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

Raphael Corrêa Medeiros (corresponding author)
Department of Agronomic and Environmental Sciences,
Federal University of Santa Maria,
Linha 7 de Setembro, BR 386, Km 40,
98400-000,
Frederico Westphalen – Rio Grande do Sul,
Brazil
E-mail: *medeiroscg@yahoo.com.br*

Luiz Antonio Daniel
Hydraulics and Sanitation Department,
São Carlos School of Engineering,
University of São Paulo,
Av. Trabalhador São-Carlense, 400,
13566-590,
São Carlos – São Paulo,
Brazil

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 anthroponotic 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.* 1989; Ludwig *et al.* 1999; 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 ($4 \pm 1 °C$) 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 mm^2 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).

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

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)
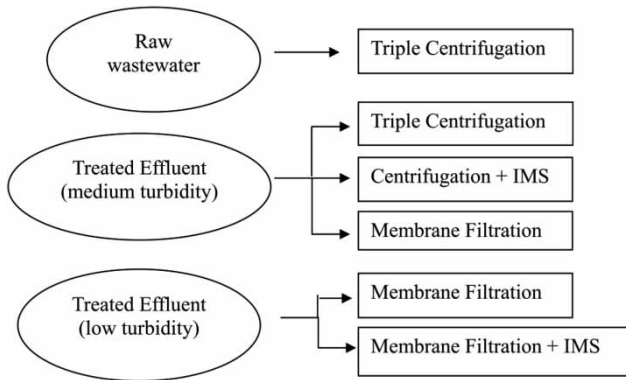
Figure 1 | General scheme of recovery assays of (oo)cysts with the ColorSeed ® kit.

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 $1,500 \times g$ 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 residual volumes of the two 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 $1,500 \times g$ for 15 minutes. Again, the supernatant was removed, leaving a volume of approximately 0.5 mL in the centrifugation tube. This volume was placed in a vortex mixer, and a pipette was used to transfer it into a microcentrifugation tube (1.5 mL capacity). The centrifugation tube was washed with 0.5 mL of deionized water, and this rinse water was also added to the microcentrifugation tube. The final volume of the microcentrifugation tube was close to 1.0 mL.

The third centrifugation was also performed at $1,500 \times g$ for 15 minutes. Again, the supernatant was removed, leaving just 0.5 mL in the microcentrifugation tube. This residual volume was again placed in a vortex mixer and then left overnight in a refrigerator at a temperature ranging from 5 to 10 °C.

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 $1,500 \times g$ 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.

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® (oo) cysts were inoculated. After manual homogenization, the samples were filtered using a vacuum pump with a flow of 4 L min^{-1} and a pressure of −500 mm Hg.

After filtration, the membrane was removed from the mounted filter and transferred to a Petri dish containing 5 mL of the elution solution (Tween 80, 0.1% v/v). Scraping with a plastic stick (present in Merifluor® kit) was performed for 10 minutes. At intervals of approximately 3 minutes, the 5 mL volume that was applied was removed, and a rinse was twice performed with 5 mL of Tween 80

solution (0.1%) for a total of 15 mL, which was transferred to a centrifugation tube.

The first centrifugation was performed at $1,050 \times g$ 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 $1,050 \times g$ 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 $1,050 \times g$ 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 3 minutes. The volume was equally divided into four centrifugation tubes and centrifuged at $1,500 \times g$ 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 (oo)cysts, 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).
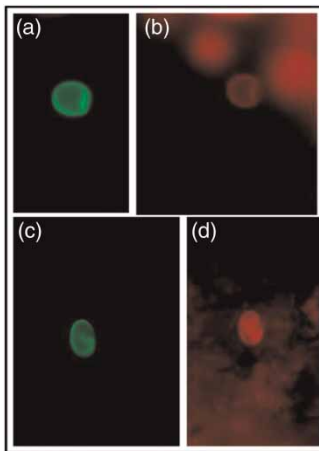

Figure 2 │ *Cryptosporidum* oocyst ((a) and (b)) and *Giardia* cyst ((c) and (d)) marked with Texas Red (ColorSeed®). Filter for fluorescein isothiocyanate (FITC) (450–490 nm) in (a) and (c); green block (510–550 nm) in (b) and (d).

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

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

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

Table 2 │ Statistical analysis for comparison of methods for (oo)cysts recovery in wastewater ($P < 0.05$ was considered statistically significant)

Trials	Subject analyzed	*Giardia* (*P*-value)	*Cryptosporidium* (*P*-value)
TC × TC	High × medium turbidity	0.0633	0.6202
TC × MF	Best method for medium turbidity	0.0141	0.2070
MF × C + IMS		0.0789	0.0984
TC × C + IMS		0.0041	0.0097
MF × MF	Medium × low turbidity	0.0033	0.2528
MF × MF + IMS	IMS step for low turbidity	0.0288	0.1662

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

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.

