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Short Communication

Perkinsus sp. infecting oyster *Crassostrea rhizophorae* (Guilding, 1828) on the coast of Bahia, Brazil

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ABSTRACT

This study investigated the occurrence of the protozoan *Perkinsus* in the oyster *Crassostrea rhizophorae* on the coast of Bahia State, Brazil. The oysters (n = 900) were collected in February–March and July–August 2010. The Ray's fluid thioglycollate medium (RFTM) analysis of gills and rectum revealed hypnospores of *Perkinsus* sp. with a high mean prevalence (63%). The infection intensity varied from very light to advanced. The polymerase chain reaction confirmed *Perkinsus* in 87.2% of the RFTM-positive oysters. Histological analysis showed trophozoites and schizonts phagocytized by hemocytes, mainly in the intestine and the stomach epithelium.

1. Introduction

Protozoan parasites can significantly impair the health of natural and cultivated populations of bivalves (Bower et al., 1994). Perkinsiosis is a disease caused by protozoa of the genus *Perkinsus*, comprising six species currently considered valid: *P. marinus*; *P. olseni* (=*P. atlanticus*), which can cause mass mortalities; and *P. chesapeaki* (=*P. andrewsi*); *P. mediterraneus*, *P. honshuensis* and *P. beihaiensis* (Villalba et al., 2004; Choi and Park, 2010). The oyster *Crassostrea rhizophorae* proved to be susceptible to artificial infection with *P. marinus* (Bushek et al., 2002).

Until recently the only record of a representative of the genus *Perkinsus* in waters close to Brazil was *P. olseni* infecting the bivalve *Pitar rostrata* in Uruguay (Cremonte et al., 2005). In Brazil, Sabry et al. (2009) recorded the first case of *Perkinsus* infecting the oyster *C. rhizophorae* from the coast of Ceará State, which revealed a close molecular identity with *P. beihaiensis*. The authors did not find *Perkinsus* in the same oyster species from Santa Catarina State, southern Brazil; this state is the largest producer of the Japanese oyster *C. gigas* in Brazil, and is located close to Uruguay. In addition, da Silva et al. (2012; in preparation) have recently reported *Perkinsus* spp. in oysters *C. gasar* (=*C. brasiliana*) from Sergipe State, northeast Brazil.

Considering the wide geographic distribution of *C. rhizophorae*, including the entire Brazilian coast (Rios, 2009), the reports of *Perkinsus* sp. infecting this oyster species in the northeastern region of Brazil, and the increasing interest in oyster farming in Bahia State, this study investigated the presence and impact of the protozoan *Perkinsus* sp. on the oyster *C. rhizophorae* in this region.

2. Materials and methods

Sampling of *C. rhizophorae* was carried out at two sites in the Maraú River estuary: MA-I (13°57′96″S; 39°00′03″W) and MA-II (14°06′55″S; 39°02′08″W); and one site in the Graciosa River estuary: GRA (13°28′76″S; 39°05′56″W) (Fig. 1). Oysters were collected (n = 150/site) in two seasons of the year 2010, summer (February–March) and winter (July–August). Water temperature and salinity were recorded in each day of sampling.

Gills and rectum of each oyster (n = 900) were removed and incubated in Ray's fluid thioglycollate medium (RFTM), with added penicillin (100 U/ml), streptomycin (100 μg/ml) and nystatin (100 U/ml) antibiotics (Ray, 1966). The tissues were incubated for seven days in the dark at room temperature. Subsequently the tissue fragments were macerated with a scalpel, stained with Lugol's solution and observed under a light microscope for the presence of *Perkinsus* sp. hypnospores. The infection intensity was determined by counting the hypnospores and classifying the oysters into four levels (very light, light, moderate and advanced) of infection intensity according

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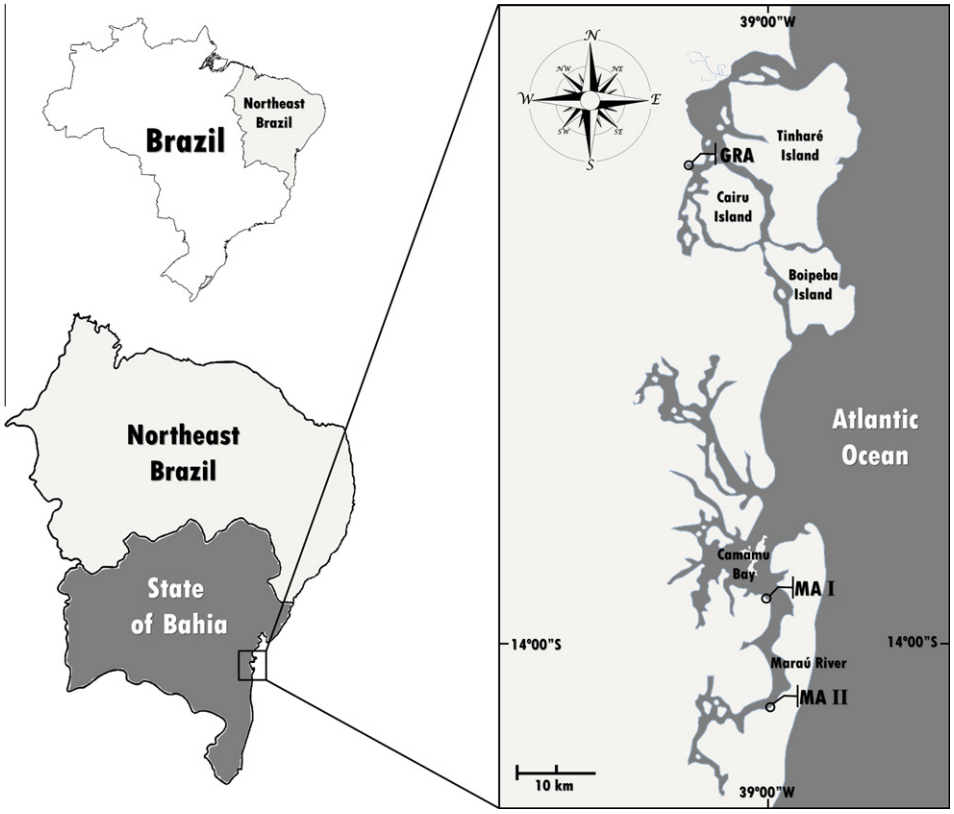

