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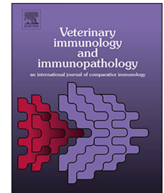

Research paper

# Antibody-mediated bacterial killing of *Ichthyobacterium seriolicida* in Japanese amberjack

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ABSTRACT

*Ichthyobacterium seriolicida* is the causative agent of bacterial hemolytic jaundice (BHJ) in Japanese amberjack, *Seriola quinqueradiata*. Fish recovering from BHJ acquire protective immunity against reinfection. In this study, fish were passively immunized to determine whether serum antibody is involved in protection against BHJ. The susceptibility of *I. seriolicida* to the bactericidal activity of Japanese amberjack serum was also investigated. In passive immunization tests, significantly lower mortality was noted in fish that received convalescent serum. Bacteria were killed when exposed to convalescent serum but not serum from naïve fish. Electron microscopic analyses showed that *I. seriolicida* cells were morphologically altered by reaction with convalescent serum. Naïve fish serum became bactericidal upon addition of purified IgM from convalescent serum. Involvement of the classical complement pathway in the bactericidal mechanism was confirmed because bactericidal activity was lost upon heating convalescent serum or chelation treatment using EDTA. Convalescent fish serum thus protects against reinfection by *I. seriolicida* via humoral immunity mediated by activation of the classical complement pathway.

## 1. Introduction

*Ichthyobacterium seriolicida* is a bacterium of the family Flavobacteriaceae that causes bacterial hemolytic jaundice (BHJ) in Japanese amberjack, *Seriola quinqueradiata* (Takano et al., 2016). BHJ-affected fish are characterized by a yellow coloration of the skin and muscles caused by hemolysis induced by *I. seriolicida* (Sorimachi et al., 1993; Maeno et al., 1995). Outbreaks of BHJ have been reported in various locations in western Japan since the 1980s (Sorimachi et al., 1993; Matsuyama et al., 2017), with resulting losses of 5 to 20% of the cultured Japanese amberjack stocks (Sorimachi et al., 1993). As such, BHJ poses a serious risk to the cultured Japanese amberjack industry in Japan, highlighting the need for an efficacious vaccine.

Although it has been demonstrated that survivors of natural infection with *I. seriolicida* achieve significant protection, the role of specific antibodies in mediating that protection remains unclear. Vaccine strategies are generally directed toward eliciting an adaptive immune response, mainly by inducing increased production of protective antibodies. To evaluate the feasibility of a potential vaccine, therefore, it is critical to clarify the role of antibodies in the protective mechanism. Passive immunization experiments have demonstrated the importance of specific antibodies in providing protection against bacterial challenge in fish (Harrell et al., 1975; Ooyama et al., 1999, 2002; Akhlaghi, 1999; Shelby et al., 2002; Marquis & Lallier, 1989; Spence et al., 1965; Olsen 1991; Shelby et al., 2002, LaFrentz 2003, Pasnik et al., 2006; Kato et al., 2015). In this study, we employed a passive immunization approach to assess the role of humoral immunity in providing protection from *I. seriolicida* challenge. We also investigated the susceptibility of *I. seriolicida* to serum bactericidal components.

## 2. Materials and methods

### 2.1. Ethics statement

This work met all relevant ethical standards for experimentation and research integrity. Fish handling, husbandry, and sampling

*Abbreviations:* BHJ, bacterial hemolytic jaundice; L15 medium, Leibovitz's L15 culture medium; BCM, bacterial culture medium; CS, convalescent serum; NS, normal serum; OD600, optical density at 600 nm; PBS, phosphate-buffered saline; TEM, transmission electron microscopy; SEM, scanning electron microscopy; EDTA, ethylenediaminetetraacetic acid

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methods were approved by the Institutional Animal Care and Use Committee of the Japanese National Research Institute of Aquaculture (IACUC-NRIA no. 28004).

### 2.2. Fish

Japanese amberjack (*S. quinqueradiata*) were bred at the Seikai National Fisheries Research Institute (Nagasaki, Japan). There was no history of BHJ. Juvenile fish were transported to the National Research Institute of Aquaculture (Mie, Japan) and then reared in plastic tanks in sand-filtered seawater. All fish experiments were performed at 25 °C. Fish were anesthetized with 2-phenoxyethanol (Wako, Osaka, Japan) before treatment.

### 2.3. Bacteria

*Ichthyobacterium seriolicida* strain JBKA-6 T (= ATCC BAA-2465 T = JCM 18228 T) isolated from Japanese amberjack was used in all experiments. Bacteria were cultured in Leibovitz's L15 culture medium (L15 medium, Sigma Aldrich, Tokyo, Japan) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Tokyo, Japan) (bacterial culture medium [BCM]) at 25 °C with continuous gyratory shaking. Log-phase cultures were diluted 5-fold with BCM and subcultured for 24 h and then used for experiments. Because the bacterium is not able to grown on plates (Takano et al., 2016), quantity of the bacterium was evaluated by optical density at 600 nm [OD600] using density meter (Biowave CO8000 Cell Density Meter, Biochrom, Cambridge, UK).

