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Antibody responses correlate with antigen dose and *in vivo* protection for oil-adjuvanted, experimental furunculosis (*Aeromonas salmonicida* subsp. salmonicida) vaccines in Atlantic salmon (*Salmo salar* L.) and can be used for batch potency testing of vaccines

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ABSTRACT

Salmon farming has increased dramatically over last thirty years and a key to the success is the introduction of protective vaccines. In Norway, almost 100% of all Atlantic salmon are vaccinated prior to sea transfer. This extensive use of vaccines demands use of a lot of resources in production and quality control of vaccines, and fish are now one of the most widely used laboratory animal species in Norway, since all batch testing today is performed by challenge experiments. With an increasing focus on the 3 R's (Replacement, Reduction and Refinement), new methods are needed.

The aim of this study was to assess the use of different vaccine evaluation methods to identify furunculosis vaccines of different "potency", using ELISA as *in vitro* assay and intraperitoneal and cohabitation challenge as *in vivo* assays. Eleven vaccines with different antigen content (0, 2, 5, 10, 20, 40, 80, 100 and 200%) and different antigen qualities were included in the study. Challenge and blood sampling for the ELISA assay were conducted 9 weeks post vaccination.

The results from this study indicated that there is a close correlation between the antigen dose in the vaccine and the antibody response against *Aeromonas salmonicida* as measured by ELISA. There is also a close correlation between the antibody response and protection for both i.p. and cohabitation challenge models. The ELISA method identified sub-potent batches better than currently used *in vivo* assay (i.p challenge) and seems to be the best method of performing a batch potency test of furunculosis vaccines particularly when taking the 3R's principles into account.

1. Introduction

Aeromonas salmonicida subspecies of *salmonicida*, the causative agent of the disease furunculosis, has for the past 20 years been successfully controlled by the use of oil adjuvanted vaccines [1,2]. Presently, 100% of all farmed Atlantic salmon (*Salmo salar* L.) in Norway, approximately 320 million fish per year, are vaccinated prior to sea transfer [3].

All batches of vaccines are tested for potency and safety before use, which requires the use of a great amount of experimental animals [4]. According to the European Pharmacopeia [5], potency tests can be performed in two ways, either by the use of a challenge test in the laboratory or by *in vitro* immunoassays. The focus in implementing the 3R's (Replacement, Reduction and Refinement) in quality control of vaccines has increased in the recent years [4,6] but replacing one evaluation method by another requires a validation study to define the correlates between the challenge test and the *in vitro* method [5,7]. The aim of a batch potency test performed in the target species is to prove that the finished product elicits an immune response that will induce a level of protection specified for the product [8]. In a previous study [9] a strong correlation between the antibody response and protection in cohabitation challenge was obtained, but to find the dose–response correlation, a study including several vaccine formulations was needed.

Several attempts have been made to identify the protective antigen in furunculosis vaccines [10,12,14,21,22], and some have

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suggested that the A-layer, a superficial layer on the cell surface, is of importance in protection [12,14,21]. We wanted to test if a bacterium is originally A-layer positive, which has lost its A-layer during sub-culturing could be revealed as sub potent in vaccine testing.

The aim of this study was to evaluate the ability of different vaccine evaluation methods to identify sub-potent furunculosis vaccines, using ELISA as *in vitro* assay and intraperitoneal and cohabitation challenge as in vivo assays.

2. Animals, materials and methods

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

2.1. Fish

1300 Atlantic salmon (*Salmo salar* L.) pre-smolts (AquaGen Standard) at an average weight of 16.2 g from VESO Vikan hatchery, N-7819 Fosslandsosen, Norway, were included in the study. The immune status of the test fish was tested prior to start of the experiment and documented seronegative against *A. salmonicida*. After arrival at VESO Vikan the fish were divided into two 500 L fiberglass tanks each containing 650 fish and acclimatised to their respective holding temperature of 12 °C over a period of 7 days. The tanks were supplied with free flowing fresh water at an average flow rate of 0.8 l kg^{-1} fish min^{-1}. Throughout the trial the fish was hand-fed a commercial feed (Ewos Micro 15) twice a day with an average daily amount corresponding to 1% of the estimated biomass.

