Efficacy of a polyvalent injectable vaccine against *Flavobacterium psychrophilum* administered to rainbow trout (*Oncorhynchus mykiss* L.)

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Running head: Efficacy of a polyvalent injectable vaccine against *Flavobacterium psychrophilum*

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Abstract

Flavobacterium psychrophilum is one of the most important pathogens affecting cultured rainbow trout (*O. mykiss*). Recent information from UK salmonid farms showed country-wide distribution of genetically and serologically divergent clones which has hampered the development of a vaccine for Rainbow Trout Fry Syndrome. The current study assessed the efficacy of an injectable polyvalent vaccine containing formalin-inactivated *F. psychrophilum* in rainbow trout. The vaccine was formulated with an oil adjuvant (Montanide ISA 760VG) or formalin killed cells alone. Duplicate groups of trout (60 ± 13 g) were given phosphate buffered saline or vaccine formulated with Montanide by intraperitoneal (i.p.) injection and challenged by intramuscular (i.m.) injection with a homologous and a heterologous isolate of *F. psychrophilum* at 525 degree days post-vaccination (dd pv). Significant protection was achieved in vaccinated fish (p=0.0001, RPS 76% homologous, 88% heterologous). Efficacy of the adjuvanted vaccine was also demonstrated by heterologous challenge at 1155 dd pv resulting in 100% protection, whereas survival in the un-adjuvanted group was not significantly different from control fish. Levels of specific antibody at 1155 dd pv, as measured by ELISA, were significantly higher in the fish vaccinated with adjuvant when compared with unvaccinated fish.

1 Introduction

Rainbow trout fry syndrome (RTFS), caused by *F. psychrophilum*, is a major cause of mortality in salmonid aquaculture and other species worldwide (Barnes, 2011). *F. psychrophilum* is a highly heterogeneous pathogen, and as a result, only one commercial vaccine (ALPHA JECT® IPNV-Flavo, a killed injectable vaccine licensed in Chile) is

55 available to control this problematic disease (Gómez, Méndez, Cascales, & Guijarro, 2014;
56 Wahli & Madsen, 2018). Treatment is still largely limited to the use of antibiotics, which has
57 led to increased levels of antibiotic resistance (Henríquez-Núñez, Evrard, Kronvall, &
58 Avendaño-Herrera, 2012; Verner-Jeffreys & Taylor, 2015), highlighting the urgent need for
59 preventative measures for control of RTFS. The rainbow trout (*O. mykiss*) industry in the UK
60 is critically dependent on the continued clinical efficacy of a single antimicrobial agent,
61 florfenicol (Verner-Jeffreys & Taylor, 2015), highlighting the need for the development of a
62 cross-protective vaccine. As outbreaks in larger trout (50-500 g) have recently been reported
63 in the UK (Iglesias, 2017) causing reduced appetite, lesions and requiring treatment with
64 antibiotics, there is also a need for a vaccine to provide longer term protection i.e. over the
65 whole production cycle.
66 A previous study in our laboratory characterised *F. psychrophilum* by genotyping and
67 serotyping a large collection of isolates gathered from clinical outbreaks in rainbow trout,
68 Atlantic salmon (*Salmo salar*) and coho salmon (*Onchorynchus kisutch*) (Ngo et al., 2017).
69 The work revealed high heterogeneity in the strains of *F. psychrophilum* and highlighted the
70 need for such studies to enable selection of vaccine candidates with the potential for
71 protection of both trout and salmon. The vaccine developed from these studies has been
72 applied to salmon via injection and trout fry by immersion vaccination, resulting in excellent
73 levels of protection (Hoare et al., 2017; Hoare, Ngo, Bartie, & Adams, 2017). As immersion
74 vaccines are currently lacking mucosal adjuvants the protection afforded by this method
75 would only cover the early life stages. Therefore an adjuvanted vaccine delivered by
76 intraperitoneal (i.p.) injection, would be the optimal method to vaccinate fish at later stages in
77 the production cycle. Oil adjuvanted vaccines are known to be strong inducers of local

inflammatory reactions followed by a specific systemic immune response (Fredriksen et al., 2013).

