Original article

Evaluation of a duplex real-time PCR in human serum for simultaneous detection and differentiation of *Schistosoma mansoni* and *Schistosoma haematobium* infections – cross-sectional study

Hagen Frickmann [a,b,1,*], Lisa-Maria Lunardon [c,1], Andreas Hahn [b], Ulrike Loderstädt [d,3], Andreas K. Lindner [e], Sören L. Becker [c,f,g], Frank P. Mockenhaupt [e], Christoph Weber [h,2], Egbert Tannich [d,i,2]

[a] *Department of Microbiology and Hospital Hygiene, Bundeswehr Hospital Hamburg, Hamburg, Germany*
[b] *Institute for Medical Microbiology, Virology and Hygiene, University Medicine Rostock, Rostock, Germany*
[c] *Institute of Medical Microbiology and Hygiene, Saarland University, Homburg/Saar, Germany*
[d] *Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany*
[e] *Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Institute of Tropical Medicine and International Health, Berlin, Germany*
[f] *Swiss Tropical and Public Health Institute, Basel, Switzerland*
[g] *University of Basel, Basel, Switzerland*
[h] *Vivantes Auguste Viktoria Klinikum, Berlin, Germany*
[i] *National Reference Centre for Tropical Pathogens, Hamburg, Germany*

ARTICLE INFO

ABSTRACT

Background: We evaluated a one-tube multiplex real-time PCR targeting DNA of *Schistosoma haematobium* complex and *S. mansoni* complex in serum samples obtained at different German diagnostic centers.
Methods: Simplex real-time PCR protocols for the detection of the multi-copy DNA-repeats *Dra1* of *S. haematobium* complex and *Sm1-7* of *S. mansoni* complex in serum were combined to a new one-tube multiplex format. The new PCR was subjected to full validation including evaluation in a diagnostic real-life setting with travelers and migrants. PCR results were compared with those of stool and urine microscopy, serology, and circulating cathodic antigen (CCA) rapid diagnostic tests in urine. Sensitivity and specificity of the diagnostic approaches were analyzed using latent class analysis (LCA).
Results: LCA assessment indicated sensitivity and specificity of 94.9% and 98.4%, respectively, for serum PCR if serology was included in the calculation, and 100% and 95.6%, respectively, if serology was not included as a parameter not necessarily associated with active infection. Agreement between the compared diagnostic procedures at genus level was fair (kappa 0.273) if serology was included and moderate (kappa 0.420) if serology was not included.
Discussion: The PCR assay proved to be highly reliable for the diagnosis of schistosomiasis in travelers and migrants.

1. Introduction

The World Health Organization (WHO) recommends serology, microscopy, or point-of-care (POC) tests that detect a *Schistosoma*-specific circulating cathodic antigen (CCA) in urine for the laboratory-based diagnosis of human schistosomiasis. Due to typical morphologies of *Schistosoma* spp. eggs, specificity and positive predictive value of microscopy are usually high, but sensitivity depends on infection intensity

and the number of eggs shed in urine or stool. Even in many high-endemicity settings, but in particular in travelers, the average infection intensity is often low, and microscopy alone may thus easily miss a considerable number of infections [1]. The latency period before *Schistosoma*-specific antibodies become detectable following infection may amount to several months and the reliability of serological assays is both species- and assay-dependent [2]. The sensitivity of the more recently introduced POC-CCA test is dependent on both parasite density and species, being most sensitive for the detection of *Schistosoma mansoni* according to a recent meta-analysis [3].

