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Field testing of a vaccine against eel diseases caused by *Vibrio vulnificus*

Belén Fouz[1], Maria Dolores Esteve-Gassent[2], Rodolfo Barrera[2], Jens Laurits Larsen[3], Michael Engelbrecht Nielsen[3], Carmen Amaro[1,*]

[1]Departamento de Microbiología y Ecología, Facultad de Biología, Universidad de Valencia, Burjassot 46100, Spain
[2]Valenciana de Acuicultura SA, Valencia 46530, Spain
[3]Section of Fish Diseases, Department of Veterinary Microbiology, KVL, 1870-C Frederiksberg, Denmark

ABSTRACT: The field results of a vaccination programme against *Vibrio vulnificus* serovar E (biotype 2) in a Spanish eel farm are reported. A total of 9.5 million glass eels were vaccinated from January 1998 to March 2000 by prolonged immersion followed by 2 subsequent reimmunisations after 12 to 14 and 24 to 28 d, respectively. The acquired protection and the immune response against serovar E were estimated over a period of 6 mo after vaccination. A similar vaccination schedule was conducted with elvers in a Danish eel farm. In this case, the acquired protection and the immune response against serovar E and the new eel-pathogenic serovars, recently described in Denmark, were evaluated over a short term. The overall results show that the vaccine against *V. vulnificus* serovar E induces a satisfactory protective immunity during the main growth period of eels (around 6 mo) with a relative percentage survival of 62 to 86% and protects them against the new eel-pathogenic serovars. Vaccination of eels by immersion seems to be the best strategy to prevent diseases caused by *V. vulnificus.*

KEY WORDS: *Vibrio vulnificus* serovar E · *Vibrio vulnificus* biotype 2 · Eel vaccines · *Vibrio* vaccines · Vaccination by prolonged immersion · Field vaccination. vibriosis

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INTRODUCTION

Disease caused by the primary pathogen *Vibrio vulnificus* serovar E (formerly biotype 2) is the main cause of mortality in eels cultured in intensive systems, causing important economic losses (Austin & Austin 1993, Biosca 1994, Dalsgaard et al. 1998, Høi 1999). The disease, in its acute form, has even provoked the closure of several farms in Europe and Canada (unpubl. data). The reason is that this serovar comprises highly virulent strains (LD_{50} around 10^2 CFU $fish^{-1}$ by the intraperitoneal route) (Biosca 1994) that can survive and be transmitted through water under favourable physicochemical conditions (Amaro et al. 1995). The pathogen is able to survive in eel blood and spread to the main body organs causing severe tissue damage (Biosca et al. 1993). The surface components specific to this serovar, lipopolysaccharide and capsule, enable cells to survive in serum (Amaro et al. 1994, Amaro et al. 1997), and proteases and haemolysins in extracellular products are responsible for tissue damage (Biosca & Amaro 1996).

Recently, 2 new serovars of the species have been isolated from diseased eel in Denmark (Høi et al. 1998), serovars O3 and O3/O4. According to the serotyping system used by Martin & Siebeling (1991), serovar E corresponds to serovar O4 (Høi et al. 1998). These new serovars produce different signs and seem to be secondary pathogens, since the source of the isolates was eel infected with either parasites or *Pseudomonas anguilliseptica* (Høi et al. 1998).

The efficacy of intensive medication to control the disease due to serovar E seems to be limited. Firstly,

*Corresponding author. E-mail: carmen.amaro@uv.es

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in the acute or sub-acute form of the disease, antibiotic treatments are of limited value. Secondly, resistant strains have been isolated and recurrent outbreaks have been registered after treatment with antibiotics (Biosca et al. 1991, Amaro et al. 1992, Biosca 1994, Høi 1999). Moreover, this practice may constitute a serious hazard to human health since *Vibrio vulnificus* serovar E is an opportunistic human pathogen (Amaro & Biosca 1996) that survives in artificial seawater microcosms for years (Marco-Noales et al. 1999). Under this scenario, application of preventative measures, such as a programmed vaccination schedule, may enhance production with minimal use of antibiotics and associated risks for bacterial resistance. In fact, the success achieved during immunisation programmes against vibriosis caused by *Listonella anguillarum* and *V. ordalii* (Austin 1983, Smith 1988) suggests that an effective vaccine against *V. vulnificus* may be the best solution. We have recently developed and tested several vaccine formulations against *V. vulnificus* serovar E under controlled laboratory conditions (Collado et al. 2000). In that work we showed that (1) capsule and extracellular products were essential protective antigens, and that (2) a triple exposure to the vaccine by prolonged immersion was needed to ensure an acceptable level of protection, with relative percentage survival (RPS) of around 70% (Collado et al. 2000). From these results, we selected a toxoid-enriched bacterin prepared with capsulated cells.

