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Transcriptomic profile of Manila clam (*Ruditapes philippinarum*) haemocytes in response to *Perkinsus olseni* infection

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ABSTRACT

Ruditapes philippinarum, one of the major contributors to the global mollusc production, is susceptible to perkinsosis, a disease caused by the protozoan *Perkinsus olseni* (Alveolata, Perkinsidae). Understanding the molecular response of Manila clam defence cells (haemocytes) to this parasite is essential to head towards controlling this disease. Here, we assembled the Manila clam haemocyte transcriptome from healthy and *P. olseni* infected clams collected from wild and experimental conditions (*in vivo* and *in vitro*) using RNA-seq. The Manila clam *de novo* transcriptome consists of 33,079 transcripts, of which 7300 were significantly annotated (E-value <1.0E−5) with the NCBI non-redundant (nr) protein database. Among these annotated transcripts, many were functionally linked to signalling, cell proliferation, cell adhesion, immune system (e.g. immune response, immune effector process, antigen processing) and response to stress and stimuli. Our study integrates previous transcriptomic information of *R. philippinarum* haemocytes with present resources, including a notable amount of new transcripts with annotation description expressed after challenge with *P. olseni* in different infection scenarios, many of them linked to stress-related processes. A preliminary analysis of differentially expressed genes (DEGs) was performed with the sequences from the pool samples of control and challenged haemocytes obtained under similar experimental conditions. A higher number of DEGs were detected in *in vitro* than *in vivo* stimulated haemocytes, denoting the more acute and different *in vitro* challenges with *P. olseni* trophozoites, zoospores and extracellular products. Most *in vitro* DEGs were down-regulated and associated with immune and stress response, likely reflecting the immune-suppression occurring at the onset of infection, while DEGs in the *in vivo* infected haemocytes were mostly related to metabolic process. The preliminary DEG analysis on haemocyte response provides some insight into the molecular mechanism of Manila clam defence cells against perkinsosis.

Statement of relevance: High Manila clam mortality rates have been reported due to perkinsosis caused by *Perkinsus olseni*, however, the molecular mechanisms of *Ruditapes philippinarum* response against perkinsosis are still not fully understood. Our study provided new data to enrich the *R. philippinarum* haemocyte transcriptome databases from previous reports, and preliminary information on haemocyte response against perkinsosis is provided.

Abbreviations: AIP, Apoptosis Inhibitor Protein; BIRC, Baculoviral Iap Repeat-Containing protein; Bp, base pair; BP, Biological Process; CC, Cellular Component; DEGs, Differentially Expressed Genes; ECP, Extracellular Product; FC, Fold Change; FDR, False Discovery Rate; FPKM, Fragments Per Kilobase of transcript per Million mapped reads; FSW, Filtered Sea Water; GO, Gene Ontology; HSP, Heat Shock Proteins; IVT, *in vitro*; IVVI, *in vivo* indoor; IVVN, *in vivo* natural; MF, Molecular Function; PE, Paired-End; RNA-seq, RNA sequencing; ROS, Reactive Oxygen Species; SOD, Superoxide Dismutase; TP, Trophozoite; ZS, Zoospore.

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1. Introduction

The Manila clam *Ruditapes philippinarum* (Adams and Reeve, 1850) is one of the most commercially important bivalves worldwide with a production of 3.9 million tons in 2013 (FAO, 2015). However, Manila clam production is threatened by diseases like those caused by the bacteria *Vibrio tapetis* (Paillard et al., 2004) and the protistan parasite *Perkinsus* spp. (Villalba et al., 2004, Waki et al., 2012). High Manila clam mortality rates have been reported in Europe (Rodríguez et al., 1994; Pretto et al., 2014) and in Asia (Choi and Park, 1997; Choi and Park, 2010) due to perkinsosis caused by *Perkinsus olseni*, and accordingly, this parasite has been included on 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/).

Several studies, especially at cytological level, have been carried out in Manila clam over the years to understand perkinsosis: histopathology as well as infection intensity and prevalence of *Perkinsus* in clam and its target tissues including gills and abductor muscle (Choi et al., 2002); pathogenicity by challenging juvenile Manila clams with *P. olseni* prezoosporangia (Shimokawa et al., 2010; Waki et al., 2012); and how haemocytes, the principal cellular defence line of mollusc against pathogens (Bayne et al., 1980; Donaghy et al., 2009), respond to *P. olseni* infection by polypeptide secretion and expression of genes such as lectins, lysozyme (Montes et al., 1995a, 1995b; Kang et al., 2006; Kim et al., 2006, 2008a, 2008b). Different *P. olseni* life stages such as trophozoite, hypnospore (a resistant dormant stage with a thick external wall) and zoospore (Auzoux-Bordenave et al., 1995), cause infection in Manila clam (Villalba et al., 2004; Waki et al., 2012). Park and Choi (2001) has pointed out that infection starts with *Perkinsus* trophozoites colonising connective tissues in the gill, mantle, labial palps, gonads and digestive gland of Manila clam, with an intense infiltration of haemocytes, targets for trophozoite multiplication and dissemination (Choi et al., 2002). In response, infiltrated cells synthesize and secrete products (e.g. a slightly glycosylated polypeptide of about 225 kDa, p225) and the haemocytes likely start the encapsulation process of the parasites (Montes et al., 1995b). Heavy infection causes tissue disruption and organ dysfunction in the host (Villalba et al., 2004; Kim et al., 2006).

