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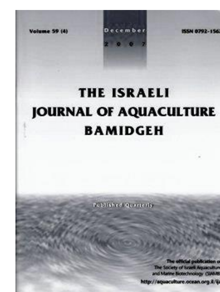

Isolation and Identification of *Tenacibaculum maritimum*, the Causative Agent of Tenacibaculosis in Farmed Sea Bass (*Dicentrarchus labrax*) on the Aegean Sea Coast of Turkey

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Key words: *Tenacibaculum maritimum*, sea bass, tenacibaculosis.

Abstract

In this paper we describe the isolation and identification of *Tenacibaculum maritimum* from infected sea bass by using bacteriological, histopathological, and molecular methods. Fish were reared in five different floating net cage farms and two hatcheries located in several regions on the Aegean Sea coast of Turkey. We observed external erythemic and erosive jaw and operculum superficial or deep ulcerative skin lesions on the head and body surfaces, gill rot, hemorrhagic and erythemic fins in infected individuals, and slight exophthalmia was also detected. In addition, internal hemorrhages and hyperemia in the visceral organs and bloody fluid in the peritoneal cavity were observed.

Bacteria isolated from the kidney and skin lesions of the infected fish produced flat, pale colored colonies on *Flexibacter maritimus* medium (FMM), marine enriched Cytophaga agar (MECA) and Marine Agar (MA) and were identified as *Tenacibaculum maritimum*, *Vibrio* sp. and motile or non-motile Aeromonas species.

Histopathologically, degeneration and liquefactive necrosis in liver, kidney, and spleen was observed in the individuals infected with *Tenacibaculum maritimum*. The identification of *T. maritimum* was performed directly from the infected fish tissue using nested polymerase chain reaction. In this study, *T. maritimum* induced mostly mixed infection with *Vibrio* sp. and motile or non-motile aeromonad species in the four fish farms resulting in 25% mortality. However, this bacterium produced an unmixed infection with the same mortality in young sea bass from only one farm.

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Introduction

Tenacibaculum maritimum, formerly, *Cytophaga marina*, *Flexibacter marinus,* and *F. maritimus*, (Wakabayashi et al., 1986; Bernardet and Grimont, 1989; Suzuki et al., 2001), the causative agent of tenacibaculosis in marine fish, was first observed in farmed red seabream, *Pagrus major,* and black seabream *Acantopagrus schlagelli*, in Japan in 1977 (Masumura and Wakabayashi, 1977; Hikida et al., 1979). Following this observation, it has also been found to cause major losses in Japanese flounder *Paralichthys olivaceus*, yellowtail *Seriola quingueradiata,* and other marine fish in Japan (Wakabayshi et al., 1986; Baxa et al., 1986); in Dover sole *Solea solea,* in Scotland Bernardet et al., 1990); in turbot in Spain and Italy (Avendano Herrera et al., 2004a; 2005; Magi et al., 2007); in Coho salmon *Oncorhynchus kisutch,* and marine reared rainbow trout *Oncorhyncus mykiss,* in Spain (Toranzo et al., 1990; Pazos et al., 1993); in Atlantic salmon *Salmo salar,* in Spain, Canada and Australia (Ostland et al., 1999; Soltani and Burke, 1994); in white sea bass *Atractoscion nobilis*, Pacific sardine *Sardinops sagax*, northern anchovy *Engraulis mordax*, and Chinook salmon *Onchorynchus tschawytscha,* in the USA; in striped trumpeter *Latris lineate*, greenback flounder *Rhomosolea tapirina*, yellow eye mullet *Aldrichetta forsteri,* and black seabream in Australia; in Senegal sole *Solea senegalensis,* in Portugal and Spain, and gilthead seabream *Sparus aurata,* (Avendano Herrera et al., 2004a, 2005); in blackspot seabream *Pagellus bogaraveo*; in wedge sole *Dicologoglossa cuneata*, and brill *Scophthalmus rhombus,* in Spain (Castro et al., 2007; Lopez et al., 2009; 2010); and in tub gurnard *Chelidonichthys lucerne,* in Italy (Magi et al., 2007).

