Mussels (*Perna perna*) as bioindicator of environmental contamination by *Cryptosporidium* species with zoonotic potential

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ABSTRACT

Sources of contamination such as animal feces runoff, organic fertilizer application, and the release of partially treated or untreated sewage can lead to the contamination of aquatic environments by *Cryptosporidium* spp. The quality of mussels as food is closely related to the sanitary conditions of the marine environment where these bivalves are found. Marine mollusks are filter feeders that are able to retain *Cryptosporidium* oocysts in their tissue, thus functioning as bioindicators. A total of 72 pooled mussel samples of the species *Perna perna* were collected at two sites (A and B) in the municipality of Mangaratiba, Rio de Janeiro State, Brazil. Sampling involved removal of 30 mussels, from each collection site every month for one year. The 30 mussels from each sampling were then allocated into three groups of 10. Two *Cryptosporidium* spp. genes (18S and GP60) were targeted for DNA amplification from the samples obtained. After purification, all of the products obtained were sequenced and phylogenetic analyses were performed. Of the 72 samples analyzed using the nested-PCR for the *18S* gene target, 29.2% were positive for the presence of *Cryptosporidium* spp. Of these samples, 52.4% were collected at site A (ie 11/21) and 47.6% at site B (ie 10/21). The *18S* genes of all the samples considered positive for *Cryptosporidium* spp. were sequenced, and the following three species were identified: *Cryptosporidium parvum*, *C. meleagridis*, and *C. andersoni*. Three distinct *C. parvum* subtypes (IIaA19G2R2; IIaA20G2R2; IIaA20G3R2) were identified using the GP60 gene. More studies to evaluate the zoonotic potential of this species should be performed as both sampling locations contain human and/or animal fecal contaminants.

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1. Introduction

Mussels of the species *Perna perna*, belonging to family Mytilidae, are native to Africa and have been introduced into South America. Currently, natural banks of the species in Brazil are located along the entire coastline (Resgalla Jr. et al., 2008).

Multiple bivalve mollusk species are considered delicacies in Brazil and in many locations worldwide. They can be harvested from natural or farmed populations (Robertson, 2007; Souza et al., 2012; Giangaspero et al., 2014). The mollusk *P. perna* typically grows faster than species from temperate climates and in Brazil are harvested from natural populations, and are economically important (Henriques et al., 2004).

These marine mollusks are filter feeders that are able to retain oocysts and cysts of various protozoa in their tissue. *Cryptosporidium* is one of the most studied of these (Gómez-Couso et al., 2004; Lucy et al., 2008; Souza et al., 2012; Giangaspero et al., 2014) because it can be responsible for outbreaks of waterborne disease (Baldurson and Karanis, 2011). The quality of mussels and other bivalves is closely related to the sanitary conditions of the marine environment where they are found (Souza et al., 2012). Improper disposal of sewage and runoff of animal and/or human feces into their habitat are potential sources of contamination of

these bivalves (Giangaspero et al., 2014).

The consumption of raw or poorly cooked mussels may result in risks to human health. If bivalves accumulate infective forms of protozoa, bacteria, or viruses in their tissue, they can remain infective until consumption unless their preparation includes a step (e.g., adequate cooking) that kills them (Pereira et al., 2004; Robertson, 2007). The use of marine bivalve mollusks is not restricted to gastronomy. In addition to its importance as food, *Perna perna* mussels have high ecological relevance because they can withstand high levels of variation in their environment (Andreu, 1976). Also, because they are able to filter high volumes of water and concentrate different microorganisms and some chemical pollutants in their tissue, they have been extensively used to monitor pollution in aquatic environments (Widdows et al., 1995; O'Connor et al., 2002; Gómez-Couso et al., 2004; Francavilla et al., 2012). Without these bivalves such monitoring would be much more time consuming, laborious, and expensive (Palos Ladeiro et al., 2013).

As a result, monitoring of these bivalves is gaining importance within the field of public health mainly because these animals act as vectors for infective protozoans (Schets et al., 2007). Molecular biology is an important tool that has been used for identifying protozoa found in mollusks with potential to cause human infection and/or disease, but few studies have attempted molecular characterization these species/subtypes (Giangaspero et al., 2014; Palos Ladeiro et al., 2014). Some studies aim to identify *Cryptosporidium* species/subtypes that can be transmitted to humans by ingesting mollusks containing oocysts. Among the species/subtypes identified by PCR in these studies, using the 18S, hsp70, and gp60 genes, *C. hominis* (IbA10G2R2, IbA9G3R2, and IeA11G3T3R1) and *C. parvum* (IIaA14G3R1, IIaA15G2R1, IIaA15G2, IIaA16G2R1, IIaA19G3R1, IIcA5G3R2) are reported (Jex et al., 2008; Xiao and Fayer, 2008; Jex and Gasser, 2010; Giangaspero et al., 2014).

