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Efficacy of a *Listonella anguillarum* (syn. *Vibrio anguillarum*) vaccine for juvenile sea bass *Dicentrarchus labrax*

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ABSTRACT: The efficacy of a commercial bivalent *Listonella anguillarum* (serotype 01 and 02) vaccine (MICROViB, Microtek International) was tested on prime- and booster-immersion vaccinated sea bass *Dicentrarchus labrax* juveniles. We carried out 2 challenge tests on the prime-vaccinated fish, 50 and 90 d after initial vaccination. A second group of fish received a booster vaccination 60 d after the prime vaccination, and were tested with a single challenge 30 d later. Relative percent survival (RPS) was 92 and 84 % (both $p < 0.01$) among the prime-vaccinated fish on the first and second challenges, respectively. The RPS of the booster-vaccinated sea bass was 100 % ($p < 0.01$). Antibody titres were tested only among 10 prime-vaccinated and 10 unvaccinated (control) sea bass, 60 d post-immunisation, and were found to rise to 1/32 in the vaccinated fish. Our results demonstrate that MICROViB immersion vaccine can effectively protect juvenile sea bass from *L. anguillarum* infection.

KEY WORDS: Immersion · Vaccination · *Dicentrarchus labrax* · *Vibrio anguillarum* · *Listonella anguillarum* · Vibriosis

INTRODUCTION

European sea bass *Dicentrarchus labrax* is one of the most farmed species in the Mediterranean (Colorni 2004). For the fish farming industry to succeed, it must produce high quality products and lower the incidence of fish disease. The major diseases affecting sea bass farming are the bacterial diseases vibriosis (Dec et al. 1990) and pasteurellosis (Bakopoulos et al. 1997), and virosis caused by nodavirus (Skliris & Richards 1999) and encephalitis virus (Sideris 1997). Vibriosis is a common disease in sea bass and has led to significant economic losses to date. It is caused by a number of species: *Listonella anguillarum* (syn. *Vibrio anguillarum*), *Vibrio ordalii*, *V. salmonicida* and *V. vulnificus* biotype 2. Classical vibriosis is caused by *L. anguillarum*, which causes haemorrhagic septicaemia in a wide variety of warm- and coldwater species (Toranzo et al. 2005).

In aquaculture, as in other areas of intense stock rearing, e.g. pigs, poultry and cattle, antimicrobial agents have been widely used to treat disease and consequently promote growth. However, the increased use of antimicrobials has led to the emergence of drug-resistant bacteria, and there are concerns regarding the degradation of antimicrobial products in marine sediments and residues in marketed fish meat (Shao 2001). A study in Denmark demonstrated high levels of individual and multiple antimicrobial resistances in *Flavobacteria* and *Aeromonads* collected from 4 Danish fish farms (Schmidt et al. 2000). With ensuing tighter government restrictions on drug use, research into vaccines has increased which, it is hoped, will provide longer lasting disease control with fewer side effects than extended chemotherapy.

As one of the most important farmed species in aquaculture, sea bass has been the focus of much immuno-

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logical research (Scapigliati et al. 2002). Gaining a deeper understanding of the sea bass immune mechanisms, therefore, will enable us to design more effective vaccines as alternatives to antibiotics. The ideal vaccine for aquaculture must be effective in preventing death, inexpensive to produce and license, provide long-term immunity and be easy to administer (Leong et al. 1997). In the present study, the efficacy of a commercial bivalent *Listonella anguillarum* immersion vaccine was tested on juvenile European sea bass.

MATERIALS AND METHODS

Fish and facilities. A total of 1500 *Dicentrarchus labrax* juveniles weighing 2.5 ± 0.2 g were transported to the Veterinary Medicine School of Thessaloniki and acclimated for a period of 30 d in 6 circular tanks with recirculating seawater. The water was recirculated once h^{-1} and passed through mechanical and biological filters. The fish were fed by timer feeders with a standard commercial food (Starter Grower Diet) at a rate of 3% d^{-1} of their body weight, 4 times d^{-1} for 15 min. Throughout the whole experiment the water temperature was 18 ± 1°C, the dissolved oxygen 8 ± 1 mg l^{-1} and the water pH 7.5 ± 0.3, unless otherwise noted.

