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Short Communication

First record of *Perkinsus marinus* infecting *Crassostrea* sp. in Rio Grande do Norte, Brazil, using real-time PCR

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ABSTRACT

A pathogen with high virulence potential in some host species, *Perkinsus marinus* remains a challenge for the ecological integrity of marine ecosystems and the health of bivalve molluscs. This study investigates the occurrence of *P. marinus* in *Crassostrea* sp. in estuaries of the Potengi River and the Guaraíras lagoon in Rio Grande do Norte, Brazil. A total of 203 oyster samples that tested positive for *Perkinsus* sp. in Ray's fluid thioglycollate medium (RFTM) were subjected to species-specific quantitiative PCR, where 61 animals (30.05 %) presented amplification graphs with a melting temperature of 80.1 ± 0.6 °C matching the positive control. This was the first record of *P. marinus* in oysters in these estuaries using qPCR as a diagnostic tool.

1. Introduction

The introduction of pathogens into disease-free areas or sanitary-controlled regions is one of the main threats to the maintenance of coastal ecosystems and the health of commercially important fishery resources. Bivalve molluscs are one of the aquatic organisms most affected by diseases caused by microorganisms, a number of these caused by protozoans of the genus *Perkinsus*, which can cause serious mortality events producing significant economic losses (Villalba et al., 2004). When they do not cause death, these protozoa can impair reproductive performance and host health generally, reducing growth and condition and weakening defenses, which favors opportunistic secondary infections (Montes et al., 2001; Lee et al., 2001; Itoïz et al., 2021). *Perkinsus marinus* is particularly pathogenic notifiable to the World Organisation for Animal Health (WOAH, 2022), having been responsible for mass mortalities of oyster populations since the 1940 s in the Gulf of Mexico (Ray, 1996). This species has been spreading along the North American coast (Ford and Chintala, 2006; Ford and Smolowitz, 2007) and in Mexico with infections in *C. corteziensis* (Escobedo-Fregoso et al., 2015; Villanueva-Fonseca et al., 2020) and *Saccostrea palmula* (Cáceres-Martínez et al., 2012).

In Brazil, the first record of *Perkinsus* sp. infecting native *C. rhizophorae* oysters was in the estuary of the Pacoti River, Ceará (Sabry et al., 2009), but without parasite species identification. From this record, new research was conducted and diagnostic techniques were improved in order to identify the species detected in Brazil. Da Silva et al. (2013) identified *P. marinus* for the first time on the Brazilian coast in *C. rhizophorae*, in the estuary of the Paraíba River, Paraíba. This protozoan has also been detected in *C. rhizophorae* of natural banks in Baia De Camamu, Bahia (Pinto et al., 2017); in *C. gasar* in the São Francisco River estuary, Sergipe (da Silva et al., 2014; Scardua et al., 2017); in the estuary of the Mamanguape River, Paraíba (Queiroga et al., 2015); and putatively infecting *C. gigas* and *C. gasar* oysters in Santa Catarina (Luz Cunha et al., 2019; Leibowitz et al., 2019).

Although it is not presented as an official diagnostic method recommended by the World Organization for Animal Health (WOAH, founded as OIE), the real-time polymerase chain reaction (qPCR) technique has been developed over the years as a fast and safe alternative to identify and quantify *P. marinus* in host tissues or in the environment (Audemard et al., 2004; Gauthier et al., 2006; Faveri et al., 2009; Marquis et al., 2020). In this study, we used the qPCR technique as a confirmatory diagnostic tool for the detection of *P. marinus* in *Crassostrea*

