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Influence of temperature on Mx gene expression profiles and the protection of sevenband grouper, *Epinephelus septemfasciatus*, against red-spotted grouper nervous necrosis virus (RGNNV) infection after poly (I:C) injection

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ABSTRACT

Influence of temperature on the susceptibility of fish against virus infection has been studied for a decade. Recent reports have been shown the effects of rearing temperatures on the fish immune system against virus infection. However, the roles of temperature in regulation of type I interferon (IFN) system has not yet been investigated. Thus, the effects of temperature on type I IFN response were investigated in this study using poly (I:C) injection in sevenband grouper and Mx gene was used as a marker for type I IFN expression. Quantitative real-time PCR (qPCR) result showed that Mx expression profiles were moderately different between temperatures. The highly up-regulated Mx transcripts at 3 h post injection (hpi) were observed in high temperatures (25 °C and 30 °C) but not in low temperatures (15 °C and 20 °C). Meanwhile, low temperatures (15 °C and 20 °C) could detect the highly up-regulated Mx transcripts at 24 hpi. Expression of Mx transcripts was also observed at 72 hpi at 15 °C. Poly (I:C)-injected fish were challenged with RGNNV after 72 and 168 hpi. At 72 hpi, 100% of fish survived at all temperatures, whereas 95% survival rate was observed at 168 hpi at 25 °C during 14 days of observation. To further verify the duration period of an antiviral state at different temperatures, qPCR and endpoint dilution assay were used to quantify the number of virus in fish challenged with RGNNV. The reduction of viral copy numbers and viral titers could be observed at 72 and 168 hpi. However, high viral copy numbers and viral titers could be detected at 168 hpi at 30 °C. These results demonstrate that temperatures influenced on the Mx expression profiles and the duration period of an antiviral state efficiently interfered with virus replication at different temperatures.

1. Introduction

Environmental conditions such as temperature have been shown to affect fish susceptibility to virus infection and fish immune system [1–6]. Increasing the water temperature from 14 °C to 20 °C has been reported to influence the cumulative mortality rates of Japanese flounder (*Paralichthys olivaceus*) infected with viral haemorrhagic septicemia virus (VHSV) [4]. Effect of temperature on fish cellular and humoral immune responses has been studied in many fish species, including channel catfish (*Ictalurus punctatus*), tench (*Tinca tinca*, L.), carp (*Cyprinus carpio*), Atlantic cod (*Gadus morhua*, L.), rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) [5–10].

Basically, the first line of fish innate immune system against virus infection is based on type I interferon (IFN) expression [11]. These major cytokines induce the expression of several interferon-stimulated genes (ISGs) which exhibit antiviral effects. Some IFN-stimulated genes which interfere with viral infection are Mx, interferon-stimulated gene 15 (ISG-15), Viperin and 2′-5′-oligoadenylate synthetase [12]. Several reports have investigated the IFN-related gene response after virus infection, including Mx gene [13–15]. Mx gene expression has been used as an indicator of IFN

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production or antiviral activity in a number of fish species [16–19]. Mx proteins are also particularly induced by poly (I:C), a synthetic double-stranded RNA that is a known IFN inducer and viral infection mimic, and they mediate the inhibition of virus infection [20–22]. Induction of Mx gene expression *in vitro* and *in vivo* in response to poly (I:C) has also been studied [22–24]. Poly (I:C) injection with observed increase in Mx gene expression has been shown to induce efficient protection from infectious salmon anemia virus (ISAV) and infectious pancreatic necrosis virus (IPNV) in Atlantic salmon and from sole aquabirnavirus in Senegalese sole (*Solea senegalensis* (Kaup)) [21,25,26]. Furthermore, Nishizawa et al. [27] demonstrated that poly (I:C) injection also offered protection of sevenband grouper against RGNNV infection related with the duration period of an antiviral state. Ohta et al. [22] also reported that the up-regulation of Mx genes of sevenband grouper after poly (I:C) stimulation. These studies indicated that Mx could be used to measure type I IFN response to virus infection. Recently, fish immune responses at different temperatures have been investigated upon virus challenge and poly (I:C) treatment [28,29]. Mx expression in zebrafish (*Danio rerio*) after poly (I:C) injection was higher at 28 °C than at 15 °C [28]. Additionally, in Japanese flounder infected by VHSV, expressions of interferon-related genes, such as ISG-15 and Mx gene, were significantly higher in fish reared at 15 °C than in fish reared at 20 °C [29]. However, the influence of temperature on type I IFN response induced by poly (I:C) and the role of type I IFN in interfering with virus replication at different temperatures have not yet been clearly investigated.

Thus, we investigated type I IFN response at different temperatures by studying Mx expression profiles using poly (I:C) as a model for mimicry of viral infection in sevenband grouper. Moreover, the duration period of an antiviral state at different temperatures was examined by observing fish mortality and the amount of virus after RGNNV challenge.

