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PARASITOLOGY

Cryptosporidium sp. in cultivated oysters and the natural oyster stock of the state of Maranhão, Brazil

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ABSTRACT: *This study detected Cryptosporidium spp. in cultivated oysters and the natural oyster stock of the state of Maranhão and determine the elective tissue(s) to examine this protozoan. For this purpose, 200 cultivated oysters were purchased from the municipality of Raposa and another 100 from Paço do Lumiar. Additionally, 100 oysters were extracted from the natural stock of the municipality of Primeira Cruz, thus making up a total of 400 oysters. They were grouped into 80 pools consisting of 5 oysters each. From each pool, the gills and visceral mass were removed to obtain 160 pools, 80 pools for the gill group and another 80 for the visceral mass group. Then, DNA was extracted from each pool using a commercial kit with modifications. Subsequently, the protozoan DNA was detected using nested polymerase chain reaction. With this technique, the DNA of the protozoan under investigation was detected in 2.5% (n = 2/80) of the pools containing gills, with 1.25% of the pools (n = 1/80) belonging to the cultivation group of oysters and the other 1.25% (n = 1/80) to the natural stock. With the results obtained in this study, it was concluded that the analyzed oysters of the genus Crassostrea, from cultivation and natural stock groups, found in the state of Maranhão, were contaminated by Cryptosporidium spp. and may become potential sources of infection in humans and other animals. In addition, the gills are the elective tissue for the study of Cryptosporidium spp. in oysters.*
Key words: *bivalve mollusc, cryptosporidiosis, nested polymerase chain reaction.*

Detecção de *Cryptosporidium* sp. em ostras de cultivo e estoque natural no estado do Maranhão, Brasil

RESUMO: *Objetivou-se com o estudo detectar Cryptosporidium sp. em ostras de cultivo e estoque natural no estado do Maranhão e determinar o(s) tecido(s) eletivo(s) para pesquisa desse protozoário. Para a realização do estudo foram adquiridas 200 ostras de cultivo do município de Raposa e 100 de Paço do Lumiar, além de 100 ostras extraídas de estoque natural do município de Primeira Cruz, totalizando 400 ostras. Estas foram agrupadas em 80 pools constituídos por cinco animais. De cada pool, as brânquias e a massa visceral foram removidas totalizando 160 pools, sendo 80 para o grupo das brânquias e 80 para o grupo de massa visceral. Na sequência, procedeu-se à extração de DNA de cada pool com a utilização de kit comercial com modificações. Posteriormente, realizou-se a detecção do DNA do protozoário por meio da técnica de Nested-PCR. Com a técnica utilizada, foi detectado o DNA do protozoário pesquisado em 2,5% (n=2/80) pools apenas de brânquias, sendo 1,25% pools (n=1/80) oriundos de cultivo e os outros 1,25% (n=1/80) de estoque natural. Com os resultados obtidos nesse estudo, conclui-se que as ostras analisadas do gênero Crassostrea sp., oriundas de cultivo e estoque natural no estado do Maranhão, estavam contaminadas por Cryptosporidium sp. e podem se reverter em fontes potenciais para seres humanos e outros animais. Para a pesquisa de Cryptosporidium sp. em ostras, as brânquias são o tecido eletivo.*
Palavras-chave: *molusco bivalve, criptosporidiose, nested-PCR.*

INTRODUCTION

Cryptosporidium spp. is an obligate intracellular protozoan that completes its biological cycle in different types of vertebrate epithelial cells, especially those of the gastrointestinal tract (THOMPSON et al., 2016; KVÁČ et al., 2016). There are descriptions of 30 *Cryptosporidium* species and genotypes that affect birds, reptiles, amphibians, fish, and mammals (LI et al., 2015; HOLUBOVÁ et al.,

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2016). Of these, 17 species (*C. parvum*, *C. hominis*, *C. canis*, *C. felis*, *C. muris*, *C. suis*, *C. meleagridis*, *C. andersoni*, *C. bovis*, *C. ryanae*, *C. baileyi*, *C. galli*, *C. molnari*, *C. scophithalmi*, *C. saurophilum*, *C. serpentis*, *and C. wrairi*) and 2 genotypes ("Cervid" and "Monkey") are related to human infection (SUNNOTEL et al., 2006; SMITH et al., 2007; FAYER et al., 2008).

