Arch Virol (2015) 160:493–498
DOI 10.1007/s00705-014-2264-3

BRIEF REPORT

The prevalence of duck hepatitis A virus types 1 and 3 on Korean duck farms

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Received: 16 August 2014 / Accepted: 20 October 2014 / Published online: 31 October 2014

Abstract This study reports the prevalence of duck hepatitis A virus (DHAV) types 1 and 3 on Korean duck farms. By RT-nested PCR assays specific for DHAV-1 or DHAV-3, DHAV-1 was detected in 9 of 157 liver samples (5.7 %) from 2 of 30 farms (6.7 %), and DHAV-3 was positive in 104 of 157 liver samples (66.2 %) from 23 of 30 farms (76.7 %). Dual infections with DHAV-1 and DHAV-3 were detected in 23 of 157 samples (14.6 %) from 5 of 30 farms (16.7 %). The data indicate that DHAV-3 infections are prevalent and that DHAV-1 reemerged in Korea, resulting in dual infections on several farms. Our data will help to establish a vaccination policy against DHAV-1 and DHAV-3 in Korea.

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Electronic supplementary material The online version of this article (doi:10.1007/s00705-014-2264-3) contains supplementary material, which is available to authorized users.

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Keywords Korea · DHAV-1 · DHAV-3 · Prevalence · Dual infection

Duck hepatitis A virus (DHAV), which belongs to the novel genus *Avihepatovirus* in the family *Picornaviridae* [1], causes a frequently fatal, contagious and rapidly spreading disease in domestic ducklings that is characterized primarily by hepatitis [2]. Based on phylogenetic and neutralization assays, DHAVs had been classified into three different sero- or genotypes: the original worldwide type DHAV-1 [3–5], the recently identified DHAV-2 in Taiwan [6], and the recently identified DHAV-3 in South Korea and China [2, 7, 8]. DHAV-1 and DHAV-2 have no cross-neutralization activity [9], whilst DHAV-1 and DHAV-3 show limited cross-neutralization activity with each other [8].

In South Korea, the occurrence of DHAV was first reported in 1985 [10]. Since then, the isolation and identification of DHAV-1 was reported in 2000 [11], and its attenuated vaccine was commercialized during the same year [12]. Although vaccination against DHAV-1 was performed in the 2000s, DHAV-like diseases have still occurred even on DHAV-1-vaccinated farms [8, 13]. Therefore, Korean duck farmers have preferred not to perform vaccination against DHAV-1. Subsequently, DHAV-3 strains were isolated and identified in Korean ducklings during 2003 and 2004 [8], which explains why vaccination against DHAV-1 in the past failed to protect against DHAV infections. During 2005 and 2006, DHAV-3, but not DHAV-1, was detected in ducklings from eight Korean commercial duckling flocks [14]. More recently, live attenuated DHAV-3 vaccine has been developed in South Korea [13].

Statistics for 2012 from the South Korean Ministry of Agriculture, Food and Rural Affairs documented that

approximately 4.89 million ducks in South Korea (44 %) are reared in Jeollanam-do Province. Since DHAV infections have been occurring frequently in South Korea, DHAV infections are believed to be one of the most devastating diseases in the Korean duck industry [4, 13, 14]. Despite the impact of DHAVs as economically important pathogens, there is a paucity of data on the current prevalence of DHAV-1 and DHAV-3 in single or in dual infections. Prior to performing vaccination nationwide with monovalent or bivalent vaccines, the exact prevalence of DHAV-1 and DHAV-3 infections on Korean duck farms should be determined. This prompted us to investigate the prevalence of DHAV-1 and DHAV-3 infections in South Korea. This paper reports the prevalence of DHAV-1 and DHAV-3 in either single infections or simultaneous infections with both types on duck farms in Jeollanam-do Province during 2012–2013.

From May 2012 to December 2013, 157 dead ducklings (4 to 21 days old) from 30 duck farms located in eight counties of Jeollanam-do Province were submitted to Jeollanam-do Livestock Sanitation Office for diagnosis (Supplementary Table 1). These ducklings showed characteristic clinical signs and gross lesions associated with DHAV infections, including lethargy, spasmodic movements, opisthotonos, and petechial and ecchymotic hemorrhages in their enlarged livers. These farms had not undergone vaccination against DHAV-1. The mortality on each farm at the time of submission varied from 0.25 % to 90.0 %, depending on the age of the birds and the duration of disease (Supplementary Table 1).

