Comparison of the performance of two spontaneous sedimentation techniques for the diagnosis of human intestinal parasites in the absence of a gold standard

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ABSTRACT

Performance evaluation of diagnostic tests is critical in the search for accurate diagnoses. A gold standard test is usually absent in parasitology, thus rendering satisfactory assessment of diagnostic accuracy difficult. Moreover, reliability (assessed by the study of repeatability) is a rarely studied characteristic of diagnostic tests. This study compared and evaluated the performance (repeatability, concordance and accuracy) of the spontaneous sedimentation technique (SST) and the Paratest for the diagnosis of *Giardia lamblia*, *Entamoeba histolytica* complex, *Blastocystis* spp., *Ascaris lumbricoides*, hookworm, *Trichuris trichiura* and *Calodium hepaticum*. Fecal samples of 143 individuals were separated into three replicates for each test. Concordance and homogeneity of the results between replicates of each test and between tests were evaluated. Proportions of positives, sensitivity and specificity were estimated using a Bayesian Latent Class Model. High repeatability of both tests was found for the detection of intestinal parasites, except for *Blastocystis* spp. and hookworm. Concordance between tests was generally high (concordance correlation coefficient, 0.72–0.88), except for *Blastocystis* spp., hookworm and *T. trichiura*. The Paratest detected more cases of *Blastocystis* spp. and fewer of hookworm than the SST. The tests were quite discordant in the detection of *T. trichiura*. A low sensitivity (39.4–49.2% for SST, 35.8–53.8% for Paratest) and a high specificity (93.2–97.2%) were found for both tests. The Paratest presented a slightly higher sensitivity for the diagnosis of *Blastocystis* spp. (53.8%), and SST did so for hookworm (49.2%). This is the first study on repeatability and accuracy (using a Bayesian approach) of two spontaneous sedimentation techniques. These results suggest underdiagnosis of little dense parasitic forms due to technical limitations in both tests. We conclude that the combined study of repeatability, concordance and accuracy is a key strategy for better evaluation of the performance of tests and is also useful for the identification of technical limitations.

1. Introduction

Intestinal parasites are, even today, major contributors to the global burden of disease, affecting especially the population living in regions in the developing world (Alum et al., 2010). Of particular importance worldwide are the soil-transmitted helminths *Ascaris lumbricoides*, hookworm and *Trichuris trichiura* and the protozoans *Entamoeba histolytica* and *Giardia lamblia* (Bethony et al., 2006; Fenwick, 2012; Harhay et al., 2010). In the Amazon region, *Calodium hepaticum* is a zoonotic helminth that has been increasingly reported as a cause of spurious infection in humans (Gonçalves et al., 2012), and *Blastocystis* spp. is a highly prevalent suspected pathogenic protozoan (Borges et al., 2009). *C. hepaticum* is also the causative agent of a rarely reported liver disease (hepatic calodiasis) found worldwide. This helminth infects the hepatic parenchyma of various mammals (rodents being the principle hosts). Hepatic infection occurs following the ingestion of embryonated eggs present in the ground or contaminated food.

In spurious infection, non-embryonated eggs are ingested (from the ground, contaminated food or liver of mammals) and directly exit with the stools without causing liver disease (Fuehrer et al., 2011; Gonçalves et al., 2012). Transmission of intestinal parasites depends on the availability of clean water, socio-economic conditions, education, personal and public hygiene practices, temperature, humidity and the survival of the environmental stages of the parasites (Alum et al., 2010).

Evaluation of the performance of diagnostics tests is critical in the search for accurate diagnostic techniques to provide adequate patient care, assess drug efficacy, monitor the effectiveness of control programs and obtain better understanding of the epidemiology of intestinal parasites (Harhay et al., 2011; Tarafder et al., 2010). In order to evaluate diagnostic tests it is important to take into account that few, if any, gold standard tests (i.e. a diagnostic test with 100% accuracy against which the sensitivity and specificity of other tests are estimated) are available in parasitology and, in particular, do not exist for the detection of intestinal parasite infection (Basso et al., 2013; Tarafder et al., 2010). Nevertheless, most studies estimating the sensitivity and specificity of tests for the diagnosis of intestinal parasites consider the results of one of two tests compared (usually the traditional test) or the combination of the results of several diagnostic tests as the gold standard (Brandelli et al., 2011; Carvalho et al., 2012; Devera et al., 2008a; Dogruman-Al et al., 2010; Glinz et al., 2010; Inês et al., 2011; Knopp et al., 2011; Levecke et al., 2011; Steinmann et al., 2012). These practices have led to biased estimations of accuracy. The use of statistical models that consider the assumption of absence of a gold standard test can overcome this problem, generating more reliable information as to the accuracy of diagnostic tests. Up to now, only three articles in the area of human intestinal parasites have presented estimations of the sensitivity and specificity of diagnostic tests using the concept of absence of a gold standard test (Booth et al., 2003; Tarafder et al., 2010; Traub et al., 2009).

