Biologicals 40 (2012) 67–71

Contents lists available at SciVerse ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Development of an antibody ELISA for potency testing of furunculosis (*Aeromonas salmonicida* subsp *salmonicida*) vaccines in Atlantic salmon (*Salmo salar* L)

Anne Berit Romstad [a,e,*], Liv Jorun Reitan [b], Paul Midtlyng [c], Kjersti Gravningen [d], Øystein Evensen [c]

[a] *Nord-Trondelag University College, Dept. of Health Science, Finn Christensensv 1, 7800 Namsos, Norway*
[b] *National Veterinary Institute, P.O. Box 750, 0106 Oslo, Norway*
[c] *Norwegian School of Veterinary Science, P.O. Box 8146 Dep, 0033 Oslo, Norway*
[d] *PHARMAQ AS, P.O Box 267, Skøyen, 0213 Oslo, Norway*
[e] *VESO Vikan, 7800 Namsos, Norway*

ARTICLE INFO

Article history:
Received 21 March 2011
Received in revised form
12 September 2011
Accepted 12 September 2011

Keywords:
Vaccination
Temperature
Antibody response
Aeromonas salmonicida
Atlantic salmon
ELISA
Protection

ABSTRACT

The study was conducted in Atlantic salmon to establish the initial and basic scientific documentation for an alternative batch potency test for salmon furuculosis vaccines. We assessed the antibody response development for *Aeromonas salmonicida* vaccines at different immunisation temperatures (3, 12 and 18 °C), by an enzyme-linked-immunosorbent assay (ELISA) 3, 6, 9 and 12 weeks post vaccination, and the correlation between antibody response and protection in cohabitation challenge experiments performed 6 and 12 weeks post vaccination. Fish immunised with a vaccine containing full antigen dose had a significant increase in antibody response after 252 day degrees and the measured values correlated well with protection after 500 day degrees. Fish vaccinated with a reduced antigen dose showed a significant lower antibody response than fish vaccinated with the full dose vaccine at all samplings, and showed a similar low relative percent survival (RPS) in the challenges. The results from this study indicate that an antibody ELISA can discriminate between vaccines of different antigen content and the method may replace challenge tests in batch potency testing of furunculosis vaccines in Atlantic salmon. An immunisation temperature of 12 °C and sampling after 6–9 weeks, seemed to be the most appropriate time for using antibody responses to confirm batch potency.

1. Introduction

Aeromonas (A.) salmonicida subspecies *salmonicida*, the causative agent of furunculosis in Atlantic salmon (*Salmo salar* L), is one of the most widely distributed pathogenic bacteria in salmonid culture [1]. For the past 15 years the disease has been successfully controlled by the use of oil-adjuvanted vaccines [2].

The European Pharmacopoeia (Ph.Eur.) defines the minimum requirements for safety and efficacy testing of veterinary vaccines and the requirements of oil-adjuvanted furunculosis vaccines for salmonids are specified in the current edition [3]. Briefly, for batch potency testing, the test vaccine and physiological saline are administered to groups of 30 fish each. The fish are kept in the same tank and challenged by injection with a virulent strain of *A. salmonicida*. Relative Percent Survival (RPS) [12] is calculated by comparison of the survival rate within the control and the test groups at 60% control mortality. However, the current monograph [3] indicates that, alternatively, a potency assay based on antibody response may be acceptable. In such cases, the correlation between antibody levels and protection after *in vivo* challenge has to be thoroughly documented [4]. Several studies have been carried out with this purpose [5,6], but many of the studies are not fully relevant as non-commercial vaccines were used. Commercial furunculosis vaccines are very efficient and most of them are based on whole bacteria. Fish generally have a low antibody response compared to homoeothermic animals [7], and a sensitive method such as an Enzyme Linked Immunosorbent Assay (ELISA) would be suitable to measure the antibody response in fish sera. In the current study, we wanted to initially establish the correlation between antibody response and protection against mortality caused by *A. salmonicida*, using cohabitation challenge experiments that closely mimic the natural route of exposure for this infection [5]. Since several different candidates for the protective antigen have been described for *A. salmonicida* [1] and most of the commercial vaccines are based on whole bacteria, we chose to use sonicated *A. salmonicida* as the coating antigen in the ELISA [5,8]. As

* Corresponding author. Nord-Trondelag University College, Dept. of Health Science, Finn Christensensv 1, 7800 Namsos, Norway. Tel.: +47 91683971.
E-mail address: anne.b.romstad@hint.no (A.B. Romstad).

1045-1056/$36.00
doi:10.1016/j.biologicals.2011.09.011

important variables when studying immune responses in poikilothermic animals are water temperature and time post vaccination (degree days: Def: water temperature in degree Celsius multiplied by number of days at that temperature) [9], this aspect was included in the study design.

The aim of this study was therefore to investigate how the antibody response against *A. salmonicida* develops by time in Atlantic salmon held in different water temperatures, and to which degree it correlates with protection following *in vivo* challenge; information which is needed for optimisation and standardisation of an antibody-based ELISA potency test procedure.

2. Materials and methods

This study was conducted in accordance with the Norwegian Regulation on Animal Experiments, which implements The European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS -123).

