**ABSTRACT**

In this work we studied the IMS purification comparing acid ($n = 12$) and heat ($n = 12$) dissociation procedures and investigated the possible losses of target organism in this step. Reagent water samples were directly inoculated with *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts (BTF Easy Seed™). Acid dissociation showed higher mean recovery efficiency and precision than heat dissociation for *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts, but there were not significant statistic differences for *Cryptosporidium* spp. oocysts. Mean recovery efficiency for both protozoa were in accordance with the acceptance criteria of Method 1623 by acid and heat dissociation. The bead–cyst/bead–oocysts dissociation procedure is fundamental for better results whereas a significant loss of organisms occurs in this step.

**Key words** | acid dissociation, heat dissociation, immunomagnetic separation, method 1623, waterborne protozoan

**INTRODUCTION**

*Cryptosporidium* spp. and *Giardia* spp. protozoa are some of the most significant waterborne contaminants in recent years (Franco 2007). Both species are widespread in Brazil and around the globe and are often found in water supply (Cantusio et al. 2010; Araújo et al. 2011; Baldursson & Karanis 2011).

These pathogens have characteristics that increase the probability of waterborne dissemination like their abundance and resistance to the environment and small cyst and oocyst size (resistance and transmission). These characteristics enable them to pass through the physical barriers of water treatment plants. The resistance to common chemical disinfectants used in the plants and low infection dose are the main factors that contribute to the occurrence of documented cryptosporidiosis and giardiasis outbreaks (Karanis et al. 2007; Baldursson & Karanis 2011).

These outbreaks show how important is to study these protozoa in water and improve current detection methods. In Brazil, drinking water Regulation # 2914/MS, published on 12 December 2011, mandates the monitoring of *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts at the intake area when the geometric average of annual concentration of *Escherichia coli* is higher than 1,000/100 mL (Brazil 2011).

The official method for detection of these pathogens in water samples is EPA Method 1623 (United States Environmental Protection Agency (USEPA) 2005), actually Method 1623.1 (United States Environmental Protection Agency (USEPA) 2012). Among the most important innovations are the new Quality Control acceptance criteria for mean recovery and precision and insertion procedures to improve the purification step by IMS, such as the washing of beads adhere to the tube, ...
the removal of magnetic materials (Fe and others), the heat dissociation and pH.

The scope of the method retains the same structure as the original and consists basically of three steps: (i) sample concentration; (ii) sample purification through immunomagnetic separation (IMS); and (iii) visualization and quantification of organisms by epifluorescence microscope of immunofluorescence assay (IFA) and a test confirming morphology using 4’,6-diamidine-2-phenylindole (DAPI) staining and differential interference contrast (DIC) microscopy.

The IMS purification step isolates the microorganisms from the debris present in the samples to further the visualization step and reduces false positives. It consists in a selective separation of Giardia spp. cysts and Cryptosporidium spp. oocysts with uniform paramagnetic microscopic spheres covered purified antibodies against the cysts and oocysts (Cantusio-Neto et al. 2013), and offers a considerable improvement in the efficiency of the method (Quintero-Betancourt et al. 2004). The bead–organism complex undergoes either acid or heat dissociation (Ware et al. 2003; United States Environmental Protection Agency (USEPA) 2012).

The IMS technique is applied to isolate many different targets of interest such as fungal/bacterial cells or spores, protozoan parasites, cellular and subcellular material, proteins and nucleic acid products (Yakub & Stadterman-Knauer 2004). This wide range of application considers different matrices including environmental, clinical, soil and food samples making the IMS one of the most versatile techniques available for target products purification from heterogeneous samples matrices (Orlofsky et al. 2013; Kanki et al. 2014; Grant & Stewart 2015).

Several factors may affect the efficiency of parasites capture and isolation through the sample by IMS such as sediment volume, magnetic material concentration and pH (Quintero-Betancourt et al. 2003; United States Environmental Protection Agency (USEPA) 2003). The pH interferes on the superficial wall cell and influences on the antigen-antibody bindings stabilization (Cook et al. 2006; Yamashiro et al. 2015).

