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Brief Report to Archives of Virology

Genotyping of Korean isolates of infectious hematopoietic necrosis virus (IHNV) based on the glycoprotein gene

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Running title: Genotyping of Korean isolates of IHNV

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The GenBank/EMBL/DDBJ accession numbers of the nucleotide sequences in this paper are AB288204 (RtPy91), AB288205 (RtJe00), AB288206 (RtGu01) and AB288207 (RtUi02).

Summary

Glycoprotein (G) gene nucleotide sequences of four Korean isolates of infectious hematopoietic necrosis virus (IHNV) were analyzed to evaluate their genetic relatedness with worldwide isolates. All Korean isolates were closely related to Japanese isolates of genogroup JRt rather than those of North American and European genogroups. It is considered that Korean IHNV has been most likely introduced from Japan to Korea by the movement of contaminated fish eggs. Among the Korean isolates, phylogenetically distinct virus types were obtained from sites north and south of a large mountain range, suggesting the possibility of more than one introduction of virus from Japan. (100 words)

Infectious hematopoietic necrosis (IHN) is one of the most important viral diseases in aquaculture facilities for salmonid fishes because outbreaks of IHN result in losses approaching 100% depending on the species and size of the fish, the virus strain and environmental conditions [18]. IHNV, the aetiological agent of IHN, belongs to the genus *Novirhabdovirus* in the family *Rhabdoviridae.* The IHNV genome is a non-segmented and single-stranded negative-sense RNA with approximately 11,000 nucleotides containing six genes in the order 3'-N-P-M-G-NV-L-5' encoding the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), non-virion protein (NV) and polymerase (L) genes, respectively [6, 9]. The G, N and NV genes have been used for analyses of IHNV evolution, diversity and phylogenetic relationship among the worldwide isolates [1, 3, 7, 10, 11, 16, 17]. Nichol et al. [10] reported that the genetic relationship of IHNV isolates correlates with the geographic origin by phylogenetic analysis with G and NV genes. Subsequently, Garver et al. [3] and Kurath et al. [7] revealed three major genogroups, denoted upper (U), middle (M) and lower (L), correlating with the geographic areas in the Pacific Northwest of North America. The genogroup U includes isolates from Alaska, British Columbia, Washington coastal watersheds and the Columbia River basin; the genogroup M includes isolates from the Columbia River basin and Idaho; and the genogroup L includes isolates from California and the southern Oregon coast. More recently, two additional genogroups for European and Japanese isolates were identified [1, 11]. It is interesting that the genogroup for European isolates shared a common source with the American genogroup M [1], while the genogroup for Japanese isolates was closely related with the American genogroup U [11]. Thus, at present, a total of five genogroups correlating with the geographic areas have been identified among worldwide isolates of IHNV.

There was no IHN occurrence in Korea before 1990, although rainbow trout (*Oncorhynchus mykiss*) eggs have been imported from North America and/or Japan to Korea since the 1960s. In 1991, the first outbreaks of IHN were recorded in hatcheries for juvenile rainbow trout and masu salmon (*O. masou*) in Kangwon Province, Korea [12]. Thereafter, a series of IHN outbreaks occurred in cultured juvenile rainbow trout in various parts in Korea. Recently, losses of cultured rainbow trout caused by IHNV in Korea were not only in juveniles, but also in commercial size fish (up to 300 g) [4], and the same phenomena have been reported in Japan [11]. It is believed that Korean IHNV may have been introduced from North America by transportation of contaminated fish eggs, but its ground is due to only one report that one Korean isolate from masu salmon was similar to North American isolates by viral protein migration patterns with SDS-PAGE and by neutralization profiles with monoclonal antibodies [12]. There is no molecular phylogeny evidence to support the hypothesis for the origin of Korean isolates. Thus, in the present study, we analyzed

glycoprotein (G) gene nucleotide sequences of four Korean isolates from cultured rainbow trout to evaluate genetic relatedness with worldwide IHNV isolates.

