Comparison of selected methods for recovery of *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts in wastewater
Raphael Corrêa Medeiros and Luiz Antonio Daniel

**ABSTRACT**

More precise methods are needed to recover *Giardia* and *Cryptosporidium* (oo)cysts from wastewater in order to advance research related to their inactivation, removal, quantification, and species differentiation. This study applied different methods to recover the maximum number of (oo)cysts from wastewater samples using ColorSeed®. Immunomagnetic separation assisted in capturing oocysts mainly in samples with medium and low turbidity. A triple centrifugation method reached recovery rates of 85% and 20%, for *Giardia* cysts and *Cryptosporidium* oocysts, respectively, in raw wastewater, and 62.5 and 17.5% in secondary-treated effluent. For low turbidity-treated effluent, membrane filtration reached 67.5% recovery for *Giardia* cysts and 22.5% for *Cryptosporidium* oocysts. Simple, quick and low-cost methods do not involve much handling of the samples and could be useful, particularly in developing countries.

**Key words** | *Cryptosporidium*, *Giardia*, recovery, turbidity, wastewater

**INTRODUCTION**

*Giardia* and *Cryptosporidium* are protozoa with a world-wide distribution and cause the most concern in terms of waterborne diseases (Baldursson & Karanis 2011; Ongerth 2013). They possess infectious (oo)cysts that are highly resilient and small enough to pass through some of the physical and chemical barriers used in water treatment procedures (Hsu & Yeh 2003; Assavasilavasukul et al. 2008). These protozoa are excreted largely in feces and may be spread through both zoonotic and anthropogenic transmission (Karanis et al. 2007).

Risebro et al. (2007) reported that, in Europe, more than half of the waterborne disease outbreaks between 1990 and 2005 were associated with these protozoa (29 outbreaks from *Cryptosporidium* and two from *Giardia*). According to Karanis et al. (2007), of the 325 reported waterborne disease outbreaks caused by pathogenic protozoa, *Giardia* was responsible for 132 (40.6%) and *Cryptosporidium* was responsible for 165 (50.8%). During the period 2004–2010, 199 waterborne protozoan parasitic outbreaks were reported (Baldursson & Karanis 2011), and in 60.3% and 35.1% of the cases, the etiological agents were *Cryptosporidium* spp. and *Giardia* spp., respectively.

Although no official data are available on outbreaks in Brazil, studies have revealed a high prevalence of these parasites mainly among children: for *Giardia*, the range is between 8.7 and 38.3% (Gross et al. 1993; Ludwig et al. 1993; Uchôa et al. 2001; Teixeira et al. 2007; Matos et al. 2008); for *Cryptosporidium*, between 1.1 and 32.4% (Oshiro et al. 2000; Pereira et al. 2002; Nascimento et al. 2009). Some potential risk factors identified in these studies were: poor sanitary conditions (no connection to sewage system, absence of a toilet in home); low family income; few years of schooling of the mother.

Thus, research is necessary to improve water and wastewater treatment techniques and to develop quick, precise, and reproducible methods to detect and count these parasites (Carey et al. 2004; Jex et al. 2008). Currently, United States Environmental Protection Agency (USEPA)-Method 1623.1 (USEPA 2012) is the standard method used to analyze water samples and is applicable to surface, underground,
treated, mineral, and recreational waters. This method uses filtration followed by immunomagnetic separation (IMS) and immunofluorescence assay (IFA) to detect (oo)cysts (Plutzer et al. 2010; USEPA 2012).

However, according to Bonatti et al. (2007), the techniques developed to count the (oo)cysts in water are generally not indicated for wastewater samples. Water and wastewater differ in the number of parasites as well as the composition and concentration of particulate matter, and researchers have sought to develop specific detection techniques that consider the complex nature of wastewater (Robertson et al. 2000; McCuin & Clancy 2005).

No exclusive method has been developed to test wastewaters. The techniques presented in the literature are based on a variety of methods, including calcium carbonate flocculation (Vesey et al. 1993), modifications of USEPA Method 1622 (USEPA 1999a) for Cryptosporidium (McCuin & Clancy 2005), sample concentration by filtration (Shepherd & Wyn-Jones 1995), centrifugation without a purification stage (Robertson et al. 2000), and membrane filtration (MF) (Franco et al. 2001, 2012).

