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Variable protein profiles in extracellular products of the protistan parasite *Perkinsus olseni* among regions of the Spanish coast

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ABSTRACT

The variability of the protein expression profiling in the extracellular products (ECPs) of *in vitro* cultured *Perkinsus olseni* deriving from 4 regions of the Spanish coast was evaluated. The regions involved were the rías of Arousa and Pontevedra (Galicia, NW Spain), Carreras River (Andalusia, SW Spain) and Delta de l'Ebre (Catalonia, NE Spain). *P. olseni in vitro* clonal cultures were produced from parasite isolates from four clams from each region. Proteins released by the *in vitro* cultured parasites were isolated and separated by two dimensional electrophoresis (2DE). Qualitative comparison of protein expression profiles in the *P. olseni* ECPs among clones from all the regions was performed with PD Quest software. Around 130 spots were counted in the gels from ECPs of *P. olseni* clones from each region, of which 23 spots were shared by clones from all the regions and various spots were representative from clones of one region (appear in every clonal culture from that region but did not in every one of the other regions). A total of 34 spots were excised from the gels and analysed for sequencing. The protein cathepsin B, involved in proteolysis, the signal recognition particle receptor subunit β, involved in protein transport through membranes, and a protein belonging to N-acetyl transferase superfamily, involved in biosynthesis, were identified in spots shared by *P. olseni* ECPs from all regions. Pepsin A precursor, involved in proteolysis; heat shock protein (HSP) 60; and phosphoserine aminotransferase, involved in biosynthesis, were representative of *P. olseni* ECPs from Ría de Arousa, while peroxiredoxin V, involved in oxidation–reduction, was representative of *P. olseni* ECPs from Ría de Pontevedra. Differences in released proteins suggest different virulence or resistance to host attack between parasites from different locations.

1. Introduction

The genus *Perkinsus* includes protistan parasites infecting molluscs over the world (Villalba et al., 2004, 2011). The most widely distributed species is *Perkinsus olseni* (Lester and Davis, 1981), which is associated with mass mortalities of carpet-shell clams *Ruditapes decussatus* in Portugal (Azevedo, 1989) and Spain (Sagristá et al., 1995) and of Manila clams *Ruditapes philippinarum* in Spain (Sagristá et al., 1995), Italy (Pretto et al., 2014), Korea (Park and Choi, 2001), Japan (Choi et al., 2002) and China (Liang et al., 2001; Wu et al., 2011). *Perkinsus marinus* has affected American oyster *Crassostrea virginica* populations in USA for more than 50 years devastating the production (Burreson and Ragone Calvo, 1996; Soniat, 1996). Both *P. olseni* and *P. marinus* are included in the list of notifiable diseases of the World Organisation for Animal Health (http://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2015/).

The importance of the extra cellular products (ECPs) of *P. marinus* in its infectivity was highlighted in previous studies (La Peyre et al., 1995; Garreis et al., 1996; Tall et al., 1999). *P. marinus* releases serine proteases that can degrade laminin and fibronectin, which are the major extracellular matrix component of stroma and basement membrane; these serine proteases can affect host immune ability by reducing oyster haemocyte mobility and lysosomal activity in oyster haemolymph (Garreis et al., 1996). The reduction of oyster haemocyte mobility due to the infection with *P. marinus* suppress the vibriocidal activity of oyster haemocytes to effectively eliminate *V. vulnificus* (Tall et al., 1999). The progression of *P. marinus* infection is inhibited by bacitracin, a protease inhibitor, which supports considering *P. marinus* extracellular proteases as virulence factors (Faisal et al., 1999). A N-glycosylated serine protease occurring in ECPs was isolated and characterised; this 41.7 kDa monomeric protease was designed as perkinsin, the major extracellular protease produced by *P. marinus in vitro* (La Peyre et al., 1995; Faisal et al., 1999). A serine protease gene with a sequence closer to family subtilisin was identified in the *P. marinus* genome (Brown and Reece, 2003). Besides serine

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proteases, high levels of acid phosphatase were found in *P. marinus* ECPs; this enzyme could dephosphorylate enzymes as NADPH oxidase, which blocks the production of reactive oxygen species (ROS) by the host (Volety and Chu, 1997). These findings led to test the occurrence of 19 hydrolytic activities in the ECPs of four *Perkinsus* spp. (*P. marinus*, *P. olseni*, *P. chesapeaki* and *P. mediterraneus*) using commercial API ZYM® kits (BioMérieux); most of these enzymes have been detected in the four species but their relative concentrations appear to be unique to each *Perkinsus* species. Thus, *P. marinus* culture supernatants showed high α-chymotrypsin activity (non-detected in *P. olseni* and *P. chesapeaki*); those of *P. olseni* high esterase, esterase lipase, and α-mannosidase; *P. chesapeaki* high alkaline phosphatase; and *P. mediterraneus* high naphthol-AS-BI-phosphohydrolase and acid phosphatase activities; α-glucosidase activity was high in the four species (Casas et al., 2002a, 2008, 2009). Furthermore, gelatine SDS–PAGE demonstrated proteolytic activity in various bands from *P. mediterraneus* and *P. chesapeaki* ECPs (Casas et al., 2008, 2009).

Variability in virulence and other physiological aspects among populations of *P. marinus* has been demonstrated (La Peyre et al., 1995; Bushek and Allen, 1996; Chu and Lund, 2006; Alemán Resto and Fernández Robledo, 2014), which is consistent with the occurrence of genetic variability among geographic strains (Reece et al., 1997, 2001; Thompson et al., 2011, 2014a, 2014b). Knowing differences in virulence is important to avoid host movements from areas with high virulent strains to areas with less virulent strains. Variability of *P. olseni* populations through the Spanish coast has been addressed using genetic markers (Vilas et al., 2011) and based on the parasite proteome (Fernández-Boo et al., 2015). Evaluation of the ECPs of the same *P. olseni* isolates as in the latter study was conducted to assess variability in potential secreted virulence factors.

