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Molecular epidemiology and genetic diversity of duck hepatitis A virus type 3 in Shandong province of China, 2012–2014

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Summary. – The infections with duck hepatitis A virus type 3 (DHAV-3) become common in eastern Asia. To better understand the molecular evolution and genetic variation of DHAV-3, a total of 482 dead Cherry Valley duckling liver samples collected from Shandong province of China during 2012–2014 were tested, and the complete P1 coding sequences of 18 DHAV-3 strains were analyzed. The detection rate of DHAV-3 was 64.5% (311/482) in clinical liver samples and 73.0% (92/126) in duckling flocks. The P1 genes of the 18 DHAV-3 isolates shared 91.9%–99.0% nucleotide similarity and 95.2%–100% amino acid similarity with those of the other 26 reference strains. Based on the P1 and VP1 gene sequences, phylogenetic analysis results indicated that the genotyping of DHAV-3 strains presented a distinct geographical distribution. Except B63 strain, all Chinese strains isolated from different host species (duck or goose) at different time were classed into the CH genotype. All Korean and Vietnamese strains belonged to the KV genotype, and all the Korean strains were clustered into KV1 subgenotype, while B63 strain and the Vietnamese strains from different host species (duck or goose) were clustered into KV2 subgenotype. Ten variable amino acid residues were highly conserved within genotypes or subgenotypes in the VP0, VP3 and VP1, respectively, which were possibly the geographic molecular markers of DHAV-3. To the best of our knowledge, this is the first study about the genetic variation of the P1 gene of different DHAV-3 strains, which will be helpful for understanding the molecular epidemiology of DHAV-3.

Keywords: duck hepatitis A virus type 3 (DHAV-3); P1 gene; VP1 gene; molecular epidemiology; geographical distribution

Introduction

Duck virus hepatitis (DVH), caused by duck hepatitis virus (DHV), is an acute, rapidly spreading, and fatal disease of young ducklings and has resulted in highly significant economic losses to the duck industry worldwide. DHV includes at least three different RNA viruses, duck hepatitis A virus (DHAV), duck astrovirus type 1 (DAstV-1) and duck astrovirus type 2 (DAstV-2), and no antigenic relationships have been found among them (Toth, 1969; Haider and Calnek, 1979; Woolcock, 2003). DHAVs had been classified into three genotypes by phylogenetic analyses: the classical genotype 1 (DHAV-1) (Kim *et al*., 2006; Ding and Zhang, 2007; Tseng *et al*., 2007), the genotype only isolated in Taiwan (DHAV-2) (Tseng and Tsai, 2007), and the genotype firstly identified in South Korea (DHAV-3) (Kim *et al*., 2007). Based on the neutralization tests, DHAVs were divided into three serotypes corresponding to the three genotypes (Kim *et al*., 2007; Tseng and Tsai, 2007).

In our previous study, DHAV-1, DHVA-3 and DAstV-1 were detected from Shandong province of China (Chen *et al*., 2014). In recent years, the mixed infections of different

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Abbreviations: DVH = duck virus hepatitis; DHV = duck hepatitis virus; DHAV = duck hepatitis A virus; DAstV = duck astrovirus; CH genotype = Chinese genotype; KV genotype = Korean and Vietnamese genotype

DHAVs become common in eastern Asia (Tseng and Tsai, 2007; Chen *et al.*, 2013; Soliman *et al.*, 2015; Lin *et al.*, 2016), causing disease prevention and control more challenging. As a new serotype of DHAV isolated in South Korea and China, DHAV-3 could cause an acute and fatal disease of ducklings (Kim *et al.*, 2007; Fu *et al.*, 2008; Xu *et al.*, 2012).

As the only member of a novel genus *Avihepatovirus* in the family *Picornaviridae*, the complete genome of DHAV is comprised of a single-stranded positive-sense RNA of approximately 7,700 nucleotides excluding the poly(A) tail at the 3' end, encapsulated in an icosahedral structure, which is composed of the structural proteins VP0, VP3 and VP1 (Kim *et al.*, 2006; Tseng and Tsai, 2007). These three structural proteins are the secondary cleavage products of a capsid precursor polyprotein (P1). According to the alignment of the capsid proteins of DHAV and other picornaviruses, several of the most variable regions are at the termini, particularly the N-terminus of VP0 and the two termini of VP1 (Johansson *et al.*, 2002; Ding and Zhang, 2007; Gao *et al.*, 2012), which indicates that some conserved functions might be performed by different capsid proteins of DHAV.

In this study, using the multiplex RT-PCR assay developed in our previous work for diagnosing DHAV-1, DHVA-3 and DAstV-1 mixed infection (Chen *et al.*, 2014), we detected 482 clinical dead ducking liver samples collected from Shandong province of China from 2012 to 2014. The complete P1 genes of 18 DHAV-3 strains isolated from different duckling flocks were selected for sequencing and analysis. This paper is the first study about the genetic variation of the P1 genes of different DHAV-3 strains, which will be helpful for understanding the molecular epidemiology of DHAV-3.

