Short communication

Immune response of gilt-head seabream (Sparus aurata) to antigens from Photobacterium damselae subsp. piscicida

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Photobacterium damselae subsp. piscicida (formerly Pasteurella piscicida) is the causative agent of pseudotuberculosis (also referred to as pasteurellosis), which is one of the most important diseases affecting the culture of gilt-head seabream (Sparus aurata) in the Mediterranean countries [1]. Several studies have reported the protection against Ph. damselae subsp. piscicida by different designs of vaccines in cultured fish [2–5]. The ability of fish vaccines to induce a protective immunity is for the most part based on experimental challenge studies and/or field experiments, but the laboratory research focused on determining which antigens are the most immunogenic in fish is very scarce [6], despite this objective it is critical to identify the individual components involved in inducing protection. This kind of data could be very useful in order to develop subunit vaccines. In this way, different studies showed that vaccines consisting of immunogenic fractions can induce higher protection than inactivated whole cell bacteria in fish [7]. Measures of the antibody response have been used to help describe the impact of physiological changes, environmental conditions and vaccination [8,9]. Antibodies in the circulation constitute an important part of the resistance against pathogenic microorganisms [10].

The objective of this study is to identify the immunodominant antigens of Ph. damselae subsp. piscicida by evaluating the antigenic specificity of the humoral response in gilt-head seabream vaccinated with a bacterin in oil adjuvant, and the potential mitogenic effect of different bacterial antigens on the leucocytes of this fish.

Gilt-head seabream of 100 g body weight were maintained in 100 l tanks of seawater at 20 °C with aeration. Twenty of these fish were immunised by intraperitoneal injection (i.p.) with 10^6 formalin-killed cells of Ph. damselae subsp. piscicida Pp8 suspended in 0.1 ml of phosphate-buffered saline (PBS) and emulsified in Freund’s incomplete adjuvant (FIA). This strain, virulent for this fish (LD_{50} for gilt-head seabream was 1.8 × 10^5 cfu g^{-1}), was isolated in our laboratory from diseased gilt-head seabream and was grown on Brain Heart Infusion Agar (OXOID) supplemented with 2% (w/v) NaCl (BHIAS) at 22 °C.

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Control fish were inoculated with PBS in FIA. Four weeks after the immunisation a group of ten vaccinated fish received a booster injection (i.p.) with \(10^6\) formalin-killed cells of \textit{Ph. damselae} subsp. \textit{piscicida} Pp8 suspended in 0.1 ml of PBS and emulsified in FIA.

Four once vaccinated, four twice vaccinated and six unvaccinated fish were anaesthetised with MS-222 and peripheral blood was sampled by caudal puncture four weeks after the first inoculation and after the booster injection. Sera from the specimens were obtained by allowing the blood to clot for 2 h at room temperature and centrifuged at 500 \(\times\) g for 30 min at 4 °C. Sera were collected and stored as single samples at \(-20\) °C until assayed (within one month). Erythrocyte-free leucocyte suspensions from blood of the unvaccinated fish were prepared by centrifugation at 500 \(\times\) g for 30 min at 4 °C using Percoll (Pharmacia) density gradient 40%. The leucocytes were collected at the interface layer, washed three times and suspended in RPMI 1640 medium (Gibco) supplemented with 100 IU penicillin ml\(^{-1}\), 100 µg streptomycin ml\(^{-1}\), 10 IU heparin ml\(^{-1}\) and 2% foetal calf serum (FCS). Leucocyte viability was determined by trypan blue exclusion test.

The extracellular products (ECPs) of \textit{Ph. damselae} subsp. \textit{piscicida} were obtained using the cellophane technique described by Liu [11]. Briefly, a sterilised cellophane sheet was placed on the surface of a BHIA plate and inoculated by spreading 0.5 ml of a 36–48 h old broth culture of the strain with a sterile swab. The plate were incubated at 22 °C for 48 h. After incubation bacterial cells were harvested off the cellophane with a minimum volume of PBS, and the cell suspension was centrifuged at 13,000 \(\times\) g for 20 min at 4 °C. The supernatant were filtered through a 0.45 µm pore size membrane filter (Millipores) and used as crude ECPs. The total protein concentration was determined following the Bradford method [12], using bovine serum albumin (Sigma) as the standard.

