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Original Article

Molecular genotyping of duck hepatitis A viruses (DHAV) in Vietnam

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Abstract

Introduction: The aim of this study was to identify the genetic characteristics and molecular genotyping of duck hepatitis A virus (DHAV) isolated in Vietnam during 2009–2013.

Methodology: Thirty duckling livers from outbreaks between 2009 and 2013 in seven provinces were collected and identified by polymerase chain reaction (PCR). Then, VP1 genes of eleven positive samples and two attenuated vaccine strains were sequenced and analyzed.

Results: Genotypic and phylogenetic analyses indicated that the 13 Vietnamese isolates were classified into two genotypes, DHAV-1 and DHAV-3. The rate of identity and homology was 91%–100% between the 10 Vietnamese and 26 global strains of DHAV-3, and 92%–100% between 3 Vietnamese and 16 strains of DHAV-1. Between the DHAV-3 and DHAV-1 strains, the divergence reached 30%. At the C-terminal of VP1 for the different strains, a hypervariable region was observed, and notably, six of the Vietnamese DHAV-3 strains in this study showed four consistent differences (at positions T184M, Q200H, K207N, and K214R) within this group that were distinct from all other DHAV-3 strains.

Conclusions: This is the first report of molecular characterization of DHAVs in Vietnam. At least two genotypes were identified, DHAV-1 and DHAV-3, with diversified clades within and between genotypes. DHAV-3 seemed to be dominant in Vietnam.

Key words: duck hepatitis A virus; VP1; genotyping; inter/intragenotypic variation; Vietnam.

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Introduction

Duck hepatitis virus (DHV) is an acute, rapidly spreading and fatal disease of young ducklings. Since it was discovered in 1945 in Long Island, New York, DHV has been identified worldwide, causing enormous loss to the livestock industry. The disease is caused by three serotypes of DHV: DHV-1, 2, and 3. DHV-1 is the most common and most virulent serotype worldwide [1,2]. Originally, DHV-1 was classified as an enterovirus [3,4]. However, recently, according to the Virus Taxonomy Ninth Report of the International Committee on Taxonomy of Viruses (ICTV), DHV-1 was classified as a member of the *Picornaviridae*, and therefore, DHV was renamed duck hepatitis A virus (DHAV). Based on phylogenetic analyses and neutralization tests, DHAVs are classified into three genotypes: DHAV-1, DHAV-2, and DHAV-3. There is no cross-neutralization between genotypes [5,6]. According to previous studies, DHAV-1 is widely distributed in China [7–9], DHAV-3 has been reported in Korea and China [5], whereas DHAV-2 has been detected only in Taiwan [6]. Limited data are available regarding DHAV viruses in other countries, including Vietnam, although severe outbreaks of DHAV have occurred in ducklings annually.

The genomic organization of DHAV is typical of a picornavirus, with a single large open reading frame (ORF) of 6,747 nucleotides for DHAV-1 and 2 and of 6,753 nucleotides for DHAV-3, flanked by 5'- and 3'-untranslated regions (UTRs) [7,10,11]. The ORF encodes a polyprotein, which is cleaved into a leader protein (L), and three structural (VP0-VP1-VP3) and eight non-structural proteins (2A1-2A2-2B-2C-3A-3B-3C-3D). The VP1 (viral protein 1) gene is a structural protein with potential antigenicity, containing epitopes recognized by B and T cells that induce protective neutralizing antibodies [12,13]. It also has the highest genetic diversity among the isolates. Phylogenetic analysis of the sequence of the VP1 gene may be used for DHAV genotyping [4]. Several methods have been established for the detection of DHAV infections, such as the indirect hemagglutination test, microneutralization assay, and enzyme-linked immunosorbent assay [14,15]. Although these

approaches have contributed to the diagnosis of DHAV infection, these methods are time consuming and lack sensitivity and specificity. Currently, direct reverse transcription polymerase chain reaction (RT-PCR) and cDNA-based PCR, which have higher sensitivity and specificity, are widely used for effective genomic and phylogenetic analysis of DHAVs.

Vietnam is a country of paddy rice cultivation and free-raising ducks/ducklings. Although duck hepatitis has been reported in Vietnam for 30 years, no molecular information regarding the genetic characteristics and genotyping analysis has been reported for DHAVs. Therefore, in the present study, we amplified the VP1 gene from 13 samples, including 11 clinical isolates collected from seven provinces and two DHAV vaccine strains currently used in Vietnam, using cDNA based-PCR. We then sequenced and compared these strains with all DHAV sequences available in GenBank. The objective of this study was to identify the genetic characteristics and genotypic forms of DHAV isolates in Vietnam for future epidemiologic and genomic studies.

