Fast Real-Time PCR assay for detection of *Tetramicra brevifilum* in cultured turbot

MERCEDES ALONSO[1], FÁTIMA C. LAGO[1], MARÍA GÓMEZ-REINO[2], JACOBO FERNÁNDEZ[3], IRIS MARTÍN[3], JUAN M. VIEITES[1] *and* MONTSERRAT ESPIÑEIRA[1]*

[1] *Research Department of Genomics and Proteomics Applied to the Marine and Food Industry, ANFACO-CECOPESCA, Vigo, 36310 Pontevedra, Spain*
[2] *Research Department of Aquaculture, ANFACO-CECOPESCA, Vigo, 36310 Pontevedra, Spain*
[3] *INSUIÑA S.L., Xove, Lugo, Spain*

(*Received 16 July 2012; accepted 8 September 2012; first published online 15 October 2012*)

SUMMARY

Global aquaculture production of turbot has rapidly increased worldwide in the last decade and it is expected to have even bigger growth in the next years due to new farms operating. The losses caused by pathogen infections have grown at the same time as the production of this species. Parasitological infections are among the main relevant pathologies associated with its culture and produce serious losses in aquaculture, reduce the growth rate in fish and may lead to unmarketable fish due to skeletal muscle abnormalities in cases with high intensity of infection. The microsporidian parasite *Tetramicra brevifilum* causes severe infections and generates major losses in farmed turbot. Infections are difficult to control due to spore longevity and its direct transmission. To facilitate the infection management, an effective tool for fast detection and identification of *T. brevifilum* is needed. This study provides a molecular methodology of fast Real-Time PCR for *T. brevifilum* detection to the aquaculture industry, useful for routine control of *T. brevifilum* at turbot farms. The method is characterized by its high specificity and sensitivity, and it can be applied to cultured turbot for parasite detection regardless of the life-cycle stage of the pathogen or the infection intensity.

Key words: aquaculture, fast Real-Time PCR, *Tetramicra brevifilum*, microsporidian, turbot disease, detection.

INTRODUCTION

Turbot, *Scophthalmus maximus*, is one of the most important farmed fish in Europe. According to the Food and Agriculture Organization production of turbot has doubled in Europe in the last decade reaching 8396 t in 2010 (FAO, 2010). A significant increase in losses, caused by pathogens, in cultured turbot has been closely associated with the fast growth of the aquacultured turbot worldwide. Diseases limit the productive potential and the quality of the aquaculture industry. Bacterial, viral and parasitological pathologies produce significant losses in aquaculture, not only by causing elevated mortalities, but also by producing physical abnormalities in the fish that might lead to unmarketable products.

Distinguished, by importance, among parasites that affect cultured turbot are those that are produced by ciliates (*Uronema marinum*, *U. nigricans*, *Philasterides dicentrarchi*) (Sterud *et al*. 2000; Padros *et al*. 2001), by mixosporidians (*Enteromyxum scophthalmi*) (Branson *et al*. 1999) and by microsporidians. Microsporidians are probably the parasites that provoke the biggest losses in farmed turbot (Person-Le, 1990; Estevez *et al*. 1992; Figueras *et al*. 1992; Lom and Dykova, 1992). Within the microsporidia, *Tetramicra brevifilum* should be considered a major threat to the culture of turbot (Estevez *et al*. 1992; Figueras *et al*. 1992). It causes big losses in the number of fish, reduces the growth rate of turbot when it progresses to the disease and when the infection is of high intensity it may lead to unmarketable fish due to the liquefaction of the skeletal muscles (Figueras *et al*. 1992).

This parasite infects the connective tissue of the muscle (Matthews and Matthews, 1980), and may also infect the liver and intestine (Estevez *et al*. 1992). Affected fish show erratic swimming behaviour, swelling of different parts of the body, darkening of the dorsal surface and overproduction of mucus. Heavily infected fish present jelly-like muscles (Figueras *et al*. 1992). Infections by microsporidians are usually difficult to control because of the longevity of the spores and by its direct transmission.

