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Isolation and Identification of Infectious Pancreatic Necrosis Virus from Rainbow Trout Cultured in Korea

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A survey was conducted to determine the prevalence of infectious pancreatic necrosis virus (IPNV) on fish farms in Korea and the epidemiology of IPNV infection in the farmed rainbow trout. In total, 43 pools of rainbow trout with apparent signs of viral infection from five provinces were obtained and analyzed. Evident cytopathic effects, including karyopycnosis and cell destruction, were observed in CHSE (chinook salmon embryo)-214 cells infected with the virus isolates. Of these, ten viral isolates were assumed to be IPNV based on biophysical properties. RNA analysis revealed that the isolates contained two-segmented RNA genomes, further indicating that the viral isolates are IPNV. Antigenic comparison of the IPNV isolates identified three distinct serological groups separable by the cross-neutralization test. Of the ten IPNV isolates, six could be classified as strain DRT, two as strain Ab, and two as strain VR299. We were not able to isolate new strain of IPNV or any isolate serologically similar to the standard strain Sp.

Key words: Infectious pancreatic necrosis virus (IPNV), Ab, DRT, VR299, rainbow trout, cross-neutralization

IPNV (infectious pancreatic necrosis virus) is an economically important fish pathogen. It is the causal agent of a contagious high-mortality disease of young, hatchery-reared salmonids (19). Survivors of epizootics may become life-long carriers and continue to shed small quantities of the virus in their feces and reproductive fluids, perpetuating the disease by either vertical or horizontal transmission. IPNV belongs to Birnaviridae and is characterized by having a single-layer protein capsid and a two-segmented, double stranded RNA (ds RNA) genome, with a protein covalently bound to the 5'-terminus (1, 2, 3, 4).

IPNV not only has serious economic consequences in trout and salmon farms world-wide, but it can also kill a number of non-salmonid fish species including striped bass, turbot, menhaden and eels. And it has been isolated from a variety of marine fish and molluscs (8, 13, 15). The classification of these viruses into serotypes is an ongoing endeavor in a number of laboratories. The original IPNV isolate is now called VR299, and two other different serotypes have been isolated which are called Ab, and Sp (9, 15). These serotypes were found to have distinctive phenotypic properties, protein and RNA profiles, and neutralization properties (13).

The first trout farm in Korea was established in 1965, but it was not until the 1980s when commercial development of salmonid farming occurred. There are currently about 200 private farms that raise salmonid fishes, particularly rainbow trout *Oncorhynchus mykiss*, scattered over several areas of the country. IPNV was presumably introduced into Korea earlier, but it only began to be isolated and identified in the laboratory in Korea recently (6, 7). First IPNV isolate reported, referred to as DRT later, was of different serological characteristics from the three major serotypes (11, 16).

The aim of this study was to investigate the epidemiology of IPNV on an endemically infected trout farms in Korea. In this report, we describe the biochemical, biophysical, and serological characteristics of the isolated virus. The results of this survey would be useful in establishing the first epidemiological map of viral salmonid diseases in Korea.

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Materials and Methods

Virus and cell culture

IPNV standard strains Ab, Sp, DRT and VR299 and the viral isolates were propagated in CHSE (chinook salmon embryo)-214 cells. CHSE-214 cells were cultured in EMEM (Eagle's minimum essential medium, Earle's salt) supplemented with 10% newborn bovine serum (NBS) and 0.075% $NaHCO_3$ at 18℃.

Virus isolation

The fish samples showing apparent clinical signs were collected during the period of January, 1994 to April, 1995 from the rainbow trout culture ponds in Korea. Pools of 1~5 frozen fishes were mixed with 5 ml of Earle's-buffer containing antibiotics, homogenized with glass tissue grinder, and stored in sealed vials at -70℃. After centrifugation of the homogenates at 2,000×g for 15 min, the supernatants were filter-sterilized (0.2 μm) and inoculated onto monolayers of CHSE-214 cells. Control (non-infected cells) and infected cultures were examined daily for 3 weeks to detect development of cytopathic effects (CPEs).

Plaque assay

The infectious titer of IPNV strains was determined by plaque assay. Confluent monolayer of CHSE-214 cells grown in 35 mm tissue culture dishes was inoculated with diluted virus stock or sample. After 1 hr adsorption at 18℃, cell monolayer was overlayed with EMEM containing 2% NBS, 0.25% agarose, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 μg/ml Fungizone. Infected cultures were incubated for 4~5 days and fixed with 10% neutral buffered formalin for 30 min. The overlay medium was removed and the cells were stained with 0.03% methylene blue to reveal the plaques.

