First isolation and characterisation of *Flavobacterium psychrophilum* from diseased rainbow trout (*Oncorhynchus mykiss*) farmed in Mexico

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Abstract

In 2015, rainbow trout (*Oncorhynchus mykiss*, weighing 4 ± 0.5 g) mortalities occurred at an aquaculture facility near the "*Nevado de Toluca*" National Park in Mexico. Affected fish showed exophthalmia, body darkening, abdominal distension, and pale gills. Pure culture, splenic isolates were recovered and biochemically- and PCR-identified as *Flavobacterium psychrophilum*. These isolates were virulent, representing a potential threat to *O. mykiss* aquaculture. This is the first study to identify and characterise *F. psychrophilum* as causative of a severe disease outbreak in Mexico-farmed *O. mykiss*.

Introduction

Flavobacterium psychrophilum is a fish bacterium that causes bacterial cold water disease (BCWD) and rainbow trout fry syndrome (RTFS), which cause substantial economic losses to the salmonid industry. Reported in many parts worldwide (see review Nematollahi et al., 2003; Barnes and Brown, 2011), including recently Argentina (Moreno et al., 2016), this pathogen is a suspected cause of aquaculture mortalities in Mexico (Ortega et al., 1998).

Rainbow trout (*Oncorhynchus mykiss*) production in Mexico is dependent on imported eggs (≈ 17.5 M in 2009), and nine Mexican states account for most trout production (≈ 9,757 t in 2013; CONAPESCA, 2013). For over a decade, BCWD and RTFS have been suspected, but isolates remain unidentified (Ortega and Valladares, 2015).

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In 2015, rainbow trout mortalities occurred at a Mexican aquaculture farm. Clinical RTFS signs were found, namely, fish mortality with exophthalmia, body darkening, abdominal distension, and pale gills. Bacteria were obtained, and this is the first study to isolate and identify *F. psychrophilum* causing RTFS in Mexican *O. mykiss*.

Materials and methods

Specimens

In January 2015, an epizootic outbreak occurred in central Mexico in *O. mykiss* (4 ± 0.5 g) farmed in concrete ponds with untreated spring water (13 ± 1ºC). Over 15 days, mortalities reached 9,000 of 18,000 total fish. All fish were originally hatched in another water source at a broodstock centre near the affected farm. Moribund fish (n = 10) were given a benzocaine overdose, transported to the lab, and subjected to post-mortem, pathological, and bacteriological exams.

Histology

Internal samples were fixed in vials with 10% buffered formalin, dehydrated, and embedded in paraffin wax using standard procedures. Each tissue was sectioned at 5 mm and haematoxylin and eosin stained (Fernando et al., 1972). Sections were observed under an Olympus BH2 light microscope.

Microbiology

Kidney and spleen scrapings were microscopically and microbiologically analysed. Slides were stained for Gram observations. Kidney and spleen bacterial samples were aseptically taken, streaked onto agar plates with Tryptone-Yeast Extract-Salt (TYES; 0.4% tryptone, 0.05% yeast extract, 0.02% anhydrous calcium chloride, 0.05% magnesium sulphate heptahydrate, and 1.2% agar, pH 7.2), and aerobically incubated at 15°C for ≤ 10 days. Representative predominant morphotype colonies were selected, streaked onto a new TYES plate to obtain pure cultures, and stored at –80°C in Criobille tubes (AES Laboratories).

Phenotyping

Isolates were phenotypically characterised (Bernardet et al., 2002). The *F. psychrophilum* type strain, NCIMB 1947^T, was comparatively used. Biochemical reactions included colony morphology and pigmentation; Gram staining; cytochrome oxidase; Congo red absorption; presence/absence of flexirubin-type pigment; catalase reaction (3% H_2O_2); and cell morphology and gliding motility. Growth was tested in TYES under aerobic conditions at 4, 15, 18, 25, and 37°C and in TYES broth supplemented with 0, 1, 2, 3, 4, and 5% NaCl. Gelatin, starch, aesculin, and Tween 80 were hydrolysed using TYES media.

All phenotype tests were aerobically incubated at 18°C and observed after 1, 4, 7, and 10 days before being recorded negative. The API ZYM system (bioMèrieux) was used following manufacturer instructions, but incubation was fixed at 18°C and read after 72 h.

In addition, antimicrobial susceptibility of the *F. psychrophilum* isolates was determined on diluted Mueller–Hinton agar and the following disk concentrations were tested (Oxoid, mg per disk): oxytetracycline (30), florfenicol (30), oxolinic acid (2) and ampicillin (10). The diameter of each zone of inhibition was determined after 68–72 h of incubation at 15°C.

