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Short communication

Vaccination experiments in the gadoid haddock, *Melanogrammus aeglefinus* L., against the bacterial pathogen *Vibrio anguillarum*

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Abstract

Vibrio anguillarum is one of the primary pathogens responsible for high levels of fish mortality in the aquaculture industry, and among gadoids O2a and b are the most common pathogenic serotypes. In this paper a variety of studies were performed to assess the optimal route by which to challenge haddock against this pathogen, and an optimal regime to vaccinate haddock. The most efficient method to challenge haddock with *V. anguillarum* in this study was immersion in a bath containing 10^7 cfu/ml, where 60% mortality was seen. Subsequent experiments showed that juvenile haddock could be protected against bacterial challenge with *V. anguillarum*, with a significant reduction in mortalities observed amongst the vaccination treatments when compared to the unvaccinated controls. However, as seen previously in cod studies, vaccination did not induce a specific antibody response.

Keywords: Haddock (*Melanogrammus aeglefinus*); Vaccine; *Vibrio anguillarum*; Gadoid

1. Introduction

During the past decades cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) fisheries have experienced a severe decline (Bolle et al., 2004; Fogarty et al., 2001). This lack of natural supply, along with the high economic importance of both cod and haddock in the fishing communities of the North Atlantic (Magnadóttir et al., 2001; Mikkelsen et al., 2004), has generated considerable interest in the farming of these gadoids. Cod has proven to be an economically viable species to culture (Magnadóttir et al., 2001; Rosenlund and Skretting, 2006), and as a consequence of this success, haddock aquaculture has followed. Intensive rearing of these and other cold-water marine species can suffer from disease outbreaks, with severe financial impacts. Atypical *Aeromonas salmonicida* and viral haemorrhagic septicaemia virus (VHSV) have been reported to affect gadoids (Magnadóttir et al., 2002), although, the serotype O2b of *Vibrio anguillarum* is the most serious pathogen described in cod to date (Larsen et al., 1994; Mikkelsen et al., 2004; Samuelsen and Bergh, 2004; Sørensen and Egidius, 1987; Sørensen and Larsen, 1986). Current licensed vaccines for this disease have been designed mainly for salmonids, which are principally affected by serotype O1 (Sørensen and Larsen, 1986) and therefore may not be suitable for gadoids. Despite the threat posed by classical vibriosis to the farming of gadoids, little research has been carried out to investigate potential vaccination against

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this pathogen, and only on cod (Arnesen et al., 2002; Espelid et al., 1991; Mikkelsen et al., 2004). Thus, the aim of the present study was to investigate the efficacy of a series of vaccine treatments against *V. anguillarum* in haddock and to establish a rigorous method of challenge to be used in such trials. In addition to this, the production of specific antibodies following vaccination was investigated.

2. Materials and methods

2.1. Cultivation of bacteria

The bacterial strain of *V. anguillarum* MT2582 (Fisheries Research Services, Aberdeen, Scotland), typed as O2, but without subtyping to a or b, was used in all trials. Since large quantities of bacteria were required, bacteria were grown for 7 days in blood Tryptone soy agar (TSA, Oxoid Ltd., UK) supplemented with 1.5% NaCl, by incubation at room temperature (22–23 °C). Bacteria were harvested from plates, and resuspended in phosphate buffered saline (PBS, pH 7.4). The concentration was measured by optical density (OD), where at 540 nm, 0.95–1.05 gives an approximate concentration of 10^9 cfu/ml (Jhingan et al., 2003). This suspension was serially diluted according to the concentrations required for the trials and, to confirm concentration accuracy, an aliquot of 10^3 cfu/ml was plated in agar plates. The bacteria used for vaccine production was inactivated by adding 1% formalin to the solution, which was removed after 24 h.

2.2. Fish husbandry

All fish used in subsequent trials were produced by intensive rearing from wild or hatchery broodstock at Viking Ardtoe Marine Laboratory, Ardtoe, Scotland (former SFIA, Marine Farming Unit) and kept under normal husbandry procedures. Individual fish batches were used for each of the trials. Fish were starved for a period of 2 days before each of the experiments. Prior to vaccination or challenge via injection, fish were sedated by immersion into water containing 0.3 ml 2-phenoxyethanol (Sigma, UK) per litre.