2. Materials and methods

2.1. Viruses

RGNNV (SGEhi00) isolated from VNN-affected sevenband grouper from Ehime Prefecture in 2000 [30] were amplified at 26 °C in E-11 cells using Leibovitz L-15 medium (Gibco) supplemented with 10% (V/V) fetal bovine serum (FBS, Takara), 100 IUml^{-1} penicillin and 100 μg ml^{-1} streptomycin. The viruses were collected from the culture supernatant by centrifugation (800 g, 10 min, 4 °C) and viral titers were performed using 96-well microplates seeded with E-11 cells by endpoint dilution assay to determine the 50% tissue culture infectious dose ($TCID_{50}$). The viral supernatant was separated into 1.5 ml tube and stored at −80 °C until use.

2.2. Poly (I:C) injection in fish

Specific-pathogen free (SPF) sevenband groupers (37.2 g) were reared in Ehime Prefectural Institute of Fisheries. The SPF fish were maintained in two tanks ($n = 80$) at each rearing temperature (15, 20, 25 or 30 °C) for 2 weeks prior the start of experiments. Fish in one of two tanks were injected intramuscularly with poly (I:C) at 100 μg 50 μl^{-1} fish^{-1}, while fish in the other tank were injected intramuscularly with 50 μl fish^{-1} of DEPC-treated water as a negative control.

2.3. Expression profiles of Mx gene in fish

Three fish of each tank were sacrificed at 3, 24, 72 and 168 hpi and head kidney samples were collected. Total RNA was extracted using RNAiso Plus (Takara) and cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystem) following the manufacturer's instruction. The cDNA was used to determine the effects of different rearing temperatures on the duration period and expression profiles of Mx gene by qPCR analysis. qPCR was carried out using THUNDERBIRD SYBR qPCR mix (Toyobo) with the consensus EF1-α and Mx primers from sevenband grouper (Table 1).

Table 1
List of primers used for qPCR.

Primer name	Sequence of oligonucleotides and direction 5′ to 3′	Accession no.
Grouper Mx-F	ATCAGCTTGGTGGTTGTTCC	(AB604608)
Grouper Mx-R	TGTGCCATCTTCAGTGCTTC	(AB604608)
Grouper EF1-α-F	TTCAAGTATGCCTGGGTGCT	(AB604609)
Grouper EF1-α-R	GGTCTGTCCGTTCTTGGAGA	(AB604609)
RGNNV CP-F	CTGCCTGATCCAACTGACAA	(JN662462)
RGNNV CP-R	CTGTTCTGCTTTCCCACCAT	(JN662462)

2.4. Evaluation of the survival rate in fish injected with poly (I:C)

At 72 and 168 hpi, thirty fish per time course were challenged by intramuscular injection of RGNNV at a dose of $10^{5.8}$ $TCID_{50}$ 50 μl^{-1} fish^{-1}. Fish were then divided into 2 groups and were maintained at 26 °C. First group ($n = 20$) was used to monitor the survival rate for 14 days, whereas the remaining group ($n = 10$) was used to determine the RGNNV infectivity as described below.

2.5. Quantification of RGNNV in fish injected with poly (I:C)

At 2 days after challenge with RGNNV, fish in the remaining group were collected to obtain brain tissues. The tissues were homogenized and analyzed to determine the RGNNV titers and absolute viral copy numbers of RGNNV. Titration of RGNNV was performed using similar weight of homogenized tissues adjusted to 1 ml with Leibovitz L-15 medium and 2 fold dilution of each sample was prepared to determine the $TCID_{50}$ by endpoint dilution assay (log_2). The cytopathic effect (CPE) was observed after inoculation of RGNNV sample into E-11 cells for 7 days. Meanwhile, the absolute viral copy numbers of RGNNV were determined by qPCR analysis of cDNA from homogenized brain tissues using the primers specific to coat protein (CP) of RGNNV (Table 1).

2.6. Statistical analysis

Statistical analysis was performed using SPSS version 17.0. Comparisons between groups in each time course were calculated by one-way analysis of variance (one-way ANOVA). Differences were defined statistically significant at $p < 0.05$.

3. Results

3.1. Effects of temperature on Mx expression profiles

Mx expression profiles in the head kidney of poly (I:C)-injected sevenband grouper reared at four different temperatures were examined using qPCR analysis. The relative Mx mRNA expression is shown in Fig. 1. Mx transcripts were up-regulated in response to poly (I:C) injection with significantly differences between temperatures. At 3 hpi, the high level of Mx transcripts was observed at 25 °C and 30 °C, which dramatically decreased at 24 hpi. On the other hand, high level of Mx transcripts at low temperatures, 15 °C and 20 °C, could be observed at 24 hpi. Meanwhile, no significant difference of Mx transcripts in all groups was observed at 168 hpi.