I. Grantee information

a. *Title*: Whole killed ISA virus vaccine: Saltonstall-Kennedy Grant Final Report
b. *Authors*: Sharon Clouthier, Eric Anderson
c. *Organization*: Maine BioTek, Inc.
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II. Abstract

The inactivation dynamics of infectious salmon anemia virus (ISAV) by β-propiolactone (BPL), binary ethylenimine (BEI), formaldehyde or heat and the antigenic and immunogenic properties of the inactivated vaccines were evaluated. Chemical treatment of ISAV with 1:6000 BPL, 1.5 % BEI or 50 mM formaldehyde abolished virus infectivity within 48 hours whereas heat treatment at 50 °C rendered the virus innocuous within 60 min. The inactivated ISAV vaccines were recognized by Atlantic salmon (*Salmo salar*) ISAV-specific antibodies and were differentially recognized by an ISAV HA-specific monoclonal antibody. The inactivated whole virus vaccines were not efficacious in vaccinated Atlantic salmon challenged by intraperitoneal injection with ISAV 700-800 degree days (12 °C) after immunization and did not elicit a measurable binding antibody response in immunized Atlantic salmon. In contrast, sera collected from convalescent fish and passively transferred to naïve fish provided 31-68% protection and Atlantic salmon that survived lethal challenge with ISA virus were 100% protected upon re-exposure to the virus. Live ISA virus was not cultured from convalescent Atlantic salmon and yet cohabitation studies with naïve Atlantic salmon indicated that live virus was still being shed from the convalescent fish.

III. Executive Summary

The first decisive stage in the production of killed viral vaccines is the inactivation of infectivity of the viral suspension with the concomitant retention of immunoprotective properties. The goal of the Saltonstall-Kennedy project was to develop an ISA virus vaccine based on this principle.

Two methods of virus amplification were identified, one of which increased the quantity of immunoreactive antigens present in each dose of vaccine. We then investigated four methods of virus inactivation: formaldehyde, binary ethylenimine, β-propiolactone and heat. The minimum time of treatment was determined by measuring the kinetics of live virus inactivation. The kinetic approach involved the systematic sampling of the vaccine after addition of the inactivation agent. Samples were removed, neutralized and tested for residual live virus at specific time points. The kinetics of virus inactivation with each treatment were presented as survival curves in which virus viability ($\log_{10} S_0/S_t$) was expressed as a function of virus incubation time with the inactivation agent where S_0 and S_t are the titers of the viral suspension before and at time t after addition of the inactivation agent. Treatment of ISA virus with each of the inactivation agents resulted in total loss of virus infectivity.

Western blot analysis was used to assess retention of immunoreactive epitopes in the inactivated virus preparations. The convalescent serum contained ISA virus-binding activity and the monoclonal antibody recognized epitopes located in the IHN virus HA protein. Retention of antigen immunoreactivity after treatment did not ensure retention of the protective and immunogenic properties of the virion. For example, no change in the immunoreactivity of heat treated virus with either the HA-specific monoclonal antibody or Atlantic salmon anti-ISA virus convalescent sera was observed and yet the relative percent survival of Atlantic salmon vaccinated with that preparation of virus was only 7%. These results indicate that Western blot analysis of the virus antigen used to prepare the vaccine can be used to monitor epitope retention and the presence of immunoreactive HA protein, but the method is not a reliable tool for predicting the immunoprotective properties of the antigen *in vivo*.

In the vaccine efficacy studies, Atlantic salmon were immunized with the various inactivated preparations of ISA virus and then challenged by intraperitoneal injection of ISA virus. We conducted three independent vaccine trials but not all of the various vaccine preparations were tested in each trial. The inactivated whole virus vaccines were not efficacious in vaccinated Atlantic salmon challenged 700-800 degree days (12 °C) after immunization and did not elicit a measurable binding antibody response in immunized Atlantic salmon. In contrast, sera collected from convalescent fish and passively transferred to naïve fish provided marginal protection and Atlantic salmon that survived lethal challenge with ISA virus were 100% protected upon re-exposure to the virus. Live ISA virus was not cultured from convalescent Atlantic salmon and yet cohabitation studies with naïve Atlantic salmon indicated that live virus was still being shed from the convalescent fish.

IV. Purpose

A. Description of problem. Maine BioTek, Inc. requested S-K funds for the above mentioned proposal which was directed to Priority A topic "Vaccines or other methods to prevent the spread of disease between farmed fish and wild fish" as outlined in NMFS S-K solicitation notice[1]. The goal of the project was to develop a safe, efficacious, inactivated infectious salmon anemia (ISA) virus vaccine.

ISA virus, an *Orthomyxovirus,* is an emerging pathogen associated predominately with disease in farmed Atlantic salmon (*Salmo salar*). Table 1 illustrates the current geographic distribution of ISA virus. The virus is among the fish pathogens listed in the Aquatic Animal Health Code of the Office International des Epizooties (OIE). As such, ISA is a notifiable disease and compulsory eradication policies are enforced worldwide.

Table 1. ISA virus geographic distribution, year diagnosed and host species

Country	Year first detected	Fish species
Norway	1984	Atlantic salmon
Canada (Atlantic Provinces)	1996-97	Atlantic salmon
United Kingdom (Scotland)	1998	Atlantic salmon
Denmark (Faroe Islands)	2000	Atlantic salmon
Chile	2001	Coho salmon
United States (Maine)	2001	Atlantic salmon
Ireland	2002	Rainbow trout

The following examples illustrate the impact of ISA virus on the Atlantic salmon aquaculture industry. On December 13, 2001, the U.S. Secretary of Agriculture issued a declaration of emergency because of ISA virus in Maine. Subsequently, 2.5 million farmed Atlantic salmon in Cobscook Bay were slaughtered with the U.S. government compensating farmers for a percentage of their losses. Losses incurred in Maine due to ISA alone in 2002 were over $12 million (US). Fish restocking in Cobscook Bay took place under conditions of strict fish health regulation and monitoring in 2002 and 2003. Nevertheless, two new outbreaks of ISA occurred in Maine during June and July 2003 resulting in the slaughter of approximately 125,000 fish from two marine sites in Cobscook Bay[2]. Neighboring salmon farmers in Canada have been dealing with ISA for a longer period of time and it is estimated that ISA has caused approximately $50 million in economic losses since 1997. ISA outbreaks in the Faroese Atlantic salmon industry have caused monetary losses of approximately DKK $250 million with concomitant reduction of shareholder equity from DKK$500 million to virtually zero[3]. Eradication policies in Norway reduced the incidence of ISA virus in the early-mid 1990's but are now deemed inadequate due to the rapid increase of ISA disease in the area. In response to the continuing threat of ISA virus, three of the world's largest salmon producers have stated that an ISA virus vaccine is needed, and will be an integral part of a comprehensive fish health management plan to gain control of ISA virus[4].

