Does each bead count? A reduced-cost approach for recovering waterborne protozoa from challenge water using immunomagnetic separation

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ABSTRACT

Giardia duodenalis and Cryptosporidium spp. are two of the most prominent aetiological agents of waterborne diseases. Therefore, efficient and affordable methodologies for identifying and quantifying these parasites in water are increasingly necessary. USEPA Method 1623.1 is a widely used and validated protocol for detecting these parasites in water samples. It consists of a concentration step, followed by parasite purification and visualization by immunofluorescence microscopy. Although efficient, this method has a high cost particularly due to the immunomagnetic separation (IMS) step, which is most needed with complex and highly contaminated samples. Based on this, the present study aimed to determine whether it is possible to maintain the efficiency of Method 1623.1 while reducing the amount of beads per reaction, using as a matrix the challenge water recommended by the World Health Organization. As for Giardia cysts, a satisfactory recovery efficiency (RE) was obtained using 50% less IMS beads. This was evaluated both with a commercial cyst suspension (56.1% recovery) and an analytical quality assessment (47.5% recovery). Although RE rates obtained for Cryptosporidium parvum did not meet Method 1623.1 criteria in any of the experimental conditions tested, results presented in this paper indicated the relevance of the described adaptations, even in challenge water.

Key words | Cryptosporidium spp. oocysts, Giardia spp. cysts, low-cost recovery methods, parasitic protozoa, recovery efficiency

HIGHLIGHTS

● The high cost of current protozoa detection methods limits their widespread use in limited settings.
● Immunomagnetic separation improves detection by cleaning the sample.
● Recovery efficiency is maintained for Giardia duodenalis with 50% less beads.
● Organisms adhering to beads after dissociation may impact recovery levels.

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INTRODUCTION

Some 2.2 billion people around the world do not have safely managed drinking water services, 4.2 billion people are deprived of safely managed sanitation services, and 3 billion lack basic handwashing facilities (WHO 2020). Waterborne diseases are considered one of the highest impact public health problems in the world and are responsible for more than 2.2 million deaths per year and many more cases of enteric infections (WHO 2020).

Almost 40% of these deaths are caused by parasitic protozoa, especially *Giardia duodenalis* and *Cryptosporidium parvum*, which are zoonotic aetiological agents responsible for more than 2.8 million cases per year of gastrointestinal infections worldwide (Squire & Ryan 2017). These infections are the second most common cause of death in early childhood (Checkley et al. 2015; Platts-Mills et al. 2015).

The repeated prevalence of these protozoa in surface water denotes significant risks to human health, especially due to their low ID50 (the number of cysts and/or oocysts needed to infect 50% of exposed people), which has been estimated to fall between 10 and 2,000 for *C. parvum* (Robert-Gangneux & Dardé 2012) and between 10 and 100 for *G. duodenalis* (Rendtorff 1979). In this context, assessing the microbiological quality of drinking water is mandatory to ensure its safety for consumption (WHO 2017).

Despite the growing trend in pathogen epidemiological investigations in developing countries (Squire & Ryan 2017), the vast majority of studies are still carried out in developed countries, where laboratories and general health infrastructure are much more accessible than those in developing countries (Snelling et al. 2007). *Cryptosporidium* is associated with moderate to severe diarrhoea and increased mortality in low-income regions (Sunnotel et al. 2006; Snelling et al. 2007), and both parasites negatively affect child growth and development (Squire & Ryan 2017). Malnutrition and HIV status are also important contributors to the increased prevalence of *Cryptosporidium* spp. and *G. duodenalis* in developing countries. Climate change and population growth are also predicted to increase both malnutrition and the recurrent prevalence of these parasites in water sources (Squire & Ryan 2017).

Over time, various methodologies have been developed to detect these organisms in water samples. The limitations in early-stage methodologies for protozoan recovery may result in a slight prevalence in surface water, for instance, leading to the incorrect assumption of low contamination (Efstratiou et al. 2007). Also, the efficiency of the critical step, that of oocyst recovery, in these methods is mostly low and extremely variable, ranging from 0 to 140% (Clancy et al. 1994; Jakubowski et al. 1996; Schaefer 2000). Because of the inconsistency of results, alternative techniques have been proposed and evaluated. Specifically, Method 1623.1, developed by the U.S. Environmental Protection Agency (USEPA), is now recognized as the accepted standard procedure for detecting *Giardia* spp. and *Cryptosporidium* spp. in water. In brief, the method consists of four steps: (1) concentration of the sample (filtration), (2) immunomagnetic separation (IMS), (3) immunofluorescent labelling (IFA), and (4) microscopic visualization of biological forms (USEPA 2012).
Regardless of the detection method employed, large volumes of water are usually required in order to increase the likelihood of detecting cysts and oocysts in the sample. However, the concentration process often leads to an accumulation of debris, such as large organic particles and algal cells, making it necessary to include a sample clarification step, which aims to separate the target organisms from this debris (McCuin et al. 2001). In this scenario, IMS is a well-established technique that employs magnetic beads coated with an antibody specific to protein targets on the surface of microorganisms such as *Giardia* spp. and *Cryptosporidium* spp., to allow their recovery from different matrices (Di Giovanni et al. 1999; Yakub & Stadterman-Knauer 2000).

