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Detection of Viruses in Farmed Rainbow Trout (*Oncorhynchus mykiss*) in Korea by RT-LAMP Assay

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The viral diseases have been the serious problem in salmonid farming, and rainbow trout is not an exception. In this study, routine surveys were conducted for detecting of viruses in farmed rainbow trout (*Oncorhynchus mykiss*) in Korea during 2009-2010. Head kidneys from individual fish were employed for virus detection by using a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay. Infectious pancreatic necrosis virus (IPNV), infectious hematopoietic necrosis virus (IHNV), and viral hemorrhagic septicemia virus (VHSV) were the target viruses in this study. 53.5% (46/86) were found to be IPNV-positive, while IHNV and VHSV showed RT-LAMP negative during examination for 2 years. Ten IPNV-positive samples were randomly selected for viral isolation and the cells showing CPEs were subjected to RT-LAMP, RT-PCR, and direct sequencing. Phylogenetic analysis showed that the rainbow trout isolate has high similarity homologies with the VR-299 strain, as previously described.

Keywords: IPNV, IHNV, VHSV, rainbow trout, RT-LAMP

Rainbow trout (*Oncorhynchus mykiss*) is an economically important aquaculture species in Korea. The annual production of rainbow trout has been continuously increased each year (Yoon, 2008). Rainbow trout is known to be the most susceptible species to infectious pancreatic necrosis virus (IPNV), infectious hematopoietic necrosis virus (IHNV), and viral hemorrhagic septicemia virus (VHSV) (OIE, 2007). These three viruses are responsible for considerable economic losses in the rainbow trout aquaculture.

In Korea, the first isolation of IPNV was reported from chum salmon (*O. keta*) (Hah *et al*., 1984), followed by several reports from rainbow trout (Lyoo *et al*., 1991; Lee *et al*., 2007). Serotyping and genetic analysis suggested that Korean IPNV strains were VR-299, Ab, and DRT strains (Hedrick *et al*., 1985; Lee *et al*., 1996; Joh *et al*., 2008).

There had been no report of IHNV in Korea prior to 1991, when the virus was detected in juvenile rainbow trout and masu salmon (*Oncorhynchus masou*) in Kangwon Province (Park *et al*., 1993). Subsequently, a series of IHNV outbreaks were recorded in rainbow trout farms across Korea. Mortality in both juvenile and market-sized fish have been recorded in rainbow trout farms in Korea (Kim *et al*., 2003). Phylogenetic analysis of all Korean IHNV isolates from rainbow trout were closely related to Japanese isolates of genogroup JRt, rather than to those of North American and European genogroups. It is believed that Korean IHNV has been most likely introduced from Japan to Korea by the movement of contaminated fish eggs (Kim *et al*., 2007).

VHSV is known to be a highly infectious virus predominantly affecting rainbow trout aquaculture, while although VHSV infection of non-salmonid species and wild populations have been reported, but natural epizootics are rare (OIE, 2007). Unlike IPNV and IHNV, VHSV had never been isolated from Asian countries until 2000 (Takano *et al*., 2001). The first isolations of VHSV in Asia were from wild olive flounder, *Paralichthys olivaceus*, in Japan (Takano *et al*., 2001), and from farmed olive flounder in Korea (Kim *et al*., 2003). Since then, it was subsequently reported in several wild marine fish species (Lee *et al*., 2007). Freshwater VHSV isolates have been known to be pathogenic in rainbow trout during infection trials with mortalities of 50-100%, while marine VHSV isolates have been shown to be non- to medium pathogenic to rainbow trout (Skall *et al*., 2004).

Loop-mediated isothermal amplification (LAMP) is a novel method of DNA amplification, which has already been applied to detection of several pathogens in aquaculture (Savan *et al*., 2005). LAMP was originally developed by Notomi *et al.* (2000), and can amplify very low numbers of target sequences to millions of copies under isothermal conditions within one hour. This method depends on auto-cycling strand displacement DNA synthesis by the *Bst* DNA polymerase large fragment with high strand displacement activity, and a set of two specially designed inner primers and two outer primers. The LAMP is highly specific because the target sequences are detected by six independent primers in the initial stage, followed by four independent primers in the later stages of the LAMP reaction. LAMP is also applicable for RNA detection by using a reverse transcriptase (RT) together with a DNA polymerase (Notomi *et al*., 2000). This technique can be carried out under isothermal conditions which can be simply achieved with a water bath or a heating block. Expensive thermal cyclers used for PCR are thus not required (Notomi *et al*., 2000).