Forecasting the short and long-term impact that ISA virus may have on the salmon aquaculture industry requires a basic understanding of ISA virus epidemiology. Fish-to-fish propagated epizootics occur at farm sites along with concomitant local amplification of virus. Shipping has been identified as the primary mechanism of ISA virus spread between farm sites at the regional and national scale (17). Local ISA virus dissemination from a farm site may also involve transport by water currents, infected escaped fish, infected wild fish or other vectors. Because knowledge about the natural history of ISA virus is incomplete, there is limited information about viral traffic between wild and farmed salmon (12, 19, 20, 23). However, Nylund et al (18) has recently described a series of hypotheses concerning the "emergence and maintenance of infectious salmon anaemia virus (ISAV) in Europe..." that, if proven true, will have a significant impact on the formulation of international management plans including those in Maine and Atlantic Canada. Nylund suggests, among other things that: "(a) ISAV is maintained in wild populations of trout and salmon in Europe; (b) ISA emerges in farmed Atlantic salmon when mutated isolates are transmitted from wild salmonids or, following mutation of benign isolates, in farmed salmon after transmission from wild salmonids; (c) transmission of ISAV from farmed to wild salmonids probably occurs less frequently than transmission from wild to farmed fish due to lower frequency of susceptible wild individuals; (d) the frequency of

[1] Department of Commerce, NOAA (2002) Financial Assistance for Research and Development Projects to Strengthen and Develop the U.S. Fishing Industry. Federal Register 67:34427-34434.
[2] OIE [www.oie.int]: Disease Information: June 20, 2003 (Vol 16, No. 25); July 4, 2003 (Vol 16, No. 27)
[3] Intrafish, 2004
[4] Intrafish, 2002

new outbreaks of ISAV in farmed salmon probably reflects natural variation in the prevalence of ISAV in wild populations of salmonids"(18). If these points are correct, ISA virus is likely enzootic in wild fish in Maine and Atlantic Canada and eradication of ISA virus will be difficult if not impossible in open water seapens. Control measures, such as efficacious vaccines would reduce the viral load in farm sites and decrease the potential for transmission of the virus between wild and farmed Atlantic salmon. In this sense, an efficacious ISA virus vaccine could form an integral component of adaptive management strategies for the salmon aquaculture industry as well for the protection and recovery of endangered Atlantic salmon in Maine.

Four different vaccine types can be considered for the control of ISA virus: whole killed virus, attenuated virus, recombinant virus antigen and DNA encoded antigen. The killed virus vaccine type is the practical choice for the ISA virus vaccine based upon USDA and EU vaccine regulatory policies and was the focus of this S-K proposal. Killed virus vaccines are often overlooked in our era of biotechnology, but the fact is that 39% (223/579) of the viral vaccines listed in the semiannual publication of veterinary biological products licensed by USDA-APHIS are whole killed preparations and 61% are live modified preparations (June, 2001). A rational choice of inactivating agents and the conditions for inactivation of virus infectivity are critical components in the manufacturing process of killed antiviral vaccines (3). Chemical inactivation agents are of two types: reticulating and alkylating. Reticulating agents include formaldehyde and glutaraldehyde and the alkylating agents include binary ethylenimine and β-propiolactone. Further improvements of whole killed viral vaccines can be made through research on the retention of immunologically important antigens during virus inactivation processes and was the focus of this S-K proposal. Maine BioTek also believed that the proposed work would provide critical information and methodologies relevant to the development of vaccines for other economically important enveloped viruses of finfish.

Maine BioTek's research and development on an ISA virus whole killed vaccine is based upon solid scientific information. Dr. Clouthier and Dr. Anderson, with funding from Sea Grant, Northeast Regional Aquaculture Consortium and Microtek International, Ltd., determined the ISA virus RNA genome segment order, nucleotide sequence and the putative encoded proteins and reported the findings in a paper entitled "Genomic organization of infectious salmon anemia virus" (6). In the study, ISA virus proteins that elicited an immune response in Atlantic salmon were identified as the ISA virus cell surface hemagglutinin glycoprotein and the type-specific nucleoprotein. The PIs also have submitted a patent enabling exclusivity in the use of the information for development of an ISA virus whole killed vaccine, recombinant vaccine and DNA vaccine. A *prototype* whole killed ISA virus vaccine was initially developed based upon Dr. Clouthier's work funded through the USDA SBIR program (Phase I) and Microtek International., Ltd. In preliminary studies, the *prototype* vaccine provided up to 80% relative survival of Atlantic salmon against ISA virus challenge ($10^{6.5}$ $TCID_{50}$) compared to unvaccinated fish. However, the relative percent survival provided by the vaccine had been inconsistent.

The action of any inactivating agent causes simultaneous modification of viral components responsible for immunogenicity and viral polynucleotides that are responsible for infectivity. The degree of modification of virion components and consequently the reduction of the infectivity as well as the immunogenicity of the viral suspension is proportional to the increase of time of treatment with the inactivating agent (5). The PIs identified epitope modification during whole killed virus vaccine preparation as a possible source of the observed variation in efficacy of the prototype ISA vaccine. This phenomenon has been observed with other whole killed viral vaccines including poliovirus (7), respiratory syncytial virus (9, 13), measles virus (8) and influenza virus (5). The most dramatic effect of inappropriate inactivation of the virus during preparation of a vaccine is the lack of recognition of an antigen by the host immune system due to chemical ablation of critical epitopes (1, 7). In addition, inactivation can result in preferential inactivation of antigenicity, a phenomenon observed with the surface glycoproteins hemagglutinin and neuraminidase of the influenza virus (11). Epitope modifications can also lead to potentiation of disease due to an imbalance in the host immune response (9). Because of the affect of chemicals on viral antigens, the choice of inactivating agent and the conditions in which it is employed are the most critical steps in the formulation of a whole killed vaccine.

Antigenic drift and shift must be considered when developing an ISA virus vaccine. Influenza virus, the protypic orthomyxovirus, undergoes antigenic shifts resulting in disease pandemics and antigenic drift that constitutes microheterogeneity within virus strains (25). Antigenic shift has not been reported for ISA virus but distinct differences in nucleic acid and amino acid sequence has been observed upon comparison of the hemagglutinin of North American and European ISA virus isolates (14). Further, ISA virus isolates from Nova Scotia, Canada, have been reported to be more closely related to European isolates than they are to viral isolates from New Brunswick, Canada and Maine (21). Consequently, Maine BioTek considered it important to explore the efficacy of the whole killed ISA virus vaccines produced using a North American isolate in a heterologous challenge with a European strain of the virus.

The purpose of the S-K project was to refine the prototype ISA virus vaccine with respect to virus inactivation, dose, formulation and heterologous protection. In addition, the research was directed at defining the role of the humoral immune response in virus clearance from ISA virus-infected Atlantic salmon.

B. Objectives of the project.

The overall goal of our Saltonstall-Kennedy project was to develop an inactivated virus vaccine for the control of ISA disease in Atlantic salmon. The work plan addressed five primary topics related to vaccine performance: preparation, formulation, potency, safety and efficacy. The specific objectives including specific tasks, sub-tasks, milestones and personnel are outlined in Table 2.

Table 2. Saltonstall-Kennedy project objectives

Task	Sub-Task	Milestone	Personnel
Virus production	Virus amplification	Well-characterized & standardized virus growth	Maine BioTek
	Antigen quantity	Standardized antigen quantity/dose	
	Antigen modification	Alternative methods of antigen treatment	
Virus production deliverable: Methods of virus amplification & modification			
Virus inactivation	Inactivation kinetics	Loss of virus infectivity	Maine BioTek
	Antigen quality	Identification of inactivation conditions for retention of immunoreactive antigens	
Virus inactivation deliverable: Inactivated & immunoreactive virus for efficacy tests			
Vaccine performance	Vaccine formulation	Antigen-adjuvant combinations	Maine BioTek
	Vaccine safety	Safety of vaccines established	Maine BioTek & DFO
	Vaccine efficacy – study 1	RPS value established for vaccine prototypes; homologous virus challenge	
	Vaccine efficacy – study 2	RPS value established for vaccine prototypes; heterologous virus challenge	Maine BioTek & DFO
	Immunological correlates of protection	Humoral immune response measured by ELISA	Maine BioTek
Vaccine performance deliverable: Vaccine prototype			

V. Approach

A. Description of work (Materials & methods; Results)

Virus and virus antigen

Virus amplification. Virus for preparation of the whole killed vaccine was the North American ISA virus isolate CCBB (6). The chinook salmon embryo cell line, CHSE-214 (16), was used to amplify the virus. Cells were maintained at 15°C in minimum essential medium (MEM) containing Hank's salts and supplemented with L-glutamine (10 mM) and 5% fetal bovine serum (all from Gibco BRL).