Materials and Methods

Clinical specimens and viruses. Liver samples from 482 dead Cherry Valley ducklings under 3 weeks old were obtained from 126 flocks in Shandong province of China between January of 2012 and December of 2014. The 126 flocks of ducklings were all not vaccinated with live DVH vaccine. The mortality of duck flocks was 20%–85%, and the sick ducklings died quickly with typical hemorrhagic hepatitis. Eighteen DHAV-3 strains (named JN1206, JN1209, LY1304, LY1305, LY1402, LY1405, LY1408, LY1417, QZ1401, QZ1403, QZ1404, WF1203, WF1210, WF1240, YT1213, YT1220, ZQ1403 and ZQ1408) isolated from different duckling flocks were selected for sequencing and analysis of the complete P1 gene (Table 1).

Primers. According to our previous study (Chen *et al.*, 2014), the primers for DHAV-1, DHVA-3 and DAstV-1 typing (DHAV-1F: 5'-GAT GTG GCA Y(T/C)GT TGT Y(T/C)AA Y(T/C)CG A- 3', DHAV-1R: 5'-CTG ATG TD(G/A/T)C CAG GR(A/G)A TTG GTC G-3', DHAV-3F: 5'-GAG CCA GAA TTG GAA TGG ACA CA-3', DHAV-3R: 5'-CAT ACT TR(G/A)C CAC CAA CTG CCA ATC-3', DAstV-1F: 5'-ATG GCC CAG AGC GGT GAA AA-3' and DAstV-1R: 5'-GCC AGG TGT CAA CAA TCA TGC-3') were used to detect and distinguish the three DHVs. Based on the sequence alignments of all DHAV-3 sequences retrieved from GenBank (http://www.ncbi.nlm.nih.gov), two pairs of primers were selected to amplify the complete P1 gene. The first pair of primers (AF 5'-CAC ACT GCC TGA TAG GGT CG-3' and AR 5'-CTG GCT TCC AAA GGT CAA TAA-3') was designed to amplify a 1225 bp fragment (nucleotides 586–1810 in the genome of DHAV-3), and the other pair of primers (BF 5'-GAC CTT TGG AAG CCA GTT TA-3' and BR 5'-CAT CAC AGG CAC GAA CAA GT-3') was designed to amplify a 1517 bp fragment (nucleotides 1795–3311 in the genome of DHAV-3). All the primers were synthesized by Sangon (Shanghai, China).

Detection of clinical specimens. All the livers of the 482 dead ducklings were analyzed by the multiplex RT-PCR method according to the previous study (Chen *et al.*, 2014). The viral RNA was extracted from the liver samples using the E.Z.N.A.™ Viral RNA Kit (Omega Bio-Tek, Doraville, USA) and reverse transcribed into cDNA using a RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada) according to the manufacturer's instructions. Then, the cDNA was tested by the multiplex RT-PCR method for differential diagnosis for mixed infection of DHVA-1, DHVA-3 and DAstV-1.

RNA extraction and P1 sequencing. The DHAV-3 strains were propagated in the allantoic cavities of 10-day-old duck embryos for 72 h at 37°C. The virus RNA was extracted from allantoic liquids of dead duck embryos. RT-PCR was then performed for amplification of selected 18 P1 genes with the two pairs of primers. The RT-PCR conditions were 50°C for 30 min, 94°C for 2 min, and then 32 cycles of 94°C for 30 s, 50°C for 35 s, 72°C for 1 min, and with a final step of 72°C for 5 min. PCR products were purified using a gel purification kit (CWBIO, China), and the purified PCR products were TA-cloned into the pMD18-T vector (TaKaRa, China) following the manufacturer's instructions, and then sent to a commercial service for sequencing (Shanghai Sangon Biological Engineering Technology & Service Co., Ltd).

Sequence analysis. The P1 sequences of 26 DHAV-3 reference strains and the VP1 sequences of other 31 DHAV-3 reference strains were retrieved from GenBank (http://www.ncbi.nlm.nih.gov, Table 1). Multiple sequence alignment was analyzed using DNAStar Clustal W program (DNAStar Inc. Madison, WI, USA). Based on P1 and VP1 nucleotide sequences and amino acid sequences of the DHAV-3 strains, the phylogenetic trees were respectively constructed by MEGA 6.0 with Neighbor-joining method (Tamura *et al.*, 2013).