Fig. 1. Map of Brazil showing the northeastern region and indicating the sampling localities on the southern coast of Bahia.

to the scale of Ray (1954) modified according to Sabry et al. (2009). The prevalence of *Perkinsus* sp. was calculated as the percentage of infected oysters at each site and season.

Gill samples from each oyster were preserved in 95% ethanol for PCR. 12 oysters from each level of *Perkinsus* sp. infection, except level 4 (eight oysters) and non-infected oysters were submitted to PCR. Briefly, one 25–90 mg subsample of gill tissue was removed from the ethanol, and total DNA was extracted with DNAzol (*Invitrogen*®) reagent according to the manufacturer's instructions. PCR reactions were performed using the primer pair PerkITS 85/750 (Casas et al., 2002) that hybridize specifically in conserved regions of the internal transcribed spacers (ITS1 and 2) and the 5.8S region of the ribosomal RNA gene complex (rRNA), exclusively for members of the genus *Perkinsus* (except for *P. qugwadi*). The positive control of the PCR reaction was DNA obtained from *P. olseni*. Amplifications were performed with 100–200 ng of genomic DNA in 25-μl reactions, using reaction conditions according to Sabry et al. (2009). Cycling parameters were as follows: denaturing at 94 °C for 10 min; 35 cycles (94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min), and a final extension at 72 °C for 10 min.

Thirty oysters per site and season ($n = 180$) were randomly selected for histopathological analysis. Briefly, a diagonal section 5 mm thick was removed and fixed in Davidson's solution (Shaw and Battle, 1957) for 24 h. The tissues were prepared according to conventional histology technique and stained with Mayer's hematoxylin and eosin (Howard et al., 2004). The histological sections were analyzed under a light microscope for the presence of *Perkinsus* sp. cells, tissue injury and host defense reactions.

3. Results

In infected oysters, the tissues incubated in RFTM showed hypnospores of *Perkinsus* sp., which were spherical cells with a diameter of 5–70 μm, stained black or bluish-black with lugol. Oysters from all three sites were infected with *Perkinsus* sp., resulting in a mean overall prevalence of 63%, and mean prevalences of 66.7% in summer and 59.3% in winter. The local prevalence of infection varied (MA-I: 65%; MA-II: 89.3% and GRA: 34.7%). Infection intensities showed the same pattern as prevalence (Table 1).

The PCR analysis produced amplicons of the expected size (~700 bp) in 36 (85.7%) of the 42 RFTM-positive samples analyzed, and detected 3 of the 12 RFTM-negative samples. PCR was more effective in confirming the cases of moderate (91.7%) and advanced (100%) infections, than the light (83.3%) and very light (75%) infections.

Trophozoites of *Perkinsus* sp. were identified in oyster tissues from all three sites and both seasons of the year. The most intensely infected organs were the intestine and the stomach, but the secondary tubules of the digestive gland and esophagus were also sporadically infected. Parasite cells were not observed in the gills. The trophozoites, generally 1–3, were found mostly in phagocytic hemocytes infiltrating the epithelium of these organs (Fig. 2A). The trophozoites were spherical and measured 5–7 μm in diameter; they had a vacuole occupying most of the cytoplasm, an eccentric nucleus, and a patent nucleolus (Fig. 2B). Schizonts were also observed (Fig. 2A and C). The trophozoites often appeared necrotic, with loss of color of the nucleus (Fig. 2A), and in other cases with signs of pyknosis and cytoplasmic acidophilia. Most oysters showed a very low degree of tissue infection and no changes in the structure of affected organs, except on three occasions in which moderate hemocytic infiltration of the connective tissue and swelling of the intestinal epithelium due to the presence of several infiltrated infected hemocytes were observed (Fig. 2A). The rate of detection of *Perkinsus* sp. by histology was very low, 38.5% of RFTM-positive oysters.