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Oncorhynchus masou Virus: Serological Relationships among Salmonid Herpesviruses Isolated from Kokanee Salmon, Masu Salmon, Coho Salmon and Rainbow Trout

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Abstract. — Serological relationships of herpesviruses isolated from salmonid fish were investigated and salmonid herpesviruses were divided into two groups. One is salmonid herpesvirus isolated from rainbow trout and streelhead trout in USA (Salmonid herpesvirus; SaHV-1) and other is SaHV-2 isolated from kokanee salmon, masu salmon, coho salmon and rainbow trout in Japan. The values of serological relationship (1/R) among SaHV-2 isolates ranged from 0.51 to 1.53 and observed high serological relationships. Between SaHV-1 and SaHV-2, the 1/R value was more than 3.16.

Introduction

Salmonid herpesvirus was first isolated from normal appearing adult stocks of rainbow trout in USA (Wolf and Taylor, 1975). This virus showed pathogenicity to rainbow trout fry and named as *Herpesvirus salmonis* by Wolf et al. (1978). Herpesvirus infection of salmonid fish in Japan was first reported by Sano (1976) on the isolation of Nerka Virus Towada Lake, Akita and Aomori Prefecture (NeVTA) from moribund kokanee salmon (*Oncorhynchus nerka*) in Towada Lake. In 1978, a herpesvirus was isolated from ovarian fluid of normally appearing mature masu salmon (*O. masou*) cultured in Otobe Salmon Hatchery in Hokkaido. This virus was named *Oncorhynchus masou* virus (OMV), based on the scientific name of the host fish and it's oncogenicity (Kimura et al., 1981a, 1981b). In 1981, a similar herpesvirus was isolated from tumor tissue of yamame (*O. masou*) cultured in Koide Branch, Niigata Prefectural Inland Fisheries Experimental Station by Sano et al. (1983). This virus was named yamame tumor virus (YTV). According to development of the aquaculture of coho salmon (*O. kisutch*), herpesviruses were isolated from pond- and pen-cultured coho salmon and tentatively named coho salmon tumor virus (CSTV) by Sano et al. (1988), *Oncorhynchus kisutch* virus (OKV-T and -M) by Horiuchi et al. (1989), coho salmon tumor virus (COTV) by Kimura and Yoshimizu (1991), and coho salmon herpesvirus (CSHV) by Kumagai et al. (1994), respectively. From 1992, mass mortality occurred among pond-cultured one year old rainbow trout. A herpesvirus was isolated from the kidney, liver and ulcerative skin lesions of affected fish, and was tentatively named rainbow trout kidney herpes virus (RKV) by Suzuki (1993). In this report, we described the serological relationships among the representative strains isolated from 4 different salmon species in Japan and USA.

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Materials and Methods

Virus strain and cell lines

Original viral name and strain number of salmonid herpesviruses (SaHV) used in this experiment are shown in Table 1. Herpesvirus strains isolated from kokanee salmon SaHV-2-1 (NeVTA), masu salmon SaHV-2-2, SaHV-2-3, SaHV-2-4, and SaHV-2-5 (OMV strain 00-7812, TyT-8101, ToT-8101 and YTV, respectively), coho salmon SaHV-2-6, SaHV-2-7, SaHV-2-8 and SaHV-2-9 (CSTV, OKV-T, OKV-M and COTV, respectively), rainbow trout SaHV-2-10 and SaHV-2-11 (RKV and RHV which was also isolated from rainbow trout in main land of Japan, Yoshimizu, 1994), and the reference strain, SaHV-1-1 (*Herpesvirus salmonis*) were propagated in the chinook salmon (*O. tschawytscha*) embryo cell line (CHSE-214) (Fryer et al., 1965) and rainbow

Table 1. Salmonid herpesviruses provided for neutralization test.