Results

Detection of clinical specimens by the multiplex RT-PCR assay

In the 482 clinical samples, 103 samples were identified as co-infection with two of the three DHVs, while 379 samples

Table 1. Summary of DHAV-3 strains used in this work

Strain	Location	Year of isolation	GenBank Acc. No.	Host	Gene
JN1206	Shandong, China	2012	KP715480	Duck	P1
JN1209	Shandong, China	2012	KP715481	Duck	P1
LY1304	Shandong, China	2013	KP715482	Duck	P1
LY1305	Shandong, China	2013	KP715483	Duck	P1
LY1402	Shandong, China	2014	KP715484	Duck	P1
LY1405	Shandong, China	2014	KP715485	Duck	P1
LY1408	Shandong, China	2014	KP715486	Duck	P1
LY1417	Shandong, China	2014	KP715487	Duck	P1
QZ1401	Shandong, China	2014	KP715488	Duck	P1
QZ1403	Shandong, China	2014	KP715489	Duck	P1
QZ1404	Shandong, China	2014	KP715490	Duck	P1
WF1203	Shandong, China	2012	KP715491	Duck	P1
WF1210	Shandong, China	2012	KP715492	Duck	P1
WF1240	Shandong, China	2012	KP715493	Duck	P1
YT1213	Shandong, China	2012	KP715494	Duck	P1
YT1220	Shandong, China	2012	KP715495	Duck	P1
ZQ1403	Shandong, China	2014	KP715496	Duck	P1
ZQ1408	Shandong, China	2014	KP715497	Duck	P1
G	Fujian, China	1999	EU755009	Duck	P1
GD	Guangdong, China	1999	GQ122332	Duck	P1
B63	Beijing, China	2008	EU747874	Duck	P1
FS	Guangdong, China	2008	EU877916	Duck	P1
SD01	Shandong, China	2008	GQ485310	Duck	P1
1v	Shandong, China	2009	GU250782	Duck	P1
C-BLZ	Beijing, China	2009	GU066822	Duck	P1
C-YCW	Beijing, China	2009	GU066824	Duck	P1
C-YCZ	Beijing, China	2009	GU066823	Duck	P1
C-YDF	Beijing, China	2009	GU066821	Duck	P1
SD02	Shandong, China	2009	GQ485311	Duck	P1
C-GY	Guangdong, China	2007	EU352805	Duck	P1
JS2010	Jiangsu, China	2010	HQ654774	Duck	P1
JT	Jilin, China	2010	JF835025	Goose	P1
SD1101	Shandong, China	2010	JQ409566	Duck	P1
B-N	Guangdong, China	2011	JX235698	Duck	P1
SD1201	Shandong, China	2011	KC993890	Duck	P1
GX12-01	Guangxi, China	2012	KC893553	Duck	P1
EY	China	2014	KP995438	Goose	P1
LS	China	2014	KP233203	Duck	P1
AP-04114	South Korea	2003-2004	DQ812093	Duck	P1
AP-03337	South Korea	2003-2004	DQ256132	Duck	P1
AP-04009	South Korea	2003-2004	DQ256133	Duck	P1
AP-04203	South Korea	2003-2004	DQ256134	Duck	P1
D11-JW-018	South Korea	2011	JX312194	Duck	P1
DN2	Vietnam	2009	JF914944	Duck	P1
KH01	Vietnam	2012	KM361879	Duck	VP1
KH02	Vietnam	2012	KM361880	Duck	VP1
LA1	Vietnam	2011	KM361881	Goose	VP1
DN1	Vietnam	2009	JF925120	Duck	VP1
NC	Vietnam	2009	JF925121	Duck	VP1

Table 2. The results of the multiplex RT-PCR assay to detect infection in suspected positive clinical samples

Infection	Individual test results		Group test results	
	Positive number	Positive rate (%)	Positive number	Positive rate (%)
DHAV-1	154	31.95	29	23.02
DHAV-3	217	46.97	43	34.13
DAstV-1	8	16.67	0	0
DHAV-1+DHAV-3	62	12.86	25	19.84
DHAV-1+DAstV-1	9	1.87	5	3.97
DHAV-3+DAstV-1	32	6.64	18	14.29
DHAV-1+DHAV-3-+DAstV-1	0	0	6	4.76
Total	482		126	

were detected as positive for only DHAV-1 (154), DHAV-3 (217) or DAstV-1 (8) (Table 2). There was no sample detected as DHAV-1, DHAV-3 and DAstV-1 co-infection. In the 126 duckling flocks, 6 (4.8%) were identified as DHAV-1, DHAV-3 and DAstV-1 mixed infection, 48 (38.1%) were identified as mixed infection with two of the three DHVs, 72 (57.1%) were single infection with one of the three DHVs (Table 2).

