Potential infectivity of the virus re-isolated from surviving Japanese amberjack (*Seriola quinqueradiata*) after experimental infection with red sea bream iridovirus

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Abstract

Red sea bream iridovirus (RSIV) is an agent causing significant economic loss in the aquaculture industry of East and Southeast Asia. Whether the virus can be isolated from fish that have survived previous infection is important information to enable assessment of the source of an infection in aquaculture facilities. In this study, an experimental infection using Japanese amberjack (*Seriola quinqueradiata*) artificially infected with RSIV was performed. The viral DNA was quantified and the virus re-isolated from individual infected fish. In the experimental infection, fish which were intraperitoneally injected with the RSIV cell culture ($10^{4.7}TCID_{50}$ $fish^{-1}$) or immersed with RSIV cell culture 10^{-4} dilution ($10^{1.7}TCID_{50}$ ml^{-1}) showed cumulative mortalities of 70% and 10%, respectively. Since RSIV was re-isolated from the pooled sample of spleen and kidneys from four of nine surviving fish after immersion infection, which appeared to have completely recovered from the disease, it is revealed that these fish were a potential source of infection of RSIV. Furthermore we also observed that RSIV could be re-isolated from the samples infected with more than 10^5 viral genome copies of total DNA mg^{-1}. This is the first study to evaluate the relationship between the quantity of viral DNA and virus re-isolation from Japanese amberjack infected with RSIV.

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Introduction

Red sea bream iridovirus (RSIV), in the genus *Megalocytivirus* (Kurita and Nakajima, 2012), is a virus causing extensive economic loss to the aquaculture industry of East and Southeast Asia (Jeong et al., 2003; OIE, 2012) and the virus has also been detected from ornamental fishes (Jeong et al., 2008; Subramaniam et al., in press). Infectious spleen and kidney necrosis virus (He et al., 2000) and turbot reddish body iridovirus (Shi et al., 2004) and other genotypes of *Megalocytivirus* also cause disease in aquaculture fish species such as mandarin fish (*Siniperca chuatsi*) and turbot (*Scophthalmus maximus*). Techniques for virus isolation from diseased fish and sequential passage of Megalocytiviruses *in vitro* can be problematic although there are many virological studies including: experimental infection trials (Jun et al., 2009; Xu et al., 2008), genetic analysis (Do et al., 2004; Kurita et al., 2002), *in vivo* immune response (Xu et al., 2010) and antigenic properties of the viral infection (Xiong et al., 2011).

Reported methods for the continuous passage of a RSIV isolate using grunt fin (GF) cells (Clem et al., 1961) and the quantification of viral DNA by real-time polymerase chain reaction (PCR) were recently published (Ito et al., 2013a). Ito et al. (2013a) also reported that viral DNA was detected from some of the surviving Japanese amberjack (*Seriola quinqueradiata*) which were immersed with the RSIV cell culture supernatant 10^{-4} dilution ($10^{1.7}TCID_{50}$ ml^{-1}) and the quantities of viral DNA in six tested organs were approximately equal to those found in the dead fish. In addition, it was demonstrated that RSIV survived for longer at 15°C than at 25°C in the environmental sea water (Ito et al., 2013b). These reports indicate that infection with RSIV may spread not only in summer when this disease is prevalent but also in winter. Therefore, whether the virus can be isolated from the infected surviving fish and potentially infect other individuals is important information to identify the source of infections and risk of horizontal transmission within aquaculture facilities. To our knowledge, however, there are limited reports on the source of infections in aquaculture facilities. In this study, to clarify the potential infectivity of RSIV re-isolated from surviving fish after infection, we performed a further experimental infection in Japanese amberjack using a similar experimental method as the previous study. Then the quantification of viral DNA was investigated and virus re-isolation was performed from individual infected fish. To determine actual infectivity of RSIV, the most suitable method would be to co-habit naïve fish with the surviving fish. However, this experimental approach is both labor intensive and expensive. If the virus infectivity could be estimated by measuring the quantity of viral genome, the method has potential use for virus surveillance.

