

Isolation of a Gram Negative Fish Pathogen from Moroccan Rainbow Trout Hatchery in Winter and its Classical Characterization

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Abstract— *Salmonidaquaculture represents one of the most important fish groups in the aquaculture industry (FAO, 2016). However, the success and sustainability of salmonid aquaculture largely depend on disease control. Although Rainbow trout is relatively sensitive to diseases, several bacterial, viral and parasitic diseases have been reported.*

However, only opportunistic pathogens whose infectivity is expressed by a decline in the fish conditions and of its natural defenses, linked to disturbances of the environment or livestock practices. Livestock is the most favorable context for the development of bacterial pathologies, that's why we have too many bacterial pathogenic species for fish; the most serious infections in our latitudes are Aeromonadaceae, Vibrionaceae, Enterobacteriaceae and Flavobacteriaceae (Noga 1996, Austin and Austin 1999, Woo and Bruno 1999).

*Moroccan aquaculture has undergone a rapid development and expansion over the last decade, to this end, Rainbow trout (*Onchorhynchus Mykiss*) have been maintained at Ain Aghbal Fish farm – Azrou-Morocco; for production, livestock and transformation activity and also for commercialization.*

*The most important bacterial freshwater pathogen affecting salmonid livestock in fish farms in Morocco is the cold-water disease as a result of affection by *Flavobacterium psychrophilum* bacteria.*

Our study aimed on approving the presence of this bacterium and to characterize it biochemically, enzymatically and also physiologically.

Keywords— *Moroccan aquaculture, Rainbow trout, *Flavobacterium psychrophilum*, bacterial pathologies, freshwater, characterization.*

I. INTRODUCTION

The cumulative mortality rates due to bacterial infections were higher, fry syndrome, spine deformities, darkening of skin color, and hemorrhages were observed in larvae after hatching, so, the present study was undertaken to investigate the presence of pathogenic bacteria in eggs in order to control the effects of bacterial contamination on the performance of the early stages of trout grown in hatchery systems, and also in juvenile stage. Bacterial, fungal, viral and parasitic diseases have been commonly reported from both wild and reared aquatic systems all over the world (Noga 1996) and they presented crucial considerations for trout production in hatchery systems. Infections result in a high fish morbidity and/or mortality, marketing problems, and associated economic losses (Austin and Austin 2007).

Although Rainbow trout is relatively sensitive to diseases, several bacterial, viral and parasitic diseases have been reported worldwide including Yersiniosis, Furunculosis, Columnaris disease, BCWD and RTFS.

The genus *Flavobacterium* was described for the first time in (1923) by Bergery et al. The taxonomic position of this bacterium has changed considerably over the past years, and it has been variously designated as *Flexibacter psychrophilus* and *Cytophaga psychrophila*, the most recent positioning being *Flavobacterium psychrophilum* (Bernardet et al. 1996).

Flavobacterium psychrophilum which is a widely distributed Gram negative bacterium, belonging to this genus, produces an acute septicaemic infection in juveniles and named rainbow trout fry syndrome (RTFS) while in adult salmonids the pathogen commonly produces extensive necrotic lesions and named bacterial cold water disease (BCWD) (Cipriano and Holt 2005), it is

of a serious importance due to the high fish mortality rate caused by his pathogen and the costs associated with its chemical treatment (Nilsen et al. 2011)

Juvenile fish are primarily affected, with high mortalities in fry. It has been recognized as a worldwide occurring pathogen in freshwater aquaculture, causing substantial economic losses (Madetoja et al.2002). In very young fish, the disease can also be associated with nervous manifestations such as erratic swimming behavior and spiral movements, (Holt et al. 1993).Although outbreaks are more prevalent in winter and spring when water temperature is below 10°C (Nematollahiet al. 2003).

In Morocco, rainbow trout, *Onchorynchus mykiss* (Walbaum), farming is an important industry with 500 tons produced per year, and infection by *F. psychrophilum* has been observed in freshwater farms since 2001.

In Ainaghbal fish farm, Azrou-Morocco the site of this study the eggs and young fish are incubated in filtered spring water, although this, high mortalities were documented in rainbow trout (*Oncorhynchus mykiss*) due to systemic bacterial cold-water disease (BCWD) in 2001. During the first month of rearing, losses of up to 72 % were observed in some batches of fish. All of these fish developed systemic BCWD within 1 to 4 wk after hatching. But there is no epidemiological study on *F. psychrophilum* in Morocco, despite the importance of CWD/ RTFS in local rainbow trout fisheries.