Four isolates of Korean IHNV, named RtPy91 (a synonym of the PRT isolate, Park et al. [12]), RtJe00, RtGu01 and RtUi02, were obtained from rainbow trout at Pyeongchang in 1991, Jecheon in 2000, Gumi in 2001 and Uiseong in 2002, respectively (Fig. 1). The isolates RtPy91 and RtUi02 were obtained from moribund juvenile fish in a population undergoing an epidemic, while the isolates RtJe00 and RtGu01 were from moribund adult fish in an epidemic (body weight 350-400 g, length 30-40 cm) [4]. These isolates were propagated in rainbow trout gonad (RTG-2), chinook salmon embryo (CHSE-214) or epithelioma papilosum cyprini (EPC) cells, which were maintained at 15°C with Eagle's MEM supplemented with 10% fetal bovine serum, 100 IU/mL penicillin G and 100 μg/mL streptomycin sulfate. The isolates used for nucleotide sequence analysis had a maximum of 3 passages in cell culture, with the exception of the older isolate RtPy91, which had approximately 9 passages.

Viral genomic RNA was extracted using TRIZOL reagent (Gibco, Gaithersburg, MD, USA) according to the manufacturer's instructions. RT-PCR was performed with primers, IHNG-F (5'-ATG GAC ACC ATG ATC ACC ACT C-3') and IHNG-R (5'-TTA GGA CCG GTT TGC CAG GT-3'), targeting the entire G gene open reading frame (ORF). Briefly, viral genomic RNA was denatured at 95°C for 5min, and then incubated at 42°C for 30min in 10 μL of RT buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl) containing 100 U of M-MLV reverse transcriptase (Takara, Shiga, Japan), 1.0 μM of IHNG-F primer, 1 mM of dNTP and 5 mM of $MgCl_2$ for reverse transcription. The synthesized cDNA was amplified in 50 μL of PCR buffer containing 0.2 μM of the PCR primers, 1.25 U of Ex-*Taq* DNA polymerase (Takara, Shiga, Japan), 0.2 mM of dNTP and 1.5 mM of $MgCl_2$ using a GeneAmp 2400 thermal cycler (Perkin Elmer, Norwalk, CT, USA) with 30 cycles (95°C for 1 min, 57°C for 1 min, and 72°C for 1 min). The PCR product was analyzed by 1.5% agarose gel electrophoresis. After purification with the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA), amplified PCR products were cloned into the pCR 2.1 vector (Invitrogen, Groningen, the Netherlands) before transformation into *Escherichia coli* TOP10 (Invitrogen, Groningen, the Netherlands) using standard protocols. Nucleotide sequences of cloned G genes were obtained with ID4 primer (5'-CTC TGG ACA AGC TCT CCA AGG-3') [8], and M-13 forward and reverse primers. Three independent clones from each isolate were used for sequence analysis, and the resulting sequences were assembled with Genetyx Win Ver. 5.1 software to identify and exclude duplicate sequences from the data set. A multiple alignment of the consensus G ORF sequences, excluding the PCR primer sequences, was constructed using Clustal X [14, 15]. This alignment was used to infer genetic relationship among the sequences with neighbor joining criteria, and a final radial tree was drawn with

software, NJplot and Unrooted [13]. The same alignment was also used to generate a maximum likelihood tree using Dnaml in the Phylip software package [2]. The determined nucleotide sequences were registered in GenBank/DDBJ as accession numbers: AB288204 - AB288207. The G gene ORF nucleotide sequences of 38 worldwide IHNV isolates in GenBank/DDBJ [1, 9, 10, 11], including another isolate from rainbow trout in Japan (isolate G4, GenBank AF244128), were used for comparative analyses. GenBank accession numbers for these comparison sequences are as in Nishizawa et al. [11].

Full length of IHNV G gene ORF sequences of 1527 nt were analyzed for the four Korean isolates. The determined G gene ORFs without the PCR primer sequences showed more than 95% identity to each other, and more than 93% identity with those sequences of 38 worldwide IHNV isolates (Table 1). A radial tree of phylogeny based on the determined G gene nucleotide sequences revealed the five major clusters described in previous studies [1, 7, 11] (Fig. 2). Three of the five clusters were for genogroups U, M and L, which were previously identified by Kurath et al. [7] for the North American isolates. The genogroups U, M and L respectively correlated to virus isolates from the upper, middle and lower portions of the geographic range of IHNV in North America. A fourth cluster was for European isolates from Germany, Italy and France [1]. As previously reported, Japanese isolates from 1971 to 1982 were included in the U genogroup cluster, while Japanese isolates from 1980 to 1996 comprised a fifth cluster designated genogroup JRt [11].