This work has straightly studied the IMS purification step comparing two dissociation procedures: acid and heat, and investigated the possible losses of target organisms in reagent water samples.

**MATERIAL AND METHODS**

**Assays**

Twenty four experiments were performed: acid dissociation \((n = 12)\) and heat dissociation \((n = 12)\).

Giardia spp. cysts and Cryptosporidium spp. oocysts were artificially inoculated into the sample at the beginning of the purification step (BTF Easy Seed™). Each sample contained 1.0 mL of BTF Easy Seed™, 2.0 mL of 0.05% Tween 20, and 7.0 mL of reagent water, for a total sample volume of 10.0 mL.

This study also considered analyzing the discharges of the magnetic particle concentrator (MPC-1 and MPC-S) and cysts and oocysts that continue adhering to the paramagnetic bead complex after dissociation steps (drops formed in the second magnet step, MPC-S).

Evaluating the MPC-1 and MPC-S magnetic concentrator residues, the exceeded volume was collected. It was processed by: (i) new IMS, separately using either acid or heat dissociation; or (ii) evaluation of 10 \(\mu\)L aliquot of the pellet after centrifuging \(1,500 \times g\) for 15 minutes). The collected material underwent for visual characterization according to the protocol.

Concerning the beads that could still be adhered to the magnet after the dissociation procedure, 50 \(\mu\)L of reagent water was added to the ‘drop’ formed after the second magnet was applied (MPC-S).

**Purification**

In the IMS purification step of the samples (United States Environmental Protection Agency (USEPA) 2003), Invitrogen GC-Combo Dynabeads® (lot 615376) was used according to the USEPA’s protocol.

**Acid dissociation**

Fifty \(\mu\)L of HCl 0.1 N were added to the centrifuge tube and vortex for 50 seconds. The sample was undisturbed
for 10 minutes at room temperature and then vortexed for 30 seconds. After that the tubes were transferred to the magnetic particle concentrator (MPC-S), and the magnetic strip was inserted.

The remaining volume of the tube was previously prepared with 5 μL of NaOH 1 N and after, carefully transferred into the IFA well, according to United States Environmental Protection Agency (USEPA) (2005).

Method 1623 (United States Environmental Protection Agency (USEPA) 2005) was used instead of 1623.1 (United States Environmental Protection Agency (USEPA) 2002) because the study experimental part was performed in 2011.

Acid dissociation (extra)

Extra acid dissociation experiments were performed according to Ware et al. (2005) to analyze acid dissociation following the same protocol described above but after the magnetic strip was inserted the remaining volume was transferred to another tube containing 50 μL of reagent water and 5 μL of NaOH 1 N. This tube was then heated for 10 minutes at 80 °C.

Heat dissociation

Fifty μL of reagent water were added to the wall of the microcentrifuge tube, which were immediately centrifuged for 50 seconds. After shaking, the microcentrifuge tube was transferred to the dry bath (Nova Ética® Thermo-block model 330 – 2D) at 80 °C for 10 minutes and then centrifuged again for 30 seconds. After, the tube was transferred to the magnetic particle concentrator (MPC-S), and the magnetic strip was inserted.

The remaining volume of the tube was carefully transferred into the IFA well (Ware et al. 2003).

Immunofluorescence assay

Samples were submitted to IFA using the Merifluor® kit (Meridian Bioscience, Cincinnati, Ohio), for observation and quantification of cysts and oocysts. The preparations were examined in a Zeiss AxioLab epifluorescence microscope (United States Environmental Protection Agency (USEPA) 2005).

Statistical analyzes

The results were statistically analyzed using the paired Student’s t-test for comparison of means (bilateral), considering a significant level of 5%, using the GraphPad Prism software (version 5.01).

RESULTS AND DISCUSSION

Acid dissociation showed better mean recovery efficiency and accuracy compared to heat dissociation for both protozoa (Table 1). Significant statistical differences were found for *Giardia* spp. cysts (*P* = 0.0065), but the same did not happened to *Cryptosporidium* spp. oocysts (*P* = 0.0572).

