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Highlights

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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,*]

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► Identification of *E. scophthalmi* by traditional microscopy methods is time consuming. ► A qPCR assay was developed as alternative diagnostic method. ► The qPCR assay allows monitoring myxozoans infections throughout their life cycle. ► The method proved to be sensitive, reproducible and reliable for detect parasite DNA. ► It will help to enhance the quality of myxosporean identification in fish intestine.

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

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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 & Dykova, 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 & 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

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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 μmesh 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:

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

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

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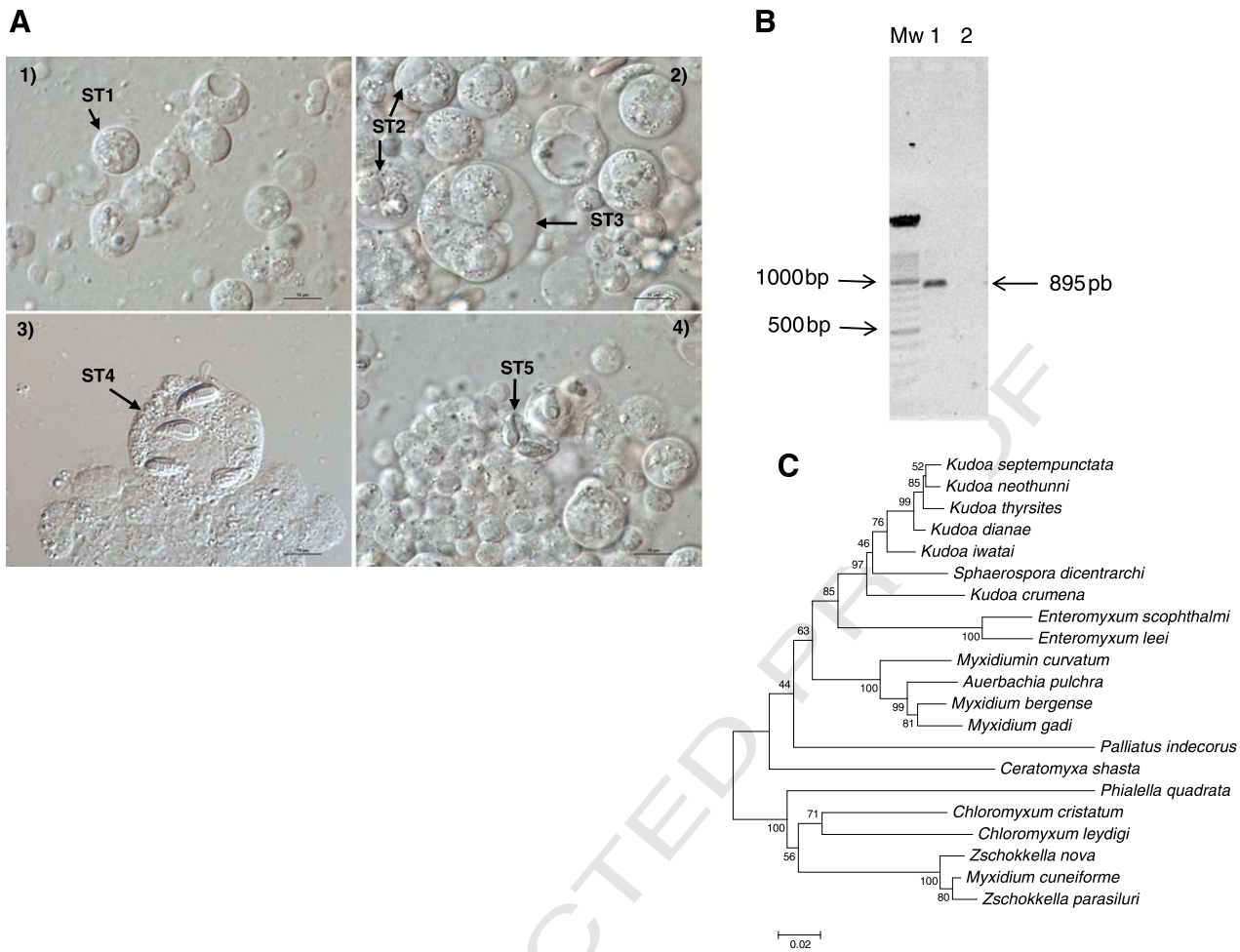