PCR

Total DNA was extracted from pure bacterial isolates using the InstaGene Matrix (Bio-Rad)

according to manufacturer instructions. DNA was stored at –20°C until PCRs. *F. psychrophilum* was confirmed using the species-specific FP1 and FP2 primers (Urdaci et al. 1998). PCR amplifications used a Mastercycler Personal Apparatus (Eppendorf) and the GoTaq Green Master Mix Kit (Promega), which included all reaction reagents, except specific primers and the DNA template. Negative controls used the same reaction mixture and sterile distilled water instead of template DNA. The products were electrophoresed in a 1.5% (w/v) agarose gel visualised with a 1/10,000 GelRed Nucleic Acid Gel Stain (Biotium) and photographed under UV light. An AccuRuler 100-bp Plus DNA RTU ladder (Maestrogen) was used as a molecular mass marker. A single 1088 bp band positively identified *F. psychrophilum*.

Genetic characterisation

In epidemiology, many genomic typing methods exist for *F. psychrophilum*. PCR assays, per Ramsrud et al. (2007), distinguished 16S rRNA alleles, which can indicate polymorphisms or genetic lineages I and II distributions (Soule et al., 2005a). Two independent PCRs were performed using CFS259-93 and ATCC 49418^T alleles-specific primers.

Restriction fragment length polymorphism (RFLP) analysis was conducted using two primer pairs (Izumi et al., 2003); 1) a degenerate universal primer pair, GYR-1 and GYR-1R, and 2) a specific primer pair for *F. psychrophilum* gyrB, PSY-G1F and PSY-G1R. Amplification cycles for denaturation, primer annealing, and primer extension were carried out using published protocols (Izumi et al., 2003). After electrophoresis, each *F. psychrophilum* was restriction enzyme digested with *Rsa*I or *Hinf*I endonucleases (New England BioLabs), according to the manufacturer's instructions. The digested solution (10 µL) was analysed by horizontal electrophoresis.

Virulence tests

F. psychrophilum Flavo-COS-042-15.1 was used to investigate bacterial virulence capacity against healthy *O. mykiss* (5-6 g). Fish samples (i.e. gills, mucus, skin, spleen, and kidney) were microscopically, bacteriologically, and PCR assessed (Urdaci et al., 1998) to ensure a pre-challenge absence of *F. psychrophilum* or other pathogens. Fish were separated in tanks (10 L, 15 fish per tank) containing aerated dechlorinated water and acclimatised for seven days. The inoculum was prepared from overnight TYES broth cultures (18°C, 120 rpm). Each experimental fish was intramuscularly injected with 0.1 mL (5 x 10^7 CFU, direct plate count). Control fish were injected with only TYES broth. All trials were conducted in a closed system at 17 ± 1°C, a pH of 7.6-7.8, and using a 12L:12D light regime. Fish were fed daily at 1.5% body weight, and tank water was changed bi-daily.

Clinical signs and dead fish were monitored/removed daily and microbiologically analysed to confirm *F. psychrophilum*-caused mortality. Kidney, spleen, and external lesions were directly streaked onto TYES plates and incubated at 18°C for one week. If pure pigmented isolate cultures were obtained, biochemical and PCR methods of identification were used.

Results and discussion

Externally, *O. mykiss* exhibited body darkening, bilateral exophthalmia, and abdominal distension. Internally, splenomegaly and located/diffuse splenic haemorrhages, some of which

were yellowish, were found (Figure 1A, B). Ascites, generally pale internal organs, adherences to the stomach and spleen, and necrosis of the abdominal wall and of the area surrounding the spleen were also recorded. These results coincide with clinical RTFS (Bustos et al., 1995).

The spleen was the most affected (Figure 2), with splenic capsule destruction; diffuse, chronic and congested necrosis; an absence of lymph cells; a predominance of splenic stromal cells; necrotizing, but not suppurative, pancreatitis and steatitis; necrotic myositis under the spleen, with muscle fibre breakage; peritonitis; and renal edema with melanomacrophage proliferation. Splenic wet mount and Gram staining microscopy from all moribund fish showed abundant long rods of Gram-negative bacteria able to flex. Pure splenic cultures of orange-yellowish colonies grew on TYES after six days from 2 of 10 specimens, while from the remaining rainbow trout colonies were recovered in mixed cultures. Two orange-yellowish bacterial isolates (Flavo-COS-042-15.1 and Flavo-COS-042-15.2), which were biochemically homogeneous and identical to NCIMB 1947^{T}, were used in biochemical and genetic assays.

Phenotypically, all isolates were Gram-negative, long, slender rods with gliding motility that produced weak cytochrome oxidase and catalase reactions. All isolates contained a cell wall-associated flexirubin-type pigment but did not absorb Congo red. Colonies grown for five days on TYES plates were bright orange-yellowish with entire edges, a raised and shiny surface, and ≈ 3 mm diameter. Growth occurred at 4-18ºC and only without NaCl. All biochemical traits are shown in Table 1.

