

RESEARCH LETTER

Characterization of ISR region and development of a PCR assay for rapid detection of the fish pathogen *Tenacibaculum soleae*

Jose R. López¹, Abdel M. Hamman-Khalifa², José I. Navas¹ & Roberto de la Herran²

¹IFAPA Centro Agua del Pino, Huelva, Spain; and ²Departamento de Genética, Facultad de Ciencias, Universidad de Granada, Granada, Spain

Correspondence: Jose R. López, IFAPA Centro Agua del Pino, Junta de Andalucía, Carretera El Portil-El Rompido s/n, 21450 Cartaya, Huelva, Spain. Tel.: +34 959 024900; fax: +34 959 024929; e-mail: lasacias@yahoo.es

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Tenacibaculosis; diagnosis; identification; disease; characterization; aquaculture.

Abstract

The aims of this work were to characterize the 16S–23S internal spacer region of the fish pathogen *Tenacibaculum soleae* and to develop a PCR assay for its identification and detection. All *T. soleae* strains tested displayed a single internal spacer region class, containing tRNA^{Ile} and tRNA^{Ala} genes; nevertheless, a considerable intraspecific heterogeneity was observed. However, this region proved to be useful for differentiation of *T. soleae* from related and non-related species. Species-specific primers were designed targeting the 16S rRNA gene and the internal spacer region region, yielding a 1555-bp fragment. Detection limit was of 1 pg DNA per reaction (< 30 bacterial cells) when using pure cultures. The detection level in the presence of DNA from fish or other bacteria was lower; however, 10 pg were detected at a target/background ratio of 1 : 10⁵. The PCR assay proved to be more sensitive than agar cultivation for the detection of *T. soleae* from naturally diseased fish, offering a useful tool for diagnosis and for understanding the epidemiology of this pathogen.

Introduction

Tenacibaculosis caused by bacteria belonging to the genus *Tenacibaculum* is one of the more devastating infectious diseases of farmed marine finfish worldwide (Hansen *et al.*, 1992; Toranzo *et al.*, 2005). *Tenacibaculum soleae* is a recently described species which has been reported causing high mortalities in commercially valuable species as Senegalese sole (*Solea senegalensis*), Wedge sole (*Dicologlossa cuneata*) and Brill (*Scophthalmus rhombus*), being virulent also for Turbot (*Scophthalmus maximus*; Piñeiro-Vidal *et al.*, 2008; López *et al.*, 2010). At present, *T. soleae* is detected from fish by cultivation and subsequent identification using biochemical and serological techniques, which are frequently inconclusive and time-consuming. Moreover, isolation from diseased fish is problematic because of the slow growth of the pathogen and the overgrowth and/or inhibition by other bacteria present within the lesions.

PCR has proved to be useful for identification and detection of bacterial pathogens from samples without any need of cultivation (Cepeda *et al.*, 2003; Gonzalez *et al.*, 2003). The gene for the 16S rRNA is widely used in

bacterial taxonomy as it contains variable stretches that have been used successfully for specific PCR primer design (Wiklund *et al.*, 2000; Del Cerro *et al.*, 2002; Oakey *et al.*, 2003). However, it has been widely shown that the internal spacer region (ISR) between the 16S and 23S rRNA genes is more variable between bacterial species than ribosomal genes themselves in both sequence and length (Barry *et al.*, 1991; Hassan *et al.*, 2003; Osorio *et al.*, 2005). Species-specific primers derived from these sequences have also been reported (Kong *et al.*, 1999; Lee *et al.*, 2002; Hassan *et al.*, 2008). In this study, we sequenced the ISR from *T. soleae* and designed species-specific primers, targeting both the 16S rRNA gene and ISR region, for its identification and detection by PCR.

