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Efficacy of a divalent and a multivalent water-in-oil formulated vaccine against a highly virulent strain of *Flavobacterium psychrophilum* after intramuscular challenge of rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Flavobacterium psychrophilum is a well-known pathogen causing significant problems in aquaculture worldwide. In recent years an increasing number of disease outbreaks caused by *F. psychrophilum* has been reported on juvenile and post smolts of rainbow trout (*Oncorhynchus mykiss*) in Norway. The current study was performed to assess the efficacy of two autogenous water-in-oil formulated vaccines containing whole cell antigens of *F. psychrophilum* to induce protective immunity against challenge. The vaccines were formulated either as multivalent (FLAVO AVM6) or divalent (FLAVO IPN) and administered by the intraperitoneal route. Intramuscular challenge with a field strain of *F. psychrophilum* was carried out 552 day degrees post vaccination, at a time when the FLAVO AVM6 and FLAVO IPN vaccinated groups had significantly higher antibody responses compared to the negative control. Results from the challenge study showed that the multivalent and the divalent vaccines had capacity to induce significant protection, with $RPS_{60} > 87\%$ and $RPS_{end} > 77.5\%$ for both vaccines. The high level of protection seen in the vaccinated groups was also reflected in the reduced ulceration rates observed at the injection site. Combining our results demonstrate that vaccination with FLAVO AVM6 and FLAVO IPN induces responses capable of protecting rainbow trout against infections with *F. psychrophilum*.

1. Introduction

Flavobacterium psychrophilum is a Gram negative bacterium causing rainbow trout fry syndrome (RTFS) and bacterial cold water disease (BCWD) in salmonids [1]. Clinical severity may vary considerably and depend mainly on strain virulence and fish size, with mortality generally being highest in fry (<5 g) and throughout the fresh water stages [2,3]. During outbreaks, ulcerations in the skin and muscle are often observed along with systemic spread of the bacteria to the spleen, head kidney and heart [4–6].

Antimicrobial therapy is still the most used practice to treat disease outbreaks caused by *F. psychrophilum*. As therapy resistance is an increasing problem, development of efficient vaccines seems to be the only foreseeable prophylactic measure to reduce the establishment, progression and spread of the disease. Immersion vaccines have so far fell short in giving sufficient and durable protecting against RTFS in salmonid fry [7]. Efficient injection vaccines might thus be crucial to protect high value species such as salmonids against *F. psychrophilum* at later stages in the production cycle. Formulations containing immunodominant/immunogenic sub-unit antigens or heat- and formalin inactivated whole bacterins, as well as DNA based vaccine delivery systems have been reported on mono- or polyvalent injection vaccines against *F. psychrophilum*. A study using heat shock proteins (Hsp) 60 and 70 from *F. psychrophilum* either as sub-unit or DNA vaccines concluded that these antigens were poor inducers of antibody responses and protective immunity [8], while using a 18 kDa outer membrane-associated OmpH-like glycoprotein has been suggested to have the capability to induce high antibody titers and significant protective immunity in rainbow trout [9–11]. However, comparing sub-unit (P25-33, a 25–33 kDa protein fraction) and inactivated bacterins (either sonicated or whole cell) administered without an oil-adjuvant demonstrated that the latter resulted in the highest level of protection and superior vaccine induced antibody responses [12]. Furthermore, cross protection between the two serotypes (Fd isolate F6A/00 and Th isolate P13-4/96) has been demonstrated after intraperitoneal administration of oil-adjuvanted vaccines followed by intramuscular challenge, suggesting that serologically different *F. psychrophilum* share protective antigen(s). For multivalent vaccines against *F. psychrophilum* it has been proposed that

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certain vaccine compositions, in terms of what bacterial antigens are included, might have inhibitory effect on the specific responses to the vaccine antigens [13].

