Specific and natural antibody response of cod juveniles vaccinated against Vibrio anguillarum

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Abstract

The purpose of the present study was to study specific and natural antibody levels in individual cod juveniles before and after being vaccinated against Vibrio anguillarum. Different vaccine preparations and vaccination regimes, i.e. bathing, dipping, i.p. injection or combination of treatments were employed and the performance of different groups to bath challenge by the bacterium tested. Antibody responses to V. anguillarum antigens in groups vaccinated by bathing and/or dipping were negligible, while responses were observed in i.p. injected fish. Fish receiving i.p. injection in addition to bathing, showed significant antibody response. Both groups showed increased levels of natural antibodies while levels were low in other groups. Fish bathed or dipped showed higher mortality when challenged than untreated fish, while fish that received a second vaccination showed the best protection. It was not ascertained whether there is a long term difference between the effects of immersion versus i.p. injection as a booster method. Levels of antibodies against V. anguillarum antigens or natural antibodies in groups with the lowest mortalities show that neither could have been used to predict protection given by the vaccines tested.

1. Introduction

Cod has for centuries been one of the most important species for fishing communities around the North Atlantic [1]. Today, with catches rapidly declining, cod is probably the marine species with the highest potential for sustainable cold water aquaculture [2].

Intensive rearing of fish, suffers from outbreaks of diseases. In cultivated cod, vibriosis, caused by Vibrio anguillarum, is one of the major bacterial diseases [3,4]. Vibriosis in cod is often associated with serotypes O2a and O2β [5,6] while serotypes O1 and O2a are most commonly isolated from salmonids. Vaccinations are the most effective way to avoid bacterial diseases and different fish species may need different vaccines, vaccination methods and vaccination schemes [7,8]. Cod has been successfully vaccinated against vibriosis and recently, vaccines adopted for use in cod, containing serotypes O1, O2a and O2β, have become available commercially. Vaccine trials have included cod of various sizes, using bathing, immersion and injection and different challenge methods [9–12].

Several studies of antibodies and antibody responses in cod have been carried out during the last two decades. Cod blood has high concentration of IgM in serum that increases by influence of environmental factors such as temperature [13] and size [14]. Cod mounts specific response to a limited number of antigens following infections and immunizations and typically individuals in a group show a wide range of responses [10,15,16]. Studies on cod immunoglobulins were reviewed recently and the authors concluded that the explanation for the lack of specificity does not appear to be due to deficiencies in their structure, organization, diversity or expression [17].

Natural antibodies are important in the first line of defence against infections [18]. They react to common antigens of pathogens and various self-antigens. They are present in sera of normal, non-immunized individuals, where they are proposed to form a link between innate and adaptive immunity [19]. Studies on natural antibodies have been carried out in a number of fish species [20] and high levels of natural or non-specific antibodies have been reported in cod [13,14]. These antibodies reacted to a variety of antigens and the strongest response was towards haptenated bovine serum albumin or TNP–BSA. Subsequently it was shown that i.p. injections with Freund’s complete adjuvant (FCA) led to increased activity against this antigen [16]. In a vaccination trial with V. anguillarum in adult cod some of the pre-immune sera contained antibodies that bound to antigens in the bacterial membrane [9] and all non-immunized control fish had antibodies that reacted with V. anguillarum in another trial [10]. A study in goldfish has shown that natural antibodies can influence results of vaccination [21].

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The aim of the present study was to study the levels of specific and natural antibody responses in individual cod juveniles before and after being vaccinated against V. anguillarum. Two different vaccine preparations and six vaccination regimes were employed. Further, the aim was to compare the performance of different groups to bath challenge by the bacterium.

2. Material and methods

2.1. Fish

The cod, of wild parentage from Icelandic waters, was hatched and reared at the Marine Institute’s Experimental Farm, Stadur, Grindavik [22]. Mean weight ± standard deviation was 1.2 ± 0.4 g at the time of bath vaccination, 10.3 ± 1.1 g at dipping, 25.7 ± 7.2 g at i.p. injection and 61.5 ± 12.7 g when challenged. From the start of the experiment and until i.p. vaccination, different groups were kept separately in 350 l tanks, 120 individuals in each. After i.p. injection and individual marking of all fish along fin margins with Visible Implant Fluorescent Elastomer dye (Northwest Marine Technology, Inc. (Salisbury, Great Britain)), all groups were transferred to one 3500 l tank. Tricine methanesulphonate, 50 mg ml⁻¹ seawater, was used for light anaesthetization during tagging, i.p. immunization, weighing and blood sampling. Nine weeks post-i.p. vaccination, the fish were transported to another facility (Sandgerdi Research Center). There, 30 fish from each group to be bath challenged were evenly distributed between six 350 l tanks, i.e. two tanks for each bath challenge and acclimatized for two weeks before challenge. Aerated borehole seawater of temperature 9 ± 2 °C and salinity 32‰, was used in both facilities and the fish were fed commercial dry pellets.

