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# Antibody profiling using a recombinant protein–based multiplex ELISA array accelerates recombinant vaccine development: Case study on red sea bream iridovirus as a reverse vaccinology model

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ABSTRACT

Predicting antigens that would be protective is crucial for the development of recombinant vaccine using genome based vaccine development, also known as reverse vaccinology. High-throughput antigen screening is effective for identifying vaccine target genes, particularly for pathogens for which minimal antigenicity data exist. Using red sea bream iridovirus (RSIV) as a research model, we developed enzyme-linked immune sorbent assay (ELISA) based RSIV-derived 72 recombinant antigen array to profile antiviral antibody responses in convalescent Japanese amberjack (*Seriola quinqueradiata*). Two and three genes for which the products were unrecognized and recognized, respectively, by antibodies in convalescent serum were selected for recombinant vaccine preparation, and the protective effect was examined in infection tests using Japanese amberjack and greater amberjack (*S. dumerili*). No protection was provided by vaccines prepared from gene products unrecognized by convalescent serum antibodies. By contrast, two vaccines prepared from gene products recognized by serum antibodies induced protective immunity in both fish species. These results indicate that ELISA array screening is effective for identifying antigens that induce protective immune responses. As this method does not require culturing of pathogens, it is also suitable for identifying protective antigens to un-culturable etiologic agents.



## 1. Introduction

Recent advances in high-throughput sequencing technologies have accelerated genome-based vaccine development, also known as reverse vaccinology (RV). One difficult step in developing effective vaccines by RV is identifying antigens capable of inducing a protective immune response. In RV approaches, genes encoding either putative cell-surface or secreted proteins that could elicit an antibody response are identified using bioinformatics. However, evaluating large numbers of candidate antigens is burdensome. Efficient screening of antigens for antibody reactivity represents a short-cut in vaccine development. One approach for screening antibody reactivity involves the use of protein arrays, which are high-throughput analytical tools that provide data on protein interactions [1–3]. Using red sea bream iridovirus (RSIV) as a model, in the present study, we developed an RV approach coupled with an ELISA-based protein array to screen for RSIV protective antigens.

Viruses in the family *Iridoviridae* are members of the nucleocytoplasmic large DNA virus group. The *Iridoviridae* exhibit icosahedral symmetry and a linear genome that varies between 150 and 303 kb in length. The family consists of five genera: *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Lymphocystivirus*, and *Megalocytivirus* [4]. The megalocytiviruses are important fish pathogens, with most outbreaks reported to date occurring primarily in east and southeast Asia [5]. The genus *Megalocytivirus* was subdivided into the following three major groups based on phylogenetic analyses of the major capsid protein and ATPase genes: RSIV, infectious spleen and kidney necrosis virus (ISKNV), and turbot reddish body iridovirus (TRBIV) [5]. Recently, scale drop disease virus has been reported as a closely related virus showing low levels of identity to *Megalocytivirus* [6].

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A formalin-inactivated virus vaccine reportedly protects fish against RSIV [7], ISKNV [8] and TRBIV [9]. In Japan, formalin-killed vaccines are commercially available for use in several fish species. The fish that had been administrated either the convalescent serum or the serum of fish that had been immunized with a commercial vaccine was shown to significantly suppress mortality associated with RSIV [10] and ISKNV [8], indicating that serum antibodies play a primary role in immunity against iridovirus infection. Therefore, the protective antigen should be identifiable by analyzing humoral immunity.

Identifying protective antigens is important in both applied research, such as the development of recombinant vaccines, and basic research to reveal the nature of viruses, such as the poorly understood megalocytiviruses. In this study, we developed a 96-well plate-based ELISA array to screen for specific protective RSIV antigens. This platform was based on a glutathione *S*-transferase (GST)-capture sandwich ELISA consisting of 72 GST-fused RSIV-derived recombinant proteins generated using wheat germ cell-free technology. Serum antibody profiles were obtained using convalescent Japanese amberjack (*Seriola quinqueradiata*). *Escherichia coli*–derived recombinant vaccines were then developed to evaluate the protective effect of antigens identified using the ELISA array. The results indicated that this method is useful for identifying protective antigens.

