Aquaculture 366–367 (2012) 122–128

Contents lists available at SciVerse ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Development of a real-time PCR assay for detection and quantification of *Enteromyxum scophthalmi* parasites in turbot intestinal samples

Carla Piazzon [a], Natalia Mallo [a], Iris Martín [b], Jacobo Fernández-Casal [b], Manuel L. Sanmartín [a], Jesús Lamas [c], José M. Leiro [a,*]

[a] Laboratorio de Parasitología, Departamento de Microbiología y Parasitología, Instituto de Investigación y Análisis Alimentarios, Universidad de Santiago de Compostela, Spain
[b] Insuiña, O Grove, Pontevedra, Spain
[c] Departamento de Biología Celular y Ecología, Facultad de Biología, Universidad de Santiago de Compostela, Spain

ARTICLE INFO

Article history:
Received 4 September 2012
Accepted 10 September 2012
Available online 15 September 2012

Keywords:
Turbot
Enteromyxum scophthalmi
Myxozoan parasite
Quantitative PCR
Diagnosis

ABSTRACT

The myxozoan parasite *Enteromyxum scophthalmi* causes severe enteritis in cultured turbot *Scophthalmus maximus*, thus generating important economic losses. At present, there are no prevention or control measures for the disease, and many aspects of the life cycle and transmission of the parasite are not yet known. In this study, a highly sensitive, reproducible and rapid quantitative (real time) polymerase chain reaction (qPCR) assay was developed to detect *E. scophthalmi* DNA. The qPCR assay targets the 28S rRNA gene of the parasite, which has a high identity (94%) with the myxosporidian *Enteromyxum leei* rRNA gene. The qPCR assay was able to detect up to 13 DNA copies, corresponding to 0.55 fg, estimating that genomic DNA has around 1450 copies of 28S rRNA gene per parasite nucleus. The mean intra- and inter-assay coefficients of variation were below 5% and no detectable amplification was observed with DNA from non-infected turbot. The assay was validated with a histological identification of intestinal content samples from experimentally infected turbot and a good correlation between both methods was observed. The results demonstrate that the qPCR assay can be applied in the diagnosis of turbot enteromyxosis and to determine the relative abundance of *E. scophthalmi* in turbot intestinal contents in health monitoring studies.

1. Introduction

Myxozoans are highly specialized metazoan parasites with a very wide range of aquatic hosts, and represent one of the most severe threats to some cultured marine fish (Álvarez-Pellitero and Sitjà-Bobadilla, 1993; Feist and Longshaw, 2006; Kent et al., 2001). In turbot, a single myxozoan species produces an intestinal infection associated with high mortality, which reaches 100% in affected stocks (Branson et al., 1999). The species has been included in the genus *Enteromyxum*, a monophyletic group of marine enteric myxozoans (Palenzuela et al., 2002; Yanagida et al., 2004), and denominated *Enteromyxum scophthalmi* (Padrós et al., 2001; Palenzuela et al., 2002). In turbot, infection by this parasite is characterised by emaciation and cachexia, caused by acute enteritis, and death of susceptible fish (Bermúdez et al., 2010; Redondo et al., 2004). Enteromyxosis spreads quickly in affected farms as a result of direct transmission among fish by viable proliferating stages released by infected animals into the water (Diamant, 1997; Quiroga et al., 2006; Redondo et al., 2004). Although it is not yet possible to culture myxozoan *in vitro*, *E. scophthalmi* can be maintained *in vivo* and used to induce experimental infections via oral routes, or through effluent or cohabitation (Bermúdez et al., 2006; Redondo et al., 2004; Sitjà-Bobadilla et al., 2006). Numerous aspects of the epidemiology, phylogeny, taxonomy, biology and transmission of turbot enteromyxosis remain unknown, making it difficult to develop methods of preventing and controlling the disease (Quiroga et al., 2006). The development of specific diagnostic methods has improved our understanding of pathogenesis, and the use of molecular techniques has provided fundamental advances in the knowledge of the evolution and biology of the phylum Myxozoa (Feist and Longshaw, 2006). Diagnosis of myxozoans is largely based on the morphological characteristics of life cycle stages, mainly mature spores (Lom et al., 1997), at light and electron microscopy level (Lom and Dyková, 1993). However, new diagnostic methods using both monoclonal and polyclonal antibodies, and lectin-based assays that allow the detection and differentiation of molecules expressed at different life cycle stages of the parasite have been developed (Sitjà-Bobadilla et al., 2004; Redondo and Álvarez-Pellitero, 2010). Molecular techniques based on PCR amplification have been adapted for diagnosis with non-lethal samples, such as intestinal contents (Fox et al., 2000), and detection of other stages of the myxozoan life cycle by targeting specific rRNA sequences, usually 18S and the ITS regions of the rRNA (Holzer et al., 2007; Kent et al., 2001; Yokoyama et al., 2010). Quantitative real-time PCR (qPCR) assays have recently

