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Research Article

Generation of Monoclonal Antibodies against Major Capsid Protein (MCP) of Nervous Necrosis Virus (NNV)

Suphattra Kamsamarn[1], Siwaporn Longyant[1,4], Saengchan Senapin[2], Akapon Vaniksampanna[3], Orapan Manajit[4] and Parin Chaivisuthangkura[1,4*]

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ABSTRACT

Viral encephalopathy and retinopathy (VER), a serious disease that affects several species of fish all over the world, is caused by nervous necrosis virus (NNV). In this study, two monoclonal antibodies (MAbs), namely 5C1 and 5C4, were generated from a mouse immunized with recombinant major capsid protein (MCP) of red-spotted grouper nervous necrosis virus (RGNNV). These MAbs displayed immunoreactivity against MCP and culture fluid of NNV-infected E-11 cell culture tested by dot blotting. Western blot analysis against recombinant MCP and the culture fluid of E-11 cells infected with NNV revealed immunoreactivity at approximately 63 and 37 kDa, respectively. Isotyping test revealed that all the MAbs were IgG2a. According to immunohistochemistry analysis, the MAbs immunoreactivities staining were found in viral assembly sites in the cytoplasm of targeted tissues such as gills and eye of NNV-infected Asian sea bass. The MAbs did not display any cross-reactivity with the recombinant capsid proteins of other fish viruses, including the infectious spleen and kidney necrosis virus (ISKNV), scale drop disease virus (SDDV), tilapia lake virus (TiLV), or other bacterial species commonly found in diseased fish. The immunoreactivity was observed when the MAbs were used for NNV detection by dot blotting in NNV-infected fish as verified by RT-PCR. These results indicated that the MAbs were useful in the development of more specific rapid and simple diagnostic technique for NNV infection in the future.

Keywords: Nervous Necrosis Virus, NNV, Major capsid protein, Monoclonal antibody

[1] Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok 10110, Thailand
[2] National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathum Thani 12120, Thailand
[3] Innovative Learning Center, Srinakharinwirot University, Bangkok 10110, Thailand
[4] Center of Excellence in Animal, Plant and Parasite Biotechnology, Srinakharinwirot University, Bangkok 10110, Thailand
*Corresponding author, email: parin@g.swu.ac.th

Introduction

The first description of viral encephalopathy and retinopathy (VER) was published in 1980s. Infections in fish have been continuously reported for the past 30 years and caused mass mortality in over 50 fish species [1]. The necrosis virus (NNV) belongs to the family Nodaviridae, a non-enveloped virus with a diameter of 25–30 nm. The genome size of NNV is approximately 4500 nucleotide (nt) and consist of two single-stranded positive-sense RNA molecules known as RNA1 (3103 nt) encoding a RNA-dependent RNA polymerase (RdRp) and RNA2 (1433 nt) encoding a 37 kDa capsid protein. Additionally, the 3'-end of RNA1 was utilized to synthesize RNA3, a subgenomic RNA that codes for the non-structural viral proteins B1 and B2, which are not packaged into virions [1]. The disease is characterized by neurological disorders associated with vacuolation and necrosis of the central nervous system. Effects of the disease may relate to species, age and temperature of the environment. The clinical signs of disease include abnormal swimming behavior, swim bladder hyperinflation, pale or dark body colour, and loss of appetite are commonly observed among NNV-affected fishes [2]. The host's developmental stage has an impact on the mortality rate. When larval stages are affected, the highest mortality often reaching 100% is observed. Whereas, in juveniles and older fish, lower losses have generally been reported [3].

Several techniques and tools for NNV diagnosis have been developed in the years since the first report of NNV to control the disease and protect NNV infection. In the early stages of an outbreak, observing the clinical signs is the most important part of diagnosis of NNV disease. After contracting NNV, the fish exhibits behaviours such as a loss of swimming activity, developing dark skin and often showing loss of equilibrium and lying down at the bottom and dying [4]. Under a light microscope, histopathological studies revealed vacuolation pyknosis and necrosis of the central nervous system, as well as basophilia of affected cells, which are the most common lesions and are remarkably consistent across species. Histopathological studies are simple and easy to detect lesions in tissue samples. Additionally, transmission electron microscopy can be used to examine the morphology of infected cells in various tissues and organs as well as virus particles. However, both techniques are less accurate and unreliable in confirming NNV infection in fish. In recent years, numerous molecular technologies have been developed, and in 1994 Muroga et al. published the first reverse transcription polymerase chain reaction (RT-PCR) protocol that targeted to the RNA2 region of the striped jack nervous necrosis virus (SJNNV) [5]. However, the existence of genetic diversity did cause some identification problems. Dalla Valle et al. published a new RT-PCR plus nested PCR assay targeted to the highly conserved region of the coat protein gene instead of the T4 region of RNA2 [6]. This technique can avoid false-negative results and requires a minimum of operational steps, reducing the risk of sample contamination. Dalla Valle et al. also developed real-time quantitative RT-PCR assays (qRT-PCR), which are highly sensitive and efficient tools for betanodavirus detection and quantification [7]. After that, Panzarin et al. developed sensitive qRT-PCRs that could detect viral titers as low as 10 TCID 50/mL [8]. Nonetheless, qRT-PCR requires more expensive equipment and operator expertise than the other PCR-based technique. This

technique is unsuitable for small laboratories to detect NNV infections for the purpose of assisting the aquaculture industry.