Tenacibaculum maritimum was first identified in diseased farmed sea bass *Dicentrarchus labrax*, along the Mediterranean coast in France (Pepin and Emery, 1993; Bernardet et al., 1994), Malta (Tabone, 1996) and Greece (Bernardet et al., 1994). Later, it was isolated from farmed gilthead seabream and sea bass on the Aegean coast of Turkey (Türk, 2006) and in sea water farmed rainbow trout on the Black Sea coast in Turkey as a component of mixed infections produced by the other pathogenic bacteria (Timur et al., 2007).

This paper describes the successful identification of *T. maritimum* strains in infected farmed sea bass using bacteriological and histopathological methods and molecular techniques.

Materials and Methods

Fish and Sampling. A total of one hundred twenty five moribund fish were obtained from five floating net cage farms and two hatcheries located on the Aegean coast of Turkey. The individuals were grouped as fry (0.5-2g), young-juveniles (5-15g), and adults (100-250g). Bacteriological, histopathological and molecular examinations were carried out during the spring and summer seasons of two years, 2008 and 2010, when the seawater temperature was higher than 15ºC. Information regarding the clinical history of the sampled fish and the outbreak of the disease was provided from farm owners.

Bacteriology. Bacterial samples taken from the kidney, liver, spleen and external skin lesions of infected fish were streaked onto *Flexibacter maritimus* medium (FMM), Marine Enrichment Cytophaga agar (MECA), Marine agar (MA), and Trypticate Soy agar (TSA) containing 1.5% NaCl. The plates were incubated at 22-24°C for 48h. To identify the morphological and physiological characteristics, a representative group of bacterial colonies were chosen (Austin and Austin, 2007). Identification of isolated *T. maritimum* was done using morphological, physiological and biochemical methods and API ZYM (Biomerieux) (Bernardet et al., 1990; Pazos et al., 1993, 1996; Cepeda and Santos, 2002). In addition, Bergey's Manual was followed for the identification of other bacterial pathogens (Holt et al., 1994).

DNA extraction from bacterial culture and tissue. Chromosomal DNA samples were extracted using Roche DNA Amplification Kit (Indianapolis, USA) for pure bacterial cultures. *Tenacibaculum maritimum* colonies were collected from the appropriate agar dishes and suspended in 1 ml of PBS. After centrifugation, the supernatants were

removed. Chromosomal DNA samples were extracted from tissue using AxyPrep Multisource Genomic DNA Miniprep Kit (Union city, USA). The concentration and quality of each DNA sample was examined spectrophotometrically at 260 nm and adjusted to a concentration of 100ng (Sarıkaya, 2008).

Polymerase Chain Reaction (PCR) and Nested-PCR amplification. The PCR amplifications were performed with the commercial kit Ready-To-Go TM PCR beads (Amersham Pharmacia Biotech), with the exception of the specific primers and DNA template. Specific primer pairs, synthesized by Boga Medical, were used for the identification of *Tenacibaculum maritimum*: primer MAR1 (5'–AATGGCATCGTTTTAAA–3') and MAR2 (5'–CGCTCTCTGTTGCCAGA–3'). PCR protocol of Toyama et al. (1996) was followed. Reference bacteria (NCIBM 2154^T, NCIBM 2153) were used for positive control, and a water sample was used for negative control.

The nested-PCR amplification was performed for the detection of *T. maritimum* in infected fish tissues as described by Avendano Herrera et al. (2004). Universal primer pairs synthesized by Boga Medical (pA 5'-AGAGTTTGATCCTGGCTCAG-3' and pH 5'-AAGGAGGTGATCCAGCCGCA-3') and first amplification was done to increase the amount of bacterial DNA in fish tissues. After the first amplification of the external primers, 1 ml of each PCR product was used as a template for the second amplification of the specific primer sets MAR1 and MAR2.