Although this technique has operational advantages and presents better results than other methods (Hsu & Huang 2007), the high cost of the immunomagnetic beads severely limits its use in limited-resource situations (Feng et al. 2011). Reducing the cost of IMS methodology is, therefore, crucial to ensure that, even in low- and middle-income countries, effective detection of pathogens in water becomes practically feasible. Such a development would lead to standardization of the methodologies across all laboratories and more consistent and reliable results worldwide. As immunomagnetic beads are a primary cost of the method, we, therefore, investigated the efficiency of the IMS method when the amount of beads per sample is serially reduced as a step towards achieving this specific goal.

**METHODOLOGY**

**Sampling**

Test water consisted in an increase of turbidity and true colour to a natural water source. In short, a 5 L groundwater sample was mixed with humic acid (20 mg L\(^{-1}\)) and kaolinite (60 mg L\(^{-1}\)) in order to reach about 40 NTU of turbidity, 250 HU of true colour and 10 mg L\(^{-1}\) of dissolved organic carbon (DOC). These characteristics represent the so-called challenge water proposed by the World Health Organization (WHO 2014) for water testing.

In our study, 5 L batches were used for each test, and these 5 L batches were divided into five samples of 1 L each.

The groundwater used in this study came from an artesian well which is fed by the waters of the Guarani Aquifer System. The well is located on the campus of the São Carlos School of Engineering, São Carlos, São Paulo, Brazil.

Specifically, for this work, prior to the beginning of the experiments, the well water was submitted to Method 1623.1 (MF + IMS + IFA) for the detection of (oo)cysts of *Giardia* spp. and *Cryptosporidium* spp. The aforementioned method was used for the analysis of all samples included in this study and is, therefore, detailed in the subsection ‘Sample processing’.

**Protozoa inoculation**

Commercially available suspensions of *G. duodenalis* (H3 isolate, 190311) and *C. parvum* (Iowa isolate, 190311; 5 × 10\(^6\) in 8 mL) (Waterborne, Inc.) were used in order to artificially contaminate the challenge water samples. Viable cysts and oocysts were shipped and stored in phosphate-buffered saline containing antibiotics at 2–8 °C and were utilized within a maximum of 60 days after receipt. Approximately 697 ± 8 cysts and 700 ± 10 oocysts were spiked together into each of four of the 1 L samples, with the remaining 1-L sample being used as a blank control (i.e., without protozoa).

Prior to the tests, the suspensions were analysed to quantify the inoculum. For that, 5 μL of each suspension was spiked on a glass slide, in triplicate, and left at room temperature for 4 h for drying. Next, the commercial kit Merilfluor™ (Meridian Diagnosis) and DAPI (4',6-diamidine-2'-phenylindole dihydrochloride) dye (USEPA 2012) were applied to the sample. Visualization was performed by immunofluorescence microscopy (Olympus® BX51). The final concentration (microorganisms/μL) was given by the average of the results observed in the three slides.

**Sample processing**

Samples were processed using Method 1623.1 (USEPA 2012) with appropriate adaptations (Medeiros & Daniel 2015; Franco et al. 2016; Sammarro Silva & Sabogal-Paz 2020) as described below.

**Sample concentration**

Vacuum pump filtration (flow rate 4 L/min) using cellulose ester membranes (47 mm diameter, 3 μm porosity,
Millipore™) was performed for concentrating the target organisms from 1 L samples (Franco et al. 2012).

After the filtration process, the material retained in the membrane was eluted by washing the membrane with 0.01% Tween 80 solution at 45 °C and scraped out using plastic handles supplied with the MeriFluor™ kit. Membrane scrapings were carried out for 5 min, in each of the directions (vertical, horizontal and diagonal) covering the entire area of the membrane (Medeiros & Daniel 2015; Sammarro Silva & Sabogal-Paz 2020).

The resulting liquid was then subjected to a double centrifugation process (1,500 × g; 10 min; room temperature) to form a pellet containing the target parasites. At the end of the process, samples were resuspended in 5 mL of appropriate kit buffer, and then subjected to IMS in order to purify the protozoa.

It is worth mentioning that throughout the filtration technique, the filter membranes may need to be replaced if they clog and interrupt the flow of the sample. The amount of membranes used depends directly on the characteristics of the study water.

**Sample purification and protozoa isolation**

The Dynabeads™ GC-Combo (Applied Biosystem) kit was applied in this step following the manufacturer’s recommendations; this kit was also used for the dissociation step, which was carried out three times using 100 μL of 10% hydrochloric acid, in each time. As part of our aim to obtain an effective but more affordable methodology, assays were performed under four different conditions with a serial reduction in the amount of beads in each. The first assay was performed according to the standard protocol of Method 1623.1, in which 100 μL of each bead type was added to the sample. For the second assay, the bead volume was reduced by 50%, and, in the third, the final amount of bead added to the sample was 25% of the standard protocol. The 4th assay was performed without any beads. Apart from these reductions, all other conditions were kept the same for each assay.

Considering the results obtained during the tests, an extra test was included in order to investigate whether the addition of double the volume of 10% hydrochloric acid (200 μL) in the standard amount of beads (100 μL) would positively influence RE.

