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Evaluation of four polymerase chain reaction primer pairs for the detection of *Edwardsiella tarda* in turbot

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ABSTRACT: *Edwardsiella tarda* is an important emergent pathogen in European aquaculture, causing several mortality events in turbot *Scophthalmus maximus* cultures in recent years. Here, we evaluated in parallel the specificity of 4 previously published pairs of primers, gyrBF1/gyrBR1, tardaF/tardaR, etfA and etfD, for the detection of 53 *E. tarda* strains isolated from different sources, as well as 18 representatives of related and unrelated bacterial species. On the basis of the obtained results, we selected the pair of primers etfD, because it was the only one that recognized all *E. tarda* strains without false positive reactions. The sensitivity of this primer set showed detection limits of 2 cells per reaction tube in the case of pure cultures and 200 cells per reaction tube in mixed cultures. With regard to the sensitivity in seeded turbot tissues (kidney, liver and mucus), the detection limit was 3×10^2 *E. tarda* cells per reaction. In experimentally infected turbot, the etfD primer set was able to detect the pathogen in internal organs even 1 d post-infection, with a dose of 0.1 cells g^{-1} of fish. In addition, this polymerase chain reaction protocol was useful for the detection of *E. tarda* in the field, and, based on the findings, we propose it as the most appropriate for accurate detection of *E. tarda* in routine diagnosis of edwardsiellosis in fish.

KEY WORDS: *Edwardsiella tarda* · PCR · Detection · Diagnosis · Turbot

INTRODUCTION

Edwardsiella tarda is a gram-negative bacterium, a member of the family *Enterobacteriaceae* and an important pathogen causing enterohaemorrhagic septicaemic disease in a variety of organisms, including amphibians, reptiles, fish, marine mammals and humans. This disease, also called edwardsiellosis, is a serious systemic bacterial disease that has a worldwide distribution in fresh and marine water, causing mortalities in important aquaculture species such as eel *Anguila japonica*, flounder *Paralichthys olivaceus* and catfish *Ictalurus punctatus*, among others (Sakazaki & Tamura 1992, Plumb 1999).

In recent years, repeated outbreaks of edwardsiellosis have been detected in several turbot *Scophthalmus maximus* cultures in Europe, causing important economic losses (Castro et al. 2006, Toranzo 2007, Castro 2008). Although classical bacteriological and serological analyses give accurate and effective identification, rapid, specific and sensitive detection techniques that allow diagnosis of edwardsiellosis could represent an important advance in the prevention of the disease.

The polymerase chain reaction (PCR) is a DNA-based method that is used for the detection of many marine pathogens (Osorio & Toranzo 2002, Toranzo et al. 2004). Although in many PCR protocols the primers employed have been designed using the 16S ribosomal RNA (rRNA) gene as a target, the application of the 16S rRNA sequence is not always advisable, since it is highly conserved. In fact, in the case of *Edwardsiella tarda*, its 16S sequence shares identity of 96 to 100% in BLAST (Basic Local Alignment Search Tool, National Center for Biotechnology Information) with other

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species of the same genus, such as *E. ictaluri* and *E. hoshinae*, or with species of different genera, such as *Pseudomonas fluorescens* and *Serratia marcescens*.

In 1998, Chen and Lai designed a pair of primers that used the haemolysin gene as a target to detect *Edwardsiella tarda*, generating an 1109 bp product in the open reading frame (ORF) II and III regions (Chen & Lai 1998). Several *E. tarda* strains were employed, most of which were isolated from infected eels and from experimentally infected tilapia and water. In 2007, Sakai et al. developed 4 primer sets (etfA, etfB, etfC and etfD) targeting the Type 1 fimbrial gene cluster of *E. tarda* to determine the presence of such fimbrial genes among the fish pathogenic and non-pathogenic strains of this bacterium isolated from Japanese fish (Sakai et al. 2007). Only 2 primer sets (etfA and etfD) showed an ability to detect *E. tarda*. Recently, Lan et al. (2008), designed a set of primers (gyrBF1 and gyrBR1) based on the sequence of the divergent region of the partial *gyrB* gene of an unusual *E. tarda* strain isolated from turbot in China. This set of primers generated a specific PCR product of 415 bp.

