

## ***Perkinsus* sp. infecting the oyster *Crassostrea rhizophorae* from estuaries of the septentrional Northeast, Brazil**

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(With 2 figures)

### **Abstract**

The mangrove oyster *Crassostrea rhizophorae* is an estuarine resource exploited by riverside communities in Northeast Brazil. Despite its socioeconomic importance, studies on the health status of this bivalve are scanty in this region. The purpose of the present study was to investigate the presence of the protozoan *Perkinsus* sp. in *C. rhizophorae* collected in August and September 2011 in three estuaries of the septentrional Northeast, Brazil: Jaguaribe (Ceará), Camurupim (Piauí) and Carnaubeiras (Maranhão) (n= 150 specimens/site). The samples were submitted to Ray's fluid thioglycollate medium (RFTM), PCR and histology assays. The RFTM assay revealed spherical, blue or bluish-black hypnospores of the genus *Perkinsus* in 50 specimens (Jaguaribe= 17.3%, Camurupim= 5.3%, Carnaubeiras= 10.6%). The intensity of the infection ranged from very light (1-10 cells per slide) to severe (more than 40 cells in each of 10 fields of the slide) for Jaguaribe; very light for Camurupim and very light to moderate (at least 40 cells observed in each of 10 fields of the slide) for Carnaubeiras. When submitted to confirmatory PCR analysis, 6 cases were confirmed (Jaguaribe=3, Camurupim=1, Carnaubeiras=2). The histology confirmed 21 cases of infection in specimens from the three estuaries. Although local collectors have reported no mortality in oyster populations that might be attributed to infection by *Perkinsus*, health surveillance of oyster populations in the septentrional region of Northeast Brazil is advisable.

**Keywords:** *Perkinsus*, *Crassostrea rhizophorae*, RFTM, PCR.

## ***Perkinsus* sp. infectando a ostra *Crassostrea rhizophorae* de estuários do Nordeste setentrional, Brasil**

### **Resumo**

A ostra-do-mangue *Crassostrea rhizophorae* é um recurso estuarino explorado por comunidades ribeirinhas do Nordeste do Brasil. Apesar de sua importância socioeconômica, estudos sobre o estado de saúde deste bivalve são escassos na região. O objetivo deste estudo foi investigar a presença do protozoário *Perkinsus* sp. em *C. rhizophorae* coletada em agosto e setembro de 2011, em três estuários da região setentrional do Nordeste brasileiro: Jaguaribe (Ceará), Camurupim (Piauí) e Carnaubeiras (Maranhão) (n = 150 espécimes/local). As amostras foram submetidas ao meio líquido de tioglicolato de Ray (RFTM), PCR e ensaios histológicos. A análise em RFTM revelou hipnósporos esféricos azuis ou preto-azulados do gênero *Perkinsus* em 50 espécimes (Jaguaribe= 17,3%, Camurupim= 5,3%, Carnaubeiras= 10,6%). A intensidade de infecção variou de muito leve (1-10 células por lâmina) a severa (mais de 40 células em cada um dos 10 campos da lâmina) para o Rio Jaguaribe; muito leve para o Rio Camurupim e muito leve a moderada (pelo menos 40 células observadas, em cada um dos 10 campos da lâmina) para o Rio Carnaubeiras. Quando submetidos à análise confirmatória por PCR, foram confirmados 6 casos (Jaguaribe= 3, Camurupim= 1, Carnaubeiras= 2). A histologia confirmou 21 casos de infecção em espécimes dos três estuários. Embora os coletores locais não tenham relatado nenhuma mortalidade em populações de ostras que pudesse ser atribuída à infecção por *Perkinsus*, é aconselhável um monitoramento sobre o estado de saúde de populações de ostras da região.

**Palavras-chave:** *Perkinsus*, *Crassostrea rhizophorae*, RFTM, PCR.

## 1. Introduction

The protozoan *Perkinsus* sp. belongs to a group of parasitic protozoa which infect mollusks worldwide, particularly bivalves. Since the first report of *Perkinsus marinus* infecting the American oyster *Crassostrea virginica* (Gmelin, 1791) in the Gulf of Mexico (Mackin et al., 1950), other species of the genus *Perkinsus* have been reported infecting several species of marine mollusks around the world (Lester and Davis, 1981; Azevedo, 1989; Blackburn et al., 1998; Villalba et al., 2004; Choi and Park, 2010). In Brazil, Sabry et al. (2009) recorded the first occurrence of *Perkinsus* sp. infecting natural populations of the oyster *Crassostrea rhizophorae* (Guilding, 1828) in the Paçoti river estuary (Ceará, Northeast Brazil).