**Microorganism visualization**

At the end of each of the three rounds of acid dissociation, 50 μL of the sample (non-adhered material) was recovered and added directly to one of the wells of the glass slide supplied with the MeriFluor™ Kit, which was previously sensitized with 5 μL of sodium hydroxide 1 M.

After the drying period of the samples on slides (4 h), (oo)cysts were stained using the commercial MeriFluor™ (Meridian Diagnosis) kit and visualized by immunofluorescence light microscopy (Olympus® BX51). As a confirmatory test, DAPI dye was added to all the samples (USEPA 2012).

**Recovery rate**

Recovery efficiency (RE) of the method is calculated according to Equation (1), where RE is the recovery rate after the complete protocol (%); C1 is the (oo)cysts enumerated in the first acid dissociation; C2 is the (oo)cysts enumerated in the second acid dissociation; C3 is the (oo)cysts enumerated in the third acid dissociation; and NP is the number of inoculated protozoa.

\[
RE = \left( \frac{C_1 + C_2 + C_3}{NP} \right) \times 100\%
\]  

**Analytical quality assay**

In order to ensure the reliability of the results obtained in this work, a test with ColorSeed™ was performed, according to Method 1623.1 (USEPA 2012). ColorSeeds™ reagent contains between 98 and 102 inactivated and permanently red-labelled Cryptosporidium spp. oocysts and Giardia spp. cysts (with a standard deviation of 2.5 or less) in 1 mL of saline solution. This examination was performed with 50% of the standard bead volume, which was statistically determined as the best option, as outlined in the ‘Results’ section. Briefly, 2 mL of 0.05% (vol/vol) of Tween 20® was added to the ColorSeed™ tube, which was vortexed and added to 1 L of challenge water, following which the sample containing the cysts and oocysts were subjected to
membrane filtration, the selected IMS protocol, and immunofluorescence for microscopic visualization. Likewise, to ensure the safety of results the Colorseed™ test with 50 μL of each bead was also performed in quadruplicate plus blank test, and the RE was determined using Equation (1).

**Statistical analyses**

Statistical analyses that led to an understanding of differences obtained by each immunomagnetic procedure were performed using PAST 3.2 software (Hammer et al. 2001), and Origin 6.0 was used for plotting results. A Shapiro–Wilk Normality test was conducted to determine if the datasets were normally distributed. Both the analysis of variance (ANOVA) and the non-parametric test of Kruskal–Wallis followed by Tukey’s pairwise and Dunn’s post hoc, respectively, were performed. Significantly different results ($\alpha = 0.05$) provided conditions to the analytical quality analysis.

**RESULTS**

**Recovery efficiency**

The cysts of *G. duodenalis* and oocysts of *C. parvum* were clearly observed against the background in all samples (Figure 1), regardless of the condition of the test, following the first and second acid dissociations. After the third round of acid dissociation, no cysts or oocysts were visualized. RE data obtained from different methodologies carried out in this study are compiled in Table 1.

As for operational purposes, it is worth mentioning that five membranes were used in each batch filtration. Figure 2 displays the obtained results for each recovery assay, considering both (a) *G. duodenalis* and (b) *C. parvum* as target organisms against Method 1623.1 minimum RE requirement for each parasite. With *G. duodenalis*, all of the tests, with the exception of the no-bead run, successfully reached the USEPA Method 1623.1 RE recommended range ($8-100\%$). With *C. parvum*, however, none of the USEPA Method 1632.1 tests was satisfactory, as all results were below $32\%$.

However, when analysing the coefficient of variation (CV) of each test, only one test (100 μL of beads) was not in accordance with the USEPA criteria for *Giardia* spp. The scenario was the opposite concerning *C. parvum*, for which only one condition (25 μL of beads) reached valid values (CV = 20%).

**Statistical analysis**

The Shapiro–Wilk test indicated that data for percent *C. parvum* recovery without adding beads did not follow a normal distribution. Although the boxplot shown in Figure 2 perhaps visually suggests that the data is normally distributed, this hypothesis was not confirmed. Therefore, *C. parvum* recoveries were analysed by non-parametric statistics. Although the Kruskal–Wallis test suggested significant

| Experimental condition | *G. duodenalis* | *C. parvum* |
|------------------------|----------------|-------------|
| 100 μL beads           | 17.4           | 7.2         |
| 50 μL beads            | 56.1           | 9.1         |
| 25 μL beads            | 19.1           | 10.3        |
| No beads               | 1.4            | 0.7         |
| 100 μL beads/200 μL acid| 11.0          | 18.8        |

Notes: Average *G. duodenalis* inoculum: 697 ± 8 cysts; Average *C. parvum* inoculum: 700 ± 10 oocysts; RE, recovery efficiency; CV, coefficient of variation. The negative control tests did not display any autochthonous protozoa. RE was calculated using Equation (1).
differences among medians of the *C. parvum* datasets and, Dunn’s *post hoc* (*p* < 0.05) pointed out that this fact was mainly due to the combinations of beads versus no beads (50, 25, and 100 μL with 200 μL acid, specifically) as significantly different (*p* < 0.05). Considering, in addition, that these results did not meet USEPA criteria, as illustrated by the dashed line in Figure 2(b), *G. duodenalis* recoveries were prioritized for the analytical quality assessment. A comparison among all experimental conditions is shown in Table 2 which shows that the 50 μL-bead dosing led to significant differences in the sample means against all of the other conditions.

