First report of *Cryptosporidium* spp. oocysts in oysters (*Crassostrea rhizophorae*) and cockles (*Tivela mactroides*) in Brazil

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ABSTRACT

The consumption of oysters and cockles, which are usually eaten raw or lightly-cooked, can cause outbreaks of human diseases, especially if these shellfish are harvested from polluted areas. In Brazil data about the occurrence of pathogens, like hepatitis A virus, in shellfish have been reported but research on natural contamination for pathogenic protozoa is still non-existent. *Cryptosporidium* oocyst contamination of oysters (*Crassostrea rhizophorae*) and cockles (*Tivela mactroides*) was evaluated during two different periods in a coastal area from São Paulo, Brazil. From June to November 2005, and from July to December 2006, 180 mollusks were harvested for tissue examination. The gills and gastrointestinal tract ($n = 36$ pools) were carefully extracted from the animals and homogenized in a tissue homogenizer by adding surfactant Tween 80 (0.1%). Immunofluorescence assays were performed and *Cryptosporidium* oocysts were detected in 50.0% of gill pools of cockles and 10.0% of gill pools of oysters. In order to evaluate seawater quality in shellfish growing areas, total levels of thermotolerant coliforms, *Escherichia coli* and enterococci were determined. This is the first time that *Cryptosporidium* oocysts were found in shellfish from the coastal region of Brazil, and to the best of our knowledge it is also the first report in Latin America and the case might be of public health importance, reflecting the extension of the contamination on seafood, requiring a need for quality control standards.

Key words | Brazil, cockles, *Cryptosporidium*, detection, oysters, seawater

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INTRODUCTION

Bivalve mollusks are benthic animals which live in close relationship with marine substratum. These shellfish have the capacity to filter large volumes of water and therefore are able to accumulate, retain and concentrate different pathogens like bacteria, viruses and protozoa (Wittman & Flick 1995; Potasman *et al.* 2002). Therefore, eating raw or improperly cooked shellfish represents a risk to health. The occurrence of protozoan parasites such as *Cryptosporidium* spp. which causes gastroenteritis in a wide range of animal and human hosts, has been reported worldwide in water samples from lakes, rivers, estuaries, ocean and sewage (Fayer 2004; Cantusio Neto *et al.* 2006). In recent times, the presence of *Cryptosporidium* oocysts was demonstrated in different species of bivalve mollusks following experimental contamination (Graczyk *et al.* 1998; Freire-Santos *et al.* 2001; Gómez-Couso *et al.* 2005).

Mussels, clams, oysters, and cockles from several coastal regions have been confirmed as being naturally contaminated with *Cryptosporidium* oocysts (Fayer *et al.* 1999; Lowery *et al.* 2001; Giangaspero *et al.* 2005; Li *et al.* 2006). Oocysts of *Cryptosporidium* were also found in commercial shellfish from 64.9% of the sites examined along the Atlantic coast, either by microscopy using direct immunofluorescence assay or molecular testing.

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(Fayer *et al.* 2003). In Brazil, data about the occurrence of hepatitis A virus in shellfish have been reported in Santa Catarina State (South Region) (Sincero *et al.* 2006) but research of natural contamination for pathogenic protozoa is still non-existent. The detection of these protozoa in shellfish has great relevance, considering that the presence of *Cryptosporidium* oocysts and *Giardia* cysts has already been reported in beaches of the São Paulo coastal region, according to CETESB- Company of Technology of Environmental Sanitation (2000). Moreover, 12,500 tons of cultured mollusks were produced in 2000, according to data from the Fishing Department (Aquaculture Panorama 2001). In this context, the aim of this study was to evaluate the natural occurrence of *Cryptosporidium* contamination in different molluskan species usually consumed in Brazil and also to verify the microbiological quality of seawater where they live and are harvested for human consumption.

MATERIALS AND METHODS

Shellfish sampling site

The "Camaroeiro" beach (23° 37' 49197" S and 45° 23' 91829"), located on the north coast of the state of São Paulo, in Caraguatatuba city, Brazil (Figure 1), was selected for the present study. This beach presents different sites of domestic sewage discharge along the coast and an estuarine environment at the confluence point of the Ipiranga River and seawater; in addition, it harbors a commercial harvesting area. A total of 120 oysters (*Crassostrea rhizophorae*) and 60 cockles (*Tivela mactroides*), between 3 and 6 cm were harvested monthly using hand tongs, during two different periods. From June to November 2005, both species were analyzed; from July to December 2006, only oysters were collected for examination, since there was a significant reduction in the natural population of cockles due to exploratory practices during this period. A specific sampling site was chosen based on its location near a sewage discharge point, where bivalves live. The shellfish were randomly selected, collected and transported under refrigerated conditions for examination at the Protozoology Laboratory, State University of Campinas (UNICAMP), Brazil.

