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Ulcer disease prophylaxis in koi carp by bath immersion with chicken egg yolk containing anti-*Aeromonas salmonicida* IgY

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ABSTRACT

Ulcer disease, caused by atypical *Aeromonas salmonicida*, is a serious concern in ornamental koi carp, because it induces skin ulceration, disfiguring ornamental fish and causing economic loses. The present study aimed to establish a novel prophylaxis with chicken egg yolk immunoglobulin, IgY, against ulcer disease and to assess its feasibility in the ornamental fish industry. Addition of egg yolk powder containing anti-*A. salmonicida* IgY to rearing water provided significant protection against an *A. salmonicida* bath infection, whereas administration of non-specific IgY did not. Consecutive immersion of fish into rearing water containing specific IgY completely prevented ulcer disease resulting from cohabitation infection, indicating that this prophylaxis could prevent infection from such type of contact. Thus, passive immunization induced by immersing fish into aquarium water containing specific IgY is a prospective prophylaxis against diseases caused by pathogens that invade the skin and gills.

1. Introduction

Koi carp (*Cyprinus carpio koi*) is a popular and economically valuable ornamental fish. The koi industry has spread worldwide, and along with the growth of the industry, outbreaks of diseases have occurred in koi farms or personal owners' ponds (Cizek et al., 2010; Dobiasova et al., 2014; Rakus et al., 2013). Ulcer disease, caused by atypical *Aeromonas salmonicida*, is the most serious disease affecting koi carp and goldfish (Goodwin and Merry, 2009; Hunt, 2006). This disease causes progressive erosion of the skin and subsequent exposure of the underlying muscle, resulting in high mortality and devastating disfigurement of ornamental fish and economic loss (Elliott and Shotts, 1980a, 1980b; Matoyama et al., 1999; Wiklund and Dalsgaard, 1998). There is no vaccine for ulcer disease in koi, and it is important to develop an effective prophylaxis.

Passive immunity is the transfer of pathogen-specific antibodies from one individual to another (Dias da Silva and Tambourgi, 2010; Hatta et al., 1993; Kovacs-Nolan and Mine, 2012; Xu et al., 2011). Antibodies are not harmful to the environment and do not have severe side effects on living organisms. Furthermore, in contrast to antibiotics, antibodies never induce development of resistant bacterial strains. Therefore, passive immunization is an ideal technique of protecting domestic animals from infectious diseases. Chicken egg yolk immunoglobulin (IgY) is a useful antibody that can be employed for passive immunization, because high titer of pathogen-specific IgY in eggs can be produced after vaccination and simple IgY extraction methods have been developed (Hatta et al., 1990). The efficacy of passive immunization against *Escherichia coli* using IgY has been observed in domestic animals such as pigs and rabbits (Kweon et al., 2000; Torche et al., 2006; Yokoyama et al., 1992). With regard to fish and shellfish, IgY has been used for passive protection in the Japanese eel (Gutierrez et al., 1993), rainbow trout (Arasteh et al., 2004; Lee et al., 2000), ayu sweetfish (Li et al., 2014), crucian carp (Jin et al., 2013), and small abalone (Wu et al., 2011) against *Edwardsiella tarda*, *Yersinia ruckeri*, *Aeromonas hydrophila*, and *Vibrio alginolyticus* infections, respectively. These studies have demonstrated that administration of IgY is effective against bacterial infectious diseases.

An effective strategy for preventing atypical *A. salmonicida* infection in koi is to block the attachment of the bacterium to the skin, because the primary sites of infection of this bacterium are the skin and gills. Therefore, in the present study, it was proposed that repeated immersion of fish in aquarium water containing antibodies to *A. salmonicida* would prevent infection of the fish by that organism. In addition, the feasibility of using this technique in the ornamental koi industry was also discussed.

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2. Materials and methods

2.1. Fish

Koi carp were obtained from Yamasaki Koi Farm of Kamihata Fish Industries Ltd. (City, Japan). Two different colored koi varieties, Ohgon and Kohaku, were used in the present study, because they are useful in cohabitation infection (described in detail in section 2.6.2). The fish were transferred to Kyushu University and acclimated for more than 2 weeks at 20 °C before use, and were fed daily with commercial food (Kyorin Food Industries, Ltd., City, Japan) at 1–2% of total body weight in each tank.

All the infection experiments were performed in accordance with the guidelines of the Animal Experiments Committee at Kyushu University.

2.2. Bacterial culture

Atypical *A. salmonicida* strain T1031 was obtained from Niigata Prefectural Inland-water Fisheries Experimental Station (Matoyama et al., 1999). The bacteria were used as antigens for immunization of hens and as pathogens for infection and were incubated at 20 °C for 36–72 h in heart infusion (HI) broth (Nissui). The colony forming units (CFU) per ml were estimated by plating serially-diluted bacterial cultures on HI agar (Nissui).

To prepare an immunogen of chicken and coated antigen for ELISA, formaldehyde was added at a final concentration of 1.0% (v/v) to the bacterial suspension and incubated for 48 h. The formalin-killed bacteria were washed twice with PBS to remove formaldehyde and resuspended in PBS. To confirm whether the bacteria were killed in this step, the suspension was seeded onto HI agar and no growth was observed.

