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Vaccination efficiency of surface antigens and killed whole cell of *Pseudomonas putida* in large yellow croaker (*Pseudosciaena crocea*)

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ABSTRACT

Large yellow croaker (*Pseudosciaena crocea*), a major marine fish aquacultured in the southeastern coastal region of China, has become endangered by the pathogen *Pseudomonas putida* in recent years. *P. putida* infections occur in low water temperatures when fish reduce food intake, thus oral antibiotic administration is not practical. Therefore, vaccination may be the only method to prevent the infection. In the present study, main surface antigens of *P. putida*, including lipopolysaccharide (LPS), outer membrane proteins (OMP), extracellular biofilm polysaccharide (EPS), and formalin-killed cell (FKC) bacterin, were prepared and the fish vaccinated. On post-immunization day 28, serum antibody titers, phagocytic responses of leukocytes, and lysozyme activities of the fish were evaluated. The efficiency of vaccination was tested by artificial challenge via intraperitoneal injection of live bacteria on post-immunization day 28 and 35, respectively. The results showed that although significant humoral and innate immune responses were elicited in all vaccination groups, the challenge produced similar poor protection in both tests, with a relative percent survival (RPS) of 0–40%. Although the EPS group showed a complete lack of protection, LPS reached the highest RPS value (40%), suggesting that LPS may be involved in protection immunity against the pathogen. Further analysis of the ultra-structures of tissues from infected fish via TEM revealed macrophage survival and intracellular replication ability of the pathogen. New strategies for development might put more emphasis on efficient clearance of intracellular bacteria. The present study is the first to report vaccination against the fish pathogen *P. putida* and the first investigation of intracellular survival of this pathogen in host macrophages.

1. Introduction

The large yellow croaker (*Pseudosciaena crocea*) is an important marine fish aquacultured in the southeastern coastal region of China and has become endangered by the pathogen *Pseudomonas putida* in recent years, which causes small white nodules in the internal organs, including the spleen and kidney, and leads to severe mortality, especially during the winter and spring [1–3]. *P. putida*, a member of genetically closely related fluorescent pseudomonads, has been reported as an opportunistic fish pathogen that infects many species, such as ayu (*Plecoglossus altivelis altivelis*), yellowtail (*Seriola quinqueradiata*), oyster toadfish (*Opsanus tau*), European eel (*Anguilla anguilla*), and rainbow trout (*Oncorhynchus mykiss*) [4–8]. Also, *P. putida* is a clinical causative agent that can induce nosocomial infections in immunocompromised patients [9,10]. To prevent outbreaks of *P. putida* infection, antibiotic and vaccine administration may be the most efficient measures; however, diseases caused by this pathogen occur at low water temperatures when fish reduce their food consumption, oral administration of medications is not always possible. Thus, vaccines may be the only option to control *P. putida* outbreaks. Nevertheless, knowledge on disease mechanisms of this bacterium is very limited and vaccine studies have not yet been performed.

Generally, in Gram-negative bacteria and in the fluorescent pseudomonas, *Pseudomonas aeruginosa*, main bacterial surface antigens, including lipopolysaccharide (LPS), outer membrane proteins (OMP), and exopolysaccharide (EPS), have demonstrated immunogenic properties and provided protective immunity to guard against pathogens [11,12] and a killed whole cell bacterin provided efficient protection against homogenous challenge. EPS usually includes secreted carbohydrates that form an extracellular capsule and biofilms; however, a preliminary experiment revealed no capsule formed by this strain, thus only biofilm EPS was targeted in this study. To identify protective *P. putida* antigens with vaccine potential, comparative analyses of immunization with these antigens should be undertaken.

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In the present study, three surface antigens LPS, OMP, EPS, and a formalin-killed cell (FKC) bacterin of *P. putida* were prepared, large yellow croakers were vaccinated, the immune response 28 days post-immunization were detected, and the relative percent of protection were evaluated. Here, we investigated infection procedures using the pathogen *P. putida*, evaluated ultra-structural changes in tissues from the challenged fish, analyzed the results, and discussed the vaccination efficiency.

2. Materials and methods

2.1. Experimental fish

Healthy experimental fish (average body weight, 50 ± 10 g, 250 ± 20 g, respectively) were obtained from net cages in Xiangshan Bay, Zhejiang Province, China.

2.2. Bacterial strains

Bacterial strain NB2011 was isolated from diseased *P. crocea*, and then physical, biochemical, and molecular markers were used to positively identify it as *P. putida* [2].

2.3. Antigen preparation

2.3.1. Preparation of FKC of P. putida

P. putida NB2011 cells were cultured in Zobell 2216E media at 28 °C for 14–16 h and then harvested by centrifugation at 6000× g for 10 min. The pellets were washed three times with sterile saline, formalin was added to the suspension to a final concentration of 0.5% (v/v), and then incubated at 28 °C for 24 h. The cell density was adjusted to a concentration of 1×10^8 cells/mL for the formalin-killed cell (FKC) bacterin prepared for fish immunization.

