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A multiplex PCR for the simultaneous detection of *Tenacibaculum maritimum* and *Edwardsiella tarda* in aquaculture

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Summary. A specific and sensitive multiplex PCR (mPCR) method was developed as a useful tool for the simultaneous detection of two important flatfish pathogens in marine aquaculture, *Tenacibaculum maritimum* and *Edwardsiella tarda*. In fish tissues, the average detection limit for these mPCR-amplified organisms was $2 \times 10^5 \pm 0.2$ CFU/g and $4 \times 10^5 \pm 0.3$ CFU/g, respectively. These values are similar or even lower than those previously obtained using the corresponding single PCR. Moreover, mPCR did not produce any nonspecific amplification products when tested against 36 taxonomically related and unrelated strains belonging to 33 different bacterial species. Large amounts of DNA from one of the target bacterial species in the presence of low amounts from the other did not have a significant effect on the amplification sensitivity of the latter. [**Int Microbiol** 2014; 17(2):111-117]

Keywords: *Tenacibaculum maritimum* · *Edwardsiella tarda* · multiplex PCR · fish pathology · aquaculture

Introduction

Fish diseases, especially those caused by Gram-negative bacteria, are a serious problem in aquaculture. At present, tenacibaculosis [2,6] and edwardsiellosis [5,8], caused by *Tenacibaculum maritimum* and *Edwardsiella tarda*, respectively, are two important bacterial diseases affecting a wide range of cultured fish species, including flatfish [2,8].

Tenacibaculum maritimum [16] is the causative agent of gliding bacterial disease (or tenacibaculosis) and it infects a wide variety of valuable marine fish species, such as turbot (*Scophthalmus maximus*), sole (*Solea solea*, *Solea senegalensis*), gilthead seabream (*Sparus aurata)*, and salmon (*Salmo salar*) [2]. The traditional culture-based method for the detection of this pathogen requires several days to weeks before results are obtained. In addition, one of the problems in the study of *T. maritimum* is the difficulty of distinguishing it from other phenotypically similar and phylogenetically related species, particularly those of the genera *Flavobacterium* and *Cytophaga* [4,16]. Therefore, in 2004 Avendaño-Herrera et al. [2] evaluated the specificity and sensitivity of two PCR methods previously described by Toyama et al. [19] and Bader and Shotts [3] in the identification of *T. maritimum* strains. They found that the former method allowed the accurate detection of *T. maritimum* in diagnostic pathology as well as in epidemiological studies of gliding bacterial disease of diseased and carrier marine fish.

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In the last decade, *E. tarda* has become an important bacterial pathogen in aquaculture. In addition, the bacterium is associated with septicemia and fatal infections in other animals, such as reptiles, birds, amphibians, marine mammals, and humans, and is thus a possible source of zoonoses [8]. Several attempts to develop methods for the rapid and accurate diagnosis of edwardsiellosis have been made, including PCR-based methods. In 2010, we published an evaluation of the specificity and sensitivity of four PCR primer pairs previously described for the detection of *E. tarda* [7]. Of these, a PCR protocol employing the gene *etfD* (which encodes the upstream region of the fimbrial gene) [15] was shown to be the most rapid and sensitive method for the accurate detection of *E. tarda* in infected fish.

Although simultaneous detection of several pathogens with a multiplex PCR (mPCR) has been widely applied to the detection of multiple viruses and bacteria in clinical specimens, this approach has not been widely used in the detection of fish pathogens [1,12,13,17]. In this work, we developed a mPCR for the rapid and economical simultaneous detection of *T. maritimum* and *E. tarda*, and in aquaculture.

Materials and methods

Bacterial strains and growth conditions. Forty-seven strains were used to evaluate the mPCR method, including six *E. tarda* strains and five *T. maritimum* strains isolated from different hosts and origins and 36 isolates of other taxonomically related and unrelated species (belonging to 33 different bacterial species) (Table 1). Reference strains of *E. tarda* and *T. maritimum* were included as positive controls. The identity of each isolate was confirmed employing biochemical tests [18] and, in some cases, using PCR-based analysis and/or serological assays.

For all experiments, the strains were routinely grown on either tryptone soy agar supplemented with 1 % (w/v) sodium chloride (TSA-1, Pronadisa, Spain), marine agar (MA; Difco, USA), or *Flexibacter maritimum* medium (FMM) [14] as appropriate for each strain. All strains were incubated at 25 °C for 24–72 h. Stock cultures were stored at –70 °C in Cryo-Bille tubes (AES Laboratory, France).