However, these pairs of primers have not yet been tested simultaneously in order to compare their specificity using a wide range of strains isolated from different hosts and geographical origins. Therefore, the aim of the present study was to evaluate the specificity and sensitivity of the 3 PCR methods described previously, in order to identify the best protocol for the detection of *Edwardsiella tarda* in fish, with special emphasis on the diagnosis of turbot edwardsiellosis.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in the primer specificity studies are listed in Table 1. This collection comprises 36 *Edwardsiella tarda* isolates from turbot in different outbreaks from 2004 to 2008, 17 *E. tarda* strains from other sources, as well as 18 isolates of other enterobacteria and marine fish pathogens (Table 1).

Strains were routinely cultured on Tripticase Soy Agar supplemented with 1% of NaCl (TSA-1; Pronadisa) and incubated at 25 or 37°C, depending on the strain, for 24 h. Before the assays, all bacterial strains were confirmed employing biochemical and serological tests (Thoesen 1994, Castro et al. 2006). Stock cultures were stored at –70°C in Cryo-Bille tubes (AES Laboratory).

DNA extraction from bacterial cultures. Chromosomal DNA was extracted employing Insta-Gene Matrix (Bio-Rad) from bacterial cultures, following the recommendations of Bio-Rad, and eluted in a final volume of 200 µl of Insta-Gene Matrix. All DNA concentrations were examined at 260 nm and adjusted to between 10 and 20 ng μl^{-1}. DNA was maintained at –30°C until used for PCR reactions. All the experiments were carried out with DNA obtained in 3 different extractions for each bacterial strain.

DNA amplification. All PCR amplifications were performed employing commercial Ready-To-Go PCR beads (Amersham Pharmacia Biotech), which included all the reagents needed for the PCR reactions with the exception of the specific primers and DNA template. Four species-specific primer pairs described by Chen & Lai (1998), Sakai et al. (2007) and Lan et al. (2008) were synthesized by Sigma-Genosys and employed in this work for the identification of *Edwardsiella tarda* (see Table 2).

One microlitre of each DNA solution and 1 µl of each primer (100 µM) were used in the amplification reactions. Reaction mixtures (25 µl) were amplified in 2 different thermal cyclers: the T Gradient Termocicler (Biometra) and the T Professional Basic (Biometra). The amplification cycles used for denaturation, primer annealing to the template and primer extension were carried out according to each published protocol. Negative controls, consisting of the same reaction mixture but with sterile distilled water instead of template DNA, were included in each batch of PCR reaction. The reproducibility of the results was assessed by repetition of the amplifications in 3 independent PCR assays.

In addition, 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′) (Edwards et al. 1989) were employed to detect the 16S rDNA in all strains.

Analysis of PCR products. Amplified products were detected by horizontal 1% (w/v) agarose gel electrophoresis for 60 min at 100 V in TAE (Tris-acetate-EDTA) 1× (0.04 M Tris, 1 mM EDTA [ethylenediaminetetraacetic acid], pH 8.0) electrophoresis buffer, visualized using 0.06 µg ml^{-1} of ethidium bromide (Bio-Rad), photographed under UV light and computer digitised (Gel Doc 100, Bio-Rad). A 50 to 2000 bp ladder (Sigma Chemical Co.) was used as a molecular mass marker. The presence of a single product of the appropriate size was considered as a positive result.

Sensitivity of the PCR. On the basis of the obtained results comparing the ability of each primer pair tested to amplify *Edwardsiella tarda* from all sources, we selected the primer pair etfD and evaluated its sensitivity with respect to *E. tarda* detection. The detection limit of this primer set was evaluated employing pure *E. tarda* and *E. tarda* cultures mixed with other fish pathogens.