Diseases caused by this pathogen have recently become one of the most crucial problems affecting salmonid culture world wide, the disease occurs in most areas of the world including USA, Canada, Chile, Australia, Japan, Korea and several European countries (Walker and Winton 2010),

According Bernarde and Browman (2006), the adherence to the gills and intestine could be the initial stage of infection by *Flavobacterium psychrophilum*, Nematollahi et al in (2003), give this ability an increasing importance because it is a characteristic of virulent strains, but little is known about the pathogenesis of *Flavobacterium psychrophilum*.

The aim of this study was to characterize *F. psychrophilum* isolates obtained from CWD/ RTFS outbreaks in Morocco using biochemical, enzymatic and antimicrobial susceptibility testing.

Material and Methods

Fertilized eggs of *Onchorynchus mykiss* are imported from France. Samples of diseased Rainbow trout, fertilized eggs and fry were collected from a fish hatchery in Azrou – Morocco in 2015 winter. Fries had a body weight between 20 and 100g.

At the time of sampling, the water temperature was noted as well as physicochemical parameters. Sampling and

water temperature were the only identifiable stress factors preceding evidence of disease (Elliott 1981). For the samples 15g of fertilized eggs and a total of 30 fries with and without eroded fins and/or tails were examined and taken for microbiological analyses.

The identification of *Fl. psychrophilum* and laboratory diagnosis of the disease it causes is traditionally based on conventional culture on agar media and taxonomic analysis (Kritihi et al. 2017).

a. Isolation and identification

Water samples were collected in sterile flacons, fertilized eggs and fish samples were taken separately from the hatchery in sterilized sampling plastic bags, classified by age, back number and the presence of lesions or damages, they were stored in 4°C and transported directly to the laboratory.

In the laboratory, water samples are filtered in sterile conditions and the filters were putted in petri dishes.

For eggs 5g were measured and made for centrifugation in centrifugation tubes containing 5ml of Cytophaga medium for 4000tr/15min, before been inoculated in 10 ml tubes containing cytophaga brought, the tubes were placed on the incubator at 14°C for up to 10 days.

Fish samples taken from internal organs (liver or kidney), damaged gill tissue and, if present, skin lesions of fries body surface were streaked onto Cytophaga agar plates using sterile loops and aseptic techniques then they were directly streaked onto Cytophaga agar plates witch composed by (0.5 g/l tryptone, 0.5 g/l yeast extract, 0.2 g/l sodium acetate, 0.2g/l beef extract with 9 g/l agar, pH 7.2-7.4) (Anacker and Ordal. 1959) 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 15°C for up to 10 days.

After incubation period, yellow-pigmented colonies were chosen and restreaked on the Cytophaga agar to obtain pure isolates.

b. Macroscopic and microscopic identification

After isolation and purification of the suspected colonies a macroscopic characterization of each once was made for confirmation using a magnifying glass.

For the Gram staining the classical method had been followed using a young bacterial culture.

For the motility test a drop of strain's young culture is placed on a coverslip that is encircled with petroleum jelly (or any other sticky material). The coverslip and drop are then inverted over the well of a depression slide. The drop hangs from the coverslip, and the petroleum

jelly forms a seal that prevents evaporation. Finally the coverslip is placed on a microscope slide, often is used in dark illumination to observe the motility of bacteria Koch (1878).

c. 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 realized using the API 20E and 20NE system (BioMereuxVitek Hazelwood, MO). Congo red reaction was tested by the method of McCurdy (1969) using 0.01mg/ml of a congo red solution.

