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Evidence for two koi herpesvirus (KHV) genotypes in South Korea

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ABSTRACT: The geographic distribution of koi herpesvirus (KHV) has recently been analyzed by polymerase chain reaction (PCR, based on the alleles of 3 domains) and sequence analysis using 3 regions of KHV genomic DNA (*Sph*I-5, 9/5, and the thymidine kinase gene). In this study, samples from 6 carp showing symptoms of KHV infection in 2008 were examined for the presence of KHV by using PCR and cell culture isolation methods. KHV was detected in 2 (Pyeongtaek and Buan) of the samples. Sequence analysis revealed that the genotype of the KHV PT-08 isolate was Asia genotype variant 1 (A1), and the genotype of the KHV BA-08 isolate was European genotype variant 4 (E4). In addition, PCR patterns and sequence analysis based on the alleles of 3 domains of an alternate KHV classification system confirmed that the genotype of the KHV PT-08 isolate was CyHV3-J, and the genotype of the KHV BA-08 isolate was CyHV3-third genotype. To our knowledge, this is the first study to demonstrate the presence of 2 genotypes of KHV (genotype A1/CyHV3-J; genotype E4/CyHV3-third genotype) in South Korea.

KEY WORDS: Koi herpesvirus · Genotype · Sequencing analysis · Korea

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INTRODUCTION

Koi herpesvirus (KHV) is a viral pathogen responsible for mass mortality among cultured cyprinids (Hedrick et al. 2000). Currently, KHV disease (KHVD) is included on the list of contagious diseases reportable to the World Organization of Animal Health (Office International des Epizooties, OIE 2009) because of its potentially harmful effects on the aquaculture industry (www.oie.int/fileadmin/Home/eng/Health_standards/aahm/2010/2.3.06_KHVD.pdf). KHV was first isolated from cultured fish in Israel and the US in 1998 (Hedrick et al. 2000). Since that time, KHV has been detected in Germany (Neukirch & Kunz 2001) and again in Israel (Perelberg et al. 2003, Ronen et al. 2003), and more recently in South Africa and the European Union countries (Haenen et al. 2004), Indonesia (Yuasa 2004, Sunarto et al. 2011), Japan (Sano et al. 2004), and Southeast Asian countries (Kurita et al. 2009).

The diagnostic methods for KHV disease have been reported previously (OIE 2009) and rely on antibody-based virus detection, DNA probe *in situ* hybridization, antibody detection, virus isolation in cell culture, polymerase chain reaction (PCR), and sequencing analysis. The OIE (2009) recommends that PCR and nucleotide sequence analysis of the PCR products are used to confirm the identity of KHV. The genetic relationship between KHVs detected in Europe and Asia has recently been analyzed by PCR and sequencing using 3 regions of KHV genomic DNA: *Sph*I-5, 9/5, and the thymidine kinase gene (Kurita et al. 2009). In addition, another genetic relationship system for KHV genotypes based on the alleles of 3 domains, 2 within Marker I (using a primer set between open reading frame [ORF] 29 and ORF 31) and 1 within Marker II (target-

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ing upstream and within the ORF133 region), has been proposed (Bigarré et al. 2009, Sunarto et al. 2011).

Herpes-like viral infections have been described in cultured common carp *Cyprinus carpio* in Korea in 1998, although the causative agent was not identified (Oh et al. 2001, Choi et al. 2004). The causative agent was finally identified as KHV in Korea in 1998 by using an *in situ* hybridization method (Lee et al. 2012). In 2010, a case of early-stage KHV infection was reported in a koi (*C. carpio koi*) broodstock commercial farm in South Korea (Gomez et al. 2011), and the infection was subsequently monitored in 2010 (Cho et al. 2011).

In the present study, samples from 6 carp that showed clinical signs of KHV infection in 2008 were examined for the presence of KHV using PCR and cell culture isolation methods. KHV was detected in 2 of the 6 samples, and the genotypes of these 2 KHV isolates were subsequently determined using PCR and nucleotide sequencing analysis.

MATERIALS AND METHODS

Fish

Diseased carp were collected in 2008 from 6 carp farms located in Pyeongtaek, Buan, Jeongeup, and Jeonju in South Korea. The common findings exhibited by the fish were severe gill lesions, which manifested as gill mottling with white patches. The gills were collected in 50 ml Corning tubes and were stored at −80°C until use.