Sequence analysis of P1 gene and VP1 gene

The P1 genes of selected 18 DHAV-3 isolates were successfully amplified by RT-PCR. P1 gene sequences of the 18 DHAV-3 strains were submitted into GenBank, and the Acc. Nos. were from KP715480 to KP715497 (Table 1). Further analysis indicated that all these P1 genes were 2199 bp long and encoded 733 amino acids, in which the VP0, VP3 and VP1 were coded by amino acids 1–256, 257–493 and 494–733, respectively. Multiple sequence alignment showed that the P1 gene sequences of the 18 strains determined in this study shared 95.9%–99.8% nucleotide sequence homology and 97.4%–100% amino acid sequence homology with each other, while they shared 91.9%–99.0% nucleotide similarity and 95.2%–100% amino acid similarity with those of the other 26 DHAV-3 reference strains. In multiple sequence alignment of VP1 protein, five other Vietnamese strains were added to the analysis (Table 1). The VP1 gene sequences of the 18 strains determined in our study shared 94.7%–100% nucleotide sequence homology and 96.2%–100% amino acid sequence homology with each other, while they shared 90.1%–100% nucleotide similarity and 90%–100% amino acid similarity with those of the other 31 reference strains.

As for P1 gene, the two DHAV-3 strains (JT strain and EY strain) isolated from geese shared 91.9%–99.2% nucleotide and 95.4%–100% amino acid sequence identities with the 42 strains isolated from ducks. Otherwise, for VP1 gene, the three DHAV-3 strains (JT, EY and LA1) isolated from geese shared 90.1%–99.6% nucleotide and 90.4%–100% amino acid sequence identities with the 46 strains isolated from ducks. It indicated that the DHAV-3 strains isolated from different hosts shared high sequence identity, which was similar to the strains entirely isolated from ducks.

Comparative analysis showed that variations occurred throughout the entire P1 protein, and there was only one high variable region (HVR) located at the C-terminus (amino acid residues 178–219) of VP1, whereas a conserved QSD (Gln-Ser-Asp) motif (amino acids 197–199) existed in the HVR of all 49 DHAV-3 strains (Fig. 1).

Phylogenetic analysis

Based on the P1 nucleotide sequences and amino acid sequences, respectively, of the DHAV-3 strains, the phylogenetic trees were constructed (Fig. 2). All of the 44 strains were clustered into two distinct genotypes: all Chinese isolates except B63 strain were included in one genotype (Chinese genotype, CH genotype), while B63 strain, all the Korean and Vietnamese strains belonged to the other genotype (Korean and Vietnamese genotype, KV genotype). The P1 gene sequences of the 37 DHAV-3 strains in CH genotype shared 94.7%–99.8% nucleotide and 95.2%–100% amino acid sequence homology with each other (Table 3). All KV strains could be further divided into two subgenotypes (KV1 and KV2), and the P1 gene sequences of the DHAV-3 strains in KV genotype shared 93.4%–99.3% nucleotide and 97.0%-99.9% amino acid sequence homology with each other (Table 3). All Korean strains were clustered into KV1, while B63 strain and the Vietnamese strain DN2 were clustered into KV2. The P1 gene sequences of the DHAV-3 strains between CH and KV genotypes shared only 91.9%–96.1% nucleotide similarity and 95.2%–97.1% amino acid similarity with each other (Table 3).

Based on the VP1 nucleotide sequences and amino acid sequences of the DHAV-3 strains, the phylogenetic trees were constructed (Fig. 3). All of the 49 strains were also clustered into two distinct genotypes: all Chinese isolates except B63 strain were included in one genotype (CH genotype), while B63 strain, all the Korean and Vietnamese strains

