of cases with microscopy-confirmed non-schistosomal infections, i.e., *Trichomonas vaginalis, Enterobius vermicularis, and Trichuris trichiura; 57.1% (4/7) controls with eosinophilia as a biomarker for helminthic often prepatent infections lacking parasitism were positive by SmMIT-LAMP while microscopy-confirmed *S. haematobium* cases remained negative indicating no cross-species reactivity. *S. mansoni* detection likely was impeded by specimen contamination [19], unfavorable storage conditions including repetitive freezing and thawing, and lack of DNA-stabilizing additive substances avoiding degradation.
SmMIT-LAMP was advanced further to fulfill WHO’s ASSURED criteria, i.e., affordable, sensitive, specific, user-friendly, robust and rapid, equipment-free, and deliverable [21,59]. A novel concentration and desiccation technology utilizing trehalose achieved reagent functionality for up to three weeks and five months at room temperature and 4 °C, respectively [60]. Fernández-Soto et al. applied a real-time species-specific LAMP to skin tissue biopsies of a patient with travel history to Uganda and contact to freshwater of the Bunyonyi Lake few weeks prior to developing thoracic pruritic painless erythematous papules; the patient presented with eosinophilia, but no parasites, cysts and ova in feces [61]. LAMP detected S. mansoni in biopsies; ectopic cutaneous ova and miracidia were found in addition to schistosomal antibodies.

Urine specimens of Zambian schoolchildren four weeks after MDA (40 mg/kg) were investigated by LAMP for the presence of the cell-free repeat Sm1–7 DNA fragment [62]. Positivity by PCR was 77.5% (86/111) versus 93.7% (104/111), and 87.4% (97/111) by LAMP depending on the DNA extraction method. The discrepancies observed by LAMP could be attributable to the extraction of membrane-based versus membrane-free DNA resulting in varying concentrations of inhibitory components. Nevertheless, PCR and LAMP performed better in detecting S. mansoni infections than Kato-Katz, i.e., ≤71.2% (79/111) by PCR and ≤85.6% (95/111) by LAMP. LAMP’s sensitivity among low infection intensities subsequent to MDA ranged from 88% (95% CI: 80–93) to 100% (95% CI: 96–100); its specificity varied from 14% (95% CI: 0.4–58) to 100% (95% CI: 59–100).

5. Assays on Multiple Schistosoma Species including Hybrids

Searches revealed the assessment of LAMP on dried urine sediments from Ghanaian participants for the presence of S. mansoni, S. haematobium, and mixed infections [63–66]. Depending on the DNA extraction method used, 88.4% (76/86) to 94.2% (81/86), and 86.0% (74/86) S. mansoni cases were found by LAMP and PCR, respectively, in addition to 83.7% (72/86) and 81.4% (70/86) S. haematobium cases, respectively (Figure 5). This confirms the prevalence of concurrent sympatric infections. Notably, LAMP and PCR revealed 5.8% (5/86) and 1.2% (1/86) false negativity for S. mansoni, respectively.

Further experiments among urine specimens of Zambian schoolchildren aged 8–16 years subsequent to MDA detected 84.6% (110/130) positivity, 96.49% sensitivity, and 100% specificity for S. mansoni, and 61.5% (80/130) positivity, 89.89% sensitivity, and 100% specificity for S. haematobium [67]. Interestingly, all samples were negative by microscopy. LAMP performed differently depending on the DNA extraction method [68]. Song et al. [14] used a two-stage nested-like rapid amplification technique consisting of a first-stage all-target RPA, and a second-stage target-specific LAMP in a microfluidic closed system. Different sample types, i.e., human whole blood, serum, and urine, were investigated without prior nucleic acid extraction during a total reaction time of 40–50 min, i.e., 10–20 min at 37 °C for RPA, and 30 min at 60–65 °C for LAMP, followed by simple visualization with fluorescent or colorimetric dyes. The hybrid RPA/LAMP was highly sensitive and specific, and robust when performed in a single- and multi-plex manner for detecting up to 16 targets inclusive of S. mansoni, S. haematobium, and S. japonicum.