Based on the modifications suggested by Robertson et al. (2000), the objective of this work was to compare different methods for the recovery of Giardia and Cryptosporidium in raw and treated wastewater with different turbidity, using marked (oo)cysts.

**MATERIALS AND METHODS**

**Sampling**

The samples for the study came from the University of São Paulo wastewater treatment plant (WWTP), located in the city of São Carlos – São Paulo State, Brazil. This WWTP has preliminary treatment equipment comprising screening apparatus, a sand and grease removal tank, and an upflow anaerobic sludge blanket reactor; and an activated sludge system (aeration tank and settling tank). Raw wastewater samples were collected after preliminary treatment and treated wastewater samples after the activated sludge system.

The samples were collected in 1-L bottles that had previously been washed, disinfected, and rinsed with an elution solution (Tween 80; 0.1% v/v). The samples were kept under refrigeration (4 ± 1°C) until testing and were analyzed no more than 24 hours after sampling.

All of the equipment that came into contact with the raw and treated wastewater, such as beakers, graduated cylinders, Erlenmeyer flasks, centrifugation tubes, microcentrifugation tubes, and pipettes, was rinsed beforehand with the elution solution. The collection flask was inverted 20 times for homogenization, and the samples were filtered through a plastic sieve with a 1.0 mm² pore size to remove coarse materials. After that, a Hach 2100P turbidimeter was used to measure the turbidity of each unspiked sample matrix prior to processing, and samples were divided into three ranges: low (0–10 nephelometric turbidity units (NTU)), medium (10–100 NTU), and high (>100 NTU).

**Recovery assays**

The recovery of the (oo)cysts by the methods was performed using a ColorSeed® kit (ColorSeed, Biotechnology Frontiers Inc., Sydney, Australia) in which marked (oo)cysts can be easily distinguished from indigenous (oo)cysts of the samples (McCuin & Clancy 2005) by changing the filter in the microscope lens. The manufacturer’s protocol was followed for the inoculation of the marked 99 ± 2.2 Cryptosporidium oocysts and 99 ± 1.4 Giardia cysts. This protocol consisted of adding 2 mL of Tween 20 solution (0.05% v/v) to the flask with the (oo)cysts; the flask was vortexed and inoculated. However, instead of performing two washes with 3 mL of water, as described in the manufacturer’s protocol, the decision was made to perform three washes with 2 mL of water, as this made homogenization easier.

After the inoculation of the ColorSeed®, the assays occurred in three different ways, as shown in Figure 1. For each recovery method four independent spiking experiments were performed. The percentage recovery efficiency (R) was calculated according to Equation (1).

$$R = \frac{\text{Number of cysts or oocysts recovered}}{\text{Number of cysts or oocysts spiked}} \times 100$$  \hspace{1cm} (1)

**Triple centrifugation method for raw wastewater**

For this method, 50 mL of raw wastewater was placed in an Erlenmeyer flask and 50 mL of Tween 80 (0.1%, v/v)
solution was added for a total of 100 mL, to which the (oo)cysts were inoculated. The sample was then homogenized for 3 minutes with a magnetic stirrer. Then, the volume was equally divided in two 50-mL centrifugation tubes, and the first centrifugation (Centrifuge 5810, Eppendorf) was performed at 1,500 × g for 15 minutes.

Next, the supernatant of each tube was removed, leaving a final volume of 5 mL, which was placed in a vortex mixer for homogenization of the pellet. The 5-mL volumes from each of the four centrifugation tubes were combined into a single tube with a 10-mL volume. The empty tube was rinsed twice with 2.5 mL of deionized water, and this rinse solution was added to the occupied tube. The final total volume in the occupied tube was 15 mL.