Cryptosporidium spp. infect via a fecal–oral transmission route through the ingestion of oocysts present in water (SMITH et al., 2007). An infected host can excrete a large number of oocysts; however, it is assumed that the ingestion of only one oocyst of this protozoan is sufficient for the development of infection in immunodeficient individuals or in those with chronic or transient immunosuppression (ROSE et al., 2002). In addition to being small and buoyant, oocysts are resistant to environmental conditions such as high temperature and salinity (SUTTHIKORNCHAI et al., 2016).

Inadequate disposal of domestic sewage and industrial effluents, in addition to the runoff of wastewater containing feces of domestic animals and humans, are potential causes of environmental contamination by *Cryptosporidium* spp. (FRANCO et al., 2012). Therefore, the use of contaminated water and its natural resources (fish, crustaceans, and molluscs) are important points to be considered by public health authorities in the study of this protozoan (GIANGASPERO et al., 2014; SUTTHIKORNCHAI et al., 2016).

Cosmopolitan distribution, sessility, and efficient filtering behavior make oysters capable of bioaccumulating pollutants and pathogens, such as protozoa present in water (SCHETS et al., 2007); therefore, it is important to use bioindicators in aquatic environments (PALOS LADEIRO et al., 2013).

Oysters are considered as delicacies worldwide and in Brazil and can be harvested from natural banks or cultivated. The state of Maranhão has a vast coastline (approximately 640 km long) that makes it suitable for oyster farming. Along the coast of Maranhão there are estuarine areas where oysters of the genus *Crassostrea* occur naturally (FRANÇA et al., 2013). In addition, the government of the state of Maranhão has developed public policies that promote the cultivation of this type of catch.

Therefore, considering that monitoring the occurrence of the oocysts of zoonotic disease-causing protozoa in oysters is important to determine whether their consumption poses a risk to human health, in addition to the fact that oysters can serve as bioindicators of water contamination. The present study detected *Cryptosporidium* spp. in cultivated and wild oysters in the state of Maranhão and determined the elective tissue(s) to study this protozoan.

MATERIALS AND METHODS

Study site and oyster (Crassostrea spp.) collection

The samples used in the study were acquired from mariculturists in the municipalities of Paço do Lumiar (02º31′S and 44º06′W) and Raposa (02º25′S and 44º06′W) and from shellfish farmers in the municipality of Primeira Cruz (2°33′S and 43°21′W), state of Maranhão, Brazil. The selection of the study site was based on the presence of oyster crops and extraction sites (Figure 1).

