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Evaluation of Three Laboratory Methods for Diagnosis of *Giardia duodenalis* in children under seven years old

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A B S T R A C T

Different methods are used for the diagnosis of giardiasis and need to be evaluated for their applicability in laboratory routine. This study evaluated the effectiveness of three different methods for detecting *Giardia duodenalis* in fecal samples from children living in a poor area. Stool samples from 89 children aged under seven years were tested for *Giardia* by direct parasitological examination (DPE), immunochromatographic test (ICG), and real-time quantitative PCR (qPCR). Positive result was obtained for 22 samples using DPE, 26 using qPCR, and 38 using ICG. The tests showed conflicting results with different sensitivities and specificities, with the ICG test having the highest sensitivity but lower specificity than the qPCR. However, the qPCR detected the parasite in seven additional samples. It is necessary to use alternative methods for *Giardia* detection. We recommend ICG for *Giardia* diagnosis when the material is insufficient for microscopic analysis, particularly in cases of clinical suspicion.

Introduction

Giardiasis is a neglected disease, despite its high prevalence in tropical and subtropical regions, particularly in places with socioeconomic inequity, poor sanitation, and poor living conditions (Savioli *et al.*, 2006). Giardiasis is more common in children than adults; they suffer from diarrhea, malnutrition, and other symptoms that can affect physical, social, and intellectual development when they do not receive adequate treatment (Robertson *et al.*, 2010).

The detection of *G. duodenalis* infection in asymptomatic patients helps reduce the spread and transmission of protozoans. Therefore, techniques with high sensitivity and specificity should be used to provide high-quality diagnoses and more reliable epidemiological studies (Vidal and Catapani, 2006).

It is well known that the methods commonly used for the diagnosis of *G. duodenalis* have low sensitivity because a number of factors make microscopic identification of parasites in stools difficult, such as intermittent cyst shedding, low parasitemia, analysis of only one sample, and laboratory ability and experience (Wolfe, 1992; Ten Hove *et al.*, 2009). Other methods are available for improving the diagnostic safety of patients. Antigen detection immunologic approaches such as the immunoenzymatic assay, direct immune-fluorescence, and immunochromatography have been useful for routine diagnostic and epidemiological investigations, with their sensitivity and specificity ranging from 85% to 100%. These tests are commercially available, easy to perform, and do not require specialized equipment or qualified personnel (Singhal *et al.*, 2015). Recently, molecular biology techniques, such as nested PCR and real-time PCR, have shown high sensitivity and specificity values compared with those of microscopy and antigen detection, some of which are capable of genotyping the parasite (Bertrand and Schwartzbrod, 2007).

Therefore, this study aimed to evaluate the efficacy of three different laboratory methods (direct parasitological examination - DPE, immune-chromatographic examination - ICG, and quantitative real - time polymerase chain reaction - qPCR) in the diagnosis of *G. duodenalis* in stool samples from children living in an area conducive to the spread of the disease.

Materials and Methods

The study was approved by the Research of Ethics Committee of Instituto Evandro Chagas/Ministry of Health by CAAE: 19866613.0.0000.0019 and CAEE: 12543519.4.0000.0019.

Stool specimen collection

In the present study, we used frozen fecal samples from 89 children aged six months to seven years who attended two government daycare centers in Ananindeua City. The specimens were collected during a survey conducted between August 2014 and June 2016. Ananindeua is a large city located in the northeastern region of Pará State and has poor sanitary conditions.

The feces were collected in screw - cap plastic containers without preservatives or disposable diapers. Fresh stool was analyzed using microscopic and immunological methods. Some of the samples were stored at -20 ℃. One gram (1 g) of solid feces, 1 mL of liquid feces, or a diaper flap (3 cm × 3 cm) was diluted to 1 - 2 ml of phosphate-buffered saline (Invitrogen), transferred to polystyrene tubes, and stored in a freezer.

In the current study, frozen samples were thawed at room temperature, retested immunologically, and tested using qPCR assays.

