International Journal for Parasitology: Parasites and Wildlife 5 (2016) 28–33

Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

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

Geisi Ferreira Mariné Oliveira [a], Melissa Carvalho Machado do Couto [b], Marcelo de Freitas Lima [c], Teresa Cristina Bergamo do Bomfim [d, *]

[a] Educacional Foundation Rosemar Pimentel, University Center Geraldo Di Biase - Barra do Piraí, Brazil
[b] Federal Rural University of Rio de Janeiro, Institute of Biology, Department of Physiological Sciences, Brazil
[c] Federal Rural University of Rio de Janeiro – Chemistry Department, Institute of Exact Sciences, Brazil
[d] Federal Rural University of Rio de Janeiro, Veterinary Institute, Department of Animal Parasitology, BR 465, Km 07, Seropédica, Rio de Janeiro 23.890-000 Brazil

ARTICLE INFO

Article history:
Received 7 August 2015
Received in revised form
4 January 2016
Accepted 13 January 2016

Keywords:
Marine bivalve
Cryptosporidium
Environmental indicator
18S
GP60

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.

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

* Corresponding author.
E-mail addresses: gfmarine@gmail.com (G.F. Mariné Oliveira), melcmcouto@gmail.com (M.C.M. do Couto), marcflima@gmail.com (M. de Freitas Lima), tcbb@superig.com.br, tcbb@ufrrj.br (T.C.B. do Bomfim).

http://dx.doi.org/10.1016/j.ijppaw.2016.01.004

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 BigDye® 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