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Development and application of *Sm1-7*-qPCR as an alternative for schistosomiasis diagnosis in low-endemic areas

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Affecting about 241.4 million people worldwide, *Schistosoma* (*S.*) *mansoni*, the main etiological agent of intestinal schistosomiasis, is one of the most prevalent diseases in the world remaining a public health issue, especially in developing countries in South America, Africa and the Caribbean. In Brazil, more than 30 million people are at risk of infection, and nearly 1.5 million are infected by *S. mansoni*[1]. Although the implemented control measures proved to be efficient and contributed to a decrease in morbidity and mortality associated with *S. mansoni* infections, they led to a new epidemiological scenario characterized by low-intensity infections and areas with low prevalence, presenting a new challenge towards the elimination and control of schistosomiasis[2].

The parasitological diagnosis of intestinal schistosomiasis is mainly performed by Kato-Katz (KK), a low-cost, relatively simple and highly specific technique recommended by the World Health Organization as a reference diagnostic method[1]. Although the technique shows high specificity, its sensitivity depends on the amount of parasite eggs present on the stool samples, presenting high performance in patients with moderate and heavy infection, but lacking in sensitivity in light infections, which contributes to the maintenance of the transmission and reinfection in those areas[3].

In this context, *Schistosoma* spp. DNA amplification techniques have been previously studied and proven to be a suitable choice for schistosomiasis diagnosis, control and further elimination. In that matter, different *S. mansoni* molecular targets were described and explored in a few different molecular technologies, such as LAMP reactions and PCR assays[4]. Although a large number of targets have been listed in the scientific literature, the most widely used is the *Sm1-7* gene, described by Hamburger *et al.*[5] because of its high repetition in the worm's genome, as well as its specificity.

Among the molecular alternatives, Real-Time PCR shows high sensitivity and specificity in low-endemicity areas, outperforming parasitological methods[3,6]. Thus, the present study aimed to present the development and evaluate the performance of a Real-Time PCR assay for the detection of *S. mansoni* DNA in stool samples collected in a low endemic area.

A cross-sectional study was conducted in the Catamarã district in Jaboatão dos Guararapes municipality, Pernambuco state-Brazil. The area, considered low endemic for schistosomiasis infections, presented a prevalence of 1.52%. Stool samples were collected in the study area during May 2023 and taken to the Schistosomiasis Reference Laboratory-Fiocruz PE, where the samples were processed by KK technique in duplicates using the Helm-Test® (BioManguinhos-Fiocruz, Rio de Janeiro, Brazil). The parasite burden was classified based on the amount of *S. manosni* eggs present on the slides according to WHO guidelines[7]. Also, the remaining fecal material was examined with a modified spontaneous sedimentation method Hoffman, Pons and Janer (HPJ)[8], in which all the sediment was observed. KK and HPJ were chosen as comparative diagnostic methods to evaluate the qPCR presented in this study.

For the qPCR assay design, new custom assay was developed

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with the aid of Thermo Fisher platform (thermofisher.com) using the highly specific 121 bp tandem repeat of *S. mansoni* described by Hamburguer *et al.*, 1991 (GenBank accession number M61098), a sequence that corresponds to 12% of the worm's genome. The sequences obtained were SmFW: 5'- CGACCAACCGTTCTATGAAAATCGT-3', SmRV: 5'-CCACGCTCTCGCAAATAATCTAAAA-3' and the probe SondaSM: 5'- [FAM]TCCGTCCAGTGGTTTC[NFQ]-3'.

For the new system standardization and limit of detection determination, three different protocols were tested, each one performed in triplicate using the positive control as a DNA template. For that, *S. mansoni* adult worms were collected and had their genomic DNA extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted DNA was further quantified, with the NanoDrop 2000 (Thermo Fisher Scientific, Massachusetts, USA), and diluted in a 10-fold serial dilution curve containing 1 ng/μL-1 fg/μL. All tested protocols were standardized to run with the Quant Studio 5 (Thermo Fisher Scientific, Massachusetts, USA) under the following conditions: initial reaction heating at 50 ℃ for 2 minutes, followed by initial denaturation of the double strand at 95 ℃ for 10 minutes to ensure that all DNA present in the reaction is in single-stranded form for the next steps. After primary denaturation, the solution undergoes a step of 40 cycles, consisting of a denaturation step at 95 ℃ for 15 seconds, followed by the annealing and extension step, which occurs at 60 ℃ for 15 seconds. After the 40 cycles, the reaction is maintained at 60 ℃ for 10 minutes for final annealing and extension. The selected protocol contained 5 μL of TaqMan® Universal PCR Master Mix 2×, 0.041× of the Forward and Reverse primers, and 0.041× of the probe. The *Sm1-7* Real-Time PCR (*Sm1-7*-qPCR) was able of amplifying up to 1 fg/μL with a corresponding Ct value of 30.74, this being considered the cut-off point to determine positive and negative samples.

