

Short communication

Pathogenicity of a Rainbow Trout Isolate RtNa-0010 of *Oncorhynchus masou* virus (Salmonid herpesvirus 2) in Salmonid and Cyprinid Fish

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ABSTRACT—Pathogenicity of a rainbow trout isolate RtNa-0010 of *Oncorhynchus masou* virus (OMV = salmonid herpesvirus 2) was investigated in salmonids and cyprinids. Immersion method was used to challenge three species of salmonid fish with RtNa-0010 and OMV reference strain OO-7812 at a dose of 100 $TCID_{50}$/mL for 60 min. Cumulative mortality of rainbow trout was 3 and 51 of 51 fish when challenged with OO-7812 and RtNa-0010 respectively. When six salmonids and three cyprinids were challenged with RtNa-0010 (10^3 $TCID_{50}$/fish) by intraperitoneal injection, mortality rates were 85%, 10% and 0% for rainbow trout, whitefish *Coregonus lavaretus maraena* and other tested fish respectively.

Key words: salmonid herpesvirus 2, *Oncorhynchus masou* virus, OMV, pathogenicity, *Oncorhynchus mykiss*, *Coregonus lacaretus maraena*

Oncorhynchus masou virus (OMV = salmonid herpesvirus 2) was isolated in 1978 from ovarian fluids of masu salmon *O. masou* in Hokkaido, Japan (Kimura *et al*., 1981a). The OMV reference strain is OO-7812. The virus has since been isolated from masu salmon at 13 hatcheries in northern Japan (Yoshimizu *et al*.,1989). OMV is pathogenic to masu salmon, chum salmon *O. keta*, kokanee salmon *O. nerka*, coho salmon *O. kisutch* and rainbow trout *O. mykiss* (Tanaka *et al*., 1984; Kumagai *et al*., 1994; Suzuki, 1993). Infected fish become anorexic and show exophthalmia and/or petechiation on the body surface. Cumulative mortality in masu, chum and kokanee salmon ranged from 80 to 100%, while in coho salmon and rainbow trout it was 29–39% (Kimura *et al*., 1983; Kumagai *et al*., 1994). For fish that survived infections, 12–100% of them developed epithelial tumours around the mouth after 4 months and this persisted for at least 1 year. This was observed in chum, coho and masu salmon, and rainbow trout (Kimura *et al*., 1981b).

In 1999, OMV disease (OMVD) caused mass mortality in marketable sized rainbow trout in Nagano Prefecture, Japan (Furihata *et al*., 2003). In rainbow trout, fish infected with epidemic isolate exhibited almost no external signs, although some fish manifested ulcerative lesions on the skin. Internally, intestinal haemorrhage and white spots on the liver were observed (Furihata *et al*., 2003; 2005). Epidemic cases in Nagano Prefecture indicate that the pathogenicity of RtNa-0010 isolated from rainbow trout is different from that of the OMV reference strain OO-7812. The aim of this study was to investigate the pathogenicity of OMV RtNa-0010 in salmonid and cyprinid Fish.

Materials and Methods

Virus strain and isolate

OMV strain OO-7812 and isolate RtNa-0010 used in this study were respectively isolated from masu salmon in 1978 and rainbow trout in 2000. The strain and isolate were inoculated to sub-confluent rainbow trout gonad (RTG-2) cells (Wolf and Quimby, 1962), which were maintained at 15°C with Eagle's minimum essential medium (MEM, Nissui) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin G (Sigma) and 100 μg/mL streptomycin sulfate (Sigma). The supernatant was then collected after 10 days and stored at –80°C until use. Infectivity titers were measured using 50% infectious dose ($TCID_{50}$) in RTG-2 cells.

Fish

The fish used in this study were rainbow trout, amago salmon *O. masou ishikawae*, Japanese char "amemasu" *Salvelinus leucomaenis leucomaenis*, Ito *Hucho perryi*, char *Salvelinus leucomaenis*, whitefish *Coregonus lavaretus maraena*, brown trout *Salmo trutta*, shinshu salmon (allotriploid; rainbow trout × rainbow trout × brown trout), common carp *Cyprinus carpio*, crucian carp (funa) *Carassius auratus*, and Japanese dace *Tribolodon hakonensis*. Species except rainbow trout were included as there are no reports on their susceptibility to OMV. All fish were reared on a commercial pellet diet and appropriate aeration was supplied during the challenge studies. Detailed information on fish used for the study is shown in Table 1.

