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Diagnostic accuracy of molecular and non-molecular assays for urogenital schistosomiasis in Zambian schoolchildren: a latent class and composite reference standard analysis

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Tables 1 to 4 are available in the supplementary files section

Diagnostic accuracy of molecular and non-molecular assays for urogenital schistosomiasis in Zambian schoolchildren: a latent class and composite reference standard analysis

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Abstract

Background

Diagnosis of urogenital schistosomiasis is hampered by the lack of a true gold standard, as urine microscopy misses light and early infections. This study aimed to compare the diagnostic performance of molecular and non-molecular assays for urogenital schistosomiasis using different reference standards.

Methods

We evaluated three molecular assays (Dra1 qPCR, a species-specific LAMP for *Schistosoma haematobium* [Sh-LAMP] and a genus-specific *Schistosoma* LAMP) alongside urine microscopy, dipstick microhaematuria and a *Sh*-TSP-2 ELISA in 89 urine samples from schoolchildren in Lusaka Province, Zambia. Performance was assessed against microscopy, latent class analysis (LCA) and a composite reference standard (CRS). Sensitivity, specificity, likelihood ratios, predictive values and overall accuracy were calculated, and pairwise agreement between tests was assessed by correlation analysis.

Results

Microscopy identified 31 infected children (34.8%; 95% CI 25.0–45.7), compared with 35 (39.3%; 95% CI 29.1–50.3) by LCA and 55 (61.8%; 95% CI 50.9–71.9) by CRS. qPCR had the highest sensitivity in all three analyses (96.4–100%). Sh-LAMP was less sensitive than qPCR, with sensitivities of 87.1%, 85.7%, and 87.3% against microscopy, LCA, and the CRS, respectively. However, it achieved the highest overall accuracy of the molecular assays against microscopy and the CRS (58.4% and 78.7%, respectively). Microscopy and microhaematuria were fully specific under LCA and CRS, although their sensitivity against the CRS was only 56.4% and 60.0%, respectively. The ELISA gave inconsistent results, and the genus-level LAMP agreed poorly with the other tests.

Conclusions

The choice of reference standard strongly influenced both accuracy and prevalence estimates, which could lead to divergent control decisions. Dra1 qPCR showed the strongest rule-out performance across reference frameworks, while the consistent sensitivity and modest infrastructure requirements of *Sh*-LAMP support its further evaluation for point-of-care testing in resource-limited settings.

Keywords: Urogenital schistosomiasis, diagnostics, qPCR, LAMP, *Schistosoma haematobium*

Background

Schistosomiasis is a chronic and often debilitating parasitic disease caused by blood flukes of the genus *Schistosoma* [1]. Globally, more than 250 million people are estimated to be infected, with approximately 90% of cases occurring in sub-Saharan Africa [2]. The disease causes an estimated 14,000 deaths annually worldwide, although its mortality burden is likely underestimated [3]. In addition, close to 800 million individuals live in areas where transmission occurs, and over 250 million people required preventive chemotherapy in 2021 [2, 4]. According to the Global Burden of Disease Study 2021, schistosomiasis accounted for approximately 1.75 million disability-adjusted life years (DALYs) worldwide in 2021 [5], although a previous meta-analysis indicated that the true burden may be several times higher [6]. Of the six *Schistosoma* species capable of infecting humans, *Schistosoma haematobium* is responsible for more than half of all infections, leading to urogenital schistosomiasis and a range of urinary and genital complications [1, 2]. Importantly, *S. haematobium* infection is strongly associated with squamous cell carcinoma of the urinary bladder, and the International Agency for Research on Cancer (IARC) classifies this parasite as a Group 1 carcinogen [7, 8].

In 2020, WHO published the new roadmap for neglected tropical diseases (NTDs), including schistosomiasis [9]. This document set out key milestones and targets to prevent, control, eliminate, or eradicate up to 20 NTDs over the next decade. In the case of schistosomiasis, WHO set as a priority objective the elimination of schistosomiasis as a public health problem from 100% of endemic countries (78 countries in total) by 2030. To this end, this roadmap highlights the importance of developing new diagnostic methods, including more sensitive and specific point-of-need tests that can be used in different scenarios [9]. Despite the recent advances in the development of new diagnostic tests and the standardisation of current ones, one of the major challenges in schistosomiasis diagnosis is the lack of a true gold-standard test with 100% specificity

and 100% sensitivity. Microscopic examination of urine to detect schistosome eggs is the gold standard test for diagnosis of *S. haematobium* infection [10]. Although this technique is simple and inexpensive [11], it cannot detect the acute stage of the disease (early infection, when no eggs are released) and is affected by day-to-day variability in egg excretion [11]. The circulating anodic antigen (CAA) has been used to diagnose *S. haematobium* infections in urine samples in low-endemic settings [12, 13]. However, it requires a basic laboratory; it cannot discriminate *Schistosoma* co-infections and can be costly when requiring highest sensitivity; the test has not been commercialised (yet).

Molecular assays, including real-time polymerase chain reaction (qPCR) and loop-mediated isothermal amplification (LAMP), are widely used for the diagnosis of schistosomiasis due to their high sensitivity and specificity [14, 15]. The qPCR assay developed specifically for *S. haematobium*, which targets Dra1, a 121 base pairs (bp) repeat sequence representing at least 15% of the parasite's genome [16], has demonstrated excellent sensitivity and specificity in samples from both endemic and non-endemic areas [15, 17]. Although qPCR generally requires specialised laboratory infrastructure, LAMP uses isothermal amplification and has potential to support diagnosis closer to the point of need [14]. However, this potential requires validation of the complete testing workflow, including specimen preparation, DNA extraction, amplification and result interpretation, under the intended conditions of use. Despite its high analytical sensitivity and minimal equipment requirements, the clinical application of LAMP for schistosomiasis remains limited by the complexity of primer design and the increased risk of contamination due to amplicon carryover [18, 19]. Moreover, further validation of both qPCR and LAMP assays across different sample types and epidemiological settings is still required to fully establish their diagnostic performance.