For viral RNA extraction, liver specimens sampled from 157 dead ducklings were homogenized in sterile 0.01 M phosphate-buffered saline (PBS, pH 7.2) using a tissue homogenizer (Percellys 24, Bertin Technologies, Seoul, Korea) and centrifuged at 14,000 × g for 5 min at 4°C. Afterwards, the supernatant was collected and stored at −80 °C until used. The viral RNA was extracted from a 200-µl starting volume of the supernatant of liver homogenate using an AccuPrep® Viral RNA Extraction Kit (Bioneer, Daejeon, Korea). The total RNA recovered was suspended in 50 µl of RNase-free water and was immediately used or stored at −80 °C until used.

A standard one-step RT-PCR assay was performed as described previously [15] using specific oligonucleotide primers that were designed from the published sequences of DHAV-1 and DHAV-3 genomes in the GenBank database. The primer pair for RT-PCR targeting 755 bp of DHAV-1 encompassed the 3' end of the VP3 region (starting from nucleotide 2075) and the 5' end of the 2A1 region (ending at nucleotide 2830) (Table 1 and Supplementary Fig. 1). The RT-PCR primer pair for DHAV-3 was designed to amplify 737 bp starting from the 3' end of the VP3 region (nucleotide 2098) and ending within the VP1 region (nucleotide 2835) (Table 1 and Supplementary Fig. 1). To increase the sensitivity and specificity of the RT-PCR, nested PCR was performed with primer pairs that were specific for sites within the first RT-PCR product (Table 1 and Supplementary Fig. 1) [15].

To obtain genomic data, RT-PCR products for a portion of the DHAV-3 VP1 region (737 bp) and nested PCR products for a portion of the DHAV-1 VP1 region (339 bp) were purified using a PureLink Quick Gel Extraction Kit (Invitrogen, Karlsruhe, Germany). Purified PCR products were ligated into pTOP TA V2 vectors using a TOPcloner TA kit (Enzynomics, Daejeon, Korea) and subcloned into DH5α competent cells (Enzynomics). Plasmids were purified using GeneAll Hybrid-Q Plasmid Rapidprep (GeneAll, Seoul, Korea) and sequenced using an ABI system 3700 automated DNA sequencer (Applied Biosystems, Foster City, USA).

Using the DNA Basic Module (DNAsis Max, Almaeda, USA), the obtained nucleotide and deduced amino acid sequences of the VP1 region were compared with those of other known DHAV strains or isolates available in GenBank (Supplementary Table 2). Phylogenetic analysis was conducted using the neighbor-joining method with 1000 bootstrapping replicates and the unweighted group method with the arithmetic mean method in the Molecular Evolutionary Genetics Analysis Version 5.1 (MEGA 5.1) program [16]. The evolutionary distances were compared by the pairwise distance method using the maximum composite likelihood model [16]. A sequence similarity search was performed for the VP1 region of DHAV-1 and DHAV-3 using the homology and distance matrix method in the DNAMAN version 6.0 program (Lynnon, Vaureuil, Canada).

Knowledge of the exact prevalence of DHAV-1 and DHAV-3 infections is a key to establishing control measures. By RT-PCR assay with a primer pair specific for DHAV-1, targeting a 755-bp fragment of the DHAV-1 VP1 region, 3 of 157 liver samples (1.9 %) from 2 of 30 farms (6.7 %) were found positive for DHAV-1 (Table 2). The nested PCR assay, targeting a 339-bp fragment of the DHAV-1 VP1 region, was performed, and 9 of 157 samples (5.7 %) from 2 of 30 farms (6.7 %) were positive (Table 2). DHAV-3 infections were detected in 66 of 157 samples (42.0 %) and 104 of 157 samples (66.2 %) from 23 of 30 farms (76.7 %) by the VP1-region-specific RT-PCR (737 bp) and nested PCR (353 bp) assay, respectively (Table 2). These results indicate that single infections of DHAV-3 prevailed on duck farms in Jeollanam-do Province, South Korea. In a previous report [14], DHAV-3 single infections were found, and DHAV-1 was not detected during 2005 and 2006 in South Korea. However, our data indicate that DHAV-1 reemerged on Korean duck farms. Interestingly, dual infections of DHAV-1 and

Table 1 Oligonucleotide primers used for the detection or sequencing of duck hepatitis A virus (DHAV)

