



Diagnosis of intestinal protozoan infections in patients in Cuba by microscopy and molecular methods: advantages and disadvantages

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ABSTRACT

Microscopy is the gold standard for diagnosis of intestinal parasitic diseases in many countries, including Cuba, although molecular approaches often have higher sensitivity as well as other advantages.

Fecal samples from 133 patients were analyzed by light microscopy and also real-time multiplex qPCR targeting *Giardia duodenalis*, *Cryptosporidium* spp., and *Entamoeba histolytica*, and, separately, *Dientamoeba fragilis*. Microscopy revealed *G. duodenalis* occurred most commonly (17 patients), followed by *Blastocystis* spp. (12 patients). In a few patients, *Entamoeba histolytica*/*E. dispar*, *Cryptosporidium* spp., and *Cyclospora cayatanensis* were identified.

Molecular analysis identified 4 more *G. duodenalis* infections and 2 more *Cryptosporidium* spp. infections; concordance between microscopy and PCR showed almost perfect agreement for *G. duodenalis* ($\kappa = 0.88$) and substantial agreement for *Cryptosporidium* ($\kappa = 0.74$). PCR indicated that *E. dispar*, rather than *E. histolytica*, had been identified by microscopy. Additionally, 16 *D. fragilis* infections were detected using molecular methods.

Although both microscopy and molecular techniques have a place in parasitology diagnostics, for parasites such as *D. fragilis*, where microscopy can underestimate occurrence, molecular techniques may be preferable, and also essential for distinguishing between morphologically similar microorganisms such as *E. histolytica* and *E. dispar*. Although in resource-constrained countries such as Cuba, microscopy is extremely important as a diagnostic tool for intestinal parasites, inclusion of molecular techniques could be invaluable for selected protozoa.

1. Introduction

Intestinal parasitoses are one of the main causes of health-service consultations in developing countries and are an important cause of morbidity worldwide. Among these, infections with some protozoa are recognized as having significant consequences on health, particularly in children (Hamdy et al., 2020). Direct consequences are usually gastrointestinal disorders, such as diarrhea, dysentery, abdominal pain, vomiting, and lack of appetite, and indirect impacts include synergistic negative effects on nutritional status, stunting, and reduced educational achievement. For instance, every year there are more than 200 million symptomatic patients with giardiasis (Mmbaga and Houtp, 2017), and

cryptosporidiosis is a leading cause of diarrhea in children younger than 5 years globally, only second after rotaviral enteritis (Mmbaga and Houtp, 2017).

Infections with *Giardia duodenalis* (syn. *Giardia lamblia* or *Giardia intestinalis*) *Cryptosporidium* spp., and *Entamoeba histolytica* are considered to be among the most common and important causes of parasite-related diarrhea in human populations. In contrast, the pathogenicities and symptoms associated with infections with *Blastocystis* spp. and *Dientamoeba fragilis* have been more controversial (Stensvold and Clark, 2016; Wong et al., 2018). Nevertheless, some evidence indicates that some sub-types of *Blastocystis* may be associated with irritable bowel syndrome (El-Badry et al., 2018; Rostami et al., 2017), or, at the very

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least, be an indicator of dysbiosis (Cifre et al., 2018). However, such associations have been more difficult to identify for *D. fragilis*. A large case-control study recently reported that both *Blastocystis* spp. and *D. fragilis* were less likely to be identified in cases with gastrointestinal symptoms ($n = 1374$ (*Blastocystis* spp. analyses); $n = 1515$ (*D. fragilis* analyses)) than in controls without such symptoms ($n = 1026$ (*Blastocystis* spp. analyses); $n = 1195$ (*D. fragilis* analyses)), where investigation for *Blastocystis* spp. infection was conducted on a sub-set (de Boer et al., 2020; Bruijnesteijn van Coppenraet et al., 2015). A study in Denmark yielded similar results (Krogsgaard et al., 2015).

Microscopy of fecal smears or concentrates, with or without staining, has been the mainstay for many years for the diagnosis of intestinal protozoan infections and remains the cornerstone of the diagnosis of parasitic infections in many routine diagnostic laboratories (WHO, 1991). However, other methods are available, including detection of parasite antigen in feces by ELISA or immunochromatography (e.g., snap tests) and molecular methods (Garcia et al., 2017).

Given the excellent sensitivities and specificities achieved by molecular methods, detection of parasite-specific DNA by PCR is fast gaining popularity. Not only is PCR simple and potentially offers a more rapid turnaround time, but it also allows discrimination between morphologically indistinguishable parasites that are of different clinical relevance (such as *Entamoeba dispar* and *E. histolytica*). In addition, multiplex PCR enables samples to be investigated for several pathogens simultaneously, and can also be used to determine relative copy numbers, although the potential for quantification is often not used in the diagnostic setting. However, due to the requirement for comparatively costly equipment and reagents, including some for which refrigeration is essential, such diagnostic assays have not been implemented in many diagnostic labs, and are particularly underused in labs in less-developed regions. Nevertheless, where facilities and resources are available, multiplexed PCR-based methods that target the most relevant gastrointestinal parasites can provide fast, reliable results and could be implemented in routine clinical diagnostics (Laude et al., 2016). However, comparison of commercial multiplex real-time PCR assays has indicated that performance may be variable, and the required sensitivities and specificities, along with lab workflow, diagnostic algorithms, and population should be considered, as well as the cost, when considering incorporating such assays into the diagnostic lab (Paulos et al., 2019).

The objective of the present study was to compare the results from microscopy-based diagnostic tests for intestinal protozoa with those obtained by real-time molecular assays (commercial multiplex for *Cryptosporidium* spp. *G. duodenalis*, and *E. histolytica* and a published PCR protocol for *D. fragilis*) among a group of patients with different gastrointestinal disorders.