Materials and methods

Bacterial strains

The strains used in this study are listed in Table 1. Together with 32 reference strains, 57 isolates obtained in our laboratory from diseased flatfish were also used. These isolates were identified based on 16S gene sequencing and

Table 1. Bacterial species and strains used in this work

Species	Strains	Source	Geographic origin
<i>Tenacibaculum soleae</i>	NCIMB 14368 ^T	Senegalese sole (<i>Solea senegalensis</i>)	Spain
	a11	Brill (<i>Scophthalmus rhombus</i>)	Spain
	a47, a50, a216, a462, a467	Wedge sole (<i>Dicologlossa cuneata</i>)	Spain
	a410, a469	Senegalese sole (<i>S. senegalensis</i>)	Spain
<i>Tenacibaculum ovolyticum</i>	LMG 13025	Atlantic halibut (<i>Hippoglossus hippoglossus</i>) eggs	
<i>Tenacibaculum maritimum</i>	CECT 4276	Fishblack sea bream (<i>Acanthopagrus schlegeli</i>)	Japan
	Lg326	Senegalese sole (<i>S. senegalensis</i>)	Spain
	a274, a388, a442, a443, a444, a461, a523	Wedge sole (<i>D. cuneata</i>)	Spain
<i>Tenacibaculum gallaicum</i>	DSM 18841 ^T	Seawater	
<i>Tenacibaculum discolor</i>	DSM 18842 ^T	Senegalese sole (<i>S. senegalensis</i>)	Spain
<i>Tenacibaculum litoreum</i>	JCM 13039 ^T	Tidal flat sediment	Korea
<i>Tenacibaculum</i> sp.	a3	Wedge sole (<i>D. cuneata</i>)	Spain
<i>Polaribacter</i> sp.	a500, a502	Wedge sole (<i>D. cuneata</i>)	Spain
<i>Flavobacterium marinotypicum</i>	CECT 578 ^T		
<i>Flavobacterium johnsoniae</i>	CECT 5015	Grass	UK
	UW 101 ^T (ATCC 17061 ^T)	Soil	UK
<i>Flavobacterium psychrophilum</i>	NCIMB 1947 ^T	Coho salmon (<i>Oncorhynchus kisutch</i>)	USA
	OSU THCO2-90	Coho salmon (<i>O. kisutch</i>)	USA
<i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i>	CECT894 ^T	Salmon (<i>Salmo salar</i>)	
<i>Aliivibrio fischeri</i>	CECT 524 ^T		
<i>Vibrio harveyi</i>	CECT 525 ^T	Amphipod (<i>Talorchestia</i> sp.)	USA
	CECT 5156	Sea bass	
	Lg 123, a10	Senegalese sole (<i>S. senegalensis</i>)	Spain
	a9, a20, a26, a82, a87, a91, a102, a106, a417	Wedge sole (<i>D. cuneata</i>)	Spain
<i>Vibrio alginolyticus</i>	CECT 521 ^T	Horse mackerel (<i>Trachurus trachurus</i>)	Japan
	CECT 436	Food	
	a134	Brill (<i>S. rhombus</i>)	Spain
	a241	Wedge sole (<i>D. cuneata</i>)	Spain
<i>Vibrio parahaemolyticus</i>	CECT 511 ^T	Human patient suffering food poisoning	Japan
<i>Vibrio campbellii</i>	CECT 523 ^T	Seawater	Hawaii
<i>Vibrio natriegens</i>	CECT 526 ^T	Salt marsh mud	USA
<i>Vibrio tubiashii</i>	CECT 4196 ^T	Hard clam (<i>Mercenaria mercenaria</i>), USA	USA
<i>Vibrio tapetis</i>	CECT 4600 ^T	Clam (<i>Tapes philippinarum</i>)	France
	a255	Wedge sole (<i>D. cuneata</i>)	Spain
<i>Vibrio splendidus</i>	CECT 528 ^T	Marine fish	
<i>Vibrio</i> spp.	a7, a29, a35, a54, a107, a256	Wedge sole (<i>D. cuneata</i>)	Spain
	a6	Senegalese sole (<i>S. senegalensis</i>)	Spain
<i>Photobacterium damsela</i> ssp. <i>piscicida</i>	CECT 5895	Sea bass (<i>Dicentrarchus labrax</i>)	
	Lg 122, a316, a319, a321, a335, a356	Senegalese sole (<i>S. senegalensis</i>)	Spain
<i>Photobacterium damsela</i> ssp. <i>damselae</i>	CECT 626 ^T	Damsel fish (<i>Chromis punctipinnis</i>)	USA
<i>Photobacterium leiognathi</i>	CECT 4191 ^T	Teleostean fish (<i>Leiognathus equula</i>)	Thailand
<i>Photobacterium angustum</i>	CECT 5690 ^T	Seawater	
<i>Photobacterium phosphoreum</i>	CECT 4192 ^T		
<i>Photobacterium</i> spp.	a185, a197	Wedge sole (<i>D. cuneata</i>)	Spain
<i>Pseudoalteromonas</i> spp.	a220, a250	Wedge sole (<i>D. cuneata</i>)	Spain
<i>Psychrobacter</i> sp.	a328	Wedge sole (<i>D. cuneata</i>)	Spain
<i>Pseudomonas fluorescens</i>	CECT 378 ^T	Water tank	UK
<i>Pseudomonas putida</i>	CECT 385	Soil	
<i>Pseudomonas anguilliseptica</i>	CECT 899 ^T	Eel (<i>Anguilla japonica</i>)	Japan
<i>Pseudomonas aeruginosa</i>	ATCC 27853		
<i>Pseudomonas baetica</i>	a390 ^T , a391, a393, a398, a399, a600	Wedge sole (<i>D. cuneata</i>)	Spain
<i>Escherichia coli</i>	ATCC 25922		

