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Passive immune-protection of small abalone against *Vibrio alginolyticus* infection by anti-*Vibrio* IgY-encapsulated feed

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A B S T R A C T

Small abalone (*Haliotis diversicolor supertexta*) is a high value-added shellfish. It however has been suffering *Vibrio alginolyticus* infections, which cause mass death of small abalone and thus great economic losses, particularly in artificial aquaculture. In this study, we attempted to treat small abalone with anti-*Vibrio* IgY to elicit a passive immunity directly against *V. alginolyticus* infections. Anti-*Vibrio* IgY was alginate encapsulated in egg powders as feed, which may avoid antibody inactivation in the gastrointestinal tract of small abalone. The feed was tested for the stability of anti-*Vibrio* IgY in a gastrointestinal mimic environment. The result showed anti-*Vibrio* IgY retained activity as high as 90% after 4 h exposure to pancreatic enzymes. Addition of 0, 5 or 10% anti-*Vibrio* IgY-encapsulated egg powders into a basal diet to form abalone diet formulae. Small abalones fed with the anti-*Vibrio* IgY formulae showed a relatively high respiratory burst activity than those without anti-*Vibrio* IgY treatments. The survival rates of small abalones fed with 5 or 10% anti-*Vibrio* IgY egg powders were in the range of 65–70% 14 days post-*V. alginolyticus* challenge (1×10^6 c.f.u.), which was significantly higher than 0% of those fed without anti-*Vibrio* IgY. The anti-*Vibrio* IgY-encapsulated formulae were thus concluded to be an effective means to prevent small abalone from *V. alginolyticus* infection, and may be practical in use in abalone aquaculture.

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1. Introduction

Small abalone (*Haliotis diversicolor supertexta*) is a large herbivorous marine snail valued as highly palatable seafood, widely cultured in Australia, China, Japan, Korea, Mexico, South Africa, and the United States [1]. However, since 2000, the mass mortality of abalone reared in grow-out ponds, settlement failure of larvae in the nursery ponds, and post-larvae abalone infected by *Vibrio* species, displayed a pattern of sudden collapse or “crash” in survival and forced many abalone farms to close [2–4].

Small abalone can be mass produced by the well-developed culture techniques of multiple-layer basket and flat-surface system. Advances in aquaculture techniques significantly increase culture density, while once the cultured objects infected the spread of disease is very often uncontrollable, which would seriously damage the entire aquaculture industry. *Vibrio* is known to be common pathogens in many ocean animals, which indeed have caused great economic losses in aquaculture industry [5,6]. *Vibrio alginolyticus* and *Vibrio parahaemolyticus* are the two most harmful pathogens in abalone aquaculture [2,7]. Once small abalone infected by *V. alginolyticus* result inevitably high mortality. The disease manifestations include less elastic, ulcerated, atrophic, darkened and cystic. Often, *V. alginolyticus*-infected larval abalones would dislodge from cultivate broad in 2-week period of time after artificial propagation [8]. Pathogenesis of the disease, however, is still unclear.

For the *V. alginolyticus* infections, current preventions use pathogenic factors, such as serine protease [9], conserved outer membrane protein [10], iron-regulated outer membrane protein [11] as antigens for vaccination. Some reports indicated that complements in humoral fluids can eradicate *Vibrio* species [12], while if such an effect occurs in abalone is not yet known as the immune system of abalone is less studied. Two immune-like cells, granulocytes and hyaliocytes [13], in abalone’s blood were reported, but their roles are not understood. Thus far, antibiotic administration in aquaculture industry is the major way to prevent the disease from occurring and as the main treatment. However, the use of antibiotics has been a great concern nowadays, even though this treatment remains effective in general.

Vaccination is also used in aquaculture industry, while its practice requires considerable managements in addition to

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significant stresses passed through recipients [13]. Passive immunization that is a way to immunize host with pathogen-specific antibodies from diseases has been an attractive approach in controlling the spread of diseases in community. In general, antibodies are environment-friendly; they have no severe side effects as well as disease resistance in low forms of lives [14]. Even more attractive is that immunoglobulin can be administered orally, because such a way can cut the cost down to a great extent. In fact, relatively inexpensive egg yolk immunoglobulins (IgY) make pathogen-specific IgY feasible in small abalone aquaculture. High titres of pathogen-specific IgY in eggs can be elicited after vaccination of hens with given antigens [15–18]. Industrially, simple IgY extraction methods have been developed [19]. For examples, given IgYs have been used in pigs [20,21] and rabbits [22] against *Escherichia coli* infections. In aquatic organisms, given IgYs have also been used in Japanese eel (*Anguilla japonica*) and rainbow trout (*Oncorhynchus mykiss*) against *Edwardsiella tarda* [23,24] and *Yersinia ruckeri* [25,26] infections, respectively. In this report, the effectiveness and practicability of anti-*V. alginolyticus* IgY used in small abalone aquaculture against *V. alginolyticus* was examined and evaluated.

2. Materials and methods

2.1. Bacterial preparation

Lyophilized *V. alginolyticus* (Microtek International, Sannichton, British Columbia, Canada, originally from FRS Marine Laboratory, Aberdeen, Scotland, UK) was rehydrated and grown in tryptic soy broth (TSB) (Difco Laboratories, Detroit MI, USA) with 1.5% NaCl at 22 °C for 24 h. Aliquots were stored in 50% (v/v) sterile glycerol at −78 °C.

