Kafkas Univ Vet Fak Derg
23 (3): 369-376, 2017
DOI: 10.9775/kvfd.2016.16695

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Sequencing and Phylogenetic Analysis Reveal the Prevalence of Duck Hepatitis A Virus Genotype-3 in Vietnam [1]

Huong Thi Thanh DOAN [1] ✉ Xuyen Thi Kim LE [1] Roan Thi DO [1]
Khue Thi NGUYEN [1] Thanh Hoa LE [1] ✉

[1] *This study was supported by the National Foundation for Science and Technology Development (NAFOSTED) of Vietnam, under grant no. 106.04-2012.60*

[1] Department of Immunology, Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, VIETNAM

Article Code: KVFD-2016-16695 Received: 29.08.2016 Accepted: 19.10.2016 Published Online: 19.10.2016

Citation of This Article

Doan HT, Le XK, Do R, Nguyen K, Le TH: Sequencing and phylogenetic analysis reveal the prevalence of duck hepatitis A virus genotype 3 in Vietnam. *Kafkas Univ Vet Fak Derg,* 23 (3): 369-376, 2017. DOI: 10.9775/kvfd.2016.16695

Abstract

Duck hepatitis A virus (DHAV) causes an acute and highly contagious disease in young ducklings worldwide. Despite the widespread use of the DHAV vaccine, many outbreaks still occur in Vietnam. In this study, we determined the full-length genome sequence of two DHAV isolates (NC and NT) obtained from infected ducks in 2009 and 2013, and compared them with the attenuated DHAV-1 vaccine strain (namely, VXXT) currently used in Vietnam. The NC and NT strains belong to the virulent DHAV-3 genotype, and their genomes consist of 7791 and 7790 nucleotides (nt), respectively. Both genomes contain one large open reading frame (ORF) of 6756 nt, encoding a polyprotein of 2251 amino acids and possess a typical picornavirus genome organization. The majority of predicted C-terminal cleavage sites in the polyprotein were Q/S or Q/G. Phylogenetic analysis of the nucleotide sequence of the full ORF revealed that all virulent Vietnamese strains are closely related to Chinese DHAV-3 strains. Noticeably, the virulent Vietnamese DHAV-3 strains had relatively low nucleotide identity with the DHAV-1 vaccine strain (73.5%). The study showed that an antigenicity-matched DHAV-3 vaccine is urgently required for use in Vietnam.

Keywords: *Duck hepatitis A virus, Genotype, Genome, Phylogenetic*

Sekans ve Filogenetik Analiz Vietnamda Ördek Hepatitis A Virüs Genotip-3'ün Prevalansını Ortaya Koymaktadır

Özet

Ördek Hepatitis A virüsü (DHAV) dünya çapında ördek palazlarında akut ve oldukça bulaşıcı bir hastalığa neden olmaktadır. DHAV aşısının yaygın kullanımına rağmen Vietnam'da hala birçok salgınlar oluşmaktadır. Bu çalışmada, 2009 ve 2013 yıllarında enfekte ördeklerden elde edilen iki adet DAHV izolatının (NC ve NT) tüm genom sekansı belirlenmiştir ve Vietnam'da mevcut kullanılan atenüe DHAV-1 aşı suşu (VXXT) ile karşılaştırılmıştır. NC ve NT suşları virulent DHAV-3 genotipine ait olup genomları sırasıyla 7791 ve 7790 nükleotid (nt)'ten oluşmaktadır. Her iki genom 6756 nt boyutunda bir büyük açık okuma çerçevesine (open reading frame ORF), sahip olup 2251 amino asit uzunluğunda bir poliprotein kodlar ve a tipik olarak picornavirus genom organizasyona sahiptir. Poliproteinde tahmini C-terminal ayrılma bölgelerinin çoğunluğu Q/S veya Q/G olarak belirlendi. Bütün ORF'nin nükleotid sekansının filogenetik analazi tüm virülent Vietnam suşlarının Çin DHAV-3 suşu ile yakından akraba olduğunu gösterdi. Virülent Vietnam DHAV-3 suşları göreceli olarak DHAV-1 aşı suşu ile düşük nükleotid benzerliğine (%73.5) sahipti. Bu çalışma antijen uyumlu bir DHAV-3 aşısının Vietnam'da acilen kullanılması gerekliliğini göstermektedir.

Anahtar sözcükler: *Ördek hepatitis A virüsü, Genotip, Genom, Filogenetik*

INTRODUCTION

Duck hepatitis virus (DHV), first described on Long Island, New York in 1949 [1], causes a rapidly spreading and often fatal disease in young ducklings [2]. Historically, three distinct serotypes of DHV (DHV-1, -2, and -3) have been described, among which there are no antigenic relationships [2-4]. Recently, according to the Ninth Report of the International Committee on Taxonomy of Viruses (ICTV), DHV-2 and DHV-3 were classified as duck astrovirus

✉ **İletişim (Correspondence)**
☎ +84 4 37564391, +84 4 37564391
✉ doantthuong74@gmail.com, imibtvn@gmail.com

type 1 (DAstV-1) and duck astrovirus type 2 (DAstV-2), respectively, whereas DHV-1 was renamed as duck hepatitis A virus (DHAV) and classified as a member of the newly defined genus *Avihepatovirus* in family Picornaviridae [5]. DHAV has three genotypes, i.e., DHAV-1, DHAV-2, and DHAV-3 [6-9], are of the most virulent variants [2,10]. Currently, DHAV-1 distributes globally, whereas DHAV-3 has been reported only in China and Korea [6,7,11,12] and DHAV-2 is limited to Taiwan [8]. Recently, the *in vivo* distribution of both DHAV-1 and DHAV-3 viruses in clinically infected ducklings was reported in Chinese flocks [13,14].