Direct parasitological examination - DPE

A direct saline/iodine wet mount was prepared by mixing a small quantity (approximately 1 mg) of fresh stool sample with a drop of saline solution and Lugol's iodine. For diarrheic specimens collected in disposable diapers, a small flap (3 cm × 3 cm) containing feces was removed and mixed with 1 mL saline solution. The smears were examined under a microscope (10 X and 40 X magnification).

Immunological test

For the antigen detection of *G. duodenalis*, a commercial ICG assay (RIDA®QUICK *Giardia*. ®R-Biopharm) was used, according to the manufacturer's protocol. This test was developed to detect *Giardia* antigens in feces. *Giardia* antigens bind to specific antibodies immobilized on a membrane support in a specific position. A positive result is indicated by a red line.

Molecular diagnosis

The genetic material of the parasite was obtained using a QIAamp DNA Stool Mini Kit (QIAGEN

GmbH. Germany), according to the manufacturer's instructions. A qPCR assay (Techne® qPCR test *G. intestinalis* ghd gene. Bibby Scientific Ltd.) was used for the detection of the DNA of *Giardia*. Master mix was composed of 10 µL, 1 µL primer/probe mixture, 1 µL primer/probe of the positive internal extraction control, 3.0 µL nuclease-free water, negative controls, and 5 µL extracted DNA to produce a total volume of 20 µL. The qPCR test was carried out using a Quantstudio™ 5 real-time PCR machine (Thermo Fisher Scientific, USA) with the following thermocycling conditions: 2 min at 95°C, 50 cycles of 10 s at 95°C, and 1 min at 60°C. According to the standard curve previously standardized with the Ct values of the six positive control points, a threshold value of 0.06 was set, with Ct 42 as the maximum positivity cut-off point for *G. duodenalis*. In this test, the *ghd* gene was used to quantify the *G. intestinalis* genome, with 100% homology for genotypes A and B.

Data analysis

The results were analyzed using statistical methods. Sensitivity and specificity were obtained using the *Chi-Square* test (BioEstat 5.0) at 95% level of significance, with DPE used as the reference test.

Results and Discussion

In this study tested 89 stool samples using the DPE, IGG, and qPCR assays. The results showed different positivity rates for giardiasis in the pediatric population. The DPE test detected 22 positive cases, with the lowest prevalence (24.7%) of giardiasis. A higher positivity rate (42.7%) was observed with ICG, with a total of 38 positive individuals. This last test showed excellent reproducibility, as all 89 samples thawed and re-examined with ICG showed the same result. The qPCR assay detected parasite DNA in 26 (29.2%) samples, seven of which were positive only in qPCR. The Ct values of the positive samples ranged from 24 to 38, with an average value of 31.

The DPE and ICG results showed agreement in 73 (82.0%) samples, 22 were positive (DPE+ and ICG+) and 51 negative (DPE- and ICG-) in both tests. In these tests, 16 (18.0%) samples gave a positive result only in ICG (DPE- and ICG+). DPE and qPCR were concordant in 71 (79.7%) samples, 15 were positive (DPE+ and qPCR+) and 56 negative (DPE- and qPCR-) using both methods. Eighteen (20.3%) samples were discordant, of which 11 were qPCR+/DPE- and 7 were qPCR-/DPE+. Table 1.

When comparing the results of ICG and qPCR assays, 63 (70.8%) samples showed concordant results; 19 samples were positive (ICG+ and qPCR+) and 44 negative (ICG- and qPCR-) in both methods. In 26 (29.2%) cases, the results were discordant, 19 were qPCR negative/ICG positive (qPCR- and ICG+) and 7 were qPCR positive/ICG negative (qPCR+ and ICG-). Table 2.

In this study compared the results of the three tests to evaluate their sensitivity and specificity. Statistical analysis showed significant differences between DPE and ICG ($p = 0.0056$) but not between DPE and qPCR ($p = 0.25$) or between ICG and qPCR ($p = 0.03$). Compared with that of the DPE assay, the sensitivity and specificity of ICG were 100.0% and 80.0%, respectively, and those of qPCR were 75.0% and 86.0%, respectively.

