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ORIGINAL ARTICLE

Improvement in cyst recovery and molecular detection of *Giardia duodenalis* from stool samples

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Abstract

Molecular detection of *Giardia duodenalis* by polymerase chain reaction (PCR) is difficult in faecal samples due to inhibitors that contaminate DNA preparations, or due to low cyst concentrations. In order to eliminate inhibitors, improve cyst recovery and molecular detection of *G. duodenalis*, different types of water, distillates (MDs), deionized (MDz), injection (MI) or Milli-Q® (MM) were used instead of formaldehyde (F) in the laboratory routine method (Ritchie). Cysts were isolated from faecal samples with low cyst concentrations (< 1 cyst/field), medium (1–2 cysts/field) or high (> 2 cysts/field). Cyst recovery was improved using all water types (MDs, MDz, MI, MM) compared to formaldehyde. At all cyst concentrations, the use of MM consistently showed the greatest recovery of *G. duodenalis* cysts . DNA samples from recovered cysts were tested for the glutamate dehydrogenase (GDH) and β-giardin (βg) genes. The use of Milli-Q® water allowed to detect both genes in all cyst concentrations, including low. The method processed with the other types of water amplified these genes at high and medium cyst concentrations. GDH and βg genes were not detected when the sample was processed with formaldehyde. These experimental results were confirmed in clinical samples. The results suggest that Milli-Q® water provides the highest cyst recovery from stool samples and, correspondingly, the highest sensitivity for detecting *G. duodenalis* by microscopy or PCR for GDH and βg genes, even at low concentration of cysts.

Keywords Giardiasis · Diagnosis · Polymerase chain reaction · Parasite load · Water purification

Introduction

Giardia duodenalis (synonyms: *G. intestinalis* and *G. lamblia*), etiological agent of giardiasis, is a protozoan that affects the gastrointestinal tract of man and domestic and wild animals [1–3] all around the world. Infection with this parasite is usually asymptomatic, but more severe symptoms, such as diarrhea/steatorrhea, intestinal malabsorption, malnutrition, and physical and cognitive impairment in children may occur [4, 5]. Fecal–oral transmission, through ingestion of cysts present in hands, contaminated water and food, is favored in environments with poor hygienic conditions [1, 6, 7].

The detection of this parasite in the laboratory routine is generally carried out using the centrifugal–sedimentation method [8], which is considered gold standard. This method is effective for the morphological identification of *G. duodenalis*, but if an epidemiological approach is the object of the study, molecular methodologies should be used. Molecular methods are able to differentiate genotypes (assemblages) of morphologically identical parasites [2, 3, 9, 10]. In this context, it is possible to verify the link between hosts and sources of infection [3, 11] and the relation between genetic diversity and clinical manifestations of the disease [12, 13].

The main molecular methods used for genotyping *G. duodenalis* are the polymerase chain reaction (PCR) associated to the restriction fragment length polymorphism (RFLP) and to the sequencing [9, 11, 14]. To improve the performance of these methods, it is necessary to eliminate inhibitors present

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in feces (complex polysaccharides, lipids, bile salts, among others) [15], and in reagents used in the preparation of the sample (formaldehyde, zinc sulfate, sucrose, among others) [16]. It is also important to use reagents and the water itself with compatible level of purity [17, 18], and seek simpler method for recovery and concentration of cysts, than, for example, sucrose gradient [19, 20].

In this study, was proposed the replacement of formaldehyde by different types of water in Ritchie method, most used in laboratory routine, in order to eliminate inhibitors, improve cyst recovery and molecular detection of *G. duodenalis* analyzing samples with low, medium and high concentration of cysts, in reconstitution experiments, validated with fecal material of patients with different parasitic loads.

Materials and methods

Collection and preservation of the samples

Human fecal samples were obtained without preservatives, maintained between 5 and 10 °C and analyzed until 24 h after collection.