Immersion challenge

For immersion challenge experiments, three

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Table 1. Mean body length and body weight of fish used in this study

Infection route	Common name	Scientific name	No. of fish/tank	Mean body length (cm)	Mean body weight (g)
Immersion	Rainbow trout	*Oncorhynchus mykiss*	60	11.1	12.0
	Japanese char	*Salvelinus leucomaenis leucomaenis*	44	2.5	0.2
	Ito	*Hucho perryi*	60	6.9	4.1
Intra-peritoneal injection	Rainbow trout	*Oncorhynchus mykiss*	20	ND*	16.8
	Amago	*Oncorhynchus masou ishikawae*	20	ND	18.4
	Whitefish	*Coregonus lavaretus maraena*	20	ND	14.7
	Brown trout	*Salmo trutta*	10	ND	25.2
	"Shinshu salmon"	(Allotriploid; *O. mykiss* × *O. mykiss* × *S. trutta*)	20	ND	34.2
	Char	*Salvelinus leucomaenis*	19	ND	41.1
	Common carp	*Cyprinus carpio*	20	ND	8.8
	Crucian carp (Funa)	*Cyprinus auratus*	20	ND	8.7
	Japanese dace	*Tribolodon hakonensis*	20	ND	7.4

*: ND: not determined.

species of fish (rainbow trout, ito and Japanese char) were used. The fish were divided into three groups of 60 fish each (for Japanese char each group had 44 fish). For each species, two of the three groups were challenged with OMV (OO-7812 or RtNa-0010 at 100 $TCID_{50}$/mL), while the third group acted as the control. This infectious dose was chosen to enable comparison with previous reports. The control groups were challenged with Hanks' BSS (Nissui). Challenges were performed using immersion exposure at 10°C for 60 min. After the immersion challenge, each tank of fish was supplied with dechlorinated tap water at 10°C, and fish mortalities were monitored daily for 21 to 65 days. All dead fish as well as sample of three rainbow trout fish (taken after 3, 6 and 9 days post-infection) were checked for infection. Kidney removed from fish was divided into two parts for cell culture isolation and PCR examination. For virus isolation, kidney samples were homogenized with nine volumes of Hanks' BSS and centrifuged (39 × *g* for 10 min at 4°C). The supernatant was inoculated onto 24-well plates seeded with RTG-2 cells and incubated at 15°C for two weeks. For CPE positive cells, further confirmation was done using PCR. DNA was extracted from the kidney of the individual fish and CPE positive samples using QuickGene SP-DT (Wako). The reaction mixture for PCR was used Ex Taq DNA polymerase (Takara) with specific set of primers of F10 primer (GTACCGAAACTCCCGAGTC) and R05 primer (AACTTGAACTACTCCGGGG), which were designed by Aso *et al*. (2001). The thermocycling profile was also performed according to Aso *et al*. (2001).

Intraperitoneal challenge

Rainbow trout, amago salmon, whitefish, shinshu salmon, common carp, funa, Japanese dace, char and brown trout were used in this experiment. For each species, fish were divided into two groups of 20 fish each (for Japanese char each group had 19 fish, while for brown trout each group had ten fish). For each fish species, one group of fish was challenged with OMV RtNa-0010 at $10^{3.8}$ $TCID_{50}$/100 μL/fish by intraperitoneal injection and the other group was injected 100 μL of MEM (Nissui) supplemented with 5% fetal bovine serum as a control. Both groups of fish were kept in 60-L tank, containing dechlorinated tap water at 9.0–13.1°C for 42 days. All dead fish and some surviving fish were tested for infection using cell culture isolation and PCR examination. For PCR examination, DNA was extracted from the kidney of the individual fish and CPE positive samples using InstaGene Matrix (BIO-RAD laboratories).

Results and Discussion

For the immersion challenge experiments RtNa-0010 showed especially high virulence in rainbow trout as compared to OO-7812 (Fig. 1). A previous report noted the low virulence of OO-7812 in rainbow trout (Kimura *et al*., 1983). Typical signs of OMVD were observed in all dead fish. OMV genome was detected from all dead fish. However, OMV was not isolated from 6 of the 51 dead fish challenged with RtNa-0010 and from 2 of 3 dead fish challenged with OO-7812. No mortality or disease signs due to OMV infection were observed in Japanese char and Ito. OMV genome was detected from rainbow trout at 3 and 6 days post-infection, however infectious particle of OMV was not detected at 3, 6 and 9 days post-infection (Table 2). This result indicates that OMV infection does not occur easily, or a considerable amount of time is necessary for the virus to multiply in the fish.