From pure cultures of 3 selected *Edwardsiella tarda* strains (2 turbot isolates and NCIMB2034), colonies were picked from TSA-1 plates, visually adjusted to

Table 1. Bacterial strains included in the present study, and the results from the specific polymerase chain reaction detection methods employed. Primer sets show number of strains detected with each primer set. NCIMB: National Collection of Marine and Industrial Bacteria (Aberdeen, UK); CECT: Colección Española de Cultivos Tipo (Valencia, Spain); ATCC: American Type Culture Collection (Rockville, USA); DSM: Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany)

Strain	Source	No. of strains	Primer sets			
			gyrBF1/gyrBR1	tardaF/tardaR	etfA	etfD
***Edwardsiella tarda* strains**						
Turbot	*Scophthalmus maximus*	36	0	0	35	36
Gilthead seabream	*Sparus aurata*	1	0	0	0	1
Japanese eel	*Anguilla japonica*	2	0	2	2	2
Catfish	*Ictalurus punctatus*	1	0	0	1	1
Striped bass	*Morone saxatilis*	2	0	0	2	2
Pacific salmon	*Oncorhynchus kisutch*	1	0	0	0	1
Angel fish	*Pterophyllum scalare*	1	0	0	0	1
Tilapia	*Oreochromis niloticus*	2	0	2	2	2
Japanese flounder	*Paralichthys olivaceus*	2	0	2	2	2
Red seabream	*Pagrus major*	3	0	3	3	3
E. tarda NCIMB2034	Unknown fish	1	0	1	1	1
E. tarda CECT849	Human faeces	1	0	1	1	1
Other bacterial strains						
Edwardsiella ictaluri ATCC33202	*Ictalurus punctatus*	1	0	0	1	0
Edwardsiella ictaluri B1.1	*Chondrostoma polylepis*	1	0	1	0	0
Edwardsiella hoshinae DSM13771	*Fratercula* sp.	1	0	0	1	0
Escherichia coli CECT433	Human	1	0	0	1	0
Enterobacter cloacae TW83/03	*Oncorhynchus mykiss*	1	0	0	0	0
Enterobacter aerogenes RPM799.1	*Scophthalmus maximus*	1	0	0	1	0
Yersinia ruckeri 1651	*Oncorhynchus mykiss*	1	0	0	1	0
Yersinia ruckeri SRG4.1	*Oncorhynchus mykiss*	1	0	0	1	0
Hafnia alvei 15/1403	*Oncorhynchus mykiss*	1	0	0	0	0
Serratia marcescens PC955.1	*Scophthalmus maximus*	1	0	0	0	0
Aeromonas salmonicida ssp. *salmonicida* ACR218.1	*Scophthalmus maximus*	1	0	0	1	0
Listonella (*Vibrio*) *anguillarum* R82	*Scophthalmus maximus*	1	0	0	0	0
Photobacterium damselae ssp. *piscicida* ATCC29690	*Seriola quinqueradiata*	1	0	0	0	0
Pseudomonas anguilliseptica CECT899	*Anguilla japonica*	1	0	0	0	0
Pseudomonas fluorescens 07/1139	*Oncorhynchus mykiss*	1	0	0	0	0
Pseudomonas aeruginosa CECT110	Unknown	1	0	0	0	0
Lactococcus garvieae SRG1.1	*Oncorhynchus mykiss*	1	0	0	0	0
Streptococcus parauberis ACC33.1	*Scophthalmus maximus*	1	0	0	0	0

Table 2. Primer sets employed in the present study for the detection of *Edwardsiella tarda*