All four Korean isolates were classified into the genogroup JRt (Fig. 2), and were thus most closely related to Japanese IHNV isolates from 1980 to 1996. The topography of the neighbor-joining tree in Figure 2 was confirmed in a maximum likelihood tree, which differed only in having slightly different branch lengths (data not shown). It has been hypothesized for many years that IHNV was introduced from North America to Japan in a shipment of contaminated sockeye salmon (*O. nerka*) eggs prior to the first Japanese IHN outbreak in 1971 [5, 19]. A recent study of IHNV in Japan suggests that after its introduction the virus has independently evolved in the Japanese rainbow trout farm environment because Japanese isolates from 1971 to 1982 were classified into North American genogroup U while the isolates from 1980 to 1996 comprised a new more divergent genogroup JRt [11]. However, the situation for Korean IHNV seemed different from Japanese isolates, because no Korean isolate was in the genogroups U, M and L for North American isolates. Moreover, the oldest Korean isolate RtPy91 shared a most common ancestor with the Japanese isolates, RtTochi86 and G4 (Fig. 2). Although this unrooted phylogenetic analysis alone cannot indicate directionality of virus traffic between Japan and Korea, the historical record shows that IHN has been in Japan since 1971 but was first found in Korea in 1991. Therefore these results suggested that Korean IHNV isolates have been most likely introduced by the movement of contaminated fish eggs from Japan to Korea, and not from North America directly. This is

supported by the fact that during the 1960s-1990s rainbow trout eggs were frequently shipped from Japan to Korea, but to our knowledge eggs were never transported from Korea to Japan.

Nucleotide diversity of the four Korean isolates was high (5%) relative to that of North American and European isolates, even though the Korean isolates were sampled from a smaller geographic area and within a more limited time period between 1991 and 2002. Moreover, within the JRt 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 that were closest to the most divergent Japanese isolate RtNag96. The RtPy91 and RtJe00 isolates originated in Pyeongchang and Jecheon, to the north of the Sobaek mountain range, while the RtGu01 and RtUi02 isolates originated in Gumi and Uiseong, to the south of the Sobaek mountain range (Fig. 1). The fish rearing environments for these two pairs of Korean isolates were completely physically separated by the mountain range. Thus, the present tree suggests that the first Korean IHNV isolate, RtPy91, most likely descended from an ancestor shared with Japanese isolates RtTochi86 or G4, and may have been transported to Jecheon to be found as the similar RtJe00 isolate in 2000. The phylogenetically distinct isolates RtGu01 and RtUi02 of Gumi or Uiseong could have arisen by evolution from the earlier IHNV in Korea represented by RtPy91, but this would have involved virus traffic across the mountain range and convergent evolution to a sequence resembling the RtNag96 isolate from Japan. Alternatively, it seems more likely that RtGu01 and RtUi02 descended from an ancestor of RtNag96 in Japan, suggesting a second introduction event from Japan to Korea.

Mention of trade names does not imply U.S. Government endorsement.

