Perkinsus beihaiensis infecting the oyster *Crassostrea rhizophorae* under cultivation and in natural stock in Camamu Bay, Bahia, Brazil

Perkinsus beihaiensis infectando a ostra Crassostrea rhizophorae em cultivo e em estoque natural na Baía de Camamu, Bahia, Brasil

Mariane dos Santos Aguiar LUZ[1]; Guisla BOEHS[1]

[1] Universidade Estadual de Santa Cruz, Programa de Pós-Graduação em Ciência Animal, Ilhéus – BA, Brazil

Abstract

This study investigated the infection of *Perkinsus beihaiensis* (Perkinsozoa) in the oyster *Crassostrea rhizophorae,* both from a long-line cultivation system and from a nearby intertidal zone of mangrove, both in the state of Bahia, northeastern Brazil. The collections were performed in October and November 2012, and in January 2013. The oysters (n = 300) were measured, examined macroscopically for signs of infection and then submitted to the following laboratory techniques: histology, Ray's fluid thioglycollate medium assay (RFTM), polymerase chain reaction (PCR) and sequencing, which confirmed the identification of the pathogen. Histological and RFTM analyses showed, respectively, a mean prevalence of 93.3% and of 69%. The infection was usually mild or very mild. There was no significant difference ($p > 0.05$) between the environments in terms of infection prevalence or severity. This is the first record of *P. beihaiensis* in the state of Bahia and the second in oysters from Brazil and South America.

Keywords: Bivalves. "Dermo". Diseases. Oyster farming. Perkinsiosis.

Resumo

Foi investigada a infecção de *Perkinsus beihaiensis* (Perkinsozoa) na ostra *Crassostrea rhizophorae* em um sistema de cultivo do tipo espinhel e em um estoque natural de ostras no manguezal adjacente ambos localizados no estado da Bahia, Nordeste do Brasil. As colheitas foram realizadas em outubro e novembro de 2012 e em janeiro de 2013. As ostras (n = 300) foram medidas, examinadas macroscopicamente quanto a sinais da infecção e submetidas às técnicas laboratoriais: histologia, ensaio em meio de cultivo de tioglicolato de Ray (RFTM), reação em cadeia da polimerase (PCR) e sequenciamento, que confirmou a identificação do patógeno. As análises histológicas e o RFTM mostraram, respectivamente, prevalência média de 93,3% e de 69%. A infecção foi geralmente leve ou muito leve. Não houve diferença significativa ($p > 0,05$) entre os ambientes em termos de prevalência ou severidade da infecção. Este é o primeiro registro de *P. beihaiensis* no estado da Bahia e o segundo em ostras do Brasil e América do Sul.

Palavras-chave: Bivalves. "Dermo". Doenças. Ostreicultura. Perkinsiose.

Correspondence to:
Guisla Boehs
Universidade Estadual de Santa Cruz, Departamento de Ciências Biológicas
Rodovia Jorge Amado, km 16
CEP 45662-900, Ilhéus, Bahia, Brazil
E-mail: gboehs@uesc.br

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Introduction

Several species of bivalve mollusks of economic value in different regions of the world can be infected by protozoa from *Perkinsus* genus. Since it was first recorded in the oyster *Crassostrea virginica,* on the coast of the United States (MACKIN et al., 1950), many studies have been conducted and aspects of this pathogen are now being well understood. This

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protozoan is highly infective at all stages of its life, and usually causes serious damage to its host, which can result in death (VILLALBA et al., 2004).

Although its existence was first described in the northern hemisphere several decades ago, this pathogen has only recently been detected in South America. *Perkinsus olseni* was the first species discovered, in *Pitar rostrata*, in Uruguay (CREMONTE et al., 2005).