**Analytical quality assessment**

Colorseed™ was used to validate the lowest IMS bead concentration that still provided an acceptable RE value. This was determined to be 50 μL of each bead suspension dissociated with two rounds of 100 μL of 10% hydrochloric acid. Under these conditions, RE reached similar values to those of a test with commercial protozoan suspensions (Table 1).

Comparing the values obtained herein with those standardized by Method 1623.1, our data were satisfactory for *Giardia* spp. regarding both RE (47.5%) and CV (4%). Concerning *Cryptosporidium* spp., RE was 17% below the value recommended by Method 1623.1, while the CV for *Cryptosporidium* spp. met the USEPA criteria (7.1%).

**Cysts and oocysts attached to the beads**

In order to verify the efficiency of the acid dissociation procedure, 50 μL of the bead suspensions obtained at the end of the IMS step were stained with MeriFluor™ and imaged using fluorescence microscopy. Figure 3 displays the image captures of the best experimental condition obtained in

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Table 2 | Statistical comparison of the experimental conditions for Giardia cyst recovery in challenge water (Tukey’s test for 95% confidence interval)

| Recovery methods compared | *p*-value |
|---------------------------|-----------|
| 100 μL versus 50 μL       | <0.0001   |
| 100 μL versus 25 μL       | 0.9889    |
| 100 μL versus no beads    | 0.0029    |
| 100 μL versus 100 μL beads/200 μL acid | 0.3927 |
| 50 μL versus 25 μL        | <0.0001   |
| 50 μL versus no beads     | <0.0001   |
| 50 μL versus 100 μL beads/200 μL acid | <0.0001 |
| 25 μL versus no beads     | 0.0011    |
| 25 μL versus 100 μL beads/200 μL acid | 0.1976 |
| No beads versus 100 μL beads/200 μL acid | 0.0953 |

Note: bold values indicate significant differences in means.
this study (50 μL of each bead and 100 μL of 10% hydrochloric acid). Visual analysis revealed that cysts and oocysts were still attached to the magnetic beads.

It was determined that, on average, 40 and 31% of the total inoculated cysts of *G. duodenalis* (697 ± 8) and oocysts of *C. parvum* (700 ± 10), respectively, remained adhered to the beads. These values are calculated according to Equation (2), where PA is the total amount of protozoan adhered to the beads after three acid dissociation procedures (%); $P_1$ is the (oo)cysts attached to the beads after the first acid dissociation; $P_2$ is the (oo)cysts attached to the beads after the second acid dissociation; $P_3$ is the (oo)cysts attached to the beads after the third acid dissociation; and NP is the number of inoculated protozoa.

$$PA = \frac{(P_1 + P_2 + P_3)}{NP} \times 100\%$$ (2)

**DISCUSSION**

The quality of water resources is a fundamental aspect of the public water supply. Although USEPA Method 1623.1 is widely used and is reported in approximately 30% of publications regarding monitoring of *Cryptosporidium* spp. and *Giardia* spp. in water (Efstratiou et al. 2017b), it still presents some limitations mainly related to its high cost. Among the steps of the method, IMS represents the highest cost, especially because there is only one supplier of magnetic beads and buffers for both protozoa (Dynabeads® GC-Combo, Life Technologies, Thermo Fisher Scientific Inc.), and therefore, the option of using alternative beads is excluded.

In the current work, RE results ranged from 0.7 to 56.1% for both protozoa (Table 1). These findings are in accordance with the literature that reported equated values to the aforementioned method for *Giardia* spp. and *Cryptosporidium* spp. (Razzolini et al. 2010, 2011; Stancari & Correa 2010; Feng et al. 2011; Ongerth 2013; Sato et al. 2013; Franco et al. 2016; Maciel & Sabogal-Paz 2016; Pinto et al. 2016). The coefficients of variation obtained (Table 1) reaffirm the high variability inherent to research with protozoa (Francy et al. 2004). Similar limitations have been reported by studies on (oo)cyst recovery with and without purification methods (Lora-Suarez et al. 2016; Maciel & Sabogal-Paz 2016; Silva & Sabogal-Paz 2021). Hence, this endorses the need of revising recovery protocols.

As indicated by the results obtained herein, in terms of RE, all tests using IMS complied with the criteria of Method 1623.1 for *G. duodenalis* but not for *C. parvum*. One of the experimental conditions, however, must be highlighted: the test performed with 50 μL of each bead solution and 100 μL of 10% hydrochloric acid yielded the highest RE for *G. duodenalis* which agrees with the values established by Method 1623.1. This condition was also statistically significant compared with the others ($p < 0.05$) and was validated by the analytical quality assessment.

Some cysts and oocysts were detected in the absence of IMS, but the RE was insignificant (Table 1).

The fact, pointed out by the results of this study, that the IMS methodology regardless of the number of beads was efficient only for the recovery of *G. duodenalis* cysts was
also reported by other researchers such as Stancari & Correa (2010), Ongerth (2013), Franco et al. (2016), and Maciel & Sabogal-Paz (2016). These studies did not obtain satisfactory results for Cryptosporidium spp. recovery either.

