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Development and application of quantitative detection method for nervous necrosis virus (NNV) isolated from sevenband grouper *Hyporthodus septemfasciatus*

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ABSTRACT

Objective: To develop the rapid and efficient quantitative detection tool for nervous necrosis virus isolated from sevenband grouper *Hyporhodus septemfasciatus.*

Methods: The viral genes of the NNV (SGYeosu08) isolated from sevenband grouper were phylogenetically analyzed. In addition, novel quantitative PCR primers based on the genomic sequence of SGYeosu08 isolate were designed and compared it with the conventional bio-assay method ($TCID_{50}$) using *in vitro* and *in vivo* samples.

Results: The phylogenetic analysis of viral genes demonstrated the relationship of SGYeosu08 with members of red-spotted grouper nervous necrosis virus (RGNNV). The qNNV_R1 primer set (R1_F and R1_R) and the qNNV_R2 primer set (R2_F and R2_R) revealed 93% primer efficiency (regression: $y = -0.2861x + 9.9401$, $R^2 = 0.9976$) and the revealed 108% primer efficiency (regression: $y = -0.3172x + 10.0611$, $R^2 = 0.9982$), respectively. Its comparison with viral infectivity calculated by $TCID_{50}$ method showed similar kinetic pattern at *in vitro* and NNV challenged fish (*in vivo*) samples.

Conclusions: Result show that this method is rapid and efficient to diagnose NNV infection compare to traditional bioassay method ($TCID_{50}$).

1. Introduction

Viral nervous necrosis (VNN), also known as viral encephalopathy and retinopathy (VER) is a severe problematic disease in the world aquaculture industry [1–3]. It has been reported from a variety of marine aquaculture species over 20 kinds including rock bream (*Oplegnathus fasciatus*), olive flounder (*Paralichthys olivaceus*), barramundi (*Lates calcarifer*), turbot (*Scophthalmus maximus*), sevenband grouper (*Hyporthodus septemfasciatus*) since its first report from bigeye trevally (*Caranx sexfasciatus*) at 1980s [1–3]. Normally, the symptoms of diseased fish appear with vacuolization and necrosis of the central nervous system and the retina and showing abnormal swimming [1]. The high mortality rate over 80% was reported from various fish species at larvae and juveniles stages [1].

Nervous necrosis virus (NNV), the causative agent of VNN, is a small non-enveloped icosahedral virus containing bi-segmented single strand positive RNA as genetic materials. RNA1 (approximately 3.1 kb in length) encodes a RNA dependent RNA polymerase for viral replication while RNA2 (1.4 kb) encrypts a viral capsid protein. It belongs to the family *Nodaviridae* and the genus *Betanodavirus*. Betanodaviruses has 4 genogroups based on the T4 region sequence of RNA2 as barfin flounder nervous necrosis virus (BFNNV), red-spotted grouper nervous necrosis virus (RGNNV), striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV) [4]. Besides, Johansen and colleagues [5] suggested new nodavirus isolates from turbot, *Scophthalmus maximus*, as a fifth genotype.

In Korea, mass mortalities caused by VNN were reported from various cultured marine fish such as sevenband grouper (*Hyporthodus septemfasciatus*), rock bream (*Oplegnathus fasciatus*), red drum (*Sciaenops ocellatus*) and olive flounder (*Paralichthys olivaceus*) since 1990 [6–8]. Furthermore, NNV has been detected from wild marine fishes in seaside of the Korea peninsula [9].

Sevenband grouper is one of the most valuable cultured fish in Korea. However, recently the outbreaks of the VNN were observed in aqua-farms of sevenband groupers during the summer and have made huge economic losses [10]. It is necessary to develop sensitive and accurate diagnostic method

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to minimize huge losses caused by NNV infection. The World Organization for Animal Health Office International des Epizooties (OIE) has recommended methods to diagnosis NNV infection such as PCR, antibody-based assay, 50% tissue culture infectivity dose ($TCID_{50}$) and so on. Recently, quantitative RT-PCR (qRT-PCR) based on the SYBR Green assay was also established with faster than traditional bio assay method ($TCID_{50}$) [11]. However, the primers were not validated for all genogroups of NNV strains [12]. Thus, in this aspect it is necessary to develop specific quantitative detection tool for Korean NNV isolate based on the complete understanding of genetic information.