Virus was amplified in either 6,300 cm^2 Cell Factories™ (Nunc) or 175 cm^2 flasks. Virus infection of each Cell Factory™ was done using a virus inoculum of $10^{3.5}$ $TCID_{50}/cm^2$ (tissue culture infective dose). Following complete cell lysis (21-30 days), the supernatant and cell debris was harvested according to the manufacturer's recommendation (Nunc). In our laboratory one Cell Factory™ was used to produce 1.2 L of virus supernatant-cell debris with a titer of approximately 10^6 $TCID_{50}$/ml. The virus antigen resulting from this method of virus amplification is referred to hereafter as the **traditional antigen** preparation.

Virus titer determination. The virus titer was calculated according to the method of Spearman (24) and Karber (10). Serial dilutions, from 10^0 to 10^{-8}, were inoculated onto cell monolayers seeded in 96-well plates. The cytopathic effect (CPE) was recorded after 15-25 days and the virus titer was determined and expressed as $TCID_{50}$/ml. A typical titer of ISA virus grown on CHSE-214 cells was between $10^6 - 10^7$ $TCID_{50}$/ml.

Virus growth curves. Growth curves of released and cell-associated ISA virus were determined using a modification of the procedure reported by Rovozzo and Burke (22). CHSE-214 cell monolayers were grown in 8-well cell culture plates at 15°C, washed with Dulbecco's PBS (D-PBS) and inoculated with ISA virus ($1x10^{7.5}$ $TCID_{50}$/ml). After a 1-

hour adsorption interval at 15°C, the monolayers were washed three times with D-PBS to remove unattached virus, fresh MEM medium was added and the wells were sealed with polyethylene film (Becton Dickinson). The plates were incubated at 15°C and samples were removed at various intervals (2 hours to 220 hours) for virus titration. At each time point, the supernatant was removed but was not clarified prior to virus titration. The monolayers were washed three times with D-PBS, scraped into 1 ml fresh MEM medium and then placed in liquid nitrogen and rapidly thawed three times prior to virus titer determination.

In general, the titer of the virus in the samples was similar in magnitude, and followed similar growth kinetics (Figure 1). The eclipse period was determined to last 42-48 hrs. Virus amplification was observed for approximately 48 hrs and a linear relationship existed between the virus titer and growth time period. These experiments led to the discovery of an important biological characteristic of ISA virus growth *in vitro*.

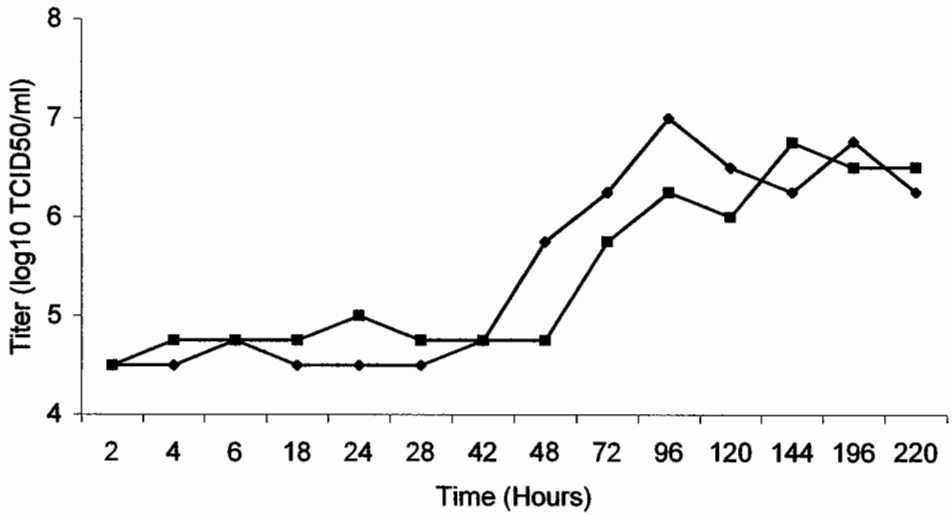

Figure 1. ISA virus growth curve. The titer of virus in intact, attached CHSE-214 cell monolayers (♦) and cell growth medium (■) was calculated according to the method of Spearman (24) and Karber (10).

Enrichment of virus antigen. Based on the virus growth curve studies, we developed a second method for producing ISA virus antigen. The virus antigen resulting from this method of virus amplification is referred to hereafter as the **enriched antigen** preparation. The enrichment is illustrated in Figure 2 which compares the relative amounts of antigen in the traditional (lane 1) and enriched (lane 2) antigen preparations.

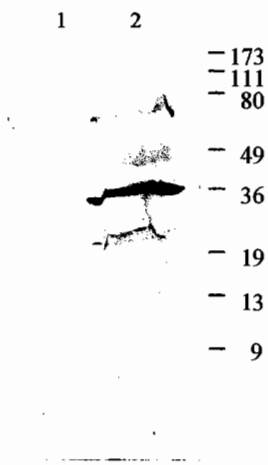

Figure 2. Western blot analysis of ISA virus with HA-specific monoclonal antibody. Lane 1, traditional; lane 2, enriched preparations of ISA virus antigen were separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) (15) and electrophoretically transferred to nitrocellulose. Immunoreactive proteins were visualized with goat anti-mouse IgG or IgM conjugated to alkaline phosphatase (Southern Biotechnology

Associates, Inc) following development with NBT/BCIP. Molecular mass standards (kDa) are indicated on the right.

Virus antigen modification. As the project progressed, we found that retention of antigen immunoreactivity after virus inactivation did not ensure retention of the protective and immunogenic properties of the viral antigen regardless of the method of inactivation, antigen dose or adjuvant. Since the antigen preparations were recognized by antibodies from Atlantic salmon that had recovered from infection, the results suggested to us that the ISA virus antigen was not being presented in a form that stimulated a measurable immune response. To investigate this possibility we modified viral antigen(s) to a form that might be more readily recognized by immune effectors. The methods we used to prepare the vaccines are referred to as modified, traditional or enriched antigen 1, 2, 3 or 4.

Virus inactivation:
Formaldehyde inactivation. Formaldehyde (37% w/w; Sigma) was added to live ISA virus, 10^6 $TCID_{50}$/ml, to a final concentration of 0.148% and the mixture was agitated for a maximum time of 24 h at 22°C. Samples for virus viability and immunoreactivity were removed at various timepoints after addition of formaldehyde.

Heat inactivation. For heat inactivation of the virus, live ISA virus (10^6 $TCID_{50}$/ml) was incubated at 50°C for 2 h. Samples for virus viability and immunoreactivity were removed at various timepoints after initiating the thermal treatment.

BEI inactivation. A freshly prepared solution of 0.1 M BEI was added to live ISA virus, 10^6 $TCID_{50}$/ml, to a final concentration of 1.5% (v/v; 2) and the mixture was agitated for 24-48 h at 37°C. Virus inactivation was terminated by the addition of sodium thiosulphate. Samples for virus viability and immunoreactivity were removed at various timepoints after addition of the BEI and after treatment with sodium thiosulphate.

BPL inactivation. BPL (Sigma) was mixed with live ISA virus, 10^6 $TCID_{50}$/ml, at 1 part BPL to 6000 parts virus solution (4, 5). The mixture was agitated for 51 h at 22°C. Virus inactivation from BPL was neutralized by the addition of sodium thiosulphate. Samples for virus viability and immunoreactivity were removed at various timepoints after addition of BPL and after treatment with sodium thiosulphate.

Test for residual live virus. To measure whether viable virus was present after chemical or thermal treatment, a portion of each antigen preparation was used to inoculate CHSE-214 cell monolayers. If there were no apparent cytopathic effects (CPE) after 21 days, the media from the primary flask was then used to inoculate a second monolayer of CHSE-214 cells. No live virus was detected and the inactivated virus preparations were used in the efficacy trials.