The present study aimed to evaluate the occurrence of *Cryptosporidium* species/subtypes in *P. perna* at two harvesting sites along the coastline of Rio de Janeiro State, Brazil, thus providing an assessment of the environmental contamination to which the mussels were exposed.

2. Materials and methods

2.1. Collection sites and sample preparation

A total of 72 mussel samples of the species *P. perna* were collected at two sites, A and B, during 2012−2013, in the municipality of Mangaratiba, Rio de Janeiro State, Brazil (Fig. 1). Both collection sites consisted of natural rocks bathed by seawater where mussels were previously observed.

Site A is an area of rocks, almost immersed in seawater, 4.5 km from Mangaratiba coast, where the water is constantly renewed due the presence of ocean currents. At this site, fishermen often remove *P. perna* mussels from the rocks for sale or for themselves. Site B is a beach with rock formations near the waterfront, in which the same mussels species (from site A) are observed. At site B usually harvest mussels from the rocks, mostly for themselves. Close to Site B the mouth of a river that flows through part of the city, and some farms and forests, all of which are potentially important sources of microorganisms. The geographical characteristics of site B may form a natural barrier to sea water renewal by the ocean currents, which may increase pathogen and pollutant occurrence in the area.

Samples were taken from each collection site once in a month (at the first week) for one year. At each sampling at each site 30 mussels were collected. Only adult individuals with mean valve length of 6 cm were used, which is considered the size for

harvesting according to Avelar (1998). These mussels were transported to the laboratory under refrigeration where the animals were randomly separated into three groups per site. Two sites monthly for a year resulted 72 samples of mussels.

After separating the groups, the mussels were processed following the method of Gomez-Couso et al. (2003). The gills and gastrointestinal tract were carefully separated after removing the mussel from the valve. In the laboratory, the material was ground and homogenized with 10 ml of distilled water using a mixer SB50 (Black & Decker, MD, EUA) and filtered through disposable sieves (Deskarplás, SP, Brazil) with a gauze overlay to remove coarse particles. After filtration the samples were centrifuged, the sediment aliquoted, and stored at approximately 4 °C for later evaluation of the presence of *Cryptosporidium* spp. using molecular tools.

2.2. Molecular diagnostics

Genomic DNA of *Cryptosporidium* spp. was extracted according to the method of Huber et al. (2007). Primers described by Fayer et al. (2010) and Sulaiman et al. (2005) were used to amplify the two target genes, the small subunit ribosomal RNA (SSU rRNA - 18S) and the 60 kDa glycoprotein (GP60) respectively, from the *Cryptosporidium* DNA fragments from the mussel s sampled. The reaction conditions and the thermal cycles adopted in this study for the first PCR and nested-PCR of both genes used were those previously described by Couto et al. (2014).

In the primary PCR and nested-PCR reactions using the 18S gene, products of approximately 1325 and 830 base pairs (bp) were expected, respectively (Couto et al., 2013), whereas in the primary PCR and nested-PCR reactions using the GP60 gene, products of approximately 650 bp and 400 bp were expected, respectively, as indicated by the bands visualized on an agarose gel (Couto et al., 2013).

The two target gene products generated from the nested-PCR reactions were visualized on a 1.2% agarose gel stained with ethidium bromide. All of the samples considered as positive for *Cryptosporidium* via 18S gene sequencing were subjected to new reactions using the GP60 gene as a diagnostic for the subtypes.

To standardize the first PCR and nested-PCR reactions for the target genes 18S and GP60, samples of *C. parvum* previously sequenced and deposited in Genbank under accession numberDQ885333 and KC291661 by Huber et al. (2007) and Couto et al. (2014), respectively. Ultrapure nuclease-free water (Promega − WI, USA) was used as negative control for the first PCR and nested-PCR reactions.

2.3. Genotypic characterization

All of the samples obtained from the nested-PCR reaction were purified using a Wizard® SV Gel and PCR Clean-Up System kit (Promega) and quantified using a spectrophotometer (Thermo - Nanodrop 2000). After purification, all of the products obtained were sequenced using the same primers from the nested-PCR amplification reaction for both target genes.

