Journal of Aquaculture Research & Development

Yardimci and Timur, J Aquac Res Development 2016, 7:2
DOI: 10.4172/2155-9546.1000408

Research Article Open Access

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

Yardimci RE* and Gülşen Timur

Istanbul University, Department of Aquaculture, Ordu Street, No: 200, Laleli, Turkey

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 preventive 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^T 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 using 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 (*Pagrus major* and *Acantopagrus 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 *Pagrus 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, corb (*Umbrina cirrosa*), sharpsnout 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

***Corresponding author:** Yardimci RE, Fisheries Faculty of Istanbul University, Department of Aquaculture, Ordu Street, No: 200, Laleli, Turkey, Tel: +902124555700, +902125140379; E-mail: etepecik@istanbul.edu.tr

Received November 25, 2015; **Accepted** December 30, 2015; **Published** February 15, 2016

Citation: Yardimci RE, Timur G (2016) Antigenic Characterisation of *Tenacibaculum maritimum* Isolates from Sea Bass (*Dicentrarchus labrax*, L.) Farmed on the Aegean Sea Coasts of Turkey. J Aquac Res Development 7: 408. doi:10.4172/2155-9546.1000408

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 NCIBM 2154*^{T} 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 10^7 cell/ml formalin killed *T. maritimum* reference strain (NCIBM 2154^T) as well as representative strains (PC 503.1, PC424, ACC13.1). The polyclonal rabbit antiserums were later obtained according to Sorensen and Larsen [30] and stored at -20°C until used in the ELISA test as a positive control.

Indirect fluorescent antibody technique

A method described by Ainsworth et al., [31], for the diagnosis of *Edwardsiella ictulari,* was used to identify the *T. maritimum* isolates with minor modifications. PBS buffer was used as negative control. 20 µl test antigens were added in well of slides. After fixation, 10 µl of Rabbit anti-*Flexibacter maritimus* antisera (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 with 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.

Serologically, 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 antiserums 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

	***T. maritimum* (NCIMB 2154^T)**	***T. maritimum* strains**
Morphology	F	F
Motility	G	G
Gram staining	-	-
Oxidase	+	+
Catalase	+	+
Flexirubine pigment	-	-
Congo Red reduction	+	+
O/129 (150 µg) Resistance	S	S
Growth on TCBS	-	-
O/F	O	O
Indole	-	-
Methyl red	-	-
Voges Proskauer	-	-
Nitrate reduction	+	+
H_2S	-	-
Arginine dehydrolase	-	-
Lysine decarboxylase	-	-
Ornithine decarboxylase	-	-
Citrate	-	-
Aesculin degradation	-	-
Gelatinase	+	+
Urease	-	-
Acid production from		
Glucose	-	-
Maltose	-	-
Mannitol	-	-
Inositol	-	-
Sucrose	-	-
Lactose	-	-
Growth at		
4°C	-	-
37°C	-	-
44°C	-	-

Table 1: A summary of morphological and phenotypic characteristics of *T. maritimum* isolates examined in the study compared to *T. maritimum* reference strain (NCIMB 2154^T).

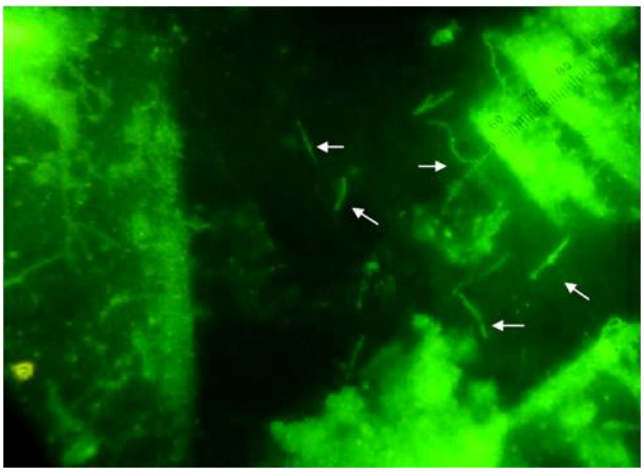

Figure 1: Filamentous T. maritimum cells (marked by arrows) located in spleen of moribund fish samples (IFAT, magnification X400).

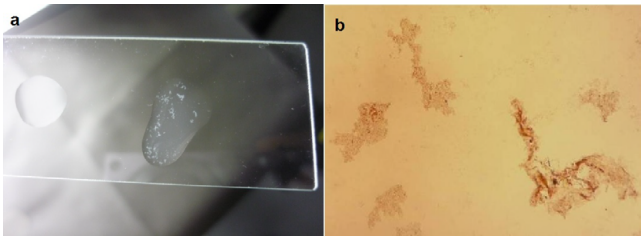

Figure 2: On slide agglutination with (a) white deposits composed of antigen-antibody complexes and (b) antigen-antibody complexes as observed under light microscope.

