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Isolation of Infectious Pancreatic Necrosis Virus from Goldfish (*Carassius auratus*) and Chum Salmon (*Oncorhynchus keta*) in Korea.

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금붕어(*Carassius auratus*)와 연어(*Oncorhynchus keta*)에서 췌장괴저 바이러스(IPNV)의 분리에 대하여

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ABSTRACT

Two viruses were isolated from kidney and spleen tissues of goldfish (*Carassius auratus*) and the ovarian fluid of chum salmon (*Oncorhynchus keta*). Both viruses replicated and produced cytopathic effect in EPC, CHSE-214, and CHH-1 cell lines at 15°C. The isolates were resistant to pH 3 and chloroform. Antiserum to infectious pancreatic necrosis virus (IPNV) serotype VR 299 neutralized the infectivity of both of the isolates. Electron microscopy showed that the particles had typical IPNV particle morphology with average diameters of 55 nm, This paper describes the first isolation of viruses infecting cultured fish in Korea.

INTRODUCTION

Today, the importance of fish culture is recognized in Korea, and the interest and investment in it has been increased. A greater number of fish per unit area and the resulting degradation of water conditions has led to more frequent outbreaks of disease. In Korea, the study of fish diseases has recently been emphasized. We report here, the first isolations of virus from goldfish and chum salmon in Korea.

The first fish cell line, Rainbow Trout Gonad (RTG-2), was developed in early 1960. About 60 kinds of fish cell lines have now been established and research on fish viruses has accelerated. As a result of work done with these cell lines, more than 20 fish viruses have been isolated and an additional 14 have been visualized by electron microscopy (Wolf and Mann, 1980; Groberg and Fryer, 1983).

Infectious pancreatic necrosis virus (IPNV) has been widely isolated and well studied (Hill, 1982). It is an unenveloped, double-stranded RNA virus. The virion is 55 nm in diameter and has an icosahedral symmetry. The virus replicates between 5°C~20°C causing a cytopathic effect characterized by granulation and the formation of spindle-shaped cells. There are three recognized serotypes (Ab, Sp, and VR 299; Pilcher and Fryer, 1980; McAllister, 1983).

MATERIALS AND METHODS

1. Cells and medium

Three cell lines were used in this study: chinook salmon embryo (CHSE-214; Nims, Fryer and Pilcher, 1970), Epithelioma papil-

losum cyprini (EPC; Tomasec and Fijan, 1971), and chum salmon heart (CHH-1; Winton and Lannan, unpublished data). The cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (MEM-10) for cell growth and 2% serum (MEM-2) for viral replication. The complete medium also contained gentamycin at a final concentration of 8μg/l.

The cells were incubated at 18°C and viruses were grown at 15°C in normal atmosphere. Milk dilution bottles (Kimax, Corning) and disposable culture bottles (Costar, Corning) were used for cell propagation. Multiwell plates (Linbro, Costar) were used for viral isolation and analysis. These plates were seeded with cells and used within 72hr.

2. Isolation of viruses

In September 1983, 60 goldfish were randomly collected from the Goldfish Hatchery, Kimpo. Samples of kidney and spleen tissues were prepared in five-fish pools and processed for virus isolation by standard methods of the American Fisheries Society: Fish Health Section (1979).

An examination of adult chum salmon at the Yang-yang recapture site was undertaken in October 1983. Eighteen semen samples and 21 ovarian fluid samples were collected from sexually mature fish and analyzed for virus content.

Kidney and spleen pools from the goldfish were homogenized with a mortar and pestle in 20 volumes of Hank's balanced salt solution (HBSS). The homogenate was centrifuged for 10 min. at 2000xg. The supernatant was diluted 1 : 5 in MEM containing an antibiotic mixture of 400μg/ml gentamicin and 400μg/ml of nystatin. Semen and ovarian fluid samples from chum samples were diluted 1 : 5 in the same antibiotic mixture. All samples were held at 4°C overnight then placed on monolayers of fish cells and incubated at 15°C.

3. Infectivity titration

The concentration of virues in the fluids of experimental cultures was measured by the end point dilution method employing monolayer cultures of cells grown in multiwell plates. Ten-fold dilutions of the virus were prepared, and each dilution was then inoculated into six wells of a 96 well plate. The plates were examined for cytopathic effect after 7 days incubation at 15°C and the 50% end point was estimated by the method of Reed and Muench (1938).

