Isolation of a New Virus from Chinook Salmon (*Oncorhynchus tshawytscha*) in Oregon U.S.A.*¹

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A new virus has been isolated from adult chinook salmon (*Oncorhynchus tshawytscha*) in the state of Oregon, U.S.A. The virus was recovered from pooled kidney and spleen tissue samples using CHSE-214 cells. Cytopathic effect (CPE) required 28 days to develop on primary isolation. The virus replicated in CHSE-214, CHH-1, KO-6, CSE-119 and FHM cells. It had an optimal growth temperature of 18°C and no virus was produced at 24°C or above.

The virus was stable in minimal essential medium (MEM) at pH 3-11 and replication was not affected in cells incubated with MEM containing 50 µg/ml iododeoxyuridine. Infectious virus was recovered from fractions of a CsCl gradient having a density of 1.20 g/cc. The agent was sensitive to chloroform and caused hemagglutination with erythrocytes from several species of fish, mammals and birds.

Electron microscopy revealed enveloped particles 125-250 nm in diameter containing a coiled nucleocapsid. Electron micrographs of freon-treated preparations showed the extracted nucleocapsid to be a helix 18 nm in diameter and up to 1000 nm in length. Based on morphology and biochemical features, the virus appears to be a member of the Paramyxoviridae.

Introduction

The use of certification examinations to control the spread of infectious diseases of fish has resulted in the discovery of several previously unknown viruses (KIMURA *et al.*, 1981; SANO, 1976; WINTON *et al.*, 1981). In recent years we have conducted viral examinations of sexually mature fish for the Oregon Department of Fish and Wildlife and for private aquaculture companies. Examinations of adult chinook salmon (*Oncorhynchus tshawytscha*) returning to the Yaquina River in the fall of 1982 and to the Trask River in 1983 have resulted in the isolation of a new virus of fish. In this paper we report our initial findings.

Materials and Methods

Cells and Medium

The virus was isolated using the chinook salmon

embryo cell line, CHSE-214 (ATCC CRL 1681). For propagating and assaying the virus we used the chum salmon heart cell line, CHH-1 (LANNAN *et al.*, 1984). Growth studies were conducted using: CHSE-214, CHH-1, rainbow trout gonad (RTG-2; WOLF and QUIMBY, 1962), atlantic salmon (AS; NICHOLSON and BYRNE, 1973), brown bullhead (BB; WOLF and QUIMBY, 1969), epithelioma papillosum cyprini (EPC; TOMASEC and FIJAN, 1977), fathead minnow (FHM; GRAVELL and MALSBURGER, 1965), kokanee salmon ovary (KO-6; LANNAN *et al.*, 1984), and coho salmon embryo (CSE-119; FRYER *et al.*, 1965). Methods for culturing fish cell lines have been described by WOLF and QUIMBY (1976). Eagle's minimal essential medium (MEM) was supplemented with 10% fetal bovine serum (MEM-10) for growing cells or 5% (MEM-5) when replicating virus. Except where noted, cells were incubated at 18°C.

Virus Isolation

The examinations of adult chinook salmon occur-

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red in the fall of 1982 at the Oregon Aqua-Foods Yaquina Bay recapture site, Lincoln County, Oregon, USA and in 1983 at the Oregon Department of Fish and Wildlife Trask River Hatchery, Tillamook County, Oregon, USA. The fish were sampled using standard methods outlined by the Fish Health Section of the American Fisheries Society (McDANIEL, 1979). Kidney and spleen tissues and ovarian fluid samples were prepared in five-fish pools, inoculated onto monolayer cultures of CHSE-214 and EPC cells, and incubated at 18°C. After 14 days, samples from these cultures were removed and inoculated onto a second set of the same cell lines. This "blind passage" was incubated for an additional 2 weeks.

Virus Replication

Monolayer cultures of CHH-1, CHSE-214, CSE-119, AS, KO-6, FHM, EPC, BB, and RTG-2 cell lines were prepared in duplicate 25 cm² flasks. The growth medium was removed and 5 ml MEM-5 added to each flask. The cultures were inoculated with virus at a low multiplicity of infection (MOI), incubated at 18°C, and observed daily for cytopathic effect (CPE). After 14 days, infectious virus in the culture fluid was assayed by calculating the 50% tissue culture infectious dose (TCID₅₀) using 96-well plates of CHH-1 cells and the method of REED and MUENCH (1938).

The optimal temperature for viral replication was measured using 75 cm² flasks of CHH-1 cells inoculated with virus at a low MOI. The cultures were incubated for 20 days at 6, 9, 12, 15, 18, 21, and 24°C. At 48 hr intervals, culture fluid was removed from the flasks at each temperature and the virus titer determined.

