Isolation of a New Rhabdovirus from Cultured Hirame (Japanese Flounder, *Paralichthys olivaceus*) in Japan

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Abstract

In March 1984, a new rhabdovirus was isolated from moribund cultured hirame (Japanese flounder, *Paralichthys olivaceus*) and from ayu *(Plecoglossus altivelis)* fry in Hyogo Prefecture. From February through May 1985, the virus was again isolated from hirame in seawater tanks in Hyogo and Kagawa Prefecture, and in Hokkaido, Japan. At temperatures between 5 and 20°C the virus replicated and induced cytopathic effects (CPE), which progressed to eventual cytolysis in susceptible cell lines, including FHM, EPC, BF-2, RTG-2, STE-137, HF-1, BB, YNK, CCO and EK-1. The CHSE-214, KO-6 and CHH-1 cell lines were refractory. Maximum infectivity of cell culture-grown virus was about $10^{9.3}$-$10^{9.8}$ TCID50/ml in FHM or EPC cell lines. The virus replicated optimally at 15 to 20°C. Virus particles were bullet shaped, 80 nm x 180 to 200 nm. The isolate was sensitive to pH 3, to diethyl ether, and to heat (50°C, 2 min.). The virus did not hemagglutinate human O type erythrocytes. Viral replication was not inhibited by 10^{-4} M 5-iodoxyuridine. Infectivity was not reduced by antisera against infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), spring viremia of carp virus (SVCV), pike fry rhabdovirus (PFRV), eel virus-American (EVA) and eel virus-European (EVX). The viral isolate was pathogenic for hirame and rainbow trout *(Salmo gairdneri)* by injection but not for chum salmon *(Oncorhynchus keta)*, coho salmon *(O. kisutch)*, masu salmon *(O. masou)*, or ayu fry by water borne exposure. From the evidence obtained thus far, this virus is a new pathogenic virus of fish, and is provisionally named HRV (hirame rhabdovirus). HRV is now considered to be an important pathogen of cultured hirame and salmonids in Japan.

Introduction

An unknown disease occurred during March 1984 among pen-cultured hirame (Japanese flounder, *Paralichthys olivaceus)* held in pens in Fukura Bay, and among cultured ayu *(Plecoglossus altivelis)* fry held in seawater tanks at the Hyogo Prefectural Fisheries Experimental Station, Hyogo Prefecture (Goric et al. 1985a, 1985b). The signs of disease in hirame were congestion of the gonads, focal hemorrhage in the skeletal muscles and fins, and accumulation of ascitic fluid. Infected ayu fry exhibited exophthalmia and petlechia on the gillcover. The following year, February to May 1985, the same disease occurred again among hirame cultured in seawater tanks at the Hyogo Prefectural Fisheries Experimental Station, Negi Island, Kagawa Prefecture and Yagishiri Island, Hokkaido. Examination of diseased fish failed to show any bacterial, fungal or parasitic agents.

However, during the course of viral examination, infectious hematopoietic necrosis virus (IHNV)-like cytopathic effect (CPE) was observed in the RTG-2 cell cultures. This agent was not neutralized with antisera against the following fish rhabdoviruses: IHNV, viral hemorrhagic septicemia virus (VHSV), spring viremia of carp virus (SVCV), pike fry rhabdovirus (PFRV), eel virus-American (EVA) and eel virus-European (EVX). Pathogenicity and virulence of the agent to the hirame was demonstrated by intraperitoneal injection (Gorie et al. 1985a).

This report describes the characteristics and pathogenicity of the new virus provisionally termed hirame rhabdovirus (HRV).

Biophysical Characterization

Biophysical characteristics of the HRV 8401H originating from diseased hirame and HRV A-8401H isolated from moribund ayu fry are summarized in Table 1. Ether treatment markedly reduced infectivity of the viruses. The BSS treated control had a titer of $10^{6.05}$ TCID 50/ml while the ether-treated suspension had less than $10^{1.80}$ TCID 50/ml. This sensitivity to ether is evidence that the virus has an essential lipid-containing envelope.

The deoxyuridine analogue IUdR was not effective in blocking viral replication. Control cultures and IUdR treated cultures had similar titers ($10^{6.05}$ TCID50/ml). The lack of inhibition by halogenated pyrimidines is evidence that the virus possesses an RNA genome.

Both isolates were inactivated by exposure to pH 3. No hemagglutination by HRV was observed with human O type erythrocytes.