In recent years an increasing number of disease outbreaks caused by *F. psychrophilum* have been recorded in rainbow trout and Atlantic salmon (*Salmo salar* L) both at hatcheries and farming sites in Norway [4;5], making the need for development of efficacious vaccines more evident. Previously we have established a challenge model using a highly virulent Norwegian field strain of *F. psychrophilum* for challenge of rainbow trout by the intramuscular route (paper in review). Using an optimized challenge protocol with discriminatory capacity between protected and un-protected fish, we here report efficacy data after vaccination of rainbow trout with either a divalent (FLAVO IPN) or a multivalent (FLAVO AVM6) injection vaccine followed by challenge with the highly virulent field strain of *F. psychrophilum*.

2. Materials and methods

2.1. Research animals and rearing conditions

Commercially bred un-vaccinated rainbow trout with an average body weight of 33.1 g (±SD 5.3 g) were obtained from Lerøy Vest, Dep. Sauvågen. The fish were acclimatized for a minimum of 1 week prior to vaccination or challenge and fed *ad libitum* with a commercial feed during all experiments. One day prior to vaccination or challenge fish were taken off feed. All studies were performed at the facilities of Bergen High Technology Center (HIB) using fresh water at a temperature of 12 °C, oxygen saturation above 70% in the outlet at all times and a daily photoperiod of 12 h. The fish had no previous disease history as a consequence of *Flavobacterium* encounter. To distinguish between the experimental groups, fish were assigned group identity either by careful cutting of the adipose fin or shortening of the maxillae. All experiments were in accordance with the *Guideline on the design of studies to evaluate the safety and efficacy of fish vaccines* (EMA/CVMP/IWP/314550/2010), the European Pharmacopeia 7.0 (5.2.7. *Evaluation of efficacy of veterinary vaccines and immunosera*) and approved by The Norwegian Animal Research Authority (NARA).

2.2. Cultivation of bacterial isolates

F. psychrophilum isolates AL20211 (challenge isolate) and AL20055 (vaccine isolate) were isolated from rainbow trout during field outbreaks in Norwegian waters in 2011 and 2009, respectively. Prior to the use of AL20211 in challenge trials or as antigens for detections of specific antibodies using the enzyme-linked immunosorbent assay (ELISA), cultivation was performed on tryptone yeast extract salts (TYES) broth and agar at 15 °C [14]. All inoculums were aliqouted and stored at −80 °C until use. Plate count quantification of bacteria was performed after thawing.

2.3. Vaccination

At vaccination, a total number of 338 rainbow trout were randomly extracted from the holding tank (500 L) and anesthetized using tricaine methane sulphonate (Tricaine Pharmaq) at 50 mg/L buffered with an equal amount of sodium bicarbonate. Vaccination was performed by intraperitoneal injection of 0.1 ml FLAVO AVM6 (autogenous multivalent vaccine batch 3430) or 0.05 ml FLAVO IPN (experimental autogenous divalent vaccine), while a non-injected group was included as a negative control (Table 1). As previous studies including an adjuvant-only group (vaccine not containing F. psychrophilum antigens) did not demonstrate protection after challenge (paper in review), an adjuvant-only group was excluded from the current study. Vaccination was carried out using a Socorex® automatic syringe with a 0.6 mm × 4 mm needle (UNIMED). FLAVO AVM6 and FLAVO IPN are two mineral oil adjuvanted (water-in-oil emulsions) vaccines containing equal amounts of formalin inactivated whole *F. psychrophilum* cells. Fish were allocated to a new holding tank and kept for 552 day degrees for induction of immunity after vaccination.

2.4. Pre-challenge of un-vaccinated fish

The susceptibility of the rainbow trout to intramuscular challenge with *F. psychrophilum* was determined by performing a pre-challenge test on a small number of un-vaccinated fish. Fifteen randomly selected fish were challenged with 0.05 ml PBS solution containing *F. psychrophilum* isolate AL20211 at concentrations equal to 1×10^7 colony forming units (CFU)/ml, 5×10^6 CFU/ml, 1×10^6 CFU/ml, 5×10^5 CFU/ml, 1×10^5 CFU/ml, 5×10^4 CFU/ml, 1×10^4 CFU/ml, 5×10^3 CFU/ml or 1×10^3 CFU/ml. The pre-challenge test was carried out in a 150 L tank for 32 days. Fifteen fish that remained un-challenged were included as controls for horizontal disease transmission.