The *Cryptosporidium* samples were sequenced using the Big-Dye® v.3.1 terminator cycling kit compatible with a 3730XL automated sequencer (Applied biosystems).

The sequences were aligned and manually adjusted using Clustal W software (Larkin et al., 2007). Then, those sequences were analyzed using MEGA 6 software (Tamura et al., 2013). The chromatograms were analyzed using CHROMAS LITE software (Technelysium, Brisbane, Australia). The BLAST platform was used to perform searches with the sequences obtained in this study through sequencing the 18S and GP60 genes from the specimens aiming to determine their identities and possible homologies and
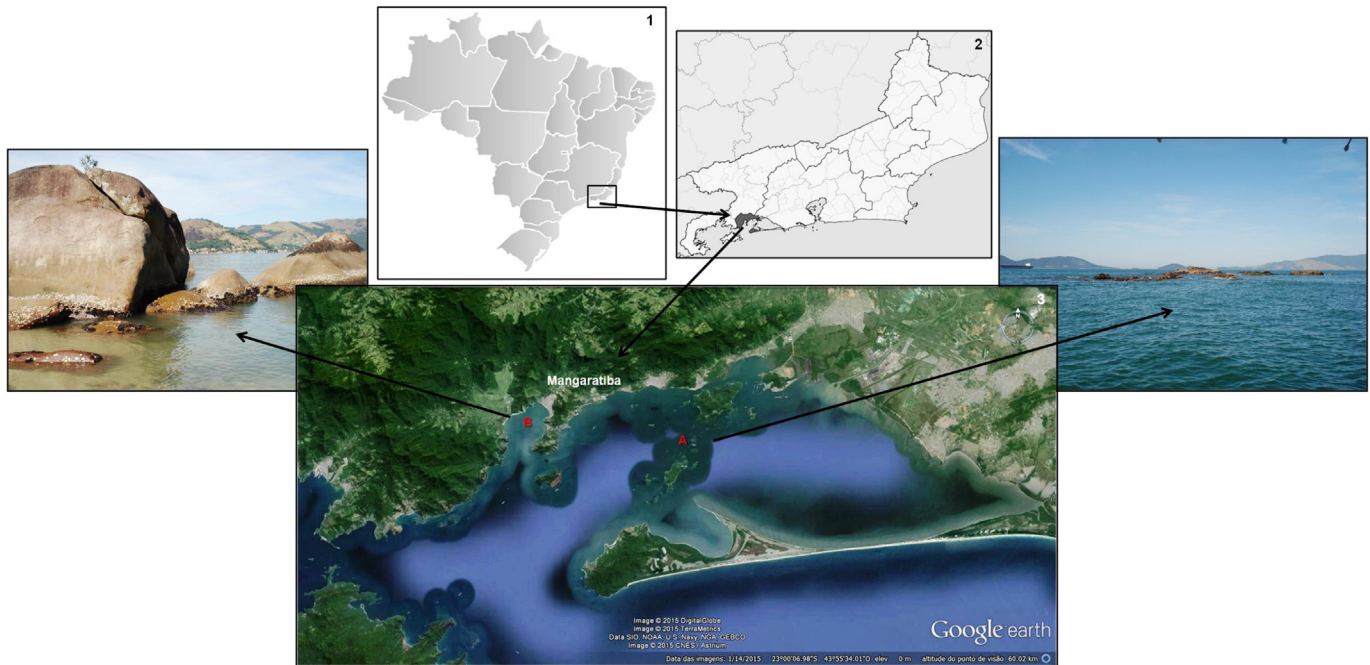
Fig. 1. Map of the studied area in the municipality of Mangaratiba, Rio de Janeiro State, Brazil. Red marker A – Collection site A; Red marker B – Collection site B; Green marker – The river known as "Rio do Saco" which leads to the ocean at collection site B. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

similarities with species previously deposited into GenBank. Phylogenetic analyses were performed using MEGA 6 software (Tamura et al., 2013) with 1000-replicate bootstrap, from which consensus trees were constructed by the Maximum Likelihood method (with complete deletion of gaps) using the Tamura-Nei model for the 18S target gene. The sequences of the present study were deposited into Genbank using the SeqIn computational program provided by the database.