Multiple PCR approaches for the diagnosis of human schistosomiasis have been introduced and have been summarized in various reviews [4–6]. For the identification of small amounts of circulating DNA in serum, as is the case at early stages of infection during acute schistosomiasis (Katayama fever), multi-copy tandem-repeat targets were chosen to ensure reliable sensitivity. PCR targets comprise the *Dra1* sequence of the *S. haematobium* complex (covering *S. haematobium*, *S. intercalatum*, and *S. guineensis*) [7,8] and the *Sm1-7* tandem-repeat sequence of the *S. mansoni complex* [9], which shows some cross-reactivity with *S. bovis* from the *S. haematobium* complex [8]. In a study comprising 38 European travelers with acute schistosomiasis, the *Sm1-7* PCR revealed superior sensitivity to serology and microscopy [10]. Following treatment, an initial reduction of cycle threshold (C_T) values can be seen corresponding to free worm DNA as a consequence of successful therapy, followed by a steady increase of C_T values afterwards [11]. A larger study comprising nearly 200 sera from travelers and migrants confirmed excellent sensitivity of both *Sm1-7* and *Dra1* PCR compared with serology and microscopy [12], wherein patients with microscopic detection of *Schistosoma* eggs were considered as "confirmed cases" and patients with negative microscopy and serology as "cases with excluded schistosomiasis". However, serology may become positive after considerable delay and shows sensitivities less than 100% depending on the *Schistosoma* species and the antigen applied [2]. Further, definite information is lacking on how long *Dra1* and *Sm1-7* PCR as well as serology remain positive after successful cure of infection, so neither parameter necessarily indicates active infection in case of positive results. DNA from residual eggs may still circulate after the pharmaceutical eradication of intravascular worms, and residual antigens may continue to promote antibody production as immunological stimuli. Indeed, even eggs in tissue can still be secreted with urine or stool following successful treatment. Accordingly, since no diagnostic approach can be considered as a "gold standard" for the diagnosis of schistosomiasis, there is a need for a test comparison in the absence of a reference standard [13–15].

Here, we describe the development and evaluation of a one-tube multiplex real-time PCR approach targeting DNA of the *Sm1-7* sequence of *S. mansoni* complex and of the *Dra1* sequence of *S. haematobium* complex, respectively, using diagnostic samples from travelers and migrants. Patients were assessed by multiple diagnostic testing approaches for schistosomiasis in different diagnostic centers. Stool and urine microscopy, serology, and POC-CCA testing in urine were carried out and serum samples were forwarded to one reference laboratory for additional multiplex real-time PCR. In the absence of a diagnostic gold standard, bio-statistical modeling using a latent class analysis (LCA) approach was employed to compare the diagnostic accuracy of the different tests.

2. Methods

2.1. Study populations

A total of 376 international travelers and African migrants coming to Hamburg, Berlin, and Homburg, Germany at presumed risk of schistosomiasis were assessed with various diagnostic approaches at the German National Reference Center for Tropical Pathogens, Bernhard Nocht Institute for Tropical Medicine, Hamburg, at the Institute for Tropical Medicine and International Health of the Charité, University Hospital Berlin and the Institute of Medical Microbiology and Hygiene, Saarland University Medical Center, Homburg between 2014 and 2018. Information on sex and age was usually available to the laboratory, while information on the countries of origin or travel was only inconsistently provided. While migrants from areas of endemicity for schistosomiasis were systematically screened, diagnostic testing for schistosomiasis in returning travelers was triggered by the physician in charge based on clinical suspicion. The exact clinical signs and symptoms leading to the initiation of such diagnostic work-up were not specifically known to the laboratory. The laboratory diagnostic tests performed were serum in-house PCR, serology with two different assays, microscopy of stool and urine as well as POC-CCA urine tests. If not all tests were carried out or not all of the aforementioned samples were provided for analysis, these samples were nevertheless considered for the analysis presented here (Supplementary material 1).

2.2. Microscopy

Stool microscopy was performed following standard procedures as described [16,17] following SAF enrichment [18]. Urine microscopy was performed with sediment from urine collected between 10 a.m. and 2 p.m.

2.3. Circulating cathodic antigen (CCA) testing

POC-CCA testing on urine samples was performed using the Schistosoma POC-CCA test manufactured by IDT Diagnostics, Cape Town, South Africa, according to the manufacturer's instructions.

2.4. Serology

Serological detection at genus level was performed in Hamburg for patients from Hamburg and in Berlin for patients from Berlin and Homburg. In Hamburg, serology comprised in-house enzyme-linked immunosorbent assay (ELISA) using *S. mansoni* cercarial antigen (cut-off: 10 arbitrary units) or in-house immunofluorescence testing using frozen sections of adult *S. mansoni* (cut-off-titer: 1:20). In Berlin, in-house ELISA (cut-off: 12 arbitrary units) or in-house immune hemagglutination assay (cut-off-titer: 1:160) were used. Possible active or previous infection was assumed if at least one assay showed a positive result.

