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Dynamic interactions between *Schistosoma haematobium*, *Schistosoma mattheei* and *Schistosoma mansoni* underscore the complex polyparasitism of intestinal schistosomiasis in southern Malawi

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Schistosomiasis is prevalent among school-aged children (SAC) in Mangochi District, Malawi, where both intestinal and urogenital forms are endemic. In 2024, we identified schistosomiasis cases predominantly associated with excretion of *Schistosoma haematobium* × *Schistosoma mattheei* ova in the faeces of two individuals from Samama village, Mangochi District. In this expanded cross-sectional study, we characterize the prevalence and species composition of *Schistosoma* infections among 247 SAC in Samama, using genus- and species-specific molecular diagnostics. We also present follow-up data from the two previous cases, showing natural mixed-species re-infection six months after treatment. Schistosomiasis prevalence among SAC was 62.3%. *Schistosoma* spp. DNA was detected in 50.6% of faecal samples and *Schistosoma* spp. ova were observed on 34.8% of urine filters. Species-specific assays detected *S. haematobium*, *S. mattheei* and *S. mansoni* DNA in 36.8%, 14.4% and 18.4% of faecal samples from children with intestinal schistosomiasis. Triple-species infections were identified in 10 children by faecal and urine testing. Notably, detection of *S. haematobium* DNA in faeces was strongly associated with *S. mattheei* co-infection ($p = 0.006$), highlighting potential cross-species interactions. Our findings underscore the need to integrate molecular diagnostics alongside routine testing strategies for enhanced surveillance of polyparasitic infections in zoonotic transmission zones across Africa.

This article is part of the Royal Society Science+ meeting issue 'Parasite evolution and impact in action: exploring the importance and control of hybrid schistosomes in Africa and beyond'.

1. Introduction

Schistosomiasis is a prevalent waterborne parasitic disease across much of Malawi, yet transmission foci vary across time and space [1]. While urogenital schistosomiasis has been known to be endemic on the Mangochi District shorelines of Lake Malawi for decades [2,3], local endemicity of intestinal schistosomiasis is more recent. The confirmation of autochthonous transmission among school-aged children (SAC) of *Schistosoma mansoni*, the primary cause of intestinal schistosomiasis in Africa [4], occurred after *Biomphalaria pfeifferi* intermediate snail hosts were first detected on the southern shores of Lake Malawi in 2017 [5]. These snails were later shown to be more widespread and naturally infected with *S. mansoni*, as revealed by targeted malacological surveillance [6–8]. Their appearance was followed by an outbreak and shift to endemicity of *S. mansoni*, alongside increasing prevalence of urogenital schistosomiasis caused by *Schistosoma haematobium* [9]. Using TaqMan probe real-time PCR assays that are not routine in schistosomiasis surveillance, both *S. mansoni* and *S. haematobium* DNA have been detected in faecal samples from Mangochi District, with *S. haematobium* DNA thought to be present in faeces due to the ectopic excretion of *S. haematobium* ova and/or cell-free DNA excretion in patients with urogenital schistosomiasis [10]. However, the TaqMan probe assays were likely confounded as they do not detect zoonotic species within the *S. haematobium* group, principally *Schistosoma mattheei*, a common parasite of local livestock that is also able to infect humans, especially where humans and livestock share water contact points [11].

Prior to 2024 in southern Malawi, zoonotic and hybrid schistosome infections—predominantly associated with *S. mattheei*—have been reported in humans, yet in these reports, the excretion of ova has been noted via the urogenital tract rather than the intestinal tract, as faecal examination was not undertaken routinely [12,13]. In 2024, we reported cases of concurrent zoonotic urogenital and intestinal schistosomiasis from two patients in Samama village, Mangochi District, herein referred to as Patient X (a 10-year-old girl) and Patient Y (a 19-year-old man). In these cases, copious atypical *S. haematobium* × *S. mattheei* ova, as well as typical *S. mattheei* ova, were predominantly excreted in the faeces. These cases showed that *S. haematobium* group species, rather than *S. mansoni*, can be the dominant contributors to *Schistosoma* ova excretion via the intestinal tract, signposting towards a potential broader shift in the landscape of intestinal schistosomiasis in southern Malawi towards multi-species infections. Additionally, they highlighted a possible deficit and diagnostic oversight in the deployment of urine-based rapid diagnostic tests for the monitoring of intestinal schistosomiasis, such as point-of-care circulating cathodic antigen (POC-CCA) urine tests—recommended to diagnose *S. mansoni* in community-wide screening [14]. POC-CCA tests detect antigens from adult *Schistosoma* worms and perform best when used to detect *S. mansoni* infections in endemic settings where the intensity of infections is high [15]. Furthermore, their superior sensitivity against Kato–Katz faecal microscopy in low-to-moderate prevalence areas led to their recommendation for mapping *S. mansoni* prevalence in regions of low-intensity infections [14,15].

For research purposes, to more accurately report the prevalence of intestinal schistosomiasis and identify the species responsible for causing disease, genus- and species-specific molecular DNA diagnostic methods can also be deployed. One recently described high-resolution melt (HRM) real-time PCR assay has enabled the rapid detection of six *Schistosoma* species (*Schistosoma bovis*, *Schistosoma curassoni*, *S. haematobium*, *S. mansoni*, *Schistosoma margrebowiei* and *S. mattheei*), both from samples containing multiple parasite ova (e.g. purified DNA extracted from urine filters), and from individual hatched miracidia, when it is also possible to detect hybridization by observing mixed nuclear DNA (nDNA) inheritance or discrepancies between nDNA and mitochondrial DNA (mtDNA) profiles [16]. Methods such as this also enable the clarification and comprehension of unexpected findings from recent studies, such as the high detection rates (26.2%) of *S. haematobium* DNA in faecal samples from SAC in Mangochi District [10].

Here, we present genus- and species-level molecular diagnostic data from urine and faecal samples provided by 247 SAC in Mangochi District, 2024. In addition, we present follow-up data from Patients X and Y in early 2025, some six months after observed praziquantel treatment for their initial hybrid intestinal and urogenital infections, highlighting putative natural re-infection and haplotype variability among *S. haematobium* × *S. mattheei* hybrids. Our aim was to elucidate species composition and hybridization patterns in intestinal schistosomiasis, thereby informing future diagnostic strategies and control measures in regions of Africa where zoonotic species from the *S. haematobium* group are co-endemic.

