Detection of *Perkinsus marinus* in the oyster *Crassostrea rhizophorae* in southern Bahia by proteomic analysis

Detecção de Perkinsus marinus na ostra Crassostrea rhizophorae do sul da Bahia por análise proteômica

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Abstract

This study reports the presence of the pathogen *Perkinsus marinus*, notifiable to the World Organization for Animal Health (Office International des Èpizooties = OIE) in the oyster *Crassostrea rhizophorae* in southern Bahia via proteomic analysis. We analyzed *Crassostrea brasiliana* from a long-line cultivation system and *C. rhizophorae* from an adjacent mangrove in Porto do Campo, Camamu Bay, Bahia, Brazil. The collections (n = 100) were performed in October 2012. In the laboratory, the oysters were measured and opened to remove the meat, which was steeped in dry ice. For extraction of proteins, adaptation of a protocol used for mussels was used, after which separation in the first dimension was taken by isoelectric focusing (IEF). The peptides were transferred to a Mass Spectrometer. The obtained spectra were analyzed with the ProteinLynx Global Server 4.2 software tool and also by MASCOT (Matrix Science) and compared to the databases of the SWISSPROT and NCBI, respectively. The identification was evidenced by beta-tubulin, *Perkinsus marinus* ATCC 50983 and protein homology code in the database NCBI = gi | 294889481. This is the first record of *P. marinus* in Bahia and the fourth in Brazil.

Keywords: "Dermo". Diseases. Oysters. Perkinsiosis.

Resumo

Este estudo relata a presença do patógeno *Perkinsus marinus*, de notificação obrigatória à Organização Internacional de Epizootias (OIE) na ostra *Crassostrea rhizophorae* no sul da Bahia, via análise proteômica. Foram analisadas as ostras *Crassostrea brasiliana* de um cultivo em espinhel e *C. rhizophorae* de um manguezal adjacente, na localidade de Porto do Campo, Baía de Camamu, Bahia. As coletas (n = 100) foram efetuadas em outubro de 2012. Em laboratório, as ostras foram medidas e abertas para a retirada da carne, que foi macerada em gelo seco. Para a extração das proteínas, foi adotada a adaptação de um protocolo utilizado para mexilhões, após o que foi realizada a separação na primeira dimensão, por focalização isoelétrica (IEF). Os peptídeos foram transferidos para um Espectrômetro de Massas. Os espectros obtidos foram analisados no software ProteinLynx Global Server 4.2 e também pela ferramenta MASCOT (Matrix Science) e comparados com os bancos de dados do SWISSPROT e do NCBI, respectivamente. A identificação foi evidenciada por meio da beta-tubulina, homologia *Perkinsus marinus* ATCC 50983 e código da proteína no banco de dados NCBI = gi|294889481. Este é o primeiro registro de *P. marinus* na Bahia e o quarto no Brasil.

Palavras-chave: "Dermo". Doenças. Ostras. Perkinsiose.

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Protozoa of the genus *Perkinsus* (Perkinsozoa) affecting bivalve mollusks in various regions of the world and can cause major economic losses depending on the species and the affected host. Among the six species currently considered valid, two (*P. marinus* and *P. olseni*), due the severity of infection, they are notifiable to the World Organization for Animal Health (VILLALBA et al., 2004). The infection caused by these protozoa is known as Perkinsiosis or also as "Dermo", in reference to the first denomination of this protozoan made in the oyster *C. virginica*, in USA (MACKIN et al., 1950). The first record of *Perkinsus* in South America was made in Uruguay (CREMONTE et al., 2005), in the case of *P. olseni*, subsequently registered in the state of Sergipe, Brazil (SILVA et al., 2014). *Perkinsus marinus* has been registered in Brazil, in the states of Paraiba (SILVA et al., 2013), Sergipe (SILVA et al., 2014) and Ceará (DANTAS NETO, 2015). The species registered in the state of Bahia is *P. beihaiensis* (LUZ; BOEHS, 2016).

