

Biochemical and Enzymatic Characterization of a Gram-negative Fish Pathogen Isolated in Morocco from Rainbow Trout and Comparison with Isolates of Other Countries and Sources

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Keywords

Flavobacterium psychrophilum • Rainbow trout • Characterization • Fish disease Isolation

1 Introduction

Some bacteria play a considerable role in the pathology of fish, they may infect fish with one or more other bacterial species, which makes them true pathogens, capable of infecting most healthy animals. However, only opportunistic pathogens whose infectivity is expressed by a decline in the fish condition and of its natural defenses, are linked to disturbances of the environment or livestock practices, creating the most favorable context for the development of bacterial pathologies. Many bacterial species are pathogenic for fish (Noga 1996; Austin and Austin 1999; Woo and Bruno 1999). The most serious infections in our latitudes are Aeromonadaceae, Vibrionaceae, Enterobacteriaceae and Flavobacteriaceae. Cold water flavobacteriosis with

Flavobacterium psychrophilum is common in freshwater aquaculture and causes very heavy losses in Salmon farms and Severe mortalities of salmonid fry throughout the world.

The aim of this study was to isolate, identify and biochemically characterize the pathological agent causing severe mortalities in Moroccan hatcheries.

1.1 *Flavobacterium* Genus Description

Flavobacterium genus was described for the first time in 1923 by Bergey et al. (1923). Bacteria of the genus *Flavobacterium* now have a number of common characteristics. They are Gram negative, rods with straight, parallel sides and rounded ends; their size is from 0.5 by 1.0–3.0 µm, with gliding motility and slow growth on solid media. Their colonies are typically translucent and pigmented yellow to orange. Usually 1–2 µm in diameter, they are convex or low convex, smooth and shiny with entire edges. Non-pigmented and occasionally opaque strains occur. They are Aerobic, having a strictly respiratory type of metabolism. Chemoorganotrophic acid, but no gases are produced from carbohydrates. Most do not ferment sugar or glucose and are Catalase-positive. They are Oxidase and Phosphatase positive, Urease and DNase negative (Nakagawa and Yamasato 1996).

Widely distributed in soil and water; Flavobacteria were isolated from a wide range of habitats. Since they are chemoorganotrophic bacteria, a number of them colonize fish in which they exert pathogenicity. This is particularly the case for *Flavobacterium branchiophilum*, *F. columnare*, *F. johnsoniae*, or *F. psychrophilum*. Others, such as *F. hydatis*, or *F. succinicans*, have been found and isolated from diseased

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fish, although their pathogenicity has not been demonstrated (Bernardet et al. 1996).

2 Materials and Methods

Fertilized eggs of Rainbow Trout (*Onchorynkus Mykiss*) are imported from France and in the hatchery, they achieved half the incubation.

The problem first apparition was after a serious number of mortalities at the fry, and the losses are estimated to be more than 80%. The events had been observed previously and no scientific study had been achieved.

Samples were collected in aseptic conditions. Diseased Rainbow trout fertilized eggs and fry were obtained from a fish hatchery in azrou - Morocco, operating with flow through tanks fed with source water. Fish had a body weight between 20 and 100 g and were held at a stocking density of 23–25 kg m⁻³. At the time of sampling, the water temperature was 10 °C. Routine grading for size 15 days prior to sampling and low water temperatures were the only identifiable stress factors preceding evidence of disease (Elliot 1981). A total of 30 fish with and without eroded fins and/or tails were examined per season and taken for microbiological analyses.

a. Isolation and identification

The growing economic importance of aquaculture in the world has led to increase interest in the rapid and reliable methods for detection and identification of bacterial fish pathogens (Nilsson and Strom 2002). The detection of *Flavobacterium psychrophilum* by conventional techniques is difficult and time-consuming. Bacteria from *Flavobacterium* genus are responsible for significant economic losses in salmonid culture (Nematollahi et al. 2003).

Water samples were collected in sterile flacons and fish samples taken from the hatchery were stoked in 4 °C and transported directly to the laboratory.