To address the growing concerns of cross-species hybridization leading to large diversity of novel inter-species and/or inter-lineages, LAMP was investigated to detect hybrids originating from S. haematobium and the phylogenetic closely-related livestock species S. bovis [3,69]. Urine specimens from healthy donors were spiked with DNA of hybrids originating from Côte d’Ivoire and Corsica/France; hybrids were confirmed previously based on mitochondrial cytochrome c oxidase (COX) and nuclear ribosomal ITS profiles [3]. Species-specific assays [70] amplified pure S. haematobium, S. mansoni, and S. bovis DNA corroborating LAMP’s high sensitivity and specificity; the latter detected also hybridale DNA of less than 0.001 nanogram though only of strains with S. bovis/S. bovis x S. haematobium profiles. Hybrids likely retrieved from an initial crossing between male S. haematobium
and female *S. bovis* leading to the introgression of *S. bovis* mitochondrial DNA into the *S. haemotobium* genome [69].

6. Conclusions
Despite the tremendous efforts to prevent and control schistosomiasis, the helminthic disease still causes large morbidity and mortality in developing countries and beyond as seen by the occurrence of inter-species and/or inter-lineages schistosomal hybrids in Europe [3]. Efforts surely are impacted by misdiagnoses and underdiagnoses of infective subjects constituting sources of ongoing parasite transmission due to limitations of conventional diagnostics [2,7,14–16]; their performance is influenced by disease status including atypical disease presentation [3,69], endemiocity/co-endemiocity levels, and chemotherapeutic treatment [1,17]. Isothermal techniques aim to overcome aforementioned limitations. Investigations on the isothermal detection of schistosomiasis among clinical specimens collected from humans revealed sensitivity reaching 96.7% and 100% for *S. japonicum* serum- and stool-based assays by LAMP and RPA, respectively; detection levels of 0.08 and 0.9 femtogram by LAMP and RPA were reported, respectively. LAMP and RPA reached up to 100% and 98.4% sensitivity when confirming *S. haematobium* among urine specimens, respectively; the detection limit was one femtogram for both techniques. LAMP urine- and stool-based assays for detecting *S. mansoni* varied in sensitivity of 88% to 100%; schistosomal DNA of 0.01 to 32 femtogram was amplified. LAMP detected hybridale DNA below 0.001 nanogram among strains with *S. bovis/S. bovis x S. haematobium* profiles. A limitation of all investigations warranting future large-scale high-throughput research is the rather small sample size reaching a few hundred participants at the maximum [27].

Cross-reaction with other *Schistosoma* species and co-endemic helminths, protozoa, bacteria and viruses was rarely seen throughout. Frimpong et al. [56] raised the issue of cross-reactivity between *S. mansoni* and *S. japonicum* likely due to DraI being a sensitive rather than specific marker. Likewise, Rosser et al. [10] discussed the possibility of cross-species amplification with species of the *S. haematobium* group possessing also the DraI region. Further research on species-specific diagnostic markers [14] including primer optimization [19,38] could address this.

LAMP and RPA operated well with ready-to-use lyophilized reagents [9]; desiccated reagents were stable for several weeks at ambient temperature [59,60]. *Bst* 2.0 Warm-Start DNA-Polymerase within LAMP eases testing due to its stability at room temperature [50]. Such properties are advantageous for settings lacking cold chain facilities. Simplified nucleic acid extraction, such as the Rapid-Heat LAMP DNA extraction method or not extracting nucleic acid at all [63] revealed the tolerability of isothermal diagnostics to potential inhibitory substances in biological samples [9,51]. The concentration of inhibitors could vary depending on levels of membrane-based versus membrane-free DNA [62]. LAMP detects not only ova DNA but also cell-free circulating DNA of parasitic lysis products [52], which is not present in plasma during active schistosomiasis, but is cleared following chemotherapeutic treatment [53]. Obtaining good-quality DNA in an inexpensive and practicable manner is highly relevant for resource-poor settings and large-scale epidemiological assessments. RPA performed optimal at 35–39 °C for 5–10 min [44,49] while LAMP required cycling conditions of 61–65 °C for up to 120 min and assay-dependent additional cycling of 5–10 min at 80 °C for enzyme inactivation [50,51]. Such operational factors certainly are advantageous over other assays requiring expensive laboratory and technical equipment. A weakness raised is the possibility of DNA degradation due to unfavorable handling and storage conditions impacting test positivity, which could be prevented by adding stabilizing substances.