Effect of sodium thiosulphate. Following chemical inactivation of live virus, the inactivating agent was neutralized with sodium thiosulphate. To test whether sodium thiosulphate influenced virus viability, live virus was incubated with the neutralizing agent (0.01 M) for 1, 4 or 24 hr at room temperature. The virus titer of the material collected was calculated according to the method of Spearman (24) and Karber (10). Serial dilutions, from 10^0 to 10^{-12}, were inoculated onto cell monolayers seeded in 96-well plates (Costar). The cytopathic effect (CPE) was recorded after 15-25 days and the virus titer was determined and expressed as $TCID_{50}$/ml. The titer of the virus was not substantially altered by the presence of sodium thiosulfate when compared to control treatments (Table 3).

Table 3. Effect of sodium thiosulphate on CHSE-214 cells and ISA virus

Treatment	Titer ($TCID_{50}$/ml)
Phosphate buffered saline + sodium thiosulphate T=0	No negative effect on cells
ISA virus at room temperature T=0	$1x10^{6.25}$
ISA virus + sodium thiosulphate at room temperature T=0	$1x10^{6.00}$
ISA virus at room temperature T=1 hr	$1x10^{5.50}$
ISA virus + sodium thiosulphate at room temperature T=1 hr	$1x10^{5.75}$
ISA virus at room temperature T=4 hr	$1x10^{5.75}$
ISA virus + sodium thiosulphate at room temperature T=4 hr	$1x10^{6.00}$
ISA virus at room temperature T=24 hr	$1x10^{6.00}$
ISA virus + sodium thiosulphate at room temperature T=24 hr	$1x10^{6.00}$

Virus inactivation kinetics. Samples of ISA virus were collected at various time points after addition of the inactivation agent and the virus titer was calculated according to the method of Spearman (24) and Karber (10). Virus titer was determined after neutralization of the inactivating agent. In control experiments, sodium thiosulfate had no effect on virus titer when compared to control treatments (Table 3). The kinetics of virus inactivation with each treatment is presented as survival curves in which virus viability ($\log_{10} S_o/S_t$) is expressed as a function of virus incubation time with the inactivation agent where S_o and S_t are the titers of the viral suspension before and at time t after addition of the inactivation agent (Figure 3). Treatment of ISA virus with either heat or BPL resulted in total loss of virus infectivity. In contrast, residual live virus was still present in preparations of ISA virus treated with BEI for up to 24 hours. ISA virus sampled after treatment with BEI, sodium thiosulphate and heat as described above was completely inactivated (not shown). These results underline the importance of verifying virus inactivation during preparation of whole killed virus vaccines.

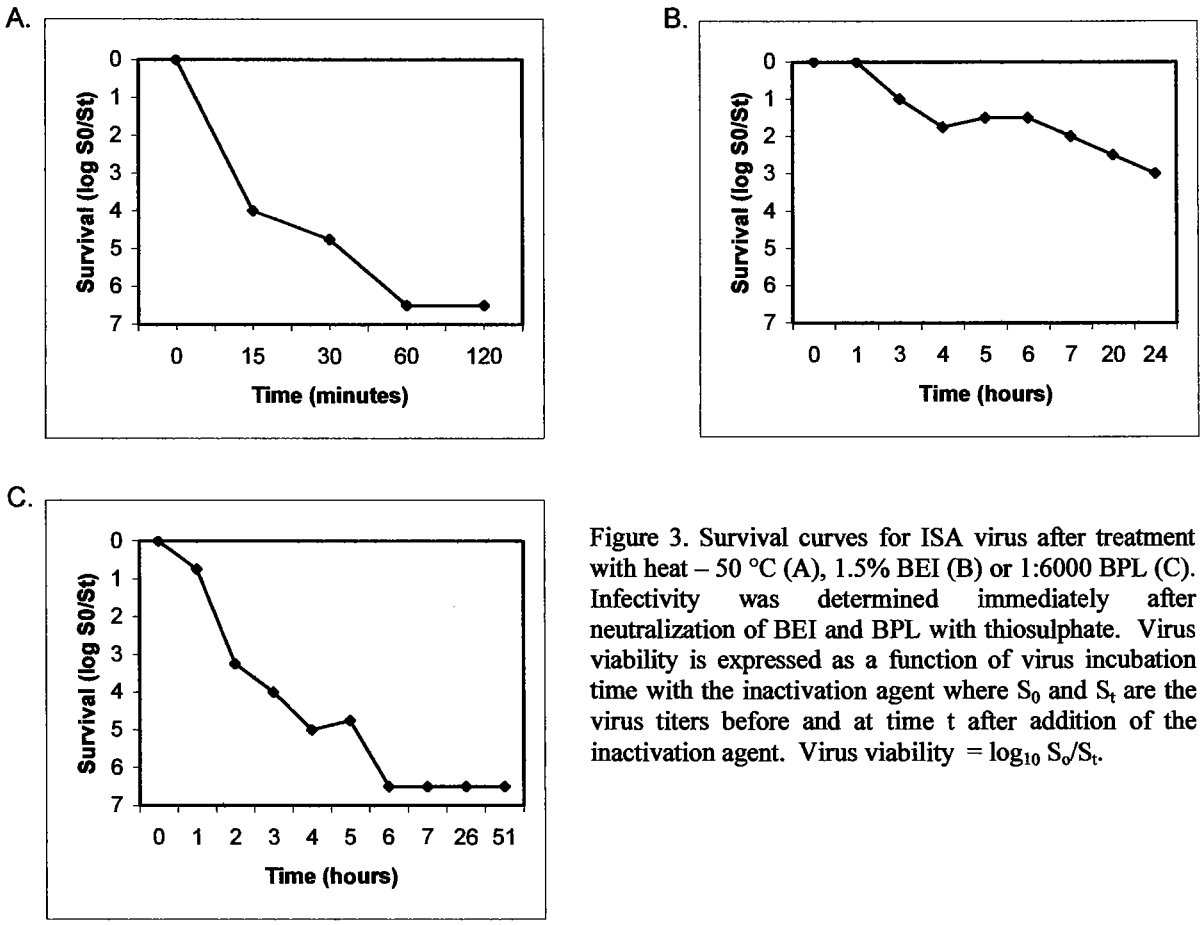

Figure 3. Survival curves for ISA virus after treatment with heat – 50 °C (A), 1.5% BEI (B) or 1:6000 BPL (C). Infectivity was determined immediately after neutralization of BEI and BPL with thiosulphate. Virus viability is expressed as a function of virus incubation time with the inactivation agent where S_0 and S_t are the virus titers before and at time t after addition of the inactivation agent. Virus viability = $\log_{10} S_o/S_t$.

Immunoreactivity of inactivated ISA virus.

Formaldehyde inactivated ISAV. We found that treatment of ISA virus with formaldehyde for 24 h did not abrogate immunoreactivity with the HA-specific monoclonal 10A3 when examined using immunohistochemical techniques. In contrast, neither monoclonal antibody 10A3 nor Atlantic salmon anti-ISA virus convalescent sera reacted with formaldehyde fixed virus antigen separated by SDS-PAGE and analyzed by the Western blot technique (Figure 4). At the present time, we believe this result is a secondary consequence of formaldehyde treatment of the antigen rather chemical ablation of the epitopes. Immediately following addition of formaldehyde to the antigen preparation, macroscopically visible clumps of protein formed. The clumps were presumably the result of chemical cross-linking. These large molecular weight aggregates were not solubilized in the denaturing and reducing sample buffer for SDS-PAGE, even in the presence of acid. However, immunoreactive material was observed in the stacking gel (not shown) which is compatible with the immunohistochemistry results. In a broader context, these

results suggest that the somewhat unpredictable efficacy of whole-killed vaccines may be a physical consequence of antigen aggregation and concomitant loss of dosage homogeneity.

