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Virulence Change of Infectious Hematopoietic Necrosis Virus against Rainbow Trout *Oncorhynchus mykiss* with Viral Molecular Evolution

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ABSTRACT—Infectious hematopoietic necrosis virus (IHNV) is the causative agent of IHN, one of the most serious viral diseases of salmonid fish. A total of five major genogroups including JRt for Asian isolates were confirmed among worldwide isolates based on glycoprotein (G) gene nucleotide (nt) sequences. The present study revealed existence of new two lineages, JRt Shizuoka and JRt Nagano, in the genogroup JRt by addition of new isolates obtained in 2006. The maximum nt diversity of G gene within JRt Shizuoka or JRt Nagano lineage was 6.3% or 3.5%, while that between JRt Shizuoka and JRt Nagano lineages was 7.0%. To evaluate influence of the evolutional divergence to virulence of IHNV, experimental challenges to rainbow trout *Oncorhynchus mykiss* were conducted by bath exposure at 10^4 $TCID_{50}$/mL of RtShiz06s and RtShiz06a (JRt Shizuoka lineage), RtNag96 and RtNag06a (JRt Nagano lineage), and ChAb76 (a representative of the genogroup U). Distinct difference was observed in IHNV virulence to rainbow trout, i.e. the highest virulence was in RtShiz06s and RtShiz06a (≥ 76% of mortalities), and subsequently in RtNag96 and RtNag06a (20–40%), but scarcely any virulence in ChAb76 (≤ 10%). Thus it was suggested that nt diversity of Japanese IHNV continued rapidly with changing its virulence in rainbow trout farm environments.

Key words: infectious hematopoietic necrosis virus, IHNV, virulence, rainbow trout, phylogeny, nucleotide diversity

Infectious hematopoietic necrosis (IHN) is one of the most serious diseases for salmonid fishes, because outbreaks of IHN result in losses approaching more than 80% depending on the species and size of the fish, the virus strain and environmental conditions (Wolf, 1988; Bootland and Leong, 1999). IHN virus (IHNV) is a member of the genus *Novirhabdovirus* in the family *Rhabdoviridae,* and consists of a linear single-strand, negative-sense RNA genome with approximately 11 k nucleotides and five structural proteins, nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), polymerase (L), moreover, a non-virion protein (NV) (Kurath *et al*., 1985; Tordo *et al*., 2005). Nichol *et al*. (1995) reported that the genetic relationship of IHNV isolates correlates with the geographic origins based on phylogenetic analysis with G and NV genes. Subsequently, Garver *et al*. (2003) and Kurath *et al*. (2003) revealed three major genogroups, denoted with upper (U), middle (M) and lower (L), correlating with the geographic areas in the Pacific Northwest of North America. More recently, two additional genogroups for European and Asian isolates were identified (Enzmann *et al*., 2005; Nishizawa *et al*., 2006; Kim *et al*., 2007). Thus, a total of five genogroups correlating with geographic ranges has been identified among worldwide isolates of IHNV (Nishizawa *et al*., 2006; Kim *et al*., 2007).

IHNV is originally enzootic in the Pacific Northwest of North America, but was spread to Asian and European countries by the transportation of IHNV-contaminated fish or fish eggs (Kimura and Yoshimizu, 1991; Winton, 1991; Bootland and Leong, 1999). The original source of Japanese IHNV isolates was introduced from Alaska, USA in the 1970s, and subsequently spread to salmon farms throughout Japan due to the inadvertent transportation of IHNV-contaminated fish eggs (Kimura and Yoshimizu, 1991; Yoshimizu, 1996; Nishizawa *et al*., 2006). The introduced virus has adapted and evolved rapidly in Japanese rainbow trout

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Oncorhynchus mykiss farm environment since around 1980 (Nishizawa *et al*., 2006), and then part of the evolved IHNV in Japan have been transported to Korea in around 1990 (Kim *et al*., 2007). In the hatcheries of salmonid fish, stable production of IHNV-free fish has been accomplished by measures for health monitoring of mature fish and the prevention of IHNV transmission, such as disinfection of eggs with iodine, and disinfection of rearing water and facilities (Yoshimizu, 2003). However, it is still difficult to prevent horizontal transmission of IHNV through water flow in rainbow trout farms. Therefore, several efficacious IHNV vaccines have been developed, e.g. killed or attenuated vaccines (Winton, 1997; Biering *et al*., 2005), recombinant vaccines with IHNV G protein (Xu *et al*., 1991; Cain *et al*., 1999) and DNA vaccines with IHNV G gene (Anderson *et al*., 1996; Kim *et al*., 2000; Lorenzen *et al*., 2002).