Diseases caused by parasites are difficult to diagnose, and a delayed histological diagnosis could aggravate the infection in the culturing tanks (Alvarez-Pellitero *et al*. 2004; Feist and Longshaw, 2008). Furthermore, the most commonly used treatments currently involve massive application of

* Corresponding author: Research Department of Genomics and Proteomics Applied to the Marine and Food Industry, ANFACO-CECOPESCA, Vigo, 36310 Pontevedra, Spain. Tel: +31 34 986 469 301. Fax: +31 34 986 469 269. E-mail: montse@anfaco.es

doi:10.1017/S0031182012001655

chemicals and antibiotics (Harikrishnan *et al*. 2010), and the use of vaccines (Lamas *et al*. 2008; Sanmartin *et al*. 2008) that do not always exist for all cases. Therefore, it is necessary to develop methodologies that can be used for routine checks and early detection of pathogens in order to prevent the establishment and the spreading of the disease. Hence, the method will reduce the overuse of chemicals and antibiotics.

Up to date, the diagnosis of the infection caused by *T. brevifilum* in turbot was based almost exclusively on histological and ultrastructural studies (Matthews and Matthews, 1980; Estevez *et al*. 1992; Figueras *et al*. 1992). Other techniques such as immunological ones, may have limited value for the use on parasites like microsporidians that have various life-cycle stages, since antigen composition or morphology may vary among different life-cycle stages (Bartholomew *et al*. 1989). In contrast, molecular methods do not depend on morphological characteristics and on the life-cycle stages (Bartholomew *et al*. 1995). One of the principal advantages of Polymerase Chain Reaction (PCR) compared to microscopical methods is the capacity of detecting early infections (Andree *et al*. 1998). In recent years, PCR techniques have been developed for the detection of different species of microsporidia. The study carried out by Leiro *et al*. (2002) is worthy of note, in which *T. brevifilum* was detected by conventional PCR using ribosomal DNA.

The relevance of the present study is the development of a molecular technique for rapid detection of *T. brevifilum* readily applicable in the aquaculture industry. This technique is based on Real-Time PCR and is of easy implementation in fish farms. Furthermore, this methodology is characterized by its high specificity and sensitivity. The method is a fast and effective tool that can be used at any stage of the life cycle of fish, regardless of the life-cycle stage of the parasite or the infection intensity.

MATERIALS AND METHODS

Sample collection, storage and DNA extraction

Juvenile turbot, *Scophthalmus maximus* (50–100 g) infected with *Tetramicra brevifilum* were provided from a local farm in the north of Galicia (Insuíña S.L, Spain). The parasite was previously identified by ultrastructural studies by experts at the farm. The infected fish were preserved and transported whole under refrigerated conditions until isolation of the parasite at the laboratory. Microsporidian spores were aseptically isolated from infected turbot by scraping xenomas (spore aggregates) from gills and fins. DNA extraction from xenomas was performed according to the protocol described by Herrero *et al*. (2011). The quality and quantity of the DNA extractions have been determined by measuring the absorbance at 260 nm and the 260/280 and 234/260 ratios using a NanoDrop™ ND-1000 spectrophotometer (Thermo Scientific) (Winfrey *et al*. 1997). DNA extractions were appropriately labelled and stored at −80 °C for subsequent tasks.

Design of a specific real-time PCR method to detect Tetramicra brevifilum

From ssrRNA (18S rRNA) sequences obtained from the NCBI database (AF364303 and EF126038) two internal primers and a probe set specific for *T. brevifilum* were designed: Parasit_Scopht F (5′-CTG AGA RAC GGC TAC CA-3′), Parasit_Scopht R (5′ TGG AGC TGG WAT TAC CG-3′); and a labelled probe, TETRAMICRA PROBE (5′-6-FAM-CAC CAG CCT TGA CCT CCA GTT ACC-BHQ1-3′). The same region was selected for use as an inhibitor control. The DNA extraction control allows amplification with *Scophthalmus maximus*. From ssrRNA (18S rRNA) sequences obtained from the NCBI database a specific probe for *S. maximus* was designed: 5′-6-FAM-CGC AAA TTA CCC ACT CCC GAC TC-BHQ-1-3′. This probe, CONTROL ROD 1, was used with the same primer pair designed for the detection of *T. brevifilum*.