2.1. Fish

3300 Atlantic salmon (*S. salar* L.) presmolts (AquaGen Standard breed) at an average weight of 15.9 g were obtained from VESO Vikan hatchery, N-7819 Fosslandsosen, Norway. The immune status of the test fish was tested prior to start of the experiment and documented seronegative for specific antibodies against *A. salmonicida*, *Vibrio salmonicida*, *Vibrio anguillarum O1* and *O2* and *M viscosa*. After arrival at the experimental facility the fish was divided into three 500L fibre glass tanks each containing 1100 fish, and acclimatised to their respective holding temperatures (3, 12 or 18 °C) over a period of 10 days. The tanks were supplied with free flowing fresh water at an average flow rate of 0.8 l per kg fish per min Throughout the trial the fish were hand-fed a commercial feed (Ewos Micro 15) twice a day with an average daily amount corresponding to1% of the biomass. Mortality was recorded daily.

2.2. Vaccines and vaccination

Two experimental vaccine formulations were produced for the study. One vaccine contained *A. salmonicida*, *Moritella viscosa* and *Infectious Pancreatic Necrosis Virus (IPNV)* antigen of the same quality and quantity as a commercially available salmon vaccine formulation (ALPHA JECT 6–2), referred to as the full dose (FD) formulation. The *A. salmonicida* antigen dose have repeatedly shown to satisfy the batch potency criteria of the furunculosis monograph. The second experimental vaccine contained the same amount of adjuvant, but only 5% of the full dose for the bacterial antigens and 10% of the IPNV component, designated the reduced dose (RD) formulation. The vaccines were produced by PHARMAQ AS, and based on a Norwegian *A. salmonicida* bacterial strain, inactivated by 0.5% formaldehyde, a mineral oil (liquid paraffin) was used as adjuvant. Two control groups were included, injected i.p. with adjuvant alone (AD) or physiological saline (PS), respectively. The fish was anaesthetised, group marked by a combination of fin clipping and ink, and inoculated by intra peritoneal (i.p.) route of injection with 0.1 ml of the test substance. 250 fish from each group (FD, RD, AD, PS) were adjoined into 3 parallel tanks (a total of 1000 fish in each tank) and kept at different temperature regimes: 3, 12, and 18 °C ± 1 °C throughout the immunisation period. From these three tanks fish were randomly selected for blood sampling or challenge.

2.3. Blood sampling

3, 6, 9 and 12 weeks post vaccination 20 fish from each group were randomly collected from each immunisation tank for blood sampling. After stunning by a blow to the head, blood was collected from the caudal vein into heparinised vacutainer tubes. The plasma was separated by centrifugation and stored at −20 °C until analysis.

2.4. Challenge

A standardised cohabitation challenge procedure [5] was employed to assess protection against furunculosis at two points in time; 6 and 12 weeks post vaccination. Thirty-two fish from each vaccine group held at 3 or 18 °C were acclimated to 12 °C ± 1 °C for two days. After acclimation these fish and the same number of fish immunised at 12° C were adjoined in a new tank for challenge. At day 1 of the challenge period, 76 fish was injected with the heterologous, virulent *A. salmonicida* strain 3175/88 and added to the tank. For the challenge carried out 6 weeks post vaccination the carrier fish were injected with 7×10^3 cfu and for the challenge carried out at 12 weeks post vaccination, 2.7×10^5 cfu of the bacterium was inoculated. Both challenges were performed in duplicate tanks at 12 °C ± 1 °C. Bacteriological examination was performed on Trypticase-Yeast-Agar (TYA) plates from 10% of the fish that died during the challenge period to verify challenge specific mortality.

2.5. Antibody analysis

The enzyme-linked immunosorbent assay (ELISA) was performed as described earlier [5,10], with minor modifications. Briefly, wells were coated with 100 μl of sonicated whole cells of *A. salmonicida* strain 3175/88(5 μg protein ml^{-1}) in 0.05 M carbonate buffer (pH 9.6). Plasma samples at three different dilutions (1:50, 1:200 and 1:1000) in duplicate wells, were incubated at 4 °C overnight. Monoclonal antibody against rainbow trout immunoglobulin, clone 4C10, was used as the secondary antibody in the ELISA [11]. From each sampling, 20 individual fish from each of the vaccinated groups and 5 individual fish from the control groups were analysed. The internal positive plasma control used for normalisation of ELISA readings across plates was produced from a pool of fish immunised with whole, formalin inactivated *A. salmonicida* strain 3175/88. The absorbance was read spectrophotometrically at 450 nm and the mean absorbance of duplicate wells was used. The variation between plates was ± 8% for the positive control test sera at 1:50 dilution. In order to compare results between plates, the results were expressed as the ratio (Rel OD) between the absorbance of the sample wells relative to positive control test sera at the same dilution. There was clear decrease of OD with increasing dilution of the test sera and a good correlation between the dilution curves of all test groups.

2.6. Statistical analysis

Statistical analyses were performed by use of SPSS 17.0 software. When comparing ELISA outcomes between the groups both Students *t*-test and by Mann–Whitney (non-parametric) p values were considered (due to a lack of normal distribution in some groups).Unless stated otherwise, p values < 0.05 were considered significant. For each challenge tank, the relative percent survival (RPS) vs saline controls was calculated according to Amend [12]

$$\left(1 - \frac{cum.\ mort.vaccinated\ group}{cum.\ mort\ in\ control\ group}\right) \times 100\%,$$

at end of trial and Chi-square tests were used to determine significance. The group–level correlation between the serological analysis and the protection after challenge was assessed by Spearman's Rank Correlation test and calculation of Pearson's R by use of SPSS 17.0.