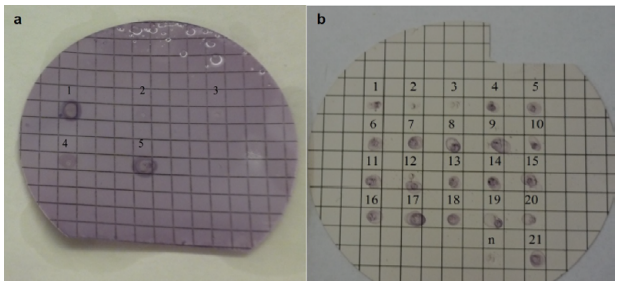

Figure 3: Dot blot assay using the antiserum obtained against Serotype O1 (a) 1: PC503.1, 2: PC424.1, 3: ACC13.1., 4: negative control, 5: T. maritimum isolate form.

diseased sea bass reared in five floating net cage farms at the Aegean Sea coasts of Turkey during 2008 and 2010. These filamentous, gram-negative, gliding bacteria are catalyse and cytochrome oxidase, Congo red absorption, nitrate reduction, gelatine hydrolysis positive, but were not producing flexirubine pigment. Biochemical homogeneity was determined among 19 *T. maritimum* isolates and when compared to other studies, a similarity was detected [1,2,4,11,12,21]. These isolates showed identical enzymatic profiles in API ZYM test kit with the previous records [6,11,12,21].

The nineteen *T. maritimum* isolates recovered from moribund European sea bass were also serotyped using the Dot-blot method. All of our *T. maritimum* isolates from different farm locations reacted only with antiserums 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.

Acknowledgements

This study was supported by Istanbul University Scientific Research Projects (project number: 2618). All protocols were approved by the Ethics Committee for Animal Experiments of the University of Istanbul. We are grateful to Prof. Alicia E. Toranzo (Universidade de Santiago de Compostela, Spain) for generously providing antisera against *T. maritimum* serotypes' O1, O2 and O3 as well as for her helpful comments.

References

1. Wakabayashi H, Hikida H, Masumura K (1986) Flexibacter maritimus sp. nov., a pathogen of marine fishes. Int J Syst Bacteriol 36: 396-398.
2. Bernardet JF, Grlmont PAD (1989) Deoxyribonucleic acid relatedness and phenotypic characteristics of Flexibacter columnaris sp. nov., nom. rev., Flexibacter psychrophilus sp. nov., nom rev., and Flexibacter maritimus Wakabayashi, Hikida & Masamura, 1986. Int J Syst Bacteriol 39: 346-354.
3. Suzukiu M, Nakagawa Y, Harayama S, Yamamoto S (2001) Phylogenetic analysis and taxonomic study of marine Cytophaga-like bacteria: Proposal for Tenacibaculum gen. nov. with Tenacibaculum maritimum comb. nov. and Tenacibaculum ovolyticum comb. nov., and description of Tenacibaculum mesophilum sp. nov. and Tenacibaculum amylolyticum sp. nov. Int J Syst Evol Microbiol 51: 1639-1652.
4. Baxa DV, Kawai K, Kusuda R (1986) Characteristics of gliding bacteria isolated from diseased cultured flounder, Paralichthys oliveceous. Fish Pathology 21: 251-258.
5. Baxa DV, Kawai K, Kusuda R (1987) Experimental infection of Flexibacter

maritimus in black sea bream (Acanthopagrus schlegeli) fry. Fish Pathology 22: 105-109.