Another important aspect during the evaluation of the performance of diagnostic tests is the repeatability of the results (Sanchez et al., 2002). Repeatability refers to the extent of agreement among repeat assessments of the same sample using the same technique in the same laboratory by the same operator (Braun-Munzinger and Southgate, 1992; White and van den Broek, 2004). Although the repeatability of a test refers to its reliability (White and van den Broek, 2004), this important characteristic has been little evaluated in studies of the performance of diagnostic tests (Charlier et al., 2005; Sanchez et al., 2002; Thomas et al., 1981).

Among the diagnostic tests based on optical microscopy those based on spontaneous sedimentation are among the least expensive and easiest to perform and enable the simultaneous detection of helminth and protozoan intestinal infections (Brandelli et al., 2011; Camacho et al., 2013; Carvalho et al., 2012; Ribeiro and Furst, 2012; Tello et al., 2012). For these reasons, in some economically underdeveloped settings their use is preferred over the tests based on centrifuge-sedimentation or centrifuge-flotation. Nevertheless, techniques based on centrifugation have demonstrated to be better in relation to those based only on spontaneous sedimentation (Carvalho et al., 2012; Gomes et al., 2004), although exceptions have been reported (Devera et al., 2008a; Tello et al., 2012).

The spontaneous sedimentation technique (SST) (also known as the Lutz technique or the Hoffman, Pons and Janer technique) is a traditional test widely used for clinical diagnosis and epidemiological surveys in Brazil and, also, Venezuela (Brandelli et al., 2011; Carvalho et al., 2012; de Souza et al., 2007; Devera et al., 2008a,b; Pinheiro et al., 2011; Santos et al., 2013; Velásquez et al., 2005). In this test, stool samples (previously preserved or diluted in water) are filtered through a gauze strip into a conical cup and subsequently submitted to sedimentation in tap water for 1 or 2 h (De Carli, 2007a). On the other hand, the Paratest (DK Diagnostics, São Paulo, Brazil) is a commercial kit for spontaneous sedimentation of preserved stool, developed with the aim of expanding new methods based on the simplification of laboratory procedures thereby improving biosecurity (Brandelli et al., 2011). The kit provides a stool container that has a cap equipped with a filter of 266 μm. This characteristic synthesizes the manipulation and examination of stool samples by performing the steps of conservation, filtration and concentration in the container itself. The amount of feces is standardized (2 g), whereas variable quantities can be used (1–5 g) in the SST (Brandelli et al., 2011; De Carli, 2007a; Hoffman et al., 1934). The Paratest is faster (15 or 30 min of sedimentation) than the SST and its compact structure allows the performance of the test in remote places. Despite the widespread use of the spontaneous sedimentation techniques, no study has evaluated their repeatability and accuracy taking into account the absence of a gold standard in the latter case.

The aim of this study was to evaluate and compare the performance (repeatability, concordance and accuracy) of two spontaneous sedimentation techniques (SST and Paratest) in the detection of infection by several pathogenic (or suspected pathogenic) intestinal parasites (G. lamblia, E. histolytica complex, Blastocystis spp., A. lumbricoides, hookworm, T. trichiura and C. hepaticum), using a Bayesian approach for the estimation of the proportion of positives, sensitivity and specificity.

2. Materials and methods

2.1. Study area and population

This study was carried out in 2009 with the collection of stool samples from children and adults from the agricultural community of Rio Pardo of the municipality of Presidente Figueiredo, located ~160 km to the north of the city of Manaus (~1°48′ S; 60°19′ W), Amazonas State, Brazil.

2.2. Field and laboratory procedures

Participants were asked to submit one fresh stool sample. The collection of samples was conducted in the households with two daily visits of the staff of the project, once in the morning and another in the afternoon. The samples were initially processed 1–3 h after collection in a local laboratory unit located in the community, as follows: firstly, thorough homogenization of each specimen was performed by stirring with a wooden spatula for at least 1 min. After homogenization, each sample was separated into three equal replicates of feces for each test. For the Paratest the replicates were deposited into three different plastic stool containers provided by the Paratest kit using a device that enables the collection of 1 g of feces. Each replicate was composed of 2 g of feces diluted in 7 ml of the preservative (5% buffered formalin pH 7.0) contained in each container. For the SST the replicates were deposited into three different containers with sodium acetate–acetic acid–formaldehyde (SAF). For each replicate of the SST, 2 g of feces (measured with the device provided by the Paratest kit) were diluted in 7 ml of SAF. In the case of a diarrheal sample, three measurements of the device provided by the Paratest kit were applied for the two tests. Samples that could not achieve the total of six replicates due to the lack of a sufficient quantity of feces were not included in the study.