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 $79 \pm 32\%$ of the *Giardia* and $81 \pm 21\%$ of the *Cryptosporidium* from raw influent and recovered $81 \pm 27\%$ of the *Giardia* and $89 \pm 10\%$ 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 $23.5 \pm 13.8\%$ of the inoculated oocysts from the raw wastewater, $36.2 \pm 11.4\%$ from the primary effluent, and $30.4 \pm 17.5\%$ from the secondary effluent; values close to those found in this work.

Montemayor *et al.* (2005) followed the procedure outlined by Shepherd & Wyn-Jones (1995), with some modifications, to analyze *Cryptosporidium* in raw wastewater and secondary effluent. The samples underwent a coarse filtration (using a filter with a 30-μm pore size) to remove particles, followed by filtration with cellulose acetate filters with a 1.2-μm pore size, centrifugation ($1,500 \times g$, 10 minutes), and subsequent IMS. With this procedure, the recovery rate was $60 \pm 18\%$. When using tertiary effluent and applying the 1623 method outlined by USEPA (1999b), the recovery rate was $33.6 \pm 20.2\%$. Flocculation with calcium carbonate (Vesey *et al.* 1993) was also used for this effluent and led to an oocyst recovery rate of $40.5 \pm 24.5\%$.

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 $2,000 \times g$ for 10 minutes. Ottoson *et al.* (2006) recorded recovery rates in raw influent of $22 \pm 1.5\%$ for *Giardia* and $15 \pm 4.6\%$ for *Cryptosporidium*. In treated wastewater, the recovery was $25 \pm 12\%$ for *Giardia* and $39 \pm 13\%$ 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 $43.7 \pm 31.6\%$ for *Giardia* and of $13.6 \pm 12.8\%$ 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*.

ACKNOWLEDGEMENTS

REFERENCES

Assavasilavasukul, P., Lau, B. L. T., Harrington, G. W., Hoffman, R. M. & Borchardt, M. A. 2008 Effect of pathogen concentrations on removal of *Cryptosporidium* and *Giardia* by conventional drinking water treatment. *Water Res.* **42**, 2678–2690.

Baldursson, S. & Karanis, P. 2011 Waterborne transmission of protozoan parasites: review of worldwide outbreaks - an update 2004–2010. *Water Res.* **45**, 6603–6614.

Bonatti, T. R., Franco, R. M. B. & Cantusio Neto, R. 2007 Comparison of two methodologies for detection of *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts in activated sludge samples from a sewage treatment plant in the city of Campinas, São Paulo State, Brazil. *J. Water Health* **5** (4), 609–614.

Carey, C. M., Lee, H. & Trevors, J. T. 2004 Biology, persistence and detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocyst. *Water Res.* **38**, 818–862.

Franco, R. M. B., Rocha-Eberhardt, R. & Cantusio Neto, R. 2001 Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in raw water from the Atibaia River, Campinas, Brazil. *Rev. Inst. Med. Trop. S. Paulo* **43** (2), 109–111.

Franco, R. M. B., Hachich, E. M., Sato, M. I. Z., Naveira, R. M. L., Silva, E. C., Campos, M. M. C., Cantúsio Neto, R., Cerqueira, D. A., Branco, N. & Leal, D. A. G. 2012 Avaliação da performance de metodologias de detecção de *Cryptosporidium* spp. e *Giardia* spp. em água destinada ao consumo humano, para o atendimento às demandas de Vigilância em Saúde Ambiental no Brasil [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]. *Epidemiol. Serv. Saúde* **21** (2), 233–242.

Gross, R., Schell, B., Molina, M. C. B., Leão, M. A. C. & Strack, U. 1989 The impact of improvement of water supply and sanitation facilities on diarrhea and intestinal parasites: a Brazilian experience with children in two low-income urban communities. *Rev. Saúde Públ.* **23** (3), 214–220.

Hsu, B. & Yeh, H. 2003 Removal of *Giardia* and *Cryptosporidium* in drinking water treatment: a pilot-scale study. *Water Res.* **37**, 1111–1117.

Jex, A. R., Smith, H. V., Monis, P. T., Campbell, B. E. & Gasser, R. B. 2008 *Cryptosporidium* - biotechnological advances in the detection, diagnosis and analysis of genetic variation. *Biotechnol. Adv.* **26**, 304–317.

Karanis, P., Kourenti, C. & Smith, H. 2007 Waterborne transmission of protozoan parasites: a worldwide review of outbreak and lessons learnt. *J. Water Health* **5** (1), 1–38.

Ludwig, K. M., Frei, F., Filho, F. A. & Ribeiro-Paes, J. T. 1999 Correlação entre condições de saneamento básico e parasitoses intestinais na população de Assis, Estado de São Paulo [Correlation between sanitation conditions and enteroparasitoses in the population of Assis, São Paulo State]. *Rev. Soc. Bras. Med. Trop.* **32** (5), 547–555.

Matos, S. M. A., Assis, A. M. O., Prado, M. S., Strina, A., Santos, L. A., Jesus, S. R. & Barreto, M. L. 2008 *Giardia duodenalis* infection and anthropometric status in preschoolers in Salvador, Bahia State, Brazil. *Cad. Saúde Pública* **24** (7), 1527–1535.

