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Impaired TLR2 and TLR7 response in olive flounder infected with viral haemorrhagic septicaemia virus at host susceptible 15 °C but high at non-susceptible 20 °C

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ABSTRACT

The olive flounder (*Paralichthys olivaceus*) is susceptible to viral haemorrhagic septicaemia virus (VHSV) at 15 °C but no mortality is observed at 20 °C even though the virus can grow profusely in vitro. Thus, we designed an experiment to better understand the immune response of olive flounder to VHSV when the host reared at 15 °C or 20 °C and infected with the virus. Olive flounder (18–22 g) reared at 15 ± 0.5 °C or 20 ± 0.5 °C were intra-peritoneally injected with VHSV ($10^{7.8}$ $TCID_{50}$/fish) and sampled ($n = 5$) for head kidney at 3, 6, 12 hpi, 1, 2, 4 and 7 dpi; similarly, mock injected control groups ($n = 5$). Real-time PCR-based absolute quantification method was followed to quantify copies of VHSV gRNA and mRNA, while the immune gene expression of the olive flounder was quantified relative to internal control, β-actin. Viral infection resulted in a cumulative mortality of 24% in olive flounder reared at 15 °C, but no mortality was recorded in the 20 °C group or control groups. TLR2 and TLR7 expression at 15 °C was enhanced during early-infection phase (3–6 hpi) and recovery phase (4–7 dpi) when viral transcription was low, but expression was significantly reduced (12 hpi–1 dpi) at peak-infection period. However, the 20 °C group showed low viral transcription and expressed high level of TLR7 and a moderately higher unchanged level of TLR2. In both the groups, TLR3 expression was unaffected. Nevertheless, expression of MDA5 and LGP2 increased significantly irrespective of rearing temperature at the time of peak infection, hence at 15 °C VHSV down-regulated expression of TLR2 and TLR7 but not MDA5 or LGP2. Comparatively, at 15 °C IRF3 expressed high but IRF7 remained very low. Interleukins (IL-1β, IL-6 and IL-8) were significantly elevated in both the groups, but quicker and for a shorter period at 20 °C. In the 15 °C group, an extended period of expression of ILs could create an unsafe prolonged inflammatory condition. The olive flounders expressed high ISGs at 15 °C but were lagging by 12 h than 20 °C group. Based on these findings, we concluded that viral-mediated disruption of TLR2 and TLR7 expression in the 15 °C group could have delayed the host interferon response and provided a window for high viral growth. However, an effective host immune response at 20 °C contained VHSV from reaching the critical limit.

1. Introduction

Viral haemorrhagic septicaemia (VHS) is one of the most dangerous viral fish diseases in temperate countries and has severely affected the production and economy of the olive flounder (*Paralichthys olivaceus*) aquaculture industry in Japan and Korea [1,2]. The disease is caused by an enveloped negative sense single-strand RNA virus called VHS virus (VHSV), which has been classified under the genus *Novirhabdovirus* of Rhabdoviridae [3]. The host, olive flounder, is a temperate fish and has an optimum growth temperature of 15 °C–25 °C [4]. The olive flounder has been shown to be susceptible to VHSV at temperatures ranging from 8 °C to 15 °C and the highest mortality rate occurs at 13 °C–15 °C but no mortality is observed above 20 °C [1,2,5]. The effect of shifting the rearing water temperature on the mortality rate of olive flounder infected with VHSV confirmed that the host do not die at 20 °C, but the same group succumbs to the disease when the rearing temperature is lowered to 14 °C immediately after infection [6]. Nevertheless, a high level of VHSV was recorded in fathead minnow (FHM) cells cultured at 15 °C or 20 °C and the viral multiplication rate was higher at 20 °C [1,5]. This confirmed that the virus can grow at 20 °C but failed to develop disease in the host. It has been reported in teleosts that the environmental temperature has a greater effect on the adaptive immunity than on innate

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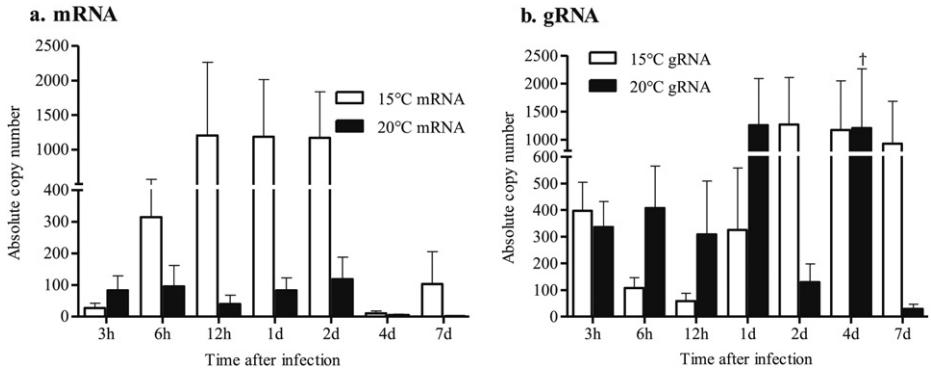

Fig. 1. Absolute viral copy number in 50 ng total RNA of olive flounder head kidney reared at 15 °C or 20 °C and infected with VHSV ($10^{7.8}$ $TCID_{50}$/fish). Mean values ($n = 5$) of mRNA (a) and gRNA (b) with standard error bar plotted against time after viral infection [15] (†: high mean value of gRNA in 20 °C group at 4 dpi is driven by one fish out of five, having very high copy number).

immunity [7]. However, stronger innate immune responses have been observed at lower temperature, which may play a major role in immune defence mechanisms [8,9]. Therefore, effect of rearing temperature on the innate immune response may not be significant but the reason for the temperature-dependent susceptibility of olive flounder to VHSV need to be resolved.