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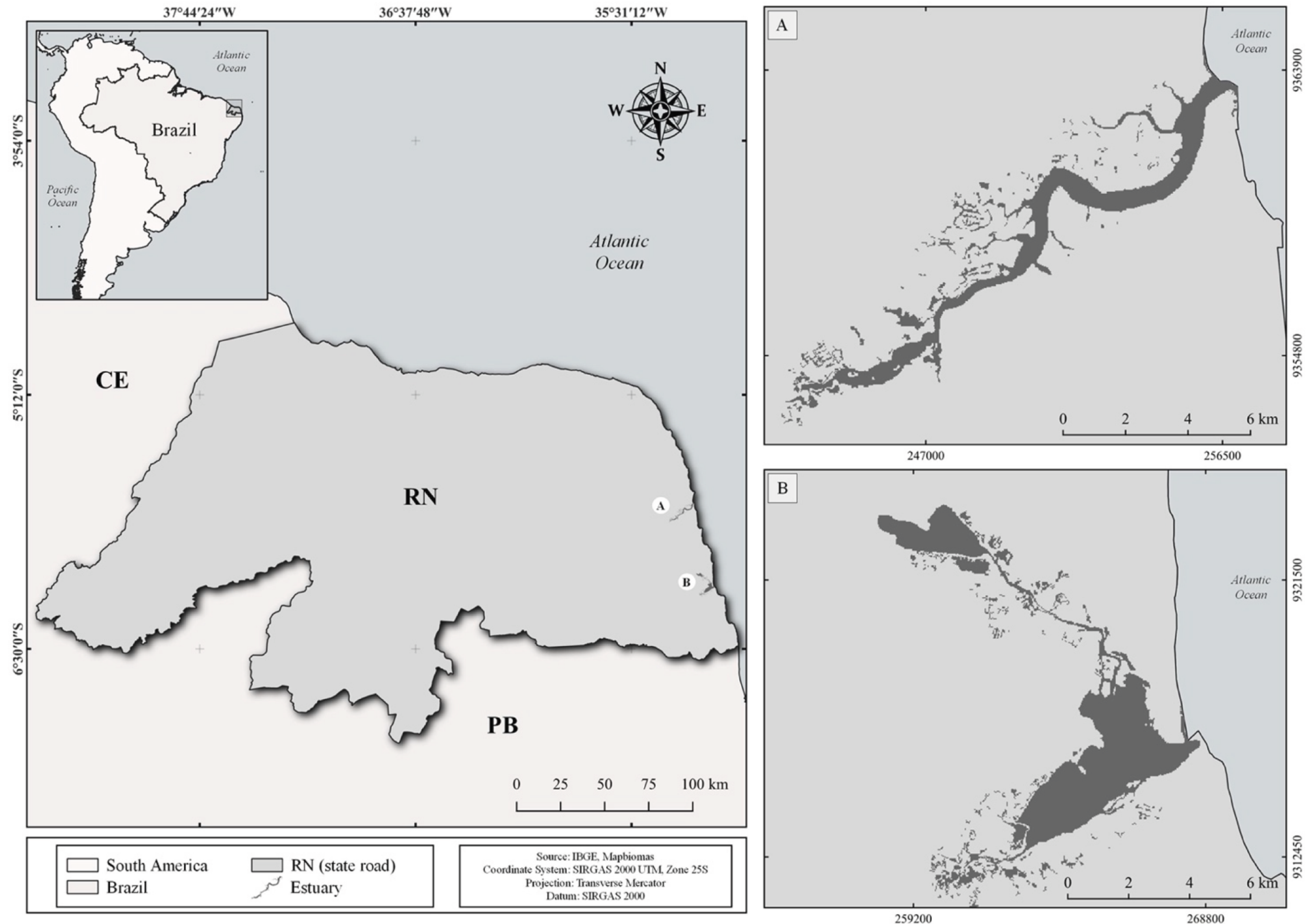

Fig. 1. Location of the estuaries sampled in the coastal region of Rio Grande do Norte, Brazil. (A) Potengi River estuary, Natal; (B) Guaraíras Lagoon estuary, Tibau do Sul.

sp. oysters from estuaries of Rio Grande do Norte, Brazil.