2.4. Cloning and sequencing of the cloned material

The cloning technique was used when it was not possible to reliably identify the specimens solely via sequencing. The material studied was cloned using the pGEM®-T Easy Vector System kit (Promega) following the manufacturer's directions and the method described by Couto et al. (2014). After linking the PCR products from GP60 gene target, the vectors were electroporated and introduced into *Escherichia coli* cells. Bacteria containing the *Cryptosporidium* DNA fragment were plated into Petri dishes containing ampicillin (100 μg/ml), X-GAL (80 μg/ml), and IPTG (0.5 mM). The Petri dishes were incubated for 24 h at 37 °C to allow the colonies to grow. Three colonies were randomly selected to be amplified using the primers from the nested-PCR reaction (of the cloned target gene) to ensure that the *Cryptosporidium* DNA was present. The PCR products were purified using the Wizard Plus System kit (Promega), before being sequenced again.

The same protocol previously described using the 18S and GP60 genes was used for sequencing the cloned samples except for the primers used. Plasmid DNA was sequenced using the universal primers M13F-pUC (GTT TTC CCA GTC ACG AC - Forward) and M13R-pUC (CAG GAA ACA GCT ATG AC - Reverse) as suggested by the manufacturer of the pGEM®-T Easy Vector System kit (Promega).

3. Results

3.1. Molecular diagnostics using the 18S gene

The presence of *Cryptosporidium* spp. was monitored in *P. perna* mussels for a period of twelve months using mussels collected at two different sites (A and B) in the municipality of Mangaratiba, Rio de Janeiro, Brazil. Of the 72 samples analyzed using nested-PCR, 29.2% (21/72) of the samples were positive for the presence of *Cryptosporidium* spp. Of these, 52.4% (11/21) of the samples were collected at site A and 47.6% (10/21) were collected at site B (Table 1).

All of the samples considered positive for *Cryptosporidium* spp. underwent 18S sequencing; it was possible to identify three species, *C. parvum*, *C. meleagridis*, and *C. andersoni*. Of the three *Cryptosporidium* species identified, 7 of 21 (33%) were *C. andersoni*, 5 of 21 (23.8%) were *C. parvum*, a species with high zoonotic potential, and 3 of 21 (14.3%) as *C. meleagridis*, which is also considered potentially zoonotic.

Cryptosporidium sequences obtained in this study by analyzing the 18S gene were deposited into GenBank under accession numbers JX141292 through JX141306. At the two sampling sites, poor-quality sequences were obtained from six samples (15, 27, 37, 47, 61, and 63), making it impossible to identify the *Cryptosporidium* species in 28.6% (6/21) of the samples (Table 1).

Distances among the taxa obtained via 18S gene analysis, and comparisons to sequences obtained from GenBank were compared using the Tamura-Nei model. This analysis revealed high similarity between the *Cryptosporidium* species found in the present study and the existing sequences in Genbank. The highest distance (0.088) was observed between the species *C. andersoni* and *C. parvum*.

Table 1
Cryptosporidium species observed in Perna perna mussels collected at sites A and B.

Months of collection	Site of collect − A				Site of collect − B			
	Samples	Nested-PCR	Species (*18S*)	Subtypes (*GP60*)	Samples	Nested-PCR	Species (*18S*)	Subtypes (*GP60*)
March	1	−			37	+	(b)	IIaA20G2R2
	2	−			38	+	*C. parvum*	IIaA20G2R2
	3	+	*C. meleagridis*	(d)	39	−		
April	4	−			40	−		
	5	−			41	−		
	6	−			42	+	*C. meleagridis*	(d)
May	7	+	*C. andersoni*	(d)	43	−		
	8	−			44	−		
	9	−			45	−		
June	10	−			46	−		
	11	−			47	+	(b)	IIaA20G2R2
	12	−			48	−		
July	13	+	*C. andersoni*	(d)	49	−		
	14	+	*C. andersoni*	(d)	50	−		
	15	+	(b)	(c)	51	−		
August	16	−			52	−		
	17	+	*C. andersoni* (a)	IIaA20G3R2 (a)	53	−		
	18	−			54	−		
September	19	−			55	−		
	20	−			56	−		
	21	−			57	+	*C. andersoni*	(d)
October	22	−			58	−		
	23	+	*C. andersoni*	(d)	59	−		
	24	−			60	+	*C. andersoni*	(d)
November	25	+	*C. parvum*	IIaA20G2R2	61	+	(b)	(c)
	26	+	*C. parvum*	(b)	62	−		
	27	+	(b)	(c)	63	+	(b)	(c)
December	28	−			64	−		
	29	−			65	−		
	30	−			66	−		
January	31	−			67	−		
	32	−			68	−		
	33	+	*C. meleagridis*	(d)	69	−		
February	34	−			70	−		
	35	−			71	+	*C. parvum*	(b)
	36	−			72	+	*C. parvum*	IIaA20G2R2/IIaA19G2R2