Electron Microscopy

Virus particles were seen in ultrathin sections of HRV infected RTG-2 cells and the kidney tissue of HRV infected hirame. The particles were bullet shaped with one rounded end and a mean diameter of 80 nm and length of 180-200 nm. These morphological and biophysical features are characteristic of viruses of the Rhabdoviridae.

Serological Characterization

The five strains of HRV used were isolated from two host species at three different locations over a two-year period. All were neutralized with anti-HRV 8401H rabbit serum. No neutralization of HRV infectivity by antisera against IHNV, infectious pancreatic necrosis virus (IPNV), *Oncorhynchus masou* virus (OMV), VHSV, SVCV, PFRV, EVA or EVX was observed. The infectivity of each control virus used was reduced over 100-fold by the homologous antiserum.

Cross neutralization tests indicated that HRV was clearly distinguishable from the five reference rhabdoviruses (Table 2). HRV was neutralized with homologous antiserum at a titer of 160 and not neutralized with the heterologous antisera tested. Anti-IHNV, SVCV, EVA and EVX rabbit sera also had the highest titer against homologous virus. Cross reactions were not recognized except between EVA and EVX.

Cell Line Susceptibility and Virus Replication

The HRV was originally isolated using RTG-2 cells. Twelve other fish cell lines (FHM, EPC, BF-2, STE-137, HF-1, BB, YNK, CCO, EK-1, CHH-1, CHSE-214 and KO-6) were also tested for HRV susceptibility. The EPC, FHM, BF-2, YNK, STE-137, BB, EK-1, CCO and HF-1 cell lines were susceptible to HRV infection. The EPC and FHM cell lines showed the greatest sensitivity, while no CPE developed in CHH-1, CHSE-214 and KO-6 cells (Table 3). These latter three cell lines were susceptible to IHNV, but the HF-1, BB, EK-1 and CCO cells were not.

As judged by virus yield (Fig. 1), the FHM and RTG-2 cells were the most efficient at replicating HRV. An incubation time of 72-120 hours was required to observe CPE in EPC, HF-1, STE-137, BF-2 cells and the virus yields were greater than $10^{8.80}$ TCID 50/ml. No CPE developed in CHSE-214, CHH-1 or KO-6 cells after 10 days incubation at 15°C.

The temperature range for viral replication was determined to be 5 to 25°C. The RTG-2 cell line was used for those experiments performed at 5 to 20°C while the EPC cell line was used at 15 to 25°C because it tolerates a higher temperature. The extent of CPE correlated with the virus titer produced over the range of 5 to 20°C. At 10°C, viral replication was slower and CPE less extensive than at 15 or 20°C. At 10°C, viral replication was slower and CPE less extensive than at 15 or 20°C. In the case of IHNV and IPNV, the optimal temperatures were 15°C and in the range of 15 to 20°C, respectively.

Pathogenicity of HRV

The pathogenicity of the virus was tested for specific pathogen-free chum salmon *(Oncorhynchus keta)*, masu salmon *(O. masou)*, coho salmon *(O. kisutch)*, rainbow trout *(Salmo gairdneri)* and ayu. Ten rainbow trout (mean weight approximately 8 g), and masu salmon (6 g) were injected intramuscularly with $10^{4.0}$ $TCID_{50}$/fish of virus, and fish were held in 20-l aquaria at 12-13°C for 21 days. Fifty chum and coho salmon, thirty masu salmon, and fifteen ayu, weighing approximately 0.2, 0.5, 0.2 and 0.5 g, respectively, were infected by the immersion method using $10^{3.0}$ $TCID_{50}$/ml. Fish were exposed to the virus at 10°C for 60 min and then held in 3-l aquaria at 12-14°C. Ayu were maintained at 16-17°C for 21 days. After 21 days, five fish, including one that died, were removed and examined for bacteria and virus.

Mortality among rainbow trout began at six days post-injection and 60% had succumbed by day 12. Hemorrhages were commonly observed in muscle and fin. The cumulative mortality of control fish was 10% in 21 days. For masu salmon 1 of 10 control fish and 0 of 10 virus injected fish died during the experiment. In coho salmon fry, no control or experimental fish died. Among chum salmon, 3 of 50 control fish and 5 of 50 virus exposed fish died. No control and 2 of 30 virus immersed masu salmon fry died during the 21-day period. Among the ayu no control or virus-exposed fish died in the experiment (Table 4).

Virus isolation was carried out using five fish from each group, composed of mortalities or fish sacrified at the end of the experiment. Virus was recovered from five individual rainbow trout (all mortality), one masu salmon fry (mortality), one masu salmon fry (sacrificed) and three coho salmon fry (sacrificed). Signs of disease were only observed in rainbow trout. All the isolates were neutralized with anti-HRV serum.