2.5. Multi-copy tandem-repeat one-tube multiplex real-time-PCR

Nucleic acid extraction from serum was performed as described previously [10] with sample volumes ranging from at least 0.2 mL up to 1 mL. In-house real-time PCR was run in a newly established one-tube multiplex assay using primers and probes as previously described for the *Dra1* sequence of the *S. haematobium* complex [7,8] and the *Sm1-7* sequence of the *S. mansoni* complex [9]. In addition, primers and a probe were added for the detection of Phocid herpesvirus DNA [19], which served as extraction/inhibition control. The PCRs were performed in 20 μL reaction volumes containing 10 μL HotStar master mix (Qiagen, Hilden, Germany), 6.0 mM total $MgCl_2$, 5×10^{-7} mol of each primer, 3×10^{-7} mol of each probe, 0.005 g/L bovine serum albumin, and 5.0 μL DNA eluate on RotorGene 6000 cyclers (Qiagen). The steps were activation at 95 °C for 15 min (minutes) followed by 40 cycles of 15 s (seconds) denaturation at 95 °C, 60 s annealing at 60 °C, and 60 s elongation at 72 °C. After this, the tubes were cooled to 40 °C for an additional 30 s before the run was finished.

Technical evaluation of the one-tube multiplex approach had been conducted in the DIN EN ISO 15189-accredited Bernhard Nocht Institute, comprising sensitivity and linearity assessment of the simplex and multiplex approaches, same as inter-assay and intra-assay variation in line with German recommendations [20]. Afterwards, the real-life

evaluation with clinical samples described here was performed.

2.6. Statistics

Sensitivity and specificity with 95% confidence intervals of PCR for *S. mansoni* complex and *S. haematobium* complex from serum, of genus-specific serology and genus-specific POC-CCA testing from urine, as well as of microscopic detection of *S. mansoni* and *S. haematobium* in stool or urine were calculated using latent class analysis (LCA) [13–15]. Agreement (kappa) between the approaches was based on the categories poor (below 0.00), slight (0.00–0.20), fair (0.21–0.40), moderate (0.41–0.60), substantial (0.61–0.80), and almost perfect (0.81–1.00) as detailed elsewhere [21]. C_T values of positive test results were assessed descriptively. All analyses were done using Stata/IC 15.1 for Mac 64-bit Intel (College Station, Texas, USA).

2.7. Data availability

All available data are presented in the manuscript, its tables, and its supplementary material file.

2.8. Ethics

Ethical clearance for the test comparisons was granted by the ethics committee of the Medical Association of Hamburg, Germany (registration number WF-011/19) in line with national laws without requirement of informed consent. Patients from Homburg and Berlin were African migrants who were enrolled during a study pertaining to the diagnostic accuracy of the POC-CCA test for detection of schistosomiasis in African migrants, and ethical approval was provided by the 'Ärztekammer des Saarlandes' in Saarbrücken, Germany.

3. Results

3.1. Study population and test results

Altogether, datasets from 376 individuals were included in the assessment with a median age of 24 (interquartile range 19–31) years and a proportion of females of 21% (n = 77/373 cases with available gender information). Numbers (including denominators) and percentages of positive tests are shown in Table 1. In short, microscopic detection of *S. mansoni* eggs was successful in 14 of 316 instances (4.4%), while *S. haematobium* eggs were seen in 11 of 303 cases (3.6%). At least one serological test for schistosomiasis was positive in 156/328 cases (47.56%); positive results of POC-CCA testing in urine were seen for 43 of 311 patients (13.83%). Positive serum PCR results for *S. haematobium* complex were obtained in 30 of 337 cases (8.90%) and for *S. mansoni* complex in 83 of 337 cases (24.63%). All data are summarized in the supplementary material 1.

3.2. Sensitivity and specificity as calculated by LCA testing and diagnostic agreement (kappa)

As shown in Table 2, LCA-based sensitivity and specificity values of

one-tube multiplex real-time-PCR targeting both *S. haematobium* complex and *S. mansoni* complex were calculated to range between 87.9% and 100%. This is the case independently of inclusion or exclusion of serology, a diagnostic approach that does not necessarily indicate active infection. While specificity of POC-CCA testing and microscopy was in a similar range, calculated sensitivity of POC-CCA ranged from 33.5 to 65.2% with better sensitivity in case of *S. mansoni* complex infections, and calculated sensitivity of microscopy from 22.4% to 57.0% with better sensitivity in case of *S. haematobium* complex infections. Diagnostic agreement parameters covered a range from poor to moderate in line with the applied categories [21] irrespective of the inclusion of serology in the assessment, although exclusion of serology allowed an increase of agreement from fair to moderate at least for the genus-wide assessment.