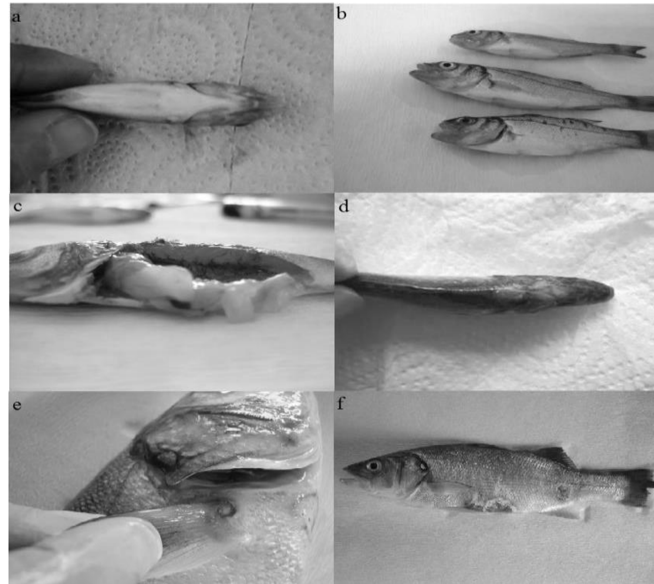
Electrophoretic detection of PCR products. Amplified products were detected by horizontal 1% (w/v) agarose gel electrophoresis for 60 min at 100 V in TAE 1X buffer (0.04 M Tris, 0.0001 M EDTA, pH 8.0). They were visualized using 0.06 μg/ml of ethidium bromide (Bio-Rad) and then photographed under UV light (Avendano Herrera et al., 2004b). 50 to 2000bp and 100 to 3000bp ladders (Sigma Chemical) were used as molecular mass markers. The presence of a single product of the appropriate size, identical to the reference strains, was considered a positive result.

Histopathology. Tissue samples from kidney, liver, spleen, intestine, heart, gill, and skeletal muscle were fixed in 10% buffered formalin, and processed for paraffin embedding. Histological sections (5μm) were stained with hematoxylin-eosin (H&E) and Masson's trichrome stains and examined microscopically (Culling et al., 1963).

Results

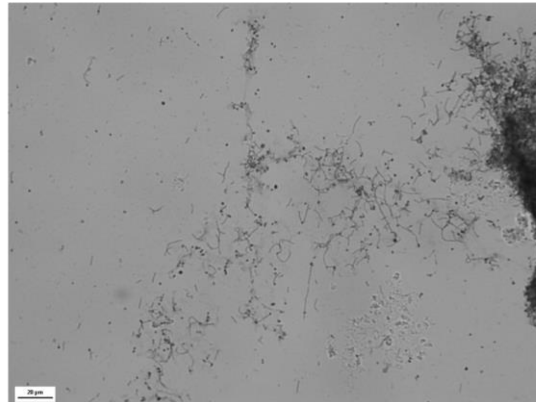
Fry samples (0.5-2g), supplied from two different hatcheries, exhibited external symptoms of erosive and erythemic jaw, slight exophthalmia, and hemorrhagic and ulcerative skin lesions on the head; internally there were lyses in visceral organs such as spleen, kidney, and liver. Hemorrhagic and ulcerative skin lesions were observed externally on the base of young sea bass fins (5-15g). Hemorrhagic, erythemic and erosive jaw, mouth and operculum, and also slight exophthalmia were detected (Fig. 1a, b). In addition, some young sea bass showed hyperemia and hemorrhaging in the visceral organs and they were exposed to lysis in liver, spleen and kidney (Fig. 1c). Some young individuals exhibited large ulcerative skin lesions on their head (Fig. 1d). Adult sea bass samples (100-250g) provided from cage farms, externally exhibited erythema and hemorrhages on their head, operculum, neck, and at the base of pelvic, pectoral, and anal fins; slight exophthalmia, erosive jaws, and multifocal hemorrhagic and ulcerative skin lesions on the body surface and caudal fin rot (Fig. 1e, f). In addition, pale liver and hemorrhagic ascites in the abdominal cavity were observed. Only one individual showed indications of hyperemia in liver and visceral fats.