In Brazil, *Perkinsus* was first recorded in Ceará, in the country's northeastern region in the oyster *Crassostrea rhizophorae* (SABRY et al., 2009). Its occurrence has more recently been recorded in the states of Bahia (BRANDÃO et al., 2013), Paraíba (DA SILVA et al., 2013) and Sergipe (DA SILVA et al., 2014), always in oysters of the genus *Crassostrea*. Additionally, *P. beihaiensis* was also registered in *Anomalocardia brasiliana* (Veneridae) from Ceará (PINHO FERREIRA et al., 2015). These studies, taken together, indicate the existence of several species of *Perkinsus* in northeastern Brazil, as reported prevalence and infection levels. However, many aspects of this pathogen need to be better understood, particularly as regards their life cycle and effects on the host. This is useful for the implementation and management of mollusks cultivation in this region.

The state of Bahia, in Brazil, has a coastline that is around 1100 km long, with several areas of estuaries and mangroves. Wild stocks are extensively fished, primarily fish, crustaceans and mollusks. Oysters are farmed, not intensively, only in a few estuaries of the state, but it is still on a small scale. Increasing production is encouraged mainly by government agencies, through various extension projects, mainly involving traditional extractive communities of the coast.

The present study aimed to investigate *Perkinsus* sp. in the oyster *C. rhizophorae*, under cultivation and in natural stock in Camamu Bay, Bahia, Brazil.

Materials and Methods

Collections and initial laboratory processing

The oysters were collected from a long-line cultivation system and from a mangrove swamp adjacent to Porto do Campo (13°57'S, 39°02'W), both in Camamu Bay, Bahia, Brazil (Figure 1). One hundred oysters were monthly examined (50 from the cultivation system and 50 from the mangrove) in October 2012, November 2012 and January 2013. In the laboratory, the oysters were measured for height (dorsal-ventral axis) (GALTSOFF, 1964): the mean height of the cultivated oysters was 5.7 ± 0.6 cm (n = 150) and for the oysters from the mangrove, 5.3 ± 0.7 cm (n = 150). The shells were subsequently opened and each oyster meat was macroscopically examined for clinical signs of protozoan of the genus *Perkinsus*, such as weight loss, pale appearance and presence of pustules, as reported previously (BOWER et al., 1994; BONDAD-REANTASO et al., 2001). There are no reports of macroscopic signs of *Perkinsus* spp. in studies on the Brazilian coast.

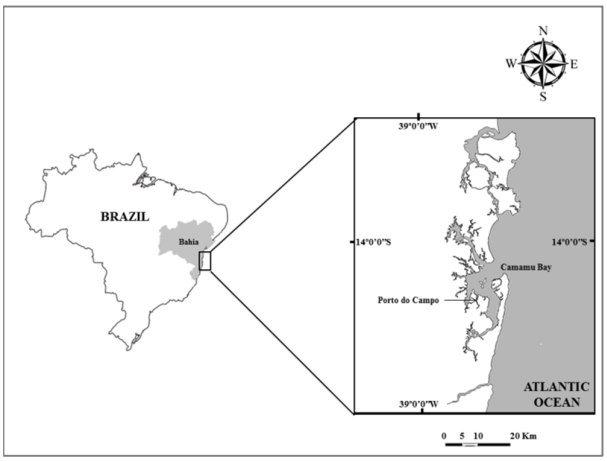

Figure 1 – Map of the study area, with the collection point in Porto do Campo, Camamu Bay, Bahia, Brazil

Histology

A 5 mm longitudinal section was removed from each oyster and subsequently fixed in Davidson's solution for 30 hours (SHAW; BATTLE, 1957). Tissues were then processed by means of classical histological technique and stained with Harris's hematoxylin and eosin (H&E) (HOWARD et al., 2004). Light microscopy was used to analyze the tissues for the presence of *Perkinsus*, tissue damage and evidence of host response. Parasite prevalence was calculated as the ratio between the number of infected oysters and the total number of oysters analyzed (BUSH et al., 1997). Chi-square test was used to compare parasite prevalence between the two environments. The significance level used was 95%.

