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Expression of infection-related immune response in European sea bass (*Dicentrarchus labrax*) during a natural outbreak from a unique dinoflagellate *Amyloodinium ocellatum*

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ABSTRACT

In the Mediterranean area, amyloodiniosis represents a major hindrance for marine aquaculture, causing high mortalities in lagoon-type based rearing sites during warm seasons. *Amyloodinium ocellatum* (AO) is the most common and important dinoflagellate parasitizing fish, and is one of the few fish parasites that can infest several fish species living within its ecological range. In the present study, *A. ocellatum* was recorded and collected from infected European sea bass (*Dicentrarchus labrax*) during a summer 2017 outbreak in north east Italy. Histological observation of infected ESB gill samples emphasized the presence of round or pear-shaped trophonts anchored to the oro-pharingeal cavity. Molecular analysis for small subunit (SSU) rDNA of *A. ocellatum* from gill genomic DNA amplified consistently and yielded 248 bp specific amplicon of *A. ocellatum*, that was also confirmed using sequencing and NCBI Blast analysis. Histological sections of ESB gill samples were addressed to immunohistochemical procedure for the labelling of ESB *igm*, *inos*, *tlr2*, *tlr4*, *pcna and* cytokeratin. Infected gills resulted positive for *igm*, *inos, pcna and* cytokeratin but negative to *tlr-2* and *tlr-4*. Furthermore, ESB immune related gene response (innate immunity, adaptive immunity, and stress) in the course of *A. ocellatum* infection using quantitative polymerase chain reaction (*qpcr*) for infected gills and head kidney was analysed. Among the twenty three immune related gene molecules tested, *cc1*, *il-8*, *il-10*, *hep*, *cox-2*, *cla*, *cat, casp9*, and *igt* were significantly expressed in diseased fish. Altogether, these data on parasite identification and expression of host immune-related genes will allow for a better understanding of immune response in European sea bass against *A. ocellatum* and could promote the development of effective control measures.

1. Introduction

Amyloodinium ocellatum (AO) is an ectoparasite protozoan belonging to the phylum Dinoflagellata and is the unique species belonging to *Amyloodinium* genus (class Blastodiniphyceae, order Blastodiniales, family Oodiniaceae) [1,2]. AO is worldwide distributed and affects brackish and sea water fish in tropical and temperate regions. Furthermore, AO is the particular dinoflagellate capable to infect elasmobranchs other than teleosts [3]. In fact, cumulative evidence shows that it has a very low host species-specificity, being isolated from four aquatic organisms Phyla: Chordata, Arthropoda [4], Mollusca [5] and Platyhelminthes [6]. The parasite represents a serious problem for both farmed and aquarium fish [7], since amyloodiniosis can lead the host to death in less than 12 h [3] with acute morbidity and mortality around 100%. However, these two parameters considerably vary on the basis of farming condition, parasite burden, fish species and season consideration [8–13]. AO biological life cycle is direct but triphasic and it can be completed in less than a week dependent on the favourable environmental factors [14]. The trophont is the parasitic stage and it is sessile and strictly anchored to host epithelia (gill or skin) through rhizoids. The parasite feeds on the host through the stomopode [14] and, after feeding (2–6 days), trophonts detach from host, becoming tomonts (cystic reproductive stage). In 2–4 days dinospores (infective stage) hatch from tomonts and actively search a new host using flagella. After the adhesion to the host, dinospores transform into trophonts within few minutes. Even if sessile, trophonts constantly turn and twist slowly, facilitating the severance of host cell fragmentation [14]. AO inflicts moderate-to-intense tissue damage associated with serious gill hyperplasia with the subsequent lamellar fusion, inflammation, hemorrhage and necrosis [8,9,11,12,15–19]. In heavy infections, death can occur in

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less than 12 h, however, mortalities have been documented also in subclinical or mild infestations as a probable consequence of osmoregulatory impairment and secondary microbial infections in damaged epithelial cells [20].

European sea bass (ESB) (*Dicentrarchus labrax*) is one of the most extensively aquacultured fish species in Mediterranean and is susceptible to *Amyloodinium ocellatum* infection [21,22]. Previous studies have indicated that after AO infection in ESB, Interleukin-1 (IL-1) and Tumor Necrosis Factor α (*tnf-α*) were activated in intestine [19]. Furthermore, peroxisome proliferator-activated receptor α (PPAR α), codifying for a protein involved in lipid metabolism, was upregulated in liver of the infected fish reared in an aquaponics system due to the need to produce energy in order to maintain homeostasis [19]. Noticeably, plasma proteome changes in gilthead head seabream (*S. aurata*) were observed as physiological response to AO and results indicated that several proteins belong to acute-phase response, inflammation, lipid transport, homoeostasis, osmoregulation, wound healing, neoplasia and iron transport were affected [23]. Moreover, the AO parasitic burden have enhanced the physiological stress response in gilthead seabream (*S. aurata*) [24]. To our knowledge, there are no further studies especially on ESB emphasizing immune gene response to AO infection through the screening of large panels of host immune related genes. Therefore, in order to improve the knowledge on the mechanisms at the basis of the host-parasite interactions with a particular attention to host immune response and tissue damages provoked by AO, histological, immunohistochemical and biomolecular analysis have been performed in the present study. Based on the current availability and functional characterization of European sea bass immune genes, we targeted genes that encompass innate/inflammatory/adaptive immune ligands, pathogen recognition receptors, antimicrobial peptides, and stress related factors. Thus, our study provides a balance between potential innate and adaptive immune mechanisms shaping amyloodiniosis pathogenesis and host response.