Vaccination. The fish were vaccinated by immersion with an inactivated whole killed *Listonella anguillarum* serotype 01 and 02 bivalent commercial vaccine (MICROViB, serial number 002-009, Microtek International). The experimental protocol is outlined in Table 1. The vaccine was diluted 1:10 in seawater, according to the manufacturer's instructions. During vaccination, the temperature of the vaccine suspension was the same as the water temperature where fish were kept (18°C). To vaccinate fish, up to 150 fish (total biomass ≤500 g) were immersed in 1 l vaccine suspension for 60 s, under constant aeration. The average weight of the fish at vaccination was 3.3 ± 0.2 g. After 60 d, 300 vaccinated fish were given a booster-vaccination as described above.

Vaccine safety test. The unvaccinated (control) fish were held in three 500 l circular tanks (1000 fish $tank^{-1}$) and the vaccinated fish in three 250 l tanks (500 fish $tank^{-1}$). Prior to the prime vaccination, a vaccine safety test was performed: 20 fish were vaccinated with a double vaccine dose and monitored for 21 d in a 100 l tank with a recirculation system.

Challenge tests. Before each challenge test, the challenge dose was pre-determined by injecting (IP) groups of 10 unvaccinated fish with selected concentrations of *Listonella anguillarum* serotype 01 strain GR 1.1 in 0.1 ml normal saline (NS) (Table 2). The *L. anguillarum* had been cultivated for 24 h at 24°C in Tryptic Soya Broth (TSB) + 1.5% NaCl. The optical density (OD) of this suspension was 0.85 at 625 nm.

We performed 2 challenge tests 50 and 30 d post-prime and post-booster vaccination, respectively. Briefly, fish received IP 0.1 ml NS with 3.3×10^6 and 2×10^6 colony-forming units (cfu) of *Listonella anguillarum* serotype 01 strain GR 1.1 at the first and the second challenge tests, respectively. For the first challenge test, vaccinated and unvaccinated fish were transferred into 50 l tanks 7 d before for acclimatization. We randomly allocated 105 vaccinated fish to 3 tanks (35 fish $tank^{-1}$) and 105 unvaccinated fish (controls) into another 3 tanks (35 fish $tank^{-1}$). The fish were starved from 1 d before the challenge until 5 d post-challenge, after which feeding took place once d^{-1} according to appetite. The water was recirculated twice h^{-1} and passed through mechanical and biological filters. We injected 20 control fish IP with 0.1 ml sterile NS in a 50 l tank with a recirculation system.

The second challenge test differed in the following way. Of the fish used, 70 had received a booster vaccination on Day 60 (2 tanks, 35 fish $tank^{-1}$), 70 had received only a prime vaccination (2 tanks, 35 fish $tank^{-1}$) and 70 were unvaccinated (2 tanks, 35 fish $tank^{-1}$). Of the unvaccinated control group, 20 fish were injected IP with 0.1 ml sterile NS in a 50 l tank with a recirculation system, in order to eliminate the possibility of mortality by handling.

