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OCCURRENCE OF *Giardia* CYSTS AND *Cryptosporidium* OOCYSTS IN ACTIVATED SLUDGE SAMPLES IN CAMPINAS, SP, BRAZIL

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SUMMARY

Giardia and Cryptosporidium have caused several outbreaks of gastroenteritis in humans associated with drinking water. Contaminated sewage effluents are recognized as a potential source of waterborne protozoa. Due to the lack of studies about the occurrence of these parasites in sewage samples in Brazil, we compared the efficiency of two procedures for concentrating cysts and oocysts in activated sludge samples of one sewage treatment plant. For this, the samples were submitted to *i*) concentration by the ether clarification procedure (ECP) and to *ii)* purification by sucrose flotation method (SFM) and aliquots of the pellets were examined by immunofluorescence. *Giardia* cysts were present in all samples (100.0%; n = 8) when using ECP and kit 1 reagents, while kit 2 resulted in six positive samples (85.7%; n = 7). As for SFM, cysts were detected in 75.0% and 100.0% of these samples (for kit 1 and 2, respectively). Regarding *Cryptosporidium*, two samples (25.0%; kit 1 and 28.5% for kit 2) were detected positive by using ECP, while for SFM, only one sample (examined by kit 1) was positive (12.5%). The results of the control trial revealed *Giardia* and *Cryptosporidium* recovery efficiency rates for ECP of 54.5% and 9.6%, while SFM was 10.5% and 3.2%, respectively. Considering the high concentration detected, a previous evaluation of the activated sludge before its application in agriculture is recommended and with some improvement, ECP would be an appropriate simple technique for protozoa detection in sewage samples.

KEYWORDS: Activated sludge; *Cryptosporidium*; *Giardia*; Ether clarification procedure; Sucrose flotation method.

INTRODUCTION

Human excrement can be a vehicle of various diseases and high concentrations of pathogens are often reported in raw sewage worldwide[8].

Giardia and *Cryptosporidium* are protozoa that produce environmental stages (cysts and oocysts, respectively) that are eliminated in the feces of the host. These parasites are a common cause of acute gastroenteritis in humans and animals[19,22], with human infection being usually acquired by direct contact between persons and by ingestion of contaminated food or water[11,30].

In the first documented waterborne outbreak of human cryptosporidiosis in the USA[6], sewage was implicated as a possible source of oocyst contamination. Another important case to be considered is the 1993 Milwaukee outbreak, which was assumed to have been caused by run-off from upstream cattle pastures but now human sewage has been recognized as the source of contamination[25].

In Brazil, only 20% of municipalities collect and treat sewage before discharging it in the environment[16]. A very frequently used procedure in secondary sewage treatment is the activated sludge system, in which aerobic microorganisms degrade the organic matter present in raw sewage and the flocculated material is easily removed during the sedimentation stage[13]. Therefore, the activated sludge is the main by-product of sewage treatment and its final disposal has been a matter of discussion in many countries[5,17,20].

Data about the occurrence and concentration of cysts and oocysts in sewage in Brazil are scarce. One of the main reasons for this may be attributed to lack of adequate methodology for concentration of these parasites in sewage samples. Recent studies included microscopic evaluation without immunofluorescence assay (IFA)[23] in sewage sludge, and the detection of cryptosporidial oocysts in raw sewage in the cities of Araras and São Paulo (state of São Paulo)[7,10,21] by calcium carbonate flocculation and membrane filter dissolution methods, both with visualization of protozoan parasites by IFA.

Since the presence of these protozoa was registered in superficial raw water of the Atibaia River, the major water source in Campinas[12], and that this river receives discharge of urban sewage[1,18] by means of

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the Pinheiros Stream, the detection of *Giardia* and *Cryptosporidium* in wastewater provides important clues for information on the environmental epidemiology of these parasites in this region. Thus, in order to determine the occurrence of *Giardia* and *Cryptosporidium* in wastewater samples, we compared the efficiency of two procedures for concentrating cysts and oocysts in activated sludge samples.

MATERIAL AND METHODS

The activated sludge samples were collected in sterilized plastic containers from the Santa Rosa sewage treatment plant (E.T.E. Santa Rosa), with a 7.79 L/minutes capacity, in Campinas. The present work was carried out in eight subsequent weeks.

