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Optimization of the Helmintex method for schistosomiasis diagnosis

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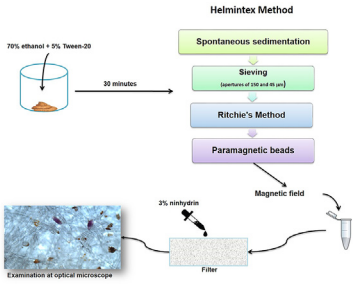
HIGHLIGHTS

- Modifications performed in the Helmintex method significantly decreased the time for completing examination.
- The treatment of fecal debris with Tween-20 significantly reduced the final sediment produced by the Helmintex method.
- Incubation with Tween 20, removal of the 75 μm aperture sieve and staining with ninhydrin resulted in improved egg recovery.

GRAPHICAL ABSTRACT



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ABSTRACT

A diagnostic test that is reliable, sensitive, and applicable in the field is extremely important in epidemiological surveys, during medical treatment for schistosomiasis, and for the control and elimination of schistosomiasis. The Helmintex (HTX) method is based on the use of magnetic beads to trap eggs in a magnetic field. This technique is highly sensitive, but the screening of fecal samples consumes lots of time, thus delaying the results, especially in field studies. The objective of this work was to determine the effects of incorporation of the detergent Tween-20 into the method in an attempt to decrease the final pellet volume produced by the HTX method as well as the use of ninhydrin to stain the *Schistosoma mansoni* eggs. We showed that these modifications reduced the final volume of the fecal sediment produced in the last step of the HTX method by up to 69% and decreased the screening time to an average of 10.1 min per sample. The use of Tween 20 and ninhydrin led to a high percentage of egg recovery (27.2%). The data obtained herein demonstrate that the addition of detergent and the use of ninhydrin to the HTX process can optimize the screening step and also improve egg recovery, thus justifying the insertion of these steps into the HTX method.

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1. Introduction

Schistosomiasis mansoni is a parasitic disease that is becoming more prevalent, despite the advances and efforts to control its spread (Chitsulo et al., 2000). A definitive diagnosis of this disease is based on the identification of the parasite eggs in the feces of

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infected individuals (Albonico et al., 2012; Levecke et al., 2014). However, most methods currently used in schistosomiasis diagnosis do not show sufficient sensitivity in areas where it has been recently introduced or where actions have been implemented to control the disease, leading to a decrease in parasite load, thus resulting in a lower number of eggs in the feces (Corachan, 2002; Noya et al., 2002).

The World Health Organization (WHO) recommends using the Kato-Katz (KK) method for schistosomiasis diagnosis (Katz et al., 1972) in field studies as it is a simple and low-cost technique. The KK method has a specificity of 100%, but its sensitivity varies with the prevalence and intensity of infection (Gray et al., 2011). According to a study conducted by Enk et al. (2008), a single sample examined by the KK method is not sufficient for the diagnosis of positive cases of schistosomiasis in low endemic areas.

A significant advance in the diagnosis of schistosomiasis in low endemic areas was the development of the Helmintex (HTX) method, which promotes the isolation of *S. mansoni* eggs based on their interaction with paramagnetic particles in a magnetic field (Teixeira et al., 2007). This technique has 100% sensitivity for egg burdens above 1.3 eggs per gram of feces (Teixeira et al., 2007), which is greater than the method currently recommended by the WHO (Caldeira et al., 2012; Pinheiro et al., 2012). Nonetheless, the HTX method requires several steps that culminate in a final sediment to be analyzed through the reading of an average of 21 slides. Due to the time required to complete the whole analysis, the method is not feasible for large-scale use or field studies.

The aims of this study were to develop a modified HTX method that includes two new steps, in order to make it more efficient and less time consuming, and to compare it to the standard HTX method. Therefore, we tested the incorporation of a nonionic detergent, Tween-20 (Helenius et al., 1979); incubation with cellulase enzyme, which is capable of hydrolyzing cellulosic materials (Lynd et al., 2002; Tolan, 2002); and the use of ninhydrin staining of the final sediment, in order to stain the *S. mansoni* eggs (Bell, 1963).

