Fish & Shellfish Immunology 44 (2015) 485–495

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Comparative evaluation of infection methods and environmental factors on challenge success: *Aeromonas salmonicida* infection in vaccinated rainbow trout

Jiwan Kumar Chettri [a], Jakob Skov [a], Rzgar M. Jaafar [a], Bjørn Krossøy [b], Per W. Kania [a], Inger Dalsgaard [c], Kurt Buchmann [a, *]

[a] Laboratory of Aquatic Pathobiology, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark
[b] Vaxxinova Norway AS, Bergen, Norway
[c] National Veterinary Institute, Technical University of Denmark, Denmark

ARTICLE INFO

Article history:
Received 8 December 2014
Received in revised form
1 March 2015
Accepted 1 March 2015
Available online 14 March 2015

Keywords:
Aeromonas salmonicida
Furunculosis
Vaccination
Side effects
Immune response
Cohabitation

ABSTRACT

When testing vaccine-induced protection an effective and reliable challenge method is a basic requirement and we here present a comparative study on different challenge methods used for infection of rainbow trout *Oncorhynchus mykiss* with *Aeromonas salmonicida*, a bacterial pathogen eliciting furunculosis. Fish were vaccinated with three different adjuvanted trivalent vaccines containing formalin killed *A. salmonicida*, *Vibrio anguillarum* O1 and O2a. These were 1) the commercial vaccine Alpha Ject 3000, 2) an experimental vaccine with water in paraffin oil adjuvant, 3) an experimental vaccine with water in paraffin oil in water adjuvant. Fish were then exposed to *A. salmonicida* challenge using i.p. injection, cohabitation in freshwater, cohabitation in saltwater (15 ppt) or combined fresh/saltwater cohabitation. Cohabitation reflects a more natural infection mode and was shown to give better differentiation of vaccine types compared to i.p. injection of live bacteria. The latter infection mode is less successful probably due to the intra-abdominal inflammatory reactions (characterized in this study according to the Speilberg scale) induced by i.p. vaccination whereby injected live bacteria more effectively become inactivated at the site of injection. Compared to cohabitation in freshwater, cohabitation in saltwater was less efficient probably due to reduced survivability of *A. salmonicida* in saltwater, which was also experimentally verified *in vitro*.

1. Introduction

Furunculosis caused by *Aeromonas salmonicida* subsp. *salmonicida* is a systemic infectious disease in salmonid aquaculture. The pioneer work in 1942 on oral vaccination of trout against *A. salmonicida* [1] has been followed by numerous relatively successful studies on injection vaccines against furunculosis with or without adjuvants [2–5]. The protection mechanisms induced are not fully elucidated but a positive correlation between protection and antibody titer indicates the involvement of protective and specific immunoglobulins [6–8] although cellular elements such as lymphocytes, macrophages and neutrophils play a significant role [9,10]. Similarly gene expression analyses of *A. salmonicida* vaccinated salmon (*Salmo salar* L.) have suggested involvement of a mixed Th1/Th2/Treg response in spleen [11]. Hepatic gene profiling of vaccinated salmon has shown a high regulation of complement factor genes suggesting that those genes may serve as markers for vaccine induced protection against furunculosis [12]. However, the main concern of oil-adjuvanted vaccines is the side effects occurring in the form of malpigmentation or intra-abdominal adhesions [4,13,14] which may be Th17 associated granulomatous lesions [15]. Therefore search for new candidate antigens and adjuvants with lower side effects but still high efficacy has priority. One of the important criteria in evaluation of candidate vaccines is survival of vaccinated fish compared to the un-vaccinated controls following infection challenge. However, the challenge method may influence the outcome of the trial [16]. In fish challenged intraperitoneally (i.p.) following i.p. vaccination the injected live pathogens will

* Corresponding author. Tel.: +45 35336728; fax: +45 35332742.
E-mail address: kub@sund.ku.dk (K. Buchmann).

http://dx.doi.org/10.1016/j.fsi.2015.03.003
1050-4648/

immediately encounter an inflammatory environment with phagocytic cells, which may inactivate many of the pathogens locally. This may lead to a high survival of challenged fish but not reflect general systemic immunity, which can protect the vaccinated fish against natural exposure to the pathogen. We have therefore evaluated if cohabitation challenge is a useful alternative to i.p. injection challenge. We tested several vaccine types and performed the challenge in various ways at 19 °C which corresponds to summer temperatures in Danish rainbow trout mariculture where furunculosis is considered the main problem [17]. In addition, we have tested protection not only in freshwater but also in 15 ppt (parts per thousand) saltwater, which is the prevailing salinity around Danish mariculture farms [18]. Vaccines tested in this study comprise a commercial oil-adjuvanted vaccine and experimental vaccines containing a Danish strain of *A. salmonicida* subsp. *salmonicida* in combination with two serotypes of *Vibrio anguillarum* and different oil emulsions as adjuvants. Further, inflammatory reactions (side-effects) in the body cavity following vaccination were evaluated in the different fish groups by using the Speilberg scale. Immune reactions in fish were described by gene expression analysis (qPCR: quantitative real time polymerase chain reaction), immunohistochemical detection of immune cells (IHC) and evaluation of serum antibody level by ELISA (enzyme-linked immunosorbent assay).

