Vaccination trials of sea bass (*Dicentrarchus labrax*) against pasteurellosis using oral, intraperitoneal and immersion methods

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Summary

Photobacterium damsela subsp. *piscicida* (Phdp) is the aetiological agent of fish Pasteurellosis, causing heavy losses in intensive mariculture plants. The present work compares the protective efficiency of five different vaccine formulation: oral, intraperitoneal, immersion, bivalent immersion (*Vibrio anguillarum*) and immersion associated with immunostimulant. Each of these vaccine formulation, containing whole cells of Phdp formalin inactivated (FKC), was administered to 100 sea-bass weighing approximately 2 g; 100 non vaccinated sea-bass were used as control. The protection against pasteurellosis was tested for 40 days after vaccination with an intraperitoneal challenge: each fish was inoculated with Phdp cells at a concentration of 2.75 x 10^4 CFU / ml. Mortality was registered in the following 14 days, vaccine protection was evaluated using a Relative Percentage Survival (RPS) index. The intraperitoneal formulation gave an excellent protection (RPS 82.4%). The most effective immersion forms was that followed by the simple immersion (RPS 23.1%) to end with the group vaccinated with the bivalent vaccine (RPS 18.7%). The protection conferred by the oral form (RPS 28.6%) is interesting for its practicality.

Keywords

Fish Pasteurellosis - *Photobacterium damsela* subsp. *piscicida* - Sea bass - Vaccine.

Introduction

Fish Pasteurellosis is a septicaemic disease. The causative agent is *Photobacterium damsela* subsp. *piscicida* (Phpd), an asporigen, gram negative coccobacillus with a bipolar colouring, both aerobic and anaerobic (8).

This disease caused heavy losses in Japanese and USA fish farms since the 1960s. The first European outbreaks were reported in the 1990s (4). This disease hits every eurialin fish species, both wild and farmed, causing heavy death rates especially in intensive fish farms and in the smallest fish.

Owing to its considerable economic impact, it has been paramount to define a control strategy against this disease, mainly based on a vaccine prophylaxis. The frequent use of chemotherapy has created drug resistance and residues in the environment. Moreover, the costly therapies with antibiotics are not always successful because of the rapid loss of appetite in the infected fish and the limited number of authorised drugs.

This paper describes the vaccination trials performed against pasteurellosis using different methods and formulations to compare their protective efficiency.

Materials and methods

Yeast strain

The vaccine was prepared with a Phdp 249/I99 field strain, isolated in Italy in sea-bass during a Pasteurellosis outbreak in the summer of 1999 Zooprophylactic Institute of North Eastern Italy,

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Padua. The first isolated strain was preserved in cryobeads at - 80°C. The same Phdp 249/I99 strain, revirulented with four consecutive *in vivo* passages on sea-bass, was also used in the experimental infection (challenge).

Fish and experimental farm

Sea-bass *(Dicentrarchus labrax)* of average weigh 2 of g, stocked in the Torre Cerrano plant of the Zooprophylactic Institute of the Abruzzo & Molise regions, were used for the experimental trials. The plant consists in 12 cylindrical 100 liters insulated tanks, filled with sea water filtered by 25, 10 and 1μ cartridge filters. The fish was acclimatised for two weeks before the experiment beginning.

Every tank, each with an internal aerator, received a water flow of 0.25 l/min during the day. The levels of oxygen, temperature and nitrites were monitored twice a day. The average temperature of unconditioned water was of 23.4° C (min 19.9° C; max 25.8° C), while the average oxygen concentration was 5.6 ppm (min 5.1 ppm; max 6.5 ppm).

The fish were fed twice a day with a commercial pellet feed, administered at a quantity of 3% of their BW/day. Feeding stopped the day prior to vaccination and the challenge.

The sea-bass average weight was 10 g at the end of the experiment.

