

First isolation of *Flexibacter maritimus* from farmed Senegalese sole (*Solea senegalensis*, Kaup) in Spain

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Abstract

The present study deals with the first isolation of *Flexibacter maritimus* from Senegalese sole, *Solea senegalensis* (Kaup), cultured in Spain. After a 24 hours shipment from one fish farm to another, a group of juvenile sole showed several external signs of disease: eroded mouth, rotten fins and shallow skin lesions or ulceration. Internally, affected specimens showed paleness of internal organs. Regardless of the use of chemical therapy, the affected group experienced almost 100% mortality. Traditional bacteriological and PCR analysis allowed the identification of the causative agent as *Flexibacter maritimus*. The pathogen was also directly detected from tissue of diseased fish by nested PCR.

Introduction

Marine flexibacteriosis is considered a potential limiting factor for the culture of economically important marine fish species causing serious mortalities in farms in many countries (Santos *et al.*, 1999). External pathological signs of this disease are eroded mouths, ulcerated skin lesions, and fins and tail rot. Infected tissues can appear pale-yellow due to the presence of large number of bacteria. Some affected fish may have shallow skin lesions or darkening of tissue between caudal and marginal fin rays, loss of epithelial surface and haemorrhage in exposed dermal tissue (Bernardet *et al.*, 1990; Alsina and Blanch, 1993; Pazos *et al.*, 1993).

Flexibacter maritimus was first described as the causative agent of marine flexibacteriosis in several cultured fish in Japan and became a common problem in that country (Hikida *et*

al. 1979; Wakabayashi *et al.*, 1986). The presence of this pathogen in Europe was first demonstrated in Scotland from a Dover sole (*Solea solea*) suffering from "black patch necrosis" (Campbell and Buswell 1982; Bernardet *et al.*, 1990). Later, this fish pathogen was isolated in Spain from turbot (*Scophthalmus maximus*) (Alsina and Blanch, 1993; Pazos *et al.*, 1993) and from salmon (*Salmo salar*) (Pazos *et al.*, 1993).

Identification of *F. maritimus* is generally based on the study of its morphological, physiological and biochemical characteristics (Bernardet *et al.*, 1990) or more recently using PCR technique (Toyama *et al.*, 1996; Bader and Shotts, 1998). The present paper reports the first description of marine flexibacteriosis in sole, *Solea senegalensis* (Kaup), cultured in a Spanish hatchery, which occurred after counting, size classification and transport. The iden-

tification of the causative organism was carried out by conventional microbiological analysis and PCR-based assays.

Materials and Methods

Fish

An apparently healthy population of juvenile sole (weight around 5-10 g) were counted and classified before shipment to another hatchery. On arrival, fish were again counted and classified. In the next 24 hours, when outbreak started, fish received chemotherapy treatment. Mortality reached 92% of the population in 15 days. The main signs of the disease were eroded mouth, skin ulcers and rotten and frayed fins. Internally, affected specimens showed paleness of liver and kidney.

Isolation and identification of bacteria

Samples from kidney and skin lesions of moribund sole were cultured on Tryptone Soy Agar (Oxoid) supplemented with 1% NaCl (TSA-1), Thiosulphate Citrate Bile Sucrose (TCBS) agar (Oxoid), and *Flexibacter maritimus* Medium (FMM) agar (Pazos *et al.*, 1996) and incubated at 25°C for 24-48h. Smears from kidney and ulcer were Gram-stained and examined using a light microscope.

Pure cultures of the isolates obtained on FMM and TSA-1 agar plates were identified using morphological, physiological and biochemical tests and API systems (API 20E and API ZYM) (Biomerieux) as previously described (Bernardet *et al.*, 1990; Pazos *et al.*, 1993; Santos *et al.*, 1993). Sensitivity to antimicrobials was evaluated by the disc diffusion method (Barry and Thornsberry, 1991) using the following chemotherapeutic agents (micrograms disc⁻¹) supplied by Oxoid: ampicillin (10), oxytetra-

cycline (30), oxolinic acid (2), streptomycin (10), chloramphenicol (30), nitrofurantoin (300), enrofloxacin (2), trimetoprim-sulfametoxazol (23,7-1,2) and the vibriostatic agent O/129-Pteridine (150). Plates were incubated at 25°C for 48 hours. Phenotypic profiles were compared to those of reference strain (NCIMB2153) obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

Serological identification was performed using slide agglutination test as previously described (Santos *et al.*, 1995). The test was carried out using both formalin-killed cells (FKC) and "O" antigens and rabbit whole cell antiserum against the reference strain of *F. maritimus* NCIMB 2153. The identity of the gliding bacterial strains isolated on FMM agar was confirmed using PCR-based analysis (Bader and Shotts, 1998).

