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Preliminary Study Into the Short Term Effects of Adjuvants on Atlantic Halibut (*Hippoglossus hippoglossus* L.).

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Abstract.
Following injection with Freunds Incomplete Adjuvant containing human gamma globulin, Atlantic halibut developed antibodies 6 weeks post-injection. Peritoneal adhesions also appeared at week 6 and increased in severity over time. Use of Montanide ISA711® as an adjuvant, again, led to antibodies being produced from week 6 but adhesion did not appear until week 10. Use of Alhydrogel® led to low level adhesions appearing at week 6 although antibodies were not detected until week 10. Whilst adjuvants may enhance the immune response, they also appear to induce potentially detrimental adhesions.

Introduction.

Immuno-prophylaxis of fish has become an integral part of effective aquaculture husbandry practices. Whilst many antigens are effective on there own, many can be enhanced in conjunction with adjuvants and immunostimulants (Anderson, 1992). The immuno-enhancing effects of oil and alum adjuvants have been studied for over 50 years and demonstrated in salmonids (Freund, 1947: Krantz *et al.* 1964, Udey and Fryer, 1978; Palmer and Smith, 1980; Horne, *et al.* 1984; Olivier *et al.*, 1985). This enhancement can occur through several routes including: elevating the production of antibodies; acting as a reservoir or depot enabling prolonged dosage; activating specific immune cell populations (Anderson, 1992). Oil emulsion vaccines are considered to give high levels of stimulation to the humoral immune system and lower levels of stimulation to the cellular immune system (Vanselow, 1987). But detrimental effects are well documented including chronic peritonitis, adhesions, granulomas and mortalities (Anderson, *et al.* 1997).

The commercial culture of Atlantic halibut (*Hippoglossus hippoglossus* L.) is expanding in both Scotland and Norway, and with it, the use of immunoprophylaxis, which is an important factor in fish husbandry. Little is known about the effect of the adjuvants commonly used in commercial vaccines on these novel aquaculture species. This project aimed to make a preliminary, short term investigation into the effects of three different adjuvant types in Atlantic halibut.

Materials and Methods.

Atlantic halibut weighing an average 110g were obtained from Marine Harvest McConnell and were kept in 1m tanks with flow through seawater at ambient temperatures (FRS Marine Laboratory, Experimental

Week	Freund's Incomplete Adjuvant (FIA)	Alhydrogel®	Montanide ISA711®	Phosphate buffered saline (PBS)
2	0	0	0	0
4	0	0	0	0
6	1	1	0	0
8	1	1	0	0
10	3	1	1	0
12	3	1	3	0

Table 1. Summary of the mean adhesion level scores for fish injected with mineral oil, non-mineral oil and alum adjuvants or standard solutions at various time post-injection. (N=2). Adhesion classification system (Midtlyng et al. 1996). Score 0 = No visible lesions, Score 1 = Slight adhesions, Score 2 = Minor adhesions, Score 3 = Moderate adhesions, Score 4 = Major adhesions, Score 5 = Extensive adhesions, Score 6 = Viscera unremovable.

Fish Production Unit, Aultbea), the fish were fed commercial salmon feed to satiation. The fish were anaesthetized with MS222 (tricane methansulphate, Sigma) at 2mg/l and given an intra-peritoneal injection with 100µl of either Freunds Incomplete Adjuvant (FIA - mineral oil type adjuvant), Alhydrogel® (Superfos AS. Denmark - aluminium salt type adjuvant) or Montanide ISA711 (ISA - non-mineral oil type adjuvant)® (Seppic, France) each contained 100µg/ml human gamma globulin (HGG)(Sigma) as a model antigen. A fourth group was given an intra-peritoneal injection with 100µl of phosphate buffered saline to act as a control. Every 14 days two fish from each group were euthanised, inspected for intra-peritoneal adhesions and scored according to the method of Midtlyng *et al.* (1996). Additionally, a blood sample was taken from the caudal sinus using a Vacuette serum separation tube and needle (Greiner). The blood was allowed to clot at room temperature for 2 hrs then centrifuged at 3000 rpm to separate the serum. Antibodies to HGG were assayed by ELISA. Briefly, a microtitre plate was coated with 100µl/well of HGG at 10µg/ml in carbonate/bicarbonate buffer (Sigma) and the plate incubated at 37°C for 1 hr. The plate was then washed twice (250µl/well) with Tris buffered saline (pH 8) containing 0.05% Tween-20 (TTBS). The plate was blocked with 5% goat serum in TTBS for 1hr at 37°C and washed as above. Sample serum (100µl) was serially diluted in TTBS and the plate incubated for 2 hrs at room temperature and washed as above. A polyclonal mouse anti-halibut IgM antibody was diluted 1in 4000 in TTBS and 100µl was added to each well followed by incubation at 37°C for 1 hr, the plate was washed as above. A goat anti-mouse IgG peroxidase conjugate (Sigma) was diluted 1in10000 in TTBS and 100µl added to each well before being incubated at 37°C for 1 hr. The plate was then washed as above. Finally, 100µl of tetramethylbenzidine substate solution (Kirkegaard and Perry) was added to each well and the plate incubated for 30 min in the dark and at room temperature before being read at 560nm in a Dias microtitre plate reader (Dynex). Controls (inclusion of nega-