In this study, we analyzed viral genes of the NNV (SGYeosu08) isolated from sevenband grouper in Korea and figured out phylogenetic relationships with previously reported strains. In addition, we newly developed a quantitative detection method for NNV based on the Korean isolate and compared it with the conventional bio-assay method ($TCID_{50}$) using *in vitro* and *in vivo* samples.

2. Materials and methods

2.1. Virus preparation

The NNV used in this study was isolated from sevenband grouper aqua-farm in Yeosu, Korea in 2008 and propagated in the striped snakehead (SSN-1) cell line. SSN-1 cells were grown at 25 °C in Leibovitz L-15 medium (Sigma Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Gibco, Gland Island, NY, USA), 150 U/mL penicillin G, and 100 μg/mL streptomycin. NNV was inoculated on a confluent SSN-1 cell monolayer and incubated at 25 °C to replicate the virus. Viral samples were aliquot in small volumes and stored at −80 °C until use.

2.2. Cloning and sequencing analysis of viral genes

Viral RNA was extracted using miRNeasy Mini Kit (Qiagen, Germany) and cDNA was synthesized using ReverTra Ace qPCR RT Kit (Toyobo, Japan) following the manufacturer's protocols. The synthesized cDNA was amplified in 20 μL of PCR mixture containing 5 μL of 10 × Ex Taq buffer, 4 μL of 2.5 mM dNTP mixture (each), 0.5 μL of Ex Taq (5 U/μL), and 20 pmol of open reading frame (ORF) primer sets in Table 1. A primer set used in this study were designed by Primer3Plus [13] based on the NNV SGYeosu08 genome sequence [14]. PCR condition was pre-denaturation at 95 °C for 5 min, 30 cycles of 1 min denaturation at 95 °C, 1 min annealing at 58 °C, and 3 min for RNA1 or 1 min for RNA2 extension at 72 °C, followed by a 5 min final extension at 72 °C. The amplified products were purified using QIAquick Gel Extraction Kit (QIAGEN) and cloned into pCR2.1-Topo vector (Invitrogen, USA). Plasmid DNA was extracted with an Accuprep Plasmid Mini Extraction Kit (Bioneer, Korea) and analyzed ORF sequence by ABI 3730 XL DNA sequencer.

2.3. Phylogenetic analysis

All sequences of other nodaviruses were obtained from GenBank database of the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/GenBank/). Sequence similarity analysis was conducted with Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/blast/) of NCBI and Pairwise Sequence Alignment Tool (http://www.ebi.ac.uk/Tools/psa/) of EMBL-EBI (the European Bioinformatics Institute) and organized by manual. The multiple sequence alignments were performed with the Clustalw2 web service (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic analyses were conducted using the MEGA6 software using a Neighbor-Joining method with 1 000 bootstrap replicates [15].

2.4. Standard and primer efficiency

Primer efficiency was examined by the quantitative PCR with 10-fold diluted plasmid DNA. The quantitative PCR was carried out in an Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer) followed by manufacturer's instructions using SYBR green mixture, AccuPower Greenstar quantitative PCR premix (Bioneer). Briefly, a 10 min pre-denaturation cycle at 95 °C, 40 cycles of 20 s denaturation at 95 °C, and a 40 s extension at 58 °C were used. The specification of the quantitative PCR was analyzed through melting curve analysis, and the baseline was determined automatically by the Exicycler Analysis Software (Bioneer).

2.5. qRT-PCR and $TCID_{50}$ method (in vitro)

One hundred microliter of $10^{5.8}$ $TCID_{50}$/mL NNV was inoculated into fourteen of 25 cm^2 culture flasks containing SSN-1 cells. The supernatant and cells were sampled immediately after virus inoculation (0 h), at 3, 6, 12, 24 h, and at 2 and 3 days from each of two culture flasks to compare the $TCID_{50}$ method and the qRT-PCR method. To measure the viral infectivity, SSN-1 cells (about 10^5 cells/well) were cultured in 96-well plates, and 50 μL of 10-fold diluted virus (10^{-1} to 10^{-8}) was inoculated onto the 96-well plates. $TCID_{50}$ value was calculated 14 days after inoculation using Reed and Muench method [16]. All samples were statistically analyzed and all the data were represented as the mean ± the standard error.

Table 1
PCR primers used in this study.