The assay's analytical specificity was determined by a Megablast search performed by the submission of the primers and probe sequences to the National Center of Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the Nucleotide collection, which resulted in no probable alignment with any organism except *S. mansoni*. Also, 1 ng/μL of purified genomic DNA of *Wuchereria bancrofti*, *Mycobacterium tuberculosis*, *Strongyloides stercoralis*, *Leishmania infantum*, *Leishmania braziliensis*, and *Escherichia coli* was submitted to the optimised protocol, and no cross-reactivity were observed.

The *Sm1-7*-qPCR was conducted according to the standardized conditions in triplicates along with an Internal Positive Control reaction (IPC) (Thermo Fisher Scientific, Massachusetts, USA), following the manufacturer's instructions, to evaluate the DNA quality and the presence of inhibitors.

To assess the true infection status of the tested patients, a combined reference method was used. Samples considered positive for schistosomiasis presented a positive result by any parasitological test (KK and/or HPJ), whereas the negative samples were those negatives for both tests.

The positivity rate, sensitivity, specificity, Cohen Kappa (to evaluate the concordance between diagnostic tests) and Fleiss' Kappa index (to evaluate the agreement between categorical classifications), area under the curve, positive and negative predictive values were estimated with a 95% confidence interval using the GraphPad Prism 8 and and R softwares (GraphPad Software, Boston, Massachusetts USA; R version 4.4.1, https://www.r-project.org/).

This project was approved by the Ethics Committee of the Aggeu Magalhães Research Institute/Fiocruz on February 27th 2023 (CAAE: 58361422.9.0000.5190/ Report number: 5.652.210). All individuals who agreed to participate in this research and signed an Informed Consent Form were included on the study.

A total of 351 patients agreed to participate in the study. All samples were diagnosed by the KK and HPJ methods, showing a positivity rate of 12.54% (44/351) for both methods. The lowest parasite load observed by KK was 12 EPG and the higher was 372 EPG. The average parasite load of the studied population was 68.21 EPG (95% *CI* 36.27-100.20). From the 38 KK positive samples, 32 of them (84.21%) were light infections, whereas 6 samples (15.79%) were classified as moderate infections.

The *Sm1-7*-qPCR detected 163 cases with a positivity rate of 46.44% (163/351). The minimum Ct of the positive samples confirmed by KK and HPJ was 19.67, while the maximum was 30.55, with an average Ct of 25.68 for *Sm1-7*-qPCR. Four samples were positive by KK, 6 for HPJ, and negative by PCR. The individuals classified with a low parasite load, by the KK, had an average Ct of (26.67±2.98) (95% *CI* 25.51-27.20), whereas individuals with moderate infections showed an average Ct of (22.92±1.71) (95% *CI* 21.12-24.71).

Considering the mean Ct of the positive samples by KK and the *Sm1-7*-qPCR, the samples were classified according to their parasite load as low or moderate by their Ct values. Samples that showed the characteristic Ct value between 23.69 and 24.63 were considered "Undefined". A total of 27 samples were classified in accordance with the *Sm1-7*-qPCR and KK, whereas 7 discordances were observed, 3 of them classified as undefined by their Ct values. The Fleiss Kappa was estimated to be 0.45 indicating a moderate agreement between KK and the *Sm1-7*-qPCR.

The *Sm1-7*-qPCR assay demonstrated variable diagnostic performance across reference standards, showing highest sensitivity (90.24%) when compared to KK, while maintaining consistently moderate specificity (56.10%-59.61%) and high negative predictive values (96.32%-97.87%) across all comparisons. In comparison to the composite reference standard, the assay showed intermediate

Table 1. Sensitivity, specificity, predictive values and Cohen's Kappa agreement between the *Sm1–7*-qPCR, and parasitological methods of Kato-Katz and Hoffman, Pons and Janer.

Reference methods	Sensitivity (95% *CI*)	Specificity (95% *CI*)	PPV (95% *CI*)	NPV (95% *CI*)	Cohen's Kappa
KK	90.24 (77.45-96.14)	58.79 (53.26-64.10)	22.29 (16.63-29.21)	97.87 (94.66-99.17)	0.211
HPJ	76.92 (57.95-88.97)	56.10 (50.69-61.37)	12.20 (8.03-18.09)	96.84 (93.28-98.54)	0.124
KK+HPJ	85.11 (72.31-92.59)	59.61 (54.03-64.95)	24.39 (18.45-31.50)	96.32 (92.59-98.2)	0.218

KK: Kato-Katz, HPJ: Hoffman, Pons and Janer.

sensitivity (85.11%) and specificity (59.61%), AUC=0.79 (Figure 1) with Cohen's Kappa values of 0.22, indicating fair agreement between the molecular test and conventional parasitological methods (Table 1).