biochemical tests. All strains were cultured aerobically at 20 °C on tryptic soya agar (TSA) made with seawater, with the exception of those belonging to *Tenacibaculum maritimum*, which were grown on *Flexibacter* medium (FMM; Pazos *et al.*, 1996).

DNA extraction

Template DNA from pure cultures was prepared by boiling bacterial colonies for 10 min in distilled water followed by centrifugation at 12 400 *g* for 1 min to sediment the cell debris. DNA from tissue samples was extracted as follows: after homogenizing 100 mg of fish tissue in TE buffer (Sigma), SDS (1%) and proteinase K (100 µg mL⁻¹) were added and the solution was incubated for 3 h or overnight at 56 °C. Thereafter, pancreatic RNase (20 µg mL⁻¹) was added and incubation was performed for 1 h at 37 °C. The solution was transferred to a phase-lock gel (Eppendorf) and the DNA was purified using the common phenol/chloroform/isoamyl alcohol procedure and finally precipitated with ethanol and dissolved in distilled water. The concentration and purity of genomic DNA were calculated from measurements of absorbance at 260 and 280 nm, recorded using a NanoDrop 1000 spectrophotometer.

16S rRNA gene and ISR region characterization

Partial 16S rRNA gene sequences were obtained using primers 20F and 1500R (Weisburg *et al.*, 1991), and ISR sequences were obtained using primers 16/23S-F and 16/23S-R (Lee *et al.*, 2002). PCR products were electrophoresed in a 1% agarose gel and purified with the kit GenElute PCR Clean-up (Sigma) following the manufacturer's instructions. The purified products were cloned in pGEM-T Easy Vector System II kit (Promega) or directly used for sequencing. Sequencing was accomplished using the kit BigDye Terminator v3.1 Cycle Sequencing (Applied Biosystems) and an ABI Prism 3130 DNA Sequencer (Applied Biosystems). The sequences were analyzed using CHROMAS LITE v2.01 and SEQMAN II (DNASTAR) programs and subjected to BLAST searches to retrieve the most closely related sequences. The presence of tRNA genes was determined using tRNAscan-SE 1.21 software (Lowe & Eddy, 1997).

Primer design

Previously reported 16S rRNA gene and ISR sequences from *T. soleae* and related species, retrieved from GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>) and those obtained in this study, were aligned by using the program CLUSTAL W (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and examined for areas of similarity and variability between dif-

ferent species and strains. On the basis of the alignments, two variable regions were chosen and a pair of primers was designed by using the PRIMER3 program (<http://frodo.wi.mit.edu/>; Rozen & Skaletsky, 2000). Primers were synthesized by Thermo Scientific (Ulm, Germany).