Detection of KHV DNA

DNA was extracted from the gills using a DNeasy Tissue Extraction Kit (Qiagen) according to the manufacturer's instructions. The extracted samples were stored at −80°C until they were used for PCR analysis for KHV DNA. The Bercovier TK primer set (forward, 5′-GGG TTA CCT GTA CGA G-3′; reverse, 5′-CAC CCA GTA GAT TAT GC-3′) was used to amplify the KHV gene (OIE 2009) in a 25 µl PCR mixture containing 10 pmol of each primer, 0.2 mM dNTP, 2 mM $MgCl_2$, and 1.25 U Ex-Taq DNA polymerase (TaKaRa). The PCR protocol was as follows: initial 5 min step at 94°C; 40 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C; and a final extension step of 10 min at 72°C. The amplified DNA was separated on 1.5% agarose gels containing ethidium bromide (0.5 µg ml^{-1}; Sigma), and the bands were visualized using a Gel Doc UV transilluminator (Bio-Rad Laboratories).

Virus isolation

The Koi fin-1 (KF-1) cell line was cultured using L-15 (Gibco Life Technologies) supplemented with 10% fetal bovine serum, 100 IU ml^{-1} penicillin G, and 100 µg ml^{-1} streptomycin sulfate. The cells were grown at 20°C for 2 wk and then seeded in 24-well tissue culture plates. The fish gills used in PCR analysis were homogenized with 9 volumes of Hank's balanced salt solution (HBSS; Invitrogen) containing 10× concentration of antibiotic-antimycotic solution (Gibco Life Technologies) and then centrifuged at 2000 × *g* (10 min at 4°C). The supernatants were filtered using a filter with a pore size of 0.45 µm (Millipore). The fluids were inoculated onto the KF-1 cells seeded in 24-well tissue culture plates, and the plates were then incubated at 20°C for observation of cytopathic effects (CPEs).

PCR for genotype analysis

The 3 genomic regions (*Sph*I-5, 9/5, and the thymidine kinase [TK] gene) and the variable regions of KHV (between ORF29 and ORF31, Marker I; ORF133, Marker II) were amplified according to the methods described by Kurita et al. (2009) and Bigarré et al. (2009), respectively. DNA was amplified using *Sph*I-5, 9/5, and TK gene primer sets in 25 µl PCR mixtures containing 10 pmol of each primer, 0.2 mM dNTP, 2 mM $MgCl_2$, and 1.25 U Ex-Taq DNA polymerase. The common PCR procedure included an initial denaturation step at 94°C for 30 s; 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min; and a final extension step lasting 7 min at 72°C. DNA was also amplified using Marker I or Marker II primer sets in 25 µl PCR mixtures containing 10 pmol of each primer, 0.2 mM dNTP, 2 mM $MgCl_2$, and 1.25 U Ex-Taq DNA polymerase. The common PCR procedure included an initial denaturation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 20 s; and a final extension step lasting 5 min at 72°C. Amplified DNA was separated on 1.5% agarose gels containing 0.5 µg ml^{-1} ethidium bromide (Sigma), and the bands were visualized using a Gel Doc UV trans-illuminator.

Cloning and nucleotide sequence analysis

Amplified PCR products were purified using a spin-column PCR purification kit (Promega) and cloned into the pGEM-T Easy vector. Plasmid DNA from the recombinant clones was extracted using a plasmid DNA extraction kit (Qiagen), and positive clones were selected using PCR, as described above. Inserts were sequenced using an ABI377 sequencer (Applied Biosystems) with T7 and SP6 primers. The resulting sequences were compared with the BLAST database (National Center for Biotechnology Information).

RESULTS

Gene detection and isolation of KHV

The KHV TK gene was detected in 2 (from Pyeongtaek and Buan samples) of the 6 carp that showed clinical signs of KHV infection in 2008, and by CPE in the KF-1 cell line (data not shown). The 2 isolates from Pyeongtaek and Buan were named PT-08 and BA-08, respectively. The locations from which the samples were obtained and characteristics of the 2 isolates are provided in Fig. 1 and Table 1.