2. Methods

(a) Study information

The Hybridization in Urogenital Schistosomiasis (HUGS) longitudinal investigation (2021–2025) was conducted among people aged 2 years and older in two rural villages in southern Malawi (Samama village, Mangochi District [S 14.418767°, E 35.220985°] and Mthawira village, Nsanje District [S 16.849802°, E 35.290041°]), with ethical approvals from The College of Medicine Research Ethics Committee, Malawi (approval no. P.08/21/3381) and the Liverpool School of Tropical Medicine Research Ethics Committee, United Kingdom (approval no. 22-028). Prior to baseline surveys in 2022, 1045 participants from a total of 2485 residents in Samama and 919 participants from a total of 3083 residents in Mthawira were randomly approached and recruited to the study [17]. After each study visit throughout the HUGS study, all participants were provided with a 40 mg kg^{-1} dose of praziquantel by a study clinician, regardless of infectious status. Although the study's primary objectives were achieved via diagnostic procedures performed on urine samples, participants of all ages were able to provide a faecal sample at each annual survey, should they wish. This sporadic faecal sampling was performed to supplement tests performed on urine samples (POC-CCA) to track intestinal schistosomiasis and to enable inspection of *Schistosoma* ova morphology in faeces, although no atypical *Schistosoma* ova were observed in faeces prior to our final surveys, which we report on here.

We performed upscaled cross-sectional faecal sampling among SAC (aged 6–15) participating in the HUGS study in Samama during our final annual follow-up survey in June–July 2024 (figure 1). SAC in Samama were selected for detailed inspection as intestinal schistosomiasis was shown to be prevalent among this group in surveys conducted prior to 2022 [9,10,18]. In June–July 2024, 524 SAC in Samama were followed up within the HUGS study, all of whom were invited to provide a faecal sample alongside a paired urine sample at their study visit. We received faecal samples from 265 SAC, of which 247 later passed internal DNA extraction and amplification control and were included in this analysis.

We also report detailed case data acquired from repeated faecal and urine sampling of Patients X and Y in January 2025, six months after treatment with praziquantel (40 mg kg^{-1}) for mixed hybrid urogenital and intestinal infections. Both patients were participants in the HUGS study and provided initial (baseline) faecal samples during our surveys in June–July 2024, along with paired urine samples. Patient X's baseline samples were provided within the SAC study. Patient Y was not included in the SAC study as he was 19 years old, but opted to provide a faecal sample for analysis within the main study. These two participants were selected for detailed inspection after numerous atypical *S. haematobium* group ova were observed in their faecal samples by microscopy, as we recently reported [19].

(b) Sample handling and DNA extraction

(i) Urine samples

In the field laboratory, prior to urine filtration, POC-CCA cassette tests (ICT International; batch no. 230515042) were used according to manufacturer's instructions for the rapid diagnosis of *S. mansoni*. Faint visual positive results were recorded as 'trace'. Any POC-CCA tests that did not present a visible control line were repeated, while 10% of tests with a visible control line were repeated for quality control. For the detection of ova-patent urogenital schistosomiasis, 10 ml of well-mixed urine was filtered by syringe across a circular nylon mesh of 1.5 cm diameter, with 20 µm pore size (Plastok [Meshes and Filtration] Ltd.). Filters were then examined by microscopy to count *Schistosoma* ova and categorize urogenital schistosomiasis infection intensity: negative, 1–9 ova per 10 ml, 10–49 ova per 10 ml and ≥50 ova per 10 ml. Microscopy-positive urine filters (on which *S. haematobium* ova were visible, occasionally alongside atypical *Schistosoma* ova) were preserved in 1 µl 100% ethanol for transport to the Liverpool School of Tropical Medicine, UK, at ambient temperature. Ethanol was evaporated from urine filters by overnight incubation at 60°C. Following this, DNA extractions were performed using the DNeasy Blood and Tissue Kit (QIAGEN), with the addition of 0.5 µl of PhHV-1 positive extraction control DNA and 0.5 g of 1.4 mm ceramic beads alongside the ATL buffer. The samples then underwent bead-beating treatment for 30 s at 3000 rpm using the MagnaLyser tissue lysis platform (Roche). Following these initial steps, DNA extraction followed the manufacturer's recommended protocol provided.

(ii) Faecal samples

Kato–Katz smears were only prepared for faecal samples of key interest following triage in the field (from POC-CCA-positive participants, 'trace' excluded), to inspect *Schistosoma* ova morphology [20]. Faecal samples from these participants were individually filtered across a 212 µm metal mesh, then applied to slides using 41.7 mg templates in duplicate. Each slide, containing both smears, was read by two independent microscopists. If *Schistosoma* ova were present, the number of ova observed across both smears was multiplied by 12 to calculate the approximate number of ova per gram of faeces. We did not use Kato–Katz faecal microscopy as a diagnostic method across the cohort due to the low sensitivity of this method in Mangochi District, a low-intensity infection area [10]. Instead, 0.2 g from the inner contents of each whole faecal sample was stored in 1.6 ml of DNA/RNA Shield™ (Zymo Research) and transported at ambient temperature to the Liverpool School of Tropical Medicine, UK, for molecular diagnostic procedures. Following centrifugation and removal of the supernatant, samples were washed in sterile phosphate-buffered saline (PBS). 1 µl of phocine herpes virus 1 (PhHV-1) was added to each sample after the PBS wash step to act as an extraction control. DNA was extracted with the QIAamp PowerFecal Pro DNA Kit (QIAGEN). As per the manufacturer's advice, we adapted the protocol to include an additional 10 minute incubation step at 65°C after the addition of lysis buffer (solution CD1), to optimize lysis of parasite ova. Samples then proceeded to bead-beating and purification as per the manufacturer's routine protocol.

(iii) Miracidial hatching (faeces and urine)

Hatching individual miracidia allows for detailed characterization of hybridization and genetic diversity within parasite populations [19,21,22]. We recently described the collection of miracidia and the extraction of their DNA after obtaining paired faecal and urine samples from Patients X and Y during the 2024 HUGS surveys [19]. We repeated paired sample collection in January 2025 (six months post-treatment) and performed the same standard protocols, storing individual hatched miracidia on Whatman FTA cards (GE Healthcare Life Sciences) following Pitchford–Visser filtration [23], and isolating DNA using a standard alkaline extraction method [24].