Fig.1 (a-f)



Following the 24 hour incubation at 22°C, *Tenacibaculum maritimum* was generally isolated from mixed infection with other pathogenic Gram negative bacteria, swabbed from the skin and visceral organs of infected fish. Flat, pale yellow colored and irregular edged and sized *T. maritimum* colonies, sampled from skin lesions and kidney of diseased fish, were observed on FMM, MECA and MA but not on TSA. *T. maritimum* colonies were found adherent to these agar media. Although they absorbed Congo red, a flexirubine pigment test produced negative result. Cells of *T. maritimum*, grown only on FMM, MECA and MA, showed pleomorphism (Fig. 2).

Fig.2. Gram negative *T. maritimum* cells in a pleomorphic shape (long filamentous, slender and spheric rods) (Gram x 1000)



The size of the bacteria ranged between 0.5x4-29μm and the bacteria displayed gliding motility. Beside *T. maritimum* colonies, most of the streak cultures of the infected fish spleen, liver, kidney and skin lesions formed cream, yellow-cream, or grey colored colonies on FMM, MECA, MA, and TSA. These pathogenic gram negative bacteria were identified as *Vibrio alginolyticus*, *V. splendidus*, *V. harveyi*, *Aeromonas achromogenes* subsp. *salmonicida*, *A. hydrophila* and *A. sobria*. Morphological and phenotypical characteristics of *T. maritimum* and other isolated pathogens are shown in Table 1.

Table 1. Morphological and phenotypic characteristics of the isolated bacteria.

	T. maritimum	*V.alginolyticus*	*V.splendidus*	*V.harveyi*	*A.hydrophila*	*A.sobria*	*A.achromogenes* subsp. *salmonicida*
Morphology	F	B	B	B	B	B	B
Motility	G	+	+	+	+	+	-
Gram staining	-	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Flexirubine pigment	-	-	-	-	-	-	-
O/129 (150ìg) Resistance	S	S	S	S	R	R	R
Growth on TCBS	-	+	+	+	+	+	+
O/F	O	F	F	F	F	F	F
Indole	-	+	+	+	+	+	+
Methyl red test	-	+	+	-	-	+	-
Voges Proskauer Reaction	-	+	-	-	+	-	+
Nitrate reduction	+	+	+	+	+	+	+
H_2S	-	-	-	-	-		-
Arginine dihydrolase	-	-	+	-	+	+	-
Lysine decarboxylase	-	+	-	+	+	+	-
Ornithine decarboxylase	-	+	-	+	-	-	-
Citrate	-	+	+	+	+	+	-
Degradation of:							
Aesculin	-	-	+	-	+	-	-
Gelatin	+	+	+	+	+	+	+
Urea	-	-	-	-	-	-	-
Acid production of							
Glucose	-	+	+	+	+	+	+
Maltose	-	+	+	+	+	+	
Mannitol	-	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-
Sucrose	-	+	-	+	+	+	+
Lactose	-	-	-	-	-	+	-
Growth at							
4ºC	-	-	+	-	+	-	-
37 ºC	-	+	+	+	+	+	+
44 ºC	-	-	-	-	-	-	-
Growth on TSA containing NaCl							
0 (w/v) NaCl	-	-	-	-	+	+	+
1.5 (w/v) NaCl	-	+	+	+	+	+	+
3 (w/v) NaCl	-	+	+	+	-	+	+
5 (w/v) NaCl	-	+	+	+	-	+	+
8 (w/v) NaCl	-	+	-	-	-	-	-
10 (w/v) NaCl	-	+	-	-	-	-	-

+: positive, -: negative, O: oxidative, F: fermentative, S: sensitive, R: resistance, G: gliding, B: basil, F: filamentous

The PCR analysis confirmed the presence of *Tenacibaculum maritimum.* MAR1 and MAR2 primers were used for the amplification of 16S rDNA isolated from *T. maritimum* and we found 1088 bp length as a unique and clear PCR product (Fig. 3 a, b).