The advancement of high-throughput sequencing technologies has allowed a rapid and cost-effective transcriptomic analysis of non-model species (Dheilly et al., 2014). As a result, *R. philippinarum* transcriptomic profiles (Milan et al., 2011; Ghiselli et al., 2012; Moreira et al., 2012) and microarray-based gene expression analyses after exposure to pathogens, including *V. tapetis* and *V. alginolyticus* (Allam et al., 2014; Moreira et al., 2014), and *P. olseni* trophozoites (Romero et al., 2015), and thermal stress (Menike et al., 2014) have been recently reported.

The molecular mechanism of *R. philippinarum* response against perkinsosis is still not fully understood; more specifically, the infection process involving the different parasite infectious forms, namely trophozoites, zoospores and extracellular products, essential to the course of the infection (Shimokawa et al., 2010; Waki et al., 2012; Romero et al., 2015; Fernández-Boo et al., 2015) needs to be investigated in depth. In the present work, we reconstructed a *de novo* transcriptome of Manila clam haemocytes exposed to *P. olseni* in natural and experimental conditions using RNA-seq on an Illumina platform. This is the first study that takes into account three *P. olseni* infectious forms involved in Manila clam infection, and provides comprehensive transcriptomic data to be evaluated and compared with the previously reported transcriptomes to enrich the knowledge on haemocyte genes and their expression in response to perkinsosis. This enriched *de novo* transcriptome was used for a preliminary evaluation of the transcriptomic response of clam haemocytes to *P. olseni* infection, and will be the basis to develop a *R. philippinarum* haemocyte microarray for further in depth analysis of gene expression in Manila clam haemocyte *in vivo* and *in vitro* infected with *P. olseni*.

2. Materials and methods

2.1. Experimental design: in vitro, in vivo challenge and wild exposure

Manila clam (*R. philippinarum*)-haemocytes were challenged with *P. olseni* in both *in vitro* (IVT) and *in vivo* (IVV) experiments (Fig. 1). Two different *in vivo* evaluations were performed: i) clams challenged with the parasite at indoor facilities (IVVI); and ii) infected clams collected from a natural bed (IVVN). Clams for IVT and IVVI experiments were harvested from a *P. olseni* infection-free natural bed in Camariñas (Galicia, NW Spain) and carried to the facilities of Centro de Investigacións Mariñas (CIMA; Spain), where the challenges were performed. A PCR test was performed to confirm that clams were free of *P. olseni* (Casas et al., 2002a). Collected clams were kept at 14–17 °C in filtered (1 μm) sea water (35‰) with aeration for about 1 week and then used either for haemolymph extraction for IVT challenge, or for IVVI challenge. For the natural exposure experiment (IVVN), adult Manila clams infected with *P. olseni* were obtained from an infected sea bed (Placeres, Ría de Pontevedra, NW Spain).

2.1.1. In vitro challenge (IVT): haemocyte vs parasite

Since the discovery of *P. olseni* in abalone (*Haliotis rubra*) in Australia by Lester and Davis (1981), there have been increasing reports on infections with this parasite in several molluscs including clams from Europe and Asia, (Azevedo, 1989; Choi and Park, 1997; Hamaguchi et al., 1998), and in oyster from Australia and Asia (Norton et al., 1993; Sanil et al., 2010). The development of *in vitro* continuous propagation techniques by Casas et al. (2002b) has opened the door for new investigation on this species using this experimental scenario.

Haemocytes were isolated from clam haemolymph for *in vitro* challenges with *P. olseni* trophozoites (TP), zoospores (ZS) and extracellular products (ECP; proteins released in the culture medium by *P. olseni*). Haemolymph was obtained from the adductor muscle using a syringe with 25 gauge needle, and then examined under light microscope to discard samples with bacterial or gamete contamination. Haemolymph was then transferred to a 1.5 mL tube and kept on crushed ice until haemocyte isolation. Haemocytes were separated from plasma by centrifuging at 800 *g* for 10 min at 4 °C. Haemocytes extracted from haemolymph samples of 10 clams were then pooled until reaching an amount of 5×10^6 cells (counted in a Malassez chamber) for each challenge.

P. olseni zoospores were obtained from parasitized carpet-shell clams (*Ruditapes decussatus*) harvested from Ría de Pontevedra and *in vitro* cultured following the procedure described by Casas et al. (2002b). *P. olseni* trophozoites were obtained from *in vitro* culture cells at the exponential growth phase (approximately 2 month cultures). Trophozoites were extracted from a volume containing 5×10^6 cells (counted in a Malassez chamber) and centrifuged at 1000 *g* for 10 min at 25 °C. For ECPs, the culture media of a 3 month *P. olseni* culture was separated from the cells by centrifugation (1000 *g*, 10 min, 25 °C), and then filtered (0.22 μm) in order to avoid cell contamination.

The challenge experiments were performed in IWAKI 6-well plates. In each well, 5×10^6 haemocytes were suspended in 2.5 mL of filtered sea water (FSW). For the challenge, 5×10^6 trophozoites and 5×10^6 zoospores obtained just before the challenge were separately suspended in 2.5 mL FSW and added into the permeable insert (0.2 μm Anopore® membrane NUNC 25 mm) in each well. For haemocyte-ECP challenge, 2.5 mL of culture media enriched with ECP were added into the inserts of the respective wells. The inserts allowed the flow of media but not of cells, hence, haemocytes and parasite cells were never in contact. For each challenge with a different parasite infectious form (TP, ZP and ECP), haemocyte samples were collected at 1 h, 8 h and 24 h, including their respective controls (without parasite cells or ECPs) in a three-replicate format. Thus, a total of 54 samples were taken (3 parasite infectious forms × 3 times × 3 replicates × 2 (control + treatment)). At each sampling time, inserts with parasite

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