2.3. Bacterial challenge

A group (1) of sixty 10-month-old naïve fish (average weight 177.5 g ± 23.53) were transported to the Challenge Unit at Marine Harvest, Lochailort (Scotland, UK) and allowed 1 week to acclimatise before challenge. Fish were divided into four experimental groups. Group 1A consisted of 10 fish challenged with an intraperitoneal injection (i.p.) of 10^5 cfu/ml of *V. anguillarum* MT2582 suspended in PBS. Group 1B consisted of 10 fish challenged via immersion in a bath containing 10^7 cfu/ml for a period of 30 min. Group 1C consisted of 10 fish challenged with an i.p. injection of 10^7 cfu/ml suspended in PBS. This group was allocated to a tank along with 30 naïve fish, which formed a cohabitation group (Group 1D).

Challenge tanks were checked regularly for mortalities and the cause of the death was verified by obtaining swabs from the head kidney from all moribund or dead fish, which were then grown on TSA + 1.5% NaCl and tested using a latex agglutination kit, Bionor™ Mono Aqua (BioNor, Norway). At the end of the experiment, head kidney swabs were obtained from all surviving fish, to check for latent *V. anguillarum* infection.

2.4. Specific antibody production post-immunisation

Two groups with 10 fish each were used in this second trial to determine specific antibody titres after vaccination on a population that had undergone a single vaccination (Group 2A) or a booster vaccination (Group 2B). Group 2A were 18-months old at the beginning of the trial (average weight 809.32 g ± 160.5) and dip-vaccinated with AquaVac® Vibrio (Schering-Plough Aquaculture, UK), an aqueous vaccine containing formalin inactivated *V. anguillarum* serotypes O1 and O2, when fish were 10 g average weight, following the normal health routine of the hatchery. Group 2B were 6-month-old unvaccinated fish (average weight 128.90 g ± 29.9).

All 20 fish were immunised by an i.p. injection of 100 μl of AquaVac® Vibrio. Prior to vaccination and at each subsequent sampling point (every 4 weeks) fish were individually anaesthetised, length and weight measured and blood sampled. Serum was obtained as described previously (Magnadóttir et al., 2002) and aliquots stored at −20 °C. Agglutination assays were carried out in 96 well, round bottom, microtitre plates (Fisher Scientific, UK). Serum samples of 50 μl were serially diluted two-fold in PBS, and 50 μl of formalin-killed bacteria (5×10^8 cfu/ml) was added to all the wells and thoroughly mixed. Plates were covered and incubated at 4 °C overnight. Antibody titres were expressed as the $\log_2$ of the maximum serum dilution giving positive agglutination. The trial was terminated 12 weeks after vaccination, a time known to give

maximal antibody titres in other fish species kept at these temperatures (Gudmundsdóttir et al., 1997).

2.5. Vaccination and bacterial challenge

A total of 700 juvenile fish (3.5 g average weight) were divided into the seven treatments (100 fish each) as detailed in Table 2. Fish were vaccinated at this time and boosted 1 and/or 2 months later, when the fish were 15 g and 35 g, respectively, or just vaccinated at month 2. All vaccines used in this trial, with the exception of Group 3F, where AquaVac® Vibrio was used, were formalin-killed *V. anguillarum* MT2582. Two months after the final vaccination, fifty fish per treatment (85 g on average) were transported to the Marine Harvest (Lochailort) challenge facilities and allocated to five tanks, each containing 10 fish per treatment group. After a week of acclimatisation, fish were challenged by immersion (10^7 cfu/ml of *V. anguillarum* MT2582 for a period of 30 min) and, as in trial 1, all subsequent mortalities tested for *V. anguillarum*. The challenge results were expressed as the relative percentage of survival (RPS) (Jarp and Tverdal, 1997) of all the mortalities which had tested positive for *V. anguillarum*. Swabs from all surviving fish were obtained to check for latent *V. anguillarum* infection. Secondary effects on growth due to vaccination were assessed throughout the trial, where weight and length was measured at every vaccination point, i.e. at monthly intervals and prior to challenge.

2.6. Statistical analysis

A χ^2 goodness-of-fit was used for the analysis of the results of trial 1 and one-way analysis of variance (ANOVA) and two-way ANOVA for trial 2. Analysis of the challenge results obtained from trial 3 was performed using the Peto test (Stallard and Whitehead, 2000) and a one-way ANOVA for the study of secondary effects on growth. In all three trials, a significance level of $p \leq 0.05$ was used.