In general, high pathogenicity of IHNV has a bias toward larvae and juveniles. However, losses due to IHN have occurred in adult and market sized rainbow trout in the past 20 years, suggesting that pathogenicity of Japanese IHNV seemed to have changed in the 1980s (Nishizawa *et al*., 2006). It was also revealed that G gene nucleotide (nt) sequence among Japanese IHNV isolates was diversified rapidly in rainbow trout farm environments (Nishizawa *et al*., 2006). Therefore, it is interesting how the evolutionary divergence influences viral pathogenicity, but this has not been elucidated scientifically. In the present study, we investigated how evolutional divergence has changed in Japanese IHNV isolates during the past 10 years by phylogenetic analysis with new seven isolates of the 2000s from rainbow trout in Shizuoka and Nagano prefectures. Moreover, four Japanese isolates being evolutionally diverged were selected for pathogenicity tests with rainbow trout to discuss virulence change of IHNV with viral evolution.

Materials and Methods

Viruses

Seven new isolates of Japanese IHNV, RtShiz06a, RtShiz06b, RtShiz06s, RtNag06a, RtNag06b, RtAichi06a and RtAichi06b, were used in this study. These new isolates and previously reported IHNV isolates in Japan and Korea were listed in Table 1. The first two letters of each isolate name indicate the host fish; i.e. Rt, Ch and Ko denote rainbow trout, chum salmon *O. keta* and kokanee salmon *O. nerka*, respectively. The following letters indicate the area of isolation; i.e. Nag, Aichi and Shiz denote Nagano, Aichi and Shizuoka prefectures in Japan, respectively. The numbers at the end of each

Table 1. List of Asian isolates of IHNV analyzed in the present study

Isolate	Host species	Year	Location		Reference
			Area	Country	
RtShiz06a	rainbow trout	2006	Shizuoka	Japan	The present study
	Oncorhynchus mykiss				
RtShiz06b	rainbow trout	2006	Shizuoka	Japan	
RtShiz06s	rainbow trout	2006	Shizuoka	Japan	
RtNag06a	rainbow trout	2006	Nagano	Japan	
RtNag06b	rainbow trout	2006	Nagano	Japan	
RtAichi06a	rainbow trout	2006	Aichi	Japan	
RtAichi06b	rainbow trout	2006	Aichi	Japan	
RtNag96	rainbow trout	1996	Nagano	Japan	Nishizawa *et al.* (2006)
AyTochi86	ayu	1986	Tochigi	Japan	
	Plecoglossus altivelis				
ChAb76	chum salmon	1976	Abashiri	Japan	
	Oncorhynchus keta				
ChYu78	chum salmon	1978	Yurappu	Japan	
RtNag82	rainbow trout	1982	Nagano	Japan	
RtNag76	rainbow trout	1976	Nagano	Japan	
KoMo71	kokanee salmon	1971	Mori	Japan	
	Oncorhynchus nerka				
RtToya80	rainbow trout	1980	Toya	Japan	
RtTochi86	rainbow trout	1986	Tochigi	Japan	
G4	rainbow trout	1992	Gifu	Japan	
RtPy91	rainbow trout	1991	Pyeongchang	Korea	Kim *et al.* (2007)
RtJe00	rainbow trout	2000	Jecheon	Korea	
RtGu01	rainbow trout	2001	Gumi	Korea	
RtUi02	rainbow trout	2002	Uiseong	Korea	

isolate designation indicate the year of viral isolation. IHNV ChAb76 isolated from chum salmon *O. keta* in Abashiri (Yoshimizu *et al*., 1989) has been used as a standard strain of Japanese IHNV in our laboratory. These Japanese isolates of IHNV within 10 passages of cell culture were stocked in –80°C until use for nt sequence analysis and pathogenicity test.

The viruses were propagated in epithelioma papillosum cyprini (EPC) cells, which were maintained at 15°C with Eagle's minimum essential medium (MEM, Gibco) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin G and 100 μg/mL streptomycin sulfate. Titration of viral infectivity was performed using with a 96-well microplates, seeded with EPC cells. After culture at 15°C for 10 days, the cytopathic effect (CPE) was evaluated to determine the 50% tissue culture infectious dose ($TCID_{50}$).

