Biologicals 42 (2014) 48–51

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Development of an *in vitro* assay based on humoral immunity for quality control of oil-adjuvant Pseudotuberculosis vaccine in Yellowtail *Seriola quinqueradiata*

Fumiya Hirano*, Saiki Imamura, Nao Nakajima, Kinya Yamamoto, Mariko Uchiyama, Hidetaka Nagai, Mayumi Kijima

National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, 1-15-1 Tokura, Kokubunji, Tokyo 185-8511, Japan

ARTICLE INFO

Article history:
Received 12 April 2013
Received in revised form
20 August 2013
Accepted 13 November 2013

Keywords:
Photobacterium damselae subsp. *Piscicida*
Vaccine
Quality control
Agglutination assay
Seriola quinqueradiata

ABSTRACT

Photobacterium damselae subsp. *piscicida* is an infectious pathogen that causes Pseudotuberculosis in Yellowtail fish. In Japan, several oil-adjuvant vaccines for Pseudotuberculosis have been approved for control of infectious diseases in aquaculture. Before distribution of an approved fish vaccine, an artificial challenge test for quality control is performed by the manufacturer and National Veterinary Assay Laboratory under Pharmaceutical Law of Japan to confirm potency. In this study, artificial challenge tests with a range of five diluted or undiluted approved vaccines was performed to determine the relationship between antigen levels and vaccine efficacy. Immunization of fish with the undiluted vaccine prevented Pseudotuberculosis. Results of artificial challenge tests demonstrated vaccine efficiency was dose dependent. Agglutination assays using immune sera were performed to determine agglutination titers, which were also dose dependent. These results suggest a link between survival rate in the artificial challenge tests and agglutination titers. Western blotting analysis identified a specific protein approximately 37 kDa in size in vaccinated fish. We confirmed antibodies were produced in vaccinated fish by immunoreactions with the approved vaccine. An agglutination assay based on humoral immunoreactions would be a useful alternative to the artificial challenge test for quality control of vaccines for aquaculture.

1. Introduction

Pseudotuberculosis, caused by *Photobacterium damselae* subsp. *piscicida*, is a common and life-threatening bacterial disease of fish including Yellowtail *Seriola quinqueradiata* and other varieties of fish, including sea bass, sole, gilt-head sea bream and turbot, in aquaculture [1–3]. Because of its wide host range, *P. damselae* subsp. *piscicida* has been the cause of great economic loss in Japan [4,5], Europe [6] and United States [7]. Based on this background, intensive studies of immune reactions against *P. damselae* subsp. *piscicida* have been performed in fish to aid vaccine development. These studies demonstrated that a variety of bacterial antigens were recognized by the fish immune system [8,9]. It was also noted that agglutination antibodies were induced in immunized or infected fish [10,11].

Two polyvalent injection type oil-adjuvanted Pseudotuberculosis vaccines have been approved for disease control in Japan since 2008 [12,13]. Before distributing these vaccines, their safety and potency must be determined by quality control. In general, quality control of fish bacterial vaccines consists of sterility tests, safety assay and potency assay. In Japan, the regulations state the potency assay must be performed by both vaccine manufacturers and the National veterinary assay laboratory (NVAL), to prevent the distribution of inefficient vaccines.

More than 100 experimental fish are required for quality control testing of fish vaccines prior to the release of one batch, since potency assays of most fish vaccines are performed by artificial challenge test. Thus, the replacement of *in vivo* assays with *in vitro* methods for the quality control of commercial batches of vaccines has the potential to reduce the numbers of fish used significantly [14]. In addition to the large numbers of fish required, the *in vivo* method has other disadvantages, such as a long experimental

Abbreviations: NVAL, National Veterinary Assay Laboratory; PVDF, polyvinylidene difluoride; i.p., intraperitoneally.

* Corresponding author. Tel.: +81 42 321 1841; fax: +81 42 321 1769.
E-mail address: humiya_hirano@nval.maff.go.jp (F. Hirano).

1045-1056/$36.00
http://dx.doi.org/10.1016/j.biologicals.2013.11.004

period. In addition, it has a lack of reproducibility, since quality control tests of fish vaccines rely on many factors such as the target animal's immune response, bacterial virulence and related factors including culture conditions. Accordingly, it is required that the government and manufacturers develop an assay that offers rapidity, simplicity, accuracy and ease of use to secure a stable supply of quality controlled vaccines to aquaculture industries. However, the *in vivo* assay is often used to assess vaccine potency because the immunoreactions of fish to vaccines are poorly understood and there is no optimal alternative method.

Therefore, the aim of this study was to investigate immunoreactions in Yellowtail fish immunized with an approved Pseudotuberculosis vaccine and to develop an *in vitro* assay based on humoral immunity as a quality control test to replace the *in vivo* artificial challenge test.

2. Materials and methods

2.1. Experimental fish

Healthy, artificially hatched Yellowtails with weights ranging from 90 to 100 g were obtained from Seikai National Fisheries Research Institute, Fisheries Research Agency (Goto, Nagasaki, Japan). The fish were maintained in 200-L or 500-L circular tanks and the water temperature was maintained at 22–25 °C. The salinity of rearing seawater was checked every day. Fish were fed Otohime EP3 (Marubeni Nisshin Feed, Tokyo, Japan) for marine fish use, throughout the experiment. The study was conducted according to the Guidelines for Animal Experiments of the NVAL.