Proteomic approaches have been used to investigate secreted proteins of both intra- and extra-cellular parasites such as *Plasmodium falciparum* (Vincensini et al., 2005), *Trichomonas vaginalis* (Kucknoor et al., 2007), *Giardia lamblia* (Ringqvist et al., 2008) and *Leishmania (Viannia) braziliensis* (Cuervo et al., 2009), with high success in the protein identification. These studies identified several proteins involved in host-parasite interaction, modulation of the immune system of the host and host cell signalling (Cuervo et al., 2009). The aim of this study was to identify the most abundant proteins included in ECPs of *P. olseni* and to evaluate variability of the protein expression profiles in ECPs through the Spanish coast, with a proteomic approach combining two dimensional electrophoresis (2DE) and mass spectrometry liquid chromatography-tandem mass spectrometry (LC-MS/MS and *de novo* sequencing).

2. Materials and methods

2.1. Production of in vitro clonal cultures

Parasites *Perkinsus olseni* were isolated from 12 carpet shell clams *R. decussatus*, 4 each from Ría de Arousa and Ría de Pontevedra in Galicia (NW Spain), and Carreras River in Huelva (Andalucía, SW Spain), and from 4 Manila clams *R. philippinarum* from Delta de l'Ebre in Catalonia (NE Spain) (Fig. 1). Environmental conditions are different among those regions; temperature ranges are 10–24 °C, 12–25 °C and 8–30 °C, while salinity ranges are 20–36 psu, 30–37 psu and 29–40 psu in the two Galician Rías, Carreras River and Delta de l'Ebre, respectively. The parasites were isolated from the gills and were allowed to proliferate *in vitro* as described by Casas et al. (2002b). The *in vitro* cultures (one per host) were cloned by limited dilution plating in 96-well culture plates (Casas and La Peyre, 2009). One monoclonal derivate of each isolate was expanded *in vitro* and used in the study. The culture

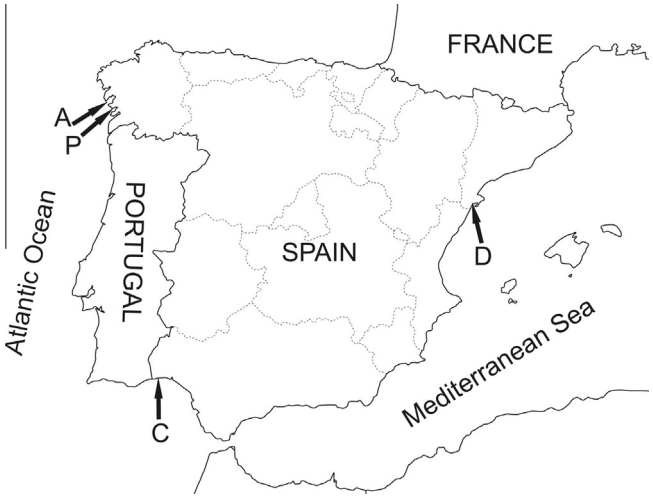

Fig. 1. Map showing the four Spanish locations where infected clams were collected: Ría de Arousa (A) and Ría de Pontevedra (P) in Galicia, Carreras River (C) in Huelva (Andalusia) and Delta de l'Ebre (D) in Catalonia.

medium JL-ODRP-2A (Casas et al., 2002b) was used in every *in vitro* culture step, from isolation to production of monoclonal cultures; the antibiotics included in the original JL-ODRP-2A were replaced with 8 mg/L chloramphenicol (Sigma–Aldrich). This culture medium does not include any protein, which makes it ideal to analyse proteins secreted from cultured cells. All cultures were repeatedly examined with inverted light microscope to assess their axenicity. The parasites were allowed to proliferate at 25 °C degrees for two months and then, when cultures were in the exponential growth phase, the culture medium was separated from cells by centrifugation (800*g*, 10 min, 25 °C), the supernatant was filtered through 0.45 μm, lyophilised and stored at −80 °C until used, while cells were reseeded (each batch of 5×10^6 cells transferred into a new flask in 5 mL of new culture medium). This process was repeated until accumulating 150 mL of supernatant from each clone, a volume previously estimated as suitable to get enough protein to perform 2DE. Fresh culture medium was prepared immediately before starting and reseeding cultures. Species identification of the cells in each clonal culture was performed by PCR followed by restriction fragment length polymorphism assay as described by Abollo et al. (2006), thus confirming that the cells from each clone corresponded to *P. olseni*.

2.2. Protein extraction

Lyophilised culture medium samples were resuspended in 4 mL of lysis buffer (8 M Urea, 2 M Thiourea, 2% 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate [CHAPS] w/v, 1% Dithiothreitol [DTT] w/v, 0.8% Ampholytes, and 100 mM phenylmethylsulphonyl fluoride, in order to neutralise the protease activity in the sample) for 2.5 h at 4 °C to extract the ECP proteins, and centrifuged (12,000*g*, 30 min, 4 °C) to remove particles. The protein concentration was determined by Lowry assay using the RC/DC Protein Assay Bio-Rad and measured in a microplate lecture Expert 96 (Asys Hitech) at 750 nm wavelength. A volume containing 400 μg of protein was purified using the 2D Clean Up kit (Bio-Rad) and resuspended in 1 mL of rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS w/v, 0.3% DTT w/v, 0.5% immobilised pH gradient [IPG] buffer).

2.3. Two dimensional electrophoresis and imaging analysis

Preliminary trials had been performed to optimise the experimental conditions for separation of *P. olseni* extracellular proteins