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Antibody Detection against Red Sea Bream Iridovirus (RSIV) in Yellowtail *Seriola quinqueradiata* Using ELISA

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ABSTRACT—FBS components in viral culture fluid interfere with the detection of specific antibody in immunized fish. In the present study, we developed a method to eliminate the interference of FBS using a model of red sea bream iridovirus (RSIV) vaccine. In an experiment using yellowtail antiserum against BSA, which is a major component of FBS in cell culture media, antibodies against BSA were completely absorbed via incubation of antiserum in 50% FBS at 25°C for 1 h. In antibody detection, ELISA with the sera of yellowtail surviving RSIV-challenge, antibodies against RSIV were detected, but those against FBS disappeared via the pre-treatment of the sera. Therefore, antibodies against RSIV are effectively detected using ELISA with the pre-treatment of fish sera.

Key words: antibody detection, ELISA, red sea bream iridovirus, yellowtail

Red sea bream iridovirus (RSIV) is the causative agent of lethal infections in over 30 marine fish species of fingerlings and market-sizes including red sea bream *Pagrus major*, yellowtail *Seriola quinqueradiata*, etc.[1,2]. RSIV is a large, icosahedral, cytoplasmic DNA virus measuring 200 to 240 nm in diameter and belongs to the genus *Megalocytivirus* in the family *Iridoviridae*. It is known that formalin-inactivated RSIV-culture fluid is effective to protect fish from RSIV disease (RSIVD)[3–5], and it is commercially available as a fish vaccine for RSIVD in Japan and Korea. Moreover, efficacy of recombinant protein vaccine, DNA vaccine and subunit vaccine has been reported[6–8]. To determine an establishment of specific immunity of the vaccinated fish, bioassay as a virus challenge test, sacrificing a lot of fish has been indispensable, and it doesn't always provide reproducible results due to fish size and viral infectivity titers. These problems seem to be complemented by immunological techniques to detect antibodies against RSIV in fish sera, but it has not been established in RSIVD so far.

Enzyme-linked immunosorbent assay (ELISA) is a useful and convenient immunological technique for detection of antigens or antibodies, particularly for routine screening involving large numbers of samples requiring high sensitivity, rapidity and low cost. Although it has been used for detection of antibodies against fish pathogenic bacteria and viruses[9–12], it was not applicable to all fish species due to low reproducibility and high background[13,14]. Recently, it was reported that fish immunoglobulin M (IgM) non-specifically adsorbed the blocking reagents on ELISA plate wells leaving a high background optical density and low reproducibility, furthermore, the non-specific adsorption of fish IgM was prevented by treatment of fish serum with 5% (w/v) skim milk solution[15]. In the case of antibody detection ELISA from the fish vaccinated with a formalin-inactivated viral culture fluid, we still have a problem relating to antibodies against fetal bovine serum (FBS) contained in the administered vaccine. In order to restrain this problem, it is necessary to purify viral particles as an ELISA antigen in methods to detect the prevalence of infectious hematopoietic necrosis (IHN) virus[16] or to remove antibodies against FBS from fish sera. However, purification of the capture antigens required complicated procedures and high cost.

In the present study, we demonstrated a procedure to reduce an antibody reaction against FBS from sera of fish immunized with RSIV-culture fluid by pretreatment of fish sera with FBS in detection ELISA for anti-RSIV antibodies.

Materials and Methods

Virus and cells

RSIV RSNag00, isolated from affected red sea bream at Nagasaki Prefecture in 2000, was cultured with grunt fin (GF) cells maintained at 25°C in basal medium Eagle (BME, Sigma) supplemented with 10% FBS, 100 μg/mL streptomycin and 100 IU/mL penicillin. Confluent GF cells were inoculated with RSIV of $10^{4.5}$ $TCID_{50}$ and cultured at 25°C for 2 weeks. After cytopathic effects (CPE) with enlarged cells appeared, the culture fluid was collected, centrifuged at 2,500 × *g* at 4°C for 5 min and stored at −80°C until use. Titration of RSIV infectivity was performed using 96-well microplates seeded with GF cells and virus titers were expressed as the 50% tissue culture infectious dose ($TCID_{50}$).

