한국어병학회지 제25권 제3호 (2012)
J.Fish Pathol., 25(3) : 143~150
www.ksfp.org
http://dx.doi.org/10.7847/jfp.2012.25.3.143

Genotype distribution of infectious haematopoietic necrosis virus (IHNV) in Korea

Seung Joo Cha, Yo Han Jung, Hyun Young Lee, Ji Yoon Jung, Hee Jung Cho and Mi Seon Park†

Aquaculture Industry Division, East Sea Fisheries Research Institute, Gangwon-Do, 210-861
1194, Haean-ro, Yeongok-Myeon, Gangneung-Si, Gangwon-Do, 210-861

Infectious haematopoietic necrosis virus (IHNV) is an important fish pathogen that infects both wild and cultured salmonids. Since the first isolation of IHNV from rainbow trout and masu salmon in 1991, a series of IHN disease outbreak has been reported in Korea. In 2011, we isolated two IHNV isolates from rainbow trout cultured in Korea. The full open-reading frame (ORF) encoding the glycoprotein (G) of them were sequenced and the amino acid sequences were phylogenetically analyzed. Phylogenetic analysis of the G revealed that both IHNV isolates were grouped into an Asian genogroup containing Korean IHNV isolates and Japanese IHNV isolates. However, based on their sequence variation, they were divided into different subgroup. While one isolate was similar to other Korean isolates, the other isolate showed a high level of similarity with Japanese isolates, suggesting the possibility of influx of new IHNV strain into Korea.

Key words : Infectious haematopoietic necrosis virus (IHNV), Glycoprotein (G), Genogroup, Phylogenetic analysis

Infectious haematopoietic necrosis virus (IHNV) is one of the most important rhabdoviral fish pathogens of both wild and hatchery-reared salmonid fish in North America, Europe, and Asia and causes extensive economic losses in fish culture facilities (Schütze *et al*., 1995; Liu H *et al*., 2011; Ammayappan *et al*., 2010). The first epidemics of IHNV occurred in sockeye salmon (*Oncorhynchus nerka*) fry at Washington and Oregon fish hatcheries during the 1950s (Liu H *et al*., 2011; Ammayappan *et al*., 2010). IHNV has a linear, single-stranded, negative-sense RNA genome of approximately 11 kb. IHNV genome contains six genes in the order 3'-N-P-M-G-NV-L-5': the nucleoprotein N, the phosphoprotein P, the matrix protein M, the glycoprotein G, the non-structural viral protein NV and the polymerase L (Park *et al*., 2010; Jonstrup *et al*., 2010). The clinical signs and histopathology have been well-documented. Young fish are more susceptible to IHNV infection and losses during acute outbreaks can reach 95% (Kim *et al*., 2007).

The diversity among IHNV isolates in the Hagerman Valley region was first reported by LaPatra, who used monoclonal and polyclonal antibodies to examine the heterogeneity of serum neutralization profiles of 106 IHNV isolates from four rainbow trout culture facilities between 1990 and 1992 (Ammayappan *et al*., 2010). Later, based on sequence analyses of the G gene, phylogenetic analyses of IHNV isolates has defined three major genetic lineages or genogroups in North America,

†Corresponding author : Mi Seon Park
Tel : +82-33-660-8601, Fax : +82-33-661-8515
E-mail : parkms@nfrdi.go.kr

which correspond with specific portions of the geographic range of the virus and are denoted as U, M and L (Ammayappan *et al*., 2010; Park *et al*., 2010; Purcell *et al*., 2009; Kurath *et al*., 2003). The U genogroup ranges from Alaska to Oregon, the M genogroup is found in rainbow trout culture in Idaho and in Columbia River basin, and the L genogroup is found in northern California and southern Oregon (Purcell *et al*., 2009). In addition, the U and M genogroup viruses reveal host-specific virulence *in vivo*, however, the molecular basis for the host-specific virulence of these genogroups is not known (Park *et al*., 2011; Penaranda *et al*., 2009). Besides these three genogroups, two additional genogroups were identified in Europe and Asia (JRt genogroup). Thus, at present, five genogroups correlating with different geographic ranges have been identified among worldwide IHNV isolates (Park *et al*., 2010; Jonstrup *et al*., 2010).

There was no IHN occurrence in Korea before 1990, but the first outbreaks of IHNV were recorded in hatcheries for rainbow trout and masu salmon (*Oncorhynchus masou*) in Kangwon Province in 1991 (Kim *et al*., 2007; Park *et al*., 1993). From that time on, IHN outbreak has been reported in rainbow trout cultured at various parts of Korea. Recently we detected two IHNV isolates from cultured rainbow trout in Korea. In this study, we analyzed the amino acid sequences of the glycoprotein (G) of the isolates to evaluate their genetic relatedness to other IHNV isolates.