On the other hand, our results obtained from disk diffusion showed an identical antibiotic susceptibility pattern for both Mexican isolates to florfenicol, oxytetracycline and ampicillin with mean inhibition zones of 59, 52 and 31 mm, respectively. In addition, as reported by other authors for *F. psychrophilum* (Henríquez-Núñez et al., 2012), none of the isolates tested generated produced any inhibition zones (<7 mm) for oxolinic acid.

For API ZYM, the detected enzyme numbers (i.e. 19) and activity levels were similar to the type strain. No enzymes were involved in carbohydrate metabolism, but were positive to alkaline phosphatase, esterase C4, lipase esterase C8, leucine arylamidase, valine arylamidase, acid phosphatase, and naphtol-AS-BI-phosphohydrolase. Thus, the two recovered Mexican isolates had classical biochemical patterns for *F. psychrophilum* (Bernardet and Grimont, 1989; Lorenzen and Olensen, 1997).

A specific PCR (Urdaci et al., 1998) identified the *F. psychrophilum* 16S rRNA gene, and isolates amplified a single 1088 bp fragment, supporting *F. psychrophilum* classification.

Typing processes are essential for understanding pathogen outbreaks, cross-transmission, geographical and host distributions, virulence, and vaccination programmes (Olive and Bean, 1999). Ramsrud et al. (2007) proposed PCRs to type *F. psychrophilum* genetic lineages (I and II) and 16S rRNA variants (6 base differences) (Soule et al., 2005a, b). Both *F. psychrophilum* isolates were positive for the CSF-259-93 allele of lineage II, and RFLP classified isolates as genotypes B and R. There is also evidence that European and Chilean *O. mykiss* isolates fit these

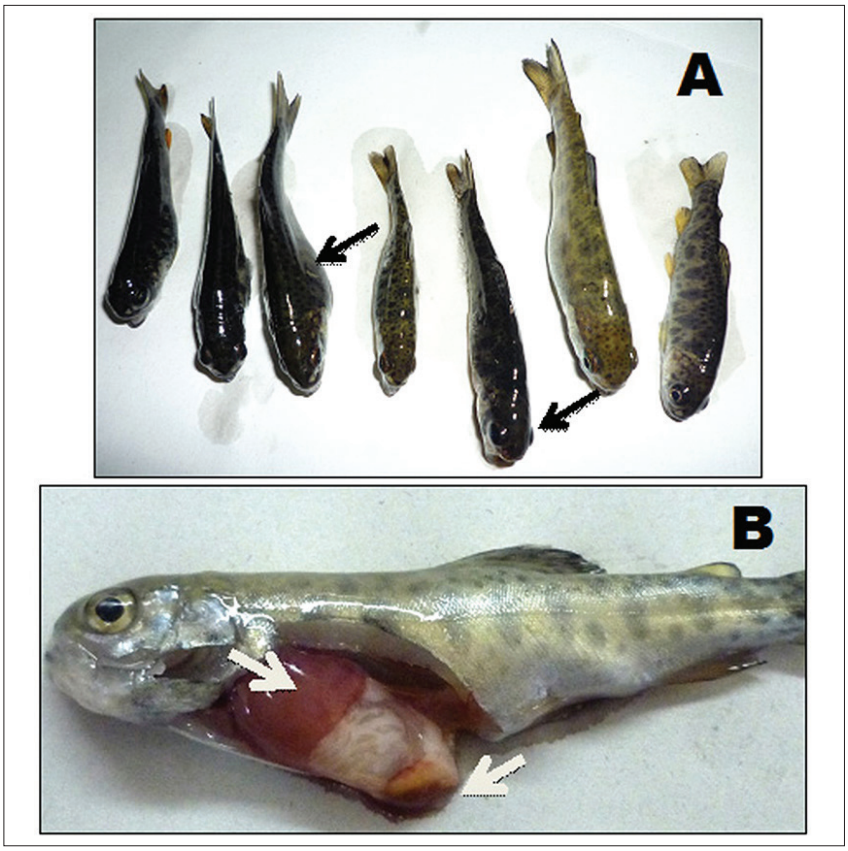

Figure 1. *O. mykiss* with (A) body darkening, bilateral exophthalmia (arrow) and abdominal distension (arrow) and (B) splenomegaly, diffuse splenic haemorrhages (arrow), and pale internal organs (arrow).