DNA extraction from bacterial cultures. Chromosomal DNA was extracted from pure bacterial using Insta-Gene Matrix (Bio-Rad, Spain), following the manufacturer's recommendations. The DNA was resuspended in a final volume of 200 µl of Insta-Gene Matrix. The concentration was determined spectrophotometrically at 260 nm and adjusted with sterile distilled water to a concentration of 10 ± 3 ng/µl. The DNA was stored at –30 °C until used for PCRs. All experiments were carried out with DNA obtained in three different extractions of each bacterial strain.

DNA amplification. All PCR amplifications were performed using Ready-To-Go PCR beads (GE Healthcare) according to the manufacturer's instructions. The species-specific primer pairs described by Toyama et al. [19] and Sakai et al. [15] were used for the mPCR and were synthesized by Sigma-Genosys.

One µl of each DNA solution and 1 µl of each primer (100 µM for *E. tarda* and 2 µM for *T. maritimum*) were used in the amplification reactions. Reaction mixtures (25 µl) were amplified in two different thermal cyclers: the T Gradient Termocicler (Biometra) and the Mastercycler Personal (Eppendorf). The PCR annealing temperatures tested ranged from 45 to 55 °C. Both the intensity of the amplicons for each targeted DNA and the absence of nonspecific bands were considered in the selection of optimal mPCR conditions. The cycling protocol was one cycle of 94 °C for 2 min, 35 cycles of 95 °C for 2 min, 45 °C for 1 min 30 s, and 72 °C for 2 min, and a final elongation at 72 °C for 7 min. Negative controls, consisting of the same reaction mixture but with sterile distilled water instead of template DNA, were included in each batch of PCRs. The reproducibility of the results was assessed by repetition of the amplifications in three independent PCR assays. As a positive control, the universal primers pA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and pH (5′-AAG GAG GTG ATC CAG CCG CA-3′ [9] were used to detect 16S rDNA in all strains.

Analysis of PCR products. Ten µl of the PCR products were separated on a 1 % (w/v) agarose gel for 60 min at 100 V in 1× TAE (0.04 M Tris, 1 mM EDTA, pH 8.0) and visualized by staining the gels with 0.06 µg ethidium bromide (Bio-Rad)/ ml. The bands were photographed under UV light and computer digitized (Gel Doc 100, Bio-Rad). A 50- to 1500-bp ladder (Fast Ruler low range DNA ladder, Fermentas, Spain) served as a molecular mass marker. The presence of a single product of the appropriate size and identical to that from the respective reference strains was considered as a positive result.

Specificity and sensitivity from bacterial cultures. The specificity of the mPCR was evaluated using genomic DNA extracted from strains belonging to different bacterial genera and species commonly found in flatfish infections (Table 1). To determine the analytic sensitivity of the mPCR, separate pure bacterial suspensions of the *T. maritimum* NCIMB2154 and *E. tarda* ACC35.1 strains were prepared to contain 10^9 cells/ml (McFarland scale 4) and were then ten-fold diluted in 0.85 % NaCl sterile saline to yield a dilution series containing 10^8 to 10^1 cells/ml. One hundred µl of each dilution was cultured on TSA-1 for *E. tarda* and on FMM for *T. maritimum*; the plates were incubated at 25 ºC. After the incubation, the colonies were counted and the bacterial concentrations of the stock cultures were calculated as CFU/ml. Known bacterial concentrations of different dilutions of both fish pathogens were mixed and the DNA was extracted using Insta-Gene Matrix, as previously described. The detection limits were determined by the presence or absence of the specific PCR products on agarose gels.

Applicability to fish tissues. The sensitivity of the mPCR in fish tissues was determined using DNA extracted from in-vitro-spiked spleen, kidney, and liver obtained from healthy sole and turbot (weight: 10–12 g). Both fish specimens were previously analyzed by bacteriological standard methods [18] to confirm the absence of pathogens that could interfere in the experiments, as described by Castro et al. [7]

Each tissue sample (mean weight: 0.1–0.2 g) was homogenized in 100 µl of PBS by repeated pipetting. Each fish sample was seeded with 100 µl of the above-described bacterial dilutions and homogenized. After incubation of the samples at 25 °C for 1 h, genomic DNA was extracted with the Easy-DNA kit (Invitrogen) by following the manufacturer's recommendations. As negative controls, DNA from fish samples "seeded" with PBS were extracted in the same manner. For mPCR, 1 µl of the purified DNA was added as the template. Detection limits were determined based on the presence or absence of PCR products in agarose gels.

