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A non-destructive method for rapid detection of *Tenacibaculum maritimum* in farmed fish using nested PCR amplification

R. Avendaño-Herrera, S. Núñez, B. Magariños and A.E. Toranzo

Departamento de Microbiología y Parasitología, Facultad de Biología & Instituto de Acuicultura, Universidad de Santiago, 15782 Santiago de Compostela, Spain.

Abstract

Marine tenacibaculosis caused by *Tenacibaculum maritimum* is an important disease in different cultured marine fish. Some of the presently available techniques for the detection of *T. maritimum* are either time consuming or lack sufficient sensitivity. In this study, we evaluated the efficacy of a nested PCR method for the detection of *T. maritimum* using non-destructive mucus samples from different cultured species of marine fish. To test this method sixty asymptomatic and symptomatic cultured fish were examined. The nested PCR assay allowed the detection of *T. maritimum* in 100% of mucus samples from farmed turbot, sole and gilthead seabream that were undergoing an epizootic of tenacibaculosis. Only 81% of the mucus samples from these diseased fish were positive by plating methods. When the nested PCR protocol was applied to different mucus samples obtained from apparently healthy fish, the detection of *T. maritimum* was positive in 15 of 29 fish tested. Nine of these fish had been diagnosed as negative for the presence of *T. maritimum* by cultivation on agar. The method presented here demonstrated that non-lethal mucus samples can be used with screening purpose and early diagnosis of marine tenacibaculosis, allowing the rapid detection of *T. maritimum* (in only 7 h) without previous isolation in culture media.

Introduction

Cultured fish are susceptible to a wide range of bacterial infections, and losses associated with the diseases subsequently make a significant impact on the quality and volume of fish produced worldwide. *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) is one of these threatening microorganisms, being the causative agent of tenacibaculosis or flexibacteriosis in a great variety of valuable marine fish species in Japan, North America, several countries in Europe and Australia (Hikida et al., 1979; McVicar & White, 1979; Devesa et al., 1989; Bernardet et al., 1990; Alsina & Blanch, 1993; Handlinger et al., 1997; Cepeda & Santos, 2002; Avendaño-Herrera et al., 2004a).

The disease is currently diagnosed by the clinical signs of the affected fish, especially severe necrotic lesions on the body surface, skin ulcers, jaw erosion, frayed fins and tail rot (Campbell & Buswell, 1982; Devesa et al., 1989; Ostland et al., 1999). However, the isolation of *T. maritimum* from fish tissues can be very difficult, due to its extremely slow growth on synthetic specific media, where the pathogen is normally overgrown by many of

the species coexisting in external samples. These facts, makes it very difficult to visualize and isolate *T. maritimum*.

Rapid detection and identification of this pathogen is crucial for effective management and disease control in sea farming. Initial attempts to detect *T. maritimum* using molecular tools were based on the design of two pairs of oligonucleotides primers for the polymerase chain reaction (PCR)-based method using the 16S rRNA gene sequence as target (Toyama et al., 1996; Bader & Shotts, 1998). Cepeda et al. (2003) published a nested PCR system for the detection of *T. maritimum* in fish tissues based on modifications of the PCR programme described previously by Bader & Shotts (1998) in order to reduce the time to achieve the diagnosis of this bacterium. However, when we used this protocol in the same conditions described by these authors with pure cultures of *T. maritimum* strains, none of the isolates tested generated an amplification signal (Avendaño-Herrera et al., 2004b).

On the other hand, we also compared the specificity and sensitivity of these two primer pairs, finding the Toyama PCR procedure the most adequate for an accurate detection of *T. maritimum* in diagnostic pathology as well as in epidemiological studies of marine tenacibaculosis (Avendaño-Herrera et al., 2004b). Although this method proved to be useful to detect *T. maritimum* acute infections in fish, the level of sensitivity was not sufficient to detect the pathogen when present in very low number in asymtomatic or carrier fish. In order to increase its sensitivity we developed a nested PCR approach, which was evaluated experimentally by seeded fish tissues, including mucus, skin, kidney and blood (Avendaño-Herrera et al., 2004b). With this method, we found an easy detection of *T. maritimum* in mucus samples with a level of sensitivity of about 10^3 CFU ml^{-1}. However, despite its potential, no application of this nested PCR protocol with mucus samples from cultivated fish was performed.

Therefore, in this study we evaluated the efficacy of a nested PCR method for the detection of *T. maritimum* using non-destructive mucus samples from different cultured species of marine fish.