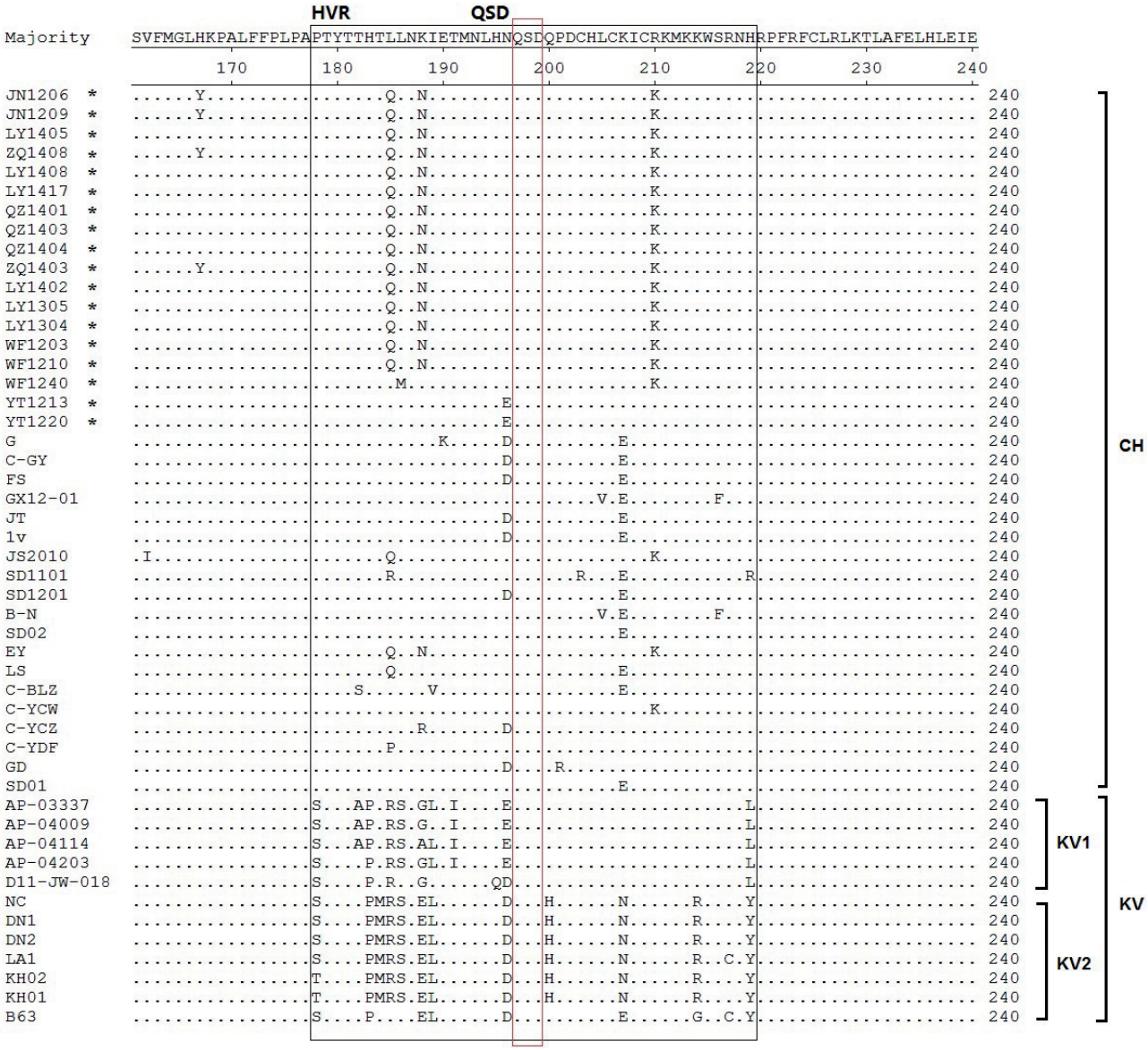


Fig. 1

Amino acids alignment based on VP1 proteins of 49 DHAV-3 strains

Isolates in this study were marked with asterisks (*). The high variable region (HVR) at the C-terminus of VP1 is shown in box and the QSD motif has been specifically marked.

belonged to the other genotype (KV genotype). The VP1 gene sequences of the 37 DHAV-3 strains in CH genotype shared 93.9%–100% nucleotide and 95.4%–100% amino acid sequence homology with each other (Table 4). Based on the VP1 nucleotide sequences and amino acid sequences, all the KV strains could also be further divided into KV1 and KV2 subgenotypes, and the VP1 gene sequences of the DHAV-3 strains in KV genotype shared 91.8%–100% nucleotide and 93.3%–100% amino acid sequence homology with each other (Table 4). All Korean strains were clustered into KV1, while B63 strain and all Vietnamese strains were clustered into KV2. The VP1 gene sequences of the DHAV-3 strains between CH and KV genotypes shared only 90.0%–95.4% nucleotide similarity and 90.0%–94.6% amino acid similarity with each other (Table 4).

The amino acids alignment showed that there were 10 variable amino acid residues highly conserved within genotypes or subgenotypes, and the 10 mutation points existed