In this study, we investigated the salmonid viruses in rainbow

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trout in Korea by using a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay, and compared the results with the nested RT-PCR assays. The positive samples were randomly selected for virus isolation and direct sequence in order to analyse the genetic relationships with other isolates from different geographical regions.

Material and Methods

Sampling

Sampling was conducted from fall 2009 to spring 2010. In total 86 juvenile rainbow trouts (body weight 0.4-0.7 kg) were collected from 4 different hatcheries in Gangwon Province. Head kidney was removed from individual fish and placed into a 1.5 ml microcentrifuge tube. Samples were transported on ice to the laboratory and processed immediately. Twenty-four individual juvenile samples were received during the fall of 2009 and 62 individual juvenile samples were processed during spring of 2010.

Viruses

VHSV and IPNV were maintained in rainbow trout gonad (RTG-2) cells and IHNV was maintained in chinook salmon embryo (CHSE-214) cells with MEM-10 (minimum essential medium supplemented with 10% fetal bovine serum (FBS), 200 mM L-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin) at 15°C. The viruses were used for positive control.

RNA extraction

100 mg of fresh kidney were homogenized in 750 μl of TRI Reagent® (Molecular Research Center), and incubated at room temperature for 5 min. Then, 200 μl of chloroform was added to the homogenate and mixed by vortex. The suspension was incubated at room temperature for 10 min and centrifuged at 12,000×g for 10 min. The upper solution was transferred into a new 1.5 ml microcentrifuge tube and the RNA precipitated by adding 200 μl of 100% isopropanol on ice for 10 min before centrifugation at 12,000×g for 10 min. The RNA pellet was washed with 70% (v/v) ethanol and centrifuged at 9,000×g for 5 min. The RNA pellet was dried at 95°C in the incubator for 2 min or until the ethanol had evaporated. To elute RNA, DEPC water (Bioneer) was added at the final concentration of 100 ng/μl and kept at -20°C until use.

Genomic viral RNA was extracted from 500 μl of viruses-infected cells culture supernatant using TRI Reagent® (Molecular Research Center) as described above and used for positive control. After elution of the RNA in DEPC-treated water, it was stored at -80°C until required.

RT-LAMP assays

RNA extracted from head kidney samples were subjected to RT-LAMP assays with three viruses-specific primer sets (Table 1). The RT-LAMP was carried out in a 25 μl of total reaction mixture containing: 2 μM each FIP and BIP, 0.2 μM each F3 and B3, 2 μM each LF, and LB primers, 1× thermopol-supplied reaction buffer, 0.6 M betaine (Sigma-Aldrich, USA), 6 mM magnesium sulfate (Sigma-Aldrich), 1 mM deoxynucleotide triphosphates (dNTPs) mix (Solgent, Korea), 8 U of *Bst* DNA polymerase (New England Biolabs), and 0.25 U of AMV (Promega) reverse transcriptase. Uninfected-cells and virus infected-cells were included as negative control and positive control, respectively. Reaction temperature and time were held at 65°C for 30 min, respectively, for detecting IPNV (Suebsing *et al*., 2011b) and VHSV, and at 63°C for 30 min for detecting IHNV (Suebsing *et al*., 2011a). The RT-LAMP products were seen as multiple bands of different size on 2% agarose gel electrophoresis.

RT-PCR assays

cDNA synthesis was conducted with 1 μg of total RNA using *M-MLV* reverse transcriptase kit (Bioneer, Korea) following the manufacturer's instructions, and subjected to PCR amplification with three viruses-

Table 1. Oligonucleotide primers for detecting salmonid viruses by reverse transcription loop-mediated isothermal amplification assay (RT-LAMP)

