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Correlation of virus replication and spleen index in rock bream iridovirus infected rock bream *Oplegnathus fasciatus*

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Rock bream iridovirus (RBIV) is a member of the *Megalocytivirus* genus that causes severe mortality to rock bream (*Oplegnathus fasciatus*) with characteristic clinical signs of spleen enlargement. In this study, we assessed spleen size and RBIV copy number patterns in RBIV-infected rock bream to determine lethal and safe levels of virus copy number/spleen index that may define disease progress. We found that rock bream infected with RBIV (1.1×10^7 virus copy number/100 μl) and held at 29, 26, 23 or 20°C exhibited significantly higher levels of spleen size compared to 17°C. In dead condition (100% mortality at 20~29°C), the spleen index (spleen weight / fish weight × 100) and virus copy number were 3.00~5.38 and 10^6~10^8/μl, respectively. Conversely, in survived condition (0% mortality at 17°C), spleen index and virus copy number was as low as not-infected control (0.34~1.22/10^0~10^1/μl, respectively). These findings suggest that spleen index can be an indicator of disease severity of RBIV disease.

Key words: rock bream iridovirus, rock bream, virus replication, spleen index, spleen enlargement

Iridoviridae is a family of large double-stranded DNA virus (120~300 nm) with an icosahedral morphology (Williams, 1996). The family includes five genera: *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Lymphocystivirus* and *Megalocytivirus*. *Megalocytivirus* cause disease in more than 50 fish species and currently threaten the aquaculture industry, causing great economic losses in Korea, Japan, China and Southeast Asia (Inouye *et al*., 1992; Nakajima and Sorimachi, 1994; Chua *et al*., 1994; Matsuoka *et al*., 1996; Miyata *et al*., 1997; Chou *et al*., 1998; Jung and Oh, 2000; He *et al*., 2002). Rock bream iridovirus (RBIV), which belongs to the genus *Megalocytivirus* (Do *et al*., 2004; Song *et al*., 2008; Kurita and Nakajima, 2012) remains an important health problem in rock bream *Oplegnathus fasciatus* (Jung and Oh, 2000).

Various indices have been used to evaluate the condition or well-being of fish, including the relative condition factor (Le Cren, 1951), relative weight (Wege and Anderson, 1978), gut index (Jensen, 1980), RNA-DNA ratios of liver and muscle (Bulow *et al*., 1981), visceral somatic index (Delahunty and de Vlaming, 1980; Adams *et al*., 1982) and liver somatic index (Edwards *et al*., 1972; Tyler and Dunn, 1976; Valtonen, 1974; Heidinger and Crawford, 1977; Bulow *et al*., 1978; Delahunty and de Vlaming, 1980; Allen and Wootton, 1982; Adams and McLean, 1985). Liver somatic index is a useful biomarker to detect the environmental stressors, and it is one of the most sensitive growth indicators (Edwards *et al*., 1972; Valtonen, 1974; Tyler and Dunn, 1976; Heidinger and Crawford, 1977; Bulow *et al*., 1978; Allen and Wootton, 1982; Adams and McLean, 1985). Liver is the meta-

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bolic organ, and fish store energy in the liver during periods of high energy intake. Most of this stored energy is in the form of glycogen (Busacker *et al*., 1990). Therefore, the relative size of the liver should be correlated to the nutritional state of the fish as well as the growth rate.

Other organ indexes except liver have not been commonly used as biomarkers for the evaluation of fish health. Spleen is one of the major filtering organs in the vascular system, removing effete blood cells and foreign agents. In humans, vertebrate and fish, the spleen is the major site of pathogen growth and disease pathology (Chinabut *et al*., 1990; Toranzo *et al*., 1991; Inouye *et al*., 1992; Santos *et al*., 1992; Fryer and Mauel, 1997; Jung and Oh, 2000; Kim *et al*., 2005; Kim *et al*., 2009; Jeffery *et al*., 2010). Hence, the spleen is frequently used in the diagnosis of damage caused by pathogen infection. Studies on fish disease such as *Flexibacter psychrophilus* (Santos *et al*., 1992), *Pasteurella piscicida* (Toranzo *et al*., 1991), *Piscirickettsia salmonis* (Fryer and Mauel, 1997), *Francesella* sp. (Jeffery *et al*., 2010), Microbacteriosis (Chinabut *et al*., 1990), Cyprinid herpesvirus 2 (Jeffery *et al*., 2007), Viral haemorrhagic septicaemia (Kim *et al*., 2009), *Trypanoplasma salmositica* (Li *et al*., 2013) and *Haemogregarina sachai* (Kirmse, 1980) have demonstrated a clear clinical sign of spleen enlargement (splenomegaly). Nearly all deaths by RBIV are accompanied by enlargement of spleen (Jung and Oh, 2000; Zhang *et al*., 2012). The simplest diagnostic method of RBIV disease is to confirm presence of abnormally enlarged cells in Giemsa-stained stamp-smear of the spleen. Basophilic enlarged cells by Giemsa stain are compactly packed with virus particles in the cytoplasm. Hence, we hypothesised that splenomegaly of rock bream can be an indicator of severity of RBIV disease.