The current study was performed to assess the efficacy of a polyvalent, whole cell injectable vaccine containing formalin-inactivated *F. psychrophilum*, with and without adjuvant (mineral oil, Montanide™) to induce protective immunity in rainbow trout (60 g) against the pathogen. Protection against a homologous and a heterologous strain was investigated in trout at 525 dd and efficacy of the vaccine against a heterologous isolate at 1155 dd (nearly twice that recommended by most vaccine manufacturers for induction of immunity to bacterial antigens). Immune responses in vaccinated and unvaccinated trout were investigated pre-challenge by ELISA and Western blot.

2 Materials and Methods

2.1 Rainbow trout

Rainbow trout eggs were supplied by AquaGen (Norway) and transported on ice to the aquarium at the Institute of Aquaculture, University of Stirling. On arrival, the eggs were subjected to iodophor surface disinfection according to the manufacturer's instructions (Buffodine, Evans Vanodine, UK). Five replicates of 10 eggs were removed and confirmed to be *F. psychrophilum*-free using a nested PCR that targets the 16S rRNA gene (Ngo et al., 2017; Toyama, Kita-Tsukamoto, & Wakabayashi, 1994). The eggs were maintained in flow-through de-chlorinated tap water at 10ºC until hatch, and thereafter maintained in a 100 L flow-through tank (5 L min^{-1}). Fry were fed to satiation daily (Inicio feed, 1.1 mm, BioMar, UK). All experimental procedures with live fish were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines (EU Directive 2010/63/EU) and were approved by the Ethics Committee of the University of Stirling.

2.2 Preparation of formalin inactivated bacteria

Two isolates of *F. psychrophilum* recovered from trout and one recovered from Atlantic salmon in the UK in 2013 were used to make a formalin killed whole cell vaccine (FKC) (AVU-1T/13, serotype Fd; AVU-2T/13, serotype Th; and AVU-3S/13, serotype FpT;) as described previously (Hoare et al., 2017). The three cultures were mixed in equal parts to form the whole cell vaccine at a final concentration of 1×10^9 colony forming units (CFU) mL−1.

2.3 Preparation of vaccine formulations and vaccination

The formalin-inactivated vaccine prepared above, was emulsified with Montanide 760VG (Seppic, France) (Montanide70:FKC30) and stored at 4 ºC. Stability of the emulsion was examined macro and microscopically for a 7 day period following emulsification.

Fish (60 g ± 13) were randomly separated into 100 L flow-through tanks with aeration at 15ºC. The experimental design of the vaccination trials is summarised in Table 1. As previous studies have shown no protection after challenge when adjuvant alone is administered (Fredriksen et al., 2013), an adjuvant-alone group was excluded from this study. Fish were anaesthetised with benzocaine (Sigma, 0.004%) and vaccinated by i.p. injection (50 µl/fish). Control groups were injected i.p. with 50 µl/fish of sterile phosphate buffered saline (PBS). Prior to challenge (525 dd and 1155 dd pv) fish were anaesthetised with benzocaine (as above) and blood was sampled from the caudal vein using a 23 G needle and syringe from three fish per duplicate group (n= 6) stored overnight at 4ºC, centrifuged for 5 min at 3000 x g for collection of serum, and stored at -20ºC until analysis.

2.4 Experimental infection of vaccinated fish

Dose response studies were conducted to determine the lethal dose 60 % (LD_{60}) for each isolate (data not shown). Challenge was conducted with both a homologous strain (AVU-2T13; 1.3 x 10^7 colony forming units (CFU/fish) and a heterologous strain of *F. psychrophilum* (AVU-1T/07; 3.6 x 10^7 CFU/fish) at 525 dd pv, and only with a heterologous strain (AVU-1T/07; 1.7 x 10^7 CFU/fish) at 1155 dd pv. The fish were maintained as above and monitored for 21 days post infection (dpi). Moribund fish or mortalities were removed and sampled by streaking head kidney, spleen and any lesions onto Modified Veggietone (MV) medium [veggitones GMO-free soya peptone (Oxoid, UK), 5gL −1; yeast extract (Oxoid, UK), 0.5 g L−1; magnesium sulphate heptahydrate (Fisher chemicals, UK), 0.5 g L−1; anhydrous calcium chloride (BHD), 0.2 g L−1; dextrose (Oxoid, UK), 2 g L−1; agar (solid medium; Oxoid, UK), 15 g L−1; pH 7.3] to confirm specific mortality. A sub-sample of colonies recovered was screened with a nested PCR (Ngo et al., 2017; Toyama et al., 1994), to confirm re-isolation of *F. psychrophilum* from the fish.