Vaccine preparation

The here reported protocol was followed to prepare the vaccinal antigen, composed of Phdp whole cells inactivated with formalin (FKC): Phdp 249/I99 field strain was cultivated in Tryptone Soya Broth (TSB, Oxford) at 2% NaCl at the temperature of 25°C. Growth was monitored by the means of a specrophotometer at 610 nm until it reached the value of optical density A_{610} nm≅0.8, corresponding to a concentration of approximately $1x10^8$ CFU/ml, according to preceding trials. Later the culture was inactivated by adding formaldehyde at the final concentration of 0.5% v/v and kept under magnetical stirring for approximately 3 hours at room temperature and then at 4° C overnight.

Subsequently, the culture was centrifuged at 12304 x g for 30 minutes at 4° C with a SORVALLRC 5C PLUS machine reovering the pellet after a double washing in PBS (pH 7.4 ± 0.2).

The number of bacteria present in the vaccinal solution was determined by counting the CFUs on plate, making 10 fold dilutions starting from 100 ml of the pre-inactivation broth culture and 50 ml inoculating on Tryptone Soya Agar (TSA Oxoid) medium plates at 2% NaCl with a spatula.

Oral vaccine

The FKC produced as described above were lyophilised (final weight 850 mg) and microincapsulated in calcium alginate microspheres coated by chitosan, obtaining a final quantity of 9.5 g of microincapsulated material containing 2.14×10^{10} FKC.

The microspheres (MSs) were prepared according to the procedures described in the WO 2005/013941 (International Patent). Briefly the MSs were prepared using the emulsion technique with 4% w/v of alginate (Protan-Biopolymers, Drammen, Norway), 0.1% w/v chitosan CL 213 (Pronova Biomedical, Oslo, Norway), 15% w/v $CaCl_2$, 20% lysozyme (LZ) (hen-egg white lysozyme chloride, SPA Milan, Italy) and 10% w/v of heat-inactivated Phdp.

Shape, mean diameter and swelling degree were determined thanks to an image analysis technique with a light microscope (Olympus BH-2, GMI, Inc., Ramsey, Minnesota, USA) equipped with a camera (CCD Camera ICD-42E; Ikegami Tsushinki Co, Japan) by means of specifically developed software (Optomax V Software, Cambridge, MA, USA). In all measurements, at least 3,000 particles were examined. Z potential (parameter that can

influence both particle stability and mucoadhesive property) was determined by photon-correlation spectroscopy (Zetasizer 3000 HSA, Malvern Instruments). Phdp loading was determined by fluorimetric techniques after FITC labelling.

Image analysis showed the MSs to have, approximately, a spherical shape with a dry diameter and swelling degree of 2.9 ± 1.1 μm and 3.5 ± 1.4 μm respectively. The Z potential was 2.1 ± 0.5. The encapsulation efficiency of Phdp was 40%.

Vaccine by immersion

The FKC pellet was resuspended in 500 ml of PBS, obtaining a final concentration of 1 x 10^9 cfu/ml, administered as described below.

Intraperitoneal vaccine

The intraperitoneal formulation was made of FKC and extracellular products (ECP). The ECP were obtained recovering the surnatant [of the bacterial culture inactivated with 0.5% formalin, sterilised by filtration through membranes with 0.45 μm pores (Millipore)] centrifuged at 12304 x g for 30 minutes at 4° C . Formalin was then neutralised by adding a solution of sodium metabisulphite 15% at a quantity of 10 ml/l of surnatant, incubating overnight at room temperature.

CPE present in the surnatant were evaluated quantifying the proteins according to the Bradford method (3), using scalar concentrations of bovine serum albumin as standard proteins (Sigma, St Louis, USA).

The intraperitoneal formulation contained 7.57 x 10^7 FKC/ml and 0.035 μg proteins/ml. To complete the preparation of the vaccine the adjuvant (Montanide, Seppic) was added at a ratio of 70:30 adiuvant:vaccine.

Vaccine administration

Ten groups of 50 sea-bass were vaccinated, while 2 groups of 50 non-vaccinated sea-bass were used as control. Each of the 5 different vaccinal forms (oral, intraperitoneal, immersion, bivalent immersion and immersion/immunostimulant) was then administered to 100 sea bass.

Oral vaccination

The oral vaccine was administered together with feed at a quantity of 8.6 mg MS/fish/day, for 5 consecutive days followed by a five day break and then by a second administration for 5 more consecutive days. Each fish received a total of 1.8 x 10^9 FKC.