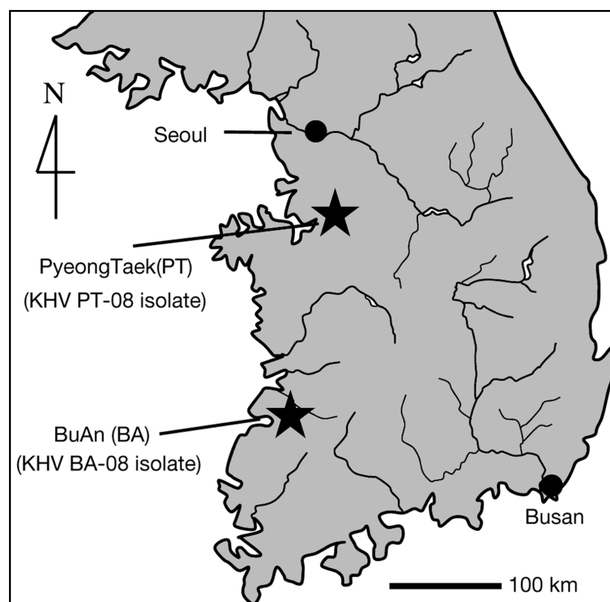

Fig. 1. Locations in Korea from which KHV-positive samples were obtained for this study

Gene sequence analysis of KHV genes (9/5 region, *Sph*I-5, and enlarged TK) in the PT-08 and BA-08 isolates

The DNA sequences of the KHV PT-08 isolate from koi revealed that the nucleotides (nts) at positions 184–187 and 212–218 of the 9/5 region were TTT T and AAA AAA, respectively. When the *Sph*I-5 region was examined, C was detected at nt 209, while AAC was not observed at nts 586–588. In the enlarged TK gene region, C, A, AA, TTT TTT T, CTT TAA AAA AAA, and AGA TAT T were detected at nts 94, 778, 849/850, 877–885, 945–956, and 961–967, respectively, while AT and CA were not observed at nts 813–814 and 957–958, respectively (Table 2). These results indicate that the KHV PT-08 isolate belongs to Asia genotype variant 1 (A1) according to the method described by Kurita et al. (2009).

The DNA sequences of the KHV BA-08 isolate from common carp revealed that nts 184–187 of 9/5 were TTT T and nts 212–218 were AAA AAA A (Table 2). C was detected at nt 209 in the *Sph*I-5 region, whereas AAC was detected at nts 586–588 (Table 2). In addition, the sequences at nts 94, 778, 813–814, 849/850, 877–885, 945–956, 957–958, and 961–967 in the enlarged TK gene region were C, G, AT, AA, TTT TTT TTT, CTT TAA AAA AAA, CA, and AGA TAT T, respectively (Table 2). These results indicate that the KHV BA-08 isolate belongs to European genotype variant (E4) according to the method described by Kurita et al. (2009).

Analysis of KHV genes based on the alleles of 3 domains in the PT-08 and BA-08 isolates

The 168 bp (Fig. 2, lane 1) and 153 bp (Fig. 2, lane 2) PCR products were amplified using the Marker I PCR primer set with DNA from the PT-08 and BA-08 isolates, respectively. The DNA sequences of the Marker I PCR product from the KHV PT-08 isolate revealed the sequences GCA GCC TCA ACC CCG GC and GAG TCG TCC ACG ATG

Table 1. Origins of 2 KHV isolates collected from fish farms in South Korea

Isolate	Sampling location	Material	Date
KHV PT-08	Pyeongtaek (PT)	Koi (gills)	15 June 2008
KHV BA-08	Buan (BA)	Common carp (gills)	08 June 2008

Table 2. Genotypes/variants of KHV samples from various countries by sequence analysis. Isolates investigated in this study are shown in bold print. TK: thymidine kinase

Genotype /variant	Country	Enlarged 9/5 region 184–187	Enlarged 9/5 region 212–218	Enlarged *Sph* I-5 region 209	Enlarged *Sph* I-5 region 586–588	Enlarged TK gene region 94	778	813–814	849–850	877–885	945–956	957–958	961–967	Source
A1	Japan, Taiwan, Philippines, Indonesia	TTTT	AAAAAA	C	–	C	A	–	AA	TTTTTTT	CTTTAAAAAAAA	–	AGATATT	Kurita et al. (2009)
	Korea, (KHV PT-08)	**TTTT**	**AAAAAA**	**C**	**–**	**C**	**A**	**–**	**AA**	**TTTTTTT**	**CTTTAAAAAAAA**	**–**	**AGATATT**	**This study**
A2	Taiwan, Indonesia	TTTT	AAAAAA	C	–	C	A	–	AA	TTTTTTTT	CTTTAAAAAAAA	–	AGATATT	Kurita et al. (2009)
E1	USA, Netherlands	TTTT	AAAAAAA	C	AAC	C	G	AT	-	TTTTTTTTT	CTTTAAAAAAAA	CA	AGATATT	Kurita et al. (2009)
E2	Netherlands	TTTT	AAAAAAA	T	AAC	C	G	AT	-	TTTTTTTTT	CTTTAAAAAAAA	CA	AGATATT	
E3		–	AAAAAAA	C	AAC	C	G	AT	-	TTTTTTTTT	CTTTAAAAAAAA	CA	AGATATT	
E4	Netherlands	TTTT	AAAAAAA	C	AAC	C	G	AT	AA	TTTTTTTTT	CTTTAAAAAAAA	CA	AGATATT	
	Korea, (KHV BA-08)	**TTTT**	**AAAAAAA**	**C**	**AAC**	**C**	**G**	**AT**	**AA**	**TTTTTTTTT**	**CTTTAAAAAAAA**	**CA**	**AGATATT**	**This study**
E5	Netherlands	TTTT	AAAAAAA	C	AAC	C	G	AT	-	TTTTTTTTT	–	–	–	Kurita et al. (2009)
E6	Israel	TTTT	AAAAAAA	C	AAC	T	G	AT	-	TTTTTTTTT	CTTTAAAAAAAA	CA	AGATATT	
E7	England	TTTT	AAAAAAA	C	AAC	C	G	AT	-	TTTTTTTT	CTTTAAAAAAAA	CA	AGATATT	