2.5 ELISA for detection of specific IgM in serum

Enzyme-linked immunosorbent assay (ELISA) was used to assess specific IgM titres to *F. psychrophilum* in serum according to (Hoare et al., 2017). The trout *F. psychrophilum* vaccine isolates were used to coat the ELISA plates (Immulon 4 HBX, UK) at 1 x10^8 CFU/mL in PBS and incubated overnight at 4°C. The dilution of fish serum used was optimised by first titrating sera from each group (1:32 to 1:1024). Fish sera samples at the optimised dilution of 1:64 in PBS were added to the wells (100 µl/well) in duplicate and incubated overnight at 4°C. The absorbance was read on a BioTek HT Synergy spectrophotometer at 450 nm.

2.6 Agglutination assay

Sera collected from each group at 1155 dd pv were serially diluted two-fold in PBS and 20 µl added to each well of a round bottom plate (Nunc™). A 20 µl sample of the homologous strain (AVU-2T13, 2 x10^8 CFU/mL, formalin-killed) was then added to each well and the plates were incubated overnight at room temperature. Agglutination was observed under 100X using an inverted microscope (Olympus CK40). The agglutination titre was calculated as the reciprocal of the highest dilution of serum showing complete agglutination of the bacteria.

2.7 SDS-PAGE and Western blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Suspensions of the *F. psychrophilum* vaccine and challenge strains were aliquoted into 1.5 ml micro-centrifuge tubes (1 ml of 2 x 10^8 CFU/mL), and centrifuged for 15 min at 3000 × g. Bacterial pellets were prepared and applied to a 12% polyacrylamide gel (Bio-Rad) gel according to [7]. Gels were stained with Coomassie Brilliant Blue (Bio-Rad) or subjected to Western blotting. Bacterial components separated by SDS-PAGE as described above, were transferred onto nitrocellulose membranes and incubated with serum taken at 1155 dd pv according to [7].

2.8 Statistical Analysis

Relative percentage survival (RPS) was calculated at 21 days post-challenge. Kaplan-Meier survival curves were generated and the log-rank test was used to compare the survival curves for the vaccinated fish and unvaccinated fish (E.L. Kaplan, 1958; Peto et al., 1977). Where there was a significant difference in mortality between replicate tanks statistical analysis was

run on individual tanks. The relative percent survival (RPS) of this trial was calculated using the following equation (Amend, 1981):

$$RPS = [1 - \frac{\text{average \% mortality of vaccinated fish}}{\text{average \% mortality of unvaccinated fish}}] \times 100$$

Specific antibody levels were analysed by one-way ANOVA followed by Welch's test.

3. Results

3.1 Vaccine Efficacy

525 dd pv: The percentage survival of the sham-vaccinated group administered PBS and challenged with the homologous strain was 40% and with the heterologous strain was 43%. The vaccinated group had significantly higher survival compared with the unvaccinated control group following homologous challenge (p = 0.0001, Figure 1 a). Following heterologous challenge there was a significant difference between control tanks so individual tanks were compared and control tank 2 had significantly higher survival compared with vaccinated tanks (p = 0.0001, Figure 1 b). The vaccine formulation containing FKC combined with Montanide ISA 760VG gave high levels of protection against both the homologous and the heterologous strains of *F. psychrophilum* (RPS of 76% and 88% respectively).