For both primers/probe sets designed, PCR was performed in a total reaction volume of 20 μl containing 100 ng DNA template, 10 μl TaqMan Fast Advanced Master Mix (Applied Biosystems), the optimized amount of primers and probe, and molecular biology grade water (Eppendorf) adjusted up to the final volume. Optimal amounts of primers and probe were evaluated by preparing a dilution series to determine the minimum concentrations giving the maximum ΔRn (normalized reporter, defined as emission intensity of reporter/Emission intensity of passive reference). First, forward and reverse primer concentrations of 50, 300, 500 and 900 nM were evaluated with a constant probe concentration of 50 nM. Following primer optimization, probe optimization was initiated using concentrations of 50–250 nM.

The reaction was performed in triplicate on DNA samples in MicroAmp Optical 96-well reaction plates (Applied Biosystems) with MicroAmp optical caps (Applied Biosystems) in the ViiA™ 7 Real-Time PCR System (Applied Biosystems). Amplification was carried out with the following cycling protocol: 95 °C for 20 s, and 40 cycles each of 95 °C for 1 s and 60 °C for 20 s.

Analytical specificity and detection limit (LDO)

The specific primers/probe set for *T. brevifilum* was tested on DNA extracts from the most important parasites affecting turbot (*Uronema* spp.,

https://doi.org/10.1017/S0031182012001655 Published online by Cambridge University Press

Enteromyxum scophthalmi, and *Philasterides dicentrarchi*) and on its host (*Scophthalmus maximus*).

To determine the sensitivity of the method, a serial dilution of *T. brevifilum* DNA was performed with turbot DNA in levels ranging from 100 ng/μl to 10 pg/μl, and the fluorescence signal was determined for the primer/probe set for *T. brevifilum*. The dilutions were prepared by a mixture of *T. brevifilum* DNA and turbot DNA at different levels until completing a final amount of 100 ng. All measurements were performed in triplicate from 3 separate samples. The sensitivity of the control primers/probe set was calculated in the same way as described above.

Application of the developed Real-Time PCR method in cultured turbot

The method was validated in infected and non-infected fish samples provided from a local farm in the north of Spain. All samples were analysed by means of classical histological techniques carried out by a pathologist from these farms. Subsequently, they were transported to the laboratory under refrigerated conditions and were analysed with the described methodology.

RESULTS AND DISCUSSION

The use of Real-Time PCR has increased over recent years. The importance of this methodology for research and parasitological diagnosis in fish and meat is reflected in a large number of published studies (Alonso *et al*. 2011, 2012). It has become a major tool for the detection of pathogens in aquaculture. However, as with any methodology for the detection of presence-absence, the risk of false-positive or false-negative results exists. The way to monitor its occurrence is by employing controls (Hoorfar *et al*. 2004; Apfalter *et al*. 2005; Lee *et al*. 2011). The present methodology, in addition to the standard controls of a PCR technique, (negative, positive and reagents blank), includes an inhibitor control. The function of this control is, in the case of a negative result in the assay, to confirm that the sample is certainly negative because it is itself indicative of the non-existence of any inhibitor substance that could block the fragment amplification. It avoids false negative results. The application of these controls in this study assures the correct development of the assay.

Design of a specific Fast Real-Time PCR method to detect T. brevifilum

In the present work, a specific fast Real-Time PCR method for authentication of *T. brevifilum* was designed. For this, from a selected internal region of the 18S rRNA, the primer/probe set for *T. brevifilum* was designed. This set generates PCR products of 162 bp. At the same time, another probe for detection of *S. maximus* with the same primer pair was designed. This was used for the detection of *S. maximus*. In this case, this set generates PCR products of 199 bp. The difference between the size of the amplified products and the same primer pair is due to length polymorphism of the 18S rRNA marker between different species (Budino *et al*. 2011; Gruebl *et al*. 2002; Kaukas and Rollinson, 1997).