Data obtained in our research also endorse the importance of two rounds of acid dissociation from the beads, corroborating the findings of Maciel & Sabogal-Paz (2016). Our protozoa recovery from challenge water also suggests that the third dissociation may be dismissed since no organisms were visualized after it, further reducing the cost of the protocol, as no labelling of an extra microscopy slide should be required for immunofluorescence.

Our revised methodology represents a significant improvement compared with those previously carried out. Particularities inherent to the matrix and the methodology itself may influence results. The WHO challenge water, which was used in this study, presents a much higher turbidity than filtered or treated water from water treatment plants (WTPs). Hence, it contains a greater amount of suspended particles, which directly impact upon this methodology performance (Kothavade 2012; Efstratiou et al. 2017a, 2017b). When there are too many solids or colloidal material in the sample, the elution process is hampered as the particles remain trapped within the membrane. This may lead to a decrease in the ratio of recovered cysts and oocysts (Franco et al. 2012), which would also explain the low recovery rates of C. parvum in the present study. Also, the high turbidity of the samples resulted in a greater number of membranes being used in the protozoa protocol, since they were quickly obstructed by the particles present in the water sample. The five membrane replacements per litre of challenge water may have facilitated the dispersion and loss of parasites, as pointed out by Franco et al. (2012) and Maciel & Sabogal-Paz (2016).

Although the loss of cysts and oocysts is observed throughout the process (Kumar et al. 2016; Pinto et al. 2016), the filtration step itself seems to have a great impact on the results. Feng et al. (2003) and Hu et al. (2004) endorse this statement reporting 92.0 and 89.0% of RE for Cryptosporidium spp. oocysts and Giardia spp. cysts, respectively, when the water sample is not filtered. However, when the filtration step is incorporated into the methodology, these authors reported that the RE declined to 18.1% for Cryptosporidium spp. and 77.2% for Giardia spp.

The slight decrease observed in the RE for G. duodenalis cysts, which consequently makes it more representative than the RE for C. parvum, – as per the results of the present study – can be attributed to the size of the organisms (Hsu & Huang 2000; Hashimoto et al. 2002; Hu et al. 2004; Franco et al. 2016). The cysts of G. duodenalis (8–12 μm) are significantly larger than the oocysts of Cryptosporidium (4–6 μm) (USEPA 2012) and, therefore, are more easily retained by the membrane. In addition, Cryptosporidium spp. oocysts have the ability to compress (Li et al. 1999), which may facilitate their passage through the filter matrix, therefore also contributing to lower recovery.

Although some authors recommend the use of membranes with smaller porosity in order to retain more (oo)cysts, the 3 μm porosity membrane has been extensively used, with favourable results (Franco et al. 2012, 2016; Medeiros & Daniel 2015; Pineda et al. 2020; Sammarro & Sabogal 2020). Additionally, as reported by Franco et al. (2012), the filtration using this kind of membrane presents a better performance in face of complex matrices, such as the one included in this study, than the filters with smaller porosities. It also redeems a generally lower cost as it would require even less replacements, which reinforces the main idea of this work, which refers to savings in material – while maintaining response reliability – in order to make the methodology more accessible and widespread.

Another variable worth pointing out in the context of non-satisfactory RE is the continued attachment of cysts and oocysts to immunomagnetic microspheres, even after two rounds of acid dissociation. Similar observations were made by Rochelle et al. (1999), Maciel & Sabogal-Paz (2016), Pinto et al. (2016), Andreoli & Sabogal-Paz (2019), and Ogura & Sabogal-Paz (2021). This suggests that the acid dissociation step proposed by Method 1623.1 is not sufficiently efficient. For G. duodenalis cysts, although the most effective condition obtained in our study used twice as much acid in relation to the amount of each bead, this alone does not seem to be a determining factor for improving the dissociation process, since by maintaining this proportion but using 100 μL of each bead and 200 μL of acid, the results were not satisfactorily proportionate.

Although the best result of this work was only for one target microorganism (i.e., G. duodenalis), the achieved
results represent a significant improvement regarding the cost–benefit of the protozoa detection protocol. The expense for processing a single water sample is approximately US $180 for all the consumables required by Method 1623.1 and considering only the Merifluor™ and Dynabeads™ kits, the cost is estimated at US $130 per sample, in which 75% of this value is due to the use of the Dynabeads™ kit (Brazilian quote in January 2020). Based on this, the expense for a single protozoan test was over US $118, significantly higher when compared with the costs of other routine assays required to monitor a water supply system.

These high costs are a limiting factor, especially in low- and middle-income countries, which usually lack the infrastructure, qualified labour and economic resources. This situation can be verified by evaluating publications on protozoa around the world. Almost 70% of the publications using Method 1623.1 are concentrated in Europe and North America, while Africa and Central/South America have only 5% (Efstratiou et al. 2017). According to Giglio & Sabogal-Paz (2018), detecting protozoa in complex matrices is expensive and limits surveillance and control programs in developing countries; thus, more research is needed to make parasite detection possible in these countries and a reduced-cost approach might assist in reaching this goal.