2. Material and methods

2.1. Biological material

Schistosoma mansoni eggs were obtained from infected mouse livers used for the maintenance of the parasite cycle in the Parasitic Biology Laboratory of the School of Biosciences, PUCRS (Ethics clearance, CEUA- 15/00443).

Human feces samples, hookworm eggs, and *Ascaris lumbricoides* eggs were obtained from a survey in the municipality of Januária, Minas Gerais, Brazil, in association with the Institute of Biological Sciences (UFMG) (MCTI, CNPq, MS-SCTIE, - Decit No. 40/2012).

Fasciola hepatica eggs were obtained from sanitary inspections of slaughterhouses provided by the Laboratory for Molecular Biology of Cestodes of the Biotechnology Center of the Federal University of Rio Grande do Sul (UFRGS). The worms were crushed and mixed with 0.9% saline solution, filtered through sieves with apertures of 150, 75, and 45 μm, and the eggs retained in the 45-μm sieve were stored in 0.9% saline solution at −20 °C.

All the stool and egg samples obtained for this study were separated in different aliquots, fixed, and maintained in 0.9% saline solution at −20 °C, 10% formaldehyde, 70% ethanol, or 100% ethyl acetate, according to each protocol used.

2.2. Standard Helmintex method

The standard HTX procedure was performed as described by Teixeira et al. (2007). Briefly, 30 g of feces were fixed in 10% formaldehyde and filtered through a sieve with an aperture of 500 μm. After consecutive washes and sedimentation, the pellet was passed through three different sieves (apertures of 150, 75, and 45 μm). The material retained on the last sieve (45 μm) was processed by the Ritchie method (1948), and 19 μL of paramagnetic particles (Bangs Labs, USA) was added to the final sediment. After incubation for 30 min, the microtubes containing the final sediment were placed in contact with a rare earth magnet (Bangs Labs, USA), and the material that did not adhere to the wall of the microtube was discarded. The samples were placed on slides, covered with a coverslip, and examined by compound optical microscopy using a 10 × magnification objective lens. The entire coverslipped area was examined.

2.3. Modified HTX method

The following modifications were made to the standard HTX method and compared against the standard method described above.

Modification 1: a) Sample fixation with 70% ethanol; b) use of filter paper as a support for staining of the final pellet with 3% ninhydrin for 15 min at 24 °C.

Modification 2: a) Sample fixation with 70% ethanol and treatment with detergent (0.5%, 2.5%, or 5% Tween-20; T-0.5, T-2.5, and T-5, respectively); b) elimination of the 75-μm sieve.

Modification 3: a) Sample fixation with 70% ethanol and treatment with detergent (T-5); b) elimination of the 75-μm sieve; c) use of filter paper as a support for staining of the final pellet with 3% ninhydrin for 15 min at 24 °C.

Modification 4: a) Sample fixation with 70% ethanol; b) elimination of the 75-μm sieve; c) sample treatment with 5 mg/mL cellulase (C-5) to reduce the sediment volume obtained in the 45-μm sieve; d) use of filter paper as a support for staining of the final pellet with 3% ninhydrin for 15 min at 24 °C.

2.4. Ninhydrin staining of eggs

For staining of the final sediment, three different concentrations of ninhydrin solution (Sigma-Aldrich) were tested: 1%, 2%, and 3% ninhydrin in 70% ethanol. For each concentration, three groups of 20 *S. mansoni* eggs in 10% formaldehyde and three groups of 20 *S. mansoni* eggs in 0.9% saline solution were placed on filter paper, covered with 500 μL of each ninhydrin solution, respectively, and incubated at 21 °C for 60 min. The eggs that had purple coloration were counted. The experiments were performed in duplicate.

From the observation that the eggs fixed with 10% formaldehyde were not stained by ninhydrin, other methods were tested. Groups of 100 S. *mansoni* eggs were seeded in stool samples, which were then fixed with 70% ethanol or 100% ethyl acetate and kept in 0.9% saline (control). The samples were processed by the standard HTX method and stained with the three ninhydrin concentrations as described above. The experiments were performed in triplicate.