Parasitological analysis and classification of the samples according to the number of cysts for the reconstitution experiments

The faecal material was processed by the method of Faust et al. [21], in order to confirm the presence of *G. duodenalis*. Each sample was classified according to the concentration of cysts following the methodology established by Uda-Shimoda et al. [22]: the number of cysts in each microscopic field was counted in the ×20 objective in a 22 × 22 mm cover slip, and samples with less than one cyst/field, 1–2 cysts/field and more than 2 cysts/field were classified as low, medium and high concentration, respectively.

Replacement of formaldehyde by different types of water in Ritchie method

One gram of feces, for each concentration of cysts (high, medium and low), was diluted in approximately 14 milliliters (mL) of 0.85% saline solution, filtered in gauze folded four times and centrifuged at 1200×*g* during 5 min. The sediment was washed two times in the same way, and we added 6 mL of different types of water: Milli-Q® Water (MM group—distilled water purified by ultrafiltration—Millipore–Bedford purification system, Bedford, MA, USA); Distilled Water (MDs group); Deionized Water (MDz group) and Injection Water (MI group), in addition to 4 mL of ethyl ether PA. After vigorous stirring, the samples were centrifuged at 1200×*g* for 2 min. As a control of the proposed modifications the classical method was also performed with 10% formaldehyde (F group, Ritchie [8]). The final sediment received the amount of each respective reagent necessary to complete 150 µL. From this volume, 50 µL were used for quantification in Neubauer's chamber and the result was computed in number of cysts/gram (g) of feces. The remaining 100 µL were stored at − 20 °C until DNA extraction. All experiments were performed in triplicate.

Molecular analysis of samples with low, medium and high concentrations of cysts

DNA of all samples was extracted using the PureLink® PCR Purification kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations and as established by Uda-Shimoda et al. [22]. The 432 base-pair (bp) fragment of glutamate dehydrogenase (GDH) gene was amplified in a semi-nested PCR reaction, and the 753 bp gene, which encodes β-giardin (βg), was amplified by standard PCR. The protocols followed the modifications described by Colli et al. [11].

The amplification products were visualized on 4.5% polyacrylamide gel, revealed by silver salts, photographed and digitally recorded.

Additional analyzes to evaluate the inhibitory effect of formaldehyde on DNA amplification

In order to confirm the inhibitory effect of formaldehyde on PCR, samples known to be positive for *G. duodenalis* were processed as follows: (1) DNA was extracted from the sediment using the QIAamp® DNA Stool mini kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations; (2) extraction by phenol–chloroform (*in house*) according to protocol established by Macedo et al. [23]; (3) washing (1×) and centrifugation of the sediment in distilled water; and (4) addition of DNA of the *G. duodenalis* trophozoite, reference strain—Portland Strain ATCC 30888 and control positive in GDH and βg genes amplification, to the DNA from the samples processed by the formaldehyde. After each procedure, the GDH and βg genes were reamplified.

Samples of patients with different parasitic loads for *G. duodenalis* processed by Ritchie method using Milli-Q® water

Samples of 30 individuals (10 for each parasite load—low, medium and high) were analyzed in triplicate by the Ritchie method using Milli-Q® water, which showed better performance in the reconstitution experiments. Clinical samples were classified in relation to parasitic load in the same way

as samples from reconstitution experiments, according to the criteria of Uda-Shimoda et al. [22].

Statistical analysis

The data were analyzed using the Software *Statistica Single User* version 13.2. The *Shapiro–Wilk* test presented a lack of normality in the data, indicating the use of non-parametric tests to compare the groups with low, medium and high concentration of cysts, using the *Kruskal–Wallis* test. The level of significance was established as 5% ($p < 0.05$).

Results

Number of cysts obtained in reconstitution experiments by Ritchie method processed by different types of water and by formaldehyde

Table 1 demonstrates that regardless of the concentration of cysts (low, medium or high), the Ritchie method using different types of water (MM, MDs, MDz, MI groups), recovered more cysts than using the formaldehyde (F). There was no significant difference between the groups, but for the MM group, the recovery of cysts was 52.9% higher in the samples with low, 38.7% in the medium and 48.5% in the samples with high concentration of cysts compared to the method processed with formaldehyde.