PCR amplification

The PCR amplifications were carried out using the commercial kit RedTaq ReadyMix (Sigma), which included all necessary reagents except the primers and DNA template. The PCR mixture consisted of reaction buffer (10 mmol L⁻¹ Tris-HCl pH 8.3, 50 mmol L⁻¹ KCl, 1.5 mmol L⁻¹ MgCl₂), 200 µmol L⁻¹ of each dNTP, 200 nmol L⁻¹ of each primer, 3 U of *Taq* DNA polymerase, template DNA, and double-distilled water up to a final volume of 50 µL. The amplification was performed in a Mastercycler gradient (Eppendorf) as follows: an initial denaturation at 94 °C for 5 min followed by 45 amplification cycles (denaturation at 94 °C for 1 min, annealing at 57 °C for 45 s, and extension at 72 °C for 1 min), and a final elongation at 72 °C for 5 min. DNA from strain *T. soleae* a47 was included as a positive control and distilled water as a negative control. PCR products were electrophoresed on a 1% agarose TBE gel stained with SYBR Safe DNA Gel Stain (Invitrogen); a 1-kb DNA ladder (Biotools) was included as a molecular weight marker.

Specificity and sensitivity of the PCR

To test the specificity of the primers in the PCR procedure, nine *T. soleae* strains, isolated from three different hosts and including the type strain, and 81 strains of other species, most of them taxonomically and/or ecologically related, were used as positive and negative controls, respectively (Table 1). For PCR amplification, 100 ng DNA template was used for each strain. The detection limit was evaluated using 10-fold serially diluted DNA, isolated from strain *T. soleae* a47, over the range 100 ng to 100 fg. Large amounts of DNA (0.5–3 µg) were also assayed. To test the sensitivity in the presence of tissue debris or other bacterial species, the same 10-fold DNA dilutions were used mixed with 1 µg of DNA from healthy Wedge sole liver or from a mixture of pure cultures of a number of marine bacteria (*T. maritimum*, *Vibrio harveyi*, *Photobacterium damsela*, *Psychrobacter* sp., and *Pseudomonas baetica*). Each assay was performed at least in duplicate.

Detection of *T. soleae* in naturally infected fish tissues

Ulcer samples from six Wedge sole with suspected tenacibaculosis caused by *T. soleae* on the basis of medical

history and the presence of filamentous bacteria in wet-mount preparations, together with samples (ulcers, liver or kidney) from four fish (one Brill, one Senegalese sole and two Wedge sole) diagnosed by culture as positive for *T. soleae*, were analyzed for the presence of the pathogen using PCR. DNA from samples was extracted as outlined above and 1 µg used for each PCR reaction.

Results

ISR region analysis

Twenty-one 16S and ISR nucleotide sequences were determined from *T. soleae* or related organism strains (accession numbers FR734188, FN433006, FN646547–FN646565). The ISR PCR products from *T. soleae* strains a11, a47, a50, a216, a410, a462, a467 and a469 were analyzed by agarose gel electrophoresis; each strain seemed to contain only one type of operon, as a single band of about 1200 bp, including partial 16S and 23S rRNA genes, was found (data not shown). Direct sequencing of ISR from some strains (a11, a47, a50, a410, a462) seemed to support this possibility; an unambiguous reading of nucleotide sequences was possible, and the sequences obtained by cloning and by direct sequencing were similar. Sequence analysis showed that *T. soleae* 16S–23S spacers were basically similar in length (586–596 bp) and belonged to a unique ISR class (ISR^{IA}), carrying tRNA genes for isoleucine (Ile) and alanine (Ala). Similarity between *T. soleae* strains ISR sequences was of 90.6–100%. The main differences between strains were due to the presence of a variable region, of approximately 90 bp and located near the 3' end, which contained

different short sequence blocks. On the basis of variation in this region, *T. soleae* ISR sequences could be grouped into two basic types, the first including those obtained from strains a11, a47, a216, and a410 (96.3–100% similarity), and the second comprising strains a50, a462, a467 and a469 (97.5–99.2%). Similarity values with other related species were clearly lower, the closest strains being *Tenacibaculum ovolyticum* LMG 13025 (85.2% similarity) and *T. maritimum* a523 (71.9%). The tRNA^{Ile} and tRNA^{Ala} genes were similar both in length (74 bp) and in nucleotide composition for all the *T. soleae* strains tested, and were also similar to those found in other species of the genus as *T. maritimum* and *T. ovolyticum*, differing only at one or two positions, or at none at all.