Chloroform sensitivity

One ml of chloroform was mixed with 2 ml of virus suspension at room temperature. The mixture was shaken for 10 min and centrifuged at 600×g for 5 min to separate the chloroform layer from the aqueous phase. Virus infectivities of the aqueous phase from the treated and control (without chloroform) samples were measured by plaque assay (17).

Virus purification

Virus was subjected to two rounds of plaque-purification and propagated on CHSE-214 cells grown in 100 mm dishes or 80 cm^2 flasks. When CPEs were extensive, the cells were scraped into the medium and subjected to centrifugation at 5,000×g for 30 min at 4℃. The supernant was saved and mixed with 9% polyethylene glycol (PEG) 6,000 in 0.5 M NaCl. The mixture was stirred overnight at 4℃, centrifuged at 1,800×g, and the pellet was resuspended in 2~3 ml TNE (0.1% Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.5). The resuspended PEG-virus pellet was ultrasonically treated for 10 to 15 sec. The resulting supernatant was then layered onto a 15~45% (W/V) linear sucrose density gradient in TNE and centrifuged at 45,000×g for 2 hr. The opaque virus-containing band was removed, diluted at least 1 : 10 with TNE, centrifuged at 90,000×g for 1.5 hr and the virus pellet was resuspended in 1 ml TNE. For some experiments the virus was further diluted with TNE.

Extraction and analysis of genomic RNA from virus isolates

One volume of fresh digestion solution (0.2 M Tris-HCl, pH 8, 10 mM EDTA, 1% SDS, 200 μg/ml proteinase K) was added to the suspension of purified virus. The mixture was incubated for 2 hrs at 50℃, and the RNA was extracted with one volume of phenol-chloroform (1 : 1) solution saturated in water. After a 10 min centrifugation at 4,000×g, the aqueous phase was extracted with a chloroform-isoamyl alcohol (24 : 1) solution. The aqueous phase was precipitated at -20℃ with absolute ethyl alcohol. The nucleic acids were pelleted by centrifugation at 15,000×g for 30 min at 4℃. The pellet was finally resuspended in TE solution (1 mM EDTA, 10 mM NaCl, 10 mM Tris-HCl, pH 8). Extracted RNA was electrophoresed in 5% polyacrylamide gels (PAGE) at 10 mA for 19 hrs.

Preparation of antisera

Serological identification of virus isolates was conducted with antisera developed in female New Zealand white rabbits injected with purified virus of IPNV reference strains (18). Prior to injection, the sera from rabbits were analysed for neutralizing antibodies to the strains of IPNV and IHNV (infectious hematopoietic necrosis virus) used in this study. None of the normal serum contained detectable antibody activity to any of the virus strains tested. Rabbits were injected by intravenously administrating 1.0 ml of the selected virus preparation (1×10^9 PFU/ml) into the marginal ear vein at weekly intervals for 3 weeks. The rabbits were bled two weeks after the last injection. The serum was collected, sterilized by filtration (0.45 m) and then stored at -20℃. All sera were inactivated at 56℃ for 30 min before use.

Cross-neutralization test

Serial two-fold dilutions of the rabbit antisera were made in EMEM, and the neutralizing antibody titers were determined by a cross-neutralization plaque assay as described previously (10). For each assay at 18℃, the reciprocal of the highest dilution of antisera that gave an 80%