Primer name	Sequences (5′–3′)	Tm (°C)	Usage
NNV08.R1.ORF.F	CATATGCGTCGCTTTGAGTTTGC	61	Gene cloning
NNV08.R1.ORF.R	GTCGACCAACACTTGAGTGCGAC	63	
NNV08.R2.ORF.F	CATATGGTACGCAAAGGTGAGAAGAA	59	
NNV08.R2.ORF.R	CTCGAGTTAGTTTTCCGAGTCAACC	58	
qNNV08.R1.for	CGAGCCATACGTCATTTCACCA	60	Quantitative PCR
qNNV08.R1.rev	CGGAACACCCAAACCAATCAA	58	
qNNV08.R2.for	AAATTCAGCCAATGTGCCCC	58	
qNNV08.R2.rev	ATTTGGCAACGACTGCACCA	58	

2.6. Challenging experiment (in vivo)

Sevenband groupers were purchased from aqua-farm which has no history of VNN occurrence. Prior to experiments, 10 fish were randomly sampled from fish stock and the brain samples were examined for betanodavirus by RT-PCR according to a previous report [4]. Total thirty five sevenband groupers (average weight = 30.9 ± 8.2 g) were reared in aquaria. Ten of thirty five fish were cultivated separately to calculate cumulative mortality. NNV at dose of $10^{3.8}$TCID$_{50}$/100 μL/fish was injected intramuscularly into the fish in aquaria and the challenged fish were daily observed for two weeks. Brain tissues of three challenged fish were collected on the 24, 48, 72, 84, 90 and 96 h after injection for measuring titration of NNV and viral copy number. The obtained tissues were homogenized with nine volumes of L-15 medium and centrifuged at 6 000 × *g* for 30 min (4 °C), and then the supernatant was used for qRT-PCR and TCID$_{50}$.

3. Results

3.1. Comparison with other NNV isolates and phylogenetic analysis

The percent of deduced amino acid sequence identities of NNV SGYeosu08 isolate with published genome sequence of NNV were determined by EMBOSS Needle pairwise sequence alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) and the results are shown in Table 2. In the result of deduced protein (RNA dependent RNA polymerase, RdRp) from RNA 1, NNV SGYeosu08 showed over 98.0% (98.0%–99.4%) of identity with other RGNNV, 88.3%–88.7% of BFNNV, 88.6% with TPNNV, 87.9% with SJNNV, respectively. In case of RNA2, NNV SGYeosu08 showed over 99.1% (99.1%–100.0%) of identity with other RGNNV, 85.5%–87.0% of BFNNV, 81.5% with TPNNV, 81.5% with SJNNV, 78.5% with TNV, respectively. RdRp encoded by RNA1 was higher conserved protein (87.9%–99.4% identities) while coat protein from RNA2 was more divergent protein that showed 78.5%–100% identities. Phylogenetic analyses of deduced protein from both viral genomes were performed to determine the relationships among betanodaviruses from various hosts. In phylogenetic analysis of deduced protein, SGYeosu08 was included in a branch of RGNNV and RGNNV isolated from Golden pompano in Malaysia and Sevenband grouper in Japan were closer to SGYeosu08 (Figure 1).

3.2. Standard and primer efficiency

The serial 10-fold dilutions of the cloned plasmid were amplified in duplicate by quantitative PCR to determine the sensitivity of the assay. The slope and R^2 of the RNA 1 primers set were −0.2861 (93% efficiency) and 0.9976, respectively. Moreover, the slope and R^2 of the RNA 2 primers set were −0.3172 (108% efficiency) and 0.9982, respectively (Figure 2). The primer set showed an equivalent efficiency and satisfactory coefficient of determination (R^2) values compared to other studies [17,18].

3.3. qRT-PCR and TCID$_{50}$ method (in vitro)

Figure 3 exposes both replication curves in NNV infectivity by the TCID$_{50}$/mL value (gray bar) and viral copy numbers calculated by qRT-PCR (black bar). In case of cell, infectivity was lower than $10^{3.1}$ TCID$_{50}$/mL until 6 h after infection and then virus replication increased rapidly after 12 h ($10^{4.0}$ TCID$_{50}$/mL), 24 h ($10^{6.1}$ TCID$_{50}$/mL), 48 h ($10^{7.5}$ TCID$_{50}$/mL) and 72 h ($10^{8.8}$ TCID$_{50}$/mL). Similarly, the viral copy number remained almost unchanged until 6 h (under $10^{2.2}$ copies/mL) and then gradually increased until 48 h. Then, About $10^{6.5}$ copies/mL were maintained until 72 h (Figure 3a). In supernatant, meanwhile, infectivity was lower than $10^{4.1}$ TCID$_{50}$/mL until 24 h after infection and then virus radically increased after 48 h ($10^{5.8}$ TCID$_{50}$/mL), 72 h ($10^{7.9}$ TCID$_{50}$/mL). Likewise, the viral copy number maintained under $10^{2.9}$ copies/mL till 24 h and then deeply improved until 48 h ($10^{4.1}$ copies/mL), 72 h ($10^{6.0}$ copies/mL) (Figure 3b). This comparison of the change in NNV infectivity and its gene copy numbers *in vitro* showed similar change patterns.