Fig. 1. A) Optical microscopy micrographs obtained under interdifferential contrast (Nomarski) of the different stages of development (ST) of *Enteromyxum scophthalmi* in the gut of naturally infected turbot, denominated states 1–5. ST1: trophozoite with one or more nuclei; ST2: trophozoite in which a primary cell contains a secondary cell, and eventually has one or more nuclei; ST3: trophozoite in a primary cell that contains one or more secondary cells; stages ST4 and ST5 are related to sporogenesis and correspond to (ST4) a plasmodium housing a sporoblast giving rise to two spores, and (ST5) the fully developed spore. B) Ethidium bromide-stained agarose gel showing a DNA fragment corresponding to the gene encoding the 28S rRNA gene of *E. scophthalmi* amplified by conventional PCR using as template DNA samples obtained from intestines of naturally infected turbot (lane 1). Lane 2 corresponds to a PCR using as template a DNA sample from uninfected turbot. MW corresponds to molecular weight markers expressed in base pairs (bp). C) Phylogenetic relationship between *E. scophthalmi* and other myxosporidian species that parasitize fish, as determined by 28S rRNA sequence analysis (with the MEGA programme). The neighbour-joining (NJ) phylogram was constructed using the Kimura-2-parameter. Number on the nodes indicates the number of times a particular branch was recorded per 100 bootstrap replications following 1000 replicates. Branches with confidence values lower than 50% were ignored. Genetic distance is represented by the scale bar.

assay, calibration range tenfold dilutions of DNA samples were run, and tested 8 times in the same experiment to validate the repeatability. The same samples were tested once a day for 5 days to determine the reproducibility and repeatability of the qPCR assay, and both intra- and inter-assay coefficients of variation (CV) were assessed. The CV values were calculated as the standard deviation (σ) divided by the mean (μ) $\times$ 100% ($CV = \sigma/\mu \times 100$) for triplicate assays (intra-assay variation) and for all nine output data (inter-assay variation). Correlation coefficients (r^2 values) were calculated for each standard curve by linear regression analysis. For the real-time assays, the amplification efficiency was calculated as $E = [10^{(-1/\text{slope})} - 1] \times 100\%$.

3. Results

3.1. Morphological and molecular diagnosis of myxozoan from turbot and phylogenetic relationships

Infections in fish naturally infected by *E. scophthalmi* were initially identified by visualization of intestinal scrapings by Nomarski interference contrast microscopy, and by observation of the different life cycle stages characteristic of this parasite (Fig. 1A). The PCR primers amplified a fragment of 895 bp of genomic DNA from myxozoan parasite *E. scophthalmi* (Fig. 1B). No amplification was obtained when genomic DNA of uninfected turbot was used as a template for the PCR (Fig. 1B, lane 2). The fragment obtained after nucleotidic sequencing of this DNA showed 94% identity with the 28S rRNA sequence of *E. leei*. Phylogenetic analysis of the 28S rRNA obtained from the myxozoans parasites isolated from the turbot confirm a high relationship with the myxozoan specie *E. leei* (Fig. 1C).

3.2. qPCR assay

3.2.1. Assay specificity and sensitivity

The specificity of the 28S rRNA products was determined by melting curve analysis between 55 and 90 °C (Eco software v3.0). The sensitivity of the qPCR was evaluated using different starting amounts of recombinant 28S rRNA from 0.1 pg (10^5 DNA copies) to 1 ng (10^9 DNA copies). Determination of the cycle threshold point (C_q) occurs during the exponential phase of the PCR cycle (Fig. 2A). A simple regression line of the C_q values for the DNA standards was plotted against the log values of their starting copy numbers, and the intestinal DNA samples were quantified (Fig. 2B). For each standard curve,

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the r^2 value, the amplification efficiency, *E* (derived from the slope of the standard curve) and the *y*-intercept value were reported; the *y*-intercept value indicates the sensitivity of the reaction: lower values indicate greater sensitivity of the qPCR amplification. The standard curve based on the pGEM-T-28S rRNA control had a slope of −3.205, a *y*-intercept value of 17.16, and efficiency of 105.14% (Fig. 2B). The minimal detectable amount of 28S rRNA using SYBR Green II was 13 DNA copies, corresponding to 0.55 fg with satisfactory test linearity (r^2=0.096) per reaction. We quantified the number of parasite nuclei per sample of intestinal contents by DAPI staining (Fig. 3, A–C). A sample of about 12.5×10^4 parasite nuclei contains approximately 181.74×10^6 copies of the 28S rRNA gene, allowing us to estimate that each parasite nucleus has in its genomic DNA about of 1450 copies of the 28S rRNA gene (Fig. 3).

