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DETECTION OF *CRYPTOSPORIDIUM* SPP. OOCYSTS IN RAW SEWAGE AND CREEK WATER IN THE CITY OF SÃO PAULO, BRAZIL

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ABSTRACT

The protozoan parasite *Cryptosporidium* has emerged as one of the most important contaminants of water, causing waterborne outbreaks of gastroenteritis worldwide. To monitor and understand the public health significance of this pathogen in environmental samples, several methods have been developed to isolate and detect *Cryptosporidium* oocysts. The purpose of this study was to perform the first investigation on the presence of *Cryptosporidium* spp. oocysts in raw sewage and creek water in the city of São Paulo, Brazil. The oocysts were concentrated by flocculation and membrane filtration. The results showed the occurrence of *Cryptosporidium* spp. in all wastewater samples analyzed, indicating a possible risk for dissemination of these pathogens in aquatic environment and in the community.

Key words: *Cryptosporidium* spp., sewage, creek waters

INTRODUCTION

The role of sewage-polluted waters in the transmission and outbreaks of microbial illness is well known. Substantial concern persists that pathogen occurrence in water supplies may be responsible for the transmission of background (endemic) levels of enteric disease (2,9,10).

Many outbreaks of giardiasis and cryptosporidiosis have been reported in the last few decades and water has been considered one of the major routes of dissemination (5). Positive findings of oocysts in untreated wastewater, filtered secondarily treated wastewater, activated sludge effluent, combined sewer overflows, groundwater, surface water, and treated drinking water indicate widespread fecal contamination (6,11).

Epidemiological evaluations on the contribution of polluted waters to cryptosporidiosis in endemic settings are not available, especially because there is no standardized technique to recover *Cryptosporidium* oocysts from environmental samples. Methods such as continuous flow centrifugation, membrane and cartridge filtration, calcium carbonate flocculation and polycarbonate membrane systems have been used to concentrate oocysts in water samples before they can be detected, but efforts to find a reliable, sensitive and practical method must be continuously pursued (3). Other considerations about current methods should be made, since these techniques could be too expensive to be adopted in developed and underdeveloped countries.

In Brazil, research about the occurrence and methods for detection of this pathogen in environmental samples is necessary, since few data are available. In addition, the new Brazilian Federal Legislation (law 1649 as of December 29th, 2000) recommends the investigation for the presence of oocysts in water.

The purpose of this study was to perform the first investigation of the presence of *Cryptosporidium* spp. oocysts in raw sewage and creek waters in the city of São Paulo using two concentration methods: Calcium Carbonate Flocculation (12) and the Membrane-Filter Dissolution Method (1).

MATERIALS AND METHODS

From July to December 1998 24 samples of raw domestic sewage and sewage-polluted creek waters were collected at two sites in the city of São Paulo: the effluent of the Edu Chaves Sewage Pumping Station (SPS) and the creek Pirajussara. The

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samples were transported in ice to the laboratory in sterile 10-liter plastic (high density polyethylene) containers.

Calcium Carbonate Flocculation was performed according to Vesey *et al.* (12). Briefly, 50 mL of 1 mol L^{-1} $CaCl_2$ and 50 mL of 1 mol L^{-1} $NaHCO_3$ were added to each container with samples of 5L. After further mixing, the pH was adjusted to 10 by the addition of 1mol L^{-1} NaOH. The solutions were left at room temperature for 4 hours to allow the formed flocs to settle. The supernatant fluid was aspirated and discarded. Care was taken not to disturb the calcium carbonate residue. The flocs were dissolved by adding 100 mL of 10% w/v Sulphamic Acid, centrifuged in 250 mL centrifuge bottles at 3000g for 15 min and the supernatant fluids discarded. The pellets were transferred to 50 mL centrifuge tubes and centrifuged at 3000g for 15 min. The supernatant fluids were decanted and the pellets combined and adjusted with elution fluid (1% Tween 80, SDS, 1%, PBS 10X, 0.001% Antifoam A).

Membrane-Filter Dissolution Method was conducted according to Aldom and Chagla (1). The 2L samples were filtered through a 142 mm-diameter, 1.2 µm-pore cellulose acetate membrane filters (Millipore). After filtration, the membrane were folded, transferred to 250 mL centrifuge bottles, and dissolved in 200 mL acetone. The dissolved membrane was successively centrifuged at 650 g for 15 min and resuspended in acetone, 95% ethanol, 70% ethanol and elution fluid.

The final concentrated samples obtained by both methods were suspended in 10 mL elution fluid and a 10 µL sample was transferred to wells of the microscope slide provided in the Merifluor Cryptosporidium, Giardia Kit (Meridian Diagnostics, Cincinnati, OH). The slides were stained according to the manufacturer's instructions. The oocysts in each well were counted with an epi-fluorescence microscope (DAS Leica DM LB / Germany) at 400X magnification and confirmed by phase contrast.

The number of oocysts detected per liter was calculated by the average of the counting of three slides: (1) number of oocysts in an analyzed drop x total mL of the pellet / volume of analyzed drop = number of oocysts in pellet; (2) number of oocysts in pellet /number of liters filtered or flocculated = number of oocysts/L.

A statistical analysis using t-student test (95% confidence level) was performed. The analysis was carried out for the data obtained by the two concentration methods.

Negative (distilled water) and positive (*Cryptosporidium* oocysts) control samples were also used throughout the study.