PCR detection of F. maritimus from tissues of diseased fish

DNA was extracted from brain, kidney, spleen and skin tissues of disease fish using Dynabeads DNA DIRECT system (Dyna) according to manufacturer's instructions, and was submitted to nested PCR amplification. Universal primers 20F and 1500R (Weisburg *et al.*, 1991) and specific primers MAR1 and MAR2 (Bader and Shotts 1998) were used in the first (PCR1) and in the second (PCR 2) steps of the nested PCR, respectively. All oligonucleotides were synthesised by Amersham Pharmacia Biotech.

The PCR amplifications were done using the commercial kit Ready-To-Go PCR beads (Amersham Pharmacia Biotech), which included all necessary reagents except primers

(1 pmol) and DNA template (0.5 µl) which were added. The reaction mixtures were amplified in a Mastercycler personal thermal cycler (Eppendorf). The cycling conditions for PCR1 were 30 cycles of denaturation (95°C for 30s), annealing (57°C for 30s), and extension (72°C for 60s). A preheating step at 95°C for 5 min and a final extension step consisting of 5 min at 72°C were carried out. The program for PCR2 consisted of a preheating step at 94°C for 10 min, then 30 cycles at 94°C for 2 min, 58°C for 2 min, 72°C for 2 min and finally held at 72°C for 4 min. The amplified products were analysed using vertical agarose electrophoresis and methylene blue staining method (VAGE/MeB) (Cepeda and Santos, 2000).

Results

Bacterial isolation and characterisation

Microscopic examination of Gram-stained smears from kidney tissue revealed the presence of Gram-negative filamentous bacteria. Only one type of colony grew on FMM plates from all internal organs examined. Colonies were flat, pale-yellow with uneven edges, presented strong adherence to the medium and adsorbed Congo red. These colony characteristics resemble those previously described for *Flexibacter maritimus* (Pazos *et al.*, 1996). The phenotypic tests showed that bacterial isolates were GRAM-negative, long slender rods with gliding motility. They produced catalase and cytochrome oxidase and reduced nitrate but no produced hydrogen sulphide nor flexirubin-type pigments. All the isolates exhibited the same enzymatic profile described for the reference *F. maritimus* strain NCIMB 2153 in API ZYM galleries (Bernardet

et al., 1990; Pazos *et al.*, 1993). A strong positive agglutination with the NCIMB 2153 antiserum was observed using the FKC antigen, but no reactivity was recorded when "O" antigen was employed. The electrophoretic analysis of PCR products showed a single band of 400 bp, as expected for a *Flexibacter maritimus* strain.

The *F. maritimus* isolates presented identical drug sensitivity pattern, being resistant to streptomycin and oxolinic acid and sensitive to all the others antimicrobials tested in this study. However, none of the antimicrobial treatments used in the fish farm were efficient in stopping the mortality, which reached almost 100% of affected population.

Occasionally, other bacterial species were recovered on FMM, TSA-1 and the selective medium TCBS from skin samples. These cultures were identified as *Vibrio pelagius* and *V. fischeri* on the basis of the results of conventional morphological, physiological and biochemical test and API 20E system.

Detection of F. maritimus in tissues of infected sole

The use of nested PCR allows the direct detection of the pathogen in the tissues of all diseased sole tested. A fragment of 400bp specific for *F. maritimus* was amplified from all the tissues analysed (spleen, kidney, brain and skin).

Discussion

Investigation of new species is one of the strategies for increasing market opportunities in the aquaculture world. Senegalese sole, *Solea senegalensis*, is a common fish in Mediterranean and southern Atlantic water, recently

reared in extensive aquacultural productions in the Northwest of Spain. Regardless of the potential economical importance of the culture of this fish species (Dinis *et al.* 1999,1992) there is few data about the susceptibility of captive Senegalese sole to fish pathogens (Rodríguez *et al* 1997; Zorrila *et al*, 1999)

In this work we present the first isolation of *F.maritimus* from cultured Senegalese sole. The phenotypic, serological and PCR-based analysis allowed us to identify the bacteria isolated from internal organs of diseased fish as *F.maritimus*. Moreover, slide agglutination assay using the thermostable "O" antigen confirmed the existence of different serological groups within *F. maritimus* species (Pazos *et al.*, 1993).

On the other hand, results from PCR assays undoubtedly support the identification of isolates as *F. maritimus*. Indeed, detection of this pathogen was possible in tissues of diseased sole such as skin ulcers, spleen, kidney and brain, without previous cultivation on agar media. The proposed nested PCR is thus a sensitive, specific and labour-saving system for diagnosis of marine flexibacteriosis compared with the traditional methods.

Further experimental studies are needed to confirm the pathogenicity of the isolates, but the detection of *F. maritimus* in juvenile sole indicates that this bacterial species should be considered as a potential risk for the culture of Senegalese sole.

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