11. Tenacibaculum group

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in

Zrncic S. (ed.).
Diagnostic Manual for the main pathogens in European seabass and Gilthead seabream aquaculture

Zaragoza : CIHEAM
Options Méditerranéennes : Série B. Etudes et Recherches; n. 75

2020
pages 97-106

11. *Tenacibaculum* group

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11.1. Aetiology of *Tenacibaculum* group

Tenacibaculosis, also known under several names, such as "black path necrosis", "saltwater columnaris disease", "marine flexibacteriosis", "eroded mouth syndrome" and "gliding bacterial disease of sea fish" (Santos *et al.*, 1999; Toranzo *et al.*, 2005) is caused by *Tenacibaculum* spp., an opportunistic Gram-negative filamentous bacteria, 0.4-0.5 µm in diameter and 1.5-30 µm long, occasionally up to 100 µm long. Most of the isolates show gliding motility on wet surfaces produce catalase and oxidase activity and cells can produce a yellow pigment, which is mainly zeaxanthin (Wakabayashi, 1986; Avendaño-Herrera *et al.*, 2006; Suzuki, 2015).

The most described species to be considered a major fish pathogen worldwide is *Tenacibaculum maritimum* which causes high mortalities and important economic losses in a wide range of wild and cultured marine fish species including seabass, seabream, sole and turbot (Toranzo *et al.*, 2005; Avendaño-Herrera *et al.*, 2006). Other *Tenacibaculum* species described associated to fish diseases in the Mediterranean are: *T. soleae* from diseased sole (*Solea senegalensis*) (Piñeiro-Vidal *et al.*, 2008a) and European seabass (*Dicentarchus labrax*)

(Castro *et al*., 2014), *T. gallaicum* from a holding tank for turbot (*Psetta maxima*) (Piñeiro-Vidal *et al*., 2008b) and sole (*S. senegalensis*), *T. dicentrarchi* from skin lesions of European seabass (*Dicentarchus labrax*) (Piñeiro-Vidal *et al*., 2012) and *T. discolor* from cultured sole (*S. senegalensis*) (Piñeiro-Vidal *et al*., 2008b).

Clinical signs of tenacibaculosis caused by *T. maritimum* are characterized by gross lesions on the body surface. This depends on the fish species and age and generally includes eroded mouth, haemorrhagic and necrotic lesions on the skin, frayed fins and tail rot and in some cases also necrosis on the gills and eyes (Toranzo *et al*., 2005; Piñeiro-Vidal *et al*., 2007). Deeper ulcerative lesions on the body sides or close to the dorsal fin have been reported in cases of *T. discolor* infection (Le Breton *et al*., 2018).

11.2. Sampling

11.2.1. Preparation and shipment of samples from fish

The genus *Tenacibaculum* can be isolated from marine samples, particularly from the surface of marine organisms, external lesions or kidney lesions by streaking directly onto agar plates (Suzuki *et al*., 2015). *T. maritimum* can be prepared for storage or transport by culturing at 20 to 25°C for 48 to 72h onto *Flexibacter maritimum* medium (FMM) or Marine agar (MA) medium (Avendaño-Herrera *et al.,* 2006).

Cultures could be then shipped on ice or temporarily preserved for several weeks refrigerated at 4°C. For longer storage, cultures can be kept in broth containing 10% glycerol at -80°C or in the gas phase of liquid nitrogen. Lyophilization may give satisfactory results (Suzuki *et al*., 2015).

Specific transport medium has been developed for skin lesion swab preservation during transport to the diagnostic laboratory (Moalic *et al.,* 2018).

11.2.2. Live fish

As these bacteria are quite sensitive and easily destroyed during transportation, live affected fish should be sent alive to the laboratory for analysis in containers half-filled with water and oxygen. Occasionally, scrapings of the lesions from dead fresh fish with typical lesions submitted for diagnostic revealed presence of *Tenacibaculum* but it is not possible to cultivate them on specific media (either specific culture media such as FMS or isolation media for *Tenacibaculum*). Bacteria were either dead or not reverifiable. In order to enable isolation of bacteria, either live moribund fish or fish and swabs from the lesions in specific transport media are requested to be submitted for diagnostic purposes.