Immunoassays based on polyclonal or monoclonal antibodies (MAbs) have higher speed, comparable sensitivity and specificity with low cost such as indirect fluorescent antibody technique (IFAT), enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry (IHC). However, the operational processes for IFAT and IHC techniques are complicated and require a lot of expertise. Additionally, ELISA has low reproducibility and a high background optical density (OD), which are caused by non-specific interactions between polyclonal antibodies and NNV particles. Therefore, it is necessary to develop a simple, more effective technique for detecting NNV that is suitable for people who are not proficient in scientific instruments and can be used in the field. MAbs can be used to investigate host-virus interactions and infection mechanisms as well as to determine the organs that a virus is targeting. In 2022, Qin et al. generated five monoclonal antibodies, which were characterized based on their reactivity and specificity to the red-spotted grouper nervous necrosis virus (RGNNV) antigen [9]. The monoclonal antibodies showed a high binding affinity to the RGNNV, as evidenced by the low dissociation constant [10] values obtained by ELISA.

In this study, the red-spotted grouper nervous necrosis virus (RGNNV) major capsid protein was cloned, expressed, and used as an immunogen for MAbs production. Two MAbs were produced and recognize the RGNNV coat protein, and they were used in numerous types of immunodetection techniques to identify NNV in Asian sea bass. These MAbs can be further developed into simple test kits, such as ELISAs for quantitative analysis and immunochromatographic strip tests that are suitable to use by farmers in cultured areas.

Materials and methods

PCR confirmation of NNV-infected fish

Total RNA was extracted from fish tissue using a High Pure viral nucleic acid kit (Roche Molecular Biochemical). The NNV was detected with RT-PCR method as published by Dalla Valle et al. [6]. A pair of primer (VNNV1: ACA CTG GAG TTT GAA ATT CA, VNNV2; GTC TTG TTG AAG TTG TCC CA) targeting the MCP gene of NNV was used. The RT-PCR conditions consisted of cDNA synthesis at 50 °C for 30 s, pre-denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 57 °C for 30 s and extension at 68 °C for 1 min then final extension at 68 °C for 5 min. The expected PCR amplicon size was 605 bp. After that, PCR product from the first step was used as a template for nested PCR with a pair of primer (VNNV3; ATT GTG CCC CGC AAA CAC and VNNV4; GAC ACG TTG ACC ACA TCA GT) and conditions consisted of initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 1 min, then final extension at 72 °C for 10 min. The expected PCR amplicon size of nested-PCR was 255 bp. The PCR amplicon was sequenced and analyzed by Blastn analysis.

Preparation of the recombinant GST-MCP protein

The PCR amplicon was initially performed using synthecized cDNA of partial coat protein gene from RGNNV (Genbank accession no.: JX402858.1) as the template with a pair of primers (NNVF-pGEX: GGA ATT CGC GAC CAC CAA GGC CGC CAA T, and NNVR-pGEX: CCT CGA GTT AGT TTT CCG AAT CAA CCC TG), designed based on the conserved region of the partial coat protein of RGNNV. These primers were added with the *EcoR*I and *Xho*I restriction sites (underlined) at their 5' ends, respectively. The PCR amplification conditions were 1 cycle of 94 °C for 2 min, 35 cycles of 94 °C for 15 s, 54 °C for 15 s, 68 °C for 15 s and 1 cycle of 72 °C for 20 s. PCR products were analyzed by 2% agarose gel electrophoresis and expected band was gel purified by using NucleoSpin® Gel and PCR Clean-up Kit (MACHEREY-NAGEL, GmbH & Co. KG, Düren, Germany). The purified PCR products were cloned into pCR™-Blunt system (Invitrogen, Thermo fisher scientific, Carlsbad, CA, USA). The ligation mixture was incubated overnight at 16 °C and then transformed into *E. coli* strain TOP10 for plasmid propagation. Next, the plasmids were digested with a restriction enzyme and the PCR amplicon was cloned into pGEX-6P-1, followed by transformation into *E. coli* strain BL21 for the expression of the recombinant GST-MCP protein.

E. coli BL21 with recombinant plasmid pGEX-MCP was cultured in Luria-Bertani (LB) broth to exponential phase and induced by adding 1 mM isopropyl β-D-1-thiogalactosidase (IPTG) for 4 h. The bacterial cells were harvested by centrifugation at 3000 *x g* for 20 min, and the pellet was resuspended in a buffer containing 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea at pH 8 and 1 mM phenylmethylsulfonyl fluoride (PMSF). The suspended solution was sonicated until a clear lysate was obtained and analyzed by SDS-PAGE and visualized after staining with Coomassie brilliant blue R-250. The induced bacterial lysate was separated by 15% SDS-PAGE. After the gel was immersed in 0.3 M KCl for 20 min, the recombinant protein band of GST-MCP was cut and immersed in 0.1% SDS for overnight. After that, the protein solution was eluted in Towbin buffer (25 mM Tris, 20% (v/v) methanol, 192 mM glycine, pH 8.3) with a Transblot apparatus (BioRad) at 50 V for 10 h in a dialysis bag. The eluted protein was then dialyzed in deionized water and concentrated using a vacuum concentrator (Savant). Protein concentration was determined by the Bradford assay [11]. The recombinant protein solutions were adjusted to 1.0 mg/mL, divided into small aliquots and stored at -70 °C.