In the present study, rock bream were artificially infected with RBIV, and RBIV copy number and spleen size were measured. Furthermore, lethal and safe levels of RBIV copy number and spleen size (index) were estimated.

Materials and Methods

Experimental infection

The RBIV was obtained from RBIV-infected rock bream in 2010 (Jung *et al*., 2014). Virus amount was quantified by quantitative real-time polymerase chain reaction (qRT-PCR). The RBIV major capsid protein (MCP) gene copies of the original virus in the supernatant preparations were 7.5×10^7/100 μl MCP gene copies, and that was suspended in phosphate-buffered saline (PBS) to 1.1×10^7/100 μl as previously described (Jung *et al*., 2014).

The RBIV-free rock bream were reared by the Fisheries Science Institute at Chonnam National University. The experimental design was identical to that explained previously (Jung *et al*., 2015). Rock bream (10.8 ± 1.5 cm, 25.1 ± 3.1 g) were used for evaluation of spleen weight at different water temperatures (29, 26, 23, 20 and 17°C) (Table 1). For viral

Table 1 Experimental details of artificial infection

Group	Infection dose/fish (virus copy number)	Days observed	Sampling point	Mortality (%)
29°C	1.1×10^7/fish	10	6, 7, 8, 9 and 10 dpi[1)]	100
26°C	1.1×10^7/fish	14	8, 9, 10, 11, 12 and 14 dpi	100
23°C	1.1×10^7/fish	15	13, 14 and 15 dpi	100
20°C	1.1×10^7/fish	26	19, 20, 21, 22, 23, 24 and 26 dpi	100
17°C	1.1×10^7/fish	150	100 and 150 dpi	0

[1)]dpi: days post infection

infection, 15 fish was intraperitoneally (i.p.) injected with 100 μl/fish containing 1.1×10^7 MCP gene copy, while the control fish was i.p. injected with the PBS (100 μl/fish) and then maintained in the aquaria containing 30 L of UV-treated seawater. To determine the RBIV replication pattern, spleen was collected from dead fish at 29, 26, 23 and 20°C and surviving fish at 17°C. Samples were stored at -80°C after being flash frozen in liquid nitrogen. Table 1 summarizes experimental conditions.

Determination of RBIV copy number in the spleen and spleen index

Genomic DNA was extracted from the whole spleen tissues (20~150 mg) of the sampled fish using an AccuPrep®Genomic DNA extraction kit (Bioneer, Korea) according to the manufacturer's instructions. Quantification of RBIV copy numbers were determined by qRT-PCR using Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer) with RBIV MCP gene specific primer set (F 5' tgcacaatctagttgagg aggtg 3' and R 5' aggcgttccaaaagtcaagg 3') according to the standard curve and method described previously (Jung *et al*., 2014). The virus copy number was expressed as viral DNA copies 1 μl of DNA of 100 μl of total DNA from a whole spleen. The detection limit level of RBIV MCP copy number was 1.0×10^1/μl. The spleen indexes were defined by the following formula:

$$\text{Spleen index} = (\text{Spleen weight (g)} / \text{Fish weight (g)}) \times 100.$$

Results

RBIV replication and spleen size at different infection condition

Dead condition (RBIV infection at 29, 26, 23 and 20°C). The virus copy number from 60 dead fish ranged from 1.11×10^7 to 9.56×10^8/μl and was regarded as 'lethal' virus copy number (Fig. 1A). The spleen weight from all of the dead fish ranged from 79 to 140 mg (average 110.3 mg) (Fig. 1B), and spleen index was in the range of 3.06 to 5.38 (average 3.65) and was regarded as 'lethal' (Fig. 1C). Fig. 1D shows cumulative mortality of fish sampled for this study (previously published by Jung *et al*., 2015).

Survived condition (RBIV infection at 17°C)

At 17°C, all the fish survived with no clinical signs. The five sampled fish at 17°C did not have enlarged spleens (range of 17 to 49 mg) and had low virus copy numbers (average 3.0×10^1/μl) at 100 dpi (Fig. 1A). The remaining survivors (10 fish) were placed in increased water temperature to 26°C; at 100 dpi, the fish did not die and had low virus copy numbers (average 5.6×10^1/μl) at 150 dpi. All the 15 surviving fish showed a spleen weight of below 50 mg against RBIV infection during the experimental period (Fig. 1B). The spleen index in survivors was in the range of 0.53 to 1.44 (average 0.84) (Fig. 1C).