Optimization of the reaction gave the best results in terms of specificity, sensitivity, efficiency and reproducibility of Real-Time PCR for the detection of *T. brevifilum*. This was possible by means of the mixture of different concentrations of primers and probe. Concentrations of 900 nM for both primers and 250 nM for the probe yielded the highest endpoint fluorescence and the lowest threshold cycle. The same optimal concentrations of primers and probe for the control set of *S. maximus* were obtained. The specificity of the Real-Time PCR system was tested with DNA from the most important parasites that affect turbot (*Uronema* spp., *Enteromyxum scophthalmi*, and *Philasterides dicentrarchi*) and with its host (*Scophthalmus maximus*) to verify the suitability and reliability of the method. No cross-reactivity was observed with any of the tested samples using the methodology herein developed.

A methodology for the detection of pathogens must be specific and it must be able to differentiate with all security whether the species of interest are present or not. This security is supplied by the specificity of the assay. The application of the developed methodology to different related species, both for their genetic proximity as for having the same host, must assure the specificity of the method. In this case the specificity was tested for species host-related to avoid interferences or cross-reactivity by the presence of other parasites.

The threshold cycle (Ct) is a basic parameter of the Real-Time PCR and it is an essential component producing accurate and reproducible data. It is defined as the fractional PCR cycle number at which the reporter fluorescence is greater than the threshold. The Ct is dependent on the starting template copy number, the efficiency of PCR amplification, the efficiency of cleavage or hybridization of the fluorogenic probe, and the sensitivity of detection (Bustin, 2004). The aim of the data analysis is to determine when target amplification is sufficiently above the background signal, by facilitating more accurate measurement of fluorescence. In all samples analysed, when 100 ng of template were used, the Ct values obtained were 21·5 ± 5·5 and 15·3 ± 1·5, for *T. brevifilum* and *S. maximus* primer/probe set respectively. For the *T. brevifilum* primer/probe set, Ct >40 corresponded to the values of non-target species (the most important parasites affecting

https://doi.org/10.1017/S0031182012001655 Published online by Cambridge University Press

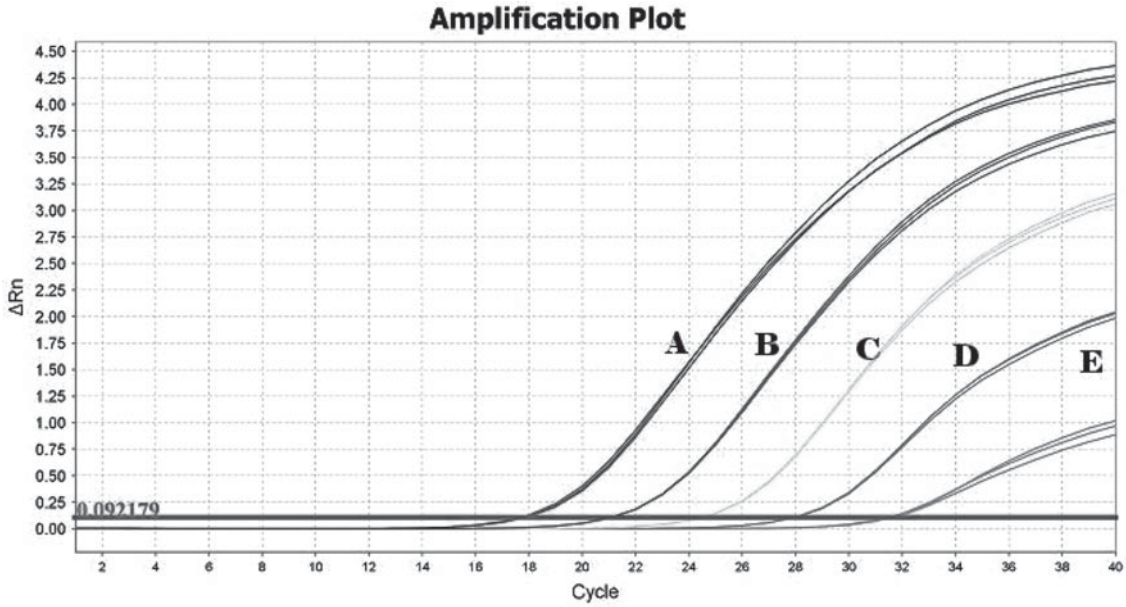

Fig. 1. Efficiency of the Real-Time PCR assay: (A) 250 ng, (B) 25 ng, (C) 2·5 ng, (D) 250 pg, and (E) 25 pg of total DNA extracted from *Tetramicra brevifilum* as template in the PCR.