3.2.2. Assay reproducibility

Both intra- and inter-assay CV values were assessed to analyze the reproducibility and repeatability of the qPCR assay. The reproducibility of the qPCR was determined by assessing the intra-assay variability (variation between replicate qPCR amplifications in the same experiment/assay plate) with high (A1) and low (A2) concentrations of *E. scophthalmi* genomic DNA, corresponding to 1 ng and 100 fg, respectively; both concentrations were tested in 10 replicates in the same assay (Fig. 4). The intra-assay variability was low for both samples, although the reproducibility was higher for the sample containing the lower concentration of DNA (Fig. 4). The inter-assay variability (i.e. to investigate variation between replicate qPCR amplifications in separate experiments/assay plates), determined by testing six positive samples in three to six separate assays, showed similar performance to intra-assay runs (Table 1A). To further test the reproducibility of the qPCR, the variation in four-fold dilutions (from 0.1 to 100 pg of *E. scophthalmi* DNA) was monitored in four/five separate PCR assays. The results obtained showed good reproducibility, with a CV ranging from 3.5% (100 pg of DNA) to 4.9% (1 pg of DNA; Table 1B).

3.3. Sample validation

Intestinal content samples collected from turbot experimentally infected with *E. scophthalmi* were tested for parasite quantification by qPCR. Microscopic examination of intestinal scrapings was also performed weekly and the intensity of infection and the changes in the mortality of infected fish was determined qualitatively (Fig. 5). The qPCR assay can detect myxozoan infection in the intestinal contents during the first week after infection. Furthermore, the qPCR accurately quantifies the progression of disease over time, showing a weekly increase in the number of parasites during the period investigated, reaching maximal values at around day 42 p.i. (Fig. 5A). The progressive increase in parasites was also detected by histological analysis, and closely matched the kinetics of the intensity of infection shown by qPCR (Fig. 5B). Although the use of conventional PCR enables detection of the parasite in the intestinal contents, the use of this technique for a precise quantification is not possible. The intensity of the amplification signal determined by conventional PCR