2.3.2. Preparation of LPS

LPS was isolated by phenol/water extraction according to the methods of Westphal & Jann (1965) [13]. The aqueous phase was dialyzed in distilled water for 4 days using a 12–14 kDa molecular mass cut-off dialysis membrane (Bio Basic, Inc. (BBI), New York, NT, USA) and then centrifuged for 15 min at 5000× g. The supernatant was lyophilized. Before use, the crude LPS extraction was dissolved in distilled water to a concentration of 0.5 mg/mL.

2.3.3. Preparation of OMP

OMP was prepared according to the methods described by Filip et al. (1973) [14] and Rahman et al. (2002) [15]. The OMP pellets were suspended in distilled water and the concentration determined using a bicinchoninic acid protein assay kit (Sangon Biotechnology Ltd., Shanghai, China) according to the manufacturer's instructions. A 0.5-mg/mL concentrated solution was prepared for vaccination.

2.3.4. Preparation of EPS

Extraction of biofilm polysaccharides was performed according to the methods described by Kives et al. (2006) [16] with some modifications. Briefly, bacteria were inoculated in Zobell 2216E media in 6-well tissue plates (BBI) and maintained in static culture to stimulate biofilm growth on the bottom of the plates. Fresh media was added every 24 h to substitute the old culture under sterile conditions. After 120 h, the plates were rinsed in saline and then scraped to remove the attached biomass, which was resuspended in saline. Pooled biofilm material from three batches was processed for 5 min in a blender (RW20; IKA Works, Inc., Wilmington, NC, USA) and then the cells were separated by centrifugation at 8000× g for 10 min. The supernatant was collected and lyophilized. EPS samples (1 mg/mL) were dialyzed against a 100-fold larger volume of distilled water using a 12–14 kDa molecular mass cut-off dialysis membrane (BBI) for 12 h at 4 °C. The EPS solution was adjusted to a concentration of 0.5 mg/mL and maintained at −20 °C for future use.

2.4. Vaccination and artificial challenge of fish

Large yellow croakers with average body weight of about 50 g were maintained at 21 ± 1 °C in tanks containing sand-filtered, ultraviolet disinfected and aerated seawater that was changed daily. Fish were fed daily with commercial dry pellets (Tianbang, Ningbo, China).

The fish were randomly divided into five groups of 50 fish each and allowed to acclimate for 14 days before the start of the experiments. The fish were intraperitoneally (i.p.) injected with 0.2 mL of antigen (FKC, LPS, OMP, EPS, and sterile saline, respectively). On post-immunization day 28, the average body weight of the croakers reached 70 g, blood was drawn from the caudal veins of five fish from each group and allowed to clot overnight at 4 °C. Blood samples were centrifuged at 3000× g for 5 min, then serum was collected and stored at −40 °C until analyzed. Also on day 0 (one day before vaccination), blood was collected from five fish and the sera was collected as a negative control for enzyme-linked immunosorbent assay (ELISA) analysis. On post-immunization day 28 and 35, 10 fish from each vaccinated groups were used for the challenge test, respectively. Each fish was i.p. injected with 0.2 mL of a *P. putida* NB2011 suspension (1×10^6 cells/mL). Taken from the saline injected control group, negative control 1 and normal control 2 were designed with 10 fish challenged with live pathogens and sterile saline, respectively. Disease symptoms and mortality of the challenged fish were recorded for the following 14 days and bacterial isolation from the infected fish and species re-identification were performed as previously described [2]. The relative percent survival (RPS) was calculated as described by Amend (1981) [17] using the formula RPS (%) = (1 − mortality of vaccinated group/mortality of control group) × 100.

Ten fish from the saline-vaccinated group were challenged with the same dose of live bacteria and samples were prepared to evaluate the ultra-structure of the tissues.

2.5. Phagocytosis assay

Staphylococcus aureus strain ATCC25923 cells were used as phagocytized particles. Briefly, the bacteria were inactivated with 0.5% formalin, washed twice, and then suspended in distilled water to a final concentration of 1×10^8 cells/mL. The phagocytic activity was evaluated as follows: 0.2 mL of anticoagulated blood was mixed with an equal volume of *S. aureus* suspension and the mixture was incubated for 60 min at 25 °C with mild shaking every 10 min. Then, 50 μL of the mixture was smeared on a glass slide and five slides were prepared for each sample. After immobilization with methanol, the smear was stained with Giemsa reagent and the number of phagocytically active leukocytes and engulfed bacterial cells were counted per 100 leukocytes by duplicate by light microscopy using an oil immersion objective. Phagocytic activities were expressed using the following formulas:

$$\text{Phagocytic percentage (PP, \%)} = (\text{phagocytic leukocytes}/\text{total leukocytes}) \times 100;$$

$$\text{Phagocytic index(PI)} = \text{numbers of bacterial cells engulfed}/\text{number of leukocytes observed}.$$