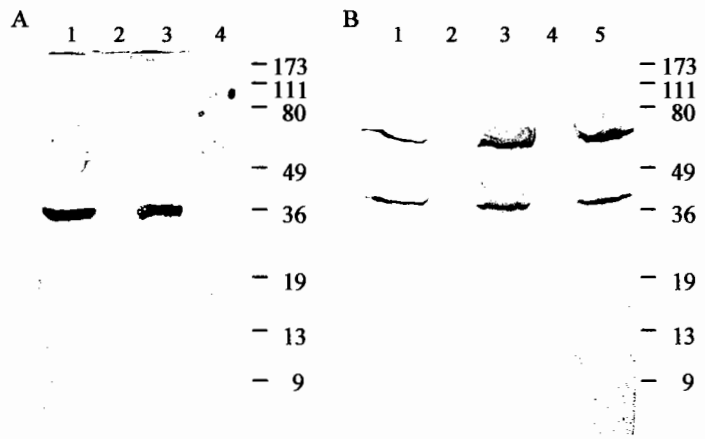

Figure 4. Immunoreactive antigens of formalin inactivated ISA virus detected by Western blot analysis with monoclonal antibody 10A3 (A) and Atlantic salmon anti-ISA virus convalescent serum (B). The proteins were separated by SDS-PAGE (15) and electrophoretically transferred to nitrocellulose. Immunoreactive proteins were visualized with goat anti-mouse immunoglobulin conjugated to alkaline phosphatase following development with NBT/BCIP. Lanes 1 and 3, untreated ISA virus antigen; lanes 2 and 4, formaldehyde treated ISA virus antigen; lane 5 (B only), whole cell lysate of CHSE-214 cells infected with ISA virus isolate CCBB. Molecular mass standards (kDa) are indicated on the right.

Heat inactivation. Heat treatment, unlike formaldehyde treatment, did not result in clumping of the antigen. Because of this the antigen was readily solubilized in denaturing and reducing sample buffer for SDS-PAGE. No difference was observed in the immunoreactivity of heat inactivated antigen when compared to untreated antigen preparations (Figure 5).

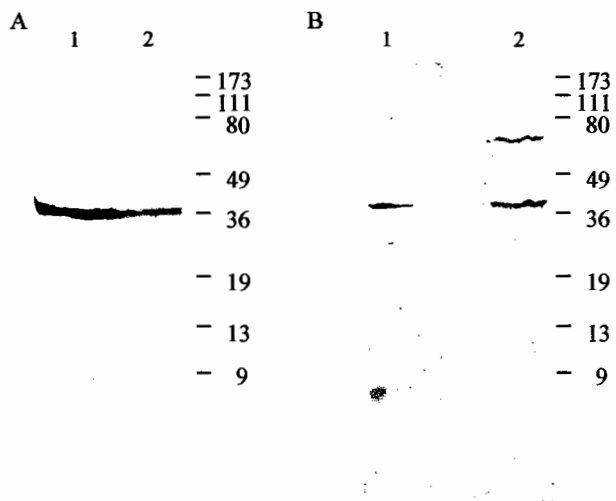

Figure 5. Immunoreactive antigens of heat inactivated ISA virus detected by Western blot analysis with mAb 10A3 (A) and Atlantic salmon anti-ISA virus convalescent serum (B). The proteins were separated by SDS-PAGE (15) and electrophoretically transferred to nitrocellulose. Immunoreactive proteins were visualized with goat anti-mouse immunoglobulin conjugated to alkaline phosphatase following development with NBT/BCIP. Lane 1, untreated ISA virus antigen; lane 2, heat treated ISA virus antigen. Molecular mass standards (kDa) are indicated on the right.

BEI inactivation. Treatment of ISA virus with BEI for 24 h did not abrogate immunoreactivity with either the HA-specific monoclonal antibody 10A3 or Atlantic salmon anti-ISA virus convalescent sera (Figure 6). However, a reduction in the immunoreactivity of viral antigens was observed after residual BEI was quenched with sodium thiosulphate (Figure 6A, 6B). Ablation was more significant with the epitopes in the 70 kDa and 42 kDa proteins recognized by the convalescent sera (Figure 6B). We found that the immunoreactivity was retained when the incubation periods following addition of sodium thiosulphate were reduced. Western blot analysis also revealed that after addition of BEI to the virus preparation, HA reactivity with the monoclonal antibody was modified relative to the untreated virus. Two immunoreactive species other than the expected 42 kDa HA were observed (Figure 6A; compare lane 2 with lanes 3 - 9): 1) 80-85 kDa immunoreactive band migrating at the approximate molecular weight of an HA dimer; and 2) 25-30 kDa truncated form of the HA. Both of these alternate forms of the HA were being detected using a HA-specific monoclonal antibody. Neither of these two species was detected by the convalescent sera suggesting that the convalescent sera does not recognize multimeric or truncated forms of the HA protein.

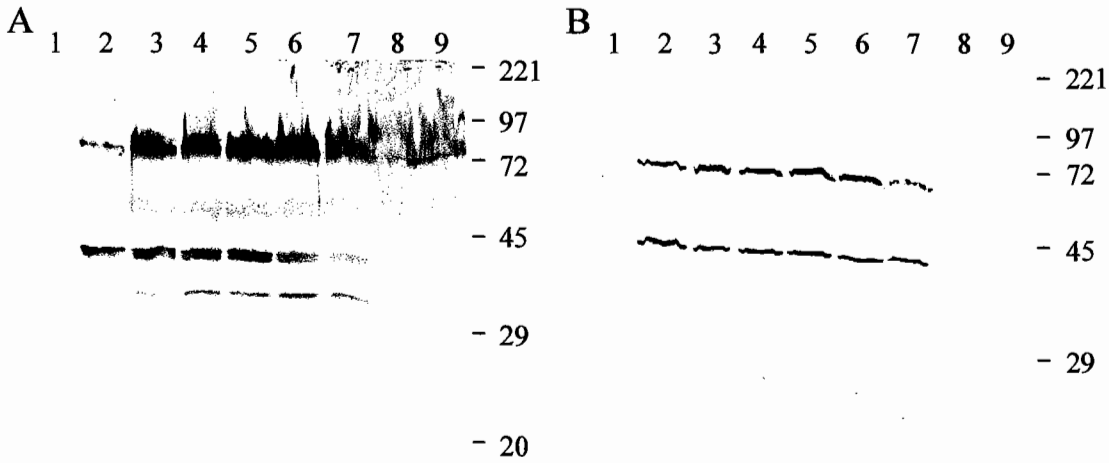

Figure 6. Immunoreactive antigens of BEI inactivated ISA virus detected by Western blot analysis with monoclonal antibody 10A3 (A) and Atlantic salmon anti-ISA virus convalescent serum (B). The proteins were separated by SDS-PAGE (15) and electrophoretically transferred to nitrocellulose. Immunoreactive proteins were visualized with goat anti-mouse immunoglobulin conjugated to alkaline phosphatase following development with NBT/BCIP. Lane 1, uninfected CHSE cells, lane 2, untreated ISA virus antigen; lane 3, BEI t = 1 h; lane 4, BEI t = 2 h; lane 5, BEI t = 3 h; lane 6, BEI t = 20 h; lane 7, BEI t = 24 h; lane 8, BEI t = 24 h, Na Thio t = 20 h; lane 9, BEI t = 24 h, Na Thio t = 20 h, 55°C t = 4 hr. Molecular mass standards (kDa) are indicated on the right.

BPL inactivation. Inactivation of ISA virus with BPL for 26 hr modified but did not eliminate immunoreactivity with either the HA-specific mAb 10A3 or Atlantic salmon anti-ISA virus convalescent sera (Figure 7). Concurrent with reduced reactivity of the 42 kDa HA protein was the appearance of the 25-30 kDa truncated HA in samples examined with mAb 10A3 (Figure 7A; compare lane 1 with lanes 2 - 6). No difference was observed between the treated and untreated samples analyzed using convalescent sera (Figure 7B).