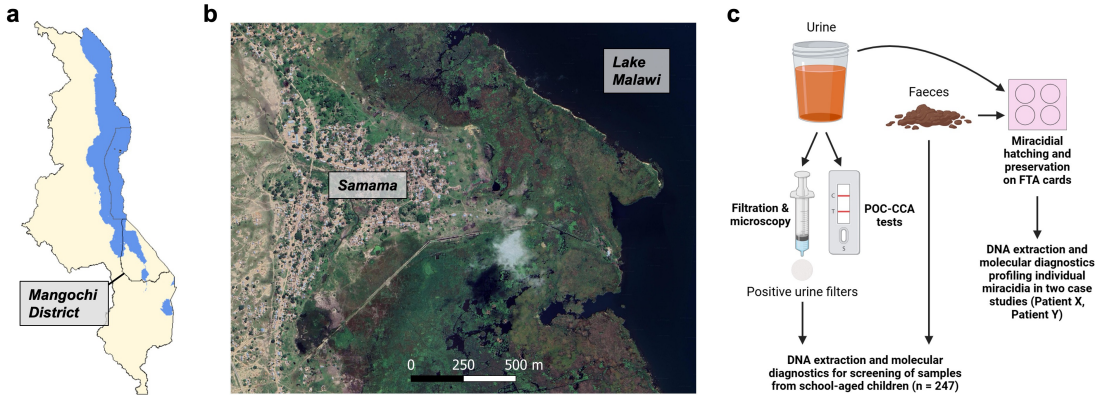

Figure 1. Sampling in Samama village, Mangochi District, Malawi for investigations into polyparasitism in intestinal schistosomiasis: (a) Map of Malawi showing the location of Mangochi District on the southern shores of Lake Malawi; (b) Satellite image (Google) showing the proximity of households in Samama village to Lake Malawi; (c) Sample collection and processing. Diagnostic data acquired from 247 SAC are presented, alongside follow-up case studies from two patients (Patient X, a 10-year-old girl, included in the SAC study; Patient Y, a 19-year-old man, not included in the SAC study). POC-CCA, point-of-care circulating cathodic antigen.

(c) Molecular diagnostics

(i) Faecal and urine samples

All DNA samples extracted from faeces and urine filters were initially screened with a duplex TaqMan probe real-time PCR assay to amplify and detect a *Schistosoma* genus-specific region of the nDNA ribosomal internal transcribed spacer 2 (ITS2) locus, alongside a locus of the PhHV-1 glycoprotein B gene, which acts as an extraction and PCR internal control [10,25]. Primer/probe sequences, reaction mix and PCR conditions are described in the electronic supplementary material, table S2a(i,ii,iii). All real-time PCR reaction sets included a positive control (DNA extracted from an adult *S. haematobium* worm from the collection at the Liverpool School of Tropical Medicine), a PhHV-1-only control (in which no faecal DNA was included in the extraction) and a template-free negative control (nuclease-free water). An ITS2 cycle threshold (Ct) value of ≤37 was considered positive for the presence of *Schistosoma* spp. DNA samples from which PhHV-1 DNA could not be detected were re-screened. When PhHV-1 DNA was not detected after re-screening (18/265 faecal samples), data from the corresponding participants were excluded. Therefore, data from 247 participants were included.

Faecal DNA samples testing positive for *Schistosoma* spp. with the genus-specific TaqMan probe real-time PCR assay underwent further purification using the Monarch PCR & DNA Cleanup Kit (New England Biolabs, USA), following the manufacturer's instructions. This cleanup step was included to ensure the removal of residual salts and other contaminants that are common in faeces and could interfere with the accuracy of subsequent HRM real-time PCR assays for species differentiation (electronic supplementary material, figure S1). Samples were then tested with an HRM real-time PCR assay that targets the mtDNA loci of six *Schistosoma* species: *S. bovis*, *S. curassoni*, *S. haematobium*, *S. mansoni*, *S. margrebowiei* and *S. mattheei* [16]. Primer sequences, reaction mix and PCR conditions are described in the electronic supplementary material, table S2b(i,ii,iii). This assay has comparable sensitivity to current species-specific (*S. mansoni*/*S. haematobium*) TaqMan probe real-time PCR assays, with the benefit of being able to detect a wider range of species, although species-specific assays lack sensitivity compared to the genus-specific TaqMan probe ITS2 real-time PCR assay employed initially [10,16]. DNA samples extracted from adult *S. bovis*, *S. curassoni*, *S. haematobium*, *S. mansoni*, *S. margrebowiei* and *S. mattheei* worms from the collection at the Liverpool School of Tropical Medicine were included in each reaction set as positive controls, as well as a template-free negative control (nuclease-free water). Because this HRM assay, to our knowledge, has not previously been used to screen faecal DNA, we also tested faecal extracts with a duplex TaqMan probe real-time PCR assay targeting *S. mansoni* and *S. haematobium* mitochondrial 16S rDNA loci [26], for comparison. Primer/probe sequences, reaction mix and PCR conditions are described in the electronic supplementary material, table S2c(i,ii,iii). Urine-filter DNA extracts were also tested with the mtDNA HRM real-time PCR assay to identify evidence of species other than *S. haematobium* associated with urogenital schistosomiasis. As *S. haematobium* ova had been identified morphologically on all ova-positive urine filters, the *S. haematobium* primer set was omitted from these reactions.

(ii) Typing individual miracidia

Upon DNA isolated from individual FTA-preserved miracidia hatched from the faeces and urine of Patients X and Y at six-month post-treatment follow-up, the above-described HRM real-time PCR assay targeting species-specific mtDNA loci of *S. bovis*, *S. curassoni*, *S. haematobium*, *S. mansoni*, *S. margrebowiei* and *S. mattheei* was used alongside a second HRM real-time PCR assay targeting the nDNA ITS2 locus [16]. Primer sequences, reaction mix and PCR conditions are described in the electronic supplementary material, table S2d(i,ii,iii). We identified hybrid miracidia by observing mixed nDNA signal (i.e. multiple peaks in the melt profiles of ITS2 PCR products) and/or discordance between the nDNA and mtDNA profiles, as was also performed on baseline samples from these patients in our initial case reports [19]. To investigate haplotype variety in hybrid miracidia typed at baseline and six-month follow-up, we performed PCR amplification of mitochondrial partial cytochrome c oxidase I (*COX1*) genes [27]. Primer sequences, reaction mix and PCR conditions are described in the electronic supplementary material,

table S2e(i,ii,iii). PCR amplicons were sequenced in the forward direction (Genewiz/AZENTA). The resulting sequences were trimmed to remove leading and trailing bases with quality scores <20. To differentiate between haplotypes within each species, partial *COX1* sequences were aligned using MAFFT (v. 7.525) to identify sites of single nucleotide polymorphisms (SNPs). Partial *COX1* sequence variants were compared to sequences in the GenBank nucleotide database and from HUGS livestock and malacological surveys [28] using the nucleotide BLAST web tool.