The objective of this study was to develop an effective vaccination programme to be used in eel culture facilities to prevent epizootics caused by *Vibrio vulnificus*. Firstly, we immunised glass eels in a Spanish eel farm during a 2 yr period, and evaluated the immune response and protection in the short and long terms. Secondly, we followed a similar vaccination schedule with elvers in a Danish eel farm and evaluated the immune response and short term protection. In this case, we also tested the cross-protection against other serovars of the species that have been associated with eel diseases (Høi et al. 1998).

MATERIAL AND METHODS

Vaccine preparations. The vaccine was prepared according to the procedure described by Collado et al. (in press). Briefly, cells and extracellular products of strains of Spanish Collection of Type Cultures (CECT) 4604 and CECT 4605, representative of the 2 clones responsible for the epizootics registered in Spain (Biosca 1994), were inactivated by formalin and subsequently heated. The toxoid-enriched bacterin, named Vulnivaccine (licensed by the University of Valencia, Spain; concession number 9701300), contained a final concentration of cells and proteins around 0.5 to 1 logarithmic units less than the original formulation (Collado et al. 2000). The quality-control protocols (sterility, lack of toxicity and absence of remaining enzymatic activities) were those outlined by Collado et al. (2000). We evaluated under controlled laboratory conditions the effectiveness of the vaccine by using 2 groups of 40 elvers (average weight 10 g) exposed 3 times at 12 d intervals for 1 h to a 1:1000 dilution of the vaccine.

Field vaccination. Upon arrival at a Spanish eel farm, glass eels *Anguilla anguilla* (approximately 0.3 g) were vaccinated by the following procedure. After quarantine, batches weighing 100 to 400 kg were vaccinated by immersion for 8 to 10 h with the vaccine diluted at 1:4000 to 1:8000 (Table 1). Water salinity was approximately 0.1% and the temperature was adjusted to 26 ± 1°C. The administration procedure was repeated twice under the same conditions at 12 to 14 d intervals. In the cases marked in Table 1, tanks with approximately 50 kg of non-vaccinated glass eels were maintained for 6 mo as control groups. Subsequently, these fish were also vaccinated and maintained in separate tanks. All groups of fish were fed a commercial diet that contained glucans (Trouvit Perla, Trouw S.A., Burgos, Spain). Fish were starved for 24 h before vaccination. A similar experiment was carried out at a Danish eel farm. In this case, 1 group containing 200 kg of elvers (weight average 2 g) (*A. anguilla*) was vaccinated under similar conditions, while a non-vaccinated group of identical size was used as a control. Water salinity and temperature were approximately 0.3‰ and 26°C, respectively.

Evaluation of immune response and protection. Fish were sampled to determine the degree of protec-

Table 1. Batches of glass eels used in the vaccination trials in Spain. *Arrival date of fish into the culture facilities; ‡Batches in which the immune response and level of protection were evaluated

Year	Date* (mo/d)	Vaccinated fish (kg tank^{-1})
1998	01/07	218‡
	02/03	167
	06/05	191‡
	09/01	192
	09/30	229
	11/20	302
1999	01/26	223‡
	03/30	205
	07/27	168‡
	08/10	148
2000	01/18	412
	03/20	389