In addition, to test the effect on the mPCR of a large amount of DNA from one pathogen in the presence of a small amount of DNA from the other, sensitivity was also determined using spiked kidney tissue from sole with different relative amounts of *T. maritimum* and *E. tarda*. Here, 100 µl of a suspension containing *ca.* 10^7 CFU/ml and prepared from one or bacterial

Table 1. Strains used in this work

Species	Strain	Source
Edwardsiella tarda	ACC35.1	*Scophthalmus maximus*
Edwardsiella tarda	HL1.1	*Scophthalmus maximus*
Edwardsiella tarda	RM288.1	*Scophthalmus maximus*
Edwardsiella tarda	ACR419.1	*Solea senegalensis*
Edwardsiella tarda	CECT489^T	Humans
Edwardsiella tarda	NCIMB2034	Fish specie
Tenacibaculum maritimum	ACC13.1	*Solea senegalensis*
Tenacibaculum maritimum	PC503.1	*Solea senegalensis*
Tenacibaculum maritimum	PC424.1	*Scophthalmus maximus*
Tenacibaculum maritimum	NCIMB2154^T	*Pagrus major*
Tenacibaculum maritimum	IEO19.1	*Solea senegalensis*
Edwardsiella ictaluri	CECT 885^T	*Ictalurus punctatus*
Hafnia alvei	05/1403	*Oncorhynchus mykiss*
Yersinia ruckeri	SAG 4.1	*Oncorhynchus mykiss*
Yersinia ruckeri	252/05	*Oncorhynchus mykiss*
Yersinia ruckeri	01 1651	*Oncorhynchus mykiss*
Escherichia coli	FV9980	Humans
Enterobacter cloacae	TM 83/03	*Scophthalmus maximus*
Enterobacter aerogenes	RPM 799.1	*Scophthalmus maximus*
Tenacibaculum aestuarii	JCM13491^T	Tidal flat sediment
Tenacibaculum ovolyticum	NBRC 15947^T	*Hippoglossus hippoglossus*
Tenacibaculum gallaicum	DSM 18841^T	Seawater
Tenacibaculum discolor	DSM18842^T	*Solea senegalensis*
Tenacibaculum litoreum	JCM13039^T	Tidal flat sediment
Tenacibaculum soleae	CECT7292^T	*Solea senegalensis*
Tenacibaculum amylolyticum	NBRC 16310^T	*Avrainvillea riukiuensis*
Tenacibaculum mesophilum	NBRC 16307^T	*Halichondria okadai*
Tenacibaculum lutimaris	DSM16505^T	Tidal flat
Tenacibaculum dicentrarchi	CECT 7612^T	*Dicentrarchus labrax*
Flavobacterium psychrophilum	PT41	*Oncorhynchus mykiss*
Pseudomonas fluorescens	ATCC 13525^T	Pre-filter tanks
Pseudomonas aeruginosa	ATCC 27853	*Oncorhynchus mykiss*
Aeromonas salmonicida ssp. *salmonicida*	ACR 215	*Scophthalmus maximus*
Aeromonas media	ATCC 33907^T	Fish farm effluent
Aeromonas hydrophila	80A1	*Oncorhynchus mykiss*
Aeromonas caviae	1.25	Humans
Vibrio ordalii	NCIMB 2167^T	*Oncorhynchus kisutch*
Vibrio splendidus	ATCC33125^T	Marine fish
Vibrio pelagius	ATCC 25916^T	Seawater
Vibrio pelagius	NCIMB 1900$^{>T}$	Seawater
Vibrio tubiashii	EX 1	*Crassostrea gigas*
Vibrio harveyi	ATCC 14126^T	*Talorchestia sp.*
Vibrio vulnificus	ATCC 29307	Humans
Vibrio nereis	ATCC 25917^T	Seawater
Vibrio anguillarum	R82	*Scophthalmus maximus*
Photobacterium damselae ssp. *damselae*	RG-91	*Scophthalmus maximus*
Photobacterium damselae ssp. *piscicida*	DI-21	*Sparus aurata*

species (*E. tarda* or *T. maritimum*) was mixed with the same volume of one of the serial dilutions (from 10^8 to 10 cells/ml) prepared from the other species. DNA extraction and the determination of detection limits were performed as described above.