RESULTS AND DISCUSSION

In Brazil, about 30 million people have no access to treated drinking water and only a limited segment of the population in the large cities has access to urban sewage system, otherwise sewage is discharged into creeks and rivers (7). Therefore, high risk of waterborne cryptosporidiosis has to be taken into consideration.

In this study, oocysts of *Cryptosporidium* spp. were detected in all 24 wastewater samples collected in the city of São Paulo. The actual number of oocysts/L was calculated as the average counts obtained by the two methods employed to concentrate oocysts. Raw sewage samples yielded 80-912 oocysts/L while in creek waters, oocysts numbers varied from 65 to 760/L (Fig. 1).

We also noted that oocysts numbers were usually higher in the Pirajussara Creek than in the domestic effluent of Edu Chaves SPS, showing the fecal contamination of these surface waters.

Moreover, these waters circulate in the metropolitan area and must be affecting the quality of local water supplies, since the presence of *Cryptosporidium* oocysts was determined in this city's groundwater (4), raw and treated water (8), drawing an apparent potential to cryptosporidiosis outbreak.

On the other hand, a number of considerations must be taken into account in the interpretation and significance of the obtained data. These are directly related to the sensitivity of the methods used to recover and detect *Cryptosporidium* oocysts from environmental samples.

In this study, for example, no statistically significant difference was observed between concentration methods using filtration or carbonate flocculation for detection of *Cryptosporidium* oocysts ($P<0.05$) (Table 1). However, the high concentration of suspended solids in the analyzed samples may be affecting the interpretation of results.

A major problem with the method of concentrating oocysts by filtration, besides its high cost, is the compaction of particles on and around the oocysts. This compaction occurs during filtration and the oocysts that are attached to other particles are incorporated into aggregates and may be missed during the detection stage. Disaggregation of these compacted particles is difficult and oocysts will be lost if a density gradient centrifugation procedure is used to clarify the sample.

The flocculation procedure described by Vesey *et al.* (12) has been reported as a more simple and gentle method. Oocysts are

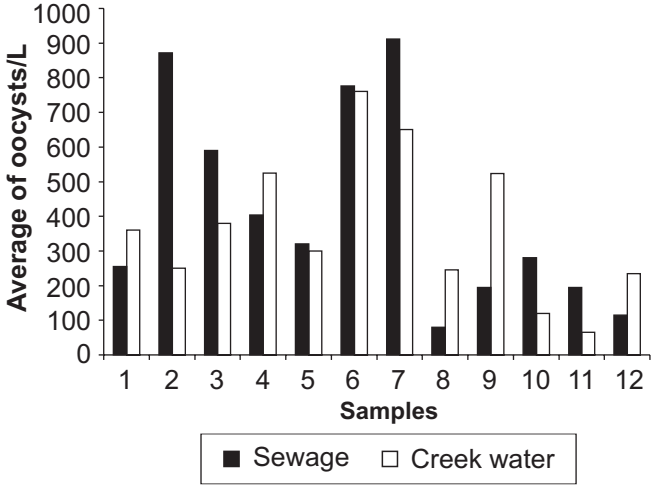

Figure 1. Levels of oocysts/L detected in raw sewage and creek waters in the city of São Paulo.

Table 1. Detection of *Cryptosporidium* spp. oocysts in raw sewage and creek water, concentrating 2L by membrane filtration and 5L by calcium carbonate flocculation.

	Number of oocysts/L			
	Raw sewage		Creek Water	
Samples	Filtration	Carbonate flocculation	Filtration	Carbonate flocculation
1	150	360	480	240
2	1200	544	300	200
3	500	680	600	160
4	280	528	150	900
5	200	440	300	300
6	1200	352	1400	120
7	1200	624	1000	300
8	160	0	150	340
9	150	240	1000	48
10	500	60	0	240
11	50	340	50	80
12	150	80	150	320
Average*	478,333	354	465	270,666
P-value	0.4058		0.1909	

* No statistically significant differences were observed between the averages.

concentrated in a dense and stable residue at the bottom of a container within 4 hours and the residue can be rapidly dissolved in sulphamic acid. This procedure concentrates particles of a wide range of sizes, resulting in a residue which may contain a greater amount of particulate matter than when filtration methods are used. However, this particulate matter is less compacted and aggregated than the one obtained by filtration.

In fact, lower recoveries of the oocysts were attributed to high turbidity in those methods (12,13), but the carbonate flocculation shows great promise to analyze samples with high concentrations of suspended solids. Further analysis might be required to improve the method and to determine its performance, before it can be routinely used.

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RESUMO

Cryptosporidium spp. em águas de esgoto

O protozoário *Cryptosporidium* é um importante patógeno contaminante de água, causador de surtos de gastroenterite em vários países. Na tentativa de compreender e monitorar o significado desse patógeno em amostras ambientais, vários métodos foram desenvolvidos para o isolamento e identificação de oocistos de *Cryptosporidium*. O objetivo deste trabalho foi realizar a primeira investigação da presença de oocistos de *Cryptosporidium* em águas de esgoto e de córrego no município de São Paulo. Os oocistos foram concentrados por floculação e por filtração em membrana. Os resultados indicaram a ocorrência de *Cryptosporidium* em todas as amostras analisadas, indicando um potencial risco de disseminação desse patógeno no ambiente aquático e também na comunidade.

Palavras-chave: *Cryptosporidium* spp., águas de esgoto, águas de córrego

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