To date, nearly 50 complete genome sequences of DHAVs have been published [7,12,15]. DHAVs have a positive single-stranded RNA genome with a single large open reading frame (ORF) of 6750 nucleotides, encoding a polyprotein of 2249 amino acids in DHAV-1 and DHAV-2 or 2251 amino acids in DHAV-3 (due to an additional 6 bp in VP1, encoding two extra amino acids).

The diversification of DHAV in recent years, and particularly the emergence of DHAV-3, may have been underestimated. It is possible that DHAV-3 exists in countries other than China and Korea; if so, the prevalence of this genotype in previously unreported locations remains unknown. Each year in Vietnam, 50 million ducks are raised in farms and in fields. The ducklings are all vaccinated with DHAV-1 vaccine(s), most commonly the VXXT vaccine strain produced by the Veterinary Vaccine Company of Vietnam (VETVACO, Hanoi, Vietnam). Nonetheless, severe annual outbreaks of DHAV still occur, suggesting an antigenic mismatch between the vaccine (DHAV-1 derivative) and the circulating virulent DHAV variants. The discovery of DHAV-3 in China and Korea [6,7] and DHAV-2 in Taiwan [8] raises questions about the existence of different genotypes of DHAVs in other countries. Therefore, in this study, we sequenced the complete genomes of two virulent DHAV strains and compared them with the vaccine strain currently used in Vietnam to determine whether DHAV-3 or DHAV-2 are occurring in this country. This is the first international report to analyze the full-length genome sequences of DHAVs in Vietnam.

MATERIAL and METHODS

Viruses Used in the Analyses

Totally 32 samples were collected including two DHAV-1 attenuated vaccine strains and 30 duckling liver clinical samples. Using primers for VP1 gene, 11 of them were identified as DHAV, and 10/11 were DHAV-3 [16]. Among them, four strains was chosen to sequence and analysis complete genome for this study, including one DHAV-1 vaccine strain (VXXT) and three field DHAV-3 strains (DN2, NC and NT) that isolated from outbreaks in Dong Nai (2009), Ho Chi Minh City (2009) and Ninh Thuan province (2013), respectively. The VXXT vaccine strain was produced by the VETVACO (Hanoi, Vietnam). The complete genome sequence of the DHAV-3 strain DN2 and the DHAV-1 vaccine strain VXXT were obtained and submitted in advance in GenBank (GenBank accession numbers JF914944 and JF914945). The complete genome sequence of the two remaining strains, DHAV-3 NC and NT, were obtained and analyzed in this study.

RNA Extraction and Reverse Transcription

Viral RNAs were extracted from samples using the RNeasy Mini Kit (Qiagen, Germany) and stored at -80°C until use. cDNA synthesis was conducted using the Maxima Reverse Transcriptase kit (Fermentas, EU) as follows: 4 µl of viral RNA (~100 ng of viral RNA), 1 µl of random hexamer primer (100 pmol/µl), 1 µl of dNTP mix (10 mM each), 5 µl of transcriptase buffer (5X), 0.5 µl of Ribolock™ RNase inhibitor (20 U), 1 µl of Maxima Reverse Transcriptase (20 U), and 8.5 µl of RNase-free water, in a final volume of 20 µl. The cDNA synthesis reaction mixture was incubated at 50°C for 60 min, and then for 5 min at 85°C to inactivate the enzyme. The cDNA samples were stored at -20°C for further use.

Primers and PCR Amplification

For preliminary detection of DHAV genotypes, three genotype-specific primer pairs were designed, based on the conserved sequences of each DHAV genotype, to specifically amplify the VP1 region *(Table 1)*. Next, five pairs of primers were designed to obtain full-length genome sequences of three DHAV-3 strains *(Table 2)*. The conditions used for amplification were as follows: initial denaturation at 94°C for 5 min; 35 cycles of 94°C/1 min, 55°C/1 min (annealing), and 72°C/2 min (extension); followed by 10 min/72°C (final extension). Negative controls (DEPC water) were included in every set of PCR. PCR products were visualized by 1% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light. After purification using the AccuPrep™ Gel Purification Kit (Bioneer Inc., South Korea), amplicons were sequenced by a commercial service (Macrogen, South Korea).