2. Materials and methods

2.1. Study population

A descriptive cross-sectional study was conducted between January and September 2019 among 133 patients either attending the Pediatric Hospital “William Soler” or for whom stool samples had been submitted to the “Pedro Kourí” Institute; both institutes are located in Havana, Cuba. Patients included in the study had gastrointestinal disturbances or there was clinical suspicion of intestinal parasitic infection. Some of the children included in the study were participating in a surveillance program for intestinal parasitic infections. Information on demographic and clinical variables (gender, age, symptoms, and some risk factors for parasitic infections) was recorded in standard epidemiological-questionnaire forms by nursing or medical staff at sample submission.

2.2. Sample analysis

2.2.1. Analysis by microscopy-based techniques

One stool sample from each patient was examined for intestinal parasites. For all samples ($N = 133$), direct wet mounts were stained with Lugol’s iodine (particularly useful for identification of *Giardia* and *Blastocystis* cysts; WHO, 1991, Wolfe, 1992; Núñez and Cordoví, 2006) and also examined following formalin-ethyl acetate concentration. A modified Ziehl-Neelsen (mZN) acid-fast stain was used for coccidian parasites such as *Cyclospora* and *Cryptosporidium* spp. (Henriksen and Pohlenz, 1981; Garcia, 2001; Walochnik and Aspöck, 2012); however, this investigation was only conducted on diarrheic samples ($n = 31$).

Fixatives were not used, and the samples were analyzed on arrival at the lab, less than an hour after collection. Each sample was analyzed independently by two experienced microscopists; for all samples there was concurrence between the results obtained by each microscopist.

2.2.2. Analysis by molecular assays for detecting intestinal parasites

DNA was extracted from all 133 stool samples using the QIAamp DNA Stool Kit (QIAGEN Inc., Valencia, California, USA) following the manufacturer’s protocol, and using approximately 200 µg from each well-mixed sample. DNA was stored frozen at $-18\text{ }^{\circ}\text{C}$ before investigation by PCR. A multiplex real-time PCR (qPCR) detection kit (VIASURE *Cryptosporidium*, *Giardia* & *E. histolytica* Real-Time PCR Detection Kit, CerTest Biotec S.L., Zaragoza, Spain) was used for molecular detection of *Cryptosporidium* spp., *G. duodenalis*, and *E. histolytica* following the manufacturer’s instructions, and including the internal control and appropriate controls provided in the kit.

For detection of *D. fragilis*, a previously published qPCR assay targeting a 77-bp fragment of the SSU rDNA gene was performed on all samples as previously described (Stark et al., 2006) using the following primers and probe: DF3 (5’GTTGAATACGTCCCTGCCCTT3’), DF4 (5’TGATCCAATGATTTACCGAGTCA3’), and Taqman probe (5’6-FAM-CACACCGCCGTCG CTCCTACCG-6-TAMRA). Briefly, amplification reactions were performed in a Rotor-Gene® 5plex equipment (Qiagen, Germany), in a volume of 25 µl with PCR buffer (HotStarTaq mastermix, Qiagen), 5 mM MgCl₂, 2 pmol of each *D. fragilis*-specific primer, 1.25 pmol of *D. fragilis*-specific XS-probe, and 5 µl of the DNA sample. Amplification consists of 15 min at 95 °C followed by 50 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. DNA extracted from a *D. fragilis*-positive sample was used as a positive control for PCR; this sample had been identified by identification of an abundance of *D. fragilis* trophozoites, and had been independently confirmed by PCR. Ultrapure water was used as template in a negative control, and negative and positive controls were included in each amplification run.

2.3. Statistical analysis

All data were entered into a spreadsheet and analyzed using EPINFO 6.04 and EPIDAT 3.1 statistical programs. Cohen’s kappa index (κ) was used to test the concordance between the results from the molecular assays and coproscopy for *G. duodenalis* and *Cryptosporidium* spp. (Cohen, 1960). To investigate associations between infections and the continuous variable (age) Mann-Whitney *U* tests were used; contingency table analyses for assessing associations with categorical variables (gender, urban/rural residence, different symptoms); *P* values below 0.05 were considered statistically significant.

3. Results

3.1. Cohort description

Of the 133 patients, 68 (51%) were female and the median age was 25 years (ranging from 2 to 78 years). The majority of the patients (121 of 133; 91%) described their homes as urban, and the others lived in a rural location. The most common symptom reported among all patients,

regardless of the result of investigation, was abdominal pain (41 patients; 31%) followed by diarrhea (31 patients; 23%), nausea (18 patients; 14%), and flatulence (16 patients; 12%).

3.2. Parasites identified using by microscopy-based and molecular techniques

From 133 patients, an intestinal parasite of relevance was identified by light microscopy-based methods in 29 (22%), with *Giardia* identified most frequently (Table 1). Of these, 21 (72%) were single infections and 8 were dual infections; both *G. duodenalis* and *Blastocystis* spp. were identified in 5 patients, *E. histolytica*/*E. dispar* complex and *Blastocystis* spp. in 2 patients, and 1 patient was infected with both *C. cayetanensis* and *Blastocystis* spp.

Using the multiplex kit for *G. duodenalis*, *Cryptosporidium* spp., and *E. histolytica*, 21 patients were found to be positive for *Giardia* (the 17 cases that were positive by microscopy and 4 others) and 5 patients were found to be positive for *Cryptosporidium* spp. (the 3 cases that were positive by microscopy and 2 others) (Table 1). Cohen's kappa index (κ) for agreement between methods for detection were 0.85 (almost perfect agreement) for *G. duodenalis* and 0.74 (substantial agreement) for *Cryptosporidium* spp., although with wide CI due to the few samples that were positive. However, as one of the two additional *Cryptosporidium* cases was not associated with diarrhea it had not been examined by mZN for coccidia. Of the 6 additional cases of *Cryptosporidium* or *Giardia* infection identified by PCR, none were identified to also be infected with other parasites, either by PCR or by microscopy.