(a) Sequence with overlapping peaks, subjected to GP60 PCR; (b) Exhibited band in the gel for 18S and/or GP60 gene; poor quality sequence did not allow identification of the species; (c) It was not possible to identify the subtype; (d) GP60 gene was not amplified.

3.2. Molecular diagnostics using the GP60 gene

All of the samples identified as positive for *Cryptosporidium* spp. in the 18S nested-PCR reactions were subjected to additional amplification reactions (primary PCR and nested PCR) for the GP60 gene. This procedure aimed to detect the possible existence of more than one *Cryptosporidium* species in the mollusks.

Of the 21 samples identified as positive for *Cryptosporidium* spp., the GP60 gene was only amplified in twelve (Table 1). The initial sequencing of the specimens using primers AL3532 and LX0029 did not allow the *Cryptosporidium* subtypes to be reliably identified, and thus, it was necessary to individualize the DNA fragments via the cloning technique. After the cloning procedure, several colonies were obtained from each sample, and three were randomly selected for new sequencing using the M13F-pUC and M13R-pUC universal primers. A total of 18 sequences were obtained after cloning; however, only 38.9% (7/18) had high enough quality to allow three different *C. parvum* subtypes (IIaA19G2R2; IIaA20G2R2; IIaA20G3R2) to be identified. All of the sequences obtained after cloning were deposited into GenBank under accession numbers KC307776 through KC307782.

4. Discussion

Few studies have addressed the role of marine bivalve mollusks as disseminators of *Cryptosporidium* to humans and animals in Brazil, and this is the first study conducted along the coastline of Rio de Janeiro State. The mollusk species *P. perna* is widely dispersed throughout the Brazilian coastline (Resgalla Jr. et al., 2008), and due to the large quantities of specimens available, they are easily obtained by extractive activities and thus extensively consumed along the studied coastline. Thus this species has the potential to become a vector of *Cryptosporidium* within the region.

Based on the results obtained using 18S sequencing, it was possible to identify three *Cryptosporidium* species in the 72 *P. perna* mussel samples studied, and the species *C. andersoni* was responsible for the highest rate of occurrence in the mollusks. Although this species is not considered to have zoonotic potential, it has already been found to parasitize cattle, especially during the post-weaning phase, in the states of Rio de Janeiro (Couto et al., 2014) and São Paulo (Sevá et al., 2010; Paz e Silva et al., 2013), Brazil. The identification of *C. andersoni* in shellfish tissue suggests that oocysts from this protozoan species were carried by agricultural runoff into rivers that flow into the ocean where the study was conducted. This is similar to the suggestion of Miller et al. (2005), who reported the presence of *C. andersoni* in mollusks along the coastal region of California, USA. The authors of the present study suggest that the presence of this protozoan species identified in *P. perna* mollusks is mainly due to the region's type of economic activity and its geography. The economy of the study site is mainly agricultural-based, where the main rural activities are cattle and poultry farming, in this order, and additionally, civil construction, ore export, and tourism (Instituto Brasileiro de Geografia e Estatística - IBGE, 2010). The study site is a coastal region where some rivers flow into the

ocean, and they most likely carry urban and rural waste.

The species with the second highest occurrence identified within the region studied was *C. parvum*, which is highly relevant to public health due to its zoonotic potential (Xiao, 2010). Some field studies conducted in Europe have reported the presence of *C. parvum* oocysts accumulated in the tissue of marine bivalve mollusks of the species *Mytilus galloprovincialis* and *Cerastoderma edule* (Gomez-Bautista et al., 2000), *Ruditapes decussatus*, *R. philippinarum*, *Venerupis pulastra*, *Dosinia exoleta*, *Oestra edulis*, *C. edule* (Gómez-Couso et al., 2003), *Chamelea gallina* (Giangaspero et al., 2005), *Mytilus edulis* (Li et al., 2006); similar results were reported by Palos Ladeiro et al. (2014) in a study conducted in the laboratory with the freshwater bivalve *Dreissena polymorpha*.

