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RNA-seq analysis of early enteromyxosis in turbot (*Scophthalmus maximus*): new insights into parasite invasion and immune evasion strategies

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ABSTRACT

Enteromyxum scophthalmi, an intestinal myxozoan parasite, is the causative agent of a threatening disease for turbot (*Scophthalmus maximus*, L.) aquaculture. The colonisation of the digestive tract by this parasite leads to a cachectic syndrome associated with high morbidity and mortality rates. This myxosporidiosis has a long pre-patent period and the first detectable clinical and histopathological changes are subtle. The pathogenic mechanisms acting in the early stages of infection are still far from being fully understood. Further information on the host–parasite interaction is needed to assist in finding efficient preventive and therapeutic measures. Here, a RNA-seq-based transcriptome analysis of head kidney, spleen and pyloric caeca from experimentally-infected and control turbot was performed. Only infected fish with early signs of infection, determined by histopathology and immunohistochemical detection of *E. scophthalmi*, were selected. The RNA-seq analysis revealed, as expected, less intense transcriptomic changes than those previously found during later stages of the disease. Several genes involved in IFN-related pathways were up-regulated in the three organs, suggesting that the IFN-mediated immune response plays a main role in this phase of the disease. Interestingly, an opposite expression pattern had been found in a previous study on severely infected turbot. In addition, possible strategies for immune system evasion were suggested by the down-regulation of different genes encoding complement components and acute phase proteins. At the site of infection (pyloric caeca), modulation of genes related to different structural proteins was detected and the expression profile indicated the inhibition of cell proliferation and differentiation. These transcriptomic changes provide indications regarding the mechanisms of parasite attachment to and invasion of the host. The current results contribute to a better knowledge of the events that characterise the early stages of turbot enteromyxosis and provide valuable information to identify molecular markers for early detection and control of this important parasitosis.

1. Introduction

Turbot (*Scophthalmus maximus*, L.) is a valuable cultured marine flatfish, whose production in 2013 accounted for over 77,000 tons, with China (67,000 tons in 2013) and the European Union (7700 tons in 2013, 11,000 in 2014) as the main producers (APROMAR, 2015). Enteromyxosis caused by *Enteromyxum scophthalmi* (Myxozoa) is a serious threat for turbot aquaculture, currently without effective therapeutic measures (Sitjà-Bobadilla and Palenzuela, 2012). The target site of this myxozoan parasite is the gastrointestinal tract, where it proliferates and spreads from the anterior intestine and pyloric caeca to other gut regions (Redondo et al., 2004). The infection leads to severe catarrhal gastroenteritis associated with a cachectic syndrome, with reduction of growth performance and high mortality rates (Bermúdez et al., 2010; Sitjà-Bobadilla and Palenzuela, 2012). Under culture conditions, the trophozoites are transmitted directly from fish to fish, which leads to a rapid spread of disease in infected tanks and

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facilities (Redondo et al., 2002; Quiroga et al., 2006; Sitjà-Bobadilla and Palenzuela, 2012). However, the disease shows a long prepatent period, with the parasite detectable in the digestive tract by histology only after several weeks in natural infections (Redondo et al., 2004; Quiroga et al., 2006). In experimental infections by effluent transmission or cohabitation, the parasite is first observed at approximately 20 days post-exposure, and at approximately 8 days after experimental per os transmission (Redondo et al., 2004; Bermúdez et al., 2006; Sitjà-Bobadilla et al., 2006; Losada et al., 2014a). Experimental infection by the oral route results in a very high and quick prevalence of infection and homogeneous lesions in recipient fish. In addition, the ingestion of trophozoites released from infected fish is thought to be the main infection route occurring in the fish farm (Redondo et al., 2002, 2004). In the early stages of infection there are no external clinical signs, histological lesions are very subtle, and the parasite is difficult to detect in conventional histological sections of the digestive tract (Quiroga et al., 2006; Bermúdez et al., 2010). In vitro, *E. scophthalmi* is able to penetrate the intestinal epithelium from the lumen as well as via the basement membrane, and the report of parasitic stages in blood smears suggests the existence of a haematic route of spread (Redondo et al., 2003, 2004; Redondo and Álvarez-Pellitero, 2010). However, a detailed understanding of entry routes and epithelial invasion strategies is lacking. We are still far from a full knowledge of the host–parasite interaction and further investigation is needed to clarify the pathogenetic mechanisms of enteromyxosis (Sitjà-Bobadilla and Palenzuela, 2012; Robledo et al., 2014), especially those acting during early stages of infection.

Whole-transcriptome analysis using RNA-seq is a suitable approach for the identification of the genes and pathways involved in host–pathogen interactions, and it is acquiring a key role in the understanding of the pathogenesis of human and veterinary diseases (Costa et al., 2013; Qian et al., 2014; Li et al., 2015). This is an essential starting point for the development of control measures, therapeutic options and genetic breeding programs. An RNA-seq analysis of turbot experimentally infected by the oral route was previously addressed, investigating the advanced stages of the disease by studying specimens at 42 days post-inoculation. That work enabled a better understanding of the genetic basis of the clinical signs and lesions which characterise the infection (Robledo et al., 2014). In this study, using a similar methodological approach, we performed a transcriptomic analysis of turbot showing very early signs of infection aimed at contributing to the current understanding of incipient enteromyxosis.