The second centrifugation was performed at 1,500 × g for 15 minutes. Again, the supernatant was removed, leaving a volume of approximately 1 mL. This volume was placed in a vortex mixer for homogenization of the pellet. The 1-mL volumes from each of the four centrifugation tubes were concentrated into a single tube, for a total volume of approximately 4 mL. The other three empty tubes were rinsed twice with 1.5 mL of deionized water. The rinse water was added to the occupied tube, and if necessary, additional deionized water was added to obtain a final volume of 15 mL. The second and third centrifugations were performed as reported above for the sample with high turbidity.

MF

First, all filtration equipment was washed with the elution solution. The membrane of mixed cellulose esters with a 3-μm pore size and 47-mm diameter (Millipore) was placed in the filtration system and was rinsed with enough of the elution solution to wet the membrane (approximately 1 mL). Then, 150 mL of the low-turbidity sample was collected into a graduated cylinder, and the ColorSeed® kit were added. The sample was homogenized for 3 minutes with a magnetic stirrer. The solution was equally divided into four 50-mL centrifugation tubes, and the first centrifugation was performed at 1,500 × g for 15 minutes.

After the centrifugation, the supernatant of each tube was removed, leaving a final volume of approximately 1 mL. This volume was placed in a vortex mixer for homogenization of the pellet. The 1-mL volumes from each of the four centrifugation tubes were concentrated into a single tube, for a total volume of approximately 4 mL. The other three empty tubes were rinsed twice with 1.5 mL of deionized water. The rinse water was added to the occupied tube, and if necessary, additional deionized water was added to obtain a final volume of 15 mL. The second and third centrifugations were performed as reported above for the sample with high turbidity.

Methods for treating wastewater

Triple centrifugation

For this method, 150 mL of secondary effluent was placed in an Erlenmeyer flask. Next, 50 mL of Tween 80 (0.1%) solution was added for a total of 200 mL, and (oo)cysts from the ColorSeed® kit were added. The sample was homogenized for 3 minutes with a magnetic stirrer. The solution was equally divided into four 50-mL centrifugation tubes, and the first centrifugation was performed at 1,500 × g for 15 minutes.

Figure 1 | General scheme of recovery assays of (oo)cysts with the ColorSeed® kit.
solution (0.1%) for a total of 15 mL, which was transferred to a centrifugation tube.

The first centrifugation was performed at 1,050 × g for 10 minutes (as reported by Franco et al. 2012), and the supernatant was then removed, leaving 5 mL in the centrifugation tube. The sample was taken to a vortex mixer, and 10 mL of deionized water was added. After the second centrifugation at 1,050 × g for 10 minutes, the supernatant was again removed. The remaining volume of 0.5 mL was passed to a microcentrifugation tube. Next, the centrifugation tube was rinsed with 0.5 mL of deionized water, and this rinse water was also transferred to the microcentrifugation tube. A third centrifugation then occurred at 1,050 × g for 10 minutes. The supernatant was again removed, and a volume of 0.5 mL remained in the microcentrifugation tube. This volume was placed in a vortex mixer and then left overnight in a refrigerator at a temperature of 5–10°C to await the detection phase of the study.

When, after filtration, IMS was used for samples with low turbidity, the entire sample was transferred from the microcentrifugation tube to a Leighton tube. After that, the microcentrifugation tube was washed three times with 1.0 mL of deionized water and this volume was also transferred to the Leighton tube. The Dynabeads manufacturer’s protocol was then followed (Dynal® CG, Combo anti-Cryptosporidium and Giardia, Invitrogen Dynal AS, Lillestrøm, Norway).

**Centrifugation followed by IMS**

The IMS was included in the centrifugation procedure in order to improve the recovery of the cysts and especially oocysts from the low-turbidity sample. In this process, 150 mL of effluent, 50 mL of Tween 80 solution (0.1%), and the (oo)cysts marked with Texas Red were homogenized in an Erlenmeyer flask for 5 minutes. The volume was equally divided into four centrifugation tubes and centrifuged at 1,500 × g for 15 minutes. The supernatant was removed, leaving 0.5 mL in each tube. These volumes were concentrated into a single tube. Each of the other three tubes was rinsed twice with 0.5 mL of deionized water. This rinse water was added to the occupied tube for a total volume of 5 mL. The tube was placed in a vortex mixer and left in a refrigerator overnight at a temperature of between 5 and 10°C.