the turbot) and negative controls. Also, in the cross-reactivity analysis, no false positive results were observed under the most severe conditions used in the test.

The accuracy of the Real-Time PCR is highly dependent on the PCR efficiency. In the developed method, it was calculated based on the slope of the standard curve obtained using DNA serial dilutions with a template of known concentration (from 250 ng to 25 pg) as templates for Real-Time PCR (Fig. 1). The efficiency (E) of the reaction was calculated according to the equation $E = (10^{(-1/\text{slope})} - 1) \times 100$ (Bustin, 2004). The amplification plot of the experiment with the primer/probe set *T. brevifilum* and *S. maximus* generated a slope of −3·34 and −3·37 and an efficiency of 99% and 98%, respectively, with a correlation coefficient of 0·999 in both primer/probe sets. These values of Ct and efficiency have demonstrated the utility of the Real-Time PCR system to detect *T. brevifilum* and *S. maximus*, which is used as control of the methodology developed, being the efficiency near to 100%. This confirms that real-Time PCR is a robust methodology to detect the presence of *T. brevifilum* in cultured turbot. The limit of detection of the developed Real-Time PCR assay reveals that at least 15 and 0·01 pg of the target DNA of *T. brevifilum* and *S. maximus*, respectively, are necessary for positive results. This difference in the limit of detection is due to the failure to isolate the parasite from fish. The DNA extraction from *Tetramicra* includes parasite and *S. maximus* DNA.

The sensitivity obtained is very important at the moment of the detection because sensitivity allows detection of the presence of *T. brevifilum* at early stages of infection, when the infection intensity is low, and regardless of the life-cycle stage of the parasite. The method permits early control measures and consequently avoids a systematic propagation of the pathogen.

Application of the developed Real-Time PCR in cultured turbot

The extracted DNA showed a good yield with optimal purity in all the products analysed (data not shown) and both molecular and classical histological techniques showed concordant results for the detection of *T. brevifilum* in the analysed samples.

The developed methodology allowed detection of *T. brevifilum* despite the life-cycle stage of the parasite, and the fact that the infection intensity was low. In addition, it provides an effective tool for the control and prevention of spreading of this pathogen in cultured turbot. The early control of pathologies improves the health and welfare of cultured fish. It also may lead to a reduction of the use of disinfecting chemicals and antibiotics in aquaculture and, as a result, it could minimize the environmental impact of the culture at the same time as increasing the profitability of the aquaculture sector. The methodology developed in this study for the detection of *T. brevifilum* can be applied to other cultures affected by this parasite, and as a sanitary control of fisheries such as in the control of *Lophius budegassa* (Maíllo *et al*. 1998). For its application to other species it will be necessary to check the specificity of the inhibitor control developed with the new host species. If the control does not obtain amplification for this host it will be necessary to design a new specific inhibitor control for the species under study. This character extends the application of this detection

https://doi.org/10.1017/S0031182012001655 Published online by Cambridge University Press

methodology, and provides a widespread tool of control for infections for this parasite.

Additional research is necessary to use the developed methodology in the water of the turbot farm. Future research should focus on aspects such as evaluating what is the best filtration system, the adequate volume of infected water, the influence of infection intensity, and other aspects relevant for the recovery of the parasite. The identification of parasites in the water of the turbot cultures could amplify the capacity of detection of this parasite and avoid the sacrifice or stress of the fish.

ACKNOWLEDGEMENTS

This work was funded by the 10MMA006E grant from the Consellería de Innovación e Industria de la Xunta de Galicia. We thank Jacobo Docal and Martín Gómez from INSUIÑA S.L. for their helpful assistance and for kindly supplying the fish and parasites samples.