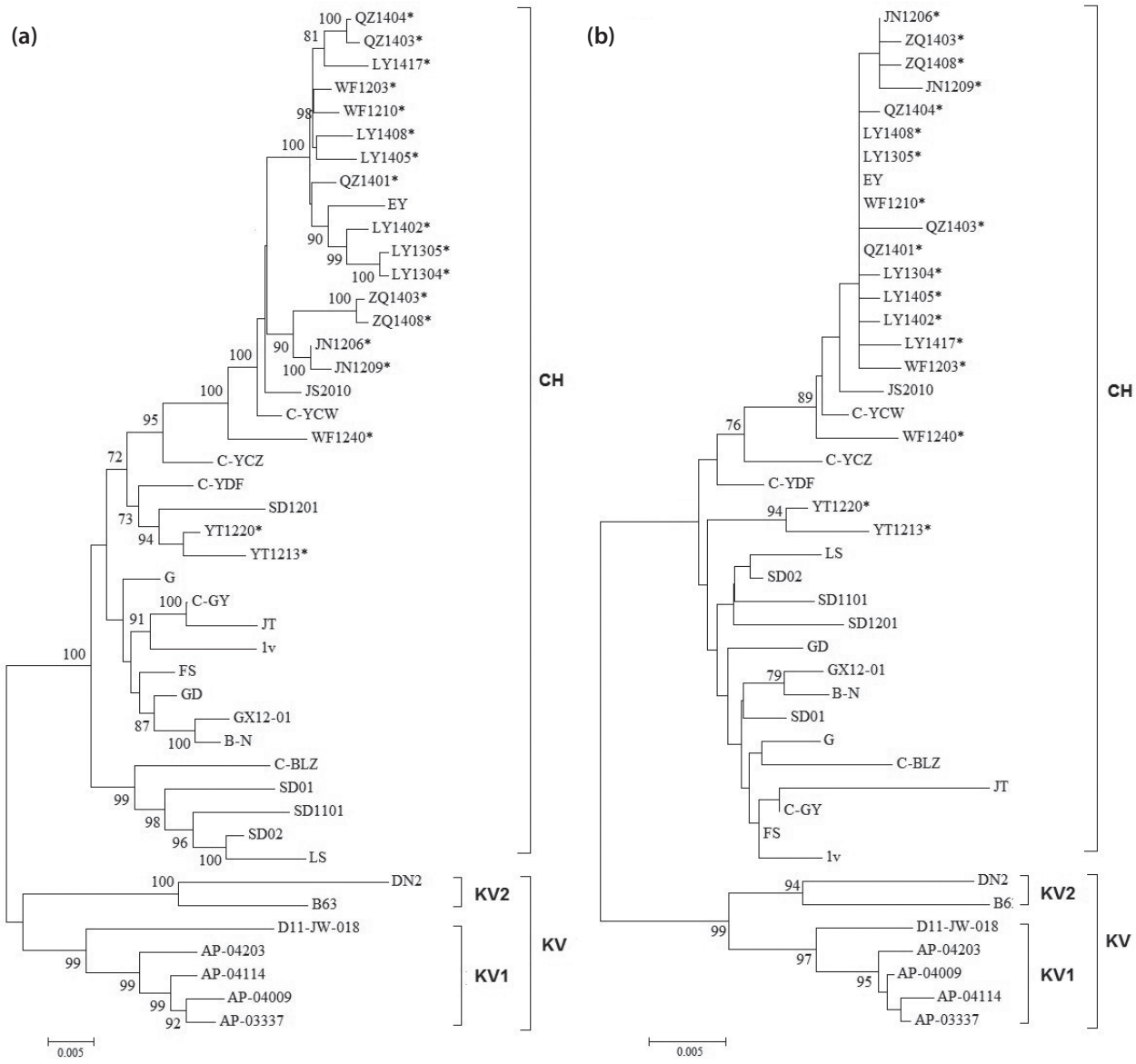
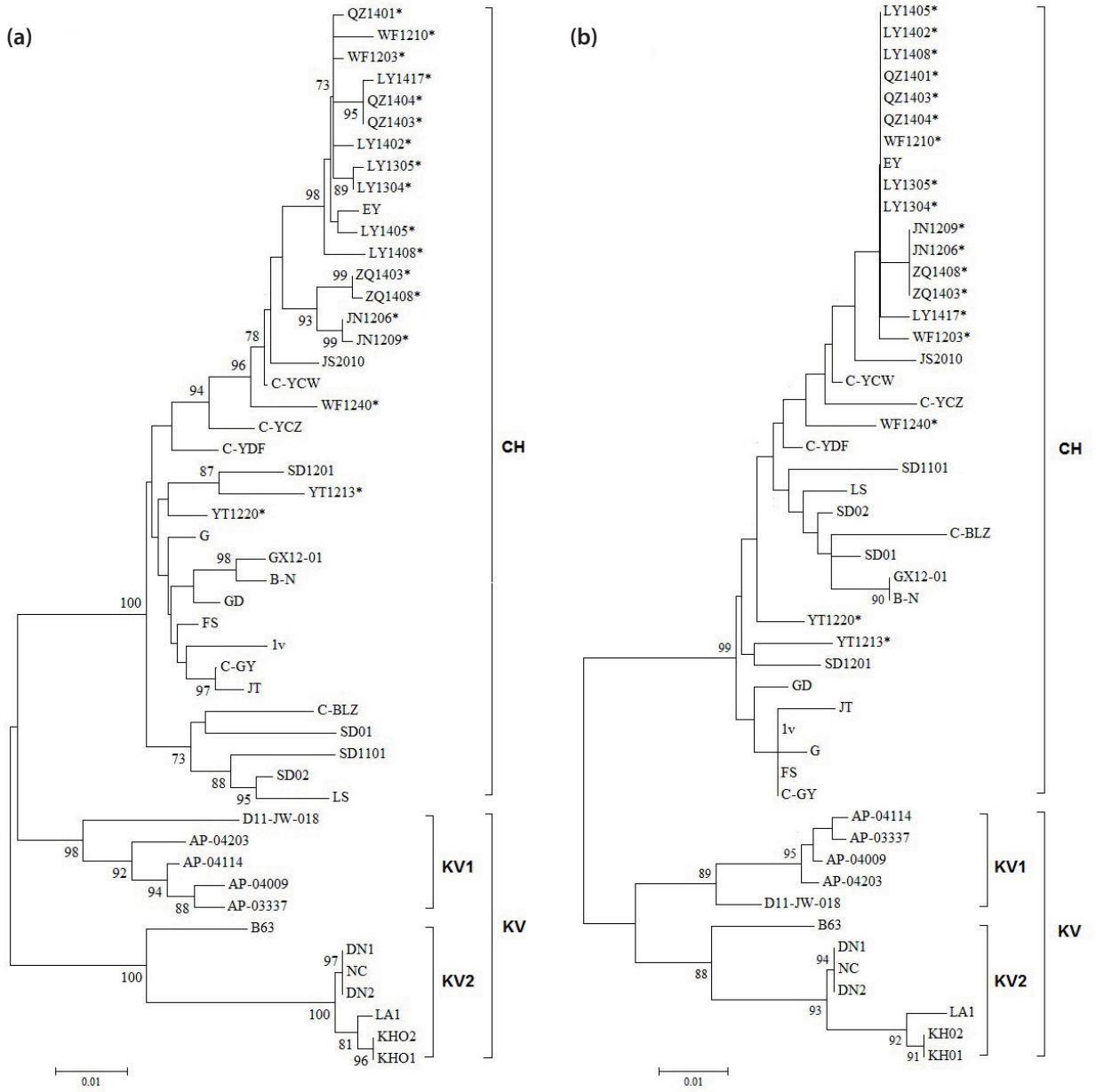