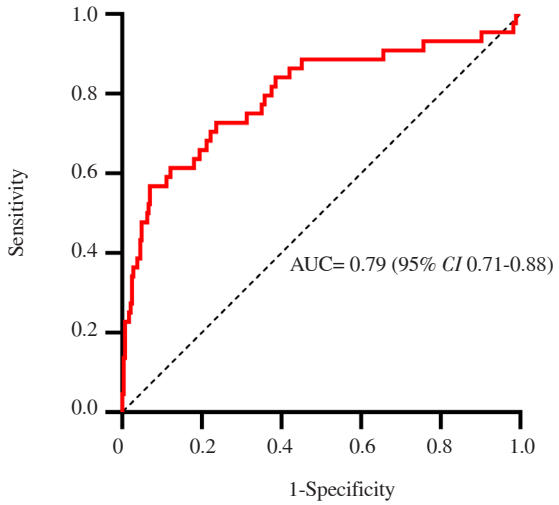

Figure 1. ROC curve Plot of the *Sm1–7*-qPCR compared to the reference method.

This study demonstrates that the *Sm1–7*-qPCR assay can accurately detect small amounts of *S. mansoni* DNA in low endemic areas. Furthermore, the new system shows promising evidence of its use as a quantitative diagnostic approach to schistosomiasis, capable of classifying the parasite load of infected individuals. The *Sm1–7*-qPCR was able to detect up to 1 fg/µL of *S. mansoni* genomic DNA, less than a single copy of the parasite's genome[9]. Also, the *Sm1–7*-qPCR did not present probable alignment with any sequence available in the NCBI database or cross-reactivity with the tested parasites or bacteria.

In the population survey carried out in this study, the positivity rate of the *Sm1–7*-qPCR (46.44%) was higher than that observed by the parasitological methods KK (12.54%), which may indicate the greater sensitivity of the assay compared to parasitological approaches. A study performed in a low endemic area, observed a positivity rate of 9.6% for qPCR in stool samples, and the corresponding positive rate was only 0.6% for KK and HPJ diagnosis[6]. Similar results were observed in another low endemic area for schistosomiais, with a positivity rate of 16.5% for qPCR and 5.5% for KK[4], demonstrating the better performance of molecular methodologies.

In comparison with the composite method, *Sm1–7*-qPCR achieved 85.11% sensitivity and 59.61% specificity, while Cohen's Kappa (0.22) suggested slight agreement between the assays. This low concordance, along with the low specificity of the new test, is likely due to the limitations of the composite reference method, which relied on techniques with inherently low sensitivity. For instance, a study using KK and HPJ as a composite reference reported a Kappa value of 0.11, reflecting minimal agreement between methods[6]. In contrast, other studies using more sensitive composite reference methods—such as 18 KK smears + Helmintex + Saline Gradient, or qPCR combined with two KK smears—achieved substantial (κ=0.71) and almost perfect agreement (κ=0.92), respectively[3,10]. These differences highlight the critical role of the reference method's sensitivity on agreement metrics and can be noticed when analyzing the *Sm1–7*-qPCR specificity.

Although the *Sm1–7*-qPCR did not achieve high agreement with the reference method, the assay showed high sensitivity, coinciding with other qPCR assays[3,6], indicating the high capacity of the molecular methods to identify truly infected individuals.

The studies mentioned above, including ours, used the same molecular target[5], but the composite reference methods applied to estimate the performance of these methods were significantly different from each other[6], highlighting the bias' induction of an imperfect gold standard[19].

This study demonstrates the usefulness of the *Sm1–7*-qPCR system for classifying infection intensity in individuals with low-to-medium parasite loads using qPCR's Ct values. While the system accurately identified light infections, it showed limited precision in the determination of low-intensity infections (Fleiss' Kappa=0.45), while other studies that also tried to quantify infections' intensity with molecular methods, found different Ct ranges[3]. These findings highlight both the potential and current limitations of *Sm1–7*-qPCR for parasite load quantification. Further validation studies across diverse endemic settings-including patients with heavy infections -are required to establish more robust Ct thresholds that better correlate with KK-derived egg counts and improve the system's classification accuracy.

The proposed *Sm1–7*-qPCR system shows potential for schistosomiasis diagnosis when combined with more specific methods (*e.g.*, multiple KK or Helmintex). Its high sensitivity

and negative predictive value reliably exclude negatives, while positives would require confirmatory testing. However, reliance on imperfect gold standards like KK not only sustains transmission in endemic areas, but also biases new diagnostic development, hindering elimination efforts[2,4]. Thus, composite reference methods combined with qPCR offer a promising solution to these limitations.

Conflict of interest statement

We declare that we have no conflict of interest.

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Authors' contributions

EP, WB, FM and EG participated in the conceptualization; EP, NC and BG participated in the data collection, analysis; EP, WJ, FM and EG contributed on data analysis, article drafting and critical revision.

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