Materials and methods

Fish samples

To evaluate the possible usefulness of the nested PCR method to detect *T. maritimum* in non-destructive samples, a total of sixty fish ranging from 10 to 300 g were obtained from turbot, sole and gilthead seabream farmed in the northwest of Spain and Portugal. Fish were sent from fish farms to the laboratory of the University of Santiago de Compostela (Spain) where they were subjected to bacteriological examination (agar cultivation and biochemical tests) and analysed by nested PCR. Fish were sent on ice and analysed within 5 h of capture. To facilitate our study, the species examined were classified into two groups: symptomatic (n = 31) and asymptomatic (n = 29) fish. The first group was defined as fish with the main signs of the diseases such as eroded mouths, skin ulcers and rotten, frayed fins, while the second group compiled fish that did not show the clinical signs of marine tenacibaculosis (Table 1).

Source	No. of samples	Detection of *T. maritimum* by Nested PCR	Culture	Microscopy
Symptomatic fish				
Turbot (*Scophthalmus maximus*)	21	21	16	21
Sole (*Solea senegalensis*)	6	6	6	6
Gilthead seabream (*Sparus auratus*)	4	4	3	4
Asymptomatic fish				
Turbot (*Scophthalmus maximus*)	29	15	6 *	9 *

* Samples also positive by nested PCR.

Table 1. Detection of *T. maritimum* in skin mucus samples from cultured marine fish, using nested PCR, agar cultivation and microscope observation.

Collection of the mucus samples

Mucus samples from fish were obtained by gently passing a sterile 10 µl plastic inoculating loop or a sterile scalpel blade along the lateral surface of individual fish (Evans et al., 2001). The mucus samples were then diluted 1:10 (v/v) in 100 ml sterile phosphate buffered saline (PBS, pH 7.4) and maintained frozen at –20°C until they were used for DNA extraction.

Microscopical and bacteriological analysis

In order to compare the efficacy of the nested PCR procedure, other standard conventional methods were carried out. For microscope observations, smear from skin samples were examined using a light microscope at 400 x magnification. At the same time, samples from skin were directly streaked onto plates containing *Flexibacter maritimus* Medium (FMM) agar (Pazos et al., 1996), which were incubated at 20°C for 72 h. After this, the colonies that exhibited the typical features for this species: flat, pale-yellow with uneven edges and strong adherence to the medium were chosen for isolation and subjected to standard phenotypical tests (Avendaño-Herrera et al. 2004a). Stock cultures of the strains were stored frozen at –70°C in Criobille tubes (AES Laboratory, France). Simultaneously, pure cultures of *T. maritimum* were scraped off the plates, resuspended in 1 ml of sterile distilled water, and the samples were maintained at –20°C until the DNA extraction.

DNA extraction

DNA was extracted using 2 different commercial systems: InstaGene Matrix (Bio-Rad, Madrid, Spain) for the pure cultures, and Dynabeads DNA DIRECT™ (Dynal) for mucus samples. In all cases, DNA purification was performed according to the manufacturer's instructions. One ml of each DNA extracted was used directly for single and nested PCR amplification and the remaining DNA sample was maintained at –20°C. All extractions included at least two positive and negative controls. The positive controls consisted of 10^5 cells from a pure culture of the *T. maritimum* strain NCIMB 2154^T and mucus seeded with the same concentration of this reference strain. Sterile distilled water and

non-inoculated mucus samples were employed as negative controls.

Nested PCR analysis

In this study a nested PCR method recently developed in our laboratory was applied to the mucus samples (Avendaño-Herrera et al., 2004b). The first round of PCR was performed according to a previously described protocol by Edwards et al., (1989) with minor modifications. This method utilizes the universal primer pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3'), but the reaction condition was shortened to 25 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 90 s and in the final cycle samples were incubated at 72°C for 5 min. A preheating cycle at 95°C for 4 min was included. All PCR amplifications were done using the commercial kit Ready-To-Go™ PCR beads (Amersham Pharmacia Biotech, Barcelona, Spain), which included all the reagents needed for the PCR reactions (buffer, nucleotides and *Taq* DNA polymerase), with the exception of the specific primers and DNA template. The reaction mixtures were amplified in a Mastercycler personal thermal cycler (Eppendorf, Hamburg, Germany). After the first amplification by the external primers was finished, 1 µl of each PCR product was used as template of the second amplification by the species-specific primer set MAR1 (5'-AATGGCATCGTTTTAAA-3') and MAR2 (5'-CGCTCTCTGTTGCCAGA-3') (Toyama et al., 1996). The samples were denatured at 94°C for 2 min, followed by 40 cycles of 94°C for 2 min, 45°C for 90 s, and 72°C for 2 min. The tubes were then held at 4°C. DNA's extracted from negative and positive controls were included in each batch of PCR reaction. Single PCR analysis was used for the detection of *T. maritimum* from pure cultures, the conditions for PCR amplification were the same as described for the second amplification.