Figure 1. here

1155 dd pv: Percentage survival in the sham-vaccinated trout injected with PBS was 29% and trout vaccinated with FKC combined with Montanide ISA 760VG had significantly higher survival compared with the unvaccinated controls, RPS of 100% (p = 0.0001, Figure 2). The vaccine formulation with FKC without adjuvant had a RPS of 25%; survival in this group was

not significantly different from the control group. Lesions at the challenge injection site were evident in all unvaccinated fish, approximately 10% of FKC vaccinated fish and were completely absent from fish vaccinated with FKC and adjuvant.

Figure 2. here

3.2 Nested PCR for detection of *F. psychrophilum*

PCR products specific for *F. psychrophilum* (1080 bp) were detected in moribund and dead fish sampled during the challenge. Conversely, the eggs tested negative by PCR (Supplementary Figure 1).

3.3 Specific antibody response

Levels of specific antibody at 1155 dd pv, as measured by ELISA against both trout vaccine strains, were significantly higher in the fish vaccinated with adjuvant when compared with unvaccinated fish (AVU 1T/13 $p = 0.01$, AVU 2T/13 $p < 0.05$) (Figure 3. a, b). Fish vaccinated with FKC alone had higher antibody levels than those of the control group, but this was not significant.

Figure 3 here

The specific IgM response of trout to vaccination was also measured by agglutination at 1155 dd pv against the homologous isolate (AVU-2T/13). The unvaccinated group and the group vaccinated with FKC alone had low titres of 8, whereas the group vaccinated with FKC and Montanide exhibited a significantly higher average titre of 144 (n= 4, $p = 0.04$) (Table 2).

3.4 SDS-PAGE and Western blot

Distinct bands ranging from 10-100 kDa were evident in the SDS-PAGE profiles of the *F. psychrophilum* strains used to prepare the polyvalent vaccine (and a heterologous strain AVU-

1T/07) following staining of gels with Coomassie (Figure 4a). The banding profiles of the strains were similar with a slightly lower weight band occurring in the heterologous strain between 20-25 kDa. In Western blotting, stronger staining was evident when *F. psychrophilum* isolates were screened with serum from the fish vaccinated with FKC and Montanide (Figure 4b (iii)) compared to that seen with serum of unvaccinated (Figure 4b (i)) or FKC only vaccinated fish (Figure 4b (ii)). A prominent band staining strongly between 20-25 kDa for all the *F. pychrophilum* strains tested was only observed in the blot using sera from fish vaccinated with FKC and adjuvant. In addition, a region of higher molecular mass antigens above 75 kDa were recognised by sera from FKC only and FKC and Montanide vaccinated fish, with stronger staining observed in fish vaccinated with adjuvant.

Figure 4 a, b. here

4. Discussion

Rainbow Trout Fry Syndrome affects juvenile rainbow trout: however recently outbreaks are occurring in larger fish (50 g+) in the UK (Iglesias, 2017). Therefore, the development of a vaccine to provide long term protection is needed. The polyvalent vaccine developed against *F. psychrophilum* in this study provided significant protection against homologous and heterologous challenge when administered to rainbow trout by i.p. injection (RPS 76-100%). The success of many injectable vaccines used in aquaculture has been attributed to the inclusion of adjuvants (Tafalla, Bøgwald, & Dalmo, 2013). Adjuvants are substances which enhance the immune response to an antigen (Awate, Babiuk, & Mutwiri, 2013) and one of the most effective adjuvants used in aquaculture is mineral oil (Rømer Villumsen, Koppang, & Raida, 2015). The present study clearly demonstrated the enhanced efficacy of the vaccine against *F. psychrophilum* when the bacterin was administered with an oil adjuvant

(Montanide™) providing protection with no mortality or development of lesions following challenge at 1155 dd pv.