Genotype	Purpose	Target gene[a]	Primer sequence (5'-3')[b]	Region (nt)	Size (bp)	Annealing T_m (°C)	Source
DHAV-1	Detection	VP1	F:TATGGAAATTTGCAGATGGCA	2075-2830	755	48	This study
			R:ATTTGGTCAGATTCAATTTCCA				
	Detection & sequencing		nF: ACACCAGCAATGGTAGCACA	2365-2704	339	50.4	This study
			nR: CTACTTCATCCCCAGACTGAT				
DHAV-3	Detection & sequencing	VP1	F: GTATGGGAACCTGCAGATGGC	2098-2835	737	50.3	This study
			R:AGCTCAAAGGCAAGTGTTTTAAGT				
	Detection		nF:GAGACTGCAAATGTGCCAATACAAGG	2184-2536	352	55.7	This study
			nR:GTGGGGCGTAGAGGTGTGACAGAAT				

[a] VP1, capsid gene

[b] F, forward primer for RT-PCR; R, reverse primer for RT-PCR; nF, forward primer for nested PCR; nR, reverse primer for nested PCR

Table 2 Detection rate of DHAV among the DHAV-suspected liver samples from duck farms (May 2012-December 2013)

Genotype(s) detected	No. of positive samples/total samples[a] (%)			No. of positive farms/total farms (%)
	RT-PCR	Nested PCR	Total positive samples (%)	
DHAV-1	3/157 (1.9)	9/157 (5.7)	9/157 (5.7)	2/30 (6.7)
DHAV-3	66/157 (42.0)	104/157 (66.2)	104/157 (66.2)	23/30 (76.7)
Coinfection of DHAV-1 & DHAV-3	9/157 (5.7) 17/157 (10.8)	23/157 (14.6) 23/157 (14.6)	23/157 (14.6)	5/30 (16.7)

[a] A total of 157 liver samples from 30 farms were tested by RT-PCR and nested PCR assays with primer pairs specific for DHAV-1 and DHAV-3. Of these, 136 samples were positive by RT-PCR and nested PCR assays

DHAV-3 were detected in 23 of 157 samples (14.6 %) from 5 of 30 farms (16.7 %) by nested PCR assays (Table 2). The prevalence of DHAV-1 and DHAV-3 in either single or dual infections in Jeollanam-do Province, South Korea, is similar to that in China [1, 7]. From these data, it is suggested that bivalent vaccination with DHAV-1 and DHAV-3 in ducklings or breeding stocks should be attempted to control both DHAV-1 and DHAV-3 infections in South Korea. Further studies should be conducted to define the interaction of these viruses and to determine the severity of mixed infections in ducklings.

DHAV infections were detected in all seasons of the year (Supplementary Table 3): 50 of 157 liver samples (31.8 %) from 10 of 30 farms (33.3 %) tested positive in spring; 42 of 157 liver samples (26.8 %) from 8 of 30 farms (26.7 %) tested positive in winter; 32 of 157 liver samples (20.4 %) from 9 of 30 farms (30.0 %) tested positive in summer, and 12 of 157 liver samples (7.6 %) from 3 of 30 farms (10.0 %) tested positive in autumn. These data also indicated that DHAV infections were more prevalent in spring than other seasons (Supplementary Table 3). Although DHAV is known to be relatively heat stable [17, 18], the exact seasonal pattern of DHAV infections is unknown. In this study, a relatively high proportion of DHAV infections were detected in the cold and hot seasons. In autumn, however, the prevalence of DHAV infections was the lowest. The reason why the prevalence of DHAV infections was the lowest in autumn is not known. Therefore, more-intense epidemiological studies throughout the world will be needed to fully understand the seasonal pattern of DHAV infections and to establish DHAV surveillance programs to prevent infections.

Using RT-PCR and nested PCR assays with primers specific for the VP1 region of DHAV-1, generation of many amplicons could be achieved by nested PCR (32 amplicons) rather than RT-PCR (12 amplicons): 9 amplicons from DHAV-1 single infection and 23 amplicons from DHAV-1 and DHAV-3 dual infections (Table 2). To compare nucleotide and deduced amino acid sequences of the many Korean DHAV-1 isolates with those of other known DHAV-1 and DHAV-3 strains or isolates, we chose and sequenced 32 nested PCR products (339 bp). Consequently, 32 Korean DHAV-1 isolates showed high nucleotide (91.3–100 %) and deduced amino acid (89.8–100 %) sequence identity to the DHAV-1 isolates, but relatively low nucleotide and deduced amino acid sequence identity to the DHAV-3 isolates, ranging from 63.4 to 69.2 % nucleotide and 65.3 to 71.4 % deduced amino acid sequence identity, respectively (Table 3). Eighty-three partial nucleotide sequences (737 bp) of DHAV-3 were obtained from Korean ducklings by RT-PCR: 66 amplicons