Physical and Chemical Tests

Thermostability of the agent was tested by incubating viral suspensions in MEM at 18, 37 and 56°C. Samples were removed at 1, 6, 24 hr and 7 days. The TCID₅₀ titer for each incubation period was determined using CHH-1 cells.

The stability of the virus at selected pH values was measured by adding 0.5 ml culture fluid from infected CHH-1 cells to 4.5 ml aliquots of MEM adjusted to pH 2, 3, 5, 7, 9, and 11. After incubation for 30 min at 25°C, the virus titer at each pH value was determined by TCID₅₀ assay.

Chloroform sensitivity was tested using the

method of FELDMAN and WANG (1961). One ml of chloroform was added to 2.0 ml of clarified culture fluid from infected CHH-1 cells. Control tubes received 1.0 ml of Hanks' balanced salt solution (BSS). The cultures were shaken for 10 min, centrifuged (2000 g, 10 min), and the virus titers determined for the BSS and chloroform-treated preparations. Infectious hematopoietic necrosis virus, known to have a lipid-containing envelope (PILCHER and FRYER, 1980), and the non-enveloped, infectious pancreatic necrosis virus (MCALLISTER, 1979) were included as controls.

Membrane filters of 1.2, 0.45 and 0.2 µ pore size were pretreated with 10% fetal bovine serum and used to filter a viral suspension. The titers before and after filtration were calculated by TCID₅₀ assay using CHH-1 cells.

The effect of the DNA virus inhibitor 5-iodo-2'-deoxyuridine (IDU) was tested by the method of KUCHLER (1977). Replicate 25 cm² flasks of CHH-1 cells containing MEM-5 or MEM-5 with 50 µg IDU/ml were inoculated with virus. *Oncorhynchus masou* virus (OMV), a herpesvirus containing a DNA genome (KIMURA *et al.*, 1981) and infectious pancreatic necrosis virus (IPNV), a birnavirus with an RNA genome (MCALLISTER, 1979) were included as controls. After incubation at 18°C for 10 days, the TCID₅₀ titers in the IDU-treated and untreated flasks were determined for each virus.

Density of the Virus in Cesium Chloride

Culture fluid from virus-infected CHH-1 cells was clarified by low speed centrifugation (2000 g, 10 min) and the virus in the supernate collected by centrifugation at 100,000 g for 1 hr in an SW 28 rotor. The viral pellet was resuspended in 0.5 ml BSS and layered onto a three-step gradient composed of 1.5 ml 40% w/w CsCl, 1.5 ml 30% w/w CsCl and 1.5 ml 20% w/w CsCl. The gradient was centrifuged in an SW 50.1 rotor at 115,000 g for 16 hr and 0.25 ml fractions collected with a probe and peristaltic pump. The refractive index of each fraction was read and the density calculated by interpolation from the International Critical Tables. The titer of infectious virus in alternate fractions was determined by TCID₅₀ assay.

Hemagglutination Assay

Culture fluid from infected CHH-1 cells was clarified by low speed centrifugation, the virus

collected by centrifugation for 1 hr at 100,000 *g*, and the pellet resuspended in BSS. Suspensions of human (A, B and O), rabbit, guinea pig, horse, sheep, pig, bovine, goat, turkey, chicken, steelhead trout, chinook, coho, and chum salmon erythrocytes were prepared in phosphate buffered saline. Rapid slide hemagglutination tests were performed by reacting 50 μ l of a 10% suspension of erythrocytes with 100 μ l of partially purified virus or with BSS. A microtiter hemagglutination test was performed by the method described in KUCHLER (1977).

Negative Staining of Virions

Culture fluid from infected CHH-1 cells was clarified by low speed centrifugation (2000 *g*, 10 min) and the virus pelleted in an SW 28 rotor at 100,000 *g* for 1 hr. The pellet was resuspended in distilled water, stained with 2% phosphotungstic acid for 1 min and examined using a Philips 300 electron microscope. In certain preparations, infected cell homogenates were first treated with Genetron (1,1,2-Trichlorotrifluoroethane) to remove cell debris.

Examination of Infected Cells

Monolayer cultures of CHH-1 cells were infected at an MOI of approximately 1.0 and the cells incubated at 18°C for 2, 3, 4, 5, or 7 days before fixation with 3% glutaraldehyde in BSS. The fixed cells were scraped from the flasks and pelleted by centrifugation at 500 *g* for 5 min. Cells were stained for 1 hr with 1% osmium tetroxide, passed through a dehydration series and embedded in Spurr's medium (SPURR, 1969). Sections were cut with an ultramicrotome, post-stained with lead citrate (REYNOLDS, 1963) and examined by electron microscopy.