Primer set	Positions (encoding protein)	Target gene	Predicted product size (bp)	Source
tardaF/tardaR				
5′-CCT TAT AAA TTA CTC GCT-3′	744–761	ORFII–ORFIII	1109	Chen & Lai (1998)
5′-TTT GTG GAG TAA CAG TTT-3′	1850–1833	(haemolysin)		
etfA				
5′-CGG TAA AGT TGA GTT TAC GGG TG-3′	2160–2182	etfA (major	415	Sakai et al. (2007)
5′-TGT AAC CGT GTT GGC GTA AG-3′	2555–2574	fimbrial subunit)		
etfD				
5′-GGT AAC CTG ATT TGG CGT TC-3′	6171–6190	etfD (fimbrial	445	Sakai et al. (2007)
5′-GGA TCA CCT GGA TCT TAT CC-3′	6596–6615	subunit)		
gyrBF1/gyrBR1				
5′-GCA TGG AGA CCT TCA GCA AT-3′	242–262	gyrB	415	Lan et al. (2008)
5′-GCG GAG ATT TTG CTC TTC TT-3′	637–656	(gyrase)		

contain 10^9 cells ml^{-1} (corresponding to McFarland Scale 4) and serially diluted in 0.85% sterile saline solution (SS) from 10^8 to 10 cells ml^{-1}.

To determine the usefulness of the etfD primers to amplify template *Edwardsiella tarda* DNA from mixed cultures, bacterial suspensions of *Listonella* (*Vibrio*) *anguillarum* (R82) and *Aeromonas salmonicida* ssp. *salmonicida* (ACR218.1) were employed. We selected these bacterial species because both are some of the most common and important pathogens in turbot aquaculture. Bacterial mixtures simultaneously including these 2 pathogens and *E. tarda* (turbot strain ACC35.1) were prepared by mixing 500 µl of each bacterial suspension, previously adjusted at a concentration of 10^7 cells ml^{-1}, and were serially diluted. All dilutions were centrifuged at 12 000 rpm for 2 min and washed twice with sterile distilled water. Extraction and amplification of genomic DNA, as well as the analysis of the PCR products, were performed as described before. Colony-forming units (CFU) were estimated by plating each dilution onto TSA-1 plates and counting the produced bacterial colonies.

Applicability to fish tissues. To determine the applicability of the etfD primer set in the detection of *Edwardsiella tarda* in fish tissues, different samples including kidney, liver, intestine, blood and mucus were obtained from healthy turbot (10 to 12 g weight), which were analysed by bacteriological standard methods (Thoesen 1994) to confirm the absence of pathogens that could interfere in the experiments. Samples of 1 g of each tissue (liver and kidney) were then homogenized respectively in 100 µl of phosphate-buffered saline (PBS, pH 7.4) by repeated pipetting. In the case of blood and mucus, the volume employed was 200 µl. Each fish sample was seeded with 100 µl of the different dilutions of the *E. tarda* turbot strain ACC35.1 and homogenized. After incubation for 1 h at 25°C, DNA extraction was performed with the Easy-DNA kit for genomic DNA isolation (Invitrogen) following the manufacturer's recommendations. DNA was maintained at –30°C until use in PCR reactions. DNA from non-seeded fish samples, and PBS were extracted in the same manner for use as negative controls.

In addition, tissues from 2 batches of 50 turbot (10 to 12 g weight) injected with *Edwardsiella tarda* strain ACC35.1 at a concentration of 0.1 ml of 10^2 CFU ml^{-1} (1 CFU g^{-1} of fish) and 10 CFU ml^{-1} (0.1 CFU g^{-1} of fish), respectively, were used to test the etfD PCR assay. The *E. tarda* strain employed in these challenges was again the isolate ACC35.1, with a LD_{50} of 1.6×10^1 cells ml^{-1} (Toranzo 2007). For the control group, the same number of fish were injected with 0.1 ml of SS and maintained at the same conditions as infected fish. Fish were maintained in 50 l aquaria with continuous aeration and water temperature of approximately 17°C. Samples of kidney, liver, spleen, intestine, blood and mucus were collected from 10 turbot before inoculation and at 1, 2 and 5 d post-inoculation, and pools of each tissue type were prepared per time point. DNA was extracted with the Easy-DNA kit for genomic DNA isolation (Invitrogen) and eluted in a final volume of 100 µl of TE buffer. Then, 1 µl of DNA was employed as template in the PCR assay. Classical bacteriological analysis, by standard plate culture techniques and further biochemical and serological identification, was performed in order to assess the ability to detect *E. tarda* by this method in the challenged fish.