Control

PBS-injected fish maintained at 29, 26, 23 and 20°C had a 100% survival rate. The fish sampled in all groups were distributed in the below detection limit level of RBIV MCP copy number (1.0×10^1/μl) (Fig. 1A). Spleen weight of all control fish was under 50 mg in the experimental period (Fig. 1B). The spleen index was in the range of 0.58 to 1.50 (average 1.13) and was regarded as 'safe' (Fig. 1C).

Discussion

Spleen acts as an important immune responsive organ against a variety of pathogens. It is crucial for the capture and destruction of pathogens and the formation of adaptive immunity (Mebius and Kraal, 2005). Spleen size of the fish is considered as a simple measurable immune parameter with a potential role in immune response against pathogens (Skarstein *et al*., 2001; Taskinen and Korter, 2002; Korter and Taskinen, 2004; Lefebvre *et al*., 2004), and European

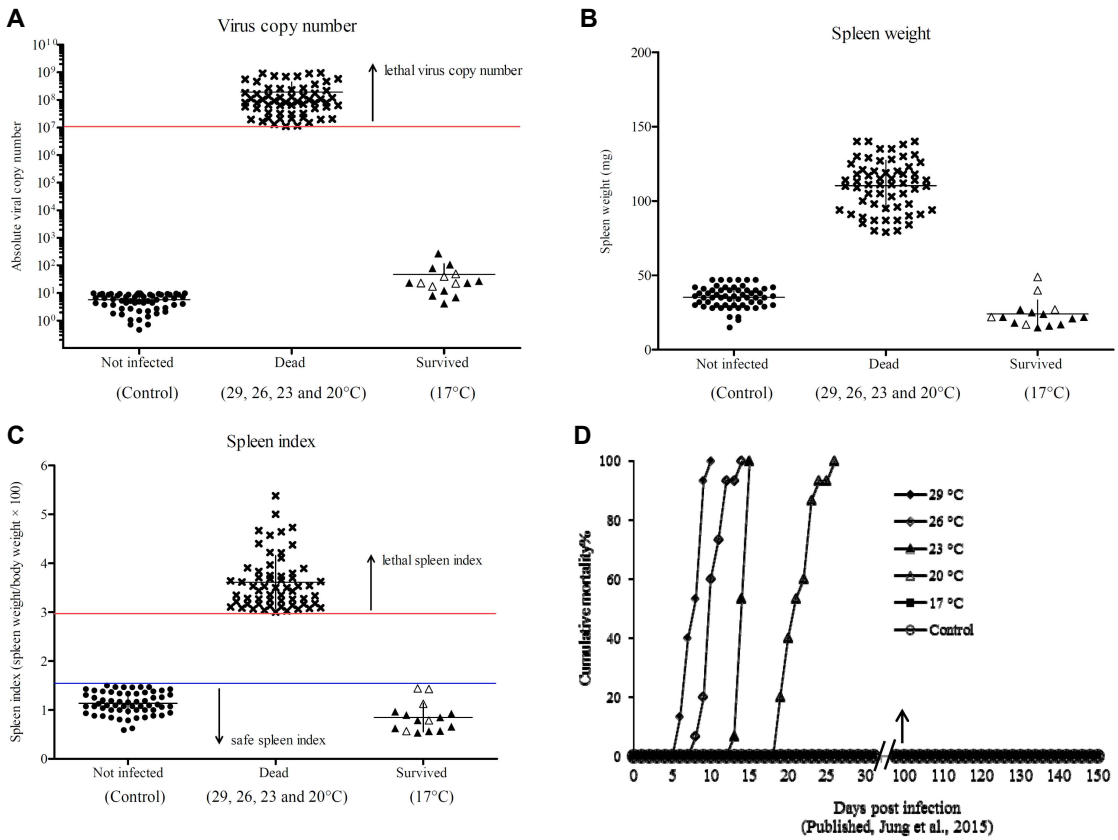

Fig. 1. Absolute major capsid protein (MCP) gene copies (A), spleen weight (B) and spleen index (C) of rock bream intraperitoneally injected with 1.1×10^7 MCP gene copy/fish at different water temperatures (29, 26, 23, 20 and 17°C). Surviving fish at 17°C were sampled at 100 (△) and 150 (▲) days post infection. The cumulative mortality (D) of rock bream previously published (Jung *et al*. 2015) is shown for reference. Virus copy number, 1.0×10^1/μl, in the PBS injected control group (A) was regarded as negative.

starlings (*Sturnus vulgaris*) with larger spleens have been shown to mount stronger immune responses (Ardia, 2005). However, RBIV-infected rock bream are characterised by enlargement of spleen (Jung and Oh, 2000), and spleen size may reflect disease status after virus infection.