11.3. Diagnostic procedures for the *Tenacibaculum* group

Diagnosis of tenacibaculosis is usually based on the isolation of the causative agent followed by morphological, biochemical, analysis of their microbial susceptibility profiles and serological characterization (Fernandez-Alvarez and Santos, 2018).

11.3.1. Presumptive diagnosis from fresh samples

Presumptive diagnosis is usually made by microscopic observation (400 x or 1000 x magnification) of the bacteria on fresh smears from scrapes the lesion or after Gram staining or MGG (May-Greenwald Giemsa) staining of the slides, using quick coloration kits (Gram stain/ RAL 555, RAL Diagnostic, Martillac, France).

11.3.2. Primary cultivation of bacteria (choice of media and isolation of strain)

Isolation of *Tenacibaculm* spp. from fish tissues is difficult due to the slow growth of the bacteria and overgrowth by other bacteria in tissue samples. The commercially available Marine agar (MA, 2216E; Difco Laboratory, Detroit, MI, USA) and *Flexibacter maritimum* medium (FMM) (Laboratorios Conda, Madrid, Spain) are considered the most efficacious media for the primary isolation of this gliding bacteria (Fernandez-Alvarez and Santos 2018). A selective medium for *T. maritimum* recovery following transportation has been elaborated to limit the growth of contaminants and to ensure good growth of the bacterial colonies (Moalic *et al.*, 2018). This medium enables the growth of different species of the *Tenacibaculum* genus.

T. maritimum is an obligate marine microorganism which does not grow on media prepared by just adding NaCl. It must be cultured in oligothrophic media elaborated with seawater (30-100% strength seawater) (Wakabayashi *et al.,* 1986. In MA (or broth) this species grows rapidly however it is difficult to recognize the typical long filamentous *T. maritimum* colonies (Toranzo, 2015). FMM is considered the most appropriate medium for the isolation of *T. maritimum* from fish tissue (Pazos *et al.*, 1996). Although when using FMM this species shows slow growth and low density, it allows a better recognition of its typical flat, pale-yellow colonies with irregular uneven edges that adhere to the medium (Toranzo, 2015). In adverse conditions these non-sporulating *Flavobacteriaceae* can produce a spheroplast-like form**,** which makes diagnosis by direct microscopic observation more difficult.

11.3.3. Screening of pure cultures

Tenacibaculum spp. belongs to a group of *Flavobacteriaceae* including pathogenic strains for marine fish. *T. marinum* is the most common representing almost 50% of the strains reported in clinical cases in the Mediterranean area (Le Breton *et al.*, 2018). All species are Gram-negative filamentous bacteria and rod-shaped, positive for catalase, oxidase and degradation of casein, non sporulating and producing a spheroplast-like form under adverse conditions.

Depending on the strains, macroscopically, on soft media, colonies appear pale to bright yellow with typical irization. Colony shapes may vary from uneven regular circles to irregular with spreading edges. For most pathogenic strains, growth will be achieved within a range of temperature from 15°C to 34°C.

Depending on the species, cells size is approximately 2 - 30 µm long x 0.5 µm wide. They can grow in a range of 5.9 to 8.6 pH in a salinity range of 20 to 30 ppt, preferably in presence of 30ppt to full-strength seawater (Wakabayashi *et al.,* 1986; Hansen *et al.,* 1992; Suzuki *et al.,* 2001; Pazos *et al.*, 1996; Moalic *et al.,* 2018).

11.3.4. Identification of the strain

11.3.4.1. API

Phenotypic homogeneity within *Tenacibaculum* species associated to fish has facilitated their identification based on their biochemical profile using miniaturized systems such as API ZYM and API 50CH (Fernandez-Alvarez and Santos, 2018).

When using API ZYM gallery it is difficult to distinguish between *T. maritimum* isolates since they are all quite homogenous, with a characteristic profile with positive results on the first 11 enzymatic reactions. All enzymes related to the metabolism of carbohydrates are absent (Avendaño-Herrera *et al.*, 2004; 2005b; 2006; Toranzo, 2015). These miniaturized systems are sufficient to identify *Tenacibaculum* at genus level but not at species level or to distinguish isolates.