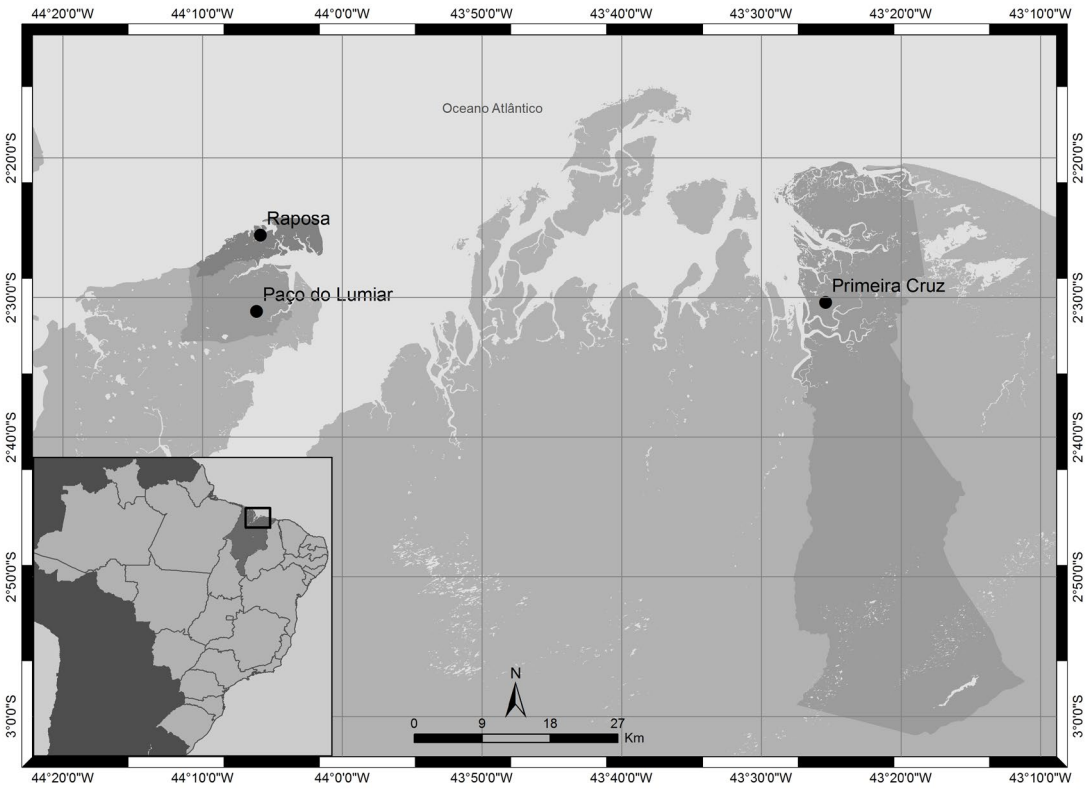
In the period from September to December 2018, 400 live oysters were acquired, 200 from the municipality of Raposa, 100 from Primeira Cruz, and 100 from Paço do Lumiar. These were grouped into 80 pools consisting of 5 animals each. In each pool, the oysters' gills and the region of the digestive glands (called visceral mass in this study) were removed. This protocol resulted in the formation of 160 pools; of which, 80 pools belonged to the gill group and 80 pools belonged to the visceral mass group. When forming pools, it was taken care that the oysters from different municipalities and of different forms of production (cultivation and natural stock) were not mixed. The sampling design of the study followed the protocol proposed by ESMERINI et al. (2010).

Sample processing

The oysters were transported alive to the Food and Water Physicochemistry Laboratory of the State University of Maranhão (UEMA) and refrigerated within 12 h after acquisition. In this laboratory, the outer surfaces of the oyster shells were washed with distilled water, and dirt [mud and barnacles (*Cirripedia*)] was removed with the aid of a brush and a sharp instrument.

Subsequently, the animals' heights (dorsoventral axis) and lengths were measured using a manual caliper, and their total mass was determined using a digital scale (0.001 g precision). Groups of five animals were formed based on similar morphometric characteristics and numbered. Subsequently, the shells of the oysters were opened using a sharp instrument, and once the shells were removed, the oysters were weighed on a scale. Next, the gills and the portions of visceral mass were excised with scissors, their weights were measured, and the pools were separately stored in labeled 2.0-mL microtubes at −20 °C.

The samples were thawed and the tissues (gills and visceral mass) of the sample groups were macerated in a crucible using a scalpel. Thus, each sample consisted of gills or visceral mass of five animals. Then, aliquots of approximately 0.5 mL were



Source: SILVA et al. (2020).

Figure 1 - Collection sites of the oysters of the genus *Crassostrea* in the state of Maranhão.

transferred to sterile 2.0 mL microtubes and stored at −20 °C until DNA extraction was performed.