Vaccine by immersion

Two vaccinal formulations by immersion were prepared:

1) Monovalent immersion

The previously described suspension of FKC in PBS was diluted 1:10 in sea water, at a final concentration of 1 x 10^8 CFU/ml. It was administered to 2 groups of 100 sea-bass. One of the groups also received a commercial immunostimulant based on seaweed extracts (Ergosan Schering-Plough), administered orally with a proportion of 0.5% of the feed for 7 consecutive days followed by a 7 day break and then by a second administration for 7 more consecutive days.

2) Bivalent immersion (Phdp + *Vibrio anguillarum*)

The Phdp suspension of FKC was mixed to the same quantity of a commercial anti *Vibrio anguillarum* vaccine (Schering-Plough) and then diluted 1:10 in sea water. The resulting vaccinal solution contained 5 x 10^7 FKC Phdp/ml + 5 x 10^7 FKC *Vibrio anguillarum*/ml.

The fish were immersed in the vaccinal solutions for 60 minutes with a ratio of 6 fish/litre of vaccine in constant aeration. Then they were cleaned of the non adhese bacterial particles with sea water and transferred to the farming tanks.

Intraperitoneal vaccination

The fish were first anaesthetised by bath with MS-

222 (Sigma, St. Louis, USA), diluted in sea water at the concentration of 5 ppm. Then they were inoculated intraperitoneally with 50 µl of the previously described formulation. Each fish received 3.79 x 10^6 FKC and 0.00177 µg of CPE.

Evaluation of vaccinal protection

Forty days after vaccination, the vaccinated groups and the control groups were challenged to evaluate the protection granted by the different vaccinal formulations. The experimental infection was performed with the intraperitoneal inoculation of 100 µl of solution in sterile PBS of Phdp bacterial cells at the concentration of 2.75 x 10^4 UFC/ml. Phdp used was the 249/I99 field strain revirulented with four *in vivo* passages on sea bass: a sea bass was inoculated with 100 µl of a Phdp 249/I99 bacterial suspension in TSB at 2% of NaCl at a concentration of 1.78 x 10^8 CFU/ml diluted 1:10 in PBS. When the fish died, its spleen was sterilely removed, then placed on a Petri plate and minced in PSB; 100 µl of this mixture were inoculated intraperitoneally in a second sea bass. When the second sea bass died, the same revirulentation passage was performed from the spleen to a third fish and then to a fourth. When the fourth fish died, its spleen was aseptically removed and minced in sterile PBS. Then it was sowed on a plate of blood agar at 2% of NaCl and incubated at 25°C. The Phdp pure colonies isolated were conserved in cryobeads at - 20° C until the experimental infection, when a microbead of the revirulented strain was added in 10 ml of TSB at 2% of NaCl and incubated at 25°C. Twenty-eight hours later, when the value of O. D. A610nm = 1, corresponding to the concentration of 3.6 x 10^8 UFC/ml, determined by counting on the plate on TSA at 2% NaCl, was reached, the culture was diluted 1:10,000 in PBS.

The mortality rate was observed for the 14 days following the experimental infection. Vaccinal protection was monstred using the Relative Percentage Survival index (RPS) calculated as described by Amend (1):

$$RPS = \frac{1-(\%\ \text{mortality rate in the vaccinated})}{\%\ \text{mortality rate in the non vaccinated}} \times 100$$

The distribution of overall mortality was calculated in every group to evaluate the significance of the differences in mortality between the various groups. The Bayesian approach was used employing Beta distribution (s+1; n-s+1) where s=number of dead fish and n=total number of fish used for the experiment.

The confidence intervals were calculated at 95% for each distribution.

Results

Phdp was isolated from sea bass in all the experimental infected groups agar blood at 2% of NaCl from inoculating spleen and anterior kidney. The identification was confirmed through seroagglutination with specific antiserum (anti Ig sea bass serum produced on rabbit, IZS of North Eastern Italy) and biochemical identification (API 20E Biomerieux).

The mortality caused by the experimental infection started 48 hours after the inoculation and finished on the 12th day (Figure 1).