Considering that the best results were obtained after the fixation of samples with 70% ethanol and 3% ninhydrin, two groups of 20 *S. mansoni* eggs fixed with 70% ethanol were processed according to modification 1 of the HTX method to the final sediment. The samples were then placed onto qualitative filter paper (5 cm × 2.5 cm; UNIFIL 24-μM pore, Brazil) and stained with 3% ninhydrin. The filter papers were placed on glass slides, moistened with 70% ethanol, and examined by light microscopy (10 × magnification). The eggs were counted and checked for staining. The experiments were performed in triplicate.

To determine the optimal time and temperature for the staining, eight groups of 20 *S. mansoni* eggs were seeded in 100 μL of the final pellet obtained by modification 1 of the HTX method. The

sediments were stained with 3% ninhydrin and exposed to different temperatures: 18 °C, 19 °C, 20 °C, 21 °C, 22 °C, 23 °C, 24 °C, and 37 °C. The samples were checked every 10 min for 60 min to verify the progress of the staining. The eggs were counted and checked for staining. The experiments were performed in triplicate.

To verify if the use of ninhydrin would result in a more efficient HTX method, two groups containing 10 samples of 30 g of feces and 100 eggs each were tested. The first group was processed according the standard HTX method, and the second group was processed according modification 1 of the standard HTX method. The sediments were analyzed using optical microscopy, and the eggs were counted and checked for staining. The time consumed to analyze the sample was also counted.

In order to verify if ninhydrin would stain other helminth eggs, 20 eggs each of *F. hepatica*, *A. lumbricoides*, and hookworm were seeded in the final sediment of the standard HTX method. The samples were stained with 3% ninhydrin, and the staining intensity was observed.

2.5. Tween 20 detergent treatment of the final fecal sediment

In order to evaluate the effect of treating the final sediment obtained from the HTX method with detergent, Tween 20 was tested at different concentrations: 0.5% (T-0.5), 2.5% (T-2.5), and 5% (T-5) in 70% ethanol. The Tween 20 samples were added to samples of 30 g of feces in a sufficient volume to cover the whole fecal sample. After incubation for 30 min, the sample was processed by modification 2 of the standard HTX method (Section 2.3 above), and the final volume was measured.

After opting for the concentration that showed a better volume reduction of the final sediment, two groups with 15 samples containing 30 g of feces each were used. Group I was processed according to the standard HTX method, and group II was fixed with T-5 and processed by modification 2 of the standard HTX method. The final sediment volume was measured for each sample.

2.6. Comparison between the standard HTX method and modification 3

In order to verify if the use of T-5 solution and ninhydrin optimizes the performance of the HTX method without reducing its sensitivity, ten sets of 100 *S. mansoni* eggs were seeded in ten samples of 30 g of feces, and the samples were processed according to modification 3 of the standard HTX method. Next, the filter papers were analyzed by optical microscopy. The numbers of generated filters as well as recovered *S. mansoni* eggs were counted and checked regarding coloration. The time spent screening the samples was also counted.

2.7. The use of cellulase to decrease the final sediment volume

The use of the enzyme cellulase (*Aspergillus niger* 9012-54-8 EEC No. 232734–4, Sigma-Aldrich, USA) was tested at three different concentrations, diluted in sodium citrate buffer (10 mM, pH 4.8; Dynamic-Brazil): 0.25 mg/mL (C-0.25), 1.2 mg/mL (C-1.2), and 5 mg/mL (C-5).

To test the effect of the enzyme, three samples of the sediment retained in the 45-μm sieve were separated and transferred to 15-mL tubes. C-0.25, C-1.2, and C-5 were added in a sufficient volume to cover the pellet. The tubes were incubated at 37 °C for 2 h. Then, the pellet was again passed through a 45-μm sieve and processed according to the last steps of the standard HTX method. The final volume of the sediment was measured and recorded.