Colonies have been tested for Gram staining, presence of flexirubin type pigment, cytochrome oxidase activity, catalase production and motility (Lorenzen et al. 1997). Strains were found to be Gram negative rod shaped, adopting gliding motility, producers of flexirubin type pigment were taken for identification and further characterization.

d. Enzymatic characterization

The ability to hydrolyze elastin and gelatin was studied by streaking each isolate on Cytophaga agar supplemented with 0.1% and 3% (w/v) of elastin and gelatin respectively (Krister and Wiklund. 2015); for the chitin and starch hydrolyze ability 5% of each element was add to the CA. For casein 20 ml of sterilized skimmed milk was add to the CA After incubation at 15 °C for 7 days, a

positive reaction was indicated by a clear zone in the surrounding turbid agar medium around the inoculum.

e. Antibiotic susceptibility test

Antibiotic susceptibility test was performed to determine the antibiotic resistant profiles of the isolates using the KirbyBauer 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 for determining 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 CytophagaAgar surface. Antibiotic disks were placed on the surface of the inoculated agar plates and then they were incubated at 14°C for 7-10 days. After incubationperiod, the antibiotic inhibition zone diameters were measured, evaluated and noted.

II. RESULTS

A total of 37isolated Gram negative, long and thin bacilli strains were isolated from samples. These isolates were then tested by some biochemical tests such as catalase, cytochrome oxidase, ONPG, H2S andglucides fermentation tests. Only 6 isolates which were found as positive for catalase and weakly positive for cytochrome oxidase, negative for ONPG, H2S Sucreoxidation/fermentation andshowed gliding movement and flexirubin type pigment production were identified as suspicious for being Flavobacterium spp bacteria, as cited by Nakagawa and Yamasato (1996).

Table.1: Biochemical and Enzymatic characteristics of *Flavobactreium psychrophilum* strains isolated from Rainbow trout (*Onchorhyncus mykiss*) in Morocco and other strains from Europe and North America.

Strains	winterisolates						Pachet al 1986	Bernardet et Kerouault 1989	Lehmann et al 1991	Holt et al 1993
	A1N201	A2N202	A3N203	A4N204	A5N205	A6N209				
Oxidase	P	P	P	P	P	P	N	P	P	N
Catalase	P	P	P	P	P	P	P	P	P	P
Casein	P	P	P	P	P	P	P	P	P	P
Gelatin	P	P	P	P	P	P	P	P	P	P
Chitin	N	N	N	N	N	N	N	N	N	N
Congo red	P	P	P	P	P	P	-	N	N	-
ONPG	N	N	N	N	N	N	-	N	N	-
Lecitin	N	N	N	N	P	N	N	P	P	N
Starch	N	N	N	N	N	N	N	N	N	N
Cit	P	P	P	P	P	P	-	-	-	-
H ₂ S	N	N	N	N	N	N	N	N	N	N

Urea	N	N	P	N	N	N	-	-	-	-
Nitrate	N	N	N	N	N	N	N	N	N	N
Sucre O/F	N	N	N	N	N	N	N	N	-	N
G.M	P	P	P	P	P	P	P	P	P	P
Flexirub	P	P	P	P	P	P	-	P	P	P

P: positive character / N: negative character / (-):no data

For the antibiotic resistant profiles of our isolates, all strains were found to be sensitive to oxytetracycline with an inhibition diameter > 8mm and to chloramphenicol with an inhibition diameter > 16mm, but resistant to

ampicillin and amoxicillin with a inhibition diameter <2mm for both of them and to neomycin with a inhibition diameter <4mm, disc diameter not include.

Table.2: The antibiotic resistant profiles of the isolates

Strains	A1N201	A2N202	A3N203	A4N204	A5205	A6209
ATB						
<u>Néomycine</u> (30µg)	<u>R</u>	<u>R</u>	<u>R</u>	<u>R</u>	<u>R</u>	<u>R</u>
<u>Oxytetracycline</u> (30µg)	<u>S</u>	<u>S</u>	<u>S</u>	<u>S</u>	<u>S</u>	<u>S</u>
<u>Chloramphenicol</u> (30µg)	<u>S</u>	<u>S</u>	<u>S</u>	<u>S</u>	<u>S</u>	<u>S</u>
<u>Amoxiciline</u> (10µg)	<u>R</u>	<u>R</u>	<u>R</u>	<u>R</u>	<u>R</u>	<u>R</u>
<u>Ampiciline</u> (10µg)	<u>R</u>	<u>R</u>	<u>R</u>	<u>R</u>	<u>R</u>	<u>R</u>

III. DISCUSSION

In the current study, we report isolation of *Flavobacterium* spp from Rainbow trout imported fertilized eggs and fries and from juveniles tanks water. Witch go with the results found by Brown et al, (1997) in their study proving that the causal agent of bacterial coldwater disease *Flavobacterium psychrophilum* is transmitted within salmonid eggs and ovarian fluid, same as *Renibacterium salmoninarum*, the causal agent of bacterial kidney disease, which could survive within salmonid eggs (Evelyn et al. 1984, Barker et al. 1991, Yousif et al. 1994). It is likely that egg transmission is a phenomenon of concern in aquaculture industry.