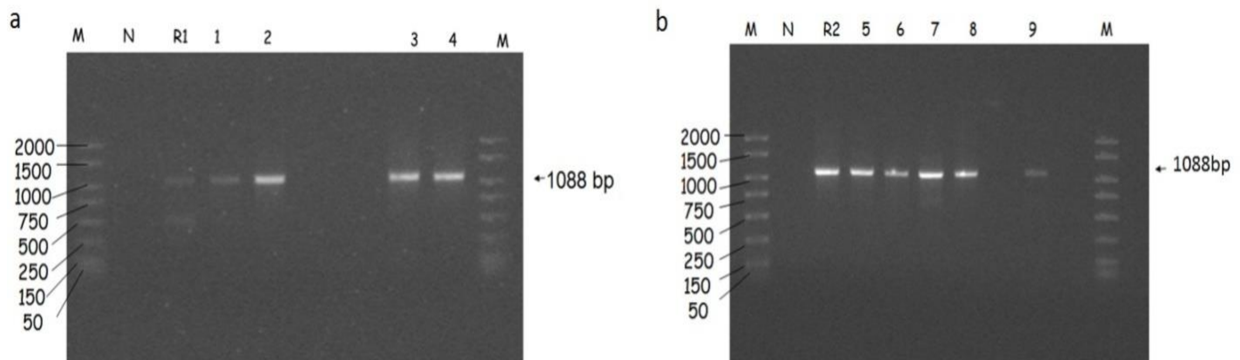


Fig.3. PCR results (M: 50-2000bp molecular weight marker, N: negative control. R1 and R2 positive control (NCIBM 2154^T and NCIBM 2153), 1-9 *T. maritimum* isolated from infected sea bass.

Nested PCR amplification protocol resulted in the positive amplification of *T. maritimum* for liver, kidney and muscle tissue samples, except for spleen tissue. All these tissues formed a unique and clear band with 1088 bp length (Fig. 4).