According to the Office International des Epizooties (OIE), two species of the phylum Perkinsozoa, *Perkinsus marinus* and *Perkinsus olseni*, are notifiable (OIE, 2012) due to the substantial mortality rates and economic losses with which they are associated around the world (Villalba et al., 2004; Choi and Park, 2010). Thus, *P. marinus* was responsible for substantial losses in populations of the American oyster *C. virginica* along the U.S. Atlantic coast and the Gulf of Mexico (Bureson and Calvo, 1996), while *P. olseni* is known to have decimated populations of the abalone *Haliotis rubra* in Australia (Lester and Davis, 1981) and of the clam *Ruditapes decussatus* in Portugal (Azevedo, 1989).

Traditionally, infections by *Perkinsus* are diagnosed with Ray's fluid thioglycollate medium (RFTM). However, the technique does not discriminate between different species of *Perkinsus* and can lead to the misidentification of *Perkinsus* spp. and some dinoflagellate species (Almeida et al., 1999). Polymerase Chain Reaction (PCR) has been used routinely to aid in the confirmation and description of different species of *Perkinsus* infecting mollusks (Penna et al., 2001). For instance, PCR was used to identify *P. olseni* infecting *R. decussatus* on the coast of Spain and in the Mediterranean Sea (Elandaloussi et al., 2009) and *Tridacna crocea* in Vietnam (Sheppard and Phillips, 2008). Molecular studies have recognized seven species of the genus *Perkinsus*: *P. marinus*, *P. olseni* (= *P. atlanticus*), *P. mediterraneus*, *P. qugwadi*, *P. honshuensis*, *P. beihaiensis* and *P. chesapeakei* (= *P. andrewsi*) (Bureson et al., 2005).

Considering the socio-economic importance of *C. rhizophorae* to riverside populations along estuaries in Northeast Brazil, the purpose of this study was to evaluate wild populations of *Crassostrea rhizophorae* with regard to the presence of pathogens of the genus *Perkinsus* using the RFTM, histology and PCR techniques. The study complements reports published by Sabry et al. (2009), Silva et al. (2013) and Brandão et al. (2013) on the occurrence of this pathogen in the states of Ceará, Paraíba and Bahia, respectively. In addition, the study makes a significant contribution to current knowledge about the distribution of *Perkinsus* along the Brazilian coast and provides subsidies for the development of sanitary measures to prevent contamination of protozoan-free areas.

## 2. Material and Methods

### 2.1. Sampling of oysters

In August and September 2011, 450 specimens of *Crassostrea rhizophorae* were collected from the roots of the red mangrove (*Rhizophora mangle*) of three estuaries in Northeast Brazil: the Jaguaribe river (04° 27' 39,21" S, 37° 47' 31,68" W) in Ceará (n=150), the Camurupim river (02° 54' 51" S, 041° 24' 58,2" W) in Piauí (n=150), and the Carnaubeiras river (02° 50' 0,08" S, 041° 57' 36,5" W) in Maranhão (n=150). The shell height of the oysters was measured in its major axis.

The tissues of each specimen were incubated in RFTM, while the gills were fixed in 95% alcohol for confirmatory PCR analysis. Thirty specimens in each collect of 150 oysters were submitted to histological assays.

Using a thermometer and a refractometer, the temperature and salinity of the water were measured at the time of sampling for the Jaguaribe river (30 °C; 30‰), the Camurupim river (27 °C; 45‰) and the Carnaubeiras river (30 °C; 18‰). The average shell height of the oysters from Jaguaribe, Cumurupim and Carnaubeiras estuaries was 58 ± 8.4mm, 50 ± 5.4mm and 50 ± 5.2mm, respectively.

### 2.2. Incubation of tissues in RFTM

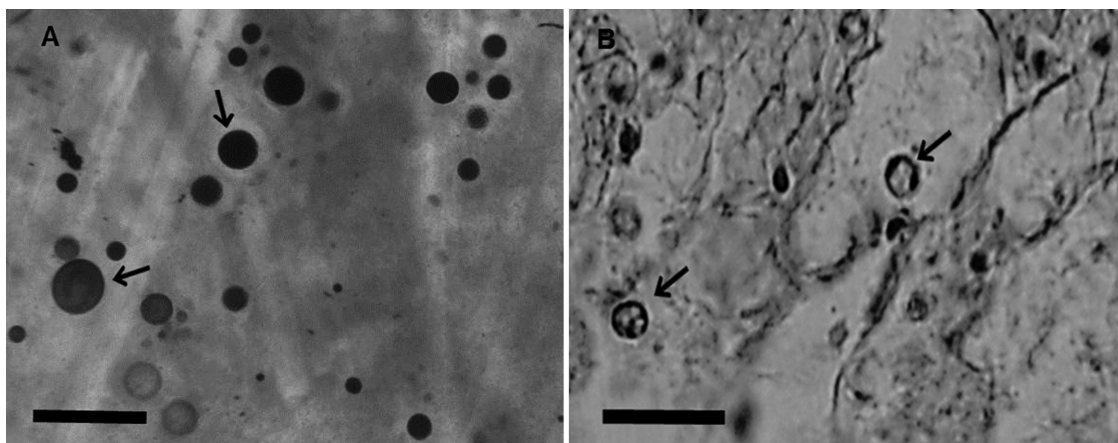
Two gill lamellae and the rectum of each specimen were incubated in RFTM (Ray, 1966) in the dark for 7 days at room temperature. Subsequently, the tissues were macerated on a slide and stained with a 3% Lugol solution. The preparation was observed under an optical microscope for the presence of spherical, blue or bluish-black hypnospores of the genus *Perkinsus*.