* Corresponding author at: Laboratorio de Parasitología, Departamento de Microbiología y Parasitología, Instituto de Investigación y Análisis Alimentarios, Universidad de Santiago de Compostela, c/ Constantino Candeira s/n, 15782, Santiago de Compostela (La Coruña), Spain. Tel.: +34 981563100; fax: +34 881816027.
E-mail address: josemanuel.leiro@usc.es (J.M. Leiro).

0044-8486/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aquaculture.2012.09.008

become a valuable tool for estimating myxozoan parasite loads and the course of infection (Cavender et al., 2004; Kallert et al., 2009; Kelley et al., 2006). The present study describes the development of a qPCR that targets the 28S sequence of the rRNA gene of the myxozoan *E. scophthalmi*, thus enabling the specific and sensitive detection and quantification of invading stages of this parasite in intestinal content samples from experimentally infected fish.

2. Material and methods

2.1. Fish

Turbot, *Scophthalmus maximus* (L.), of weight 100 g, were obtained from a local fish farm. The fish were maintained in 250 L tanks with recirculating, aerated sea water (14 °C), and subjected to a photoperiod of 12L: 12D, and fed daily with commercial pellets (Skretting, Burgos, Spain). The fish were acclimatized to laboratory conditions for two weeks before the start of the experiments. All of the experiments were carried out in accordance with European regulations on animal protection (Directive 86/609), outlined in the Declaration of Helsinki.

2.2. Parasites and infections

Specimens of *E. scophthalmi* were obtained from intestines of naturally infected turbot from a turbot farm (Galicia, NW Spain) suffering an outbreak of enteromyxosis. The infected fish were anaesthetized with benzocaine, and then the intestines were removed, placed in sterile seawater and dissected longitudinally. The intestinal mucosa was scraped with a scalpel. Intestinal scrapings were homogenised with a Pasteur pipette and filtered through a 40 μ mesh screen (Sigma-Aldrich, Spain). Filtrates were centrifuged twice at 600×*g* for 5 min and the pellet was resuspended in seawater. Parasites were counted in a Neubauer chamber and the suspension was adjusted to 5×10^6 forms/ml. Experimentally infected fish were infected orally by inoculating each fish with 0.5 ml of the suspension containing the parasites, with an automatic Cornwall BD syringe (Becton-Dickinson, USA).

2.3. Light microscopy

On days 7, 14, 21, 28 and 35 post infection, three experimentally fish were sacrificed by overexposure to anaesthetic, and segments of pyloric caeca, anterior and posterior intestine and rectum were dissected out. Intestine tissues were fixed in Bouin's fixative and embedded in paraffin. Thin sections (5 μm) were stained with haematoxylin and eosin and examined to detect the presence of myxosporean stages. Infection intensity was semi quantitatively classified from no infection (0) to severe infection (+++), according to the number of parasites present in the intestinal sections.

2.4. DAPI staining of nuclei

Samples obtained from enemas (1 mL) (see below) were washed with PBS by centrifugation, fixed in methanol for 5 min, washed again with PBS and incubated with 0.8 mg/ml of 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Sigma–Aldrich) in PBS for 15 min at room temperature. After several washes in PBS, samples were added to a haemocytometer and the number of parasite nuclei per mL was determined by fluorescence microscopy.