Experimental and natural fish infections. To determine the applicability of the mPCR protocol in infected fish, batches of five turbot and five sole (average weigh 10–12 g) were inoculated with 0.1 ml of a suspension of *E. tarda* (ACC35.1 isolate) and/or *T. maritimum* (NCIMB2154^T strain) at a concentration of 10^3 and 10^5 CFU/ml, respectively. Three batches of fish were inoculated with *E. tarda* (strain ACC35.1), three batches with *T. maritimum* (strain NCIMB2154^T), and three batches with both strains. As the negative control, three batches of fish were "infected" with sterile PBS and maintained under the same conditions as the experimentally infected fish. The fish were maintained in 50-liter aquaria with continuous aeration and a water temperature of 17 ± 1 ºC. Five days post-infection, the kidneys were collected from all turbot and sole fish and DNA was extracted as previously described. Classical bacteriological analysis by standard plate culture was also performed.

To validate the mPCR in diseased fish held in natural conditions, 30 turbot specimens ranging in weight from 50 to 100 g and showing disease symptoms were tested. The fish were collected from a rearing facility with previous natural outbreaks of edwardsiellosis and tenacibaculosis. The same number of apparently healthy fish sent to our laboratory for routine analysis were tested using the mPCR assay. Kidney samples were analyzed as previously described. Conditions for DNA extraction, PCR amplification, and PCR product visualization were the same as described above. In parallel, classical bacteriological analyses were performed to confirm the presence of *E. tarda* and/or *T. maritimum*.

Results

Specificity of the mPCR assay. The mPCR method was optimized for the simultaneous detection of two bacteria, *T. maritimum* and *E. tarda*, using specific primer pairs. The annealing temperature that produced the best amplification (in terms of band intensity and the absence of non-specific products) using the two primer pairs was 45 °C. The specificity of the method was evaluated using a DNA mixture prepared from the two target pathogens, which yielded amplification products of 1088 bp and 445 bp for *T. maritimum* and *E. tarda*, respectively. Non-specific amplifications were not observed using DNA from other taxonomically and/or ecologically related bacteria. The reproducibility of the PCR was demonstrated in that the same results were obtained in at least three independent PCR assays and using two different thermal cyclers. As expected, with the universal primers pA/pH a PCR product of the predicted size (1501 bp) was generated in all strains tested.

Sensitivity of mPCR assay. To assess the sensitivity of the method, the mPCR was performed using DNA extracted from the bacterial serial dilutions. The expected 1088-bp and 445-bp PCR products were obtained in samples containing as few as 200 and 4 cells of *T. maritimum* and *E. tarda*, respectively, per PCR tube ($2 \times 10^5 \pm 0.2$ and $4 \times 10^3 \pm 0.3$ CFU/ml; Fig. 1).

The the mPCR protocol was also tested using DNA templates from fish tissues seeded with different concentrations of the two pathogens. The results demonstrated the presence of these bacteria in kidney, liver, and spleen. The detection limits in these assays were the same, regardless of the type of tissue: $2 \times 10^5 \pm 0.2$ CFU/g for *T. maritimum* and $4 \times 10^5 \pm 0.3$ CFU/g for *E. tarda* (200 and 400 cells per PCR tube, respectively; Fig. 2). Moreover, large amounts of *E. tarda* or *T. maritimum* (10^7 CFU/ml) had no effect on the detection limit of either pathogen; the values were of the same order of magnitude as described above (10^5 CFU/g; data not shown).