2.2. Bacterium for challenge

A frozen stock, of an Icelandic isolate of V. anguillarum (F-139-03) serotype O2β recently passed through three times through cod, was inoculated onto blood agar with 1.5% NaCl (BA-NaCl) and incubated at 16 °C for 3 days. Growth was verified with BioNor agglutinating antibodies against V. anguillarum. A loopful of the growth was suspended in 1.5 ml⁻¹ phosphate buffered saline with 1% peptone and 1.5% NaCl (PBS-P-S) and distributed evenly on 150 mm BA-NaCl agar plates and incubated at 16 °C for 19 h. Then, 4.5 ml of ice cold PBS-P-S was poured over each agar plate, the growth scrapped into the buffer, harvested and placed on ice. Density measured 2.0 at 590 nm in spectrophotometer, or close to 10⁵ colony forming units (CFUs) according to previous measurements. The solution was diluted by tenfold steps to the concentrations needed to do the bath challenge. Doses were chosen according to results from previous challenge tests. CFUs were confirmed by plate counting after incubation for 3 days at 16 °C.

2.3. Vaccines and immunizations

The vaccines tested were a generous gift from Pharmaq in Norway, where they were already licensed. A special permit was issued by The Veterinary Officer for Fish Diseases in Iceland for the use of these vaccines in the current experiment. The vaccines were V. anguillarum bacterins for use in cod, containing serotypes O1α, O2α and O2β. A water-based preparation was used for bathing or dipping and a preparation containing mineral oil adjuvant formula (AJ-oil) for injection. The immunizations were carried out as described by the manufacturers, i.e. bathing for 30 min in vaccine diluted 1:200 in seawater, dipping for 30 s into vaccine diluted 1:10, injection with 0.1 ml i.p. or a combination thereof, see Table 1. Control fish were subjected to bathing, dipping and injection with PBS-P-S and one group received AJ-oil only.

| Table 1 | Experimental set up. Vaccinations by bathing, dipping, intra-peritoneal injection or combinations thereof (V), kidney samples for bacteriology (K) blood samples (S), and bath challenge (X). |
|-----------------|------------------|-------------|------------------|
| **Vaccine administration** | **Weeks from start of experiment.** | **Vaccinations (V), sampling (K, S) and challenge (X)** |
| | **0 weeks** | **6 weeks** | **15 weeks (I)** | **24 weeks (II)** |
| C: PBS control | K, S | K, S | K, S, X |
| B: bath | V | K, S | K, S, X |
| D: dip | V | K, S | K, S, X |
| BD: bath and dip | V | V | K, S, X |
| VA: adjuvanted vaccine i.p. | V | K, S, X |
| B and VA | V | V | K, S, X |

Mean weight of fish at bathing 1.2 g; dipping 10.3 g; i.p. injection 25.7 g; bath challenge 61.5 g.

a: I: blood sampling before i.p. vaccination.

b: II: blood sampling before bath challenge.

2.4. Bath challenge

Sixty fish from each group were divided between 6 tanks. Two tanks were used for each of three bacterial concentrations tested by bath challenge, 2 × 10⁵ CFU ml⁻¹, 2 × 10⁶ CFU ml⁻¹ and 2 × 10⁷ CFU ml⁻¹. The fish were bathed in 200 l seawater containing bacteria in the concentrations as stated above. After 1 h, 150 l of fresh seawater were added to each tank and bating continued for an additional hour. The seawater was oxygenated during these 2 h and subsequently the flow rate was restored to normal. The fish were observed for four weeks, hand fed twice daily, deaths recorded and dead individuals frozen.

2.5. Kidney samples for bacteriology

A total of 130 kidney samples for bacterial isolation on BA-NaCl agar were collected. Sixty samples were collected during the vaccination period at the indicated times and a total of 70 samples, 10 from each group before challenge, see Table 1. Fish that died during challenge were frozen on the day of death and kidney samples inoculated on BA-NaCl agar at the end of the experiment.

2.6. Blood sampling

On three occasions, as shown in Table 1, a total of 120 blood samples were taken from the caudal vein of 10 fish in each experimental group. The blood was allowed to clot overnight at 4 °C, spun and the serum harvested and kept at −20 °C until tested.