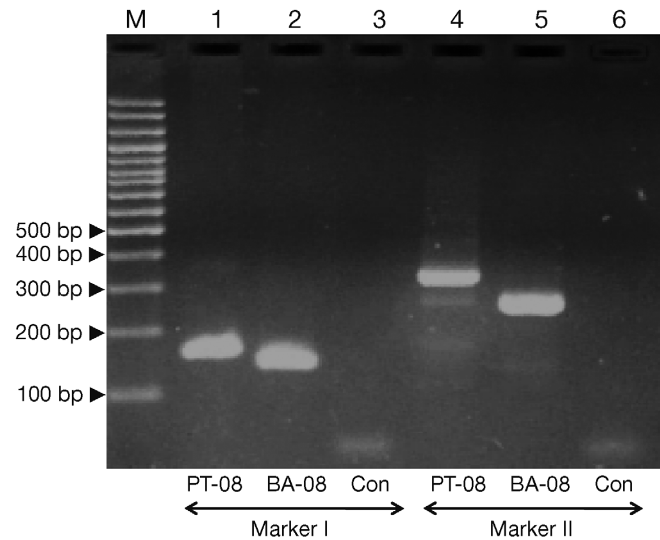

Fig. 2. Agarose gel electrophoresis of PCR products for the Marker I primer set (lanes 1, 2, and 3) and Marker II primer set (lanes 4, 5, and 6). Lanes 1 and 4: Isolate PT-08; Lanes 2 and 5: Isolate BA-08; Lanes 3 and 6: DNA sample from healthy carp as controls (con)

GTG ACA CC at nts 16–32 and positions 82–104, respectively (Fig. 3). However, the DNA sequences of Marker I from the KHV BA-08 isolate revealed nts at positions 82–104, while the nts were not observed at positions 16–32 (Fig. 3).

In addition, the PCR product of full-length 352 bp (Fig. 2, lane 4) DNA of the PT-08 isolate was observed in the Marker II region, while a PCR product of 278 bp (Fig. 2, lane 5) containing a 74 bp deletion was observed in the BA-08 isolate.

Bigarré et al. (2009) used (+) and (–) symbols to indicate the presence and absence of each of the 3 variable domains, respectively. Thus, the genotype allele of PT-08 isolate was confirmed as $I^{++}II^{+}$, because 168 bp (Fig. 2, lane 1) and 352 bp (Fig. 2, lane 4) were detected by PCR using Marker I and II primer sets, respectively. Additionally, the genotype allele of isolate BA-08 was confirmed as $I^{-+}II^{-}$, because 153 bp (Fig. 2, lane 2) and 278 bp (Fig. 2, lane 5) were detected by PCR using Marker I and II primer sets, respectively. Thus, it was confirmed that the PCR patterns of PT-08 and BA-08 isolates are $I^{++}II^{+}$ and $I^{-+}II^{-}$, respectively (Fig. 3).