Virus	Primer	Position	Sequence (5′→3′)
IHNV	F3[a]	110-127	CAGCCAAACCGTCCAACC
	B3[b]	299-316	TCGTTTCCGACCGACAGG
	FIP[c]	179-200/TTTT/128-145	CAGAGTGCATCCCTCGGGGTAGTTTTCGACACCGCAAGCGAATC
	BIP[d]	220-241/TTTT/269-288	ATGCCTCTCAACTGAGATGCCCTTTTATGTGGGATAGGCAATCAGC
	LF[e]	158-177	TGAAGAGCGGGTTTGACCAG
	LB[f]	243-267	AGGATCTTCGATGATGAGAATAGGG
IPNV	F3	1348-1367	AGAGGCATCAGAAAAGTGGC
	B3	1531-1548	ATAGCTTCCTGCCTCGGA
	FIP	1432-1453/TTTT/1374-1391	TGGTCTTGGTGAGGTCCCCAATTTTTCGTGCTGTCAACGCTCTT
	BIP	1465-1484/TTTT/1507-1524	GGACGCTACCTGTCACACGCTTTTGGCCCATGAGTCCATGAC
	LF	1438-1459	CTATAAGGGGAGCCGCCAT
	LB	1508-1539	CGGAGGCCGCTACCATGAT
VHSV	F3	579-596	AACATCACCCTGCCCAAC
	B3	776-795	CAGGTCGGTCTTGATCCATT
	FIP	642-663/TTTT/599-618	AAGCGTTTCTGAGGTAGGGCAATTTTACTGGCAAGGAGTCTACTGG
	BIP	694-715/TTTT/740-757	CACAGGGTGGTCAAGGCAATCGTTTTCCGTGCATGCCATTGTGA
	LF	625-641	TGGGCCTGAGGTGTAGCG
	LB	716-733	TTGCGGGTCACCACCCCT

[a] F3= outer primer
[b] B3= outer primer of complementary sequence
[c] FIP= forward inner primer
[d] BIP= backward inner primer
[e] LF= loop forward primer
[f] LB= loop backward primer

Table 2. Oligonucleotide primers for detecting salmonid viruses by nested RT-PCR assays

Virus	Primer	Orientation	Position	Sequence (5′→3′)
IHNV	EXT1	Sense	563-583	AGAGATCCCTACACCAGAGAC
	EXT2	Antisense	1235-1255	GGTGGTGTTGTTTCCGTGCAA
	INT1	Sense	623-643	TCACCCTGCCAGACTCATTGG
	INT2	Antisense	1085-1105	ATAGATGGAGCCTTTGTGCAT
IPNV	P1	Sense	1403-1425	AGAGATCACTGACTTCACAAGTGAC
	P2	Antisense	1738-1761	TGTGCACCACAGGAAAGATGACTC
	P3	Sense	74-90	CAACACTCTTCCCCATG
	P4	Antisense	225-241	AGAACCTCCCAGTGTCT
VHSV	VHSVF3	Sense	898-918	GATCAGGTCCCCCARRTCNGT
	VHSVR1	Antisense	450-471	TTCTTTGGAGGGCAAACNATH
	VHSVF4	Sense	539-588	GTACCCKTTCTTCCCCGAAC
	VHSVR2	Antisense	739-758	GTAGCRCCGRTCCAGTAGAC

Table 3. The prevalence of salmonid viruses in rainbow trout *Oncorhynchus mykiss* by RT-LAMP and nested RT-PCR assays

Year	Number of samples	Prevalence					
		IHNV		IPNV		VHSV	
		Nested RT-PCR	RT-LAMP	Nested RT-PCR	RT-LAMP	Nested RT-PCR	RT-LAMP
2009	24	0 (0.0%)	0 (0.0%)	5 (20.8%)	6 (25.0%)	0 (0.0%)	0 (0.0%)
2010	62	0 (0.0%)	0 (0.0%)	35 (56.5%)	40 (64.5%)	0 (0.0%)	0 (0.0%)
Grand total	86	0 (0.0%)	0 (0.0%)	40 (46.5%)	46 (53.5%)	0 (0.0%)	0 (0.0%)