2.7. Serology

ELISA tests. Cod sera were diluted 1:100 and added to 96 well microtrays (Maxisorp, Nunc) coated with sonicated preparation of V. anguillarum, protein concentration 10 μg ml⁻¹, followed by mouse antibodies to cod IgM, alkaline phosphatase conjugated goat antibody to mouse Ig and the color developed [23]. When measuring natural antibodies, the plates were coated with 5 μg ml⁻¹ TNP–BSA containing 15.8 residues per BSA molecule. In both ELISAs the value of the blank was detracted from the reading and the final value for each sample was the mean of OD readings from two wells.

2.8. Statistics

The Kruskal–Wallis test was used to test for normality of the distribution of ELISA values and a t-test for comparisons of means between groups. The programmes used were InStat and StatView.
3. Results

3.1. Challenge

Two of three bath challenge doses induced very little death. For fish challenged with the highest dose, $2 \times 10^7$ CFU ml$^{-1}$, accumulated death in the PBS control group was 20%, Fig. 1. In groups either bathed or dipped there was 50% accumulated death, with bathed fish dying off faster than dipped. Accumulated death was 15% or less in other groups, Fig. 1. Means of antibody responses to *V. anguillarum* and TNP–BSA before challenge are shown in Table 2 for comparison to challenge results. Evidently neither antibody responses against *V. anguillarum* nor TNP–BSA ensured survival after challenge since group BD with 5% mortality showed negligible antibody responses while in group B–VA with no mortality the highest antibody responses were observed.

3.2. Bacterial isolations

*V. anguillarum* was not isolated from any of the 130 samples taken at various times before challenge, but the bacterium was isolated from 83% of samples from fish that died during challenge.

3.3. Antibody responses following vaccinations

Antibody responses to *V. anguillarum* antigens in controls and groups receiving bathing, dipping, or both treatments were negligible both at 15 or 24 weeks after the start of the experiment (Fig. 2a). Fish receiving vaccination i.p. showed raised antibody levels but comparison with controls did not show significant differences. On the other hand samples from fish receiving i.p. injection in addition to bathing, II–B–VA, showed significantly raised mean, $p < 0.05$, when compared to controls before challenge. Adjuvant alone raised the antibody mean slightly but insignificantly compared to controls. Groups receiving i.p. vaccination showed raised antibody responses to TNP–BSA while other groups showed negligible changes in their response to TNP–BSA during the time of the experiment (Fig. 2b).

In Fig. 3, responses to *V. anguillarum* and TNP–BSA are shown for every individual in four groups. There are no examples of high responses in controls and bathed individuals. Four out of 10 fish receiving i.p. vaccination showed moderate responses (OD$_{405}$ 0.5–1.0) to *V. anguillarum* antigen preparation, while the same number of fish bathed and vaccinated i.p. showed high responses (OD$_{405}$ > 1.0). Order of samples on the X-axis is always the same so individual responses to either antigen can be compared within each group. Antibodies to TNP–BSA in majority of individuals in these two groups showed raised levels.

4. Discussion

In the present study, levels of specific and natural antibody responses in individual cod fry from groups subjected to different

**Table 2**

<table>
<thead>
<tr>
<th>Vaccine-groups</th>
<th>Accumulated death %</th>
<th>Mean ELISA values of antibodies against</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>V. anguillarum</em></td>
</tr>
<tr>
<td>C: PBS control</td>
<td>20</td>
<td>0.126</td>
</tr>
<tr>
<td>B: bath</td>
<td>50</td>
<td>0.131</td>
</tr>
<tr>
<td>D: dip</td>
<td>50</td>
<td>0.094</td>
</tr>
<tr>
<td>BD: bath and dip</td>
<td>5</td>
<td>0.058</td>
</tr>
<tr>
<td>A: AJ-oil adjuvant i.p.</td>
<td>10</td>
<td>0.270</td>
</tr>
<tr>
<td>VA: adjuvated vaccine i.p.</td>
<td>15</td>
<td>0.474</td>
</tr>
<tr>
<td>B and VA</td>
<td>0</td>
<td>0.846</td>
</tr>
</tbody>
</table>

**Fig. 1.** Accumulated death percentage in seven groups of vaccinated cod juveniles and controls receiving PBS, subjected to bath challenge with $2 \times 10^7$ CFU ml$^{-1}$ *V. anguillarum* of serotype O2iI for 2 h and observed for four weeks.

**Fig. 2.** Antibodies against a) *V. anguillarum* and b) TNP–BSA in groups of cod following one or two vaccinations against *V. anguillarum*, showing median and range of ELISA readings of cod sera, 10 fish in each group. The line within each box is the median the boxes show 25–75% percentiles, the whiskers 10–90% percentiles and circles the extreme values. I and II represent sampling times as shown in Table 1; C: PBS control group; B: bathed group; D: dipped group; BD: group bathed and dipped; A: adjuvant administered i.p.; VA: adjuvated vaccine administered i.p.
vaccination schemes were measured. This is the first study addressing antibody profiles of cod groups where vaccination starts with small fry. Further, performance of the different groups when subjected to bath challenge was assessed.