Field validation. A total of 80 diseased turbot ranging from 50 to 200 g coming from natural outbreaks in 3 different rearing facilities, as well as a similar number of apparently healthy fish sent to our laboratory for routine analysis, were tested using the etfD PCR assay. Tissue samples (kidney, spleen, liver and intestine), as well as blood and mucus, were analysed. Conditions for DNA extraction and PCR amplification were the same as described above. In parallel, classical bacteriological analyses were performed in order to confirm the presence or absence of *Edwardsiella tarda*.

RESULTS

PCR specificity

To test the specificity of each pair of primers for *Edwardsiella tarda* identification, DNA extracted from a collection of 71 strains, including *E. tarda* and non–*E. tarda* isolates were used in PCR reactions with each primer pair (Table 1). When the primers tardaF/tardaR were employed, the expected amplification product was obtained in only 21% of the strains used in the present study. In fact, only strains isolated from Japanese eel and flounder, tilapia, red seabream and the reference strain CECT 849 from human faeces generated the 1109 bp PCR-fragment, and no isolate from turbot *Scophthalmus maximus* showed the expected amplification product. Moreover, non-specific amplification was observed with DNA template from *E. ictaluri*. With regard to the primers etfA, the expected 415 bp band was not amplified in 4 *E. tarda* strains isolated from turbot, gilthead seabream, Pacific salmon and angel fish. In addition, these etfA primers produced non-specific amplification with non–*E. tarda* strains. PCR amplification with the set of primers gyrBF1/gyrBR1 did not yield PCR products in any of the strains tested. In contrast to the other 3 PCR primer pairs tested, the primer set etfD identified all the *E. tarda* isolates, and no cross-amplification with other bacterial species was detected. Based on these results, only the primer pair etfD was selected for subsequent studies.

Reproducibility of PCR was demonstrated, since the same results were obtained in, at least, 3 independent PCR assays for each primer set tested and using 2 different thermal cyclers. As expected, the universal primers pA/pH yielded a PCR product of the predicted size (1501 bp) in all strains tested (data not shown).

Determination of PCR sensitivity from pure and mixed cultures

The sensitivity of the etfD primer pair was determined by amplification of the DNA extracted from different bacterial suspensions (10^8 to 10 CFU ml^{-1}) of *Edwardsiella tarda* strains in pure and mixed cultures. The expected 445 bp PCR product was obtained with samples containing as low as 2 cells per PCR reaction tube ($2 \times 10^3 \pm 0.2$ CFU ml^{-1}) in the case of pure cultures (Fig. 1a) and 200 cells per PCR reaction tube ($2 \times 10^5 \pm 0.2$ CFU ml^{-1}) in the case of mixed cultures (Fig. 1b).

Determination of PCR sensitivity from seeded tissues and experimentally infected fishes

The application of the etfD PCR protocol to DNA templates obtained from fish tissues seeded with different concentrations of *Edwardsiella tarda* showed that this pathogen can be detected in kidney, liver and mucus at a detection level of 3×10^2 CFU per reaction tube (3×10^5 CFU g^{-1} fish tissue or ml^{-1} of mucus) (Fig. 1c). When blood was employed, the detection limit was 3×10^5 CFU per tube of reaction (3×10^8 CFU ml^{-1} of blood).

The PCR assay was also applied to kidney, liver, spleen, intestine, blood and mucus samples obtained from experimentally inoculated turbot with doses of 1 and 10 CFU fish^{-1}. *Edwardsiella tarda* was detected by PCR from the internal organs of all infected fish, even 1 d post-inoculation and with the lower concentration of bacteria inoculated, but not from blood or mucus (Table 3). No amplification was observed in tissues from the PBS-inoculated fish used as negative controls.

