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Assessment of the diagnostic performance of four methods for the detection of *Giardia duodenalis* in fecal samples from human, canine and feline carriers

Flávia Fernandes de Mendonça Uchôa[a,*], Adriana Pittella Sudré[b], Sabrina Destri Emmerick Campos[a], Nádia Regina Pereira Almosny[c]

[a] *Post Graduate Program in Veterinary Clinics and Animal Reproduction, School of Veterinary Medicine, Fluminense Federal University (UFF), Vital Brasil Filho st., 64, Vital Brasil, Niterói, RJ, Brazil*

[b] *Department of Microbiology and Parasitology, Biomedical Institute, Fluminense Federal University (UFF), Professor Hernani Melo st., 101/212A, São Domingos, Niterói, RJ, Brazil*

[c] *Department of Clinics and Surgery, School of Veterinary Medicine, Fluminense Federal University (UFF), Rua Vital Brasil Filho st, 64, Vital Brasil, Niterói, RJ 24230-340, Brazil*

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ABSTRACT

Enteric parasitic diseases including giardiasis are of public health concern. Different methods are available for the diagnosis of this parasitic infection in fecal samples such as the identification of protozoan cysts and trophozoites by light microscopy, detection of specific antigens by ELISA, and amplification of DNA fragments by PCR. The present study aimed at assessing the performance of four laboratory tests for the detection of *Giardia duodenalis* in fecal specimens from three different host species with a previous diagnosis of giardiasis; canine, feline and human patients provided new stool samples to be retested for *Giardia* before initiating treatment with antiprotozoal drugs. For this purpose, triplicate fecal specimens from 54 humans, 24 dogs and 18 cats living in the city of Niterói, RJ, southeast Brazil, were analysed by light microscopy, ELISA, immunochromatography, and nested PCR. The centrifugal-flotation method detected *Giardia* cysts in 89.6% (86/96) of the fecal samples. The protozoan parasite was detected via immunochromatography in 87.5% (84/96) of these samples. *Giardia* was detected by ELISA in 69.8% (67/96) of the stool specimens from carriers with a previous diagnosis of *Giardia* infection. *Giardia* was detected by PCR in only 39.6% (38/96) of the fecal specimens. Based on these findings, we suggest that, among the four assays that were used in this study, the zinc sulphate flotation technique (Faust et al., 1939) is the best diagnostic assay in terms of sensitivity and specificity to detect *G. duodenalis* on serially collected samples from dogs, cats and humans.

1. Introduction

Giardia duodenalis (also known as *G. lamblia*) has been associated with numerous outbreaks and epidemics in the most diverse hosts (Mekaru et al., 2007; Palm et al., 2003; Painter et al., 2015). Rapid and accurate diagnostic methods are of paramount importance for researchers when planning and conducting epidemiological surveys and are also useful when implementing disease control measures in a population within a particular geographical area (Caccio, 2004). There are a number of techniques available for the diagnosis of giardiasis in fecal samples. Protozoan cysts and trophozoites can be recognized under the light microscope (coprological examination), specific antigens can be detected by ELISA, and DNA fragments amplified by PCR (Babaei et al., 2011; Koehler et al., 2014). Selection of appropriate diagnostic tests depends on the availability of equipment, reagents and experienced technicians, laboratory turnaround time, and cost (Ndao, 2009). Intermittent shedding of cysts in feces, low numbers of cysts in stool specimens, and asymptomatic infections are the hallmarks of giardiasis and make the diagnosis challenging. As a result, *Giardia* infection is underdiagnosed in all hosts and the prevalence of the disease underestimated (Leib and Zajac, 1999; Ignatius et al., 2012; Painter et al., 2015). The objective of the present study was to assess the performance of classical and modern approaches for the diagnosis of *Giardia duodenalis* infection in fecal samples from dogs, cats, and humans including conventional microscopy by coprological examination using the zinc sulphate flotation technique (Faust et al., 1939), detection of antigens by ELISA and immunochromatography, and detection of DNA by Nested-PCR.

* Corresponding author.
E-mail addresses: flavia_uchoa@yahoo.com.br (F.F.d.M. Uchôa), nadiaalmosny@id.uff.br (N.R.P. Almosny).