2.2. Chickens

Four-week old female leghorns were used. After disinfecting the wings with 70% ethanol, each animal intramuscularly received 200 μg formalin-inactivated *V. alginolyticus*. Priming vaccination antigens were mixed with the complete Freund's adjuvant. The eggs were collected two weeks after priming. Hens were boosted 3 times with the antigens but containing the incomplete Freund's adjuvant at two-week interval. IgY activities were compared for pre- and after-immunized yolks. Egg yolk was separated from egg white. The egg yolk was diluted with 0.1 M PBS in 1:4, and kept at 4 °C 12 h for subsequent titration.

2.3. Preparation of IgY

IgY preparation was performed according to the method published earlier with some modifications [20]. Briefly, egg yolk was separated from the egg white. The egg yolk was then diluted 4 times in 0.1 M phosphate-buffered saline. After 12 h storage at 4 °C, the suspension was filtered through 25 μm pore size membrane filter to removed debris and yolk membrane. The filtrate, containing the water-soluble fraction of egg yolk, was dried to powders by spray-heat machine at 105 °C and then stored at 4 °C till to make sheet diets. Nonspecific IgY egg powders were extracted from pre-vaccination eggs. Nine weeks after the first vaccination, the eggs were used to provide egg powders containing anti-*V. alginolyticus* IgY. Coating procedure was in accordance to the method described previously [27]. Briefly, IgY powders were added to 2% (w/v) sodium alginate (Sigma, St. Louis, MO, USA) solution at a loading rate of 25% (w/w) and dissolved with 0.5% $CaCl_2$ (w/v, final concentration). By stirring (500 × *g*) at 4 °C for 30 min, the alginate coated IgY was spray-dried at 105 °C.

2.4. The stability assays of anti-Vibrio IgY

For the time stability test, either alginate encapsulated or non-encapsulated IgY solutions were incubated at 37 °C at time intervals of 0, 1, 2, 4 h. For the heat stability test, IgY solutions were incubated at 0, 5, 10, 20, 40, 60, 70, 80, and 90 °C for 10 min. The heat-treated IgY was cooled in an ice bath. For the stability in gastrointestinal conditions, a simulated gastric fluid (SGF) and a simulated intestinal fluid (SIF) were prepared for the stability of the encapsulated or non-encapsulated IgY. These two solutions were prepared according to pharmacopoeia [28]. SGF (3.2 mg/mL pepsin in 0.03 M NaCl at pH 1.2) was added to IgY to give a ratio of 1:250 in terms of enzyme to substrate. SIF consisted of 10 mg/mL of pancreatin in 0.05 M KH_2PO_4, pH 7.5. The SGF was added to the IgY to give an enzyme to substrate ratio of 1:20. To simulate the physiological conditions of gastrointestinal, first 2 h of dissolution were carried out in SGF and the rest of the time in SIF at intervals of 0, 1, 2, 4 h. Antibody activity, defined as the ability of the anti-*Vibrio* IgY was measured by ELISA. Antibody activity was represented as percentage when divided by the control.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Antibody-capture ELISA was performed to determine the specific anti-*V. alginolyticus* IgY content of the samples. Buffers were prepared according to Kummer et al. [29], except that the blocking buffer consisted of 1% skimmed milk solids in PBS. Briefly, each well of the 96 well polystyrene plates (Corning Costar, Cambridge, MA) was coated with 50 μL of *V. alginolyticus* lysate (100 ng/well) in a coating buffer and put into hood for air-dry overnight. Blocking was performed by adding 200 μL of a blocking buffer in each well, and then incubated in 37 °C for 2 h. The wells were washed with PBS/T (0.05% Tween 20 in PBS), and underwent serial dilutions from spray-heat dried powders or sheet pellets. The plates were incubated for 12 h at 4 °C. After three washings with PBS/T, 50 μL of horseradish peroxidase-conjugated goat anti-IgY Fc (1:1000; R&D Systems, Minneapolis, MN) was added, mixed and incubated at 37 °C for 1 h. Plates were washed with PBS/T. 50 μL of an *o*-phenylenediamine dihydrochloride solution (Sigma, St. Louis, MO) was added. After 30 min of incubation at 37 °C, 50 μL of 3 N HCl was added to stop the reactions. The colour intensity was read by an ELISA plate reader (model 550, Bio-Rad, Mississauga, ON) with a 490 nm filter.

2.6. Preparation of the feed containing anti-Vibrio IgY

Formulae added 5 or 10% egg powders (anti-*V. alginolyticus* IgY or nonspecific egg powder as control) into basal diets (20% crud fish powder, 28% soy beans powder, 24.5% sodium alginate, 4% mineral substrate, 2.3% Vit. mix, 0.1% Vit. E, 3% corn oil, 17.5% dextrin, and 0.6% choline). They were dissolved in neutral 0.01 M phosphate-buffered saline and extruded into sheet-form diet pellets. Sheet diets were dried under a stream of ambient temperature air. Marine oil (3% pellet weight) was then sprayed onto the pellets to reduce leaching of additives into water before uptake by abalone.

2.7. Extraction of IgY from feed

Specific and nonspecific diet formulae were stored at 4 °C for use. One gram of each diet was grinded to powders and soaked in 10 mL of PBS with 1% BSA, and incubated overnight at 4 °C. The samples were vortexed and centrifuged at 20,000 × *g* 4 °C for 20 min. The supernatant was collected to measure IgY contents by ELISA.

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