2. Materials and methods

Oysters of the *Crassostrea* genus (n = 1200) were collected in the estuaries of the Potengi River (S05°45′51.48″, W035°12′42.16″), and the Guaraíras lagoon (S06°12′38.62″, W035°08′08.27″) in the state of Rio Grande do Norte (Fig. 1). The collections were carried out in 2019, March and September in Potengi and April and October in Guaraíras, a total of 300 oysters/month/estuary. Seawater temperature and salinity were measured during sampling. The average height of the oysters was 52.94 ± 8.65 mm and 64.45 ± 8.28 mm respectively in the Potengi River and the Guaraíras Lagoon estuaries. The temperature and salinity of the water ranged from 25 to 26 °C and 8 to 34‰ in the Potengi River and from 26 to 27 °C and 1 to 36‰ in the Guaraíras lagoon. The oysters were collected manually in natural banks from the roots of *Rhizophora mangle* and were then transported to the laboratory. Rectum and gill tissues of each animal were incubated in Ray's fluid thioglycollate medium (RFTM), in the presence of antibiotics (penicillin G/streptomycin, 100 U ml^{-1}/μg^{-1}) and antifungal (nystatin, 100 U ml^{-1}) for 7 days in the dark at room temperature. Then the tissues were processed and observed by optical microscopy to verify the presence or absence of *Perkinsus* sp. (Ray, 1954). Infection intensity was established according to a modified semiquantitative Mackin scale (Sabry et al., 2009) and mean infection intensities and standard deviations (±SD) were calculated. Simultaneously, tissue samples from gills were preserved in 95 % alcohol and stored at −20 °C for DNA extraction and confirmatory molecular diagnosis.

From the samples positive by the RFTM method, 203 were selected and subjected to total genomic DNA extraction, following the protocol proposed by Dantas Neto et al. (2020) using DNAzol kit™ (Invitrogen, USA), according to the manufacturer's instructions. For the confirmatory diagnosis of *P. marinus*, we used the primers of Audemard et al. (2004), with reactions in a total volume of 10 μL, including 5 μL of Platinum™ SYBR™ Green qPCR SuperMix-UDG (Invitrogen, USA), 0.3 μL of primer F PmarITS-70F (5′CTTTTGYTWGAGWGTTGCGAGATG3′), 0.3 μL of R PmarITS-600R primer (5′CGAGTTTGCGAGTACCTCKAGAG3′), 0.1 μL of ROX reference dye, 1 μL of sample DNA and 3.3 μL of ultrapure water (Invitrogen, USA). The qPCR reactions were performed using an ABI 7500 Real Time PCR System (Applied Biosystems, USA). The thermocycling conditions were 50 °C for 2 min and 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s, per Livak and Schmittgen (2001). After the qPCR cycle, the samples were subjected to the melting cycle. The results were obtained using the 7500 System SDS software (Applied Biosystems, USA).