As previously mentioned, the high cost of the methodology does not fall exclusively onto the IMS, but it is, in fact, the main expense. The Merifluor™ kit, additional use of DAPI, the epifluorescence microscope and all the necessary infrastructure to carry out the method are direct contributors to its enhancement. However, none of the aforementioned items/reagents can be removed from the global protocol without causing its mischaracterization and most likely loss of results. In this sense, we opted for the careful optimization of one of the methodological steps as an attempt to generate financial savings. The cost reduction in the IMS procedure is reflected by the increased durability of the kit, which, according to our results, can be used in 100 samples instead of 50, as recommended by the manufacturer. Therefore, the alternative offered by our study (50 μL of beads), which complies with the USEPA criteria at least for G. duodenalis sp. and C. parvum, is crucial to more effectively evaluate the water quality in developing countries having a direct impact on public health. However, this will continue to be extremely challenging, not least because scientists in developing countries face lower absolute levels of funding and must often pay far too expensive and unsustainable costs for consumables and equipment.

Further studies are recommended to improve the organism-bead dissociation process, seeking to increase the protozoa detection protocol performance in the sample purification phase.

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CONFLICTS OF INTEREST

The authors hereby declare previous originality check, no conflict of interest and open access to the repository of data used in this paper for scientific purposes.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

REFERENCES

Andreoli, F. C. & Sabogal-Paz, L. P. 2019 Coagulation, flocculation, dissolved air flotation and filtration in the removal of Giardia spp. and Cryptosporidium spp. from water supply. Environmental Technology 40, 654–663. https://doi.org/10.1080/09593330.2017.1400113.

Checkley, W., White Jr., A. C., Jaganath, D., Arrowood, M. J., Andreoli, F. C. & Sabogal-Paz, L. P. 2015 A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for Cryptosporidium. The Lancet Infectious Diseases 15, 85–94. https://doi.org/10.1016/S1473-3099(14)70772-8.

Clancy, J. L., Gollnitz, W. D. & Tabib, Z. 1994 Commercial labs: how accurate are they? Journal of American Water Works Association 86, 89–97. https://doi.org/10.1002/j.1551-8833.1994.tb06198.x.

Di Giovanni, G. D., Hashemi, F. H., Shaw, N. J., Abrams, F. A., LeChevallier, M. W. & Abbaszadegan, M. 1999 Detection of Infectious Cryptosporidium parvum oocysts in surface and filter backwash water samples by immunomagnetic separation and integrated cell culture-PCR. Applied and Environmental Microbiology 65, 3427–3432. https://doi.org/10.1128/AEM.65.8.3427-3432.1999.

Efstatiou, A., Ongerth, J. & Karanis, P. 2017 Waterborne transmission of protozoan parasites: review of worldwide outbreaks – an update 2011–2016. Water Research 114, 14–22. https://doi.org/10.1016/j.watres.2017.01.056.

Efstatiou, A., Ongerth, J. & Karanis, P. 2017b Evolution of monitoring for Giardia and Cryptosporidium in water. Water Research 123, 96–112. https://doi.org/101016/j.watres.2017.06.042.

Feng, Y. Y., Ong, S. L., Hu, J. Y., Song, L. F., Xiao, L. T. & Ng, W. J. 2003 Effect of particles on the recovery of Cryptosporidium oocysts from source water samples of various turbidities. Applied and Environmental Microbiology 69, 1898–1903. https://doi.org/10.1128/AEM.69.4.1898-1903.2003.

Feng, Y. Y., Zhao, X., Chen, J., Jin, W., Zhou, X., Li, N., Wang, L. & Xiao, L. 2011 Occurrence, source, and human infection potential of Cryptosporidium and Giardia spp. in source and tap water in Shanghai, China. Applied and Environmental Microbiology 77, 3609–3616. https://doi.org/10.1128/AEM.00416-11.

Franco, R. M. B., Hachich, E. M., Sato, M. I. Z., Naveira, R. M. L., Silva, E. C., Campos, M. M. C., Cantusio Neto, R., Cerqueira, D. A., Branco, N. & Leal, D. A. G. 2012 Performance evaluation of different methodologies for detection of Cryptosporidium spp. and Giardia spp. in water for human consumption to meet the demands of the environmental health surveillance in Brazil. Epidemiologia e Servicos de Saúde 21, 233–242.

Franco, R. M. B., Branco, N., Amaro, B. C. T., Neto, R. C. & Fiuza, V. R. S. 2016 Cryptosporidium species and Giardia genotypes detected in surface water supply of Campinas, southeast Brazil, by molecular methods. Journal of Veterinary Medicine and Research 3, 1053–1059.

Francy, D. S., Simmons, O. D., Ware, M. W., Granger, E. J., Sobsey, M. D. & Schaefer, F. W. 2004 Effects of seeding procedures and water quality on recovery of Cryptosporidium oocysts from stream water by using U.S. Environmental Protection Agency Method 1623. Applied and Environmental Microbiology 70 (7), 4118–4128. doi:10.1128/AEM.70.7.4118-4128.2004.

Giglio, G. L. & Sabogal-Paz, L. P. 2018 Performance comparison of three methods for detection of Giardia spp. cysts and Cryptosporidium spp. oocysts in drinking-water treatment sludge. Environmental Monitoring and Assessment 190, 686. http://doi.org/10.1007/s10661-018-7057-9.