Detection of fragments of GDH and βg genes in samples with different concentrations of *G. duodenalis* cysts

From reconstitution experiments, samples with high and medium concentration of cysts showed the 432 bp fragment of the GDH gene in all groups processed with different types of water (MM, MDs, MDz, MI). In the samples with low concentration of cysts, the DNA was only amplified when Milli-Q® water and Distilled water were used (Fig. 1a). The same result occurred for the βg gene in samples with high and medium concentration of cysts, but in samples with low concentration of cysts, the 753 bp fragment was only amplified when the material was processed with Milli-Q® water (Fig. 1b). Regardless of cysts concentration, the DNA of *G. duodenalis* was not amplified for the genes analyzed when samples were processed with formaldehyde (F).

The inhibitory effect of formaldehyde was not eliminated, even with several attempts performed, since no DNA fragment was amplified, regardless of cysts concentration (low, medium and high).

Table 1 Number of cysts recovered according to the concentrations and groups tested in reconstitution experiments

Concentration of cysts	Groups	n	Mean	±	Standard deviation	*p**
Low	MM	3	5354.3	±	1160.0	0.4029
	MDs	3	4177.0	±	1826.4	
	MDz	3	2968.7	±	2592.5	
	MI	3	3927.0	±	1787.5	
	F	3	2521.0	±	2257.5	
Medium	MM	3	9625.0	±	2912.8	0.434
	MDs	3	7729.3	±	3250.4	
	MDz	3	7271.0	±	2158.3	
	MI	3	7062.7	±	2518.2	
	F	3	5896.0	±	2470.6	
High	MM	3	29375.0	±	6387.2	0.2674
	MDs	3	23541.7	±	6562.6	
	MDz	3	20885.3	±	4329.1	
	MI	3	21666.7	±	7637.6	
	F	3	15114.7	±	8721.1	

MM group with replacement of Formaldehyde by Milli-Q® water, *MDs* formaldehyde by distilled water, *MDz* formaldehyde by deionized water, *MI* formaldehyde by injection water, *F* formaldehyde 10%

n: number of samples analyzed (triplicate)

Mean: results computed in number of cysts/g of feces

**Kruskal–Wallis* test not significant considering significance level of 5%

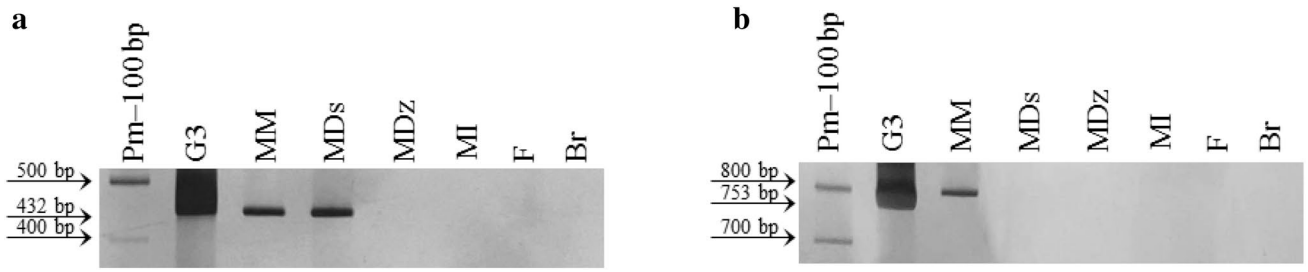

Fig. 1 Amplification 432 base-pair (bp) fragment of glutamate dehydrogenase (GDH) gene (**a**) and 753 bp fragment of β-giardin (βg) gene (**b**) extracted from *Giardia duodenalis*, from fecal material of reconstitution experiments with low concentrations of cysts, processed with Milli-Q®, Distilled, deionized and injection water and with formaldehyde, visualized in polyacrylamide gel 4.5%. Pm–100 bp: molecular weight 100 bp DNA ladder; G3: positive control—DNA *G. duodenalis* trophozoite of reference strain (Portland Strain ATCC 30888); Br: negative reaction control—without DNA; MM: group with replacement of formaldehyde by Milli-Q® water; MDs: formaldehyde by distilled water; MDz: formaldehyde by deionized water; MI: formaldehyde by injection water; F: formaldehyde 10%

Validation of the Ritchie method with Milli-Q® water in samples of patients with different parasitic loads of *G. duodenalis*

For all (30/100%) samples with low, medium and high parasitic load, the GDH (Fig. 2) and βg genes were amplified, validating the method with Milli-Q® water.