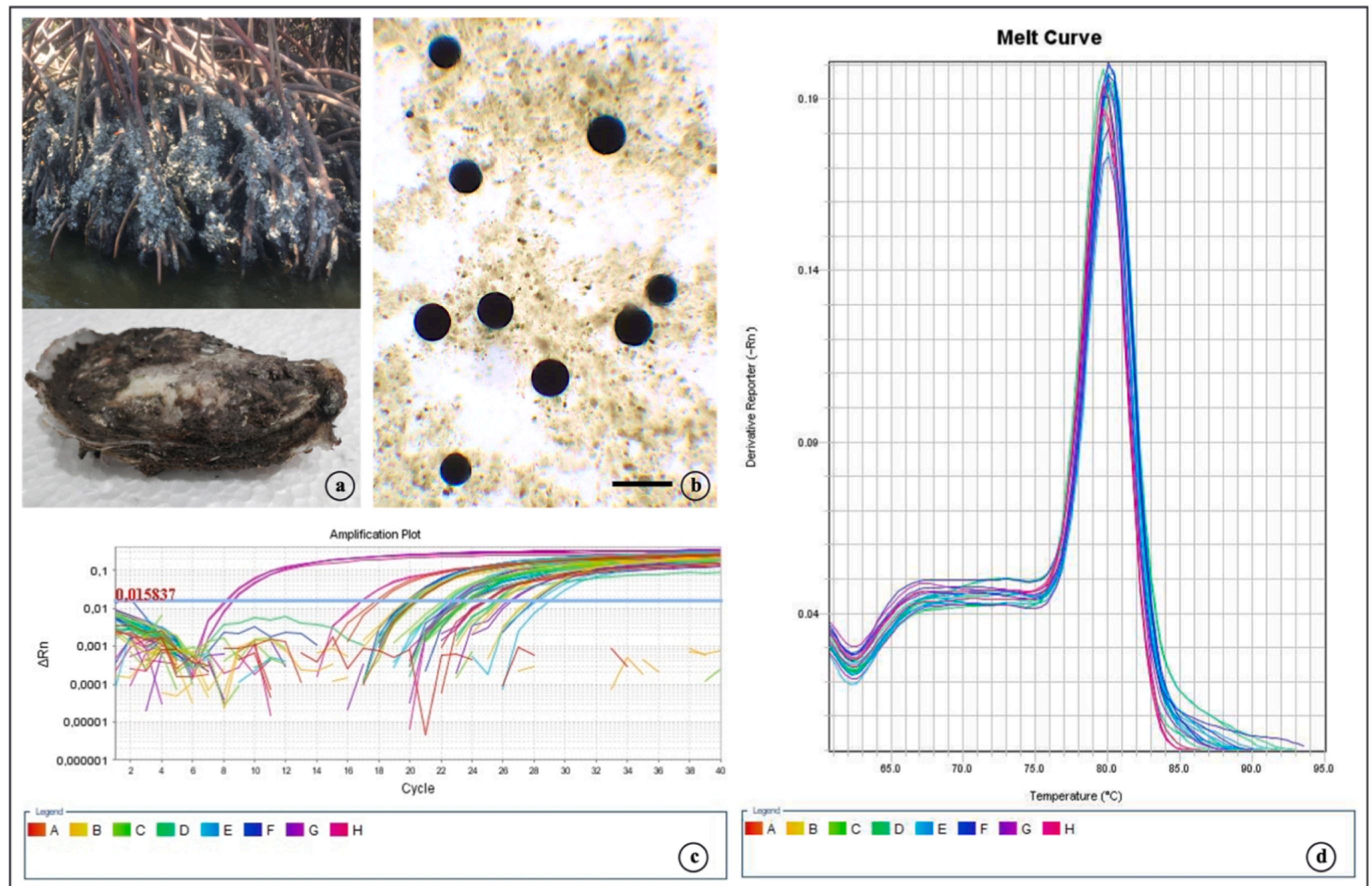

Fig. 2. (a) *Crassostrea* sp. collected from the root of *Rhizophorae mangle*; (b) hypnospores in gill and rectum tissues after incubation by RFTM and stained by lugol (2 %) (Scale bar = 100 µm); (c) amplification profile of fragments in the ITS region of *Perkinsus marinus* in the positive control and tested samples (A–H); (d) melting curve of positive control and samples (D–H).

3. Results

RFTM assays showed the presence of *Perkinsus* sp. infecting tissues of 308 *Crassostrea* sp. Oysters, of which 106 (17.66 %) were collected in the Potengi River and 202 (33.66 %) in the Guaraíras Lagoon. Mean infection intensities in the Potengi River were generally light, increasing from 2.40 ± 0.78 in March to 2.69 ± 1.22 in September. In Guaraíras lagoon the intensities were similarly light, 2.17 ± 1.01 in April and 2.54 ± 1.08 in October. The 203 selected RFTM-positive samples (n = 88, Potengi River; n = 115, Guaraíras Lagoon) were submitted to qPCR technique to identify *P. marinus*. Results showed 61 positive samples (27 = Potengi River; 34 = Guaraíras lagoon) by species-specific qPCR PmarITS70/600 producing amplified fragments of expected size with dissociation curve corresponding to the positive control and an observed melting temperature of 80.1 ± 0.6 °C (Fig. 2). The qPCR results showed a prevalence of animals infected with *P. marinus* of 30.68 % (27/88) and 29.56 % (34/115) in the analyzed samples from the Potengi river and Guaraíras lagoon estuaries, respectively.