(d) Statistical analyses

Statistical analyses were performed in R (v. 4.4.3). Data visualizations were performed using the R/CRAN *ggplot2* (v. 3.5.1) and *UpSetR* (v. 1.4.0) packages. All prevalence estimates generated from molecular diagnostic tests were calculated as proportions with 95% confidence intervals using the Wilson score method with continuity correction, implemented via R's 'prop.test' function. Sensitivity, specificity, positive predictive and negative predictive values of POC-CCA tests compared to molecular diagnostics were calculated using the R/CRAN *epiR* (v. 2.0.83) package. Upon data from faecal samples testing positive for *Schistosoma* DNA using the genus-specific *Schistosoma* spp. ITS2 real-time PCR, a Wilcoxon rank-sum test was used to compare ITS2 real-time PCR Ct values between samples with species-specific signal detected by mtDNA HRM real-time PCR and samples from which no species-specific mtDNA loci could be amplified and detected [29].

We conducted logistic regression to investigate the detection of *S. haematobium* DNA in faeces among all participants infected with *S. haematobium* (detected in the urine and/or the faeces). By restricting the analysis to *S. haematobium*-infected individuals, the model examined (co-)infection factors that might influence migration of *S. haematobium* worms to the mesenteries after infection. The binary outcome variable was the presence or absence of *S. haematobium* DNA in faeces. *Schistosoma mattheei* co-infection, *S. mansoni* co-infection and urogenital schistosomiasis infection intensity were included as explanatory variables, adjusting for participant age and sex. Household clustering was not accounted for, as the model investigates within-host parasite migration in infected individuals rather than factors associated with infection risk. All categorical predictors were treated as factor variables. Age was included as a continuous covariate. Odds ratios (ORs) and 95% confidence intervals (CIs) for explanatory variables were calculated using the 'glm' function, specifying a binomial family with a logit link. Statistical significance was defined as $p < 0.05$. We checked for multicollinearity using the 'vif' function in the R/CRAN *car* (v. 3.1.3) package. Model diagnostic plots were generated using the base R 'plot' function to assess residuals and leverage statistics. Generalized variance inflation factor (VIF) values indicated no concerning multicollinearity, no highly influential points were identified, and residuals demonstrated consistency with model assumptions (see data and code package).

3. Results

(a) Prevalence and aetiology of schistosomiasis among school-aged children

The mean age of the 247 SAC from whom urine and faecal samples were analysed was 10.0 (range = 6–15). Of these children, 124 were male and 123 were female. We determined the overall prevalence of schistosomiasis to be 62.3% (95% CI = 56.0–68.4%) by detecting *Schistosoma* spp. DNA either in participants' faeces or on urine filters from participants with ova-patent urogenital schistosomiasis. Of the 154 *Schistosoma*-infected children, 68 had intestinal schistosomiasis only, 29 had urogenital schistosomiasis only and 57 had mixed intestinal and urogenital infections. Among all SAC, the prevalence of intestinal and urogenital schistosomiasis was 50.6% (125/247) [95% CI = 44.2–57.0] and 34.8% (86/247) [95% CI = 29.0–41.2], respectively. *Schistosoma* infection was undetectable in faeces and urine in 93/247 participants by our methods.

From faecal samples, we detected *S. haematobium*, *S. mattheei* and *S. mansoni* DNA using the mtDNA HRM real-time PCR assay. We did not detect *S. bovis*, *S. curassoni* or *S. margrebowiei* DNA from any samples. *Schistosoma mattheei* DNA was detected at similar rates in faeces and urine among the cohort of SAC (7.3 and 6.9%, respectively), and was detectable in both the urine and faeces of 5/30 participants with *S. mattheei* infections (table 1). Genus-specific *Schistosoma* spp. ITS2 real-time PCR Ct values were significantly lower, indicating higher-intensity intestinal infection, in samples where we could achieve species-level identification by mtDNA HRM real-time PCR ($p < 0.001$) (figure 2a). From *Schistosoma*-positive faecal samples, *S. haematobium*, *S. mattheei* and *S. mansoni* mtDNA was detected in 36.8% (46/125), 14.4% (18/125) and 18.4% (23/125), respectively. Faecal mtDNA HRM real-time PCR results for *S. haematobium* and *S. mansoni* were comparable to the duplex species-specific TaqMan probe real-time PCR assay targeting *S. mansoni* and *S. haematobium* mitochondrial 16S rDNA loci [26] that has previously been used to screen DNA extracted from faecal samples for these two species alone [10]. All *S. haematobium* results were the same by both assays. The only discrepancy noted was one positive *S. mansoni* result by HRM real-time PCR that was not detected by the TaqMan probe assay.

Either by morphological identification of *S. haematobium* ova on urine filters, or by detection of *S. haematobium*, *S. mansoni* or *S. mattheei* mtDNA in DNA extracts, we were able to identify a *Schistosoma* species in 77.3% (119/154) of participants with schistosomiasis, including in all 86 ova-patent urogenital infections by microscopy (*S. haematobium*) ± molecular DNA detection of other *Schistosoma* spp., and in 52.8% (66/125) of intestinal infections by molecular DNA detection. When combining faecal and urinary diagnostic data, we detected triple-species infections between *S. haematobium*, *S. mattheei* and *S. mansoni* in 10 participants (figure 3). Among participants with intestinal schistosomiasis from which a species-specific signal was obtained, the species combinations identified specifically in the faeces were: *S. haematobium* only = 29/66, *S. mattheei* only = 8/66, *S. mansoni* only = 11/66, *S. haematobium* and *S. mattheei* = 6/66, *S. haematobium* and *S. mansoni* = 8/66, *S. mattheei* and *S. mansoni* = 1/66, *S. haematobium*, *S. mattheei* and *S. mansoni* = 3/66.