Oyster and cockle processing

At the laboratory, the shellfish were shucked and the parasitological analysis was conducted as described by Gómez-Couso *et al.* (2003), with modifications. Each sample consisted of the pooled gills and gastrointestinal tracts from ten mollusks and a total of 24 pools of oysters and 12 pools of cockles were examined. The gills and gastrointestinal tract (GI) were excised from each animal and placed in a tissue homogenizer containing Tween 80 (0.1%) and distilled water (2:1). The tissue pools were homogenized and the lipids were extracted by addition of diethyl ether. Then, the tube was vigorously hand agitated for 30 seconds and centrifuged at 1250 x *g* for 5 minutes in

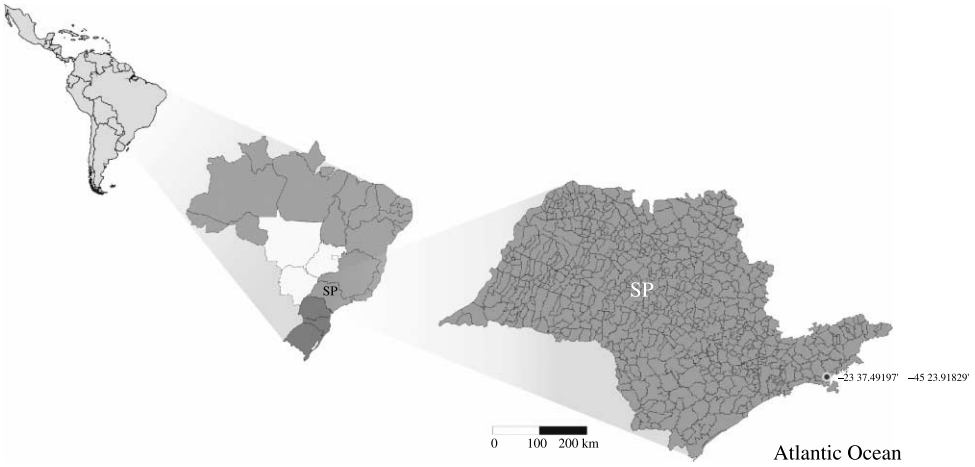

Figure 1 | Camaroeiro beach and shellfish harvesting site in Caraguatatuba, (SP), Brazil.

PBS (0.04M pH 7.2). The supernatant was aspirated and the pellet was placed in a microcentrifugue tube.

Detection of *Cryptosporidium* oocysts

A direct immunofluorescence antibody test (IFAT) was used to detect *Cryptosporidium* oocysts in the pellet. For this, samples were vortex mixed, and aliquots of each pellet were pipetted onto slides. The slides were air-dried, fixed with methanol for ten minutes and then stained with a commercially available kit, Merifluor® (Meridian Bioscience, Cincinnati, Ohio), which uses a specific monoclonal antibody conjugated with fluorescein isothiocyanate (FITC) against an epitope of *Cryptosporidium* oocyst wall for protozoan visualization and quantification with epifluorescence microscopy. The criteria used to consider specimens positive for oocysts were based on their size (4 -6μm), shape and their pattern and intensity of immunofluorescence staining (i.e., bright apple green fluorescence of the oocyst wall). Phase-contrast microscopy was used for complete diagnosis and allowed us to confirm the morphology of the parasite, as well as DAPI (4',6-diamino-2-phenylindole), a nucleic acid stain which fluoresces blue and shows the position and number of nuclei in the oocyst. The number of oocysts detected in each naturally contaminated shellfish was calculated as the total oocyst number detected on the examined aliquots, multiplied by tissue homogenate total volume and divided by the number of shellfish that were being tested.

Bacterial analysis

Samples of seawater were collected monthly in flasks previously decontaminated, and the most probable number (MPN) procedure was carried out to enumerate thermotolerant coliforms, *Escherichia coli* and enterococci according to methodology recommended by *Standard Methods for the Examination of Water & Wastewater* (2005). In accordance with the federal legislation resolution CONAMA (National Environmental Council) N° 274 (2000), beach water quality can be classified as proper or improper for recreational purposes. The water is categorized as improper if at least one of these indicators is verified: (i) more than 2500 thermotolerant coliforms/100 ml; (ii) more than 2000 *Escherichia coli*/100 ml; (iii) more than 400 enterococci/100 ml.

RESULTS AND DISCUSSION

The occurrence of diseases transmitted by shellfish consumption has been increasing worldwide. Many outbreaks associated with bivalve consumption have been reported, especially associated with the ingestion of oysters, followed by clams and mussels (Potasman *et al.* 2002).

Due to the scarcity of data for pathogenic protozoa in seafood on our country, a total of 180 shellfish were analyzed during two different periods of sampling. Oocysts of *Cryptosporidium* were found in both bivalve species analyzed under natural conditions. In the first period they were detected in three of the six gill pools of cockles with a mean concentration of 60 oocysts per cockle. In the second period, oocysts of *Cryptosporidium* were detected in one of twelve gill pools of oysters with an estimated concentration of 12 oocysts per oyster.

The detected oocysts were in conformity with standard fluorescence detection criteria. Phase-contrast microscopy carried out simultaneously at 600x magnification confirmed the characteristic morphology of the parasite and nuclei were visualized with DAPI. *Cryptosporidium* oocysts were not found in any gastrointestinal tract homogenates pools ($n = 6$ for cockles and 12 for oysters) analyzed during the twelve months of sampling.