Results

Isolation of Virus

The CHSE-214 and EPC cells used in the virus assay remained normal in appearance during the initial 14 day incubation. Samples from these cultures were removed and inoculated onto a second set of the same cell lines. On day 14 of this "blind pass", cytopathic effect began to appear in the cultures of CHSE-214 cells containing kidney and spleen homogenates. Cultures containing ovarian fluid samples and all EPC cells remained normal.

Samples were removed from the CHSE-214 cells showing CPE, passed through a 0.45 μ membrane filter and subcultured on CHSE-214 cells where CPE reappeared after approximately 10 days. Early cytopathology was composed of a cluster of 3–10 cells becoming refractile and rounded. With further incubation, CPE became generalized, involving the entire cell sheet (Fig. 1). Later, cells detached from the surface of the flasks and floated free in the medium.

Virus Replication

Of the cell lines tested, the virus replicated and produced CPE in CHH-1, CHSE-214, KO-6 and CSE-119 cells. In these cell lines the average titers ranged from 3.2×10^5 to 5.2×10^6 TCID₅₀/ml. The FHM cell line produced a low titer of virus (3.0×10^2 /ml) without CPE. When CHH-1 cells were infected at a low MOI and incubated at selected temperatures, viral synthesis was optimal at 18°C but occurred from 6–21°C (Table 1). No virus was produced at 24°C and cytopathic effect was only observed in cultures incubated between 15–21°C. These data suggest that the host range of the virus is confined to cold blooded animals.

Physical and Chemical Features

The virus remained viable after exposure to 18°C for 7 days, but was completely inactivated after 6 hr at 56°C or 24 hr at 37°C (Table 2). No reduction in titer occurred after a freeze-thaw cycle at –20 or –70°C.

Infectious virus passed membrane filters with pore sizes of 1.2, 0.45 or 0.2 μ . The unfiltered suspension contained 3.2×10^5 TCID₅₀/ml. Passage through a 1.2 μ filter reduced the titer to 6.3×10^4 . After the 0.45 μ membrane, the titer was 3.2×10^3 and following the 0.2 μ filter, it was only 3.2×10^1 TCID₅₀/ml. These results might be expected if infectious particles had an irregular shape or varied in size.

The virus was not affected by incubation for 30 min in MEM-5 at pH 3, 5, 7, 9 or 11. However, no infectious virus remained after exposure to pH 2 for 30 min.

Chloroform completely inactivated the virus (Table 3). Control suspensions treated with BSS had a titer of 3.2×10^5 TCID₅₀/ml. Inactivation of the virus by chloroform indicates the complete virion has an envelope containing essential lipids.

Fig. 1. Cytopathic effect produced in a monolayer culture of the chum heart cell line (CHH-1) by the virus isolated from chinook salmon. A Normal cell monolayer. B Infected culture.

Table 1. Replication of the virus isolated from chinook salmon in the chum heart cell line (CHH-1) incubated at seven selected temperatures

Temperature (°C)	$\log_{10}$ TCID ₅₀ /ml at days post-infection									
	2	4	6	8	10	12	14	16	18	20
6	0.0	0.0	0.0	0.0	2.5	3.4	3.8	3.5	4.0	4.6
9	0.0	0.0	0.0	0.0	3.5	3.5	4.3	3.8	4.5	4.8
12	0.0	0.0	0.0	2.8	3.4	3.6	3.8	5.6	4.8	4.8
15	0.0	0.0	0.0	2.6	4.5	4.8	5.0	6.0	5.8	5.8
18	0.0	0.0	2.3	3.3	4.8	5.3	4.7	5.5	7.0	5.6
21	0.0	2.0	2.0	2.6	3.8	4.3	5.3	5.5	5.5	5.5
24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 2. Stability of the virus isolated from chinook salmon exposed to three selected temperatures in minimal essential medium

Temperature (°C)	Titer (TCID ₅₀ /ml) ^a			
	1 hr	6 hr	24 hr	7 days
18	ND ^b	ND	3.2×10^6	3.2×10^6
37	1.0×10^7	4.0×10^5	0.0	0.0
56	1.0×10^4	0.0	0.0	0.0

^a Initial titer was 2.0×10^6 .^b Not determined.**Table 3.** Stability of the virus isolated from chinook salmon to treatment with chloroform for 30 min

Virus	Titer (TCID ₅₀ /ml)	
	Treated	Untreated
Chinook salmon isolate	0.0	3.2×10^5
Infectious pancreatic necrosis virus ^a	1.0×10^9	6.3×10^8
Infectious hematopoietic necrosis virus ^b	0.0	2.5×10^6

^a Control virus lacking essential lipids.^b Control virus containing essential lipids.