Culturing in Ray's fluid thioglycollate medium - RFTM

Two gill lamellae and the rectum together from each oyster (n = 300) were incubated in Ray's fluid thioglycollate medium (RFTM), with the use of antibiotics (penicillin and streptomycin) and antifungal medication (nystatin) (RAY, 1963). The medium was incubated for seven days in the dark, at room temperature (20-25°C). Following this period, the tubes were opened and the oyster tissue fragments were removed. These were then macerated and subsequently stained in 3% iodine solution for observation under an optical microscope (OIE, 2009). The infection intensity was calculated according to the scale developed by Ray (1954) and modified by Sabry et al. (2009):

- Nil infection: zero hypnospores on the whole slide (100 x);
- Very mild infection: up to 10 hypnospores on the whole slide (100 x);
- Mild infection: 11-100 hypnospores on the whole slide (100 x);
- Moderate infection: at least 40 hypnospores observed in 10 different fields (400 x);
- Severe infection: more than 40 hypnospores observed in 10 different fields (400 x).

Molecular tools

A polymerase chain reaction (PCR) on 23 RFTM-culture positive oysters and 6 negative oysters were performed. Subsamples of 25-70 mg from the digestive gland and gills, preserved in 95% ethanol, were subjected to total DNA extraction, using DNAzol (Invitrogen®), following the manufacturer's protocol. The primer pair PerkITS85/750 (CASAS et al., 2002) was used for the PCR reactions: these specifically hybridized in conserved regions of the internal transcribed spacers (ITS1 and 2) and the 5.8S region of the ribosomal ribonucleic acid (rRNA) gene complex, which are exclusive to members of the genus *Perkinsus* (except for *P. qugwadi*). The PCR reactions were performed in a total volume of 12.5 µl, containing 100-200 ng of genomic DNA, 1x PCR buffer concentrate, 1.5 mM of $MgCl_2$, 0.2 mM of each nucleotide, 0.8mM primers and one unit of Taq DNA polymerase. The positive control used in the PCR was DNA from *P. beihaiensis* (provided by R. Sabry and M. Dantas, Labomar, Ceará, Brazil). Nuclease-free water was used as the negative control. The protocol included DNA denaturation at 94°C for 10 minutes; 35 cycles of amplification at 94°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute; followed by a final extension at 72°C for 10 minutes. The PCR products were separated in 1.5-2% agarose gel, in Tris-acetic acid-EDTA (TAE) buffer, stained with ethidium bromide and viewed under UV light.

Samples were sequenced by ACTGene Análises Moleculares (Porto Alegre, RS, Brazil) using an automatic sequencer (AB 3500 Genetic Analyzer) equipped with 50 cm capillaries and POP7 polymer (Applied Biosystems).

The sequences were aligned with those of other species of *Perkinsus* contained in GenBank, using the Mega 6.0 and Bioedit 5.0.9 software. ITS rRNA gene sequences from this study and those from GenBank (n = 30) were used for the phylogenetic analysis. The sequences from GenBank that were used were: *P. chesapeaki* (= *P. andrewsi*) AY876302 and AY876311; *P. olseni* (= *P. atlanticus*) AF441207, AF441209, AF441211, EF204082 and EF204083; *P. marinus*

AY295180, AY295188, AY295194 and AY295197; *P. mediterraneus* AY487834 and AY487835; *P. honshuensis* DQ516697 and DQ516698; *P. beihaiensis* EU068080 to EU068088, EF204068 and EF526436, from China; and JN054739 and JN054741 from India; and *P. beihaiensis*-like JX502842 from Brazil. The outgroup taxon was *P. qugwadii* AF151528.

The sequences obtained during this study were submitted to GenBank with the access number KP300873.

Neighbor-joining phylogenetic trees and distance matrices were produced using the Mega 6.0 software, based on alignment with sequences that are publicly available at GenBank.