The prevalence of infection by *Perkinsus* was defined as the total number of infected animals divided by the total number of oysters collected in each estuary (Bush et al., 1997). The intensity of the infection was estimated according to the Mackin scale (Ray, 1954).

### 2.3. Detection of *Perkinsus* by polymerase chain reaction (PCR)

Tissues testing positive for *Perkinsus* in the RFTM assay were preserved in 95% alcohol for PCR analysis. DNA extraction was achieved with a DNAzol® kit (Invitrogen) following the manufacturer's directions, while PCR was performed with the primer set Perk ITS 85/750 (Casas et al., 2002) which specifically hybridizes with conserved regions of the internal transcribed spacers (ITS) from the rRNA gene complex, unique to members of the genus *Perkinsus* (except for *Perkinsus qugwadi* incertae sedis). DNA extracted from cells of *Perkinsus beihaiensis* was used as positive control. In the negative control, the DNA was replaced by nuclease-free water.

Each 25µL reaction contained 50-100ng DNA template, 1x proprietary PCR buffer, 1.5mM MgCl<sub>2</sub>, 0.2mM of each dNTP, 0.8mM of each primer, and 1 unit of Taq DNA polymerase (Invitrogen). The protocol included DNA denaturation at 94 °C for 10 min and 35 cycles of amplification at 94 °C (1 min), 55 °C (1 min) and 72 °C (1 min), followed by a final extension at 72 °C for 10 min.



**Figure 1.** Histological sections showing cells of *Perkinsus* infecting the oyster *Crassostrea rhizophorae*. (A) *Perkinsus* sp. hypnospores in gill tissues of *C. rhizophorae* after incubation in RFTM and stained with Lugol's iodine solution. Scale bar = 50 $\mu$ m. (B) Trophozoite of *Perkinsus* sp. (arrow) in connective tissue near the digestive gland. Scale bar = 20 $\mu$ m.

The PCR products were separated by electrophoresis on 1.5% standard TAE agarose gel containing ethidium bromide.

#### 2.4. Histological sections

The gills, gonads and digestive gland of 30 specimens from each collect of oysters (n=150) were excised and fixed in Davidson's solution (Shaw and Battle, 1957) for 24 hours. Following embedding in histological paraffin at 60 °C, 5 $\mu$ m sections were prepared and stained with Harris' hematoxylin and eosin (H & E) (Howard et al., 2004) and analyzed under an optical microscope. The observed prevalence of *Perkinsus* was defined as the number of infected specimens divided by the total number of analyzed oysters (Bush et al., 1997).

### 3. Results

#### 3.1. Detection of *Perkinsus* sp. by RFTM assay

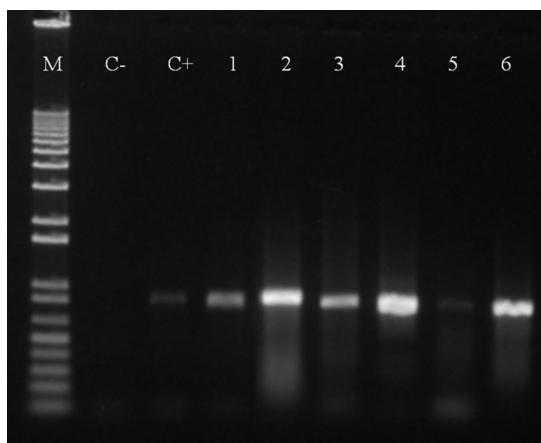
Fifty oysters from the three collects tested positive for perkinsiosis in RFTM (50/450). The enlarged, spherical cells of the pathogen (5-55 $\mu$ m in diameter) stained black in Lugol (Figure 1A). The prevalence of infected oysters was 17.3% (Jaguaribe), 5.3% (Camurupim) and 10.6% (Carnaubeiras) (Table 1). The intensity of the infection was very light to severe (Jaguaribe), very light (Camurupim) and very light to moderate (Carnaubeiras).