Nucleotide Sequencing and Computational Analysis

Nucleotide sequences were identified using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST). Multiple nucleotide alignment of the Vietnamese and previously available DHAV sequences was performed using GENEDOC 2.7 (http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html). A phylogenetic tree was constructed using the MEGA 6.06 package, based on the neighbor-joining method with 1000 bootstrap replicas [17]. DNA contigs were assembled to generate the complete genome sequence in MacVector 8.2, and predicted polyprotein cleavage sites were determined using GENEDOC 2.7 with reference data from a previous analysis [7]. The DHAV sequences obtained in

this study were deposited in GenBank with the accession numbers KU860089 and KU860090. The GenBank accession numbers of the complete ORF sequences, sample codes, and geographical origins are shown in *Fig. 1*.

RESULTS

Sequence Analysis of the Whole Genomes of Four Vietnamese DHAV Strains

The VP1 region was chosen for preliminary species identification using three pairs of genotype-specific primers *(Table 1)*. All collected samples were subjected to PCR-coupled sequencing and BLAST-based genotype confirmation. The VP1 nucleotide sequence revealed that the field isolates (NC and NT strains) were of genotype 3 (DHAV-3). The cDNAs of these two strains were converted and used as templates for coupled primer-walking PCR and sequencing to obtain the complete genome nucleotide sequence. Primers are listed in *Table 2*.

The sizes of the complete genomes of the NC and NT strains (preliminarily classified as DHAV-3 by nucleotide analysis of VP1) were 7791 and 7790 nucleotides, respectively, including the 18-nucleotide poly-adenine (polyA) tail at the 3′ end *(Table 3)*. Each genome consisted of one large ORF flanked by 5′ and 3′ untranslated regions (UTRs). The complete ORF sequences were used in a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST), providing further confirmation that the NC and NT virulent strains were of the DHAV-3 genotype. Comparative sequence analyses revealed that both strains had typical picornavirus genome organization: 5′UTR–L–VP0–VP3–VP1–2A1–2A2–2B–2C–3A–3B–3C–3D–3′UTR, with a 3′ tail of 18 adenine nucleotides (polyA). Details of genomic features for each strain, including sequential gene arrangement, gene clusters, and characteristics of individual genes, are provided in *Table 3*.

All three Vietnamese DHAV-3 strains, including NC, NT, and DN2 (GenBank accession number JF914944), had an ORF of 6756 nt, a 5′UTR of 652 nt, and a 3′UTR of 366-369 nt. The DHAV-1 VXXT strain (GenBank accession number JF914945) had an ORF of 6750 nt, with a VP1 gene that was 6 nt shorter than in DHAV-3 strains (714 versus 720 nt in DHAV-3 viruses); a 5′UTR of 626 nt (26 nt shorter); and a 3′UTR of 315 nt (51 nt shorter) *(Table 3)*.

The three Vietnamese DHAV-3 strains had similar base compositions: adenine, 27.49-27.68%; uracil, 29.37-29.45%; guanine, 22.13-22.47%; and cytosine, 20.35-20.76%. The base composition of the DHAV-1 VXXT strain was as follows: adenine, 29.1%; uracil, 28%; guanine, 22.7%; and cytosine, 20.2%.

Analysis of the polyprotein sequence revealed that the sequential protein order was the same as in all picornavirus

Table 1. *Genotype-specific primers used for amplication of VP1 region for preliminary discrimination of DHAV-1, DHAV-2 and DHAV-3*

Primer Name	Sequence (5′-3′)	Amplicon Size (kb)	Identification of Genotype*
DHAV1F	GCCCCACTCTATGGAAATTTG	~ 0.8	DHAV-1
DHAV1R	ATTTGGTCAGATTCAATTTCC		
DHAV2F	CACCACTTGGAGGAAATCAACAG	~ 0.8	DHAV-2
DHAV2R	CCACCTGATGTTTTGTTGTGAGAG		
DHAV3F	ATGCGAGTTGGTAAGGATTTTCAG	~ 0.8	DHAV-3
DHAV3R	GATCCTGATTTACCAACAACCAT		

** **DHAV:** Duck hepatitis A virus*

Table 2. *List of primer pairs designed to obtain complete nucleotide sequence of the Vietnamese DHAV-3 viruses*

Primer Name	Sequence (5′-3′)	Nucleotide Positions	Amplicon Size (kb)
DK1F	TTTGAAAGCGGCTGTGGTGTAG	1-21	~ 1.7
DK1R	GAGAGCAAAAGTTGGCATTCG	1745-1725	
DK2F	GTGGGTGATTTTCAGTGGGC	1653-1672	~ 1.7
DK2R	AACCAACCTCGGTAAGTGAGCACG	3413-3390	
DK3F	TGGAATCACTTGTTCGTGCC	3286-3305	~ 1.7
DK3R	AGACTGCCATCCCTCATTGC	4987-4986	
DK4F	GCGTAGGTTTCCAATCCGAC	4892-4911	~ 1.8
DK4R	GCTAACAGATTGTCCCACTCAGC	6691-6669	
DK5F	TTTGACTACACAGTTGCGTTCC	6563-6585	~ 1.2
DK5R	AGGGTGGGGAGGAATAGTAAAG	7781-7760	

