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Characterization of the Infectious Pancreatic Necrosis Virus (IPNV) isolated from Pan-Cultured Rainbow Trout in Korea

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한국산 양식송어에서 분리된 전염성 췌장괴저 바이러스의 특성

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ABSTRACT : Infectious Pancreatic Necrosis Virus (IPNV) is one of the most important pathogens for inland fish farming and had been first reported in Korea from returning adult chum salmon (*Oncorhynchus keta*) at hatcheries on the east coast.

During the past years, several viruses identified as IPNV were isolated not only from chum salmon, but also from gold fish (*Carassius auratus*), eel (*Anguilla japonica*), and rainbow trout (*Salmo gairdneri*). An isolate, coded DRT, from fingerlings of pan-cultured rainbow trout in Daechung Dam showed different serotype from three known reference serogroups of IPNV such as VR-299, Sp, and Ab. Antisera to three of these serotypes, however, partially neutralized the infectivity of this isolate. Anti-Sp type was rather effective than either anti-VR-299 or anti-Ab, implying DRT could be more closely related to Sp. DRT has been purified and its RNA genome segments were compared showing that the isolate does not belong to any of known serogroups even with some common antigenicity.

KEY WORDS □ IPNV, Serotype, Fish pathogen

Since 1982, we have investigated the distribution of pathogenic viral agents among several species of inland pan-cultured fish. Infectious Pancreatic Necrosis Virus (IPNV) was the first isolate, and several viruses identified as IPNV has been reported afterwards not only from chum salmon (*Oncorhynchus keta*) but also gold fish (*Carassius auratus*), eel (*Anguilla japonica*), and rainbow trout (*Salmo gairdneri*) (Hah *et al.*, 1984). Most of the viruses isolated were known to be antigenically related to the reference strain of VR-299 on the basis of cross neutralization test and comparison of polypeptide and RNA genome patterns on polyacrylamide gel electrophoresis(Hah *et al.*, 1984; Hedrick *et al.*, 1985). Only one IPNV isolate ever reported, referred to as DRT later, was of different serological characteristics. No Infectious Hematopoietic Necrosis Virus (IHNV) has yet been isolated in Korea.

IPNV was first isolated from trout (Wolf *et al.*, 1960) and belongs to the family of Birnaviridae (Dobos *et al.*, 1979 ; Brown, 1986) with the characteristics of medium sized, unenveloped, icosahedral animal viruses which have bisegmented double-stranded RNA genomes. By 1971, two other serologically different IPNV strains were isolated from rainbow trout (Jørgensen and Kehlet, 1971) establishing three reference serogroups of VR-299, Sp, and Ab (Macdonald and Gower, 1981). Several IPNV isolates, however, had been reported to be immunologically or biochemically different from aforementioned serogroups (Hill and Way, 1983 ; Macdonald *et al.*, 1983 ; Hatori *et al.*, 1984 ; Ishiguro *et al.*, 1984; Caswell-Reno *et al.*, 1986).

IPNV is an important pathogen causing an acute, contagious disease in juvenile rainbow trout and brook trout (Wolf and Quimby, 1971; Pilcher and Fryer, 1980). Early in 1987, wide spread epidemics occurred at all rainbow trout hatcheries in Korea causing the death of more than ten million fries. Viruses isolated from moribund fish of these hatcheries were identified as IPNV. The geological distribution of IPNV in Korea has been changing every

year gradually spreading throughout whole country. Prevention of IPNV is extremely urgent inasmuch as chum salmon is still under a supervised rehabilitation program by the Korean Government and rainbow trout is of greatly increasing commercial value.

MATERIALS AND METHODS

Cells

The fish cell line, RTG-2 (kindly provided by Dr. John L. Fryer, Oregon State University, Corvallis, OR) was grown at 18℃ as monolayers in Eagle's MEM with Earle's salts supplemented with 10% Fetal Bovine Serum (Flow Laboratories, North Ryde, N.S.W., Australia) and Penicillin-Streptomycin (50 IU/ml and 50μg/ml, respectively; Flow Laboratories).

Viruses

The IPNV isolates were from the kidney and spleen of moribund fish after gross mortality of pan-cultured rainbow trout fingerlings of Daechung Dam, according to the standard methods of the American Fisheries Society Fish Health Section, 1979. Briefly, supernatant of kidney and spleen homogenates from five-fish pool was preincubated at 4℃ for 16hr before infection to RTG-2 monolayer in the presence of antibiotic mixture of gentamicin and nystatin (400μg/ml each). An isolate, coded DRT, among many viruses recovered has been selected after cross neutralization test with reference antisera.