The gastrointestinal tract homogenates we analyzed were more difficult to examine than gill homogenates, due to the presence of thick layers on slides, and this may thus explain the complete absence of *Cryptosporidium* oocysts in this tissue. Therefore, oocysts may have gone undetected because of masking. Moreover, Graczyk *et al.* (1997*a*,*b*) demonstrated the internalization of *Cryptosporidium parvum* oocysts by monolayers of hemocytes in native oysters (*Crassostrea virginica*) and found that there was a loss of fluorescence in the oocyst wall as the time of contact increased, and also pointed out that it disintegrated with time. The oocysts that remained unaltered may be retained principally by the gills, because the pores of the gills and the oocysts are of similar sizes.

Microbiological indicators of seawater were detected in high levels, and the majority of samples were not in

accordance with standard legislation. Thermotolerant coliforms ranged from 9.0×10^2 to 3.3×10^4, *Escherichia coli* from 4.0×10^2 to 4.0×10^3 and enterococci ranged from 2.0×10^2 to 4.0×10^3 MPN per 100 ml of seawater.

In several months, more than one microbiological indicator was present at higher levels than permitted, as October (2005) when thermotolerant coliforms attained levels of 3.3×10^4 and *E. coli* 4.0×10^3 MPN per 100 ml. The microbiological data obtained from seawater indicated poorer water quality at the oysters and cockles harvesting site. The elevated level of faecal indicators shows high levels of faecal contamination and may thus explain the number of positive bivalves with *Cryptosporidium* oocysts. However, many other authors have not found a direct and specific correlation between faecal indicator coliforms counts in water with *Cryptosporidium* (Gómez-Couso *et al.* 2003; Gómez-Couso *et al.* 2004; Graczyk *et al.* 2007; Schets *et al.* 2007).

Previous studies have shown the contamination of shellfish by *Cryptosporidium* oocysts in North America (Fayer *et al.* 2003; Miller *et al.* 2005) and in Europe (Gomez-Bautista *et al.* 2000; Li *et al.* 2006; Schets *et al.* 2007). The present survey represents the first report of the occurrence of *Cryptosporidium* oocysts in two different species of bivalve mollusks in Brazil and in Latin America to date, and indicates the existence of widespread contamination of this waterborne pathogen.

Although this protozoan parasite does not reproduce in bivalve tissues, it can accumulate in high quantity, and oocysts retained in mollusk tissues may remain infectious (Fayer *et al.* 1997; Freire-Santos *et al.* 2001; Graczyk *et al.* 2007). Furthermore, *Cryptosporidium* oocysts can survive in marine waters from several days up to one year, according to experimental data (Tamburrini & Pozio 1999; Freire-Santos *et al.* 2000) and their long-term survival in the environment could increase the hazard of infectious forms being filtered and removed by mollusks, posing a threat if commercialized and consumed.

In Caraguatatuba, São Paulo state, only 32.0% of domestic sewage is collected and treated (SABESP 2006), and in Brazil discharge of effluents is done preferentially into rivers, contaminating and depreciating the value of the water and other environments it flows to (IBGE 2000).

In this study, the number of oocysts found under natural conditions in these bivalve mollusks is sufficient to cause an infection, if they were viable and found to be species infectious for humans. The mean infectious dose for *Cryptosporidium parvum*, a zoonotic species, is around 10 oocysts (Cooperative Research Centre for Water Quality & Treatment (2005)). Furthermore, it is thought that the development of an infection through the ingestion of a single oocyst can occur in people with immunological impairments posing a threat to immunocompromised populations (Rose *et al.* 2002).

It is a well-known fact that different species of shellfish act as a biological indicator of animal and human fecal pollution in different environments, where they live or are cultivated (Giangaspero *et al.* 2005; Miller *et al.* 2005); with our findings, however, the *Tivela mactroides* cockle emerges as a new species able to remove waterborne pathogens from contaminated areas very effectively and must be added to this extensive list.

It is noteworthy that in Camaroeiro beach, oyster and cockle fishing is an exploratory practice, where the local population harvests large quantities of animals for their subsistence, without any supervision of federal organizations or competent environmental agencies. Considering that in Brazil the Federal Legislation- resolution CONAMA N° 357 (National Environmental Council 2005) contemplates only the analysis of fecal coliforms in waters where mollusks are cultivated, it is necessary to include monitoring of this protozoan.

CONCLUSIONS

- The present study leaves no doubt that *Cryptosporidium* is present in oysters and cockles from the north coast of São Paulo State (Brazil). *Cryptosporidium* can be life-threatening, and the ingestion of seafood, particularly oysters, could be involved in the epidemiology of food borne diseases whenever they are consumed in a raw form.
- In developed nations, people with immunological deficiencies are more aware of the risks associated with the consumption of raw foods and are therefore more likely to avoid this type of food. That is not the case for the Brazilian population, at least for poorer individuals. In this context, our report shows valuable information.

- Future works must focus on the detection of this protozoan in beach water, and the correlation of salinity, pH, temperature and precipitation with *Cryptosporidium* at an estuarine area.

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