No cases were found to be positive for *E. histolytica* by PCR, suggesting that those cases previously identified as potential *E. histolytica* cases by microscopy were actually *E. dispar*.

Although *Dientamoeba fragilis* was not detected using microscopy, qPCR identified 16 positive samples (as illustrated by the results from 10 randomly-selected positive samples in Fig. 1). Replicates produced the same results. In seven of these patients (44%), no other intestinal parasites were detected by microscopy or PCR. In four patients of the

Table 1
Comparison of identification of intestinal parasites by microscopy-based and molecular-based methods among 133 patients with gastrointestinal symptoms.

Intestinal parasites identified ^a	Number of positive cases identified			Proportion infected (95% CI)
	By microscopy	By PCR	In total (microscopy and/or PCR)	
<i>Giardia duodenalis</i>	17	21	21	15.8 (10.5–23.0)
<i>Cryptosporidium</i> spp.	3	5	5	3.8 (1.4–8.7)
<i>Entamoeba histolytica</i> / <i>E. dispar</i>	4	0 ^c	4	3.0 (0.9–7.7)
<i>Blastocystis</i> spp. ^b	12	0	12	9.0 (5.1–15.2)
<i>Cyclospora</i> <i>cayetanensis</i> ^{a, b, c}	1	0	1	0.8 (<0.01–4.6)
<i>Dientamoeba fragilis</i>	0	16	16	12.0 (7.4–18.8)
Total infected with at least one intestinal protozoan parasite^{ad}	29	38	42	31.6 (24.3–39.9)

^a Modified Ziehl-Neelsen (particularly relevant for identifying *Cryptosporidium* spp. and *Cyclospora cayetanensis*, was used only on diarrheic samples ($n = 31$), but all samples were examined by other light microscopy approaches and by PCR ($N = 133$).

^b *Blastocystis* spp. and *Cyclospora cayetanensis* not investigated by PCR.

^c *Cyclospora cayetanensis* only investigated by modified Ziehl-Neelsen in diarrheic samples ($n = 31$).

^d Due to co-infections (see text), total infected with at least one intestinal protozoan parasite does not equal sum of number of positive cases identified.

^e PCR only detects *Entamoeba histolytica*.

D. fragilis-positive patients, co-infection with *Blastocystis* spp. was identified and in four patients, coinfection with *G. duodenalis* was identified by both microscopy and PCR. In one *D. fragilis*-positive sample, co-infection with *E. histolytica*/*E. dispar* was identified by microscopy. Thus, in all those samples in which *Blastocystis* spp. cysts were detected by microscopy, another intestinal protozoan was also identified.

3.3. Demographic and clinical associations with the most frequently intestinal parasites

Although no associations between parasitic infections and gender or with urban/rural residence were identified, there were associations between age and some parasitoses. In those patients in which *Giardia* infection was detected ($n = 21$, including those identified only by molecular methods, and irrespective of co-infections) the median age was 14 years. This is significantly younger than the median age of the whole cohort. Similarly, the median age of those in which *Blastocystis* spp. was identified ($n = 12$, irrespective of co-infections) was significantly lower than that of the whole cohort, being only 9 years. However, if those patients who were infected with both *Giardia* and *Blastocystis* were removed from the analysis, then the median age of the *Blastocystis* cases ($n = 7$) was 22 years and no longer significantly different from that of the whole cohort. In contrast, the median age of the *Giardia* cases ($n = 15$) remained significantly lower at 8 years. The median age of patients in which *D. fragilis* was diagnosed ($n = 16$, regardless of co-infection) did not differ significantly from the median age of the entire cohort.

Regarding symptoms, Table 2 provides an overview of the most frequently reported symptoms and their occurrence in patients in whom *Giardia*, *D. fragilis*, *Cryptosporidium* spp. and/or *Blastocystis* spp. were detected, and also patients in whom no positive findings (protozoan parasites or commensals) were recorded. Investigations of associations between symptoms and specific infections were hampered by the relatively low numbers of positive samples and that several positive samples were with dual infections.

Those patients in which no protozoan infection was detected were less likely to report abdominal pain than those patients in which *Giardia* or *D. fragilis* were identified. Similarly, patients in which no protozoan infection was detected were also less likely to report diarrhea than those patients with *Giardia* infection, and were also less likely to suffer from flatulence. Although *Cryptosporidium* infection was associated with diarrheal symptoms, it was not possible to identify statistically significant associations within this dataset. As previously noted, *Cryptosporidium* was detected by PCR in one patient without diarrhea, which therefore was not analyzed by mZN.

4. Discussion

Protozoan intestinal infections are recognized as an important cause of morbidity in developing countries, with children the most vulnerable population (Harhay et al., 2010). Transmission of intestinal protozoa is mainly through the fecal-oral route, following ingestion of the infectious stages, often with contaminated water and food. Some intestinal protozoa are host-specific, but for others, such as some *Cryptosporidium* species, zoonotic transmission may also occur. Previous survey-type studies in Cuba, particularly among children, have identified *G. duodenalis* and *Blastocystis* spp. as occurring most commonly, but *Cryptosporidium* spp., *Cyclospora*, and *E. histolytica*/*dispar* have also been reported (Vital et al., 1999; Mendoza et al., 2001; Núñez et al., 2003; Cañete et al., 2012). *D. fragilis* infection has not previously been reported in Cuba, although there are anecdotal reports from the 1940s, but this could be associated with lack of sensitive diagnostic tests. It is thus particularly relevant that use of molecular methods indicated that *D. fragilis* infection occurs relatively commonly in Cuba. This suggests a need for further investigation, particularly regarding whether *D. fragilis* infection is associated with clinical symptoms.