The two sedimentation techniques were processed and examined in the Leonidas e Maria Deane Institute (Fiocruz, Manaus) by an experienced laboratory technician. The delay in time from stool sample processing to microscopic reading ranged from 3 to 17 days. The Paratest was carried out according to the manufacturer's instructions. In brief, each container with the diluted feces was

moderately shaken, the sediment exit (located in the cap) was taken off and the container was inverted and placed onto a polystyrene tray for the spontaneous sedimentation of the fecal suspension. Thirty minutes later, two drops of the sediment from each container were placed on a slide and stained with lugol for subsequent microscopic examination. The SST was based on Lutz (1919) and Hoffman et al. (1934). Each replicate of conserved feces was filtered through gauze folded twice and the filtrate was received in a 125 ml polystyrene conical cup. Tap water was added to the filtrate up to a volume of 3/4 of the cup. The suspension was allowed to stand for 2 h, and after this period, part of the sediment was collected with a pipette, and one drop was placed on a slide and stained with lugol for subsequent microscopic examination.

2.3. Statistical analysis

The number of positive results and their percentages for each intestinal parasite were calculated separately for each replicate of the tests. The number (and percentages) of discordant results between tests were calculated for each pair of replicates compared. These descriptive analyses were also performed with the combination of the results of the replicates of each test and considering a positive result when at least one of the three replicates was positive. This latter information is referred to herewith as a "combined result".

The Cochran Q test was used to test intra-test homogeneity, that is, that assessed if the percentage of positive results was the same among the three replicates of each test. A generalization of the kappa index (Broemeling, 2009) (here named "kappa index for repeatability measure (Kappa IRM)") was calculated for the assessment of diagnostic agreement among the three replicates of each test. The McNemar test was used to test the homogeneity (same percentage of positive results) of the results between the two tests. The kappa index (here named "kappa index between tests (Kappa IBT)") was calculated to assess the diagnostic concordance between the SST and the Paratest (between each pair of replicates compared and between the combined result of each test). Kappa measures were interpreted as follows: <0, poor agreement; 0–0.20, slight agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; and 0.81–1.0, almost perfect agreement (Landis and Koch, 1977). The concordance correlation coefficient (CCC) was used to obtain an overall measure of agreement between the SST and the Paratest taking the results of the three replicates into account.

A Bayesian latent class approach (Joseph et al., 1995) was used to obtain estimates for the sensitivity and specificity of the two techniques and the proportion of positives for each intestinal parasite. The conditional dependence between the two tests was estimated using a fixed parameter (Dendukuri and Joseph, 2001).

The proportion of positives was assumed to follow a Beta prior distribution with alpha and beta parameters equal to 1 (noninformative distribution). Informative prior distributions were used for sensitivity and specificity. Taking into account the results obtained by Gomes et al. (2004) and Brandelli et al. (2011), we assumed that the sensitivity of the SST and the Paratest should be between 30% and 60% and the specificity, between 90% and 100%. Thus, the parameters for the Beta prior distribution for sensitivity were alpha equal to 19.35 and beta 23.65, and for specificity 71.25 and 3.75, respectively.

Three different chains were run from different starting points to assess convergence to ensure robust estimation. Model convergence was assessed using Gelman and Rubin convergence statistics. The first 5000 iterations were discarded as burn-in and the next 1500 iterations by chain were used to obtain a sample of the marginal posterior density for each parameter (proportion of positive cases, sensitivity and specificity).

The median and the 95% credible interval of these samples were used as point and interval estimation of the parameter. The model was fitted with the WinBUGS 1.4 software (Spiegelhalter DJ, Thomas A, Best NG, 2004. WinBUGS version 1.4.; http://www.mrc-bsu.cam.ac.uk/bugs/winbugs/contents.shtml).

2.4. Ethics statement

This study was approved by the Ethics Committee in Investigation of the Oswaldo Cruz Foundation (Protocol 384/07 of 20/08/2007). Written Informed consent was obtained from all the study participants.

3. Results

3.1. Parasitological findings by diagnostic method

A total of 143 stool samples were subjected to the analysis of three replicates by each test. The cumulative percentages of positive cases (considering the results of the replicates of each test) as well as the total percentages of positive cases observed are presented by intestinal parasite in Fig. 1. The examination of more than one replicate of the same stool sample by the SST did not represent an increment in the percentage of positives encountered for *G. lamblia*, *E. histolytica* complex, *A. lumbricoides*, *T. trichiura* and *C. hepaticum*. The examination of the three replicates by the SST (in comparison to only one replicate) led to an increase in the percentage of positives for *Blastocystis* spp. (18.9% to 26.6%) and hookworm (14.7% to 21.7%).

In relation to the results obtained with the Paratest, increases were also observed in the percentage of positives for hookworm (6.3% to 11.9%), being even more pronounced for *Blastocystis* spp. (23.8% to 39.2%). Using the combined result of the two tests together, we calculated the total of the positive cases observed for each intestinal parasite. On considering this combined result, an increase was observed in the percentage of positive cases of *Blastocystis* spp. (43.4%) and hookworm (25.9%), and, to a lesser extent, of *T. trichiura* (9.8%).

