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Pathogenicity of the Virus Isolated from Brain of Abnormally Swimming Salmonid

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Pathogenicity of the new virus isolated from the brain of coho salmon *Oncorhynchus kisutch* was investigated by waterborne and intramuscular inoculation to coho salmon, masu salmon *O. masou*, iwana *Salvelinus pluvius*, steelhead trout *O. mykiss*, and ito *Hucho perryi*. Typical disease signs of spinning swimming and lethargic behavior were observed in the experimentally infected fishes. The cumulative mortality of coho salmon fry for 60 days bathed with the virus reached 6 to 34%. In intramuscular injection of the virus, cumulative mortality of coho salmon fry reached 51 to 63%. The virus also showed pathogenicity to masu salmon, steelhead trout and iwana. Virus antigen was detected by indirect fluorescent antibody test (IFAT) in the kidney, brain and blood cells of the infected fish.

Key words: pathogenicity, coho salmon, masu salmon, iwana, steelhead trout, ito, virus

Recently, a new virus has been isolated from the brain of coho salmon, *Oncorhyncys kisutch*, rainbow trout, *O. mykiss*, iwana, *Salvelinus pluvius*, and ayu, *Plecoglossus altivelis*, and the ovarian fluid of masu salmon, *O. masou*, cultured in the northern parts of Japan. The affected fish showed abnormal swimming and lethargic behavior. The isolated virus was found not to be related to any known fish viruses. The virus had been detected from both juvenile and adult salmonid, and it is suspected that the virus is widespread in cultured salmonid of northern Japan (Oh *et al.*, 1995).

From these findings, it is necessary to confirm its pathogenicity for the elucidation of the transmission route among salmonids and for the establishment of prevention methods against this disease. This study was undertaken to determine the pathogenicity of this virus to several salmonids by waterborne and intramuscular infections.

Materials and Methods

Virus used

The virus strain BrCo-9221 isolated from brain of diseased coho salmon and passed 12 to 15 times in the CHSE-214 cell line, was used in this study. The virus was replicated in the CHSE-214 cell lines with MEM containing 10% of FBS (MEM-10) at 15°C for 7 days. The culture fluid was filtered using a Millipore filter HA (0.45 μm) and stocked at −80°C until use. The infectivity of the virus was determined by the method described by Reed and Muench (1938).

Experimental fish

Coho salmon, masu salmon, ito, iwana, and steelhead trout were used in this investigation. The average body weights of fishes used in this experiment were as follows: coho salmon, 0.15 and 1.5 g; masu salmon, 0.2 and 1.5 g; ito, 3.0, 3.5 and 12 g; iwana, 15 g; steelhead trout, 3.0 and 12 g. Coho and masu salmon were cultured in our laboratory, and ito, iwana and steelhead trout were provided from Nanae Fish Culture Experimental Station, Faculty of Fisheries, Hokkaido University.

All the fishes used in this experiment were maintained in plastic tanks with running dechlorinated city water. Prior to inoculation, none of the fishes showed signs of disease and neither pathogenic bacteria nor viruses were detected.

Waterborne infection

For waterborne infection, the stock virus (virus infectivity was $10^{7.3}$ $TCID_{50}$/m*l*) was diluted and sus-

pended in 2 liters of dechlorinated city water. The virus infectivity in the aquaria was adjusted to $10^{2.0}$ and $10^{3.2-3.5}$ $TCID_{50}$/ml. Coho salmon and masu salmon were exposed to two different concentrations of virus for 1 h. Fifty fish were used in each suspension. Twenty ito having an average weight of 3.0 g were exposed to a virus suspension of $10^{3.5}$ $TCID_{50}$/ml.

Intramuscular injection

For intramuscular injection, the stock virus was diluted to 10 and 1000 fold with MEM-10. One hundred μl of the diluted virus was injected into the muscle between the lateral line and dorsal fin.

Coho salmon (1.5 g average weight) were injected with the virus of $10^{3.5}$ and $10^{5.0}$ $TCID_{50}$/fish, and masu salmon (1.5 g average weight) were injected with $10^{3.2}$ and $10^{5.1}$ $TCID_{50}$/fish. Thirty-three fish were used in each of the four experiments. Twenty iwana (15 g average weight) were treated with $10^{3.0}$ and $10^{5.3}$ $TCID_{50}$/fish. Each of the 20 steelhead trout (3 g average weight) was treated with $10^{3.5}$ $TCID_{50}$/fish and 12 g fish were treated with $10^{3.2}$ and $10^{5.0}$ $TCID_{50}$/fish. Ito with an average weight of 3.5 g were injected with the virus of $10^{3.2}$ and $10^{5.8}$ $TCID_{50}$/fish, and fish with an average weight of 12 g were treated with $10^{5.8}$ $TCID_{50}$/fish. Each test group consisted of 20 fish. In control fish group, the same number of fish for each tested species were treated the same without the virus.