Preparation of viral sample from RGNNV-infected E-11 cells

For viral sample preparation, 1 mL of culture fluid from RGNNV-infected E11 cells (E11 is a clone of the SSN-1 cell line, fibroblast cells derived from striped snakehead, *Ophicephalus striatus*) from Center of excellence for shrimp molecular biology and biotechnology (CENTEX shrimp) provided by Dr. Saengchan Senapin was centrifuged at 20000 *x g* at 4 °C for 2 h. The precipitant was then resuspended in 50 µL of phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4) at 4 °C. The protocol was adapted from Taweesak Klinkong [12]. The suspended

solution was obtained, analyzed using SDS-PAGE, and then visualized following Coomassie brilliant blue R-250 staining.

Immunization

All experimental procedures involving animals were approved by the Srinakharinwirot University Animal Care and Use Committee, Thailand with the approval number COA/AE-001-2565. Four Swiss mice were immunized intraperitoneally (i.p.) with 100 µL of 1 mg/mL purified GST-MCP mixed with 15 µL of RGNNV-infected E11 cell culture fluid, emulsified with an equivalent volume of Freund's complete adjuvant (FCA, Sigma). After two weeks, the mice were injected with a similar mixture using Freund's incomplete adjuvant (Sigma) instead of FCA. Booster immunizations were then given three times at two-week intervals. One week after the fourth immunization, the mice's antisera were collected by collecting blood from the orbital sinus and centrifuging at 3500 *x g* for 10 min. The antisera were tested against lysates of *E. coli* containing pGEX-MCP, viral samples from cell culture fluids, and homogenates from uninfected and RGNNV-infected Asian sea bass (*Lates calcarifer*) using Western blotting. The mouse that showed the best immunoreactivity was further immunized with the same proteins mixed with incomplete Freund's adjuvant three days before hybridoma production.

Monoclonal antibody production

The cell fusion protocol used in this study was modified from the method firstly described by Kohler and Milstein (1976). In brief, spleen cells were collected from the immunized mouse and fused with P3X myeloma cell line using 50% polyethylene glycol (PEG; Sigma, USA). The cells were plated onto 15 microculture plates (96 wells/plate). After two weeks, the culture fluids from hybridomas clone were selected by dot blotting against lysate of pGEX-MCP (GST-MCP) mixed with culture fluid of RGNNV-infected E11 cells as a positive control and mixture of lysate of pGEX-6P-1 (GST) and culture fluid of uninfected E11 cells as a negative control. Western blotting was then used to further screen the hybridomas that had positive results. Then, using the limiting dilution method, the positive clones for dot blotting and Western blotting were recloned three times and stored in liquid nitrogen. Mouse immunoglobulins class and subclass were determined using the sandwich ELISA technique and the Mouse MonoAb ID Kit (HRP) (Zymed, San Francisco, CA, USA).

Monoclonal antibody characterization

Specificity testing

For dot blotting, the lysates of *E. coli* harboring pGEX-MCP, GST- infectious spleen and kidney necrosis virus (ISKNV), and GST- scale drop disease virus (SDDV) and *E. coli* containing pET15b His-Tilapia lake virus (TiLV), and culture fluid of RGNNV-infected and −uninfected E11 cells were applied at

1 µL/spot to each square marked on nitrocellulose membranes. The membranes were incubated at 60 °C for 10 min, blocked with 5% blotto (5% nonfat dry milk and 0.5% Triton X-100 in PBS) for 5 min, then washing three times with PBS and incubated with hybridoma culture fluid diluted to 1:20 in 1% blotto for 4 h. After washing with PBS, the membranes were incubated with horseradish peroxidase labeled, goat anti-mouse, gamma immunoglobulin heavy and light chain specific antibody (GAM-HRP, Bio-Rad) at 1:1,500 dilution for 3 h. After an intensive washing with a blocking solution, the membranes were developed using immunoperoxidase method. The developed process involved incubating the membrane in a substrate solution containing 0.03% diaminobenzidine (DAB), 0.006% hydrogen peroxide, and 0.05% cobalt chloride in PBS for 5 min [13].

Western blotting process was used for the second screening of MAbs, the lysates of *E. coli* containing pGEX-6P-1 (GST), pGEX-MCP, purified recombinant GST-MCP protein, culture fluid of uninfected E11 cells, culture fluid of RGNNV-infected E11 cells, homogenates of RGNNV-uninfected fish and RGNNV-infected fish were used as antigens and were separated with 15% SDS-PAGE [14] at 70 V for 2 h. The proteins were transferred onto a nitrocellulose membrane using a Transblot apparatus. (BioRad). After blocking the membranes for 10 min with a 5% blocking solution, it was incubated for 5 h at RT with a hybridoma-conditioned medium at 1:20 dilution or mouse anti-MCP antiserum. After washing with 0.1% blocking solution, the membrane was incubated with GAM-HRP at a dilution of 1:2500 for 3 h and processed as described in dot blotting method.

Cross-reactivity testing

The cross-reactivity of MAbs was determined using the dot blotting assay. Heat-killed bacteria suspension was adjusted to a concentration of 10^8 CFU/mL (1 µL/spot). The bacteria are *Aeromonas caviae*, *Aeromonas hydrophila*, *Aeromonas sobria*, *Aeromonas veronii*, *Flavobacterium columnare*, *Pseudomonas aeruginosa*, *Photobacterium damselae*, *Streptococcus agalactiae*, *Streptococcus iniae*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. The lysate of *E. coli* containing GST-MCP were used as a positive control. All antigens were spotted onto nitrocellulose membranes and blocked with 5% blotto for 5 min. After being washed with PBS and incubated with fluid from a hybridoma culture, the reaction was developed using immunoperoxidase method as described above.