3.2. Proportion of positives and test sensitivity and specificity

The highest estimated proportions of positive cases were encountered for *Blastocystis* spp. (63.8%; CrI 46.1–83.5), hookworm (31.8%; CrI 15.0–50.9) and *A. lumbricoides* (23.2%; CrI 9.7–40.1). In general the sensitivity was similar in both tests and for almost all the intestinal parasites evaluated, except for *Blastocystis* spp. and hookworm. In these cases, the sensitivity varied from 42.8% to 46.4% in the SST and from 43.4% to 45.6% in the Paratest. The Paratest presented a slightly higher sensitivity for the diagnosis of *Blastocystis* spp. (53.8%; CrI 43.2–65.3) in comparison to the SST (39.4%; CrI 30.3–49.9) and the SST presented a slightly higher sensitivity for hookworm (49.2%; CrI 36.0–62.4) in comparison to the Paratest (35.8%; CrI 25.0–51.4). The specificity was high (between 93.2% and 97.2%) and similar in both tests and for all the intestinal parasites evaluated (Table 1).

3.3. Repeatability

In both tests, the concordance between replicates was almost perfect (Kappa IRM value superior to 0.93) with homogeneity of the results of replicates for *G. lamblia*, *E. histolytica* complex, *T. trichiura* and *C. hepaticum*. However, one exception was *A. lumbricoides*, in which almost perfect concordance was observed in both tests but with heterogeneity in the SST (Cochran Q, *P* = 0.011). For the detection of *Blastocystis* spp., the SST and the Paratest presented substantial intra-test concordance (Kappa IRM = 0.79 and
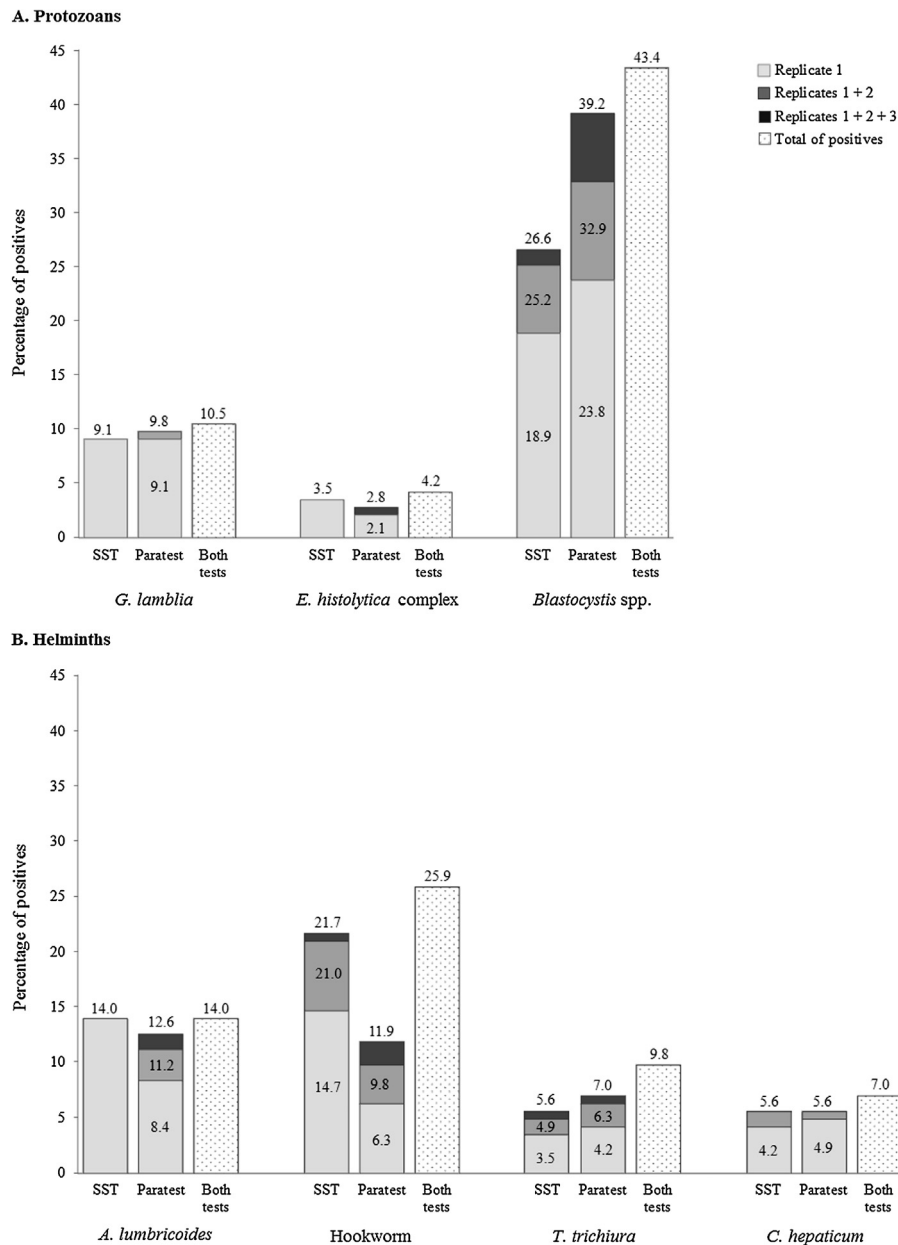
A. Protozoans

B. Helminths

Fig. 1. Cumulative percentages of positive cases by test and total percentages of positives, by intestinal parasite.