These samples (n = 8) were filtered through a 1 mm^2 plastic sieve to remove large debris and the filtrate was split into two aliquots, further concentrated by the ether clarification procedure (ECP) and sucrose flotation method (SFM), according to ROBERTSON *et al.* (2000)[26], with modifications. Briefly, for ECP, aliquots of filtrate were diluted (1:3) in 1% Tween 80 solution and the pellets were retrieved by double centrifugation (15 min; 1500 x *g*) and further concentrated by using ether centrifugal sedimentation (ECP) (10 min; 1500 x *g*), after previous hand agitation (30 sec). For SFM, after double centrifugation, saturated sucrose solution (s.g. = 1.20) was added to the pellets and once more centrifuged during 15 min (1500 x *g*). The superficial layer (3 mL) was collected and transferred to a clean tube. This procedure was repeated once again. The final washed concentrate samples were resuspended in 1.0 mL with distilled water and duplicate 5 µL aliquots were examined using two different fluorescent monoclonal antibody tests (kit 1: *Merifluor kit, Meridian Bioscience, Cincinnatti, Ohio* and kit 2: *Crypto/Giardia-Cel I. F. Test, CELLABS PTY LTD, Australia*), according to the manufacturer's instructions, with the purpose of enumerating cysts and oocysts in the analyzed samples. Simultaneously, a confirmatory test was performed by inclusion of fluorogenic vital dye DAPI (4', 6'-diamidino-2-phenylindole, Sigma Chemicals) for visualization of morphologic characters (nucleus, axonemes or suture). These preparations were observed through a Zeiss Axiolab epifluorescence microscope, with excitation and barrier filters equipped with appropriate filter blocks for FITC and DAPI viewing (400 x).

A control trial was performed to evaluate recovery efficiency of the methods employed. For this, samples of effluent sewage previously determined negative by ECP and SFM methods were seeded with estimated numbers of *Giardia* cysts (4.4 x 10^3) and *Cryptosporidium* oocysts (5.4 x 10^4), using the well-slide counting technique[9]. These samples were analyzed by the same procedures described above. The detected cysts and oocysts in all samples are in conformity with standard fluorescence detection criteria[15].

The estimate of the number of cysts and oocysts/L (x) was calculated by the following formula[3,9]:

$$x = \left[\frac{\text{nº of (oo)cysts x } 10^6}{\text{vol. of sample in well}} \times \frac{\text{vol. of pellet (1 mL)}}{\text{vol. of sample (mL)}} \right]$$

Statistical analyses were carried out using the SAS (Statistical Analysis System) software. ANOVA was used to make comparisons among variables, using the GLM (general linear models) procedure[29].

RESULTS

When using ECP, *Giardia* cysts were present in all activated sludge samples (n = 8; 100.0%) from ETE Santa Rosa, which were examined with kit 1 reagents (Table 1), while kit 2 resulted in six positive samples (85.7%; n = 7). In turn, when using SFM, G*iardia* cysts were detected in 75.0% (n = 8) and 100.0% (n = 7) of these samples (for kit 1 and 2, respectively). Regarding cryptosporidial oocysts, two samples (25.0% kit 1; and kit 2: 28.5%) were detected positive by using ECP, while for SFM, only one sample (examined by kit 1) was positive (12.5%) (Table 2).

Analysis of variance (p = 0.05) showed a significant difference between the methodologies for *Giardia* cyst recovery, with ECP being the most efficient. For *Cryptosporidium* detection, no significant differences were found between the procedures. Moreover, for both protozoa, there was no significant difference between the monoclonal antibody kits (Table 1).

A higher number of *Giardia* cysts/L was observed in samples stained with kit 1 (4.0 x 10^4 to 1.2 x 10^6) when compared to samples stained with kit 2 reagents (4.0 x 10^4 to 4.4 x 10^5) (Table 1). For *Cryptosporidium*, a higher number of oocysts/L were observed with kit 1 (4.4 x 10^4 to 8.0 x 10^4) than with kit 2 (4.0 x 10^4) (Table 2).

Higher recovery efficiency was obtained in control trials using ECP (*Giardia* and *Cryptosporidium* recovery efficiency rates of 54.5% and 9.6%, respectively) than by SFM (10.5% for *Giardia* and 3.2% for *Cryptosporidium*).