Table 3. *Sequential gene arrangement in clusters, comparison of the genomic features and amino acid residues at predicted C-terminal cleavage sites for NC, NT, DN2 (DHAV-3) and VXXT (DHAV-1) strains*

Gene/Region	Size (Nucleotide)[a]				Predicted C-terminal Cleavage Sites (Amino Acid)			
	DHAV3		DHAV1		DHAV3		DHAV1	
	NC	NT	DN2	VXXT	NC	NT	DN2	VXXT
5'UTR	652	652	652	626	-	-	-	-
ORF	6756	6756	6756	6750				
3'UTR	366	366	369	315				
PolyA	18	18	18	18				
Complete size	7791	7790	7789	7703				
Polyprotein	2251	2251	2251	2249	-	-	-	-
L	**30**	**30**	**30**	**30**	**L/G** (30–31)	**L/G**	**L/G**	**L/G** (30–31)
VP0	226	226	226	226	Q/G (256–257)	Q/G	Q/G	Q/G (256–257)
VP3	237	237	237	237	Q/G (493–494)	Q/G	Q/G	Q/G (493–494)
VP1	240	240	240	238	E/S (733–734)	E/L	E/L	E/S (733–734)
P1	**703**	**703**	**703**	**701**	**E/S**	**E/L**	**E/L**	**E/S**
2A1	20	20	20	20	NPG/P (753–754)	NPG/P	NPG/P	NPG/P (749–752)
2A2	285	285	285	285	Q/S (1038–1039)	Q/S	Q/S	Q/S (1036–1037)
2B	119	119	119	119	Q/S (1157–1158)	Q/S	Q/S	Q/S (1155–1156)
2C	333	333	333	333	Q/G (1490–1491)	Q/G	Q/G	Q/S (1488–1489)
P2	**757**	**757**	**757**	**757**	**Q/G**	**Q/G**	**Q/G**	**Q/S**
3A	93	93	93	93	Q/S (1583–1584)	Q/S	Q/S	Q/S (1581–1582)
3B	34	34	34	28	Q/G (1617–1618)	Q/G	Q/S	Q/S (1615–1616)
3C	181	181	181	187	Q/G (1798–1799)	Q/G	Q/G	Q/G (1796–1797)
3D	453	453	453	453	-	-	-	-
P3	**761**	**761**	**761**	**761**	-	-	-	-

[a] ***Size:*** *Number of nucleotides (genome) or amino acids (proteins); numbers in parentheses indicate position of amino acid residues at the possible predicted C-terminal cleavage sites. Only positions of sites in the NC (DHAV-3) and VXXT (DHAV-1) strains are indicated; these sites are the same in other DHAV-3 strains (NT and DN2)*

genomes, with four major clusters: L, P1 (VP0–VP3–VP1), P2 (2A1–2A2–2B–2C), and P3 (3A–3B–3C–3D). Except for VP1 in the DHAV-1 VXXT strain, which was two amino acids shorter, the other clusters (L, non-structural P2, and P3) and individual genes were the same sizes in the Vietnamese DHAV-1 and DHAV-3 strains examined in this study *(Table 3)*. Eleven protease-cleavage sites were identified in the polyprotein in all four Vietnamese strains and two reference strains isolated in China, resulting in generation of 12 independent proteins (i.e., L, VP0, VP3, VP1, 2A1, 2A2, 2B, 2C, 3A, 3B, 3C, and 3D). The majority of predicted C-terminal cleavage sites in the polyprotein were Q/S or Q/G, with the exception of L/G between L and VP0; E/L or E/S between VP1 and A1; and NPG/P between 2A1/2A2 *(Table 3)*.

Sequence Comparison

Table 4 shows a comparison between the nucleotide and amino acid sequences of the Vietnamese NC strain and those of other Vietnamese strains (NT, DN2, VXXT) and the genotype-reference GD and C80 strains from China. The NC Vietnamese genome had nucleotide identity of 93.8-99.0% (ORF) or 94.0-98.8% (complete genome) with other DHAV-3 strains (i.e., NT, DN2, and reference GD), but much lower identity with the DHAV-1 VXXT vaccine (73.5%, ORF) and C80 (73.6%, ORF) reference strains. The amino acid similarity was higher: 98.6-99.4% within DHAV-3 strains and 83.2-83.6% between DHAV-3 and DHAV-1 strains *(Table 4)*.

Next, we compared the complete ORF sequences of the three Vietnamese DHAV-3 isolates (NC, NT, and DN2; 6756 nt) and that of the DHAV-1 vaccine strain VXXT (6750 nt), as well as their deduced amino acid sequences, with 44 other DHAV sequences available in GenBank (16 DHAV-1, 2 DHAV-2, and 26 DHAV-3). The GenBank accession numbers, codes of samples, and geographical origin used in this study are shown in *Fig. 1*.