The mortality in the control groups 14 days after the experimental infection was of 91%.

The average mortality in the vaccinated sea bass was of 58.4%, corresponding to a RPS of 35.84%.

Mortality in the groups vaccinated by simple immersion, bivalent immersion and immersion with immunostimulant was corresponding 70%, 74% and 67% respectively (Table I); the RPS amount was of 23.1%, 18.7% and 26.4%, respectively.

Mortality in the groups vaccinated by intraperitoneal

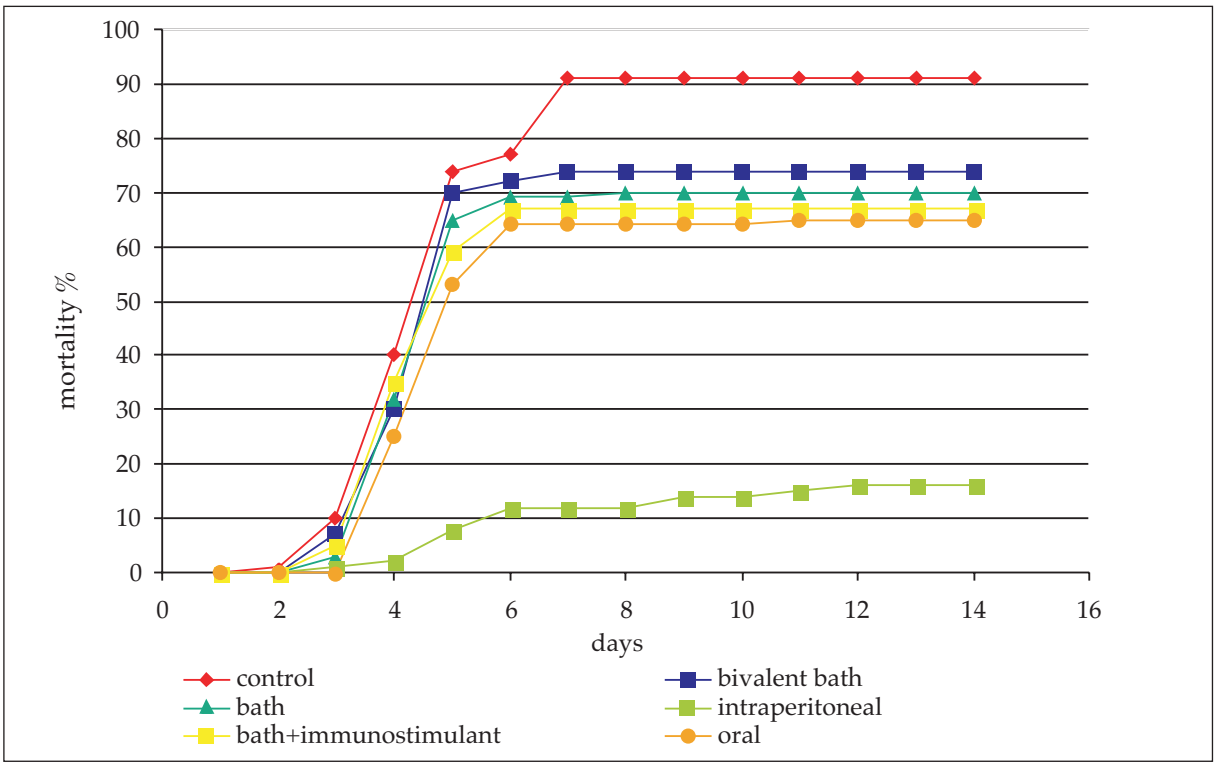

Figure 1
Mortality post-challenge

vaccination was 16%, resulting in a RPS of 82.4%, while mortality in the orally vaccinated group was 65% with a RPS of 28.6%.

Mortality in the control groups was statistically higher than mortality in each vaccinated group ($p<0.05$) (Figure 2). If we compare the different vaccinal formulations, only the protection granted by the intraperitoneal vaccination is significantly higher than the other vaccinal forms, whose RPS values are equivalent.

Discussion

Our results agree with those reported by Fabris *et al.* (7) who, in intraperitoneal vaccination trials with FKC + ECP on larger sea bass (15-20 g) obtained an excellent protection (RPS 87.5%).