Table 2
Comparison of nucleotide or deduced amino acid sequence within betanodavirus.

Type	Isolation source	Country	RNA1			RNA2		
			Accession no.	L (nt)	Deduced amino acid (%)	Accession no.	L (nt)	Deduced amino acid (%)
RGNNV	Redspotted grouper (*Epinephelus akaara*)	China	EF558368	3 103	98.9	EF558369	1 433	99.1
	Golden pompano (*Trachinotus blochii*)	Malaysia	GQ904198	3 024	99.3	GQ904199	1 363	100
	European seabass (*Dicentrarchus labrax*)	Tunisia	FJ789783	3 104	99.1	FJ789784	1 433	99.1
	Dragon grouper (*Epinephelus lanceolatus*)	China	AY721616	3 103	99.4	AY721615	1 433	100
	Sevenband grouper (*Hyporthodus septemfasciatus*)	Japan	AB373028	3 105	99.4	AB373029	1 433	100
	Greasy grouper (*Epinephelus tauvina*)	Singapore	AF319555	3 103	99.0	AF318942	1 433	99.1
	Barramundi (*Lates calcarifer*)	Australia	GQ402010	2 998	99.2	GQ402011	1 017	99.1
	Olive flounder (*Paralichthys olivaceus*)	Korea	FJ748760	3 104	98.6	DQ864760	1 088	100
	Gray mullet (*Mugil cephalus*)	Korea	–			DQ116038	1 017	100
	White star snapper (*Lutjanus stellatus*)	Taiwan	–			AY835642	1 433	99.7
	Senegal Sole (*Solea senegalnesis*)	Spain	FJ803911	3 051	98.0			
BFNNV	Barfin flounder (*Verasper moseri*)	Japan	EU826137	3 100	88.3	EU826138	1 433	87.0
	Atlantic cod (*Gadus morhua*)	Norway	EF617330	3 100	88.3	EF617329	1 433	86.4
	Atlantic halibut (*Hippoglossus hippoglossus*)	Norway	AJ401165	3 100	88.7	–		
	Haddock (*Melanogrammus aeglefinus*)	Canada	–			AY547549.1	1,367	85.5
TPNNV	Tiger puffer (*Takifugu rubripes*)	Japan	EU236148	3 112	88.6	EU236149	1 422	81.5
SJNNV	Striped Jack (*Pseudocaranx dentex*)	Japan	AB056571	3 107	87.9	AB056572	1 421	81.5
TNV	Turbot (*Scophthalmus maximus*)	Norway	–			AJ608266	1 417	78.5