Previous studies using inactivated *F. psychrophilum* in conjunction with oil adjuvants have demonstrated protection, usually associated with increased levels of specific IgM in the serum of vaccinated fish (Fredriksen et al., 2013; Hoare et al., 2017; Högfors, Pullinen, Madetoja, & Wiklund, 2008; Madetoja et al., 2006). The protection in the current study appears to be at least partly mediated by specific serum antibodies (IgM) as shown by ELISA, agglutination and Western blotting. Serum antibodies from fish vaccinated with the polyvalent vaccine recognised a heterologous strain of *F. psychrophilum* by Western blotting. A significantly stronger reaction was observed in the Western blot when sera from fish vaccinated with adjuvant was applied with bands staining between 20-25 kDa not observed in blots incubated with un-adjuvanted sera. An additional region >75 kDa was recognised by sera from both groups of vaccinated fish with stronger staining in the adjuvanted group. These two regions appear to be the targets of specific antibodies following vaccination.

Previous studies have identified different molecular weight fractions (18–28, 41–49, and 70–100 kDa) as immunogenic molecules of *F. psychrophilum* by Western blotting, using convalescent rainbow trout serum (Crump, Perry, Clouthier, & Kay, 2001; Högfors et al., 2008; Benjamin R. LaFrentz, Lindstrom, LaPatra, Call, & Cain, 2007). These proteins have potential to be the basis for a recombinant vaccine to provide cross-protection against *F. psychrophilum*. One such study, using convalescent rainbow trout serum, found an immuno-reactive proteinase K-resistant band with an apparent molecular mass of 17 kDa (Crump et al., 2001) and suggested the highly immuno-reactive band is likely to be the predominant component of the thick slime layer seen on the surface of these bacteria, possibly LPS. Whereas, the high molecular weight fractions are suggested to be part of the glycocalyx of *F.*

psychrophilum (Benjamin R. LaFrentz et al., 2007). Further studies are needed to assess the characteristics of the immunogenic band between 20-25 kDa observed in the present study with regard to a potential recombinant vaccine target which could be cross-protective. Trials to date that have assessed the efficacy of fractions to protect trout against laboratory challenge have shown whole cell formulations to provide greater or equal protection (Aoki, Kondo, Nakatsuka, Kawai, & Oshima, 2007; Högfors et al., 2008; B R LaFrentz, LaPatra, Jones, & Cain, 2004; Rahman et al., 2002). Studies such as on the potential of the outer membrane fraction and membrane vesicle rich supernatant from the stationary phase culture supernatants of *F. psychrophilum* have been shown to induce protective immunity in rainbow trout and ayu (*Plecoglossus altivelis*) (Aoki et al., 2007; Rahman et al., 2002), however both were less effective than whole cell formulations. In the present study, the combination of FKC of three strains of *F. psychrophilum* and an oil adjuvant induced protection (up to 1155 dd) against a heterologous strain of *F. psychrophilum* in rainbow trout. Further large-scale studies, are needed to determine the efficacy of the developed vaccine against a range of heterologous strains from different geographical regions. In addition, studies on the innate and cellular immune response could provide more insight into the mechanisms of protection provided by the adjuvanted vaccine.

Conclusions

The adjuvanted, polyvalent vaccine tested in this study gave excellent protection in rainbow trout (RPS of 100 %) against a heterologous strain of *F. psychrophilum*. The protection correlated with systemic IgM responses as observed by ELISA, agglutination and Western blotting.

Conflict of interest

The authors declare that they have no competing interests.

Authors Contribution

Conceived and designed the experiments: RH, SJJ, AA. Developed the vaccine: TPHN, KLB, RH, SJJ, AA. Carried out the vaccination and challenge: RH, SJJ, TPHN. Analysed the data: RH, SJJ. Wrote the paper: RH, SJJ, KT, AA.

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Table 1. Experimental design of vaccination trials.

Degree days post-vaccination	Groups	No. Fish (replicate tanks)	Innoculum (µl i.p.)	Challenge strain i.m. (Dose:CFU/fish)
525	Control (unvaccinated)	15 (2)	50 µl PBS	Homologous AVU-2T13 (1.3×10^7)
	Vaccine + Montanide	15 (2)	50 µl FKC:Montanide	Homologous AVU-2T13 (1.3×10^7)
	Control (unvaccinated)	15 (2)	50 µl PBS	Heterologous AVU-1T/07 (3.6×10^7)
	Vaccine + Montanide	15 (2)	50 µl FKC:Montanide	Heterologous AVU-1T/07 (3.6×10^7)
1155	Control (unvaccinated)	14 (2)	50 µl PBS	Heterologous AVU-1T/07 (1.7×10^7)
	Vaccine (FKC)	14 (2)	50 µl FKC	Heterologous AVU-1T/07 (1.7×10^7)
	Vaccine & Montanide	14 (2)	50 µl FKC:Montanide	Heterologous AVU-1T/07 (1.7×10^7)