Table 1. Sampling history of rainbow trouts used in this study

Pool No.	Location	Source of eggs	Sampling date	No. of fish	Temperature of water(℃)
1	Kangwon/Mi-tan	Self-hatching	95/2	5	12
2	Chungbuk/Kum-ga	Kangwon	95/2	5	14
3	Chungbuk/Kum-ga	Japan	95/2	2	14
4	Chungbuk/Kum-ga	Cheju	95/2	5	13
5	Chungbuk/Kum-ga	japan	95/2	5	13
6	Chungbuk/Choong-ju	Self-hatching	95/2	5	13
7	Chungbuk/Chochiwon	Kyongbuk	95/2	5	12
8	Kangwon/Pyong-chang	Self-hatching	95/3	5	14
9	Kangwon/Pyong-chang	Kangwon	95/3	5	14
10	Kangwon/Jeong-seon	Self-hatching	95/3	5	12
11	Chungbuk/Kum-ga	Kangwon	95/2	5	13
12	Kangwon/Young-woul	Japan	95/3	1	14
13	Chungbuk/Kum-ga	Kangwon	95/2	5	13
14	Kangwon/Mi-tan	Self-hatching	95/2	2	12
15	Kangwon/Mi-tan	Self-hatching	95/2	5	12
16	Kangwon/Chun-chon	Kangwon	95/3	5	7
17	Kangwon/Jeong-seon	Self-hatching	95/3	5	12
18	Chungbuk/Choong-ju	Kangwon	95/2	5	13
19	Chungbuk/Choong-ju	Kangwon	95/3	5	14
20	Chungbuk/Choong-ju	Kangwon	95/2	5	12
21	Kangwon/Pyong-chang	Japan	95/3	5	14
22	Chungbuk/Je-chon	Self-hatching	95/3	2	13
23	Chungbuk/Je-chon	Kangwon	95/3	2	12
24	Kangwon/Chun-chon	Kangwon	95/3	5	10
25	Chungbuk/Gui-san	Kangwon	95/3	1	14
26	Chungbuk/Dan-yang	Kangwon	95/4	5	14
27	Chungbuk/Dan-yang	Kangwon	95/4	5	14
28	Chungbuk/Dan-yang	Kangwon	95/4	5	14
29	Chungbuk/Dan-yang	Kangwon	95/4	5	14
30	Kangwon/Pyong-chang	Kangwon	95/4	1	13
31	Kangwon/Pyong-chang	Self-hatching	95/4	5	14
32	Kangwon/Pyong-chang	Kangwon	95/4	5	12
33	Kangwon/Pyong-chang	Kangwon	95/4	3	12
34	Kangwon/Pyong-chang	Kangwon	95/4	5	12
35	Kyongbuk/Mun-kyong	Self-hatching	95/4	5	14
36	Kangwon/Young-woul	Kangwon	95/3	5	14
37	Kangwon/Young-woul	Kangwon	95/3	5	14
38	Kangwon/Young-woul	Kangwon	95/3	5	14
39	Kyounggi/Yang-pyong	Self-hatching	95/4	5	12
40	Chungnam/Sintanjin	Kangwon	95/1	5	11
41	Kyongbuk/Young-yang	Self-hetching	95/4	5	12
42	Kangwon/Pyong-chang	Kangwon	95/4	5	12
43	Chungbuk/Kum-ga	Japan	95/2	5	14

reduction in the number of virus plaques per 0.1 ml of inoculum was recorded as the antibody end-point titer (5).

Results and discussion

Identification of virus infection

Husbandry and disease history on a rainbow trout farm where IPNV had occurred in successive batches of fingerlings were documented. Samples were collected from diseased fish and from fish known to be free of IPNV during 1994 and 1995. Water temperature data were also recorded for each farm (Table 1). Most of the 43 pooled samples were obtained from fish groups at rainbow trout farms with epizootics causing high mor-

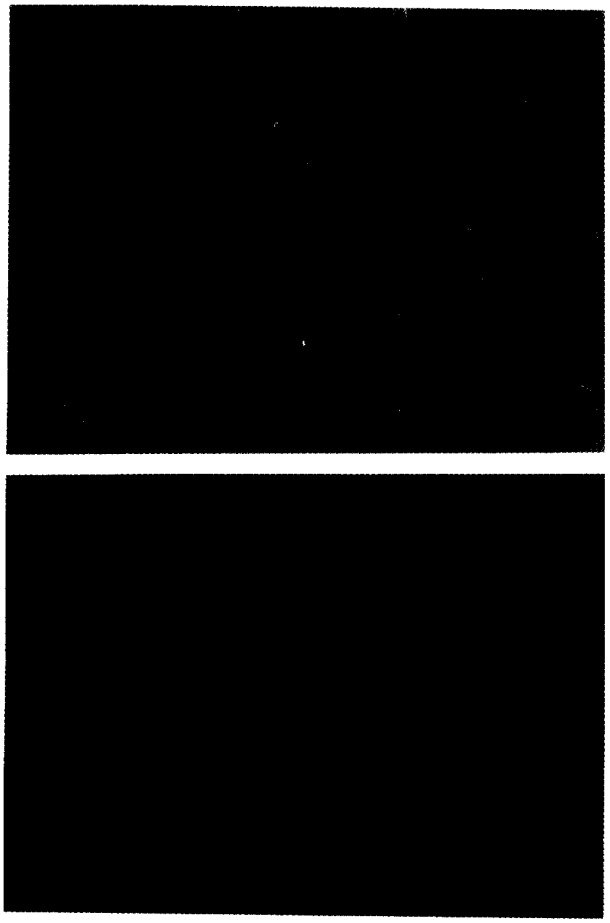

Fig. 1. Cytopathic effects produced in chinook salmon embryo cell ine (CHSE-214) by the isoated virus strain 1 at 72 hrs postinoculation. A: Uninfected cell monolayer, B: Rounding-up and detachment of monolayer after infection with IPNV isolates, Magnification, ×100.