3.3. C_T-value assessment as stratified by microscopic and CCA testing results

Comparing C_T-values of positive PCR results with microscopy and CCA-testing indicated lower mean C_T-values (i.e. suggesting higher infection intensity) in microscopically positive as compared to microscopically negative samples. Similar observations were made between CCA-positive and CCA-negative tests (Table 3).

4. Discussion

In case of delayed diagnosis, e.g., in migrants, severe clinical courses of schistosomiasis can be observed even in non-endemic European settings [22], defining a need for reliable diagnostic options. The study was performed to assess the diagnostic performance characteristics of a one-tube multiplex real-time PCR targeting multi-copy tandem-repeat sequences for the simultaneous detection and differentiation of circulating DNA from *S. haematobium* complex and *S. mansoni* complex in serum of travelers and migrants in a non-endemic setting. The results indicate superior sensitivity and specificity of serum PCR compared with results from microscopy, POC-CCA, and serology that were performed in parallel. This holds true for the observed values in LCA assessment but is, admittedly, weakened by the calculated 0.95 confidence intervals.

The observed superiority of highly sensitive PCR compared with investigator-dependent microscopy is well known and has been shown earlier [10,12]. Higher sensitivity of microscopy for detection of *S. haematobium* than for *S. mansoni* results from the more complex stool matrix in which *S. mansoni* eggs are usually present, while microscopic detection of *S. haematobium* eggs in urine sediment requires less experience. In line with this, lower C_T-values indicating higher abundance of target DNA were observed in PCR from serum samples of microscopically positive patients, in whom higher pathogen loads are expected.

POC-CCA shows better sensitivity compared with stool microscopy as demonstrated in a systematic review [23]. Our assessment confirmed limited sensitivity compared with PCR and likewise lower sensitivity of POC-CCA for *S. haematobium* than for *S. mansoni* as described earlier [3]. However, we were able to demonstrate a relatively high specificity of the POC-CCA test in the assessed travelers and migrants, similar to results obtained in Switzerland [24]. Even more pronounced than for PCR,

Table 1
Numbers of positive and negative results by genus and species complex as obtained from the 376 assessed individuals.

	Schistosoma spp. (non-Asian species)		*S. haematobium* complex		*S. mansoni* complex	
	Samples tested (N)	Frequency (%)	Samples tested (N)	Frequency (%)	Samples tested (N)	Frequency (%)
Serum PCR positives	337	102 (30.3%)	337	30 (8.9%)	337	83 (24.6%)
Microscopy positives	318	23 (7.3%)	303	11 (3.6%)	316	14 (4.4%)
Serology positives	328	156 (47.6%)	328	156 (47.6%)	328	156 (47.6%)
Circulating cathodic antigen (CCA) test positives	311	43 (13.8%)	311	43 (13.8%)	311	43 (13.8%)
Samples negative in all tests	248	125 (50.4%)	240	124 (51.7%)	246	127 (51.6%)
Samples positive in any test	382	209 (54.7%))	381	193 (50.7%)	382	199 (52.1%)

Table 2

Sensitivity and specificity as calculated by latent class analysis (LCA) as well as Fleiss' kappa by genus and species complex with and without inclusion of serological results.