Results and Discussion

Macroscopic and tissue analyzes

Macroscopically, no clinical signs suggestive of the presence of *Perkinsus* sp. was observed. The tissue analysis showed trophozoites, sized between 3 and 10 μm (mean: 6.8 ± 2.2 μm; n = 30), in the epithelium of the digestive gland, stomach and intestine and in the mantle. Schizonts (dividing trophozoites) were also observed in these same places, and they measured between 2 and 6 μm (mean: 4.2 ± 1.8 μm; n = 30).

Both schizonts and trophozoites occurred mostly in the stomach. Infiltration and phagocytic hemocytes were observed in areas with highest concentrations of the pathogen. The presence of hemocytes phagocytizing *Perkinsus* sp. indicates a defense response from the host. In *Tapes philippinarum* infected by *Perkinsus* sp. in Korea, despite encapsulation, there were cases of atrophy in the digestive epithelium caused by the pathogen (LEE et al., 2001), which, however, was not observed in this study.

There was no statistical difference (p = 0.16; n = 300) in the prevalence of *Perkinsus* sp. among oysters from the mangrove or the cultivation. The mean prevalence was of 93.3% (Table 1).

Table 1 – Prevalence of *Perkinsus beihaiensis* in *Crassostrea rhizophorae* shown through using Ray's fluid thioglycollate medium (RFTM) and histological techniques in a mangrove environment (n = 150) and in cultivation (n = 150) – Camamu, Bahia, Brazil – 2012 and 2013

	Mangrove		Cultivation	
	RFTM	Histology	RFTM	Histology
October 2012	74%	92%	50%	100%
November 2012	82%	100%	46%	86%
January 2013	76%	94%	88%	88%

Results from culturing in RFTM

Tissues from the rectum and the gills incubated in RFTM showed *Perkinsus* sp. at the hypnospore stage. The cells measured between 5 and 75 μm in diameter. The mean prevalence was 69% in both gill lamellae and rectum (Table 1). This value was higher than that found in the state of Ceará (Brazil) (5.78%) (SABRY et al., 2009) and slightly higher than that found of *Perkinsus* sp. on the southern coast of Bahia (63%) (BRANDÃO et al., 2013).

With regard to the environment, the prevalence was similar among the oysters from the mangrove and from the cultivation, thereby indicating that confinement apparently did not have any negative influence on this pathogen's occurrence. Infection in mangroves was, in most cases, mild (level I) and mild-light (level II). In the cultivation, the infection most common was nil infection (level 0) or mild-light (level II) (Figure 2).