In this study, we sought to compare the diagnostic performance of three molecular methods (qPCR and two distinct LAMP assays) and non-molecular (urine microscopy, microhaematuria and ELISA) tests for urogenital schistosomiasis in urine samples in Zambia. To address the limitations of microscopy as an imperfect reference standard, we applied both latent class analysis (LCA) and composite reference standard (CRS) approaches. Our findings contribute to the ongoing validation and standardisation of molecular diagnostics for urogenital schistosomiasis in reference laboratory settings.

Methods

Ethics

The study received ethical clearance from the Levy Mwanawasa Medical University Research Ethics Committee (LMMU-REC 000054/23), and authorization to carry out the research was granted by the National Health Research Authority (NHRA0001/23/04/2023). Before the investigation began, additional approval was obtained from both the Lusaka Province Health Office and the Ministry of Education. Parents or legal guardians of all participating children provided informed consent, and assent was requested from the children themselves when applicable. Participation was voluntary, with clear communication to families that they were free to withdraw at any point without any negative consequences. Strict measures were taken to maintain participant confidentiality and privacy. For children who tested positive for schistosomiasis, their names were shared with the Ministry of Health's Neglected Tropical Diseases Unit so that appropriate follow-up could be arranged. All procedures were performed according to the ethical guidelines of the 2013 Declaration of Helsinki.

Study area and population

This exploratory diagnostic accuracy study used samples from a previously published cross-sectional study conducted in 2023 among 208 school-aged children in Chongwe and Kafue districts, Lusaka Province, Zambia [20].

The study setting, participant recruitment and urine sample collection are described in the primary study [20]. Of the 208 participants in the primary study, 89 were included in the present analysis based on the availability of extracted DNA remaining after the primary study was completed. The remaining DNA was used for comparative evaluation of molecular and non-molecular diagnostic tests for urogenital schistosomiasis. The sample size was therefore determined by the number of samples included from the parent study rather than by an independent prospective sample size calculation for diagnostic accuracy. Urine samples were collected and kept at 4 °C in Zambia before being shipped frozen to the Parasitology Reference and Research Laboratory at the Instituto de Salud Carlos III (Spain).

Parasitological and biochemical analysis

Urine samples were examined for both visible (gross) haematuria and *S. haematobium* eggs. Microhaematuria was assessed using Hemastix® reagent dipsticks (Bayer, Leverkusen, Germany), following the manufacturer's guidelines, with results categorized as negative, trace, 1+, 2+, or 3+. *S. haematobium* eggs were detected using the urine filtration technique [21]. In brief, each sample was thoroughly mixed, and 10 mL of urine was drawn into a syringe and pushed through a nucleopore membrane filter with a pore size of 20 μm. The filter was subsequently transferred onto a microscope slide and examined under 10x and 40x objectives to identify and quantify *S. haematobium* eggs. Eggs were manually counted, and infection intensity was expressed as the number of eggs per 10 mL of urine.

169

170 **Recombinant protein production**

171 The recombinant *Sh*-TSP-2 protein was produced and purified following previously
172 established procedures [22]. Briefly, *Escherichia coli* BL21 (DE3) cells carrying the expression
173 plasmid were grown overnight at 37 °C with shaking at 200 rpm in 10 mL of Luria broth containing
174 50 µg/mL kanamycin (LBkan). The overnight culture was then diluted 1:100 into 500 mL of fresh
175 LBkan and incubated at 37 °C with agitation until the culture reached an OD 600 between 0.5 and
176 1.0. Protein expression was induced by adding 1 mM isopropyl-beta-D-thiogalactopyranoside
177 (IPTG), and cultures were allowed to continue shaking for a further 24 h. After induction, bacterial
178 cells were collected by centrifugation at 8,000 *g* for 20 minutes at 4 °C, resuspended in 50 mL of
179 lysis buffer (50 mM sodium phosphate pH 8, 40 mM imidazole, 300 mM NaCl), and subjected to
180 three freeze-thaw cycles followed by sonication on ice. The lysate was centrifuged at 20,000 *g* for
181 20 min at 4 °C, and the clarified supernatant was stored at –80 °C.

182 Purification of recombinant proteins was performed at 4 °C on an ÄKTA Pure UPC FPLC
183 system (GE Healthcare, Chicago, IL, USA) using Ni^{2+}-based immobilized metal affinity
184 chromatography (IMAC). Protein samples were diluted fourfold in lysis buffer, passed through a
185 0.45 µm filter, and loaded onto a 1 mL His-Trap IMAC column (GE Healthcare) pre-equilibrated
186 with lysis buffer at a flow rate of 1 mL/min. After washing the column with 10 column volumes of
187 lysis buffer, bound proteins were eluted using a linear imidazole gradient ranging from 100 to
188 500 mM. Fractions containing the purest target protein were combined and exchanged into PBS
189 using Amicon Ultra-15 centrifugal concentrators with a 3 kDa molecular weight cut-off. The
190 identity of the purified proteins was verified by SDS-PAGE followed by Western blotting using
191 anti-His monoclonal antibodies (Invitrogen, Carlsbad, CA, USA).

192

193

194 **ELISA assays**

195 Nunc™ MicroWell™ 96-well Medisorp™ plates (Thermo Fisher Scientific, Waltham, MA,
196 USA) were coated overnight at 4 °C with Sh-TSP-2 at a concentration of 2 µg/mL prepared in
197 0.1 M Na_2CO_3/$NaHCO_3$ buffer (pH 9.6). After the coating step, plates were rinsed with PBS
198 containing 0.05% Tween-20 (PBST) and then blocked for 2 h at room temperature (RT) using
199 100 µL of PBST supplemented with 5% skimmed milk. Once blocking was complete, 50 µL of
200 urine samples diluted 1:10 in PBST were added to the wells and incubated overnight at 4 °C. The
201 next day, plates were washed again with PBST before adding 100 µL of goat anti-human IgG
202 conjugated to HRP (Sigma, MA, USA), diluted 1:5,000 in PBST, followed by incubation for 1 h at
203 RT. After a final series of PBST washes, colour development was performed using TMB substrate,
204 and the reaction was stopped by adding 0.5 M sulfuric acid. Absorbance was then read at 492 nm
205 using a Multiskan FC microplate reader (Thermo Fisher Scientific).