2.2. Bacterial strains and culture conditions

Two strains of *P. damselae* subsp. *piscicida*, F36 and IJF197, were used in this study. Strain F36 was isolated from Yellowtail in Oita, Japan by Dr. Y. Fukuda, Fisheries Research Institute, Oita Prefectural Agriculture, Forestry and Fisheries Research Center. Strain IJF197 was isolated from Yellowtail in Nagasaki, Japan by Merck Sharp and Dohme (MSD) Animal Health, Ibaraki, Japan, and donated to our laboratory for the assessment of vaccine potency. Both strains were cultured on 1.5% NaCl added-trypticase soy broth (Difco, Detroit, MI, USA) for 18 h at 25 °C.

For the Agglutination assay, strain F36 was inactivated with 0.5% (w/v) formalin (Wako, Osaka, Japan) at 4 °C for 48 h, and washed with phosphate-buffered saline (PBS) 3 times. The antigen concentration was adjusted to approximate optical density (OD) of 0.6 using an absorption spectrophotometer (Ultrospec 2100 pro, GE Healthcare UK Ltd, Buckinghamshire, England) at a wavelength of 600 nm. Strain F197 was also inactivated with 0.5% (w/v) formalin and washed with PBS 3 times. Formalin-killed bacterial samples of F36 and IJF197 were also used for western blot analysis. For the challenge test, Strain IJF197 was used at 8.0×10^2 colony forming units per fish.

2.3. Vaccine

The vaccine used in this study was an inactivated injection-type polyvalent Pseudotuberculosis vaccine with an oil adjuvant (MSD Animal Health).

2.4. Artificial challenge test and obtain immune serum

To obtain diluted vaccines, the vaccine lot was diluted with an oil adjuvant diluent (MSD animal Health, Ibaraki, Japan) containing Montanide™ ISA 763 AVG (Seppic, Paris, France) and PBS at ratio of approximately 7:3. A total of 140 fish were divided into 7 groups, and intraperitoneally (i.p.) injected with 0.1 ml of either undiluted vaccine, ten-fold diluted vaccines (from 1:10 to 1:100,000), or PBS at 22 °C. Three weeks after the vaccination, 8–11 fish from each group were selected randomly and blood was collected. Serum was obtained for the determination of *P. damselae* subsp. *piscicida* agglutinating titers. Serum samples were incubated at 45 °C for 20 min to inactivate complement, and then stored at −20 °C until used. Part of the fish sera from fish immunized with the undiluted full-dose vaccine was used for western blot analysis. The remaining ten fish per group were then challenged i.p. with *P. damselae* subsp. *piscicida* strain IJF197 at 25 °C, and the survival rates were monitored for 14 days.

2.5. Agglutination assay

Agglutinating titers were determined in 96-well micro-plates. Serial twofold dilutions of aliquots (25 μl) of each serum were performed in sterile PBS. The same volume (25 μl) of antigen solution (formalin-killed bacterial samples of F36) was then added to each well. The plate was incubated at 25 °C for 2 h, and then overnight at 4 °C. The agglutination antibody titer was determined by the highest dilution of a sample that caused complete agglutination of each antigen.

2.6. Preparation of rabbit anti-Yellowtail immunoglobulin (Ig)

Rabbits were immunized twice at two weeks interval with ammonium sulfate-precipitated Yellowtail Ig. Blood samples were collected from rabbits 3 days after the 2nd inoculation, incubated at 37 °C for 1 h and then at 4 °C for more than 1 h. To obtain rabbit anti-Yellowtail Ig sera, samples were centrifuged at 3000 rpm for 10 min and incubated at 56 °C for 20 min (to inactivate complement). Samples were then pooled and stored at −20 °C until used. Rabbit anti-Yellowtail Ig serum was absorbed with the formalin-killed F36 strain, 3 times at 37 °C before use in western blot analysis, to avoid non-specific reactions between rabbit serum and bacterial antigens.

2.7. Detection of immunoreactions in Yellowtail by SDS-PAGE and western blot analysis

For western blot analysis, whole bacterial lysates of the F36 strain, or whole bacterial lysates of the IJF197 strain were mixed with sample buffer and SDS-PAGE was performed with 15% slab gels as previously described [15]. The gel was transferred to polyvinylidene difluoride (PVDF) membranes and western blot was performed. After 3 washes with PBS containing Tween-20 (PBST), serum from Yellowtail immunized with the Pseudotuberculosis vaccine was used as a primary antibody and rabbit anti-Yellowtail Ig was used as secondary antibody. After five washes with PBST, horseradish peroxidase-conjugated mouse anti-rabbit IgG (Rockland Immunochemicals, Inc., PA, USA) was used to detect specific antigens in immune serum. The membrane was visualized using 3, 3-diaminobenzidine tetrahydrochloride and cobalt chloride.

2.8. Statistical analysis

Correlation coefficients were used to evaluate the correlation between antibody titers and survival rates of the artificial challenge test with the undiluted and diluted vaccines. Statistical analysis of the protective efficacy of the vaccines was performed by Fisher's exact test. Values of $P < 0.05$ were considered statistically significant.