Mixed group challenge. There was also a 100 l tank into which were placed 105 fish (35 controls, 35 prime-vaccinated and 35 booster-vaccinated). The different fish groups were identified by marking (cutting) their

Table 1. *Dicentrarchus labrax*. Outline of vaccine trial. Juvenile sea bass were vaccinated by injection (IP) with a commercial *Listonella anguillarum* vaccine. Average weight of fish at vaccination = 3.3 g. Fish were subsequently challenged by injection (IP) with live *L. anguillarum*: 3.3×10^7 cfu ml^{-1} in challenge test 1 and 2.0×10^7 cfu ml^{-1} in challenge test 2. Unvaccinated fish were used as controls (n = number of fish)

Group	Day 1 Prime vaccination	Day 50 Challenge test 1	Day 60 Booster vaccination	Day 90 Challenge test 2
Unvaccinated (control)	–	n = 105	–	n = 105
Prime-vaccinated	n = 500	n = 105	–	n = 105
Booster-vaccinated	–	–	n = 300	n = 105

Table 2. *Dicentrarchus labrax*. Determination of dose for challenge with *Listonella anguillarum* (n = number of fish). 1st pre-challenge: A–D, challenged fish groups; E, control (unvaccinated) fish group. 2nd pre-challenge: A–C, challenged fish groups; D, control (unvaccinated) fish group. Temperature: 15 ± 1°C, dissolved oxygen: 9.6 ± 0.2 mg l^{-1}, pH: 7.6 ± 0.1. NS: normal saline

Group	n	IP dose (cfu in 0.1 ml)	Cumulative mortality 24 h	48 h
1st pre-challenge				
A	10	1.5×10^8	10/10	10/10
B	10	7.5×10^7	6/10	8/10
C	10	1.5×10^7	5/10	8/10
D	10	7.5×10^6	4/10	6/10
E	5	NS	0/50	0/50
2nd pre-challenge				
A	10	2×10^7	8/10	10/10
B	10	2×10^6	2/10	5/10
C	10	2×10^5	0/10	0/10
D	5	NS	0/5	0/5

opercula. The mortality was observed daily for 15 d post-challenge.

Bacteriological examination. Moribund fish bacteriological samples were taken from the liver. Bacteria were grown on TSA + 1.5% NaCl and identified by biochemical tests (West & Colwell 1984, Barrow & Feltham 1993).

Agglutinating antibody titre assays. On Day 60 post-prime vaccination, 10 vaccinated and 10 unvaccinated randomly selected fish were sampled for plasma antibodies. Blood samples were taken from the caudal vein. The syringes were heparinised to avoid blood coagulation. The fish were removed after blood sampling. Blood samples were centrifuged at $250 \times g$ for 15 min at 20°C and the supernatant was used to estimate the antibody titre by the method of plate agglutination.

An agglutination assay was set up, using 96-well microtiter plates (Flow). Plasma was serially diluted in saline and 0.05 ml was added to each well, together with 0.05 ml of *Vibrio anguillarum* antigen suspension. The antigen was prepared by heat inactivating (50°C, 45 min) bacteria from the same batch as used in the challenge test. A positive and a negative (saline) control were included in each assay (Baytest kit, LADS-Vibriosis, Bayer®). The plates were incubated at 4°C for 48 h. The antibody titre was the reciprocal of the last 2-fold dilution giving full agglutination.

Statistical analysis. The efficacy of the vaccine was assessed by calculating the cumulative mortality of vaccinated and unvaccinated fish. A chi-squared test was used to determine statistical differences in mortality between vaccinated groups and the unvaccinated control group (Zar 1999). Probabilities lower than 0.05 were considered significant. Relative percent survival (RPS) (Amend 1981) of vaccinated fish was calculated as follows: RPS = 1 – (% mortality in test group/% mortality in control group) × 100.

RESULTS

Vaccine safety test

No mortalities or abnormalities were observed in the tested fish during the 21 d observation period following the double dose vaccination.

Challenge tests

The challenge dose was pre-determined by injecting groups of 10 fish with selected concentrations of *Listonella anguillarum* serotype 01 strain GR 1.1 (Table 2). In challenge test 1, the mortality in control group started on Day 2 and reached a maximum cumulative mortality of 75% 7 d post-challenge. In the vaccinated fish groups, the maximum cumulative mortality achieved was 6% (92% relative percent survival, RPS) ($p < 0.01$) on Day 6 (Table 3). No mortality was observed in the 20 injection control fish injected with sterile NS.