Additionally, *C. parvum* and *C. hominis* have been found together in the tissues of marine bivalves of the species: *D. exoleta*; *R. philippinarum*; *V. pullastra*; *Venerupis rhomboideus*; *Venus verrucosa*; *M. galloprovincialis* and *O. edulis* (Leoni et al., 2007). Another study conducted in the USA and Canada also found *C. parvum* together with *C. hominis* in gill samples from oysters and mussels (Fayer et al., 2003), although descriptions regarding the identification of *C. parvum* subtypes are scarce.

Giangaspero et al. (2014) examined mollusks of species *M. galloprovincialis*, sold in markets in Foggia, Italy, and reported three *C. parvum* subtypes belonging to the IIa subtype family (IIaA15G2R1, IIaA15G2, and IIaA14G3R1) considered to have high zoonotic potential. In the present study, it was also possible to identify three *C. parvum* subtypes belonging to the IIa subtype family (IIaA20G3R2, IIaA20G2R2, IIaA19G2R2). Although the specimens described belong to a subtype family that is considered zoonotic, the authors cannot suggest that these pose a high risk to the human population because infection in people have not yet been observed, and further studies are required.

Two of the subtypes identified in the present study (IIaA20G2R2, IIaA19G2R2) have already been reported by Couto et al. (2014) as infecting calves during the pre-weaning phase in Rio de Janeiro State, Brazil, reinforcing the authors' theory that the oocysts were most likely carried by runoff to rivers that flow into the study site. This is similar to the observations in Koompapong and Sukthana (2012), who suggested that contamination of aquatic environments by *C. parvum* is related to the presence of wild animals, livestock, and human dwellings surrounding the riverbeds.

The species *C. meleagridis* had the lowest percentage of occurrence in the present study. This species typically parasitizes birds, but it poses a potential risk to human health (Xiao, 2010). To date, the presence of *C. meleagridis* in the tissue of marine bivalve mollusks has been reported only by Fayer et al. (2003), and thus, the present study is the second to describe such occurrence and the first to report the presence of this species in South American shellfish. As previously stated, the authors have suggested that the presence of oocysts of this *Cryptosporidium* species in the tissue of *P. perna* mollusks may be related to aquatic environmental contamination similar to that which most likely occurred for *C. andersoni*. Poultry farming is one of the main sources of revenue for the municipality in this study region (IBGE, 2010). Therefore, it is assumed that the environmental contamination is most likely due to fecal matter being carried into riverbeds that cross the region and flow into the ocean. Additionally, the presence of marine birds, such as seagulls, along the coastline may also help to spread *C. meleagridis* in the aquatic environment. This was also suggested by Koompapong and Sukthana (2012) because *C. meleagridis* was found in seawater samples in Thailand after the seagull migration period. Besides that, another feasible hypothesis for the presence of *C. meleagridis* in marine mollusks' tissue is the possibility of runoff water with human sewage in the study area. The presence of this *Cryptosporidium* species in sewage was previously reported by Li et al. (2012) as the second most prevalent species in wastewater from China.

In addition to concern regarding possible human infection via improper consumption of marine bivalves, they have another important role that should be highlighted. Shellfish such as *P. perna* may be used as bioindicators of environmental contamination. Due to the mussels' filtering ability and consequent accumulation of oocysts in their tissue, they facilitate environmental monitoring. The use of mollusks for this purpose increases every day and has been reported by multiple authors (Miller et al., 2005; Lucy et al., 2008; Giangaspero et al., 2009; Francavilla et al., 2012; Palos Ladeiro et al., 2014). The use of bivalves provides detection and identification of pathogens in aquatic environments easier than does filtration, and also reduces the time required and costs for the testing (Palos Ladeiro et al., 2013).

There have been no published studies of mussels as bioindicators of aquatic environmental contamination conducted in Brazil until now. More studies using the marine bivalves are required to evaluate the zoonotic potential of the *Cryptosporidium* species in Brazil, and to evaluate the zoonotic potential of the *Cryptosporidium* species found, especially if *P. perna* mussels from the collection sites are consumed raw or partially cooked. This would provide an assessment of the role of the mussels as bioindicators of environmental contamination. Additionally, the nested-PCR technique was able to detect *Cryptosporidium* spp. oocysts in mussel tissue, and thus, it is indicated as a qualitative detection method.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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