Antigenic Characterisation of *Tenacibaculum maritimum* Isolates from Sea Bass (*Dicentrarchus labrax, L.*) Farmed on the Aegean Sea Coasts of Turkey

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**Abstract**

Tenacibaculosis, caused by *Tenacibaculum maritimum*, can result in severe mortalities of several marine fish species and thus represents a major challenge in Mediterranean aquaculture. Serological knowledge about this pathogen is required to develop effective preventative measures (vaccination). For this purpose, nineteen *T. maritimum* isolates, recovered between 2008 and 2010 from diseased European sea bass (*Dicentrarchus labrax, L.*) farmed at the Aegean Sea Coasts of Turkey, were characterised. All isolates produced flat, irregular, pale yellow colonies after incubation at 22–24°C for 48 hours, displayed pleomorphism with gliding motility with a size ranging between 4–20 × 0.5 µm and were otherwise biochemically identical to the *T. maritimum* NCIMB 2154 reference strain. The specific fluorescence appearance of the *T. maritimum* isolates were revealed by Indirect Fluorescent Antibody Technique (IFAT) which was also used to detect the bacterium in tissue samples. The presence of antibodies in the blood sera of the diseased fish against this pathogen was detected by agglutination and Enzyme-Linked Immuno Sorbent Assay (ELISA). Dot-Blot testing identified all *T. maritimum* isolates as serotype O1. To our knowledge, this is the first report on O1 serotype *T. maritimum* isolates from sea bass farmed in Turkey.

**Keywords:** *Tenacibaculum maritimum*; Cultured sea bass; *Dicentrarchus labrax*; IFAT; ELISA; Serotyping; Dot-blot testing

**Introduction**

*Tenacibaculum maritimum* is the causative agent of tenacibaculosis in marine fish [1-3]. Since the first recognition of *T. maritimum* infection in farmed red and black sea bream (*Papus major* and *Acanotopagrus schlagelli*) with high mortality in Japan, the presence of *T. maritimum* has become increasingly apparent in other marine fish species in Japan, USA, Canada, Australia, UK, France, Spain, Malta, Italy, Greece and Turkey [1,4-18].

This bacterium is difficult to distinguish from other phylogenetically and phenotypically similar species. In previous studies, serological methods such as slide agglutination, IFAT and ELISA were used for the identification of *T. maritimum* [5,7,13,19-21]. Although the bacterium is biochemically homogeneous, different O-serogroups, which seem to be related to the host species, could be detected by Avendano-Herrera et al., [21]. At least three groups of *T. maritimum* isolates from marine fish were distinguished [20]. These groups are associated with the host origin: group 1 comprises the strains isolated from sole (*Solea senegalensis* and *S. solea*), group 2 consists of the isolates from sea bream and sea bass, group 3 corresponds to the turbot isolates. These three groups of isolates could also be distinguished by randomly amplified polymorphic DNA-PCR [21]. By this methodology, the first group comprised all strains isolated from sole and gilthead sea bream, the second comprised the isolates from yellowtail (*Seriola quinqueradiata*), Atlantic salmon (*Salmo salar*) and turbot (*Scophthalmus maximus*) and the third group is formed by one isolate from *Papus major* and one from *Solea solea* [21].

It is important to determine the predominant *Tenacibaculum* serotype and different serotypes distribution to be able to develop effective preventive measurements like vaccines. In a serological characterization study of *T. maritimum* isolates, from farmed tub gurnard (*Chelidonichthys lucernus*) and wild turbot, carried out in Italy, it was determined that the isolates belonged to serotype O3 [16]. Castro et al., [22] reported that, by an old typing scheme, turbot isolated strains of the bacterium belonged to serotype O2 in Spain. However, this needed revaluation according to the authors as they detected also serotype O3 in turbot and sole in the same study.

In Turkey, *T. maritimum* was isolated from farmed gilthead sea bream and sea bass at the Aegean sea coast [18-21,23-25] and from farmed rainbow trout in sea water of the Black Sea coast in a mixed infection case with other pathogen bacteria [26]. In our previous study, we described the isolation and identification of *T. maritimum* from infected sea bass by bacteriological, histopathological, and molecular methods [27]. *T. maritimum* isolates have also been detected in seven different fish species including sea bream, sea bass, meagre (*Argyrosomus regius*), turbot, cob (*Umbrina cirrosa*), sharpnose sea bream (*Diplodus puntazzo*) and snappers (*Sparus pagrus*) in Turkey [28]. Until now, serological studies have never been carried out for this bacterial pathogen in Turkey. The aim of this study was to characterise isolates of *T. maritimum* from cultured sea bass (*Dicentrarchus labrax* L.) in Turkey.

**Material and Methods**

**Bacterial strains**

*T. maritimum* isolates were examined in this study. They were

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isolated from diseased European sea bass reared in five floating net cage farms at the Aegean Sea coasts of Turkey between 2008 and 2010. Conventional bacteriological tests and API ZYM test kits were used for biochemical identification of these strains. The *T. maritimum* NCIMB 2154<sup>T</sup> reference strain was included as a positive control.