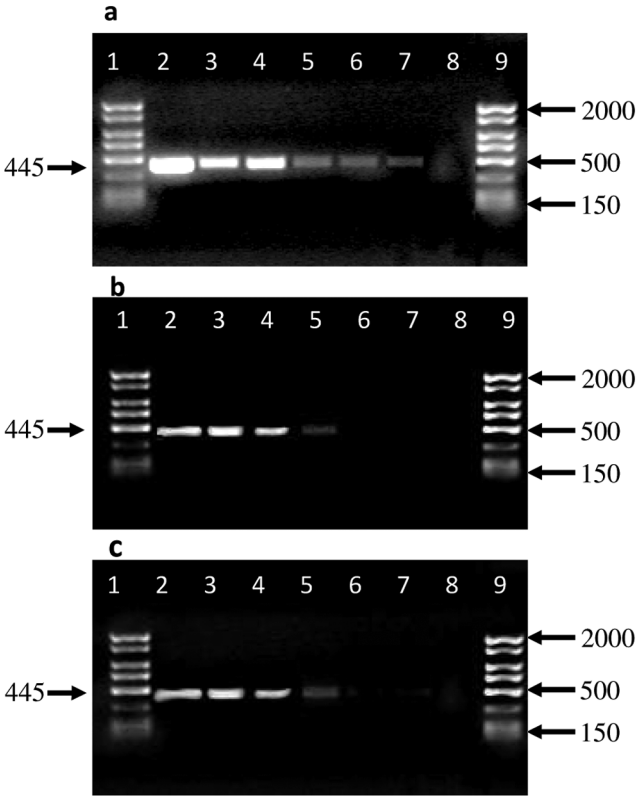

Fig. 1. Sensitivity of the polymerase chain reaction protocol using purified DNA from serial dilutions of (a) a pure culture of *Edwardsiella tarda* strain ACC35.1, (b) mixed cultures and (c) from seeded samples of kidney. Lanes 1 and 9: AmpliSize Molecular Ruler (50 to 2000 bp ladder; Sigma); (a,b) Lanes 2 to 8: dilutions ranging from 2×10^8 CFU (colony-forming units) ml^{-1} (Lane 2) to 2×10^2 CFU ml^{-1} (Lane 8). (c) Lanes 2 to 8: dilutions ranging from 3×10^8 CFU ml^{-1} (Lane 2) to 3×10^2 CFU ml^{-1} (Lane 8). Numbers on the left indicate the specific amplified product in basepairs

Table 3. Detection of *Edwardsiella tarda* in experimentally inoculated (at doses of 1 and 100 colony-forming units [CFU] fish^{-1}) and in naturally infected turbot by polymerase chain reaction (PCR) and by classical bacteriological analysis using standard culture methods (C) (+: positive detection; –: negative detection; dpi: days post-infection). All apparently healthy fishes showed negative results for PCR amplification and classical bacteriological analysis

Samples	Experimental infection												Naturally infected samples	
	1 CFU fish^{-1}						10 CFU fish^{-1}							
	1 dpi		2 dpi		5 dpi		1 dpi		2 dpi		5 dpi			
	PCR	C	PCR	C	PCR	C	PCR	C	PCR	C	PCR	C	PCR	C
Kidney	+	–	+	–	+	–	+	–	+	–	+	–	+	+
Liver	+	–	+	–	+	–	+	–	+	–	+	–	+	+
Intestine	+	–	+	–	+	–	+	–	+	–	+	–	+	+
Spleen	+	–	+	–	+	–	+	–	+	–	+	–	+	+
Blood	–	–	–	–	–	–	–	–	–	–	–	–	+	+
Mucus	–	–	–	–	–	–	–	–	–	–	–	–	–	–

By classical bacteriological analysis, *E. tarda* colonies were not detected on agar plates when samples from internal organs, blood and mucus were plated.