DNA extraction

DNA extraction and polymerase chain reaction (PCR) were performed at the molecular biology laboratory, a part of the multiuser graduate laboratory (LAMP) at UEMA. The Wizard® genomic DNA purification kit (Promega) was used to extract DNA from the collected tissue samples (gills and visceral mass) according to the manufacturer's instructions, with some modifications related to the amount of sample (approximately 100 mg) and the procedure to break the oocyst wall [0.5 mm glass spheres were used at a ratio of 1:1 and 400 µL of Tris–ethylenediaminetetraacetic acid (TE), followed by homogenization in a vortex mixer for 10 min at 2800 rpm].

The extracted DNA was quantified using a spectrophotometer; absorbance was measured at 260 nm, and the 260/280 nm ratio was used to determine the purity of the samples (SAMBROOK & RUSSEL, 2001). Next, the concentration of the samples was adjusted to approximately 200 ng/µL using TE (pH 8.0), and the DNA was stored at −20 °C until nested PCR (n-PCR) was performed.

n-PCR

Detection of *Cryptosporidium* spp. DNA was performed according to the protocol proposed by SILVA et al. (2013). The amplification of fragments of the 18S rRNA subunit gene (611-bp fragment, GenBank number: AF108862) was performed using the n-PCR technique and the SHP1 (5′ ACC TAT CAG CTT TAG ACG GTA GGG TAT 3′) and SHP2 (5′ TTC TCA TAA GGT GCT GAA GGA GTA AGG 3′) primers in the first amplification and SHP3 (5′ ACA GGG AGG TAG TGA CAA GAA ATA ACA 3′) and SSU-R3 primers (5′ AAG GAG TAA GGA ACA ACC TCC A 3′) in the second amplification. In the first amplification, 25 µL of solution containing

3.0 µL of DNA sample, 12.5 µL of GoTaq® colorless master mix (Promega®), 7.5 µL of ultrapure water, and 10 pmol of each oligonucleotide were used.

Reactions were performed using the EP gradient thermal cycler (Eppendorf®) under the following conditions: initial denaturation at 94 ºC for 3 min, followed by 39 cycles at 94 ºC for 45 s; 56 ºC for 45 s; and 72 ºC for 60 s, with final extension at 72 ºC for 7 min. In the second amplification, the same conditions were used, replacing the oligonucleotides with the appropriate pair and using 3.0 µL of the product obtained from the first amplification. A positive control (DNA extracted from oocysts isolated from dog feces provided by the Universidade Estadual Paulista, Araçatuba Campus, from cultivation) and a negative control (water) were added to each amplification batch.

Electrophoresis and record of the amplification products

Aliquots of 5.0 µL of the samples amplified in n-PCR were homogenized with 5.0 µL of 5X sample buffer [glycerol (50%), bromophenol blue (0.125%), xylene cyanol (0.125%), and TE (pH 8.0)] and subjected to electrophoresis on 1.5% agarose gel containing 4 µL of 10 mg/mL ethidium bromide making up a final concentration of 0.4 mg/mL agarose gel. Electrophoresis was performed in a horizontal vat with 1X Tris–borate–ethylenediaminetetraacetic acid buffer solution (TBE), pH 8.0, for 45 min at 120 V. The bands were visualized under ultraviolet light, and digital images were recorded with the L-PIX Image EX software (Loccus Biotecnologia, Brazil).

RESULTS AND DISCUSSION

To our knowledge, this is the first study conducted in the state of Maranhão for the detection of *Cryptosporidium* spp. DNA in oysters. *Cryptosporidium* spp. was detected in tissue homogenates from the gills alone after molecular analysis of 160 pools were conducted (Figure 2).

In total, two gill pools (2.5%) were positive for the protozoan, one from the municipality of Primeira Cruz (n = 1/80; 1.25%) and the other from Paço do Lumiar (n = 1/80; 1.25%). The oysters from the former were obtained from the natural bank and those from the latter were obtained from suspended longline cultivation.