2.5. Challenge procedures

Intramuscular challenge was performed in 4 parallel 150 L tanks with a minimum of 25 fish/group/tank (Table 2). Vaccinated groups and negative controls were challenged with *F. psychrophilum* isolate AL20211 at a concentration of 1×10^5 CFU/ml suspended in 0.05 ml PBS. Similar to the pre-challenge, injection was performed using 0.5 ml BD Micro-Fine™ U-100 insulin syringes (BD Medical). The challenge dose was placed in the muscle approximately 1 cm on the dorsal site of the lateral line, slightly posterior to the dorsal fin. After challenge, fish and tanks were tended and monitored on a daily basis by the test facility and environmental parameters were recorded every morning. Based on mortality, relative percentage survival (RPS) was calculated at the time point corresponding to 60% mortality in the control group (RPS_{60}) while RPS at endpoint (RPS_{end}) was calculated at the time point when mortality had ceased in the control group. The challenge study was terminated at the time when mortality had ceased in all experimental groups for a minimum of 3 consecutive days.

2.6. Sampling of blood and registration of ulceration

Blood samples were collected from the caudal vein of 10 fish/group at the time of challenge using 4 ml vacutainers containing lithium heparin (Becton Dickinson). Plasma was separated from whole blood by centrifugation at 1500 × *g* at 18 °C for 15 min and stored at −20 °C until analysis.

Deceased fish were continuously investigated for disintegration of the surface of the skin at the site of injection. Records were made in a binary manner (present or not present) with ulcers seen as major swellings or lesions/skin disintegration registered as positives. At the end of the experiment all survivors were accounted for and scored.

2.7. ELISA

ELISA was performed on 96-well polystyrene microtiter plates (Nunclon™ Δ Surface). Wells were coated with 100 μl antigen (*F. psychrophilum* isolate AL20055). All wells were washed 3 times in PBST (PBS containing 0.05% Tween 20) between every incubation step (BioTek ELx405). Unspecific binding was blocked with 5% dry milk in PBST for 1 h at room temperature. Plasma from rainbow trout was added in two-fold dilutions from 1:25 to 1:102,400 in PBST with 1% dry milk and incubated over night

Table 1
Summary of experimental groups, vaccine content and allocation of fish during challenge.

Experimental group	Formulation	Antigenic content[a]	Vaccine volume injected (ml)	Fish per tank during challenge			
				Tank 1	Tank 2	Tank 3	Tank 4
Negative control	–	None	Not injected	25	25	25	27
FLAVO IPN	W/O	*F.psy*, *IPNV*	0.05	25	25	25	27
FLAVO AVM6	W/O	*A.sal*, *L.ang* O1, *L.ang* O2, *M.vis*, *V.sal*, *F.psy*	0.1	25	26	26	27

[a] *A.sal* – *Aeromonas salmonicida*, *L.ang* O1 – *Listonella anguillarum* O1, *L.ang* O2 – *Listonella anguillarum* O2, *M.vis* – *Moritella viscosa*, *V.sal* – *Vibrio salmonicida*, *F.psy* – *Flavobacterium psychrophilum*, IPNV – infectious pancreatic necrosis virus.

at 4 °C. *F. psychrophilum* specific antibodies were detected using 4C10 mouse-anti-trout monoclonal antibodies [15] (Norwegian Veterinary Institute, Oslo) diluted 1:3800 in PBST/1% dry milk for 1 h, followed by incubation with rabbit–anti-mouse alkaline phosphatase immunoglobulin (1:500 in PBST/1% dry milk) for 1 h (Dako). Nitrophenyl phosphate (NPP) was used as substrate at a concentration of 1.0 mg/ml and the reaction was stopped after 30 min. Reading of optical density (OD) was carried out using a BioTek EL 800 plate reader at OD_{405} with the Gen 5 data analysis software. All results were expressed as the dilution factor at which the OD_{405}-reading was 0.5.