2.2. Vaccines

Eleven different furunculosis vaccines varying in antigen content from adjuvant-only up to 200% antigen content (double the standard dose of a commercial furunculosis vaccine) were included in the study (Table 1). The adjuvant volume was kept constant in all vaccines. Nine of the vaccines contained an A-layer positive isolate of *A. salmonicida* while two vaccine preparations were made with an A-layer negative variant, at 5% and 100% antigen content. The A-layer negative *A. salmonicida strain* was originally A-layer positive when isolated from Atlantic salmon, but lost its A-layer through sub-culturing. The vaccines were produced by PHARMAQ AS, and were based on the same bacterial strains (except for the A-layer negative strains), same adjuvant system and produced according to the same procedures as their commercial vaccines. In addition, one group was injected with physiological saline and used as saline control.

2.3. Vaccination

The vaccines were blinded before use, and eleven groups of 108 fish in each group were anaesthetised, marked by fin clipping and ink, and injected i.p. with 0.1 ml of the vaccine per fish. The saline control group was injected i.p. with 0.1 ml of 0.9% NaCl per fish. The groups were randomly divided in 4 tanks, two challenge tanks with 32 fish per group and two sampling tanks with 22 fish per group. Blinding was first lifted after the completion of the challenge trials.

2.4. Challenge

Standardised cohabitation [10,19] and intraperitoneal (i.p.) challenge procedures [19] were employed 9 weeks post vaccination. In the cohabitation challenge 50 fish (shedders) were injected with a lethal dose of *Aeromonas salmonicida* strain 3175/88, 1×10^4 cfu/fish, and added to the tanks at day 1 of the challenge.

For i.p. challenge each fish was injected with a dose of 3×10^1 cfu/fish (expected mortality of 60–80%). Both challenges were performed in tanks at 12 ± 1 °C. Feeding, care and registration of mortality were carried out daily. Relative Percentage Survival was calculated at the end of each challenge [11]. Bacteriological examination was performed on TYA (trypticase-yeast-agar) plates from 10% of the fish that died during challenge to verify the cause of death

2.5. Blood sampling

Six and 9 weeks post vaccination 22 fish from each group were netted out from the immunisation tanks 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 further analysis.

2.6. Side effect study

After blood sampling 6 and 9 weeks post vaccination all fish were examined for intraperitoneal adhesions according to Speilberg scale [12], with 0 as lowest observation indicating no visual adhesion and 6 as highest where extensive adhesions in the peritoneal cavity are observed. The adhesions were recorded using a modified continuous scale, Speilberg visual analogue scale [13], where the adhesions are scored visually on a continuous scale and afterwards presented as a number with one decimal. The continuous scale is believed to give a better ability to perform statistical analysis of the data observed.

2.7. Antibody quantification

The enzyme-linked immunosorbent assay (ELISA) was performed as described [10,14], with minor modifications. *A. salmonicida* strain 3175/88 was cultured in BHIB (brain heart infusion broth) and washed three times in PBS. The resuspended pellet was kept on ice while being sonicated at least for 10 min. After protein determination the sonicated solution was used for coating the wells, 100 μl per well (5 μg protein ml^{-1}) in 0.05 M carbonate buffer (pH 9.6) and incubated overnight. Plasma samples at three different dilutions (1:50, 1:200 and 1:1000) were incubated at 4 °C overnight, followed by a monoclonal antibody against rainbow trout immunoglobulin, clone 4C10 [15], that is known to cross react with Atlantic salmon Ig. The internal positive plasma control used on all 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. 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 pool at the same dilution. All samples were analysed 9 weeks post vaccination, but only 16 fish in 6 A-layer positive groups were analysed 6 weeks post vaccination.

2.8. Statistical analysis

Comparison of levels of antibodies between the groups were performed by comparison of means by use of SPSS 17.0/19.0. Relative Percentage Survival (RPS) was calculated at 60 control mortality and at end of challenge according to Amend 1981[11]: $(1 - (\text{cum. mort. vaccinated group}/\text{cum .mort in control group})) \times 100\%$. According to Pharm Eur [5], furunculosis vaccines with $RPS_{60} < 85\%$ fails the test. Correlation between the serological analysis (mean Rel OD), antigen content in the vaccine and protection