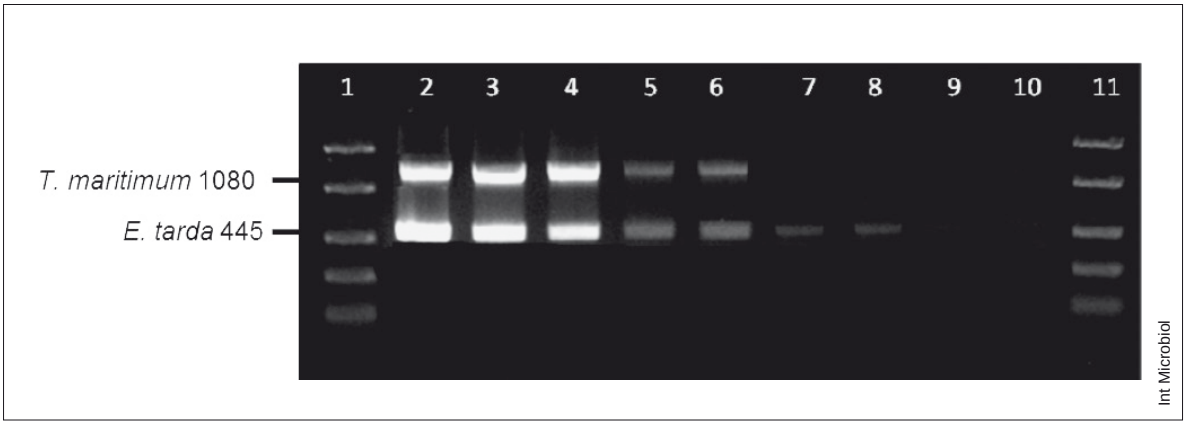

Fig. 1. Sensitivity of the mPCR protocol as determined using purified DNA from serial dilutions of mixed cultures of *Edwardsiella tarda* ACC35.1 and *Tenacibaculum maritimum* NCIMB2154 strains. Lanes 1 and 11: Fast Ruler low range DNA ladder (50–1500 pb); lanes 2–10: dilutions ranging from 10^9 to 10^1 cells/ml from *E. tarda* and *T. maritimum*. Numbers on the left indicate the specific amplified product in bp.

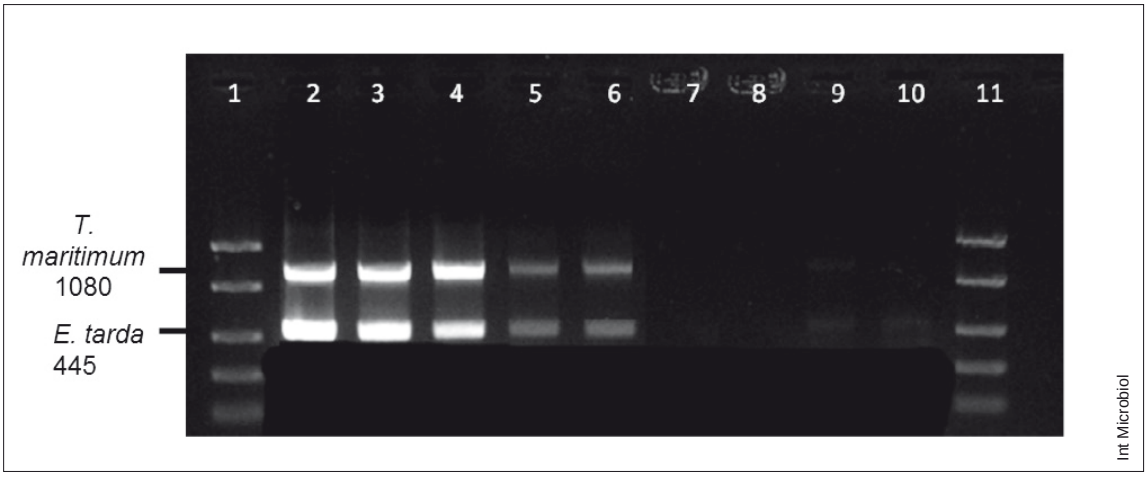

Fig. 2. Sensitivity of the mPCR protocol using purified DNA from serial dilutions of kidney samples seeded with *Edwardsiella tarda* ACC35.1 and *Tenacibaculum maritimum* NCMIB 2154. Lanes 1 and 11: Fast Ruler low range DNA ladder (50–1500 pb); lanes 2–10: dilutions ranging from 10^9 to 10^1 cells/ml (lane 8) from *E. tarda* and *T. maritimum*. Numbers on the left indicate the specific amplified product in bp.

Experimental and natural fish infections. The mPCR was also applied to kidney samples from experimentally infected fish. Using this technique, *E. tarda* and *T. maritimum* were detected even in fish that had not yet developed the disease. In fact, after 5 days, none of the killed turbot or sole showed any clinical symptoms and colonies from either of the two pathogens were not detected when the internal organs were plated on agar plates. By contrast, the mPCR was able to detect one or both pathogens (depending on the inoculation trials) in all experimentally infected fish (Fig. 3). As expected, negative controls produced no amplifications. Among the samples obtained from naturally infected fish, *T. maritimum* was detected in kidney samples, producing the specific 1088 bp product, whereas the *E. tarda* specific band (445 bp) was not observed. The mPCR results were confirmed by the isolation of filamentous colonies in FMM plates, which were identified as *T. maritimum* by classical biochemical methods as well as by specific PCR [2]. Consistent with the mPCR results, *E. tarda* was not detected, neither by growth in TSA-1 nor by specific PCR [7]. Also, in apparently healthy turbot neither of the pathogens could be isolated and the PCR amplifications were negative.