2.8. Statistical analysis

Data processing was performed using Microsoft Excel, while GraphPad PRISM 5 was used to generate graphs and analyze for statistical differences. Ulceration rates and antibody responses were analyzed using the non-parametric one-way ANOVA test in addition to two-tailed, unpaired *t*-tests to compare two experimental groups. The two-tailed Fisher's exact test was used to determine differences in the registered mortality. In all cases a *p*-value of <0.05 was considered significant.

3. Results

3.1. Pre-challenge of un-vaccinated rainbow trout

Accumulated mortality in the pre-challenge ranged from 77.8% to 100% (results not shown). In the groups challenged with bacterial concentrations ranging from 1×10^4 CFU/ml to 1×10^7 CFU/ml mortality was the highest (86.7%–100%) and first registered at days 8–11, while the lower concentrations (1×10^3 CFU/ml and 5×10^5 CFU/ml) showed delayed mortality starting at days 12–14 with 77.8% and 86.7% accumulated mortality, respectively. Mortality progression was similar between groups, irrespective of challenge concentration.

3.2. Antibody responses against F. psychrophilum

Blood plasmas were collected from 10 rainbow trout per experimental group at the time of challenge (552 day degrees post vaccination) and analyzed for specific antibodies against *F. psychrophilum* using ELISA. Results showed that fish vaccinated with FLAVO AVM6 ($OD_{405} = 880 \pm 169$) and FLAVO IPN ($OD_{405} = 3240 \pm 873$) had significantly higher antibody responses compared to the non-injected control group ($OD_{405} = 30 \pm 5$) (Fig. 1). The two vaccinated groups had antibody levels that were not statistically different.

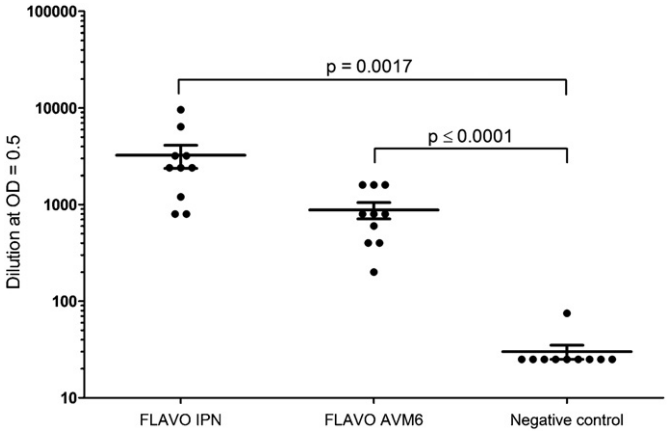

Fig. 1. Specific antibody responses against *F. psychrophilum* isolate AL20055 prior to challenge 552 day degrees post vaccination ($n = 10$).

3.3. Challenge of vaccinated rainbow trout

Survival rates from the challenge study were presented as a Kaplan–Meyer plot, as shown in Fig. 2. Acute mortality started in the non-injected control group at day 9, while it was delayed by 3 days in the vaccinated groups starting at day 12. This delayed onset of mortality was reflected in the high RPS_{60} values in the vaccinated groups (Table 2). Accumulated mortality in the control group reached the same levels (>90%) in the main challenge and the pre-challenge and both showed an acute progression. At the time of challenge termination, accumulated mortality in the FLAVO AVM6 and FLAVO IPN vaccinated groups were extremely statistically significant ($p < 0.0001$) compared to the negative control

Table 2
Relative percent survival (RPS) and accumulated mortality in all tanks after intramuscular challenge of experimental groups with *F. psychrophilum* strain AL20211. RPS values were estimated by comparing the FLAVO AVM6 and FLAVO IPN vaccinated rainbow trout to the negative control.