Strain number	Original name	Fish species	Organ	Reference
SaHV-2-1	NeVTA	Kokanee	Hole body	Sano (1976)
SaHV-2-2	OMV 00-7812	Masu	Ovarian fluid	Kimura et al. (1981a)
SaHV-2-3	OMV TyT-8101	Masu	Tumor tissue	Yoshimizu et al. (1988)
SaHV-2-4	OMV ToT-8101	Masu	Tumor tissue	Yoshimizu et al. (1988)
SaHV-2-5	YTV	Yamame	Tumor tissue	Sano et al. (1983)
SaHV-2-6	CSTV	Coho	Tumor tissue	Sano et al. (1988)
SaHV-2-9	COTV	Coho	Tumor tissue	Kimura and Yoshimizu (1991)
SaHV-2-7	OKV-T	Coho	Liver, Kidney	Horiuchi et al. (1989)
SaHV-2-8	OKV-M	Coho	Liver, Kidney	Horiuchi et al. (1989)
SaHV-2-10	RKV	Rainbow	Kidney	Suzuki (1993)
SaHV-2-11	RHV	Rainbow	Kidney	Yoshimizu (1994)
SaHV-1-1	*H. salmonis*	Rainbow	Ovarian fluid	Wolf and Taylor (1975)

trout gonad cell line (RTG-2) (Wolf and Quimby, 1962) supplied with a minimum essential medium with 10% fetal bovine serum, 100 IU/ml of penicillin-G (Sigma) and 100 μg/ml of streptomycin sulfate (Sigma). *H. salmonis* was grown at 10 °C and other strains were cultured at 15 °C. For preparation of stock virus, cytopathic effect (CPE) was observed, and then the cultured supernatant was stocked in -80 °C until use. NeVTA, YTV, and CSTV were kindly provided by Dr. Fukuda, Tokyo University of Fisheries.

Preparation of antisera

The method described by Eaton et al.(1991) was followed to produce the antisera with some modification. Briefly, after CPE development, the cells and culture fluid were collected and centrifuged at 280 g for 20 min. Cell associated-virions were released by adding small drops of distilled water onto the pellets (Kumagai et al., 1994) and centrifuged at the same speed to remove cell debris. The supernatant was precipitated by adding 7.0% (w/v) polyethylene glycol 6,000 (PEG-6,000) and 2.5% (w/v) NaCl. After centrifugation (3,000 g, 30 min), the pellet was resuspended in 1/10 volume of PBS (pH 7.4) and centrifuged again at the same speed to remove the PEG. The supernatant was laid over 30% PBS+sucrose cushion and virion was sedimented by centrifugation at 35,000 g for 2 hours. The pellet resuspended in 1/100 volume of PBS (pH 7.4) was dialyzed overnight at 4°C against the same buffer.

New Zealand white rabbits were injected subcutaneously in the back with partially purified antigens suspended in Freund's complete adjuvant in 1:1 proportion that was mixed completely. After four weeks following the initial inoculation, each rabbit was given a 1 ml intravenous booster in it's ear. For one week later, a second intravenous booster was given. After an additional week the antibody titer was checked and the rabbit was then bled. All collected bloods were kept one hour at 37°C and stored overnight at 4°C. The collected serum was centrifuged at low speed to remove resting blood cells, inactivated at 56°C for 30 min and distributed into 1.0 ml aliquots and stored at -80°C until used.

Neutralization test

Cross-neutralization tests among the twelve herpesvirus strains and their respective antiserum were conducted according to the method described by Hedrick et al. (1987). Briefly, the lowest dilution of each antiserum (1:10) was made by Hank's Balanced Salt Solution (HBSS) in test tubes and diluted twofold serial dilution with multichannel pipette to four replicate rows in the 96 well plates. To each well containing a diluted antiseum an additional 50 μl of virus suspension containing a 100 tissue culture infectious dose ($TCID_{50}$) was added and later confirmed by back titration. The viruses and the diluted antisera were incubated at 20°C for 1.5 to 2 hours and mixed every 15 minutes with a micro-mixer. A suspension of CHSE-214 cells (0.1 ml/well) containing 5% fetal bovine serum was then added in each well with a multichannel pipette. The plate was sealed with a plate seal, and SaHV-1-1 was incubated at 10 °C and other strains were incubated at 15 °C.

About one week later, the plates were scored. At the same time the back titer of the virus was confirmed infectivity, and the 50 % endpoints for neutralization was calculated according to the method of Reed and Muench (1938). Each neutralization titer was obtained after finishing a duplicate neutralization test.

Results

Cross-neutralization test

The results of a cross-neutralization test are shown in Table 2. Herpesvirus strains isolated from kokanee salmon, masu salmon, coho salmon, and rainbow trout in Japan reacted strongly with each other in the cross-neutralization test. ND_{50} values of these strains proved from 1:30 to 1:240. However, antiserum to SaHV-1-1 reacted below the level of detection (serum dilution less than 1:10) with the eleven herpesviruses from Japan.