4. Physical and chemical characterization

Chloroform sensitivity of the virus was determined by a modification of the method of Feldman and Wang (1961). Two ml of chloroform was added to 2ml of cell-free supernatant from an infected cell culture. A control tube of supernate received 2ml of HBSS. These mixtures were shaken for 10 min. Both the chloroform treated and control cultures were centrifuged at 600xg for 5 min. Infectious virues in the aqueous phase of the chloroform treated sample and in the HBSS control was assayed by $TCID_{50}$ analysis.

Stability to pH 3 was tested by incubation of a virus suspension in MEM adjusted to pH 3. Control virus was suspended in MEM at pH 7. After 30min incubation at 15°C, the virus concentrations were determined.

5. Neutralization tests

Antisera to VR-299, SP and AB IPNV were obtained from the Department of Microbiology, Oregon State University, Covallis, Oregon.

Neutralization tests were made in multiwell plates with anti-VR-299, anti-SP and anti-AB serum as described by Okamoto et al. (1983). Antisera were diluted with MEM-0 at pH 7.6 in two-fold series and a titre of 320 to 650,000 ND_{50} of neutralizing antibody (0.05ml/well) was provided on multiwell plate. Freshly harvested (not frozen) viruses were diluted with MEM-0 to give 100 $TCID_{50}$ per 0.05ml and 0.05ml of the virus suspension was added to each of the wells of the multiwell plate. The virus suspension and antisera were incubated

for 1hr at 20°C with constant mixing. A 0.1ml volume of MEM-10 containing CHSE-214 cells (1×10^5cells/ml) was then inoculated into each well of the multiwell plate. The multiwell plates were sealed and incubated at 15°C for 7 days.

Neutralizing antibody titres (ND_{50}/0.05ml) are expressed as the reciprocal of the highest dilution of antiserum protecting 50% of the inoculated cell cultures.

6. Electron microscopy

Virus from infected monolayers of EPC cells was harvested, by centrifugation at 2000g for 20 min to remove cell debris and the virions were pelleted by centrifugation at 115,000g for 1hr. Virus pellets were resuspended in three volumes of distilled water and stained for 1 min with 3% phosphotungstic acid (PTA) pH 6.0. One drop of the mixture was placed on a carbon coated mesh. The meshes were then dried and examined with a Hitachi H-500 electron microscope.

RESULTS

1. Isolation of the viruses

After 7 days incubation at 15°C, one of the cell cultures inoculated with material from a goldfish kidney and spleen pool began to show several focal areas of CPE. Supernate from infected cells was harvested after CPE was complete and inoculated onto fresh cells following a 1 : 100 dilution. The same CPE was obtained and a second 1 : 100 dilution and subculture again gave similar results. Cytopathic effect was again observed after a third subculture was passed through a 0.22um filter. This isolate was designated G5.

Cell lines inoculated with chum salmon ovarian fluid samples also began to show CPE after 6 days at 15°C. Subsequent subcultures showed similar CPE. This isolate was harvested and designated YCO11.

The virus isolates G5 and YCO11 were propagated in EPC, CHH-1, and CHSE-214 cells. Freezing and thawing treatment did not change the titer of the viruses, so stocks were preserved at −20°C.

The plaque morphology and cell lysis patterns of the G5, and YCO11 isolates were similar to each other and to those of IPNV. Infected cells showed granulation and many spindle shapes (Figure 1).

2. Physical and chemical characterization

Chloroform treatment did not reduce the infectivity of the viruses (Table 1). These results indicate the viruses lack an essential lipid component or envelope. In addition, the viruses G5 and YCO11 were not inactivated by exposure to pH 3 (Table 2).

Table 1. Effect of chloroform on the infectivity of viruses from goldfish and chum salmon.

Virus	Virus Titer($TCID_{50}$)	
	Chloroform	BSS
G5	$10^{5.5}$	$10^{6.5}$
YCO 11	$10^{5.3}$	$10^{6.5}$
IPNV*	$10^{5.3}$	$10^{6.5}$
IHNV**	0	$10^{6.0}$

* IPNV is an unenveloped virus.
** IHNV is an enveloped virus.

Table 2. The effect of pH3 on the infectivity of viruses from goldfish and chum salmon.

Virus	Virus Titer ($TCID_{50}$)	
	pH3	pH7
G5	$10^{7.0}$	$10^{6.6}$
YCO 11	$10^{5.7}$	$10^{7.0}$

*: Sample viruses were incubated for 30min in MEM-0 at pH 3 and pH7.