REFERENCES

Alonso, M., Herrero, B., Vieites, J. M. and Espineira, M. (2011). Real-time PCR assay for detection of *Trichinella* in meat. *Food Control* **22**, 1333–1338.

Alonso, M., Lago, F. C., Herrero, B., Vieites, J. M. and Espiñeira, M. (2012). Molecular characterization of microalgae used in aquaculture with biotechnology potential, *Aquaculture International* **20**, 847–857 (DOI: 10·1007/s10499-012-9506-8).

Alvarez-Pellitero, P., Quiroga, M. I., Sitja-Bobadilla, A., Redondo, M. J., Palenzuela, O., Padros, F., Vazquez, S. and Nieto, J. M. (2004). *Cryptosporidium scophthalmi* n. sp (Apicomplexa : Cryptosporidiidae) from cultured turbot *Scophthalmus maximus*. Light and electron microscope description and histopathological study. *Diseases of Aquatic Organisms* **62**, 133–145.

Andree, K. B., MacConnell, E. and Hedrick, R. P. (1998). A nested polymerase chain reaction for the detection of genomic DNA of *Myxobolus cerebralis* in rainbow trout *Oncorhynchus mykiss*. *Diseases of Aquatic Organisms* **34**, 145–154.

Apfalter, P., Reischl, U. and Hammerschlag, M. R. (2005). In-house nucleic acid amplification assays in research: How much quality control is needed before one can rely upon the results? *Journal of Clinical Microbiology* **43**, 5835–5841.

Bartholomew, J. L., Rodriguez, R. J. and Aradawa, C. K. (1995). Development of a DNA-probe for the myxosporean parasite *Ceratomyxa-Shasta*, using the Polymerase Chain-Reaction with arbitrary primers. *Diseases of Aquatic Organisms* **21**, 215–220.

Bartholomew, J. L., Smith, C. E., Rohovec, J. S. and Fryer, J. L. (1989). Characterization of a host response to the myxosporean parasite, *Ceratomyxa-Shasta* (Noble), by histology, scanning electron-microscopy and inmunological techniques. *Journal of Fish Diseases* **12**, 509–522.

Branson, E., Riaza, A. and Alvarez-Pellitero, P. (1999). Myxosporean infection causing intestinal disease in farmed turbot, *Scophthalmus maximus* (L.), (Teleostei : Scophthalmidae). *Journal of Fish Diseases* **22**, 395–399.

Budino, B., Lamas, J., Pata, M. P., Arranz, J. A., Sanmartin, M. L. and Leiro, J. (2011). Intraspecific variability in several isolates of *Philasterides dicentrarchi* (syn. Miamiensis avidus), a scuticociliate parasite of farmed turbot. *Veterinary Parasitology* **175**, 260–272.

Bustin, SA. (2004). *A-Z of Quantitative PCR*. La Jolla, CA, USA.

Estevez, J., Iglesias, R., Leiro, J., Ubeira, F. M. and Sanmartin, M. L. (1992). An unusual site of infection by a microsporean in the turbot *Scophthalmus maximus*. *Diseases of Aquatic Organisms* **13**, 139–142.

FAO (2010). FAO Fisheries and Aquaculture Technical Paper 500/1. *World Aquaculture* 2010.

Feist, S. W. and Longshaw, M. (2008). Histopathology of fish parasite infections – importance for populations. *Journal of Fish Biology* **73**, 2143–2160.

Figueras, A., Novoa, B., Santarem, M., Martinez, E., Alvarez, J. M., Toranzo, A. E. and Dykova, I. (1992). *Tetramicra brevifilum*, a potential threat to farmed turbot *Scophthalmus maximus*. *Diseases of Aquatic Organisms* **14**, 127–135.

Gruebl, T., Frischer, M. E., Sheppard, M., Neumann, M., Maurer, A. N. and Lee, R. F. (2002). Development of an 18S rRNA gene-targeted PCR-based diagnostic for the blue crab parasite *Hematodinium* sp. *Diseases of Aquatic Organisms* **49**, 61–70.