Serological relationship

Serological relationships (1/R) described by Hedrick et al. (1987) were calculated from results of cross-neutralization tests shown in Table 2. The formula of 1/R value for serological relationships are shown below (Archetti and Horsfall. 1950).

$$R = \sqrt{\frac{\text{virus 1 with antiserum 2}}{\text{virus 1 with antiserum 1}} \times \frac{\text{virus 2 with antiserum 1}}{\text{virus 2 with antiserum 2}}}$$

In this formula, 1.0 indicates complete homology, and greater or less values indicate the increase of serological difference. The results of calculation of the 1/R values for twelve viruses are shown in Table 3. Almost all herpesviruses isolated from Japan showed similar values, which were between 0.51 to 1.53. The value of 0.51 was between SaHV-2-9 and SaVH-2-10, and the value of 1.53 was between SaHV-2-9 and SaHV-2-2. But the values of between SaHV-1-1 and Japanese strains showed more than 3.16.

Discussion

The data from cross-neutralization tests demonstrated that the presence of two distinct serological groups in fish salmonid harpesvirus was correlated as reported by Hedrick et al. (1987). They examined neutralization test with 5 strains such as SaHV-1-1 (*H. salmonis*) and steelhead herpesvirus (SHV, Hedrick et al., 1986) isolated from North America, and SaHV-2-1 (NeVTA), SaHV-2-2 (OMV 00-7812), and SaHV-2-5 (YTV) isolated from Japan. High 1/R values were observed between the strains isolated in USA and Japan. Eaton et al. (1991) distinguished the difference of the DNA homologies of the two different areas and then proposed that there was two herpesvirus groups in the salmonid herpesvirus, such as salmonid herpesvirus type 1 included *H. salmonis* (SaHV-1-1) and SHV, and salmonid herpesvirus type 2 included NeVTA (SaHV-2-1), OMV (SaHV-2-2), and YTV (SaHV-2-5). But they did not conduct the study to use the strains isolated from coho salmon and rainbow trout. However, a common antigen may exist between salmonid herpesvirus type 1 (SaHV-1) and type 2 (SaHV-2). Hayashi et al. (1993) reported that a monoclonal antibody against

Table 2. The results of serum cross-neutralization test of herpesviruses isolated from different salmon species.

Fish species	SaHV-2 Strain	Antisera								
		SaHV-2-2	-3	-4	-5	-1	-9	-8	-10	SaHV-1-1
Masu	SaHV-2-2 (OMV 00-7812)	50	60	70	35	40	30	35	50	<10
	-3 (OMV TyT-8101)	30	60	70	50	80	40	50	40	<10
	-4 (OMV ToT-8101)	50	80	60	40	80	70	30	40	<10
	-5 (YTV)	100	50	120	160	60	80	80	60	<10
Kokanee	SaHV-2-1 (NeVTA)	120	160	240	240	120	70	240	70	<10
Coho	SaHV-2-9 (COTV)	35	35	35	50	35	50	40	40	<10
	-6 (CSTV)	60	120	100	40	70	70	70	100	<10
	-8 (OKV-M)	120	70	120	50	100	60	60	80	<10
	-7 (OKV-T)	60	60	60	40	70	35	40	40	<10
Rainbow	SaHV-2-10 (RKV)	40	40	12	60	50	140	60	60	<10
	-11 (RTY)	50	30	60	60	40	30	60	40	<10
Rainbow	SaHV-1-1 (*H. salmonis*)	<10	<10	<10	<10	<10	<10	<10	<10	20

Table 3. Serological relationships of herpesviruses isolated from different salmon species using 1/R value calculated from results of serum cross-neutralization study in Table 2.