206

207 **DNA extraction and purification**

208 DNA was isolated using the Quick-DNA Urine Kit (Zymo Research, Irvine, CA, USA)
209 according to the manufacturer's guidelines. In summary, 70 µL of Urine Conditioning Buffer was
210 added to each 1 mL urine sample, followed by 10 µL of Clearing Beads. The mixture was then
211 vortexed briefly and centrifuged at 3,000 *g* for 15 min. After this initial clarification step, the
212 Tough-to-Lyse Samples workflow was applied. The supernatant was carefully poured off, leaving
213 approximately 200 µL of pellet. This pellet was transferred to a ZR BashingBead Lysis Tube (0.1
214 & 0.5 mm beads), and 600 µL of BashingBead Buffer was added. Samples were then processed on
215 a Vortex-Genie® 2 at maximum speed for 5 min. Tubes were centrifuged at 10,000 *g* for 1 min, and

400 µL of the resulting lysate was loaded onto a Zymo-Spin III-F Filter, followed by another spin at 10,000 *g* for 1 min.

To the filtrate, 1,200 µL of Genomic Lysis Buffer was added, and the mixture was passed through a Zymo-Spin IC-S Column. The column was then washed sequentially with 200 µL of Urine DNA Prep Buffer and with 700 µL and 200 µL of Urine DNA Wash Buffer. After washing, columns were placed into clean 1.5 mL tubes, and DNA was eluted by adding 50 µL of molecular-grade deionized water directly onto the membrane, followed by centrifugation at 14,000 *g* for 1 min. The yield and purity of the extracted DNA were evaluated using a NanoDrop One spectrophotometer (Thermo Fisher Scientific).

An aliquot of approximately 20 µL from each of the 89 extracted DNA samples was sent to the Parasitic and Molecular Immunology Group laboratory at CIETUS (Faculty of Pharmacy, University of Salamanca) for molecular analysis using the *Schisto*-LAMP assays.

qPCR assay

qPCR assays targeting the *Dra1* tandem repeat sequence [17] were carried out in duplicate. Each 20 µL reaction mixture contained 5 µL of extracted DNA, 10 µL of Quantimix Easy master mix (Biotools, Madrid, Spain), and 1 µL of a PrimeTime® Standard qPCR Assay. The assay provided primers at final concentrations of 500 nM each: forward primer (5′–GATCTCACCTATCAGACGAA–3′) and reverse primer (5′–TCACAACGATACGACCAAC–3′) along with a hydrolysis probe at 300 nM (FAM-TGTTGGTGGAAGTGCCTGTTTCGCAA-IowaBlack Fluorescent Quencher). Extraction controls (positive and negative control samples) and no-template controls were included to support cut-off determination. Amplification was performed in a Rotor-Gene Q real-time thermocycler (Qiagen, Hilden, Germany) using the following program: an initial denaturation step at 95 °C for

2 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s. Samples were considered positive when amplification was detected within the predefined threshold cycle (Ct) range. Samples with discordant amplification results between duplicate reactions were subjected to repeat testing.

Schisto-LAMP assays

LAMP assays were performed using the reaction mixtures and specific primer sets previously described elsewhere for detection of species-specific *S. haematobium*, based on the ribosomal intergenic spacer (IGS) (*Sh*-LAMP) [23], and a genus-specific LAMP assay designed on the internal transcribed spacer 1 (ITS-1) for the simultaneous detection of different species, including *S. mansoni*, *S. haematobium*, *S. intercalatum*, and *S. bovis* (LAMP-G) [24]. The reactions were carried out using previously described conditions, with the exception of the final reaction volume, which was reduced from 25 µL to 15 µL. Briefly, LAMP reaction mixtures (15 µL) contained 40 pmol each of FIP and BIP primers, 5 pmol each of F3 and B3 primers, 0.4 µM of LF primer (LAMP-G only; Table 1), 1.4 mM of each dNTP (Bioron), 1× Isothermal Amplification Buffer -20 mM Tris-HCl (pH 8.8), 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Tween 20 (New England Biolabs Ltd., Ipswich, MA, USA)- supplemented with 6 mM $MgSO_4$ and 8 U of *Bst* 2.0 WarmStart DNA polymerase (New England Biolabs Ltd.) with 2 µL of template. *Schisto*-LAMP reactions were performed in 0.5 mL tubes that were incubated in a heat block at 65 °C for 60 min and then heated at 80 °C for 5–10 min to stop the reaction.

To facilitate sample handling and minimize the risk of cross-contamination, samples were processed in batches of 10–12 samples. Every reaction run included negative controls (non-DNA water control) and positive amplification controls (*S. haematobium* genomic DNA from the CIETUS sample collection).

Detection of LAMP products

LAMP products were visually detected by adding 2 µL of SYBR Green I fluorescent dye (1:10 dilution of a 10,000× stock; Invitrogen®) to each reaction tube post-amplification. Positive reactions exhibited a clear colour change to green, whereas negative controls remained orange. To confirm some of the colorimetric results, LAMP products (3–5 µL) were resolved on 1.5% agarose gels stained with Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH, Dueren, Germany). Successful amplifications were verified by the characteristic ladder-like banding pattern on the gel.