Analysis of PCR products

Amplified products were detected by horizontal 1% (w/v) agarose gel electrophoresis for 60 min at 100 V in TAE 1X (0.04 M Tris, 0.0001M EDTA, pH 8.0) electrophoresis buffer, visualized using 0.06 µg ml^{-1} of ethidium bromide (Bio-Rad) and photographed under UV light and computer digitized (Gel Doc 100, Bio-Rad). A 50-2000-bp ladder (Sigma Chemical Co, St. Louis, MO, USA) was used as a molecular size marker. The presence of a single band of 1088 base pair (bp) was considered as a positive result.

Results and discussion

The need for fast, specific and sensitive molecular tools for the detection of important bacterial pathogens in cultivated marine fish using non-destructive samples is particularly crucial in the context of sea-farming (Arias et al., 1995; Osorio et al., 1999; Bader et al., 2003).

In this work, we tested whether *T. maritimum* could be detected directly in field conditions utilizing skin mucus as a non-destructive sampling procedure by using a nested PCR assay. The application of this protocol gave positive amplification to *T. maritimum* for mucus samples obtained from all symptomatic fish tested in this study, regardless of the fish host (Table 1). All of them rendered a unique and clear PCR band of the expected 1088 bp length (Figure 1),

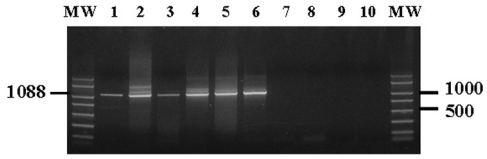

Figure 1. Detection of *T. maritimum* from mucus samples obtained from symptomatic (lanes 2, 4, and 5) and asymptomatic (lanes 3, 6, 7, 8 and 9) fish by nested PCR. Lanes: MW, AmpliSize Molecular Ruler (50-2000-bp ladder, Sigma); 1, positive control (DNA extracted from mucus seeded with *Tenacibaculum maritimum* NCIMB 2154^T); 2 to 9, DNAs extracted from mucus samples; 10, negative control (no DNA). Numbers on the right indicate the position of molecular size marker in kb. Numbers on the left indicate the size of the specific amplified products in bp.

while the negative control did not yield any amplification product. Microscopic examination of smears from skin lesions of these symptomatic fish revealed the presence of abundant long, thin, rod-shaped bacteria with the ability to flex. However, the microbiological analysis on FMM plates of external skin failed in the recovery of this bacterium in 1 and 5 samples of gilthead seabream and turbot respectively (Table 1). This failure can be explained by the growth inhibition of *T. maritimum* by other bacterial species present in the samples (Pazos et al., 1996).

When the nested PCR protocol was applied to different mucus samples obtained from apparently healthy fish, the detection of *T. maritimum* was positive in 15 of 29 asymptomatic fish tested (Table 1), yielding the expected 1088 bp fragment. In the other fish as well as in the negative controls no amplification was obtained. When all fish from this group were examined by microscopy, a few gliding bacteria were observed in skin scraped over large areas of the body in only 9 fish. Attempts to culture this gliding bacterium from the body surface was successful in 6 of the 29 cases analyzed, and the remaining fish were diagnosed as negative for the presence of *T. maritimum* by classical plate-culturing methods. It is important to note that all of the samples, which were positive by microscopical and bacteriological analysis, were also positive in the nested PCR.

The results presented in this work demonstrated clearly that the nested PCR applied to mucus samples from symptomatic fish was effective for the rapid detection of *T. maritimum* in the field, avoiding the possible false negative results obtained when only plate culture procedure is employed. These results are in agreement with the observation previously reported by Avendaño-Herrera et al., (2004b) when experimentally seeded fish tissues were employed. In the case of asymptomatic fish, it is important to note that the positive results reflected a high probability of infection, because a few days post-analysis these groups of fish showed disease signs followed by mortalities in the farms.

The effectiveness of the use of mucus samples for rapid diagnosis of marine tenacibaculosis could be explained by the primary site of infection of the pathogen, which is the body surface. In fact, it has been demonstrated that the skin mucus does not contain compound that inhibit the growth of *T. maritimum,* and therefore the localization of the bacteria is more likely within the mucus layer (Magariños et al., 1995; Handlinger et al., 1997).

In summary, from a practical point of view, we consider that the nested PCR approach is very useful for mucus screening purpose and, therefore, one of the strongest tools for early diagnosis of marine tenacibaculosis with a positive economic impact on marine fish culture. This non-destructive method allows the rapid detection of *T. maritimum* (in only 7 h) without previous isolation in culture media. This protocol can be useful for further understanding of the epidemiology of *T. maritimum* in order to elucidate possible transmission routes.

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References

Alsina M & Blanch AR (1993). First isolation of *Flexibacter maritimus* from cultivated turbot (*Scophthalmus maximus). Bulletin of the European Association of Fish Pathologists* **13,**157-160.