The values obtained in this study were compared with IPR (initial precision recovery) established by Method 1623, using reagent water as matrix (Cantusio-Neto et al. 2011). The acceptance criteria values in this method are 24–100% for both protozoa, and relative standard deviation (RSD) lower than 55% for *Cryptosporidium* spp. oocysts and 49% for *Giardia* spp. cysts (United States Environmental Protection Agency (USEPA) 2005).

In January 2012 Method 1623 was revised and updated by version 1623.1, which contains changes in the Quality Control values of mean recovery efficiency for *Giardia*.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Minimum, mean and maximum recovery efficiency values (%) and relative standard deviation (RSD) for Cryptosporidium spp. oocysts and <em>Giardia</em> spp. cysts by either acid or heat dissociation in the IMS purification phase in reagent water artificially inoculated with BTF Easy Seed™</th>
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<tr>
<td><strong>Cryptosporidium spp.</strong></td>
<td><strong>Giardia spp.</strong></td>
</tr>
<tr>
<td>Minimum</td>
<td>Mean</td>
</tr>
<tr>
<td>Acid (%)</td>
<td>38</td>
</tr>
<tr>
<td>Heat (%)</td>
<td>13</td>
</tr>
</tbody>
</table>
spp. (27–100%) cysts and Cryptosporidium spp. oocysts (38–100%) and Precision (as maximum relative standard deviation) lower than 37% for oocysts and 39% for cysts (United States Environmental Protection Agency (USEPA) 2012).

Mean recovery efficiency and the RSD values obtained in this work for both protozoa by acid dissociation were within the acceptance criteria of Method 1623 Analytical Quality Control (United States Environmental Protection Agency (USEPA) 2005). However, the RSD by heat dissociation were not in accordance with the criteria. The great difference between minimum and maximum values shows the lower accuracy for heat dissociation (Table 1).

Concerning Method 1623.1 the results obtained for both protozoa by acid dissociation remain within the acceptance criteria. However, using heat dissociation, the mean recovery for Giardia spp. cysts was lower and the RSD for both protozoa were higher compared to the criteria.

The dissociation procedure of the purification step in the Method 1623 must be performed twice (United States Environmental Protection Agency (USEPA) 2005). Most oocysts were recovered in the first reaction; however, the second reaction was very important due to an increase of mean recovery for Cryptosporidium spp. oocysts, in acid dissociation (11.0%) and heat dissociation (15.9%), according to Table 2.

Nevertheless, most Giardia spp. cysts were recovered in the second reaction by heat dissociation. The time required for bead–cyst dissociation was longer than the 10 minutes used in the first reaction, which was provided in the second one (Table 2).

Considering acid dissociation most of Giardia spp. cysts were recovered in first reaction as observed for Cryptosporidium spp. oocysts. The second reaction confirmed the importance with an increase of 28% in mean recovery efficiency (Table 2).

The mean recovery of organisms varies widely in the current methods established for protozoa detection (Cantusio-Neto et al. 2010). This variability is due to losses throughout sample processing, from concentration to the immunofluorescence reaction step. The differences between commercially available kits for cysts and oocysts detection need to be taken into consideration and also the differences in the characteristics of the water matrix (Warnecke et al. 2003; Quintero-Betancourt et al. 2004; Hu et al. 2004).

In this study reagent water samples were used to investigate the sensitivity of dissociation procedures, acid and heat, totally free from interfering contaminants as recommended by the IPR (United States Environmental Protection Agency (USEPA) 2005). Reagent water sample makes the visualization clearer and the confirmation easier in opposite to environmental sample. The 4′,6-diamidino-2-phenylindole (DAPI) is a nuclear stain used for visualization cysts and oocysts internal structures as an additional criteria to the phase contrast microscopy to confirm the identification (Cantusio-Neto 2008) and its use is questioned by some authors who argue that it increases background fluorescence, hindering the detection of organisms (Chauret et al. 1999). Therefore, confirmatory morphology tests with DAPI were not applied is this case. Studies with environmental samples are in progress and they will have important contributions to a better understanding of the step improvement.