Infectious hematopoietic necrosis virus, the enveloped control, was also inactivated by chloroform. Infectious pancreatic necrosis virus which lacks essential lipids was unaffected.

Treatment of cells with iododeoxyuridine did not affect viral synthesis (Table 4). Replication of the DNA virus control (OMV) was completely blocked

Table 4. Replication of the virus isolated from chinook salmon in the chum heart cell line (CHH-1) with growth medium containing $50 \mu\text{g/ml}$ 5-iodo-2'-deoxyuridine

Virus	Titer (TCID ₅₀ /ml)	
	Treated ^a	Untreated ^b
Chinook salmon isolate	4.0×10^7	4.0×10^6
Infectious pancreatic necrosis virus ^c	4.0×10^9	2.0×10^9
<i>Oncorhynchus masou</i> virus ^d	0.0	6.3×10^5

^a Cells were grown and virus replicated using minimal essential medium containing 5% serum (MEM-5) and $50 \mu\text{g/ml}$ 5-iodo-2'-deoxyuridine.^b Cells were grown and virus replicated in MEM-5 without iododeoxyuridine.^c Control virus with RNA genome.^d Control virus with DNA genome.

while the RNA virus control (IPNV) was not. The lack of inhibition by halogenated thymidine analogs is considered presumptive evidence that a virus possesses an RNA genome (REVOZZO and BURKE, 1973; KUCHLER, 1977).

Density of the Particles

Infectious virus was recovered from fractions near the center of the CsCl equilibrium gradient. The virus did not form a visible band and the titer was low. When infectious virus was assayed by TCID₅₀ and plotted against the density of each fraction, a peak of infectivity was found in fractions having a density of 1.20 g/ml .

Fig. 2. Electron micrograph of negatively stained nucleocapsids showing the helical structure.

Fig. 3. Electron micrograph of a thin section of infected CHH-1 cells showing enveloped particles (arrows) containing coiled nucleocapsids.

Fig. 4. Electron micrograph of a thin section through an infected CHH-1 cell showing many nucleocapsids in the cytoplasm.

Hemagglutination

The virus caused rapid hemagglutination of erythrocytes from 11 of the 14 species of mammals, birds and fish tested. Agglutination was not observed using sheep, goat or bovine cells. The end points of the hemagglutination titers were similar for the 11 types of reacting cells. Agglutination of the fish erythrocytes was particularly rapid.

Virus Morphology

Negatively stained preparations of Genetron-treated and partially purified cell culture fluid revealed helical nucleocapsids approximately 18 nm in diameter and up to 1000 nm in length (Fig. 2). The nucleocapsid appeared to be a left-handed helix containing a 5 nm central pore. The capsomeres slanted slightly toward one end.

Thin sections of CHH-1 cells infected for 72 hr showed enveloped viral particles that varied in size between 125 and 250 nm. The virions were not regular in shape and could be seen to contain coiled nucleocapsids (Fig. 3). The particles were present in groups and appeared to have matured by budding

through the cell membrane. Areas containing large numbers of nucleocapsids were observed in the cytoplasm of infected cells (Fig. 4).

Discussion

The isolation and characterization of this virus has been hindered because the agent produces CPE slowly and the titers obtained from cultured cells are low. While the virus has been isolated in consecutive years from fish in two separate river systems, the origin and distribution of the agent is not known. The extended length of time required to produce viral CPE on initial isolation may have allowed the virus to go undetected until now. Morphologically and chemically, both isolates appear the same. Serological analysis in progress will test their relatedness.

The isolates were obtained from infected adult chinook salmon showing no signs of gross pathology. Attempts were made to recover the virus from progeny of the Yaquina Bay stock without success. Pathogenicity trials are in progress using four spe-

cies of salmonid fish and both isolates of the virus.

The results reported here indicate the virus is an enveloped particle of approximately 125–250 nm in diameter containing a coiled helical nucleocapsid. The genome of the agent is believed to be RNA. The virus hemagglutinates the erythrocytes of a number of animals including fish. It replicates in cell lines derived from Pacific salmon at an optimal temperature of 18°C and is stable at pH 3–11. The infectious virion has a density of 1.20 g/ml in CsCl.

We believe that this agent is a previously undescribed virus of fish. Chemical and morphological features suggest that the virus is a member of the family Paramyxoviridae (CHOPPIN and COMPANS, 1975; KINGSBURY, 1977). Further work will be required before precise taxonomic placement of this new fish virus can be made.

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