After defining which cellulase concentration led to a greater reduction of the final sediment volume, two groups of 15 samples containing 30 g of feces were separated. Group I was processed according to the standard HTX method. Group II was processed without the 75-μm sieve and the addition of the C-5 cellulase solution to the pellet retained on the 45-μm sieve. The sediment was incubated at 37 °C for 2 h. The pellet was then treated as described previously. The final volume of the sediment was measured.

2.8. Comparison between the standard HTX method and modification 4

In order to analyze the recovery of the eggs in the final sediment after treatment with the C-5 solution, 100 *S. mansoni* eggs were seeded in ten samples of 30 g of feces. The samples were processed according to modification 4 of the standard HTX method. The number of generated filters was noted; in addition, the recovered *S. mansoni* eggs were counted and checked regarding coloration. The time consumed to screen the samples was counted.

2.9. Identification criteria for S. mansoni eggs in the preparation stained with ninhydrin

Fig. 1 illustrates the findings used as criteria for identification of *S. mansoni* eggs in preparations stained with ninhydrin: (a) size (approximately 150 μm); (b) shape; (c) lateral spicule; (d) sharp delimitation of the shell; (e) purple/blue color (ninhydrin staining); and (f) empty space between the shell and internal content (the miracidium). Size, lateral spicule, and shell delimitation should be considered “major” criteria. At least two major criteria and one minor criterion (color, shape, or empty space) are required to establish diagnosis, but extensive evaluation should be performed to validate the proposed “major” and “minor” classification criteria.

2.10. Statistical analysis

The statistical analysis was performed using analysis of variance (ANOVA) and the Tukey test for comparison between the standard HTX groups and the different HTX modification methods ($p \leq 0.05$), while the paired Student's t-test was used for comparison between the standard HTX method and those that used Tween-20 or cellulase.

3. Results

3.1. The efficiency of S. mansoni egg staining using ninhydrin varied according to the fixative agent and the temperature

The eggs were colored purple when ninhydrin was used at all three concentrations (1%, 2%, and 3%) at 21 °C, although variations in the staining time occurred (Table 1). In eggs treated with 3% ninhydrin, the purple coloration could be observed after 27 min (Fig. 1). The samples did not stain when 10% formaldehyde was used.

The time required to stain the eggs with 3% ninhydrin decreased as the temperature increased. At 37 °C, the eggs became colored at 10 min. However, at 24 °C, purple coloration was achieved at 15 min. Therefore, since 24 °C is similar to room temperature, it was chosen as the temperature for further experiments.

F. hepatica and hookworm eggs also were stained with 3% ninhydrin.

3.2. Staining of the final pellet with ninhydrin and standard HTX processing led to a reduction in reading times but a decreased egg recovery

The samples produced by the standard HTX method presented a

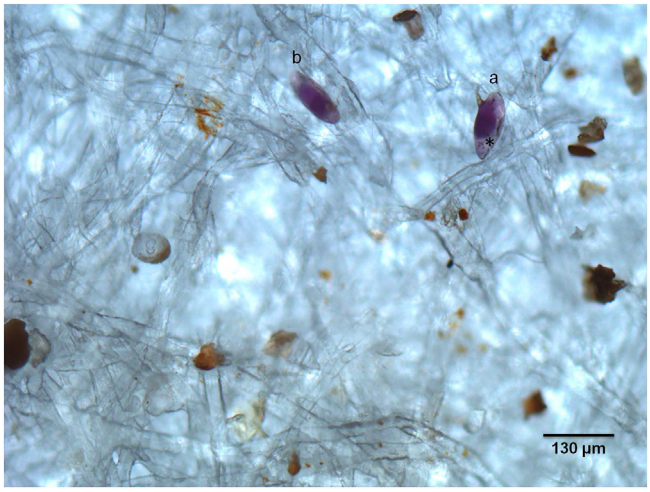

Fig. 1. *S. mansoni* eggs in human fecal sediment stained with 3% ninhydrin (100 × magnification), illustrating the identification criteria: size, purple color, shape, spine, well-defined wall (arrow head), and the internal empty space (asterisk). One egg (a) presents all criteria, while the image of another egg (b) lacks the typical shape and a visible spine. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

recovery of 20% of the total eggs seeded in the initial stool sample when the entire sample was examined. An average of 20 slides were prepared and examined in 6 h and 26 min. Modification 1 of the HTX method, which was carried out with the addition of ninhydrin to the final sediment, presented a total of 13.2% of eggs stained in the entire sample, and 4 filters were prepared and examined in 23 min (Figs. 2–4).