2.5. Samples and DNA extraction

Samples of the intestinal contents were collected by means of enemas, enabling the infection to be tracked in individual fish without killing them. Feeding was withheld the day before administration of the enemas, carried out by injecting 0.5 ml of PBS into the fish rectum through a cannula attached to a 1 ml-syringe. For each enema, about 0.2 ml of intestinal contents was obtained. The intestinal content samples were centrifuged at 600×*g* for 5 min and the pellet was resuspended in 0.2 ml of PBS. Parasite numbers per sample were estimated in a Neubauer chamber, and the DNA was extracted and purified with a DNeasy Blood & Tissue kit (Qiagen, UK) and eluted in a final volume of 0.2 ml according to the manufacturer's recommendations. DNA samples were stored at −20 °C until PCR or qPCR analysis.

2.6. Conventional and quantitative real-time PCR (qPCR)

The 28S ribosomal DNA sequence of *E. scophthalmi* was targeted for PCR and qPCR assays. The primers were designed on the basis of a partial sequence of 28S large subunit ribosomal RNA gene of *Enteromyxum leei* (NCBI accession number FJ428227; Fig. 1C), described by Bartosova et al. (2009). PCR amplifications were performed as previously described (Budiño et al., 2011). A region of 895 bp of 28S rRNA gene of *E. scophthalmi* was amplified with forward/reverse primers: 5′-ACCTCCACTCAGGCAAGATTA-3′/5′-GATGGTGAACTATGCATGAGC-3′ (F/R ELS28S). The PCR products were purified by Microcon-PCR (Millipore, USA) and cloned in the pGEM-T Easy vector (Promega, USA) using the kit and instructions supplied by the manufacturer, as previously described (Leiro et al., 2002). Sequencing products were precipitated with sodium acetate/ethanol and separated on an ABI PRISM 377 DNA Sequencer™ (Applied Biosystems, USA).

qPCR amplifications were performed with an Eco Real-Time PCR system (Illumina, USA) and a 10-μl reaction volume containing 5 μl of the reagent from an Kappa SYBR FAST qPCR kit (KappaBiosystems, USA), 0.5 mM of each primer, and 10 ng of template DNA. After polymerase activation, a denaturing cycle of 95 °C was performed for 5 min, and then 40 cycles were run with denaturation for 30 s 95 °C, annealing for 45 s at 57 °C, and extension for 60 s at 72 °C. To establish the coefficient of correlation of the PCR assay, a series of tenfold dilutions of the genomic DNA was prepared, and each dilution was run in 4 replicate experiments. For data analysis, the melting curve and cycle threshold (C_q) values were selected as the evaluation parameters. To verify that the primer pair only produced a single product, a dissociation protocol was added after thermocycling; dissociation of the PCR products was determined between 65 °C and 95 °C. The readout of the reaction with melting temperatures of 80 °C to 85 °C, a dF/dT fluorescence value above 2, and a C_q value below the C_q of the detection limit were used to validate a positive reaction. Samples with a C_q value higher than the C_q value of the positive control alone (diluted twofold) were subjected to further testing in a qPCR after tenfold dilution. The assay included a no-template control and a standard curve of five serial dilutions points (tenfold dilution) of a linearised plasmid cloned with 895 bp of 28S rRNA gene of *E. scophthalmi*. The copy number for the linearised plasmid DNA was calculated as follows:

$$\text{Copynumber} = 6.023 \times 10^{23}(\text{copies/mol}) \times \text{concentration of standard } (\text{g/μl})/\text{MW}(\text{g/mol}).$$

2.7. Molecular analyses

Alignment and consensus of the study sequences was performed with Clustal W software (Larkin et al., 2007) and edited with the Jalview Multiple Alignment Editor V1.8. Sites containing gaps were excluded. Phylogenetic trees were constructed with the MEGA programme, by the neighbour-joining (NJ) method applied to the Kimura two-parameter correction model (Kimura, 1980) by bootstrapping with 1000 replicates (Felsenstein, 1985).

2.8. Lower limit of detection and repeatability and reproducibility of assays

To establish external standard curves for the quantification of *E. scophthalmi* and to determine the limit of detection of the qPCR