Sequence analysis

Viral genome RNA was extracted from the infected cells using an RNA extraction kit (Isogen, Nippon Gene) according to the manufacturer's instructions. The extracted viral genome was subjected to reverse transcriptase (RT)-PCR amplification for the G gene open reading frame (ORF) with primers HG (–31:–12) (5′-AGA ACG CAA CTC GCA GAG AC-3′) and HG (1602:1622) (5′-GTG GGG AGG AAG TGA AGA TTG-3′), which were designed by Nishizawa *et al*. (2006). For reverse transcription, extracted RNAs were preheated at 95°C for 5 min, and then incubated at 42°C for 30 min in 10 μL of PCR buffer (10 mM Tris-HCl, pH 8.3 and 50 mM KCl) containing 100 U of M-MLV reverse transcriptase (Takara), 1.0 μM of HG (–31:–12) primer, 1 mM of each dNTP and 5 mM of $MgCl_2$. After incubation at 99°C for 10 min, targeted 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), 0.2 mM of each dNTP and 1.5 mM of $MgCl_2$ using a thermal cycler (Astec PC-806) programmed for 1 cycle at 72°C for 10 min and 95°C for 2 min, then 30 cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and finally 72°C for 5 min. The amplified products were analyzed by 1.0% agarose-TAE (40 mM Tris-acetate, pH 8.0 and 1 mM EDTA) gel electrophoresis.

After being purified with a PCR purification kit (Stratagene), amplified products from viral genomes were subjected to nt sequence analysis using an ABI PRISM dye terminator sequencing chemistry (Applied Biosystems) with ID4 primer (5′-CTC TGG ACA AGC TCT CCA AGG-3′) (Miller *et al*., 1998) and the PCR primers, HG (–31: –12) and HG (1602:1622), according to the manufacture's instructions. Triplicate PCR products originating from independent RT reactions were sequenced for each isolate. The resulting sequences were assembled with the software DNASIS (Hitachi) to identify and exclude duplicate sequences from the data set. Based on a single representative of each sequence, which were registered with the DNA data bank of Japan (DDBJ) as accession numbers B510192-B510198, a multiple alignment of the sequences was constructed using Clustal X (Thompson *et al*., 1994; 1997) to infer genetic relationships among sequences

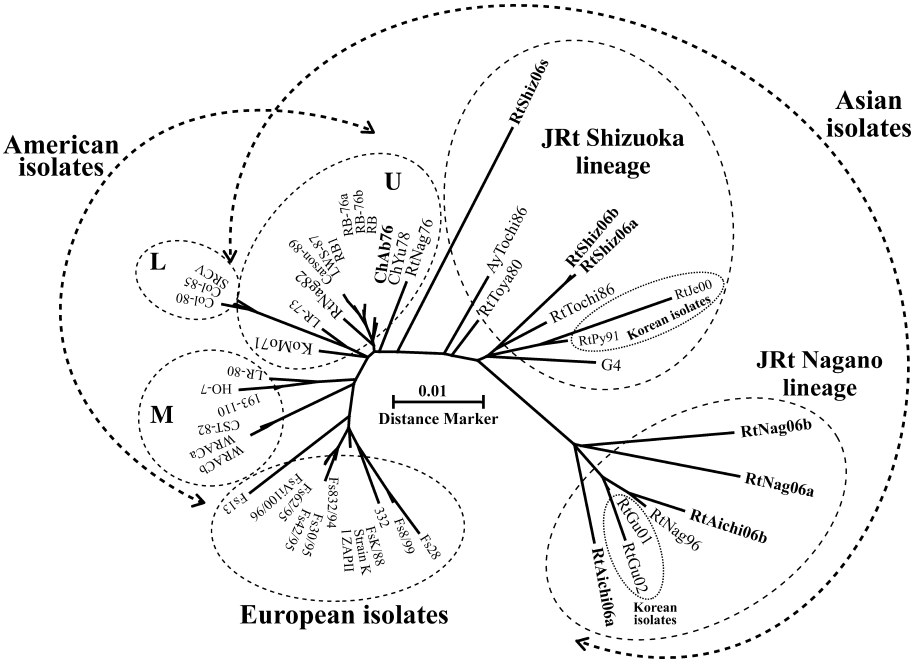

Fig. 1. Molecular phylogenetic tree showing the genetic relationships among 49 isolates of infectious hematopoietic necrosis virus (IHNV) based on nucleotide sequences of the glycoprotein gene open reading frame (ORF). The distance marker refers to the expected number of substitutions per site.