Primer design and specificity

A pair of primers to identify *T. soleae*, forward G47F (5'-ATGCTAATATGTGGCATCAC-3'), and reverse G47R (5'-CGTAATTCGTAATTAACCTTTGT-3'), were designed at the 5' region of the 16S gene and of the ISR, respectively (Fig. 1), flanking a 1555-bp fragment.

The specificity was tested experimentally as indicated using pure cultures from target and non-target strains (Table 1). All *T. soleae* strains produced a clear PCR band of the expected size (1555 bp). A phantom band of about 750 bp was sometimes also visible. Conversely, no PCR product was detected from non-target species (Fig. 2).

Sensitivity

The detection limit of the PCR assay, when purified DNA of *T. soleae* was used as template, was as little as 1 pg in

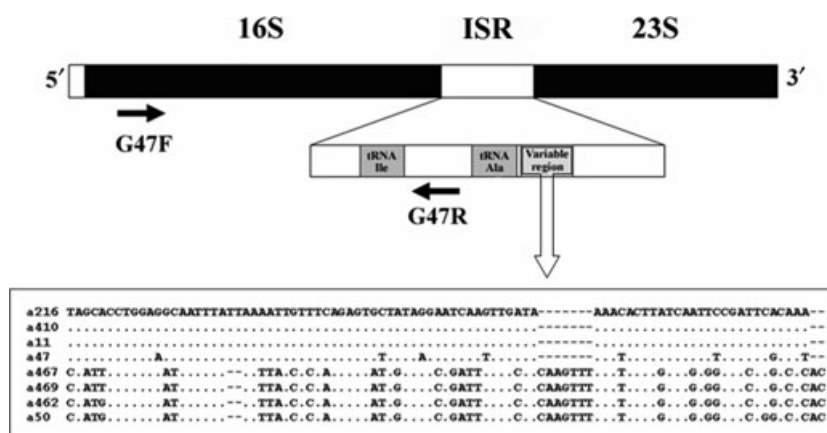


Fig. 1. Diagrammatic representation of a *T. soleae* rRNA operon, showing the relative locations of primers G47F and G47R, the tRNA genes and the ISR intraspecific variable region. Primers are represented as black arrows. Primer G47F was designed at the beginning of the 16S gene and corresponds to nucleotides 137–156 in the sequence with GenBank accession no. AM989478. Primer G47R was designed within the 16S–23S intergenic spacer, between the two tRNA genes present, and corresponds to nucleotides 193–214 in the sequence with GenBank accession no. FN646550.

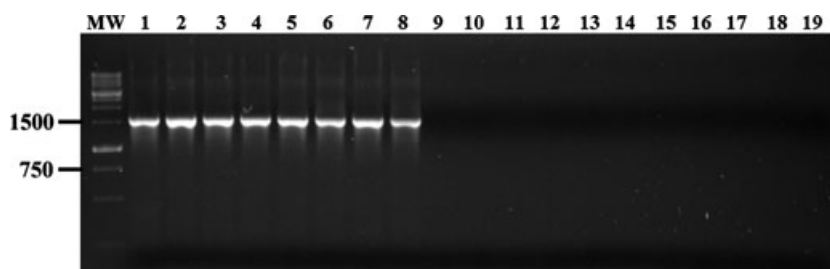


Fig. 2. Profile of PCR using primers G47F and G47R and DNA templates from *T. soleae* and some non-target related species. MW: 1-kb ladder molecular weight marker. Lanes 1–8: *T. soleae* strains NCIMB 14368^T, a11, a47, a50, a216, a410, a462 and a467, respectively. Lanes 9–19: *Tenacibaculum* sp. a3, *T. ovolyticum* LMG 13025, *T. maritimum* strains CECT 4276, Lg326 and a443, *Polaribacter* sp. a502, *Flavobacterium psychrophilum* strains NCIMB 1947 and OSU THCO2-90, *Flavobacterium johnsoniae* strains CECT 5015 and UW101, and *Flavobacterium marinotipicum* CECT 578, respectively. Numbers on the left indicate the position of molecular size marker in bp. Only *T. soleae* strains showed the expected 1555-bp band.