tion and the magnitude of specific immune response as follows: (1) in laboratory experiments, 12 d after vaccination; (2) in the Spanish eel farm, 15 d and 1, 2, 4 and 6 mo after vaccination; and (3) in the Danish farm, 12 d after each single vaccine dose. To calculate RPS (1 – [% mortality in vaccinated fish/% mortality in controls] × 100) (Amend 1981), groups of 40 fish from both vaccinated and control groups were challenged with the strain CECT 4604 following the methodology described by Amaro et al. (1995). The infective dose was equivalent to approximately 10 times LD_{50}. To evaluate the immune response, serum and surface mucus samples (pooled from 6 to 8 fish) of vaccinated and unvaccinated fish were tested for antibody titres and bactericidal or bacteriostatic effect. Because of the small size of the eels, serum was sampled only from animals weighing more than 2 g. Antibody titres against whole cells of serovar E (CECT 4604) were determined by an indirect ELISA according to Collado et al. (2000), and bactericidal or bacteriostatic activities were measured as the survival percentage of the same strain in these fluids after 0, 1 and 3 h of incubation according to the procedure described by Amaro et al. (1999). To determine cross-protection against the other serovars, the RPS and the immune response of Danish eels against the strains 95-8-6 of serovar O3 (Høi et al. 1998) and 95-8-161 of serovar O3/O4 (Høi et al. 1998) were evaluated by using the method described above.

Evaluation of protection against vibriosis in the field. When mortality due to *Vibrio vulnificus* in the experimental tanks was suspected, moribund fish were subjected to laboratory investigations to confirm the diagnosis. Moribund fish were necropsied and bacteriologically analysed according to Amaro et al. (1992). Bacterial strains were identified using the API20E (Biomerieux, France) system and this was confirmed serologically with sera against serovar E (Amaro et al. 1992). Both vaccinated and control groups of animals were given medication to avoid economic losses.

Finally, the presence of *Vibrio vulnificus* serovar E cells in water from affected tanks was monitored by indirect immunofluorescence. Volumes of 1 l of water were fixed with 2% formalin (v/v), pre-filtered for debris elimination and filtered onto black 0.2 µm pore sized polycarbonate filters (Millipore Corp., Bedford, MA, USA). Immune staining with antibodies against strain CECT 4604 was as described by Marco-Noales et al. (2000).

RESULTS

In laboratory experiments performed with Vulnivaccine, RPS after bath challenge with serovar E strain CECT 4604 was around 85% (Table 2). The antibody response against serovar E was moderate or poor, with mean antibody titres of 200 and 16 in sera and mucus, respectively (Table 2). A bactericidal effect was detected in mucus but not in serum (Table 2).

Afterwards, a vaccination programme of glass eels was established in a Spanish farm from 1998 to 2000 (Table 1). As shown in Table 3, RPS ranged from 62 to 86% (Table 3). The immune response was evaluated in parallel. As glass eels were small, serum was not sampled to measure its bactericidal activity or specific antibody titres. As expected, strain CECT 4604 was able to survive, and even multiply, in samples of mucus from

Table 2. Effectiveness of Vulnivaccine under laboratory conditions. *Vaccine was licensed under the name Vulnivaccine; †Mean ± SD (n = 3) of relative percentage of survival (RPS) between vaccinated and unvaccinated fish challenged by bath with strain CECT 4604 (Amaro et al. 1995); ‡Antibody titres against bacterial strain CECT 4604 were determined by ELISA (Collado et al. 2000); §Bactericidal and bacteriostatic activities were measured as the percentage survival of strain CECT 4604 after 3 h incubation in mucus or serum. +: survival percentage <50%; –: survival percentage >100%

Vaccine	RPS† (%)	Antibody titre in‡		Bactericidal activity in§	
		serum	surface mucus	serum	surface mucus
Vulnivaccine*	84.6 ± 5.2	200	16	–	+
Control	–	<50	<2	–	–

Table 3. Immune response and level of protection obtained in glass eels vaccinated by prolonged immersion in a Spanish farm. *Mean ± SD of weight of vaccinated fish; †RPS between vaccinated and unvaccinated fish challenged by bath with strain CECT 4604 (Amaro et al. 1995); ‡Antibody titres against bacterial strain CECT 4604 were determined by ELISA according to Collado et al. (2000); §Bactericidal activity was measured as the percentage survival of strain CECT 4604 after 3 h incubation in surface mucus. +: 60 to 90; ++: 30 to 60; +++: 5 to 30