specific primer sets (Table 1). The specific-primers EXT1-EXT2 (one-step PCR) and INT1-INT2 (nested PCR) were used for amplification of 672 and 462 base pairs (bp) targeting the IHNV G protein (Troyer and Kurath, 2003). P1-P2 (one-step PCR) and P3-P4 (nested PCR) primers were employed for amplification of 359 and 168 bp targeting the IPNV VP2/NS junction gene (Suzuki *et al*., 1997). VHSVR1-VHSVF3 (one-step PCR; Stone *et al*., 1997; Dixon *et al*., 2003) and VHSVR2-VHSVF4 (nested PCR; *self-designed*) primers were used for amplification of 468 and 200 bp targeting the VHSV G protein. 5 μl of cDNA was mixed with each of the three virus specific-primers (Table 2) and transferred into the lyophilized *AccuPower*® PCR PreMix tube (Bioneer), then made to the reaction volume of 20 μl with DEPC-treated water (Bioneer). PCR amplification was conducted with an initial cycle 95℃ for 5 min and followed by 30 cycles of 95℃ for 30 sec, 50℃ (55℃ in the case of VHSV) for 30 sec and 72℃ for 60 sec. A final extension step was conducted at 72℃ for 7 min. A nested PCR amplification was carried out using 5 μl of the first PCR products mixing with each of the nested specific-primers (Table 2), transferred into *AccuPower*® PCR PreMix tube (Bioneer) and made up to a final volume of 20 μl with DEPC-treated water. PCR reaction conditions were the same as described above. The PCR products were analyzed by 2.0% agarose gel electrophoresis.

Virus isolation

Head kidney tissue homogenate with 500 μl of MEM were filtered with HA membrane (0.45 μm; Millipore, USA) and inoculated onto RTG-2 and CHSE-214 cells seeded in 24-well tissue culture plates (2 wells/sample). Inoculated cells were incubated at 15℃ for 7 days, and cells showing cytopathic effect (CPE) were subjected to RT-LAMP, RT-PCR, and sequence analysis.

Nucleotide sequence analysis

Ten positive samples were randomly selected for virus isolation and determined RNA extraction as mentioned above. RNAs extracted from cell cultured were subjected to PCR amplification. The PCR

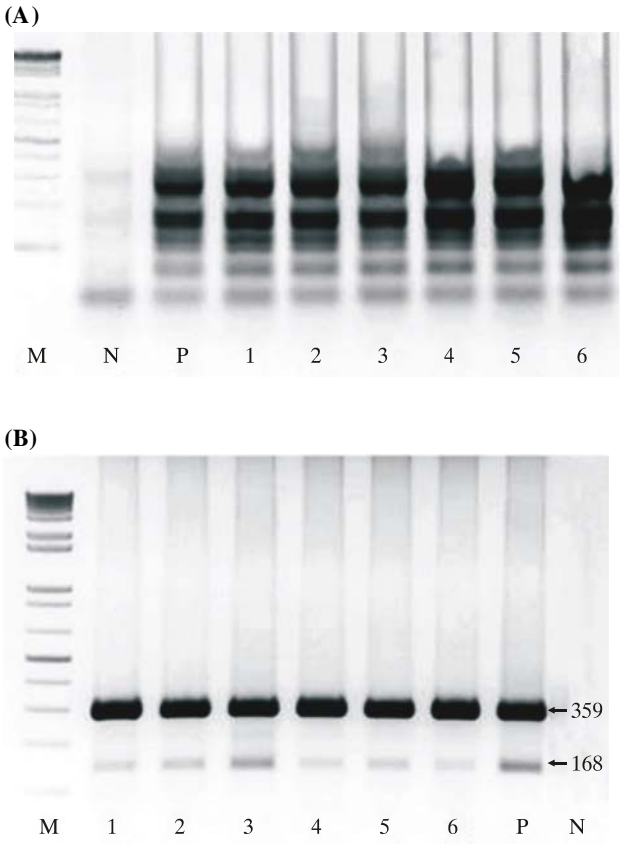

Fig. 1. Detection of IPNV in rainbow trout field samples by the RT-LAMP (A) the RT-LAMP products were seen as multiple bands of different size, and the nested RT-PCR (B) showed the amplification of 359 bp and 168 bp. Lanes: M, 100 bp DNA marker; 1 to 6, juvenile samples; P, 10 fg of RNA extracted from IPNV-infected RTG-2 cells; N, negative control.

products were purified with the *AccuPrep*® Gel Purification kit (Bioneer) and subjected to nucleotide sequence analysis using an ABI PRISM 3730*xl* DNA Analyzer (PE Applied Biosystems, USA) available at National Instrumentation Center for Environmental Management, Seoul National University (Seoul, Korea). The sequences were aligned with sequences available in the GenBank database (National Center for Biotechnology Information, NCBI) according to the CLUSTAL W by the software Molecular Evolutionary Genetics Analysis (MEGA) version 4 (Tamura *et al*., 2007). The nucleotide sequence was registered to GenBank under accession number JF774415.