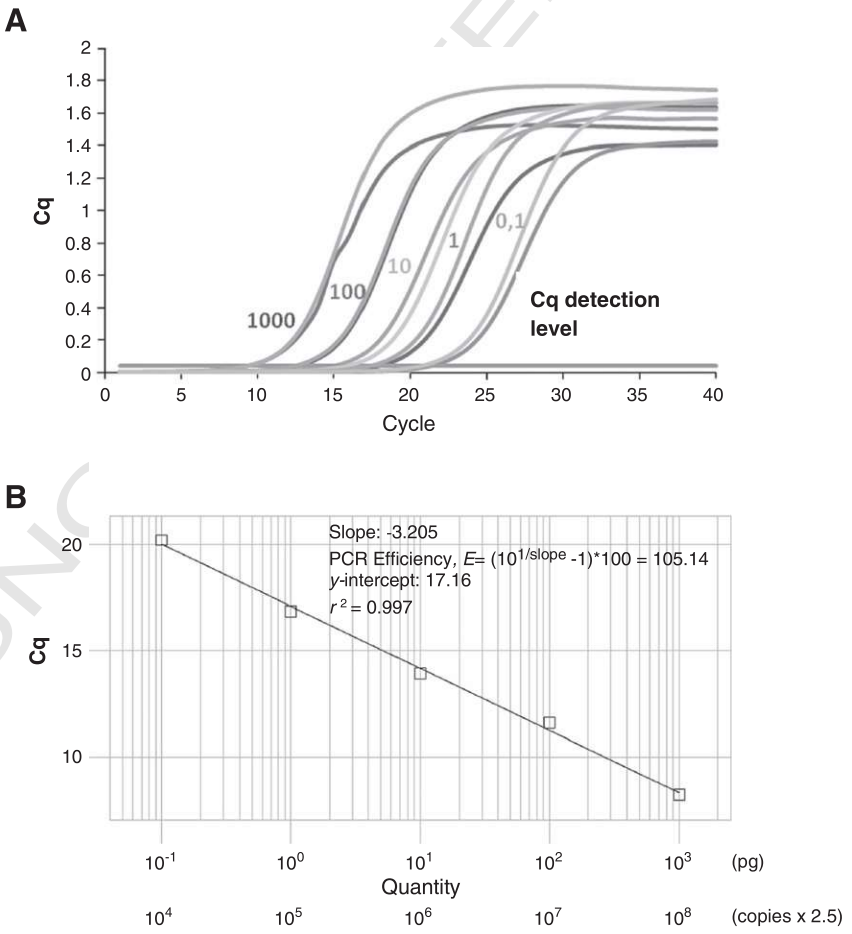

Fig. 2. Qualitative qPCR amplification from known amounts of template DNA to construct standard curves for quantification of unknown samples. A) Linear plot of the increase in florescence against cycle number of DNA standard ranging from 0.1 to 1000 pg with corresponding C_t values for each of the amplified standards. B) Simple linear regression of the C_t values from standard plotted against the log of the initial DNA concentration of plasmid containing the 28S rRNA amplicon.

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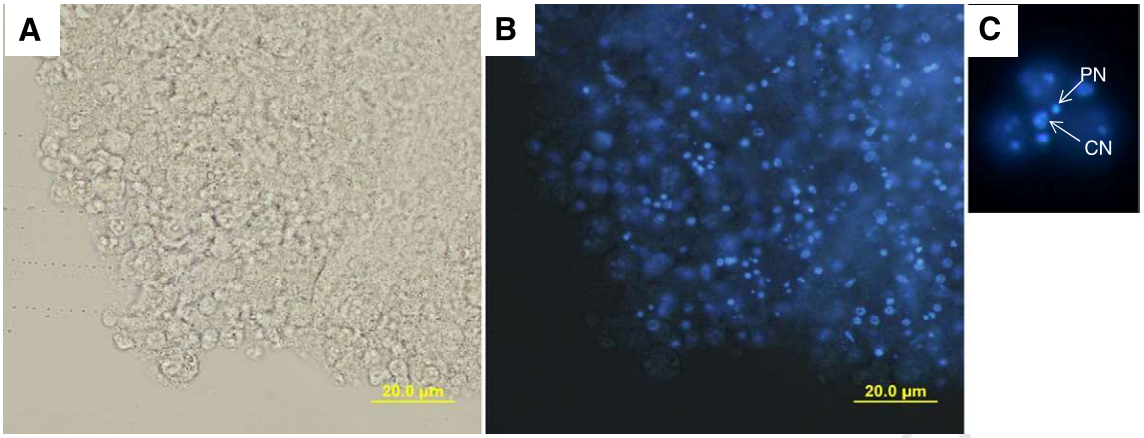
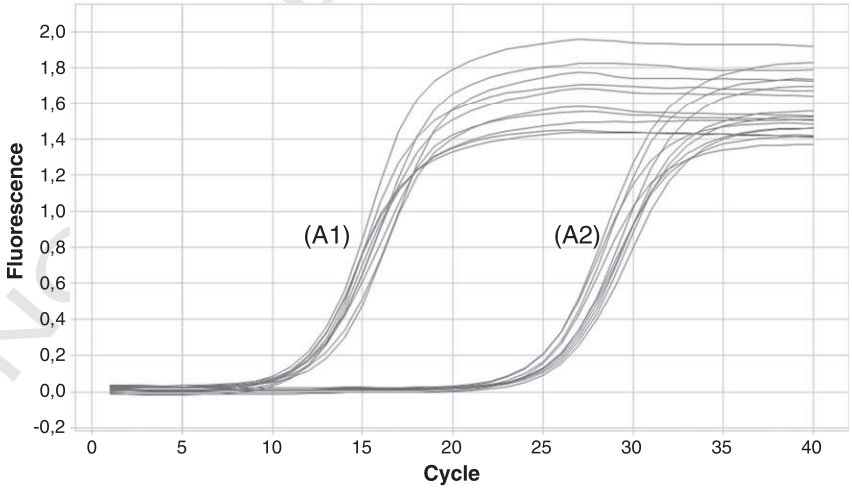


Sample (dilution)	Cq	pg	pgx dilution	Mean	SD
1/10	15.911	9.246	92.46	174.75	67.72
1/10	15.273	14.638	146.38		
1/100	17.875	2.246	224.6		
1/100	17.809	2.356	235.6		