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2. Materials and methods

The studied population included 54 humans, 24 dogs and 18 cats living in the city of Niterói, RJ, southeast Brazil who attended local private clinical laboratories. These individuals had a previous diagnosis of giardiasis i.e. fecal samples from these patients were positive for *Giardia*, and they provided new stool specimens to be retested for *Giardia* as part of this study before treatment with antiprotozoal drugs was started. Study participants received screw-cap plastic vials and instructions for serial sample collection, storage and submission of stool samples. Three fecal samples (with a minimum of 4 g each) were collected from each individual and refrigerated for a maximum of 24 h after sampling and until reception and processing at a local diagnostic laboratory. Patients were selected according to their availability and willingness to provide samples for research purposes before commencing anti-*Giardia* therapy. As intermittent shedding of cysts in stools can make the microscopic diagnosis of giardiasis difficult and may result in false negatives, triplicate fecal samples were collected in order to increase the accuracy and sensitivity of this method.

First, all samples were washed. This initial step is briefly described as follows: an aliquot of approximately 4 g of feces was mixed in 20 ml of distilled water and then this solution was filtered through sieve and gauze and poured into 2 glass tubes with capacities up to 10 ml. These tubes were centrifuged for 2 min at 640*g* and the supernatant discarded. Then the sediment from one of these tubes was subjected to zinc sulphate flotation technique (Faust et al., 1939), which consisted of resuspending the sediment in 7 ml of 33% zinc sulphate solution (density 1200) (Synth®) and centrifuging at 640*g* for 2 min. The liquid formed a convex dome (meniscus) which was collected with a platinum inoculation loop, transferred to a glass slide, stained with Lugol's solution (Synth®), covered with a coverslip, and screened under a light microscope (Nikkon®) under low power (100×) magnification and high power (400×) magnification.

Positive samples were divided into three groups during tabulation of the results and were graded on a scale from + to +++ according to the average number of cysts per microscopic field as follows: *Giardia* sp. + (up to 2 cysts per field), *Giardia* sp. ++ (3–5 cysts per field) and *Giardia* sp. +++ (> 5 cysts per field). The sediment remaining in the tube was aliquoted and then frozen at −20 °C for further immunological and molecular analyses. Only 1 sample out of the triplicate set of samples available, from each patient that was positive for *Giardia* by light microscopy, was selected to be tested by the three other diagnostic techniques, i.e. ELISA, immunochromatography and nested PCR. The selection was based on the 3-scale grading system described above and an aliquot from the sample with the highest number of cysts on the slide was chosen for further tests. For those patients whose samples were negative for *Giardia* in the zinc sulphate method, the aliquot with the highest volume of sediment was selected after centrifugation, since this same aliquot would later be subjected to other diagnostic techniques.

Frozen sediment samples were tested for *Giardia* with an immunochromatographic assay (Alere Inc., Waltham, Massachusetts, USA). This assay was performed according to Costa et al. (unpublished data) and Uchôa et al. (2017a) with minor modifications. The Giardia Stool Antigen Detection Microwell ELISA (IVD Research Inc., Carlsbad, California, USA) commercial kit was used for the immunoenzymatic assay according to the instructions provided by the manufacturer. Microplates were washed in the 405TM TS Microplate Washer (Bio-Tek Instruments Inc., Winooski, Vermont, USA) and the spectrophotometric reading was carried out on a Testline ELx800 ELISA reader (Bio-Tek Instruments Inc., Winooski, Vermont, USA) with a 450 nm filter.

QIAampFast DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used to extract DNA from fecal samples according to the protocol published by Adamska et al. (2010) with slight modifications. Briefly, 200 µl of sediment were subjected to 3 cycles of boiling and freezing in liquid nitrogen for 2 min in each temperature to induce lysis of protozoan cysts and release of nucleic acids. From this step onwards, manufacturer's instructions were followed except for the incubation and DNA recovery steps that were slightly modified as follows: incubation in proteinase K was extended to 2 h at 56 °C and the extracted DNA was eluted in 50 µl of buffer in order to increase DNA concentration.

The extracted DNA was subjected to a nested PCR protocol that targets fragments of the genes coding for the β-giardin protein (β-g) and the triose phosphate isomerase (*tpi*) enzyme. The primers used in the external reaction for β-g were described by Cacciò et al. (2002), and amplified a DNA fragment of approximately 753 base pairs (bp). A fragment of approximately 511 bp was amplified in the nested reaction with the primers described by Lalle et al. (2005). The second nested-PCR protocol amplified a fragment of approximately 532 bp of the tpi gene with the primers described by Sulaiman et al. (2003). For both genes, PCR was performed under the same reaction conditions described by Sudre et al. (2014). DNA was also subjected to semi-nested PCR in order to amplify a fragment of approximately 432 bp of the gene encoding the glutamate dehydrogenase (gdh) enzyme; primers and reaction conditions were the same as was used by Read et al. (2004).