2. Materials and methods

2.1. Experimental design

The experimental setup and sampling were as previously described (Robledo et al., 2014). Briefly, infection was achieved by the oral route (Redondo et al., 2002) and tissue samples were collected in Bouin's fluid and RNAlater (Qiagen, Germany) for histological techniques and RNA-seq, respectively. A histological evaluation was performed, and infected turbot were classified into three groups (slightly, moderately and severely infected) according to the histopathological grading described by Bermúdez et al. (2010). For RNA-seq analysis, spleen, head kidney and pyloric caeca from three control (CTRL) and three *E. scophthalmi*-infected (recipient, RCPT) fish at 24 days post-inoculation were used. The three RCPT fish were selected by histology among those graded as slightly infected and numbered (infected turbot 1, 2 and 3). RNA aliquots from the samples of RCPT fish were sequenced individually, while samples from CTRL fish were pooled by organ, resulting in three RCPT and one CTRL sample per organ.

2.2. Immunohistochemistry

Immunohistochemical detection of *E. scophthalmi* was performed on sections from different regions of the digestive tract (oesophagus, stomach, pyloric caeca, anterior, middle and posterior intestine) to confirm the presence of the parasite. Thin sections (3 μm) were placed on slides treated with silane to improve section adherence and dried overnight at 37 °C. After deparaffination (two 5 min washes in xylene) and rehydration (graded alcohol series), the endogenous peroxidase activity was inhibited by incubating the slides with peroxidase-blocking solution (Dako, Denmark) for 40 min. A 2 h incubation at room temperature was performed with a polyclonal antibody against *E. scophthalmi* (Estensoro et al., 2014) (diluted 1:50,000). The secondary antibody conjugated with peroxidase was the anti-rabbit EnVision + System Labelled Polymer-HRP (Dako) for 30 min, followed by development with diaminobenzidine (Dako). All incubations were performed in humid chambers and three 5 min washes with 0.01 M PBS were carried out between all subsequent steps. Sections of severely infected turbot were used as positive controls. In the sections included as negative controls, the primary antibody was replaced by antibody diluents.

2.3. RNA-seq and differential expression analysis

Some of the procedures and methodologies employed were described previously (Robledo et al., 2014). Briefly, RNA extraction was performed using the RNeasy mini kit (Qiagen, Germany) with DNase treatment and RNA quality and quantity were evaluated in a Bioanalyzer (Bonsai Technologies, Spain) and in a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies Inc., Delaware, US), respectively. The samples were barcoded and prepared for sequencing by the Wellcome Trust Centre for Human Genetics (Oxford, UK) and sequenced on an Illumina HiSeq 2000 as 100 bp paired-end reads. All the data files have been deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) database under the project ID PRJNA300347; as well the generated transcriptome sequences and their annotation have been deposited in Mendeley Data (https://data.mendeley.com/) and can be accessed using doi: 10.17632/3vhc8py3cv.2. Quality filtering and removal of residual adaptor sequences was conducted using Trimmomatic v.0.32 (Bolger et al., 2014). The recently assembled turbot genome (Figueras et al., 2016) was used as a reference for read mapping. Filtered reads were mapped to the genome using Tophat2 v.2.0.11 (Kim et al., 2013) which leverages the short read aligner Bowtie2 v.2.2.3 (Langmead and Salzberg, 2012) with a maximum intron length of 20 kb. HTSeq-count (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html) was used to extract the raw reads from the mapping files and differentially expressed genes were obtained using EdgeR (Robinson and Oshlack, 2010) with a False Discovery Rate (FDR) corrected *P* value of 0.05. The differentially expressed (DE) genes were identified and annotated using Blast2GO v.2.7.0 (Conesa et al., 2005) with an *E*-value cutoff of E^{-6}. Enriched Gene Ontology (GO) terms for each organ were identified by comparing the DE genes against the full transcriptome using Blast2GO Fisher's exact test ($P < 0.05$, FDR corrected). Furthermore, in this study, Kyoto Encyclopedia of Genes and Genomes (KEGG, Kanehisa et al., 2016) enrichment was assessed using KOBAS 2.0 (Wu et al., 2006) ($P < 0.05$, FDR corrected) with the draft turbot genome annotation as background. Those reads from pyloric caeca samples which did not align against the genome, both from turbot of this study (at 24 days post-inoculation) and from a previous study with parasitised turbot at 42 days post-inoculation (Robledo et al., 2014), were extracted and de novo transcriptome assembly was carried out using ABySS (version 1.3.7; Simpson et al., 2009) with

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