	Schistosoma spp. (non-Asian species)		*S. haematobium* complex		*S. mansoni* complex	
	Assessment including serological results					
	Sensitivity (0.95 Confidence interval)	Specificity (0.95 Confidence interval)	Sensitivity (0.95 Confidence interval)	Specificity (0.95 Confidence interval)	Sensitivity (0.95 Confidence interval)	Specificity (0.95 Confidence interval)
Circulating cathodic antigen (CCA) test	0.355 (0.239, 0.489)	0.925 (0.880, 0.953)	0.334 (0.135, 0.614)	0.873 (0.828, 0.907)	0.405 (0.271, 0.555)	0.922 (0.879, 0.951)
Serum PCR	0.949 (0.821, 0.987)	0.984 (0.077, 1.)	0.933 (0.648, 0.991)	1 (0, 1)	0.959 (0.766, 0.994)	0.973 (0.601, 0.999)
Microscopy	0.290 (0.188, 0.419)	1 (0, 1)	0.554 (0.313, 0.772)	1 (0, 1)	0.224 (0.128, 0.362)	1 (0,1)
Serology	0.873 (0.639, 0.964)	0.677 (0.611, 0.737)	0.701 (0.488, 0.852)	0.543 (0.486, 0.598)	0.937 (0.480, 0.996)	0.652 (0.588, 0.711)
Fleiss' Kappa (0.95 Confidence interval)	0.273 (0.198, 0.331)		0.064 (0.019, 0.130)		0.241 (0.184, 0.302)	
	Assessment excluding serological results					
Circulating cathodic antigen (CCA) test	0.412 (0.208, 0.652)	0.921 (0.879, 0.950)	0.351 (0.142, 0.640)	0.872 (0.828, 0.906)	0.652 (0.349, 0.867)	0.918 (0.876, 0.947)
Serum PCR	1 (0, 1)	0.956 (0.139, 1)	1 (0, 1)	1 (n.e.)	1 (0, 1)	0.879 (0.780, 0.937)
Microscopy	0.326 (0.149, 0.572)	0.992 (0.968,0.998)	0.570 (0.326, 0.783)	0.997 (0.976, 1)	0.379 (0.183, 0.626)	0.996 (0.973,0.999)
Fleiss' Kappa (0.95 Confidence interval)	0.420 (0.307, 0.485)		0.064 (0.019, 0.130)		0.238 (0.170, 0.318)	

n.e. = non-estimable.

Table 3

Cycle threshold (C_T) values of serum PCR targeting *S. haematobium* complex and *S. mansoni* complex as associated with positive and negative results in other test approaches.

	Serum PCR targeting *S. haematobium* complex		Serum PCR targeting *S. mansoni* complex	
	Number (N)	C_T value (mean ± standard deviation (SD))	Number (N)	C_T value (mean ± standard deviation (SD))
Microscopy positive	10	31.0 (3.3)	12	29.0 (3.5)
Microscopy negative	6	33.0 (2.8)	38	31.4 (3.1)
Serology positive	14	31.9 (2.7)	57	30.8 (3.6)
Serology negative	7	30.9 (3.9)	8	29.9 (3.1)
CCA-positive	4	31.3 (3.0)	18	28.4 (2.3)
CCA-negative	8	32.6 (2.8)	27	32.7 (2.7)

CCA = Circulating cathodic antigen. Low cycle threshold values indicate a high abundance of target DNA and vice versa.

lower C_T-values and thus higher abundance of target DNA were associated with positive POC-CCA tests.

Serology is not indicative for active infection [2,25] and, accordingly, agreement between PCR, microscopy, and POC-CCA testing was better if serological parameters were not included in the assessment. Altogether, serology is only a weak parameter in potentially confirming other test results in this study, because the methods were inconsistently applied throughout the study and depended on the study site.

The test comparison was based on LCA, a variant of structural equation models, which estimates non-observed variables as the disease status over observed variables, i.e., the results of diagnostic tests, as recently detailed [15]. Such indirect methods are a remaining option of estimating test characteristics in the absence of a reliable gold standard as it is the case for the diagnosis of schistosomiasis. Also as detailed elsewhere, gold standard-based comparisons are often associated with a false sense of certainty, because firstly, reference methods with close-to-100% sensitivity and specificity are de facto unavailable and secondly, "new methods with higher diagnostic accuracy cannot demonstrate their superiority over gold standards since the method set as gold standard has the ideal diagnostic accuracy by definition" [15]. Admittedly, indirect methods like LCA provide estimations and no "absolute truth". As even an assay with the highest sensitivity within a comparison

of different tests necessarily has a limit-of-detection, LCA may have slightly overestimated the sensitivity of PCR. Nevertheless, high sensitivity of PCR can be expected as suggested by previous evaluation studies with the simplex assays [7–9]. Further, the high variation between the compared diagnostic approaches as indicated by low kappa values makes severely differing performance of the approaches likely. In contrast, the striking difference of calculated specificity of PCR, depending on whether or not serological results were included in the calculation, suggests an underestimation of PCR specificity if serological results are neglected. This is why serology was intentionally included in one of the calculations, because positive serology is sometimes the only detected parameter in cases of active schistosomiasis. This is although positive serology may be absent, e.g., in case of early infections, or still positive in spite of previous successful therapy, making this test result neither necessary nor sufficient by itself to set the diagnosis of active schistosomiasis in a context-free setting.