The McNemar's test at a significance level of 5% was applied to the results of each of the diagnostic tests in order to analyse the degree of disagreement between them. The present study was approved by the Research Ethics Committee of the School of Medicine/Antônio Pedro University Hospital (CAAE 44055615.0.0000.5243) and by the Ethics Commission on Animal Experimentation of the Fluminense Federal University (UFF), Niteroi, RJ, Brazil (number 643).

3. Results and discussion

The zinc sulphate flotation technique (Faust et al., 1939) was able to detect *Giardia* cysts in fecal specimens from 86 (89.6%) individuals - humans, dogs and cats - with a previous diagnosis of giardiasis and in which positive stool samples were retested for *Giardia*. In human patients, 92.6% (50/54) of the samples were positive for *Giardia* by light microscopy. *Giardia duodenalis* was found in 79.2% (19/24) of feces from canine patients via light microscopic examination; 94.4% (17/18) of the fecal samples from feline patients were positive for *Giardia* on coprological examination. The results of the microscopic examination of fecal samples from the three host species included in this study are shown on Table 1.

The zinc sulphate flotation technique provided satisfactory results in terms of sensitivity for the detection of *Giardia* cysts in stool specimens. Serial sampling may have increased the sensitivity of this diagnostic test. Due to the pattern of intermittent fecal cyst shedding, examination of triplicate samples would boost the sensitivity of this test for the diagnosis of giardiasis. Other authors that used the same sampling scheme (triplicate sampling) obtained results similar to ours (Hiatt et al., 1995; Cartwright, 1999; Hanson and Cartwright, 2001; Jacobs et al., 2001; Decock et al., 2003; Duffy et al., 2013; Uchôa et al., 2017b). In the present study, a large number of human and feline samples were positive for *Giardia* by Faust's method. The efficacy of sample collection may explain the large number of positives in the coprological

Table 1
Results of the microscopic examination of fecal samples (zinc sulphate flotation technique) from the three host species (dogs, cats and humans) included in this study. Samples were graded according to the average number of cysts per microscopic field.

	Zinc sulphate flotation technique (Faust et al., 1939)				
	Negative	"*Giardia* sp. +"	"*Giardia* sp. ++"	"*Giardia* sp. +++"	Total
Humans	4	49	0	1	54
Dogs	5	11	7	1	24
Cats	1	16	1	0	18
Total	10	74	8	2	94

examination. Dogs often live in multipet households with large outdoor areas, and these animals are allowed to be together in the same patio space. Sampling errors and bias are expected in such environments as age and origin of the stool specimens that are collected in those areas could be unknown. The sampling scheme that we asked participants of this study to follow may not have been properly accomplished in such cases. Cats usually live indoors and defecate inside litter boxes so chances for sample mix-up are minimal.

Although the zinc sulphate flotation technique is considered the gold standard method for the detection of *Giardia duodenalis* in fecal samples from a wide variety of host species (Hiatt et al., 1995; Decock et al., 2003; Mundim et al., 2003; Tangtrongsup and Scorza, 2010) and it was carried out on triplicate samples in this study, false negative results still occurred in 10 cases, possibly due to the intermittent pattern of shedding of *Giardia* cysts in the feces. Intermittent shedding of cysts in feces has been pointed out as the major obstacle to the diagnosis of giardiasis in all animal species (Hiatt et al., 1995; Rocha et al., 1999; Jacobs et al., 2001; Mundim et al., 2003; Bartmann and Araujo, 2004). Cyst shedding in feces may cease and then restart after a long period of time as reported by other authors (Smith and Wolfe, 1980; Flanagan, 1992; Hiatt et al., 1995; Doğruman et al., 2006; Brincker and de Araujo, 2009; Jahan et al., 2014). False negative results may also occur due to morphologic deterioration of cysts and trophozoites, which precludes identification of protozoan stages in fecal samples by light microscopy as reported by Weitzel et al. (2006).