For the intraperitoneal injection challenges, RtNa-0010 showed highest virulence in rainbow trout. Mortalities were 85% in rainbow trout and 10% in whitefish, while no mortality was observed for four salmonid and three cyprinid fish species (Table 3). In the case of rainbow trout, typical signs of OMVD were observed in all dead fish. Mortality in whitefish was 2 of 20 fish and typical signs of OMVD were observed in both dead fish. Although OMV was isolated from only one of the dead fish, the virus genome was detected from both dead fish. Neither mortality nor any disease signs due to OMV infection were observed in amago salmon, Japanese char, brown trout, Shinshu-salmon, common carp, funa and Japanese dace. Our results and previous reports indicate that pathogenicity of OMV strains differs depending on the species from which the strain was initially isolated. CSH9003 isolated from marine cultured coho salmon caused high mortality in coho salmon and lower mortality in masu salmon, while no mortality was observed in rainbow trout (Kumagai *et al*., 1995). Comparative analysis of OMV genome is necessary to understand these variations in pathogenicity or infectivity.

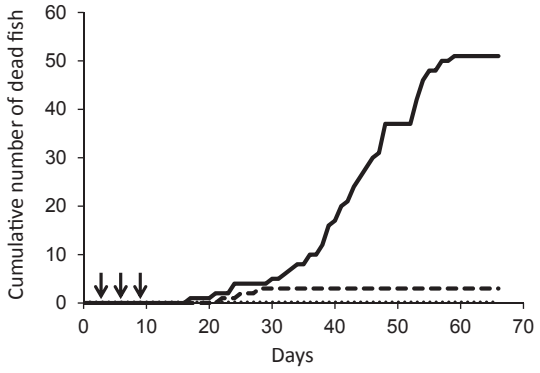

Fig. 1. Cumulative number of dead rainbow trout exposed to OMV by immersion. Fish were exposed to each OMV strain/isolate at 100 $TCID_{50}$/mL for 60 min and then the fish were reared at 5–14°C for 65 days. The control group was treated the same way as infection groups but exposed to a virus-free medium. Arrows represents sampling time points.
——: RtNa-0010, – – –: OO-7812, ••••••: Control.

Virus was isolated or detected by PCR from dead whitefish. This result indicates that fish outside the genus *Oncorhynchus* are susceptible to OMV. Whitefish is originally distributed widely in cooler parts of Europe, North America and Russia, and was only recently brought to Japan (Amano *et al*., 1988). The susceptibility of this fish to OMV raises the possibility of virus existing outside Japan. So although OMVD outbreaks have only been reported in Japan, worldwide surveillance are strongly recommended. Since whitefish is susceptible to OMV, combined aquaculture with rainbow trout, or sharing of water resources must be done with caution. Quarantine controls from Japan to OMV free county are highly recommended in order to prevent a world-wide pandemic.

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Table 2. Detection of OMV genome by PCR and OMV isolation from rainbow trout after virus exposure

Strain/Isolate	3 days		6 days		9 days	
	PCR positive/ examined	CPE positive/ examined	PCR positive/ examined	CPE positive/ examined	PCR positive/ examined	CPE positive/ examined
OO-7812	1/3	0/3	2/3	0/3	0/3	0/3
RtNa-0010	0/3	0/3	1/3	0/3	0/3	0/3

Table 3. Mortality of fish (six salmonid fish and three cyprinid fish) after intraperitoneal challenge with OMV RtNa-0010

Fish species	Dead fish/ total fish challenged	CPE positive dead fish/ total dead fish	PCR positive dead fish/total dead fish	CPE positive surviving fish/ tested fish	PCR positive surviving fish/ tested fish	Dead fish/ total fish in control group
Rainbow trout	18/20	17/18	N/A	0/1	0/1	0/20
Amago salmon	0/20	N/A**	N/A	0/10	0/5	0/19
White fish	2/20	1/2	2/2	0/9	0/5	0/20
Char	0/19	N/A	N/A	0/9	0/5	0/19
Brown trout	0/10	N/A	N/A	0/5	0/5	0/10
Shinshu salmon (Allotriploid; Rt × Rt × Bt*)	0/20	N/A	N/A	N/A	N/A	N/A
Common carp	4/20	0/4	0/4	0/8	0/5	6/20
Crucian carp (Funa)	0/20	N/A	N/A	0/10	0/5	0/20
Japanese dace	1/20	0/1	0/1	0/9	0/5	1/20

* rainbow trout × rainbow trout × brown trout.
** not applicable.

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