Table 3 Comparison of nucleotide and deduced amino acid sequences of the VP1 gene of the isolated Korean DHAV strains with those of other strains

Genotype	Strain	% identity			
		32 Korean DHAV-1[a]		83 Korean DHAV-3[b]	
		nt	aa	nt	aa
DHAV-1	DHAV-1/duck-wt/KOR/DHV-HS/1994	99.0-100	96.9-100	65.1-67.8	69.4-70.4
	DHAV-1/duck-wt/KOR/DHV-HSS/1995	91.3-92.3	93.9-96.9	67.8-69.2	67.3-69.4
	DHAV-1/duck-vs/USA/5886/2007	95.0-95.7	93.9-96.9	65.8-68.1	67.3-69.4
	DHAV-1/duck-vs/UK/H/2007	92.6-93.6	91.8-94.9	63.8-65.4	68.4-70.4
	DHAV-1/duck-wt/USA/DRL-62/1962	98.3-99.3	96.9-100	65.1-66.8	69.4-71.4
	DHAV-1/duck-wt/USA/R85952/1955	95.0-96.0	91.8-94.9	65.1-67.5	68.4-70.4
	DHAV-1/duck-wt/CHN/H/1993	93.6-94.6	94.9-96.9	64.1-66.1	68.4-70.4
	DHAV-1/duck-wt/CHN/GZ/2003	95.0-96.3	90.8-95.9	64.1-66.8	66.3-68.4
	DHAV-1/duck-wt/CHN/GHZ04/2004	92.3-93.3	91.8-94.9	65.1-68.1	68.4-70.4
	DHAV-1/duck-wt/CHN/C-DXX/2005	93.0-94.0	90.8-93.9	65.8-67.1	67.3-69.4
	DHAV-1/duck-wt/CHN/GFS06/2006	91.6-92.6	89.8-92.9	65.4-68.1	68.4-70.4
	DHAV-1/duck-wt/CHN/SG/2007	95.0-96.3	90.8-95.9	64.1-66.8	65.3-67.3
	DHAV-1/duck-wt/CHN/SY0812/2008	94.3-96.0	89.8-94.9	64.4-67.1	66.3-68.4
	DHAV-1/duck-wt/CHN/SH/2009	95.0-96.0	91.8-94.9	65.4-67.1	68.4-70.4
	DHAV-1/duck-vs/CHN/ZJ-A/2010	94.3-96.0	89.8-94.9	64.4-67.1	66.3-68.4
	DHAV-1/duck-wt/CHN/HB02/2011	91.6-92.6	91.8-94.9	64.8-66.4	67.3-69.4
	DHAV-1/duck-wt/Taiwan/03D/2003	91.3-92.3	93.9-96.9	63.4-65.1	68.4-70.4
	DHAV-1/duck-vs/Vietnam/ VXXT/2007	98.3-99.3	94.9-98.0	65.8-68.5	69.4-71.4
	DHAV-1/duck-wt/Vietnam/ GL08/2008	98.3-99.3	94.9-98.0	65.8-68.5	69.4-71.4
DHAV-2	DHAV-2/duck-wt/Taiwan/04G/2004	59.4-61.3	58.3-61.5	64.4-66.1	70.8-71.9
	DHAV-2/goose-wt/Taiwan/90D/1990	59.0-62.0	58.3-61.5	64.7-66.1	70.8-71.9
DHAV-3	DHAV-3/duck-wt/KOR/AP-04114/2003	64.2-69.5	68.4-72.4	93.8-95.7	92.0-96.0
	DHAV-3/duck-wt/KOR/AP-04203/2003	64.5-70.5	69.4-73.5	93.4-94.8	94.0-98.0
	DHAV-3/duck-wt/KOR/AP-04009/2003	64.4-69.4	68.4-72.4	93.8-95.7	94.0-98.0
	DHAV-3/duck-wt/KOR/AP-03337/2003	63.6-69.1	68.4-71.4	93.4-95.4	93.0-97.0
	DHAV-3/duck-wt/KOR/ D11-JW-018/2011	62.2-67.1	68.4-72.4	95.7-99.7	97.0-100
	DHAV-3/duck-wt/CHN/GD/1999	65.6-70.5	70.4-71.4	91.5-93.4	90.0-93.0
	DHAV-3/duck-wt/CHN/C-YCZ/2005	69.5-70.1	68.4-72.4	90.8-92.1	89.0-92.0
	DHAV-3/duck-wt/CHN/C-PSY/2006	69.5-70.1	68.4-72.4	90.8-92.1	89.0-92.0
	DHAV-3/duck-wt/CHN/C-LX/2007	70.1-70.8	68.4-72.4	91.8-92.8	89.0-92.0
	DHAV-3/duck-wt/CHN/FS/2008	65.6-70.5	68.4-72.4	91.5-93.4	90.0-93.0
	DHAV-3/duck-wt/CHN/SD02/2009	70.5-71.5	68.4-72.4	88.9-90.8	90.0-93.0
	DHAV-3/duck-wt/CHN/SD1101/2010	66.6-71.5	69.4-73.5	88.9-90.8	91.0-94.0
	DHAV-3/duck-wt/CHN/B-N/2011	69.5-70.5	68.4-72.4	90.5-91.1	90.0-93.0
	DHAV-3/duck-wt/CHN/JS2/2012	70.5-71.1	69.4-73.5	89.5-91.1	88.0-91.0
	DHAV-3/duck-wt/CHN/JNA/2013	70.8-71.5	68.4-72.4	91.1-92.8	90.0-93.0
	DHAV-3/duck-wt/Vietnam/DN2/2009	64.2-69.1	68.4-72.4	90.5-92.8	92.0-96.0
	DHAV-3/duck-wt/Vietnam/NC/2009	68.5-69.1	68.4-72.4	89.8-92.5	92.0-96.0