Seven out of 10 stool samples negative for *Giardia* cysts by light microscopy were positive for *Giardia* antigens by immunochromatography as shown in Table 2. Statistical comparison between light microscopy and immunochromatography showed no statistically significant disagreement between the results obtained with each laboratory method (p = .8036). Immunochromatography performed better than the zinc sulphate flotation technique in canine samples in the diagnosis of giardiasis as 95.8% (23/24) of these specimens were positive in the former assay. However, the difference between the results obtained in each of the two diagnostic tests were not statistically significant (p = .1250).

Immunochromatography performed well in the detection of *Giardia* in fecal samples from all three species studied. Similar results have been reported by other authors (Garcia and Garcia, 2006; Papini and Cardini, 2006; Mosallanejad et al., 2010). Most of the performance assessments of laboratory tests that have been published over the years emphasize that immunological assays are better than conventional microscopy for the diagnosis of *Giardia duodenalis* infection. However, in our study there were no statistically significant disagreements between immunochromatography and light microscopy in any set of samples from dogs, cats or humans as shown in Table 2. Discrepancies between the results of our study and the results of studies published by other authors may be attributable to the fact that in most of the studies only one sample was collected and tested as opposed to the methodology of our study in which triplicate samples were collected and available for testing.

Table 3
Diagnostic performance of four methods for the detection of *Giardia duodenalis* in fecal samples from human, canine and feline carriers.

	Zinc sulphate flotation technique		Immunochromatography	ELISA	PCR
Humans	+	50	47	35	30
	–	4	7	19	24
Dogs	+	19	23	19	7
	–	5	1	5	17
Cats	+	17	14	13	1
	–	1	4	5	17
Total	+	86	84	67	38
	–	10	12	29	58

False negative results may occur if only one fecal sample is examined by light microscopy decreasing the sensitivity of this diagnostic method.

Regarding the ELISA assay, 69.8% of samples were positive for *Giardia*. The *Giardia* antigen detection was possible in 64% (35/54) of human samples, 79.2% (19/24) of dog samples and 72.2% (13/18) of cat samples as shown in Table 3. It is possible to detect *Giardia* coproantigen by a rapid antigen test (fecal ELISA antigen test) even in the absence of cysts in the feces. This antigen is released in fecal material regardless of the presence or absence of intact protozoan stages in stool samples as reported by Strand et al. (2008). In addition, the immunological test was performed on fecal sediment and that may have increased the concentration of the antigen in samples with low numbers of cysts. Many studies comparing the performance of a number of laboratory diagnostic techniques show that ELISA is more sensitive than conventional microscopy for the detection of parasitic infections (Decock et al., 2003; Cirak and Bauer, 2004; Silva et al., 2016).

In the present study, there was no statistically significant difference between the performance of the ELISA and the performance of the conventional microscopy in the diagnosis of giardiasis on fecal specimens except for the human samples (p = .0003). In humans, the number of false negatives in the ELISA was high which demonstrates lower sensitivity of this diagnostic test on samples from this host as shown in Table 2. This difference may stem from the fact that the numbers of parasite cysts in human samples in our study were low as previously discussed. Interestingly, ELISA performed well on fecal samples from dogs and cats in the diagnosis of giardiasis even though this assay was originally developed for human use. It is worth noting that although the ELISA and immunochromatography kits that are used

Table 2
Comparison of the results of the microscopic examination by the zinc sulphate flotation technique (Faust et al., 1939) of fecal samples from the three host species (dogs, cats and humans) included in this study with the results from the other three diagnostic methods (ELISA, immunochromatography and PCR) for the detection of *Giardia duodenalis* in carriers.

		Immunochromatography			ELISA			PCR		
		+	−	P-Value	+	−	p-Value	+	−	p-Value
Zinc sulphate flotation technique	Humans									
	+	44	6	p = .5078	34	16	p = .0003	29	21	p < .0001
	−	3	1		1	3		1	3	
	Dogs									
	+	19	0	p = .1250	17	2	p = 1.000	7	12	p = .0005
	−	4	1		2	3		0	5	
	Cats									
	+	14	3	p = .2500	13	4	p = .1250	1	16	p = .0000
	−	0	1		0	1		0	1	
	Total									
	+	77	9	p = .8036	64	22	p = .0003	37	49	p < .0030
	−	7	3		3	7		1	9	

Table 4
Comparison of the results of the immunochromatography on fecal samples from the three host species (dogs, cats and humans) included in this study with the results from ELISA and nested-PCR for the detection of *Giardia duodenalis* in carriers.