DAPI=**12.5x10^4** of total nuclei of the parasite in the sample
1000 pg=1.04x10^9 copies of 28S rRNA gene
174.75 pg=181.74x10^6 copies of 28S rRNA gene
Number of copies/nucleus =181.74 x 10^6 / 12.5 x 10^4= **1453.92**

Fig. 3. Microphotograph of intestinal scrapings of turbot infected with *E. scophthalmi* and viewed under phase contrast (A) and fluorescence (B) microscopy after staining with DAPI (B). Figure C shows a detail of host cell (CN) and parasite (PN) nuclei stained with DAPI (arrows). The table below shows the data obtained after quantification of DNA by qPCR in picograms (pg) and the estimation of the number of gene copies per nucleus of the parasite. SD: standard deviation.



Sample concentration	Assaysnumber	Cq (Mean±SD)	CV (%)
1000pg (A1,high)	10	8.659±0.398	4.598
0.1pg (A2,low)	10	2.587±0.531	2.458

Fig. 4. Intra-assay variability of threshold cycle value (C_q), calculated from the coefficient of variation (CV; standard deviation (SD)/mean × 100%), of a standard curve for two concentrations of plasmid DNA containing the 28S rRNA gene fragment of *E. scophthalmi*: 1000 pg (A1, high concentration) and 0.1 pg (A2, low concentration), and obtained by qPCR in 10 replicates.

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Table 1
Inter-assay of qPCR variability in threshold cycle value (C_q), calculated from the coefficients of variation (CV) for several samples of DNA from *E. scophthalmi* in different tests (A), or by use of different concentrations of parasite DNA as standard (cloned DNA fragment of 28S rRNA) and assay by qPCR in different tests (B). SD: standard deviation.

(A)

Sample (number)	Assay number	Cq (Mean ± SD)	CV (%)
20C	4	22.73 ± 0.44	1.93
19	6	24.92 ± 0.68	2.72
29	5	21.53 ± 0.78	3.64
32	5	19.37 ± 1.04	5.38
43	3	19.75 ± 0.29	1.46
46	3	22.09 ± 0.39	1.76

(B)

Sample concentration (pg)	Assay number	C_q (Mean ± SD)	CV (%)
100	5	11.49 ± 0.40	3.49
10	4	14.76 ± 0.67	4.53
1	5	17.06 ± 0.84	4.94
0.1	5	20.12 ± 0.76	3.78

was not proportional to the level of infection observed; for example, the amplification signal was higher in week 3 than in week 4 or 5, when the intensity of infection was clearly higher (Fig. 5C). The higher levels of parasites quantified by qPCR in week 5 and 6, also coincided with periods of increased fish mortality (Fig. 5D).