3.3. The use of tween 20 detergent in the fixation step led to a reduction in the final sediment volume produced by the HTX method

Modification 2 of the HTX method led to a reduction of 13± 6% ($p = 0.011$) of the final sediment volume with T-0.5, 38± 3% ($p < 0.0001$) with T-2.5, and 54± 5% ($p < 0.0001$) with T-5, compared with the final sediment volume of the standard HTX method.

The use of T-5 solution in 15 samples led to a significant ($p < 0.0001$) reduction in the final sediment volume with the modified method, with a mean volume reduction of 69% compared to the standard method (Fig. 5).

Table 1
Mean time used to stain S. mansoni eggs with ninhydrin.

Ninhydrin solution	Ninhydrin	Coloration	Average time for staining (min)
Formaldehyde 10%	1%	–	–
	2%	–	–
	3%	–	–
Saline 0,9%	1%	+	51
	2%	+	43
	3%	+	27
Ethanol 70%	1%	+	52
	2%	+	40
	3%	+	24
Ethyl acetate 100%	1%	+	54
	2%	+	37
	3%	+	29

Results represent the average of three triplicate: negative staining (–); positive staining (+).

3.4. The use of cellulase led to a reduction in the final sediment volume produced by the HTX method

The addition of cellulase to the sediment retained in the 45-µm sieve showed a volume reduction of 14.7% with the use C-0.25, 27.9% with C-1.2, and 41.7% with C-5, when compared to the final sediment volume produced by the standard HTX method.

The addition of C-5 to 15 samples led to a significant ($p < 0.0001$) reduction in the final sediment volume with the modified method, with a mean volume reduction of 48% compared to the standard method (Fig. 6).

3.5. Detergent addition and ninhydrin staining improved the performance of the HTX method

With the addition of the detergent Tween 20 and ninhydrin to the stool samples containing *S. mansoni* eggs, a 27.2% recovery of the seeded eggs was obtained. The final sediment was examined in 10 min by screening an average of 2.6 filter papers per sample (Figs. 2–4).

3.6. Cellulase treatment of the HTX final sediment decreased the recovery of S. mansoni eggs

The use of cellulase showed a recovery of 11.2% of the eggs, while the standard HTX method showed a recovery of 20%. The average number of filter papers used to screen the samples was 3.3, and an average of 12 min was needed to analyze each sample (Figs. 2–4).

4. Discussion

It is well known that efforts to control schistosomiasis are hindered by the lack of sensitive diagnostic tools (Sandoval et al., 2006; Spear et al., 2012). The control of this parasite relies on mass drug administration in endemic areas in an attempt to alleviate morbidity of individuals affected by this disease (Geary, 2012). However, effective therapy has the potential to turn those areas into low endemic areas, where the infected population will present a low worm burden when examined, making detection of the