Both cysts and oocysts in positive samples showed apple-green fluorescence, although higher intensity was observed against the

Table 1
Number of *Giardia* cysts/L detected in activated sludge samples from Santa Rosa Sewage Treatment Plant (Campinas, Brazil) using two commercially available monoclonal antibodies* and two techniques (ECP and SFM) for enumeration and visualization of protozoan parasites

	Technique			
	ECP		SFM	
Sample	Kit 1	Kit 2	Kit 1	Kit 2
I	$2.0x10^5$	-	0	-
II	$8.0x10^4$	0	$1.6x10^5$	$8.0x10^4$
III	$3.6x10^5$	$1.6x10^5$	$8.0x10^4$	$1.6x10^5$
IV	$2.8x10^5$	$8.0x10^4$	$1.2x10^5$	$2.0x10^5$
V	$1.2x10^6$	$3.6x10^5$	$8.0x10^4$	$2.4x10^5$
VI	$8.4x10^5$	$3.2x10^5$	$6.0x10^5$	$4.0x10^4$
VII	$8.8x10^5$	$3.2x10^5$	0	$1.2\ x10^5$
VIII	$8.0x10^5$	$4.4x10^5$	$4.0x10^4$	$4.0x10^4$
Mean	$5.8x10^5$	$2.4x10^5$	$1.35x10^5$	$1.25x10^5$
SD	$4.0x10^5$	$1.6x10^5$	$1.95x10^5$	$7.8x10^4$

* Kit 1: *Merifluor kit, Meridian Bioscience, Cincinnatti, Ohio;* Kit 2: *Crypto/Giardia-Cel I.F. Test, CELLABS PTY LTD, Australia;* (ECP): concentration by the ether clarification procedure; (SFM): purification by sucrose flotation method; 0: absence; -: not determined.

Table 2
Number of *Cryptosporidium* oocysts/L detected in activated sludge samples from Santa Rosa Sewage Treatment Plant (Campinas, Brazil) using two commercially available monoclonal antibodies* and two techniques (ECP and SFM) for enumeration and visualization of protozoan parasites

	Technique			
	ECP		SFM	
Sample	Kit 1	Kit 2	Kit 1	Kit 2
I	$4.0x10^4$	-	0	-
II	$4.0x10^4$	0	0	0
III	0	$4.0x10^4$	0	0
IV	0	0	0	0
V	0	$4.0x10^4$	0	0
VI	0	0	0	0
VII	0	0	$8.0x10^4$	0
VIII	0	0	0	0
Mean	$1.0x10^4$	$1.14x10^4$	$1.0x10^4$	0
SD	$1.8x10^4$	$1.95x10^4$	$2.8x10^4$	0

* Kit 1: *Merifluor kit, Meridian Bioscience, Cincinnatti, Ohio;* Kit 2: *Crypto/Giardia-Cel I.F. Test, CELLABS PTY LTD, Australia;* (ECP): concentration by the ether clarification procedure; (SFM): purification by sucrose flotation method; 0: absence; -: not determined.

background and other interfering material for oocysts when using kit 2 reagents, making their identification very easy when compared with the counterpart sample processed by kit 1; in contrast, cysts showed brighter green fluorescence when kit 1 staining was performed (Fig. 1a; 1b).

DISCUSSION

Environmental pollution is becoming a global matter nowadays and issues like water contamination, lack of safe water or disposal of residues are problems that can lead to serious public health consequences. In Latin America, subterraneous and superficial water contamination due to deficiency of wastewater treatment systems is escalating[14]. In Campinas, only 14% of domestic sewage is treated[27].

Due to the sample type examined in this study, which contains a great number of particulate materials, and because methodologies using filtration can be inadequate for these samples (due to rapid filter blockage)[10,26], we evaluated the applicability of two methods (ECP and SFM) for protozoa detection in activated sludge samples and a higher recovery was obtained when using ECP instead of SFM, which was also confirmed in control trials. Our results corroborate those from ROBERTSON *et al*.[26], who concluded that ether clarification for detection of *Giardia* cysts yields consistently higher recoveries than sucrose flotation when the raw sewage samples were examined. We can infer that major drawbacks of SFM derive from cyst and oocyst losses during the sucrose purification phase.

Both parasites were found in this study but *Giardia* prevailed with a higher number of positive samples than *Cryptosporidium,* and at higher concentrations; these data agree with results reported by BUKHARI *et al*.[2] and CARRARO *et al*.[4].

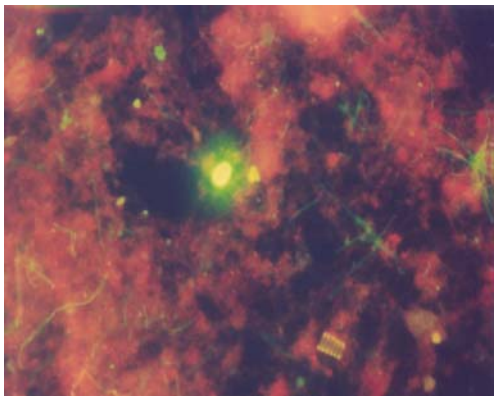

Fig. 1 - *Giardia* cyst, stained with kit 1 reagents, present in activated sludge samples of Santa Rosa ETE, Campinas, Southeast Brazil. 500x.