Pairwise comparison of the ORF nucleotide sequences of the 48 strains revealed 91-99% identity between the 16 DHAV-1 strains, 92-100% identity between the 26 DHAV-3 strains, and 100% identity between the two DHAV-2

Table 4. *Comparison of the nucleotide and amino acid sequences of the Vietnamese NC strain to those of other Vietnamese strains (NT, DN2, VXXT) and the genotype-reference Chinese GD (DHAV-3) and C80 (DHAV-1) strains*

Genes/Regions	Length (DN2 Strain)		Nucleotide Identity (%)					Amino Acid Homology (%)				
	nt	aa	DHAV-3			DHAV-1		DHAV-3			DHAV-1	
			NT	DN2	GD	VXXT	C80	NT	DN2	GD	VXXT	C80
5'UTR	652	-	97.1	94.9	98.3	70.7	68.9	-	-	-	-	-
L	90	30	96.7	97.8	97.8	80	77.8	100	100	96.7	86.7	83.3
VP0	678	226	97.1	93.2	97.9	70.8	71.8	99.1	98.2	99.1	78.8	79.2
VP3	711	237	97.5	93.4	99.4	70.5	70.0	99.6	96.6	99.6	79.7	79.7
VP1	720	240	97.8	92.6	99.0	71.2	71.5	97.5	93.3	99.2	76.2	76.7
2A	915	305	97.3	93.4	99.0	69.3	69.3	97.4	96.1	99.3	74.4	75.4
2B	357	119	96.9	95.5	98.9	80.4	80.1	98.3	97.5	99.1	91.6	92.4
2C	999	333	98.2	93.7	99.1	76.7	76.3	99.4	98.2	99.4	91.9	91.9
3A	279	93	98.2	93.5	99.3	67.0	68.1	97.8	93.5	98.9	72.0	74.2
3B	102	34	99.0	95.1	100	70.6	71.6	100	100	100	79.4	79.4
3C	543	181	96.9	93.5	99.1	75.9	77.1	98.9	98.3	100	89.5	89.5
3D	1362	454	97.3	94.9	99.0	75.6	75.5	98.7	99.1	99.8	88.1	88.3
3'UTR	366	-	97.5	96.1	97.9	76.2	75.9	-	-	-	-	-
Structural P1	2109	703	97.4	93.1	98.8	71.3	71.5	98.7	96.0	99.3	78.2	78.5
Non-structural P2	2271	757	97.6	93.9	99.0	73.9	74.1	98.4	97.2	99.3	84.8	85.3
Non-structural P3	2286	762	97.4	94.4	99.1	74.6	75.1	98.6	98.3	99.7	86.1	86.5
ORF	6756	2252	97.5	93.8	99.0	73.5	73.6	98.6	97.2	99.4	83.2	83.6
Complete genome	7791	2252	97.2	94.0	98.8	72.8	72.8	-	-	-	-	-

nt: *nucleoties; aa: amino acid, Reference strain GD: accession numbers GQ122332; strain C80: accession numbers DQ864514*

strains (data not shown). Among the four Vietnamese strains (NC, NT, DN2 of DHAV-3; VXXT of DHAV-1), the highest nucleotide identity (98.4-98.6%) was observed between the Vietnamese NT and Chinese 12-01 (GenBank: KC893553) and B-N (JX235698) strains, and between the Vietnamese NC and Chinese DHAV-3 SD1201 strains (KC993890). The Vietnamese vaccine strain VXXT had 99% identity with the Korean DHAV-1 DRL62 (DQ219396) strain.

Phylogenetic Analysis of DHAV Genotypes

Based on the full ORF nucleotide sequences, a phylogenetic tree was constructed to examine the relationships between four Vietnamese and 44 other DHAV strains of all three genotypes *(Fig. 1)*. The tree revealed three clear genetic groups representing the DHAV-1, DHAV-2, and DHAV-3 genotypes. The DHAV-3 genotype was represented by 21 isolates from China and Korea and three isolates from Vietnam. These 24 DHAV-3 isolates could be divided into three clades (Clades 1, 2, and 3). Almost all Chinese isolates (15/16) grouped into Clade 1, along with two Vietnamese isolates (NC and NT). Of those, the NC isolate was most closely related to two Chinese strains, SD1201 and G, and the NT isolate was most closely related to the B-N and 1201 strains. All five Korean DHAV-3 strains were closely related within Clade 2. Clade 3 contained the Chinese B-63 and Vietnamese DN2 strains *(Fig. 1)*. The average genetic similarity within DHAV-3 Clades 1, 2, and 3 was 94%, 93%, and 93.5%, respectively.

The DHAV-1 genotype contained 19 different strains, divided into four main subgroups (Clades 1–4). The Vietnamese vaccine strain VXXT was clustered in Clade 3 with two Korean strains, the DRL62 and HS isolates. Clades 1, 2, and 4 comprised 19 Chinese DHAV-1 isolates *(Fig. 1)*; Clade 2 consisted of the Taiwanese strain O3D (GenBank number DQ249299) and four Chinese strains (DQ864514, DQ886445, HQ232302, and GQ130377) *(Fig. 1)*. The average nucleotide sequence similarity within clades 1, 2, 3, and 4 was 96-99%.