3. Results and discussion

3.1. Bacterial challenge

Due to the lack of research on haddock vaccination and pathogen susceptibility, in this study it was necessary initially to establish an appropriate method to deliver a bacterial challenge. Mortalities started to occur on day 6 post-challenge, slowly progressing until reaching a peak on day 23 with 60% mortality registered in Group 1B (Fig. 1A). All mortalities tested positive for *V. anguillarum*, while surviving fish tested negative. When statistical analysis was carried out, a significant difference between Group 1B and the rest of the groups ($\chi^2 = 6.54$, $p = 0.01$) was found. In agreement with other reports (Gildberg and Mikkelsen, 1998; Balfry et al., 2001; Jhingan et al., 2003), high mortalities were registered amongst fish challenged via immersion, a method that mimics natural infection. This proves that while injection challenge can have advantages as a delivery route for bacterial infection in many circumstances (Espelid et al., 1991; Lönnström et al., 2001; Magnadóttir et al., 2002), it is still possible to obtain a high level of mortality using a bath immersion. Cohabitation with infected fish (Group 1D), another method where natural infection is mimicked, was also tested in this trial but resulted in only 10% mortality and may have been also influenced by the low mortality of the challengers, Group 1C (10%). It seems likely that if the number of fish succumbing to infection is low, the cohabitants would have a lower chance of developing the disease. Conversely, when the mortality of the diseased fish or challengers is high, it is possible to reach a good level of mortality in non-vaccinated fish, as observed previously for salmon (Midtlyng et al., 1996). Challenge via immersion is highly appropriate for haddock, which are extremely sensitive to stress (Thorsen et al., 2003; Martin-Robichaud and Berlinsky, 2004), since it lowers the stress levels produced by i.p. injection, as fish do not have to be anaesthetised or handled to the same degree.

3.2. Specific antibody production post-immunisation

Both populations maintained a relatively stable weight and length throughout sampling (see Table 1). The antibody titres of the immunised populations showed a similar trend, with a slight induction of specific antibody with titres averaging two or less (see Table 1), and never reaching a value above a titre of four (obtained in only one fish from Group 2A). Statistical analysis showed a significant difference between the antibody titre ($\log_2$) prior to vaccination and after immunisation in both Group 2A ($p = 0.001$) and Group 2B ($p = 0.014$). However, the actual increase in antibody response was in all cases very low. When the titres of both populations were compared, no significant difference was found ($p = 0.585$). The study of specific antibody production in fish after vaccination could provide an alternate method of validating a vaccination trial without the use of a bacterial challenge. However,