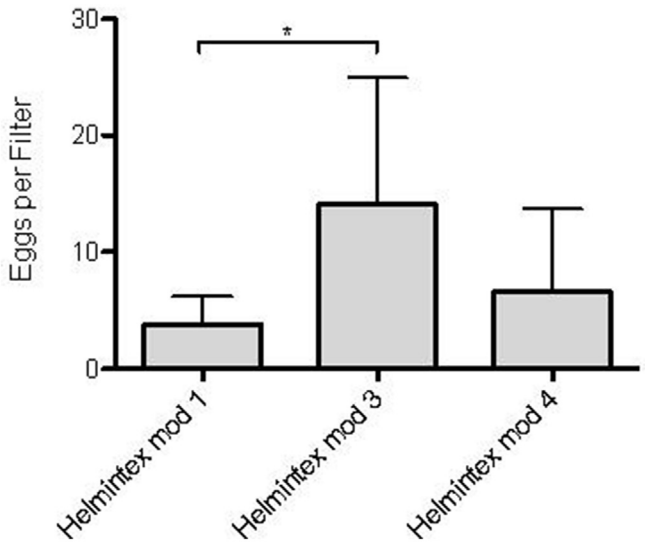

Fig. 2. Mean number of eggs per filter paper together with standard deviations shown as error bars for each modified Helmintex method. * indicates a significant difference ($p < 0.05$).

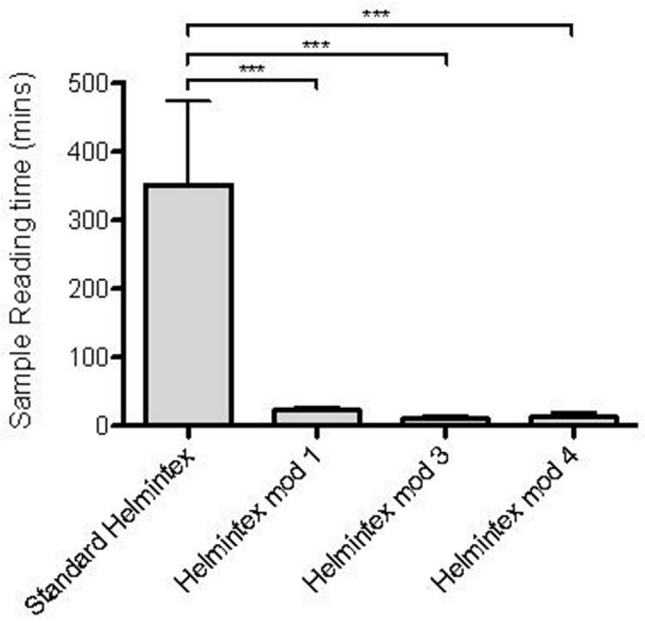

Fig. 3. Mean time required to read a sample with standard deviations shown as error bars for each modified Helmintex method. *** indicates a significant difference ($p < 0.0001$).

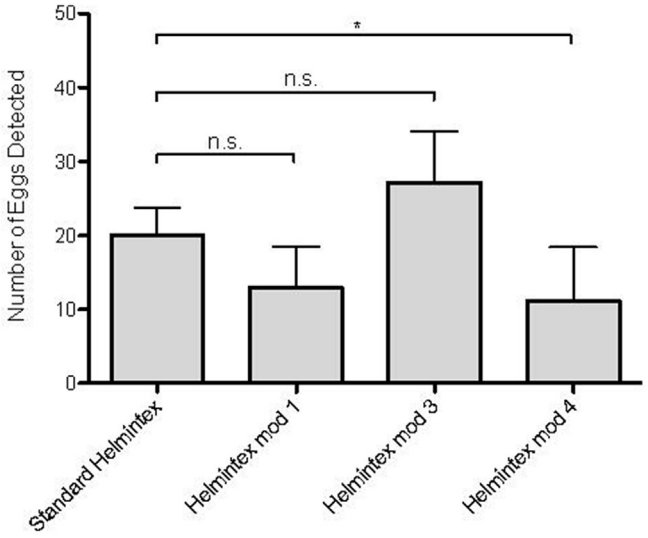

Fig. 4. Means and standard deviations of the total number of eggs detected in 10 samples of seeded feces for each modified Helmintex method. ANOVA indicates that the means are significantly different. Tukey's multiple comparison test was used to determine whether the difference is significant from the standard Helmintex method; n. s. indicates not a significant difference; * indicates a significant difference ($p < 0.05$).