McCuin, R. M. & Clancy, J. L. 2005 Methods for the recovery, isolation and detection of *Cryptosporidium* oocysts in wastewaters. *J. Microbiol. Methods* **63**, 73–88.

Montemayor, M., Valero, F., Jofre, J. & Lucena, F. 2005 Occurrence of *Cryptosporidium* spp. oocysts in raw and treated sewage and river water in north-eastern Spain. *J. Appl. Microbiol.* **99**, 1455–1462.

Nascimento, W. R. C., Cavalcanti, I. M. F., Irmão, J. I. & Rocha, F. J. S. 2009 Presença de *Cryptosporidium* spp em crianças com diarréia aguda em uma creche pública de Recife, Estado de Pernambuco [Presence of *Cryptosporidium* spp. in children with acute diarrhea in a public daycare center in Recife, State of Pernambuco] *Rev. Soc. Bras. Med. Trop.* **42** (2), 175–178.

Ongerth, J. E. 2013 The concentration of *Cryptosporidium* and *Giardia* in water - the role and importance of recovery efficiency. *Water Res.* **47**, 2479–2488.

Oshiro, E. T., Dorval, M. E. C., Nunes, V. L. B., Silva, M. A. A. & Said, L. A. M. 2000 Prevalência do *Cryptosporidium parvum* em crianças abaixo de 5 anos, residentes na zona urbana de Campo Grande, MS, Brasil, 1996 [Prevalence of *Cryptosporidium parvum* among children of less than 5 years of age in the urban zone of Campo Grande, Mato Grosso do Sul State, Brazil, 1996]. *Rev. Soc. Bras. de Med. Trop.* **33** (3), 277–280.

Ottoson, J., Hansen, A., Bjorlenius, B., Norder, H. & Stenstrom, T. A. 2006 Removal of viruses, parasitic protozoa and microbial indicators in conventional and membrane processes in a wastewater pilot plant. *Water Res.* **40**, 1449–1457.

Pereira, M. G. C., Atwill, E. R., Barbosa, A. P., Silva, S. A. & García-Zapata, M. T. A. 2002 Intra-familial and extra-familial risk factors associated with *Cryptosporidium parvum* infection among children hospitalized for diarrhea in Goiânia, Goiás, Brazil. *Am. J. Trop. Med. Hyg.* **66** (6), 787–793.

Plutzer, J., Ongerth, J. & Karanis, P. 2010 *Giardia* taxonomy, phylogeny and epidemiology: facts and open questions. *Int. J. Hyg. Environ. Health* **213**, 321–333.

Risebro, H. L., Doria, M. F., Andersson, Y., Medema, G., Osborn, K., Schlosser, O. & Hunter, P. R. 2007 Fault tree analysis of the causes of waterborne outbreaks. *J. Water Health* **5** (Suppl.), 1–18.

Robertson, L. J., Paton, C. A., Campbell, A. T., Smith, P. G., Jackson, M. H., Gilmour, R. A., Black, S. E., Stevenson, D. A. & Smith, H. V. 2000 *Giardia* cysts and *Cryptosporidium* oocysts at sewage treatment works in Scotland, UK. *Water Res.* **34** (8), 2310–2322.

Shepherd, K. M. & Wyn-Jones, A. P. 1995 Evaluation of different filtration techniques for the concentration of *Cryptosporidium* oocysts from water. *Water Sci. Technol.* **31** (5–6), 425–429.

Teixeira, J. C., Heller, L. & Barreto, M. L. 2007 *Giardia duodenalis* infection: risk factors for children living in sub-standard settlements in Brazil. *Cad. Saúde Pública.* **23** (6), 1489–1493.

Uchôa, C. M. A., Lobo, A. G. B., Bastos, O. M. P. & Matos, A. D. 2001 Parasitoses intestinais: prevalência em creches comunitárias da cidade de Niterói, Rio de Janeiro – Brasil [Intestinal parasitism: prevalence in day-care centers of Niteroi city, Rio de Janeiro – Brazil]. *Rev. Inst. Adolfo Lutz* **60** (2), 97–101.

USEPA – US Environmental Protection Agency 1999a Method 1622: *Cryptosporidium* in Water by Filtration/IMS/FA. Office of Water. EPA 821-R-99-061.

USEPA – US Environmental Protection Agency 1999b Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. Office of Water. EPA 821-R-99-006.

USEPA – US Environmental Protection Agency 2005 Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. Office of Water. EPA 815-R-05-002.

USEPA – US Environmental Protection Agency 2012 Method 1623.1: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. Office of Water. EPA 815-R-12-001.

Vesey, G., Slade, J. S., Byrne, M., Shepherd, K. & Fricker, C. R. 1993 A new method for the concentration of *Cryptosporidium* oocysts from water. *J. Appl. Bacteriol.* **75**, 82–86.