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Antisera against fish IgM

Rabbit antiserum against yellowtail IgM was prepared using a method described by Kim *et al.*[15]. Briefly, purified yellowtail IgM was mixed with Freund's incomplete adjuvant and injected subcutaneously into New Zealand white rabbits. A booster dose was administrated 4 times at 1 wk intervals. A blood sample was taken for antibody production analysis and then the final bleeding was performed.

Preparation of fish sera for ELISA

Immunization with bovine serum albumin fraction V (BSA)

Four yellowtail with 100 g of mean body weight (MBW) were reared in an aquarium with 300 L flowing UV-sterilized seawater at 25 ± 1°C, and labeled with pectoral fin chopping individually. The fish were injected intraperitoneally with 1.0 μg/100 μL/fish of BSA solution in phosphate buffered saline (PBS), and approximately 100 μL of blood was collected from each fish at 0, 3, 7, 14 and 21 days post injection. Sera were collected by centrifugation (2,000 × *g*, 10 min, 4°C) and stored at –20°C until use.

Immunization with RSIV-culture fluid

Seventy yellowtail with 48.5 g of MBW were reared in two separated aquaria with 300 L flowing UV-sterilized seawater at 25 ± 1°C (n = 50 and 20 in each). The fifty fish in the first aquarium were intramuscularly inoculated with 100-fold dilution of the RSIV-culture fluid at a dose of $10^{2.5}$ $TCID_{50}$/100 μL/fish, while the twenty fish in the remaining aquarium were inoculated with the same volume of 100-fold dilution of BME supplemented with 10% FBS. Fish in each group were reared for an additional 15 days to monitor the mortalities. Sera from survivors in each group were collected and subjected to detection of antibodies against RSIV using an ELISA system as described below.

Immunization with formalin-inactivated RSIV-culture fluid

A total of 120 yellowtail (MBW = 21.9 g) were reared in 2 aquaria (n = 60 in each) with 500 L flowing UV-sterilized seawater at 25 ± 1°C. Fish from each aquarium were injected intraperitoneally with 100 μL of formalin-inactivated RSIV prepared by adding formalin (0.3% v/v) and incubation at 4°C for 10 days according to the method of Nakajima *et al.*[3], or BME was injected for control fish. At 0, 4, 7, 10, 14 and 21 days post injection, 10 fish from each group were randomly selected to be sacrificed for blood samples, and the sera were subjected to detection of antibodies against RSIV.

Adsorption of anti-BSA IgM using FBS

Fish antiserum against BSA was diluted 40-folds with 5% skim milk solution containing different concentration of FBS (75, 50, 25, 12.5 and 6.25% [v/v]) and incubated at 25°C for 1 h or 16 h, because BSA is a main component of FBS. The treated sera were subjected to antibody detection ELISA to compare the remaining reactivity of anti-BSA IgM.

Antibody detection ELISA

Procedure of antibody detection ELISA was performed according to the modified method of Kim *et al.*[15] and Nishizawa *et al.*[17]. Briefly, RSIV-culture fluid was diluted 10-folds with distilled water as an antigen for detection of antibodies against RSIV, while 100-folds diluted FBS was prepared as the antigen for antibodies against BSA. Antigen solution was loaded to ELISA plate wells at 50 μL/well and fixed by drying at 25°C overnight. After being washed three times with phosphate buffered saline (PBS, pH 7.5) containing 0.05% Tween 20 (T-PBS), ELISA plate wells were blocked with 5% skim milk in PBS at 25°C for 1 h.