6. Chen ME, Henry-Ford D, Groff JM (1995) Isolation and Characterization of Flexibacter maritimus from Marine Fishes of California. Journal of Aquatic Animal Health 22: 7-11.
7. Ostland VE, La Trace C, Morrison D, Ferguson HW (1999) Flexibacter maritimus associated with a bacterial stomatitis in Atlantic salmon smolts reared in net-pens in British Columbia. J Aqua Anim Health 11: 35-44.
8. Mc Vicar AH, White PG (1982) The prevention and cure of an infectious disease in cultivated juvenile Dover sole Solea solea (L.). Aquaculture 26: 213-222.
9. Powell M, Carson J, Van-Gelderen R (2004) Experimental induction of gill disease in Atlantic salmon Salmo salar smolts with Tenacibaculum maritimum. Diseases of Aquatic Organisms 61: 179-185.
10. Schmidtkel L, Carson J, Howard T (1991) Marine Flexibacter infection in Atlantic salmon. Characterization of the putative pathogens. Proceedings of the Saltas Research Review Seminar.
11. Bernardet JF, Kerouault B, Michel C (1994) Comparative study on Flexibacter maritimus strains isolated from farmed seabass (Dicentrarchus labrax) in France. Fish Pathology 29: 105-111.
12. Pazos F, Santos Y, Nunez S, Toranzo AE (1993) Increasing occurence of Flexibacter maritimus in the marine aquaculture of Spain. FHS/AFS Newsletter 21: 1-2.
13. Pazos F (1997) Flexibacter maritimus: estudio fenotípico, inmunológico y molecular. Tesis doctoral, Universidad Santiago de Compostela, Spain.
14. Tabone J (1996) Isolation and characterization of the fish pathogen Flexibacter maritimus from cultured sea bass Dicentrarchus labrax L, B.Sc. Thesis, University of Malta, Malta.
15. Salati F, Cubadda C, Viale I, Kusuda R (2005) Immune response of sea bass Dicentrarchus labrax to Tenacibaculum maritimum antigens. Fisheries Science 71: 563-567.
16. Magi GE, Avendano-Herrera R, Magarinos B, Toranzo AE, Romalde JL (2007) First reports of flexibacteriosis in farmed tub gurnard (Chelidonichthys lucernus L.) and wild turbot (Scophthalmus maximus) in Italy Bull. Eur Ass Fish Pathol 27: 177-184.
17. Kolygas MN, Gourzioti E, Vatsos IN, Athanassopoulou F (2012) Identification of Tenacibaculum maritimum strains from marine farmed fish in Greece. Veterinary Record 170: 623.
18. Türk N (2006) Fish Disease, Flexibacteriosis. Aquaculture and Fisheries 2: 53-54.
19. Arenas J, Mata M, Santos Y (2003) Evaluation of an enzyme-linked immunosorbent assay for serological typing of Tenacibaculum maritimum. European Association of Fish Pathologist 11th International Conference of 'Disease of Fish and Shell Fish'.
20. Avendano-Herrera R, Magarinos B, Romalde JL, Toranzo AE (2003) An update on the antigenic diversity of Tenacibaculum maritimun strains isolated from marine fishes. FHS/AFS Newsletter 31: 24-26.
21. Avendano-Herrera R, Magarinos B, Lopez-Romalde S, Romalde JL, Toranzo AE (2004) Phenotyphic characterization and description of two major O-serotypes in Tenacibaculum maritimum strains from marine fishes. Diseases of Aquatic Organisms 58: 1-8.
22. Avendano-Herrera R, Magarinos B, Morinigo MA, Romalde JL, Toranzo AE (2005) A novel O-serotypes in Tenacibaculum maritimum strains isolated from cultured sole (Solea senegalensis). Bull Eur Ass Fish Pathol 25: 70-74.
23. Van-Gelderen R, Carson J, Nowak B (2009) Effect of extracellular products of Tenacibaculum maritimum in Atlantic salmon, Salmo salar L. Journal of Fish Diseases 32: 727-731.
24. Castro N, Magarinos B, Nunez S, Toranzo AE (2007) Reassessment of the Tenacibaculum maritimum serotypes causing mortalities in cultured marine fish. Bull Eur Ass Fish Pathol 27: 229-233.
25. Şen E (2007) Sea Bass (Dicentrarchus labrax) on a research Flexibacter maritimus infection in fish. M.Sc. T. C. Istanbul University, Institute of Science and Technology.
26. Timur G, Timur M, Akaylı T, Korun J (2007) Survey Study Of Pathologies Affecting Farmed Sea Bass (Dıcentrarchus labrax L. 1758) And Marine Cultured Rainbow Trout (Oncorhynchus Mykiss) in Turkey. 13th international conference of the EAFP "Diseases of fish and shellfish", Grado-Italy.
27. Yardımci RE, Timur G (2015) Detection of Tenacibaculum maritimum, the Causative Agent of Tenacibaculosis in Farmed Sea Bass (Dicentrarchus labrax) on the Aegean Sea Coast of Turkey. The Israeli Journal of Aquaculture - Bamidgeh 67: 1172-1182.
28. Avsever ML, Türk N, Ün C, Didinen BI, Tunalıgil S (2015) Detection of Tenacibaculum maritimum from Seven Different Cultured Marine Fish in Turkey. The Israeli Journal of Aquaculture-Bamidgeh, 6 pages, accepted manuscript.
29. Toranzo AE, Baya AM, Roberson BS, Barja JL, Grimes DJ et al. (1987) Specificity of slide agglutination test for detecting bacterial fish pathogens. Aquaculture 61: 81-97.
30. Sorensen UB, Larsen JL (1986) Serotyping of Vibrio anguillarum. Appl Environ Microbiol 51: 593-597.
31. Ainsworth AJ, Capley G, Waterstr Eet P, Munson D (1996) Use of monoclonal antibodies in the indirect fluorescent antibody technique (IFA) for diagnosis of Edwardsiella ictulari. Journal of Fish Diseases 9: 439-444.
32. Lorenzen E, Karas N (1992) Detection of Flexibacter pschrophilus by immunofluorescence in fish suffering from fry mortality syndrome: A rapid diagnostic method. Diseases of Aquatic Organisms 13: 231-234.
33. Knappskog DH, Rodseth OM, Slinde E, Endresen C (1993) Immunochemical analyses of Vibrio anguillarum strains isolated from cod, Gadus morhua L, suffering from vibriosis. Journal of Fish Diseases 16: 327-338.
34. Cipriano RC, Pyle JB, Starlıper CE, Pyle SW (1985) Detection of Vibrio anguillarum antigen by dot blot assay. Journal of Wildlife Diseaes 21: 211-218.
35. Toranzo AE (2015) Tenacibaculosis of farmed fish in Southern Europe. Tenacibaculum maritimum Workshop Maritime Centre, British Columbia, Canada.