OLIVEIRA et al. (2016) evaluated 72 samples of mussels (*Perna perna*) from the municipality of Mangaratiba, state of Rio de Janeiro, and detected the DNA of *Cryptosporidium* spp. in 29.2% of the samples analyzed using n-PCR. According to ROBERTSON (2007) and GUIGUET LEAL & FRANCO (2008), the occurrence of this parasite in

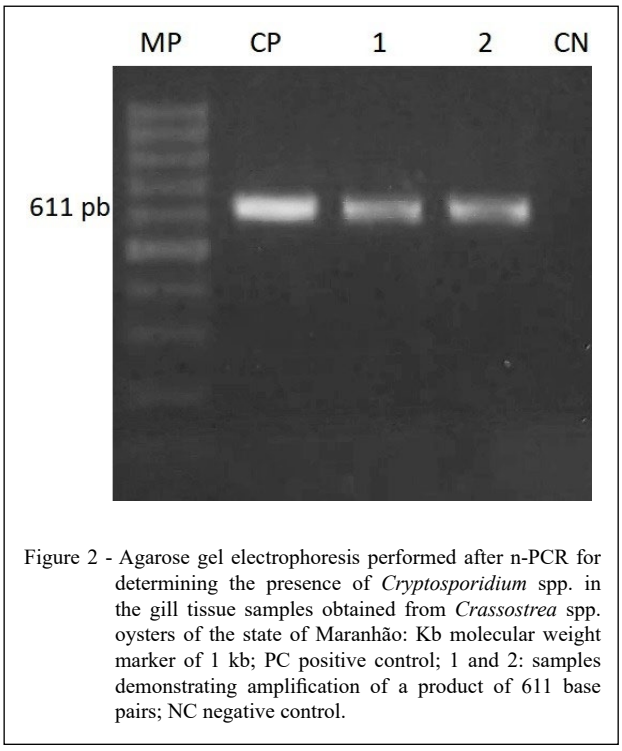


Figure 2 - Agarose gel electrophoresis performed after n-PCR for determining the presence of *Cryptosporidium* spp. in the gill tissue samples obtained from *Crassostrea* spp. oysters of the state of Maranhão: Kb molecular weight marker of 1 kb; PC positive control; 1 and 2: samples demonstrating amplification of a product of 611 base pairs; NC negative control.

edible bivalve species has already been documented in European countries (Portugal, Spain, Italy, France, the Netherlands, England, Ireland, and Northern Ireland), North America (USA and Canada), Oceania (New Zealand and Australia), and Africa (Egypt).

In Brazil specifically, contamination by *Cryptosporidium* spp. oocysts observed in the tissue homogenates of gills of *C. rhizophorae* and vongoles (*Tivela mactroides*) was first documented in 2008, with frequencies of 10% and 50%, respectively (GUIGUET LEAL et al., 2008).

Molecular techniques have been widely used for conducting research on the diagnosis and detection of *Cryptosporidium* spp. over the past 25 years (SMITH et al., 2006; THOMPSON and ASH, 2015), and n-PCR is a very sensitive molecular assay based on the amplification of a sequence present in a previously amplified fragment (RIBEIRO et al., 2015).

The detection of *Cryptosporidium* spp. oocysts in environmental or animal tissue samples is considered a challenge although n-PCR is sensitive in terms of detecting this protozoan. According to DUMÈTRE & DARDÉ (2003), there are a variety of techniques for breaking the walls of protozoan oocysts that include in vitro excystation, digestion with proteinase K, grinding with glass beads, and the use of thermal shocks. However, there is no standard protocol related to the storage temperature or number of freeze/thaw cycles to be employed. In the present study, glass spheres were used, a protocol that is similar to that used by OLIVEIRA et al. (2016), with the exception of the use of thermal shocks. Differences in the procedures used for DNA extraction may influence the results obtained in different studies.