Materials and methods

Cell line

GF cells were purchased from ATCC®. Cell lines were maintained in basal medium Eagle (BME; MP Biomedicals) supplemented with 10% FBS (Equitech-Bio) and antibiotics (100 U penicillin ml^{-1} and 100 mg streptomycin ml^{-1}). The cell lines and virus infected cells were cultivated at 25°C.

Virus

RSIV KagYT-96 isolate (Ito et al., 2013a) was used in this study. Eight passages of the supernatant of viral culture in GF cells was aliquoted into 3.6ml cryo tubes (Nunc®, Thermo Fisher

Scientific) and stored at -85°C until use in this experiment. A vial was thawed and titrated on 96-well black plates using mAb M10 (Nakajima and Sorimachi, 1995) as detailed in Ito et al. (2013a).

Fish

Japanese amberjack for this experimental infection were bred from broodstock with no history of RSIV disease at the Goto Laboratory, Seikai National Fisheries Research Institute. The juvenile fish were transported to NRIA, and were reared in 1ton fiber reinforced plastic (FRP) tanks.

Experimental infection

Thirty juvenile Japanese amberjack (initial body weight 38.5 ± 8.90g) were used. The fish were divided into three groups of ten fish each. Fish from group one were intraperitoneally (IP) injected with 0.1 ml of supernatant of the aforementioned virus cell culture at $10^{5.7}TCID_{50}\,ml^{-1}$. The second group was immersed for 1h in the supernatant of RSIV cell culture 10^{-4} dilution ($10^{1.7}TCID_{50}\,ml^{-1}$) with sand-filtered seawater. The third group was injected with culture medium without virus as the negative control. The experimental design and final fish size of each group in the experiment is summarized in Table 1. Fish in all groups were held in 60 L tanks at 25.0°C and fed a commercial diet (Saki-Hikari®, Kyorin) twice a day and mortality was observed for 28 days. A pooled sample of spleen and kidney was aseptically dissected from every deceased and surviving fish. The samples were analyzed by virus isolation in GF cell culture, for RSIV DNA by conventional PCR and RSIV DNA was quantified by real-time PCR as described below.

Detection of viral DNA from fish by PCR

The PCR was performed using specific primers to RSIV; 1-F and 1-R (Kurita et al., 1998; OIE, 2012). From samples of pooled spleen and kidney material, the total DNA was extracted using Gentra Puregene Tissue Kit (Qiagen). The reaction mixture for PCR was KOD FX (Toyobo) and used according to the manufacturer's protocol. The thermocycling profile was performed according to published protocols (OIE, 2012).

Real-time PCR for quantitation of viral DNA

The quantitation of viral DNA of collected samples was determined according to the methods described in Ito et al. (2013a). Briefly, the primers used were: the forward primer; ivMCP186F (5'- CGGCCAGGAGTTTAGTGTGACT -3') and LUX™ (Light Upon eXtension) fluorogenic primer FAM-labelled (Life Technologies) reverse primer: ivMCP288R-FAM (5'-CGTCCGCTGTTCTCCTTGCTGGAC(FAM)G -3'), which corresponded to nucleotide po-

Table 1. Experimental design, and mean ± SD of final total length and body weight of fish used in this study.

Group (Infection route/Negative control)	No. of samples	Total length (cm)	Body weight (g)
Intraperitoneal injection	10	17.3 ± 1.84	53.1 ± 11.5
Immersion (10^{-4})	10	16.9 ± 1.97	51.6 ± 12.6
Negative control	10	16.3 ± 1.62	50.9 ± 9.80

sitions 186-288 in RSIV MCP (major capsid protein) gene. The thermocycling profile was performed according to published protocols (Ito et al., 2013a). The quantified RSIV DNA in the sample was shown as the number of copies per mg total DNA.

Virus re-isolation from infected fish

The virus re-isolation from infected fish was determined according to the methods described in Ito et al. (2013a). Briefly, homogenates of the pooled sample of spleen and kidney were prepared with approximately 50-times volume of BME and passed through a 0.45 μm filter. The filtrate (500 μl) was then inoculated into a 25 cm^2 cell culture flask which included a suspension of GF cells (approximately 6 x 10^5 cells ml^{-1}) in 10ml of BME.

Statistical analysis

Fisher's exact test was used to compare cumulative mortality among each infection group and negative control group. Statistical significance was determined to be at $P<0.01$.