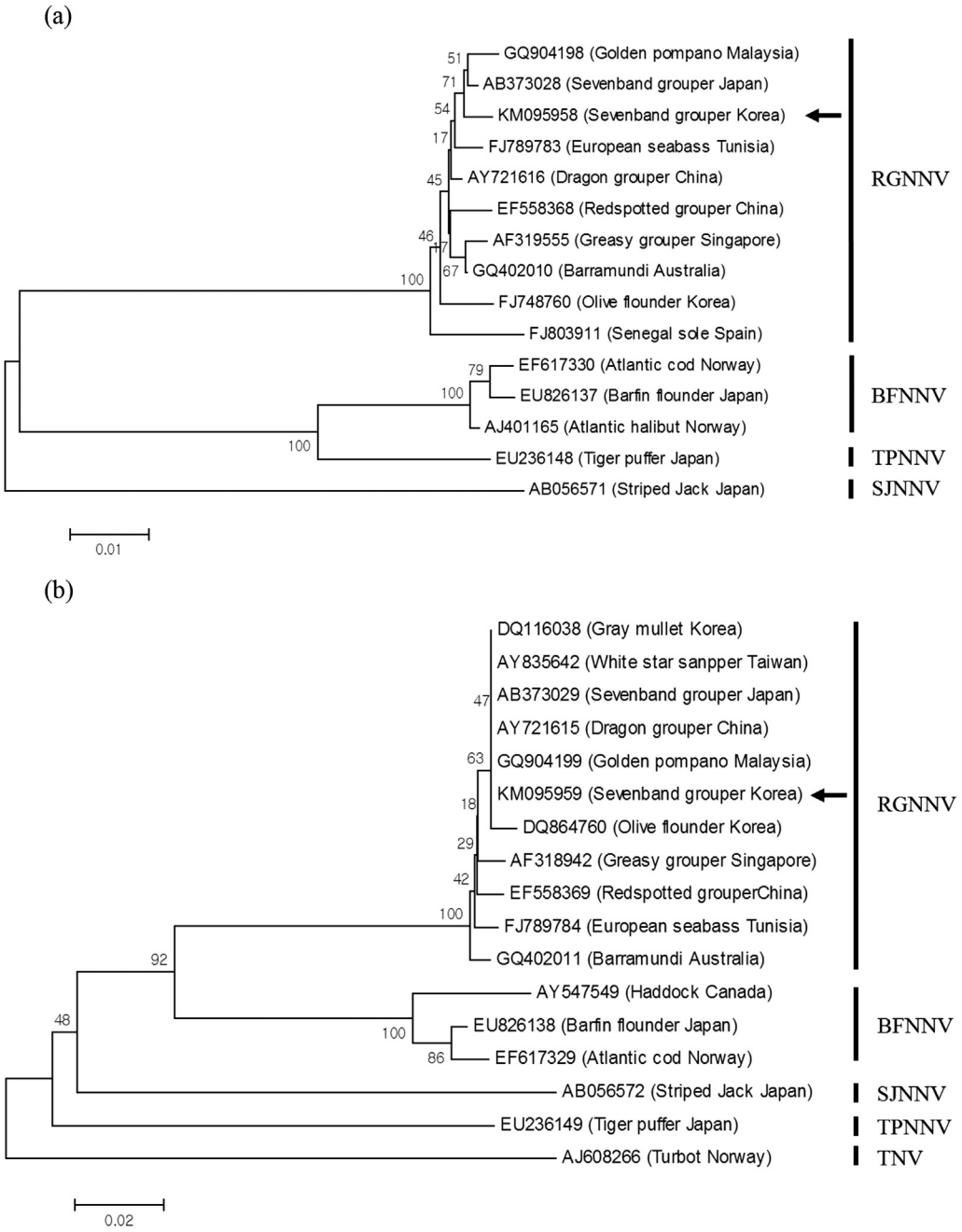

Figure 1. Phylogenetic analysis of RNA1 (a) and RNA2 (b) of NNV SGYeosu08 isolate. Phylogenetic analysis was conducted by the neighbor-joining method (1 000 bootstrap) by MEGA 6.0 [15].

3.4. qRT-PCR and $TCID_{50}$ method with NNV challenged sevenband grouper (in vivo)

The challenged fish died from day 3 after infection and cumulative mortality was estimated as 100% (Figure 4a). Entire diseased fish showed abnormal swimming behavior and darkening body color. A total of 18 fish samples (three fish from each sampling time) were used to determine average viral copy numbers/mL and $TCID_{50}$/mL and these results are in Figure 4b. NNV was under detection limit on the 24 hours after injection (H.A.I.), but the virus rapidly multiplied on the 48 H.A.I. The NNV infectivity on the 72, 84 and 90 H.A.I were $10^{5.0}$, $10^{4.0}$, $10^{5.8}$ $TCID_{50}$/mL, respectively. Meanwhile, two fish were died on the 96 H.A.I. thus it recorded the highest titer of $10^{8.6}$ $TCID_{50}$/mL. Equally, the viral copy number was not detected on the 24 H.A.I. but went up to $10^{4.0}$ copies/mL on the 48 H.A.I. The NNV copy numbers on the 72, 84, 90 and 96 H.A.I were $10^{4.0}$, $10^{4.0}$, $10^{4.9}$ and $10^{7.2}$ copies/mL, respectively. Figure 4c represents the copy numbers of NNV *versus* the infectivity ($TCID_{50}$/mL) for individual samples. From these results, the changes of NNV infectivity in challenged fish were comparable to that of the copy numbers.