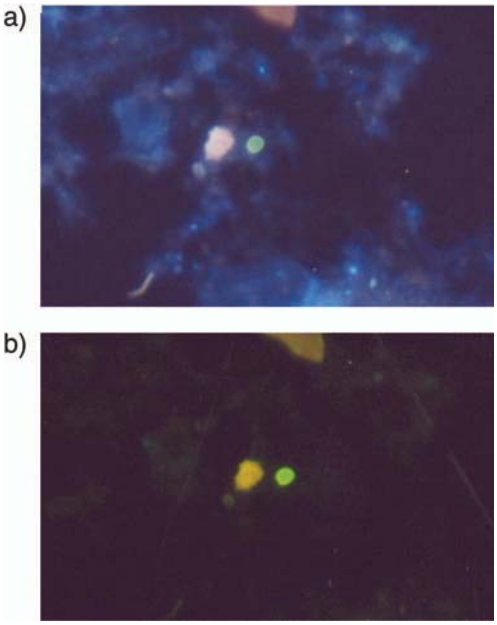

Fig. 2 - *Cryptosporidium* oocysts: *a)* stained with DAPI; *b)* stained with kit 2 reagents, present in activated sludge samples of Santa Rosa ETE, Campinas, Southeast Brazil. 500x.

Cyst and oocyst concentrations in raw and treated sewage appear to be highly variable: about 100-9200 cysts/L and 1-560 oocysts/L in samples of raw sewage or 2-898 cyst/L and 1-120 oocyst/L in treated sewage samples[7,24], and up to 1.6×10^{10} oocysts/L may be daily discharged from a treatment plant[31]. The high concentration of parasites observed in this study may be explained by the fact that the activated sludge process tends to concentrate them in the flocculated material[28].

Although the statistical tests had not shown a significant difference between the kits, we observed better fluorescence quality for *Cryptosporidium* oocysts when kit 2 was used; as a result, the choice of antibody could be a personal choice.

The high concentration of cysts and oocysts found in activated sludge reinforces the necessity of residue evaluation before its final disposal in agriculture. Of all methods used in this study, ECP was the

most efficient in recovering *Giardia* and *Cryptosporidium* from activated sludge. However, other studies about the viability of cysts and oocysts found (through animal infectivity experiments) are necessary to evaluate the risks to Public Health, before choosing this type of reuse.

RESUMO

Ocorrência de cistos de *Giardia* e oocistos de *Cryptosporidium* em amostras de lodo ativado em Campinas, SP, Brasil

Giardia e *Cryptosporidium* causaram vários surtos epidêmicos de gastroenterite, associados à água potável. Efluentes de esgoto contaminados foram incriminados como uma fonte potencial de cistos e oocistos. Uma investigação foi conduzida para verificar a presença de cistos de *Giardia* e oocistos de *Cryptosporidium* em amostras de lodo ativado de uma Estação de Tratamento de Esgoto. Para isto as amostras foram submetidas: *i)* a concentração pelo processo de clarificação com éter (ECP) e *ii)* método de purificação por flutuação em sacarose (SFM) e, as alíquotas dos sedimentos foram examinadas por imunofluorescência. Cistos de *Giardia* estiveram presentes em todas as amostras avaliadas (100,0%; n = 8) quando utilizado ECP e kit 1, enquanto o kit 2 resultou em 6 amostras positivas (85,7%; n = 7). Para SFM, cistos de *Giardia* foram detectados em 75,0% e 100,0% destas amostras (para kit 1 e 2 respectivamente). Considerando os oocistos de *Cryptosporidium*, duas amostras (25,0%; kit 1 e 28,5% kit 2) foram positivas usando-se ECP enquanto para SFM, apenas uma amostra (examinada pelo kit 1) foi positiva (12,5%). Os resultados do experimento-controle revelaram que as taxas de recuperação para *Giardia* e *Cryptosporidium*, quando utilizado ECP foi de 54,5% e 9,6% e para SFM, foi de 10,5% e 3,2%, respectivamente. Considerando a detecção de alta concentração desses protozoários, é recomendada a avaliação prévia do lodo ativado antes de sua aplicação na agricultura e, com alguma melhora, ECP pode ser uma técnica apropriada e simples para a detecção de protozoários em amostras de esgoto.

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