The IMS purification step is very frail and complex, probably the main cause for cyst and oocyst loss. The complete dissociation of the bead–cyst and bead–oocyst complex is fundamental, so the choice of dissociation procedure (acid or heat) is very important.

Regarding the residues from the Lyton tube (MPC-1 magnetic concentrators), no cysts or oocysts were found in this material.

Evaluating the beads that remain adhered to the magnet after dissociation in the ‘drop’ formed in the second magnet phase (MPC-S), the analyzed material still contained 10% cysts and 5% oocysts for both dissociation procedures. This is one of the main causes of reduced recovery of organisms (Figure 1).

Heat dissociation was proposed by Ware et al. (2003) as an alternative to improve acid dissociation procedure for Cryptosporidium spp. oocysts in water, although it is also used for Giardia spp. cysts. They showed oocyst mean

**Table 2 | Mean recovery efficiency (%) for Cryptosporidium spp. oocysts and Giardia spp. cysts of the first and second dissociation reaction, using both acid or heat dissociation in the IMS purification step in reagent water artificially inoculated with BTF Easy Seed™**

<table>
<thead>
<tr>
<th></th>
<th>Cryptosporidium spp.</th>
<th>Giardia spp.</th>
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<tbody>
<tr>
<td></td>
<td>1ª dissociation</td>
<td>2ª dissociation</td>
</tr>
<tr>
<td>Acid (%)</td>
<td>51.5</td>
<td>5.7</td>
</tr>
<tr>
<td>Heat (%)</td>
<td>34.6</td>
<td>5.5</td>
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recovery in reagent water samples of 71% through heat dissociation and 41% through acid dissociation. The authors claimed that in an acid medium the reaction pH is altered reducing antibody binding and fluorochrome expression. However, in the acid dissociation experiments performed by Ware et al. (2003), after incubating the sample in 50 μL of HCl 0.1 N (0.1 mol H⁺/L) for 10 minutes at room temperature as specified in Method 1623, Ware and colleagues transferred the oocysts isolated from the beads by the magnetic bar to another tube with 5 μL of NaOH 1 N and 50 μL of reagent water and incubated for more 10 minutes at 80 °C. This extra 50 μL of reagent water which is not required in Method 1623 reduces by half the H⁺ concentration (0.05 mol H⁺/L) and may have contributed to the pH alteration of the reaction and the action of the antibodies (Cook et al. 2006) that are directed and bound to the oocyst epitopes. This modification to the Method 1623 procedure may have resulted in a lower recovery rate for acid dissociation compared to heat dissociation of the Cryptosporidium spp. oocysts obtained by Ware et al. (2003).

In the present study extra acid dissociation experiments were performed adding more 50 μL of reagent water such as Ware et al. (2003) experiments. The means recovery efficiency obtained in these experiments were 37% for Giardia spp. cysts and 32% for Cryptosporidium spp. oocysts, lower than showed in Table 1, which follows Method 1623. Thus, the potential variation of pH promoted by acid dissociation according to Ware et al. (2003) probably did not occur or did not compromise the performance when the acid dissociation protocol meets the USEPA.

**CONCLUSION**

Acid dissociation was more efficient than heat dissociation for Giardia spp. cysts and Cryptosporidium spp. oocysts although there were no statistically significant difference between the dissociation procedures for Cryptosporidium oocysts. The experiments have also showed that the bead-cyst and bead–oocyst dissociation step are fundamental for better results, whereas a significant loss of organisms may occurs in this phase, specifically including the cysts and oocysts that remain adhered to the beads in the ‘drop’. The increase in incubation time for more than 10 minutes may contribute for better results in the heat dissociation procedure.

**REFERENCES**

Genotypic characterization of Cryptosporidium hominis


Cantusio-Neto, R. 2008 Methods studies: calcium carbonate flocculation and adaptation of membrane filtration and immunomagnetic separation techniques for *Cryptosporidium* and *Giardia* detection in water samples. PhD thesis. Biology Institute, State University of Campinas, Campinas, São Paulo, Brazil.


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