Yellowtail sera, diluted 40-folds with PBS containing 5% skim milk, were incubated at 25°C for 1 h, and loaded in duplicate ELISA plate wells at 50 μL/well. After being washed three times with T-PBS, the ELISA plate wells were incubated with rabbit antiserum against yellowtail IgM, diluted 500-folds with PBS containing 5% skim milk at 25°C for 30 min, followed by incubation with horseradish peroxidase (Dako)-conjugated swine serum against rabbit Ig, diluted 1,000-folds at 25°C for 30 min. The ELISA plate wells were washed three times with T-PBS, and loaded with 50 μL of substrate solution (1.0 mg/mL o-phenylenediamine, 0.03% H_2O_2, 100 mM Na_2HPO_4, 50 mM citric acid) to be incubated at 25°C for 30 min. After color development, the reaction was stopped with 2 N H_2SO_4 and the absorbance at 492 nm (OD_{492}) was read using a microplate reader (MTP-300, Corona).

Results and Discussion

A time-dependent change of antibody production was monitored in fish (Fig. 1). Antibodies against BSA were detected from sera 7 days after the injection, peaked at 14 days and decreased 21 days after the injection. Although the immunized fish showed a similar pattern of antibody response to BSA antigen, difference was observed in ELISA values among sera. This could be due to an individual difference among fish response to antigen. From these results, it is confirmed that specific antibodies are detected well by the antibody detection ELISA system based on the previous method[15,17]. The fish serum #2 collected 14 days after BSA-injection was selected and subjected to the following experiments for absorption of antibodies against BSA by FBS because it shows the highest ELISA values.

The anti-BSA fish serum (serum #2 in Fig. 1) was

treated with different concentrations of FBS to compare the remaining reactivity of anti-BSA IgM (Fig. 2). ELISA values of the sera without FBS treatment was more than 1.27, while the values decreased by increasing FBS concentration, and was less than 0.07 by treatment with more than 50% FBS. No significant difference was observed in the ELISA values between 1 h and 16 h incubations. In the case of fish vaccination with viral culture fluid, antibodies against not only viral antigens but also FBS included in culture medium will be induced, and an anti-FBS reaction in sera could be an impediment for detection of specific antibodies against virus antigens. This impediment is overcome using purified viral particles as ELISA antigens, but high cost and complicated procedures are required for viral purification. In the present study, it was demonstrated that treatment of fish sera in 50% FBS solution at 25°C for 1 h was enough to absorb the antibodies against BSA even if the fish antiserum contained high levels of antibodies against BSA.

Next, yellowtail were injected with RSIV-culture fluid to detect antibodies against RSIV (Fig. 3). The fish injected with RSIV began to die 11 days after the inoculation and the cumulative mortality was 55%, while no mortality was observed in the mock-challenged fish. Sera of every five survivors from RSIV- and mock-challenged groups were treated with FBS and subjected to antibody detection ELISA against RSIV. ELISA values of the sera from fish surviving RSIV-challenge ranged from 0.13 to 0.36 (average: 0.21), but those of fish surviving mock-challenge were less than 0.01 (average: 0.01). It was confirmed that ELISA values of the sera treated with FBS were clearly lower than those of the sera without FBS-treatment in both RSIV- and mock-challenged fish (data not shown), suggesting that antibodies against FBS in the fish sera could be completely absorbed by the FBS-treatment. It is therefore considered that the ELISA values observed in the RSIV-injected fish were due to antibodies against RSIV, demonstrating that the antibodies against RSIV were produced in the surviving fish and it could be detected using the present ELISA system.