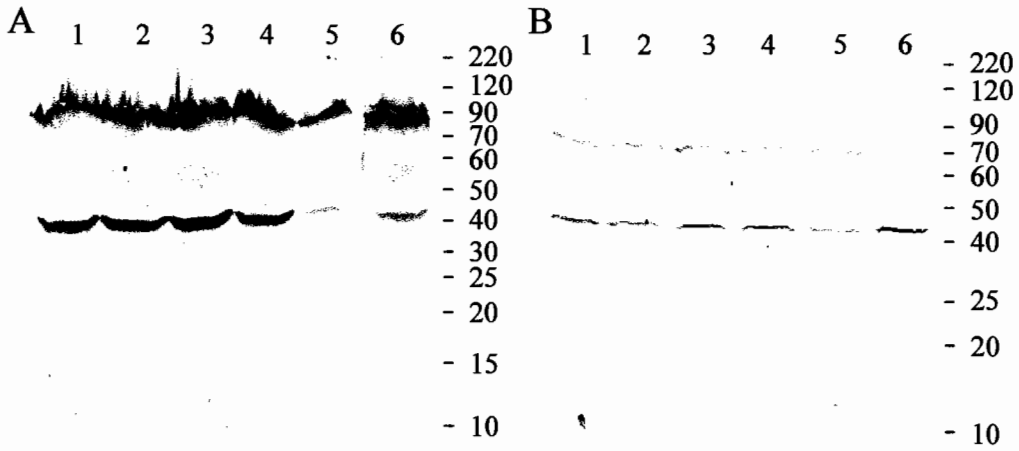

Figure 7. Immunoreactive antigens of BPL inactivated ISA virus detected by Western blot analysis with monoclonal antibody 10A3 (A) and Atlantic salmon anti-ISA virus convalescent serum (B). The proteins were separated by SDS-PAGE (15) and electrophoretically transferred to nitrocellulose. Immunoreactive proteins were visualized with goat anti-mouse immunoglobulin conjugated to alkaline phosphatase following development with NBT/BCIP. Lane 1, untreated ISA virus antigen; lane 2, BPL t = 1 h; lane 3, BPL t = 2 h; lane 4, BPL t = 3 h; lane 5, BPL t = 26 h; lane 6, BPL t = 24 h, Na Thio t = 5 h. Molecular mass standards (kDa) are indicated on the right.

Antigen modification. Modification of the enriched or traditional ISA virus antigen preparations using the methods 1, 2, 3 or 4 did not abrogate immunoreactivity with either the HA-specific monoclonal antibody 10A3 or Atlantic salmon anti-ISA virus convalescent sera. Western blot analysis did reveal that after treatment of the virus antigen, HA reactivity with the monoclonal antibody or the convalescent sera was modified relative to the untreated virus. Immunoreactive species other than the expected 42 kDa HA were observed including truncated forms of the HA (data not shown). Recognition of the truncated forms of the HA by the convalescent sera was a significant finding that we had not anticipated since truncated forms of the HA generated after BEI or BPL treatment of the virus were not recognized by convalescent sera. The reactivity of the modified antigen by the convalescent sera suggested that the antigen was present in a form that might elicit a protective humoral immune response in vaccinated Atlantic salmon.

Vaccine efficacy studies

The *in vivo* efficacy studies were conducted at the Department of Fisheries and Oceans Biological Station, St. Andrews, New Brunswick, Canada. We tested the efficacy, potency, safety, virus clearance and humoral immune response elicited by the inactivated virus preparations.

Vaccine formulation. Immediately prior to vaccinating fish, the inactivated virus preparations were mixed with adjuvant to form a stable emulsion.

Fish husbandry and vaccination. Specific pathogen free Atlantic salmon (50-75g) were obtained from the Huntsman Marine Science Center, New Brunswick, Canada and held at 10-12°C in fresh water. Two weeks prior to vaccination, fish were anesthetized in 100 µg/L of tricaine methane sulfonate (TMS) and microchip transponders were implanted in the peritoneal cavity of the Atlantic salmon. The tags facilitated our ability to track fish throughout the study. While in the freshwater life stage, duplicate groups of 25 Atlantic salmon (30-60 g) were vaccinated by intraperitoneal injection with 0.2 ml of the vaccine. An additional five fish per group were vaccinated but not challenged. After vaccination, the fish were acclimated to salt water (30 ppm) over the course of 5 days and held at 12°C in salt water for the remainder of the trial. Fish were challenged 700-800 degree days (dd) after vaccination.

Challenge virus preparation. Virus for the homologous challenge study was the North American ISA virus isolate CCBB (6). The chinook salmon embryo cell line, CHSE-214 (16), was used to amplify the virus. Cells were

maintained at 15°C in minimum essential medium (MEM) containing Hank's salts and supplemented with L-glutamine (10 mM) and 5% fetal bovine serum (all from Gibco BRL).

Virus infection of CHSE cells seeded in a 6,300 cm^2 Cell Factory™ (Nunc) was done using a virus inoculum of $10^{3.5}$ $TCID_{50}/cm^2$ (tissue culture infective dose). Following complete cell lysis (21-30 days), the supernatant and cell debris were harvested according to the manufacturer's recommendation (Nunc).

Challenge virus titer determination. The virus titer of the material collected from the Cell Factory™ was calculated according to the method of Spearman (24) and Karber (10). Serial dilutions, from 10^0 to 10^{-8}, were inoculated onto cell monolayers seeded in 96-well plates (Costar). The cytopathic effect (CPE) was recorded after 15-25 days and the virus titer was determined and expressed as $TCID_{50}$/ml. The virus in the cell culture supernatant-cell debris had a titer of 10^6 $TCID_{50}$/ml.

Condition of virus challenge. Vaccinated Atlantic salmon were challenged with 10^6 $TCID_{50}$ live ISA virus (CCBB isolate) administered by IP injection 700-800 degree days (dd) after vaccination.

Measures of vaccine performance

a. *Safety.* For the safety test, fifty-five fish per vaccine were injected intra-peritoneally (IP) with 0.2 ml of the vaccine combined with adjuvant. Fish in two control groups were mock vaccinated with either saline or adjuvant. The vaccinated fish were then placed into holding tanks where they were observed daily for any mortality related to the vaccine for at least 21 days post vaccination. No mortalities were observed. As such, these fish were then challenged with ISA virus 700-800 dd after vaccination to determine the efficacy of the vaccines.
b. *Virology: Confirmation of mortality due to ISA virus.* The cause of fish mortality in the efficacy study was confirmed to be ISA by live virus culture. A portion of the gill, liver, spleen and head kidney was removed asceptically, placed in phosphate buffered saline (1:5 w/v), homogenized, clarified and inoculated onto cell monolayers in 24-well plates to confirm that ISA virus was the cause of death.
c. *Relative percent survival (RPS).* The results of the vaccine efficacy trials are presented as cumulative percent mortality and expressed in terms of relative percent survival (1 - % mortality vaccinates/% mortality negative control) x 100.
d. *Humoral immune response.* Sera samples were collected at the time of challenge from vaccinated Atlantic salmon and analyzed for the presence of ISA virus-specific antibodies by ELISA.

Vaccine efficacy study 1

This study was designed to measure the efficacy and safety of heat, formaldehyde, BEI or BPL inactivated ISA virus vaccines administered to Atlantic salmon by intraperitoneal injection. The fish were immunized in freshwater and then acclimated to saltwater and were challenged approximately 800 degree days (dd) after immunization. The results of the vaccine trial are presented in Table 4 and Figure 8.

Table 4. Vaccine efficacy

Vaccine	Dose[a]	% mortality[c]	RPS[b]
ISAV, heat/ FCA	Dose 1	70	7
ISAV, heat/ FCA	Dose 2	76	0
FCA-1	-	75	-
Saline-1	-	65	-
ISAV, formalin/ FCA	Dose 1	60	0
ISAV, formalin/ FCA	Dose 2	60	0
ISAV, BEI-1/ FCA	Dose 1	62	0
ISAV, BEI-1/ FCA	Dose 2	60	0
ISAV, BEI-3/ FCA	Dose 1	74	0
ISAV, BPL/ FCA	Dose 1	60	0
ISAV, BPL/ FCA	Dose 2	64	0
FCA-2	-	60	-
Saline-2	-	72	-
Convalescent fish from previous trial[d]	-	0	100
Cohabitation	-	71% naive/0% convalescent	-

[a] Dose 1 > Dose 2

[b] RPS is reported relative to the mortality of fish in the saline control group
[c] 2 x 25 vaccinated fish were challenged and used for efficacy determination; 5 fish were bled for vaccinated/no challenge sera collection at 700 dd.
[d] 21 convalescent fish from a previous trial were co-incubated with 21 naïve fish from the current trial.