Of note is the capability of isothermal diagnostics presented to detect minimal infection levels as reported subsequent to chemotherapeutic treatment, including MDA [47], and among individuals presenting as apparently healthy. This is alarming as such subjects are likely overlooked by conventional diagnostics and constitute an unrecognized source promoting ongoing pathogen spread [9]. LAMP revealed increasing negative conversion rates
reaching 83.0% at nine months post-treatment of *S. japonicum* infections while immunore-
sponses remained low throughout [47]. Additionally, LAMP’s sensitivity to *S. mansoni*
subsequent to MDA among Zambian schoolchildren reached 100% (95%CI: 59–100) [62].
Likewise, LAMP detected *S. mansoni* and *S. haematobium* with 96.49% and 89.89% sensitivity,
respectively, subsequent to MDA while microscopy remained negative throughout [67].
Given the recent advances in schistosomal vaccine candidates [8] and the lack of consensus
in standardized parameters assessing protection in humans impeded by the lack of diag-
nostic tools as per WHO’s ASSURED criteria suggests to consider expanding isothermal
detection techniques to the field of vaccinology.
In summary, LAMP and RPA reached species-dependent sensitivity up to 100% and detec-
tion in the femtogram range within clinical applications for diagnosing human schistosomi-
asis; hybridale DNA was found in nanogram amounts. This is advantageous for revealing
trace infection levels subsequent to chemotherapeutic treatment and among apparently
healthy individuals. The possibility of inter-species and intra-clade schistosomal cross-
reactivity warrant further research for optimized diagnostic markers and primers. The test
reagents’ stability at ambient temperature, and tolerability to potential inhibitors permitting
simplified nucleic acid extraction are favorable in particular for resource-poor settings and
large-scale investigations; added stabilizing substances may avoid DNA degradation ame-
liorating test positivity. Cycling conditions of 5–10 min at 35–39 °C for RPA, and up to 120
min at 61–65 °C for LAMP with additional assay-dependent 5–10 min at 80 °C for enzyme
inactivation allow simple cycling equipment; amplicons are easily visible also with just
the naked eye. It is worthwhile to consider expanding isothermal techniques beyond just
diagnostic purposes and the effectiveness of chemotherapeutics to the field of vaccinology
given the recent advances for assessing parasitological trial endpoints indicating protection
from the blood-feeding flukes.

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References
1. Avendaño, C.; Patarroyo, M. Loop-Mediated Isothermal Amplification as Point-of-Care Diagnosis for Neglected Parasitic
Infections. *Int. J. Mol. Sci.* 2020, 21, 7981. [CrossRef]
2. Diego, J.G.-B.; Fernández-Soto, P.; Febrer-Sendra, B.; Crego-Vicente, B.; Muro, A. Loop-Mediated Isothermal Amplification in
through vaccination Microorganisms. *Microorganism* 2021, 9, 1465. [CrossRef]
6. Patwary, F.K.; Archer, J.; Sturt, A.S.; Webb, E.L. Female Genital Schistosomiasis: Diagnostic Validation for Recombinant DNA-
8. Panzner, U.; Excler, J.L.; Kim, H.J. Recent advances and methodological considerations on vaccine candidates for human
schistosomiasis Front. *Trop. Dis.* 2021, 2, 719369. [CrossRef]
2015, 9, e0003963. [CrossRef]
10. Rosser, A.; Rollinson, D.; Forrest, M.S.; Webster, B.L. Isothermal Recombinase Polymerase amplification (RPA) of Schistosoma
haematobium DNA and oligochromatographic lateral flow detection. *Parasites Vectors* 2015, 8, 446. [CrossRef]
Clinical Assessment of a Portable, Isothermal Recombinase Polymerase Amplification (RPA) Assay for the Molecular Diagnosis
of Urogenital Schistosomiasis. *Molecules* 2020, 25, 4175. [CrossRef]


49. Sun, K.; Xing, W.; Yuanuyuan, W.; Fu, W.; Wang, Y.; Zou, M.; Luo, Z.; Xu, D. Recombinase polymerase amplification combined with a lateral flow dipstick for rapid and visual detection of *Schistosoma japonicum*. *Parasites Vectors* 2016, 9, 476. [CrossRef]

50. Kane, R.A.; Rollinson, D. Comparison of the intergenic spacers and 3′ end regions of the large subunit (28S) ribosomal RNA gene from three species of Schistosoma. *Parasitology* 1998, 117, 235–242. [CrossRef]


