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Reducing background optical density in enzyme-linked immunosorbent assay for detecting nervous necrosis virus (NNV)-specific IgM by immobilizing fish sera

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ABSTRACT

Enzyme-linked immunosorbent assay (ELISA) for detecting fish antibodies is problematic due to its low reproducibility and high background optical density (OD). In this study, we investigated problematic sources using nervous necrosis virus (NNV, a fish pathogenic virus belonging to the genus *Betanodavirus*) and sevenband grouper (*Hyporthodus septemfasciatus*) as a model for detecting specific fish IgM through ELISA. It was revealed that both fish IgM and mammalian immunoglobulins were non-specifically adsorbed to purified NNV particles. This could be the problematic source of the high background OD and low reproducibility in ELISA. ELISA values of naïve fish IgM non-specifically adsorbed to NNV particles immobilized onto ELISA plate wells ranged from 0.09 to 0.15 (high background OD). However, ELISA values of NNV antigens non-specifically adsorbed to naïve fish IgM immobilized onto ELISA plate wells were all < 0.03 (low background OD). Thus, we developed a sandwich ELISA by immobilizing fish sera. NNV-specific antibodies could be indirectly detected by detecting NNV antigens captured by fish IgM immobilized onto ELISA plate wells using anti-NNV serum. When anti-NNV and naïve fish sera were subjected to such sandwich ELISA, ELISA values of anti-NNV fish sera ranged from 0.24 to 2.48. Its reproducibility was sufficiently secured based on results obtained from five experiments performed on different days. Conversely, ELISA values of naïve fish sera were all < 0.02, suggesting that background OD was completely regulated. The present sandwich ELISA does not require antiserum against fish IgM, meaning that NNV-specific antibodies are detectable from any fish species using only one antiserum against NNV.

1. Introduction

Enzyme-linked immunosorbent assay (ELISA) is a useful and quantifiable technique for detecting antigens, particularly for routine screening involving large numbers of samples requiring high sensitivity, rapidity and low cost. ELISA is also widely used for the detection of specific antibodies against pathogens because it is important to determine if immunity has been established in vaccinated animals or animals with a history of infectious diseases. Several studies have used ELISA for antibody detection in aquatic animals (Jorgensen et al., 1991; Yoshimizu et al., 1992; Dixon et al., 1994; LaPatra, 1996; Höglund and Pilström, 1994, 1995; Watanabe et al., 1998; Swain and Nayak, 2003; Okuda et al., 2006; Kim et al., 2007, 2008, 2009; Takami et al., 2010). However, the use of ELISA for fish antibody detection can be problematic sometimes due to its low reproducibility, especially when microorganisms are used as antigens. This is partly due to high background optical density (OD) caused by non-specific reactions between fish antibodies and antigens (Olesen et al., 1991; Höglund and Pilström, 1994, 1995; Knopf et al., 2000; Kibenge et al., 2002; Guo and Woo, 2004). We have encountered such problem in ELISA for the detection of specific antibodies against nervous necrosis virus (NNV), a fish pathogen belonging to genus *Betanodavirus* within family *Nodaviridae* (Schneemann et al., 2005; Costa and Thompson, 2016), especially for the detection of specific IgM in fish immunized with cultured NNV suspension inactivated by formalin. Kim et al. (2007) have reported that fish IgMs are non-specifically adsorbed to blocking reagents for ELISA plate wells and that such non-specific adsorption can be suppressed by pretreatment of fish sera with a skim milk solution. Choi et al. (2014) have reported that binding of NNV antigens to ELISA plate wells can be inhibited by components in cell culture medium such as fetal bovine serum (FBS), amino acids and/or salts. Such inhibition for binding of NNV antigens can be improved by diluting cultured virus suspension ≥300-fold with distilled water. Although NNV-specific antibody detection ELISA has been improved by following those procedures, satisfactory level in background OD with reproducibility has not been achieved. Recently, it has been reported that antigenicity of

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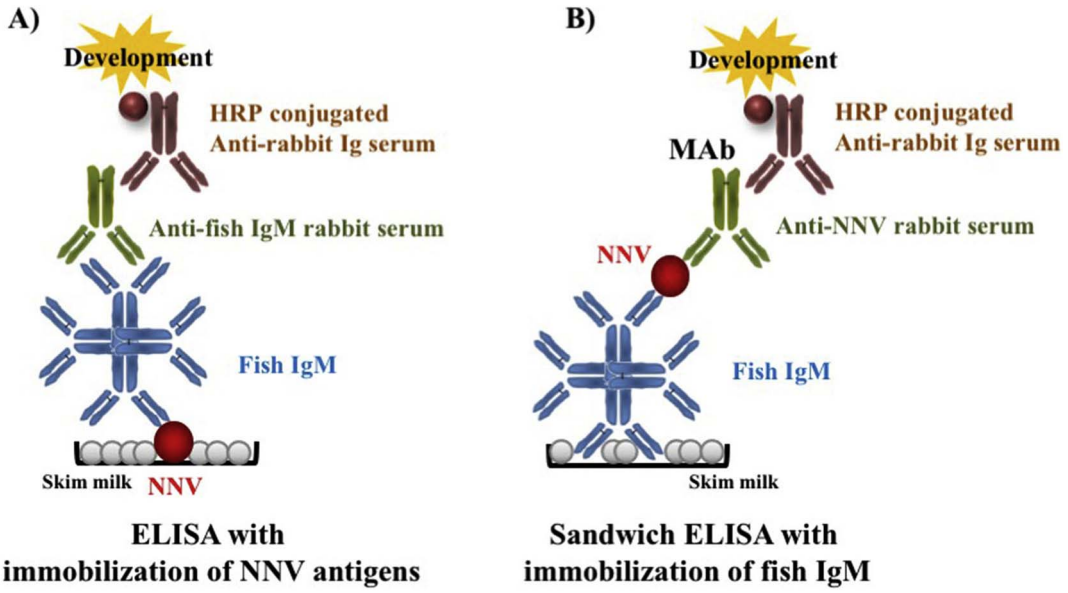