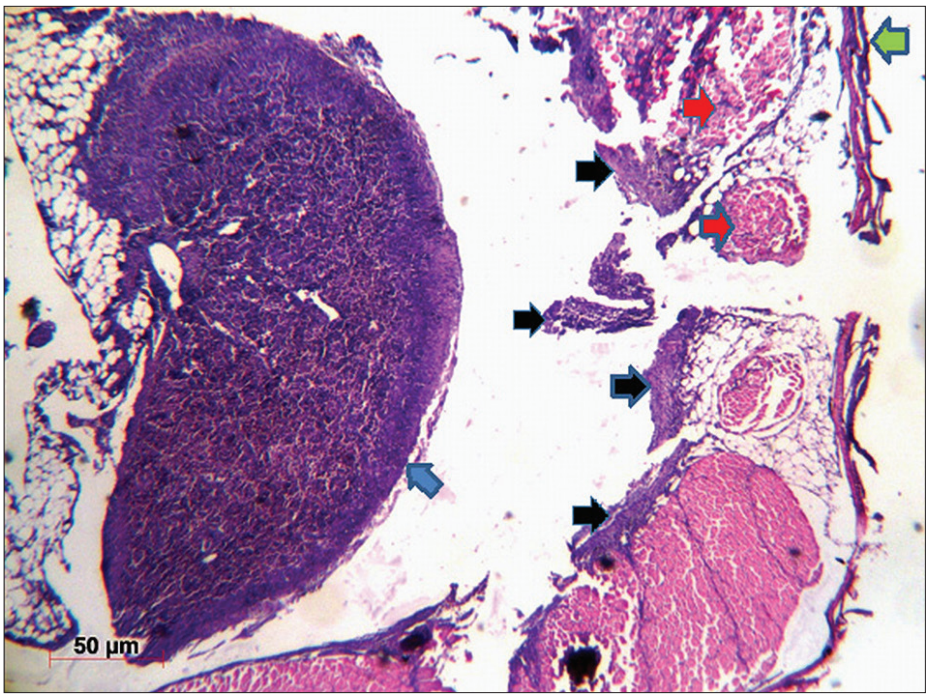

Figure 2. Representative H&E-stained *O. mykiss* tissues. Transversal splenic tissues with fibronecrotic splenitis (blue arrow), necrotic steatitis (black arrows), and necrotic myositis of the abdominal muscle (red arrows). Green arrow indicates epidermal tissue. Bar = 50 mm.

Table 1. Phenotypic and biochemical properties of Mexican isolates. +, Positive; –, Negative, and w, weakly positive.

Characteristics	**Flavo-COS-042-15.1**	**Flavo-COS-042-15.2**	**NCIMB 1947^T**
Gram	–	–	–
Morphology	long rods	long rods	long rods
Motility	gliding	gliding	gliding
Catalase	w	w	w
Oxidase	w	w	w
Flexirubin pigment	+	+	+
Congo red adsorption	–	–	–
Growth at (°C)			
4	+	+	+
10	+	+	+
15	+	+	+
18	+	+	+
22	–	–	–
37	–	–	–
Growth with NaCl			
0	+	+	+
1	–	–	–
3	–	–	–
5	–	–	–
7	–	–	–
10	–	–	–
Hydrolysis of			
Aesculin	–	–	–
Gelatine	+	+	+
Tween 80	+	+	+
Starch	–	–	–
Genotypes using			
PCR-RFLP	B/R	B/R	B/S
16S rRNA allele	CSF-259-93	CSF-259-93	Both*

* Positive for ATCC 49418 and CSF-259-93 alleles (Soule et al., 2005b)

genotypes (Izumi et al., 2003; Valdebenito and Avendaño-Herrera, 2009), suggesting Mexican *F. psychrophilum* might have originated from fish and eggs imports. Genotypes B and S and both alleles (ATCC 49418^T and CSF-259-93) were found in the type strain (Izumi et al., 2003; Valdebenito and Avendaño-Herrera, 2009).

F. psychrophilum Flavo-COS-042-15.1 caused 100% mortality. In the first 48 h, *O. mykiss* suffered skin darkening and scale loss. By day 5, deep, ulcer-like wounds were present on the lateral side and inoculation site (data not shown). On day 8, the first mortality occurred, and 100% mortality was reached on day 10. The control group had no mortalities.

F. psychrophilum Flavo-COS-042-15.1 was recovered from kidney, spleen, and lesion samples, but the lesion produced mixed cultures, which could be due to direct environment contact. Biochemical and PCR assays confirmed all isolates as *F. psychrophilum* (Table 1), supporting pathogenicity.

As occurring in other countries and in fish farming worldwide, the increasing or the intensification of aquaculture activities in Mexico have been accompanied by the appearance of new diseases in Mexico such as Francisellosis in tilapia (Ortega et al., 2016). This is the first study to identify and characterise *F. psychrophilum* as a causative outbreak agent in Mexican *O. mykiss* aquaculture. Severe clinical signs and mortalities indicate isolate virulence. Thus, *F. psychrophilum* represents a relevant potential risk for local *O. mykiss* farming, and future studies should establish clinical significance for trout aquaculture in Mexico.

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