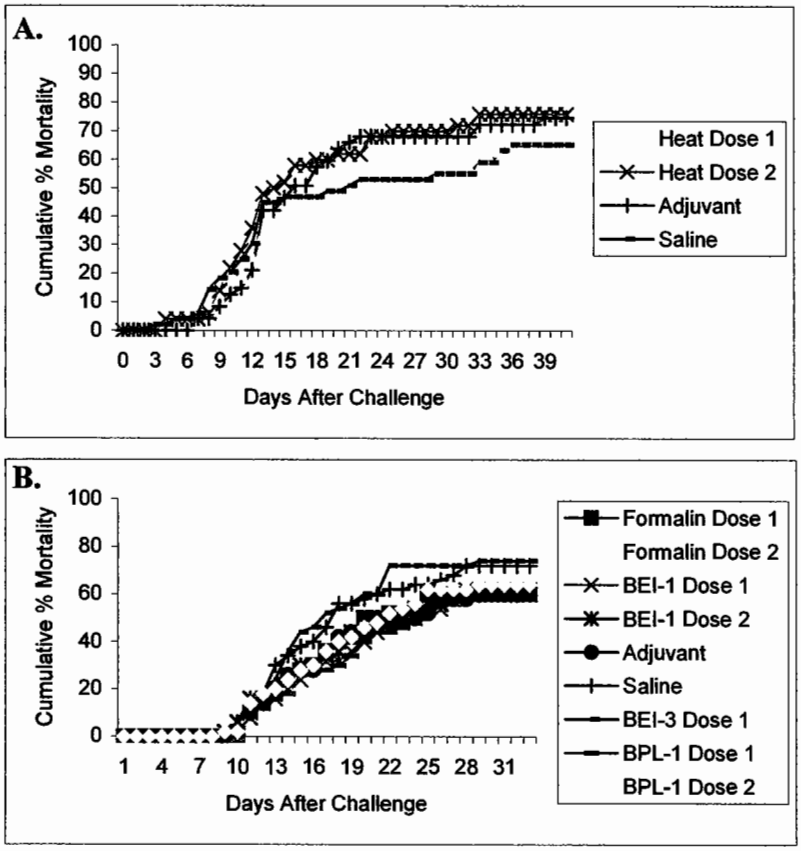

Figure 8. Cumulative percent mortality curve for Atlantic salmon vaccinated with two doses of heat treated (Panel A), formalin-, BEI- or BPL-treated (Panel B) ISA virus and challenged with live ISA virus. Fish in the control groups were mock vaccinated with either adjuvant or saline. Virus in BEI-3 was inactivated with BEI as described above but the incubation periods following addition of thiosulphate were reduced to preserve the antigen while ensuring complete virus inactivation.

Vaccine efficacy study 2

This study was designed to measure the efficacy, safety and antibody response of the modified traditional or enriched antigen vaccines 1, 2 or 3 in Atlantic salmon. The fish were immunized in freshwater and then acclimated to saltwater and were challenged approximately 800 degree days (dd) after immunization. The results of the vaccine trial are presented in Table 5 and Figures 9 and 10.

Table 5. Vaccine efficacy

Vaccine	% mortality[a]	RPS[b]
Traditional ISAV/FCA	61	0
Modified traditional antigen 1/ FCA	43	0
Modified traditional antigen 2/ FCA	47	0
Modified traditional antigen 3/ FCA	59	0
Enriched ISAV/ FCA	52	0
Modified enriched antigen 1/ FCA	54	0
Modified enriched antigen 2/ FCA	45	0
Modified enriched antigen 3/ FCA	53	0
Modified enriched antigen 4/ FCA	42	0

FCA	41	2
Saline	42	-

[a] 2 x 25 vaccinated fish were challenged and used for efficacy determination; 5 fish were bled for vaccinated/no challenge sera collection at 700 dd.
[b] RPS is reported relative to the mortality of fish in the saline control group

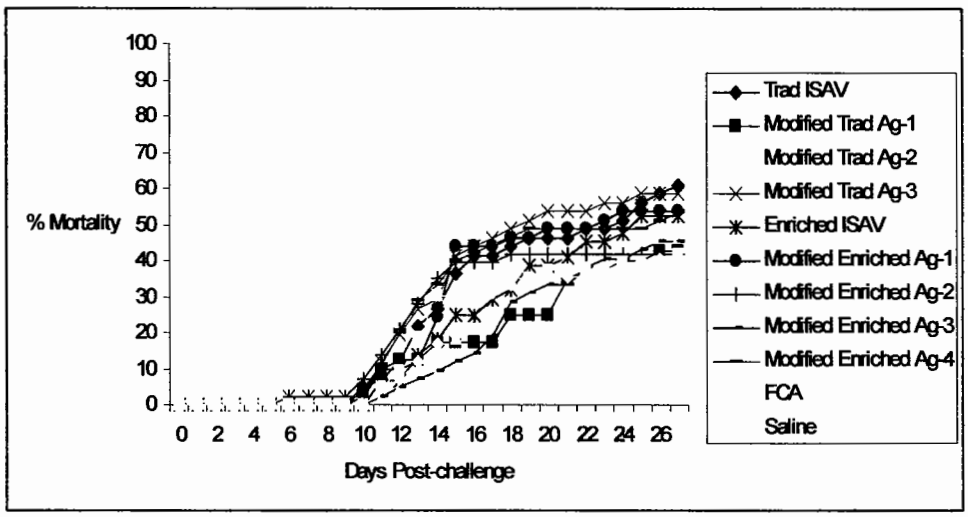

Figure 9. Cumulative percent mortality curve for Atlantic salmon vaccinated with modified traditional or enriched ISAV antigen and challenged with live ISA virus. Fish in the control groups were mock vaccinated with either adjuvant or saline.

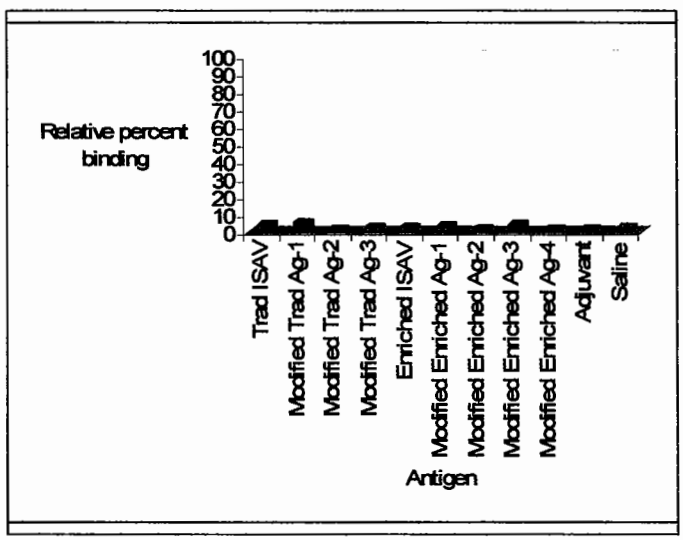

Figure 10. ISA virus-specific antibodies in immunized Atlantic salmon. Sera was collected from vaccinated Atlantic salmon approximately 800 dd after immunization and analyzed by enzyme linked immunosorbant assay (ELISA). The relative level of ISA virus-specific binding antibodies present in the sera is expressed as a percent of the $OD_{405\ nm}$ value obtained using positive control sera. Sera containing ISA-virus binding antibodies typically have a relative percent of 20 or greater.

