Journal of Fish Diseases (1997) **20,** 343–350

Evaluation of cross protection by vaccines against atypical and typical furunculosis in Atlantic salmon, *Salmo salar* L.

B K Gudmundsdóttir and S Gudmundsdóttir

Institute for Experimental Pathology, University of Iceland, Keldur, Reykjavík, Iceland

Abstract

In Iceland, farmed salmonids are vaccinated against *A. salmonicida* ssp. *achromogenes* (Asa), which causes atypical furunculosis and is endemic in local waters. Classical furunculosis, caused by *A. salmonicida* ssp. *salmonicida* (Ass), was not diagnosed in this country until June 1995. In the present study, protection in experimental challenges against atypical and classical furunculosis in Atlantic salmon vaccinated with an autogenous Asa bacterin (Iceland Biojec.OO, IBOO), a commercial furunculosis vaccine (Biojec.1500), or a mixture of both vaccines was compared. The results showed that both vaccines gave protection against an injection challenge with Asa. However, better protection was obtained with the IBOO (homologous) vaccine. Infection of Asa by cohabitation could not be established in fresh water. Fish vaccinated with Biojec.1500 or with both vaccines simultaneously were equally well protected against Ass in a cohabitation challenge. On the other hand, no protection against classical furunculosis was achieved in fish vaccinated by IBOO alone.

Introduction

Atypical *Aeromonas salmonicida* strains cause significant disease problems in various wild and farmed fish species, and have a world-wide geographical distribution (Austin & Austin 1993). In Icelandic aquaculture, the most harmful bacterial pathogen is *A. salmonicida* ssp. *achromogenes*. The disease has been diagnosed in wild salmonids and Atlantic cod, *Gadus morhua* L., and halibut, *Hippoglossus hippoglossus* L., is also susceptible to infection. Atlantic salmon, *Salmo salar* L., is the most susceptible species, with mortalities up to 30% in land-based tanks containing brackish water (salinity 0.3–2%), where geothermal water is used to warm sea water to 8–10°C. Disease signs vary and fish may die without obvious lesions. Haemorrhage is seen in more advanced cases, and blood filled pustules and even open lesions develop. The bacterium can be isolated from both the ulcers and internal organs.

Classical furunculosis, caused by *A. salmonicida* ssp. *salmonicida*, was diagnosed for the first time in Iceland in June 1995 in wild Atlantic salmon caught from a river (Ellidaár) in Reykjavík. In the following months, the disease became epidemic in salmon and brown trout, *Salmo trutta* L., in the river, and was also detected in salmon from another river and one ocean ranching farm (Gudmundsdóttir, Gudmundsdóttir, Magnadóttir & Helgason 1996).

Typical (ssp. *salmonicida*) and atypical *A. salmonicida* strains have been reported to share cell-associated antigens such as the A-layer protein, the LPS component, iron-regulated outer membrane proteins and porins (Evenberg, Verslius & Lugtenberg 1985; Pyle & Cipriano 1986; Chu, Cavaignac, Feutrier, Phipps, Kostrzynska, Kay & Trust 1991; Hirst & Ellis 1994; Lutwyche, Exner, Hancock & Trust 1995), but exotoxins produced by typical and atypical strains, including ssp. *achromogenes,* are of a different nature (Ellis 1991; Austin & Austin 1993; Gudmundsdóttir 1996).

All *A. salmonicida* vaccines commercially available

Correspondence *Bjarnheidur K. Gudmundsdóttir, Institute for Experimental Pathology, University of Iceland, Keldur, v/Vesturlandsveg, IS-112, Reykjavík, Iceland*

are produced from typical *A. salmonicida* (ssp. *salmonicida*) strains for the prevention of furunculosis in salmonids. In Iceland, commercial furunculosis vaccines have not yet been licensed as typical strains are a recent problem and still restricted to one part of the country, but since 1992, fish farmers have vaccinated farmed salmonids by injection with an autogenous *A. salmonicida* ssp. *achromogenes* bacterin produced by a commercial vaccine producer (Alpharma N.W. Inc., Bellevue, WA, USA). As *A. salmonicida* ssp. *achromogenes* is endemic in Icelandic waters and isolated strains are found to be a homogeneous group according to their biochemical activity and virulence properties (Gudmundsdóttir *et al.* 1996), the autogenous bacterin is licenced for use in farms throughout the country.