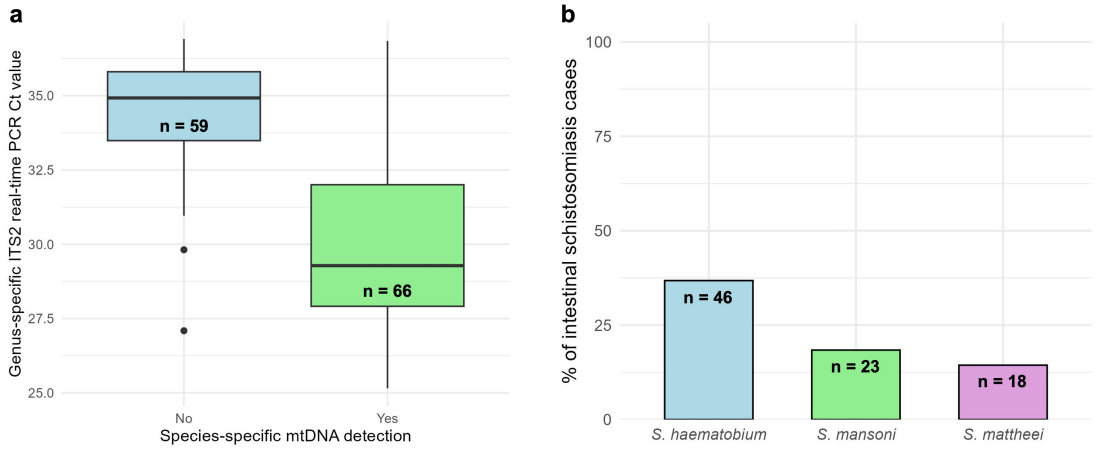

Figure 2. Molecular identification of *Schistosoma* species DNA in intestinal schistosomiasis. (a) Box plot of genus-specific *Schistosoma* spp. ITS2 real-time PCR Ct values plotted for cases with and without species-specific mtDNA detection using HRM real-time PCR; (b) percentage of intestinal schistosomiasis cases in which species-level identification was achieved by mtDNA HRM real-time PCR, stratified by *S. haematobium*, *S. mansoni* and *S. mattheei*. Lower Ct values indicate higher parasite DNA concentrations. Percentages in (b) are calculated as a proportion of *Schistosoma* spp. PCR-positive faecal samples. Ct = cycle threshold.

Table 1. Prevalence of intestinal and urogenital schistosomiasis among school-aged children. Genus-level diagnostic results were obtained by *Schistosoma* spp. ITS2 real-time PCR; species-level detection was performed with mtDNA HRM real-time PCR. 95% CI = 95% confidence interval.

sample ($n = 247$)	*Schistosoma* spp. ITS2 real-time PCR		mtDNA HRM real-time PCR					
			S. haematobium		*S. mansoni*		*S. mattheei*	
	count	prevalence (95% CI)	count	prevalence (95% CI)	count	prevalence (95% CI)	count	prevalence (95% CI)
faeces	125	50.6% (44.2–57.0)	46	18.6% (14.1–24.2)	23	9.3% (6.1–13.8)	18	7.3% (4.5–11.5)
urine	86	34.8% (29.0–41.2)	86	34.8% (29.0–41.2)	15	6.1% (3.6–10.0)	17	6.9% (4.2–11.0)
TOTAL (urine and/or faeces)	154	62.3% (56.0–64.8)	106	42.9% (36.7–49.4)	34	13.8% (9.8–18.8)	30	12.1% (8.5–17.0)

(b) Point-of-care circulating cathodic antigen tests lack sensitivity when *S. mansoni* is not the dominant cause of intestinal schistosomiasis

Compared to the 50.6% (125/247) of faecal samples from SAC testing positive for *Schistosoma* spp. DNA by the genus-specific *Schistosoma* spp. ITS2 real-time PCR assay, the percentage of urine samples testing positive by POC-CCA was markedly lower: 15.0% (37/247) if 'trace' results were considered positive, or 6.9% (17/247) if 'trace' results were considered negative. *Schistosoma* ova were observed by microscopy in duplicate Kato–Katz smears performed on samples provided by 3/17 SAC with positive POC-CCA tests: one sample contained lateral-spined *S. mansoni* ova (approx. 12 per g), while two samples (one of which was from Patient X) contained terminal-spined *S. haematobium* group ova (approx. 24 and approx. 84 per g). Both with 'trace' results considered positive and negative, the sensitivity of POC-CCA tests was ≤30% when compared to the genus-specific *Schistosoma* spp. TaqMan probe ITS2 real-time PCR assay for the diagnosis of intestinal schistosomiasis without species-level identification, and when compared to *S. mansoni*-specific results acquired by testing faecal DNA extracts with the mtDNA HRM real-time PCR assay (table 2). Even with 'trace' results considered positive, POC-CCA tests only detected 20% of intestinal schistosomiasis caused by all *Schistosoma* species.

Among six *S. mansoni* mono-infected SAC (where *S. mansoni* DNA was detected in faeces but other *Schistosoma* species were not detected in faeces or urine), 1/6 was POC-CCA-positive, 1/6 'trace' and 4/6 negative. Meanwhile, POC-CCA tests performed poorly for the detection of urogenital schistosomiasis among 25 *S. haematobium* mono-infected SAC without intestinal schistosomiasis (3/25 positive, 0/25 'trace' and 22/25 negative). POC-CCA performance was also poor among 13 SAC with *S. haematobium* and *S. mattheei* co-infections when *S. mansoni* DNA was not detectable (3/13 positive, 0/13 'trace' and 10/13 negative).

(c) *Schistosoma mattheei* co-infection is associated with *S. haematobium* DNA detection in faeces

Schistosoma haematobium DNA was detected only 1.87 times more commonly in the urine than the faeces, from the total of 106 *S. haematobium* cases (figure 4a). Among these confirmed cases, the number of *Schistosoma* spp. ova per 10 ml urine was 0 in 20/106, 1–9 in 41/106, 10–49 in 26/106 and ≥50 in 19/106. Of the SAC from whom *S. haematobium* DNA was detected in the faeces, urogenital schistosomiasis was not detected by urine filtration in 20/46, while 16/46 had light urogenital infections (1–49 ova per 10 ml urine) and 10/46 had heavy urogenital infections (≥50 ova per 10 ml urine). A logistic regression model