Rearing condition and observation of the infected fish

After these treatments, each of the experimental and control group of fish were reared in individual aquaria of 3 liter with running dechlorinated city water. Water temperature varied between 12 to 15°C during the 60 (or 30) days of observation. Fish were fed daily with commercial trout crumbles and pellets (Nihon-Nosan Kogyo Co., Ltd.). The disease sign, i.e., changes of behavior, abnormal spinning swimming and external lesions on the skin and eye were observed, and mortality was recorded daily. Dead fish were collected and stocked at −80°C.

Virus reisolation

The brain of dead and moribund fish (iwana, steelhead trout and ito) or the whole body of dead and moribund fish (coho and masu salmon) were taken for virus reisolation. For virus reisolation and measurement of virus infectivity, the whole fish or the brain was homogenized with HBSS using a stomacher (Organo). The homogenates (1 : 10) were filtrated with a Millipore filter HA (0.45 μm), and diluted with HBSS. A monolayer of CHSE-214 cells was prepared in 96 well plates (Falcon) and incubated at 15°C, for 24 h. After inoculation of the filtrates, the plates were incubated at 15°C for 7 days and virus infectivity was measured as described above.

The filtrates were then inoculated to CHSE-214 cells which were grown on a cover glass inserted in 24 well plates (Falcon). The cells were maintained at 15°C for 24 h. The cover glass was taken out and fixed with acetone. Viral antigens were detected with an indirect fluorescent antibody test (IFAT) using antiserum against the virus strain BrCo-9221 (1 : 160), and FITC-conjugated swine anti-rabbit immunoglobulins (Wako). Uninfected CHSE-214 cells and infected CHSE-214 cells with the virus (strain BrCo-9211) were used as negative and positive controls.

Sixty and/or 30 days after infection, the surviving fish (showing no disease sign) were provided for the same experiment as described above.

Detection of viral antigens and measurement of the virus infectivity in the internal organs

Forty iwana having an average weight of 15 g were injected intramuscularly with the virus of $10^{4.3}$ $TCID_{50}$/fish and maintained as described above. Every day, two fish were used to detect the viral antigen and other two fish were tested to measure the virus infectivity until 10 days after injection.

Direct stamps of blood, kidney and brain tissues of infected fish were prepared on slide glass and fixed with acetone. Indirect fluorescent antibody test (IFAT) was carried out as described above. Virus infectivities of blood, kidney, spleen, brain, eye, and liver of injected fish were also measured using micro titer plates.

Results

Cumulative mortality of artificially infected fish and virus reisolation

During 15 to 20 days after the infection by different infection methods and challenge dose, the infected fish showed marked disease signs, such as abnormal swimming (spinning or upside-down), lethargic behavior, pop eye and anorexia, before they

died. The virus was reisolated and virus antigen was detected by IFAT from all moribund and dead fish, as well as from all survived fish. Daily mortality, cumulative mortality and virus infectivity were as follows.

Coho salmon: In coho salmon infected with the virus of $10^{2.0}$ $TCID_{50}$/m*l* by waterborne, the cumulative mortality was 6% for 0.15 g fish, and 8% for 1.5 g fish, respectively (Table 1). For fish with the virus of $10^{3.2}$ $TCID_{50}$ per m*l*, cumulative mortality for 60 days reached 24 and 34% in 0.15 g fish and 1.5 g fish, respectively. On the other hand, higher cumulative mortalities were observed by i.m. injection. Cumulative mortality for 60 days for 1.5 g fish injected with the virus of $10^{3.5}$ and $10^{5.0}$ $TCID_{50}$ per fish reached 51 and 63%, respectively. In waterborne infection, many moribund fish were observed during 35 to 50 days post infection. On the other hand, in intramuscular injection the mortality gradually increased from the 20 days after injection. Virus infectivity of the dead and survived fish were $10^{5.30}$ to $10^{8.05}$ $TCID_{50}$/g and $10^{2.80}$ to $10^{7.30}$ $TCID_{50}$/g, respectively, at days post-infection (Table 1).