Discussion

Currently, aquaculture is one of the fastest growing food production systems in the world. In Europe, both turbot,

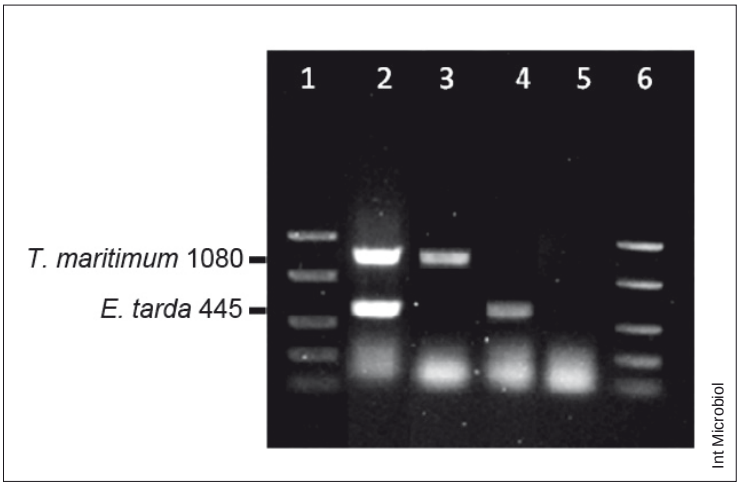

Fig. 3. Results obtained in the detection of experimentally infected fish by mPCR. Lanes 1 and 6: Fast Ruler low range DNA ladder (50–1500 pb); lane 2: fish infected simultaneously with *Edwardsiella tarda* and *Tenacibaculum maritimum*; lane 3: fish infected with *T. maritimum* only; lane 4: fish infected with *E. tarda* only; lane 5: negative control. Numbers on the left indicate the specific amplified product in bp.

the most commonly produced flatfish, and Senegalese sole (*Solea senegalensis*), because of its relatively fast growth and highly appreciated meat, are commercially important species. Under intensive aquaculture conditions, healthy-looking fish without clinical signs may carry pathogens, posing a serious risk for the spread of diseases among fish populations. The bacterial diseases tenacibaculosis (*Tenacibaculum* sp.) and edwardsiellosis (*E. tarda*) may result in high mortalities in turbot and sole. The rapid detection of these pathogens would allow for their effective control. PCR-based methods are one of the best tools for the diagnosis of these and other fish bacterial diseases because of their specificity, sensitivity, and rapid performance. In this study, we optimized an mPCR protocol for the simultaneous diagnosis of tenacibaculosis and edwardsiellosis in flatfish.

The detection limits obtained from cultures of *T. maritimum* and *E. tarda* in the present study, in which the two PCR primer pairs were simultaneously used in a unique PCR protocol, were similar or even lower than those previously obtained by our group [2,7]. In those studies, the detection limit for *E. tarda* in mixed bacterial cultures was of the order of 10^5 CFU/ml [7], while in the current work the sensitivity was of the order of 2 log-units. These values are within the same order of magnitude or even lower than those reported by other authors for *E. tarda* detection [10,11]. For *T. maritimum*, the only published molecular diagnostic method is the PCR previously evaluated by our group, in which the sensitivity was between 1.6×10^4 and 1.1×10^5 CFU/ml [2]. Therefore, our newly developed mPCR provides a powerful tool for the accurate detection of *E. tarda* and *T. maritimum* from bacterial cultures.

The detection limits obtained from DNA extracted from fish tissues were approximately 10^5 CFU/g for both pathogens. These values were comparable to those previously reported [2,7] for the individual PCR protocols of *T. maritimum* and *E. tarda* [2,7] and to those previously described in other studies of the simultaneous detection of important fish pathogens [1, 12,13,17]. mPCR is generally thought to be less sensitive than single PCR because of competition for reaction reagents, especially if the assays differ in their amplification efficiencies or one or more of the target organisms is present in high numbers [17]. However, we found that large amounts of DNA from one of the two fish pathogens did not significantly alter the amplification sensitivity of DNA from the other pathogen, as the detection limits were within the same order of magnitude as those obtained previously [2,7]. Finally, the applicability of this technique was also demonstrated in experimentally and naturally infected fish. The speed, simplicity, sensitivity, and specificity of the mPCR developed in this study and the importance of these target pathogens in marine aquaculture make this protocol a very useful tool for the early and simultaneous detection of *T. maritimum* and *E. tarda* in fish cultures.

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Competing interests. None declared

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