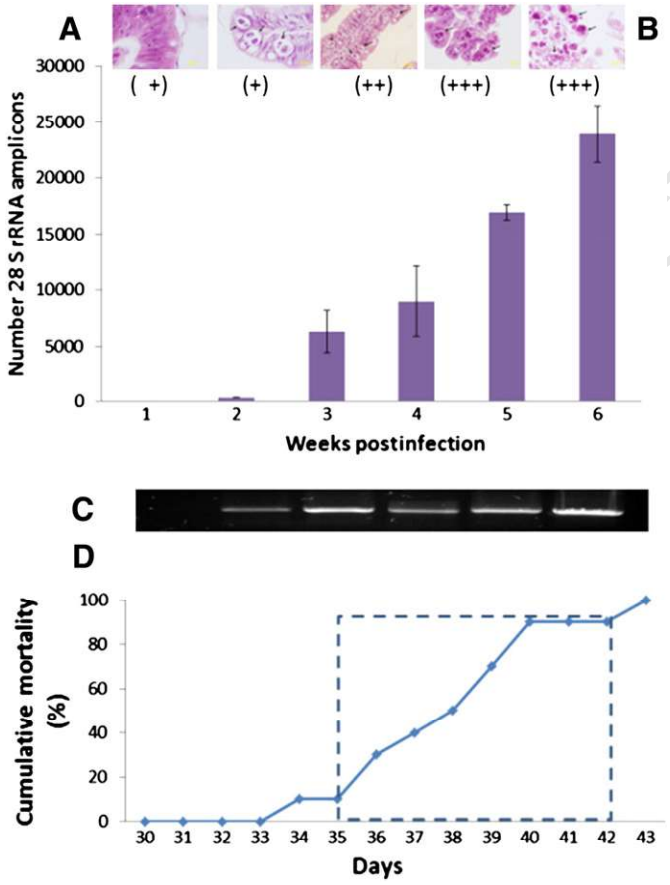

Fig. 5. A) Real-time quantitative PCR (qPCR) validation showing the amplification kinetics in intestinal samples collected weekly from experimentally infected fish with *E. scophthalmi*. B) Histological diagnosis of five selected intestinal samples from experimentally infected fish containing parasites at various stages of development. (+) indicates the qualitative levels of intensity of infection. C) Analysis of changes in the kinetics of infection by conventional PCR. D) Cumulative mortality curve for fish experimentally infected with *E. scophthalmi*, and those from which the intestinal contents were obtained for carrying out the PCR and qPCR assays. The box indicates the days on which the maximum fish mortality was detected.

4. Discussion

Diagnosis of myxozoan infections is complicated by the variable morphology of presporogonic stages of the parasite in the host (Hallett and Bartholomew, 2006). Microscopic examination of the intestinal contents from naturally and experimentally infected fish, which were used to obtain parasite DNA, revealed the existence of typical developmental stages described for *E. scophthalmi* by Palenzuela et al. (2002) and Redondo et al. (2004). In the present study, we describe the development of a new molecular qPCR assay for the detection and quantification of the myxozoan *E. scophthalmi* in intestinal content samples from infected turbot. We evaluated the specificity, sensitivity and repeatability of the assay. Validation of the *E. scophthalmi* qPCR assay revealed good sensitivity and repeatability. We designed a primer pair based on the nucleotide sequence of 28S rDNA gene of the myxozoan *E. leei*, a species that is closely related to *E. scophthalmi* in terms of morphology, histopathology and life cycle (Álvarez-Pellitero et al., 2008; Branson et al., 1999; Palenzuela et al., 2002; Sitjà-Bobadilla et al., 2007). The amplicon amplified by conventional PCR with the primer pair F/R ELS28S shows 94% nucleotide homology with a fragment of 28S rRNA gene of *E. leei* (Bartosova et al., 2009). No amplification was observed for host or non-*E. scophthalmi* DNA, suggesting that both primers amplified only the parasite target sequences. A reference standard (the pGEM-T-28S plasmid) was developed and used to calibrate qPCR values to *E. scophthalmi* 28S rRNA copy number. The qPCR enabled detection of 13 copies of 28S rRNA corresponding to 0.55 fg DNA; these detection levels were similar to those reported in other studies using the qPCR assay (Herrero et al., 2011; Wang et al., 2003). rRNA genes are organised in tandem repeats with about 1000 copies per cell in myxozoans and there is an implicit assumption that the same number of copies of the ribosomal genes per nucleus is present in all parasite stages, although their number is quite variable between different myxozoan species (Hallett and Bartholomew, 2006). In our study we have estimated that the 28S rRNA gene in the development stages of *E. scophthalmi* has about 1500 copies per parasite nucleus.

It has been suggested that a coefficient variation (CV) greater than 20% produces a significant loss of precision in quantitative assays (Reed et al., 2002). Intra-assay variation quantifies the error within a single assay when the same template is run multiple times on the same plate with the same reagents; inter-assay variation is quantified among results from two separate assays on either the same or different days (Wong and Medrano, 2005). In this study, intra- and inter-assay CVs ranged between 2 and 5%, indicating the high repeatability of the qPCR assay, with values similar to those obtained in other qPCR assays developed for quantification of parasites (Monis et al., 2005), including myxozoans (Cavender et al., 2004; Jorgensen et al., 2011; Kelley et al., 2006).

Although the Myxozoa are well known organisms, many questions remain about their origin, phylogeny and life cycle (Canning and Okamura, 2004; Kent et al., 2001). The qPCR assay developed in the present study enabled us to monitor the infection in experimentally infected turbot throughout the entire life cycle of the parasite. The results of the validation demonstrated a significant association between histologically detectable infections and high qPCR levels in the assay. The results obtained by qPCR were confirmed by conventional PCR, although this is a not an accurate method for quantifying the intensity of parasitism during experimental infection. On the other hand, the highest levels of infection (between 5 and 6 weeks after infection) detected by qPCR also coincided with the period of highest mortality of infected fish.

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