Post-vaccination time (mo)	Avg weight of fish (g)*	RPS†(%)	Antibody titre in mucus‡	Bactericidal activity in mucus§
0.5	0.34 ± 0.005	82 ± 5	4	+
1	0.35 ± 0.038	72 ± 4	4	+
2	0.44 ± 0.066	70 ± 8	4	++
4	1.03 ± 0.30	75 ± 4	8–16	++
6	1.98 ± 1.017	80 ± 6	16	+++

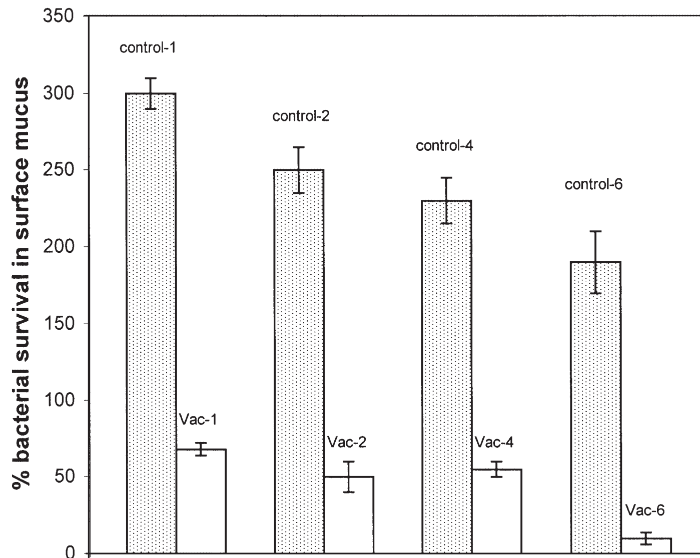

Fig. 1. Survival of strain CECT 4604 of *Vibrio vulnificus* serovar E (O4) after 3 h incubation in surface mucus obtained from glass eels immunised (Vac) or not (control) with Vulnivaccine by prolonged immersion in a Spanish farm. Samples were taken 1, 2, 4 and 6 mo after vaccination. Mean values ± SD (n = 3) are shown

unvaccinated glass eels (Fig. 1). However, the growth of this strain was inhibited by mucus from vaccinated glass eels, reaching the strongest inhibitory effects 6 mo after vaccination (Table 3, Fig. 1). The antibody response was poor, with titres ranging from 4 to 16, with the highest values detected 5 to 6 mo after vaccination (Table 3).

Two outbreaks due to *Vibrio vulnificus* serovar E were registered in the Spanish farm during the study period: in July 1998 2 weeks after an increase in water temperature (up to 30°C) and nitrite concentrations, and in December 1999 after an increase in nitrite and pH (from 5.5 to 7). The first outbreak affected only non-vaccinated fish and was controlled by treatment with oxolinic acid in water (8 ppm for 5 d followed by 2.5 ppm for 10 d). The second episode affected fish vaccinated in 1998 but not those vaccinated in 1999. The problem was controlled by 3 baths with tetracycline (25 ppm) at intervals of 4 to 5 d. In both cases, water salinity was around 0.15 to 0.2% and *V. vulnificus* serovar E was not detected in water by immunofluorescence before antibiotic treatments. We could not compare mortality rates between vaccinated and control groups because both were medicated.

In the vaccination experiments performed in Denmark, elvers were also well protected against *Vibrio vulnificus* serovar E, showing RPS around 85% after 12 d of the vaccination schedule (Table 4). The mucus antibody titres were similar to those observed in mucus of glass eels and was also bactericidal (Table 4). In this case, the immune response in serum from vaccinated fish could be analysed because elvers weighed more than 2 g, and we found a clear bactericidal effect together with high antibody titres against serovar E (Table 4).

The cross-protection of vaccinated elvers against serovars O3 and O3/O4, recently associated with eel disease, was also evaluated in Denmark. None of the new serovars was virulent by bath immersion (LD_{50} higher than 10^8 CFU ml^{-1}), so that we could not calculate the RPS. However, when mucus and serum were tested, they showed bacteriostatic or bactericidal effects against both serovars (Figs 2 & 3).