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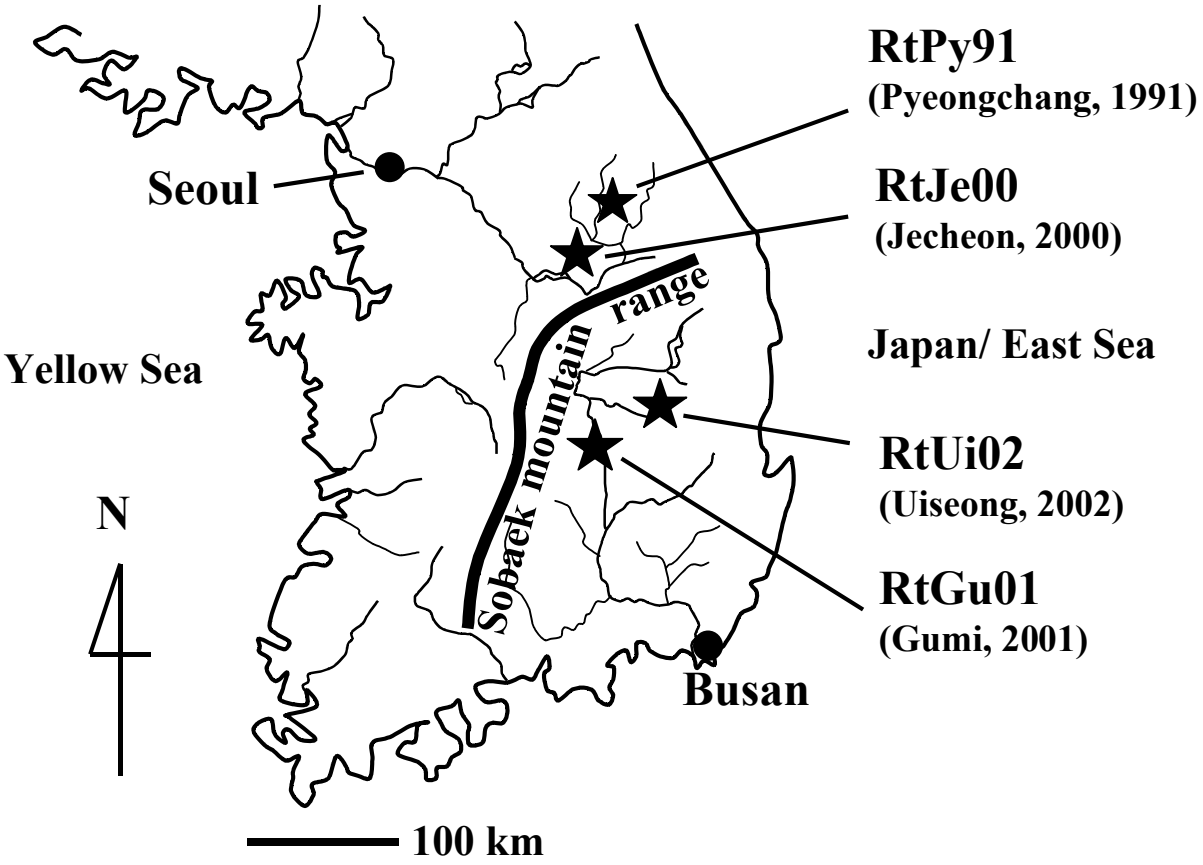
FIGURE LEGENDS

Fig. 1. Sampling location and years of Korean isolates of IHNV. Stars indicate sites of origin for the four Korean IHNV isolates, and thick line indicates mountain range.

Fig. 2. Molecular phylogenetic tree showing the genetic relationships among 42 isolates of infectious hematopoietic necrosis virus (IHNV) based on the nucleotide sequence of the glycoprotein open reading frame sequences. Bootstrap values at 1000 times of construction are shown at major nodes in the tree. Distance marker refers to the expected number of substitutions per site.

Figure

Fig. 1.
Kim et al.