Methodology

Clinical samples containing viruses

Thirty clinical samples from duckling livers were collected from outbreaks in seven provinces (Hung Yen, Hanoi, Sai Gon, Dong Nai, Khanh Hoa, Long An, and Ninh Thuan) in Vietnam between 2009 and 2013 (Table 1). The RNA was directly extracted from the samples for further molecular study. In some cases, the supernatants from the sample suspensions were propagated in 10-day-old embryonated specific pathogen-free (SPF) duck eggs, and allantoic fluid was collected 72 hours following inoculation. The fluids containing viruses were stored at -80°C for RNA extraction. Two attenuated vaccine strains currently used in Vietnam, namely VXXT, produced by VETVACO, the Veterinary Vaccine Company (Hanoi, Vietnam) and VXAC, produced by the Veterinary Vaccine Research Centre (Hanoi, Vietnam), respectively, were also included for molecular investigation and genotyping.

RNA extraction and reverse transcription

Viral RNA was extracted from samples using the RNAeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The total RNA was eluted in 30 μL of elution buffer and stored at -80°C until use. The cDNA synthesis reaction was performed using the Maxima Reverse Transcriptase Kit (Fermentas, Vilnius, Lithuania) as follows: 4 μL of viral RNA, 100 pmol random hexamer primer, 1 μL of dNTP mix (10 mM each), 5 μL of 5X transcriptase buffer, 20 U Ribolock Rnase inhibitor, 20 U Maxima Reverse Transcriptase, and 8.5 μL of RNase-free water, in a final volume of 20 μL. The RT reaction mixture was incubated at 50°C for 60 minutes and then for 5 minutes at 85°C to inactivate the enzyme. The cDNA was stored at -20°C until further use.

Primers and PCR amplification of the VP1 gene

Three pairs of primers were designed to specifically amplify the VP1 gene based on the conserved sequences of each DHAV genotype (Table 2). The conditions used for the amplification were as follows: initial denaturing at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes. The positive control and negative control were included in each PCR experiment. The PCR products were then separated by 1% agarose electrophoresis, stained with ethidium bromide, and photographed under UV light. The expected amplicon size was approximately 800 bp of the DNA region for the entire VP1 gene.

Nucleotide sequencing and computational analysis

Strong bands of approximately 800 bp identified by positive amplification were purified using a Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany) and used directly for sequencing (Macrogen, Seoul, Korea). The nucleotide sequences were identified using the basic local alignment search tool (BLAST) on the National Center for Biotechnology Information (NCBI) database website (http://www.ncbi.nlm.nih.gov/BLAST). A multiple nucleotide alignment of Vietnamese and published DHAV sequences was produced using GENEDOC version 2.7 [16]. A phylogenetic tree was constructed using MEGA6.06 [17] using the neighbor-joining method with 1,000 bootstrap replicas. The DHAV sequences obtained in this study were deposited in GenBank under the following accession numbers: JF925119 to JF925122, JF914944, JF914945, JF957697, and KM361878 to KM361883. The GenBank accession numbers of the VP1 gene sequences, their hosts, codes of samples, and geographical origin used in this study are shown in Table 1.

Table 1. List of DHAV strain VP1 sequences used in this study and their host and geographical origin (samples directly used in this study are bolded).