with neighbor joining criteria, and a final radial tree was drawn with the software NJplot and Unrooted (Perrière and Gouy, 1996). The G gene ORF nt sequences of 49 worldwide IHNV isolates indicated in Fig. 1 were used for comparative analyses. Deposited nt sequences of IHNV isolates in DDBJ were used for comparison purposes: ChAb76 (AB250927), ChYu78 (AB250928), KoMo71 (AB250929), RtNag76 (AB250930), RtNag82 (AB250931), RtNag96 (AB250932), AyTochi86 (AB250933), RtTochi86 (AB250934), RtToya80 (AB250935), RB (M16023), RB-76a (U15170), RB-76b (L40880), RB1 (U50401), LWS-87 (L40879), Carson-89 (L40872), LR-73 (L40877), SRCV (L40881), Col-80 (L40873), Col-85 (L40874), LR-80 (L40878), HO-7 (L40876), 193-11 (L40871), WRACa (L40883), WRACb (L40882), CST-82 (L40875), G4 (AF244128), StrainK (X73872), λ ZAPII (X89213), Fs13 (AY331658), FsVi-100/96 (AY331666), Fs62/95 (AY331664), Fs42/95 (AY-331663), Fs30/95 (AY331662), Fs832/94 (AY331661), FsK/88 (AY331665), Fs8/99 (AY331660), Fs28 (AY-331659), and 332 (AY331657) (Koener *et al.,* 1987; Gilmore and Leong, 1988; Kim *et al.,* 1994; Morzunov *et al.,* 1995; Nichol *et al.,* 1995; Schutze *et al.,* 1995; Enzmann *et al.,* 2005; Nishizawa *et al.,* 2006; Kim *et al*., 2007).

Virulence of IHNV isolates to rainbow trout

Five isolates of IHNV, RtShiz06s, RtShiz06a, RtNag06a, RtNag96 and ChAb76 representing JRt Shizuoka lineage, JRt Nagano lineage and genogoup U were used for the experimental challenge tests (Fig. 1). The experimental challenges were duplicated using rainbow trout juveniles with 0.8 g of mean body weight. Fish were divided into six tanks by 50 fish, respectively, and were challenged with the five different isolates of IHNV at 10^4 $TCID_{50}$/mL or with MEM_{10} (control) by bath exposure at 10°C for 60 min. After the virus challenge, fish in each tank were reared with flowing spring water at 10°C (72 cycles of water exchange per day), and fish mortalities were monitored daily for additional 21 days. During the experiments, the fish were satiated with a commercial dry-pellet diet.

Results and Discussion

PCR products with approximately 1.7 kb in size corresponding to the target region were amplified from seven isolates of Japanese IHNV used in the present study, and those amplified products were confirmed to contain a single ORF with 1,527 bases encoding 508 residues of amino acids for G protein (data not shown). Pairwise comparisons of nt diversities and a radial tree of phylogeny among Japanese and worldwide IHNV isolates based on nt sequences of G gene ORF was shown in Table 2 and Fig. 1, respectively. As described in the previous studies (Garver *et al*., 2003; Kurath *et al*., 2003; Enzmann *et al*., 2005; Nishizawa *et al*., 2006), five major genogroups correlating with geographical origins of the isolates were confirmed; three of the five genogroups were identified as upper (U), middle (M), lower (L) for North American isolates, the forth genogroup was for European isolates sharing a common source with the genogroup M, and the fifth genogroup denoted with JRt was for Asian isolates sharing a common source with the genogroup U.

