A novel O-serotype in *Tenacibaculum maritimum* strains isolated from cultured sole 
(*Solea senegalensis*)

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Abstract

*Tenacibaculum maritimum* was consistently isolated from tenacibaculosis outbreaks affecting sole farms in Portugal and south of Spain in the last two years. These new *T. maritimum* isolates could not be assigned within the two major serotypes (O1 and O2) already described. Rabbit antiserum was prepared against one Portuguese sole isolate to examine the antigenic relationships between the isolates from sole using microtitre agglutination tests, dot blot assay and immunoblotting of lipopolysaccharides. Serological characterization of the recent sole isolates demonstrated that they belong to a novel O-serotype named O3, allowing us to extend the serological scheme for this fish pathogen. This information is useful for epizootiological and vaccination studies.

Tenacibaculosis or flexibacteriosis is recognized as an important infectious disease in marine fish since 1979 (Hikida et al., 1979; McVicar and White, 1979). The presence of the etiological agent *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) was first demonstrated by Bernardet et al. (1990) and since then, this pathogen has spread among a wide variety of host species, producing significant losses in cultured fish such as turbot (*Scophthalmus maximus*), Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus kisutch*), sea bass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata*) (Devesa et al., 1989; Pazos et al., 1993; Bernardet et al., 1994; Ostland et al., 1999) and, lately, in sole (*Solea solea* and *S. senegalensis*) (Cepeda and Santos, 2002; Avendaño-Herrera et al., 2004).

In previous work we analysed the antigenic diversity of *T. maritimum* from sole, gilthead sea bream and turbot, which allowed us to propose a O-serotyping scheme for *T. maritimum* composed by two major serotypes (O1 and O2) mainly associated with the host species (Avendaño-Herrera et al., 2004). However, since the middle of 2003, new outbreaks of tenacibaculosis have occurred in sole farmed in Portugal and south of Spain. These new *T. maritimum* isolates could not be assigned within the serotypes already described. We report here the existence of a

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new serotype within this fish pathogen, which was proposed as serotype O3.

Twelve *T. maritimum* strains isolated from sole in Portugal and south of Spain were used in the present study. The bacterial strains were confirmed as *T. maritimum* using the PCR-based analysis described by Toyama et al. (1996). The bacteria were routinely cultivated on *F. maritimus* Medium (FMM) (Pazos et al., 1996) at 24°C for 72 h. Stock cultures were maintained frozen at –70°C in Criobille tubes (AES Lab., France).

The antigenic analysis were carried out using the thermostable antigens of each strain obtained after heat killing the bacterial suspensions (10^9 cells ml⁻¹) in phosphate buffered saline (pH 7.4) at 100°C for 60 min, washed once in the same saline solution and maintained at 4°C until required. The Portuguese sole strain ACC13.1 was selected and used to obtain immune serum in rabbit according to the methods described by Sørensen and Larsen (1986). The serological analysis were performed with the unabsorbed and absorbed rabbit serum. For the absorption, the serum against isolate ACC13.1, as well as antisera against strains PC503.1 from sole (serotype O1) and PC424.1 from turbot (serotype O2) (Avendaño-Herrera et al., 2004), were mixed with the antigens of the heterologous isolates and incubated overnight at 4°C. This process was repeated twice to ensure a complete absorption of common antigens.

The agglutination titers of the antiserum ACC13.1 were measured against the suspensions of O-antigens from the homologous and heterologous strains (Stevenson and Daly, 1982). The serological characterization of the twelve *T. maritimum* isolates was performed employing the dot blot assays as described by Cipriano et al. (1985) with the unabsorbed and absorbed antiserum

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<td>PC424.1 (O2)</td>
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<td>Turbot strain PC424.1</td>
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<td>12 sole isolates</td>
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* Parenthesis indicate the serotype.

+++, strong and immediate reaction; +, delayed positive reaction; -, negative reaction. Asterisks indicate different result detected with absorbed and unabsorbed antiserum.

Table 1. Results of microagglutination test and dot blot assay with O-antigens and rabbit antisera raised against the *T. maritimum* isolates.
obtained against the ACC13.1 isolate and each representative of serotypes O1 and O2.

In order to evaluate the antigenic variability among strains, we characterized the isolates analyzing their lipopolysaccharides (LPS) following the procedures of Hitchcock and Brown (1983). Samples were examined by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE; Laemmli, 1970) using 12% acrylamide in the resolving gel and 4% acrylamide in the stacking gel. LPS components separated by SDS-PAGE were transferred onto nitrocellulose membrane by electrophoretic blotting according to procedures of Towbin et al. (1979), and treated for immunological analysis as outlined above for the dot blot assay.

The results of microtitre agglutination tests using unabsorbed serum showed the highest titres with the homologous strains, providing evidence of antigenic specificity (Table 1). When the dot blot assays were performed, the present sole isolates studied showed strong reaction with the antiserum raised against the Portuguese sole strain (Figure 1a). Although weak cross-reactions with the serum against the serotype O2 were observed, they fully disappeared when the O2 antiserum was absorbed with the heat stable O-antigen of the heterologous Portuguese sole strain, confirming that all the new sole isolates correspond to a distinct \textit{T. maritimum} serological group (Table 1).

The immunoblot of the LPS clearly assured the existence of this novel serological group within \textit{T. maritimum}, which was distinguished without the necessity to use absorbed antiserum in agreement with our previous findings (Avendaño-Herrera et al., 2004). This serotype named O3 comprised all the sole strains previously untypable, which showed a strong immunological reaction of their O side chains when the unabsorbed serum raised against the sole isolate ACC13.1 was used (Figure 1b). As expected no reaction with the antiseras raised against the representative of serotypes O1 and O2 was detected (Figure 1b).

Figure 1. Dot blot assay (a) and immunoblot of the LPS (b) of the isolates of \textit{T. maritimum}, using the antiserum raised against the Portuguese sole isolate ACC13.1. In 1a, strain ACC13.1 (dot 4) and saline solution (0.85% wt/vol NaCl)(dot 24) were used as positive and negative controls respectively. Dots: 1 and 5 to 8, serotype O1 strains; 2 and 9 to 12, serotype O2 strains; 3, serotype O1/O2 strain; 13 to 23, new serotype O3 isolates. Lanes in 1b: 1, serotype O1 strain (PC503.1); 2, serotype O2 strain (PC424.1); 3 to 6, new serotype O3 isolates.
Until now the majority of *T. maritimum* isolated from sole in northwestern of Spain belonged to serotype O1 (Avendaño-Herrera et al., 2004). This antigenic heterogeneity would warrant further investigation because serologically distinct *T. maritimum* strains may be associated with different outbreaks. Moreover, often sole farms in the Iberian peninsula do not posses the whole fish cycle, importing or moving fish from other areas, which suggest the possible coexistence of *T. maritimum* serotypes O1 and O3 in a same farm. Further studies are necessary to elucidate this question. Therefore, our finding here must be taken into account in order to consider the incorporation of the new serotype O3 antigens in the vaccine formulation against tenacibaculosis in sole culture.

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**References**