Table 1
Median value and 95% credible intervals of proportion of positives, sensitivity and specificity of SST and Paratest by intestinal parasites, as estimated by Bayesian analysis.

Protozoans/ Helminths	Tests	Proportion of positives		Sensitivity		Specificity	
		Median	CrI 95%	Median	CrI 95%	Median	CrI 95%
G. lamblia	SST	14.7	4.4–29.1	44.5	32.5–57.7	96.4	92.6–98.8
	Paratest			45.6	33.7–59.0	96.1	92.2–98.6
E. histolytica complex	SST	5.0	0.9–13.0	44.4	30.9–59.0	96.9	93.9–98.9
	Paratest			43.4	29.7–57.9	97.2	94.5–99.0
Blastocystis spp.	SST	63.8	46.1–83.5	39.4	30.3–49.9	95.7	90.2–98.7
	Paratest			53.8	43.2–65.3	94.7	87.6–98.4
A. lumbricoides	SST	23.2	9.7–40.1	46.4	35.0–58.5	96.1	91.9–98.6
	Paratest			44.6	33.4–56.5	96.6	92.5–98.9
Hookworm	SST	31.8	15.0–50.9	49.2	36.0–62.4	93.2	85.7–98.1
	Paratest			35.8	25.0–51.4	96.3	91.9–98.9
T. trichiura	SST	9.5	2.3–21.2	42.8	28.8–57.6	96.5	92.9–98.8
	Paratest			44.2	30.1–59.2	95.6	91.7–98.5
C. hepaticum	SST	8.3	1.9–19.1	44.2	31.4–58.3	96.6	93.3–98.8
	Paratest			44.2	30.9–58.3	96.5	93.2–98.8

SST = spontaneous sedimentation technique; CrI = credible interval.

Table 2
Concordance (repeatability) and homogeneity between replicates of SST and of Paratest by intestinal parasite.

Protozoans/ Helminths	Tests	Kappa IRM		Cochran Q
		Median	(CI 95%)	P value
G. lamblia	SST	1.00	0.97–1.00	0.368
	Paratest	0.98	0.95–1.00	0.717
E. histolytica complex	SST	0.98	0.95–1.00	0.050
	Paratest	0.99	0.96–1.00	0.607
Blastocystis spp.	SST	0.79	0.70–0.86	0.355
	Paratest	0.76	0.68–0.84	0.017
A. lumbricoides	SST	0.96	0.92–0.98	0.011
	Paratest	0.94	0.89–0.97	0.459
Hookworm	SST	0.76	0.66–0.84	0.003
	Paratest	0.88	0.81–0.93	0.305
T. trichiura	SST	0.95	0.96–0.98	0.368
	Paratest	0.93	0.87–0.97	0.273
C. hepaticum	SST	0.99	0.96–1.00	0.223
	Paratest	0.97	0.94–0.99	0.472

SST = spontaneous sedimentation technique; Kappa IRM = kappa index for repeatability measure; CI = confidence interval.

0.76, respectively). Moreover, the results of replicates were heterogeneous only in the Paratest (Cochran Q, $P = 0.017$). Regarding hookworm, the two tests did not present the same results. The Paratest presented almost perfect concordance (Kappa IRM = 0.88) and the SST lower concordance (Kappa IRM = 0.76) since the results of the replicates were heterogeneous in the SST (Cochran Q, $P = 0.003$) (Table 2).

3.4. Concordance between tests

For each intestinal parasite, the results of the kappa IBT were concordant with the general measure given by the CCC. The concordance between tests for the diagnosis of G. lamblia and C. hepaticum was almost perfect (CCC of 0.88 and 0.81, respectively) and was substantial for E. histolytica complex and A. lumbricoides (CCC of 0.72 and 0.78, respectively). For the diagnosis of Blastocystis spp., hookworm and T. trichiura the concordance was fair or slight (CCC of 0.38, 0.17 and 0.10, respectively) and the results of the tests were heterogeneous for Blastocystis spp. and hookworm (McNemar test, $P < 0.001$ and $P = 0.009$, respectively) (Table 3).

4. Discussion

In the present study we analyzed the repeatability and estimated the accuracy of two spontaneous sedimentation techniques applied in the diagnosis of intestinal parasite infections using a Bayesian approach for the first time.

The study of repeatability showed that the SST and the Paratest presented high repeatability for the detection of nearly all the intestinal parasites evaluated. Low repeatability was observed in the two tests only for the diagnosis of Blastocystis spp. and hookworm, and this may be explained by the different percentages of positive cases between replicates or because the positive cases did not correspond to the same individuals in the replicates.