4. Discussion

This is the first record of occurrence of *P. marinus* in native oysters of the *Crassostrea* genus in the estuaries of the coast of Rio Grande do Norte, Brazil, using qPCR assays as confirmatory diagnosis. In this study, the biometrics of the oysters only aimed to verify the size of the animals that would be submitted to analyses without relating the size to the presence or absence of the pathogen. The abiotic factors might not have influenced the occurrence of the protozoan considering that it was detected in different environmental conditions.

Currently, qPCR has been an important tool for monitoring *Perkinsus* sp. (Umeda and Yoshinaga, 2012; Cui et al., 2018) and is also used for investigations of co-infection involving other notifiable species by means of duplex qPCR (Itoïz et al., 2021). The qPCR technique has a greater sensitivity to assess prevalence and intensity of infections, speed of testing, ability to identify and distinguish species and feasibility of using previously stored samples (Gauthier et al., 2006; Faveri et al., 2009). As already observed in RFTM assays (Gauthier and Fisher, 1990; Rodríguez and Navas, 1995) and PCR (Marsh et al., 1995), qPCR can also allow a non-invasive diagnosis of *Perkinsus* sp. through the hemolymph without sacrificing the animal (Ríos et al., 2020), and analysis of serial samples over time from the same animal. Audemard et al. (2004) had previously demonstrated that qPCR could also be a powerful tool to investigate the abundance of free stages of *P. marinus* in the environment, allowing for detection of infectious and non-infectious cells, alive or dead, in the water column when assay parameters are optimized using DNA extraction kits for stool samples.

In this study, the prevalence of *Perkinsus* sp. as detected by RFTM was 17.66 % in the Potengi River and 33.66 % in the Guaraíras Lagoon, and the samples sent for molecular diagnosis showed prevalences of *P. marinus* of 30.68 % and 29.56 % in Potengi and Guaraíras, respectively. In Brazil, da Silva et al. (2013) recorded 83.33 % prevalence in PCR assays of the *Perkinsus* genus and identified restriction patterns

corresponding to *P. marinus* in the restriction fragment length polymorphism analysis (PCR-RFLP) in all samples considered. Infections by *P. marinus* in native oysters from the estuaries of Paraíba and Sergipe presented prevalence of 48 % and 64 % in the PCR-RFLP, respectively (Queiroga et al., 2015; Scardua et al., 2017). da Silva et al. (2014) recorded a prevalence of 66.66 % of *P. marinus* in *C. gasar* oyster samples using species-specific ITS primers. In *C. gigas* and *C. gasar* oysters in Santa Catarina, there was a 7 to 100 % and 55 to 100 % prevalence, respectively, of *Perkinsus* sp. in the PCR assays, and subsequently *P. marinus* was identified by Sanger sequencing (Luz Cunha et al., 2019; Leibowitz et al. 2019). The prevalences of *P. marinus* observed only in samples submitted to qPCR were 30.68 % in Potengi and 29.56 % in Guaraíras. Marquis et al. (2020) reported prevalences of up to 68 % in *C. virginica* oysters from coastal Maine, USA, using this same technique. Despite the prevalences registered in both estuaries, no mortality was observed that could be associated with the occurrence of *P. marinus* during the assessed period. Other studies on molluscs from the Brazilian coast also did not show mortalities in infected populations.

In recent years, the records of *P. marinus* in bivalve molluscs in Brazil were based on molecular diagnostics through conventional PCR and PCR-RFLP, except for the work of Pinto et al. (2017) who used proteomics as a diagnostic tool and da Silva et al. (2014) who performed fluorescence *in situ* hybridization (FISH) as a complementary technique to PCR. Quantitative qPCR assays have been developed to detect and quantify cells of *P. marinus* in oyster tissue because they have greater sensitivity when detecting infected oysters diagnosed by RFTM as not infected (Gauthier et al., 2006; Faveri et al., 2009). In this study, qPCR was shown to be efficient for the detection of *P. marinus* in *Crassostra* sp. estuaries of Rio Grande do Norte, Brazil, using ITS primers recommended for official diagnosis through conventional PCR (WOAH, 2021). The use of this technique for the diagnosis of notifiable pathogens is important because it contributes to accelerate decision-making in the face of mortality events in populations of cultivated bivalves and natural banks.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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