Three reference serogroups of IPNV, VR-299, Sp, and Ab, were originally from Dr. John L. Fryer and passaged in RTG-2 thereafter in our laboratory.

Purification of Virus

Viruses were purified according to the method described by Dobos *et al.* (1979) with slight modification. Infected cells were harvested in culture media with rubber scraper after severe CPE appears in the monolayer cultures. Cells were further disrupted by sonication (Lab-Line System, Lab-Line Instruments, Melrose Park, IL) with the maximum setting of 3×30 sec on ice. After removal of cellular debris, viral suspension was adjusted to the final concentration of 0.5M NaCl. Viral particles were recovered from precipitate with 9% (w/v) polyethyleneglycol (m.w. 8.000, Sigma, St. Louis, MO) followed by centrifugation at 12,000rpm for 30min (Sorvall RC5C, Welmington, DE). Pellet was resuspended in TNE buffer (pH7.5) and layered on top of the discontinuous CsCl gradient of 40%, 30%, and 20%. Virus band was collected with a syringe after ultracentrifugation at 35,000 rpm for 16hr using SW50.1 rotor (Beckman Instrument L7-55, Palo Alto, CA). Viral suspension was loaded on 15～50% sucrose density gradient centrifugation at 35,000rpm for 2hr in SW50.1 rotor. Virus was further purified through the second CsCl gradient when necessary. Total amount of virus recovered was determined by the formular of Smith *et al.* (1969), where optical density unit at 260nm equals to either 2.1×10^{12} particles or 200μg of virus.

Neutralization test with specific antisera

Neutralization with serotype specific antisera was carried out as described by Okamoto *et al.* (1983). In brief, antisera was serially diluted in two fold with EMEM (pH7.6) in 96 multiwell plate (Falcon 3072, Becton Dickinson, Oxnard, CA). Antisera were applied to each well at the titer of 200 to 102,400 ND_{50} (Neutralizing Dose 50) in 50μl. Fresh virus of 100 $TCID_{50}$ also in 50μl was added for the evaluation of neutralization. Plates were incubated at 20℃ for 1hr with continuous shaking. One tenth ml of RTG-2 cells at 1×10^5cells/ml was plated into each well and further incubated at 18℃ for 7 days. Neutralization titer of the antisera was expressed in the reciprocal of the highest dilution of antisera protecting 50% of the cells inoculated.

Time course study of neutralization was performed as described by Macdonald and Gower (1981) with slight modification. Antisera at 2×10^2 ND_{50} of final titer was added to 1×10^5 $TCID_{50}$ in the 200μl reaction mixture incubated at 24℃. Each aliquot of 25 μl was diluted 100 fold in ice-cold HBSS at 0.5, 1, and 2min after the addition of serogroup specific antisera. The survival rate estimated by $TCID_{50}$ analysis had been expressed as the fraction of number of infectious viral particles to the number in the beginning.

Electron Microscopy

Purified virus suspension in 2.5% Phosphotungstic acid(pH 6.0) was replicated on a carbon film grid for negative staining. After draining off the excess liquid, the replica was examined with a Hitachi H-500 electron microscope.

Extraction of double stranded RNA genome segments

Viral particles were digested at 65℃ for 2hr with proteinase K (Sigma, St. Louis, MO) at the final concentration of 1mg/ml prepared in the reaction buffer (10mM Tris pH7.6, 5mM EDTA pH8.0, and 1% SDS). Double stranded genomic RNA segments were purified by phenol : chloroform extraction, and precipitated with 2 parts of absolute ethanol containing 2.5M ammonium acetate. RNA preparation was further dried in a speedvac concentrator(Savant Instruments, Farmingdale, NY). Resuspended viral RNA in 10μl of TE buffer was stored at -20℃ until use.

RESULTS AND DISCUSSION

Morphology of the isolate DRT in Electron Microscopy

Viral particles recovered from the monolayer cultures of RTG-2 with severe CPE had been observed under an electron microscope with negative staining technique. CPE appeared clearly on three day cultures of RTG-2 at 18℃ (Figure 1). Electron

Fig. 1. *Cytopathic effect developed by the isolate DRT strain of IPNV.*
Normal RTG-2 cells (left) were infected with DRT and incubated for 3 days at 18℃. Typical IPNV CPE appeared (right).

Microscopy revealed that DRT was non-enveloped, and of cubical symmetry with approximate diameter of 55nm (Figure 2). Particles of IPNV lack inner layer structure which characteristically appears in closely related reovirus (Loh *et al.*, 1965).