#### 3.2. Detection of *Perkinsus* sp. by PCR

The 50 specimens testing positive for *Perkinsus* sp. in the RFTM assay were submitted to confirmatory PCR analysis. Six cases were confirmed (Jaguaribe n=3, Camurupim n=1, Carnaubeiras n=2) (Table 1 and Figure 2). The primer pair PerkITS produced amplicons of expected size (850 bp) in all the samples, confirming the presence of *Perkinsus* sp. (Figure 2).

#### 3.3. Histopathological analyzes

The histological analysis confirmed 21 cases of the 50 cases detected by RFTM (Table 1). Trophozoites of *Perkinsus* were detected in the gills and connective tissues



**Figure 2.** Molecular diagnosis of *Perkinsus* sp. in 1.5% agarose gel. Detection of the rDNA ITS region of *Perkinsus* sp. by PCR. M: molecular marker (1 Kb Plus, Invitrogen), C-: negative control (ultra-pure water); C+: positive control; lanes 1 (CE), 2 (CE), 3 (PI) 4 (MA), 5 (PI) and 6 (CE): positive samples.

**Table 1.** Prevalence (%) of infection by *Perkinsus* sp. in 450 specimens of the oyster *Crassostrea rhizophorae* collected in three estuaries in Northeastern Brazil: Jaguaribe (Ceará), Camurupim (Piauí) and Carnaubeiras (Maranhão). The number of *Perkinsus*-positive oyster per sample is shown in parenthesis for each diagnostic technique.

Estuary	RFTM (%)	Histology (%)	PCR (%)
Jaguaribe	17.3 (26/150)	33.3 (10/30)	11.5 (3/26)
Camurupim	5.3 (8/150)	20 (6/30)	25.0 (2/8)
Carnaubeiras	10.6 (16/150)	16.6 (5/30)	6.2 (1/16)

around the digestive gland of the oysters. The trophozoites were spherical, with a vacuole occupying most of the cytoplasm (Figure 1B). No damage or hemocyte infiltration was detected in the tissues infected by the pathogen.



#### 4. Discussion

In this study we report the occurrence of the protozoan *Perkinsus* sp. infecting the oyster *Crassostrea rhizophorae* in three estuaries of the states Ceará, Piauí and Maranhão, Northeast Brazil. *Perkinsus* sp. was detected by RFTM assay, a sensitive and reliable diagnostic technique, and subsequently submitted to confirmatory molecular diagnostics and histology.

The average prevalence of *Perkinsus* sp. infecting the oysters collected for this study was relatively low (up to 17.3% by RFTM). This is consistent with the absence of records of mortality among these oyster populations which might be associated with the parasite. However, the values found were higher than the prevalence of 6.7% recorded by Sabry et al. (2009) in oysters from Pacoti river, another estuary in Ceará. On the other hand, despite this difference, Brandão et al. (2013) reported a mean prevalence of 63% for *Perkinsus* sp. in *C. rhizophorae* populations from Bahia and Silva et al. (2013) prevalence of 92% for *Perkinsus* sp. and up to 100% for *Perkinsus marinus* in the same species from Paraíba, two other states in Northeast Brazil.

Only 6 of the 50 cases detected by RFTM assays were confirmed by PCR, probably because most infections were very light and/or because the amount of gill tissue retrieved from each specimen for DNA extraction was too small to ensure the detection of the pathogen. In a discussion on the occurrence of false-negative PCR results in the detection of parasites in bivalves, Burreson (2008) reached the same conclusion. Other authors have reported discrepancies between RFTM and PCR results (Reece et al., 2008; Sabry et al., 2009), and other causes of sampling errors can compromising the accuracy of PCR assays, including the effects of focal or localized lesions (Burreson, 2008). Histological sections of oyster tissues contained cells of *Perkinsus* sp. with a vacuole occupying most of the internal volume of the cytoplasm, an eccentric nucleus and prominent nucleoli, as described by Sabry et al. (2009, 2013) and Brandão et al. (2013). These authors detected changes in oyster tissues and organs infected by *Perkinsus* sp. in addition to phagocytosed trophozoites. In this study, no such changes were observed, possibly because of the lightness of the infection, but further studies are needed to better understand the effects of this pathogen on the host.

The results of the three detection methods employed in the present study (RFTM assay, histology and PCR) unequivocally confirm the occurrence of *Perkinsus* infecting native populations of *C. rhizophorae* in three estuaries in Northeastern Brazil. This is the first report of *Perkinsus* from coastal regions in northern South America. Although no *Perkinsus*-related mortality has been reported for this region, health surveillance of oyster populations in Northeast Brazil is advisable, especially when the identity of the species remains uncertain.

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