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Short communication

Intramuscular challenge of rainbow trout (*Oncorhynchus mykiss*) with two Norwegian field strains of *Flavobacterium psychrophilum*

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ABSTRACT

In recent years there has been an increasing occurrence of *Flavobacterium psychrophilum* infections in farmed salmonids in Norway. The current study describes two field isolates of *F. psychrophilum* collected from farmed rainbow trout (*Oncorhynchus mykiss*) fingerlings and post smolts in Norway. Virulence of the two isolates was tested *in vivo* by intramuscular (IM) and/or intraperitoneal (IP) challenge of disease free, un-vaccinated rainbow trout. The isolates were concluded to be highly virulent compared to a reference isolate as they yielded high mortality after IM challenge even at low challenge doses. The more virulent of the two isolates was further used to establish a challenge model to evaluate the efficacy of vaccines against infections with *F. psychrophilum*. Three groups were included in the vaccination-challenge study; a vaccinated group given a 6 antigen (Ag) component vaccine containing *F. psychrophilum* antigens (6 Ag/*F.psy*$^{+}$), a control vaccinated group administered a similar 5 antigen component vaccine without *F. psychrophilum* antigens (5 Ag/*F.psy*$^{-}$), and a non-injected negative control group. Results from the IM challenge demonstrated that 1) our challenge model is able to discriminate between protected and unprotected experimental groups and 2) that the vaccine induced protection is specific against *F. psychrophilum* as mortality in the 5 Ag/*F.psy*$^{-}$ group was equally high as in the negative control, while the 6 Ag/*F.psy*$^{+}$ induced a high level of protection ($RPS_{60} = 86.7\%$). The present study is one of the first to describe protection against *F. psychrophilum* infections induced by a multicomponent injection vaccine.

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Flavobacterium psychrophilum is the etiological agent causing epizootics of rainbow trout fry syndrome (RTFS) and bacterial cold water disease (BCWD) in farmed salmonids. *F. psychrophilum* cells are Gram negative, flexible rods [7,18] with proteolytic activity [13]. Disease severity generally depend on the size of the fish [17], host species [14] and bacterial serotype and strain. Of seven serotypes so far described, the serotypes Th and Fd have been linked to disease outbreaks in rainbow trout (*Oncorhynchus mykiss*) [7,12]. Typical clinical signs of *F. psychrophilum* on salmonids include severe necrotic skin lesions and erosion of the peduncle, with chronically infected fish displaying systemic distribution of *F. psychrophilum* to vascularized tissues [21].

Compared to European aquaculture, there have so far been very few reports on mortality associated with *F. psychrophilum* in the Norwegian farming of salmonids. However, during 2008 *F. psychrophilum* was detected at 16 farm sites with rainbow trout and Atlantic salmon (*Salmo salar L*) both at summer (15–16 °C) and at winter (3 °C) temperatures. This was a drastic increase in the number of systemic infections compared to previous years, with only two outbreaks reported in 2004 and one in 2007 [15,16] Mortality in these cases were typically higher in fry than in smolts [19]. In later years, disease on post smolts of rainbow trout has been a recurring challenge in West-Norway in the period from May to August linked to high levels of ice melting reducing sea water salinity. *F. psychrophilum* is able to survive in brackish water [10] and this has been suggested a new niche for more virulent strains of the pathogen [16].

With development of a challenge model for *F. psychrophilum* as the main objective, two Norwegian field strains have been isolated and their virulence tested *in vivo* by intramuscular injection in vaccinated and/or un-vaccinated rainbow trout. The strains were isolated from two different outbreaks, one in fresh water and one in sea water. Isolate AL20055 (Th + Fd serotype) affected fingerlings at 30–40 g in fresh water resulting in approximately 10% accumulated

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mortality. Disease outbreak occurred in June 2009 at a water temperature around 15 °C, just after vaccination and relocation of fish. Isolate AL20211 (Th serotype) was isolated from a sea site located in the Osterøy area in the county of Hordaland, Norway in August 2011. At this farm site, increased mortality was registered in the period from mid June until August with accumulated mortality reaching 8% in the same period. Additionally, salmonid alphavirus (SAV, causative agent for pancreas disease) was detected in the end of May while *F. psychrophilum* was detected about one month later. It is uncertain to what extent each pathogen contributed to the overall mortality at the farm site. Light microscopy and smears of the isolated bacteria in potassium hydroxide (KOH) revealed rod-like, Gram negative bacteria. DNA isolation followed by polymerase chain reaction (PCR) further identified the isolates as *F. psychrophilum*. P13-4/96, an isolate characterized as Th serotype isolated from rainbow trout in Finnish waters during 1996 [9] was used for comparison of virulence in the *in vivo* studies to be described, as it has been widely used in previous challenge tests resulting in high mortality on rainbow trout of similar size [3,8]. Cultivation of the three *F. psychrophilum* isolates was carried out using tryptone yeast extract salts (TYES) broth and agar at 15 °C [4].

