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

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ABSTRACT

Giardia spp. and *Cryptosporidium* spp. are recognized worldwide as highly infectious protozoan parasites that can cause severe gastrointestinal disease in humans and animals. The detection of these pathogens in activated sludge samples becomes interesting since there is an increasing trend for the use of sewage sludge (biosolids) in agriculture. A total of 22 samples were collected and evaluated by means of Centrifugal – Concentration, followed or not followed by a purification process (ether clarification and sucrose flotation). Student t tests for comparison of the two procedures indicated a higher recovery rate of *Giardia* cysts with Centrifugal – Concentration; with regard to *Cryptosporidium* oocysts, no significant differences were found between the two methods, as only two samples were positive. The Centrifugal – Concentration procedure was shown to be the simplest and cheapest to perform, as emphasized by the efficiency recovery results.

Key words | Centrifugal – Concentration, *Cryptosporidium*, *Giardia*, recovery, sewage sludge

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INTRODUCTION

Giardia spp. and *Cryptosporidium* spp. are recognized worldwide as highly infectious protozoan parasites that can cause severe gastrointestinal disease in humans and animals (Armon *et al.* 2002; Rose 2002). In recent years, the presence of these parasites has been reported usually in superficial waters and sewage samples (Armon *et al.* 2002), in many countries.

Sewage sludge is a by-product of activated sludge treatment; it contains an elevated mixture of organic and inorganic compounds and, because of this, a growing trend for the application of this treated sewage residue in agricultural land zones has emerged. During wastewater treatment by this process, cysts and oocysts are sedimented in the sludge. Meanwhile, the presence of these protozoa has implications concerning sewage sludge reuse (Armon *et al.* 2002). Consequently, it is important to quantify the

presence of (oo)cysts associated with the practice of sludge application in agriculture and more accurate detection and quantification methods are necessary.

This study was designed to assess the occurrence of *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts in activated sludge samples and to compare two methods of (oo)cysts concentration: Centrifugal – Concentration procedure (CC) and Centrifugal – Concentration followed by a purification and clarification technique (CC/PC).

MATERIALS AND METHODS

Sampling site

The city of Campinas is located in the southeastern region of Brazil ($22°53'20''$/W $47°04'40''$), with a population of

about 1 million inhabitants (IBGE 2000). The Samambaia Sewage Treatment Plant, situated in a suburb of the eastern region of the city, serves a population of 40,000 (SANASA 2006) and receives only domestic inputs with an average outflow of 98 L/s.

Sample collection

Activated sludge samples ($n=22$) were collected every fifteen days (January to November 2003) in plastic PVC bottles, previously decontaminated and washed with 0.1% Tween-80 elution solution. All the samples were immediately sent to the laboratory for analysis.

Sample concentration and purification

Each sample was vigorously homogenized and filtered through a plastic sieve with $1.0\,mm^2$ pore size. Afterwards, it was divided into two aliquots for parasitological analysis, using two different concentration methods (described below), adapted from Robertson *et al.* (2000). The modifications included the initial sample volume effectively submitted to centrifugation (5 ml in this study), initial sieving performed with a plastic mesh instead of muslin and the introduction of vortex mixing steps during sample processing and previously to aliquot removal for immunofluorescence assay (IFA).

Centrifugal – Concentration (CC)

The 5 ml aliquot was submitted to continuous centrifugation after dilution with 10 ml of elution solution (1500 × g; 15 min) during the deceleration phase. The supernatant was discharged and a pellet of 1 ml was left at the bottom of the tube and an aliquot of the resulting sediment (after vortex mixing during 2 minutes; Phoenix® Model AP 56) was analyzed by IFA.

Centrifugal – Concentration followed by purification and clarification (CC/PC)

The second one was also obtained through the initial process of centrifugation with elution solution. After supernatant discharge, the resulting sediment was successively submitted to the sucrose solution purification step

and ethylic ether clarification. The tube was brought to volume (15 ml) with sucrose-saturated solution (specific gravity = 1.2), vortex homogenized (2 min) and centrifuged (1,500 × g, 15 min). The supernatant was collected, placed in clean tubes and washed three consecutive times with distilled water (dH$_2$O) (1,050 × g, 10 min). Finally, the supernatant was discharged, the pellet resuspended to 1 ml in dH$_2$O and later analyzed. The residual sediment that remained at the tube bottom (after superficial layer collection) was defatted by adding 5 ml of refrigerated ethylic ether. After vigorous manual shaking (30 sec), the tube content was brought to volume with dH$_2$O, homogenized by vortex mixing and centrifuged again (1,050 × g, 10 min). The supernatant, including fat and ethylic ether were aspirated out and the resulting sediment was resuspended to 1 ml with dH$_2$O and analyzed as described below.