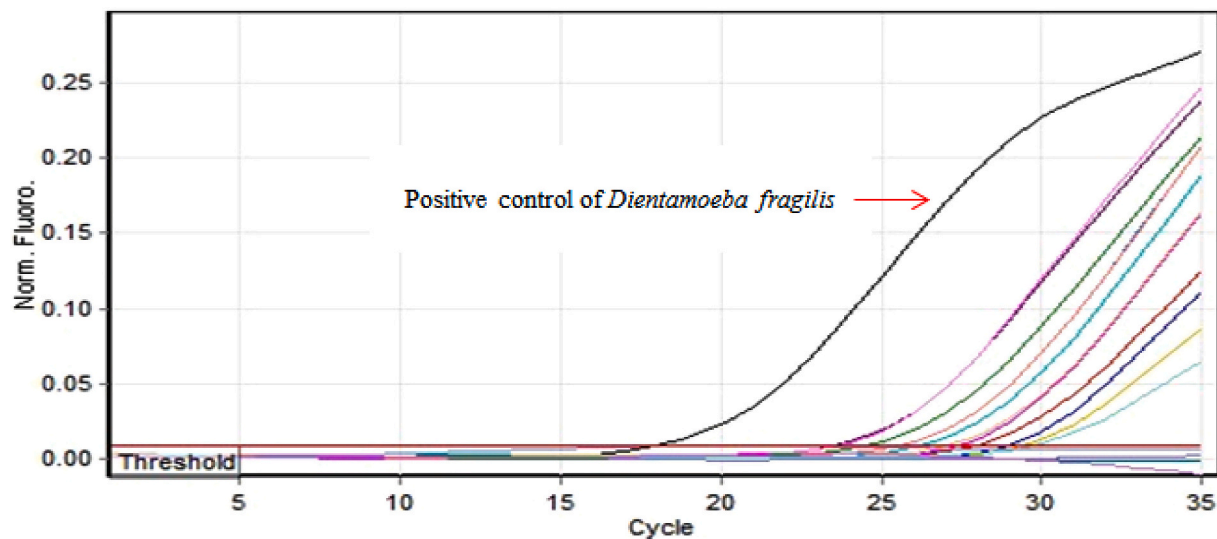


Fig. 1. Detection of *D. fragilis* in stool samples by real-time PCR following the protocol of Stark et al., 2006. Positive control (black curve) and representation of positive samples identified. The negative control did not amplify and is running below the red threshold (cut-off) line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

The occurrence of the four predominant symptoms recorded in association with specific identified protozoal infections or no infections identified.

	Abdominal pain (n = 41)	Diarrhea (n = 20)	Nausea (n = 18)	Flatulence (n = 15)
All <i>Giardia</i> infections (n = 21)	14/21 (67%)	9/21 (43%)	5/21 (24%)	11/21 (52%)
<i>Giardia</i> as mono-infection (n = 11)	8/11 (73%)	6/11 (55%)	1/11 (9%)	5/11 (45%)
All <i>D. fragilis</i> infections (n = 16)	10/16 (63%)	4/16 (25%)	2/16 (13%)	7/16 (44%)
<i>D. fragilis</i> as mono-infection (n = 7)	5/7 (71%)	2/7 (29%)	1/7 (14%)	4/7 (57%)
All <i>Cryptosporidium</i> infections ^a (n = 5)	1/5 (20%)	4/5 (80%)	1/5 (20%)	0/5 (0%)
All <i>Blastocystis</i> infections ^b (n = 12)	5/12 (42%)	3/12 (25%)	5/12 (42%)	3/12 (25%)
No protozoal infection identified ^c (n = 72)	15/72 (21%)	13/72 (18%)	9/72 (13%)	0/72 (0%)

^a No other parasites were identified in any of the samples where *Cryptosporidium* spp. was detected.

^b All the samples in which *Blastocystis* spp. was detected were also found to contain other parasites (see section 3.2).

^c Samples in which commensals were found (e.g., *Entamoeba coli* and *Endolimax nana*) are not included in the “no protozoal infection” group.

Diagnosis of intestinal parasitic infections in Cuba is generally by microscopic examination of stool samples, primarily using direct wet mounts or formalin ethyl concentration, with staining with Lugol's iodine and also mZN. More sensitive methods, such as immunofluorescence for *Cryptosporidium* and *Giardia*, are not used, reflecting the lack of equipment and reagent resources.

The sensitivity and specificity of coproscopy for detection of protozoan parasites is sometimes poor, and requires prolonged hands-on work by an experienced microscopist, particularly when excretion levels are low. Therefore, molecular assays are increasingly being used in diagnostic labs, with multiplex panels considered to be of particular value (Parčina et al., 2018). Various molecular diagnostic panels are now commercially available from different suppliers, with others under development or in the process of validation. In addition, molecular approaches can be used provide more information regarding particular

species or subtypes, which may simply not be possible to determine by microscopy-based methods.

However, molecular methods also have some disadvantages, often requiring refrigeration capacity for reagents along with expensive equipment and consumables. This is particularly so for multiplex panels, which provide simplicity and rapid turnaround times, but at a high cost. In addition, molecular assays only detect those pathogens for which the appropriate primers are used – other organisms of potential relevance will not be detected and additional analyses will be required should other parasites or other pathogens be suspected.