Next, the IMS was performed. First, the volume in the centrifugation tube was transferred to a Leighton tube. Then, the centrifugation tube was rinsed twice with 2.5 mL of deionized water, and this rinse water was also placed in the Leighton tube. The Dynabeads manufacturer’s protocol was then followed (Dynal®, CG, Combo anti-Cryptosporidium and Giardia, Invitrogen Dynal AS, Lillestrøm, Norway).

**Negative control assays**

A negative control assay was performed between each recovery assay to ascertain whether there was contamination. In these cases, instead of wastewater sample, the entire procedure was performed with deionized water, absent of protozoan cysts. All control assays were negative for the occurrence of (oo)cysts.

**Detection and identification**

The detection and identification of the (oo)cysts was performed by IFA with a Merifluor® kit (Meridien Bioscience Diagnostics, Cincinnati, OH, USA). The sample in the microcentrifugation tube was placed in a vortex mixer for 2 minutes and inverted three times for homogenization. Then, 50 μL of the sample was removed with a micropipette that had been previously washed with an elution solution (Tween 80, 0.1%) and transferred to the well slide in the Merifluor® kit.

After drying at room temperature, the sample was fixed with absolute methanol (20–50 μL) for 10 minutes. Then, a drop of detection fluid and a drop of counter stain, both from the Merifluor® kit, were applied.

The slide was placed in a dark, humid chamber at 37°C for 30 minutes. For washing, 100 μL of deionized water was added to the well slide, which was tilted to 45° on a clean paper towel, with the long edge down. A drop of the mounting medium present in the Merifluor® kit was added to the well, and a cover slip was applied.

The count of the (oo)cysts in the wells was always performed within a maximum time period of 7 days (USEPA 2012) using an epifluorescence microscope (Olympus BX51) with a magnification of 300–800×. The cysts and oocysts were identified by their size, shape, fluorescence, and morphology corresponding to the control parasite.
suspension (the positive control in the Merifluor® kit). A green filter block was used to visualize the Texas Red stains imparted by ColorSeed®.

Statistical analysis

The statistical analysis was performed using STATISTICA 7.0 software (StatSoft Inc. 2004). Significant differences among the methods in terms of mean recovery were determined using one-way analysis of variance with the application of Student’s t-test based on the null hypothesis of equal mean recovery for all of the methods. A value of $P < 0.05$ was considered statistically significant.

RESULTS

The use of ColorSeed® in the recovery assays provides greater certainty because the cysts and oocysts are marked and differentiated from those that are intrinsic to the samples. Changing the filter in the microscope lens allows for the clear differentiation of the inoculated (o)ocysts, as observed in Figure 2.

The results from the recovery assays and the statistical analysis are presented in Tables 1 and 2, respectively.

The triple centrifugation (TC) method provided the highest mean recovery rate for secondary effluent with medium-turbidity samples for both protozoa (assays 1–4, in Table 1).

<table>
<thead>
<tr>
<th>Trials</th>
<th>Subject analyzed</th>
<th>Giardia (P-value)</th>
<th>Cryptosporidium (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC × TC</td>
<td>High × medium turbidity</td>
<td>0.0633</td>
<td>0.6202</td>
</tr>
<tr>
<td>TC × MF</td>
<td>Best method for medium turbidity</td>
<td>0.0141</td>
<td>0.2070</td>
</tr>
<tr>
<td>MF × C + IMS</td>
<td>Medium turbidity</td>
<td>0.0041</td>
<td>0.0984</td>
</tr>
<tr>
<td>TC × IMS</td>
<td>Medium × low turbidity</td>
<td>0.0033</td>
<td>0.2528</td>
</tr>
<tr>
<td>MF × IMS</td>
<td>IMS step for low turbidity</td>
<td>0.0288</td>
<td>0.1662</td>
</tr>
</tbody>
</table>

The rate of recovery of the cysts reached 85% for the high-turbidity effluent and 62.5% for the medium-turbidity effluent, but the difference between these rates was not significant ($P < 0.05$). Similarly, there was no significant difference in the rate of recovery of the oocysts from the high-turbidity (20.0%) and medium-turbidity (17.5%) effluent ($P < 0.05$), as shown in Table 2.