Virus titers in tissues of infected rainbow trout were determined for kidney and spleen pools, liver and pancreas pools, heart, intestine, airbladder and muscle. The virus was recovered from all tissues examined. Kidney and spleen pools had the highest titer, reaching $10^{6.05}$ to $10^{6.90}$ $TCID_{50}$/ml.

Discussion

The virus isolated from cultured hirame and ayu appears to be a previously unreported pathogen of marine fish. No evidence of a similar agent is noted among recent listings of fish viruses.

Fish rhabdoviruses, such as IHNV, VHSV, SVCV, PFRV, EVA and EVX are well known. Swim bladder inflammation (SBI) virus proved to be morphologically and serologically identical to SVCV (Bachman and Ahne 1973, 1974). HRV was not neutralized with the antisera against IHNV, VHSV, SVCV, PFRV, EVA or EVX. Cross neutralization tests indicated that HRV was clearly distinguishable from these reference rhabdoviruses.

HRV shows IHNV-like CPE with eventual lysis of FHM, EPC, BF-2, RTG-2, STE-137, HF-1, BB, YNK, CCO and EK-1. However, the CHSE-214, CHH-1 and KO-6 cell lines, all with an epitheloid morphology and derived from salmonid fish, were refractive to HRV. The pattern of this cell susceptibility was clearly different from IHNV and VHSV, and distinguishable from the rhabdoviruses of cod ulcus syndrome (Jensen et al. 1979) or crab (Johnson 1984).

Although the rhabdoviruses isolated from warmwater fish (SVCV, SBIV, PFRV, EVA, EVX), the virus isolated from grass carp (Ahne 1975) and from a North American cichlid (Malsberger and Lautenslager 1980) replicate at 25°C or above, HRV did not replicate at 25°C. A comparison between HRV and *Rhabdovirus salmonis* (Osadchaya and Nakonechnaya 1981) could not be made because of the limited information available.

The pathogenicity and virulence of HRV for hirame was observed following intraperitoneal injection into 100 to 250 g fish (Gorie et al. 1986). The cumulative mortality was 20%, but mortality was not observed at 15°C or [illegible]. The mortality of hirame in natural outbreaks in Hokkaido was 25% (1984) and 7.2% (1985). For Kagawa Prefecture it was 3.3% and greater than 90% in Hokkaido. The size of fish affected was 100 to 700 g. This high mortality among naturally infected hirame observed in Hokkaido may be caused by the lower rearing temperatures in the winter.

HRV was lethal for rainbow trout and the cumulative mortality was 60%. The signs of HRV infection in rainbow trout were similar to those of VHS, with hemorrhaging in muscles commonly seen. Histopathologically, hematopoietic cells were pyknotic or necrotic in hirame (Kimura et al., unpublished data). Castric and de Kinkelin (1984) have reported that VHSV is pathogenic for sea bass and turbot, both marine species. The gross signs and histopathological data reported indicate a condition similar to that of HRV-infected hirame. In this study, chum, coho and masu salmon and ayu fry did not experience high mortality when exposed to HRV by immersion method, but HRV was isolated from dead and surviving fish. A longer observation period may have resulted in a higher mortality.

Evidence suggests HRV is a new and pathogenic fish virus which is considered to be an important pathogen of cultured hirame and salmonids in Japan.

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Table 1. Biochemical and biophysical characterization of hirame rhabdovirus.

Characteristics	log. $TCID_{50}$/ml HRV 8401 H	HRV A-8401 H
Control	6.05	5.06
Ether treatment, 5°C, 18 h	< 1.80	< 1.80
pH 3.0, 3 hr	4.05	4.30
60°C, 2 min	< 0.80	< 0.80
60°C, 1 min	< 0.80	< 0.80
IUDR 50 μg/ml*	6.05	6.30
Haemagglutination of human O type erythrocytes*	negative	negative

*See text

Table 2. Cross neutralization test of the HRV and IHNV, VHSV, SVCV, EVA, EVX and PFRV