The aim of this study was to compare protection against furunculosis and atypical furunculosis in Atlantic salmon vaccinated with a commercial furunculosis vaccine (Biojec.1500) and the autogenous *A. salmonicida* ssp. *achromogenes* bacterin (Iceland Biojec.OO).

Materials and methods

Fish

Fingerlings with an average weight of 25 g, which were offspring of wild Atlantic salmon from an Icelandic river, were used in the study. The fish came from a fish hatchery (Lækur Ölfusi) where *A. salmonicida* infection has never been diagnosed. The fish were spot marked with Alcian blue and acclimatized for one week before vaccination. During the experiment the fish were kept in 200-l tanks with continuously flowing (1 l min^{-1}) well water, at 10 ± 2°C, and fed commercial dry pellets (Vextra mini 1.6 mm, EWOS), 2% of body weight per day, with an automatic feeder.

Bacteria

Two *A. salmonicida* strains were used in the study. *A. salmonicida* ssp. *achromogenes*, isolate S24–92, originating from diseased Atlantic salmon fingerlings from an Icelandic fish farm (Vogavík) and *A. salmonicida* ssp. *salmonicida*, isolate V341–95, isolated from wild Atlantic salmon from a river in Iceland (Ellidaár). Both isolates were Gram-negative, non-motile, facultatively anaerobic, short rods producing cytochrome oxidase, catalase and brown pigment, and agglutinating monoclonal antibodies to *A. salmonicida* (BIONOR Aqua). The two isolates possessed two well known cell-associated virulence factors, LPS (lipopolysaccharide) and A-layer, and secreted proteases comparable with those of the type strains, NCMB 1110 for ssp. *achromogenes* and NCMB 1102 for ssp. *salmonicida* (Gudmundsdóttir 1996). The isolates differed in their sensitivity to ampicillin and cephalothin, β-haemolysis on blood agar, hydrolysis of aesculine and arginine, production of indole, production of acid from sucrose, mannitol and salicin, and production of gas from glucose.

Vaccines and immunizations

The vaccines used were an autogenous bacterin from *A. salmonicida* ssp. *achromogenes,* strain M108–91 (Iceland Biojec.OO, IBOO), and a furunculosis vaccine (Biojec.1500). Both vaccines contained mineral oil adjuvant (Alpharma N.W. Inc.).

Each of four vaccine-groups consisted of 480 fingerlings. The experimental design is summarized in Table 1. Prior to vaccination or control injection, the fish were anaesthetized by immersion in p-aminobenzoate at a concentration of 40 mg l^{-1}. The vaccines and the control injections of saline were administered intraperitoneally (i.p.). After vaccination, fish from each group were equally distributed into 12 tanks containing 40 fish in each. Eight tanks, located in one room in the aquarium, were used for challenge by isolate S24–92 (*A. salmonicida* ssp. *achromogenes*) and four tanks in another room for challenge by isolate V341–95 (*A. salmonicida* ssp. *salmonicida*). There was no exchange of materials between the two rooms.

Challenge tests

The isolates used for challenge were kept in brain heart infusion broth (BHI, Oxoid) supplemented with 15% glycerol at –80°C, following the primary isolation from diseased fish. For preparation of the challenge inocula, the isolates were cultivated on blood agar at 15°C for 3 days. Bacterial colonies were suspended in PBS and the density adjusted by spectrophotometer until the absorbance of the cell suspension at 600 nm measured 1.00 (OD_{600}) against a substrate blank. This stock solution was diluted by tenfold steps to the required concentration, which was based on results from pre-

Table 1 Experimental design*

		Number of fish challenged					
		Asa by injection			Asa by cohabitation	Ass by cohabitation	
Injected with:	Dose (ml)	40 000 CFUs	4000 CFUs	2000 CFUs	15% cohabitation	9% cohabitation	11% cohabitation
PBS	0·2	80	80	80	80	80	80
IBOO	0·2	80	80	80	80	80	80
Biojec.1500	0·2	80	80	80	80	80	80
IBOO + Biojec.1500	0·1 + 0·1	80	80	80	80	80	80