Results

Experimental infection

Cumulative mortality curves of experimental infection are shown in Figure 1. The fish infected with the virus showed cumulative mortalities of 70 and 10% after IP injection and immersed in RSIV cell culture, respectively. Mortality in the IP injected group was observed between 10-15 days after injection. One of ten immersed fish died at 15 days post exposure (dpe). Mortality in the IP injected group was significantly higher compared with the negative control group ($P=0.002$). However mortality in the immersed group was not significantly different from the negative control group.

Detection of viral DNA from fish by PCR

Results of detection of RSIV DNA by PCR are shown in Table 2. The RSIV DNA was detected

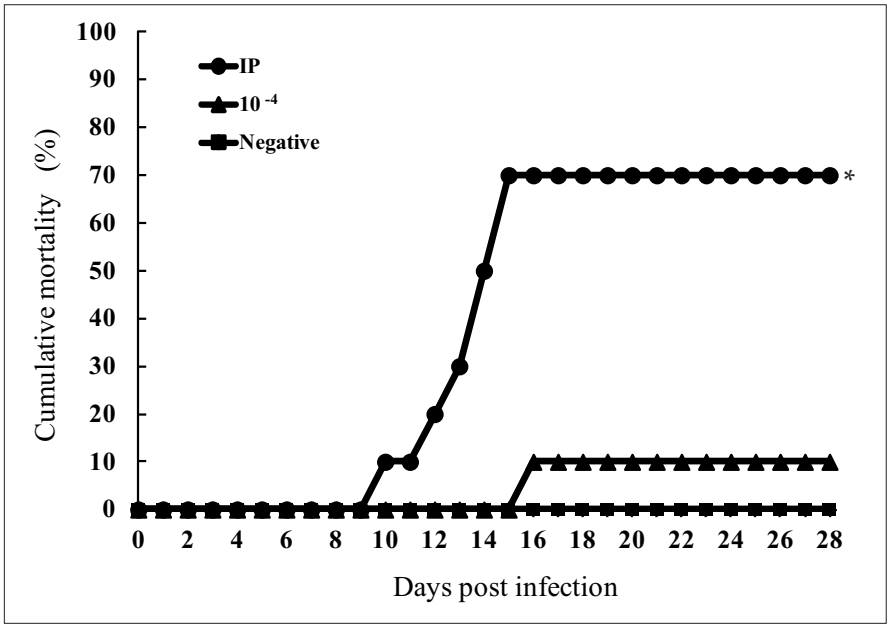

Figure 1. Cumulative mortality curves for Japanese amberjack experimentally infected with RSIV: (●) intraperitoneally injected with RSIV, (▲) immersion (10^{-4} diluted) infection with RSIV, (■) negative control. Asterisk symbol: the mortality rate was significantly different from the control ($P<0.01$ by Fisher's exact test)

Table 2. Results of detection of viral DNA by PCR, quantity of viral DNA and virus re-isolation from the pooled sample of spleen and kidney of Japanese amberjack infected with RSIV. (+; positive, -; negative)

Group	Individual no.	Dead/ Surviving	PCR	MCP copies/ DNA mg	Virus re-isolation
IP [*1] injection	1	Dead	+++[*2]	2.38.E+06	+
	2	Dead	+++	5.18.E+07	+
	3	Dead	+++	3.20.E+08	+
	4	Dead	+++	5.55.E+07	+
	5	Dead	+++	1.60.E+08	+
	6	Dead	+++	2.72.E+08	+
	7	Dead	+++	2.84.E+08	+
	8	Surviving	++	1.18.E+02	-
	9	Surviving	+	2.46.E+01	-
	10	Surviving	++	1.16.E+02	-
10^{-4} Immersion	1	Dead	+++	5.81.E+08	+
	2	Surviving	-	UD [*3]	-
	3	Surviving	-	UD	-
	4	Surviving	+++	4.03.E+06	+
	5	Surviving	-	UD	-
	6	Surviving	-	UD	-
	7	Surviving	+++	4.65.E+05	+
	8	Surviving	-	UD	-
	9	Surviving	+++	1.01.E+08	+
	10	Surviving	+++	6.99.E+07	+
Negative control	1	Surviving	-	UD	-
	2	Surviving	-	UD	-
	3	Surviving	-	UD	-
	4	Surviving	-	UD	-
	5	Surviving	-	UD	-
	6	Surviving	-	UD	-
	7	Surviving	-	UD	-
	8	Surviving	-	UD	-
	9	Surviving	-	UD	-
	10	Surviving	-	UD	-