Five Japanese isolates, KoMo71, ChAb76, ChYu78, RtNag76, RtNag82, obtained before around 1980, were located in the genogroup U, because sources of those isolates could be transferred from Alaska, North America with contaminated fish eggs (Nishizawa *et al*., 2006). The remaining isolates of Japanese IHNV obtained after 1980 up to 2006 constructed the genogroup JRt, and in which two lineages, JRt Shizuoka and JRt Nagano, appeared newly. The JRt Shizuoka lineage was composed of RtShiz06s, RtShiz06a, RtShiz06b, AyTochi86, RtToya80, RtTochi86, G4 and two Korean isolates (RtPy91, RtJe00), while the JRt Nagano lineage included RtNag96, RtNag06a, RtNag06b, RtAichi06a, RtAichi06b and two Korean isolates (RtGu01, RtUi02) (Fig. 1). The maximum nt diversity within the Shizuoka and Nagano lineages were 6.3% and 3.5%, respectively, and that within Japanese isolates were 7.0%, which were observed between RtShiz06s and RtNag06a (Table 2). Formerly, the maximum nt diversity within the Japanese iso-

Table 2. Pairwise comparison of genetic diversities among Japanese and worldwide isolates of IHNV based on nucleotide sequences of glycoprotein gene open reading frame

Isolate	Genogroup (lineage)	Range of nucleotide sequence diversity (%)						
		Japanese			North American			European
		JRt Shiz.	JRt Nag.	U	U	L	M	
Japanese	JRt Shizuoka	≤ 6.3	2.7–7.0	1.6–4.6	1.8–4.5	3.0–5.3	2.8–5.1	2.6–5.7
	JRt Nagano		≤ 3.5	3.7–5.8	3.8–5.7	4.9–6.5	4.7–6.4	4.6–7.0
	U			≤ 1.6	0.5–1.7	2.0–2.7	1.5–2.7	1.5–3.2
North American	U				≤ 1.5	1.8–2.7	1.8–2.4	1.6–3.2
	L					≤ 0.7	3.0–3.6	2.7–4.0
	M						≤ 2.8	1.6–3.3
European								≤ 3.0

lates collected before 1996 was 4.5% (Nishizawa *et al*., 2006), and it was confirmed that the nt diversity within Japanese isolates increased by 2.5% for the past 10 years, indicating that Japanese IHNV still continued to evolve rapidly in Japanese rainbow trout farm environments. The nt diversity between RtShiz06s and RtNag06a was 7.0%, while those between ChAb76 and RtShiz06s or RtNag06a were 3.7% and 5.3%, respectively. Moreover, the isolation of RtShiz06s and RtNag06a were 30 years later from that of ChAb76. It was thus calculated that RtShiz06s and RtNag06a might be diverged in around 1986, which coincide with when pathogenicity of Japanese IHNV to a big size of rainbow trout seemed to have changed. This data strongly support the previous presumption that source of Japanese IHNV introduced into Japan in the early 1970s was settled and evolved rapidly at rainbow trout farm environments (Nishizawa *et al*., 2006), and as a result of this, the diverged two lineages correlating with the geographical ranges, JRt Shizuoka and JRt Nagano lineages, are considered to appear in Japanese farm environments (Fig. 1). Watersheds of Shizuoka and Nagano prefectures were completely separated by mountain range, but rainbow trout surviving IHN have been transferred frequently between farms of both prefectures. It is entirely surprising that JRt Shizuoka and Nagano lineages have been preserved, suggesting that the settled IHNV in each watershed might evolve stably without influence of the transferred IHNV.

As mentioned above, pathogenicity of Japanese IHNV seemed to be changed in the 1980s, and it was confirmed that Japanese IHNV still continue to evolve in rainbow trout farm environments. It was thus interesting how the evolutional divergence within Japanese IHNV isolates influenced their virulence in rainbow trout (Nishizawa *et al*., 2006). In the next experiments, we selected five isolates of Japanese IHNV, RtShiz06s and RtShiz06a, RtNag96 and RtNag06a, and ChAb76, representative of the JRt Shizuoka lineage, JRt Nagano lineage, and the genogroup U, respectively, for their pathogenicity tests with rainbow trout (Fig. 2). In the experiment A, the challenged fish began to die at 7–12 days after virus exposure, and cumulative mortalities of fish challenged with RtShiz06s, RtShiz06a, RtNag96, RtNag06a and ChAb76 were 92%, 86%, 44%, 30% and 10%, respectively (Fig. 2A). The similar tendency was reproduced by the experiment B, i.e. cumulative mortalities of fish challenged with RtShiz06s, RtShiz06a, RtNag96, RtNag06a and ChAb76 were 86%, 76%, 44%, 20% and 0%, respectively (Fig. 2B). No mortality was observed in control groups of both experiment A and B. Furthermore, in different challenge tests with rainbow trout (14 g of mean body weight), cumulative mortality of fish challenged with RtShiz06s at 10^5 $TCID_{50}$/fish was 72%, but that with ChAb76 at the same dose was zero (data not shown). These distinct differences observed in virulence of Japanese IHNV isolates against rainbow trout, i.e. the strongest virulence was in RtShiz06s and RtShiz06a belonging to JRt Shizuoka lineage, and subsequently RtNag96 and RtNag06a of JRt Nagano lineage, but scarcely any virulence in ChAb76 belonging to genogroup U, suggest that virulence of Japanese IHNV seems to have increased over the 30 years. Similar change of IHNV virulence to rainbow trout was also observed in North American isolates (Troyer *et al.*, 2000). The virulence change of Japanese isolates was not correlated with divergence of G gene, because nt distances from ChAb76 to low-virulent isolates, RtNag96 and RtNag06a (4.3% and 5.3%, respectively), were farther than those from ChAb76 to high-virulent isolates, RtShiz06s or RtShiz06a (3.7% and 3.3%, respectively). And as this cause, we speculate that the change of IHNV virulence could be regulated not only by G protein, a major protective antigen.

