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Upper Temperature Limits for the Multiplication of Betanodaviruses

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ABSTRACT—Among the four types of betanodaviruses, redspotted grouper nervous necrosis virus (RGNNV) has the highest optimum temperature (25–30°C) for its multiplication. We tested 16 RGNNV isolates for their temperature sensitivity in cultured cells and demonstrated that their upper temperature limits ranged from less than 30°C to 35°C. At the temperatures over the upper limits, viral RNA replication was inhibited similarly. These results indicate that temperatures mainly affect RNA replication or earlier virus multiplication processes. The incompetence of betanodaviruses at 37°C suggests their avirulence in human.

Key words: betanodavirus, upper temperature limit, temperature sensitivity, replicative competence, virion stability

Pathogenic microorganisms have to adapt to or survive host environments, including nutrition, ionic strengths, temperatures, and defense systems, etc. Temperature is one of the most important environmental factors and controls directly the physiological states of pathogens. Microorganisms that reside internally homeothermal aminals should have narrow optimum temperatures for their growth because of the stable host temperatures. In contrast, fish pathogens need to adapt relatively wide ranges of temperatures since fish are poikilothermal animals, of which body temperatures can be changed depending on the environmental conditions.

Betanodaviruses, members of the family *Nodaviridae*, are the causative agents of a highly destructive disease of hatchery-reared larvae and juveniles of a variety of marine fish. In some species, adult and mature fish have also been sufferred from the disease. The disease, designated as viral nervous necrosis (VNN) or viral encephalopathy and retinopathy, has spread worldwide to more than 30 marine fish species from 14 families[1,2]. The viruses localize in the brains, spinal cords, and retinas of the affected fish. Recently, the viruses were detected from apparently healthy wild marine fish[3,4].

Betanodaviruses are nonenveloped, spherical viruses with a bipartite positive-sense RNA genome RNA1 (3.1 kb) and RNA2 (1.4 kb), which encode an RNA-dependent RNA polymerase (protein A) and the coat protein (CP), respectively[5,6]. Recently, we characterized a subgenomic RNA3 (0.4 kb), which encodes protein B2 having a suppressor function for post-transcriptional gene silencing[7]. The viruses can be classified into the four types, designated striped jack nervous necrosis virus (SJNNV), barfin flounder nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV), and redspotted grouper nervous necrosis virus (RGNNV), based on similarities in the partial RNA2 sequences[8]. RGNNV has a broad host range and causes VNN among a variety of warm water fish species, particularly groupers and sea bass[1]. The optimal temperatures for multiplication of RGNNV are 25–30°C which are relatively higher than those of SJNNV (20–25°C), TPNNV (20°C), and BFNNV (15–20°C)[9]. However, little is known about betanodavirus multiplication at over 30°C[10–12]. To know upper temperature limits for betanodavirus multiplication is important for a possible control of VNN by elevating rearing water temperatures and for securing us safety to eat raw fish. Therefore, in this study, we cultured several RGNNV isolates at over 30°C and determined thieir upper temperature limits for multiplication. Base on the data obtained in this study, we also refer to possible mechanisms that regulate temperature sensitivity in betanodaviruses.

Materials and Methods

Viruses and cells

RGNNV isolates were collected from around the world and used in this study (Table 1). They were confirmed to belong to the RGNNV type by sequencing their variable regions in RNA2[8]. The E-11 cell line[9] was grown at 25°C in Leibovitz's L-15 medium (Invitrogen) supplemented with 5% fetal bovine serum. For preparing inoculums, all the viruses were cultured at 25°C using E-11 cells and culture supernatants were stored at –80°C until use.

Temperature sensitivity tests of the viruses

E-11 cells grown in a 6-well plate (Iwaki) were inoculated with each of the viruses at a multiplicity of infection (MOI) of 1.0 at 25°C. One hour after inoculations, the inoculum was replaced by L-15 medium (2 mL per well) and the cells were incubated at 30°C, 32°C, 35°C, or 37°C. Fifty micro liter culture supernatant then was collected 0, 36, 72, and 120 h after incubation and its viral titer was measured by determining the 50% tissue cul-

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Table 1. Multiplicative competence of RGNNV isolates at different temperatures