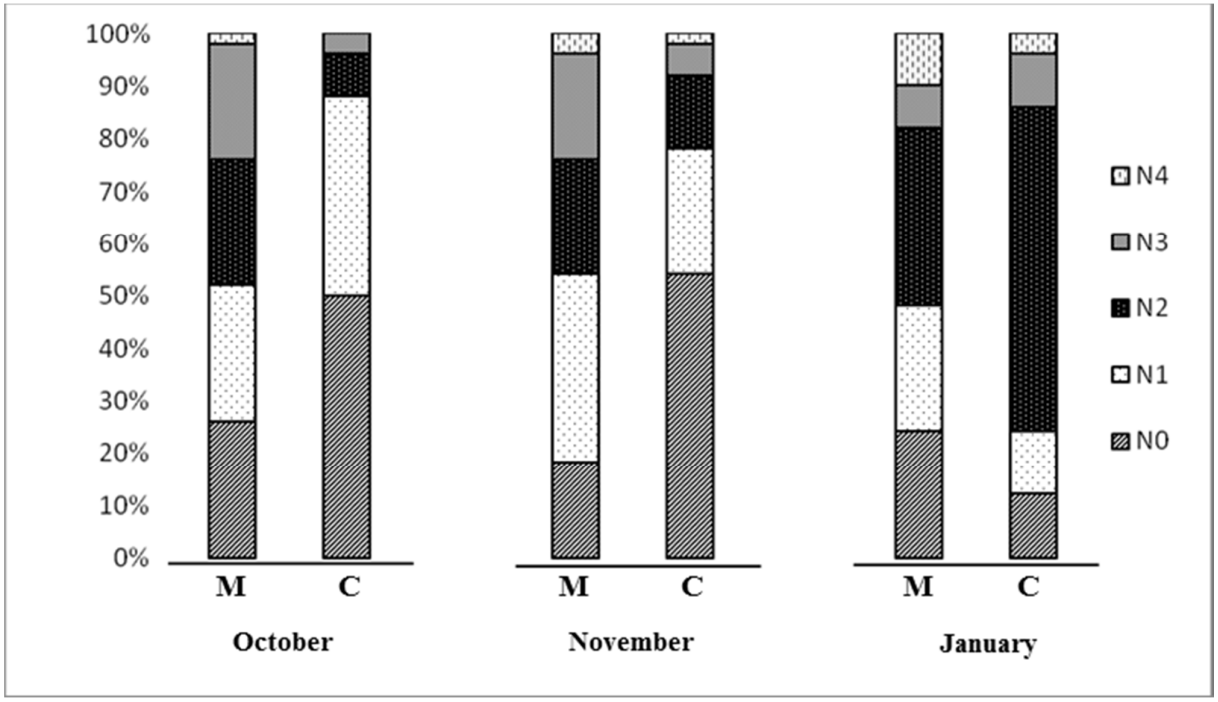

Figure 2 – Intensity levels of *Perkinsus beihaiensis* infection in *Crassostrea rhizophorae* from the mangrove (M) and the cultivation (C) (n = 300) Description of the different levels of parasite intensity: Nil infection: zero hypnospores on the whole slide (100 x); Very mild infection: up to 10 hypnospores on the whole slide (100 x); Mild infection: 11-100 hypnospores on the whole slide (100 x); Moderate infection: at least 40 hypnospores observed in 10 different fields (400 x); Severe infection: more than 40 hypnospores observed in 10 different fields (400 x) (UESC-LMM, 2012-2013)

Diagnosis by means of molecular tools

Among the total RFTM-positive samples that were subjected to PCR, 47.83% were positive and produced amplicons of the expected size (700 bp). The five rRNA gene sequences obtained in this study, which were analyzed using the Basic Local Alignment Search Tool (BLAST) (NCBI), showed 99% similarity with *Perkinsus beihaiensis*-like sequences from Ceará, Brazil (SABRY et al., 2009, 2013) and China (MOSS et al., 2008) and 99-100% similarity with *P. beihaiensis* sequences from India (SANIL et al., 2012). Neighbor-joining phylogenetic analysis showed support capacity values of 100% for confirming the species as being *P. beihaiensis* (Figure 3).

Efficiency of laboratorial techniques

All RFTM-culture negative samples were also negative using PCR, 47.83% were positive using both techniques and 52.17% were positive using RFTM and negative using PCR. Very mild and mild infection levels were numerically represented by 1-10 and 11-100 hypnospores observed on the entire slide, respectively. At these levels, the protozoa may be unevenly distributed in the organ tissue, meaning that the fragment (25-50 mg) used for PCR might not contain the parasite. In comparing RFTM and histological techniques, although the first is considered more sensitive than the second (OIE, 2006), the histology showed higher prevalence of *P.*

beihaiensis, and a similar result was observed previously (SABRY et al., 2013). These differences were most likely due to the use of only some organs (gills and rectum) in RFTM and PCR techniques and a greater number of organs in histology. Tissue analysis showed a higher prevalence of the parasite in the stomach, which was not used in RFTM and PCR techniques. We suggest conducting further investigation on the distribution of the parasite in the host, as well as using the same organs in different techniques.

In conclusion, despite the high prevalence, apparently *P. beihaiensis* is not causing severe damage to *C. rhizophorae*, both on-farm and in natural stock, which is due to low levels of infection. However, we suggest that studies and monitoring of *Perkinsus* spp. should continue in relation to oysters and other bivalves in northeastern Brazil.