Staining for immunofluorescence

Both methodologies examined 5 µl aliquots of the final sediment, removed immediately after sample vortex mixing (two minutes) and inversion of the microcentrifuge tube three times (for sample homogenization purposes), using the Merifluor kit (Meridian Bioscience Diagnostics, Cincinnatti, Ohio) in accordance with the manufacturer's instructions. The (oo)cysts considered were those that fluoresced bright apple green when viewed under light with a wavelength of 450–490 nm and that presented the characteristic morphological features.

Positive control trials

Positive control trials were carried out to evaluate the sensitivity of the methodology. *Giardia* spp. and *Cryptosporidium* spp. suspensions provided by the Merifluor® kit as positive staining controls were pooled for use in spiking controls. Therefore, an estimated number of *Giardia* cysts (magnitude order: 10^2) and *Cryptosporidium* oocysts (magnitude order: 10^3) determined by the well slide counting technique were seeded in the activated sludge samples (previously determined negative by the IFA assay). The samples were allowed to stand for two hours and then analyzed by the same procedures described above.

Data analysis

In order to estimate the number of (oo)cysts per liter (X), the following equation was used:

$$X = \frac{\alpha \times \beta}{\gamma \times \delta} \tag{1}$$

where α is the number of (oo)cysts multiplied by 10^6, β is the total sediment volume (ml), γ is the sediment volume in the well (μl) and δ is the sample volume (ml).

In the case of the CC/PC method, the number of (oo)cysts visualized in the sample corresponded to the total of separately visualized organisms in the steps of sucrose flotation and ether clarification. For comparison between different procedures (CC and CC/PC) and different periods (January to June and July to November), Student t tests were carried out using the Excel package ($H_0 : \mu_{cc} \geq \mu_{cc/pc}$; $H_a : \mu_{cc} < \mu_{cc/pc}$ and $H_0 : \mu_{cc} > \mu_{cc/pc}$; $H_a : \mu_{cc} < \mu_{cc/pc}$, respectively) (Zar 1999). A p-value of < 0.05 was considered significant.

Levels of (oo)cyst recovery efficiency (Y) were calculated in the following way:

$$Y = \frac{\alpha \times 100}{\beta} \tag{2}$$

where α is the number of recovered (oo)cysts and β, the total number of (oo)cysts inoculated in the sample (5 ml).

RESULTS AND DISCUSSION

Data on sludge microbiological quality are scarce in developing countries (Jiménez et al. 2002) due to the lack of a standardized methodology for the recovery of these protozoa in sewage (Clancy & McCuin 2003). The techniques which have been traditionally developed and optimized for enumeration of *Giardia* spp. and *Cryptosporidium* spp. in water are generally not suitable for (oo)cysts enumeration in sewage; the great difficulty is related to the large concentration of particulate material present in sewage (McCuin & Clancy 2005). Thus, the critical point is the adequate separation and recovery of these parasites from the matrix rich in particles.

When CC was used, all the samples (100%, $n = 22$) were positive for *Giardia* spp. cysts, while in relation to

CC/PC, the positive rate was 95.4% (21 samples). The presence of *Cryptosporidium* spp. oocysts was registered in two samples (9.0%) using CC technique and no oocysts were detected when the CC/PC protocol was used.

