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Protective oral vaccination against infectious salmon anaemia virus in *Salmo salar*

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ABSTRACT

Infectious salmon anemia (ISA) is a systemic disease caused by an orthomyxovirus, which has a significant economic impact on the production of Atlantic salmon (*Salmo salar*). Currently, there are several commercial ISA vaccines available, however, those products are applied through injection, causing stress in the fish and leaving them susceptible to infectious diseases due to the injection process and associated handling. In this study, we evaluated an oral vaccine against ISA containing a recombinant viral hemagglutinin-esterase and a fusion protein as antigens. Our findings indicated that oral vaccination is able to protect Atlantic salmon against challenge with a high-virulence Chilean isolate. The oral vaccination was also correlated with the induction of IgM-specific antibodies. On the other hand, the vaccine was unable to modulate expression of the antiviral related gene Mx, showing the importance of the humoral response to the disease survival. This study provides new insights into fish protection and immune response induced by an oral vaccine against ISA, but also promises future development of preventive solutions or validation of the current existing therapies.

1. Introduction

Infectious salmon anaemia (ISA) is a systemic disease affecting salmonids, mainly Atlantic salmon (*Salmo salar*). The causative agent of the disease is the infectious salmon anaemia virus (ISAV). Similar to other orthomyxovirus such as influenza, ISAV is an enveloped virus and has in its membrane a mushroom shaped projection protein. These correspond to the hemagglutinin-esterase protein responsible for both viral attachment and release (HE) [1], while fusion activity is found in another protein (protein F) [2].

The mortality associated with the disease varies from 10 to 95% [3]. The major clinical findings corresponds to pale gills, exophthalmos, edema and hemorrhage [4]. On post-mortem examination, primary findings include functional abnormalities in several organs due to endothelial damage [3].

The development of intraperitoneal (IP) oil-based vaccines containing inactivated ISAV has been one of the leading strategies used to prevent losses caused by the disease, in fact all the vaccines used in Canada and United States employ this strategy [5]. However, scientific reports describing the mode of action and efficacy of these vaccines are scarce. While the vaccine development has focused on inactivated vaccines a few researchers have developed alternatives based on recombinant antigens and DNA vaccine technology [5,6].

The innate antiviral defense system in teleost is based on the production of interferon (IFN) [7,8], which represents the first line of defense against viral infection. IFN type I is produced by any nucleated cell, and type II is produced by specialized immune cells, the IFN-II takes part in the adaptive response whereas IFN-I is a major mediator of the innate immune response [9]. A fast signaling pathway induces expression of a series of proteins including Mx with direct and indirect antiviral properties [10]. Although antiviral defense is initiated by IFN-induction against infectious pancreatic

Abbreviations: DD, degree days; ISAV, infectious salmon anaemia virus; IPNV, Infectious pancreatic necrosis virus; RPS, relative percentage survival; ARR, absolute risk reduction; NNT, number necessary to treat; RN, recombinant; NR, non-recombinant; EN, encapsulated; NE, non-encapsulated; IP, intraperitoneal; DPC, days post-challenge.

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necrosis virus (IPNV) both *in vitro* and *in vivo*, the mechanism is not fully elucidated [11,12]. In addition, the protective role of Mx, which has been demonstrated against IPNV, has not been described in ISAV [13–16].

The aim of this work is to evaluate *in-vivo* different formulations containing various components of a recombinant oral vaccine against ISA and its effect in experimental challenges against a Chilean highly virulent isolate.

2. Material and methods

2.1. Fish maintenance

Disease free Atlantic salmon average weight 40 g were maintained at Centrovet animal facilities in a fresh water recirculation system (Santiago, Chile). Before trials, fish were acclimated to controlled environment during 2 weeks in 1 m^3 tanks at a density of 15 kg/m3, and a water exchange rate of 1 m^3/hour. Water condition for acclimation and trials were: 12.1 °C (±0.6), pH 7.4 (±0.3) and oxygen saturation 80–120%. Two weeks after acclimation fish were placed on 100 L tanks in groups of 55 at a density of 50 kg/m3 where the experiments were conducted. Fish were fed ad libitum (Golden Activa 2.0 mm, Biomar).

2.2. Vaccine and virus

The vaccine was developed using recombinant DNA technology in *Saccharomyces cerevisiae*, the conserved regions of surface proteins of the virus HE and protein F from different ISAV isolates were cloned as synthetic nucleotide-optimized genes for yeast on an expression vector (DNA 2.0, USA). Yeast expressing the antigens were subjected to disruption using a cell disintegrator (DYNO®-MILLS, GlennMills), and ISAV protein-containing fractions were encapsulated in a cationic polysaccharide matrix (MicromatrixTM, Advanced BioNutrition, Columbia, MD, USA), according to procedure described elsewhere [17,18]. Feeds were formulated with five different components (Table 1) including control groups.