Reference standard calculation

Given the well-known limitations of microscopy as an imperfect reference method, two complementary analytical strategies were used to estimate the diagnostic accuracy of the molecular assays. First, latent class analysis (LCA) was performed using the *randomLCA* package in R v4.4.1 [25]. LCA integrates the results of several diagnostic tests within a probabilistic modelling framework to estimate both disease prevalence and test performance when no perfect reference exists. In this model, *S. haematobium* infection status was treated as an unobserved (latent) binary variable with two categories: "infected" and "non-infected". Sensitivity and specificity for each assay were derived by quantifying the relationship between the latent infection class and the observed outcomes of the diagnostic tests. Participants were assigned to the latent class with the highest posterior probability (i.e., > 0.5). Classification was unambiguous: all posterior probabilities of infection were either < 0.03 or > 0.98, so any threshold within this range would have yielded the same result. Second, we constructed a composite reference standard (CRS) adapted from previously published criteria [26, 27]. Under this approach, participants were

classified as infected if they had (i) a positive microscopy result; or (ii) a negative microscopy result but at least three positive results among the five remaining index tests (qPCR, *Sh*-LAMP, LAMP-G, microhaematuria and ELISA). Conversely, individuals were considered non-infected when microscopy was negative and fewer than three of these five tests yielded positive results. We used a threshold of three positive results rather than the two concordant results applied in previous schistosomiasis studies [27]. Three of the five index tests are DNA-based assays that detect the same target material and are therefore likely to be positive together (conditional dependence), so with a two-test threshold, any two molecular assays alone could define infection. Under that rule, 79/89 participants (88.8%; 95% CI 80.3–94.5) would have been classified as infected, compared with 34.8% by microscopy. Requiring at least three of the five tests corresponds to a majority rule, a recognised way of combining several imperfect tests [28, 29]. It reduces, but does not eliminate, the influence of the molecular assays on the reference classification.

Statistical analysis

Diagnostic performance metrics, including sensitivity, specificity, likelihood ratios, predictive values and overall accuracy, were calculated for each test using the MedCalc diagnostic test calculator [30]. As implemented in MedCalc, 95% confidence intervals were exact (Clopper–Pearson) for sensitivity, specificity and accuracy, logit-based for predictive values and log-based for likelihood ratios. Prevalence estimates are reported with exact (Clopper–Pearson) 95% confidence intervals. Pairwise associations between the binary (positive/negative) test results were evaluated using Pearson correlation analysis (equivalent to the phi coefficient for binary data) in GraphPad Prism version 10.2 (GraphPad Software, Boston, MA, USA). Correlation coefficients (r) were used to assess the strength and direction of associations between tests. All p-values were generated using two-tailed tests, with statistical significance set at $p < 0.05$.

Use of large language models

During the preparation of this manuscript, the authors used Claude (Anthropic; Claude Sonnet 5.5 and Claude Opus 5.5) for the purposes of English language editing, revising the wording of the Methods and Discussion, and independently verifying the diagnostic accuracy and correlation calculations. The authors have reviewed and edited the output and take full responsibility for the content of this publication.

Results

Participant flow and study sample

Of the 208 children enrolled in the primary study, 89 (42.8%) had residual urine DNA extracts available for LAMP testing and were included in the diagnostic accuracy analysis (Figure 1).

Diagnostic performance of qPCR and LAMP assays using microscopy as the reference standard

Among the 89 participants analysed, 31/89 (34.8%; 95% CI 25.0–45.7) were positive by microscopy, whereas 58/89 (65.2%; 95% CI 54.3–75.0) showed no detectable eggs in urine (Additional file 1: Table S1). Diagnostic performance parameters (including sensitivity, specificity, positive and negative likelihood ratios (PLR and NLR, respectively), positive and negative predictive values (PPV and NPV, respectively), and overall accuracy) were calculated using microscopy as the reference method (Table 2). The qPCR assay showed the highest sensitivity (100%; 95% CI [88.8–100.0]), followed by microhaematuria (93.5%; 95% CI [78.6–99.2]), the species-specific *Sh*-LAMP assay (87.1%; 95% CI [70.2–96.4]) and LAMP-G (74.2%; 95% CI [55.4–88.1]). Conversely, specificity was low (<50%) across all molecular tests, whereas

microhaematuria exhibited high specificity (93.1%; 95% CI [83.3–98.1]). PLR values ranged from 1.3 to 1.5 for the three molecular assays. NLR was zero for qPCR, reflecting a high rule-out capacity, whereas Sh-LAMP and LAMP-G showed NLRs of 0.30 and 0.60, respectively. PPV ranged from 41.1% to 45.0% for qPCR, *Schisto*-LAMP assays, and ELISA, whereas microhaematuria yielded a substantially higher PPV of 87.9% (95% CI [73.7–94.9]). NPV reached 100% (95% CI [79.4–100]) for qPCR and exceeded 75% for the remaining molecular assays. Among the molecular assays, overall accuracy was highest for the *Sh*-LAMP assay at 58.4% (95% CI [47.5–68.8]), whereas microhaematuria reached 93.3% (95% CI [85.9–97.5]).

Diagnostic performance of qPCR and LAMP assays using LCA and CRS as reference standards

For the LCA, we estimated each participant's probability of belonging to the latent "infected" or "non-infected" group based on the six diagnostic tests included in the dataset. Several latent class models specifying different numbers of classes were explored, and model fit was evaluated using the Bayesian Information Criterion (BIC). The two-class model without random effects demonstrated the optimal balance between goodness-of-fit and parsimony, yielding the lowest BIC value (619.84). Consequently, this two-class solution was selected to estimate posterior class-membership probabilities for each diagnostic pattern and categorize participants into latent "infected" and "non-infected" groups.

Based on the LCA model, 35/89 participants (39.3%; 95% CI 29.1–50.3) were classified as positive for urogenital schistosomiasis (Additional file 1: Table S1). Among the evaluated assays, qPCR demonstrated the highest sensitivity (100%; 95% CI [90.0–100]), followed by microhaematuria (94.3%; 95% CI [80.8–99.3]), microscopy (88.6%; 95% CI [73.3–96.8]), *Sh*-LAMP assay (85.7%; 95% CI [69.7–95.2]), and LAMP-G assay (74.3%; 95% CI [56.7–87.5])

(Table 3). Specificity reached 100% (95% CI [93.4–100.0]) for both microscopy and microhaematuria, whereas molecular tests showed lower specificity values, ranging from 29.6% to 44.4%. The species-specific *Sh*-LAMP assay yielded the highest PLR (1.54; 95% CI [1.17–2.03]), while qPCR showed the lowest NLR. PPV values for the molecular tests were comparable, ranging from 46.4% to 50.0%. NPV was highest for qPCR (100%; 95% CI [79.4–100]), followed by microhaematuria (96.4%; 95% CI [87.5–99.0]) and microscopy (93.1%; 95% CI [84.3–97.1]).