In terms of survival, the strongest bath challenge dose used in the present study was suboptimal, since accumulated death in the control group was only 20%. Interestingly the groups bathed or dipped showed less survival than the control group with bathed individuals dying faster than dipped and with both groups reaching 50% accumulated death on day 21 after challenge. The cod juveniles in the current study were 1.2 g at the time of bathing which is too small for a long time protection to be established according to a recent paper [11]. In that same paper dipping at 5 g resulted in long-term protection in an experiment where 95% of controls died, which is in contrast with the results reported here. Difficulties in the establishment of bath-challenge models are well known. Sometimes observed death of controls is too high and benefits of vaccinations are in danger of being underestimated while too low accumulated death of untreated controls can obscure a real difference between types of vaccines and vaccination modes [24]. The manufacturers of the vaccines tested recommended two vaccinations and according to our results, bathing followed by either dipping or injection, can give good results. AJ-oil alone did not induce significant protection in the current experiment. This is in agreement with another study where frys receiving AJ-oil only were not protected against bath challenge while i.p. challenge of the same group resulted in significant protection [12]. Due to the set up of the experiment, there were 24 weeks between bathing and challenge, 18 weeks between dipping and challenge while 9 weeks passed between injection and challenge.

Antibody responses in the vaccinated groups demonstrate that only individuals receiving adjuvated vaccine i.p. show raised antibodies to *V. anguillarum*. There were higher individual readings as well as higher mean in the B–VA group (bathed prior to i.p. vaccinations).
come into contact with immune sera reacted with bacterial antigens possibly because the following second immunization. In that study some of the non-mainly to LPS antigens and there was no increase in antibody levels administered to 1–3 kg cod, inducing moderate antibody response, immersion versus i.p. injection as a booster method. The conditions of the experiment, but it was not ascertained vaccination schedules in cod. The same was true for some of the PBS injected of immune response genes was demonstrated 3–7 days after i.p. vaccination [26]. Other methods of vaccination were not mediated antibacterial activity in samples from cod, 3 days after rum in cod. Other possibilities are already being contemplated in goldfish [21].

In conclusion, antibodies against V. anguillarum antigens or antigens from related V. anguillarum. In another study [9], bacterins in FCA were administered i.p. in AJ-oil adjuvant evoke more antibody responses in cod than bacterins administered in FCA or FIA.

In comparison to fish of wild origin, all test groups showed low levels of natural antibodies [13,14]. The explanation is probably both the young age and clean environment of the test groups. Fish that received V. anguillarum antigens i.p. were the only ones to show some rise in antibodies against TNP–BSA during the trial period. Antibodies to TNP–BSA have not been measured previously in sera of cod vaccinated against V. anguillarum but FCA, regardless of the antigen used, induced increase in antibodies to TNP–BSA in cod [16]. In the present study, the group receiving AJ-oil only, showed lower response towards TNP–BSA than fish in the groups receiving bacterin in AJ-oil. This indicates an influence of the bacterial antigens in raising antibodies reacting with TNP–BSA as was the case with FCA, an oil adjuvant containing bacterial antigens. In the current study natural antibody levels were very low when the vaccinations started and hence less likely to influence the results than could have been the case in older fish with higher levels of natural antibodies as reported recently for vaccination trial in goldfish [21].

The present results suggest that antibody measurements are not the way to assess protection by vaccines against V. anguillarum in cod. Other possibilities are already being contemplated and a recent paper reports that there was enhanced serum-mediated antibacterial activity in samples from cod, 3 days after i.p. vaccination [26]. Other methods of vaccination were not investigated. In the same study, some upregulation in expression of immune response genes was demonstrated 3–7 days after immunization. The same was true for some of the PBS injected control fish. Looking at the present results it can be inferred that methods for studies on cellular immunity are needed in order to reveal the mechanisms explaining the observed susceptibility of fish either bath or dip vaccinated as well as the protection obtained in fish receiving both treatments. Further information on the cod immune responses is needed in order to develop successful vaccines and answer the question on a method other than challenge that could be used to study efficacy of vaccines/vaccination schedules in cod.

In conclusion, antibodies against V. anguillarum antigens or against TNP–BSA could not have been used to predict protection in a bath challenge. A booster vaccination had positive effects under the conditions of the experiment, but it was not ascertained whether there is a long-term difference between the impact of immersion versus i.p. injection as a booster method.

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