*Key: Asa, *A. salmonicida* ssp. *achromogenes*; Ass, *A. salmonicida* ssp. *salmonicida*; CFUs, colony forming units; PBS, phosphate buffered saline; IBOO, an autogenous Asa bacterin; Biojec.1500, a commercial furunculosis vaccine.

challenge tests. Challenge cultures were checked for purity and colony forming units (CFUs) confirmed by plate counting. Challenge was performed 70 days post-vaccination.

Challenge with *A. salmonicida* ssp. *achromogenes* was performed by an intramuscular (i.m.) injection of isolate S24–92, dissolved in 0.1 ml PBS. Two tanks of fish each received doses of 2×10^3 CFUs, 4×10^3 CFUs and 4×10^4 CFUs per fish. In the cohabitation challenge with *A. salmonicida* ssp. *achromogenes*, 28 salmon were injected i.m. with 4×10^3 CFUs per fish of isolate S24–92 and were added to the fish in each of the two tanks (15% cohabitation).

In the cohabitation challenge with *A. salmonicida* ssp. *salmonicida*, 20 salmon were injected i.m. with 2×10^4 CFUs per fish of isolate V341–92 and were added to the fish in each of two tanks (11% cohabitation), and 16 salmon were injected i.m. with 2×10^3 CFUs per fish and added to the fish in each of the other two tanks (9% cohabitation).

Dead fish were removed and recorded daily for 4 weeks. The cause of death was verified by reisolation of the bacteria from kidney samples.

Calculation and statistical analysis

Relative percentage survival (RPS) and mean days to death (MDD) were calculated using the following formulae:

RPS = [1 – (per cent mortality in vaccinated fish/per cent mortality in control fish)] × 100.

MDD = [Σ (number of mortalities × number of days post-challenge)]/total number of mortalities.

A chi-squared test was used to evaluate the significance of the differences in mortality between the control group (PBS-injected) and the vaccinated groups, and between the different vaccinated groups. The MDD results were analysed using Student's *t*-test.

Results

Challenges with *A. salmonicida* ssp. *achromogenes* (Asa)

Accumulated mortality in the control groups (PBS) of fish challenged by i.m. injection of Asa reached a maximum of 100% after 14 days with a challenge dose of 4×10^4 CFUs (Fig. 1A), 95% after 14 days with a challenge dose of 4×10^3 CFUs (Fig. 1B) and 81% after 16 days with a challenge dose of 2×10^3 CFUs (Fig. 1C). The onset of death was somewhat delayed in all vaccinated groups (Fig. 1) and the mortality was significantly lower than in the control group ($P < 0.007$). However, the only significant difference from the control MDD value was obtained in the IBOO group challenged with 4000 CFUs ($P = 0.0373$) (Table 2). When mortalities in all three challenges were compared, the protection evoked by IBOO was better than that evoked by Biojec.1500 ($P = 0.0187$). Furthermore, the IBOO + Biojec.1500 vaccine was more efficient than the Biojec.1500 vaccine ($P = 0.0447$), but the difference in protection in the IBOO- and IBOO + Biojec.1500-vaccinated fish was not significant ($P = 0.7214$). The highest RPS value (49%) was obtained when IBOO vaccinated fish were challenged with the lowest dose causing 81% mortality of control fish (Table 2).