A limitation of the study is that the different diagnostic methods could not be consistently applied for every patient, which represents a deviation from the STARD reporting standards for diagnostic accuracy studies [26]. On the other hand, it was specifically this approach that allowed comparative testing in the non-endemicity setting where schistosomiasis is rarely diagnostically considered.

Another limitation of the PCR approach is the fact that only African *Schistosoma* species are covered by the duplex-PCR and it does not include Asian *Schistosoma* species such as *S.japonicum* or *S. mekongi* [8].

Thirdly, our study is limited by the unavailability of clinical information on most of the included patients, comprising symptoms and assumed stage of disease, as well as on the specific reasons that led to diagnostic work-up for suspected schistosomiasis.

5. Conclusions

In conclusion, we have introduced a one-tube multiplex real-time PCR assay targeting the *Dra1* sequence of *S. haematobium* complex and the *Sm1-7* sequence of *S. mansoni* complex in serum. In spite of the abovementioned limitations, high sensitivity and specificity compared with alternative diagnostic approaches have been demonstrated with travelers and migrants from a non-endemic setting in Germany, suggesting the use of serum PCR as a diagnostic approach well suited for the diagnosis of schistosomiasis due to species other than the *S. japonicum* complex in the population of travel returnees and migrants. As a consequence, the duplex serum PCR could potentially be recommended as a diagnostic standard approach in case of suspicion of non-*S. japonicum*-complex-associated schistosomiasis in travel medicine.

Funding

CRediT authorship contribution statement

Hagen Frickmann: Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing. **Lisa-Maria Lunardon:** Methodology, Validation, Investigation, Writing – review & editing. **Andreas Hahn:** Methodology, Conceptualization, Software, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Ulrike Loderstädt:** Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Andreas K. Lindner:** Validation, Methodology, Investigation, Resources, Writing – review & editing. **Sören L. Becker:** Methodology, Validation, Investigation, Resources, Writing – review & editing, Funding acquisition. **Frank P. Mockenhaupt:** Methodology, Validation, Investigation, Resources, Writing – review & editing. **Christoph Weber:** Methodology, Validation, Investigation, Resources, Writing – review & editing, Funding acquisition. **Egbert Tannich:** Methodology, Conceptualization, Validation, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tmaid.2021.102035.

References

[1] Schwarz NG, Rakotozandrindrainy R, Heriniaina JN, Randriamampionona N, Hahn A, Hogan B, et al. *Schistosoma mansoni* in schoolchildren in a Madagascan highland school assessed by PCR and sedimentation microscopy and Bayesian estimation of sensitivities and specificities. Acta Trop 2014;134:89–94.

[2] Hinz R, Schwarz NG, Hahn A, Frickmann H. Serological approaches for the diagnosis of schistosomiasis - a review. Mol Cell Probes 2017;31:2–21.

[3] Ochodo EA, Gopalakrishna G, Spek B, Reitsma JB, van Lieshout L, Polman K, et al. Circulating antigen tests and urine reagent strips for diagnosis of active schistosomiasis in endemic areas. Cochrane Database Syst Rev 2015;3:CD009579.

[4] Abath FG, Gomes AL, Melo FL, Barbosa CS, Werkhauser RP. Molecular approaches for the detection of *Schistosoma mansoni*: possible applications in the detection of snail infection, monitoring of transmission sites, and diagnosis of human infection. Mem Inst Oswaldo Cruz 2006;101(Suppl 1):145–8.

[5] Utzinger J, Becker SL, van Lieshout L, van Dam GJ, Knopp S. New diagnostic tools in schistosomiasis. Clin Microbiol Infect 2015;21:529–42.

[6] Pomari E, Piubelli C, Perandin F, Bisoffi Z. Digital PCR: a new technology for diagnosis of parasitic infections. Clin Microbiol Infect 2019;25:1510–6.

[7] Hamburger J, He-Na, Abbasi I, Ramzy RM, Jourdane J, Ruppel A. Polymerase chain reaction assay based on a highly repeated sequence of *Schistosoma haematobium*: a potential tool for monitoring schistosome-infested water. Am J Trop Med Hyg 2001;65:907–11.