2. Materials and methods

2.1. Fish

Rainbow trout eggs (6000) were obtained from Rakkeby trout hatchery (Jutland, western Denmark) and hatched out (7 °C) in a recirculated pathogen-free facility at Salmon hatchery Bornholm, Nexø, Denmark. Fish were then kept in 700 L tanks in municipal tap water at 11–13 °C with daily feeding (1.5% of fish biomass) using pelleted feed (BioMar A/S, Denmark) [19].

2.2. Vaccines

Experimental vaccines were prepared based on Danish isolates of *A. salmonicida* subsp. *salmonicida* (090710-1/23) and *V. anguillarum* O1 and O2a, recovered and diagnosed from a disease outbreak in a mariculture farm according to previous studies [20,21]. Experimental vaccines included formalin killed *A. salmonicida* bacteria with a concentration of 6.8×10^8 CFU/ml and 3.4×10^4 CFU/ml for each of the *V. anguillarum* O1 and O2a isolates. Paraffin oil was applied as adjuvant and prepared as water in oil (70% oil and 30% water phase) or water in oil in water formulation (35% oil and 65% water phase). The water phase contained 20% aluminum hydroxide. The commercial vaccine applied was Alpha Ject 3000 (Pharmaq AS, Norway), which contains *A. salmonicida* subsp. *salmonicida* and *V. anguillarum* O1 and O2a in unspecified concentrations with a paraffin oil adjuvant.

2.3. Vaccination

Rainbow trout in Danish farms receiving vaccination against furunculosis have already been vaccinated against enteric red mouth disease (ERM caused by *Yersinia ruckeri* biotype 1 and 2). Therefore, in the present study ERM immersion vaccination was used as a background vaccination, on top of which experimental vaccines with the Danish strain of *A. salmonicida* were administered. Thus, each fish was subjected to two or three vaccinations: First a basic immersion vaccination against ERM was given (all groups received this vaccination). Secondly immersion vaccination against furunculosis was given but only to one group. Thirdly, injection vaccination with multivalent vaccines containing *A. salmonicida* and *V. anguillarum* bacterins was provided with different adjuvant combinations (Table 1).

2.3.1. Basic immersion vaccination of fish

When fish reached a mean body weight of 5–6 g, 5000 of the fish received a basic 30 s dip vaccination against ERM by using AquaVac® Relera™ (MSD Animal Health) (bacterin diluted 1:10). This was done to mimic the general immunoprophylactic status in the Danish aquaculture farms, where all fish are vaccinated against enteric red mouth disease at this early time point in their life. A total of 1000 fish were kept as un-vaccinated controls. One month later a sub-group of 1000 fish (out of 5000 fish: size 8–12 g) received an additional basic 30 s immersion vaccination against *A. salmonicida* by immersing fish in a diluted (1:10) bacterin (a non-adjuvanted experimental bacterin consisting of formalin-killed *A. salmonicida*: (0.5×10^9 CFU/ml) (Group 2)). This was done to investigate if a primary immersion would enhance the response when fish later were injection vaccinated.

2.3.2. Injection vaccination of fish

Two and a half month post *A. salmonicida* dip vaccination, fish (now with a size of 20–23 g) were divided into five different groups in duplicate (1000 fish per group; 500 per replicate) for injection vaccination (i.p. injected with 0.1 ml of vaccine in all cases). Table 1 shows vaccination methods and content of vaccines. The different vaccinated groups were as follows: 1) *Group 1*: ERM immersion vaccination only (serving as control group and designated ERMdip). 2) *Group 2*: ERM immersion plus *A. salmonicida* immersion followed by injection with a trivalent experimental vaccine containing *A. salmonicida* and *V. anguillarum* in water in oil in water adjuvant (ERMdip + FUROdip + VibFUROwow). 3) *Group 3*: Fish were given the same combination of vaccines as group 2 except that no dip vaccination against furunculosis was applied (ERMdip + VibFUROwow). 4) *Group 4*: This group was vaccinated in the same manner as group 3 but with water in oil adjuvant (ERMdip + VibFUROwo). 5) *Group 5*: ERM immersion plus i.p. injection with a commercial oil-adjuvanted vaccine Alpha Ject 3000 (ERMdip + Alpha Ject 3000).

2.4. Challenge experiments

Four comparative challenge experiments at varying environmental conditions (but always at 19 ± 1 °C) were conducted to evaluate the protective effect of vaccines (Table 2). Fish were challenged (3, 4, 5 and 6 months post vaccination) in duplicate fish tanks (volume 100 l) by i.p. injection (0.1 ml volume) (conducted only in freshwater) or by cohabitation (tested both in fresh and saltwater). Cohabitation challenges in the two experiments (2nd and 3rd challenges) were performed by adding 15 naïve donor fish i.p. injected with bacteria (6×10^5 CFU/fish) (shedders) into each challenge tank containing vaccinates ($n = 25$). In the third challenge trial, salinity in tanks with vaccinates was gradually increased to 15 ppt (5 ppt per day) by adding Marine SeaSalt (Tetra, Germany) whereupon infected donor fish (shedders) were added. Due to the low disease transmission induced by this procedure in all groups a fourth challenge was performed. Firstly, subgroups of donor fish were injected with three dosages of bacteria in order to prolong the transmission period of donor fish (shedders). Secondly, salinity was increased only after all shedders had been removed from the tanks (following transmission of their bacteria) (Table 2). Donor fish were injected with three different doses (5 fish/dose) of bacteria (6×10^3 CFU/fish, 6×10^4 CFU/fish and 6×10^5 CFU/fish). Morbidity was recorded by inspection at 2 h intervals (for up to 3 weeks) and moribund fish were instantly removed (euthanized in a lethal