To conduct the experiments presented here, a total number of 270 healthy, unvaccinated rainbow trout at 36.6 g (±SD 7.6 g) were transported from a commercial farm (Lerøy Vest, Dep. Bjørsvik smolt) and acclimatized to laboratory conditions at the facilities of Industrilaboratoriet i Bergen (ILAB) at Bergen High Technology Center (HIB) for one week prior to any further handling. Samples from spleen and head kidney from 5 fish were confirmed negative for *F. psychrophilum* by QPCR screening performed at an ISO-certified commercial test laboratory (PATOGEN ANALYSE AS, Norway) prior to vaccination and challenge. Enzyme linked immunosorbent assay (ELISA) also confirmed that the fish did not have serum antibodies specific for *F. psychrophilum*. Both studies presented here were performed in fresh water at 12 °C with oxygen saturation >70% (in the outlet) and continuous lighting.

Choice of challenge isolate, route of administration and challenge dose are critical factors to achieve a challenge model with discriminatory capacity between protected and un-protected fish. Experimental challenge of rainbow trout with *F. psychrophilum* is mainly performed by bath or injection. At the early life stages (<5 g) bath challenge is the preferred method as rainbow trout still are susceptible to infection and the challenge mimics the natural route of infection [1,6]. Larger rainbow trout (>5–10 g) is normally less susceptible to experimental challenge with *F. psychrophilum* and challenge is subsequently performed by intramuscular (IM) injection [2]. In the present study, 15 rainbow trout in each challenge group were pre-challenged either by the IM or the intraperitoneal (IP) route with 0.05 ml *F. psychrophilum* (AL20211) at doses of 5.0×10^7, 5.0×10^6 or 5.0×10^5 CFU diluted in phosphate buffered saline (PBS) (results not shown). IP challenge causing mortality has been demonstrated by others [11] but was unsuccessful in the current study as no fish died post challenge even at the highest

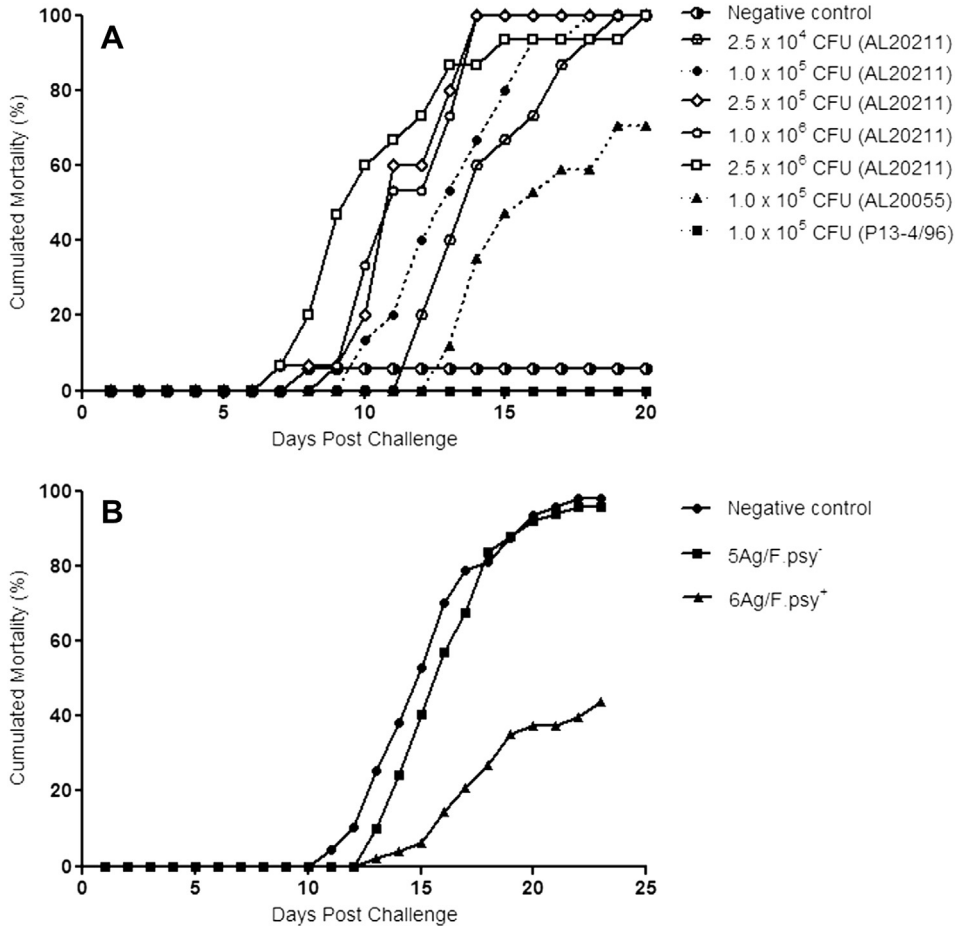

Fig. 1. Accumulated mortality in rainbow trout (*Oncorhynchus mykiss*) after intramuscular challenge with *Flavobacterium psychrophilum*. 1A) Virulence test with two Norwegian field strains (AL20055 and AL20211) and a Finnish isolate (P13-4/96) of *F. psychrophilum*. The dashed lines indicates the three different isolates at equal challenge doses ($n = 15$/challenge group). 1B) Accumulated mortality after intramuscular challenge of vaccinated (6 Ag/*F.psy*$^+$), mock-vaccinated (5 Ag/*F.psy*$^-$) and a negative control using the most virulent *F. psychrophilum* strain AL20211 at 5×10^3 CFU/injection. Each graph represents average mortality from two tanks with 25 fish/experimental group ($p < 0.0002$).

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