Epitope overlapped testing

The epitope of overlapping among MAbs was detected by indirect ELISA. Briefly, the microculture plates were coated with 50 µL of 0.5 µg/mL purified recombinant GST-MCP protein and incubated in humidified box at 4 °C overnight. Then the wells were washed four times with 200 µL of 0.5% blotto, and then incubated with 50 µL of 5% blotto at room temperature for 30 min. Then, 50 µL of diluted MAbs (1:20 in 5% blotto) was added to the wells and incubated at RT for 4 h. After washing four times with 200 µL of 0.5% blotto, 50 µL of diluted 1:1500 in 1% blotto of secondary antibody tracer (HRP conjugated Goat anti-mouse IgG) was added in wells and incubated at RT for 4 h. After washing four times with 200 µL of 0.5% blotto and 200 µL of PBS, the 100 µL enzyme substrate (1 mg/mL of *o*-phenylenediamine and 0.006% H_2O_2 in citrate buffer pH 4.5) was then added, and incubated for 5 min at room temperature. Reactions were stopped by addition of 100 µL per well 1 N H_2SO_4 and plates were read at 490 nm by a ELISA reader. The optical density (OD) in the well containing two different MAbs is higher than in the well containing each MAbs, indicating that the two MAbs recognize various antigen epitopes.

Immunohistochemistry

Naturally RGNNV-infected fish samples were processed for indirect immunoperoxidase staining using MAbs that were specific to NNV at 1:100 dilution. Then, GAM-HRP was diluted 1:1000 and incubated with each slide for 2 h after washing four times with PBS. Immunoperoxidase method was visualized by incubating for 5 min in PBS with 0.03% DAB and 0.006% hydrogen peroxide. The preparations were counterstained with hematoxylin and eosin Y (H&E), dehydrated in a graded ethanol series, cleaned in xylene, and mounted in Permount. Brown colouring was used to show that pink cytoplasm had positive immunoreactivity.

Comparative sensitivity detection of monoclonal antibodies using dot blotting and PCR in RGNNV-infected fish samples.

The purified recombinant GST-MCP protein, originally at a concentration of 35 µg/mL, was diluted in a 10-fold serial dilution with PBS. Meanwhile, the culture fluid from RGNNV-infected E11 cells was diluted 2 folds with PBS. The resulting samples were then spotted onto nitrocellulose membranes and subjected to dot blotting using NNV-specific MAbs. The lowest concentrations of GST-MCP protein and RGNNV-infected E11 cell culture fluid that exhibited reactivity with the MAbs, visibly detectable to the naked eye following immunoperoxidase staining were considered as sensitivity.

To confirm the status of ten homogenates of normal fish and ten homogenates of RGNNV-infected fish, the fish samples were subjected to PCR analysis. The homogenates were boiled with 10% SDS for 1.30 min to denature the antigens, which were then used to assess the specificity of the MAbs through dot blotting. The purpose of the antigen process was to evaluate the effectiveness of MAbs in minimizing non-specific or background signals.

For the PCR analysis, viral nucleic acid was extracted from the culture fluid of RGNNV-infected E11 cells and diluted 10 folds before being used as a template for PCR. The sensitivity of the NNV-

specific MAbs was compared to that of the RT-PCR assay specific to the MCP gene, as previously described by Dalla Valle et al. [6].

Results

Major capsid protein gene expression

The expression of the GST-MCP recombinant protein was induced using IPTG, and its success was confirmed by SDS-PAGE stained with Coomassie Brilliant Blue R-250 (Figure 1A, lanes 1 to 4). Following the hybridoma production from the mice with the highest immunoreactivity, two MAbs (5C1 and 5C4) were generated. These MAbs could detect a protein of approximately 63 kDa in the lysate of *E. coli* containing pGEX-MCP plasmid and purified recombinant GST-MCP protein when tested with Western blotting (Figure 1 B and C, lanes 3 and 4). Both MAbs could bind to 37 kDa proteins in the culture fluid of RGNNV-infected E11 cells and homogenates of RGNNV-infected fish, where the molecular mass corresponded to the MCP of RGNNV (Figure 1 B and C, lanes 6 and 8). Conversely, the MAbs demonstrated no immunoreactivity to the lysate of *E. coli* strain BL21, GST, culture fluid of uninfected E11 cells, or homogenates of uninfected fish. The putatively degraded GST-MCP protein in bacterial lysate was observed as lower immunoreactive bands (Figure 1 B and C, lane 3).