Data shown in this study might shed light on the potential role of the fertilized eggs importation in spreading *Flavobacterium* spp infection from an area to another even the difference on climate.

During the last decade, the productivity of the aquaculture industry is much intensified. Currently, it is a major economic activity in many countries (FAO. 2007). Mass production on fish farms can expose fish to stress conditions that can cause infections by pathogens including *Flavobacterium psychrophilum* (Wakabayashi 1991). Bacteria from *flavobacterium* genus are responsible for significant economic losses in salmonid culture (Nematollahi et al. 2003). That has led to increase

interest in the rapid and reliable methods for detection and identification of bacterial fish pathogens (Nilsson and Strom 2002). In other side, Michel and 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; this was the case in our study because we got non-separated bacterial culture especially for the affected and eroded fins in fry's samples.

Phenotypically, on the modified Cytophaga agar medium our isolates produced colored colonies varied from bright yellow to orange with 2-3 mm in diameter with thin spreading margins. isolated strains were then identified as *Flavobacterium psychrophilum* spp using conventional techniques, they are Gram-negative, long bacilli, produced flexiruben upon addition of 20% KOH (colonies turned brown orange), motile by gliding, very weakly catalase positive, cytochrome oxidase positive, gelatin test positive, and non-agarolytic.

Bernardet and Kerouault (1989) and Holt et al (1993) note that *F psychrophilum* grows best in Shieh's and TYES media which suggests that medium composition is important when testing growth characteristics at the limits of the physiological range for the species, this justified our choice for the first time to work with the TYES medium, in order to limited the study to the isolation of this bacterium.

For our strains no growth was observed on TSA plates at 15°C or 20°C or in the presence of NaCl concentrations above 1.5% and this is consistent with reports made by Holt et al (1993). Although Bernardet and Kerouault (1989) were not able to grow their isolates in this NaCl concentration. The Moroccan isolates showed an optimal growth on 15°C and there was no growth at temperatures above 30°C, same results were found by Bernardet and Kerouault 1989; Lehmann et al. 1991; and Holt et al. 1993.

The phenotypic, biochemical and growth characters determined in this study of 6 isolates of *Flavobacterium psychrophilum* are in good agreement with published data for this taxon (Pacha 1968; Bernardet and Kerouault 1989; Lehmann et al. 1991; Holt et al. 1993).

Performing the oxidation and/or fermentation tests using glucose as the only source of carbon revealed that the isolates were negative. They were also not able to use sucrose, starch or glucose in their basal media. Results of BioMerieux Api 20NE rapid test strip inoculations were consistent with the biochemical reactions of *Flavobacterium psychrophilum* (Cipriano and Holt 2005)

Our isolates have too many different characteristics, besides the sampling origins and seasons, we found heterogeneity in some biochemical characters, that could be explained by the presence of too many species of *Flavobacterium* bacteria not only one. So to confirm which *Flavobacterium* specie or species are responsible of the huge loss in our hatchery a PCR analysis must be conducted.

Furthermore, it should not be ignored that unconscious use of antibiotics in fish farms may lead to inhibit the bacterial growth for the sensitive species belonging to the banal flora including *Flavobacterium* bacteria. 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. (Duchaud et al. 2018).

The results in the present study also showed that all strains are sensitive to chloramphenicol, some of them to oxytetracycline but they are resistant to ampicillin, amoxicillin and neomycin.

IV. CONCLUSION

In conclusion, we report that bacteria from the *Flavobacterium* genus are isolated from rainbow trout imported fertilized eggs and fries 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.

Furthermore, the long-term natural cohabitation between clinically infected and non-infected fries or between the

fries and the un-hatched eggs in the same tank or in the same water suggests a potential for the spread of *Flavobacterium* spp bacteria. Because it is difficult to assess the impact of the water transmission of the infection and its subsequent effects on salmonid fish population in the fish farm in Azou-Morocco. Further work is required to characterize the virulence determinants of the Moroccan isolates and compare them with other virulent strains of *Flavobacterium psychrophilum* from other geographic regions.

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