Cross Neutralization with serogroup specific antisera

Neutralization test was performed to evaluate the serologically different characteristics of the isolate DRT. Using three different serogroup specific antisera developed in NZW rabbits, neutralization titer of DRT has been estimated. The titers were 660, 1,600, and 1,600 with anti-VR-299, anti-Sp, and anti-Ab, respectively. As shown in Table 1, such cross

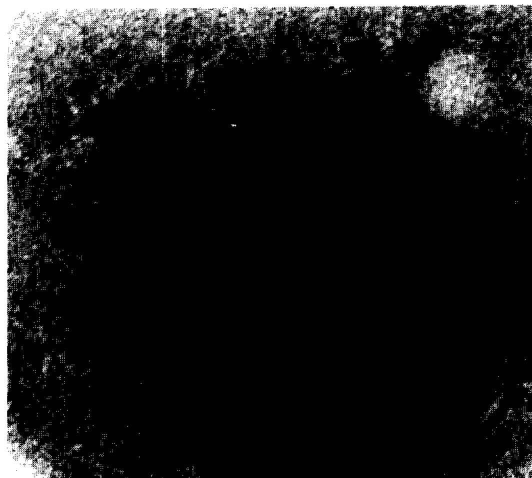

Fig. 2. *Electron Microscopy of DRT strain of IPNV.*
Purified viral particles were prepared in 2.5% phosphotungstic acid and negatively stained for examination. DRT was non-enveloped and of icosahedral structure with approximate diameter of 55nm.

neutralization titers strongly imply that the isolate and the such cross neutralization titers strongly imply that the isolate and the other reference strains are serologically distinct.

The neutralization titer anti-VR-299, especially, indicates the isolate DRT could be quite different from VR-299 than from either Sp or Ab. The initial neutralization rate in the presence of excess antibody is one of the most sensitive methods for the detection of antigenic differences between serologically different viruses (McBride, 1959 ; McMichael *et al.*, 1975 ; Macdonald and Gower, 1981). The time course study of neutralization of DRT with three diffenent serotype secific antisera showed DRT is rather closely related to Sp type (Figure 3). Since most of the IPNVs isolated in Korea had been identified to be antigenically related to VR-299 (Hah *et al.*, 1984), which is prevalent in North America (Wolf and Quimby, 1971), the distinct serotype of DRT seems to be unusual as the other serogroups, Sp and Ab,

Table 1. *Cross neutralization of infectivity for four different serogroups of IPNV with specific antisera, anti-VR-299, anti-Sp, and anti-Ab.*

Virus	$TCID_{50}$	anti-VR-299	anti-Sp	anti-Ab
VR-299	400	4,160	400	200
Sp	140	200	3,200	200
Ab	50	1,040	1,600	37,960
DRT	100	660	1,600	1,600

Viral particles were preincubated for 1hr with excess amount of serogroup specific antibodies before inoculation of RTG-2 cells. Serum titer were evaluated as the reciprocal of 50% antiserum endpoint dilution which could neutralize the amount of virus at $TCID_{50}$ estimated at the time of experiment.

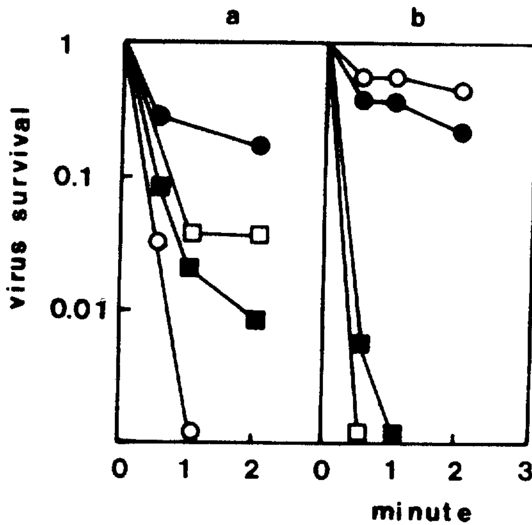

Fig. 3. *Time course study of Neutralization with two different serotype specific antisera, anti-VR-299 and anti-Sp.*
Viral suspension was estimated the infectivity at proper intervals after the addition of antisera, anti-VR-299 (a) and anti-Sp (b). Four different serogroup of IPNV showed different kinetics of neutralization;VR-299 (●), Sp (□), Ab (○), and DRT (■).

have been frequently found in Europe (Jørgensen and Kehlet, 1971).