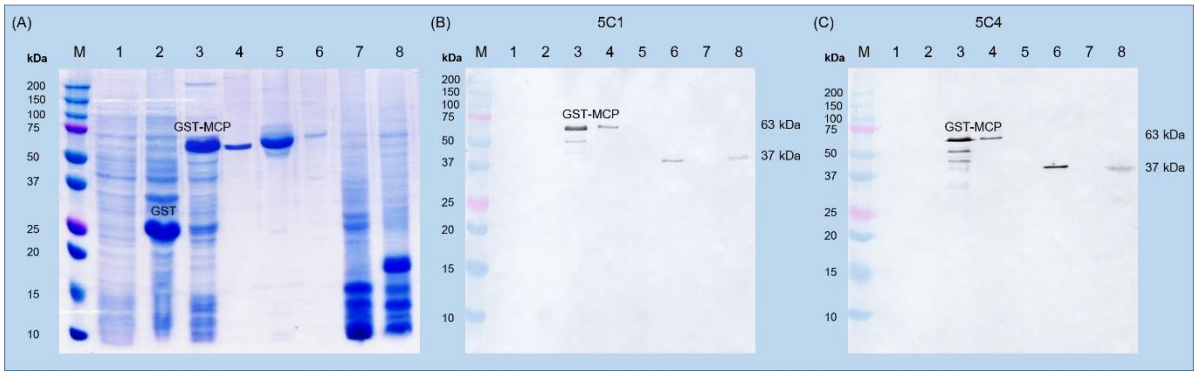

Figure 1 SDS-PAGE (A) and Western blotting of monoclonal antibodies specificity (B and C). The nitrocellulose membrane was treated with MAb 5C1 (B) and 5C4 (C). Lane: 1; lysate of *Escherichia coli* strain BL21; Lane 2: lysate of *E. coli* containing pGEX-6P-1 plasmid (GST); Lane 3: lysate of *E. coli* containing pGEX-MCP plasmid; Lane 4: purified GST-MCP protein; Lane: 5 culture fluid of uninfected E11 cell; Lane 6: culture fluid of RGNNV-infected E11 cells; Lane 7: homogenate of uninfected fish; Lane 8: homogenate of RGNNV-infected fish; Lane M: standard protein marker.

Monoclonal antibody characterization

The positive clones were screened using culture fluid of uninfected E11 cells, culture fluid of RGNNV-infected E11 cells, lysate of *E. coli* containing pGEX-6P-1 plasmid (GST), lysate of *E. coli* containing pGEX-MCP plasmid by dot blotting. Two clones (5C1 and 5C4) were selected for their ability to bind to culture fluid of RGNNV-infected E11 cells and GST-MCP (Figure 2, row a). An isotyping test revealed that all MAbs were IgG2a isotypes. Epitope overlapping was determined using

indirect ELISA, and the results indicated that all MAbs may be binding to the same epitope, as there was no increase in OD. values for each combination of MAbs when compared to a single MAbs. To determine specificity of MAbs against *E. coli* containing GST-ISKNV, GST-SDDV and His-TiLV. All of the MAbs developed in this study had no cross-reactivity with recombinant proteins from other virally infected fish (Figure 2, row b). Furthermore, it was observed that all MAbs exhibited no immunological cross-reactivity with twelve bacterial species that are commonly encountered in fish. (Figure 2, rows c to e). As a result, these MAbs have the potential to detect RGNNV in fish samples.

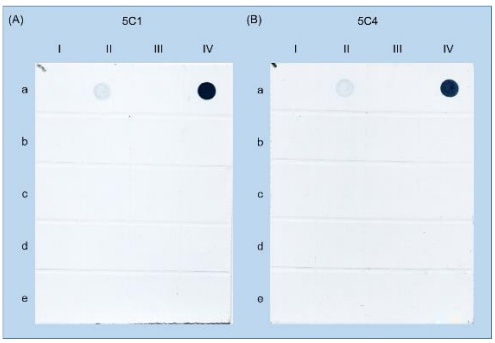

Figure 2 Specificity and cross-reactivity of monoclonal antibodies (MAbs) were tested by dot blotting. Culture fluid of uninfected and RGNNV-infected E11 cells, lysates from *E. coli* and bacterial lysate was at approximately 10^8 CFU/mL were spotted on a nitrocellulose membrane and treated with MAbs 5C1 (A) and 5C4 (B).

Row a. culture fluid of uninfected E11 cells (I), culture fluid of RGNNV-infected E11 cells (II), lysate of *E. coli* containing pGEX-6P-1 plasmid (GST) (III), lysate of *E. coli* containing pGEX-MCP plasmid (IV).

Row b. lysates of *E. coli* containing GST-ISKNV (I), GST-SDDV (II), Histidine (His) (III), His-TiLV (IV).

Row c. *Aeromonas caviae* (I), *A. hydrophila* (II), *A. sobria* (III), *A. veronii* (IV).

Row d. *Flavobacterium columnare* (I), *Pseudomonas aeruginosa* (II), *Photobacterium damselae* (III), *Streptococcus agalactiae* (IV).

Row e. *Streptococcus iniae* (I), *Vibrio cholerae* (II), *V. parahaemolyticus* (III), *V. vulnificus* (IV).

Immunohistochemical analysis of MAb specificity.

Dot blotting and Western blotting from the screening process revealed that MAb 5C4 displayed a stronger immunoreaction compared to that of 5C1. Consequently, MAb 5C4 was chosen for subsequent immunohistochemical analysis. The immunohistochemical analysis utilizing MAb 5C4 revealed immunoreactivity within the cytoplasm of infected cells in targeted organs, such as gill and eye of RGNNV-infected fish. This immunoreactivity presented as a distinct staining pattern, as depicted in Figure 3

(column I, rows A and B). Characteristic VNN lesions with swollen cells containing viral particles were seen in the gills as depicted in Figure 3 (columns I and II, row A), and there was a slight vacuolation in the retinal layers of the eye, as shown in Figure 3 (columns I and II, row B). Additionally, no cross-reactivity was observed when the MAbs were tested against tissues from uninfected fish, as illustrated in Figure 3 (column III).