		ELISA			PCR		
		+	−	p-Value	+	−	p-Value
Immunochromatography	Humans						
	+	32	15	p = .0075	29	18	p = .0001
	−	3	4		1	6	
	Dogs						
	+	19	4	p = .1250	7	16	p = .0000
	−	0	1		0	1	
	Cats						
	+	10	4	p = 1.0000	1	13	p = .0002
	−	3	1		0	4	
	Total						
	+	32	15	p = .0030	29	18	p < .0001
	−	3	4		1	6	

Table 5
Results of the PCR amplification reactions of the different target genes used on fecal samples from the three hosts (dogs, cats, and humans) studied for the detection of *Giardia* DNA.

	DNA amplification										
	Negative	1 gene				2 genes				3 genes	Total
		β-g	*tpi*	*gdh*	Total	*β-g* + *tpi*	*β-g* + *gdh*	*tpi* + *gdh*	Total		
Humans	24	8	6	5	19	2	2	1	5	6	30
Dogs	17	6	0	0	6	0	1	0	1	0	7
Cats	17	1	0	0	1	0	0	0	0	0	1
Total	58	15	6	5	26	2	3	1	6	6	38

in the diagnosis of giardiasis are designed for one particular host species, these assays may perform well in fecal samples from multiple hosts as humans, dogs, cats and other mammals are infected with the same *Giardia* species (Sprong et al., 2009; Sotiriadou et al., 2013).

Other authors have used ELISA for the diagnosis of giardiasis in dogs, cats and humans. There are few studies about the use of this enzyme immunoassay on feline samples, and in most of these studies the ELISA was considered highly sensitive and highly specific for the diagnosis of *Giardia* infection in feces of any of these three hosts (Behr et al., 1997; Rocha et al., 1999; Decock et al., 2003; Garcia et al., 2003; Mekaru et al., 2007; Papini et al., 2013; Sadaka et al., 2015). Mircean et al. (2012) claim that both the sensitivity and specificity of ELISA for the diagnosis of canine giardiasis in fecal samples are low. These authors obtained results similar to ours in terms of the diagnostic performance of this immunoenzymatic assay. The comparison between the results obtained in each immunological technique (ELISA versus immunochromatography) is shown in Table 4.

The less than satisfactory results of the ELISA in the diagnosis of giardiasis when compared with the results obtained by immunochromatography can be attributed to the dilution of the fecal antigen as high concentrations of dilution buffer are necessary to perform this assay. Besides this drawback of the assay, another problem is that ELISA plate washing which is a step required to prevent accumulation of fecal waste may interfere with test performance too. It is interesting to mention that the sensitivity of different immunodiagnostic methods may vary, especially in those cases when stool specimens with low parasite loads are analysed (Rishniw et al., 2010). The correlation between the reaction intensity of ELISA and parasite load in the samples analysed has been previously described by other authors (Addiss et al., 1991; Vidal and Catapani, 2005). Rishniw et al. (2010) highlight the fact that immunological assays may give false-negative results if antigen concentrations in the samples are low. In the present study, the majority of the stool specimens tested, especially those from humans, had few parasite stages. Lower fecal antigen concentration could be explained by the fact that many of these fecal specimens contained only a single cyst on the glass slides screened under the light microscope.

Currently there is a strong trend in research and diagnostics towards the use of molecular methods, and these methods have been considered by many as the most sensitive and most specific tests for the diagnosis and routine public health surveillance of a large number of infectious and parasitic diseases. In contrast to the results obtained in previously published studies (Ghosh et al., 2002; Verweij et al., 2003, Boadi et al., 2014; Silva et al., 2016) PCR was able to detect only 39.5% (38/96) of *Giardia* carriers; 55.5% (30/54) were humans, 29.1% (7/24) dogs, and 6% (1/18) cats as shown in Table 3. In the present study, fecal samples were considered positive for *Giardia* when at least one of the target genes studied was amplified from these stool specimens. The number of targeted genes successfully amplified by PCR in stool samples from dogs, cats and humans as shown in Table 5.

The use of multiple gene loci is advantageous in the diagnosis of giardiasis by PCR due to variations in the discriminatory power of these genes. The sensitivity of the PCR reactions is increased by using a more conserved locus, which is represented by the gene encoding the *β-g* protein, and by two gene loci with higher polymorphism represented by the genes encoding tpi and gdh enzymes (Ryan and Cacciò, 2013). Sudre et al. (2014) highlight the fact that PCR may provide false negative results due to a low concentration of *Giardia* cysts in fecal samples. In the present study, the small number of parasite cysts present in fecal samples from all three species studied, especially those from humans, may have contributed to the low efficiency of PCR in the diagnosis of *Giardia* infection.