The concordance between tests was high (substantial or almost perfect concordance) except for the diagnosis of Blastocystis spp., hookworm and T. trichiura. The low concordance encountered for Blastocystis spp. is explained by the fact that the Paratest detected more positive cases than SST, thereby making the Paratest the best test for the detection of this protozoan. On the other hand, the lack of concordance obtained in the diagnosis of hookworm was due to the detection of less positive cases by the Paratest, thereby making the SST better in this case. Regarding T. trichiura, the result of low concordance was in contrast with the finding of homogeneity of the

test results. However, the interpretation of these results together indicates that the tests were completely discordant in the detection of positive cases, that is, when a test was positive for T. trichiura eggs the other was negative, and vice versa.

Parasitic forms present a heterogeneous distribution in stool samples (De Carli, 2007b). Homogenization of a stool prior to sample processing has been suggested as a procedure to overcome this problem and improve the diagnostic accuracy (Krauth et al., 2012). Up to now, the usefulness of homogenization by stirring has only been demonstrated for Schistosoma japonicum and Schistosoma mansoni in reducing intra-sample variation of egg location and of fecal egg counts, respectively (Krauth et al., 2012; Ye et al., 1998). For A. lumbricoides, T. trichiura and hookworm this procedure was not helpful (Krauth et al., 2012; Ye et al., 1997). In our study, the homogenization was performed for each stool sample before separation into replicates. The result of high repeatability of both tests for the diagnosis of almost all the intestinal parasites studied means that the homogenization contributed to obtain replicates with similar qualitative content of parasitic forms. On the other hand, the low repeatability of both tests for Blastocystis spp. and hookworm detection suggests that this result could have been influenced by the heterogeneous distribution of their forms in the stools, which, in these cases, could persist even after the homogenization of stools.

We believe that technical characteristics of the two techniques may also explain the low repeatability of the tests. During the process of the two tests, sediment is generated by the action of gravity, being characterized by a heterogeneous distribution of parasitic forms, which is dependent on the settling velocity of these forms in the liquid media (Sengupta et al., 2011). The homogenization of the entire sediment before the collection of aliquots for microscopic evaluation is not carried out in either of these tests. However, in the SST, aliquot collection is influenced by the handling of the technician since the aliquots may be removed with the pipette from any part of the sediment. Since handling is a known external factor of variation in diagnostic tests (Sanchez et al., 2002), we believe that some variability in our results conferred by this factor may be expected. This problem is minimized in the Paratest because the sediment generated is not directly manipulated by the technician. In this test, the two drops analyzed by microscopy are almost always the first of the most posterior end of the sediment that are eliminated through the exit located in the cap of the container.

The intestinal parasites that did not have reproducible results in the study of repeatability are characterized by presenting parasitic forms of small size (Blastocystis spp., the diameter of which may vary between 2 and 200 μm) (Tan, 2008) and low specific density (hookworm, with a specific density of approximately 1.055 in zinc sulphate solution) (Sawitz et al., 1939). Size and density are important characteristics for the determination of the dynamics of sedimentation of parasitic forms in liquid media. According to some authors, the sedimentation of particles in water is expected to follow Stokes' law which implies that the settling velocity depends on particle size, difference in density between particles and water, and water viscosity (Medema et al., 1998; Sengupta et al., 2011). Shuval (1978) reported that the settling velocity of hookworm (0.39 m h^{-1}) in clean water is slower than that of Ascaris (0.65 m h^{-1}) and Trichuris (1.53 m h^{-1}) and as such the eggs of hookworm are one of the last in settle, suggesting that their eggs probably occupy the most anterior part of the sediment. Information about the settling velocity of Blastocystis spp. is not available in the literature. However, we believe that its forms are also little dense (and probable settle slowly) since microscopic examination shows that any capillary action produced in the slide easily produces the displacement or flotation of these forms (Gonçalves, personal communication). The low repeatability of the SST for the diagnosis of hookworm and Blastocystis spp. could be explained by the previously mentioned

Table 3
Concordance and homogeneity between SST and Paratest by pair of replicates and by combined result of the tests for each intestinal parasite.