Fig. 2

Phylogenetic (neighbor-joining) trees based on DHAV-3 P1 nucleotide sequences (a) and amino acid sequences (b)
The values at the forks indicate the percentage of trees, in which this grouping occurred after bootstrapping the data (1000 replicates; shown only when >70%). The scale bar shows the number of substitutions per base. Isolates in this study were marked with asterisks (*).

Table 3. The nucleotide and amino acid sequence identity of P1 protein between DHAV-3 strains of different genotype and subgenotype

Genotype or subgenotype	Nucleotide similarity (%)			Amino acid similarity (%)		
	CH	KV1	KV2	CH	KV1	KV2
CH	94.7-99.8	92.9-96.1	91.9-94.7	95.2-100	95.4-97.1	95.2-96.9
KV1	92.9-96.1	96.4-99.3	93.4-96.3	95.4-97.1	98.5-99.9	97.0-98.9
KV2	91.9-94.7	93.4-96.3	96.3	95.2-96.9	97.0-98.9	97.1



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Phylogenetic (neighbor-joining) trees based on DHAV-3 VP1 nucleotide sequences (a) and amino acid sequences (b)
The values at the forks indicate the percentage of trees, in which this grouping occurred after bootstrapping the data (1000 replicates; shown only when >70%). The scale bar shows the number of substitutions per base. Isolates in this study were marked with asterisks (*).

Table 4. The nucleotide and amino acid sequence identity of VP1 protein between DHAV-3 strains of different genotype and subgenotype

Genotype or subgenotype	Nucleotide similarity (%)			Amino acid similarity (%)		
	CH	KV1	KV2	CH	KV1	KV2
CH	93.9-100	91.9-95.4	90.0-95.4	95.4-100	91.7-94.6	90-94.6
KV1	91.9-95.4	95.7-99.2	91.8-94.2	91.7-94.6	97.1-99.6	93.3-99.6
KV2	90.0-95.4	91.8-94.2	95.4-100	90-94.6	93.3-99.6	95.4-100

Table 5. Ten variable amino acid residues highly conserved within genotypes or subgenotypes in DHAV-3 P1 protein

Amino acid regions of P1	Amino acid variation points	CH(37)*	KV(12)	
			KV1(5)	KV2(7)
VP0 (1-256)	164	Y(36)/N(1)	F(5)	F(2)
	210	D(37)	E(5)	E(2)
VP3 (257-493)	485	A(20)/G(17)	N(5)	N(2)
	492	N(37)	D(5)	D(2)
VP1 (494-733)	528	L(37)	F(5)	F(7)
	590	M(37)	T(5)	T(7)
	616	N(37)	S(5)	S(7)
	671	P(37)	S(5)	S(5)/T(2)
	676	H(37)	P(5)	P(7)
	712	H(36)/R(1)	L(5)	Y(7)

not only in VP1 but also in VP0 and VP3 (Table 5), which indicated that the genetic variation of P1 proteins was consistent with their genotypes.