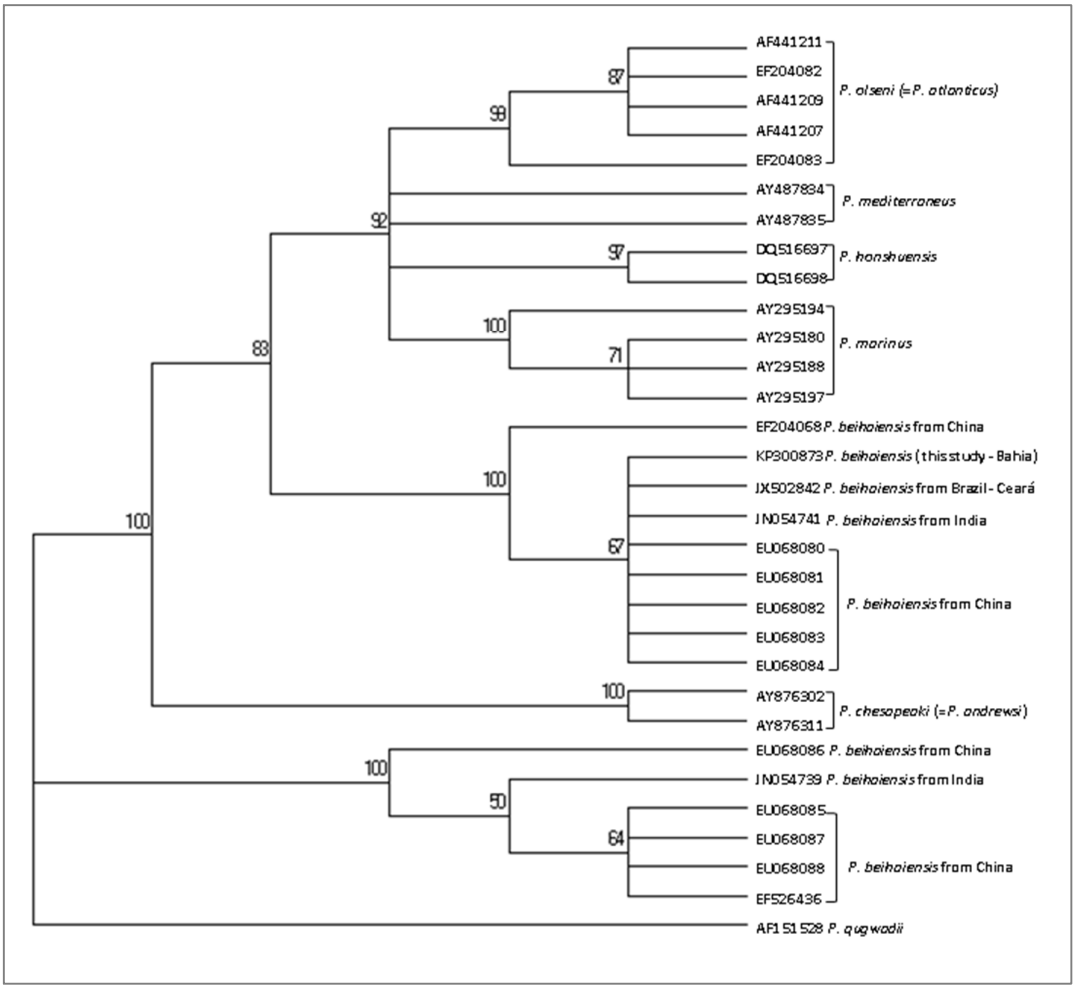

Figure 3 – Phylogenetic analysis by means of the neighbor-joining method with bases in 601 bp sequences for species of *Perkinsus*. The numbers near the branches represent support after a bootstrap of 1000 replications (only bootstrap values greater than 60 are shown). (UESC-LMM, 2012-2013)

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