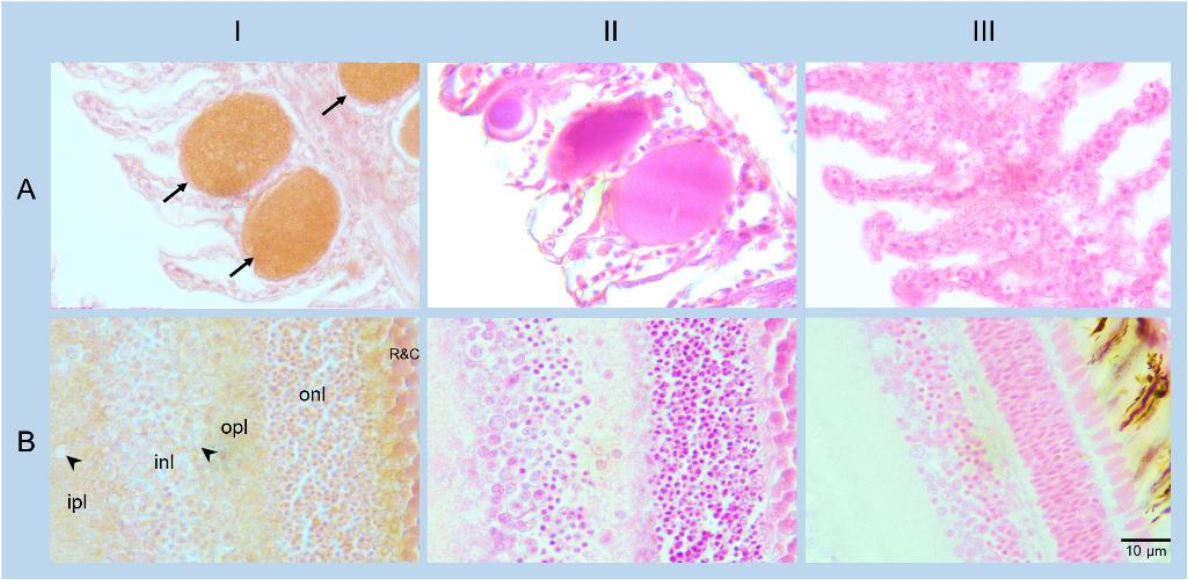

Figure 3 Immunohistochemical analysis was conducted to evaluate the specificity of MAbs. Column I: RGNNV-infected fish tissues treated with MAb 5C4 displayed strong immunoreactivities in infected cells by brown staining in the cytoplasm of the gills (A) (black arrow) and eye (B). Additionally, the presence of vacuolation was detected (black arrow head). Column II: the RGNNV-infected fish tissues, and Column III: the uninfected fish tissue were H&E staining reactions. R&C, rods and cones; onl, outer nuclear layer; opl, outer plexiform layer; inl, inner nuclear layer; ipl, inner plexiform layer [15]. Scale bar = 10 µm.

Comparative sensitivity detection of monoclonal antibodies using dot blotting and PCR in RGNNV-infected fish samples.

The culture fluid of RGNNV-infected E11 cells used for sensitivity detection by dot blotting was used for nucleic acid extraction in the instance of NNV detection by RT-PCR. The PCR product at 605 bp was found at a 10^{-7} dilution (Figure 4A). To determine sensitivity using a dot blot test, the MAbs namely, 5C1 and 5C4 had detection sensitivity against culture fluid of RGNNV-infected E11 cells and purified recombinant GST-MCP protein at 1:128 and 0.273 µg/mL, respectively (Figure 4B and 4C). Moreover, the sensitivities of all MAbs were inferior to that of PCR.

To validate the efficiency of the MAbs for NNV-infected fish detection by dot blotting compared to RT-PCR. Dot blotting was performed using homogenates from both uninfected fish and RGNNV-infected fish, as confirmed by RT-PCR according to the protocol described in section 2.1. The inspection

found that all NNV-infected fish samples demonstrated positive results by RT-PCR (Figure 5A) and dot blotting assay by using MAbs 5C1 and 5C4 (10/10) (Figure 5B), while the NNV-uninfected fish samples gave negative results (10/10) (Figure 5).

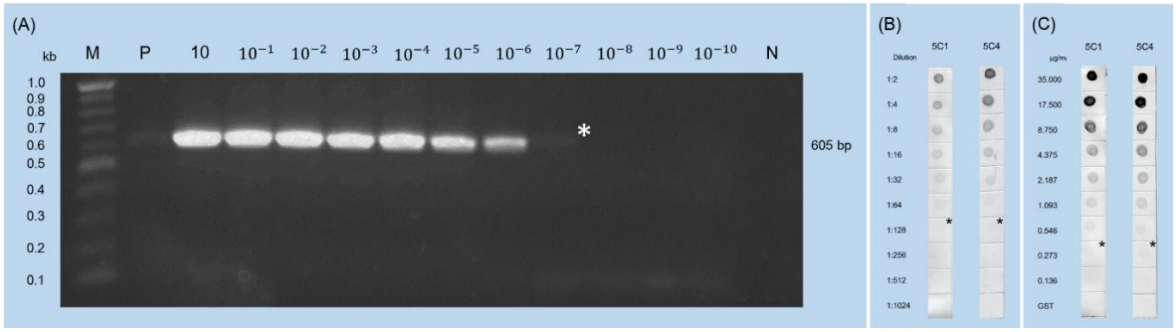

Figure 4 Sensitivity comparison between RT-PCR and dot blotting detection of RGNNV. The culture fluid of RGNNV-infected E11 cells was ten-fold serially diluted and used in RT-PCR analysis (A). For dot blotting, culture fluid of RGNNV-infected E11 cells (B) and purified recombinant GST-MCP protein (C) were serially diluted and spotted onto a nitrocellulose membrane, where they were processed with MAbs 5C1, and 5C4. Lane M: DNA markers; lane N: negative control; lane P: positive control; * the lowest detection limit.

