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Temperature-dependent viral replication and antiviral apoptotic response in viral haemorrhagic septicaemia virus (VHSV)-infected olive flounder (*Paralichthys olivaceus*)

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ABSTRACT

The olive flounder (*Paralichthys olivaceus*) shows a high rate of mortality to viral haemorrhagic septicaemia virus (VHSV) in the winter and spring but has zero mortality over 20 °C. In this experiment, we studied the effect of rearing temperature on viral replication, viral transcription and antiviral apoptotic immune response in VHSV-infected olive flounder by real-time polymerase chain reaction. Olive flounder were given intra-peritoneal injections of VHSV ($10^{7.8}$ $TCID_{50}$/ml) and were reared at 15 °C or 20 °C. Five fish were randomly sampled for head kidney at 3, 6 and 12 h post-infection (hpi) and 1, 2, 4 and 7 days post-infection (dpi). Total RNA extracted from the tissue was reverse transcribed and used as template for real-time PCR. In the 15 °C group, the number of viral gRNA copies peaked after 2 dpi and remained high through 7 dpi, while in the 20 °C group, the copy number was at the highest at 1 dpi but drastically declined at later stages. Viral mRNA levels in the 15 °C group gradually increased starting at 3 hpi to reach their maximum value at 12 hpi and remained high until 2 dpi, whereas the other group showed much lower copy numbers that were undetectably low at 4 and 7 dpi. Type II IFN expression increased as the viral copies increased and the 20 °C group showed quicker and stronger expression than the 15 °C group. The MHC class I and CD8 expression was high in both the groups at early stage of infection (3–6 hpi) but at later stages (2–7 dpi) in 15 °C group expression reduced below control levels, while they expressed higher to control in 20 °C group. The expression of granzyme in 15 °C fish showed a single peak at 2 dpi, but was consistently expressing in 20 °C fish. Individuals expressed very high levels of perforin expressed very high levels of caspase 3. In 15 °C fish, TNFα, FasL and p53 expressed significantly higher than 20 °C only at initial stages of infection (3–6 hpi). Caspase 3 expression found to be low in 15 °C fish whereas it was significantly elevated in 20 °C group. Interestingly individual fish with high caspase 3 expression contained very low viral RNA. Thus, from our experiment, we can conclude that an effective apoptotic immune response in VHSV-infected olive flounder plays a crucial role in the survival of the host at higher temperatures.

1. Introduction

The olive flounder (*Paralichthys olivaceus*), a temperate marine fish that is distributed along the Western Pacific coast, is a major aquaculture species for the Korea and Japan [1,2]. However, since 2001 Korean olive flounder aquaculture industry has been experiencing wide-spread mortality due to viral haemorrhagic septicaemia virus (VHSV) infection during the winter and spring, but shows no mortality in the summer season [3,4], indicating a relationship between antiviral immunity and rearing temperature. Sano et al. [5] demonstrated the effect of water temperature on the mortality of VHSV-infected olive flounder. They found that the fish do not die at 20 °C, but the same group succumbs to the virus at 15 °C. This phenomenon of temperature-dependent pathogenicity has been reported for infectious haematopoietic necrosis virus (IHNV) or VHSV-infected salmonids [6–8], VHSV-infected Japanese eel (*Anguilla japonica*) [9], VHSV-infected bluegills (*Lepomis macrochirus*) [10] and spring viraemia of carp virus (SVCV)-infected common carp (*Cyprinus carpio*) [11].

Upon viral infection, the host induces expression of interferon (IFN) to stimulate antiviral immune responses. Both type I and type II IFNs are effective activators of major histocompatibility complex

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(MHC) class I, which presents processed antigen to CD8+ T cells (cytotoxic T lymphocyte – CTL) [12,13]. Activated CTLs kill the infected cell by releasing cytolytic granules (such as granzyme and perforin) or activating death receptors to initiate apoptosis of the target cell [14,15].

An immediate apoptotic effect upon viral infection should limit viral replication and stop or reduce viral spreading. However, some viruses have developed mechanisms to block or delay early apoptosis so that they can achieve maximum replication [14,16]. Though the recombinant Mx protein was very efficient in controlling the VHSV in in vitro condition [17], VHSV-infected olive flounder containing high viral copies at 15 °C expressed high level of Mx transcript but toll-like receptor 7 (TLR-7) and TLR-2 were under-expressed [18]. The non-virion (NV) protein of VHSV is reported to inhibit apoptosis at an early infectious stage to allow effective viral recruitment [19]. A VHSV NV knockout mutant failed to cause disease in olive flounder at 15 °C [20,21] confirming that VHSV has an inhibitory mechanism on the host immune system.

Based on our previous results [18] and the importance of an apoptotic immune defence mechanism, we designed this study to investigate the expression kinetics of apoptosis-associated genes in VHSV-infected olive flounder maintained at two different temperatures. We developed VHSV infection in olive flounder at 15 °C and 20 °C to study the apoptotic immune response and the viral replication at early infectious as well as later recovery period.