The oysters of the genus *Crassostrea* (Ostreidae) are important extractive resources around the Brazilian coast and its farming is already practiced in several places. On the coast of Bahia, the cultivation of these oysters is practiced on a small scale, mainly involving traditional extractive communities in the Bay of Iguape (Salvador) and in the municipalities of Valença, Taperoá, Camamu and Maraú, located further south. Monitoring of the diseases is already done in some of these crops (COVA et al., 2015; LUZ; BOEHS, 2015).

Oysters for this study were collected in October 2012, wherein *C. brasiliana* from a long-line cultivation system and *C. rhizophorae* in an adjacent mangrove, both located in Porto do Campo, Camamu Bay, Bahia, Brazil (13°57'S, 39°02'W). The oysters were placed in buckets containing a small amount of seawater and transported to the State University of Santa Cruz (UESC), where they were immediately processed. The specimens were measured on the dorsal-ventral axis (= height), according Galtsoff (1964), then opened with a knife for removal of soft tissues (flesh) and sample preparation for extraction of proteins, which included maceration on dry ice. For this procedure, the protocol used by Diz and Skibinski (2007) for protein extraction of *Mytilus edulis* and *M. galloprovincialis* (Mytilidae) was adapted. After that, separation in the first dimension was taken by isoelectric focusing (IEF). Separated peptides were transferred to a Mass Spectrometer (Micromass Q-TOFmicro, Waters) and ionized in a capillary voltage of 3000V, fragmented in positive mode with selection of the minimum relative intensity of 10 counts. The obtained spectra were analyzed with the ProteinLynx Global Server 4.2 software (WATERS) as well by tool MASCOT (Matrix Science) and compared to the databases of the SWISSPROT and NCBI, respectively.

The identification of oysters was done by DNA sequencing. The results showed that the oysters farming were *C. brasiliana* and mangrove oysters were *C. rhizophorae*. The mean height of *C. rhizophorae* was 4.65 ± 0.74 cm (n = 50) and of *C. brasiliana* was 5.43 ± 0.47 cm (n = 50).

The pathogen, which was evidenced only in *C. rhizophorae*, could be identified through the presence of beta-tubulin, *Perkinsus marinus* ATCC 50983 homology with molecular weight of 50685 Daltons, Iso Electric Point = 4.73, Score (score of protein) = 102 and protein code in the database NCBI gi =|294889481.

The primary objective of this study was to map the proteins of the oysters of the genus *Crassostrea*, as this knowledge is important both for the management of natural stocks as of oysters in cultivation. The laboratory tools typically used for detection of the

genus *Perkinsus* in marine mollusks are: tissue analysis (histology), culturing in Ray's fluid thioglycollate medium (RFTM) and polymerase chain reaction (PCR). These three techniques allow the secure identification of this protozoan in the genus level, and perform the calculation of prevalence (= number of individuals infected/number of individuals collected). Histology and RFTM also enable the evaluation of the severity of the infection. According to previous studies (e.g., SILVA et al., 2014; LUZ; BOEHS, 2016), based on international protocol (OIE, 2009), to obtain the identification of the species, should do the following DNA sequencing of the PCR and perform phylogenetic analysis. Although all the above techniques are labor intensive, they are usually used together for monitoring this important pathogen worldwide. In a study conducted by Luz and Boehs (2016) in the same places, *P. marinus* was not detected by the techniques mentioned. The detection of *P. marinus* through proteomics proved to be in this case more sensitive than molecular tools conventionally used, indicating that perhaps this pathogen is present in low prevalence and/ or low number in the tissues in the study region. The detection probably was only possible because pools of oysters were used in the proteomic analysis, which probably promoted a concentration of the beta-tubulin, *Perkinsus marinus* ATCC 50983. Then, proteomics can be used as an additional tool in the detection of this pathogen.

This is the first record of *P. marinus* in Bahia and the fourth in Brazil, all in northeastern Brazil.

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