The average values of (oo)cysts per liter determined in this study (Tables 1 and 2) corroborate those presented by Santos et al. (2004), who had also compared two other methodologies: ether clarification (*Giardia*: 8.0×10^4 cysts/L to 1.3×10^6 cysts/L; *Cryptosporidium*: 0 to 4×10^4 oocysts/L) and sucrose flotation (*Giardia*.: 8×10^4 cysts/L to 1.2×10^6 cysts/L; *Cryptosporidium*: of 0 to 8×10^4 oocysts/L) separately. In this study, the combined procedure (CC/PC) included the ether clarification and sucrose flotation steps. Other studies in Brazil which have examined raw and treated sewage samples

Table 1 | Number of (oo)cysts per liter in activated sludge samples, collected at Samambaia STP, Campinas, Brazil Southeastern Region, determined by Centrifugal – Concentration (CC) and Centrifugal – Concentration followed by purification and clarification (CC/PC), for the first period (January to June)

Technique	CC		CC/PC	
Sample	*Cryptosporidium* spp.	*Giardia* spp.	*Cryptosporidium* spp.	*Giardia* spp.
1	0	1.6×10^5	0	1.2×10^5
2	0	4.0×10^4	0	0
3	0	2.0×10^5	0	8.0×10^4
4	0	2.0×10^5	0	1.6×10^5
5	0	8.0×10^4	0	4.0×10^4
6	0	3.2×10^5	0	8.0×10^4
7	0	3.6×10^5	0	1.6×10^5
8	0	1.6×10^5	0	1.6×10^5
9	4.0×10^4	2.4×10^5	0	2.4×10^5
10	0	2.4×10^5	0	2.4×10^5
11	0	1.2×10^5	0	1.6×10^5
Mean	3.6×10^3	1.9×10^5	0	1.3×10^5
SD*	1.1×10^3	9.1×10^4	0	7.2×10^4

*SD: Standard deviation.

Table 2 | Number of (oo)cysts per liter in activated sludge samples, collected at Samambaia STP, Campinas, Brazil Southeastern Region, determined by Centrifugal – Concentration (CC) and Centrifugal – Concentration followed by purification and clarification (CC/PC), for the second period (July to November)

Technique	CC		CC/PC	
Sample	*Cryptosporidium* spp.	*Giardia* spp.	*Cryptosporidium* spp.	*Giardia* spp.
12	0	3.6×10^5	0	1.6×10^5
13	4.0×10^4	3.6×10^5	0	4.4×10^5
14	0	4.0×10^5	0	4.0×10^4
15	0	4.4×10^5	0	2.4×10^5
16	0	2.0×10^5	0	2.8×10^5
17	0	2.4×10^5	0	4.8×10^5
18	0	4.4×10^5	0	2.8×10^5
19	0	3.2×10^5	0	2.4×10^5
20	0	2.0×10^5	0	2.4×10^5
21	0	2.0×10^5	0	2.4×10^5
22	0	2.4×10^5	0	8.0×10^4
Mean	3.6×10^3	3.0×10^5	0	2.4×10^5
SD*	1.1×10^3	9.6×10^4	0	1.3×10^5

*SD: Standard deviation.

revealed a higher density variation of these organisms per liter: Dias Júnior (1999) observed 2,222 cysts/L and 472 oocysts/L in effluent samples and Farias *et al.* (2002) reported 80 to 912 oocysts/L in raw sewage.

Considering the (oo)cyst recovery efficiency rate achieved by the combined procedure (CC/PC), in the first period of this study (Table 1) a modification was introduced with the aim of improving the method: all the supernatant volume was collected in the flotation stage in sucrose saturated solution, instead of only the 3 ml on the surface. Thus, the results were analyzed separately for the first (January to June) and the second periods (July to November) and the Student t test was used to evaluate whether such a change meant better (oo)cyst recovery.

The number of (oo)cysts/L and the average value are shown in Table 1, for the first period (*Giardia* spp. range: 0–3.6×10^5 cysts/L; *Cryptosporidium* spp. range: 0–4.0×10^4 oocysts/L) and in Table 2, for the second period (*Giardia* spp. range: 4.0×10^4–4.8×10^5 cysts/L; *Cryptosporidium* spp. range: 0–4.0×10^4 oocysts/L), considering both procedures together.

The average value for the number of *Giardia* spp. cysts recovered by CC was higher when compared to the CC/PC, in the first period (for $t_{0,05(1),20} = 1.725$) (Zar 1999). In relation to the detection of *Cryptosporidium* spp. oocysts, there was no significant difference between CC and CC/PC because only one sample was positive (CC).

The average value of the number of cysts recovered by CC was greater than those recovered by CC/PC, in the second period (for $t_{0.05(1),20} = 1,725$) (Zar 1999). There was no significant difference between CC and CC/PC, with regard to the detection of oocysts, because only one sample was positive (CC).