Hammer, Ø., Harper, D. A. T. & Ryan, P. D. 2001 PAST: paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4, 1–9. http://palaeo-electronica.org/2001_1/past/issue1_01.htm.

Hashimoto, A., Kunikane, S. & Hirata, T. 2002 Prevalence of Cryptosporidium oocysts and Giardia cysts in the drinking water supply in Japan. Water Research 36, 519–526. https://doi.org/10.1016/S0043-1354(01)00279-2.

Hsu, B. M. & Huang, C. 2000 Recovery of Giardia and Cryptosporidium from water by various concentration, elution, and purification techniques. Journal of Environmental Quality 29, 1957–1959. https://doi.org/10.2134/jeq2000.00472425002900050028x.

Hsu, B. M. & Huang, C. 2007 IMS method performance analyses for Giardia in water under differing conditions. Environmental Monitoring and Assessment 131, 129–134. https://doi.org/10.1007/s10661-006-9462-8.

Hu, J., Feng, Y., Ong, S. L., Ng, W. J., Song, L., Tan, X. & Chu, X. 2004 Improvement of recoveries for the determination of protozoa Cryptosporidium and Giardia in water using Method 1623. Journal of Microbiological Methods 57, 321–325. https://doi.org/10.1016/j.mimet.2004.04.013.

Jakubowski, W., Boutros, S., Faber, W., Fayer, R., Ghiorse, W., LeChevallier, M., Rose, J., Schaub, S., Singh, A. & Stewart, M. 1996 Environmental methods for Cryptosporidium.
Kothavade, R. J. 2012 Potential molecular tools for assessing the public health risk associated with waterborne Cryptosporidium oocysts. *Journal of Medical Microbiology* 61, 1039–1051. https://doi.org/10.1099(mm.0.043518-0.

Kumar, T., Abd Majid, M. A., Onichandran, S., Jaturas, N., Andiappan, H., Sallibay, C. C. & Phirtyasamith, S. 2016 Presence of Cryptosporidium parvum and Giardia lamblia in water samples from Southeast Asia: towards an integrated water detection system. *Infectious Disease of Poverty* 5, 3–15. https://doi.org/10.1186/s40249-016-0095-z.

Lora-Suarez, F., Rivera, R., Triviño-Valencia, J. & Gomez-Marin, J. E. 2016 Detection of protozoa in water samples by formalin/ether concentration method. *Water Research* 100, 377–381. doi:10.1016/j.watres.2016.05.038.

Maciel, P. F. & Sabogal-Paz, L. P. 2016 Removal of Giardia spp. and Cryptosporidium spp. from water supply with high turbidity: analytical challenges and perspectives. *Journal of Water and Health* 14, 369–78. https://doi.org/10.2166/wh.2015.227.

McCuin, R. M., Bukhari, Z., Sobrinho, J. & Clancy, J. L. 2001 Recovery of Cryptosporidium oocysts and Giardia cysts from source water concentrates using immunomagnetic separation. *Journal of Microbiological Methods* 45, 69–76. https://doi.org/10.1016/S0167-7012(01)00250-0.

Medeiros, R. C. & Daniel, L. A. 2015 Comparison of selected methods for recovery of Giardia spp. cysts and Cryptosporidium spp. oocysts in wastewater. *Journal of Water and Health* 13, 811–818. https://doi.org/10.2166/wh.2015.228.

Ogura, A. P. & Sabogal-Paz, L. P. 2021 Detection and alkaline inactivation of Cryptosporidium spp. oocysts and Giardia spp. cysts in drinking-water treatment sludge. *Journal of Water Process Engineering* 40, e101939. http://doi.org/10.1016/j.jwpe.2021.101939.

Ongerth, J. E. 2015 ICR SS protozoan data site-by-site: a picture of Cryptosporidium and Giardia in U.S. surface water. *Environmental Science and Technology* 47, 10145–10154. https://doi.org/10.1021/acs.est.0c027503.

Pineda, C. O., Leal, D. A. G., Fiuela, V. R. D. S., Jose, J., Borelli, G., Durigan, M., Pena, H. F. J. & Bueno Franco, R. M. 2020 Toxoplasma gondii oocysts, Giardia cysts and Cryptosporidium oocysts in outdoor swimming pools in Brazil. *Zoonoses and Public Health* 67, 785–795. http://doi.org/10.1111/zph.12757.

Pinto, D. O., Urbano, L. & Cantusio Neto, R. 2016 Immunomagnetic separation study applied to detection of Giardia spp. cysts and Cryptosporidium spp. oocysts in water samples. *Water Supply* 6, 144–149. http://doi.org/10.2166/ws.2015.121.

Platts-Mills, J. A., Babji, S., Bodhidatta, L., Gratz, J., Haque, R., Havit, A. & Shakoor, S. 2015 Pathogen-specific burdens of community diarrhoea in developing countries: a multisite birth cohort study (MAL-ED). *The Lancet Global Health* 3, e564–e575. https://doi.org/10.1016/S2214-109X(15)00151-5.