Field validation

In the case of the samples obtained from naturally infected turbot coming from 3 different rearing facilities, *Edwardsiella tarda* was detected in all internal organs and blood, producing the specific etfD PCR product (445 bp). However, no amplification was observed when the mucus samples of these fish were employed (Table 3). In all cases of infected fish, *E. tarda* was isolated on TSA-1 plates using homogenates from all internal organs and blood, but not from mucus, and was identified by biochemical and serological tests. Neither isolation of the pathogen, nor PCR amplification occurred when the apparently healthy turbot were analysed. The total time for the PCR procedure, including DNA extraction from samples, amplification and gel electrophoresis, was shorter than 6 h.

DISCUSSION

Edwardsiella tarda is an important emerging bacterial pathogen in turbot *Scophthalmus maximus* culture causing high and rapid mortalities in this fish species (Castro et al. 2006, Toranzo 2007). Therefore, there is an urgent need to develop a sensitive and accurate method for the fast detection of this bacterium, in both infected and carrier fish. Moreover, the application of this technique to environmental samples could be of great importance to determine reservoirs of *E. tarda*.

DNA-based methods have been developed in recent years for the fast and sensitive diagnosis of pathogens of many aquatic species (Toranzo et al. 2005). In the present study, we evaluated in parallel the effectiveness of the primers tardaF and tardaR from Chen & Lai (1998), 2 selected sets of primers (etfA and etfD) from Sakai et al. (2007) and the pair of primers gyrBF1/gyrBR1 from Lan et al. (2008) in order to assess the best PCR protocol to identify and detect *Edwardsiella tarda* from both pure and mixed cultures, as well as in fish and environmental samples. For this, we employed a collection of *E. tarda* strains with a wide range of host and geographical origins, as well as a collection of related and unrelated bacterial isolates. The obtained results demonstrated that only the primer pair etfD was specific for *E. tarda* detection. The lack of amplification in all strains employed in the present study when the primers gyrBF1 and gyrBR1 were used is explained by the fact that the sequences of these primers, published by Lan et al. (2008), show 4 and 2 mismatches, respectively, with the *gyrB* sequence reported for *E. tarda* NCIMB2034 (GenBank accession number EU259314.1). These mismatches together would be enough to prevent amplification of the *gyrB* gene in *E. tarda* strains whose sequence differs from that of the unusual strain LTB-4.

Sensitivity for the primer set etfD was about 2 and 200 CFU per tube of reaction in the case of pure and mixed cultures, respectively, and 300 CFU in seeded tissues and mucus. The sensitivity obtained for the etfD PCR assay for *Edwardsiella tarda* is comparable to those obtained for other bacterial fish pathogens (Osorio et al. 1999, Romalde et al. 2004, Avendaño-Herrera et al. 2004). However, the detection limit in blood was in the order of 3×10^5 CFU per tube of reaction, which could be considered a poor value for its applicability as a non-destructive diagnostic procedure for the detection of carrier fish of *E. tarda*.

In addition, when experimental infection of turbot was developed, we could detect *Edwardsiella tarda* by PCR from the internal organs of inoculated fish, but not from the mucus or blood. Based on this, we propose the kidney as a target organ for the detection of carrier animals of *E. tarda*. The failure to detect the bacterium by conventional microbiological culture could be due to the period of study (5 d). In fact, we have previously demonstrated that mortalities caused by *E. tarda* in turbot inoculated with doses $<10^4$ CFU ml^{-1} start on Day 12 post-inoculation (Toranzo 2007).

On the other hand, the analysis of naturally infected turbot received in our laboratory showed that, when the infection is already present in fish, PCR and bacteriological protocols allow the detection of *Edwardsiella tarda* from all samples tested except mucus. The negative PCR detection in this type of sample could be explained by recent studies in our laboratory in which the antibacterial activity of mucus against *E. tarda* was demonstrated (data not shown).

In summary, we propose the PCR protocol employing the primer set etfD (Sakai et al. 2007) as a rapid and sensitive method for the accurate detection of *Edwardsiella tarda* in infected fish.

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