### 2.4. Serum

Convalescent serum (CS) was obtained from survivors of artificial infection with *I. seriolicida* as follows. A suspension of *I. seriolicida* (0.1 mL; OD600 = 0.07 in BCM) was injected intravascularly into Japanese amberjack (body weight 125-197 g, n = 30) via the caudal vein using a 25-gauge syringe. Fish were maintained in 500-L tanks. Death of challenged fish was observed from 5 to 9 days after injection. The cumulative mortality rate was 60%. Surviving fish (n = 12) were bled via the caudal vein at 3 weeks after challenge. In order to prepare Japanese amberjack normal serum (NS), blood was drawn from fish (body weight 118-201 g; n = 10) that received an intravascular injection of 0.1 mL of BCM 3 weeks prior to blood sampling. Blood was allowed to clot by incubating for 1 h at room temperature, and serum was obtained by centrifuging the clotted blood at 400 × *g* for 20 min at 4 °C. The supernatant was collected and centrifuged again at 20,000 × *g* for 5 min to remove debris. Finally, the serum was filtered with a 0.45-μm filter unit (Merck Millipore, Darmstadt, Germany), divided into small aliquots, and stored at −80 °C until use. Samples of pooled CS and NS collected from 12 and 10 fish, respectively, were used for passive immunization tests, electron microscopy, and purification of serum IgM. Individual serum samples were used for the serum bactericidal assay.

### 2.5. Passive immunization

Japanese amberjack (body weight 88-115 g; n = 5 for each group) were injected intraperitoneally with 1 mL of pooled NS or CS. One day after serum injection, fish were challenged with an intravascular injection of *I. seriolicida* suspension in BCM (OD600 = 0.05) via the caudal vein using a 25-gauge syringe. Groups of fish were maintained in 160-L tanks. Cumulative mortality was recorded for 10 days. Because it is difficult to isolate live *I. seriolicida* from dead fish (Y. Fukuda, personal communication), the presence of the bacteria in dead fish was confirmed by PCR analysis (Mitsui et al., 2004). Briefly, DNA was extracted from the spleen of dead fish using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. PCR assays specific for *I. seriolicida* were performed using KOD Fx (TOYOBO, Osaka, Japan), based on the original previously described protocol (Mitsui et al., 2004).

### 2.6. Electron microscopic analysis of serum-treated I. seriolicida

A total of 38 mL of log-phase bacterial culture (OD600 = 0.05) were incubated without shaking with 2 mL of pooled NS or CS for 5 min at 25 °C. Cells were then pelleted by centrifugation at 9,000 × *g* for 5 min and re-suspended in 500 μL of phosphate-buffered saline (PBS). Bacteria were fixed with 2% paraformaldehyde and 2% glutaraldehyde in cacodylate buffer (pH 7.4) for 1 h, stored in 2% glutaraldehyde in cacodylate buffer (pH 7.4) for 18 h, and then post-fixed with 2% osmium tetroxide for 3 h. For transmission electron microscopy (TEM), cells were dehydrated using an ethanol series and embedded in epoxy resin (Quetol-812, Nissin EM, Japan). Thin sections (70 nm) were prepared and stained with 2% uranyl acetate and lead citrate and examined using a JEOL JEM 1200EX (Jeol, Tokyo, Japan) transmission electron microscope. For scanning electron microscopy (SEM), cells were dehydrated using an ethanol series, which was then substituted with butyl alcohol, critical-point dried, sputter-coated with osmium tetroxide, and examined using an S-800 s (Hitachi, Tokyo, Japan) scanning electron microscope.

### 2.7. Analysis of serum bactericidal activity

For some experiments, serum complement was inactivated by heating at 45 °C for 30 min (Sakai, 1981). To block activation of the classical and alternative complement pathways, an equal volume of 8 mM ethylenediaminetetraacetic acid (EDTA)-PBS was added to CS 30 min before analysis. Inactivation of complement by heating or EDTA supplementation was previously verified using an erythrocyte hemolysis test with rabbit erythrocytes and serum obtained from Japanese amberjack immunized with rabbit erythrocytes (data not shown).

### 2.8. Purification of IgM for use in the serum bactericidal assay

Japanese amberjack IgM was purified from a pooled NS and CS sample using a Protein A mini kit (Pro-Chem, MA) according to the manufacturer's protocol. Eluted IgM was dialyzed against PBS and then stored at −80 °C until use. The dialyzed protein was electrophoresed on SDS-PAGE under reducing conditions, stained with Coomassie Brilliant Blue (EzStain Aqua, ATTO, Tokyo, Japan), to confirm the purification of IgM.

### 2.9. Serum bactericidal assay

The bactericidal activity of serum was measured using samples from 10 naïve and 10 convalescent fish. A culture of log-phase *I. seriolicida* (OD600 = 0.06) was centrifuged at 9,000 × *g* for 5 min. The cell pellet was then washed once with L15 medium and re-suspended in a small volume of L15 medium to adjust the OD600 to 0.85. The serum bactericidal assay was carried out in 1.5-mL plastic tubes. For the assay, 30 μL of NS, CS, or heat-inactivated CS or PBS was added along with 30 μL of PBS to 60 μL of I. seriolicida suspension, such that the serum was diluted to a final concentration of 25%. To assay the inhibitory effect of EDTA, 60 μL of EDTA-supplemented CS (described in the previous section) was added to 60 μL of *I. seriolicida* suspension. For some heat-inactivated CS samples, 30 μL of pooled NS and 60 μL of *I. seriolicida* suspension were mixed. To evaluate the role of serum IgM in the bactericidal activity, 30 μL of IgM (200 μg/mL) purified from NS or CS was added to 30 μL of NS, and then 60 μL of *I. seriolicida* suspension was added and mixed. The reaction mixtures were then incubated at 25 °C for 2 h with periodic shaking. Live cells were stained using a LIVE/DEAD *Bac*Light bacterial viability kit (Invitrogen, CA) according to the manufacturer's protocol. To remove unreacted fluorescent dye, cells were washed twice with PBS by centrifugation at 9,000 × *g* for

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