In the work described here, for both *Giardia* and *Cryptosporidium* a few positive additional samples were found by molecular methods (four more for *Giardia* and two for *Cryptosporidium*). However, the concordance between results from microscopy and PCR was good, being considered almost perfect agreement for *G. duodenalis* ($\kappa = 0.85$) and substantial agreement for *Cryptosporidium* spp. ($\kappa = 0.74$). A study in Côte d'Ivoire found only moderate diagnostic agreement ($\kappa = 0.47$) between microscopy (formalin-ether concentration) and a commercially available multiplex PCR gastrointestinal pathogen panel for detection of *G. duodenalis* (Becker et al., 2015). Moderate diagnostic agreement ($\kappa = 0.424$) was also found for *G. duodenalis* detection in a study from Timor-Leste and Cambodia using microscopy (zinc sulphate centrifugal flotation) and an in-house multiplex PCR (Llewellyn et al., 2016). In addition, a study from Venezuela (Incani et al., 2017) found only moderate concordance between microscopy and a multiplex PCR ($\kappa = 0.4$). These less-substantial agreements found in these three studies (Becker et al., 2015; Llewellyn et al., 2016; Incani et al., 2017) compared with ours may reflect that in these other studies the microscopy for *Giardia* cysts did not involve any staining; Lugol's iodine (as used in our study) improves identification (Wolfe, 1992).

As also pointed out by the authors of the study in Côte d'Ivoire (Becker et al., 2015), it is important to be aware that, from a clinical perspective, hypersensitivity of a molecular method may not always be highly useful. Positive results may require independent confirmatory methods to discriminate active infection from asymptomatic shedding of nucleic acids. For example, in our study, one of the additional *Cryptosporidium* results was associated with a patient who was not experiencing diarrhea. This may reflect asymptomatic infection, but could also perhaps indicate that this positive finding was not associated with infection, but rather with detection of DNA from low-level passage of non-infectious oocysts. This could be due to the *Cryptosporidium* being of a species that does not infect humans (the multiplex used does not

specify which species it detects) or because the oocysts were inactivated prior to ingestion. The only symptom recorded in this patient was nausea, which could be associated with *Cryptosporidium*, but could also be associated with another pathogen or condition. Indeed, as only those samples in which diarrhea was reported were examined by mZN for coccidia, this sample was only investigated by molecular means. Therefore, the kappa index does not provide a good representation regarding the similarity of the methodological aspects for *Cryptosporidium* detection as only the microscopy-based investigation included this symptom in the methodological algorithm. It should be noted that due to the lack of analysis of the samples for other (e.g., bacterial or viral) pathogens, we cannot exclude that these may have been responsible for the symptoms in at least some of the patients.

Regarding the other three protozoa investigated in this study, *Entamoeba* spp. were identified by microscopy (*E. dispar/histolytica*), but as there were no positive results in the multiplex PCR for *E. histolytica*, it is assumed that all the microscopy-positive results actually indicated *E. dispar*. Again, similar findings were reported in the study from the study in Côte d'Ivoire (Becker et al., 2015). Furthermore, a previous study from Cienfuegos Province, Cuba using microscopy and an in-house duplex qPCR found that of *E. histolytica/dispar* infections identified by microscopy, the majority were due to *E. dispar* (Núñez et al., 2001). Given that *E. dispar* is considered a non-pathogenic commensal and *E. histolytica* an important pathogen that can cause, in addition to intestinal symptoms, an invasive, severe, extra-intestinal disease, there seems little point in using a diagnostic method that cannot distinguish between pathogenic and non-pathogenic species. Although various articles (e.g., Hamzah et al., 2010; Lau et al., 2013) suggest that the World Health Organization endorsed PCR as the method of choice way back in 1997 (WHO, 1997), reading of this document does not actually indicate this. However, it does state that confirmation of the two distinct species is a major accomplishment. Thus, it is clear that using a technique that distinguishes between these species is important and of clinical relevance.

As tools for molecular detection of *Blastocystis* spp. were not used, infection with this protozoan was only identified using traditional microscopy-based methods; the rate of occurrence was lower than previously reported in Cuba (14%–38%) (Núñez et al., 2003; Cañete et al., 2012). In the current study, *Blastocystis* spp. infection was only identified in association with other protozoal infections, and therefore it was not possible to determine whether there was an association with a particular symptom spectrum. Indeed, given that *Blastocystis* is often found more commonly in healthy individuals than those with gastrointestinal complaints, the potentially pathogenic role of this parasite in human infections remains controversial (Clark et al., 2013; Andersen and Stensvold, 2016). Nevertheless, a recent retrospective study from Switzerland involving a large number of patients found a correlation between *Blastocystis* spp. detection and abdominal pain, but not diarrhea or other symptoms, and, as in our study, also noted a high degree of coinfection (Légeret et al., 2020). Furthermore, some isolates of *Blastocystis*, notably ST7, have been associated with disruption of the gut microbiota, potentially resulting in a pathogenic effect (Yason et al., 2019); investigation of *Blastocystis* sub-types was not in the remit of the present study.

Although *D. fragilis* was detected in 16 samples by PCR, this parasite was not detected by microscopy, neither as trophozoites nor the cyst form, which was first identified a few years ago (Stark et al., 2014). Indeed, as noted by Stensvold and Nielsen (2012), formal-ether concentration would not be expected to be appropriate for detecting *D. fragilis* trophozoites, although may have enabled identification of cysts. In general, even when staining is used, identification of *D. fragilis* in stool samples is considered difficult; the trophozoite stage can easily be misidentified because their staining is pale and their nuclei may appear similar to those of *Endolimax nana* or *Entamoeba hartmanni* (<https://www.cdc.gov/dpdx/dientamoeba/index.html>).