Outer (OM) and cytoplasmic membrane (CM) preparations were obtained following the techniques described by Al-Harbi and Austin [13]. Briefly, the cells were washed three times in PBS, and suspended in 10 mM Tris–HCl buffer (pH 8.0) containing 0.3% NaCl. The cells were disrupted by sonication (ten pulses of 30 s at 50 W). Cell debris was removed by centrifugation at 100,000 \(\times\) g for 1 h at 4 °C. CM was solubilised selectively with sodium lauryl sarcosinate (Sigma) by the method of Filip et al. [14]. OM was sedimented by centrifugation at 100,000 \(\times\) g for 1 h at 4 °C and the supernatant (containing CM) dialysed against distilled water for 24 h at 4 °C with 6–8 kDa molecular weight cut-off membrane (Spectrum) and stored at \(-20\) °C. OM was washed three times in PBS and stored at \(-20\) °C.

Lipopolysaccharide (LPS) preparations were obtained following the methodology described by Chandan et al. [15]. Ten ml of the cell suspension (\(10^9\) cfu ml\(^{-1}\)) in PBS were mixed with 0.1 ml of 0.5 M ethylenediaminetetraacetate (EDTA) in PBS (pH 7.2). The mixture was autoclaved at 121 °C for 10 min, cooled to room temperature and centrifuged for 10 min at 10,000 \(\times\) g. The supernatant was dialysed against distilled water using a Spectrum/Per membrane (molecular weight cut-off 6–8 kDa) (Spectrum) and freeze-dried. The extract was suspended in 1 ml of H\(_2\)O for chemical analyses. The concentrations of LPS were determined following the technique described by Keller and Nowotny [16] using \textit{Escherichia coli} LPS as the standard.

O-antigens (Ag-O) were obtained following the technique described by Edwards and Ewing [17]. Microbial cells were pelleted by centrifugation at 1600 \(\times\) g for 25 min at 4 °C, suspended at 10% (v/v) in PBS, heat-killed by placing in a boiling water bath for 60 min and then twice centrifuged at 8000 \(\times\) g for 10 min at 4 °C. The pellet was resuspended in ethanol at room temperature for 24 h. Then, the suspension was centrifuged at 3000 \(\times\) g for 10 min and washed twice in acetone. The pellet was dried overnight at room temperature, and crushed. The antigen-O was stored at 4 °C until required for use. In the experiment, the antigen-O was resuspended in PBS at 1 mg ml\(^{-1}\), and was maintained in a boiling water bath for 2 h with shaking.

Extracellular material (EM) preparations were obtained following the technique described by Bonet et al. [18]. Cells of the microorganism were centrifuged at 6000 \(\times\) g for 20 min, and the supernatants discarded. The cell pellets were suspended in 1 N KOH and extraction was carried out by stirring for 4 h at room
temperature. The cell residue was separated by centrifugation at 8000×g for 30 min at 10 °C, and the clear KOH extract was precipitated by addition of three volumes ethanol. Then, the precipitate was diluted in distilled water and lyophilised and stored at 4 °C until use.