DISCUSSION

Recently, Lee et al. (2012) reported that the mass mortality of carp observed in 1998 was associated with KHV infection, and the virus was introduced

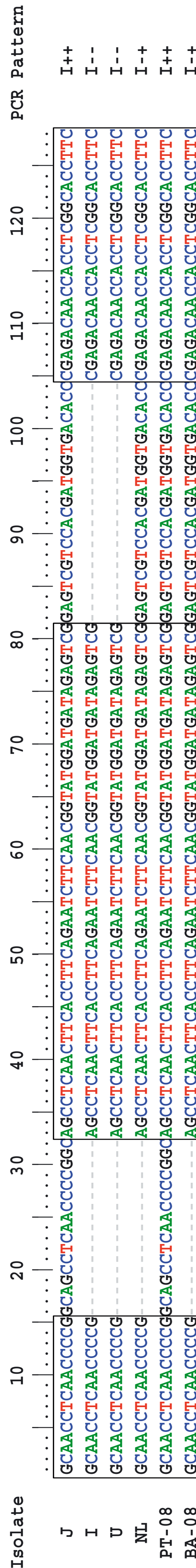

Fig. 3. Alignment and classification of Marker I alleles in this study. The alignment includes sequences of the 2 isolates PT-08 and BA-08. J: Japan, I: Israel, U: US, NL: Netherlands

into carp farms in South Korea in 1998 or earlier. However, the identification of KHV was not reported until 2010 in South Korea (Cho et al. 2011). In the present study, samples from 6 carp showing gross lesions consistent with KHV infection in 2008 were examined by PCR using a KHV TK primer set and virus isolation. KHV was detected by PCR in 2 samples (PT-08 and BA-08), and by CPE in the KF-1 cell line (data not shown). These results confirmed the presence of KHV in the carp fisheries of South Korea in 2008 (Fig. 1).

For genotype analysis, the DNA sequences of the KHV PT-08 isolate from koi are displayed in Table 2 where they are compared with other known isolates. The total nucleotide sequences of the *Sph*I-5, 9/5, and TK genes of the KHV PT-08 isolate revealed 100 % identity with KHV genes (GenBank accession numbers: AB375381, AB375384, and AB375390, respectively). Thus, the genotype of the KHV PT-08 isolate in this study was found to be Asia genotype variant 1 (A1), thereby confirming the presence of the Asian type of KHV in South Korea. Additionally, the allele of PT-08 isolate was also confirmed as I^{++} II^{+}, specifically, the CyHV3-J genotype in Asia. The geographical distribution of the A1 genotype has previously been reported by Kurita et al. (2009) to include Japan, Taiwan, and Indonesia. In fact, all koi were imported to South Korea from Japan until 2008; therefore, the KHV PT-08 strain may have come from Japan, although evidence for this hypothesis is circumstantial.

Despite the geographical location in Asia, the genotype of the KHV BA-08 isolate from common carp in South Korea was revealed to be a European genotype variant (E4), according to the method described by Kurita et al. (2009). The total nucleotide sequences of the KHV BA-08 *Sph*I-5, 9/5, and TK genes revealed 100 % identity with KHV genes (GenBank accession numbers AB375379, AB375382, and AB375387, respectively). In addition, the PCR pattern of the BA-08 isolate was confirmed as $I^{-+}II^{-}$. This result indicates that the isolate is related to another genotype distinct from the CyHV3-I, U, and J genotypes. It was reported that KHV strains in the Netherlands included several genotypes (E1–E5) and several alleles ($I^{--}II^{-}$, CyHV3-U/I genotype; $I^{++}II^{+}$, CyHV3-J genotype; $I^{-+}II^{-}$, third genotype; Bigarré et al. 2009, Kurita et al. 2009). Thus, it was confirmed that our BA-08 isolate was an identical genotype to one of the Netherland isolates (E4 genotype variant and third genotype allele). Until 2008, common carp were not imported from European countries including the Netherlands; rather, most common carp were

imported from China. However, there is no evidence for KHV transfer from China, so the virus may have been introduced by fish from a third country or fish purchased by travelers. KHV inspection has been conducted internationally since 2004 (Haenen et al. 2004), and KHV has been listed by the OIE as a reportable disease since 2006 (Taylor et al. 2010). Therefore, the spread of the disease associated with this virus was not internationally controlled before 2004.

In South Korea, KHV quarantine for import or export of carp was conducted under the purview of the Quality Control of Fishery Products Act until 21 December 2008. In the case of export, carp were inspected for KHV only at the request of recipient nations. Since 22 December 2008, the Aquatic Animal Diseases Control Act has been enacted and enforced in South Korea, applicable to all fish-related infectious diseases listed by the OIE. The South Korean government conducts disease surveillance and carries out preventive activities in affected areas in South Korea under the purview of this Act. Specifically, carp should be exported after biannual monitoring and confirmed to be in a disease-free state (Cho et al. 2011). Therefore, enhanced surveillance and control measures are expected to have a positive effect in controlling infectious disease, including KHV, in South Korea.

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