[8] Cnops L, Soentjens P, Clerinx J, Van Esbroeck M. A *Schistosoma haematobium*-specific real-time PCR for diagnosis of urogenital schistosomiasis in serum samples of international travelers and migrants. PLoS Neglected Trop Dis 2013;7:e2413.

[9] Wichmann D, Panning M, Quack T, Kramme S, Burchard GD, Grevelding C, et al. Diagnosing schistosomiasis by detection of cell-free parasite DNA in human plasma. PLoS Neglected Trop Dis 2009;3:e422.

[10] Wichmann D, Poppert S, Von Thien H, Clerinx J, Dieckmann S, Jensenius M, et al. Prospective European-wide multicentre study on a blood based real-time PCR for the diagnosis of acute schistosomiasis. BMC Infect Dis 2013;13:55.

[11] Fuss A, Mazigo HD, Mueller A. Evaluation of serum-based real-time PCR to detect *Schistosoma mansoni* infection before and after treatment. Infect Dis Poverty 2020; 9:74.

[12] Guegan H, Fillaux J, Charpentier E, Robert-Gangneux F, Chauvin P, Guemas E, et al. Real-time PCR for diagnosis of imported schistosomiasis. PLoS Neglected Trop Dis 2019;13:e0007711.

[13] Qu Y, Tan M, Kutner MH. Random effects models in latent class analysis for evaluating accuracy of diagnostic tests. Biometrics 1996;52:797–810.

[14] Hahn A, Schwarz NG, Frickmann H. Comparison of screening tests without a gold standard-A pragmatic approach with virtual reference testing. Acta Trop 2019;199: 105118.

[15] Hahn A, Podbielski A, Meyer T, Zautner AE, Loderstädt U, Schwarz NG, et al. On detection thresholds-a review on diagnostic approaches in the infectious disease laboratory and the interpretation of their results. Acta Trop 2020;205:105377.

[16] Nazer H, Greer W, Donnelly K, Mohamed AE, Yaish H, Kagalwalla A, et al. The need for three stool specimens in routine laboratory examinations for intestinal parasites. Br J Clin Pract 1993;47:76–8.

[17] Hiatt RA, Markell EK, Ng E. How many stool examinations are necessary to detect pathogenic intestinal protozoa? Am J Trop Med Hyg 1995;53:36–9.

[18] Marti H, Escher E. SAF–an alternative fixation solution for parasitological stool specimens. Schweiz Med Wochenschr 1990;120:1473–6.

[19] Niesters HGM. Quantitation of viral load using real-time amplification techniques. Methods 2001;25:419–29.

[20] Rabenau HF, Kessler HH, Kortenbusch M, Steinhorst A, Raggam RB, Berger A. Verification and validation of diagnostic laboratory tests in clinical virology. J Clin Virol 2007;40:93–8.

[21] Landis JR, Koch GG. The measurement of observer agreement of categorical data. Biometrics 1977;33:159–74.

[22] Salas-Coronas J, Vázquez-Villegas J, Lozano-Serrano AB, Soriano-Pérez MJ, Cabeza-Barrera I, Cabezas-Fernández MT, Villarejo-Ordóñez A, Sánchez-Sánchez JC, Abad Vivas Pérez JI, Vázquez-Blanc S, Palanca-Giménez A, Cuenca-Gómez JA. Severe complications of imported schistosomiasis, Spain: a retrospective observational study. Trav Med Infect Dis 2020;35:101508.

[23] Kittur N, Castleman JD, Campbell CH, King CH, Colley DG. Comparison of *Schistosoma mansoni* prevalence and intensity of infection, as determined by the circulating cathodic antigen urine assay or by the kato-katz fecal assay: a systematic review. Am J Trop Med Hyg 2016;94:605–10.

[24] Marti H, Halbeisen S, Bausch K, Nickel B, Neumayr A. Specificity of the POC-CCA urine test for diagnosing *S. mansoni* schistosomiasis. Trav Med Infect Dis 2020;33: 101473.

[25] Volkman T, Clifford V, Paxton GA. *Schistosoma* serology after praziquantel treatment of *Schistosoma* infection in refugee children resettled in Australia: a retrospective analysis. Trav Med Infect Dis 2020;37:101680.

[26] Cohen JF, Korevaar DA, Altman DG, Bruns DE, Gatsonis CA, Hooft L, et al. STARD 2015 guidelines for reporting diagnostic accuracy studies: explanation and elaboration. BMJ Open 2016;6:e012799.