In challenge test 2, booster-vaccinated and unvaccinated fish were challenged 30 d post-booster vaccination. Mortality in control groups started on Day 2 and reached a maximum cumulative mortality of 44% on Day 8. No mortalities were obtained in the booster-vaccinated group (100% RPS) ($p < 0.01$). The cumulative mortality in the prime-vaccinated group reached 7% (84% RPS) ($p < 0.01$) (Table 4). Protection was significantly higher in the booster-vaccinated group than in the prime-vaccinated group ($p < 0.05$). No mortalities were observed in the control group injected with sterile NS.

Table 3. *Dicentrarchus labrax*. Mortality and RPS (relative percent survival; percentage of fish per group that survived) of anti-*Listonella anguillarum* immersion vaccinated sea bass juveniles challenged (IP) 50 d post-prime vaccination. Mortality in replicate groups: number of dead fish per group of 35 fish. *$p < 0.01$

Treatment	Mortality in replicate groups	Average mortality (%)	RPS
Vaccinated challenged	1/35, 2/35, 3/35	6*	92
Unvaccinated challenged	29/35, 24/35, 26/35	75*	–
Unvaccinated NS	0	0	100

Table 4. *Dicentrarchus labrax*. Mortality and RPS (relative percent survival; percentage of fish per group that survived) of anti-*Listonella anguillarum* immersion vaccinated sea bass juveniles challenged (IP) 30 d post-booster vaccination

Treatment	Mortality in replicate groups (n)	Average mortality (%)	RPS
Booster-vaccinated challenged	0/35, 0/35	0[a]	100
Prime-vaccinated challenged	2/35, 3/35	7[b]	84
Unvaccinated challenged	16/35, 15/35	44[c]	–
Unvaccinated NS	0	0	100

[a–c]and[b–c]: $p < 0.01$; [a–b]: $p < 0.05$

Table 5. *Dicentrarchus labrax*. Mortality amongst unvaccinated, prime-vaccinated and booster-vaccinated sea bass juveniles in mixed tank, following injection (IP) challenge with live *Listonella anguillarum*, 30 d post-booster vaccination

Treatment	Mortality per group	Mortality (%)	RPS
Booster-vaccinated	0/35	0[a]	100
Prime-vaccinated	1/35	3[b]	93
Unvaccinated	14/35	40[c]	–

[a–c] and [b–c]: $p < 0.01$; [a–b]: $p < 0.05$

Mixed group challenge

A mixed group challenge was carried out in which unvaccinated and vaccinated fish were contained within the same tank. Survival of fish in the mixed tank were as follows: no mortalities were observed in the booster-vaccinated group (100% RPS) ($p < 0.01$), 3% mortality occurred in the prime-vaccinated group (93% RPS) ($p < 0.01$) and 40% mortality occurred in the unvaccinated control group (Table 5).

Gross pathology

The dead and moribund fish showed signs of external haemorrhaging at the base of the fins, around the anus and in the skin. The opercula were raised and the mouth intensely widened. Internal symptoms observed were haemorrhaging of the peritoneum, intestines and liver, swollen swim bladder and splenomegaly.

Agglutinating antibodies

Plasma antibodies were tested at 60 d post-prime vaccination. Anti-*Listonella anguillarum* antibodies were detected only in vaccinated fish, with agglutinating antibody titres ranging from $\frac{1}{4}$ to $\frac{1}{32}$ (Table 6).

DISCUSSION

In the present study, immersion vaccination of juvenile sea bass was shown to be highly effective at eliciting a protective immune response against *Listonella anguillarum*, the causative agent of classical fish vibriosis. High vaccine efficacy was achieved following single vaccinations (93% RPS), and supplemental booster-vaccinations provided 100% RPS.