## 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 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. Cell and virus culture

Grunt fin (GF) cells [11] were cultured at 25 °C in basal medium Eagle (BME, Gibco-BRL, Grand Island., NY) supplemented with 10% fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS), 100 IU/ml of penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 25 mM HEPES. The virus used for this study, RSIV strain RIE12-1, was isolated from diseased red sea bream using GF cells. Confluent GF cells grown in 75-$cm^2$ flasks were inoculated with virus suspension at 25 °C. Infected cells were continuously cultured until cytopathic effect was complete. Subsequently, the supernatant was collected and centrifuged at 2000 × *g* for 20 min. The viral suspension was stored in 1-ml aliquots at −80 °C and used for all experiments. Virus titer was determined using the 50% tissue culture infective dose ($TCID_{50}$) method [12].

### 2.3. Fish

Japanese amberjack (*S. quinqueradiata*) were bred at the Seikai National Fisheries Research Institute (Nagasaki, Japan) and National Research Institute of Aquaculture (Ooita, Japan). Greater amberjack (*S. dumerili*) were purchased from A-marine Kindai (Wakayama, Japan). There was no history of RSIV at either institute. Juvenile fish were transported to the National Research Institute of Aquaculture (Mie, Japan) and then reared in plastic tanks in sand-filtered seawater. Fish were anesthetized with 2-phenoxyethanol before treatment.

### 2.4. Serum preparation

A total of 20 Japanese amberjack (61.1 ± 14.0 g mean body weight) were injected intraperitoneally (i.p.) with RSIV culture fluid at a dose of $10^{4.3}$ $TCID_{50}$/100 μl/fish and reared in seawater at 25 °C. As a control, 10 Japanese amberjack were administered the same volume of BME supplemented with 10% FBS. Fish in each group were reared for an additional 21 days to monitor mortality. Serum was collected from survivors in each group and stored at −80 °C until use.

### 2.5. Antibody titration

ELISA antibody titer was measured at room temperature according to the modified method of Kwon (2010) [13]. RSIV culture supernatant and GF cell culture supernatant as a control were precipitated using polyethylene glycol. The precipitate was dissolved in carbonate-bicarbonate buffer (0.015 M sodium carbonate, 0.035 M sodium bicarbonate, pH 9.6) and adsorbed onto the wells of black 96-well plates (Thermo Scientific, Waltham, MA, USA). The wells were then blocked with 5% skim milk in Tris-buffered saline (TBS). To adsorb antibodies against FBS, Japanese amberjack serum was diluted 100-fold with TBS containing 50% FBS and 2.5% skim milk, incubated for 1 h on an orbital shaker, and then loaded in triplicate ELISA plate wells. After washing manually three times with TBST, anti-Japanese amberjack IgM monoclonal antibody (MAb) (500 ng/ml) [10] was added to each well and incubated for 1 h. The plate wells were again washed three times with TBST, and horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Southern Biotech, Birmingham, AL) was added to each well. Chemiluminescence signals were detected using HRP chemiluminescence substrate solution (Roche Diagnostics, Mannheim, Germany) on an Arvo Sx 1420 multilabel counter (Wallac, Turku, Finland). ELISA values are expressed as the ratio of the relative light units (RLU) of the culture supernatant of RSIV-infected GF cells to the RLU of the culture supernatant of uninfected GF cells, after subtraction of the RLU of the blank wells. Each value is the mean of triplicate measurements.

The neutralizing titer of Japanese amberjack serum was determined by incubating 2-fold serial dilutions of heat-inactivated serum with RSIV (100 $TCID_{50}$/ml) at 25 °C for 1 h, followed by incubation with GF cells for 8 days. The cytopathic effect was monitored by microscopic observation, and the dilution needed to produce 50% neutralization was calculated according to the Behrens-Karber's method.

### 2.6. Genomic analysis of RSIV strain RIE12-1

Virus genomic DNA extracted from the supernatant of a culture of RSIV strain RIE12-1 was used as the template for PCR amplification of entire regions of the virus genome using primers designed with reference to the genome sequence of RSIV genotype-I (accession number AB104413) to form a fragment of approximately 6 kbp, with approximately 1-kbp overlap at each end (Supplemental Table 1). PCR amplification was performed using KOD-plus-neo DNA polymerase (Toyobo, Osaka, Japan). The PCR products were electrophoresed on an agarose gel and extracted using a commercial kit (Promega, Madison, WI, USA). A mixture consisting of equal parts of each fragment was sequenced using a 454 GS FLX system (Roche Diagnostics). The resulting sequences were assembled using Newbler, ver. 2.9. Open reading frames (ORFs) were predicted using Gene Mark (http://exon.gatech.edu/GeneMark/), with default parameters.

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