When the three methods used for medium-turbidity effluent are compared (assays 2–4, in Table 1), the TC method provided a better recovery rate for Giardia statistically ($P < 0.05$) than MF or centrifugation with IMS. However, in the recovery of Cryptosporidium oocysts, the TC method only provided a better performance when compared to the centrifugation with the addition of the IMS stage and no significant difference was found between the TC and MF techniques, as can be seen in Table 2.

Table 1 | Recovery rates of ColorSeed® Giardia cysts and Cryptosporidium oocysts in raw and treated wastewater

<table>
<thead>
<tr>
<th>Trial</th>
<th>Turbidity (NTU)</th>
<th>Method (# replicates)</th>
<th>% Cyst recovery (mean ± SD)</th>
<th>% Oocyst recovery (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>165</td>
<td>TC (4)</td>
<td>85.0 ± 12.9</td>
<td>20.0 ± 8.2</td>
</tr>
<tr>
<td>2</td>
<td>23.8</td>
<td>TC (4)</td>
<td>62.5 ± 15.0</td>
<td>17.5 ± 5.0</td>
</tr>
<tr>
<td>3</td>
<td>23.8</td>
<td>MF (4)</td>
<td>35.0 ± 5.8</td>
<td>12.5 ± 5.0</td>
</tr>
<tr>
<td>4</td>
<td>23.8</td>
<td>C + IMS (4)</td>
<td>27.5 ± 4.1</td>
<td>7.0 ± 2.6</td>
</tr>
<tr>
<td>5</td>
<td>6.5</td>
<td>MF (4)</td>
<td>67.5 (± 12.6)</td>
<td>22.5 (± 15)</td>
</tr>
<tr>
<td>6</td>
<td>6.5</td>
<td>MF + IMS (4)</td>
<td>47.8 (± 5.7)</td>
<td>35.3 (± 6.1)</td>
</tr>
</tbody>
</table>

TC, triple centrifugation; MF, membrane filtration; C, centrifugation; IMS, immunomagnetic separation; SD, standard deviation.

Table 2 | Statistical analysis for comparison of methods for (o)ocysts recovery in wastewater ($P < 0.05$ was considered statistically significant)

<table>
<thead>
<tr>
<th>Trials</th>
<th>Subject analyzed</th>
<th>Giardia (P-value)</th>
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TC, triple centrifugation; MF, membrane filtration; C, centrifugation; IMS, immunomagnetic separation.
Also, Table 1 shows the influence of turbidity in the test for MF (assays 3 and 5) with higher percentages of recovery in low turbidity, showing statistical differences for the recovery of *Giardia* cysts; however, this was not true for *Cryptosporidium* oocysts (*P > 0.05*).

In addition, the *Giardia* cysts were more easily recovered than the *Cryptosporidium* oocysts from raw wastewater (*P = 0.0001*). This finding was also true for the medium-turbidity effluent, where significant differences for recovery of cysts and oocysts were found among the three methods (*P = 0.0013* for TC; *P = 0.0011* for MF; and *P = 0.0002* for centrifugation followed by IMS). The same was also confirmed with low-turbidity effluent (*P = 0.0238*).

**DISCUSSION**

It is difficult to interpret and compare the data presented by various studies of pathogenic protozoa because of the different procedures, sampling methods, concentrations, and epidemiological status of the populations (Montemayor et al. 2005; Ongerth 2013).

Robertson et al. (2000), using a ‘small volume, no purification step’ method, recovered 79 ± 32% of the *Giardia* and 81 ± 21% of the *Cryptosporidium* from raw influent and recovered 81 ± 27% of the *Giardia* and 89 ± 10% of the *Cryptosporidium* from treated effluent. The TC method reached recovery rates very close to their study only for *Giardia* cysts.

McCuin & Clancy (2005) modified the 1622 method (USEPA 1999a) for the recovery of *Cryptosporidium* oocysts in wastewater. Using a smaller sample volume (250 mL) and ColorSeed®, these researchers recovered 23.5 ± 13.8% of the inoculated oocysts from the raw wastewater, 36.2 ± 11.4% from the primary effluent, and 50.4 ± 17.5% from the secondary effluent; values close to those found in this work.