tality in fingerlings. Each pool was made from 5 fingerlings with body weights under 2 g, 2~3 fingerlings with body weights heavier than 2 g, or 1 adult fish. Altogether 189 fishes were collected for this study. Virus samples were prepared by mixing frozen pool samples with 5 ml buffer and grinding the mixture.

Although samples were obtained from fish showing signs of viral infections, it was not certain whether the fish were actually infected and were harboring viruses. To confirm virus infection following experiments were performed. First, small amount of samples were inoculated onto confluent monolayer of CHSE-214 cells and the development of IPNV-specific CPEs was monitored. Twenty two out of 43 samples produced prominent CPEs. The CPE initiated from losing the fusiform shape and the rounding up of CHSE-214 cells, followed by the granulation of cytoplasm, karyopycnosis and finally cell lysis (Fig. 1). Develpoment of clear CPEs was iden-

Table 2. Infectivity of the virus isolates in CHSE-214 cell line

Pool No.	Virus titer (PFU/ml)
1	1.1×2^{5}
2	1.0×2^{2}
7	7.0×2^{8}
8	≤2
9	≤2
10	≤2
15	3.6×2^{4}
17	≤2
21	2.4×2^{5}
23	≤2
26	≤2
28	1.6×2^{6}
29	1.2×2^{6}
30	≤2
31	≤2
32	≤2
36	≤2
37	≤2
38	≤2
40	≤2
43	≤2

Virus isolates showing prominent CPEs on CHSE cells were serially 2-fold diluted and inoculated onto the confluent monolayer of CHSE-214 cells. Overlay medium was added and the number of plaques was counted at 3 days after inoculation.

tifiable at different times after sample inoculation, and the time required for the development of CPE appeared to be dependent on the amount of infectious virus present in the sample. As shown in Table 2, 22 samples positive for CPE development were harboring different amount of infectious virus. Sample No. 7 contained as many as 7×2^{8} PFU/ml of infectious virus and the CPE developed 3 days after inoculation. On the other hand, 15 samples contained infectious virus less than the detection limit of our method for plaque titration. Although containing few infectious virus, all 15 samples produced CPE on CHSE-214 cells when inoculated cells were incubated longer (up to 10 days).

Most of IPNV seemed to be isolated from samples of farms rearing fish in water temperature between 12~14°C. This matches with water temperature appropriate to IPNV life cycle and there was no outbreak of disease when the water temperature was below 12°C. There is a close relationship between water temperature and the occurrence of specific viral disease of fish. If water temperature is too low there might be low infection probability of IPNV but it gives negative growth effect on rainbow trout. And if the water temperature is raised it would provide a better condition for several aquatic

Table 3. The effect of chloroform treatment on the infectivity of the virus isolates

Pool No.	Virus titer (PFU/ml)	
	Non-treated group	Chloroform treated
1	7.4×10^{5}	1.2×10^{5}
2	2.8×10^{3}	4.4×10^{2}
7	1.9×10^{4}	1.5×10^{4}
8	1.9×10^{9}	-*
9	1.9×10^{6}	-
10	8.9×10^{6}	-
15	1.6×10^{2}	1.2×10^{2}
17	5.7×10^{5}	-
21	4.6×10^{9}	4.9×10^{3}
23	5.5×10^{9}	-
26	6.2×10^{2}	1.6×10^{2}
28	4.2×10^{5}	3.5×10^{5}
29	4.6×10^{5}	2.1×10^{4}
30	3.1×10^{3}	7.1×10^{2}
31	2.6×10^{7}	-
32	2.5×10^{6}	1.3×10^{6}
35	6.5×10^{6}	-
36	6.9×10^{6}	-
37	5.1×10^{6}	-
38	3.8×10^{6}	-
40	1.4×10^{5}	-
43	2.0×10^{7}	-

* : plaque formation was not detected

diseases such as IHNV infection. In fact, based on our observation half of the IPNV infection was detected at the water temperature of 14°C, and at the same water temperature two thirds of IHNV infection was also detected (see below).