3. Neutralization tests

Neutralization tests performed with antisera to three known groups of IPNV showed that the G5 and YCO-11 isolates were related to the VR-299 serogroup (Table 3).

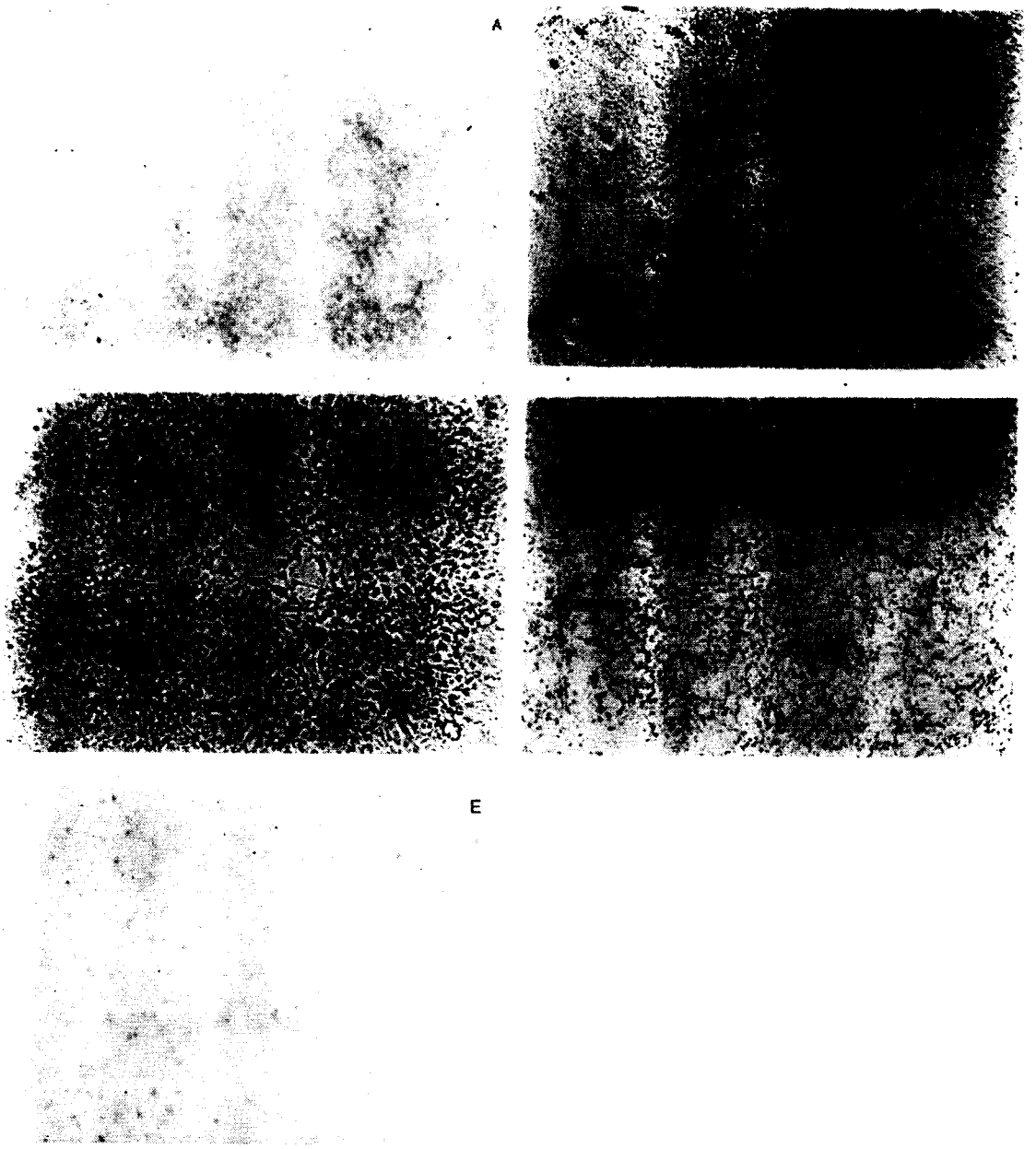

Fig. 1. A. Phase contrast micrograph of uninfected EPC cells (600×)
B. Plaque morphology of YCO 11 in EPC cells (150×)
C. Plaque morphology of YCO 11 in EPC cells (600×)
D. Cell lysis by YCO 11, EPC cells (150×)
E. Cell lysis by G5 in CHH-1. Giemsa stain (600×)

Table 3. Neutralization test of viruses isolated from goldfish (G5) and chum salmon (YCO-11).