Masu salmon: Mortality in masu salmon experimentally infected with waterborne and i.m. injection showed a similar tendency to that of the coho salmon. The cumulative mortality was 8% for 0.2 g fish, and 14% for 1.5 g fish immersed with the virus of $10^{2.0}$ $TCID_{50}$/m*l*, and cumulative mortality of the fish immersed with $10^{3.5}$ $TCID_{50}$/m*l* were 30% for 0.2 g fish and 26% for 1.5 g fish, respectively. In i.m. injection, cumulative mortalities reached 54% in fish

Table 1. Mortality and virus infectivity in the coho salmon artificially infected with the virus strain BrCo-9221

Body weight (g)	Infection		No. of test fish	No. of dead fish						Virus infectivity*3		Cumulative mortality (%)
	Method*1	Dose*2		Days post infection						Dead	Surviving	
				10	20	30	40	50	60			
0.15	WB	2.0	50	0	0	1	0	2	0	5.30–5.80	2.80–5.05	6
		3.2	50	0	0	0	0	9	3	5.55–6.05	3.80–6.55	24
		MEM 10	50	0	1	0	0	0	0	—*4	—	2
1.5	WB	2.0	50	0	0	1	1	0	2	5.30–7.55	4.05–7.05	8
		3.2	50	0	1	2	6	8	0	6.05–7.30	5.10–6.80	34
		MEM 10	50	0	0	0	0	0	0		—	0
	IM	3.5	33	0	1	4	2	8	2	5.55–7.80	4.05–7.30	51
		5.0	33	0	7	3	1	8	2	5.55–8.05	4.05–6.80	63
		MEM 10	33	0	0	0	0	0	0		—	0

*1 WB: Waterborne, IM: Intramuscular injection, *2 Log $TCID_{50}$/m*l* or fish, *3 Log $TCID_{50}$/g of fish, *4 Not detected.

Table 2. Mortality and virus infectivity in the masu salmon artificially infected with the virus strain BrCo-9221

Body weight (g)	Infection		No. of test fish	No. of dead fish						Virus infectivity*3		Cumulative mortality (%)
	Method*1	Dose*2		Days post infection						Dead	Surviving	
				10	20	30	40	50	60			
0.2	WB	2.0	50	0	0	1	2	1	0	4.50–5.80	3.05–4.80	8
		3.2	50	1	1	4	5	2	2	4.05–6.30	4.05–5.05	30
		MEM 10	50	2	0	0	0	0	1	—*4	—	6
1.5	WB	2.0	50	0	0	1	0	3	3	4.55–7.05	3.80–7.05	14
		3.5	50	0	1	0	2	6	4	5.55–7.55	5.05–6.80	26
		MEM 10	50	0	0	0	0	0	0		—	0
	IM	3.2	33	0	1	3	2	4	8	6.30–8.05	6.05–6.55	54
		5.1	33	0	4	4	4	4	1	6.05–8.05	3.80–7.05	51
		MEM 10	33	0	0	0	0	0	0		—	0

*1 WB: Waterborne, IM: Intramuscular injection, *2 Log $TCID_{50}$/m*l* or fish, *3 Log $TCID_{50}$/g of fish, *4 Not detected.

injected with the virus of $10^{3.2}$ $TCID_{50}$/fish, and 51% in those injected with $10^{5.1}$ $TCID_{50}$/fish. All of the tested moribund and survived fish showed virus infectivity similar to that found in the coho salmon (Table 2).

Iwana, steelhead trout and ito: Iwana and steel-

Table 3. Mortality and virus infectivity in the iwana, steelhead trout and ito artificially infected with the virus strain BrCo-9221