Virus	Virus titer Log $TCID_{50}$ /well	HRV 8401 H	IHNV HV-1[a]	IHNV H[b]	VHSV K[c]	VHSV A[d]	SVCV H	SVCV K	PFRV K	EVA H	EVX H
HRV 8401 H	1.75	100	< 17	< 42	< 42	< 42	< 42	< 42	< 17	< 42	< 42
IHNV HV-1	3.00	< 13	90	ND[e]	ND	ND	ND	ND	ND	ND	ND
IHNV H	1.75	< 17	ND	141	< 42	< 42	< 42	< 42	< 17	< 42	< 42
SVCV H	2.00	< 11	ND	< 42	< 42	< 42	141	ND	< 17	< 42	< 42
EVA H	1.55	< 17	ND	< 42	< 42	ND	< 42	< 42	ND	≧ 281	≧ 281
PFRV K	2.25	< 17	ND	< 42	ND	ND	ND	ND	≧ 112	ND	ND

[a] Provided by the Ministry of Agriculture and Fisheries
[b] Provided by Dr. B. J. Hill
[c] Provided by Dr. P. de Kinkelin, antiserum against VHSV was polyvalent
[d] Provided by Dr. W. Ahne
[e] Not determined
[f] ND_{50} = 50% Neutralization titers against 100 $TCID_{50}$ virus

Table 3. Comparison of cell line susceptibility to HRV and IHNV infection

Abbreviation	Cell line Source		Cell morphology	HRV 8401H CPE[1]	HRV 8401H Titer[2]	IHNV CHAB CPE	IHNV CHAB Titer
RTG-2	Rainbow trout	Gonad	F[3]	+	8.30	+	4.30
EPC	Carp	Epithelioma	E[4]	+	7.30	+	8.30
FHM	Fathead minnow	Caudal trunk	F	+	8.80	+	8.80
BF-2	Bluegill	Fin	F	+	8.55	+	4.55
YNK	Yamame	Kidney	F	+	8.30	+	4.55
STE-137	Steelhead trout	Embryo	E	+	8.05	+	4.80
CHSE-214	Chinook salmon	Embryo	E	–	< 2.80	+	6.55
CHH-1	Chum salmon	Heart	E	–	≦ 2.05	+	8.30
KO-6	Kokanee salmon	Embryo	E	–	< 2.80	+	4.80
HF-1	Hirame	Fin	E	+	8.80	–	< 2.80
88	Brown bullhead	Caudal trunk	F	+	8.30	–	< 2.80
EK-1	Eel	Kidney	E	+	7.80	–	< 2.80
CCO	Channel catfish	Ovary	F	+	4.55	–	< 2.80

[1] Cytopathic effect
[2] Log $TCID_{50}$/ml
[3] Fibroblast
[4] Epithelioid

Table 4. Comparison of mortality in rainbow trout, masu salmon, coho salmon, chum salmon and ayu artificially infected with HRV

Species of fish	Method of infection	Experiment	Number of fish employed	Number of dead: 3 days after exposure	8	9	12	21	Cumulative mortality (%)
Rainbow trout (8 W 6.0 g)	IP[1]	Test	10	0	1	2	3	0	60
		Control	10	0	1	0	0	0	10
Masu salmon (8 W 6.0 g)	IP	Test	10	0	0	0	0	0	0
		Control	10	0	0	0	0	1	10
Coho salmon (8 W 0.5 g)	IS[2]	Test	60	0	0	0	0	0	0
		Control	60	0	0	0	0	0	0
Chum salmon (8 W 0.2 g)	IS	Test	50	1	3	1	0	0	10
		Control	50	1	1	0	0	1	8
Masu salmon (8 W 0.2 g)	IS	Test	30	2	0	0	0	0	7
		Control	30	0	0	0	0	0	0
Ayu (8 W 0.5 g)	IS	Test	15	0	0	0	0	0	0
		Control	15	0	0	0	0	0	0

[1] Intraperitoneal injection, injecting dose 1.0×10^6 $TCID_{50}$/fish
[2] Immersion method, injecting dose 1.0×10^7 $TCID_{50}$/ml 60 min 10°C