Virus($TCID_{50}$)**	Antiserum*		
	Anti-VR-299	Anti-SP	Anti-AB
VR-299 (158)	61, 440***	1, 920	<320
SP (155)	640	20, 480	<320
AB (199)	2, 037	1, 280	17, 065
G5 (100)	30, 720	800	480
YCO-11 (160)	35, 840	960	320

* Antiserum in serial two-fold dilutions was incubated with each virus for 1 hr prior to addition of CHSE-214 cells.

** Amount of virus incubated with each dilution of antiserum.

*** Reciprocal of the dilution of antiserum at which 50% of the wells were protected from viral induce cytopathic effect.

4. Electron microscopy

Electron microscopy of negatively stained virions of the YCO11 and G5 siolates showed particles with hexagonally icosahedral symmetry and average diameters of 55 nm (Fig. 2). The particles were similar to those reported for IPNV.

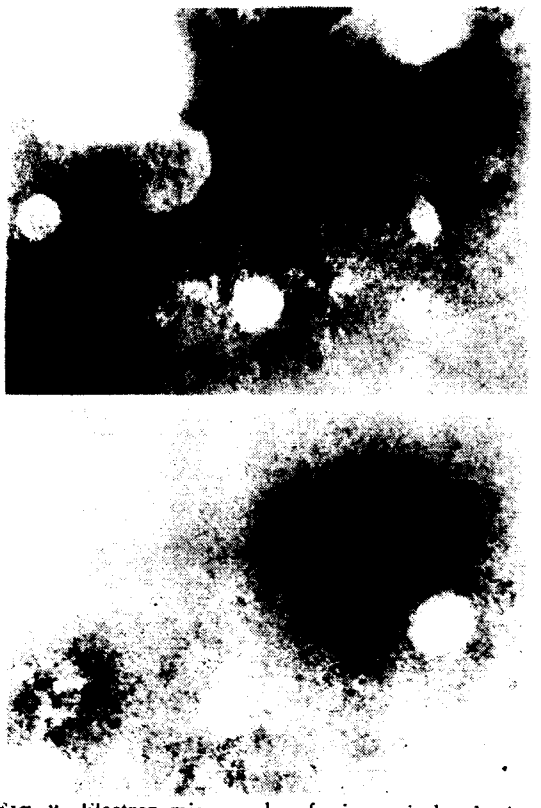

Fig. 2. Electron micrographs of viruses isolated. A. (240, 000×) from chum salmon (YCO11) and B. (320, 000×) from gold fish (G5).

DISCUSSION

We isolated viruses from the kidney and spleen tissues of goldfish and the ovarian fluid of chum salmon. The viruses replicate in EPC, CHSE-214, and CHH-1 cells at 15°C. They were resistant to pH 3 and chloroform. Electron microscopy revealed icosahedral particles with diameters of 55nm. These physical and chemical characteristics of the viruses are similar to those of IPNV (McAllister, 1983). Neutralization of G5 and YCO11 by antiserum confirmed the relationship of these two new isolates to IPNV serotype VR299.

Infectious pancreatic necrosis virus commonly infects salmonid fish in North America, Europe and Japan. Rrecently, the virus has been isolated from other species of fish (Adair and Ferguson, 1981; Hedrick et al., 1983). This is the first appearance of IPNV in Korean chum salmon. These results suggest that IPNV may be widely distributed in cultured fishes in Korea (both salmonid and nonsalmonid). We believe these two viruses to be the first isolations of IPNV from any fish on the east coast of Asia. Future research is needed to determine the origin of these isolates and their impact on the successful rearing of both anadromous and fresh water fish in Korea.

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적 요

금붕어(Carassius auratus)의 콩팥 및 지라조직과 연어(Oncorhynchus keta)의 난소액에서 각각 바이러스가 분리되었다. 두 바이러스는 모두 EPC, CHSE-214, CHH-1 영구 계대 세포 안에서 증식하여 세포변화 현상(cytopathic effect)을 보였다. 이들은 pH3 클로로포름에 대해 저항성을 지녔다. 이들 바이러스의 감염성은 감염성 췌장괴저 바이러스(IPNV) 원형형 VR-299의 항혈청에 의해 중화되었다. 또 그 전자 현미경적 구조는 평균 구경 55nm의 IPNV의 특성적 형태를 보였다. 이 논문에서 한국내 양식 어류에 감염하는 바이러스의 최초분리를 보고한다.

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