Viral infection results in a coordinated activation of immune responses in the host. Viral nucleic acids and glycoprotein, which are pathogen-associated molecular patterns (PAMP), induce host pattern recognition receptors (PRR), such as toll-like receptors (TLRs) and retinoic acid-inducible gene-I (RIG-I) like receptors (RLRs) to initiate an innate immune response [10]. Viral nucleic acids have been reported to be detected by cytoplasmic PRRs such as melanoma differentiation-associated protein 5 (MDA5) and Laboratory of genetics and physiology 2 (LGP2), while endosomic TLR7 detect the ssRNA of viruses [11]. TLR2 is a membrane bound receptor known to detect various microbial components, including the envelope glycoproteins of viruses [12]. These receptors induce activation of inflammatory factors and interferon (IFN) stimulated genes (ISGs) to create a non-specific antiviral condition by enhancing production of cytokines and control viral growth. They have also been shown to activate other immune cells and adaptive immunity [11–13].

The present study was conducted in continuation of our previous work. At a culture temperature of 15 °C, olive flounder infected with VHSV were shown to under-express TLR2 and TLR7 (relative to internal control, β-actin) when viral transcription was high [14]. VHSV infection caused high level of caspase-3 expression in the olive flounder at 20 °C but no response at 15 °C [15]. The viral transcription (mRNA) was highly elated in the olive flounder at 15 °C from 6 h postinfection (hpi) to 2 days postinfection (dpi) but it was comparatively lower at 20 °C; however, at 4–7 dpi both the groups recorded very low viral transcription. High viral genomic RNA (gRNA) was noticed at 1–7 dpi in 15 °C group, while it was high only at 1 dpi in 20 °C group (Fig. 1) [15]. Therefore, the expression of the TLRs at 20 °C needs to be examined. Positive response of MDA5 and LGP2 in olive flounder infected with VHSV has been reported [16,17] but their expression kinetics in olive flounder at host susceptible or non-susceptible temperatures is not studied. Even though TLR7 was under-expressed, we found a high level of expression of IFN regulatory factors (IRFs), interleukins (ILs) and ISGs in olive flounder after VHSV infection when reared at 15 °C [14]. Thus, we hypothesized the reason would be activation of PRRs other than TLR2 or TLR7. Hence, we continued our investigation to understand the immune gene kinetics of TLRs, RLRs, IRFs, ILs and ISGs in VHSV-infected olive flounder reared at 15 °C or 20 °C and they were compared with the viral gRNA and mRNA at the respective time point.

2. Materials and methods

2.1. Experimental infection

Olive flounder (18–22 g) were brought from a local farm and maintained at two temperatures, 15 ± 0.5 °C and 20 ± 0.5 °C, in our indoor rearing facility. The fish were acclimatised to their respective rearing condition for 7 days prior to experiments. Ten randomly selected olive flounder were sacrificed to diagnose for the presence of VHSV by nested polymerase chain reaction (PCR)

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	Reference
β-actin	HQ386788	131	cctcttccagccttcattc	tggttcctccagatagcac	56	2.0979	[14]
VHSV N	EF079895	138	atctggaggcaaagtgcaag	ccatgaggttgtcgttgttg	62	2.0221	[14]
MDA5	HQ401014	133	acgagcgacctcttgatttg	agcgtcaccacgaagttttg	60	2.0795	
LGP2	HM070372	110	gatgatgcagatgatccaagactaca	ctcgctgctctaaaatcaccacat	60	1.9977	
TLR2	AB109393	120	gctacatctgcgactctcct	cacagggacacgaacaaatc	58	1.8974	[14]
TLR3	AB109394	122	aacgcctggttcatcaagtg	cgaatgtcgaagtgcaagag	58	1.9199	
TLR7	HQ845984	97	cctgggaaatctggaagaac	tttgagggaggagaaactgc	62	2.0290	[14]
IRF3	GU017418	156	acaccatgaaccagagcaac	tgtccaaaagtgtccctgtg	62	2.0354	[14]
IRF7	GU017419	123	tctgatctgtcggcactttc	ccgaacacggagttaatgag	58	1.8257	[14]
IL-1β	AB070835	110	aaagaagcatcaccactgtct	ctactcaacaacgccacctt	56	1.9473	[14]
IL-8	AF216646	105	tccgtgggtgaagagagt	attagggtcgtgttgagttgt	56	2.0955	[14]
IL-6	DQ884914	119	actgatcggcctcatcaaag	tcttcctgtggaaagtgctg	62	1.9146	[14]
IFN type 1	AB511962	88	caggtgtcaaatgcatcagc	tggaatcctcctcaaacagg	62	2.0254	[14]
ISG-15	AB519717	135	gctgtatgacaacggtcagc	ctcaggaagacctggatgg	60	1.9417	[14]
Mx	AB110446	159	tcactggatttcccaacctc	tgtcactcaaactgctgctg	62	2.0376	[14]

[a] Ta: annealing temperature.