Discussion

In this study, we detected DHAV-1, DHAV-3 and DAstV-1 in clinical liver specimens from 126 different duckling flocks in Shandong province of China during 2012 to 2014. Among the 482 clinical liver samples, 225 (46.7%) samples were positive for DHAV-1 infection, 311 (64.5%) samples were positive for DHAV-3 infection, and 49 (10.2%) were positive for DAstV-1 infection (Table 2). Otherwise, of the 126 duckling flocks, 65 (51.6%) were positive for DHAV-1 infection, 92 (73.0%) were positive for DHAV-3 infection, and 29 (23.0%) were positive for DAstV-1 infection. The results showed that DHAV-3 was the most predominant DHV serotype in Shandong province of China in recent years, which was consistent with our previous studies (Chen *et al*., 2013, 2014; Lin *et al*., 2016).

As has been shown in other members of the *Picornaviridae*, such as foot and mouth disease virus (FMDV) and encephalomyocarditis virus (EMCV), VP1 is considered as the most abundant external capsid protein and contains the primary neutralization epitope (Costa-Mattioli *et al*., 2002; Kim *et al*., 2006). Earlier studies suggested that the expressed FMDV capsid precursor protein could be folded properly to maintain the functional discontinuous epitopes (Saiz *et al*., 1994; Biswal *et al*., 2015). Analysis of the capsid proteins of DHAV indicated that some conserved functions might be performed by different capsid proteins in DHAV (Ding and Zhang, 2007). In DHAVs, VP1 is the most external and dominant of viral surface proteins and has the highest genetic diversity amongst different isolates (Kim *et al*., 2006, Tseng *et al*., 2006; Ding and Zhang, 2007; Gao *et al*., 2012; Xu *et al*., 2013).

The genetic variation and molecular evolution of DHAV-3 VP1 have been studied (Xu *et al*., 2013; Ma *et al*., 2015). Although the rate of nucleotide substitution for DHAV-3 VP1 genes was about three times faster than that for DHAV-1 VP1 genes, no detectable changes were found in viral neutralization properties within the same serotype (Ma *et al*., 2015). In this study, we sequenced and analyzed the complete P1 genes of 18 DHAV-3 strains isolated from Shandong province of China from 2012 to 2014. The results showed that the P1 genes of all DHAV-3 strains shared 91.9%–99.8% nucleotide similarity and 95.2%–100% amino acid similarity with each other. Based on the P1 sequences of the 44 DHAV-3 strains and VP1 sequences of the 49 DHAV-3 strains, the phylogenetic trees indicated that the genotyping of DHAV-3 strains presented a distinct geographical distribution. Except B63 strain, all Chinese strains isolated from different host species (duck or goose) at different time were classed into the CH genotype, while all Korean and Vietnamese strains belonged to the KV genotype (Fig. 2 and 3). The amino acids alignment showed that ten variable amino acid residues existing in the VP0, VP3 and VP1 were highly conserved within genotypes or subgenotypes (Table 3), which indicated that the ten variable amino acid residues were possibly the geographic molecular markers, and were beneficial in further research on the epidemiological investigation of DHAV-3.

Interestingly, as the only Chinese strain divided into KV genotype, the B63, was isolated in China in 2008, but until now, its source still remained unclear. It gives us the indication that we should pay more attention to detection of the genetic variations of DHAV-3. Recently, DHAV-3 was detected in geese, which showed new epidemiological characteristics of DHAV (Liu *et al*., 2011). The three DHAV-3 strains (JT, EY and LA1) isolated from geese in China and Vietnam sharing high sequence identity with the strains isolated from ducks reminded us to prevent the cross-infection between duck and goose in the control of duck viral hepatitis.

According to the amino acid alignment, different from the conserved SGD (Arg-Gly-Asp) motif of DHAV-1, an identical QSD motif (amino acids 689–691) existed in the HVR of all 49 DHAV-3 strains (Fig. 1). Previous research of picornaviruses suggested that the conservative RGD (Arg-Gly-Asp) motif played a critical role in viral infectivity and the mutations at the motif had significant effect on viral growth (Rossmann *et al.*, 1985; Boonyakiat *et al.*, 2001; Rieder *et al.*, 2005). In our recent study we showed that there were no significant differences in the viral load in the same organ regardless whether the ducklings were single-infected of co-infected by DHAV-1 and DHAV-3. This indicates that the co-infection of the two viruses had no effect on the *in vivo* viral loads (Lin *et al.*, 2016). More studies should be done to examine whether the QSD motif in DHAV-3 and the SGD motif in DHAV-1 interacted with different cell receptors in the infected ducklings.

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