To complement the microscopy-based and LCA analyses, a CRS was used to categorize infection status. Based on the CRS criteria, 55/89 participants (61.8%; 95% CI 50.9–71.9) were classified as infected and 34/89 (38.2%; 95% CI 28.1–49.1) as non-infected (Additional file 1: Table S1). Evaluated against the CRS, qPCR demonstrated the highest sensitivity (96.4%; 95% CI [87.5–99.6]), followed by *Sh*-LAMP (87.3%; 95% CI [75.5–94.7]) and LAMP-G (78.2%; 95% CI [65.0–88.2]) (Table 4). Conversely, microscopy and microhaematuria showed markedly lower sensitivities (56.4% and 60.0%, respectively), although both achieved 100% specificity (95% CI [89.7–100.0]). Specificity among molecular assays ranged from 41.2% (qPCR) to 64.7% (Sh-LAMP), while ELISA reached 67.6%. The *Sh*-LAMP assay yielded the highest PLR (2.47; 95% CI [1.55–3.94]), whereas qPCR provided the lowest NLR. PPV was highest for microscopy and microhaematuria (both 100%), while molecular assays showed PPVs ranging from 72.6% to 80.0%. In contrast, qPCR achieved the highest NPV (87.5%; 95% CI [62.9–96.7]), followed by the *Schisto*-LAMP assays. Overall diagnostic accuracy was highest for the Sh-LAMP assay (78.7%; 95% CI [68.7–86.6]), followed by qPCR and microhaematuria, which both showed identical accuracy of 75.3% (95% CI [65.0–83.8]).

Correlation among diagnostic methods

Correlation analysis revealed variable levels of agreement among the evaluated diagnostic tests (Figure 2). Microscopy showed a strong positive correlation with microhaematuria (r = 0.85; $p < 0.001$). Weak-to-moderate positive correlations were observed between microscopy and qPCR (r = 0.34; $p = 0.001$), as well as between microscopy and the *Sh*-LAMP assay (r = 0.31; $p = 0.003$).

qPCR demonstrated a moderate positive correlation with microhaematuria (r = 0.36; $p < 0.001$) and a weak positive correlation with ELISA (r = 0.24; $p < 0.05$). In contrast, both *Schisto*-LAMP assays (*Sh*-LAMP and LAMP-G) generally showed low correlation with the other diagnostic methods; only *Sh*-LAMP showed a weak but significant association with microhaematuria (r = 0.29; p = 0.007), whereas the association between LAMP-G and microhaematuria was not significant (r = 0.16; p = 0.14). Specifically, LAMP-G showed minimal non-significant correlation with qPCR (r = 0.06) and microscopy (r = 0.17), indicating limited concordance. Finally, apart from its association with qPCR, ELISA displayed weak, non-significant correlations with the other tests, including inverse, negligible relationships with *Sh*-LAMP (r = –0.02) and LAMP-G (r = –0.06).

Discussion

Given the public health burden of schistosomiasis across 78 endemic countries, the WHO roadmap for NTDs highlights the need for sensitive and specific point-of-care diagnostics adaptable to diverse epidemiological settings [9]. To address this priority, we compared the diagnostic performance of three molecular assays (qPCR, *Sh*-LAMP, and LAMP-G) against microscopy for detecting urogenital schistosomiasis in a Zambian school-aged children cohort, alongside microhaematuria and ELISA. To account for the recognized limitations of microscopy as an imperfect reference standard, two independent statistical modelling approaches (LCA and CRS) were applied.

Across all analytical frameworks, qPCR consistently achieved the highest sensitivity, confirming its diagnostic value for ruling out infection in low-parasite-burden or subclinical cases. However, qPCR also exhibited reduced specificity (a pattern shared, to a lesser extent, by both LAMP assays). In contrast, microscopy and microhaematuria maintained high specificity across all frameworks but showed markedly reduced sensitivity, particularly against the CRS (56.4% and 60.0%, respectively). This apparent reduction in molecular specificity is unlikely to reflect poor analytical performance *per se*, but rather represents an inherent limitation of using microscopy, or microscopy-anchored composite standards, as the comparator benchmark. Because Dra1-based qPCR targets a highly repetitive genomic sequence [16], it can detect parasite burdens too low to produce eggs visible by urine filtration, as well as residual or degraded circulating DNA following successful treatment or during early, pre-patent infections. Indeed, an ITS-based real-time PCR has been shown to remain positive in Kenyan children up to 18 months post-praziquantel treatment at time points when microscopy had reverted to negative [31]. This supports the interpretation that many qPCR-positive/microscopy-negative discordant results in our study may represent subclinical or residual infections rather than false-positive errors. Consistent with this, our qPCR sensitivity estimates align with previous reports describing near-complete sensitivity of Dra1-based qPCR in Zanzibari urine samples across varying infection intensities [15] as well as comparable performance across three qPCR protocols in imported-infection settings [32]. The lower specificity observed here compared to those series likely stems from the higher background transmission intensity in our Zambian study population, where a greater proportion of subclinical or residual infections would inflate the apparent false-positive rate when measured against an egg-based reference. Finally, environmental DNA contamination of urine samples or, for LAMP assays, amplicon carry-over contamination [18, 19], may further contribute to apparent false positives that actually represent true infections missed by microscopy.

Microhaematuria, meanwhile, represents a downstream clinical consequence of tissue pathology induced by egg deposition. Its high specificity relative to microscopy thus carries an inherent circularity, as both indicators are tightly coupled to established, patent infections rather than early or low-intensity stages [33, 34]. By contrast, the ELISA assay detects host IgG antibodies directed against the recombinant *Sh*-TSP-2 antigen, reflecting cumulative exposure rather than active infection. Because these antibodies persist for months or even years following spontaneous clearance or praziquantel treatment, serological signals align imperfectly with egg- or DNA-based definitions of current infection. This immunological persistence explains the modest and inconsistent performance of ELISA across all three reference standards in our studied cohort.