2.3. Preparation of egg yolk powder containing anti-A. salmonicida IgY

Two egg-laying hens approximately 50 weeks old were used in this study. The hens were intramuscularly injected with 0.25 ml of formalin-killed *A. salmonicida* (1.0×10^9 CFU/hens) mixed with an equal volume of incomplete Freund's adjuvant into four sites at muscles under their wing. After primary immunization, two additional booster injections were administered at 2-week intervals. The eggs were collected daily from the immunized hens. The egg yolks were pooled, freeze-dried, and the egg yolk powders were stored at 4 °C. Control egg yolk powder was prepared by following the procedure described above.

2.4. Detection of A. salmonicida-specific IgY

The *A. salmonicida*-specific IgY antibodies were detected by ELISA. The concentration of formalin-killed bacteria was adjusted to 5×10^8 CFU/ml with 50-mM sodium carbonate buffer (pH 9.6). Fifty microliters of the suspension was added to a 96-well ELISA plate H Type (Sumitomo Bakelite, Japan) and incubated for 1 h at 37 °C. Then, the plate was incubated with 100 µl/well of blocking buffer [1% collagen peptides in Tris-buffered saline (TBS; pH 7.4) containing 0.05% Tween 20 (TBS-T)] overnight at 4 °C. The egg yolk samples were diluted 10,000-fold with TBS-T, and 50 µl of each dilution was added to the well and incubated for 1 h at 37 °C. Subsequently, 100 µl/well of 10,000-fold diluted alkaline phosphatase-conjugated rabbit IgG against chicken IgY (Zymed, CA, USA) was added and incubated for 1 h at 37 °C. Between each step, the wells were washed four times with TBS-T. For color development, 100 µl of the substrate solution (1 mg/ml p-nitro phenyl phosphate in diethanolamine buffer; pH 9.5) was added to the wells and incubated for 20 min at 37 °C. The reaction was stopped by adding 50 µl/well of 2-N NaOH and the absorbance was measured using a microplate reader at 405 nm (Bio Rad Model-550).

To determine the concentration of *A. salmonicida*-binding IgY in the egg powder, a standard curve was constructed using purified IgY, which was purified as previously described (Hatta et al., 1990). For this estimation, we used an ELISA protocol different from the one previously described. This protocol was based on a method proposed in an earlier study (Somamoto et al., 2014) but was modified to incorporate HRP-conjugated anti-chicken IgY rabbit antibodies (Anaspec, Inc.) for the detection of chicken IgY.

2.5. Microscopic observations

The binding of specific IgY to *A. salmonicida* was observed under a fluorescent microscope. The concentration of formalin-killed *A. salmonicida* was adjusted to 5×10^7 CFU/ml with PBS. Immune egg yolk powder was suspended in PBS and mixed with the bacterial suspension at a final concentration of 100 µg/ml. After incubation for 1 h at room temperature, the bacterial suspension was washed three times in PBS. Subsequently, FITC-conjugated anti-chicken IgY rabbit antibodies (Sigma) were added and incubated for 1 h at room temperature. The bacterial suspension was then washed three times in PBS and samples were observed the under fluorescent microscope.

2.6. Design of infection experiments

The protective effects of bath immersion in rearing water containing IgY were evaluated as follows by two different challenge tests.

2.6.1. Protective effect of egg yolk powder following bath infection

The protective effect of IgY on the fish against the bacteria was assessed by bath infection with *A. salmonicida*. A total of 100 fish weighing 2.1–4.2 g were kept in five 2-l tanks (20 fish/ tank), and 0.5, 0.2, and 0.1 g/l immune egg yolk powder (high, medium, and low dose, respectively) and 0.5 g/l non-immune egg yolk powder were added to each tank. The control group comprised fish immersed in water without egg yolk powder. The fish were left in the water containing the egg yolk powder for 60 min. In each tank, fish were exposed to 0.9×10^6 CFU/ml *A. salmonicida* for 1 h at 20 °C. Subsequently, they were transferred to 45-l tanks and the number of dead fish was recorded daily for 20 days.

2.6.2. Protective effect of egg yolk powder following cohabitation with infected fish

To visually distinguish between "pre-infected fish" and "cohabited fish," two different colored fish varieties, Kohaku and Ohgon, were used in the cohabitation experiment. Kohaku and Ohgon, weighing 3.4–7.0 g each, were used as pre-infected and cohabited fish, respectively. Ohgon were kept at 20 °C in three 60-l tanks and then immersed in tanks with egg yolk powder containing *A. salmonicida*-IgY for 60 min before cohabitation with pre-infected fish. The concentrations of egg yolk powder per rearing tank were 0, 0.1, and 0.05 g/l for the first experiment and 0, 0.025, and 0.0125 g/l for the second experiment. To obtain pre-infected fish, Kohaku were immersed with 1.0×10^7 CFU/ml *A. salmonicida* suspension for 60 min. After infection, the pre-infected fish were transferred to each tank containing the egg yolk powder-treated fish (cohabited fish). The fish were reared in a closed water circulating system. The same dose of egg yolk powder was added every three days and one-third of the water in each tank was changed every seven days during the experiment. The number of dead fish was recorded daily for 40 days, and the mortalities of pre-infected fish and cohabited fish were separately calculated. When clinical signs were observed at 40 days post-infection (dpi), the mortalities were recorded until 45 dpi. The