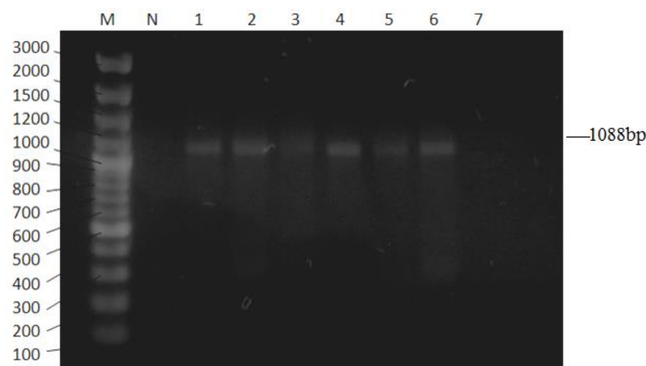
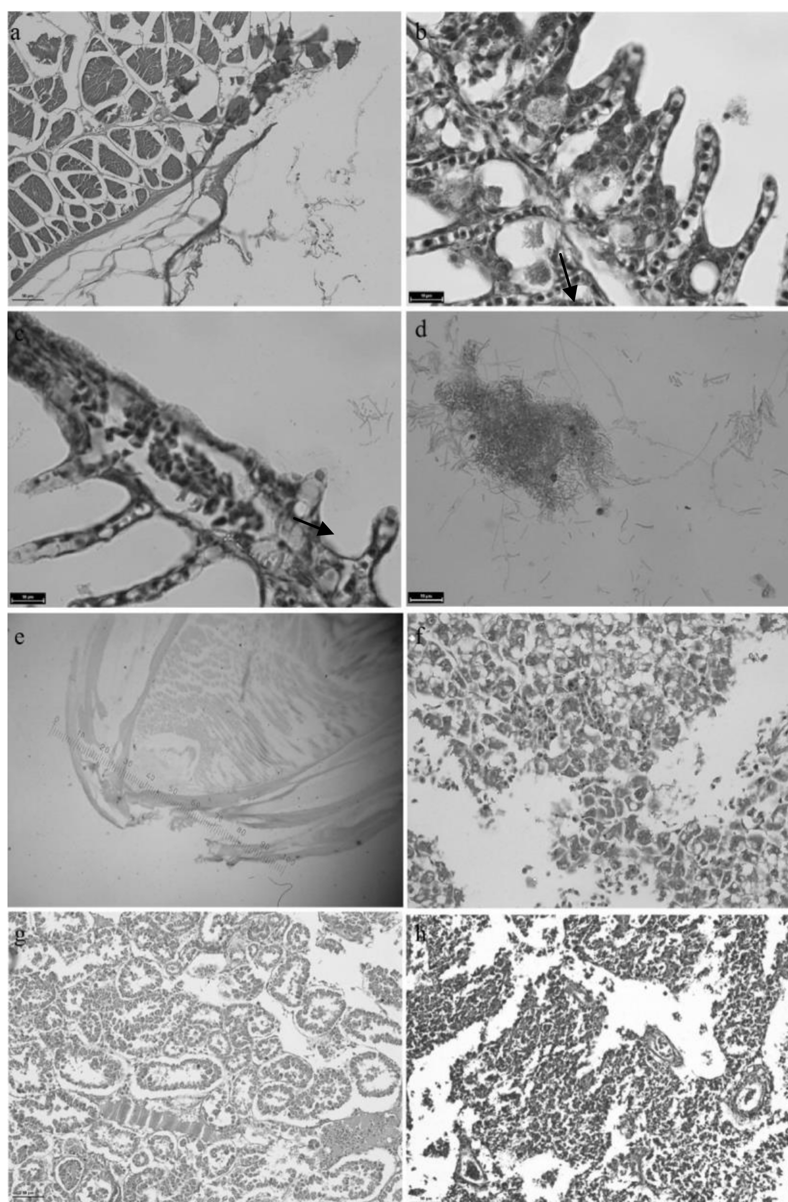


Fig.4. Detection of *T. maritimum* from infected fish tissue by nested PCR (M: 100-3000bp molecular weight marker, N: negative control. 1: muscle tissue sample, 2: kidney tissue sample, 3: liver tissue sample, 4: kidney tissue sample, 5: kidney tissue sample, 6. liver tissue sample,
7. spleen tissue sample.

Histopathologically, sea bass individuals infected with *Tenacibaculum maritimum* generally displayed superficial skin lesions with the complete loss of epithelial layers exposed to the collagen fibers of the dermis or deep ulcerative skin lesions (Fig. 5a), hemorrhagic, and erosive gill filaments with free or adherent filamentous bacteria (Fig. 5b, c), accumulation of the filamentous bacteria within the abdominal fluid (Fig. 5d), erosive jaws (Fig. 5e), multifocal liquefactive necrosis and hemorrhage in the affected liver (Fig. 5f), multifocal liquefactive necrosis, periglomerular edema, and tubular necrosis, hemorrhage, and depletion of the interrenal hemopoietic tissue in kidney (Fig. 5g) and multifocal liquefactive necrosis in spleen (Fig. 5h). However, mixed infected sea bass displayed similar histopathological findings and additionally multifocal melanomacrophage centers; deposition of hemosiderin pigments were observed in the affected spleen and kidney tissues.

Fig.5. (a-h)



Discussion

The presence of *Tenacibaculum maritimum* has become more prevalent in many other marine fish species in countries such as Japan, USA, Canada, Australia, UK, France, Spain, Portugal, Malta, Italy, Greece, and Turkey since the first diagnosis of *T. maritimum* infection in cultured red and black seabream with a high mortality rate in Japan in 1977 (McVicar and White, 1979; 1982; Wakabayshi et al., 1986; Baxa et al., 1986; Devesa et al., 1989; Toranzo et al., 1990; Pepin and Emery, 1993; Pazos et al., 1993; Bernardet et al., 1994; Tabone, 1996; Avendano Herrera et al., 2004a; Turk, 2006; Şen, 2007; Timur et al., 2007).