Montemayor et al. (2005) followed the procedure outlined by Shepherd & Wyn-Jones (1993), with some modifications, to analyze *Cryptosporidium* in raw wastewater and secondary effluent. The samples underwent a coarse filtration (using a filter with a 50-μm pore size) to remove particles, followed by filtration with cellulose acetate filters with a 1.2-μm pore size, centrifugation (1,500 × g, 10 minutes), and subsequent IMS. With this procedure, the recovery rate was 60 ± 18%.

When using tertiary effluent and applying the 1623 method outlined by USEPA (1999b), the recovery rate was 33.6 ± 20.2%. Flocculation with calcium carbonate (Vesey et al. 1993) was also used for this effluent and led to an oocyst recovery rate of 40.5 ± 24.5%.

The values found in the present study were superior for *Giardia* cysts, but very close for *Cryptosporidium* oocyst recovery, in comparison to the results reported by Ottoson et al. (2006), who used the 1623 method (USEPA 2005) with some modifications. These modifications included a 50–100 mL volume for raw wastewater and a 300–600 mL volume for treated effluent and centrifugation of the sample at 2,000 × g for 10 minutes. Ottoson et al. (2006) recorded recovery rates in raw influent of 22 ± 1.5% for *Giardia* and 15 ± 4.6% for *Cryptosporidium*. In treated wastewater, the recovery was 25 ± 12% for *Giardia* and 39 ± 13% for *Cryptosporidium*.

The MF method employed fewer steps to concentrate the sample than the centrifugation method followed by IMS; however, the scraping of the membrane may not have reached all of the (oo)cysts that permeated the membrane during filtration. According to Carey et al. (2004), the recovery of (oo)cysts in the filtration step may have been affected by the high turbidity of the sample, which made filtration of the raw wastewater impractical because of the rapid clogging of the filter. However, filtration may be used for effluents (Robertson et al. 2000).

Bonatti et al. (2007) modified the method described by Robertson et al. (2000) by using 5 mL of sample with the centrifugation stage alone. This method, which involved less handling of the sample and a relatively small cost, resulted in a recovery rate of 43.7 ± 31.6% for *Giardia* and of 13.6 ± 12.8% for *Cryptosporidium*, which represent values that are somewhat smaller than those found with the TC method.

Wastewater has a complex composition with countless interfering elements, making it possible that the poor recovery rate seen in this study with the method using IMS resulted from the large degree of handling of the sample, the various transfers from one tube to another, and the many necessary steps involved in the procedure. These activities all increase the loss of the (oo)cysts (Robertson et al. 2000; Bonatti et al. 2007).

In this context, TC proved to be a promising method because it was quick, inexpensive, and simple. However, this technique has a high limit of detection, which may
not be favorable when the number of (oo)cysts in the sample is low (Robertson et al. 2000), such as in treated effluent. Moreover, for this kind of effluent the MF method could be more effective, and according to Franco et al. (2012), also low-cost and fast compared to centrifugation followed by the IMS technique.

**CONCLUSION**

This study demonstrated a method consisting of three centrifugations followed by immunofluorescence microscopy that may be applied to raw sewage and to treated effluent, mainly for the recovery of *Giardia* cysts. *Cryptosporidium* oocysts were more difficult to recover than *Giardia* cysts in complex matrices such as wastewater, whether treated or not. The IMS assists in capturing mainly oocysts in samples with medium and low turbidity, and helps visualize the withdrawal of much of the particulate matter present in the sample. However, it is a laborious step, with many details, and is costly. The use of a ColorSeed® kit provided greater reliability in the assay results, in which little handling of the sample led to the recovery of as much as 85% of the *Giardia* cysts and 20% of the *Cryptosporidium* oocysts. Thus, these pathogenic protozoa can be monitored in wastewater with high, moderate, and low turbidity by the TC proposed and MF which are easy, quick, and low-cost, and extremely important in developing countries such as Brazil with a high prevalence of *Giardia*.

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