2. Materials and methods

2.1. Experimental infection

VHSV (F1Wa05 strain) cultured in fathead minnow (FHM) cells was stored at −80 °C. Olive flounder (18–22 g) collected from a local farm were divided into two groups and were maintained at 15 ± 0.5 °C and 20 ± 0.5 °C in a controlled indoor rearing facility. The fish were acclimatised to their respective rearing condition for 7 days prior to infection. A nested polymerase chain reaction (PCR) of 10 randomly selected individuals using VG1-VD3 and VD5-VD3 primers (VG1: 5′-atggaatggaacacttttttc-3′ VD5: 5′-tcccgctatcagtcaccag-3′and VD3: 5′-tgtgatcatgggtcctggtg-3′) [22] confirmed that the fish were initially free of VHSV. Sixty fish per group were intraperitoneally injected with 100 μl of viral suspension ($10^{7.8}$ $TCID_{50}$/fish). A similar mock injection was given to control fish for both the 15 °C and 20 °C groups. We randomly selected five fish/sample at 3, 6 and 12 h post-infection (hpi), and 1, 2, 4 and 7 day post-infection (dpi) from both the 15 °C and 20 °C groups. The head kidneys of fish were aseptically dissected, flash frozen and stored at −80 °C until they were used for total RNA extraction.

2.2. Total RNA extraction and reverse transcription

Total RNA was extracted using RNAiso Plus (Takara Bio Inc, Japan), following standard protocols. The dried RNA pellet was dissolved in RNase-DNase free water (Sigma–Aldrich, USA) and was aliquoted and stored at −20 °C until further use. The RNA was checked for quality by 1% agarose gel electrophoresis and was quantified using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, USA). One microgram of total RNA treated with DNase I (Amplification grade; Invitrogen Life Technologies, USA) was reverse transcribed using moloney murine leukaemia virus (M-MLV) reverse transcriptase (Bioneer, Korea) using an oligo dT primer (Invitrogen Life Technologies, USA) as recommended by the manufacturer. The resulting cDNA was stored at −20 °C.

The cDNA of VHSV genomic RNA (gRNA) was synthesised by reverse transcribing 1 μg of total RNA with specific sense primer for the VHSV N gene (5′-gtatcataaaagatgatgagttatgttacagggg-3′). This cDNA was used for absolute quantification of mature virus (negative strand genome). To quantify the viral N gene transcript (positive sense mRNA), cDNA was synthesised using oligo dT primers (the same mentioned above).

2.3. Primers

Primers for partial sequencing of caspase 3 (sense primer- 5′-*gtcccacagcttcagatacagc*-3′, antisense primer 5′-*gacgtggagacgacatcag*-3′; product size: 712 bp) were designed from our cDNA library following earlier described methods [18]. The deduced amino acid sequence showed close homology with the caspase 3 from the fire clownfish (*Amphiprion melanopus*) (92%), mandarin fish (*Siniperca chuatsi*) (91%), Atlantic salmon (*Salmo salar*) (73%), chicken (*Gallus gallus*) (66%) and human (*Homo sapiens*) (65%), confirming that the obtained sequence is caspase 3 of the olive flounder. Primers for real-time PCR were designed using Primer3-Plus. The primer efficiency was determined as previously described [18]. The details of the primers used for real-time PCR expression study are given in Table 1.

2.4. Quantitative expression of immune genes and viral RNA

Real-time PCR was carried out in an *Exicycler*™ 96 Real-Time Quantitative Thermal Block (Bioneer, Korea) using SYBR Green *AccuPower*® PCR PreMix (Bioneer, Korea). One microlitre of five-fold diluted cDNA was used as the PCR template (performed in duplicate). The reaction program was set according to the supplied manual. The relative expression level was calculated based on Pfaffl's normalization method [23] using the equation

Table 1
Detailed information on the primers used for real-time PCR.

Target gene	GenBank acc. no	Product length	Sense primer	Antisense primer	Ta[a]	PCR efficiency
β actin	HQ386788	131	cctcttccagccttcattc	tggttcctccagatagcac	56	2.0979
VHSV N RNA	EF079895	138	atctggaggcaaagtgcaag	ccatgaggttgtcgttgttg	62	2.0221
Type II IFN	AB435093	126	ctacaagcggcgatatgatg	ggaggttctggatggttttg	64	2.0807
MHC Class I	AB126921	148	tctccctcctctccagtcagc	gctcatctggaaggtcccgtcat	58	1.9694
CD8	AB082957	108	taagggcaacactaacacagg	atgaggaggaggagaaggag	56	2.0980
TNFα	AB040449	113	aaacacctcacgtccatca	gcgtcctcctgactcttct	56	1.9888
Perforin	AB084905	100	aacaacaacaacaacccacac	Tgtccctgtcccacactt	56	1.9325
Granzyme	AB191196	121	attccaggcagctcataaa	gtcttggtttccttcacaga	58	1.9217
FasL	AB206381	128	gagcaaatcgagggacgaga	ttcctttctccagcgtgacc	64	1.9674
p53	EF564441	159	cgaggaaagcagcaccaaga	ccccgaccgacaataggaag	58	1.9558
Caspase 3	JQ394697[b]	115	acatcatgacacgggtgaac	tccttcgtcagcattgacac	58	1.9146

[a] Ta: annealing temperature.
[b] Sequenced in this study.