PBS: phosphate buffered saline; FKC: formalin-killed cells; i.p.: intra-peritoneal; i.m.: intra-muscular, CFU: colony forming units

Table 2. Agglutination titre of serum from trout unvaccinated (injected with PBS) and vaccinated (injected with FKC, or FKC emulsified with Montanide) at 1155 degree days post-vaccination.

Fish no.	Unvaccinated	FKC	FKC:Montanide
1	<8	<8	128
2	<8	<8	64
3	<8	8	256
4	<8	<8	128

FKC: formalin-killed cells

Figure 1. Percentage survival of rainbow trout vaccinated by intraperitoneal injection with *Flavobacterium psychrophilum*-formalin killed cells (FKC) adjuvanted with Montanide ISA 760VG (FKC and Montanide) and challenged at 525 degree days post-vaccination (dd pv) by intramuscular injection with (A) a homologous strain of *F. psychrophilum* (RPS 76%); (B) a heterologous strain of *F. psychrophilum* (RPS 88%). Controls were given sterile phosphate buffered saline by intraperitoneal injection. The survival rates from each replicate tank following challenge are presented (n = 15; p = 0.0001).

Figure 2. Percentage survival of rainbow trout vaccinated by intraperitoneal injection with *Flavobacterium psychrophilum* formalin killed cells (FKC) with and without adjuvant (Montanide ISA 760VG) and challenged at 1155 degree days post vaccination (dd pv) by intramuscular injection with a heterologous strain of *F. psychrophilum*. FKC only (RPS 25%); FKC & Montanide (RPS 100%). Control fish received sterile phosphate buffered saline by intraperitoneal injection. The survival rates from each replicate tank following challenge are presented (n = 14; p = 0.0001).

Figure 3. Specific antibody (IgM) levels to *F. psychrophilum* in vaccinated rainbow trout at 1155 days post vaccination. (A) to homologous strain AVU-2T/13; (B) to homologous strain AVU-1T/13. Antibody levels were significantly higher in the fish vaccinated with adjuvant when compared with unvaccinated fish [AVU 1T/13 p = 0.01, AVU 2T/13 $p < 0.05$]. Mean values are shown as blue dots. Groups that do not share a letter are significantly different, (n = 6).

Figure 4. SDS-PAGE of *Flavobacterim psychrophilum* strains. (a) Whole cell lysates from vaccine strains (Lane 1: AVU-1T/13, Lane 2: AVU-2T/13, Lane 3: AVU-3S/13) and a heterologous strain (Lane 4: 171/07) were separated by SDS-PAGE and stained with Coomassie stain. Arrows indicate high intensity bands at 10-15, 20, 37-50, 100 and 150-250 kDa. (b) Western blot analysis of a heterologous strain (Lane 1:171/07) and vaccine strains (Lane 2: AVU-1T/13, Lane 3: AVU-2T/13, Lane 4: AVU-3S/13). Whole cell lysates were separated by SDS-PAGE, blotted onto nitrocellulose and reacted with serum from (i) unvaccinated (control) fish, (ii) FKC vaccinated fish or (iii) FKC & Montanide vaccinated fish. Serum was a pool from two fish from each treatment group, with a titre of 512, taken 11 wpv. Arrows indicate high intensity bands between 20-25 kDa. Molecular mass standards (kDa) are indicated on the left.

Supplementary Figure 1. Nested PCR for detection of *F. psychrophilum* in colonies recovered from moribund/mortalities post-challenge. 1% agarose gel showing second round PCR products. M: Ladder, Lane1-16: bacterial DNA recovered from fish, (-) negative control, (+): positive control.