Week	Freunds Incomplete Adjuvant (FIA)	Alhydrogel(r)	Montanide ISA711(r)	Phosphate buffered saline (PBS)
2	-	-	-	-
4	-	-	-	-
6	+	-	+	-
8	+	-	+	-
10	+	+	+	-
12	+	+	+	-

Table 1. Summary of the mean adhesion level scores for fish injected with mineral oil, non-mineral oil and alum adjuvants or standard solutions at various time post-injection. (N=2). Adhesion classification system (Midtlyng et al. 1996). Score 0 = No visible lesions, Score 1 = Slight adhesions, Score 2 = Minor adhesions, Score 3 = Moderate adhesions, Score 4 = Major adhesions, Score 5 = Extensive adhesions, Score 6 = Viscera unremovable.

tive control sera, omitting block and/or antibodies) were included to monitor non-specific reactions. Results were considered positive if the absorbance was at least double that of the negative control sera.

Results.

The results for the appearance of adhesions are given in table 1. These show that low level adhesion started to appear in the FIA and Alhydrogel® injected fish 6 weeks after vaccination and in the Montanide ISA711® group 10 weeks after vaccination. Whilst the adhesions present in the Alhydrogel® group did not appear to increase in severity, those that appeared in the groups injected with the oil adjuvants did become more severe with time reaching a score of 3, indicating moderate adhesions, before the end of the experiment. No adhesions were apparent in the control group. The results of the antibody analysis are given in table 2. These show that the groups injected with the oil adjuvants produced a positive antibody titre 6 weeks post vaccination which was 4 weeks before the group injected with the aluminium salt adjuvant.

Discussion.

The commercialisation of the culture of Atlantic halibut has been achieved at considerable economic cost. High mortality levels, especially in larval fish, has lead to the animals have a high value. The scarcity of animals suitable for broodstock programs has meant that they too have a considerable value. Consequently, farmers place considerable importance on protecting their investment by the use of antibiotics and immuno-prophylaxis. However, if a vaccine should prove to be detrimental to the health of the animal then the farmer is faced with the dilemma of what further action to take to protect his investment. There is an alternative route for injecting Atlantic halibut in the gonadal septum, but this may affect the fecundity of potential broodstock fish.

There are studies on the susceptibility to, and the efficacy of vaccination against, typical and atypical *Aeromonas salmonicida* (Hjeltnes, *et al.*

1995; Bricknell, *et al.* 1999; Ingilæ *et al.* 2000). Although susceptible to both typical and atypical *A. salmonicida,* the levels required to induce mortality are higher than those for Atlantic salmon (*Salmo salar* L.) (Bricknell, *et al.* 1999). However, vaccination with atypical strains in FIA showed high levels of protection with 90% relative percent survival in a homologous challenge and high antibody titres (Ingilæ, *et al.* 2000). The study by Inglæ *et al.* (2000) also included a comparison study on spotted wolffish (*Anarhichas minor* L.) that involved vaccines employing both FIA and a vegetable-animal oil adjuvant. Unfortunately, the authors did not include the non-mineral oil adjuvanted vaccine in the halibut trial, which would have allowed a further comparison with the work presented here, and there was no indication of any detrimental effects from the adjuvanted vaccines.

A field study of various vaccine types showed that all three adjuvant types, as used in the current study, induced adhesions over a nine month period but that the adhesions associated with the mineral oil adjuvant persisted for a much longer time than those associated with alum type adjuvants (Midtlyng, 1996). It has also been shown that adjuvanted vaccines can increase the risk of impaired growth rates (Midtlyng and Lillehaug, 1998).

The results from the present study would suggest that adjuvant type affects the induction time of the antibody response and it was noted that the alum adjuvant resulted in a longer induction time than either of the oil adjuvants. Yet, when looking at the detrimental effects, the severest adhesions were seen with the oil adjuvants rather than the alum adjuvant.

The limitations of this study are obvious and the authors hope to repeat the study over a much longer time period and include more suitable control groups. Yet the adverse effects of these adjuvants were obvious even in this limited sample and over the short time scale.

Whilst in general the benefits of vaccination outweigh the detrimental effects of adjuvanted vaccines, the increased value of individual fish and the lower overall production numbers associated with halibut farming must lead to a more considered approach to immunoprophylaxis.

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