In the two tanks where challenge with Asa was

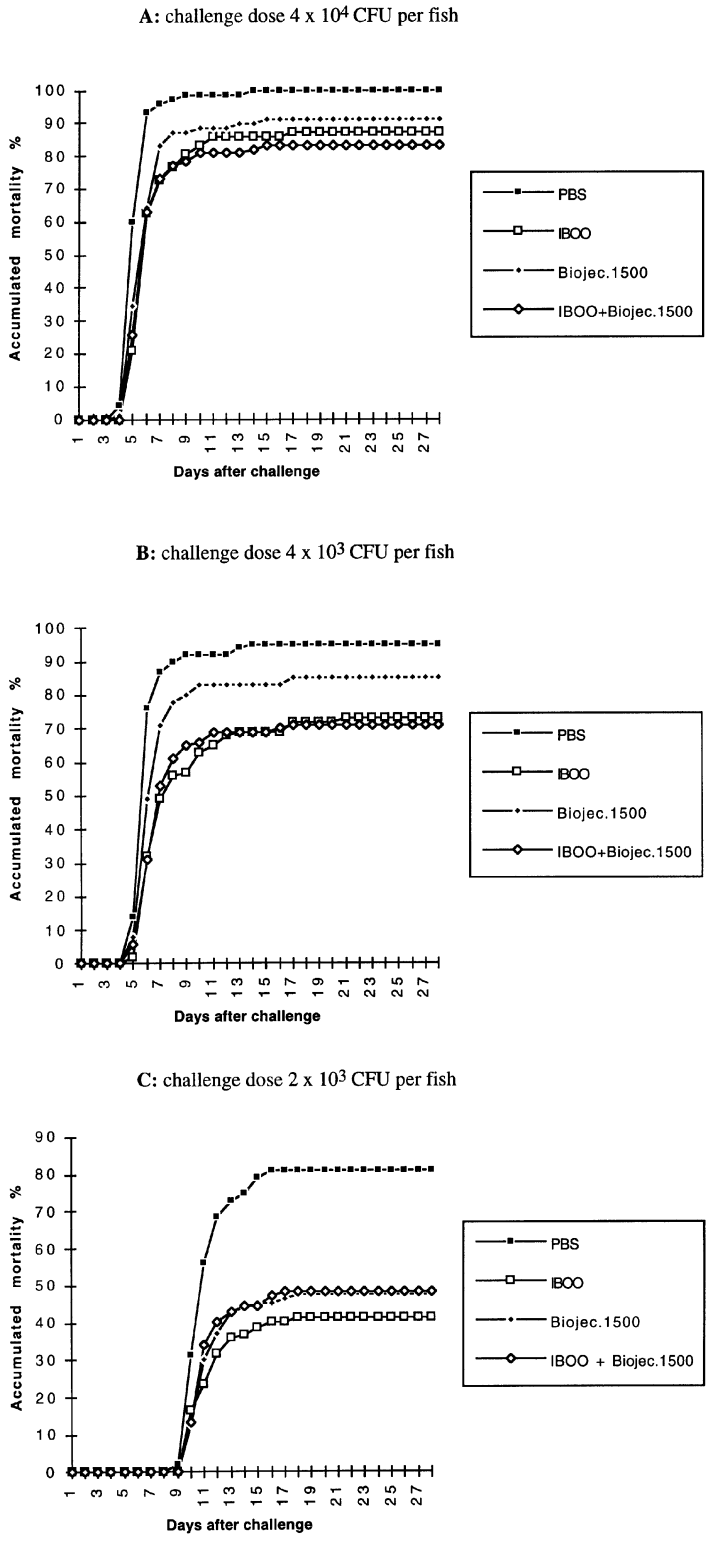


Figure 1 Accumulated mortalities of Atlantic salmon challenged 70 days post-vaccination by intramuscular injection with three different doses (A, B and C) of *A. salmonicida* ssp. *achromogenes*, strain S24–92; 80 fish divided between two tanks were in each treatment group.

Table 2 Relative per cent survival (RPS) and mean days to death (MDD) of Atlantic salmon challenged by i.m. injection of *Aeromonas salmonicida* ssp. *achromogenes*, strain S24-92, 70 days post-vaccination (80 fish were in each treatment group); the *P*-value is from comparison with the PBS-controls*