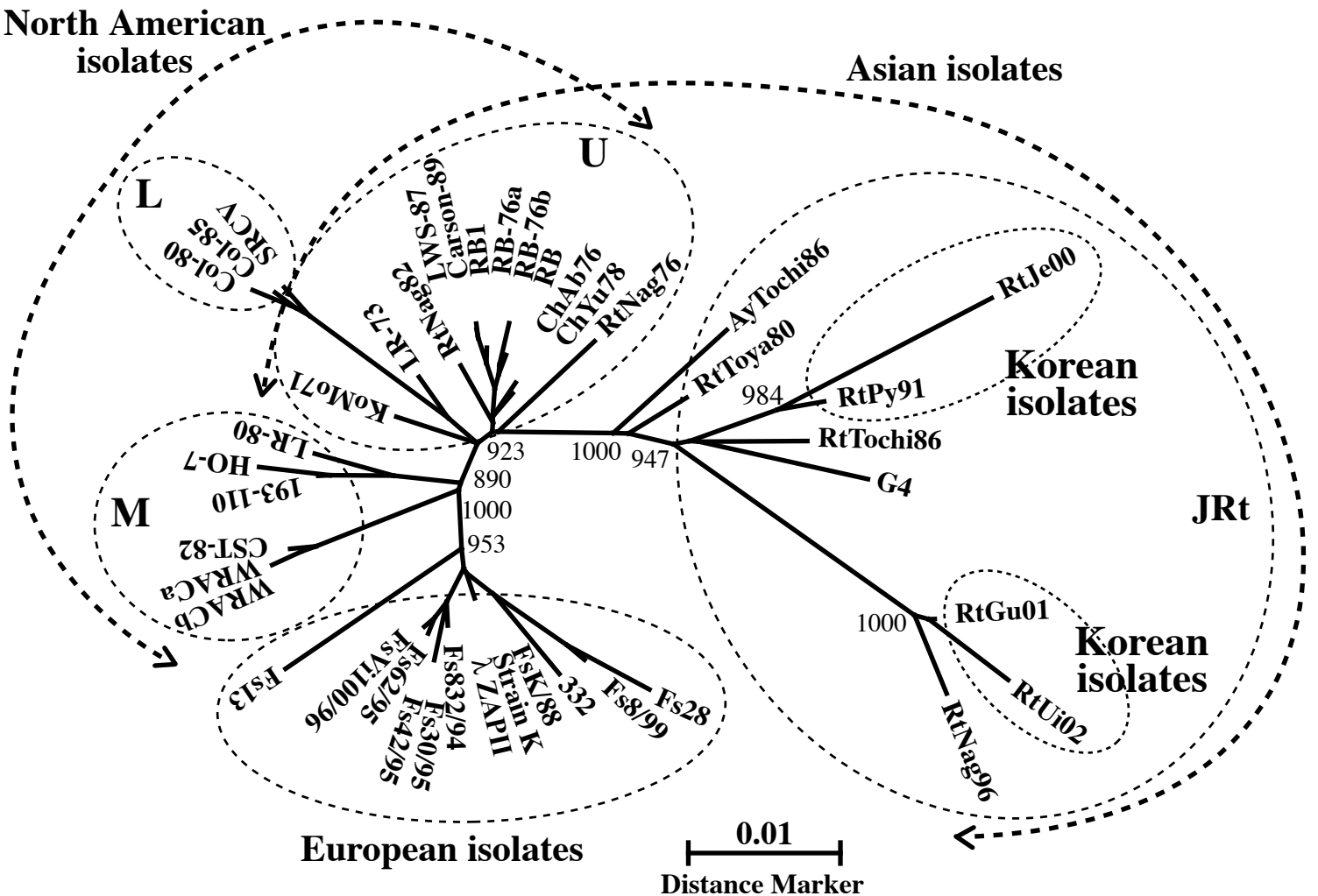

North American isolates
Asian isolates
L
U
M
JRt
Korean isolates
Korean isolates
European isolates
Col-80
Col-85
SRCV
LR-73
RtNag82
LWS-87
Carson-89
RB1
RB-76a
RB-76b
RB
ChAb76
ChYu78
RtNag76
KoMo71
LR-80
HO-7
193-110
CST-82
WRACa
WRACb
Fs13
FsVi100/96
Fs62/95
Fs42/95
Fs30/95
Fs832/94
λ ZAPII
Strain K
FsK/88
332
Fs8/99
Fs28
AyTochi86
RtToya80
RtJe00
RtPy91
RtTochi86
G4
RtGu01
RtUi02
RtNag96
923
890
1000
953
1000
947
984
1000
0.01
Distance Marker

Table 1. Pairwise comparisons of nucleotide sequence identities of the 1485 nt glycoprotein gene ORFs, without the PCR primer sequences, among Korean (A), and Japanese, North American and European isolates of IHNV (B)

A

Isolates	Korean isolates			
	RtUi02	RtGu01	RtPy91	RtJe00
RtUi02	100	99.3	96.3	95.0
RtGu01		100	96.9	95.2
RtPy91			100	98.1
RtJe00				100

B

Isolates	Geno group	Range of nucleotide sequence identities (%)						
		Korean	Japanese		North American			European
			JRt	U	U	L	M	
Korean		95.0-99.3	94.7-99.3	95.0-97.3	95.1-97.2	94.1-95.9	94.3-96.3	93.8-96.6
Japanese	JRt		96.0-98.5	95.3-98.4	95.2-98.3	94.3-97.0	94.5-97.3	94.1-97.4
	U			98.4-99.5	98.4-99.5	97.3-98.0	97.3-98.4	96.8-98.4
North American	U				98.5-99.9	97.3-98.3	97.5-98.3	96.8-98.4
	L					99.3-99.5	96.3-97.0	95.9-97.3
	M						97.2-99.8	96.6-98.4
European								97.0-100