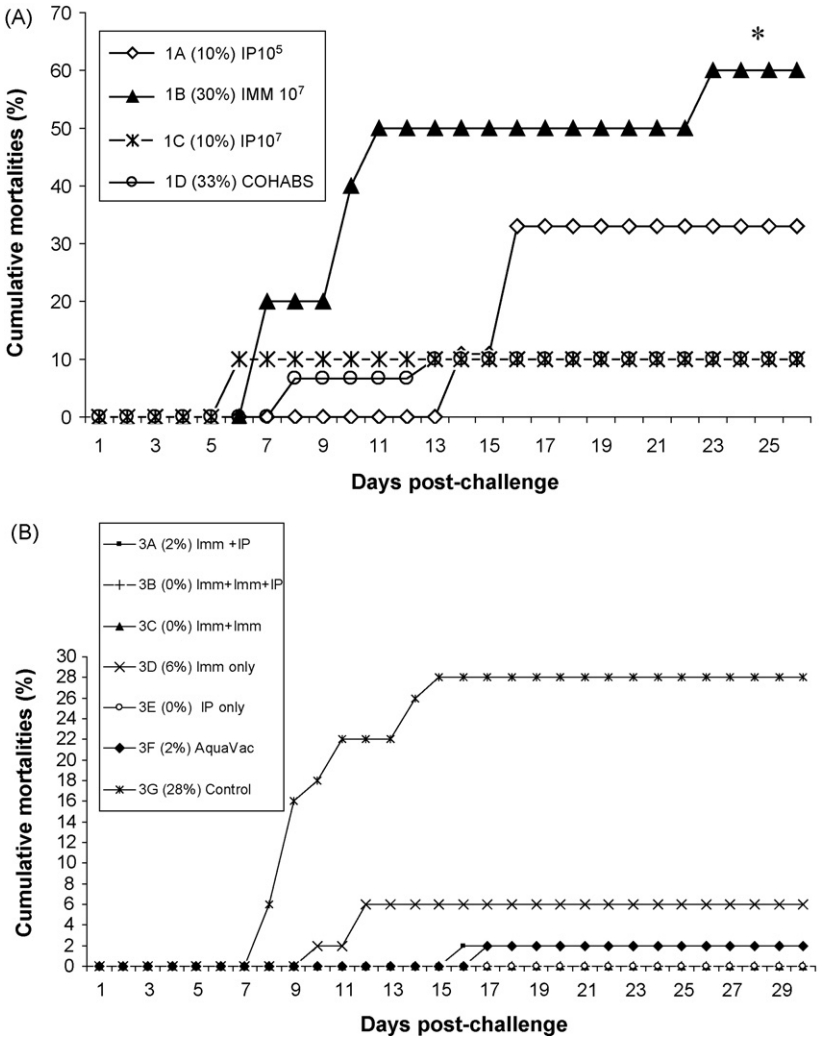

Fig. 1. (A) Cumulative mortalities of juvenile haddock, *Melanogrammus aeglefinus* in trial 1, following bacterial challenge with *Vibrio anguillarum* (MT2582) delivered by: intraperitoneal injection with 10^5 cfu/ml (1A); immersion in 10^7 cfu/ml for 30 min (1B); intraperitoneal injection with 10^7 cfu/ml (1C); cohabitation (1D). *Significant difference for $p \leq 0.05$. (B) Cumulative mortalities post-challenge in trial 3. Treatments were as follows: (3A) immersion at 3.5 g and i.p. injection boost at 35 g; (3B) immersion at 3.5 g, immersion boost at 15 g, i.p. injection boost at 35 g; (3C) immersion at 3.5 g and immersion boost at 15 g; (3D) immersion at 3.5 g; (3E) i.p. injection at 35 g; (3F) i.p. injection commercial vaccine (AquaVac® Vibrio) at 35 g; (3G) control fish (not vaccinated). Fish were 85 g on average when challenged. Final mortalities seen in each group are given in brackets after the symbol code.

we show that as previously observed in cod (Espelid et al., 1991; Magnadóttir, 1998, 2000), haddock do not appear to produce a substantial antibody response post-immunisation either with an individual vaccination dose or with a booster, indicating that this may be a characteristic inherent within the Gadoid family. In order to determine the cause of this lack of specific antibody production, researchers have investigated the effect of different antigens on the adaptive immune system of cod (Espelid et al., 1991; Pilström and Petersson, 1991; Magnadóttir et al., 2001), but have as yet failed to elicit a specific antibody response in cod. However, it has been reported recently that some individuals in a cod population, have a low increase in the antibody responses after immunisation with *V. anguillarum*, which lasted up to 44 weeks post-immunisation (Lund et al., 2006; Lund et al., in press). To date, no reason for this low or lack of specific antibody response has been found, although some authors believe that the higher concentration of IgM in gadoids in comparison with other teleosts (Magnadóttir, 1998) could provide the answer (Arnesen et al., 2002).

Table 1
Trial 2: weight, length and antibody titre of two populations of haddock (Groups 2A and 2B) i.p. vaccinated with AquaVac® Vibrio

Weeks	Procedure	Haddock group							
		Group 2A (i.p. injection)				Group 2B (immersion vaccination + i.p. boost)			
		Weight ± S.E. (g)	Length ± S.E. (mm)	Fish losses	Antibody titre ± S.E. (log_2)	Weight ± S.E. (g)	Length ± S.E. (mm)	Fish losses	Antibody titre ± S.E. (log_2)
0	Immunisation, blood sampling and measurements	809.32 ± 50.7	357.68 ± 8.2	–	0	128.90 ± 7.9	193.20 ± 8.3	–	0
4	Blood sampling and measurements	777.20 ± 64.7	351.40 ± 8.7	–	1.12 ± 0.39	130.90 ± 9.1	201.70 ± 3.5	1	0.57 ± 0.21
8	Blood sampling and measurements	752.60 ± 53.8	349.30 ± 8.8	–	1.62 ± 0.57	181.78 ± 11.4	232.56 ± 3.8	1	1.14 ± 0.43
12	Blood sampling and measurements	854.30 ± 71.1	363.60 ± 10.9	–	1.62 ± 0.57	214.14 ± 11.2	236.29 ± 5.1	1	1.71 ± 0.64

Values are means of fish sampled ± standard error (S.E.), $n = 10$.