Razzolini, M. P., Silva Santos, T. F. & Bastos, V. K. 2010 Detection of Giardia and Cryptosporidium cysts/oocysts in waterbodies and drinking water sources in Brazil urban areas. *Journal of Water and Health* 8, 399–404. https://doi.org/10.2166/wh.2009.172.

Razzolini, M. P., Weir, M. H., Matte, M., Matte, G. R., Fernandes, L. N. & Rose, J. B. 2011 Risk of Giardia infection for drinking water and bathing in a peri-urban area in São Paulo, Brazil. *International Journal of Environmental Research* 21, 222–234. https://doi.org/10.1080/09693727.2010.533367.

Rendtorff, R. C. 1979 The experimental transmission of Giardia lamblia among volunteer subjects. In: Waterborne Transmission of Giardiasis (W. Jakubowski & J. e. Hoff, eds). US Environmental Protection Agency. Environmental Research Centre, Cincinnati, Ohio, pp. 64–81. EPA-600/9-79-001.

Robert-Gangeux, F. & Dardé, M. L. 2012 Epidemiology of and diagnostic strategies for toxoplasmosis. *Clinical Microbiology Reviews* 25, 64–296. https://doi.org/10.1128/CMR.05013-11.

Rochelle, P. A., De Leon, R. & Johnson, A. 1999 Evaluation of immunomagnetic separation for recovery of infectious Cryptosporidium parvum oocysts from environmental samples. *Applied and Environmental Microbiology* 65, 841–845. https://doi.org/10.1128/AEM.65.2.841-845.1999.

Sammarro Silva, K. J. & Sabogal-Paz, L. P. 2020 Giardia spp. cysts and Cryptosporidium spp. oocysts in drinking water treatment residues: comparison of recovery methods for quantity assessment. *Environmental Technology* 41, 1–10. https://doi.org/10.1080/09593330.2020.1723712.

Sato, M. I. Z., Galvani, A. T., Padula, J. A., Nardocci, A. C., de Souza Lauretto, M., Razzolini, M. T. P. & Hachich, E. M. 2023 Assessing the infection risk of Giardia and Cryptosporidium in public drinking water delivered by surface water systems in Sao Paulo State, Brazil. *Science of the Total Environment* 442, 389–396. https://doi.org/10.1016/j.scitotenv.2021.09.077.

Schaefer, F. W. 2001 Can we believe our results? In: *Cryptosporidium: The Analytical Challenge* (M. Smith & K. C. Thompson, eds). Royal Society of Chemistry, Cambridge, UK, pp. 155–161.

Silva, H. G. & Sabogal-Paz, L. P. 2020 Filter backwash water and floated residue containing pathogenic protozoa: detection method and treatment alternatives. *Water, Air, & Soil Pollution* 251, 1–11. doi:10.1007/s11270-020-04515-z.

Silva, K. J. S. & Sabogal-Paz, L. P. 2021 Ferric sulphate flocculation as a concentration method for Giardia and Cryptosporidium in filter backwash water. *Water Practice and Technology*, 1–9. doi: 10.2166/wpt.2021.021.

Snelling, W. J., Xiao, L., Ortega-Pierres, G., Lowery, C. J., Moore, J. E., Rao, J. R., Smyth, S., Millar, B. C., Rooney, P. J., Matsuda, M., Kenny, F., Xu, J. & Dooley, J. S. 2007 Cryptosporidiosis in developing countries. *Journal of Infection in Developing Countries* 1, 242–256.

Squire, S. A. & Ryan, U. 2017 Cryptosporidium and Giardia in Africa: current and future challenges. *Parasites and Vectors* 10, 1–32. https://doi.org/10.1186/s13071-017-2111-y.
Stancari, R. A. & Correa, M. 2010 Occurrence of *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts in water sources and public water supplies. *Revista do Instituto Adolfo Lutz* 69, 453–460.

Sunnotel, O., Lowery, C. J., Moore, J. E., Dooley, J. S. G., Xiao, L., Millar, B. C., Rooney, P. J. & Snelling, W. J. 2006 *Cryptosporidium*. *Letters in Applied Microbiology* 43, 7–16. https://doi.org/10.1111/j.1472-765X.2006.01936.x.

USEPA. Method 1623.1:2012 *Cryptosporidium and Giardia in Water by Filtration/IMS/FA*. United States Protect Agency. US Environmental Protection Agency, Office of Water.

World Health Organization (WHO) 2014 *International Scheme to Evaluate Household Water Treatment Technologies*. Geneva, Switzerland: WHO.

World Health Organization (WHO) 2017 *Guidelines for Drinking-Water Quality: Fourth Edition*. Geneva: WHO.

World Health Organization (WHO) 2020 1 in 3 People Globally Do Not Have Access to Safe Drinking Water. UNICEF, WHO. Available from: https://www.who.int/news-room/detail/18-06-2019-1-in-3-people-globally-do-not-have-access-to-safe-drinking-water-unicef-who (accessed 20 September 2020).

Yakub, G. P. & Stadterman-Knauer, K. L. 2000 Evaluation of immunomagnetic separation for recovery of *Cryptosporidium parvum* and *Giardia duodenalis* from high-iron matrices. *Applied of Environmental Microbiology* 66, 628–3631. https://doi.org/10.1128/aem.66.8.3628-3631.2000.

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