Antibody titres in gilt-head seabream serum against whole cells, subcellular fractions and ECPs of *Photobacterium damselae* subsp. *piscicida* Pp8 were determined in gilt-head seabream by using an indirect enzyme-linked immunoadsorbent assay (ELISA). Wells of the microtitre plates (Costar E.I.A. plates) were coated with 50 µl of a suspension of 10^8 cfu ml⁻¹ of the bacterial strain or with a solution of antigenic preparations (20 µg of protein ml⁻¹), and incubated overnight at 4 °C. Then, the wells were washed three times with PBS supplemented with 0.05% of Tween 20 (PBS-T), blocked for 2 h at room temperature with 300 µl of PBS-T supplemented with 0.25% (w/v) of bovine serum albumin (PBS-T-BSA), and washed three times with PBS-T. Antigen-coated wells were incubated at room temperature for 2 h with 50 µl of serial dilutions of fish serum in PBS-T-BSA, washed three times with PBS-T, and incubated for 2 h at room temperature with 50 µl of rabbit anti gilt-head seabream immunoglobulin serum diluted in PBS-T-BSA (1:500). Finally, the wells were incubated for 1 h at room temperature with peroxidase-conjugated anti-rabbit immunoglobulin (Sigma) diluted in PBS-T-BSA (1:5000), washed three times with PBS-T, and then 50 µl of the developing solution (sodium acetate 0.1 M, pH 6 with 1% tetramethylbenzydine solution (Sigma) and hydrogen peroxide) were added and incubated in the dark at room temperature for 15 min. The reaction was stopped by adding 50 µl 1 M H₂SO₄. Absorbance values were determined using a microplate reader at 450 nm. Serum from non-immunised gilt-head seabream treated in the same way as the serum from vaccinated fish was used for negative controls. The reaction was considered positive in those wells with an optical density higher than that obtained for negative controls.

Leucocytes were cultured in flat-bottomed microtitre plates with 5×10⁵ cells per well. Each well was added with 100 µl RPMI 1640 medium with antibiotics, heparin and 10% FCS. Antigens were used at the following concentrations: formalin-killed *Photobacterium damselae* subsp. *piscicida* (10⁶ whole cells ml⁻¹), 10 µg ml⁻¹ of ECPs, EM, OM, 50 µg ml⁻¹ of Ag-O and CM, 100 µg ml⁻¹ of LPS. 2.5 µg ml⁻¹ of phytohaemagglutinin (PHA) and 12.5 µg ml⁻¹ of LPS of *Escherichia coli* (Sigma) were used as positive controls. All tests were carried out in triplicate. Cultures were incubated for 3 days at 20 °C. At 24 h prior to harvest, 0.5 µCi of [³H] thymidine (Amersham) was added to each well, the liquid was removed and the cells were washed four times with HBSS. The cells were harvested on to fibreglass paper and washed four times, and the incorporated radioactivity was measured in a liquid scintillation counter. The proliferative responses were expressed as counts per minute (cpm).

Results were analysed by an ANOVA and Student’s *t*-test using a Statistical Software Statview for the Macintosh (SAS Institute Inc.). Differences were considered statistically significant at *P*<0.01.

The serum antibody titres to antigenic preparations of *Photobacterium damselae* subsp. *piscicida* are summarised in Table 1. In this study, it has been demonstrated that the vaccination of gilt-head seabream with a single injection of formalin-killed *Photobacterium damselae* subsp. *piscicida* produced a humoral immune response after four
weeks against different antigenic preparations of this microorganism. Four weeks after vaccination the fish assayed showed significant increases \((P<0.01)\) of the serum antibody titres for all antigenic preparations assayed with respect to those obtained for unvaccinated fish, the highest titres of antibodies being those obtained against OM, CM, Ag-O and ECPs. This result is in concordance with the data recorded by Santarem and Figueras [19], who reported a peak antibody titre in turbot \((	extit{Scophthalmus maximus})\) 28 days after immunisation with O-antigen from \textit{Ph. damselae} subsp. \textit{piscicida}. In this study, the advantage of a booster vaccination has been demonstrated to achieve high levels of antibodies against the different antigenic preparations. These results are in agreement with those data reported by Magarin˜o se ta l. [4], who recommended an initial gilt-head seabream vaccination for \textit{Ph. damselae} subsp. \textit{piscicida} and then a booster vaccination. In the group of fish that received a booster vaccination four weeks after the first immunisation, significant increases \((P<0.01)\) of the serum antibody titres to bacterin, ECPs, OM and LPS were observed with respect to the titres detected with only one vaccination. These significant increases were detected three and four weeks after the booster. In contrast, the serum antibody titres for Ag-O, EM and CM were not higher than those obtained in the first immunisation. The booster vaccination improved significantly \((P<0.05)\) the titres of antibodies against LPS, but not those detected against EM. This increase of the titre of antibodies against LPS could be a factor to consider in a future vaccine because there are data that suggest the addition of LPS to a chloroform-killed vaccine against \textit{Ph. damselae} subsp. \textit{piscicida} stimulated the production of serum antibodies and improved the effectiveness of the vaccine [5].