No	Genotype	Country	Code of samples	Isolated host	GenBank
1	DHAV-1	China	SY2	Duck	EF407858
2	DHAV-1	China	SY4	Duck	EF407860
3	DHAV-1	China	SY5	Duck	EF407861
4	DHAV-1	China	AV2111	Duck	EF442073
5	DHAV-1	China	ZI07	Duck	EF502168
6	**DHAV-1**	**Vietnam/Hanoi**	**GL08**	Duck	**JF925122**
7	**DHAV-1**	**Vietnam**	**VXXT (vaccine)**	Duck	**JF914945**
8	**DHAV-1**	**Vietnam**	**VXAC (vaccine)**	Duck	**JF957697**
9	DHAV-1	Korea	HS	Duck	DQ812094
10	DHAV-1	Korea	DRL-62	Duck	DQ219396
11	DHAV-1	China	E53	Duck	EF151313
12	DHAV-1	China	HN	Duck	EU395438
13	DHAV-1	China	FS	Duck	EU395438
14	DHAV-1	China	Vac	Duck	EU395440
15	DHAV-1	China	SH	Duck	EF502171
16	DHAV-1	China	GFS99	Duck	FJ496344
17	DHAV-1	China	GZ	Duck	EU888310
18	DHAV-1	China	R	Duck	EF585200
19	DHAV-1	China	X	Duck	FJ496343
20	DHAV-2	Taiwan	04G	Duck	EF067923
21	DHAV-2	Taiwan	90D	Duck	EF069724
22	DHAV-3	China	C-HRY	Duck	FJ626665
23	DHAV-3	China	C-NXC	Duck	FJ626669
24	DHAV-3	China	C-PSY	Duck	FJ626670
25	DHAV-3	China	CYCZ	Duck	GU066823
26	DHAV-3	China	CLX	Duck	FJ626667
27	DHAV-3	China	C-PJK	Duck	KC191694
28	DHAV-3	China	C-YZC	Duck	FJ626673
29	DHAV-3	China	FS	Duck	EU877916
30	DHAV-3	China	G	Duck	EU755009
31	DHAV-3	China	JN-6	Duck	KC191688
32	DHAV-3	China	JN-12	Duck	KC191689
33	DHAV-3	China	SD01	Duck	GQ485310
34	DHAV-3	China	SD1101	Duck	JQ409566
35	DHAV-3	China	VF-40	Duck	KC191686
36	DHAV-3	China	YT-63	Duck	KC191693
37	DHAV-3	China	CYCW	Duck	GU066824
38	DHAV-3	China	CYDF	Duck	GU066821
39	DHAV-3	China	GD	Duck	GQ122332
40	**DHAV-3**	**Vietnam/Hung Yen**	**BM**	Duck	**JF925119**
41	**DHAV-3**	**Vietnam/Hung Yen**	**HY**	Duck	**KM361878**
42	DHAV-3	China	GD1	Duck	EU289393
43	DHAV-3	China	B-N	Duck	JX235698
44	DHAV-3	China	YT-BX	Duck	KC191694
45	**DHAV-3**	**Vietnam/Ninh Thuan**	**NT**	Duck	**KM361883**
46	**DHAV-3**	**Vietnam/Ninh Thuan**	**NT2**	Duck	**KM361882**
47	DHAV-3	Korea	AP03337	Duck	DQ256132
48	DHAV-3	Korea	AP04009	Duck	DQ256133
49	DHAV-3	Korea	AP04203	Duck	DQ256134
50	DHAV-3	Korea	AP04114	Duck	DQ812093
51	DHAV-3	China	B63	Duck	EU747874
52	**DHAV-3**	**Vietnam/Dong Nai**	**DN1**	Duck	**JF925120**
53	**DHAV-3**	**Vietnam/Dong Nai**	**DN2**	Duck	**JF914944**
54	**DHAV-3**	**Vietnam/Saigon**	**NC**	Duck	**JF925121**
55	**DHAV-3**	**Vietnam/Khanh Hoa**	**KHO1**	Duck	**KM361879**
56	**DHAV-3**	**Vietnam/Khanh Hoa**	**KHO2**	Duck	**KM361880**
57	**DHAV-3**	**Vietnam/Long An**	**LA1**	Duck	**KM361881**

Note: Genotype for Vietnamese isolates, DHAV-1 and DHAV-3, are listed based on the results of genetic characterization in this study.