Challenge dose (CFUs per fish)	PBS	IBOO	*P*-value	Biojec.1500	*P*-value	IBOO + Biojec.1500	*P*-value
RPS							
40 000	(0% survival)	12	0·0002	8	0·0066	13	0·0001
4000	(5% survival)	23	0·0001	11	0·0625	25	<0·0001
2000	(19% survival)	49	<0·0001	41	0·0002	41	0·0002
MDD							
40 000	5·56	6·54	0·2813	6·18	0·2193	6·34	0·2840
4000	6·31	7·88	0·0373	6·85	0·2524	7·24	0·0725
2000	11·26	11·61	0·4960	6·59	0·4714	11·46	0·4789

*Key: PBS, phosphate buffered saline; IBOO, an autogenous Asa bacterin; Biojec.1500, a commercial furunculosis vaccine.

attempted by 15% cohabitation, all injected fish died within a week. Cohabitation infection was not established as no other mortalities occurred in these tanks in the following 6 weeks.

Challenges with *A. salmonicida* ssp. *salmonicida* (Ass)

Twenty-eight days after 11% cohabitation challenge with Ass, mortality in the control group had reached 96% (Fig. 2A) and 72% in the tanks with 9% cohabitation (Fig. 2B). The results from both challenge experiments showed that mortalities of fish vaccinated either with Biojec.1500 or IBOO + Biojec.1500 were significantly lower ($P < 0.0001$) than for the IBOO-vaccinated fish or the control groups. In the lower challenge, the IBOO-vaccinated salmon died later than the fish in the control groups ($P < 0.0001$), but in the more effective challenge, the difference was not significant ($P = 0.4271$) (Table 3). This was the only notable difference obtained between these groups as mortalities of the IBOO-groups were not statistically different from the control groups ($P = 0.4516$). The highest RPS value (70%) was obtained in the fish vaccinated with IBOO + Biojec.1500 in a challenge with 72% mortality of controls (Table 3). In the challenge resulting in 96% mortality of controls, the highest RPS value (44%) was also in the IBOO + Biojec.1500 group, but the difference from mortalities in the Biojec.1500 group was not statistically significant ($P = 0.4050$).

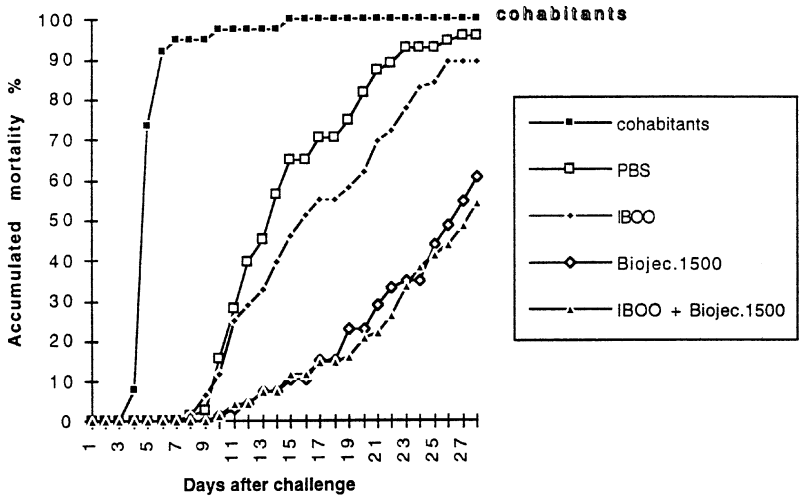
Discussion

The results from this study showed that both an autogenous *A. salmonicida* ssp. *achromogenes* (Asa) bacterin, IBOO, and a commercial furunculosis vaccine, Biojec.1500, evoked protective immunity against an injection challenge of atypical furunculosis in Atlantic salmon. Furthermore, fish vaccinated with Biojec.1500 or with both vaccines simultaneously were equally well protected against classical furunculosis. Protection against co-habitation infection of classical furunculosis was not achieved in fish vaccinated by IBOO alone.