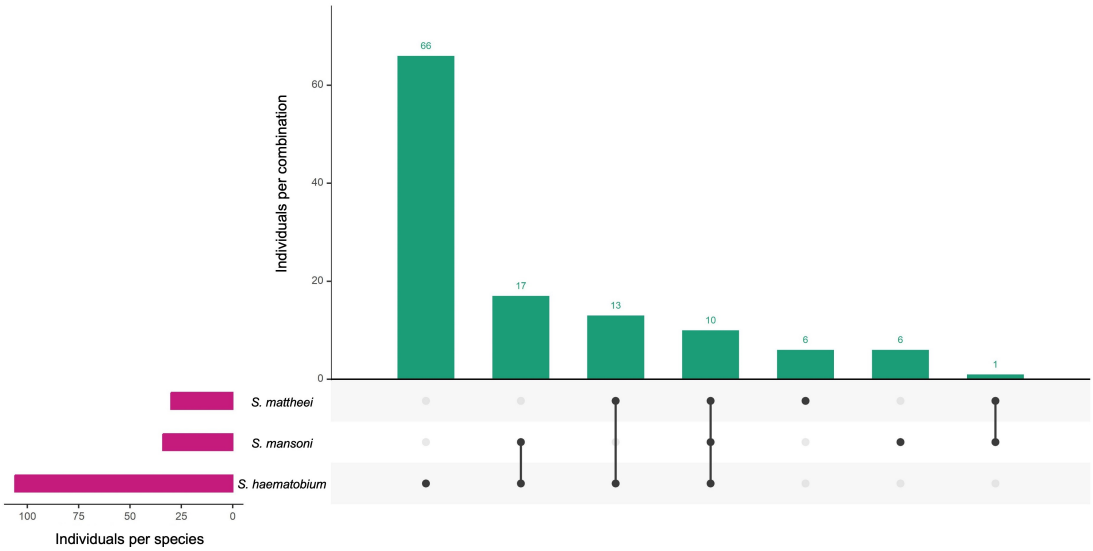

Figure 3. Co-infection counts between *S. haematobium*, *S. mansoni* and *S. mattheei* among 119 participants from whom we achieved species-level identification in faeces and/or urine. A solid black dot indicates infection in the species combination matrix. No individuals appear in more than one combination group. To the left of species names are counts per species, which show the number of individuals infected with each.

Table 2. Sensitivity, specificity, positive predictive and negative predictive values of POC-CCA tests compared to faecal *Schistosoma* spp. ITS2 real-time PCR and mtDNA HRM real-time PCR. PPV, positive predictive value; NPV, negative predictive value.

reference test	index test	sensitivity (95% CI)	specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
ITS2 real-time PCR (*Schistosoma* spp.)	POC-CCA ('trace' +)	20% (13–28)	90% (83–95)	68% (50–82)	52% (45–59)
	POC-CCA ('trace' −)	9% (4–15)	95% (90–98)	65% (38–86)	50% (44–57)
mtDNA HRM real-time PCR (*S. mansoni*)	POC-CCA ('trace' +)	30% (13–53)	87% (81–91)	19% (8–35)	92% (88–96)
	POC-CCA ('trace' −)	22% (7–44)	95% (91–97)	29% (10–56)	92% (88–95)

constructed to assess factors associated with the high rates of *S. haematobium* DNA detection in faeces showed that *S. mattheei* co-infection was significantly associated. Specifically, the odds of detecting *S. haematobium* DNA in the faeces of participants infected with *S. haematobium* were 4.7 times greater in cases of co-infection with *S. mattheei* ($p = 0.006$), after adjusting for *S. mansoni* co-infection, urogenital schistosomiasis infection intensity, age and sex (table 3). The detection of *S. haematobium* DNA in faeces was not significantly associated with *S. mansoni* co-infection nor urogenital infection intensity, but the odds of its detection were 2.4 times higher in boys than girls ($p = 0.047$) and were higher in younger children ($p = 0.023$). *Schistosoma mattheei* DNA was identified from urine filters only in the presence of *S. haematobium* co-infection. Meanwhile, in all seven cases of *S. mattheei* infection without detectable *S. haematobium* co-infection, *S. mattheei* DNA was identified from faecal samples, but not urine (figure 4b).

(d) Two patient case studies highlight recurrent hybrid infection and livestock-associated schistosome lineages

When we re-sampled the faeces and urine of Patient X and Y six months following praziquantel treatment, we found them to be infected with the same *Schistosoma* species: *S. haematobium* (both patients), *S. mattheei* (both patients) and *S. mansoni* (Patient Y only). Again, we identified *S. haematobium* × *S. mattheei* hybrid miracidia and found their relative abundance to be higher in the faeces than the urine (figure 5a). We found evidence of natural re-infection following treatment, shown by changes in mtDNA profiles among *S. haematobium* × *S. mattheei* hybrid miracidia: at baseline, 58 of the 61 *S. haematobium* × *S. mattheei* miracidia typed from stool and urine contained *S. mattheei* mtDNA, yet at follow-up, 26 of the 31 *S. haematobium* × *S. mattheei* miracidia typed contained *S. haematobium* mtDNA (figure 5b).

Using partial *COX1* sequences obtained from *S. haematobium* × *S. mattheei* miracidia hatched from the faeces and urine of these two patients, we identified four *S. mattheei* haplotypes (H1–H4), with SNPs observed at three sites. The dominant haplotype (H1) was observed in 43/52 *S. haematobium* × *S. mattheei* hybrids with *S. mattheei* partial *COX1* sequences (electronic supplementary material, table S1). All *S. mattheei* haplotypes were also detected in our livestock and snail surveys, including all four of the most common haplotypes in cattle [28]. Additionally, we detected five *S. haematobium* haplotypes among *S. haematobium* × *S. mattheei* miracidia, all belonging to *S. haematobium* haplogroup 1—the common cause of human urogenital schistosomiasis across mainland Africa [30]. All partial *COX1* sequences aligned to references from the same species detected by the mtDNA HRM real-time PCR assay.

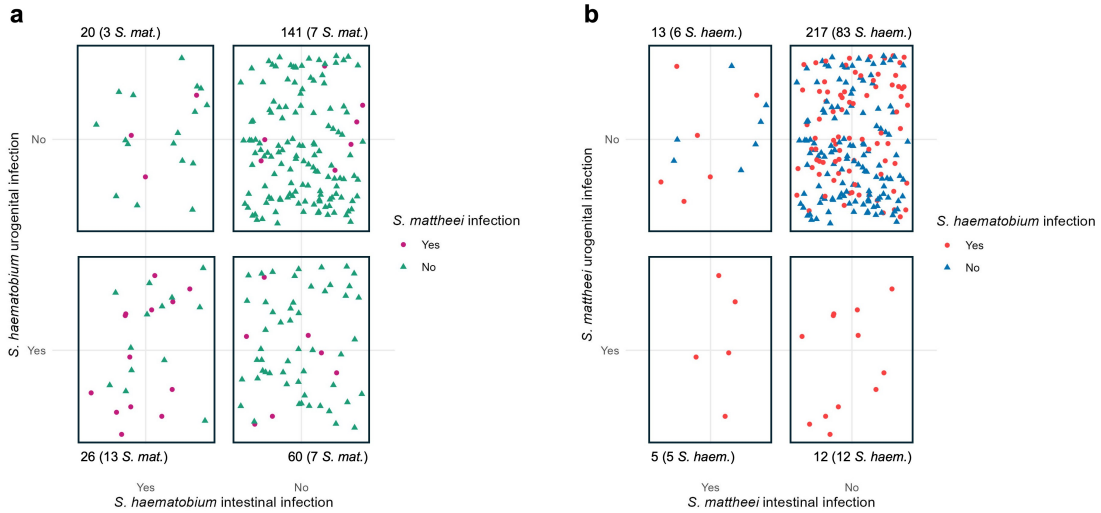

Figure 4. Jitter plots showing DNA excretion within the *S. haematobium* group. (a) *S. haematobium* infection status stratified by infection site, with points coloured and shaped by *S. mattheei* infection status; (b) *S. mattheei* infection status stratified by infection site, with points coloured and shaped by *S. haematobium* infection status. Each point represents a participant. Panel borders distinguish the possible infection combinations. Points within each panel are scattered randomly to enable visualization of individual cases. 'Yes' indicates confirmed infection. Each panel contains a total count and a co-infection count.