Injection is widely accepted as the most effective method of vaccine delivery in fish. The majority of vaccines are delivered in oil-based adjuvants, which slow the release of antigen and result in a prolonged inflammatory response (Evensen et al. 2005). However, injection of oil-based vaccines can induce several side effects, which include the formation of granulomas, inflammation and pigmentation, and can lead to downgrading of fish (Mutoloki et al. 2004, Afonso et al. 2005, Haugland et al. 2005). Injection vaccination is also associated with handling stress and is labour-intensive. Immersion vaccination, on the other hand, requires minimal handling and thus avoids excess stress and labour.

In this study, the uptake of *Listonella anguillarum* antigens by fish following immersion vaccination was demonstrated to be very effective. According to Ototake et al. (1996) the skin and gills are the major sites of antigen uptake after immersion immunisation, and the antigen remains at the site of uptake with only small amounts being transported to the head kidney and the spleen. Activation of complement and induction of specific humoral immunity probably take place to a great degree locally in the gills and mucus. Immersion immunisation induces higher numbers of antibody-secreting cells in the gills, compared to the head kidney, spleen and intestine (dos Santos et al. 2001).

Table 6. *Dicentrarchus labrax*. Agglutinating antibody titres of prime-vaccinated and unvaccinated fish. Blood samples were taken from 10 vaccinated and 10 unvaccinated fish 60 d post-prime vaccination. Agglutinating antibody titres were determined for each of 10 fish group^{-1}

Group	Agglutinating antibody titre									
Unvaccinated	0	0	0	0	0	0	0	0	0	0
Vaccinated	1/32	1/8	1/4	1/16	1/16	1/8	1/8	1/8	1/8	1/16

Vervarcke et al. (2005) demonstrated that immersion immunisation of African catfish *Clarius gariepinus* with *L. anguillarum* O2 antigens induced higher levels of mucosal antibodies than by IP injection, which conversely gives rise to higher levels of circulating antibodies.

Although agglutinating antibody titres measured in this study were low (see Table 6), the protection afforded by the immersion vaccine was very high. This result suggests that the cellular immune response may have played a greater role in inducing immunity following the immersion vaccination. Vervarcke (2004) also showed protection against *Listonella anguillarum* by immersion vaccination, despite low levels of circulating antibodies. These results, and ours, indicate that circulating antibody titres, although a good indication, do not necessarily correlate with protection.

Scapigliati et al. (2002) tested serum antibody levels of sea bass 1 yr after vaccination by immersion with the same commercial vaccine used in this study. The fish were found to have detectable levels of circulating anti-vibrio Ig. In an *in vitro* assay, it was shown that leucocytes from fish that had received the vaccine were able to produce specific antibodies 1 yr later, thus confirming that immersion vaccination induced B-cell memory in sea bass (Scapigliati et al. 2002).

The IP route of pathogen administration was used in our experiment because we have found it to be a more reliable and reproducible method of infection for vaccine efficacy studies. In the natural environment however, *Listonella anguillarum* usually enters into the fish through the mouth, intestine, gills and skin injuries. These natural routes of infection are countered by both passive and active protection of the fish. Active protection is provided by local cellular and humoral immunity, both specific and non-specific. Therefore, fish vaccinated in the field might be expected to show an even higher degree of protection than obtained in our study, due to additional protective local immunity of the natural routes of entry of the pathogen, which are bypassed during the IP injection challenge.

In summary, immersion vaccination of juvenile sea bass with a bivalent *Listonella anguillarum* serotype 01 and 02 vaccine resulted in a high efficacy (93 to 100% RPS). The high level of protection obtained by immersion vaccination in this study is comparable with the protection seen after IP vaccination. Bowden et al. (2002) showed that in Atlantic halibut *Hippoglossus hippoglossus* L., both IP injection and immersion vaccination against *Vibrio anguillarum*, provided effective vaccination routes with near 100% survival in both challenged groups. Although the vaccine MICROViB was developed for salmonids, excellent protection was obtained in sea bass at 2 challenge points following IP injections of *L. anguillarum*.

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