Challenges by cohabitation fish infected with *A. salmonicida* ssp. *salmonicida* (Ass) were effective. Asa infection was not achieved using the cohabitation challenge model in fresh water, although the Asa strain (S24–92) used for challenge was highly virulent. In Iceland, Asa outbreaks in salmon are primarily a problem in brackish water. Our experiments to establish an Asa cohabitation or immersion challenge model in fresh water have repeatedly failed. Introduction of sea water into the challenge system has resulted in an Asa infection of salmon fingerlings, but only fresh water was available in the facilities used for this study. This indicates that there are important differences between these two subspecies of *A. salmonicida* regarding virulence factors necessary for adherence and invasive capacities in fresh water. Since an adequate cohabitation challenge model was not available, an i.m. challenge was used for the detection of protective immunity against Asa. This route of infection produced acute disease with clinical signs and disease propagation similar to that observed during a natural infection.

Both vaccines conferred protection against an i.m. challenge with Asa, indicating the presence of important protective antigens shared by strains of both subspecies. However, the IBOO vaccinated fish were better protected.

A: Cohabitants, injected with 2 x 10^4 CFU, were 11% of total fish



B: Cohabitants, injected with 2 x 10^3 CFU, were 9 % of total fish

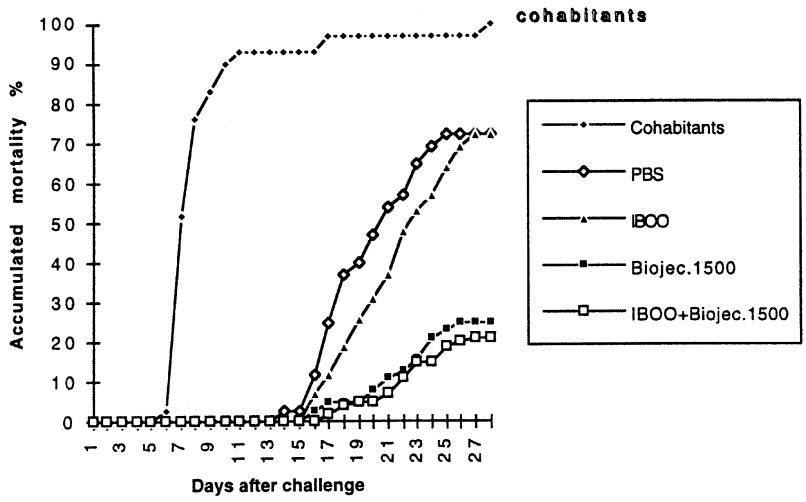


Figure 2 Accumulated mortalities of Atlantic salmon challenged 70 days post-vaccination by cohabitation (A and B) with *A. salmonicida* ssp. *salmonicida,* strain V341–95; 80 fish divided between two tanks were in each treatment group.

Fish vaccinated with Biojec.1500 or with both vaccines simultaneously were protected against Ass infection in the cohabitant challenge model, but fish receiving the IBOO vaccine only were not protected. If shared antigens are important for cross-protection, it is a matter of consideration why immunity to Asa did not protect against an Ass infection. In a preliminary study using fish vaccinated in the field, we found some indication that immunity induced by IBOO vaccine conveyed protection against an Ass challenge (Gudmundsdóttir *et al.* 1996). It is possible that

Table 3 Relative per cent survival (RPS) and mean days to death (MDD) of Atlantic salmon challenged by cohabitation 70 days post-vaccination with *A. salmonicida* ssp. *salmonicida*, strain V361-95 (80 fish were in each treatment group); the *P*-value is from comparison with the PBS-controls*

PBS	IBOO	*P*-value	Biojec.1500	*P*-value	IBOO + Biojec.1500	*P*-value
RPS						
(4% survival)	7	0·2117	36	<0·0001	44	<0·0001
(28% survival)	0	–	65	<0·0001	70	<0·0001
MDD						
14·84	16·38	0·4271	21·40	0·0014	21·22	0·0101
20·29	22·15	<0·0001	22·65	<0·0001	23·12	<0·0001

*Key: PBS, phosphate buffered saline; IBOO, an autogenous Asa bacterin; Biojec.1500, a commercial furunculosis vaccine.

constant exposure to the bacterium under field conditions boosts, and consequently improves, the immune response.