Materials and Methods

Fish

Juvenile and fry were obtained from 10 culture facilities in Kangwon Province in 2011 (Table 1). Spleen and kidney tissues were isolated from fish and stored at-80℃ until RNA extraction.

Table 1. Sampled fishes for virus isolation

Location of sampling sites	Date of sampling	Species	Size (cm)	Prevalence
Yangyang	Feb, May, Jul, Oct 2011	Rainbow trout	2.50～17.5	N.D
		Cherry salmon	2.29～14.2	N.D
		Chum salmon	4.10～5.83	9%
Chuncheon A	Feb, May, Sep, Oct 2011	Rainbow trout	6.78～30.3	N.D
Chuncheon B	Feb, May, Sep, Oct 2011	Rainbow trout	16.3～33.5	N.D
Pyeongchang A	Mar, May, Aug 2011	Rainbow trout	9.8～27.5	25%
Pyeongchang B	Mar, May, Sep 2011	Rainbow trout	3.5～29.9	33%
Pyeongchang C	Mar, May, Aug 2011	Rainbow trout	7.7～36.5	21%
Pyeongchang D	Mar, May, Aug, Oct 2011	Rainbow trout	7.0～27.5	42%
Jeongseon A	Mar, May, Sep, Oct 2011	Rainbow trout	8.7～22.5	N.D
Jeongseon B	Mar, May 2011	Rainbow trout	3.4～23.2	90%
Yeonwol	Feb, May, Aug 2011	Rainbow trout	14.5～39.5	11%

* Prevalence in result is presented as number of positive sample/number of examined sample; N.D, non detection

RNA extraction

Tissues were homogenized in individual tubes and RNA was extracted by TRIzol reagent (Gibco, USA) according to the manufacturer's instructions.

RT-PCR amplification

cDNA was synthesized from total RNA extracted from tissues using 0.5μg oligo-dT primer (Invitrogen, The Netherlands), ~500ng RNA sample, 10mM dNTP and 200 units of SuperScript™ RT (Invitrogen, The Netherlands). To detect IHNV gene, RT-PCR was conducted using the cDNA as a template, Taq DNA polymerase (Enzynomic, Korea) and PCR primers: 5'-TCAAGGGGGGAGTCCTCGA-3'; 5'-CACCGTAC TTTGCTGCTGCTAC-3'. The second round of PCR was performed using 2μl PCR product and PCR primers: 5'-TTCGCAGATCCCAACAACAA-3'; 5'-GCGCACA GTGCCTTGGCT-3'. Cycling conditions were 95℃ 5 min, 24 cycles: 95℃ 30 s, 50℃ 30 s, 72℃ 1 min; followed by 72℃ 7 min, 4℃ hold.

To obtain full ORF (open reading frame) of IHNV G gene, RT-PCR was performed using SuperScript™ II Reverse Transcriptase (Invitrogen, The Netherlands) and Ex-Taq polyermase (TaKaRa, Japan) and primers: IHNV-G-1U, 5'-CGGGATCCACCAAAACAATGGAC ACC-3'; IHNV-G-1D, 5'-CCGCTCGAGGGACCGGT TTGCCAGGTGG-3'. Cycling conditions were 94℃ 5 min, 35 cycles: 94℃ 30 s, 58℃ 30 s, 72℃ 1 min 30 s; followed by 72℃ 7 min, 4℃ hold. The RT-PCR products were purified and cloned into a pGEM-T easy vector (Promega, USA).

Sequence and Computer-assisted analysis

The nucleotide sequences and the deduced amino acid sequences of the full-ORF of G gene were compared with the GenBank/EMBL databases using BLAST. Multiple sequence alignments were performed using the Clustal W multiple alignment algorithm. Construction of a phylogenetic tree was drawn with Bosque software Ver. 1.8.1 and Neighbor-Joining method with 1000 strap replicates. The IHNV G gene ORF nucleotide sequences of 44 worldwide IHNV isolates in GeneBank/DDBJ were used for comparative analyses.

Results and Discussion

To determine whether or not the IHNV was the causative agent of epizootics, PCR was performed using primers specific for IHNV supported by Manual of diagnostic tests for aquatic animals (OIE, 2010). We found that the PCR amplified the IHNV G gene in 2 fish samples collected at 2 regions in Kangwon Province (Fig. 1) and they were named as PcKw11 and PcOj11, respectively. In order to determine the genetic characteristics of the isolates, a full-length ORF of glycoprotein (G)