[a] The nucleotide and deduced amino acid sequence identities of partial VP1 genes (339 bp) among the 32 Korean DHAV-1 isolates were 98.3-100 % and 94.9-100 %, respectively

[b] The nucleotide and deduced amino acid sequence identities of partial VP1 genes (737 bp) among the 83 Korean DHAV-1 isolates were 96.2-100 % and 95.7-100 %, respectively

from DHAV-3 single infections and 17 amplicons from DHAV-1 and DHAV-3 dual infections (Table 2). Therefore, we compared their nucleotide and deduced amino acid sequences with those of other known DHAV-1 and DHAV-3 strains or isolates. Eighty-three Korean DHAV isolates shared high nucleotide and deduced amino acid

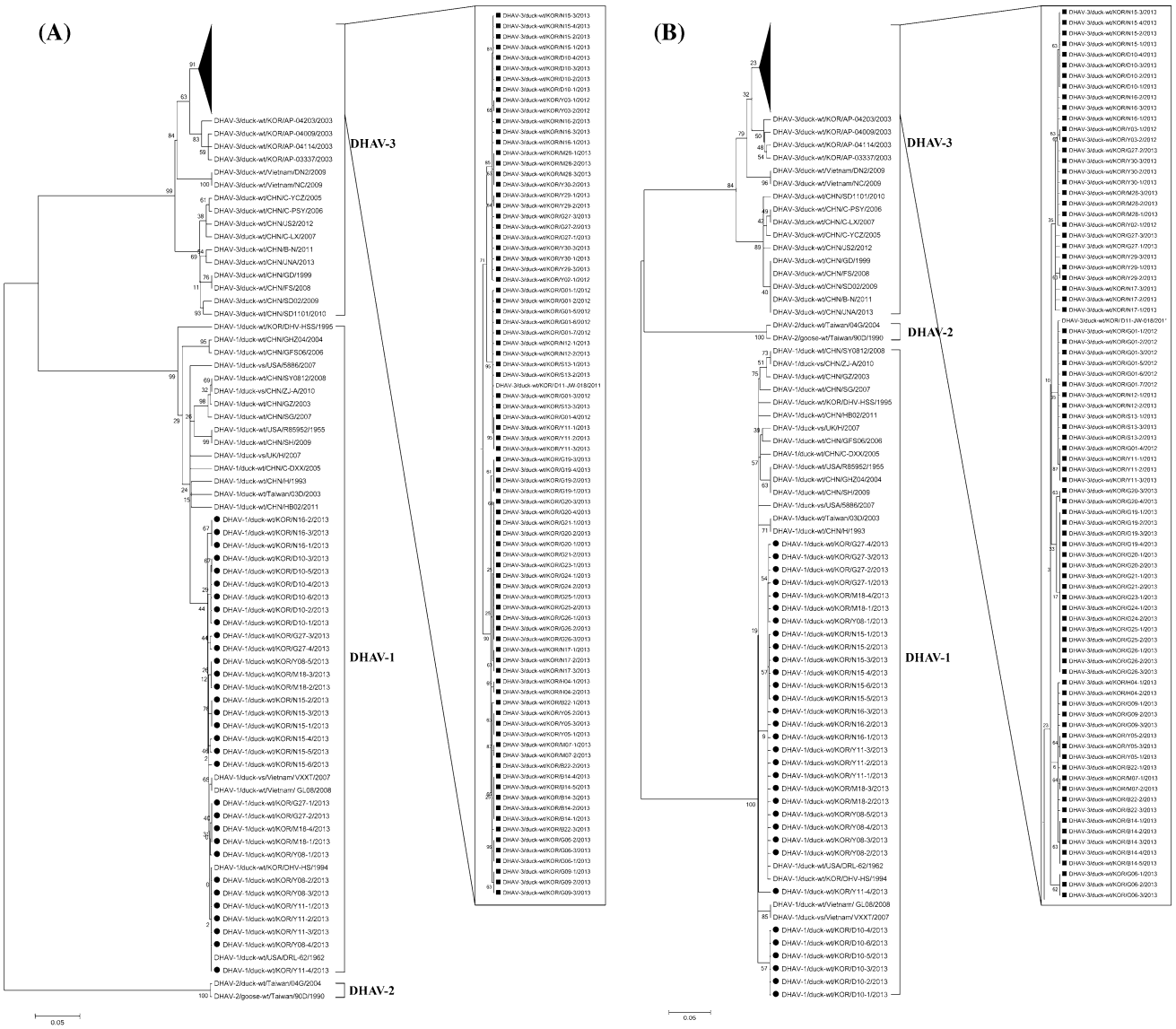

Fig. 1 Phylogenetic trees based on partial nucleotide (A) and deduced amino acid (B) sequences of the VP1 region of 115 Korean DHAV isolates, indicating their genetic relationships to other known DHAV strains or isolates. The overlapping 339-nucleotide sequences of the VP1 region between the Korean DHAV-1 (339 bp) and DHAV-3 (737 bp) were selected and compared (see Supplementary Fig. 1). Trees were generated using the neighbor-joining method with 1000 bootstrapping replicates in the MEGA 5.1 program. The bar indicates the genetic distance, and the black circle and the black square indicate the Korean isolates. Reference sequences used in the analysis were obtained from the GenBank database (Supplementary Table 2)

sequence identity with DHAV-3 strains or isolates, ranging from 88.8 to 99.7 % nucleotide and 88.0 to 100 % deduced amino acid sequence identity (Table 3). However, they had low nucleotide (62.2–71.5 %) and deduced amino acid (68.4–73.5 %) sequence identity to DHAV-1 strains or isolates. Both Korean DHAV-1 and DHAV-3 isolates showed the lowest nucleotide and deduced amino acid sequence identity to the DHAV-2 isolates (Table 3).