Isolate	Originated host	Country	Viral titer (TCID50/mL)[b]				
			0 h	120 h			
				30°C	32°C	35°C	37°C
SGMie95	Sevenband grouper (*Epinephelus septemfasciatus*)	Japan	$10^{4.2}$	$10^{7.2}$	$10^{7.3}$	$10^{5.4}$	$<10^{2.5}$
F03-146	Orange-spotted grouper (*Epinephelus coioides*)	Hong Kong	$10^{4.4}$	$10^{6.9}$	$10^{6.8}$	$10^{5.6}$	$<10^{1.9}$
SBGre96	European sea bass (*Dicentrarchus labrax*)	Greece	$10^{3.9}$	$10^{7.6}$	$10^{6.9}$	$10^{5.9}$	$<10^{2.2}$
BAAus94	Barramundi (*Lates calcarifer*)	Australia	$10^{3.9}$	$10^{7.5}$	$10^{7.4}$	$10^{5.1}$	$<10^{1.9}$
HG0002	Humpback grouper (*Chromileptes altivelis*)	Indonesia	$10^{3.4}$	$10^{7.4}$	$10^{7.7}$	$10^{4.0}$	$<10^{1.8}$
SGWak97	Sevenband grouper (*Epinephelus septemfasciatus*)	Japan	$10^{4.0}$	$10^{7.3}$	$10^{6.7}$	$<10^{2.5}$	$<10^{1.8}$
JSOit98	Japanese sea bass (*Lateolabrax japonicus*)	Japan	$10^{3.9}$	$10^{7.4}$	$10^{6.6}$	$<10^{1.8}$	$<10^{1.8}$
KGOit97	Kelp grouper (*Epinephelus moara*)	Japan	$10^{4.0}$	$10^{7.1}$	$10^{7.0}$	$<10^{2.0}$	$<10^{1.8}$
KGNag02	Kelp grouper (*Epinephelus moara*)	Japan	$10^{4.6}$	$10^{7.7}$	$10^{6.7}$	$<10^{3.1}$	$<10^{1.9}$
BTKag01	Bluefin tuna (*Thunnus thynnus*)	Japan	$10^{4.0}$	$10^{7.9}$	$10^{6.8}$	$<10^{1.8}$	$<10^{1.8}$
SEEhi04	Striped jack (*Pseudocaranx dentex*)	Japan	$10^{3.6}$	$10^{7.2}$	$10^{7.0}$	$<10^{3.3}$	$<10^{1.8}$
HG9901	Humpback grouper (*Chromileptes altivelis*)	Indonesia	$10^{3.8}$	$10^{6.8}$	$10^{6.4}$	$<10^{2.4}$	$<10^{1.8}$
BGTha99	Brownspotted grouper (*Epinephelus chlorostigma*)	Thailand	$10^{4.3}$	$10^{7.6}$	$10^{6.9}$	$<10^{3.5}$	$<10^{1.8}$
JFHir92	Japanese flounder (*Paralichthys olivaceus*)	Japan	$10^{4.4}$	$10^{6.1}$	$10^{4.5}$	$<10^{2.2}$	$<10^{1.9}$
RGOka94	Redspotted grouper (*Epinephelus akaara*)	Japan	$10^{4.8}$	$10^{7.3}$	$<10^{2.6}$	$<10^{1.8}$	$<10^{2.0}$
WSBUS99A	White sea bass (*Atractoscion nobilis*)	USA	$10^{3.0}$	$<10^{1.8}$	$<10^{1.8}$	$<10^{1.8}$	$<10^{1.8}$
SJNag93[a]	Striped jack (*Pseudocaranx dentex*)	Japan	$<10^{2.7}$	$<10^{2.4}$	$<10^{2.2}$	$<10^{1.8}$	$<10^{1.8}$

[a]; An SJNNV isolate as a negative control.
[b]; E-11 cells were inoculated with each of the isolates and viral titers of the culture supernatants were measured at the indicated periods.

ture infectious dose ($TCID_{50}$)/mL as described in Reed and Muench[13]. For the titration, E-11 cells were inoculated with the collected samples and incubated at 25°C for 10 days. Experiments were duplicated independently and obtained data were averaged.

Northern hybridization experiment

E-11 cells were inoculated with a standard RGNNV isolate, SGWak97 (Table 1) at an MOI of 1.0. The cells then were washed once with Hanks' balanced salt solution (Nissui) and were homogenated with 250 μL ISOGEN (Nippon gene) to prepare total RNA according to the manufacturer's instruction. Obtained RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water and used for Northern hybridization experiments.