Protozoans/ Helminths	Tests	SST+		P+		SST+ P−		SST− P+		Kappa	McNemar	CCC
		N	%	N	%	N	%	N	%	IBT	P value	(CI 95%)
G. lamblia	SST1/P1	13	9.1	13	9.1	2	1.4	2	1.4	0.83	1.000	0.88
	SST2/P2	12	8.4	12	8.4	1	0.7	1	0.7	0.91	1.000	(0.85,0.91)
	SST3/P3	12	8.4	12	8.4	1	0.7	1	0.7	0.91	1.000	
	SSTc/Pc	13	9.1	14	9.8	1	0.7	2	1.4	0.88	1.000	−
E. histolytica complex	SST1/P1	5	3.5	3	2.1	3	2.1	1	0.7	0.49	0.625	0.72
	SST2/P2	2	1.4	2	1.4	0	0.0	0	0.0	1.00	1.000	(0.67,0.77)
	SST3/P3	2	1.4	3	2.1	0	0.0	1	0.7	0.80	1.000	
	SSTc/Pc	5	3.5	4	2.8	2	1.4	1	0.7	0.66	1.000	−
Blastocystis spp.	SST1/P1	27	18.9	34	23.8	11	7.7	18	12.6	0.40	0.265	0.38
	SST2/P2	21	14.7	40	28.0	4	2.8	23	16.1	0.45	<0.001	(0.28,0.47)
	SST3/P3	23	16.1	47	32.9	8	5.6	32	22.4	0.27	<0.001	
	SSTc/Pc	38	26.6	56	39.2	6	4.2	24	16.8	0.53	<0.001	−
A. lumbricoides	SST1/P1	20	14.0	12	8.4	8	5.6	0	0.0	0.72	0.008	0.78
	SST2/P2	17	11.9	15	10.5	3	2.1	1	0.7	0.86	0.625	(0.73,0.83)
	SST3/P3	14	9.8	13	9.1	3	2.1	2	1.4	0.80	1.000	
	SSTc/Pc	20	14.0	18	12.6	2	1.4	0	0.0	0.94	0.500	−
Hookworm	SST1/P1	21	14.7	9	6.3	17	11.9	5	3.5	0.20	0.017	0.17
	SST2/P2	16	11.2	7	4.9	13	9.1	4	2.8	0.21	0.049	(0.09,0.24)
	SST3/P3	6	4.2	4	2.8	6	4.2	4	2.8	-0.03	0.754	
	SSTc/Pc	31	21.7	17	11.9	20	14.0	6	4.2	0.36	0.009	−
T. trichiura	SST1/P1	5	3.5	6	4.2	5	3.5	6	4.2	-0.04	1.000	0.10
	SST2/P2	3	2.1	3	2.1	2	1.4	2	1.4	0.32	1.000	(0.03,0.18)
	SST3/P3	2	1.4	2	1.4	2	1.4	2	1.4	-0.01	1.000	
	SSTc/Pc	8	5.6	10	7.0	4	2.8	6	4.2	0.41	0.754	−
C. hepaticum	SST1/P1	6	4.2	7	4.9	0	0.0	1	0.7	0.92	1.000	0.81
	SST2/P2	8	5.6	6	4.2	3	2.1	1	0.7	0.70	0.625	(0.77,0.85)
	SST3/P3	7	4.9	5	3.5	2	1.4	0	0.0	0.83	0.500	
	SSTc/Pc	8	5.6	8	5.6	2	1.4	2	1.4	0.74	1.000	−

SST = spontaneous sedimentation technique; SST+ = positives for SST; P+ = positives for Paratest; SST+ P− = positives for SST and negatives for Paratest; SST− P+ = negatives for SST and positives for Paratest; N = number of cases; % = percentage of cases; Kappa IBT = kappa index between tests; CCC = concordance correlation coefficient; CI = confidence interval; SST1, SST2 and SST3 = results of SST for replicates 1, 2 and 3, respectively; P1, P2 and P3 = results of Paratest for replicates 1, 2 and 3, respectively; SSTc = combined result of SST; Pc = combined result of Paratest.

handling problem. The pipette introduced into the sediment to collect an aliquot is usually introduced into the most posterior part of the sediment and almost never at the more superficial part, where the majority of little dense forms are probably located. This means that during the microscopic evaluation there is probably a lack of representativeness of the most anterior part of the sediment. In the Paratest, the low repeatability also encountered for *Blastocystis* spp. and hookworm detection may be explained in the same way. Since the most posterior part of the sediment is always the first accessed, the most anterior portion is probably underrepresented in the diagnosis. Furthermore, the time of sedimentation suggested by the kit (15–30 min) may not be sufficient for satisfactory sedimentation of the little dense eggs of hookworm and the possible little dense forms of *Blastocystis* spp.

The dynamics of sedimentation of the parasitic forms of protozoans and helminths in water or preservative liquids of stool remains little studied. The settling velocity is also dependent on other characteristics of the parasites, such as the capacity of some parasitic forms to adhere to surfaces and suspended matter (Gaspard et al., 1994; Sengupta et al., 2011). Some authors have reported that high particle concentrations in wastewater typically results in flocculation of the particles (Droppo, 2001) and this may lead to attachment and entrapment of eggs of helminths or cysts and oocysts of protozoans to these flocs (Medema et al., 1998), affecting the settling velocity of the parasites.

We observed that although both tests presented low repeatability in the diagnosis of *Blastocystis* spp., the Paratest detected more positive cases. The better results of the Paratest were expected because it is known that forms of this protozoan are lysed in contact with water (Amato Neto et al., 2003; Stenzel and Boreham, 1996). We believe that although the feces were previously conserved in SAF, it was not determinative to avoid the lyses of some

forms of *Blastocystis* spp. during processing of the SST. Some authors have previously reported the detection of *Blastocystis* spp. in feces conserved in formaldehyde and subsequently submitted to spontaneous sedimentation in water (Eymael et al., 2010; Velásquez et al., 2005), sometimes with a lower proportion of detection in comparison with other tests (Eymael et al., 2010). On the other hand, although both tests also presented low repeatability in the diagnosis of hookworm, the SST detected more cases thereby suggesting the possible presence of some technical problems in the Paratest in relation to hookworm detection. Beyond the previously discussed problem about the settling of parasites with a low specific density, the capacity of adherence of eggs could be another characteristic producing limitations of the test. The ability of eggs to adhere to the plastic that composes the filter of the Paratest and also their ability to attach to flocculated particles from the fecal suspension should be evaluated. These characteristics could contribute to hampering the passage of eggs through the filter of the test. Brandelli et al. (2011) emphasized that the filtration system of the Paratest could be a major factor contributing to the false-negative results observed for larvae in their study.