Investigations and discussion on the use of real-time PCR assays for

D. fragilis (Gough et al., 2019) have indicated contentious issues regarding both detection and potential pathogenicity of *D. fragilis* (some of which also should also be considered for other protozoan parasites, including those discussed here such as *Giardia*, *Cryptosporidium*, and *Blastocystis*). The authors (Gough et al., 2019) suggest that multiple false positives may occur, and they recommend a specific assay (Genetic Signatures EasyScreen) as a screening tool, and also amplicon sequencing, neither of which are appropriate for our laboratory and situation. As C_T values in our study were relatively early (23.5–29.5) and the detection was probe based, we feel confident that these were not false positives, taking into account the information presented by (Gough et al., 2019). However, it should be noted that the analytical performance of the PCR method used (Stark et al., 2006) was not investigated here. Furthermore, other investigations of this PCR setup have indicated that the forward primer and probe are non-specific (Verweij et al., 2007), and further optimization would be necessary before considering implementation into our current diagnostic lab routines. Sequencing of positive amplicons (although quite short at only 77 bp) could provide further strength to the study, but was not possible. Reports of *D. fragilis* in Cuba are scant, as permanent stains of fixed fecal smears are not routinely implemented and therefore this is the first report of this parasite identified by molecular tools in Cuba. However, as with *Blastocystis*, the pathogenic and clinical role of *D. fragilis* infection is controversial. Although gastrointestinal symptoms associated with *D. fragilis* infection have been described in the literature, it is also identified in healthy subjects without abdominal symptoms (Wong et al., 2018). Due to the low number of patients in our study, the frequency of co-infections with other protozoa, and the absence of information on infections with bacterial or viral pathogens, it is difficult to draw any firm conclusions regarding *D. fragilis* as an etiological agent of gastrointestinal symptoms. However, the association we identified between infection with *D. fragilis* and symptoms of abdominal pain and flatulence, but not diarrhea, suggest the possible role of this agent in these symptoms and indicate the need for larger, more focused studies to investigate this aspect in greater depth. Indeed, some previously reported case histories describe similar associations of *D. fragilis* infection with abdominal pain and flatulence (e.g., Halkjær et al., 2015; Norberg et al., 2003). Thus, this study indicates that this protozoan should not be ruled out in the differential diagnosis of gastrointestinal disturbance caused by intestinal parasitic infections.

5. Conclusions

Our study supports implementation of molecular tools into the parasitology diagnostic laboratory, even in under-resourced settings. Such tools are particularly important for detecting protozoa that are challenging to identify by microscopy (e.g., *D. fragilis*). Molecular tools are also essential for distinguishing between morphologically identical species that are of differing clinical relevance (e.g., *E. histolytica* and *E. dispar*).

Although often more sensitive than microscopy, the disadvantages of excess sensitivity of molecular methods should, as previously noted, also be taken into account. In addition, the value of maintaining competence and experience in microscopy techniques in the diagnostic laboratory should not be overlooked.

Thus, in our experience, molecular and traditional techniques have overlapping, but complementary, roles in diagnosing protozoan infections resulting in clinical manifestations in patients.

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Transparency declarations

There are no conflicts of interest to declare.

Ethical statement

The study was approved by the Ethics Commission of IPK and the Pediatric Hospital William Soler (Project Code: 20009).