Identification and serotyping of IPNV isolates

Although we aimed to isolate and identify IPNV from infected fishes, IHNV could be isolated with equal possibility since the pathological appearance on infected fishes and the CPE were not clearly distinguishable between IPNV and IHNV. Thus, further experiments were needed to identify IPNV from the 22 virus-positive samples. For this purpose virus samples were propagated to a large quantity for physicochemical analysis.

One of the major difference between IPNV and IHNV is that IHNV has an envelope while IPNV does not (3, 14). Since IHNV envelope contains lipid, chloroform treatment would significantly reduce the infectivity of IHNV, but the infectivity of IPNV would not be much affected (3). As shown in Table 3, 10 out of 22 virus-positive samples were resistant to chloroform treatment and could be identified as IPNV (samples 1, 2, 7, 15, 21,

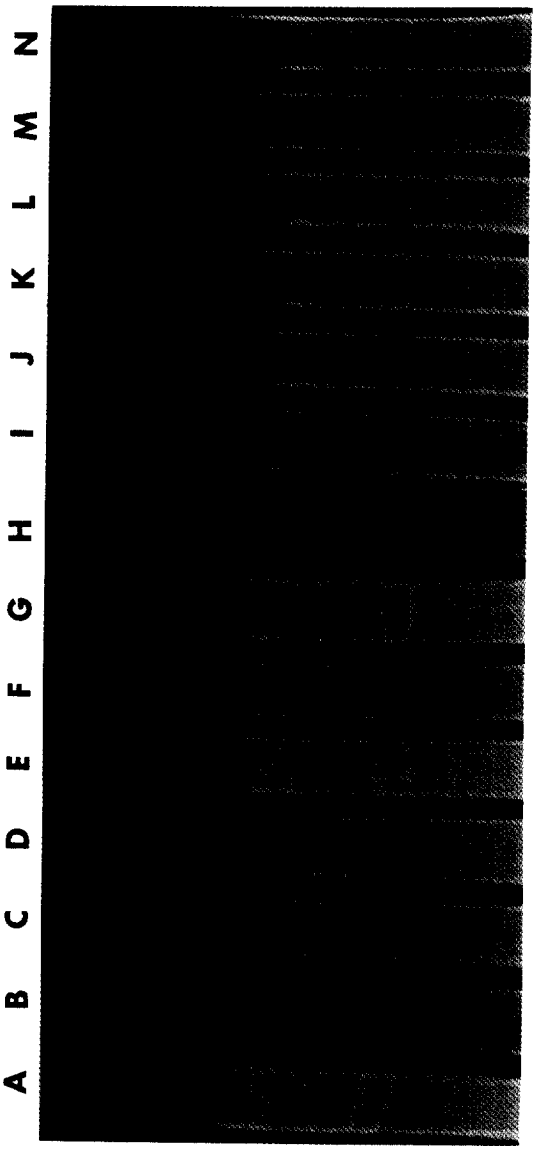

Fig. 2. Polyacrylamide gel electrophoretogram of genomic RNA from IPNV isolates. Electrophoresis was performed at 10 mA for 19 hrs. Lane A, IPNV DRT; Lane B, IPNV Sp; Lane C, IPNV VR-299 Lane D, IPNV Ab; Lane E, isolate 1; Lane F, isolate 2; Lane G, isolate 7; Lane H, isolate 15; Lane I, isolate 21; Lane J, isolate 26; Lane K, isolate 28; Lane L, isolate 29; Lane M, isolate 30; Lane N, isolate 32.

26, 28, 29, 30, and 32). There was no significant difference in virus titer of these isolates before and after chloroform treatment. Virus isolate 21 was an exception in that there was a significant drop in virus titer after chloroform treatment, still retaining some viral infectivity. It was assumed that the sample 21 was mixed-infected with IPNV and IHNV. This assumption was confirmed by seropositivity of the isolate 21 to both anti-IPNV and anti-IHNV antibodies (data not shown).