4. Discussion

In this study, we have shown that the NNV SGYeosu08 isolated from Korea was included in RGNNV group from both viral gene (RNA1 and RNA2). Since Panzarin *et al*

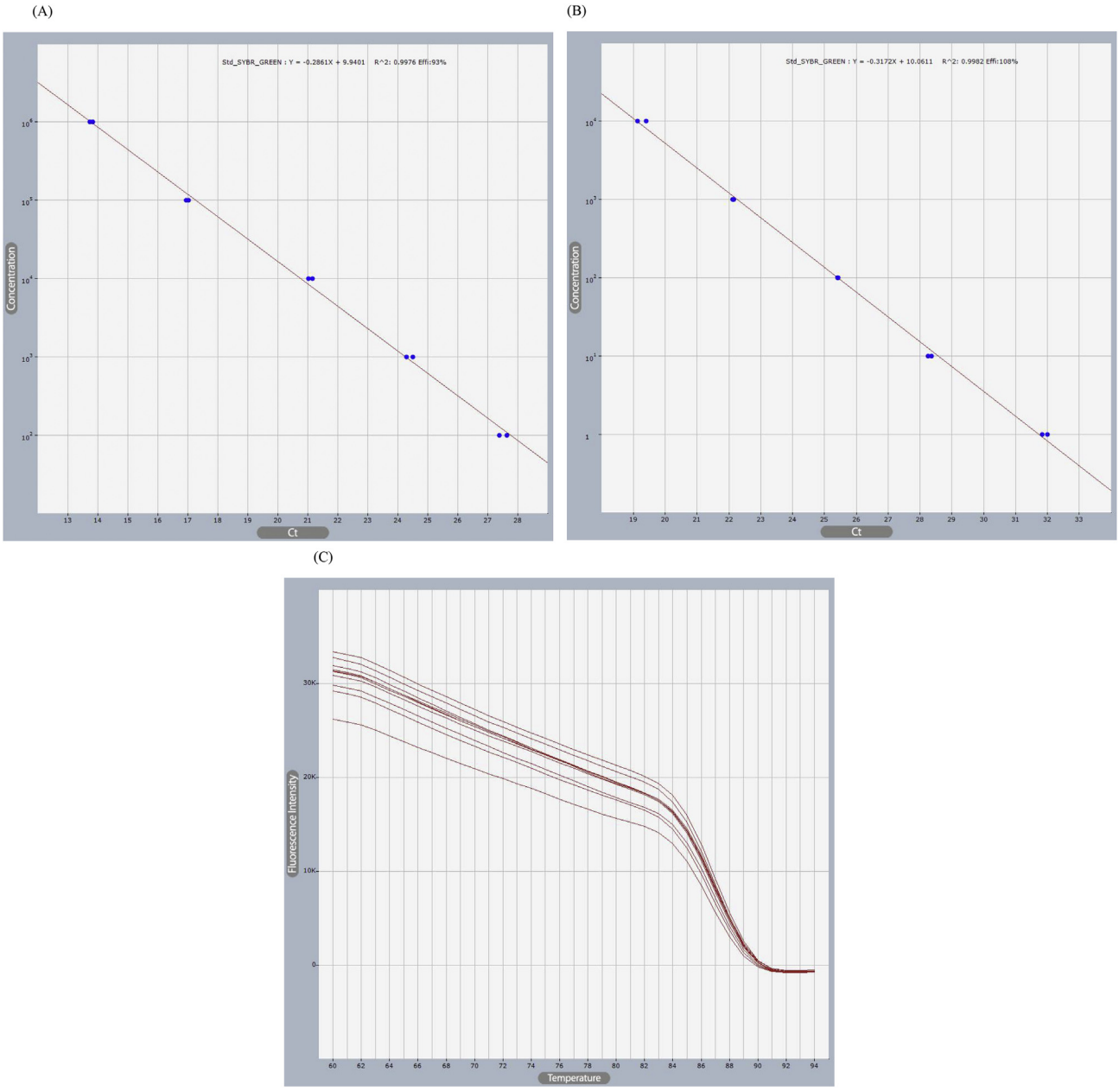

Figure 2. Quantitative PCR standard curve of RNA1 (A), RNA2 (B) and melting curve (C). The plasmid DNA harboring NNV genes were diluted by 10-fold and amplified in duplicate by quantitative PCR.

demonstrated the presence of the RGNNV/SJNNV reassortants harboring the RNA1 of the RGNNV and the RNA2 of SJNNV, or opposite combination in Southern Europe [19] it is necessary to analyze both NNV genes. Based on the complete genome sequence, the deduced coat protein sequence was more diverse than RNA dependent RNA polymerase compared to other betanodaviruses. This is similar result that of viral hemorrhagic septicemia virus (VHSV), RNA polymerase protein is the highest conserved protein among six encoding proteins even it is the largest gene [20]. Therefore, RdRp gene might be more appropriate target gene to develop diagnostic tool for certifying all genotypes whereas previous studies were developed mainly using a coat protein gene [18,21].