11.3.4.2. Mass spectrometry (MALDI-TOF)

Recently, the application of a novel proteomic fingerprinting approach based matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) coupled with Mass-UP software (López-Fernández *et al*., 2015) has proven to be a useful and reliable tool for the identification and classification of isolates of *Tenacibaculum* at the species level (Fernández-Alvarez *et al.,* 2017). MALDI-TOF-MS technique was able to identify, regardless of the culture media (FMM or MA) used, eight genus-specific peaks for *Tenacibaculum* and at least one species-specific peak in *T. maritimum*, *T. soleae*, *T. dicentrarchi* and *T. ovolyticum*. However, mass spectra for *T. discolor* and *T. gallaicum* were very similar and no species-specific peaks could be detected (Fernández-Alvarez *et al*., 2017).

The stability of the MALDI-TOF spectra found even under different cultivation conditions is because protein mass fingerprinting mainly represents conserved ribosomal proteins together with several housekeeping and structural proteins which are abundant and relatively independent of the culture or external conditions (Welker, 2011; Singhal *et al*., 2015).

This research suggests that the MALDI-TOF-MS technique could be a good complementary approach to 16S rRNA sequencing and have an even higher discriminating potential than analysis of the 16S rRNA gene (Fernández-Alvarez *et al.,* 2017).

11.3.4.3. PCR

Species specific PCRs targeting a particular fragment of 16S rRNA gene have been designed for the following *Tenacibaculum* spp.:

T. maritimum		
Forward primer	**MAR1**	5-AATGGCATCGTTTTAAA-3’
Reverse primer	**MAR2**	5´-CGCTCTCTGTTGCCAGA-3´
Expected amplified product is 1088 bp (Toyama *et al*., 1996)		
Forward primer	**Mar 1**	5’-TGTAGCTTGCTACAGATGA-3’
Reverse primer	**Mar 2**	5’- AAATACCTACTCGTAGGTACG-3’
Expected amplified product is 400bp (Bader and Shotts 1998)- able to detect pure and mixed cultures of *T. maritimum*		

T. solae		
Forward primer	**Sol-Fw**	5’-TGCTAATATGTGGCATCACAA-3’
Reverse primer	**Sol-Rv**	5’-CAACCCATAGGGCAGTCATC-3’
Expected amplified product is 248 bp - able to detect pure and mixed cultures of *T. solae* (Garcia-González *et al*., 2011).		
Forward primer	**G47F**	5′-ATGCTAATATGTGGCATCAC-3′
Reverse primer	**G47R**	5′-CGTAATTCGTAATTAACTTTGT-3′
Designed at the 5′ region of the 16S gene and of the ISR respectively flanking a 1555 bp fragment. Able to successfully identify pure and mixed cultures of *T. soleae* and from tissues of infected fish (López *et al*., 2011).		

T. dicentrarchi		
Forward primer	**Tenadi Fw**	5’-ATACTGACGCTGAGGGAC-3’
Reverse primer	**Tenadi Rv**	5-TGTCCGAAGAAAACTCTATCTCT-3’
Expected amplified product is 284 bp product. Able to successfully identify pure and mixed cultures of *T. dicentrarchi* and from tissues of infected fish (Avendaño-Herrera *et al*., 2017).		

Simple PCR methods are only capable of detecting acute infections. However, when the pathogen is present in low numbers, such as in asymptomatic or carrier fish, PCR has low sensitivity (Cepeda and Santos, 2002; Avendaño-Herrera *et al*., 2006).

Nested-PCR methods to detect *T. maritimum* have been developed and proven to be more sensitive (Cepeda and Santos, 2002; Cepeda *et al*., 2003; Avendaño-Herrera *et al*., 2004 b, c) however, they are also more expensive and time-consuming since they require two rounds of PCR (Fernández-Alvarez and Santos, 2018).