Previous studies have indicated that the extracellular products (ECPs) and the capsule of \textit{Ph. damselae} subsp. \textit{piscicida} play an important role in the virulence of this microorganism [20,21]. It is interesting to remark that it has been possible to detect high levels of circulating antibody against ECPs four weeks after the immunisation, when fish were inoculated only with washed formalin-killed cells of the pathogenic microorganism. This result is in concordance with the results observed by Boesen et al. [6], who also reported high levels of circulating serum antibody against ECPs of \textit{Vibrio anguillarum} after inoculation of rainbow trout specimens with formalin-killed cells of this bacterial species. These antibodies could be synthesised against some of the ECPs that were associated with the bacterial surface, in this way, Magariños et al. [4] demonstrated a similar pattern of enzymatic and hydrolytic activities detected in the ECPs and the whole cells of \textit{Ph. damselae} subsp. \textit{piscicida}. However, Magariños et al. [3] carried out vaccination trials on gilt-head seabream against \textit{Ph. damselae} subsp. \textit{piscicida} using two vaccine formulations, a whole-cell bacterin and a toxoid enriched whole-cell vaccine, and they reported that only the toxoid-enriched vaccine conferred protection against \textit{Ph. damselae} subsp. \textit{piscicida}, perhaps because the most virulent products are in higher concentration in the extracellular products and they are not associated with the bacterial cell.

There is no agreement on the correlation between the level of serum antibodies and protection against pasteurellosis. Several studies have reported that serum antibodies play an important role in this protection and low levels of agglutinating antibodies have been associated with prolonged epizootics of pasteurellosis in yellowtail [22]. On the contrary, Magariños et al. [3] found no correlation between the level of agglutinating antibodies and protection in vaccinated seabream. However, high levels of antibodies against OM have been correlated with protection against several fish pathogens such as \textit{Cytophaga}-like [13] and \textit{Flavobacterium psychrophilum} [23]. The titres obtained with soluble and particulated (bacterin) antigens after the booster are similar, and these results are not in agreement with other data that reported that the immune response is more pronounced against soluble than particulated antigens [13].

Leucocytes from unvaccinated fish were stimulated in vitro with the antigenic preparations, and the proliferative responses were compared with that of leucocytes not stimulated with the same preparations (Fig. 1). A mitogenic effect of the \textit{Ph. damselae} subsp. \textit{piscicida} antigens has been demonstrated for LPS, OM and Ag-O, whereas the other antigenic preparations induced low stimulation, although these differences were not-significant statistically \((P>0.01)\) due to variations among individual fish. These results are in agreement with those obtained by Boesen et al. [6], who reported in the case of the immune response of rainbow trout to \textit{Vibrio anguillarum} serogroup O1, proliferative responses for preparations of the
bacterial cell envelope, but low stimulation for ECPs. In contrast, these authors observed an elevated proliferative response for formalin-killed bacteria, whereas in this study a low stimulation has been reported, although in the Boesen’s study the differences observed in the proliferative responses elicited by different antigenic preparations were also statistically non-significant.

In conclusion, the results of this study suggest that the OM preparation was one of the most immunodominant antigens assayed which produced an immunological response, and this information could be useful to design prophylactic strategies. The ability of the outer membrane fraction (OM) to produce a high immunological response is consistent with other reports in rainbow trout, goldfish and ayu vaccinated against *Aeromonas salmonicida* and *F. psychrophilum* [23,24].

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References