DPE is the most common test used by public health services for the diagnosis of intestinal parasites in fecal samples. Sometimes, it is the only technique used in communities farther away from urban centers, where government investment in public health is low. DPE is considered a rapid, easy-to-perform, and inexpensive technique that requires a small quantity of feces for analysis. In this study, in addition *to G. duodenalis* (positivity of 24.7%), other intestinal parasites, such as *Ascaris lumbricoides* (6.7%), *Trichuris trichiura* (4.5%), and *Ancylostoma* spp. (1.1%), were detected using this method, which shows the importance of using this technique despite its low sensitivity. This method is useful in areas with poor sanitation conditions and where intestinal parasitosis is a common problem, particularly during childhood.
Currently, methods for antigen detection in feces

have shown good performance in the diagnosis of intestinal protozoa. They are rapid, simple, and easy to perform; allow the use of a single fecal and frozen sample; are efficient in epidemiological studies; and permit simultaneous processing of multiple specimens (Vidal and Catapani, 2006; Wilke and Robertson, 2009; Symeonidou *et al.*, 2020). In this study, the ICG method demonstrated 100% reproducibility in long-term storage of frozen samples, showing that antigen-based tests can be used when fecal samples are inappropriate for microscopic analysis.

In the present study, the parasite was detected most frequently by ICG, with a high prevalence (42.7%) in the test. Compared to that of the microscopic method, ICG showed high sensitivity and specificity (100.0% and 86.0%, respectively) and significant differences between the tests (p = 0.0056). These results highlight the importance of using ICG in epidemiological studies and clarifying its etiology in cases where the diagnosis of giardiasis is inconclusive.

Similar studies using other immunological tests have shown the importance of these techniques for the diagnosis of *G. duodenalis*. According to Hanson and Cartwright (2001), an immunoenzymatic assay (ELISA) can increase the probability of detecting the parasite, especially in asymptomatic cases where intermittent shedding of cysts and low parasitemia reduce the sensitivity of microscopy. Berne *et al.*, (2014) demonstrated that ELISA is more effective than the centrifugal concentration technique fluctuation, and the chance of detecting positive specimens is three times higher than that of the microscopic method. More recently, Oreby *et al.*, (2019) showed that the ELISA method was able to detect more positive cases of *G. duodenalis* than microscopy in children with and without symptoms suggestive of gastrointestinal disease. These findings show that the immunological method has a higher sensitivity and specificity than microscopy, corroborating the data observed in the present study. Techniques based on DNA identification are also used for the detection and genotyping of *G. duodenalis* and are important for the study of zoonotic genotypes of the parasite. In this study, the positivity rate for *G. duodenalis* using real-time qPCR was 29.2% (26/89). Although this technique detected a lower number of positive samples than ICG, qPCR identified parasites that were not observed using the other methods (DPE and ICG). The low positivity of qPCR compared to ICG does not support other studies that concluded that real-time PCR was more effective in detecting the parasite (Verweij *et al.*, 2003; Schuurman *et al.*, 2007; Alharbi *et al.*, 2020; Puebla *et al.*, 2020). According to Puebla *et al.*, (2020), molecular techniques have the disadvantage of identifying specific pathogens and not detecting other species present in feces and must be used as a complementary technique to microscopic diagnosis.

The low sensitivity of the molecular test in the present study may be related to factors such as the quality of the extracted DNA, PCR inhibitors in the feces, composition of the feces, low diversity of *G. duodenalis* genotypes detected by the technique, and insufficient storage and preservation of the samples (Bertrand and Schwartzbrod, 2007; Wilke and Robertson, 2009; Kuk and Cetinkaya, 2012).

According to Kuk and Cetinkaya (2012), the poor quality of the DNA obtained after extraction can be due to the degradation of the genetic material by the DNAse contained in the frozen feces. The fecal samples used in the present study were frozen (-20 ºC) after DPE and ICG assays were performed for qPCR analysis. It is likely that during the thawing process, the quality of *Giardia* DNA affected the qPCR results. In addition, Sudre *et al.*, (2014) concluded that a low concentration of *G. duodenalis* cysts in fecal samples can contribute to false-negative qPCR results.