Fig. 1. Illustrations of enzyme-linked immunosorbent assay (ELISA) with immobilization of NNV antigens or fish sera for the detection of NNV-specific antibodies.

cultured NNV suspension can be vary due to changes in aggregation state of NNV particles and coat proteins (CPs) depending on using buffers with different ionic strength (Gye and Nishizawa, 2016a). This might also be one of the sources contributing to problems encountered in antibody detection using ELISA.

In the present study, using NNV as a model, we investigated sources for problems encountered in the detection of sevenband grouper (*Hyporthodus septemfasciatus*) IgM using ELISA. Furthermore, we developed a sandwich ELISA by immobilizing sevenband grouper sera to improve problems in specificity and background OD.

2. Materials and methods

2.1. Purification of standardized NNV suspension and purified NNV particles

NNV SgNag05 isolate belonging to RG genotype (Nishizawa et al., 1995, 1997) was cultured in SSN-1 cells at 25 °C. SSN-1 cells were maintained with Leibovitz's L-15 medium (Gibco) containing 10% (v/v) fetal bovine serum (FBS, Hyclone), 150 IU/ml of penicillin G, and 100 μg/ml of streptomycin (L-15_{10}). Cultured NNV was centrifuged at 12,000 × *g* for 20 min at 4 °C. The resulting supernatant was harvested and stocked as standardized cultured NNV suspension. Purification of NNV particles was performed using the cultured NNV suspension following published method (Gye and Nishizawa, 2016b). Briefly, NNV suspension was dialyzed in a tube made of Biotech cellulose ester (CE) membrane with molecular weight cut off (MWCO) of 10^6 (Spectrum Laboratories) in 15 mM Tris-HCl (pH 8.0) for 10 days. The dialyzed NNV suspension was subjected to anion-exchange chromatography using Hi-trap Q column (GE Healthcare). NNV antigens in the column were then eluted with a step-gradient of NaCl (500, 600, 700, and 1,000 mM in 15 mM Tris-HCl, pH 8.0). Fractions for chromatogram peak eluted with 700 mM NaCl were collected and concentrated by centrifugal ultrafiltration (10^4 MWCO, Vivaspin, Sartorius) according to the manufacturer's instruction. Concentrated NNV particles were adjusted to approximately 100 μg/ml in 15 mM Tris-HCl (pH 8.0) using ultraviolet absorption spectrometry (NanoDrop 1000, ThermoFisher Scientific) and stocked at − 80 °C until use (purified NNV suspension). Infectivity titers for both of the cultured NNV and purified NNV suspensions were $10^{9.3}$ $TCID_{50}$/ml, although the amount of NNV antigens contained in the cultured NNV suspension was 30-fold higher than that in the purified NNV suspension.

2.2. Rabbit antisera against NNV and sevenband grouper IgM

Two different rabbit antisera were used in this study: anti-NNV rabbit serum and anti-sevenband grouper IgM. Purification of sevenband grouper IgM was performed using IgM purification kit (ThermoFisher Scientific) according to the manufacturer's instruction. The purified NNV particles and fish IgM suspension were respectively emulsified with Freund's incomplete adjuvant (Sigma) and subcutaneously injected into New Zealand white rabbit. The rabbit was re-injected intravenously with the purified NNV or IgM suspension three times with 10-day interval as boosters. Final bleeding was performed at 3 days after the 4th injection.

2.3. Immunization of sevenband grouper with NNV

The cultured NNV suspension was mixed with the same volume of 1% formaldehyde solution and incubated at 25 °C for 2 days to inactivate NNV. The inactivated NNV suspension was intramuscularly injected to eight sevenband grouper (means body weight: 132 g) twice with a 10-day interval at 100 μl/fish. Fish were reared in aquaria with 50 L of seawater at 27 °C with 24 turnovers/day. At 7 days after the 2nd immunization, fish were sacrificed to obtained anti-NNV sevenband grouper sera. As control, eight of fish without immunization were also sacrificed to obtained naïve fish sera.

2.4. Ethics statement

Experiments using rabbits and fish were carried out in strict accordance with recommendations of the Institutional Animal Care and Use Committee of Chonnam National University (Approval No: CNU IACUC-YS-2015-3 and CNU IACUC-YS-2015-4, respectively).

2.5. Enzyme-linked immunosorbent assay (ELISA) for detecting NNV-specific sevenband grouper IgM

2.5.1. ELISA with immobilization of NNV antigens

The procedure of ELISA with immobilization of NNV antigens is illustrated in Fig. 1A. Two different NNV antigens (purified NNV and cultured NNV suspensions) and two negative controls (L-15_{10} and PBS) without NNV antigens were used. Purified NNV suspension was diluted 10-fold with sterilized deionized water (DIW). Cultured NNV suspension and L-15_{10} were diluted 300-fold with DIW. Diluted antigens were