Other PCR-based methods are: 1) PCR-enzyme linked immunoabsorbent assay (PCR-ELISA) (Wilson *et al*., 2002), 2) reverse transcriptase polymerase chain reaction-enzyme hybridization assay (RT-PCR-EHA) (Wilson and Carson, 2003), 3) DNA microarray probe (Warsen *et al*., 2014). These assays have been able to detect *T. maritimum* from pure cultures but their effectiveness to separate different *Tenacibaculum* species and to detect the pathogen from infected fish tissues have not yet been examined (Fernández-Alvarez and Santos, 2018). Recently, a real-time PCR method, targeting a 164bp fragment of the 16S rRNA gene, has been developed for the detection and quantification of *T. maritumum* in fish and seawater samples (Fernández-Alvarez *et al*., 2019).

11.3.4.4. Typing of the bacteria

Serological typing methods

The identification of serotypes and antigenic characterization are key for diagnostics and epidemiology studies towards a successful vaccine development (Fernández-Alvarez and Santos, 2018).

Three different host-specific major O-serotypes have been identified in *T. maritimum* causing mortalities in cultured marine fish: serotype O1 include strains isolated from gilthead seabream, serotype O2 isolated from turbot, serotype O1 and O3 from sole and serotype O3 and recently also O2 from salmon (Avendaño-Herrera *et al*., 2004; 2005c; Le Breton, 2019).

Other *Tenacibaculum* spp. pathogenic to fish also show antigenic heterogeneity. Lack of cross-reaction between *T. maritimum*, *T. soleae* and *T. discolor* and the existence of at least two serotypes (O1 and O2) in *T. soleae* strains and one in *T. discolor* (serotype O1) have recently been confirmed (Fernández-Alvarez *et al.,* 2018).

Molecular typing methods

Several methods have been developed for the identification and genotyping of bacteria. Ribotyping enables discrimination at the species and sub-species level through DNA fragmentation of *T. maritimum* strains into five different rRNA gene restriction patterns (P1 to P5). These restriction patterns correspond to the four serotypes described for the species. It has a good reproducibility but it is not able to discriminate the strains based on the host source or geographic isolation (Pazos, 1997).

RAPD-PCR is a rapid and easy technique, which not only reveals patterns coinciding with the O-serotypes of *T. maritimum* but also enables the strain to be separated into different groups according to the host species. RAPD-PCR permits *T. maritimum* to be discriminated from *T. discolor*, *T. gallaicum* and *T. soleae* (Piñeiro-Vidal, 2008). The major constraint of the RAPD-PCR technique is that it is difficult to reproduce patterns in different laboratories and compare isolates tested on different days (Fernández-Alvarez and Santos, 2018).

ERIC-PCR and REP-PCR although they display very clear distinct genetic profiles for the different *Tenacibaculum* species and strains from the same species, do not provide correlation between the genetic profiles and the serotypes, host or geographical location of isolation (Fernández-Alvarez *et al*., 2018).

Multi-locus sequence analysis (MLSA) combines PCR and automated DNA sequencing and successfully allows the genetic differentiation of *Tenacibaculum* species, being able to clearly discriminate between *T. maritimum*, *T. gallaicum*, *T. soleae*, *T. discolor*, *T. dicentrarchi* and *T. ovolyticum* (Habib *et al*., 2014). It seems a reliable tool for epidemiological studies and monitoring tenacibaculosis in marine environments (Fernández-Alvarez and Santos, 2018).

11.3.5. In vitro susceptibility testing

For in *in vitro* antimicrobial susceptibility testing two methods have been recommended: 1) using FMM agar and broth and diluting 0.3% Mueller-Hinton Agar (DMHA) (Avendaño-Herrera *et al*., 2005a) or 2) using broth prepared with natural or artificial seawater with or without supplementation with 5% fetal calf serum (CLSI, 2006).