2. Material and methods

2.1. Fish sampling

Tissues samples from the infected fish and non-infected fish (gills and head kidney) were collected from ESB juveniles (mean weight 45 g) in a farm (lagoon-type rearing site) located in the delta area of the Po River (Porto Viro-RO, Italy), during a severe AO outbreak at the end of July 2017. The disease episode was characterized by high morbidity and mortality (100% within 7 days after the outbreak identification) and the etiology was microscopically confirmed by trophonts identification in gill biopsies. Clinical symptoms were dyspnea, superficial swimming, flushing, anorexia, and lethargy (in the advanced stage of the disease). ESB were reared in a 400 m^3 raceway supplied with brackish water (7‰ salinity and 28–30 °C). As a control, organs from ESB not infected by AO (asymptomatic and negative to the gill biopsy observation), reared in a different raceway of the same farm, were sampled for this study.

Before immersion in RNA later® Solution (Ambion®, https://www.thermofisher.com), gills and head kidney from infected and non infected fish (n = 5/group) were hygienically dissected and cutted to ≤0.5 cm in any single dimension. Tissue samples were placed into 1 ml of RNA-later (Ambion®, Life technologies), kept at 4 °C for 24 h and stored at −80 °C prior to RNA extraction and PCR analysis.

For histology and immunohistochemistry 2–3 gill arches deriving from 10 infected and from 5 healthy ESB were fixed in 4% buffered formaldehyde and Bouin's solution (Bio-Optica, Milano, www.bio-optica.it), embedded in paraffin, sectioned, and stained with Haematoxylin-Eosin, PAS-Alcian blue and Masson's trichrome, or submitted to immunohistochemical protocol.

2.2. Amyloodinium ocellatum isolation

Amyloodinium ocellatum was collected from infected ESB based on [25] with some modifications. AO trophonts were detached by placing moribund ESB in a clean 1 L plastic jar containing 0.5 L of freshwater for 2–3 min. Then, fish were removed from the jar, the water salinity was adjusted to 20 ppt by addition of 0.5 L of 40 ppt salt water and the jar content poured into a glass jar through a plastic funnel lined with two layers of 100 µm nylon filter mesh, to remove large debris. The filtrate was set aside for 15–20 min to allow sedimentation and transformation of trophonts into tomonts; the saltwater overlay was removed and tomonts transferred in sterile 50 ml tubes. Tomonts were washed twice by centrifugation for 10 min at 150 × *g*, saltwater overlay removal and re-suspension in sterile saltwater (20 ppt). Successively, 2 ml of the tomonts suspension in sterile saltwater were gently layered onto 2 ml of Percoll® (Sigma-Aldrich, https://www.sigmaaldrich.com/) in 15 ml tubes and tomonts purified by centrifugation for 10 min at 180 × *g*. The supernatant was removed and the pelleted tomonts were washed three times with 15 ml of sterile saltwater, then stored at −20 °C until further use. The thawed pellet was disrupted with a disposable pestle. The DNA was extracted from the homogenate using the DNeasy Tissue Kit (Qiagen, https://www.qiagen.com) according to the manufacturer's instructions.

2.3. Polymerase chain reaction (PCR) and sequencing

Amyloodinium ocellatum – specific primers AO18SF (5′-gaccttgccc-gagaggg-3′) and AO18SR (5′-ggtgttaagattcaccacactttcc-3′) were used for PCR amplification of a 248 bp segment of the 3′ end of the SSU rDNA gene [26]. PCR amplification of the AO DNA using the AO18SF/R primer set was carried out in a final volume of 50 µL according to [26]. PCR reaction was performed using a Bio-Rad thermocycler (Bio-Rad Laboratories Inc., CA, USA, http://www.bio-rad.com/) with a reaction mixture containing 100 ng DNA, 100 ng of each primer, and 1.25 Units HotStart Taq (Invitrogen, USA, https://www.thermofisher.com/) under the following conditions: initial denaturation at 94 °C for 15 min followed by 35 cycles of initial denaturation (94 °C for 1 min), annealing (58 °C for 1 min), extension (72 °C for 1 min), and a final elongation (72 °C for 5 min). Subsequently, 5 µL of the PCR product were analysed by 2% agarose gel electrophoresis stained with ethidium bromide and visualiszed with an UV transilluminator. PCR product molecular weight was determined using a 100 bp DNA ladder (Thermo Fisher Scientific, Pittsburgh, PA, USA). To identify the type of AO isolate used in the present study, PCR products were then purified using a QIAquick Purification kit (Qiagen, https://www.qiagen.com/it/) and directly sequenced using the AO18SF/R primer set (https://www.eurofinsgenomics.eu/). Using a BLAST search, the sequences obtained were compared with those published in NCBI GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The multiple sequence alignment was performed using the CLUSTALW (www.ebi.ac.uk/clustalw2).

2.4. Immunohistochemistry

Immunohistochemistry was performed on gills samples in order to label some inflammation related antigens during ESB response to amyloodiniosis. For this purpose, 4 µm histological sections of ESB gills fixed in Bouin's were addressed to immunohistochemical procedure using a HRP-based anti-rabbit or anti-mouse kit (EnVisionTM FLEX, K8009, K8021 and K8023 - Dako, Agilent). Briefly the slides were routinely dewaxed and rehydrated and all incubations were performed at room temperature (RT) in a humid chamber. Tissue endogenous peroxidase was inactivated by slides immersion in H_2O_2 for 30 min at RT, antibodies aspecific binding was blocked with 1:20 normal goat serum (30 min), then the procedure included an antigen retrieval treatment (10 min at 90 °C) (High pH, K8004; or Low pH, K8005 - Dako, Agilent) and a 2 h incubation with primary antibodies specific for the