Regarding the isothermal amplification methods, the species-specific *Sh*-LAMP assay was more sensitive than the LAMP-G assay across all evaluation frameworks, in line with previous evaluations of species-specific LAMP targets [14]. However, their specificities were identical against microscopy and LCA (43.1% and 44.4%, respectively) and differed only marginally against the CRS (64.7% vs 61.8%), indicating that the advantage of *Sh*-LAMP lies in sensitivity rather than specificity. Our *Sh*-LAMP results are broadly consistent with a recent meta-analysis of point-of-care tests for *S. haematobium* in Africa, which reported pooled LAMP sensitivity and specificity of 94.7% and 66.4%, respectively [35]. That review identified the choice of reference standard as a major source of variation in reported specificity, an effect clearly demonstrated in our study, where *Sh*-LAMP specificity varied from 43.1% (versus microscopy) to 64.7% (versus CRS). This highlights that diagnostic accuracy estimates must be evaluated strictly relative to the reference benchmark used.

A notable feature of our analysis was the substantial divergence in estimated infection prevalence between the two reference frameworks: 35 participants (39.3%; 95% CI 29.1–50.3) were classified as infected by LCA, compared to 55 (61.8%; 95% CI 50.9–71.9) under the CRS.

Such discrepancies are expected, as the two approaches rely on fundamentally different mathematical and biological assumptions. Similar divergences between LCA and CRS have been reported for other infectious diseases lacking a gold standard, such as tuberculosis [36], although not universally (e.g., some schistosomiasis studies applying both methods reported close agreement [27]). Indeed, the CRS combines multiple diagnostic assays to overcome the sensitivity limitations of microscopy. However, three of the five index tests in our CRS are DNA-based assays that may share conditional dependence and tend to be positive together, so a CRS built from them is prone to inflating estimated prevalence [26]. For this reason, we required at least three of five positive results rather than two; with two, 88.8% of participants would have been classified as infected. Even under this stricter rule, eight microscopy-negative participants were classified as infected on the basis of the three molecular assays alone, so the CRS prevalence of 61.8% is probably still an overestimate. By contrast, LCA estimates test accuracy and latent infection status without predefining a rule for case confirmation [25, 27]. This divergence highlights uncertainty relevant to schistosomiasis surveillance and programme evaluation. In representative surveys, differences arising from the diagnostic framework may affect treatment decisions, surveillance strategies and assessments of progress towards the WHO 2030 elimination targets [9]. Diagnostic test selection and interpretation should therefore reflect programme objectives, epidemiological context and the diagnostic method underpinning the applicable treatment thresholds.

Taken together, the correlation profiles reflect the biological diversity of the evaluated diagnostic markers, supporting a multi-assay diagnostic approach for *S. haematobium*. Microscopy correlated strongly with microhaematuria ($r = 0.85$), confirming the expected association between egg excretion and urinary morbidity. qPCR displayed moderate concordance with microhaematuria ($r = 0.36$) and a weak association with ELISA ($r = 0.24$), demonstrating partial overlap between molecular burden, serology, and clinical pathology.

In contrast, the lack of correlation between LAMP-G and all other diagnostic modalities (including qPCR) suggests technical rather than biological explanations, such as primer cross-reactivity, amplification artifacts, or reduced analytical sensitivity inherent to genus-level primer sets [18]. Amplicon sequencing in future work will help elucidate these discordant results. Lastly, the weak correlations observed for ELISA stem from its target compartment (systemic IgG response rather than excreted parasite products) and the known long-term persistence of antibodies after parasite clearance [37].

Several limitations should be considered when interpreting these findings. First, only a single urine sample was collected per participant, precluding assessment of the well-documented day-to-day variability in egg excretion [11]. This single-sample approach likely underestimated the true diagnostic sensitivity of microscopy and may partly explain its poor performance when evaluated against the CRS; repeated sampling over consecutive days, as previously recommended [31, 38], would provide a more robust microscopy benchmark. Second, confirmatory sequencing of LAMP amplicons was not performed; consequently, non-specific amplification cannot be fully ruled out as a potential contributor to the lower specificity observed for both LAMP assays. Third, the CRS incorporates the index tests under evaluation, which can inflate their apparent accuracy (incorporation bias); for instance, when the CRS was rebuilt without *Sh*-LAMP (requiring three of the four remaining tests), *Sh*-LAMP specificity fell from 64.7% to 46.8%. Moreover, because three of the five tests contributing to the CRS are molecular, the CRS is weighted towards DNA-based detection, which favours the molecular assays when they are evaluated against it. Likewise, the LCA assumes conditional independence between tests, which is unlikely to hold fully for the DNA-based assays or for microscopy and microhaematuria. Fourth, although our sample size (n = 89) was sufficient to demonstrate clear differences in diagnostic performance, it constrained the precision of certain metrics, as reflected in the relatively wide confidence intervals for specificity

and predictive values. Fifth, participants were recruited from two districts in Lusaka Province, Zambia; therefore, the generalisability of these findings to other transmission settings, broader geographical regions, or specific age cohorts requires independent confirmation. Furthermore, the analysed samples represented a subset of a larger parent cohort rather than a population specifically recruited for the present diagnostic accuracy study. Consequently, selection bias cannot be completely excluded, and the findings should be interpreted within the context of the sampling strategy employed. Finally, while LCA and CRS both overcome the constraints of relying solely on microscopy, neither represents an absolute gold standard, and residual misclassification cannot be entirely ruled out.