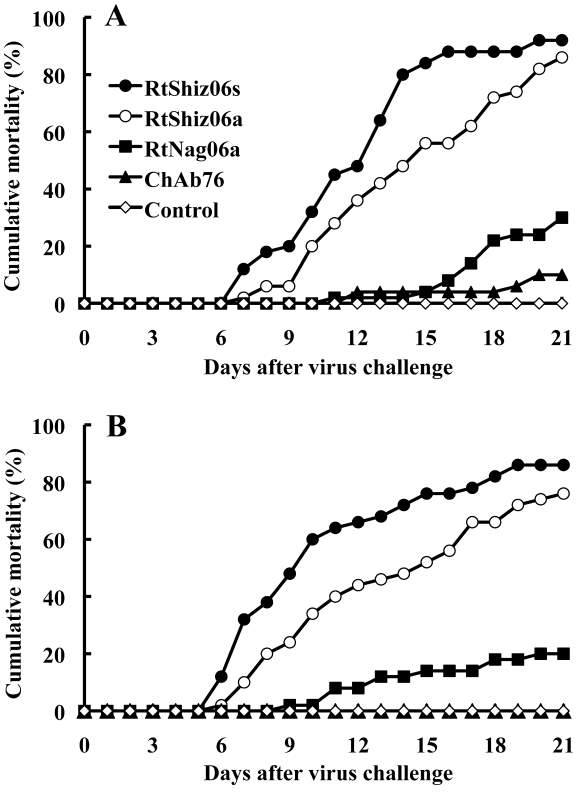

Fig. 2. Time dependent change of cumulative mortality of rainbow trout challenged by bath exposure with RtShiz06s, RtShiz06a, RtNag96, RtNag06a, ChAb76 and MEM10 (control). The experimental challenges were duplicated in experiments A and B.

In pathogenicity tests performed in the 1980s, ChAb76 and RtNag76 showed more than 70% of cumulative mortalities to masu salmon and rainbow trout, respectively (data not shown). ChAb76 was also considered to be virulent to rainbow trout, because it was almost identical (nt diversity: 0.7%) with RtNag76 (Fig. 1). However, low or no virulence to rainbow trout was

observed in ChAb76 in the present results (Fig. 2). The ChAb76 used in the present test has been stored in –80°C until use, in order to save culture-passages within 10 times expecting that no change in virulence of ChAb76 during the storage occurs. Therefore, it is considered that the absence or lowness of ChAb76 virulence to rainbow trout in the present experiments (Fig. 2) could be caused in host fish factor, for example decreasing of fish susceptibility to IHNV by natural selection of survivors in IHN during past a quarter of a century ago. Actually, surviving rainbow trout from IHN were commonly selected to culture in Japanese farms because efficacious vaccine for IHN has not been marketed in Japan.

In conclusion, the present study demonstrated that nt diversity of Japanese IHNV still continued with changing its virulence in rainbow trout farm environments. Rainbow trout seemed to be naturally selected to be resistant to IHNV, but IHN in rainbow trout farms is still in endemic, suggesting that the diversification of Japanese IHNV may be promoted by the conventional method repeating natural selection of surviving fish from IHN.

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