It is difficult to explain the discrepancy in protective capacity of these two vaccines. One explanation could be that important protective antigens, reported to be shared by typical and atypical strains, like the iron-binding outer membrane proteins or porins (Hirst & Ellis 1994; Lutwyche *et al.* 1995), are only present in the Ass bacterin, but not the autogenous Asa. Alternatively, the quality of the vaccine preparations regarding the relative amounts of shared antigens could explain the difference. Although both vaccines were prepared by the same vaccine producer and contain the same adjuvant, it is not known if they were produced under exactly the same culture conditions.

In the Ass challenge with 4% survival of control fish, the protection induced by Biojec.1500 resulted in an RPS value of 36%. In the Asa challenge, where 5% of the controls survived, the RPS value of the Biojec.1500 vaccinated fish was 11%, indicating that the Biojec.1500 vaccine is better suited for inducing protection against Ass than Asa. In another study, we found that extracellular products of Asa elicited better protection than whole bacteria in a homologous challenge of actively immunized Atlantic salmon. The protection strongly correlated with the detection of antibodies directed against a toxic metallo-caseinase, AsaP1, in fish sera (Gudmundsdóttir & Magnadóttir 1997). The difference in production of extracellular toxins by the two *A. salmonicida* subspecies used in this study has been demonstrated and only strains of ssp. *achromogenes* are known to produce the AsaP1 toxin (Gudmundsdóttir 1996). Therefore, we believe that an improvement could be gained by introduction of AsaP1 into the Asa vaccine.

According to the results of this study the furunculosis vaccine might be used for protection against *A. salmonicida* strains of both ssp. *salmonicida* and *achromogenes*. However, before this can be recommended with confidence further studies on protection in the field are needed.

Acknowledgments

This work was supported by the Icelandic Research Council, A. L. Pharma Inc. and Stofnfiskur hf. The authors also wish to thank Dr Gudmundur Georgsson for his help.

References

Austin B. & Austin D. A. (1993) Aeromonadacea representatives (*Aeromonas salmonicida*). In: *Bacterial Fish Pathogens*: *Diseases in Farmed and Wild Fish*, 2nd edn (ed. by L. M. Laird), pp. 86–170. Ellis Horwood, London.

Chu S., Cavaignac S., Feutrier J., Phipps B. M., Kostrzynska M., Kay W. W. & Trust T. (1991) Structure of the tetragonal surface virulence array protein and gene of *Aeromonas salmonicida. The Journal of Biological Chemistry* **266,** 15 258–15 265.

Ellis A. E. (1991) An appraisal of the extracellular toxins of *Aeromonas salmonicida* ssp. *salmonicida. Journal of Fish Diseases* **14,** 265–277.

Evenberg D., Verslius R. & Lugtenberg B. (1985) Biochemical and immunological characterization of the cell surface of the fish pathogenic bacterium *Aeromonas salmonicida. Biochimica et Biophysica Acta* **815,** 233–234.

Gudmundsdóttir B. K. (1996) Comparison of extracellular proteases produced by *Aeromonas salmonicida* strains, isolated from various fish species. *Journal of Applied Bacteriology* **80,** 105–113.

Gudmundsdóttir B. K., Gudmundsdóttir S., Magnadóttir B. & Helgason S. (1996) Research in bacterial diseases of salmonid fish. *Læknabladid, The Icelandic Medical Journal* **82,** 72–77.

Gudmundsdóttir B. K. & Magnadóttir B. (1997) Protection of

Atlantic salmon (*Salmo salar* L.) against an experimental infection of *Aeromonas salmonicida* ssp. *achromogenes. Fish & Shellfish Immunology*, **7,** 55–69.

Hirst I. D. & Ellis A. E. (1994) Iron regulated outer membrane proteins of *Aeromonas salmonicida* are important protective antigens in Atlantic salmon against furunculosis. *Fish & Shellfish Immunology* **4,** 20–45.

Lutwyche P., Exner M. M., Hancock R. E. W. & Trust T. J. (1995) A conserved *Aeromonas salmonicida* porin provides protective immunity to rainbow trout. *Infection & Immunity* **63,** 3137–3142.

Pyle S. W. & Cipriano R. C. (1986) Specificity of lipopolysaccharide antigen of *Aeromonas salmonicida. Microbios Letters* **31,** 149–155.