Table.1 Comparison of the microscopic assay (DPE) with ICG and qPCR for *Giardia duodenalis* in 89 fecal samples from children under seven years old.

Test	DPE* (+)	DPE (-)	Total
ICG (+)	22	16	38
ICG (-)	-	51	51
Total	**22**	**67**	**89**
qPCR (+)	15	11	26
qPCR (-)	7	56	63
Total	**22**	**67**	**89**

*DPE: direct parasitological examination. **ICG: immunochromatographic test. ***qPCR: real-time quantitative PCR. Fonte: authors.

Table.2 Comparison of ICG and qPCR assay used to detect *G. duodenalis* in 89 fecal samples from children under seven years old.

Test	ICG* (+)	ICG (-)	Total
qPCR (+)**	19	7	26
qPCR (-)	19	44	63
Total	**38**	**51**	**89**

*ICG: immunochromatographic test. **qPCR: real-time quantitative PCR. Fonte: authors.

Although Schuurman *et al.*, (2007) and Jothikumar *et al.*, (2021) concluded that a single *Giardia* cyst is sufficient to obtain DNA and perform qPCR detection, it is possible that the amount of genetic material obtained from disposable diaper samples is insufficient for molecular detection.

The molecular findings of this study are not unique. Real-time PCR (RT-PCR) used by Verweij *et al.*, (2003) found no *G. duodenalis* DNA in two samples containing *G. lamblia* cysts. In contrast, RT-PCR identified *Giardia* in all positive fecal samples detected by antigen testing. These researchers concluded that RT-PCR is as specific and sensitive as an immunological test. Gotfred-Rasmussen *et al.*, (2016) found more positive results in qPCR and immunofluorescence assays than in two routinely used parasitological techniques.

Despite the high expense of most tests based on antigen and genetic material detection, these findings underscore the importance of molecular and immunological tests in *Giardia* diagnosis. It should be noted that these tests only detect specific pathogens, which limits their use in laboratory routines. Contrary to these studies, Uchoa *et al.*, (2018) observed much better results in the parasitological (zinc sulfate flotation) and immunological (immunoenzymatic and immunochromatography) tests than in the nested-PCR and concluded that the parasitological method performed better than the other tests for the detection of *G. duodenalis* when using serial samples. Although molecular and immunological approaches are considered the gold standard tests for several pathogens, simple and inexpensive tests should be used for the routine diagnosis of intestinal parasites.

In this study also cannot exclude the possibility that other assemblages, non-A and non-B, were present in samples positive in the other tests because the molecular technique used detects only assemblages A and B, which are commonly found in humans. Non-A-B genotypes occur at a low frequency in the human population. Fantinatti *et al.*, (2016) identified assemblage E in 34% of children in the day care center of the slum of Rio de Janeiro, Brazil. This

indicates that assemblage E can be present at high frequencies in some Brazilian populations. Therefore, genotyping is essential to identify cases not detected by qPCR.

The immunoassay (ICG) is the most effective method for the detection of *G. duodenalis* infection in insufficient or frozen specimens. We recommend adopting an immunological test for complementary diagnosis of giardiasis in cases where the sample is unsuitable for microscopic examination, especially in patients with clinical suspicion, owing to its greater sensitivity and specificity and low cost compared to molecular testing.

Although ICG is effective, the use of microscopic methods increases the chance of detecting giardiasis and other intestinal parasites that share the same route of transmission, providing a more reliable result in epidemiological investigations in areas with poor sanitation.

Finally, considering the positivity in at least one of the tests, the prevalence of giardiasis was high (50.6%), which shows that giardiasis remains an important public health problem in the poor areas of Brazil.

Author´s contribution

All authors participated in the work substantially. Their roles are described below: MCMS, LSS, and JDAPM: conceived and designed the study; HAT, ALMS, PSL, and DMT: performed the experiments; MCMS, and HAT: analyzed the data and wrote the paper.

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Declaration of competing interest

The authors declare that there is no conflict of interest.

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