Interestingly, despite the small number of cysts that were seen in human stool specimens by conventional microscopy, the set of samples from this particular host had the highest number of positive results by PCR. Such results can be attributed to the physical characteristics of the samples as observed during DNA extraction of the parasite. In humans, stool samples were free of contaminants such as dirt, hair, clay, grass and sand. On the other hand, these contaminants were often found in fecal samples from the other hosts, especially cats, and may have adversely affected laboratory analysis of these specimens. According to

Hawash (2014), since a fecal sample is a very complex material, DNA degradation may ensue in this specimen, and polymerase activity may be impaired as well making it difficult to retrieve genetic material from inside the parasite cysts. In addition, stool specimens from animals are often highly contaminated with soil organic matter or sand from a cat litter box. These contaminants may dilute target DNA in the midst of nucleic acids from fecal microorganisms and from the animal host itself making DNA extraction quite difficult (Wilke and Robertson, 2009; Adamska et al., 2010; Stroup et al., 2012; Kuk and Cetinkaya, 2012). These contaminants may also interfere by hindering the release of DNA from cysts and therefore reducing the efficiency of the purification process during the extraction procedure. These PCR inhibitors would lead to false-negative results during DNA amplification (Nantavisai et al., 2007; Babaei et al., 2011).

In contrast to the results of our study, in a study published by Traub et al. (2004), a higher number of positive results for *Giardia* were obtained by PCR on fecal samples compared to the lower number of stool specimens that were positive for *Giardia* by the zinc sulphate flotation technique. However, these authors examined only one fecal sample by conventional microscopy. Like Traub et al. (2004), Read et al. (2004) were also able to amplify *Giardia duodenalis* DNA from fecal samples that were negative for this protozoan infection by light microscopy, and reported that the amplification of *Giardia* DNA in stool specimens containing even a single trophozoite may still be possible. In the present study, only one fecal sample was negative for *Giardia* by light microscopy and positive for this protozoan parasite by PCR as shown in Table 2. These findings may be explained by the fact that the heterogeneous distribution of cysts in a fecal sample would result in higher concentrations of genetic material in the aliquot used for DNA extraction. This uneven distribution of cysts throughout the fecal sample would occur even in a stool specimen with very low numbers of cysts as reported by Boadi et al. (2014).

Paz e Silva et al. (2012) obtained positive results for *Giardia* by PCR in stool specimens that were negative for this parasite by light microscopy and vice-versa. This variability in results may stem from the fact that these were samples with low numbers of protozoan cysts as suggested by these authors. These findings are similar to ours and have also been reported by Palmer et al. (2008) and Lebbad et al. (2010). Verweij et al. (2003) claim that stool samples that are negative for *Giardia* by PCR and positive for *Giardia* by conventional microscopy are samples in which a small volume of fecal material was used to obtain DNA for PCR. These authors believe that the fact that DNA extraction was done on samples with an insufficient volume of fecal matter may have decreased the concentration of nucleic acid in the specimens tested by PCR and thus decreased the sensitivity of this molecular assay.

4. Conclusion

Since a definitive diagnosis of giardiasis can be challenging due to a number of factors including intermittent shedding of cysts in feces and low numbers of cysts in stool specimens, if a test comes back negative, it is recommended that the fecal sample be tested by a different diagnostic test in order to completely rule out *Giardia duodenalis* infection. In this context, the use of a combination of diagnostic methods seems to be a good strategy in the diagnosis of this protozoan disease. Based on our findings and comparing our results with the results of other studies previously published by other authors, we suggest that the zinc sulphate flotation technique is the best laboratory test for the diagnosis of giardiasis. This method is a low cost diagnostic test that can detect multiple parasitic infections and performs well when serial fecal samples are tested. Immunological techniques, especially immunochromatography, can be used as alternative diagnostic methods but these are expensive, sensitive and specific tests that do not detect enteric parasites other than *Giardia*. Therefore, immunological assays should be used in those cases in which light microscopy yields negative results. PCR technique allows molecular characterization of *Giardia duodenalis* isolates which are of paramount importance in epidemiological studies. However, PCR was not able to detect light infections in feces of asymptomatic carriers with low parasitic loads especially animals.

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