The DHAV-2 genotype was represented by only two strains from Taiwan (04G and 90D), which were 100% similar *(Fig. 1)*.

DISCUSSION

To date, several DHAV sequences from Korea, China, and Taiwan have been published [7,11,12,18,19]. In this study, we reported for the first time in an international journal the complete genome sequences and analysis of three DHAV-3 strains isolated from outbreaks in Vietnam, including DN2 (isolated in Dong Nai, 2009), NC (isolated in Ho Chi Minh

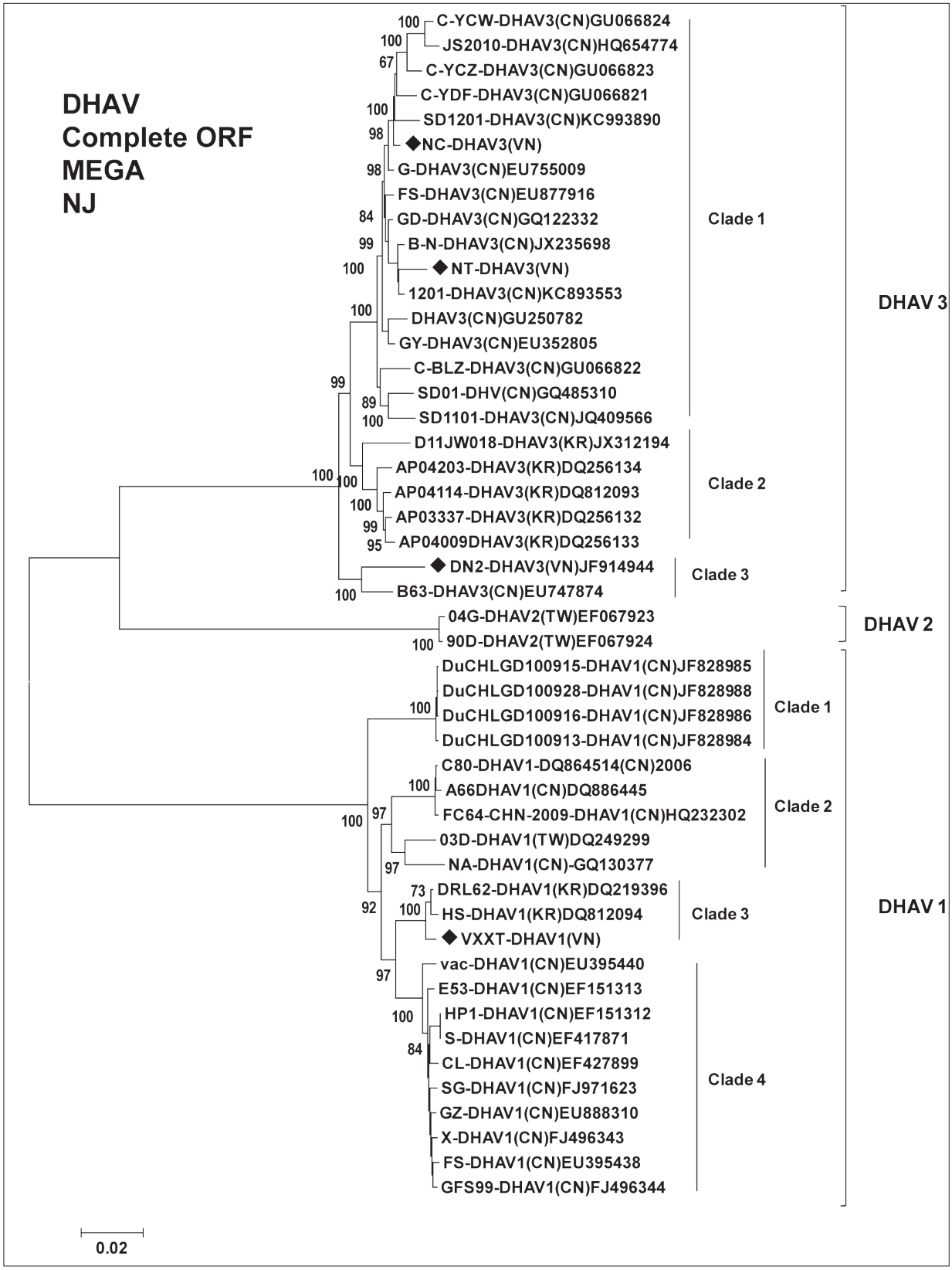

Fig 1. Phylogenetic tree showing the relationships between 4 Vietnamese and 44 DHAV strains of all three genotypes based on analysis of the full Open Reading Frame (ORF) nucleotide sequences. The phylogenetic tree was constructed with MEGA6.06 package using the neighbor-joining method [17] with bootstrap values of 1000 replicas (shown at each branch). Scale bar at bottom indicates the number of nucleotide substitutions per site. The isolates in this study are marked with bold letters and are indicated by a diamond symbol (♦). The accession numbers are given at the end of each sequence, where applicable. International country codes (https://countrycode.org/) in brackets; eg. VN, Vietnam; CN, China; TW: Taiwan; KR, South Korea.