[*1]; Intraperitoneal
[*2]; Number of plus signs shows brightness of amplicons after electrophoresis
[*3]; UD, Under the detection limit (one copy of RSIV DNA per well)

from the pooled samples of spleen and kidney from all deceased and surviving fish in the IP injected group. Viral DNA was detected from the only dead fish and 4/9 surviving fish in the immersed RSIV group at 28 dpe. The viral DNA was not detected from any fish within the negative control group.

Sequential quantification of RSIV DNA from fish by real-time PCR

The values of RSIV MCP gene quantification from the pooled sample of spleen and kidneys of all dead fish in the IP injected group were 2.38.E+10^{6}-3.20.E+10^{8} copies mg DNA^{-1} (Table 2). The values of the viral DNA of the surviving fish at 28 dpe in the IP injected group were lower than the dead fish (10^{1}-10^{2} copies mg DNA^{-1}). The value of the viral DNA of the one dead fish in the immersed RSIV group was 5.81.E+10^{8} copies mg DNA^{-1}, a value almost equal to the dead fish of the IP injected group. Moreover the values of the viral DNA of 4/9 fish in the immersed RSIV group were 4.65.E+10^{5}-1.01. E+10^{8} copies mg DNA^{-1} and these values were equivalent to the range of values of the dead fish. From 5/9 immersed surviving fish and all fish of the negative control group, the values of RSIV DNA were below the detection limit (1 copy RSIV DNA/well) of the assay.

Virus re-isolation from infected fish

The virus was re-isolated from the spleen and kidney pooled sample of all dead fish in the IP injection group (Table 2). However, the virus was not re-isolated from the surviving fish in the IP injection group although all the samples showed positive for RSIV in both conventional PCR and real-time PCR. The virus could be re-isolated from the one dead fish in the immersed RSIV group. Moreover the virus re-isolation succeeded from 4/9 surviving fish at 28 dpe in the immersed RSIV group. RSIV was not re-isolated from any samples dissected from immersed surviving fish that screened negative by the conventional or real-time PCR assays and from any of the negative control fish.

Discussion

This is the first study to evaluate the relationship between the quantity of viral DNA and the virus re-isolation from Japanese amberjack infected with RSIV. Cumulative mortalities of the IP injected and immersed 10^{-4} diluted RSIV cell culture groups in this study were lower than our previous study (Ito et al., 2013a). It is known that the susceptibility of juveniles to RSIV disease is generally higher than adults (OIE, 2012). This phenomenon could explain the difference in resistance of the smaller sized fish used in the previous study (Ito et al., 2013a).

It was previously reported that the viral DNA could be detected from several organs of 2/5 surviving Japanese amberjack which had recovered from RSIV disease (Ito et al., 2013a). Also in this study, the viral DNA could be detected from the pooled spleen and kidney samples of 4/9 immersed surviving Japanese amberjack, and the detected copies of genome were in the same range as that of the dead fish in the IP injected group.

The number of copies of viral genome in 4/9 immersed surviving fish was 10^{5}-10^{6} DNA mg^{-1} and the remaining five were below the detection limit, although the viral genome copy number of the IP injected surviving fish was 10^{1}-10^{2} DNA mg^{-1}. These results indicate that the viral kinetics of the infected fish may differ by the route of infection or the dose. A further study is needed to demonstrate this hypothesis.

In this study virus was re-isolated from Japanese samberjack infected by immersion. This result demonstrates that apparently healthy fish that have recovered from RSIV infection have the potential to transmit virus to naïve fish and are a potential source of new infection.

The re-isolation of virus only from tissues with high genome copy numbers highlights the possible lack of sensitivity of the GF cell line and its suitability for virus surveillance. The development of a more sensitive cell line for RSIV isolation is one of next studies that should be investigated.

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