DISCUSSION

The laboratory vaccination assays showed that Vulnivaccine was effective in protecting elvers against *Vibrio vulnificus* serovar E. The RPS values were higher than those previously obtained with other toxoid-enriched bacterins (Collado et al. 2000). Since the main modification in the formulation of Vulnivaccine was the reduction in concentration of both cells and

Table 4. Immune response and level of protection obtained in the short term in elvers vaccinated by prolonged immersion in a Danish eel farm. *Mean ± SD of the weight of vaccinated fish; †Mean ± SD of the RPS between vaccinated and unvaccinated fish challenged by bath with strain CECT 4604 (Amaro et al. 1995); ‡Mean ± SD of antibody titres against bacterial strain CECT 4604 (determined by ELISA); §Bactericidal activity was measured as the percentage survival of strain CECT 4604 after 3 h incubation. +: 80 to 90; ++: 40 to 80; +++: 5 to 40. ND: not done

Time after first vaccination (d)	Average weight of eels (g)*	RPS† (%)	Antibody titres in‡		Bactericidal activity in§	
			serum	mucus	serum	mucus
0	1.90 ± 0.85	ND	800 ± 200	ND	ND	ND
12	2.09 ± 0.95	ND	5700 ± 1500	ND	ND	ND
24	2.39 ± 0.81	ND	7000 ± 800	ND	ND	ND
36	2.93 ± 1.20	83 ± 4	9300 ± 1000	3 ± 1	+++	+

toxoids, lower concentrations of antigens seem to improve the immune response in eel. In agreement with the results obtained by Collado et al. (2000) we detected a bactericidal effect in mucus of immersion-vaccinated elvers but not in serum. Thus, the bactericidal effect of mucus prevented disease in 1 of the first steps, probably the colonisation.

When experiments with Vulnivaccine were carried out with both glass eels and elvers in fish farms, vaccinated fish were well protected against *Vibrio vulnificus* serovar E, showing RPS close to those found in elvers vaccinated in the laboratory. In the case of glass eel, the protective effect lasted at least 6 mo after vaccination. A similar degree of protection has also been reported in Baltic salmon smolts, 4 mo after immersion vaccination with a triple bacterin containing formalin-killed *Listonella anguillarum*, *Aeromonas salmonicida* and *Yersinia ruckeri* (Buchmann et al. 1997). The protection conferred by Vulnivaccine can be extended to the new pathogenic serovars since we detected a bactericidal effect in mucus and serum of farmed vaccinated fish against serovars O3 and O3/O4. The immune response against serovar E in the short term in mucus from glass eels and elvers was similar to that found in elvers vaccinated in the laboratory, since they showed low antibody concentrations and a bactericidal effect. This effect persisted in glass eels, reaching the highest level 6 mo after vaccination. Similar titres have been obtained by other authors, who rarely detected specific antibodies in skin mucus from immunised fish (Cobb et al. 1998). The immune response in serum from glass eel could not be evaluated because their small size made sampling difficult. When field trials were carried out with elvers, we sampled serum and detected a clear bactericidal effect and high antibody titres against serovar E. Compared with the results obtained in laboratory vaccination, the specific immune response in serum was higher. One of the reasons may be that the feed intake under laboratory conditions is much lower due to the stress associated with the transportation and adaptation to the new maintenance conditions (unpubl. obs.). This result suggests that the feed is very important to the development of an optimal immune status in eel.

The bactericidal effect detected in vaccinated fish in farms was directly related to high titres of specific antibodies in serum but not in mucus. Perhaps low concentrations of antibodies are sufficient to activate the complement in mucus or, more probably, we underestimated antibody titres because in our ELISA we use anti-eel serum immunoglobulin as a secondary antibody. In any case, our results support the important role of antibodies in the defence of eel against *Vibrio vulnificus* serovar E. In fact, circulating antibodies have been established as the principal protective immune response against extracellular bacteria in higher vertebrates (Abbas et al. 1991). These antibodies may also be present in mucus and act as a primary line of defence against *V. vulnificus*, as has been suggested for other pathogens (Rombout et al. 1986, Austin & McIntosh 1988, Itami 1993).