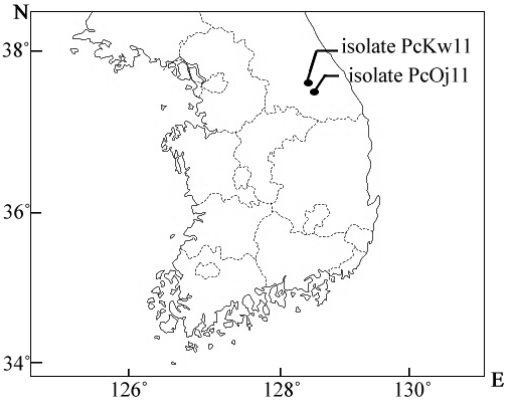

Fig 1. Location of fish farms where epizootics occurred and IHNVs was isolated

of IHNV was amplified from these samples by PCR. Sequence analysis of the glycoprotein full-length ORFs revealed that the G proteins shared 93% to 99% amino acid sequence identity to each other into identitied by Korea (Table 3).

In order to determine the relationship between isolates of Korea IHNV and previously reported IHNVs, we compared the glycoprotein G sequence of IHNV from 2 Korean isolates to those of 44 other IHNVs available in GeneBank including 6 Korean isolates and 13 Japanese isolates (Table 2). A phylogeny tree based on the determined G gene nucleotide sequence revealed the five major genogroups described in previous studies (Kim *et al*., 2007; Nishizawa et al., 2006) (Fig. 2). Among the 5 genogroups, 3 coincided with the North American genogroups U, M and L, as previously proposed by Kurath et al (Kurath *et al*., 2003). The other two are genogroup for European isolates and genogroup for Asian isolates. As previously reported, Japanese isolates from 1971 to 1982 were included in the U genogroup cluster, while Japanese isolates from 1980 to 1996 comprised an Asian genogroup (Kim *et al*., 2007; Nishizawa et al., 2006).

Table 2. Viral isolates and GeneBank accession numbers for glycoprotein sequences in Table 3 and Fig 1.

Virus isolate	GeneBank accession no.	Country	Virus isolate	GeneBank accession no.	Country
RtPy91	AB288204	Korea	LR-80	L40878	USA
RtWanJu09	HM021723	Korea	193-11	L40871	USA
RtJe00	AB288205	Korea	WRAC	L40882	USA
RtGu01	AB288206	Korea	HO-7	L40876	USA
RtUi02	AB288207	Korea	SRCV	L40881	USA
ChYa07	FJ230851	Korea	Col-85	L40874	USA
RtTochi86	AB250934	Japan	Col-80	L40873	USA
RtToya80	AB250935	Japan	LR-73	L40877	USA
G4	AF244128	Japan	lambda ZAPII	X89213	USA
AyTochi86	AB250933	Japan	strain K	X73872	-
RtShiz06a	AB510193	Japan	FsK/88	AY331665	Germany
ChYu78	AB250928	Japan	Dau832-94	EU676209	Germany
ChAb76	AB250927	Japan	Dau819-96	EU676208	Germany
RtShiz06b	AB510194	Japan	Dau32-97	EU676202	Germany
RtNag82	AB250931	Japan	CH-118-02	EU676197	Switzerland
RtNag76	AB250930	Japan	FsVi100/96	AY331666	Germany
KoMo71	AB250929	Japan	Fs62/95	AY331664	Germany
RtNag96	AB250932	Japan	Fs42/95	AY331663	Germany
RtAichi06a	AB510197	Japan	Fs832/94	AY331661	Germany
RB-76	L40880	USA	Dswego	EU676226	Germany
LWS-87	L40879	USA	Fs30/95	AY331662	Germany
Carson-89	L40872	USA	332	AY331657	Germany
RB-76	L40880	USA	Fs8/99	AY331660	Germany

Table 3. Comparative analysis of the glycoprotein of IHNVs. Homology is given as percentage of amino acid sequence identities of the IHNV glycoprotein gene ORF among Korea See Table 2 for explanations of isolate abbreviations and GeneBank accession numbers for protein sequences.

Isolates	Korean isolates							
	PcKw11	RtUi02	RtGu01	ChYa07	RtPy91	PcOj11	RtJe00	RtWanJu09
PcKw11	100	99.0	99.0	98.0	96.0	93.0	93.0	94.0
RtUi02		100	99.0	98.0	96.0	94.0	94.0	94.0
RtGu01			100	98.0	96.0	94.0	94.0	95.0
ChYa07				100	96.0	93.0	93.0	94.0
RtPy91					100	97.0	96.0	94.0
PcOj11						100	93.0	95.0
RtJe00							100	95.0
RtWanJu09								100