Experimental group		Challenge tank				Average
		Tank 1	Tank 2	Tank 3	Tank 4	
Negative control	Acc.mort[a]	88.5	100.0	92.3	96.2	94.3
FLAVO IPN	RPS_{60}	93.3	93.3	86.7	80.0	88.3
	RPS_{end}	82.6	88.5	75.0	72.0	79.5
	Acc.mort	15.4	11.5	23.1	26.9	19.2
FLAVO AVM6	RPS_{60}	93.3	86.7	86.7	93.3	90.0
	RPS_{end}	87.0	69.0	66.4	87.5	77.5
	Acc.mort	11.5	31.0	31.0	12.0	21.4

[a] Acc.mort – accumulated mortality.

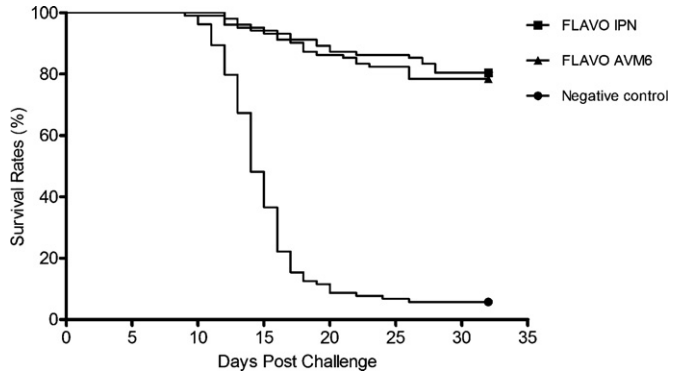

Fig. 2. Survival rates after intramuscular challenge of vaccinated (FLAVO AVM6 or FLAVO IPN) and un-vaccinated (negative control) rainbow trout with *F. psychrophilum* isolate AL20211. Each graph represents the average results for 4 parallel tanks holding about 25 fish/challenge group ($p < 0.0001$).

(Fig. 1 and Table 2). Although tank differences were observed, the average RPS_{60}, RPS_{end} and accumulated mortality were similar between the FLAVO AVM6 and FLAVO IPN vaccinated groups.

3.4. Registration of ulceration

Generally, fish that died after intramuscular challenge with *F. psychrophilum* had visible focal ulcerations limited to the injection site. Ulcerations were seen as necrotic skin lesions and in some cases the muscle erosion had resulted in exposure of the spinal cord. Most survivors were found to have the surface of the skin intact, however minor swellings were observed in some cases. In the control group ulcerations were found on 72.5% of the fish (Fig. 3). Ulceration rates were significantly lower ($p < 0.0005$) in the FLAVO IPN and FLAVO AVM6 vaccinated groups from where 12.7% and 15.7% of the fish were found to have ulcerations at the injection site, respectively.

4. Discussion

The present study demonstrates that the multivalent FLAVO AVM6 and the divalent FLAVO IPN vaccines containing inactivated whole cell antigens of *F. psychrophilum* were able to protect rainbow trout against bacterial cold water disease. Intramuscular challenge was performed approximately 8 weeks post vaccination at which the fish had reached a size ranging from 60 to 80 g. This route of challenge was chosen as other infection models where *F. psychrophilum* infection is established by more natural means (e.g. immersion or cohabitation) are difficult to reproduce with adequately high mortality [16], partly because of increased resistance to experimental infections at this fish size. The pre-challenge was performed on un-vaccinated rainbow trout using a field strain (AL 20211) of *F. psychrophilum* for which previous studies have demonstrated high virulence (paper in review). End point mortality on un-vaccinated fish in the pre-challenge and main challenge was consistently above 88.5% demonstrating the reproducibility of our challenge model. Furthermore, the negative (unchallenged) control group cohabited with the challenged fish did not show any mortality during the pre-challenge. This implied that the challenge pressure contributed by horizontal transmission of *F. psychrophilum* could be considered insignificant and that the main cause of mortality was the intramuscularly injected challenge dose.