Vaccine efficacy study 3

This study was designed to measure the efficacy, safety and antibody response of the modified traditional or enriched antigen vaccines 1, 2, 3 or 4 in Atlantic salmon. The fish were immunized in freshwater, acclimated to saltwater and then challenged approximately 700 degree days (dd) after immunization. The results of the vaccine trial are presented in Table 6 and Figures 11 and 12.

Table 6. Vaccine efficacy

Vaccine	% mortality[a]	RPS[b] (saline)	RPS[c] (adjuvant)
Traditional ISAV/ oil-in-water adjuvant	52	31	0
Modified traditional antigen 1/ oil-in-water adjuvant	43	43	17
Modified traditional antigen 2/ oil-in-water adjuvant	52	31	0
Modified traditional antigen 3/ oil-in-water adjuvant	70	7	0
Enriched ISAV/ oil-in-water adjuvant	59	21	0
Modified enriched antigen 1/ oil-in-water adjuvant	54	28	0
Modified enriched antigen 2/ oil-in-water adjuvant	61	19	0
Modified enriched antigen 3/ oil-in-water adjuvant	61	19	0
Modified enriched antigen 4/ oil-in-water adjuvant	52	31	0
Oil-in-water adjuvant	52	-	-
Saline	75	-	-

[a] 2 x 25 vaccinated fish were challenged and used for efficacy determination; 5 fish were bled for vaccinated/no challenge sera collection at 700 dd.
[b] RPS is reported relative to the mortality of fish in the saline control group
[c] RPS is reported relative to the mortality of fish in the adjuvant control group

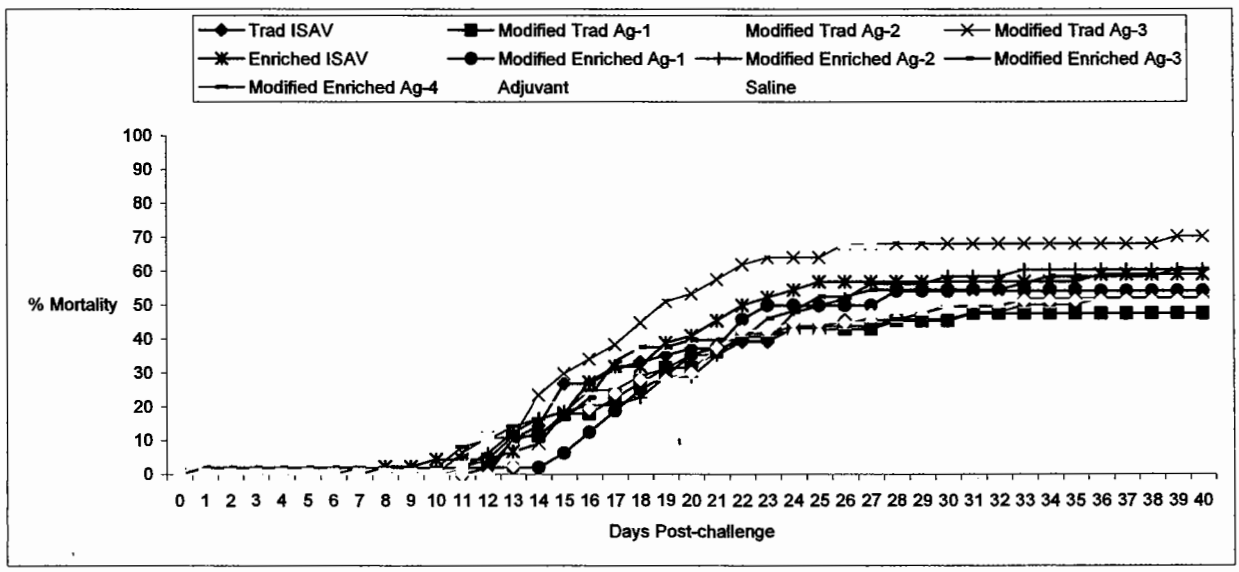

Figure 11. Cumulative percent mortality curve for Atlantic salmon vaccinated with modified traditional or enriched ISAV antigen and challenged with live ISA virus. Fish in the control groups were mock vaccinated with either adjuvant or saline.

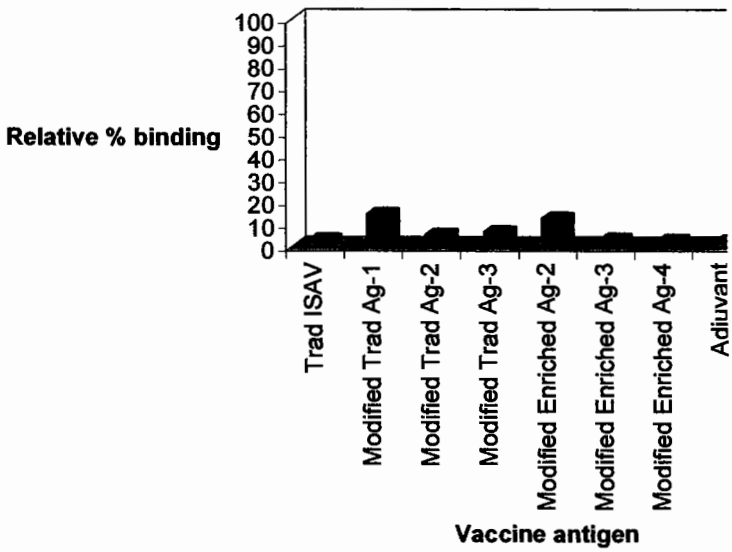

Figure 12. ISA virus-specific antibodies in immunized Atlantic salmon. Sera was collected from vaccinated Atlantic salmon approximately 700 dd after immunization and analyzed by enzyme linked immunosorbant assay (ELISA). The relative level of ISA virus-specific binding antibodies present in the sera is expressed as a percent of the $OD_{405\ nm}$ value obtained using positive control sera. Sera containing ISA-virus binding antibodies typically have a relative percent of 20 or greater.

Passive immunization study

This study was designed to examine the effectiveness of passive immunotherapy for the control of ISA in Atlantic salmon. Three groups of fish (45 g) were immunized by IP injection with 0.2 ml convalescent sera, naïve sera or saline 24 hours prior to exposure to challenge with ISAV. Cumulative percent mortality and relative percent survival were calculated and are presented in Table 7 and Figure 13

Table 7. Passive immunization trial

Vaccine	% mortality	RPS
saline	62	-
Naïve sera	32	48.4
Convalescent sera	22	64.5

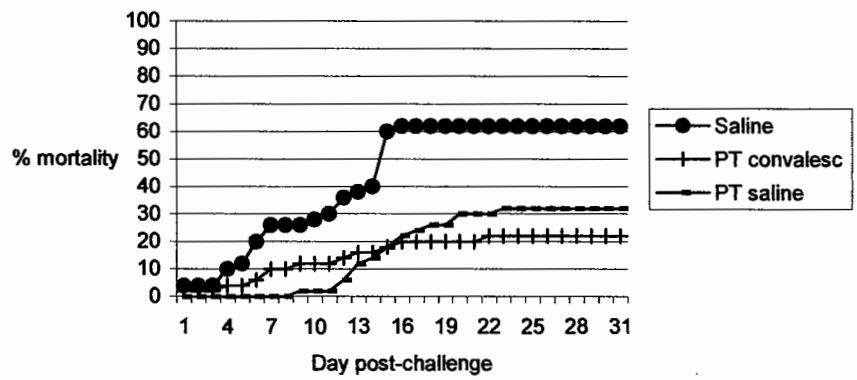

Figure 13. Cumulative percent mortality for Atlantic salmon inoculated by intraperitoneal injection with convalescent sera collected from fish that had survived exposure to ISA virus. Fish in the control groups were mock vaccinated with saline or with naïve sera.

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