Conclusions

Our results show that diagnostic assay choice depends on the epidemiological goal and the desired balance between sensitivity and specificity. qPCR remained the most sensitive assay across all analytical frameworks and appears particularly useful for excluding infection because of its consistently high sensitivity and negative predictive value. Meanwhile, the *Sh*-LAMP assay provides a balanced diagnostic profile and low infrastructure requirements, positioning it as a promising point-of-care candidate for resource-limited field settings [35], in direct alignment with the WHO 2030 roadmap elimination targets [9]. Future research should prioritize longitudinal evaluations before and after praziquantel administration to assess assay performance relative to parasitological cure, incorporate amplicon sequencing for molecular validation, and extend field testing to larger, geographically diverse cohorts alongside formal cost-effectiveness analyses to inform national elimination strategies.

List of abbreviations

BIC: Bayesian Information Criterion

bp: base pairs

CAA: circulating anodic antigen

CI: confidence interval

CRS: composite reference standard

Ct: threshold cycle

DALYs: disability-adjusted life years

ELISA: enzyme-linked immunosorbent assay

HRP: horseradish peroxidase

IARC: International Agency for Research on Cancer

IGS: intergenic spacer

IMAC: immobilized metal affinity chromatography

IPTG: isopropyl-beta-D-thiogalactopyranoside

ITS-1: internal transcribed spacer 1

LAMP: loop-mediated isothermal amplification

LAMP-G: genus-specific *Schistosoma* LAMP

LCA: latent class analysis

NLR: negative likelihood ratio

NPV: negative predictive value

NTDs: neglected tropical diseases

OD: optical density

PBS: phosphate-buffered saline

PBST: PBS containing 0.05% Tween-20

PCR: polymerase chain reaction

PLR: positive likelihood ratio

PPV: positive predictive value

qPCR: real-time polymerase chain reaction

RT: room temperature

SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Sh-LAMP: species-specific *Schistosoma haematobium* LAMP

Sh-TSP-2: *Schistosoma haematobium* tetraspanin-2

TMB: 3,3′,5,5′-tetramethylbenzidine

WHO: World Health Organization

Supplementary information

Additional file 1: Table S1.xlsx. Infection status of participants according to microscopy, latent class analysis and the composite reference standard.

Declarations

Ethics approval and consent to participate

The study was approved by the Levy Mwanawasa Medical University Research Ethics Committee (LMMU-REC 000054/23), and authorization to carry out the research was granted by the National Health Research Authority (NHRA0001/23/04/2023). Parents or legal guardians of all participating children provided informed consent, and assent was requested from the children themselves when applicable. All procedures were performed according to the 2013 Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MMM and JS conceived the study. MMM and BCV performed the experiments. JM and MMM collected the samples. JS, MMM, PFS and AM prepared the first draft. MJP, DC and NL critically reviewed the manuscript. All authors read and approved the final manuscript.

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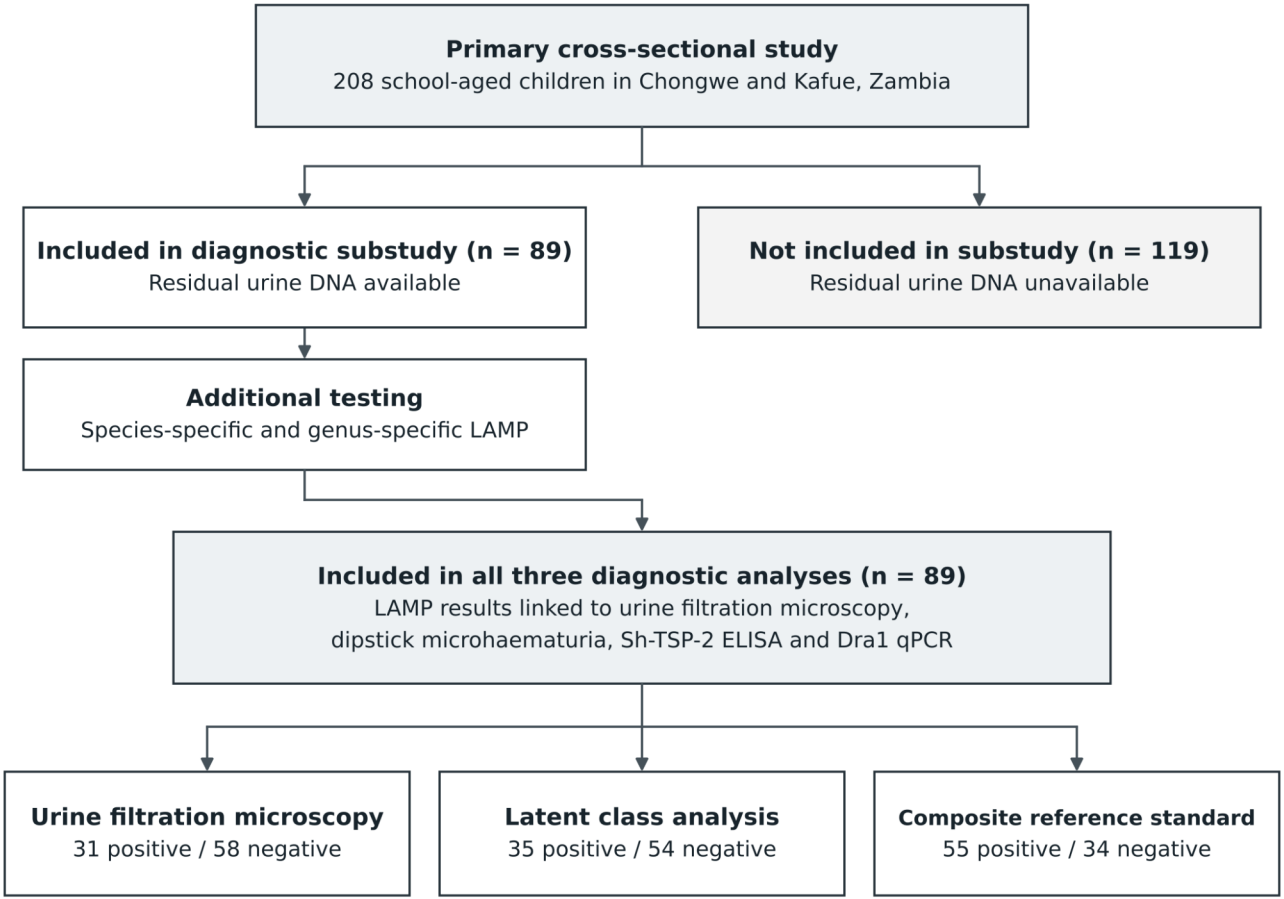
Figure legends