Results

Virus detection

The prevalence of viruses in rainbow trout is summarized in Table 3. Only the occurrence of IPNV in rainbow trout was recorded at 53.5% (46/86) by the RT-LAMP during the two years monitoring (Fig. 1). Particularly, 25.0% (6/24) samples were IPNV-positive in 2009 and the prevalence was increased to 64.5% (40/62) in 2010. On the other hand, IHNV and VHSV were not detected in any of the samples tested.

To compare the detection results of the RT-LAMP with those of the nested RT-PCR assays, the results revealed the decreased prevalence of IPNV at 46.5% (40/86; Table 3) by the nested RT-PCR, in particular showed 20.0% (5/24) and 56.5% (35/62) IPNV-positive in 2009 and 2010, respectively. None of the rainbow trout samples were positive with IHNV and VHSV.

Virus isolation

Ten IPNV-positive samples by RT-LAMP were randomly selected for virus isolation. Tissue homogenates with MEM were inoculated onto RTG-2 cells. The CPE was observed in all of ten samples, and found to be IPNV-positive by both RT-LAMP and RT-PCR (data not shown). The RT-PCR pro-

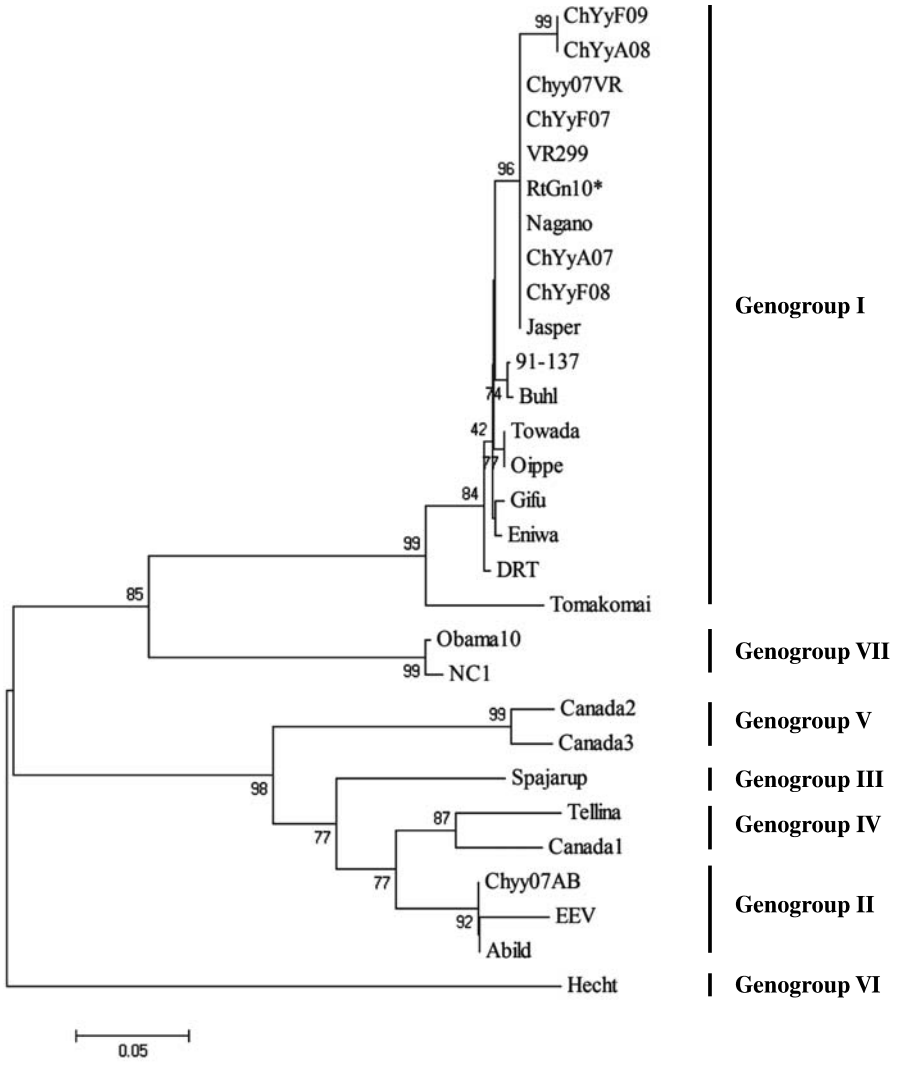

Fig. 2. Molecular phylogenetic tree based on nucleotide sequence of the VP/NS junction region among 29 worldwide isolates of IPNV. Bootstrap values from 1,000 replicates are shown at major nodes. Bar, 0.05 replacement nucleotides per site.