Discussion

In this study, we proposed the replacement of formaldehyde by different types of water in a parasitological method widely used for the diagnosis of *G. duodenalis* [8] in order to eliminate inhibitors, improve cyst recovery and molecular detection of this parasite in clinical samples, since there are parasites morphologically identical, but belong to different genotypes [3, 9–11, 24, 25]. As a strategy, we used reconstitution experiments performed with samples with low, medium and high numbers of cysts and validation with clinical samples of individuals with low, medium and high parasitic load. The samples were processed using Ritchie method with formaldehyde, and replacing the formaldehyde by Milli-Q®, distilled, deionized and injection water. The modification that provided the best result was replacement of formaldehyde by Milli-Q® water, allowing the parasitological and molecular detection of *G. duodenalis* in samples of patients even at low parasite load.

In reconstitution experiments, it was verified that, regardless of cysts concentration, the method processed with different types of water (MM, MDs, MDz, MI groups) recovered more cysts than the method with formaldehyde (F). Density difference between water and formaldehyde may justify these results. The density of formaldehyde (> 1 g/cm^3) may interfere with sedimentation capacity of cysts, especially in low concentration, indicating the use of water in centrifugal–sedimentation methods, regardless of the purification treatment.

No significant difference was observed in the method processed with different types of water, indicating that the

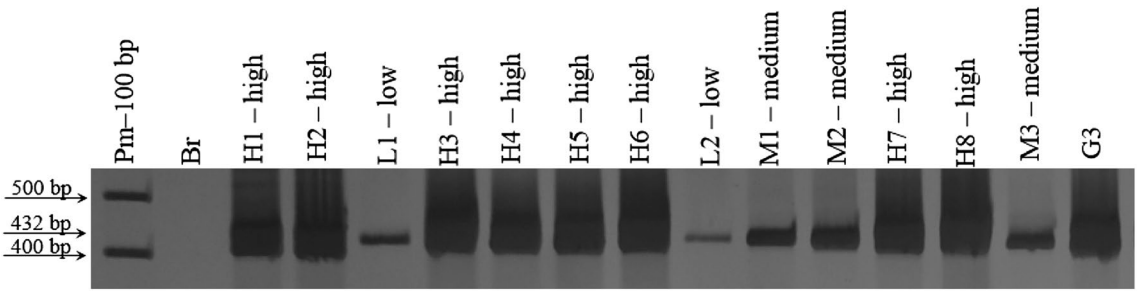

Fig. 2 Amplification 432 base-pair (bp) fragment of glutamate dehydrogenase (GDH) gene extracted from *Giardia duodenalis*, from fecal material of patients with high, medium and low parasitic load, processed with Milli-Q® water, visualized in polyacrylamide gel 4.5%. Pm–100 bp: molecular weight 100 bp DNA Ladder; G3: positive control—DNA *G. duodenalis* trophozoite of reference strain (Portland Strain ATCC 30888); Br: negative reaction control—without DNA; samples H1, H2, H3, H4, H5, H6, H7 and H8: patients with high parasitic load; samples M1, M2 and M3: patients with medium parasitic load; samples L1 and L2: patients with low parasitic load

purification did not interfere in the centrifugal-sedimentation process. However, it was evident that Milli-Q® water recover more cysts in relation to Ritchie method performed with formaldehyde at all concentrations tested, including samples with low concentration of cysts. The results indicate the importance of the modification performed and that it did not interfere in the microscopic examination of the parasite, as questioned by Stojecki et al. [16].