Fish Body weight	Infection		No. of test fish	No. of dead fish						Virus infectivity*3		Cumulative mortality (%)
				Days post infection								
	Method*1	Dose*2		10	20	30	40	50	60	Dead	Surviving	
Iwana												
(15 g)	IM	3.0	20	0	2	4				5.30–6.80	3.05–5.80	30
		5.3	20	0	4	4				5.55–7.05	3.80–6.05	40
		MEM 10	20	0	0	0					—*4	0
Steelhead trout												
(3 g)	IM	3.5	20	1	2	4				NT*5	NT	35
		MEM 10	20	0	0	0				NT	NT	0
(12 g)	IM	3.2	20	1	3	3				5.55–7.55	4.05–5.30	35
		5.0	20	0	4	5				4.80–8.05	4.05–6.05	45
		MEM 10	20	0	0	1				—	—	5
Ito												
(3 g)	WB	3.5	20	0	1	0	0	0	0	6.80	3.05–5.05	5
		MEM 10	20	0	0	0	0	0	0		—	0
(3.5 g)	IM	3.2	20	0	0	0	0	0	0		4.05–6.05	0
		5.8	20	0	0	0	0	0	0		3.55–5.80	0
		MEM 10	20	0	0	0	0	0	0		—	0
(12 g)	IM	5.8	20	0	0	0	0	0	0		4.80–6.30	0
		MEM 10	20	0	0	0	0	0	0		—	0

*1 WB: Waterborne, IM: Intramuscular injection, *2 Log $TCID_{50}$/m*l* or fish, *3 Log $TCID_{50}$/g of fish, *4 Not detected, *5 NT: Not tested.

Table 4. Detection of the virus antigen by indirect fluorescent antibody test (IFAT) and measurement of the virus infectivity in iwana artificially infected with the virus strain BrCo-9221 by intramuscular injection*1

Tissue	Days post injection						
	0	1	2	3	5	7	10
IFAT test							
Kidney	0/2*2	1/2	2/2	2/2	2/2	2/2	2/2
Blood	0/2	0/2	0/2	0/2	1/2	2/2	2/2
Brain	0/2	0/2	0/2	0/2	0/2	2/2	2/2
Virus infectivity							
Kidney	—*3	4.30	5.30*4	5.80	5.80	6.05	6.80
Spleen	—	4.05	5.80	5.55	5.30	5.80	6.30
Blood	—	—	—	—	3.50	3.80	3.05
Brain	—	—	—	—	4.30	6.30	6.55
Eye	—	—	—	—	—	3.30	3.80
Liver	—	—	—	3.30	3.30	3.30	4.80

*1 Dose: $10^{4.3}$ $TCID_{50}$/fish, *2 Number of posotive fish/number of fish examined, *3 —: Not detected, *4 Log $TCID_{50}$/g of tissue, limit of detect is $\leqq 2.3$.

head trout were treated only by intramuscular injection and observed for 30 days. In iwana, cumulative mortality for the fish injected with the virus of $10^{3.0}$ $TCID_{50}$/fish reached 30%, and 40% for those injected with $10^{5.3}$ $TCID_{50}$/fish, respectively. Steelhead trout of 3.0 g injected with the virus of $10^{3.5}$ $TCID_{50}$/fish showed cumulative mortality of 35% for 30 days. Twelve gram steelhead trout injected with $10^{3.2}$ $TCID_{50}$/fish and those injected $10^{5.0}$ $TCID_{50}$/fish showed cumulative mortality of 35% and 45%, respectively. In ito, only one fish died by waterborne infection ($10^{3.5}$ $TCID_{50}$/m*l*) and no fish died by i.m. injection. Iwana and steelhead trout showed virus infectivity similar to that found in the coho salmon. In ito, no fish showed any disease signs. However, all of the survived fish showed virus infectivity of $10^{3.05}$ to $10^{6.30}$ $TCID_{50}$/g in the brain (Table 3).

Detection of virus antigen and virus infectivity in the internal organ of infected iwana

The results of the indirect fluorescent antibody test (IFAT) to detect the virus antigens and viral infectivity measurement in the internal organs of infected iwana are shown in Table 4.

Virus antigens were first detected in the kidney 1 day post-infection, and then detected in the blood and brain samples on 5 and 7 days post-infection. In the kidney, positive fluorescence was observed particularly on big cells that were determined to be macrophages (Fig. 1a). The neuron fibers in the brain tissue and the cytoplasm of erythrocytes of infected fish were also positively stained (Fig. 1b and 1c).

The virus infectivity first became measurable in the kidney and spleen 1 day post-infection. Viral infectivity in the kidney was $10^{4.30}$ $TCID_{50}$/g of tissue, and continuously increased up to 10 days post-infection. At 10 days post-infection, the viral infectivity measured $10^{6.80}$ $TCID_{50}$/g of tissue. The virus was isolated from the brain of infected fish 5 days post-infection, the viral infectivity being $10^{4.30}$ $TCID_{50}$/g. After 10 days, the infectivity increased to $10^{6.55}$ $TCID_{50}$/g. Blood, eye and liver tissues also showed the presence of the virus, but the viral infectivity was low as $10^{3.05}$ to $10^{4.80}$ $TCID_{50}$/g of tissue after 10 days.