Bakopoulos *et al.* (2) did not reach any protection when the vaccine was tested with an experimental intraperitoneal infection. Gravningen *et al.*1998 report that a trial performed with a commercial immersion bivalent vaccine (*V. anguillarum* and Phdp) obtained a RPS of 73.68 (9).

The high protection granted by the ECP was also described in previous studies (13, 14). The ECP are toxic, their 72 hours LC50 is 1.8 mg of proteins/g BW when administered by intraperitoneal route to a turbot (*Scophtalmus maximus*), while a citotoxical effect can already be registered at the concentrations of 0.9 µg of proteins/mg BW (13).

In our study the bivalent vaccination with *Vibrio anguillarum* did not show any better results than the other immersion vaccination techniques. Therefore the cross protection provided by *V. anguillarum* against other aethiological agents, *Aeromonas salmonicida* (15) and *Streptococcus* sp. (18) was not remarkable against *Photobacterium damsela* subsp. *piscicida*, as confirmed by other studies (11).

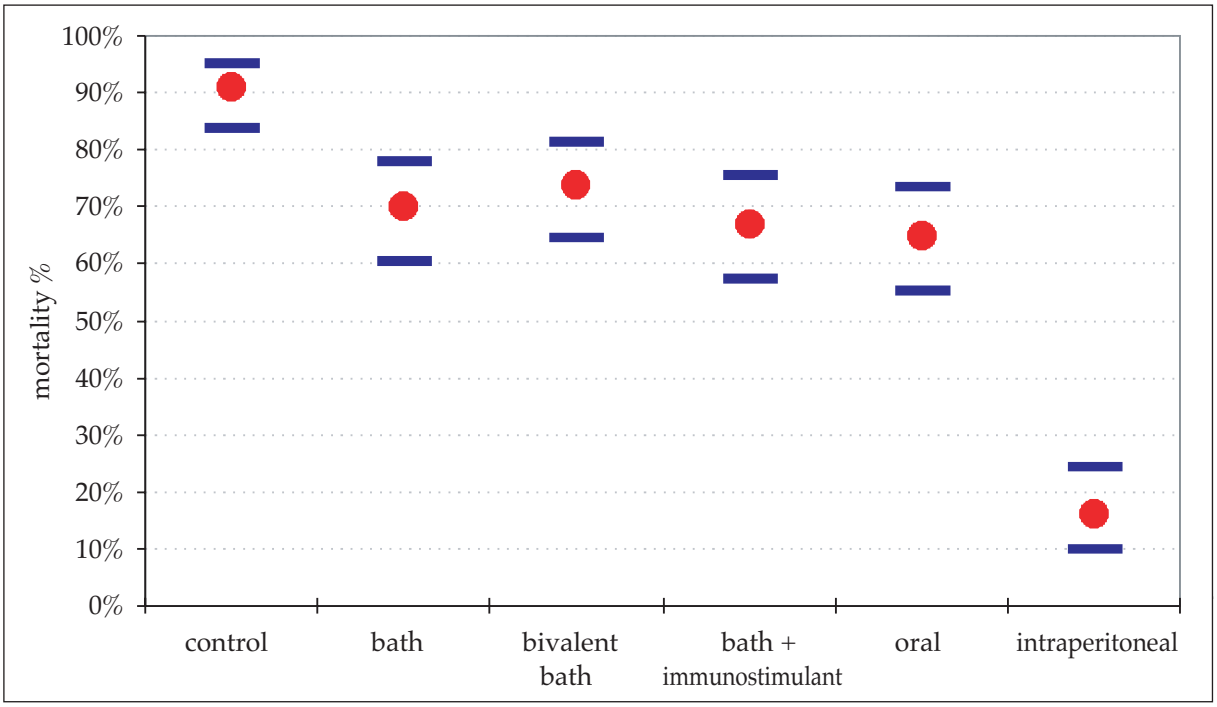

Figure 2
Mortality percentage and confidence limits

Table I
RPS, mortality and confidence limits

Groups	RPS%	Mortality %	l.c.l.	u.c.l.
Control	-	91	83.8	95.1
Bath	23.1	70	60.4	78.1
Bivalent bath	18.7	74	64.6	81.6
Bath + immunostimulant	26.4	67	57.3	75.4
Oral	28.6	65	55.2	73.6
Intraperitoneal	82.4	16	10.1	24.4

l.c.l. = lower confidence limit; u.c.l. = upper confidence limit.