The average *Giardia* spp. number of cysts recovered by CC/PC in the second period was greater when compared to the first one and this difference was significant (for $t_{0.05(1),22} = 1.717$) (Zar 1999). The recovery efficiency levels for each period are shown in Table 3.

The high concentration of cysts found in activated sewage samples can be explained by the fact that giardiasis is considered one of the main intestinal parasitic diseases of man, particularly in developing countries (Thompson 2000). It is endemic in Brazil: prevalence rates range from between 13.8 and 63.3% for children (Rocha *et al.* 1999). Another reason for the high concentrations found, could be the fact that in Campinas only 37% of the sewage was treated and discharged into the water bodies, contributing to source contamination (SANASA 2006). With respect to *Cryptosporidium* spp., the lesser positive rate found in this study could be related to the marked seasonality shown by cryptosporidiosis in Brazil associated with great rainfall periods (Rose *et al.* 2002). Other problems which could have affected recovery efficiency are oocyst adsorption onto particulate matter by means of interactions such as hydrophobicity, surface charges characteristic of the particle and the oocyst, ionic strength and matrix pH (Chesnot & Schwartzbrod 2004).

Table 3 | Recovery efficiency means following positive control trials with *Giardia* spp. and *Cryptosporidium* spp., for both periods

Period	Method	Protozoan	Mean recovery (%)	SD[c]	RSD[d]
I	CC[a]	*Giardia*	5.55	0.77	13.87
		Cryptosporidium	1.25	1.7	136
	CC/PC[b]	*Giardia*	0	0	0
		Cryptosporidium	0	0	0
II	CC	*Giardia*	43.7	31.6	72.31
		Cryptosporidium	13.6	12.8	94.11
	CC/PC	*Giardia*	19.7	17.6	89.34
		Cryptosporidium	6.3	10.5	166.66

[a]CC: Centrifugal – Concentration.
[b]CC/PC: Centrifugal – Concentration followed by purification and clarification.
[c]SD: Standard deviation.
[d]RSD: Relative standard deviation.

In control trials, most studies involve the addition of (oo)cysts to samples in quantities which are several orders of magnitude higher than background levels (10^4 and to 10^5) (Massanet-Nicolau 2003). In this study, the purpose was to assess the recovery procedure potentials with inoculation of these resistance forms in the order of 10^2 for cysts and 10^3 for oocysts, a more realistic condition. Thus, the goal of this study was to minimize organism losses using minimal manipulation in the first procedure (Centrifugal – Concentration) and to maximize impurity removal with the combination of two stages of purification (sucrose flotation and ether clarification) in the same procedure; therefore, we obtained lower percentages of recovery with the sucrose flotation step due to the higher viscosity characteristic of this solution which contributes to the loss of (oo)cysts.

Chesnot & Schwartzbrod (2004) conducted research with ether and found that increased centrifugation and washing steps could jeopardize recovery. In the case of CC/PC, the results obtained in the first period suggested that supernatant aspiration, during the sucrose flotation stage, constituted a critical step. The modified procedure resulted in greater recovery efficiency, with regard to *Giardia* cysts, which was noted in the higher average number of cysts attained in the second period (Table 2); there was no difference for oocysts due to the low number of positive samples. The recovery efficiency means obtained showed that the introduction of modifications in the sucrose flotation protocol brought about improvement for the second period (Table 3).

The simplicity of the proposed procedure (Centrifugal – Concentration) and the recovery efficiency obtained suggest that this method is preferable due to its low cost and manipulation level. Methods such as IMS (immunomagnetic separation) have been proposed for sewage samples (Iacovsky et al. 2004), but it is too expensive for routine work in Latin America. It would be interesting to carry out further studies to develop methodologies which are not so costly and could be applied in routine laboratory procedures.

CONCLUSIONS

- The great amount of particulate matter makes it difficult to standardize a technique for *Giardia* cysts and *Cryptosporidium* oocysts recovery from sewage samples.
- The proposed technique of Centrifugal – Concentration was shown to be an interesting alternative for its simplicity, low manipulation level and acceptable recovery efficiency.
- Future works must focus on the possibility of developing a technique which allows adequate separation of the particles from the matrix, the critical point for sewage samples. A possibly helpful step would be to change the elution solution by the addition of TRIS, a salt which might facilitate adequate separation of *Cryptosporidium* oocysts from the matrix.