We report a low sensitivity of the SST (39.4–49.2%) and the Paratest (35.8–53.8%) in the diagnosis of some intestinal parasites. The Paratest was more sensitive (53.8%) for the diagnosis of *Blastocystis* spp. and the SST for hookworm detection (49.2%). For parasites obtaining the same sensitivity in both tests, we also found a good concordance between the tests for their detection, except for *T. trichiura*. So far, previously published data about the accuracy of the SST and the Paratest are unreliable since the authors defined the gold standard as the combination of the results of two or more tests or considered the SST as the gold standard (with 100% of accuracy). The widespread use of the SST in Brazil has made it frequently and erroneously considered as the gold standard

test in many studies. These practices are responsible for the high variability of the reports of the sensitivity of SST encountered in the literature, with values ranging from 38.4% to 100% (Brandelli et al., 2011; Devera et al., 2008a; Gomes et al., 2004). Regarding the Paratest, the only data available reported 33% and 55% of sensitivity for the diagnosis of eggs/larvae and cysts, respectively (Brandelli et al., 2011). In our study, the specificity of the two tests was high, as previously reported, although the 100% specificity reported by some authors is unreal if considering the possibility of occurrence of false positives (Tarafder et al., 2010).

To date, only one article has estimated the sensitivity and specificity of a centrifuge-sedimentation technique (Danish Bilharziasis Laboratory (DBL) technique) for the diagnosis of a human intestinal parasite using a Bayesian method. The DBL-technique presented a sensitivity ranging from 65% to 78% for the diagnosis of *S. japonicum* from some non-human mammals, and a high specificity (92.6–99.1%), when only one stool sample was considered (Carabin et al., 2005). Since our study was based on the analysis of a single stool sample, the day-to-day variation in the output of parasitic forms was not assessed, thus, the estimations of sensitivities could have been higher if more samples had been analyzed.

Low sensitivity of diagnostic tests for the detection of hookworm infection may be related to rapid degeneration of hookworm eggs over time. The sensitivity is influenced by delays in time between stool production and the processing of samples in the laboratories (Dacombe et al., 2007; Knopp et al., 2008; Krauth et al., 2012) and, between the processing of samples and microscopic reading when using the Kato-Katz technique (Knopp et al., 2008; Tarafder et al., 2010; WHO, 1994). A decrease in sensitivity of almost 50% for the detection of hookworm was reported with the formol-ether concentration method when preservation with formalin was delayed by more than 3 h (Dacombe et al., 2007). In our study the time from stool collection in households until their processing (preservation) was of up to 3 h. However, the delay in the processing was probably higher if considering that was not possible for us ascertain the time of stool production by the participants. Because of the difficulties of the participants to deliver the stools to the field laboratory, samples were collected in the households by two daily visits of the staff of the project. The delay in time between stool production and processing in the field laboratory probably contributed, to some extent, to the low sensitivity encountered by both tests in hookworm detection. This aspect could also be true for *Blastocystis* spp., since the vacuolar form of this protozoan is fragile (Stenzel and Boreham, 1996).

When considering the analysis of three replicates for each test, the increment obtained in the percentage of positives of *Blastocystis* spp. and hookworm indicates that the processing of three replicates contributed to better diagnosis. This last result coincides with that of low repeatability of both tests for *Blastocystis* spp. and hookworm detection. Thus, the great variability between the results of the replicates led to a great increase in the percentage of positives observed when considering the cumulative results of the replicates. Regarding *T. trichiura*, on the detection of different positive cases by the two tests, the combined use of the two tests provided a better diagnosis. However, despite the general improvement of the diagnosis in these cases, the low sensitivity reported for both tests indicates an important underestimation of the total number of positive cases.

5. Conclusions

In this study we report an overall high repeatability for the SST and the Paratest (except for *Blastocystis* spp. and hookworm by both tests) and high concordance between the two tests (except for the diagnosis of *Blastocystis* spp., hookworm and *T. trichiura*). Low

sensitivity and high specificity were encountered by both tests. We conclude that the combined study of repeatability, concordance and accuracy (in the absence of a gold standard test) is a key strategy for better evaluation of the performance of tests and is also useful for the identification of technical limitations, providing opportunities for the generation of proposals for technical improvements. Additional studies on the dynamics of sedimentation of diverse parasitic forms in liquid media are needed in order to improve the traditional tests currently used for the diagnosis of intestinal parasites. Moreover, for tests based on sedimentation, the standardization of homogenization of the total sediment before the collection of aliquots for microscopic evaluation should be implemented in all situations.

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