To prepare probes specific for RGNNV RNA1 and RNA2, first, two plasmids pRG1BS1 and pRG2BS2 were constructed as cDNA templates, respectively. For pRG1BS1 construction, pSG1TK5[14] was digested with *Cla*I and *Eco*RI and the resulting 0.3 kb viral cDNA fragment was inserted into pBluescript II KS (–) (Stratagene). Similarly, for pRG2BS2 construction, pSG2TK13[14] was digested with *Bam* HI and *Eco*RI and the resulting 0.4 kb fragment was ligated into pBluescript II KS (–). To prepare probes for positive-sense RNA1 and RNA2, pRG1BS1 and pRG2BS2 were linearized with *Sal* I and *Bam* HI, respectively, and transcribed with T7 RNA polymerase (Takara). To prepare probes for negative-sense RNA1 and RNA2, pRG1BS1 and pRG2BS2 were linearized with *Eco*RI and transcribed with T3 RNA polymerase (Roche). Northern hybridization experiments were performed as described in Iwamoto *et al.*[14] using DIG-labelled probes.

Results

Multiplicative competence of the RGNNV isolates at over 30°C

The 16 RGNNV isolates were cultured at over 30°C and their culture supernatants were titered periodically. The viral titers at 120 h post-inoculation are listed in Table 1 because the titers at the time well represented viral multiplicative competence at each temperature (data not shown). When the viruses were cultured at 30°C, all the isolates except WSBUS99A multiplied well and gave the titers ranged from $10^{6.1}$ to $10^{7.9}$ $TCID_{50}$/mL (Table 1). At 32°C, all the isolates excluding WSBUS99A and RGOka94 multiplied and produced $10^{4.5}$ to $10^{7.7}$ $TCID_{50}$/mL of viruses. Among these 14 positive viruses, JFHir92 showed the relatively low viral titer $10^{4.5}$ $TCID_{50}$/mL at 32°C though this virus showed better proliferation at 30°C. At 35°C, SGMie95, F03-146, SBGre96, BAAus94, and HG0002 multiplied and gave the titers from $10^{4.0}$ to $10^{5.9}$ $TCID_{50}$/mL. These viral titers at 35°C were approximately one to two orders of magnitude less than those obtained at 30°C and 32°C (Table 1). Notably, none of the isolates tested produced detectable progeny viruses at 37°C. Degradation of virions was indicated by the diminished viral titers at 120 h post-inoculation (Table 1). There seems to be no distinct correlation between the temperature sensitivities and the locations of the countries from which the viruses were isolated (Table 1).

Effects of temperatures on viral RNA replication

To address the mechanisms underlying the temperature sensitivity, viral RNA replication of an RGNNV isolate (SGWak97) was examined primarily at different temperatures. As shown in Fig. 1A, viral RNAs replicated well at 30°C and 32°C from 36 to 120 h post-inoculations. At 35°C, the level of viral RNA replication was maximum at 36 h post-inoculation, which was thereafter decreased gradually. Viral RNA replication was not detected at 37°C even though the RNA blots was exposed for a long period (Fig. 1B). Replication of both positive- and negative-sense RNAs was inhibited by elevating temperatures. These results on replicative competence were compatible to the viral titer data in Table 1. One prominent observation was that the amounts of positive-sense and negative-sense RNA2 were relatively lower than those of RNA1 and RNA3 when the virus was exposed to the incompatible high temperature 35°C (Fig. 1).