As the geological distribution of IPNV of Korea has been changing (Hah *et al.*, 1984), such an unusual appearance of new serotype of DRT could be a sign of another severe epidemic of inland fish farms. IPNV is reported to be highly virulent to rainbow trout (Pilcher and Fryer, 1980), and IPNV was the major pathogen for most of the great loss of fingerlings of rainbow trout in Korea (Hah *et al.*, 1984; Hedrick *et al.*, 1985).

Analysis of double-stranded genomic RNA of DRT

IPNV has two segments of double stranded genomic RNA (Dobos, 1976; Macdonald and Yamamoto, 1977). The size of genomic RNAs of DRT has been analyzed using 5% polyacrylamide gel electrophoresis (Figure 4). When genomic RNAs from the other serogroup strains were compared, DRT had RNAs of very similar size with those of VR-299 and Sp. Migration of these RNAs on 1.5% agarose gel, however, showed two segments of DRT were smaller than each segment of RNA from the other strains (Figure 5).

Three different serogroup strains have characteristic RNAs and polypeptides (Hedrick et al., 1983; Macdonald et al., 1983). DRT seems to encode different polypeptides, while the absolute number of

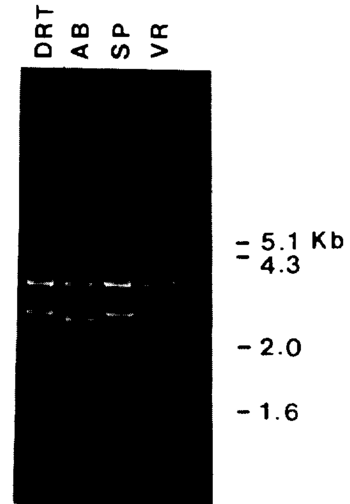

Fig. 4. *Double stranded genomic RNA analysis on polyacrylamide gel electrophoresis.*
Genomic RNAs from four strains of IPNV with different serotype were purified by phenol : chloroform extraction after proteinase K digestion at 65℃ for 2hr. On 5% polyacrylamide gel electrophoretogram, only genomic RNA segments from Ab were distinguished. The other segments were of very similar size.

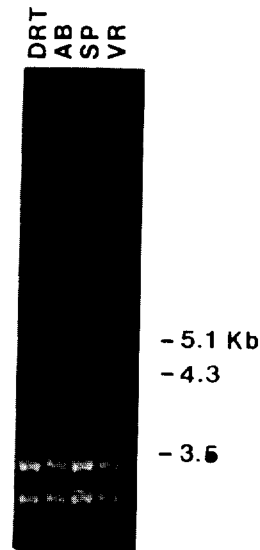

Fig. 5. *Double stranded genomic RNA segment analysis on agarose gel electrophoresis.*
RNAs were separated on 1.5% agarose gel electrophoresis in TBE buffer (pH8.0) at 100V. Each segment of four different strains of IPNV showed distinct migration pattern.

peptides is not clear as yet. The major capsid protein which might be of an important role for infection was referred to as VP2. VR-299 and Sp have a protein VP4 which is a cleavage product of VP3 (Hedrick *et al.*, 1983 ; Huang *et al.*, 1986). As the size of VP2 from DRT capsid is slightly different from the other VP2 (data not shown), DRT could be using modified molecules for infection and thus have a distinct pattern of multiplication inside host cells. Analysis of genome is absolutely required at present for the exact understanding of the nature of different serotypes and their function of viral proteins in different serogroups of IPNV.

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적 요

1983년 우리나라에서 처음으로 전염성췌장괴저 바이러스가 분리되기 시작한 이래 여러가지 종류의 내수면 양식 물고기로부터 이 바이러스가 검출되어 왔다. 세계적으로 인정되고 있는 세가지 표준혈청형 즉 VR-299, Sp, 그리고 Ab형에 대한 중화항체 실험으로, 우리나라에서 발견되는 전염성췌장괴저 바이러스는 주로 VR-299형임이 확인되어 왔으나, 대청댐에서 양식된 송어로부터 발견된 한 주의 바이러스는 독특한 혈청형을 보이고 있어 그 특성을 조사하였다.

이 바이러스로부터 단백질과 핵산을 분리하여 세가지 표준 혈청형 바이러스의 그것과 비교하여 본 결과, 크기에 있어 분명한 차이가 보였고, 중화항체를 이용한 감염저지 실험으로 Sp형과 비교적 가까운 연관관계를 가지는 것으로 밝혀졌으나, 세가지 표준 혈청과는 뚜렷이 구분되는 새로운 혈청형으로 판정되었다.

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