a 50- μ L reaction volume. A 100-fg template could sometimes be detected, although this product was extremely weak and not always reproducible. Conversely, large DNA amounts gave positive results, showing that the optimum template concentration was from 2 μ g to 100 ng (Fig. 3). When DNA extracted from fish tissues was seeded with different concentrations of *T. soleae* DNA and used as template, the detection limit was of 10 pg of *T. soleae* DNA in 1 μ g of fish DNA. Thus, the assay was capable of detecting one *T. soleae* genomic copy among 10^5 copies from fish tissues. Similar results were found when this assay was made with DNA from mixed cultures of marine bacteria instead of from fish tissues.

Detection of *T. soleae* in naturally infected fish tissues

Results obtained with naturally infected fish samples indicated that the proposed protocol was more sensitive than agar cultivation for detecting *T. soleae*. When the samples used were from fish suspected of suffering tenacibaculosis by *T. soleae*, three of the six fish tested proved positive by PCR. Although filamentous bacteria had been observed in these samples by microscopy, none grew in culture medium, presumably because of inhibition or overgrowth by

environmental bacteria. On the other hand, when fish diagnosed by culturing as positive for *T. soleae* were used, all four samples gave positive results.

Discussion

Because of their specificity, sensitivity and rapid performance, PCR-based methods constitute one of the strongest tools for bacteria diagnosis, and specific protocols have been developed for many major bacterial pathogens in aquaculture (Toyama *et al.*, 1996; Wiklund *et al.*, 2000; Pang *et al.*, 2006; Beaz-Hidalgo *et al.*, 2008). PCR constitutes a useful tool not only for detecting pathogens in diseased fish, but also in asymptomatic carriers, in the environment, or for selecting pathogen-free egg stocks.

In this study, we developed a PCR protocol against *T. soleae*, an emerging pathogen in marine aquaculture whose identification is tedious and time-consuming, requiring prior isolation of the bacteria and the utilization of phenotypic tests, which require days or weeks to perform. The PCR assay described here is specific and sensitive, enabling quicker and easier identification of the pathogen.

The 16S rRNA gene and the ISR region were selected as primer targets to take the greatest advantage of these

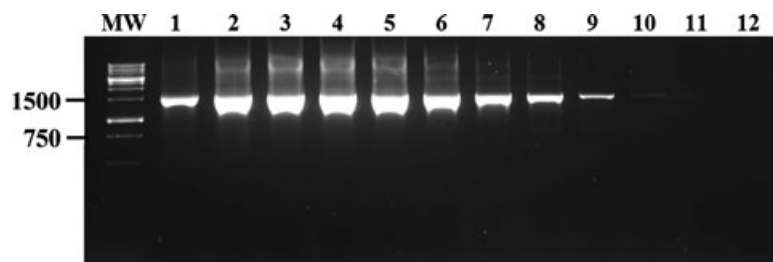


Fig. 3. Detection limit of the PCR protocol with primers G47F and G47R, determined with different dilutions of purified DNA of *T. soleae*. MW: 1-kb ladder molecular weight marker. Lanes 1–11: 3, 2, 1 μ g, 500, 100, 10, 1 ng, 100, 10, 1 pg and 100 fg of DNA, respectively; lane 12, negative control. Numbers on the left indicate the position of molecular size marker in bp.

two DNA regions. Although 16S rRNA gene is highly conserved in eubacteria and contains only small regions of variation, the vast database of sequences available makes finding and comparison with close relatives feasible. The ISR region, in comparison, is generally more variable than rRNA genes, allowing better discrimination of closely related species, even among strains of the same species (Lee *et al.*, 2002; Osorio *et al.*, 2005). However, few sequences are available from the Flavobacteriaceae species. This is the first report of ISR sequences from *Tenacibaculum* species, namely *T. soleae*, *T. maritimum* and *T. ovolyticum*, which will facilitate the identification of other specific primers for Flavobacteriaceae species.

