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Time-course study of injection site inflammatory reactions following intraperitoneal injection of Atlantic cod (*Gadus morhua* L.) with oil-adjuvanted vaccines

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Abstract The inflammatory response of Atlantic cod (*Gadus morhua* L.) following vaccination with oil-based vaccines has not been previously characterized in any detail. In this study, groups of Atlantic cod were intraperitoneally injected with commercial oil-adjuvanted vaccines ALPHA JECT 3000 (AJ 3000) and AJ 6-2. A water-based vaccine ALPHA MARINE™ *Vibrio* (AVM), an experimental liposome vaccine and physiological saline (placebo) were also included for comparison. Histopathological changes at the injection sites were evaluated semi-quantitatively at 1, 2, 4, 8, 12, 16, 20 and 25 weeks post-vaccination (p.v.), parallel with the examination of vaccine antigen retention. Gross intra-abdominal lesions were only examined at 12 and 25 weeks. The results show that the onset of inflammation in all vaccinated groups was rapid to develop, with intense cellular infiltrations predominated by mononuclear cells especially in groups injected by oil-based vaccines. Inflammation induced by AVM and liposome vaccines resolved within 12 weeks. In contrast, oil-adjuvanted vaccines produced mild, persistent but ultimately decreasing reactions. Persistent antigens were observed in oil-based and liposome vaccines. The results show that the cod inflammatory response is similar to other bony fish species. The findings also suggest that cod has an efficient innate immune system that is able to rapidly remove or sequester antigens from the injection site leading to the down-regulation of inflammation. Oil-adjuvanted vaccines appear to be well-tolerated by this species and show promise as a possible approach for disease control.

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Introduction

The biggest problem of farmed cod at the moment is classical vibriosis caused by *Vibrio anguillarum* [1]. It affects all stages of production [2—4]. Another disease likely to be important is atypical furunculosis whose aetiology is atypical variants of *Aeromonas salmonicida* [4—6]. Atypical *A. salmonicida* has been isolated from diseased wild cod [7] and farmed wild cod in Iceland [8] and also from farmed cod fry in Norway [4]. Recently, a new disease entity affecting cod has been described caused by infection with a bacterium belonging to the genus *Francisella* [9].

Vibriosis is currently controlled by vaccination using commercially available aqueous vaccines [3,4]. The vaccines are administered either by dip or by intraperitoneal injections. Juveniles have to be protected as early as possible [3] and since aqueous vaccines offer short-term protection, booster vaccinations are required in order to prolong the period of protection.

The threat by atypical *A. salmonicida* adds a new impetus to cod disease control. Vaccines containing *A. salmonicida* antigens are required either singularly or in combination with *Vibrio* antigens. It is known from the studies of salmon that furunculosis vaccines need to be adjuvanted with oil in order to be effective [10]. Indeed oil-adjuvanted vaccines have already been tested in cod but over a short-term duration following vaccination [4,11]. A problem associated with oil-based formulations is that they induce injection site lesions that may persist up to harvest [10,12,13]. Indications are that such side effects occur in cod as well [4,11].

Interestingly, the cod immune system differs from other bony fish species. Cod has a higher concentration of natural immunoglobulins (IG) in the blood and little or no increase in the antibody response is observed following vaccination with hapten carriers or bacteria [14—16]. Furthermore, the estimated size and organization of gene segments and the expression and organization of IG gene repertoire are also different [17]. However, little is known about the inflammatory response following vaccination besides one article [18] whose focus was on the phagocytic capacity of head kidney macrophages. Inflammation plays an instructive role to the adaptive immune response [19,20]. On the other hand, it may result in the induction of unacceptable side effects at the injection site as already mentioned. The purpose of the present study was to characterize the inflammatory reaction in cod following vaccination with oil-adjuvanted vaccines and also to evaluate the suitability of using oil-based vaccines.

Materials and methods

Animals

Approximately 1500 Atlantic cod (*Gadus morhua* L.) juveniles weighing about 5 g each were procured from Cod Culture Norway and transferred to EWOS experimental facilities at Lønningdal where the study took place. The rearing temperature was about 10 °C at the start of the experiment. In the first 2 months, the temperature fell to 4 °C where after it rose to 6 °C over the remaining duration of the experiment. The fish were fed on commercial cod feed.

Randomisation and vaccination

When the fish reached an average weight of 113 g, about 1200 juveniles were randomised into 6 groups, each with 200 fish. Only 5 of the 6 groups were included in the present study. Fish in each group were reared in 2 randomly selected parallel tanks (100 fish in each).

The injection preparations used in the present experiment are given in Table 1. Emulsification of the antigens with adjuvant was done using a homogenizer with a standard emulsification stator/rotor connected to an emulsior screen. The oil-based antigen preparation was formulated as water-in-oil (w/o), where the water phase (containing bacterial antigens) was dispersed into an oil phase (continuous phase containing emulsifiers and stabilizers). The potency of the vaccines was according to specifications given in European Pharmacopoeia monographs for *A. salmonicida* and *V. anguillarum*, RPS >80 and 90, respectively, and for *Moritella viscosa* the potency was RPS >60 and 60% control mortality in the non-vaccines (according to internal standards of PHARMAQ). Sterility, free formaldehyde, inactivation, stress, viscosity and droplet size tests were all performed and standardised on blended bacterin according to standard procedures in the laboratory. Liposomes were made according to the classical film method. Briefly, lipids were dissolved in a chloroform/methanol (2:1 v/v) mixture and the organic solvent was removed by rotary-evaporation. Glass beads were added and the films were hydrated in water above phase transition temperature (N2-atmosphere). The vaccine was blended at a potency equal to what was described above for *A. salmonicida*.

Vaccination was done as previously described [13] with minor modifications. Briefly, the fish were anaesthetised using Finquel (Argent Laboratories) at a dosage of 20 g L^{-1}. Injection preparations (0.1 ml) were administered intraperitoneally approximately 0.7 mm anterior and slightly to the right of the anal pore. Self-refilling syringes (Socorex) and 0.6 × 3 mm needles (Unimed) were used.

Marking of the fish

Four weeks following vaccination, the fish were anaesthetised as described above and marked using Visible Implant Fluorescent Elastomer (Northwest Marine Technology, USA). After another 5 days, all the fish were pooled into 2 large tanks where they were reared up to 25 weeks.

Sample collection and evaluation of intra-abdominal lesions

Samples for the evaluation of inflammatory changes were collected at 0, 1, 2, 4, 8, 12, 16, 20 and 25 weeks p.v. Randomly selected fish caught by dip netting were first anaesthetised and sorted into groups. Ten fish from each group were then sacrificed by a sharp blow to the head. Organ samples of pyloric caeca (injection site), spleen and head kidney not exceeding 1 × 1 × 1 cm were collected and stored in 10% phosphate buffered formalin for a minimum