Fish species	SaHV-2 Strain	Antisera								
		SaHV-2-2	-3	-4	-5	-1	-9	-8	-10	SaHV-1-1
Masu	SaHV-2-2 (OMV 00-7812)	1.00	1.30	0.92	1.42	1.12	1.53	0.85	1.22	>3.16
	-3 (OMV TyT-8101)		1.00	0.80	1.00	1.22	1.47	0.85	0.67	>3.47
	-4 (OMV ToT-8101)			1.00	1.21	0.94	1.41	1.00	0.83	>3.47
	-5 (YTV)				1.00	0.84	1.33	0.70	1.19	>5.69
Kokanee	SaHV-2-1 (NeVTA)					1.00	1.39	0.83	0.89	>4.89
Coho	SaHV-2-9 (COTV)						1.00	0.96	0.51	>3.16
	-8 (OKV-M)							1.00	0.83	>3.47
Rainbow	SaHV-2-10 (RKV)								1.00	>3.47
Rainbow	SaHV-1-1 (*H. salmonis*)									1.00

SaHV-2-2 reacted with 3 different salmonid herpesviruses such as strain H-83 isolated from masu salmon, SaHV-2-2 (OMV 00-7812) and SaHV-1-1 (*H. salmonis*) by western blot analysis but did not have any neutralizing activity.

All salmonid herpesvirus strains isolated from a different salmon species in Japan showed strong relationships among them in the serological test, regardless of isolated organs that are coelomic fluid, tumor tissue, kidney, and liver, and area, because the value of 1/R is located between 0.5 and 1.5. This results make it possible that these strains isolated from salmon species in Japan are able to include the same group and belong to salmonid herpesvirus 2 (SaHV-2). Also, this results suggest that there was a presumed original strain and it spread to other areas. Many of the phenotypic properties of each herpesvirus from salmonid fish have been described. These include their pathogenicity, host and cell line specificity, optical growth temperatures, and size and morphology of the virion. Recently, an indirect fluorescent antibody test (IFAT) using anti NeVTA nucleocapsid rabbit antiserum was reported by Kumagai et al. (1995). But they did not report on the comparison of the sensitivity against different strains and the neutralization ability. Serologically, SaHV-2-1 (NeVTA), SaHV-2-2 (OMV 00-7812), and SaHV-2-5 (YTV) were confirmed to be the same virus by Hedrick et al. (1987), and a herpesvirus strain H-83 and 6 strains of SaHV-2 isolated from ovarian fluid and tumor tissue of masu salmon in Japan were confirmed as the same virus by DNA restriction endonuclease cleavage analysis by Hayashi et al. (1987, 1989) and Guo et al. (1991). From the results of comparison of the DNA homologies, SaHV-2-2 (OMV 00-7812) and SaHV-2-5 (YTV) were classified as the same virus, but SaHV-2-1 (NeVTA) was similar yet distinct from these two viruses (Eaton et al.,1991). Serological test is a more or less rough method to compare between each strain, but these results showed that SaHV-1 is completely different from SaHV-2 but individual strains of SaHV-2 are not significantly different and is able to infect the genus Oncorhynchus. However, many things remain to be explored on the characteristics of salmonid herpesvirus, such as epitope mapping with monoclonal antibody to explore antigen difference, oncogenicity with relation to other herpesvirus, etiology to prevent and eradicate viral disease, and evolution among the strains isolated from different salmon species.

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Oncorhynchus masou virus: 我が国のヒメマス，サクラマス，ギンザケ，ニジマスから分離されたサケ科魚類のヘルペスウイルスの血清学的関連性

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日本のサケ・マス由来のヘルペスウィルス（Salmonid Herpesvirus 2; SaHV-2 と呼称）の代表株12 株および米国由来株（SaHV-1）を対象に家兎抗血清を作製し，血清学的性状を比較検討した.作製した家兎抗血清の交差中和試験のND_{50} 値は，SaHV-2の抗血清とホモの各株とのND_{50}値が1:30から1:240の範囲であったが，SaHV-1とSaHV-2との間でのND_{50}値は1:10以下であった．得られたND_{50}値を基に血清学的な相関関係を比較するために比較計数1/Rを求めると，SaHV-2間では51～153を示し，SaHV-1とSaHV-2の間では3.16以上の値を示した.これらの結果から日本のサケ科魚類由来ヘルペスウイルスは,米国由来株とは明らかに異なっていたが,日本由来株間では魚種や分離臓器（体腔液，腫瘍，腎臓，肝臓），地域に関係なく血清学的に極めて近縁であることが明らかになった．以上の結果は日本のサケ科魚類由来株をSalmonid Herpesvirus 2とすることを裏づける結果となり，これらのウイルスをSalmonid Herpesvirus -2（SaHV-2）と呼称することを提案する．