Harikrishnan, R., Jin, C. N., Kim, M. C., Kim, J. S., Balasundaram, C. and Heo, M. S. (2010). Effectiveness and immunomodulation of chemotherapeutants against scuticociliate *Philasterides dicentrarchi* in olive flounder. *Experimental Parasitology* **124**, 306–314.

Herrero, B., Vieites, J. M. and Espiñeira, M. (2011). Duplex real-time PCR for authentication of anglerfish species. *European Food Research and Technology* **233**, 817–823.

Hoorfar, J., Malorny, B., Abdulmawjood, A., Cook, N., Wagner, M. and Fach, P. (2004). Practical considerations in design of internal amplification controls for diagnostic PCR assays. *Journal of Clinical Microbiology* **42**, 1863–1868.

Kaukas, A. and Rollinson, D. (1997). Interspecific variation within the 'hypervariable' region of the 18S ribosomal RNA gene among species of Schistosoma Weinland, 1858 (Digenea). *Systematic Parasitology* **36**, 157–160.

Lamas, J., Sanmartin, M. L., Parama, A. I., Castro, R., Cabaleiro, S., de Ocenda, M. V. L., Barja, J. L. and Leiro, J. (2008). Optimization of an inactivated vaccine against a scuticociliate parasite of turbot: Effect of antigen, formalin and adjuvant concentration on antibody response and protection against the pathogen. *Aquaculture* **278**, 22–26.

Lee, S. G., Lee, S. H., Park, S. W., Suh, C. I., Jheong, W. H., Oh, S. and Paik, S. Y. (2011). Standardized positive controls for detection of norovirus by reverse transcription PCR. *Virology Journal* **8**, 1–8.

Leiro, J., Iglesias, R., Parama, A., Aragort, W. and Sanmartin, M. L. (2002). PCR detection of *Tetramicra brevifilum* (Microspora) infection in turbot (*Scophthalmus maximus* L.) musculature. *Journal of Parasitology* **124**, 145–151.

Lom, J. and Dykova, I. (1992). Fine-structure of Triactinomyxon early stages and sporogony – myxosporean and actinosporean features compared. *Journal of Protozoology* **39**, 16–27.

Maíllo, P. A., Amigó, J. M., Baena, R., Salvadó, H. and Gracia, M. P. (1998). *Tetramicra brevifilum* (Matthews and Matthews, 1980) (Microsporida: Tetramicriidae) in a new fish host, *Lophius budegassa* (Spinola, 1807) in Spain. *Journal of Parasitology Research* **84**, 208–212.

Matthews, R. A. and Matthews, B. F. (1980). Cell and tissue-reactions of turbot *Scophthalmus maximus* (L) to *Tetramicra Brevifilum* gen N, SP-N (Microspora). *Journal of Fish Diseases* **3**, 6495–515.

Padros, F., Zarza, C. A. and Crespo, S. (2001). *Infecciones por ciliados histiófagos en acuicultura marina: Aspectos hitopatológicos*, Instituto Canario de Ciencias Marinas.

Person-Le, J. R. (1990). Sole and turbot culture. In *Aquaculture* (ed. Barnabé, G.), pp. 687–734. Ellis Horwood, New York, USA.

Sanmartin, M. L., Parama, A., Castro, R., Cabaleiro, S., Leiro, J., Lamas, J. and Barja, J. L. (2008). Vaccination of turbot, *Psetta maxima* (L.), against the protozoan parasite *Philasterides dicentrarchi*. effects on antibody production and protection. *Journal of Fish Diseases* **e31**, 135–140.

Sterud, E., Hansen, M. K. and Mo, T. A. (2000). Systemic infection with Uronema-like ciliates in farmed turbot, *Scophthalmus maximus* (L.). *Journal of Fish Diseases* **23**, 33–37.

Winfrey, M. R. R., Rott, M. A. and Wortman, A. T. (1997). *Unraveling DNA: Molecular Biology for the Laboratory*. Prentice Hall, New York, USA.