ducts of all the IPNV-positive samples from RTG-2 cells was sequenced using P1 and P2 primers (Suzuki *et al*., 1997) targeting the IPNV VP2/NS junction gene. The highly conserved sequences were found among all ten samples, and the detected virus was named as RtGn10 (GenBank accession no. JF774415). The entire 310-nt VP2/NS junction gene showed 99% identity with the VR-299 strain (GenBank accession no. AF343572) and was the most closely related to the Korean IPNV isolates from chum salmon (100% identity; GenBank accession no. HQ638081 to HQ63804). Phylogenetic analysis revealed 7 genotypes, as reported previously (Nishizawa *et al*., 2005), and our rainbow trout isolate fell into genotype I (Fig. 2).

Discussion

IHNV, IPNV, and VHSV are highly infectious viral diseases of salmonid species, especially for rainbow trout, held under intensive rearing conditions. In Korea, there has been no recent systematic survey on three viruses in rainbow trout. RT-LAMP assay is a simple and rapid gene amplification technique for early detection pathogens in field, which can be achieved less than one hour under isothermal condition by using a simple heating block or a water bath. We successfully developed the RT-LAMP to detect IHNV (Suebsing *et al*., 2011a) and IPNV (Suebsing *et al*., 2011b). According to these studies, the sensitivity of LAMP assay is higher than the conventional PCR assay. Therefore, the investigation of three viruses was conducted in rainbow trout by using our developed RT-LAMP assays, and compared the detection results with the nested RT-PCR assays.

In this study, only IPNV was detected in rainbow trout by both the RT-LAMP and the nested RT-PCR and the sensitivity of RT-LAMP assay for detecting virus with field samples was higher than the nested RT-PCR assay, indicating that the RT-LAMP assay is more appropriate for early field diagnosis. In addition, the RT-LAMP assay can achieve the result within one hour including gel electrophoresis, whereas the nested RT-PCR required at least 4 h to be completed.

IPNV has a wide geographical distribution, occurring in most major salmonid-farming countries and it is believed to be spread through the importation of salmonid fishes and their eggs (Reno, 1998). In Korea, IPNV epidemics occurred at most rainbow trout hatcheries, resulting in the death of more than ten million fries in the 1980s (Hah *et al*., 1984; Hedrick *et al*., 1985). Three strains, DRT, Ab and VR-299, were identified by serological technique (Lee *et al*., 1996). In the present study, one strain of IPNV, named RtGn10, was recorded in rainbow trout and showed high similarity with the VR-299 strain. The VR-299 strain was reported in rainbow trout in Korea in the 1985 and 1996 (Hedrick *et al*., 1985; Lee *et al*., 1996). It belongs to genogroup I, the most frequently isolated from rainbow trout in the North America and Japan (Nishizawa *et al*., 2005).

IHNV and VHSV have a serious economic impact in salmonid farms worldwide (OIE, 2007). The occurrence of IHNV in Korea was firstly reported from juvenile rainbow trout and masu salmon and afterward the number of outbreaks from rainbow trout were frequently reported (Kim *et al*., 2003, 2007). However, all of rainbow trout samples were found to be IHNV-negative in this study. VHSV is well known for causing mortalities in rainbow trout in the European aquaculture industry (Jørgensen, 1992) and has a widespread distribution in a variety of freshwater and marine fishes (Mortensen *et al*., 1999). The first isolations of VHSV in Korea were from farmed olive flounder (Kim *et al*., 2003). Since then, the high frequency of VHSV infection has been occurred from wild marine fish species in Korea (Lee *et al*., 2007). However, there has been no report of VHSV from salmonid fishes in Korea, including in this study.

In the present study, we investigated the presence of three major viruses in rainbow trout in Korea and found that IPNV is thought to be the dominant virus in rainbow trout in Korea. However, a further study is required with more samples from other parts of Korea in order to understand the infection pattern in rainbow trout in Korea aquaculture. It is important to detect these viruses for successful farm management and to prevent the spread of fish viruses in aquaculture industry.

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