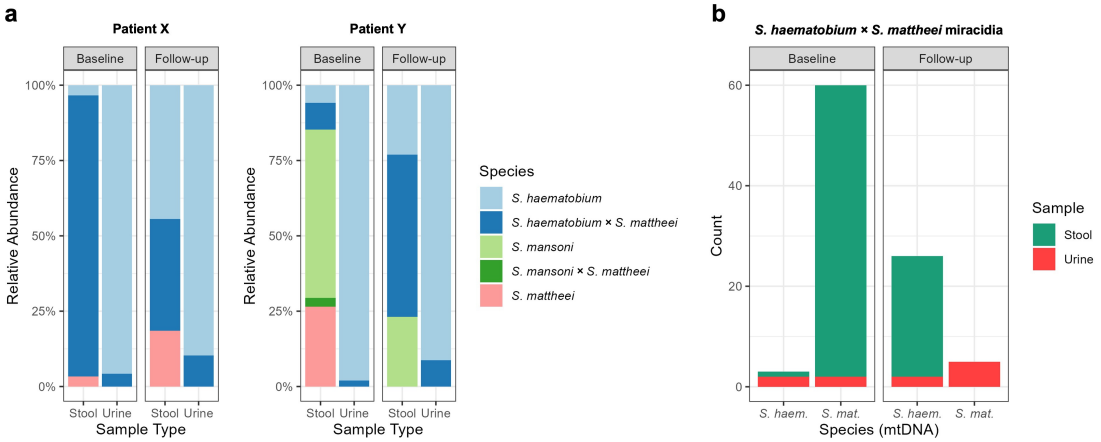

Figure 5. Characterizing miracidia hatched from the faeces and urine of two patients with repeated *S. haematobium* × *S. mattheei* infections (Patients X and Y) using HRM real-time PCR assays. (a) Relative abundance of different *Schistosoma* spp. miracidia; (b) mitochondrial DNA inheritance counts among *S. haematobium* × *S. mattheei* hybrid miracidia.

Table 3. Adjusted odds ratios, 95% confidence intervals and *p*-values from a multivariable logistic regression model assessing factors associated with the detection of *S. haematobium* DNA in the faeces of participants infected with *S. haematobium*. Adjusted odds ratios are exponentiated model coefficients; 95% CI = 95% confidence interval.

predictor variable		adjusted odds ratio (aOR)	95% CI	*p*-value
age (per year)		0.83	0.69–0.97	0.023
sex	female	1	—	—
	male	2.39	1.03–5.77	0.047
S. mattheei infection	no	1	—	—
	yes	4.72	1.61–15.37	0.006
S. mansoni infection	no	1	—	—
	yes	1.79	0.56–5.85	0.323
urogenital schistosomiasis infection intensity (ova per 10 ml urine)	<10	1	—	—
	10–49	0.53	0.16–1.57	0.260
	≥50	0.87	0.22–3.28	0.840

4. Discussion

This study provides new insights into the complex aetiology of intestinal schistosomiasis in southern Malawi, revealing a dynamic polyparasitic landscape in which *S. haematobium* group species may be significant contributors to intestinal disease. Traditionally, *S. mansoni* has been considered the primary agent of intestinal schistosomiasis in Africa [4]. Meanwhile, *S. haematobium* is considered to predominantly cause urogenital disease [4], including male genital schistosomiasis (MGS) [31] and female genital schistosomiasis (FGS) [32]. However, the detection of *S. haematobium*, *S. mattheei* and *S. mansoni* DNA in 36.8%, 14.4% and 18.4% of *Schistosoma*-positive faecal samples in our study challenges these assumptions and suggests a broader aetiological role for the *S. haematobium* group in intestinal schistosomiasis. These findings are supported by the case studies of Patients X and Y, from which copious *S. haematobium* group miracidia were hatched from atypical ova in stool at two time points.

The morphological [19] and genetic analyses of ova and miracidia obtained from Patients X and Y illustrate the surprising frequency with which ova typed as typical *S. haematobium* can be excreted alongside *S. haematobium* × *S. mattheei* hybrids in faeces, despite their canonical association with urogenital pathology in Malawi and across Africa more broadly. The wider detection of *S. haematobium* DNA in the faeces of SAC and its strong association with *S. mattheei* co-infection suggests that adult worm pairing between these two species can lead to the migration of heterospecific schistosome pairs [33,34] to the intestinal mesenteries, where first-generation hybrid ova are laid and excreted via the faeces, as seen in Patients X and Y. A second, non-mutually exclusive explanation may lie in historical introgression. If *S. haematobium* populations in Mangochi District have undergone repeated hybridization events with *S. mattheei* over many generations, it is plausible that alleles influencing mesenteric migration have become fixed or more common within local *S. haematobium* populations, with *S. mattheei* heritage in such populations being potentially undetectable by assays that target few genomic loci. Such 'ancient' hybridization events between *S. haematobium* and *S. bovis* have been characterized [35], but not between *S. haematobium* and *S. mattheei*. This could explain the excretion of *S. haematobium* ova in the faeces of participants with *S. haematobium* mono-infections. Contamination of faecal samples with urine is not likely to be responsible for this finding, as urogenital schistosomiasis infection intensity was not associated with finding *S. haematobium* DNA in faeces, while we identified *S. haematobium* DNA in the faeces of 20 participants from whom no *Schistosoma* ova were identified in urine samples. Moreover, we extracted DNA from the inner contents of faecal samples, rather than the external surface, to guard against contamination.