The use of molecular biology in the diagnostic process hampers the pretreatment of the samples because it is a methodology that is prone to inhibitors [15]. The method of isolation and purification of cysts by sucrose solution is often used and effective for application in molecular methods [19, 20, 26], however, it requires extensive work to recover cysts, making it very toilsome. In contrast, the Ritchie method concentrates the cysts quickly eliminating the excess of fecal debris and fats [8]. Previous studies have already proposed alterations in the Ritchie method, or similar formaldehyde–ether methodologies, in order to increase the sensitivity and recovery of parasitic structures [27, 28], to decrease the toxicity of the method [29], and to detect the DNA of the parasite by PCR [11]. However, none of these authors proposed modifications to detect DNA of *G. duodenalis* in clinical samples with low parasite load and using two molecular markers.

In the reconstitution experiments there was a difference between the markers when samples with low concentration of cysts were processed. The GDH fragment was amplified when the samples were processed with Milli-Q® water and distilled water and βg only when Milli-Q® water was used. The quality of reagent water, purified water, is verified by its resistivity and conductivity (quantity of ionic contaminants), total organic carbon (quantity of CO_2), microbiological agents and endotoxins [17], being these characteristics acquired by different purification processes (filtration, deionization, distillation, ultrafiltration/nanofiltration, reverse osmosis, among others). Evaluation of nanofiltration membranes used in the compaction process after passing Milli-Q® water and deionized water showed greater physical–chemical changes, amounts of total organic carbon and biological content in compacted membranes with deionized water [30]. These results show the importance of the purification process, being ultrafiltration, used in Milli-Q® water, an efficient technology in the removal of crucial elements that significantly hinder from molecular analyzes, such as endotoxins, nucleases (DNAses and RNAses) and proteases that are able to catalyze the hydrolysis of DNA and RNA making these molecules unstable [17, 18, 31]. Our results highlight the relevance of improving the methods for detecting *G. duodenalis*, aiming the analysis of samples with low numbers of cysts and the molecular marker to be used. These results also reinforce the importance of the quality and purity of the reagent when molecular methodologies are used, and a greater sensitivity of the GDH gene, corroborating with the literature [11, 31]. The modification using Milli-Q® water, associated with the use of molecular markers for the GDH gene, may be the most appropriate choice for researches in regions with predominance of individuals with low parasitic load.

Another result of the present work, which corroborates with those previously discussed, is that in all samples processed with formaldehyde, the DNA of the parasite could not be amplified, even when several attempts in order to eliminate the inhibitory effects of formaldehyde were made. Wilke and Robertson [32] and Stojecki et al. [16] have already demonstrated that the reagents used in the pre-treatment of the sample, including formaldehyde, can interfere with the efficiency and analysis of the result. Differently, Lass et al. [33], demonstrated that it is possible to detect DNA of *G. duodenalis* in samples preserved in formaldehyde for a short period of time (about one month) after numerous washes and a physical process for cysts disruption. Lee et al. [34] were successful in samples fixed for longer periods (10 years or more) after rehydration of the sample with alcohol, inhibition of the activity of DNAses and removal of proteins. These formaldehyde elimination processes are more laborious and expensive. In addition, in an experiment conducted in our laboratory, it was observed that in samples with low parasitic load subjected to several washes, cyst loss can reach 100% (data not shown). The degradation of DNA caused by formaldehyde still limits the PCR process, because only small fragments of the gene can be amplified [34].

The modified method with Milli-Q® water was validated in samples with low, medium and high parasitic load by the amplification of GDH and βg genes in all samples tested. Molecular detection offers advantages over conventional methods [35], since it is able to detect the parasite also in samples of patients with low parasite load; it is more specific; and can differentiate the genotypes of the parasite. Thus, it is possible to establish the focus and dynamics of transmission, and the prevalence and prophylaxis involving the main zoonotic genotypes [1–3, 11].

We be concluded that, using Milli-Q® water, cyst recovery is the highest from stool samples. It improved detection of *G. duodenalis* by microscopy or PCR for GDH and βg genes, even in samples with low concentration of cysts.

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Author contributions The experiments were designed by RCB, CRdA and MLG. Material preparation, data collection and analysis were performed by RCB, CMC, LILR, ÉCF, SM and MLG. The first draft of

the manuscript was written by RCB and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Human Ethics Committee of the Faculdade Integrado of Campo Mourão (Paraná/Brasil) under registration number 1.594.078 and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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