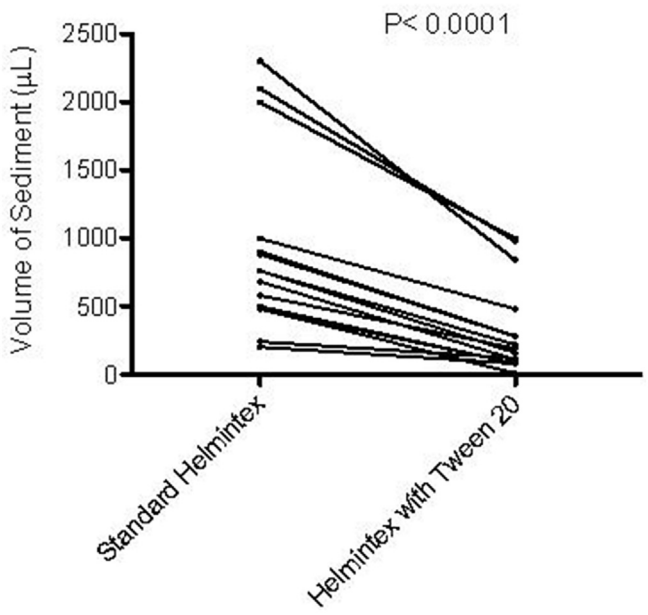

Fig. 5. Sediment volumes at the final step using the standard Helmintex method compared to the Hemintex method with Tween 20 (modification 2) for 15 samples of seeded feces. The Student's paired *t*-test indicates a significant ($p < 0.0001$) reduction in the final sediment volume with the modified method, with a mean volume reduction of 69± 15% compared to the standard method.

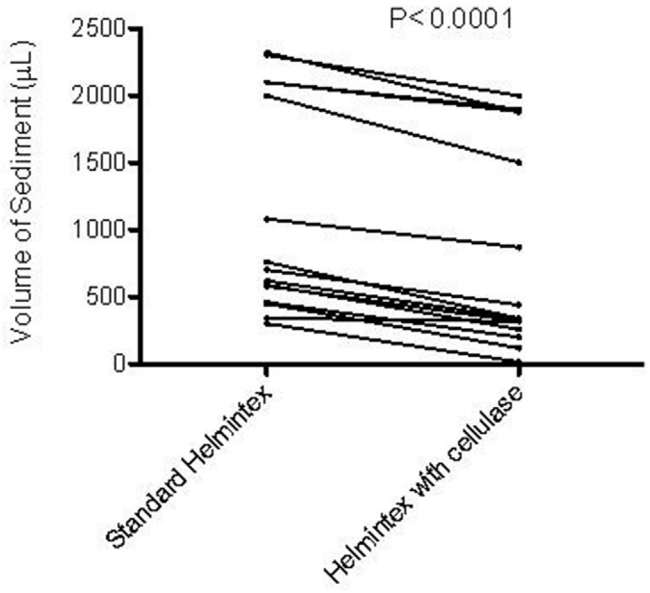

Fig. 6. Sediment volumes at the final step using the standard Helmintex method compared to the Hemintex method with cellulase (modification 4) for 15 samples of seeded feces. The Student's paired *t*-test indicates a significant ($p < 0.0001$) reduction in the final sediment volume with the modified method, with a mean volume reduction of 48± 26% compared to the standard method.

parasite in those individuals very difficult (Spear et al., 2012). Therefore, a reliable and very sensitive new gold standard technique applicable in field studies is urgently needed.

The WHO recommends the KK method for epidemiological studies, but it lacks sensitivity for lower egg burdens (Ebrahim et al., 1997; Zhanga et al., 2009). In individuals excreting fewer than 100 eggs per gram of feces, the estimated sensitivity is 60% (Alarcón de Noya et al., 1992; Noya et al., 2002).

Recent studies have shown that the sensitivity of the HTX method is superior to that of the KK method (Caldeira et al., 2012; Pinheiro et al., 2012). Data presented by Teixeira et al. (2007) show that the HTX method recovers 30 times more eggs than the KK method. This is a huge improvement in parasitological diagnostics, and the HTX method currently stands as one of most sensitive methods in the validation stage.