In vitro studies on the susceptibility of *T. maritimum* to various chemotherapeutic agents indicate that strains isolated from different host species and geographical regions exhibit a similar pattern, with susceptibility to penicillins, erythromycin, tetracyclines, trimethoprim, potentiated sulfonamides and fluoroquinolones, and resistance to colistin, kanamycin, neomycin and the quinolones, oxolinic acid and flumequine (Soltani *et al.,* 1995, Avendaño-Herrera *et al.,* 2004, 2005a). However, field results might be different even when the isolated bacteria are highly sensitive (*in vitro*) to a chemotherapeutant used for treating the condition (Cepeda and Santos, 2002).

T. gallaicum, *T. discolor, T. soleae* and *T. dicentrarchi* also show to be susceptible to amoxicillin, florfenicol and oxytetracycline and resistant to oxolinic acid. Resistance to flumequine, enrofloxacin, oxytetracycline and trimethoprim-sulfamethoxazole has also been detected in some strains of *T. gallaicum*, *T. discolor* and *T. soleae* (Avendaño-Herrera *et al*., 2008).

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Photos

Fig. 11.1. ***Tenacibaculum maritimum*** **lesion in** ***D. labrax*****. a) Infected fish in the cage showing extensive skin lesions with whitish to yellowish mucus ; b) lesions on the trunk ; c) gill focused lesions and hemorrhages ; d) lesion with hemorrhages on the dorsal part of the head.**

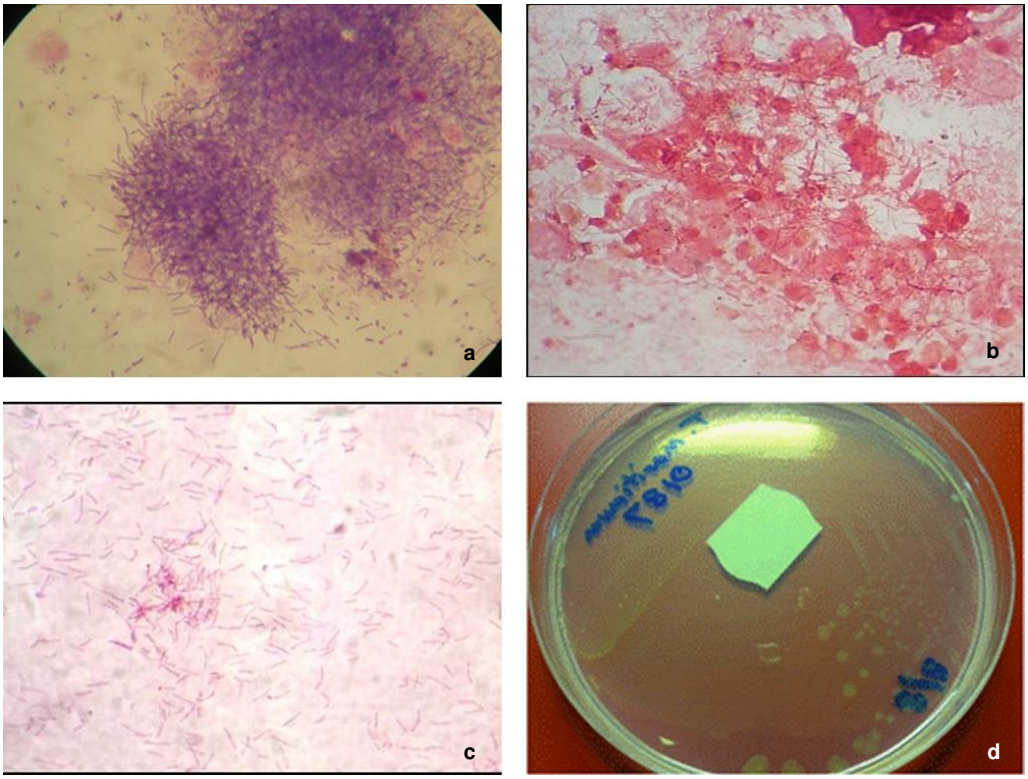

Fig. 11.2. a) and b) Fresh smear Gram staining (RAL555 staining) 400 x microscopic observation of *Tenacibaculum* sp. from scraping of skin lesions; c) stained smears obtained from pure colonies; d) colonies grown on the FMM agar.