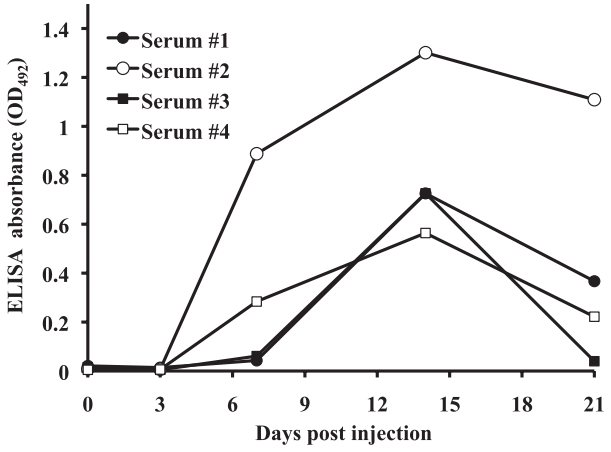

Fig. 1. A time-dependent change of antibodies against BSA in yellowtail. Fish were injected with 1.0 μg of BSA at day 0. FBS was used as an ELISA antigen.

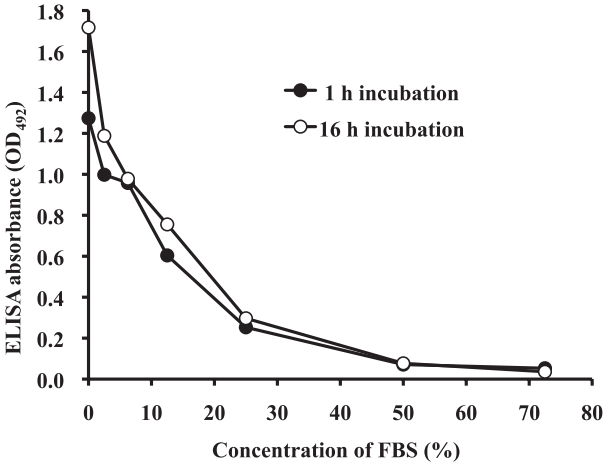

Fig. 2. Reduction of ELISA values of antibodies against FBS in yellowtail serum by FBS-treatment. Yellowtail serum against BSA was incubated with various concentrations of FBS for 1 h or 16 h. FBS was used as an ELISA antigen.

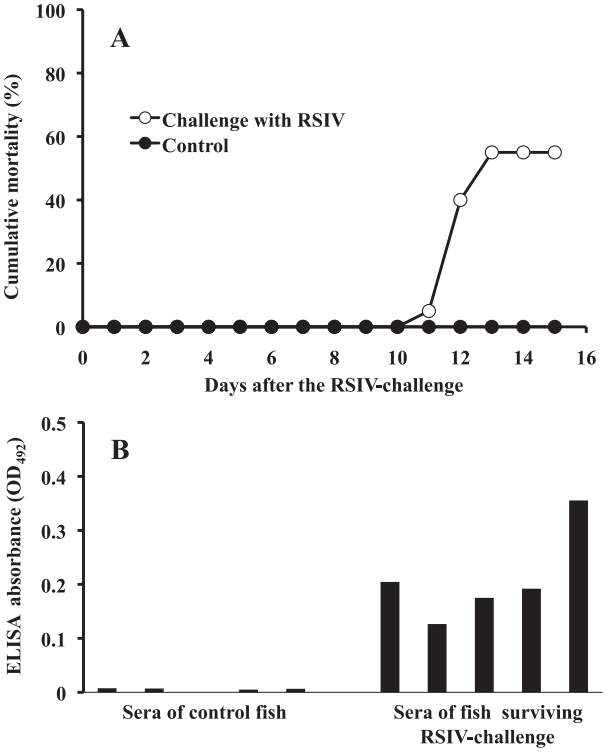

Fig. 3. Cumulative mortality of fish challenged with red seabream iridovirus (RSIV)-culture fluid (A) and ELISA values of antibodies against RSIV in sera of survivors from RSIV-challenge (B). Fish was injected intramuscularly with RSIV at $10^{2.5}$ $TCID_{50}$/fish or basal medium eagle (BME) at 100 μL/fish. Sera were collected from survivors 15 days after the RSIV-challenge.