Nevertheless, the HTX method presents some disadvantages. For example, it is difficult to perform in field studies because laborious processing is necessary. Another disadvantage is the time required to analyze the numerous slides generated from the last

step of the method.

Taking into consideration these difficulties, the present study aimed to implement solutions that could improve and optimize the performance of the HTX method by reducing the final sediment volume and staining the eggs to facilitate their visualization in the final sediment.

Bell (1963) proposed staining *S. mansoni* eggs with ninhydrin. Ninhydrin is an oxidizing agent that reacts with α-amino acids at a pH between 4 and 8, generating a compound with a purple color (Plummer and Plummer, 1988). However, combining the HTX method and Bell's method would add to the processing time because Bell's original process requires a 12-h incubation with ninhydrin solution (Bell, 1963). In order to reduce this processing time, modifications of the original methods were evaluated. In this study, it was shown that 3% ninhydrin staining of *S. mansoni* eggs is accomplished within 15 min at room temperature. The use of ninhydrin also significantly reduced the time for complete screening of samples by microscopy from an average of 6 h–23 min.

In addition, we evaluated the specificity of ninhydrin staining using eggs from other parasites that have a similar size to those of *S. mansoni*, which would likely be present in the HTX final sediment. The observation that *F. hepatica* and *Ancylostoma* sp. eggs were also stained does not affect the application of ninhydrin in the process as the morphological characteristics are still preserved and *S. mansoni* eggs are easily recognized when compared to other eggs of the same size.

In an attempt to reduce the final sediment volume produced by the HTX method, the addition of cellulase and Tween 20, separately, was tested. In general, the cellulase produced by filamentous fungi presents its best activity under acidic conditions (pH 3.6–5.0) and at high temperatures such as 54 °C (Castro, 2010). In the present study, the incubation with cellulase was done at 37 °C and pH 4.8, leading to a 48% reduction of the final sediment volume. It is possible that incubation of the sediment with the enzyme at higher temperatures could result in an even lower volume. However, the conditions used here were chosen to avoid shrinkage of the miracidium, minimize structural distortions of the eggs, and reduce egg hatching, all of which could ultimately render identification of *S. mansoni* eggs difficult (Bell, 1963).

Tween 20, also known as polyethylene glycol sorbitan monolaurate, is a nonionic detergent that is able to break protein–lipid and lipid–lipid interactions; it is often used as a cleaning agent during enzyme-linked immunosorbent assay procedures (Helenius et al., 1979). When this detergent was applied to the initial steps of the HTX method, a significant reduction in the final sediment volume resulted, producing a volume 69% lower than that of the standard HTX method. These results showed that Tween 20 was more effective than cellulase at reducing the final sediment volume. The sediment produced after the addition of Tween 20 was laid out on an average of 2.6 filter papers per sample, and its examination by microscopy was completed in an average of 10 min.

Sieving steps are sites for retention of eggs, especially if the eggs adhere to clumps of fecal debris. These clumps are usually seen by microscopy of untreated sediments. The removal of the 75-μm sieve step in combination with the use of Tween-20 (modification 3 of the HTX method) led to the highest egg recovery, compared to either the standard HTX method or other modified methods. This improvement could be due to the fact that some eggs might get trapped in the 75-μm sieve during the standard HTX procedure.

The data presented in this work will pave the way for further investigations. One consideration to be made could be to double the amount of feces initially used in the method, enabling the HTX method to analyze a larger sample from each patient. In this way, the HTX method could halve the limit of the detection of eggs per gram of feces, thus further improving its sensitivity.

In summary, the modifications of the HTX method evaluated (5% Tween 20, removal of the 75-μm sieve, and ninhydrin staining) significantly improved the recovery of *S. mansoni* eggs by 27.2% while decreasing the examination time by microscopy. Although the modified HTX method is still a complex and labor-intensive method, its high sensitivity and specificity supports its use as the gold standard for evaluation of other diagnostic tools for schistosomiasis. This modified HTX method may also have a role in field applications, like cure control and as one criterion for transmission interruption certification. Other modifications are also currently being studied.

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