In the laboratory, water samples were filtered in sterile conditions and the filters were put in petri dishes. Fish samples taken from internal organs (liver, kidney, rate and spleen), damaged gill tissue and, if present, skin lesions of fish body surface were streaked onto Cytophaga agar (CA, 0.05% tryptone, 0.05% yeast extract, 0.02% sodium acetate, 0.02% beef extract with 0.9% agar, pH 7.2–7.4) (Anacker and Ordal 1959) plates using sterile loops and aseptic techniques were directly streaked onto CA plates and incubated at 14 °C for up to 10 days.

Eroded fins and tails were sampled by scraping the margin of the lesion with a sterile scalpel blade; the collected material was then inoculated onto Cytophaga agar plates and incubated at 14 °C for up to 10 days. After the incubation period, yellow-pigmented colonies were chosen and restreaked on the CA to obtain pure isolates. Colonies were tested for Gram staining, presence of flexirubin type pigment, cytochrome oxidase activity, catalase production and motility (Lorenzen et al. 1997). Gram negative rod shaped, gliding motility, production of flexirubin type pigment were taken for identification and further characterization.

a. Biochemical characterization

Catalase activity was determined by the coverslip method of Taylor and Achanzar (1972) and by adding several drops of 3% (v/v); oxidase reaction was determined by DrySlide Oxidase (Difco Laboratories, Detroit, MI, USA). Other biochemical tests were achieved using the API 20E system[®] (BioMereux Vitek Hazelwood, MO). Congo red reaction was tested by the method of McCurdy (1969) using 0.001% (w/v).

b. Antibiotic susceptibility test

Antibiotic susceptibility test was performed to determine the antibiotic resistant profiles of the isolates using the Kirby Bauer disc diffusion method (Bauer et al. 1966). Antibiotic discs (Oxoid, England) of neomycin (30 µg), oxytetracycline (30 µg), chloramphenicol, (30 µg), amoxicillin (10 µg), ampicillin (10 µg), were used to determine the resistance profiles. Briefly, Cytophaga Broth (CB) was used to prepare bacterial suspensions. The turbidity of suspensions was adjusted as Mac Farland 0.5 and 100 µl of aliquots were spread over CA surface. Antibiotic disks were placed on the surface of the inoculated agar plates and the plates were incubated at 14 °C for 7–10 days. After incubation period, the antibiotic inhibition zone diameters were measured and the results were evaluated and noted.

3 Results and Discussion

A total of 73 gram negative, long and thin bacilli showing gliding movement and flexirubin type pigment production were isolated from the samples. These isolates were then tested by some biochemical tests such as catalase, cytochrome oxidase, ONPG, H₂S and glucides fermentation tests. A total of 36 (49.31%) isolates were found as positive

for catalase and weakly positive for cytochrome oxidase, negative for ONPG, H₂S and glucides fermentation were identified as suspicious for being as *Flavobacterium*.

Our isolates have too many different characteristics, besides the sampling origins and seasons, we found heterogeneity in some biochemical characters. So to confirm which *Flavobacterium* specie or species are responsible of the huge losses in our hatchery a PCR analysis had to be conducted.

Michel et al. (1999) have reported that in some cases, isolation was not possible from infected tissues due to the presence of viable but non-cultivable cells. Furthermore, it should not be ignored that unconscious use of antibiotics in fish farms may lead to inhibit the bacterial growth. Several studies have been performed to determine the antibiotic resistance profiles of *Flavobacterium* bacteria in various regions all over the world and quite variable profiles have been observed. The results in the present study also showed that all strains are sensitive to oxytetracycline and chloramphenicol, but resistant to ampicillin, amoxicillin and neomycin.

4 Conclusion

Bacteria from the *Flavobacterium* genus were isolated for the first time in a semi-arid country, in North Africa—Morocco. We could identify them biochemically and we still need to identify them by PCR and ARN16S.

All strains were found to be sensitive to oxytetracycline and chloramphenicol, but resistant to ampicillin, amoxicillin and neomycin.

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