The greater the damage of sevenband grouper industry, more sensitive and accurate detection method depending on the genotype is required to minimize enormous damages caused by NNV infection. We developed new quantitative RT-PCR tool based on its genetic information, moreover applied it to virus replication kinetics and demonstrated that it is comparable to traditional bio-assay method ($TCID_{50}$). This is the first evaluation study of the changes in NNV replication kinetics and its gene copy numbers *in vitro* and *in vivo*. Interestingly, in *in vitro* experiment, inside of SSN-1 cell the virus replicated gradually from the beginning of experiment while the virus kept start level until 24 h then rapidly rising at 48 h in the supernatant. This result gives a simple hint that the NNV was released out from cells after 24 h post infection. By the way, minimal viral replication times were approximately 13 min for T7 bacteriophages [22], 1.2 days for HIV [23], and 30 h for duck hepatitis B virus [24].

In the challenged experiment, the NNV SGYeosu08 isolate showed high mortality up to 100% within 6 day. The viral infectivity and copy numbers in the brain tissue were increased along to the mortality rate. Although the morality rate was 50%

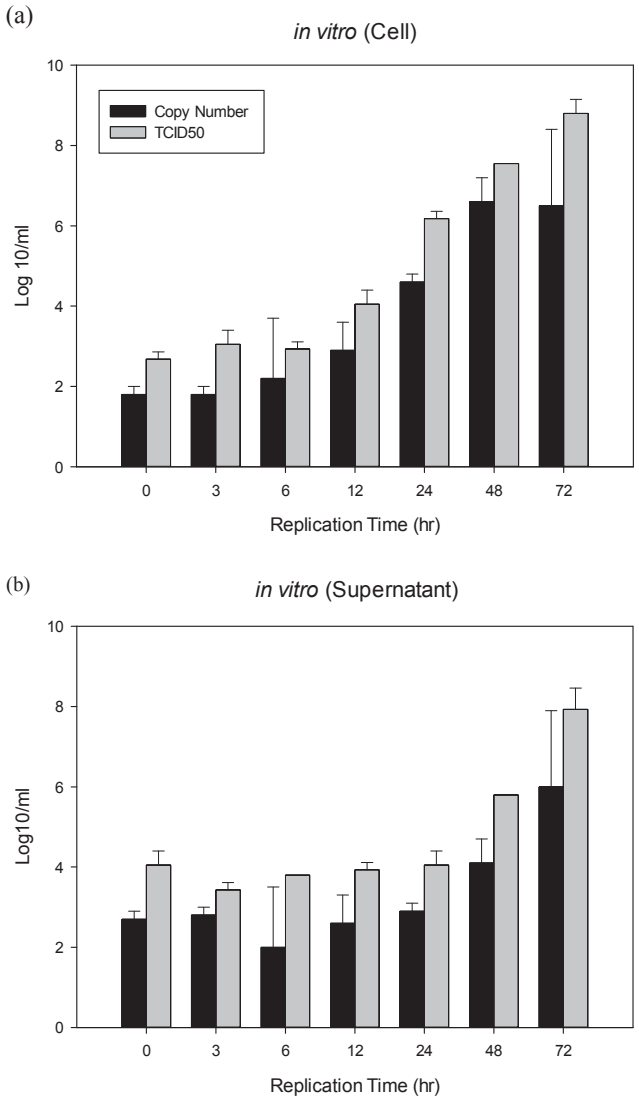

Figure 3. Comparison of NNV titer ($TCID_{50}$/mL) and copy number in SSN-1 cell (a) and supernatant (b) (*in vitro*). Black bar indicates log values of the copy number and gray bar indicates log values of NNV titer ($TCID_{50}$/mL).