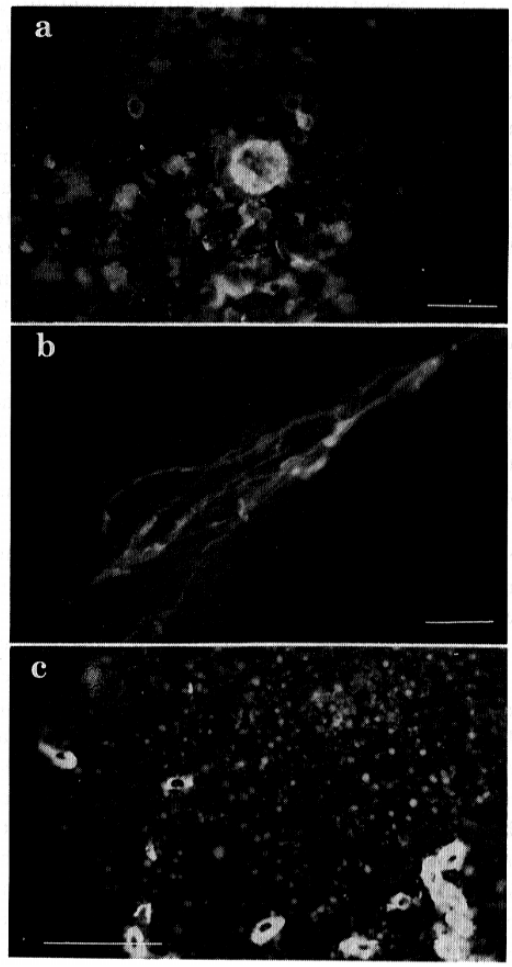

Fig. 1. Detection of the virus antigen by IFAT in imprinted fish tissues of iwana infected with the virus strain BrCo-9221.
a: Cytoplasm of macrophage in kidney. b: Neuron fibers in brain. c: Cytoplasm of erythrocytes in blood. Scale bar = 50 μm.

Discussion

Results from this study indicate that the virus isolated from the brain of coho salmon is a pathogenic agent and shows pathogenicity, not only to the original host species, but also to masu salmon, iwana and steelhead trout, although it has little pathogenicity to ito.

The cumulative mortality observed in waterborne infection was lower than that in intramuscular infection. However, the viral infectivity of infected fish, not only dead and/or moribund fish but also survived fish, was not significantly different between waterborne and intramuscular infection.

As the present virus manifested persistent infection (PI) in some salmonid fish cell lines (Oh *et al.*, 1995), it is suspected that the PI had developed in the tissues of surviving fish. The persistent infection of this virus *in vitro* requires further study. In infectious pancreatic necrosis virus (IPNV), natural persistent infection on brook and rainbow trout has been described by Billi and Wolf (1969), Yamamoto (1975) and Reno *et al.* (1978). Transmission of IPNV from mature fish to their progeny is known, and the treatment with iodine does not completely inhibit the virus transmission, resulting eggs to become contaminated with IPNV (Wolf *et al.*, 1963, Bullock *et al.*, 1976). The same virus of present one has been isolated from the ovarian fluid of normal mature masu salmon (Oh *et al.*, 1995). If embryos originated from the parental fish infected with this virus are infected with this virus and become persistently infected, this virus may produce a serious problem in salmon propagation. Further studies on vertical transmission and the persistent infection *in vivo*, is important for the prevention of transmission.

The virus antigen was first detected in the kidney only 1 day after injection and then observed in the blood and brain. Virus infectivity became measurable first in the kidney and spleen and then in the liver. In the brain of infected fish, the virus infectivity was observed at 5 days post-infection, and the infectivity increased until the 10th day after infection. The virus infectivity in the brain slowly increased, and the infected fish showed abnormal spinning swimming and died. On that time, infectivity had reached to $10^{6.30}$ $TCID_{50}$/g in the brain. These results suggest that the primary target organ of this virus is the kidney and spleen, and the virus replicates in these organs and spreads through the blood vessels to other organs. Finally, the virus may attack the brain tissue and cause the abnormal swimming behavior. Histopathological study is necessary to verify this hypothesis.

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