City, 2009), and NT (isolated in Ninh Thuan province, 2013), as well as the DHAV-1 attenuated vaccine strain (VXXT) currently used for vaccination in Vietnam.

DHAVs were first detected in Vietnam in the 1980s. Some local reports based on epidemiological studies assumed that all DHVs in Vietnam were of the DHAV-1 genotype. However, none of the collected DHVs has been molecularly genotyped. In addition, despite efforts to understand the causes of this disease, no previous study has genetically characterized DHAV in Vietnam. Although a vaccine is used nationwide, severe outbreaks still occur in many areas throughout the country. In this study, we showed that the VXXT vaccine strain currently used in Vietnam is of the DHAV-1 genotype, whereas all three clinical samples collected from different provinces were of the DHAV-3 genotype. Our study of the complete genomes also revealed relatively low identity at the nucleotide (73%) and amino acid (83.1-83.6%) levels between strains of these two genotypes. This may explain the limited protection conferred by DHAV-1 vaccines against the circulating virulent DHAV-3 strains. Previous immunity studies in Taiwan and South Korea indicated that no cross-neutralization occurs in hosts infected with viruses of different genotypes [8,18].

Phylogenetic analysis of the complete ORFs confirmed that DHAVs in Vietnam were of two genotypes, DHAV-1 and DHAV-3. Noticeably, the Vietnamese vaccine strain (VXXT) was very closely related to two Korean strains (DRL62 and HS), forming a separate subgroup (Clade 3). This observation suggests that the VXXT strain may have originated in Korea. Our results also confirmed previous studies showing that the Chinese DHAV-1 strains, along with the Vietnamese strains used in this study, are highly diverse, forming three subgroups (Clades 1, 2, and 4) with nodal support (100%) in the phylogenetic tree [11].

Within the DHAV-3 genotype, all five Korean strains formed one subgroup (Clade 2). Clade 1 included most strains from China and two strains from Vietnam (NC and NT). The Vietnamese DN2 strain was closely related to the Chinese B-63 strain and formed a completely new subgroup, Clade 3. A previous study [15] reported a gene rearrangement between DHAV-1 and DHAV-3 in the Chinese DHAV-3 B-N strain, in which the 100 nt before the initiator codon has high identity with the corresponding sequence of DHAV-1. In this study, we demonstrated that the B-N strain was most closely related to another Chinese strain, 1201, and the Vietnamese strain NT. Moreover, we also paid special attention to the 5'UTR regions. Although the sequences of this region exhibit low similarity (66-72%) between genotypes 1, 2, and 3, all of them have some conserved regions, especially at 64 nt at the C-internal of the regions. In addition, the 34 amino acids at the N-terminus and the 24 amino acids from positions 65 to 88 of VP1 are extremely conserved among all genotypes. This information provides insight into the molecular features of DHAVs and hints at a transitional state between DHAV-1 and DHAV-3.

Our molecular investigation suggests that DHAV-3 viruses are predominantly spread in Vietnam. This is consistent with a previous study in China showing that DHAV-3 is more widespread than DHAV-1 [13], although other publications argued that DHAV-1 remains the most prevalent genotype in China [11,20]. Additionally, mixed infection with DHAV-1 and DHAV-3 in Chinese flocks has been in identified at various rates: 57.7% in one study [13] and 12% in another [14]. To date, however, we have not observed any mixed infections with these two genotypes in Vietnam. To clarify this issue further, more samples will be needed.

In summary, we report here the complete genome sequences of four DHAV samples: one attenuated vaccine of DHAV-1 genotype and three virulent strains of the DHAV-3 genotype currently circulating in Vietnam. These results will improve our understanding of the epidemiology and evolution of DHAVs in Vietnam. In addition to confirming the existence of DHAV-3 reported in previous studies, our observations emphasize the distribution of DHAV-3 viruses outside China and Korea and the significance of exploring DHAV-3 viruses in other countries where duck hepatitis is endemic and prevalent in ducklings. In light of these findings, an antigenicity-matched DHAV-3 vaccine is urgently required for use in Vietnam.

Acknowledgments

This study was supported by the National Foundation for Science and Technology Development (NAFOSTED) of Vietnam, under grant no. 106.04-2012.60 to Doan HTT. We also express our thanks to the National Key Laboratory of Gene Technology (Institute of Biotechnology, Vietnam) for providing equipment and facilities. The authors also extend thanks to colleagues for their kind provision of the materials used in this study.

REFERENCES

1. Levine P, Fabricant J: A hitherto-undescribed virus disease of ducks in North America. *Cornell Vet,* 40, 71-86, 1950.

2. Woolcock PR: Duck hepatitis. **In,** Saif YM, Barnes HJ, Glisson JR, Fadly AM, McDougald LR, Swayne DE (Eds): Diseases of Poultry. 11th edn., 343-354, Iowa State University Press, Ames, 2003.