In order to confirm the nucleotide- and deduced-amino-acid-based genotyping of these Korean DHAV-1 and DHAV-3 isolates, phylogenetic analysis of the partial nucleotide and deduced amino acid sequences of 32 DHAV-1 (339 bp) and 83 DHAV-3 (737 bp) were performed with those of other known DHAV-1 and DHAV-3 strains or isolates. Alignments indicated that 32 Korean isolates belonged to the DHAV-1 genotype (Supplementary Fig. 2), whereas 83 Korean isolates clustered with the DHAV-3 genotype (Supplementary Fig. 3). To compare sequence data between the Korean DHAV-1 and DHAV-3, the overlapping nucleotide sequences (339 bp) between Korean DHAV-1 (339 bp) and DHAV-3 (737 bp) (Table 1 and Supplementary Fig. 1) were selected and analyzed

phylogenetically. Phylogenetic analysis of the overlapping nucleotide (Fig. 1A) and deduced amino acid (Fig. 1B) sequences sharply divided 32 and 83 Korean isolates into DHAV-1 and DHAV-3, respectively. These data demonstrate nucleotide- and deduced- amino-acid-based genotyping of Korean DHAV-1 and DHAV-3.

In conclusion, this study demonstrated that DHAV-3 infections were prevalent on duck farms in Jeollanam-do Province and that DHAV-1 has reemerged in South Korea. In addition, dual infections of DHAV-1 and DHAV-3 were not uncommon. These results are especially relevant for future vaccination policy in South Korea.

Acknowledgments This study was supported by a grant from the Korea Basic Science Institute (C33730) and Chonnam National University (2012), Republic of Korea.

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