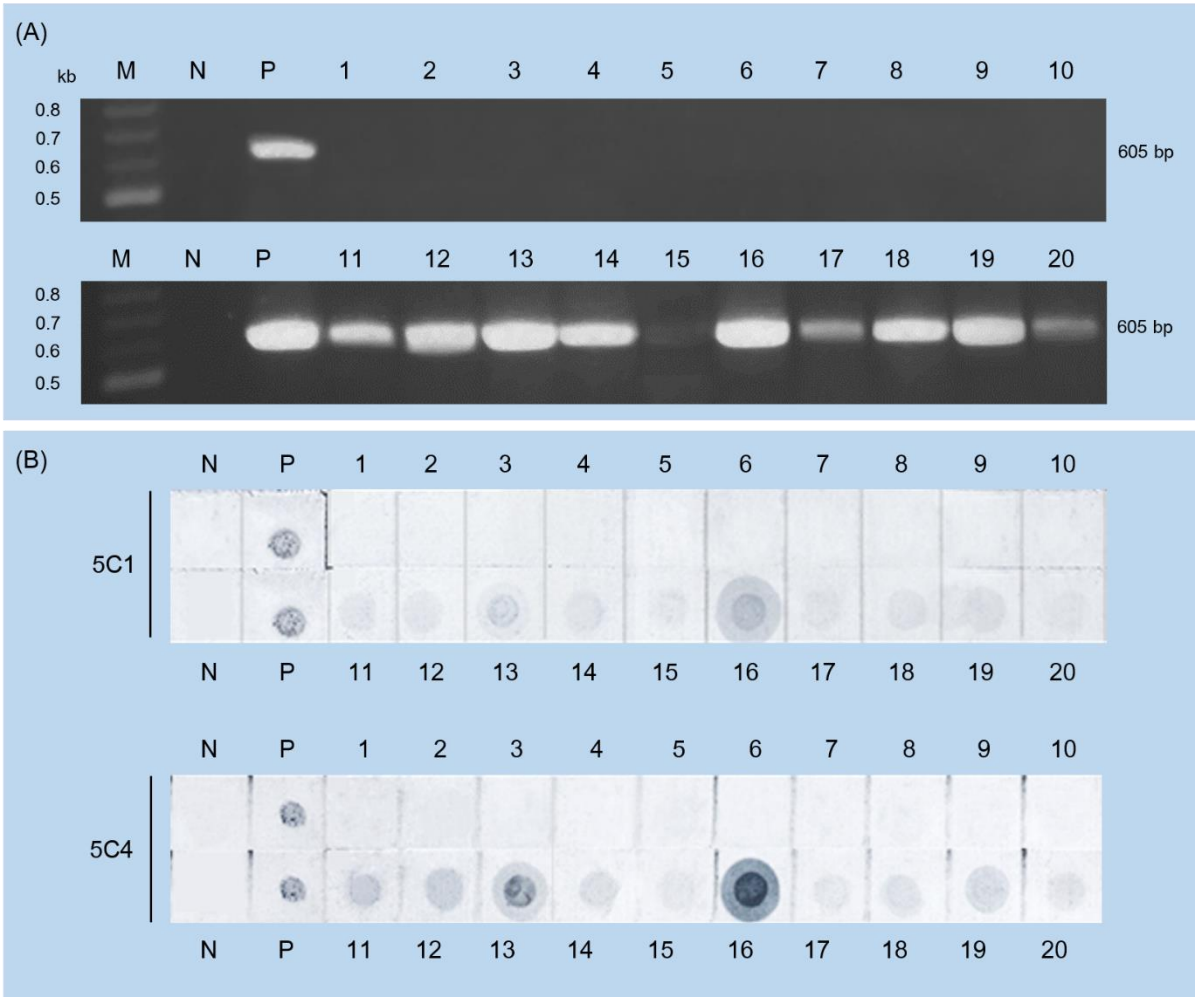

Figure 5 Comparison between RT-PCR and dot blotting analysis for RGNNV detection in the RGNNV-infected fish samples. The RT-PCR study of tissue homogenates from ten uninfected fish samples (No. 1-10) and ten RGNNV-infected fish samples (No. 11-20) revealed the presence of a positive band at 605 bp (A). Dot blotting was used to evaluate the effectiveness of the MAbs in detecting RGNNV in both uninfected (No. 1-10) and RGNNV-infected fish homogenates (No. 11-20) (B). Lane M: DNA marker; lane P: positive control; lane N: negative control.

Discussion

Aquaculture is a crucial aspect of the world's food supply, providing an ever-increasing global population. However, this industry is facing the effects of climate change, including rising water temperatures, changes in water pH and salinity, and reduced rainfall in fertile regions. These environmental changes have led to increased disease incidence in aquatic animals, resulting in significant fish mortality rates that could potentially impact the world's food supply [16]. One of the most significant challenges faced by aquaculture is the spread of NNV infections, which are causing severe problems worldwide. RGNNV, in particular, has a broad host range, infecting up to 120 species of freshwater and

marine fish [1]. Furthermore, RGNNV has been found in shrimp and squid, which serve as reservoir hosts [17].

