Journal of Microbiological Methods 145 (2018) 73–78

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

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

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ARTICLE INFO

Keywords:
ELISA
Faust technique
Giardia duodenalis
Immunochromatography
PCR

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.

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https://doi.org/10.1016/j.mimet.2018.01.001
Received 12 October 2017; Received in revised form 4 January 2018; Accepted 8 January 2018
Available online 09 January 2018
0167-7012/

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