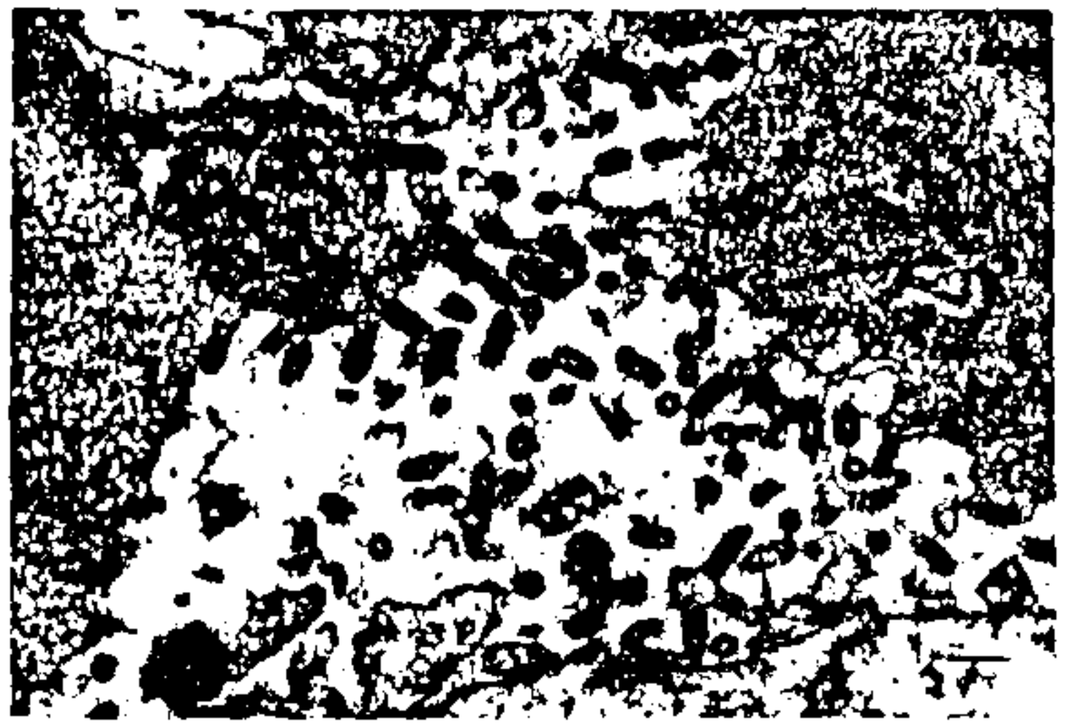

Fig. 1. Electron micrograph showing large numbers of bullet shaped virus particles in RTG-2 cell infected with HRV. Stained with 2% uranyl acetate, bar = 200 nm.

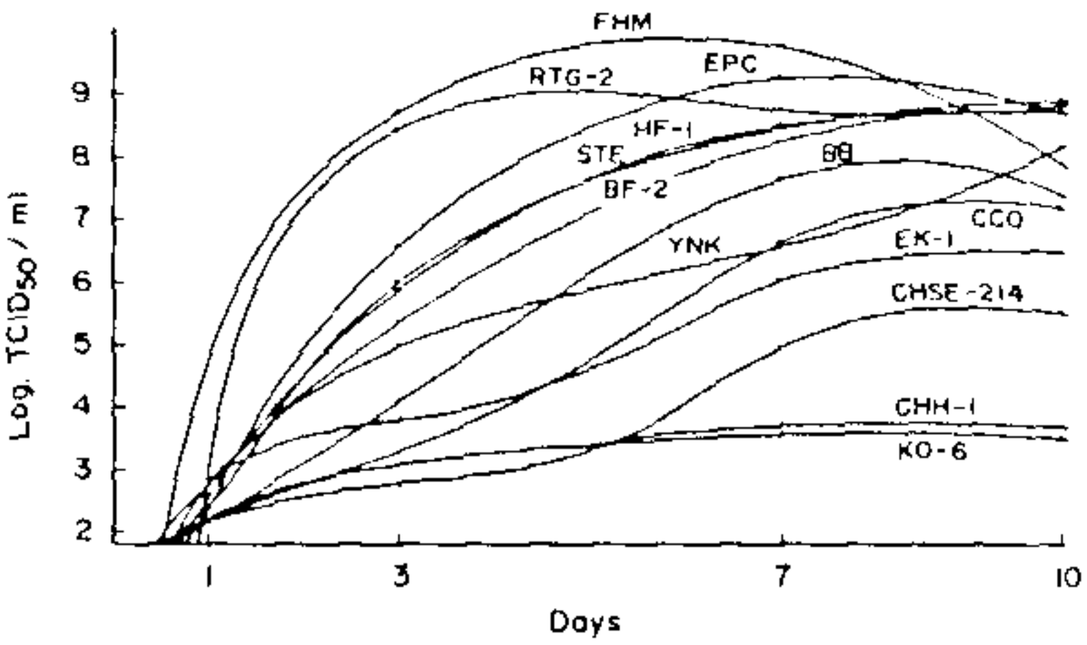

Fig. 2. Replication of HRV in selected fish cell lines incubated at 15°C, M.O.I. = 0.1.