3. Haider SA, Calnek BW: *In vitro* isolation, propagation, and characterization of duck hepatitis virus typee III. *Avian Dis*, 23, 715-729, 1979. DOI: 10.2307/1589748

4. Toth TE: Studies of an agent causing mortality among ducklings immune to duck virus hepatitis. *Avian Dis,* 13, 834-846, 1969. DOI: 10.2307/1588590

5. Knowles NJ, Hovi T, Hyypia T, King AMQ, Lindberg AM, Pallansch MA, Palmenberg AC, Simmonds P, Skern T, Stanway G, Yamashita T, Zell R: Picornaviridae. **In,** King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (Eds): Virus taxonomy: Classification and nomenclature of viruses: ninth report of the International Committee on Taxonomy of Viruses. 855-880, Elsevier, San Diego, California, 2012.

6. Kim MC, Kwon YK, Joh SJ, Lindberg AM, Kwon JH, Kim JH: Molecular analysis of duck hepatitis virus type 1 reveals a novel lineage close to the genus Parechovirus in the family Picornaviridae. *J Gen Virol,* 87, 3307-3316, 2006. DOI: 10.1099/vir.0.81804-0

7. Ding C, Zhang D: Molecular analysis of duck hepatitis virus type 1. *Virology*, 361, 9-17, 2007. DOI: 10.1016/j.virol.2007.01.007

8. Tseng CH, Tsai HJ: Molecular characterization of a new serotype of duck hepatitis virus. *Virus Res,* 126, 19-31, 2007. DOI: 10.1016/j.virusres.2007.01.012

9. Fu Y, Pan M, Wang X, Xu Y, Yang H, Zhang D: Molecular detection and typing of duck hepatitis A virus directly from clinical specimens. *Vet Microbiol*, 131, 247-257, 2008. DOI: 10.1016/j.vetmic.2008.03.011

10. Jin X, Zhang W, Zhang W, Gu C, Cheng G, Hu X: Identification and molecular analysis of the highly pathogenic duck hepatitis virus type 1 in Hubei province of China. *Res Vet Sci*, 85, 595-598, 2008. DOI: 10.1016/j.rvsc.2008.01.001

11. Gao J, Chen J, Si X, Xie Z, Zhu Y, Zhang X, Wang SJ, Jiang S: Genetic variation of the VP1 gene of the virulent duck hepatitis A virus type 1 (DHAV-1) isolates in Shandong province of China. *Virol Sin,* 27, 248-253, 2012. DOI: 10.1007/s12250-012-3255-8

12. Xu Q, Zhang RH, Chen LL, Yang L, Li JX, Dou PF, Wang H, Xie ZH, Wang Y, Jiang SJ: Complete genome sequence of a duck hepatitis A virus type 3 identified in Eastern China. *J Virol*, 86, 13848, 2012. DOI: 10.1128/JVI.02651-12

13. Chen LL, Xu Q, Zhang RH, Yang L, Li JX, Xie ZJ, Zhu YL, Jiang SJ, Si XK: Improved duplex RT-PCR assay for differential diagnosis of mixed infection of duck hepatitis A virus type 1 and type 3 in ducklings. *J Virol Methods,* 192, 12-17, 2013. DOI: 10.1016/j.jviromet.2013.04.012

14. Lin SL, Cong RC, Zhang RH, Chen JH, Xia LL, Xie ZJ, Wang Y, Zhu YL, Jiang SJ: Circulation and *in vivo* distribution of duck hepatitis A virus types 1 and 3 in infected ducklings. *Arch Virol,* 161, 405-416, 2016. DOI: 10.1007/s00705-015-2648-z

15. Wei CY, Su S, Huang Z, Zhu WJ, Chen JD, Zhao FR, Wang YJ, Xie JX, Wang H, Zhang G: Complete genome sequence of a novel duck hepatitis A virus discovered in Southern China. *J Virol*, 86, 10247, 2012. DOI: 10.1128/JVI.01643-12

16. Doan HTT, **Le XTK, Do RT, Hoang CTM, Nguyen KT, Le TH:** Molecular genotyping of duck hepatitis A viruses (DHAV) in Vietnam. *J Infect Dev Ctries*, 10, 988-995, 2016. DOI: 10.3855/jidc.7239

17. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S: MEGA 6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol,* 30, 2725-2729, 2013. DOI: 10.1093/molbev/mst197

18. Kim MC, Kwon YK, Joh SJ, Kim SJ, Tolf C, Kim JH: Recent Korean isolates of duckhepatitis virus reveal the presence of a new geno- and serotype when compared to duck hepatitis virus type 1 type strains. *Arch Virol,* 152, 2059-2072, 2007. DOI: 10.1007/s00705-007-1023-0

19. Wang L, Pan M, Fu Y, Zhang D: Classification of duck hepatitis virus into three genotypes based on molecular evolutionary analysis. *Virus Genes,* 37, 52-59, 2008. DOI: 10.1007/s11262-008-0233-1

20. Li J, Bi Y, Chen C, Yang L, Ding C, Liu W: Genetic characterization of duck hepatitis A viruses isolated in China. *Virus Res,* 178, 211-216, 2013. DOI: 10.1016/j.virusres.2013.10.007