Fish were fed with vaccine-formulated feed prepared in a final concentration equivalent to 6 mg vaccine/fish/day for 10 days, as described previously [17]. For the experimental challenge, virulent ISAV isolate (HPR7b strain) isolated from a field outbreak (X Region, Chile) was used. The virus was expanded in cell line SHK-1 according to Eliassen et al. [18].

2.3. Experimental design and samples

The challenges were conducted in Centrovet animal facility (Santiago, Chile). 55 fish per group were stocked in 100 L tank, at a density of 22 kg/m^3. Experimental design comprised 12 groups of 55 fish per tank, 6 groups challenged and 6 groups unchallenged (performed in duplicate and in separate tanks). The transmission model was IP, since it is highly reproducible and reliable method in efficacy evaluation [19]. 450° days (DD) post vaccination the challenged groups were inoculated by IP injection, 0.2 mL of ISAV (3×106 $TCID^{50}$/fish). The non-challenged group was inoculated with 0.2 mL of L-15 medium.

Mortality was recorded daily until day 53 post challenge and necropsy was performed as a diagnosis method of the disease according to characteristic lesions [20,21] and confirmed by PCR analysis according to Mikalsen et al. [22]. Blood and kidney tissue were sampled from 3 fish per group at different sampling times (pre-vaccination, post vaccination and 150, 300, 500, 630 and 740 DD post vaccination) for antibody evaluation and gene expression respectively. Kidney tissue were stored at −20 °C in RNA Later (Ambion, California, USA) and the blood was centrifuged for serum extraction and stored at −20 °C. Sampling and monitoring of ISAV-induced mortality were performed in parallel experimental groups in order to avoid influences of stress in mortality due to handling.

The trials were performed in accordance with the Chilean legislation for animal experimentation under the manual “Bioethical aspects of animal experimentation” issued by the National commission of scientific and technological research.

2.4. Antibody ELISA

Nunc Maxisorp (Nunc, Roskilde, Denmark) plates were activated with 5 μg of an equimolar mixture of vaccine antigens in bicarbonate buffer, pH 8.5. The plates were blocked with PBS containing 1% BSA and test sera diluted 1:50 were added and incubated at 4 °C overnight. The following day the plates were washed with PBS and incubated with monoclonal mouse anti-salmon (dilution 1:500) IgM isotype IgG1 (BiosChile, IGSA, Chile) for 1 h at 30 °C. The plates were then washed again and incubated at 30 °C with horseradish peroxidase-conjugated goat anti-mouse (dilution 1:1000) IgG (KPL, Maryland, USA). Serum antibody titers were determined using 3,3′,5,5′-tetramethylbenzidine as a chromogenic substrate and H_2SO_4 to stop the reaction. Values were obtained by measuring the absorbance at 450 nm. Sera from experimentally ISAV-infected and from healthy non-immunized fish were used as positive and negative controls, respectively and all sera were tested in triplicates.

2.5. RNA extraction and cDNA synthesis

RNA was extracted from kidney tissues using the kit AxyPrep Multisource Total RNA (Axygen, Massachusetts, USA) according to the manufacturer's instructions. For reverse transcription 1 μg of template RNA was used (ImProm-II, Reverse Transcription,

Table 1
Immunization treatments, vaccines and controls used in this study.

Treatment	Details
Encapsulated recombinant antigen (Vaccine)	Freeze dry extract of recombinant ISAV antigens expressed in *Sacharomyces cerevisiae* and encapsulated in polymeric matrix (Micromatrix™)
Empty microcapsule	Freeze dry extract that contains the polymeric matrix (Micromatrix™) without antigen.
Recombinant yeast non encapsulated (Yeast RN NE)	Freeze dry extract of recombinant ISAV antigens expressed in *Sacharomyces cerevisiae* non-encapsulated.
Non recombinant yeast non encapsulated (Yeas NR NE)	Freeze dry extract of wild type *Sacharomyces cerevisiae* non-encapsulated.
Non recombinant yeast encapsulated (Yeast NR EN)	Freeze dry extract of wild type *Sacharomyces cerevisiae* encapsulated in polymeric matrix (Micromatrix™)
Control	Oil coated feed.

NR: Non recombinant, RN: Recombinant, NE: Non Encapsulated, EN: Encapsulated, Micromatrix: Commercial name of the cationic polysaccharide matrix used for encapsulation of antigens.