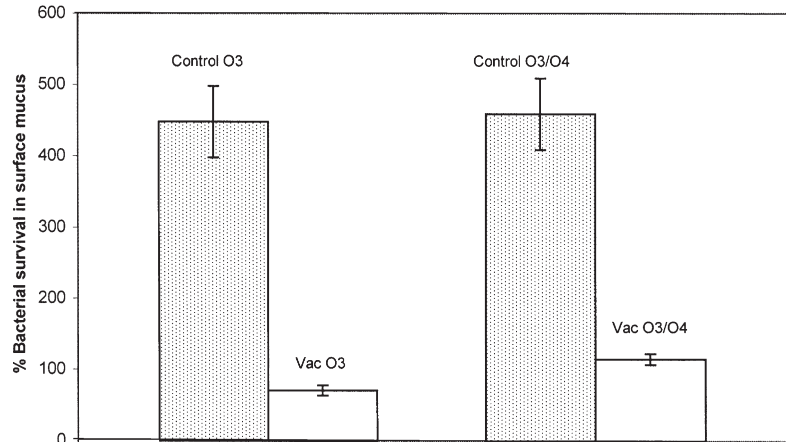

Fig. 2. Survival of strains 95-8-6 serovar O3 and 95-8-161 serovar O3/O4 of *Vibrio vulnificus* after 3 h incubation in surface mucus obtained from elvers immunised (Vac) or not (Control) with Vulnivaccine by prolonged immersion in a Danish farm. Mean values ± SD (n = 3) are shown

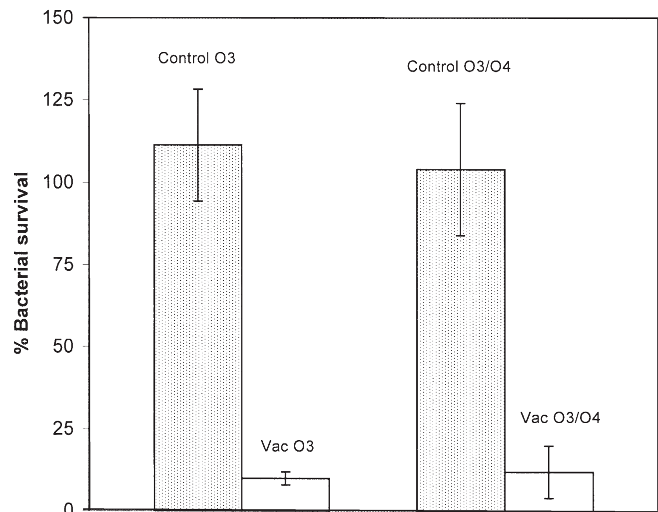

Fig. 3. Survival of strains 95-8-6 serovar O3 and 95-8-161 serovar O3/O4 of *Vibrio vulnificus* after 3 h incubation in serum obtained from elvers immunised (Vac) or not (Control) with Vulnivaccine by prolonged immersion in a Danish farm. Mean values ± SD (n = 3) are shown

Finally, 2 outbreaks due to *Vibrio vulnificus* serovar E were registered in the Spanish farm during the study period. Although we could not compare mortality rates between vaccinated and control groups, it should be emphasised that the disease did not affect the animals vaccinated within the previous 6 mo. These results were quite satisfactory and confirmed that Vulnivaccine effectively protected eels against natural disease due to *V. vulnificus* serovar E for at least 6 mo following vaccination. The pathogen was not detected in water by immunofluorescence during the outbreaks. This result may indicate that the bacterium was not present in water, as has already been suggested by other authors (Arias 1998) who did not succeed in isolating it from water tanks during outbreaks. In theory, the salinity (0.15 to 0.2%) was too low to favour the survival and the spread of *V. vulnificus* (Amaro et al. 1995), and the disease may have been transmitted by direct contact between animals. However, it is also possible that the bacterium was present in low numbers or associated with particulate material. In this case, the isolation methods would need to be modified to isolate the bacterium.

In summary, our results show that Vulnivaccine, delivered by prolonged immersion, induces a protective response to *Vibrio vulnificus* serovar E (serovar O4) in vaccinated glass eels and elvers on a large scale. This vaccine may also protect against serovars O3 and O3/O4. As the vaccine induces protection in fish during the main growth period, vaccination of glass eels by immersion on delivery to the culture facilities may be the best strategy to prevent *V. vulnificus* diseases. Work on eel protection by oral vaccination is in progress.

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