Declaration of Competing Interest

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References

- Andersen, L.O., Stensvold, C.R., 2016. *Blastocystis* in health and disease: are we moving from a clinical to a public health perspective? *J. Clin. Microbiol.* 54 (3), 524–528. <https://doi.org/10.1128/JCM.02520-15>.
- Becker, S.L., Chatigre, J.K., Gohou, J.P., Coulibaly, J.T., Leuppi, R., Polman, K., Chappuis, F., Mertens, P., Herrmann, M., N'Goran, E.K., Utzinger, J., von Müller, L., 2015. Combined stool-based multiplex PCR and microscopy for enhanced pathogen detection in patients with persistent diarrhoea and asymptomatic controls from Côte d'Ivoire. *Clin. Microbiol. Infect.* 21 (6), 591.e1–10.
- Cañete, R., Díaz, M.M., Avalos, García R., Laúd, Martínez P.M., Manuel, Ponce F., 2012. Intestinal parasites in children from a day care centre in Matanzas City, Cuba. *PLoS One*. 7(12), e51394. <https://doi.org/10.1371/journal.pone.0051394>.
- de Boer, M.D., Schuurs, T.A., Vermeer, M., Ruijs, G.J.H.M., van der Zanden, A.G.M., Weel, J.F., 2020. Bruijnesteijn van Coppenraet LES. Distribution and relevance of *Dientamoeba fragilis* and *Blastocystis* species in gastroenteritis: results from a case-control study. *Eur. J. Clin. Microbiol. Infect. Dis.* 39 (1), 197–203. <https://doi.org/10.1007/s10096-019-03710-z>.
- Bruijnesteijn van Coppenraet, L.E., Dullaert-de Boer, M., Ruijs, G.J., van der Reijden, W. A., van der Zanden, A.G., Weel, J.F., Schuurs, T.A., 2015 Jun. Case-control comparison of bacterial and protozoan microorganisms associated with gastroenteritis: application of molecular detection. *Clin. Microbiol. Infect.* 21 (6) <https://doi.org/10.1016/j.cmi.2015.02.007>, 592.e9–19.
- Cifre, S., Gozalbo, M., Ortiz, V., Soriano, J.M., Merino, J.F., Trellis, M., 2018. *Blastocystis* subtypes and their association with irritable bowel syndrome. *Med. Hypotheses* 116, 4–9. <https://doi.org/10.1016/j.mehy.2018.04.006>.
- Clark, C.G., van der Giezen, M., Alfellani, M.A., Stensvold, C.R., 2013. Recent developments in *Blastocystis* research. *Adv. Parasitol.* 82, 1–32. <https://doi.org/10.1016/B978-0-12-407706-5.00001-0>.
- Cohen, J., 1960. A coefficient of agreement for nominal scales. *Educ. Psychol. Meas.* 20, 213–220.
- El-Badry, A.A., Abd El Wahab, W.M., Hamdy, D.A., Aboud, A., 2018. *Blastocystis* subtypes isolated from irritable bowel syndrome patients and co-infection with *Helicobacter pylori*. *Parasitol. Res.* 117 (1), 127–137. <https://doi.org/10.1007/s00436-017-5679-4>.
- García, L.S., 2001. Intestinal protozoa (coccidia and microsporidia). In: García, L. (Ed.), *Diagnostic Medical Parasitology*, 4th ed. American Society of Microbiology, Washington.
- García, L.S., Arrowood, M., Kokoskin, E., Paltridge, G.P., Pillai, D.R., Procop, G.W., Ryan, N., Shimizu, R.Y., Visvesvara, G., 2017. Practical guidance for clinical microbiology laboratories: laboratory diagnosis of parasites from the gastrointestinal tract. *Clin. Microbiol. Rev.* 31 (1) <https://doi.org/10.1128/CMR.00025-17> e00025-17.
- Gough, R., Ellis, J., Stark, D., 2019. Comparison and recommendations for use of *Dientamoeba fragilis* Real-time PCR assays. *J. Clin. Microbiol.* 57 (5) e01466-18.
- Halkjær, S., Stensvold, C.R., Petersen, A.M., 2015. *Dientamoeba fragilis* infection as cause of severe abdominal discomfort and flatulence. *Ugeskr. Laeger* 177 (2A), 30–31.
- Hamdy, D.A., Abd El Wahab, W.M., Senosy, S.A., Mabrouk, A.G., 2020. *Blastocystis* spp. and *Giardia intestinalis* co-infection profile in children suffering from acute diarrhoea. *J. Parasit. Dis.* 44 (1), 88–98.
- Hamzah, Z., Petmitr, S., Mungthin, M., Leelayoova, S., Chavalitshewinkoon-Petmitr, P., 2010. Development of multiplex real-time polymerase chain reaction for detection of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* in clinical specimens. *Am. J. Trop. Med. Hyg.* 83 (4), 909–913. <https://doi.org/10.4269/ajtmh.2010.10-0050>.
- Harhay, M.O., Horton, J., Olliaro, P.L., 2010. Epidemiology and control of human gastrointestinal parasites in children. *Expert Rev. Anti-Infect. Ther.* 8, 219–234.
- Henriksen, S.A., Pohlenz, J.F., 1981. Staining of cryptosporidia by a modified Ziehl-Neelsen technique. *Acta Vet. Scand.* 22 (3–4), 594–596.
- Incáni, R.N., Ferrer, E., Hoek, D., Ramak, R., Roelfsema, J., Mughini-Gras, L., Kortbeek, T., Pinelli, E., 2017. Diagnosis of intestinal parasites in a rural community of Venezuela: advantages and disadvantages of using microscopy or RT-PCR. *Acta Trop.* 167, 64–70.
- Krogsgaard, L.R., Engsbro, A.L., Stensvold, C.R., Nielsen, H.V., Bytzer, P., 2015. The prevalence of intestinal parasites is not greater among individuals with irritable bowel syndrome: a population-based case-control study. *Clin. Gastroenterol. Hepatol.* 13 (3) <https://doi.org/10.1016/j.cgh.2014.07.065>, 507–513.e2.
- Lau, Y.L., Anthony, C., Fakhrruzazi, S.A., Ibrahim, J., Ithoi, I., Mahmud, R., 2013. Real-time PCR assay in differentiating *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* infections in orang Asli settlements in Malaysia. *Parasit. Vectors* 6 (1), 250. <https://doi.org/10.1186/1756-3305-6-250>.