First, absolute MCP gene copy and spleen weight were evaluated from the dead fish obtained from the RBIV-injected group and maintained higher than 20°C. A threshold (dangerous limit) was then set up for the virus copy number and spleen index indicating possible virus copy number and spleen index, which causes fish death. The virus copy range was 10^7 to 10^8/μl (10^9~10^{10}/100 μl/whole spleen weight 79~140 mg), and the lethal ratio of the spleen index due to RBIV infection was 3.06 to 5.38. A similar virus copy range and spleen index of dead rock bream against megalocytivirus was observed in the range of 2.03×10^7/mg and 4.85 ± 1.06, respectively (Jin et al. 2011).

In addition, control and surviving fish had very low virus copy at below 10^1/μl (detection limit level, below 10^3/100 μl/whole spleen weight 20~47 mg) and a similar range of spleen index (0.59~1.50 in the control group and 0.34~1.22 in the surviving group). These levels were regarded as safe. A contrasting observation has been reported by Hadidi *et al*., (2007)

that enlarged spleen (approximately 65 mg) and high index (1.4) was observed in an *Flavobacterium psychrophilum*-resistant rainbow trout *Oncorhynchus mykiss* group, while reduced spleen weight (approximately 30 mg) with low index (0.7) was observed in a susceptible group. Hence, spleen size and RBIV copy number in this study suggest that the spleen size (spleen enlargement due to virus infection) is not correlated with RBIV disease resistance but severity of the disease.

This was evident from our supplementary data that previously published in shown the reference (Jung *et al*., 2017). In fixed water temperatures of higher than 23°C, rock bream mortality was extreme at 100% (Jung *et al*., 2014; Jung *et al*., 2015; Jung *et al*., 2016). For this reason, water temperature shifting from 23°C to 17°C was tried in order to obtain survivors and detail the RBIV replication effect on the rock bream spleen index and evaluate the spleen index for a recovery stage from RBIV infection (Jung *et al*., 2017). Rock bream infected with RBIV and held for 7, 4 and 2 days at 23°C before the water temperature was reduced to 17°C had mortality rates of 28% (group A1), 0% (group A2) and 0% (group A3), respectively (Jung *et al*., 2017). In groups A1, A2 and A3, the acute stage of RBIV infection was from 7 to 22 dpi; when virus replication reached peak at 20 to 22 dpi (average range of 10^5~10^7/μl), spleen weight and spleen index reached their highest (average range of 108~125 mg and 2.97~3.76, respectively) (Supplementary Fig. 1). Furthermore, no mortality occurred from 30 to 100 dpi, and these fish showed no clinical signs of RBIV. This time period was regarded as a recovery stage from infection. It was evident that gradual decrease of virus copy numbers (average 10^7 reduced to 10^1/μl) accompanied with gradual reductions of spleen weight and spleen index (average 94 to 27 mg and 2.79 to 0.84, respectively) (Supplementary Fig. 1). This indicates that the spleen size of RBIV-infected rock bream was positively co-related with virus replication. A similar observation has been reported for malaria-infected mouse (i.e. enlarged spleen reduced to its normal size at several weeks after primary malaria infection in the mouse) (Stevenson and Kraal, 1989; Weiss, 1989; Achtman, 2003).

Use of the size of immune system organs as an index of investment in the immune system is a common approach, and the spleen is of particular interest. The spleen is a relatively small but critical organ that is involved in the production of lymphocytes that are used to fight against infections (John, 1994). Use of the size of the spleen as a proxy measure of immunological activity has been widespread, particularly in birds (Møller, 1997; Møller *et al*., 1998; Shutler *et al*., 1999), mammals (Cowan *et al*., 2009; Schulte-Hostedde and Elsasser, 2011) and fish (Korter and Taskinen, 2004; Lefebvre *et al*., 2004; Skarstein *et al*., 2001; Taskinen and Korter, 2002), under the assumption that a larger spleen produces and stores more lymphocytes than a smaller spleen (Nunn, 2002); hence, it can be induced disease resistance. Although RBIV-infected rock bream are characterised by enlargement of spleen, it may not be due to the increment of lymphocytes but expansion of virus-infected cell size or number; it can be concluded that rock bream spleen enlargement is not related to disease resistance and that spleen size reflects RBIV disease status.

This study clearly showed spleen size (index) has a strong positive relationship with mortality and virus copy number, and suggests the possibility of using spleen index as an indicator for the severity of RBIV disease.

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