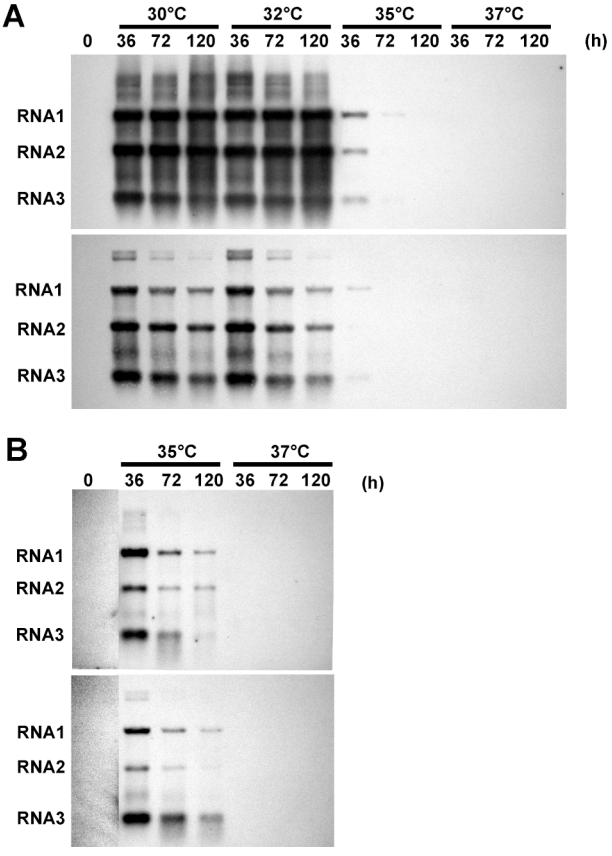

Fig. 1. Replicative competence of SGWak97 at different temperatures. E-11 cells were inoculated with SGWak97 and cultured at the indicated temperatures. Total RNA was isolated from the inoculated cells and used for Northern hybridization experiments to detect positive-sense (upper panel) and negative-sense (lower panel) viral RNAs. Blots were exposed to X-lay films for 15 min (A) and 5 h (B). Data are representative of three independent experiments.

Discussion

Upper temperature limits for the multiplication of the 16 RGNNV isolates were evaluated based on the viral titers of the culture supernatants and the replicative competence at different temperatues. Although most of the viruses adapted to 32°C, only five isolates multiplied at 35°C showing relativelly lower viral titers than those at 30°C and 32°C. Furthermore, at 37°C, no evidence of virus multiplication was obtained and the virus particles were significantly unstable in all the viruses tested. These results indicate that the upper temperature limit for the multiplication of betanodaviruses is 35°C. However, it remains to be addresssed whether E-11 cells still retain host factors at 37°C, which are necessary for betanodavirus multiplication.

Curiously, WSBUS99A did not multiply even at 30°C though this virus belongs to RGNNV that prefers the highest temperatures among the four types of betanodaviruses[9]. When RNA2 sequences were compared among the 16 RGNNV isolates, the WSBUS99A RNA2 sequence was genetically distant from those of the other viruses (unpublished data). These results suggest that WSBUS99A changed its RNA2 sequence to adapt such low temperatures. However, in the other 15 viruses, their upper temperature limits for the multiplication were varied and ranged from 30°C to 35°C though their RNA2 sequences were closely similar to each other. Thus, RNA1 as well as RNA2 may control the temperature sensitivities of betanodaviruses.

Temperature sensitivities are controlled based on the activities of viral RNA replicases in dengue virus[15], soil-borne wheat mosaic virus[16], and bacteriophage ø12[17]. The correlation between the levels of virus multiplication and viral RNA replication at any of the temperatures tested in this study suggests that temperature sensitivities in betanodaviruses also are controlled in a replicase-dependent manner. However, there still are possibilities that virus multiplication processes earlier than viral RNA replication (e. g., virion attachment onto the cell surface, uncoating, and initial translation of viral proteins) or virion stability could regulate the temperature sensitivities. Thus, more experiments are required to address which of the virus multiplication processes regulate temperature sensitivities in betanodaviruses. A higher priority experiment is to evaluate replicase activities of the RGNNV isolates at the different temperatures used in this study to see their correlation with the virus multiplication levels.

Several viral diseases were controlled experimentally by elevating temperature of rearing water in yellow tail[18], carp[19], and coho salmon[20]. Very recently, RGNNV infection of humpback grouper *Cromileptes altivelis* fingerlings was reduced significantly by elevating rearing water temperature to 35°C[21]. This result is compatible with our findings that most of the RGNNV iso-

lates did not multiply at 35°C. Based on our results, rearing water temperature at 37°C looks more effective to control VNN though this temperature also would be stressful to fish.

Betanodaviruses have been identified from many wild marine fish species caught around culture facilities[3] and in oceanic regions[4] as well as from diseased cultured fish[1]. These facts mean that we would take betanodaviruses orally with high possibilities when we eat raw marine fish. However, the upper temperature limits for virus multiplication observed in this study and the strict host specificity of the viruses[11,14] would prevent us human from betanodavirus infections.

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