ACKNOWLEDGEMENTS

REFERENCES

Armon, R., Gold, D., Brodsky, M. & Oron, G. 2002 Surface and subsurface irrigation with effluents of different qualities and presence of *Cryptosporidium* oocysts in soil and on crops. *Wat. Sci. Tech.* **46**(3), 115–122.

Chesnot, T. & Schwartzbrod, J. 2004 Quantitative and qualitative comparison of density-based purification methods for detection of *Cryptosporidium* oocysts in turbid environmental matrices. *J. Microbiol. Methods* **58**, 375–386.

Clancy, J. L. & McCuin, R. M. 2003 Occurrence of *Cryptosporidium parvum* in wastewaters in the U.S. Water Quality Technology Conference, Philadelfia, USA.

Dias Júnior, O. 1999 *Occurrence of Giardia sp Cysts and Cryptosporidium sp Oocysts in Superficial Waters and Sewage from Araras City – SP* (Ocorrência de cistos de Giardia sp e oocistos de *Cryptosporidium* sp em águas superficiais e esgoto no município de Araras – S.P). PhD Thesis, Mackenzie University.

Farias, E. W. C., Gamba, R. C. & Pellizari, V. H. 2002 Detection of *Cryptosporidium* spp. in raw and creek water in the city of São Paulo. *Brazil. Braz. J. Microbiol.* **33**, 41–43.

Iacovsky, R. B., Barardi, C. R. M. & Simões, C. M. O. 2004 Detection and enumeration of *Cryptosporidium* sp. oocysts in sewage sludge samples from the city of Florianópolis (Brazil) by using immunomagnetic separation combined with indirect immunofluorescence assay. *Waste Manage. Res.* **22**, 171–176.

IBGE – Brazilian Institute for Geography and Statistics (Instituto Brasileiro de Geografia e Estatística) 2000 *National Research of Basic Sanitation*. www.ibge.gov.br/estatistica/populacao/condicadevida/pnsb/default.shtm (Accessed in January, 2006).

Jiménez, B., Maya, C., Sánchez, E., Romero, A., Lira, L. & Barrios, J. A. 2002 Comparison of the quantity and quality of the microbiological content of sludge in countries with low and high content of pathogens. *Wat. Sci. Tech.* **46**(10), 17–24.

Massanet-Nicolau, J. 2003 New method using sedimentation and immunomagnetic separation for isolation and enumerations of *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts. *Appl. Environ. Microbiol.* **69**(11), 6758–6761.

McCuin, R. M. & Clancy, J. L. 2005 Methods for the recovery, isolation and detection of *Cryptosporidium* oocysts in wastewaters. *J. Microbiol. Methods* **63**(1), 73–88.

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. *Wat. Res.* **34**(8), 2310–2322.

Rocha, M. O., Mello, R. T., Guimarães, T. M. P. D., Toledo, V. P. C. P., Moreira, M. C. C. G. & Costa, C. A. 1999 Detection of a *Giardia lamblia* coproantigen by using a commercially available immunoenzimatic assay, in Belo Horizonte, Brazil. *Rev. Inst. Med. Trop. S. Paulo* **41**(3), 151–154.

Rose, J. B., Huffman, D. E. & Gennaccaro, A. 2002 Risk and control of waterborne cryptosporidiosis. *FEMS Microbiol. Rev.* **26**, 113–123.

SANASA – Sanitation and Water Supplying Society (Sociedade de Abastecimento de Água e Saneamento S/A) (www.sanasa.com.br). Accessed in February, 2006.

Santos, L. U., Bonatti, T. R., Cantusio Neto, R. & Franco, R. M. B. 2004 Occurrence of *Giardia* Cysts and *Cryptosporidium* oocysts in activated sludge samples in Campinas, SP, Brazil. *Rev. Inst. Med. Trop. S. Paulo* **46**(6), 309–313.

Thompson, R. C. A. 2000 Giardiasis as a re-emerging infectious disease and its zoonotic potential. *Int. J. Parasitol.* **30**, 1250–1267.

Zar, J. H. 1999 *Biostatistical Analysis*, 4th edition. Prentice Hall, New Jersey, USA.

Available online May 2007