**“O” antigen preparation and immunization of rabbits**

“O” antigen preparation was performed as described by Toranzo et al., [29]. The density in each bacterial suspension was adjusted to 3 McFarland and boiled for 1 hour at 100°C for preparation of “O” antigens stored at 4°C until their use in Dot-Blot testing. Rabbits were immunized intravenously with 10<sup>7</sup> cell/ml formalin killed antigens stored at 4°C until their use in Dot-Blot testing. Rabbits were 3 McFarland and boiled for 1 hour at 100°C for preparation of “O” antigen preparation. PBS buffer was used as negative control. 30 µl test antigens were added in well of slides. After fixation, 10 µl of Rabbit anti-*Flexibacter maritimus* antiserum (Microtek RFM01) diluted 400 × in PBS were added and incubated for 30 min at 37°C. Thereafter, slides were treated with 1:80 dilution of FITC labelled with goat anti-rabbit IgG for 30 min at 37°C, washed with PBS for three times and stained with 0.1% Evans blue for 30 min at 37°C. Finally, 100 µl of 25% glycerol solution (including 2.5 g DABCO) was added before slides were analysed under the fluorescent microscope. The IFAT procedure described by Lorenzen et al., [32] was also used for the detection of *T. maritimum* strains directly in fish tissues.

**Slide agglutination test and enzyme linked immunosorbent assay**

Blood samples were collected from the caudal artery of moribund fish. The antisera which had been stored at -20°C were used in slide agglutination test. This test was performed with a small amount of bacterial colonies mixed with several drops of serum obtained from fish samples. PBS buffer was again used as negative control [29] while immunized rabbit serum served as positive control. ELISA was performed as described by Knappskog et al., [33]. Monoclonal anti-European sea bass IgM marked HRP (Aquatic diagnostics CO1) was used and PBS was included as a negative control.

**Dot-blot analysis**

The dot-blot analysis was performed as described by Cipriano et al., [34]. Rabbit sera were obtained from the University of Santiago de Compostela (Microbiology and Parasitology Department). Antisera against serotypes O1, O2 and O3 were prepared from representative strains PC 503.1, PC 424.1 and ACC 13.1, respectively as previously described by Avendano et al., [21].

**Results**

All bacterial isolates produced flat, irregular, pale yellow colonies after incubation at 22-24°C for 48 hours on MA and FMM. The bacteria showed pleomorphism with gliding motility within a size range 4-20 × 0.5 µm and reacted positive in the cytochrome oxidase and catalase tests, but did not produce flexirubine pigments. Morphological and phenotypical characteristics of the *T. maritimum* isolates are shown in Table 1. These isolates exhibited identical enzymatic profiles in API ZYM tests to the reference strains in the test kit database.

Sero logically, IFAT was used for the identification of *T. maritimum* strains and to show the specific fluorescence appearance of *T. maritimum* cells through microscopy. *T. maritimum* cells were detected in spleen, kidney and liver tissues of moribund fish samples using IFAT (Figure 1).

The slide agglutination test demonstrated positive reaction against fish antiserum (Figures 2a and 2b). Although these fish antisera were cross absorbed with other *Tenacibaculum* sp., specific monoclonal anti-European sea bass IgM marked HRP (Aquatic diagnostics CO1) was used in ELISA. The presence of antibodies in the blood sera of the diseased fish, against this pathogen, was also detected by ELISA and slide agglutination.

All isolates showed strong reaction only with the antiserum raised against the serotypes O1 (strains PC 503.1) in the dot-blot assays. It was therefore concluded that all *T. maritimum* isolates recovered from moribund sea bass samples were serotype O1 (Figures 3a and 3b).

**Discussion**

In this study, nineteen *T. maritimum* strains were isolated from
European sea bass were also serotyped using the Dot-blot method. All of our *T. maritimum* isolates from different farm locations reacted only with antisera against the sole isolate PC 503.1 (serotype O1). Avendano et al., [20] originally noted that *T. maritimum* isolated from sea bream and sea bass from Spain reacted only with antiserum obtained against PC 424.1 (serotype O2). However, since then they have concluded that serotype O1 is the most predominant in Spanish reared sea bream and seabass, while serotype O2 is most common in turbot. In sole O1 and O2 serotypes are predominant but O2 isolates are increasing in numbers. In NW Spain the first isolates of this bacterium belonged to serotype O2, but recent isolates have also been found to be O3 [35].

In this study, IFAT was used for the detection of *T. maritimum* strains in fish tissues and identify bacterial cells. Specific fluorescence appearance of the bacteria cells of *T. maritimum* isolates were revealed as it was made previously by Powell et al., and Van-Gelderen et al., [9,23]. Baxa et al., [5] detected this pathogen in all tissues of black sea bream fry by using FAT technique, however this pathogen was only isolated from skin surface with culture methods. IFAT was used for the confirmation of recovery of this pathogen from gills following experimental inoculation by Powell et al., [9]. In this study, IFAT was also congruently used for the detection of *T. maritimum* in all tissue imprints of fish samples that *T. maritimum* was not isolated with culture methods. In this study, besides slide agglutination technique, the presence of antibodies in the blood sera of the moribund fish against this pathogen was also detected by using ELISA so recording false positive reactions was avoided. Both of these techniques have a short analysis time, and much less amounts of serum is used. Taken together, serological techniques proved to be more sensitive, rapid and efficient than the conventional bacteriological methods in the detection of *T. maritimum* in the moribund fish tissues and serum.

In conclusion, this first serotyping of Turkish *T. maritimum* isolates from sea bass revealed that they all belonged to serotype O1 and it suggest this serotype to be the predominant one in Turkey. However, further studies are needed to confirm this finding in order to produce effective vaccines against this pathogen in Turkey.

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