Several factors are related to the identification of *Cryptosporidium* spp. in bivalve molluscs, such as the tissue used (LEAL & FRANCO, 2008), the cultivation environment with the presence of wild and domestic animals (KVÁČ et al., 2016), and rainfall index associated with scarcity of basic sanitation (GIANGASPERO et al., 2014; SUTTHIKORNCHAI et al., 2016).

With regard to the tissue used, some studies describe the gill as the elective tissue for the investigation of protozoa in oysters because of the filter feeding mechanism that retains light particles in the gills (ESMERINE et al., 2010; PUTIGNANI et al., 2011), which was confirmed in the present study.

With regard to the cultivation environment, when the oysters were acquired it was possible to capture a photographic record of the proximity of the oyster extraction location to a breeding site of domestic animals (pigs) and with waste (Figure 3). In this sense, it is important to highlight that oysters reflect the conditions in which they live, which is why they are considered bioindicators of environmental contamination. The cohabitation of several species is a potential cause of environmental contamination by *Cryptosporidium* spp. through the drainage of wastewater with feces from these animals to the extraction and cultivation sites.

It can be assumed that the marine environment where the sampled oysters were extracted and cultivated is contaminated with

Figure 3 - Environmental conditions of the oyster (*Crassostrea* spp.) cultivation site in the municipality of Paço do Lumiar, Maranhão. (A) movement of domestic animals and (B) deposition of solid waste.

Cryptosporidium spp. oocysts and; therefore, the protozoan moves from the terrestrial environment to the marine environment. It is also speculated that the high rainfall in the state of Maranhão during the rainy season, associated with the scarcity of basic sanitation, is a source of infection by *Cryptosporidium* spp. in oysters in the municipalities of Primeira Cruz and Paço do Lumiar.

Although the viability of oocysts has not been confirmed through the isolation of *Cryptosporidium* spp. in mice, the consumption of raw or lightly cooked oysters of the genus *Crassostrea*, which is common on the coast of Maranhão, may be a source of transmission of *Cryptosporidium* spp. in humans. Moreover, there is a lack of regulatory apparatuses that regulate the maximum permissible values of pathogenic protozoa in oysters as a way to ensure the quality and safety of this catch for human consumption.

FAYER et al. (1999) conducted a study on the presence of *Cryptosporidium parvum* in oysters harvested from the Chesapeake Bay for human consumption and, in the three collection periods, found oocysts corresponding in size and shape to those of the investigated protozoan. With regard to the results of infectivity studies, the researchers reported that in 3 of the 16 tested sites, in the three collection periods, oocysts produced detectable infections in mice and concluded that the low rate of infectivity may reflect the small number of oocysts administered to each mouse or the lack of infectivity due to age or unknown environmental effects. Therefore, it is essential that oysters are correctly and completely cooked for ensuring the safety of the consumers of this delicacy.

CONCLUSION

Under the conditions described in this experiment, DNA from *Cryptosporidium* spp. was detected in the analyzed oysters, thereby leading to the conclusion that cultivated oysters of the genus *Crassostrea* from those from the natural stock of the state of Maranhão may be contaminated by *Cryptosporidium* spp. Moreover, the gills were found to be the elective tissue for the study of this protozoan.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest. The funders had no role in study design, sample collection and laboratory processing, data analysis and interpretation, manuscript writing, or in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

The authors equally contributed to the manuscript "Detection of *Cryptosporidium* spp. in cultivated oysters and the natural oyster stock of the state of Maranhão, Brazil." All authors critically reviewed the manuscript and approved of the final version.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

Studies with invertebrates do not require approval by an institutional ethics committee according to the precepts of law no. 11,794 of October 8, 2008; decree no. 6,899 of July 15, 2009; and the rules issued by the National Council for the Control of Animal Experimentation. The provisions apply to animals belonging to phylum Chordata, subphylum Vertebrata.

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