Further verification that the 10 viral isolates are IPNV

Table 4. Cross-neutralization tests of ten IPNV isolates with antibodies to four reference IPNV strains, Ab, Sp, DRT, and VR-299

Antibody to to	Virus isolates													
	Ab	Sp	DRT	VR299	1	2	7	15	21	26	28	29	30	32
IPNV Ab	**5120**	5120	160	1280	**1280**	640	**2560**	40	1280	1280	320	320	160	160
IPNV Sp	2560	**10240**	80	5120	320	160	160	80	1280	640	80	160	320	160
IPNV DRT	80	320	**640**	2560	640	**1280**	640	**1280**	640	**5120**	**640**	**640**	160	**640**
IPNV VR299	320	2560	160	**10240**	640	**1280**	640	80	**5120**	2560	80	160	**1280**	320

was achieved by identifying two RNA segments characteristic of IPNV from the viral isolates. Viral genomic RNAs were obtained from purified particles of the 10 viral isolates and subjected to 5% polyacrylamide gel electrophoresis (PAGE). As shown in Fig. 2, two segments of RNAs were visualized for each viral isolate. The mobility of RNA segments from the viral isolates was comparable to those from reference strains of IPNV, Ab, VR 299 and DRT. For example, RNA gel patterns shown in the lanes I and M of Fig. 2 were similar to that of the lane C. Thus, based on the size of genomic RNA, isolates 21 and 30 could be classified as VR299. Similarly, RNA patterns of the isolates 1 and 7 were similar to that of the strain Ab, and the other 6 isolates were similar to the strain DRT. However, none of the RNA was similar to the RNA of the strain Sp. It is eviden from the RNA gel patterns that there is a significant divergence in the size of the RNAs among different IPNV isolates. These differences can be exploited to aid the classification of IPNV isolates and can be used as a supplement to serological typing.

The neutralizing titer of the antisera against homologous and heterologous viruses was determined in order to examine the serological similarity among the reference and isolated strains of IPNV. The results shown in Table 4 indicate that the virus isolates 1 and 7 were serologically similar to IPNV Ab strain, isolates 21 and 30 were similar to IPNV VR299 strain and six others were similar to IPNV DRT strain. But none of the 10 isolates examined in this study showed significant similarity with Sp serologically. Thus, serologic study further support the data obtained from genomic RNA analysis shown in Fig. 2.

The bisegmented dsRNA genome is characteristic of IPNV (1, 4) and the two RNA segements are different in size among the different strains of IPNV (8, 12). In general the migration rate of the large RNA segments of IPNV in PAGE is as follows: VR-299>Sp>Ab. The migration rate for the small RNA segments is the fastest for Sp followed by VR-299 and Ab. The sizes of the two RNA segments of the strain DRT are slightly greater than those of VR-299 (16). Analysis of the genomic RNAs of the 10 IPNV isolates on PAGE revealed that 6 of them are similar to IPNV DRT, 2 are similar to VR-299, and the other 2 are similar to Ab. These results are confirmed by serologic analysis using antibodies to the 4 reference strains of IPNV. In the presence of excessive antibodies, initial rate of neutralization was used as a sensitive indicator to tell the antigenic differences among distinct groups of viruses. Our data obtained by using antisera against four standard strains of IPNV are interesting since 60% of the IPNV isolates were identified as DRT strain. Similar study by Park *et al.* (1989) reported that most of IPNV isolates were VR-299. This discrepancy might be due to the similarities among the capsid proteins of DRT and those of VR299 (16). Our data on cross-neutralization also revealed that our antisera against IPNV DRT was highly reactive to VR299 while the antisera to VR299 was not as much reactive to DRT (Table 4). Thus, conclusive result could be obtained with more specific monoclonal antibodies to DRT or VR299 which are not reactive to other strains of IPNV. Another possibility is that DRT had replaced VR299 during the past several years. If VR299 is the original strain and DRT is a new strain, herd immunity to VR299 forced the disappearance of VR299 while it was not effective to DRT. Therefore, each IPN outbreak might have selected DRT.

Previous studies have shown that IPNV is the dominant viral fish pathogen in Korea (6, 7, 16) but this study suggests that IHNV is at least as prevalent as IPNV and is distributed all over the country. Furthermore, as evidenced by chloroform treatment (Table 3), mixed infection of IHNV and IPNV was detected. Mixed infection of two diseases with similar clinical signs sometimes results in much more severe clinical consequences compared to single infection. From the beginning of 1990's clinical signs and mortality of IHNV infection has become more severe than IPNV and thus has become serious economical damage to aquaculturists in Japan. Therefore more studies on IHNV are needed because IHNV infection also tends to increase in Korea as suggested in this study.

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