- Laude, A., Valot, S., Desoubeaux, G., Argy, N., Nourrisson, C., Pomares, C., Machouart, M., Le Govic, Y., Dalle, F., Botterel, F., Bourgeois, N., Cateau, E., Letterier, M., Le Pape, P., Morio, F., 2016. Is real-time PCR-based diagnosis similar in performance to routine parasitological examination for the identification of *Giardia intestinalis*, *Cryptosporidium parvum*/*Cryptosporidium hominis* and *Entamoeba histolytica* from stool samples? Evaluation of a new commercial multiplex PCR assay and literature review. *Clin. Microbiol. Infect.* 22 (2), 190.e1–190.e8.
- Légeret, C., Rüttimann, C., Furlano, R.I., Ruf, T., Poppert, S., Fankhauser, H., Köhler, H., 2020. *Blastocystis* in Swiss children: a practical approach. *Eur. J. Pediatr.* 179 (6), 979–984. <https://doi.org/10.1007/s00431-020-03599-3>.
- Llewellyn, S., Inpankaew, T., Nery, S.V., Gray, D.J., Verweij, J.J., Clements, A.C., Gomes, S.J., Traub, R., McCarthy, J.S., 2016. Application of a multiplex quantitative PCR to assess prevalence and intensity of intestinal parasite infections in a controlled clinical trial. *PLoS Negl. Trop. Dis.* 10 (1), e0004380.
- Mendoza, D., Núñez, F.A., Escobedo, A., Pelayo, L., Fernández, M., Torres, D., Cordoví, R. A., 2001. Intestinal parasitic infections in 4 child day-care centers located in San Miguel del Padrón municipality, Havana City, 1998. *Rev. Cubana Med. Trop.* 53 (3), 189–193.
- Mmbaga, B.T., Houpt, E.R., 2017. *Cryptosporidium* and *Giardia* infections in children: a review. *Pediatr. Clin. N. Am.* 64, 837–850.
- Norberg, A., Nord, C.E., Evengard, B., 2003. *Dientamoeba fragilis*—a protozoal infection which may cause severe bowel distress. *Clin. Microbiol. Infect.* 9, 65–68.
- Núñez, F.A., Cordoví, R.A., 2006. Manual de Técnicas Básicas para el diagnóstico de las Parasitosis Intestinales UNICEF. La Habana, Cuba: IPK.
- Núñez, F.A., González, O.M., Bravo, J.R., Escobedo, A.A., González, I., 2003. Intestinal parasitosis in children admitted to the pediatric teaching Hospital of Cerro, Havana City, Cuba. *Rev. Cubana Med. Trop.* 55 (1), 19–26.
- Núñez, Y.O., Fernández, M.A., Torres-Núñez, D., Silva, J.A., Montano, I., Maestre, J.L., Fonte, L., 2001. Multiplex polymerase chain reaction amplification and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* DNA from stool samples. *Am. J. Trop. Med. Hyg.* 64, 293–297.
- Parčina, M., Reiter-Owona, I., Mockenhaupt, F.P., Vojvoda, V., Gahutu, J.B., Hoerauf, A., Ignatius, R., 2018. Highly sensitive and specific detection of *Giardia duodenalis*, *Entamoeba histolytica*, and *Cryptosporidium* spp. in human stool samples by the BD MAX™ enteric parasite panel. *Parasitol. Res.* 117 (2), 447–451. <https://doi.org/10.1007/s00436-017-5720-7>.
- Paulos, S., Saugar, J.M., de Lucio, A., Fuentes, I., Mateo, M., Carmena, D., 2019. Comparative performance evaluation of four commercial multiplex real-time PCR assays for the detection of the diarrhoea-causing protozoa *Cryptosporidium hominis*/*parvum*, *Giardia duodenalis* and *Entamoeba histolytica*. *PLoS One* 14 (4), e0215068. <https://doi.org/10.1371/journal.pone.0215068>.
- Rostami, A., Riahi, S.M., Haghghi, A., Saber, V., Armon, B., Seyyedtabaei, S.J., 2017. The role of *Blastocystis* sp. and *Dientamoeba fragilis* in irritable bowel syndrome: a systematic review and meta-analysis. *Parasitol. Res.* 116 (9), 2361–2371. <https://doi.org/10.1007/s00436-017-5535-6>.
- Stark, D., Beebe, N., Marriott, D., Ellis, J., Harkness, J., 2006. Evaluation of three diagnostic methods, including real-time PCR, for detection of *Dientamoeba fragilis* in stool specimens. *J. Clin. Microbiol.* 44, 232–235.
- Stark, D., García, L.S., Barratt, J.L., Phillips, O., Roberts, T., Marriott, D., Harkness, J., Ellis, J.T., 2014. Description of *Dientamoeba fragilis* cyst and precystic forms from human samples. *J. Clin. Microbiol.* 52 (7), 2680–2683. <https://doi.org/10.1128/JCM.00813-14>.
- Stensvold, C.R., Clark, C.G., 2016. Current status of *Blastocystis*: a personal view. *Parasitol. Int.* 65 (6 Pt B), 763–771. <https://doi.org/10.1016/j.parint.2016.05.015>.
- Stensvold, C.R., Nielsen, H.V., 2012. Comparison of microscopy and PCR for detection of intestinal parasites in Danish patients supports an incentive for molecular screening platforms. *J. Clin. Microbiol.* 50 (2), 540–541.
- Verweij, J.J., Mulder, B., Poell, B., van Middelkoop, D., Brienen, E.A.T., van Lieshout, L., 2007. Real-time PCR for the detection of *Dientamoeba fragilis* in fecal samples. *Mol. Cell. Probes* 21 (5–6), 400–404.
- Vital, M.G., Orihuela, J.L., Orihuela, M.E., 1999. Parasitismo intestinal en círculos infantiles. *Rev. Cub. Med. Gen. Integr.* 15, 266–269.
- Walochnik, J., Aspöck, H., 2012. Protozoan pathogens: Identification. In: eLS. John Wiley & Sons, Ltd, Chichester. <https://doi.org/10.1002/9780470015902.a0001949.pub3>.
- WHO (World Health Organization), 1991. Basic Laboratory Methods in Medical Parasitology. World Health Organization, Chidini, PL (Ed.). https://www.who.int/malaria/publications/atoz/9241544104_part1/en/.
- WHO (World Health Organization), 1997. Amoebiasis. *Wkly Epidemiol. Rec.* 72, 97–100. <https://www.who.int/docstore/1997/wer/1997/wer7214.pdf>.

- Wolfe, M.S., 1992. Giardiasis. Clin. Microbiol. Rev. 5 (1), 93–100. <https://doi.org/10.1128/cmr.5.1.93>.
- Wong, Z., Faulde, K., Robinson, J.L., 2018. Does *Dientamoeba fragilis* cause diarrhea? A systematic review. Parasitol. Res. 117, 971–980.

- Yason, J.A., Liang, Y.R., Png, C.W., Zhang, Y., Tan, K.S.W., 2019. Interactions between a pathogenic *Blastocystis* subtype and gut microbiota: *in vitro* and *in vivo* studies. Microbiome. 7 (1), 30. <https://doi.org/10.1186/s40168-019-0644-3>.