Tenacibaculum soleae strains ISR showed only minor size variations in length and belonged to a single ISR class, containing tRNA^{Ile} and tRNA^{Ala} genes. The presence of a single ISR class is frequent in bacteria. For example, the analysis of the ISR region of 155 bacterial strains belonging to a variety of taxa, carried out by Stewart & Cavanaugh (2007), revealed that only 41% of the strains had two or more ISR classes. In the same study, the presence of tRNA^{Ile} and tRNA^{Ala} genes was also common, being detected in 48% of the ISR sequences obtained by these authors; nevertheless, its frequency varied depending on the bacterial taxa, being absent, for example, in *Actinobacteria*. However, in Flavobacteriaceae, the tRNA^{Ile}-tRNA^{Ala} combination appears to be dominant, being present in different genera of the family, such as *Flavobacterium* or *Cellulophaga* (Figueiredo *et al.*, 2005; Welker *et al.*, 2005; Holmfeldt *et al.*, 2007; Ford, 2008), as well as in all the *Tenacibaculum* and *Polaribacter* strains tested by our group. ISR intraspecific variation in *T. soleae* was of 0–9.4%, a lower value than that reported by Stewart & Cavanaugh (2007) when comparing sequences from the same species and ISR class (0–12.1%). Differences between *T. soleae* ISR sequences were due mainly to the absence/presence of distinct sequence blocks, as reported by other authors for a variety of bacterial species, including fish pathogens such as *Photobacterium damsela* (Gürtler & Barrie, 1995; Chun *et al.*, 1999; Osorio *et al.*, 2005; Stewart & Cavanaugh, 2007). On the other hand, ISR sequences proved useful for differentiating *T. soleae* from related species, displaying lower interspecific similarity values than obtained with 16S rRNA gene. For example, the similarity of *T. soleae* a47 and *T. ovolyticum* LMG 13025 was 97.7% when 16S rRNA gene sequences were compared, but only 85.2% with ISR sequences. In this sense, it is important to note that although the ISR region generally displays greater nucleotide divergence than 16S rRNA gene, this is not always the case. In fact, Stewart & Cavanaugh (2007) noted that the ISR region was less discriminating than 16S rRNA gene for 24% of the strains tested.

The specificity of the proposed PCR protocol was validated in nine *T. soleae* strains and 81 strains belonging to other species, most of these from marine environments, including several common fish pathogens. No cross-reactions with any of the non-target organisms were observed. The sensitivity experiments showed a detection limit with DNA extracted from pure cultures of 1 pg DNA/PCR tube, equivalent to 30 or fewer bacterial cells, a result that agrees with those described with other PCR methods designed for bacterial fish pathogens (Del Cerro *et al.*, 2002; Mata *et al.*, 2004; Romalde *et al.*, 2004; Hong *et al.*, 2007). The isolation of *T. soleae* from diseased fish is in many cases unsuitable due to the slow growth of the pathogen and overgrowth or inhibition by other faster growing bacteria present within the lesions. Thus, the usefulness of the proposed PCR protocol to detect the bacteria from mixed cultures and fish tissue samples was also tested. The results from seeding DNA extracted from fish tissues or from a mixture of bacterial cultures confirmed the sensitivity of the method (10 pg of *T. soleae* DNA was detected at a target/background ratio of 1: 10⁵), although as expected the detection level was lower than that with pure cultures, probably due to the presence of some PCR inhibitor. It has been reported that high levels of non-target DNA, constituents of bacterial cells, and different compounds found in animal tissues can have an adverse effect on PCR (Wilson, 1997; Becker *et al.*, 2000). When naturally infected fish were subjected to the PCR assay, positive results were recorded for all the confirmed cases, and in half of the suspected cases in which cultures failed to detect the bacteria. The PCR-assay was therefore more sensitive than agar culturing for detecting *T. soleae* from tissue samples, offering a useful tool for rapid diagnosis and examination of the epidemiology of this pathogen.

In summary, the present study reports the first PCR protocol suitable for identifying this pathogen from pure or mixed cultures, as well as for detection from fish tissue samples.

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