While our data suggest that *S. mansoni* is present in a minority of intestinal schistosomiasis cases in our study setting, its potential to participate in hybrid pairings remains. In Patient Y, we observed molecular evidence of *S. mansoni* participation in a hybrid cross with *S. mattheei*, underscoring the rare but possible interaction between *S. haematobium* group species and *S. mansoni*. This is consistent with known mating dominance patterns, where *S. haematobium* group males tend to outcompete *S. mansoni* males, reducing the likelihood, but not precluding, the formation of *S. mansoni*-involved hybrid pairings [36,37]. However, similar inter-group pairings involving *S. bovis* × *S. mansoni* crosses have been shown to produce parthenogenetic offspring carrying only the maternal nuclear genotype, thereby restricting downstream compatibility with intermediate snail hosts [38]. Mixed-species pairs within the *S. haematobium* group were observed far more commonly than *S. mansoni* and *S. mattheei* pairing in Patient Y, from whom triple infections with *S. haematobium*, *S. mansoni* and *S. mattheei* were detected both in mid-2024 and early 2025. In both Patients X and Y, the sex-species profiles of *S. haematobium* × *S. mattheei* hybrids changed following treatment and natural re-infection. In such pairings, the age of each infection is thought to influence cross-species mating dominance [34]. In mid-2024, *S. haematobium* × *S. mattheei* miracidia from both patients predominantly inherited *S. mattheei* mtDNA, while *S. haematobium* mtDNA was more commonly detected at follow-up in early-2025. This shift in both patients after six months points towards constantly meandering micro-epidemiological features and environmental pressures that influence downstream human infection and mating patterns within the definitive host [39].

Our findings have important implications for schistosomiasis surveillance and clinical management. POC-CCA tests, widely used to detect *S. mansoni*, demonstrated reasonable correspondence with overall *S. mansoni* prevalence at the population level, as they also did in the recent study of Archer *et al.* in Mangochi District [10]. However, individual-level concordance was poor, and in this setting, where *Schistosoma* ova excretion in the faeces appears to be predominantly driven by *S. haematobium* group worm pairings, the test is not appropriate to diagnose intestinal schistosomiasis. Additionally, variability in POC-CCA batch performance limits its diagnostic value [40]. Our selective Kato–Katz preparations yielded only one light *S. mansoni* case by microscopy from 17 samples provided by POC-CCA-positive SAC. These results indicate that the poor performance of POC-CCA in the cases of six children determined to have *S. mansoni* mono-infections by molecular diagnostics may have been at least partially a result of low *S. mansoni* infection intensity. Inspection of *Schistosoma* ova morphology gave us the first indication that *S. haematobium* group hybrids may be causing intestinal schistosomiasis [19], but reliance on Kato–Katz faecal microscopy alone can result in false negative results, particularly when ova outputs are low [10,15]. Our study highlights the need to incorporate sensitive molecular diagnostics into both surveillance and clinical settings. While species-level typing may remain too labour-intensive and unnecessary for current routine clinical diagnostics, the use of species-specific diagnostics in clinical research is needed to characterize the complications and treatment outcomes associated specifically with zoonotic and hybrid infections. Importantly, it remains unknown whether *S. haematobium* group hybrid infections cause intestinal or hepatosplenic morbidity comparable to *S. mansoni* [41]. Similar knowledge gaps remain in the management of urogenital schistosomiasis caused by hybrid species [42]. Meanwhile, the absence of a TaqMan probe assay for *S. mattheei* remains a critical diagnostic gap for more precise surveillance of zoonotic schistosomiasis. Development and integration of such an assay in epidemiological and clinical research will be essential to achieve schistosomiasis elimination [43].

Our study does have several limitations. First, species-specific diagnostic methods target mtDNA loci; mtDNA is maternally inherited, leading to a potential underestimation of species-level prevalence as paternal ancestry may be masked in

mixed-species pairings that excrete ova via the intestines. Additionally, the lower sensitivity of current species-level diagnostics when compared to genus-specific options is demonstrated in our results: we could not achieve species-level identification in almost 50% of faecal samples from which *Schistosoma* spp. DNA was detected. Next, miracidial profiling here targeted only one locus in each of the nDNA and mtDNA, supplemented by partial mitochondrial *COX1* sequences in hybrids. This restricts our ability to detect ancient hybridization or deep introgression, which would require the investigation of more genomic loci [35]. It is also important to note that by focusing molecular typing solely on urine filters from participants with ova-patent urogenital infections, our study may underestimate the true prevalence of urogenital schistosomiasis. This limitation is compounded by the broader challenge in *Schistosoma* infection research of accurately quantifying adult worm burdens and understanding the true sex ratios of *Schistosoma* species in human hosts, as ova-patent infections only reflect situations where both male and female worms are present and paired. Infections with unpaired worms, especially single-sex infections, remain undetected by ova-based diagnostics [44]. This biological complexity may obscure the full picture of transmission dynamics and species distribution, potentially biasing our interpretation of infection epidemiology.

In conclusion, this study underscores the emerging role of zoonotic and hybrid schistosomes in driving intestinal schistosomiasis in southern Malawi. These findings reveal fundamental gaps in current diagnostic tools and reinforce the need for a One Health approach to surveillance—one that accounts for livestock reservoirs, human–livestock water contact and the diagnostic limitations of tools developed for a simplified species landscape.

Ethics. This study received ethical approvals from The College of Medicine Research Ethics Committee, Malawi (approval no. P.08/21/3381) and the Liverpool School of Tropical Medicine Research Ethics Committee, United Kingdom (approval no. 22-028).

Data accessibility. Data and code used to carry out analyses in R are available from GitHub [45].

Supplementary material is available online [46].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. A.M.O.: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing—original draft, writing—review and editing; L.J.C.: data curation, formal analysis, investigation, methodology, supervision, validation, visualization, writing—review and editing; D.L.: data curation, investigation, project administration, software, writing—review and editing; P.M.: data curation, investigation, project administration, writing—review and editing; G.N.: data curation, investigation, project administration, writing—review and editing; A.J.: data curation, investigation, writing—review and editing; S.J.: data curation, investigation, writing—review and editing; R.C.: data curation, investigation, writing—review and editing; S.R.: data curation, investigation, writing—review and editing; P.C.: data curation, investigation, writing—review and editing; D.R.K.: data curation, investigation, writing—review and editing; S.A.K.: data curation, investigation, methodology, project administration, writing—review and editing; E.J.LaC.: conceptualization, data curation, investigation, supervision, writing—review and editing; A.P.R.: conceptualization, investigation, methodology, resources, supervision, writing—review and editing; J.M.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, writing—review and editing; J.R.S.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualization, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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