In the sera periodically sampled from the fish administered formalin-inactivated RSIV-culture fluid, no antibody against RSIV was detected in those sera (data not shown). It was reported that protection against RSIVD was observed by the administration of formalin-inactivated RSIV corresponding to $10^{3.5}$ $TCID_{50}$/fish in red sea bream and $10^{6.5}$ to $10^{7.7}$ $TCID_{50}$/fish in other marine fishes[3–5], and commercial RSIV-vaccines contained more than $10^{8.4}$ $TCID_{50}$ according to an attached description. Unfortunately, RSIV-culture fluid used in the present study contained $10^{5.5}$ $TCID_{50}$/mL, which was quite lower than that of the previous studies and commercial vaccines. Thus this may be why no antibody against RSIV was induced in the immunized fish up to the detectable level for ELISA.

It is finally concluded that the antibody reaction to FBS in sera of fish immunized with RSIV-culture fluid was able to be absorbed by the treatment of the sera in 50% FBS, and antibodies against RSIV were detected in fish sera using the present ELISA system, even if the fish were administered RSIV-culture fluid. The present ELISA system could be expected to contribute to an immunological study on cultured viruses in host fishes.

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References

1) Matsuoka, S., K. Inouye and K. Nakajima (1996): *Fish Pathol.*, **31**, 233–234. 2) Kawakami, H. and K. Nakajima (2002): *Fish Pathol.*, **37**, 45–47. 3) Nakajima, K., Y. Maeno, J. Kurita and Y. Inui (1997): *Fish Pathol.*, **32**, 205–209. 4) Nakajima, K., Y. Maeno, A. Honda, K. Yokoyama, T. Tooriyama and S. Manabe (1999): *Dis. Aquat. Org.*, **36**, 73–75. 5) Nakajima, K., T. Ito, J. Kurita, H. Kawasaki, T. Itano, Y. Fukuda, Y. Aoki, T. Tooriyama and S. Manabe (2002): *Fish Pathol.*, **37**, 90–91. 6) Park, S. J., H. J. Seo, J. H. Son, H. J. Kim, Y. I. Kim, K. H. Kim, Y. K. Nam and S. K. Kim (2005): *J. Microbiol. Biotechnol.*, **15**, 873–879. 7) Caipang, C. M., I. Hirono and T. Aoki (2006): *Fish Shellfish Immunol.*, **20**, 597–609. 8) Tamaru, Y., M. Ohtsuka, K. Kato, S. Manabe, K. Kuroda, M. Sanada and M. Ueda (2006): *Biotechnol. Prog.*, **22**, 949–953. 9) Lorensen, K. and P. F. Dixon (1991): *Dis. Aquat. Org.*, **11**, 99–103. 10) Dixon, F. D., A. M. Hattenberger-Baudouy and K. Way (1994): *Dis. Aquat. Org.*, **19**, 181–186. 11) LaPatra, S. E. (1996): *Annu. Rev. Fish Dis.*, **6**, 15–28. 12) Watanabe, K., S. Suzuki, T. Nishizawa, K. Suzuki, M. Yoshimizu and Y. Ezura (1998): *Fish Pathol.*, **33**, 445–446. 13) Olesen, N. J., N. Lorensen and P. E. V. Jørgensen (1991): *Dis. Aquat. Org.*, **10**, 31–38. 14) Knopf, K., K. Naser, M. H. T. van der Heijden and H. Taraschewski (2000): *Dis. Aquat. Org.*, **43**, 39–48. 15) Kim, W. S., T. Nishizawa and M. Yoshimizu (2007): *Dis. Aquat. Org.*, **78**, 55–59. 16) Yoshimizu, M., Y. Hori, Y. Yoshinaka, T. Kimura and J. A. Leong (2001): In "OIE conference of risk analysis in aquatic animal health". OIE, Paris, pp. 237–241. 17) Nishizawa, T., I. Takami, Y. Kokawa and M. Yoshimizu (2009): *Dis. Aquat. Org.*, **83**, 115–122.