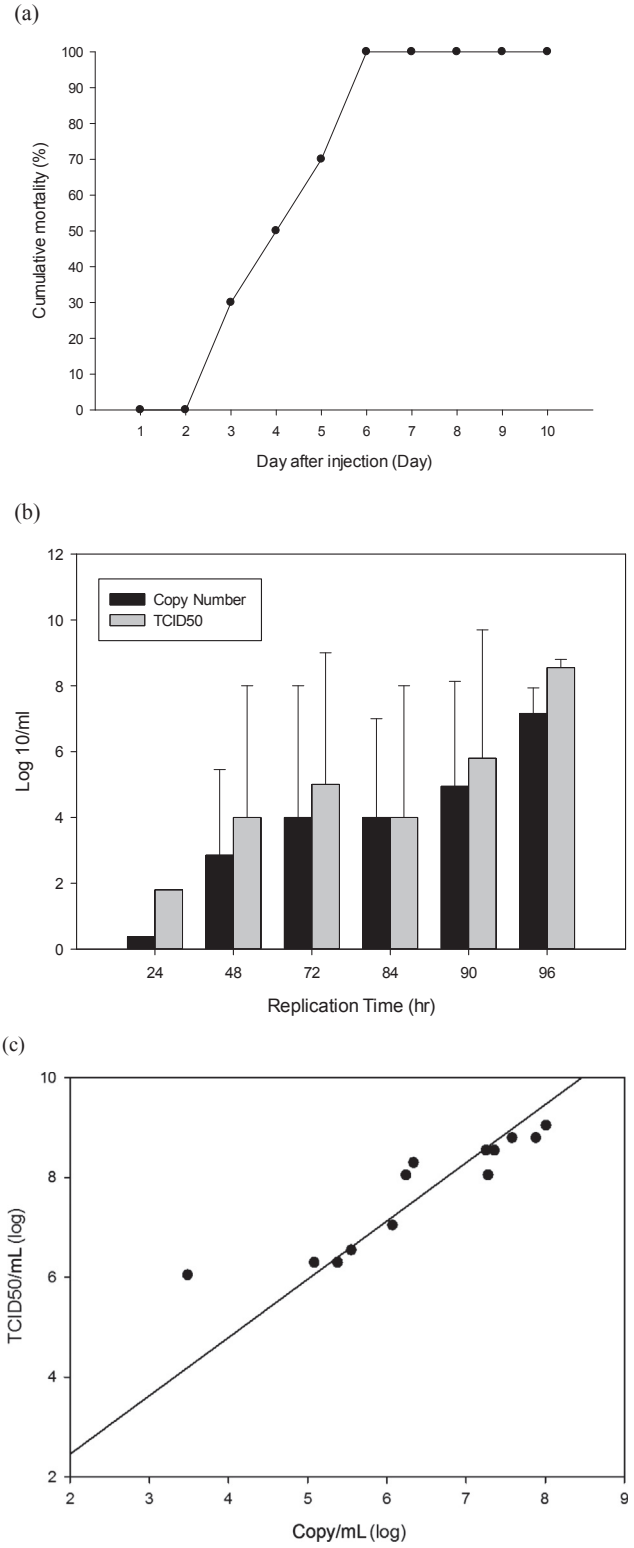

Figure 4. NNV challenge test and comparison of NNV titer ($TCID_{50}$/mL) and copy number (*in vivo*). (a) Mortality of challenged fish; (b) Black bar indicates average log values of the NNV copy number and gray bar indicates average log values of NNV titer ($TCID_{50}$/mL); (c) The copy numbers of NNV determined from qRT-PCR *versus* those from NNV titer ($TCID_{50}$/mL) for individual samples.

at 96 H.A.I (4th day), the viral replication was reached approximately maximum value in fish.

In case of enveloped virus, it shows more high value of the infectivity compare to the copy numbers in *in vitro* experiments while the copy numbers was higher than that of the infectivity in *in vivo* trials [25]. In contrast, there was no significant difference on the results between *in vitro* and *in vivo* experiments in this study.

Hick and Whittington also estimated both viral copy number and viral infectivity ($TCID_{50}$) with NNV diluted in cell culture media. In their study, the number of viral copies was strongly correlated with the number of $TCID_{50}$ [18]. Similarly, in case of brain tissue from our study, most samples were correlated intensely. However, few samples were out of regression. For example, one sample had about 6 Log $TCID_{50}$/mL but it showed different copies. Thus, extra studies with more field samples are required to find accurate reasons what causes the differences between *in vivo* results.

In conclusion, the qRT-PCR assay developed in this study has great advantages such as high sensitivity and low-time consuming work to detect NNV *in vitro* and *in vivo*. This tool will be very useful for rapid detection of NNV in fish of aqua-farms and researches to understand the relationship between virus replication and occurrence of NNV.

Conflict of interest statement

We declare that we have no conflict of interest.

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