RGNNV pathogenesis generally corresponds with central nervous system (CNS) damage, as evidenced by severe cellular vacuolation and neuronal degeneration in various parts of the nervous system, including the retina, brain, spinal cord, and peripheral ganglia [18]. The first reports of VNN in brown-spotted groupers (*Epinephelus malabaricus*) and orange-spotted groupers (*Epinephelus coiodes*) were published in Thailand in 1995 and 1996, respectively. Subsequently, a viral agent was successfully isolated from red-spotted grouper fry (*Epinephelus coioides*) in southern Thailand, which exhibited clinical symptoms of viral nervous necrosis (VNN). Fish infected with the disease were collected from both farms and the wild. The findings demonstrated that all samples were categorized under RGNNV [19]. Since there have been reports of outbreaks in the past 30 years, a number of methods have been suggested to identify NNV. These methods include virus isolation in cell cultures, light and electron microscopy, immunology assays such as ELISA and immunofluorescence, as well as molecular assays such as PCR, RT-PCR, and real-time RT-PCR. All of these techniques, however, have some drawbacks, such as the need for expensive instruments, skilled employees, and high costs, and may not be suitable for field detection [1].

In this study, we can generate two monoclonal antibodies (MAbs) that bind to RGNNV when evaluated with dot blotting, Western blotting, and immunohistochemistry (IHC). The IHC signals were found in the cytoplasm of target cell organs including gills and eye. The IHC pattern corresponds to the previous reports that were affected by other strains of NNV, such as reassortant RGNNV/SJNNV via incubation with rabbit polyclonal antiserum anti-SJNNV [20], RGNNV-infected European seabass (*Dicentrarchus labrax*) treated with anti-RGNNV monoclonal antibody [21]. Although it is considered that all of the developed MAbs can bind to the same epitope, the presence of multiple MAbs can increase the probability of selecting suitable ones for developing immunological assays. According to the study, the sensitivity of MAbs 5C1 and 5C4 was found to be lower than that of the RT-PCR method. However, these MAbs can still be utilized in various immunoassays such as ELISA, which is capable of screening a large number of samples for disease diagnosis and outbreak monitoring. Additionally, dot blot-based methods are best suited for use in smaller laboratories [22].

In this study, we utilized two types of antigens, purified GST-MCP and RGNNV-infected E11 cell culture fluid for the comprehensive detection of linear and conformational epitopes on viral particles presented in fish homogenate solution. However, the similar conformational epitopes in fish proteins were a result of high background level in dot blot assays. To address this issue, a denaturing reagent was added to the fish homogenate solution, which reduced the problems and provided more consistent signals [23]. Therefore, the sample was treated with SDS solution and boiled for 1.30 min to denature the protein. Since the late 1990s, there has been development of monoclonal antibodies (MAbs) specific to nervous necrosis virus (NNV). Nishizawa et al. (1995) successfully generated MAbs specifically targeted to NNV. Through Western blot analysis, they identified the 42 kDa coat protein of the striped jack nervous necrosis virus (SJNNV) and provided evidence of the MAbs ability to neutralize SJNNV

[24]. Furthermore, MAbs were produced to target the yellow grouper nervous necrosis virus (YGNNV). Western blot analysis confirmed the recognition of the 42 kDa coat protein of YGNNV by these MAbs. Subsequent experiments demonstrated that the MAbs exhibited a higher neutralization index (NI) value, ranging from 6.5 to 4.5 ($\log_{10}$ NI), indicating their effectiveness in inhibiting YGNNV infection in cell culture. These findings highlight the potential utility of these MAbs in preventing and combating NNV infections [25]. In addition to SJNNV and YGNNV, MAbs specific to grouper nervous necrosis virus (GNNV) were developed. These MAbs exhibited sensitivity in ELISA systems, with a detection limit of 2.5 ng per well of purified GNNV protein or 6.5×10^4 TCID50 per well of GNNV culture fluid from cultured cells [26]. In 2022, the researchers successfully generated monoclonal antibodies (MAbs) against the capsid protein (Cp) gene of the RGNNV. These MAbs recognized RGNNV virions specifically, making them suitable for the development of a rapid detection method [9]. Furthermore, the MAbs specifically targeted linear epitopes on the capsid protein, allowing for the effective detection and labelling of both intracellular and free RGNNV virions. However, their ability to neutralize RGNNV infection was found to be limited [27]. To facilitate the detection of nervous necrosis virus (NNV), a simple and rapid method has been developed using MAbs. A lateral flow immuno-chromatographic strip assay specific to NNV, specifically the RGNNV genotype, has been successfully developed. The assay exhibits an impressive limit of detection, capable of identifying as low as 105.05 $TCID_{50}$/100 μL, with no cross-reactivity observed towards other fish viruses. To validate the practicality of this assay, field-level testing was conducted using brain tissue samples from RGNNV-infected and non-infected sevenband grouper (*Hyporthodus septemfasciatus*). The results demonstrated exceptional performance, with a specificity of 100% and a sensitivity of 94.92%, yielding a high diagnostic effectiveness of 96.81%. These findings highlight the efficacy and reliability of the lateral flow immuno-chromatographic strip assay as a valuable tool for NNV detection in farm-level [2]. The continuous development of MAbs has significantly enhanced the efficiency and sensitivity of pathogen detection. However, in comparison to the previously reported MAbs, the MAb generated in this study appears to be less effective in pathogen detection.

In conclusions, the study successfully generated NNV-specific MAbs and validated their specificity through dot blot, Western blot, and immunohistochemistry assays. The results indicate that the MAbs can be utilized to detect NNV infection in field samples, but they have inferior sensitivity compared to RT-PCR. Therefore, further research is required to develop more MAbs with higher sensitivity for the monitoring NNV infection through immunological examination.

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