The oral formulation gave good protection (RPS 28.6%), thanks both to the small dimensions of the granules (5) and to the chitosan covering of the antigen which, as other authors specify (10, 16, 17) protects against the inactivation of the antigen operated by digestion. It is an encouraging result, especially if we consider that the vaccine was administered naturally, i.e. with feed. It is the first time this result has been obtained, aside from studies where the antigen was administered by oral or anal intubation (2, 6, 12). This technique makes it easier to uptake the antigen, but it cannot be practised in the farms. The oral formulation obtained therefore a good result, slightly better

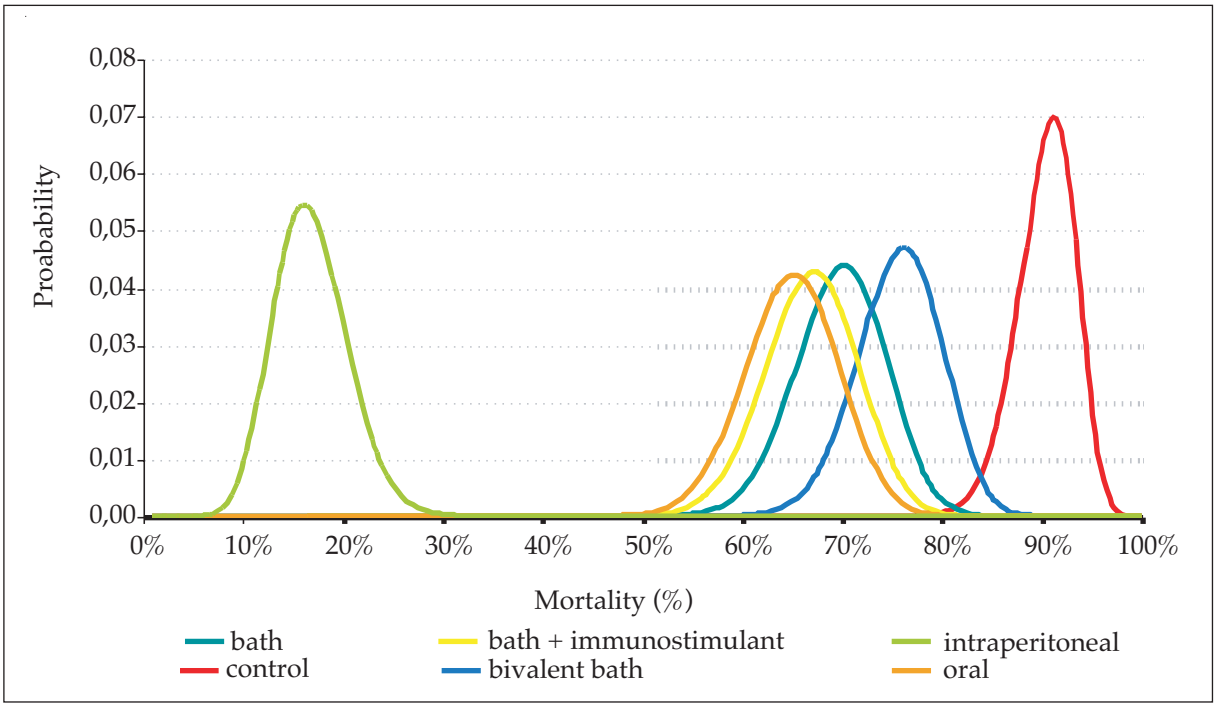

Figure 3
Beta distribution of mortality percentage

than the other formulations for immersion. We are therefore encouraged to further our studies to evaluate the absorption and validity of different dosages and less expensive encapsulation techniques. Oral vaccination is preferable than other administration techniques, because it is not at all stressfull for the animals and because it allow mass prophylaxis without further labour costs.

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