3.3. Vaccination regimes—secondary effects and bacterial challenge

To determine if, as in cod, protection can be conferred after vaccination despite the lack of specific antibody production, a vaccination experiment was performed. Measurements taken throughout the trial showed no significant difference (length, $p = 0.947$; weight, $p = 0.792$), indicating no growth related side effects due to vaccination. The nature of the bacterial challenge carried out 2 months after last vaccination was not particularly severe, with the highest mortality (28%) seen in the control Group 3G, and with the remaining groups ranging between 0 and 6% mortality (Fig. 1B). Thus, as in the case of cod (Espelid et al., 1991; Mikkelsen et al., 2004), a high protection (RPS of 100–78.6%) was seen in all vaccinated fish, which was statistically significant when vaccinated groups were individually compared with the control (Table 2) and also when all vaccinated groups were compared with the control ($\chi^2 = 95.13$, $p < 0.001$). Three groups of vaccinated fish showed the highest level of protection reaching an RPS of 100%, where the initial immersion vaccination was followed with one or two boosters (3C and 3B, respectively) or where an i.p. injection was administered when fish were 35 g (3E). It is well known (Nordmo and Ramstad, 1997) that the efficacy of vaccination is related to the strength of the challenge, with lower mortalities registered in vaccinated fish when control mortalities are also low. This is reflected in the present results, where the mortality of the control was lower than anticipated making it difficult to determine which of these regimes was the most effective. If this had not been the case and had the challenge been more severe, a larger difference might have been observed between the mortalities seen in the different vaccinated fish groups. However, even though the difference between RPS among the vaccinated treatments was not statistically supported (Peto test, $p > 0.05$), it is interesting to note that the group with the highest level of mortality among the vaccinated treatments was the one in which fish were vaccinated at 3.5 g with a single immersion (6%). This may suggest that a booster vaccination, immersion or i.p., is necessary to maximise protection. Indeed, it has been reported previously that a single immersion does not confer protection as high as that seen in fish boosted either by i.p. injection or immersion (Thorburn and Jansson, 1988; Angelidis et al., 2006).

In conclusion, our results are in agreement with previous reports in cod where a good level of protection is conferred after vaccination against *V. anguillarum*,

Table 2
Trial 3: summary of the vaccination protocol and challenge results

Group	Vaccination protocol			Challenge results		
	Delivery	Month	Vaccine	RPS (%)	χ^2-Value	p-Value (Peto test)
3A	Immersion at 3.5 g i.p. boost at 35 g	0 2	MT2582	92.8	14.13	<0.001
3B	Immersion at 3.5 g Immersion at 15 g i.p. boost at 35 g	0 1 2	MT2582	100	16.95	<0.001
3C	Immersion at 3.5 g Immersion at 15 g	0 1	MT2582	100	16.95	<0.001
3D	Immersion at 3.5 g	0	MT2582	78.6	9.67	<0.05
3E	i.p. at 35 g	2	MT2582	100	16.95	<0.001
3F	i.p. at 35 g	2	100 μl AquaVac® Vibrio	78.6	8.91	<0.05
3G	n.a.	n.a.	Control	–	–	–

Immersion: immersion in a bath containing 10^8 cfu/ml of formalin-killed *Vibrio anguillarum* for a period of 60 s; i.p.: intraperitoneal injection with 100 μl of a vaccine containing 10^9 cfu/ml of formalin-killed *V. anguillarum* (except Group 3F). Relative percentage of survival (RPS) and summary of the Peto test performed with this data in vaccinated treatment groups compared to the control group (3G). All results are significant for $p \leq 0.05$.

although this does not correlate with a high level of specific antibody production (Espelid et al., 1991; Lund et al., 2006; Schrøder et al., 2006). Much still remains to be discovered about gadoid immunity, especially for haddock, which should be the focus of future research. Investigations into the length of protection obtained with different methods of vaccination or the effects of immunostimulation on the early development stages, would give a better indication of the optimal strategy to follow to improve fish health. This is essential for the future of haddock aquaculture, to enable fish to be protected from vibriosis and other potentially lethal diseases.

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