Arias CR, Garay E & Aznar R (1995). Nested PCR method for rapid and sensitive detection of *Vibrio vulnificus* in fish, sediments and water. *Applied Environmental Microbiology* **61,** 3476-3478.

Avendaño-Herrera R, Magariños B, López-Romalde S, Romalde JL & Toranzo AE (2004a). Phenotypic characterization and description of two major O-serotypes in *Tenacibaculum maritimum* strains isolated from marine fishes. *Diseases of Aquatic Organisms* **58,** 1-8.

Avendaño-Herrera R, Magariños B, Toranzo AE, Beaz R & Romalde JL (2004b). Comparative evaluation of species-specific polymerase chain reaction primer sets for the diagnosis of *Tenacibaculum maritimum* infection. *Diseases of Aquatic Organisms* (in press).

Bader JA & Shotts EB (1998). Identification of *Flavobacterium* and *Flexibacter* species by species-specific polymerase chain reaction primers to the 16S Ribosomal RNA gene. *Journal of Aquatic Animal Health* **10,** 311-319.

Bader JA, Shoemaker, CA & Klesius PH (2003). Rapid detection of columnaris disease in channel catfish (*Ictalurus punctatus*) with a new species-specific 16-S rRNA gene based PCR primer for *Flavobacterium columnare*. *Journal of Microbiological Methods* **52,** 209-220.

Bernardet JF, Campbell AC & Buswell JA (1990). *Flexibacter maritimus* is the agent of 'black patch necrosis' in Dover sole in Scotland. *Diseases of Aquatic Organisms* **8**, 233-237.

Campbell AC & Buswell JA (1982). An investigation into the bacterial aetiology of 'black patch necrosis' in Dover sole, *Solea solea* L. *Journal of Fish Diseases* **5,** 495-508.

Cepeda C & Santos Y (2002). First isolation of *Flexibacter maritimus* from farmed Senegalese sole (*Solea senegalensis,* Kaup) in Spain. *Bulletin of the European Association of Fish Pathologists* **22,** 388-391.

Cepeda C, García-Márquez S & Santos I (2003). Detection of *Flexibacter maritimus* in fish tissue using nested PCR amplification. *Journal of Fish Diseases* **26**, 65-70.

Devesa S, Barja JL & Toranzo AE (1989). Ulcerative skin and fin lesions in reared turbot, *Scophthalmus maximus* (L). *Journal of Fish Diseases* **12,** 323-333.

Edwards U, Rogall T, Bloecker H, Emde M & Boettger EC (1989). Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Research* **28,** 1942-1946.

Evans JJ, Shoemaker CA & Klesius PH (2001). Distribution of *Streptococcus iniae* in hybrid striped bass (*Morone chrysops x Morone saxatilis*) following nare inoculation. *Aquaculture* **194,** 233-243.

Handlinger J, Soltani M & Percival S (1997). The pathology of *Flexibacter maritimus* in aquaculture species in Tasmania, Australia. *Journal of Fish Diseases* **20,** 159-168.

Hikida M, Wayabayashi H, Egusa H & Masumura K (1979). *Flexibacter* spp. A gliding bacterium pathogenic to some marine fishes in Japan. *Bull. Jpn. Soc. Sci. Fish.* **45,** 421-428.

Magariños B, Pazos F, Santos Y, Romalde JL & Toranzo AE (1995). Response of *Pasteurella piscicida* and *Flexibacter maritimus* to skin mucus of marine fish. *Diseases of Aquatic Organisms* **21,** 103-108.

McVicar AH & White PG (1979). Fin and skin necrosis of cultivated Dover sole, *Solea solea* (L). *Journal of Fish Diseases* **2,** 557-562.

Osorio CR, Collins MD, Toranzo AE, Barja JL & Romalde JL (1999). 16S rRNA gene sequence analysis of *Photobacterium damselae* and nested PCR method for rapid detection of the causative agent of fish Pasteurellosis. *Applied Environmental Microbiology* **65,** 2942-2946.

Ostland VE, LaTrace C, Morrison D & Ferguson HW (1999). *Flexibacter maritimus* associated with a bacterial stomatitis in Atlantic salmon smolts reared in net-pens in British Columbia. *Journal of Aquatic Animal Health* **11,** 35-44.

Pazos F, Santos Y, Macias AR, Núñez S & Toranzo AE (1996). Evaluation of media for the successful culture of *Flexibacter maritimus*. *Journal of Fish Diseases* **19,** 193-197.

Toyama T, Kita-Tsukamoto K & Wakabayashi H (1996). Identification of *Flexibacter maritimus, Flavobacterium branchiophilum* and *Cytophaga columnaris* by PCR targeted 16S Ribosomal DNA. *Fish Pathology* **31,** 25-31.