In this study, *T. maritimum* was isolated as a component of a mixed infection in association with *Vibrio* spp. or motile and non-motile aeromonads (Hadlinger et al., 1997; Power et al., 2004; Timur et al., 2007; Lopez et al., 2009), except in juvenile fish from a particular farm with the same (25%) mortality rate. The incidence of tenacibaculosis outbreak increased during winter or spring (Bernardet et al. 1994), however, in our study, the outbreaks were observed in spring and summer seasons at sea bass farms when the water temperature rose to 15ºC (McVicar and White, 1979; 1982; Avendano-Herrera et al., 2006a).

Fry, juvenile, or adult sea bass samples infected with *T. maritimum* generally had erythemic and erosive jaw and mouth, erythemic head and neck, hemorrhages at the base of the fins, ulcerative skin lesions, and tail rods as previously reported for various marine fish (Devesa et al., 1989; Bernardet et al., 1994; Ostland et al., 1999; Cepeda and Santos, 2002). In general, the loss of epithelial surfaces in fish provides a portal for the entry of other pathogens. In addition, the slow growth of the pathogen and inhibition of it by other bacterial species makes isolation of bacteria from infected fish difficult.

Tenacibaculum maritimum was isolated only from affected fish skin lesions and kidney streaked on FMM, MECA and MA. They produced flat pale yellow colored colonies having irregular edges, and did not produce colonies on TSA containing 1.5 % NaCl as previously described (Alsina and Blanch, 1993; Pazos et al., 1996; Avendano-Herrera et al., 2004a; Magi et al., 2007). *T. maritimum* was also found rather adherent to FMM (Avendano-Herrera et al., 2006b). These filamentous gram-negative gliding bacteria reacted positively to catalyse and cytochrome oxidase, Congo red absorption, nitrate reduction, gelatine hydrolysis, but reacted negatively to flexirubine pigment, indol, Voges Proskauer, Methyl red test, acid production from carbohydrates, and production of H_2S (Hikida et al., 1979; Baxa et al., 1986; Wakabayashi et al., 1986; Bernardet and Grimont, 1989; Bernardet et al., 1990; 1994; Alsina and Blanch, 1993; Pazos et al., 1993; Avendaño-Herrera et al., 2004a).

In our study, results of the PCR assays helped distinguish *T. maritimum* from the related species *Flavobacterium branchiophilum* and *F. columnare*, as well as from several other pathogenic bacteria in infected individuals (Toyama et al., 1996; Suzuki et al., 2001; Avendano-Herrera et al., 2004b). The use of nested PCR assays enabled direct identification of the pathogen in infected tissues (muscle, kidney, and liver).

Histopathologically, in skin lesions long slender rods or filamentous bacteria adherent to the eroded skin surface were detected and they extended deep into the connective tissues or muscle tissues (Hadlinger et al., 1997; Bernardet, 1998). We also detected eroded hemorrhagic gill filaments having filamentous bacteria adherent to gill lamellae as described by Powell et al. (2004) in Atlantic salmon smolts experimentally infected with *T. maritimum.* In addition to these findings, colonization of filamentous bacteria has been observed within the abdominal fluid in this study. These histopathological findings and nested PCR results confirmed that *T. maritimum* causes systemic diseases in sea bass (Alsina and Blanch, 1993; Cepada and Santos, 2002; Avendano-Herrera et al., 2004b; 2006b).

Our study describes the successful identification of *Tenacibacullum maritimum* strains in infected farmed sea bass using bacteriological, histopathological, and molecular methods. *T. maritimum* was generally involved in mixed infections together with *Vibrio* sp. and motile or non-motile *Aeromonas* species in studied fish, but the bacteria was also found to induce an unmixed infection in young sea bass with the same mortality rate (25%) from one farm.

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