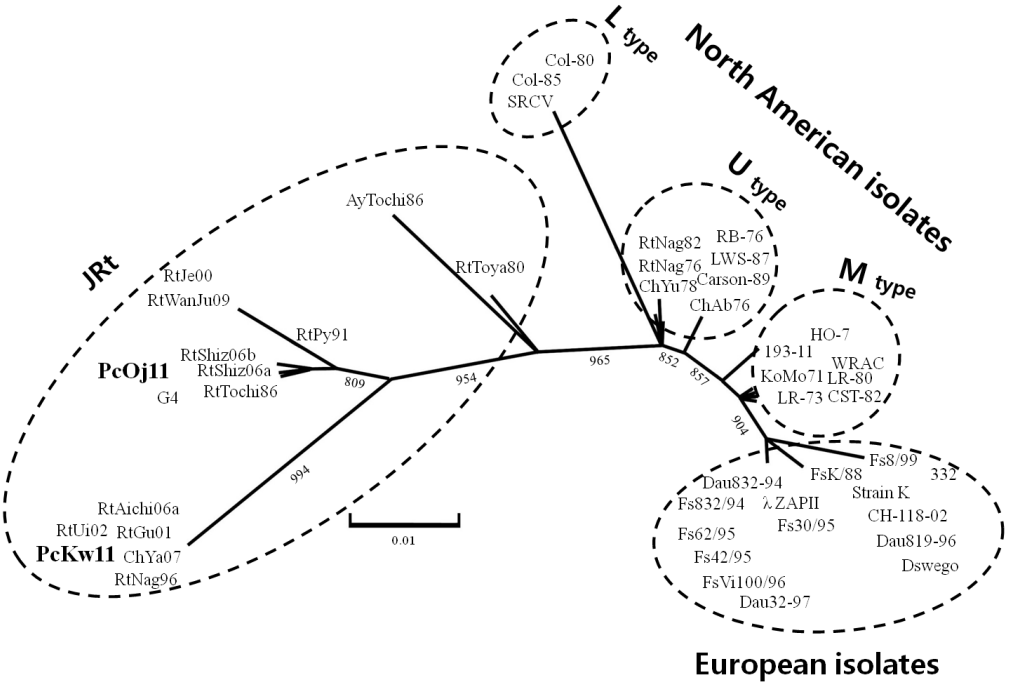

Fig 2. Phylogenetic analysis of IHNV glycoprotein (G). Molecular phylogenetic tree showing the genetic relationships among 44 isolates of IHNV based on the amino acid sequence of the glycoprotein. The numbers indicate the percentage bootstrap support for each node from 1000 replicates. The distances are proportional to the relative sequence deviations between individual amino acid sequences. The phylogenetic analyses were carried out with the Clustal W program.

Both Korean isolates (PcKw11, PcOj11) were classified into the Asian genogroup which contains all Korean isolates and several Japanese isolates (Fig. 2). It has been reported that the IHNV has independently evolved in the Japanese rainbow trout farm environment because Japanese isolates from 1971 to 1982 were classified into North American U genogroup, while the isolates from 1980 to 1996 comprised a divergent Asian genogroup (Nishizawa et al., 2006). According to Kim *et al (2007),* within the Asian genogroup, the Korean isolates fell into two different phylogenetic subgroups: the first included the RtPy91 and RtJe00 isolates, which shared a closest common ancestor with Japanese isolates RtTochi86 and G4; the second was the RtGu01 and RtUi02 isolates, which were closest to the Japanese isolate RtNag96. Our results suggest that, of the two Korean isolates, PcKw11 showed high similarity with three Korean isolates RtUi02, RtGu01, and ChYa07 (≥ 98% amino acid identity) (Fig. 2 and Table 3). However, the other isolate PcOj11 showed relatively low similarity with Korean isolates (93%～97%). Instead, this isolate showed high similarity with Japanese isolates RtShiz06a, RtShiz06b, and RtTochi86 (94%～97%) (Fig. 2 and Table 3), indicating that this isolate shared a common ancestor with these Japanese isolates. Kim et al. (Kim et al., 2007) suggested that the fish-rearing environments was completely physically separated by the mountains and the phylogenetically distinct isolates RtGu01 and RtUi02 have arisen by evolution from the earlier IHNV in Korea represented by RtPy91. However, our phylogenetic analysis suggests that, besides the diversification within Korea, an influx of new IHNV strain into Korea has occurred. Considering the frequent transport of rainbow trout eggs in Korea, it is possible that PcOj11 isolate has already spread among fish farms in Korea.

Here, we have phylogenetically analyzed 2 IHNV isolates from rainbow trout cultured in Korea. Both isolates fell within the Asian genogroup. While one isolate has the same ancestor with pre-existing IHNV strain, the other seems to be the result of influx of new IHNV strain. IHNVs appear to adapt easily to other fish-farming region. Thus, the prevention of further spread to other regions and other fish species cultured in Korea is urgent.

Acknowledgements

This research was supported by NFRDI(RP-2012-AQ-103).

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Manuscript Received : September 28, 2012
Revised : November 6, 2012
Accepted : November 8, 2012