Figure 1. Participant selection and diagnostic classification. Of 208 school-aged children enrolled in the primary study in Chongwe and Kafue districts, Zambia [20], 89 were included because residual urine DNA extract was available; the remaining 119 were excluded because no residual DNA remained. Species-specific (*Sh*-LAMP) and genus-specific (LAMP-G) loop-mediated isothermal amplification results were linked to the urine filtration microscopy, dipstick microhaematuria, *Sh*-TSP-2 enzyme-linked immunosorbent assay (ELISA) and Dra1 quantitative PCR (qPCR) results for the same participants. Microscopy, latent class analysis and the composite reference standard classified 31, 35 and 55 of the 89 participants as positive, respectively.

Figure 2. Pairwise correlations among diagnostic tests for urogenital schistosomiasis. Heatmap of Pearson correlation coefficients (r; equivalent to the phi coefficient for binary results) between qPCR, microscopy, *Sh*-LAMP, LAMP-G, microhaematuria, and ELISA results across all 89 participants. Colour intensity corresponds to the strength and direction of the correlation, from strong positive (dark blue, $r = 1.0$) to strong negative (dark red, $r = -1.0$); the diagonal represents each test's perfect self-correlation. Asterisks denote statistically significant correlations ($p < 0.05$).


Primary cross-sectional study
208 school-aged children in Chongwe and Kafue, Zambia
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Residual urine DNA available
Not included in substudy (n = 119)
Residual urine DNA unavailable
Additional testing
Species-specific and genus-specific LAMP
Included in all three diagnostic analyses (n = 89)
LAMP results linked to urine filtration microscopy,
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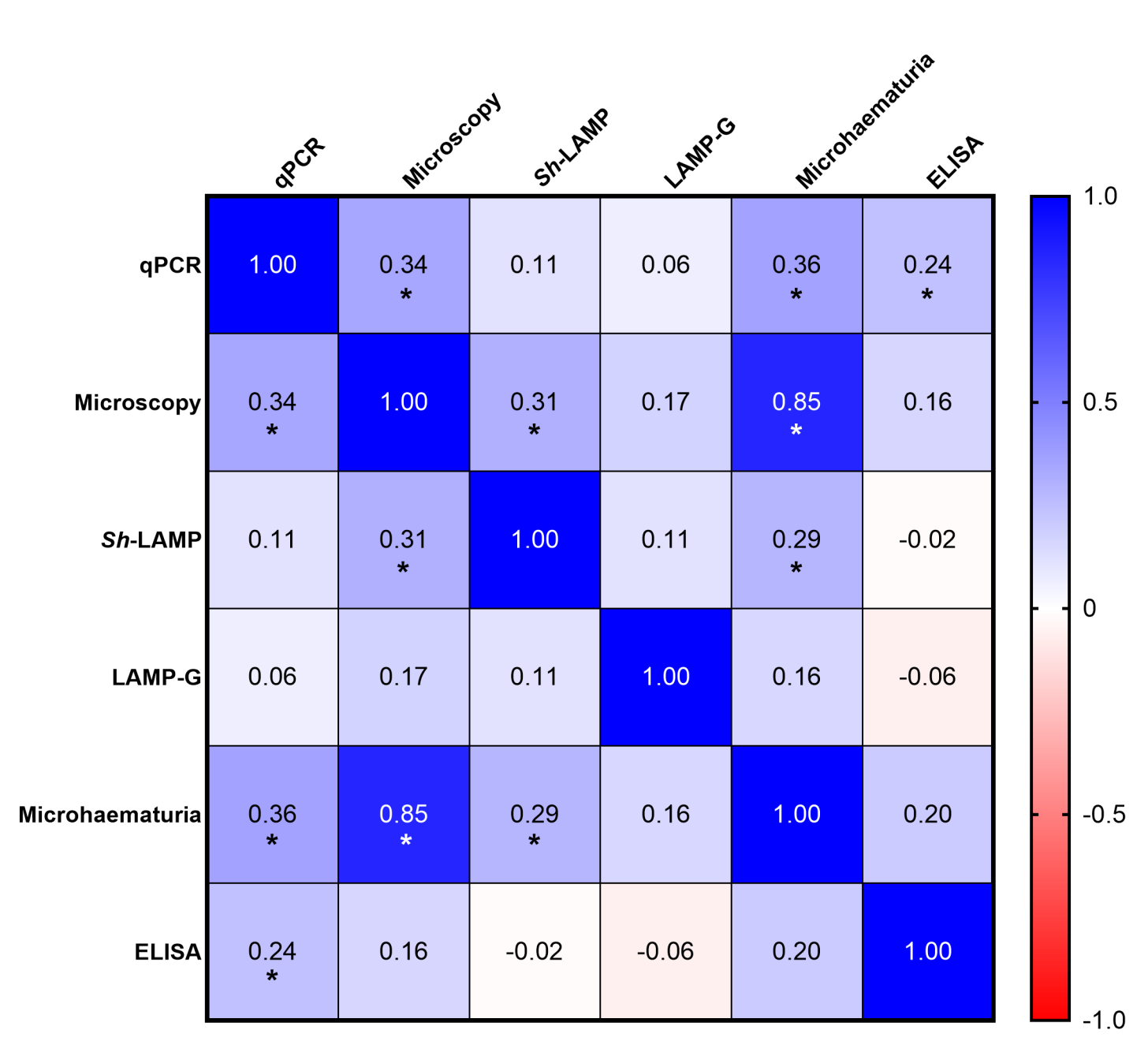
Urine filtration microscopy
31 positive / 58 negative
Latent class analysis
35 positive / 54 negative
Composite reference standard
55 positive / 34 negative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Supplementary Files

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