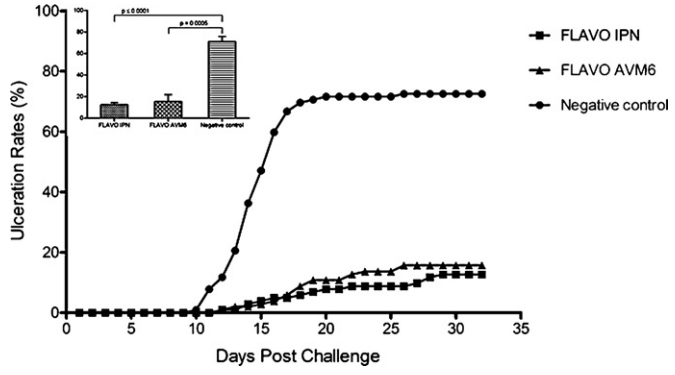

Fig. 3. Ulceration rates at the site of challenge injection. Each line displays the average of 4 parallel tanks with 25 fish/experimental group/tank. The column bar graph shows the accumulated results including standard deviation and statistical significance.

Oil-adjuvanted vaccines are known to be strong inducers of inflammatory and humoral responses in the form of antigen specific antibodies [17,18]. For inactivated *F. psychrophilum* bacterins, protection has been demonstrated both for oil-adjuvanted [19,20] and non-formulated vaccines [12]. Moreover, specific antibodies have been found 7 months post vaccination in field trials [19]. In our study, significantly higher antibody titers were measured 552 day degrees post vaccination in the FLAVO AVM6 and FLAVO IPN vaccinated groups compared to the un-vaccinated control group. Even though fish in the FLAVO IPN vaccinated group showed about 3.7 times higher average antibody titer compared to the FLAVO AVM6 vaccinated group, the antibody levels could not be related to the survival rates as both vaccines were able to induce the same level of protection against the challenge. This suggests that the level of antibodies induced by both vaccines had reached a certain threshold needed for protection to be conferred against this route of challenge.

Skin and muscle ulcers are typical clinical sign of *F. psychrophilum* infections [21]. Not only may ulcers and necrotic tissues result in reduced animal welfare, but also contribute to significant down classification of the filet. Proteolytic activity (gelatin, casein and cartilage collagen degradation) related to muscle necrosis could be a possible virulence factor for *F. psychrophilum* [22] as tissue dissolution frees nutrients that may further support and amplify bacterial growth. This may also privilege other pathogens to establish infections as an important portal of entry has been breached. In the current study ulceration during disease progression was related to the efficacy of the vaccines as observed in the challenge tests and was almost equally rated in the FLAVO AVM6 (15.7%) and FLAVO IPN (12.7%) vaccinated groups while ulcers were found in 72.5% of the un-vaccinated control group post challenge. Irrespective of experimental group, ulcers were registered in 60-70% of the fish that died due to challenge. Taken together with the finding that 94.3% of the fish in the negative control group died as a result of the intramuscular challenge this underlines the heavy infection pressure represented by this route of challenge. In addition, the pre-challenge revealed the highly opportunistic nature of *F. psychrophilum* in muscle tissue as challenge concentrations as low as 1×10^3 CFU/ml, corresponding to 50 bacteria being injected per fish, could bring about 77.8% accumulated mortality. The fact that about 20% of the fish in the vaccinated groups died after challenge could thus have been a result of the heavy infection pressure shifting the host-pathogen balance too far in favor of the pathogen, rather than the ability of the vaccines to induce protection.

The present study is to our knowledge one of the first to demonstrate protective immunity induced by a multi- or divalent water-in-oil formulated vaccine against *F. psychrophilum*. From our results we conclude that vaccination with FLAVO AVM6 or FLAVO IPN leads to production of specific antibodies that contribute to protect rainbow trout against ulcerations and mortality caused by acute BCWD.

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