Results

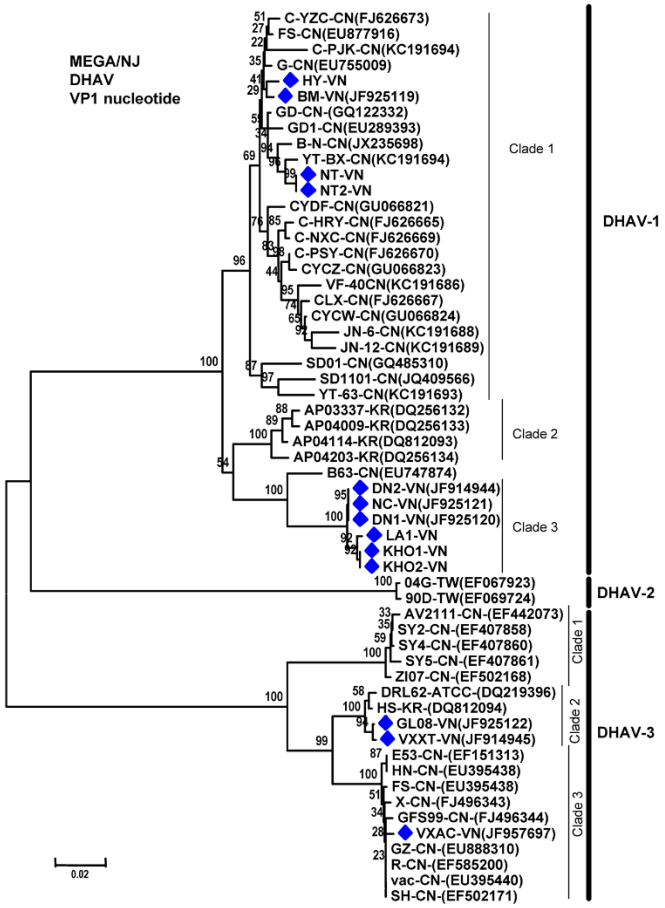
DHAV infection in ducks from Vietnam

Three primer pairs were used to screen for the presence of DHAV genotypes 1, 2, and 3 from the infected duckling samples. Of the total 30 DHAV field samples, 11 PCR products were clearly visualized on agarose gels, indicating the presence of DHAV in the samples. Of the 11 PCR-positive cases, one (GL08 sample) was successfully amplified using the DHAV-1 primers, and 10 amplicons (DN1, DN2, NC, BM, HY, KHO1, KHO2, NT, NT2, LA1) were specifically positive with the DHAV-3 primers. No samples were positive for DHAV-2. Both of the two attenuated DHAV vaccine strains from Vietnam, VXXT and VXAC, were positive for DHAV-1. Based on the sequencing results, the length of the VP1 gene for all DHAV-3 strains was 720 nt, six nucleotides longer than that sequence obtained from the DHAV-1 strains (714 bp).

Sequence analysis of the VP1 gene

The complete VP1 gene sequences of 13 Vietnamese isolates were aligned with 44 other DHAV sequences for all three genotypes, including 16 DHAV-1, 2 DHAV-2, and 26 DHAV-3, available in GenBank. There are ten DHAV-3 strains, all of which are field samples, and three DHAV-1 strains, including one field sample and two vaccine strains. The results of the sequence comparison showed that for nucleotide and amino acid, there was 91%–100% identity between the 10 Vietnamese samples and 36 strains of DHAV-3, and 92%–100% between the 3 Vietnamese samples and 19 strains of DHAV-1. This indicated that intragenotypic divergences within the DHAV-1 and DHAV-3 genotypes, both at the nucleotide and amino acid sequence levels, were between 0% and 9%, except for DHAV-2, in which the two known strains of Taiwanese origin were 100% identical. However, intergenotypic variation between DHAV-3 and DHAV-1 and DHAV-2 reached over 30%. These variations of 29%–30% were also observed between the ten Vietnamese

Figure 1. The phylogenetic tree was produced with MEGA6.06 using the neighbor-joining method [17] with bootstrap values of 1000 replicates (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 square symbol (♦). For the strain abbreviations, see Table 1. The accession numbers are given in bracket at the end of each of sequence, where applicable. VN, Vietnam; CN, China; TW: Taiwan; KR, South Korea.

DHAV-3 virulent isolates and the two currently used DHAV-1 attenuated vaccine strains. Therefore, identity between these two strains for DHAV-2 was 100%, whereas between these DHAV-2 strains and any DHAV-1 and DHAV-3 group did not exceed 71%. The

Table 2. Primers used for the VP1 gene of DHAV for genotyping.

Genotypes	Primer name	Sequences (5'-3')	Nucleotide position	Target gene (size [bp])
DHAV-1	DHAV1F	GCCCCACTCTATGGAAATTTG	2064-2084[a]	VP1 (714)
	DHAV1R	ATTTGGTCAGATTCAATTTCC	2810-2830[a]	
DHAV-2	DHAV2F	CACCACTTGGAGGAAATCAACAG	1949-1971[b]	VP1 (714)
	DHAV2R	CCACCTGATGTTTTGTTGTGAGAG	3004-3027[b]	
DHAV-3	DHAV3F	ATGCGAGTTGGTAAGGATTTTCAG	2130-2150[c]	VP1 (720)
	DHAV3R	GATCCTGATTTACCAACAACCAT	2920-2943[c]	

[a] Oligonucleotide positions refer to those in the published complete DHAV-1 sequence (GenBank accession number DQ864514); [b] Oligonucleotide positions refer to those in the published complete DHAV-2 sequence (GenBank accession number EF067923); [c] Oligonucleotide positions refer to those in the published complete DHAV-3 sequence (GenBank accession number DQ256133).

13 Vietnamese DHAV strains in this study shared 70% identity for nucleotides and 68% for amino acids with any of the strains of the other genotypic groups. Of these, the identity between the ten Vietnamese DHAV-3 virulent isolates and the two DHAV-1 attenuated vaccines was only 70%–71% for both the nucleotides and amino acids. These findings suggest the DHAV-1 vaccine currently used in Vietnam against the circulating and dominant DHAV-3 viruses may not provide the best protection because of mismatched antigenicity.

Regarding the variability of amino acid sequences in the 36 DHAV-3 strains compared, it was revealed that there was a hypervariable region at the C-terminal of VP1 among the different strains. Interestingly, six of the Vietnamese DHAV-3 strains (DN1, DN2, KHO1, KHO2, LA1, and NC) showed four consistent differences within this group but distinct from all other DHAV-3 strains. The mismatched positions are T184M, Q200H, K207N, and K214R (Table 3).

Phylogenetic analysis of DHAV genotypes

The phylogenetic tree based on the VP1 nucleotide sequences was constructed for all 57 DHAV strains, including the 13 strains of Vietnamese origin. The tree revealed three clear genetic groups representing genotypes for DHAV-1, DHAV-2, and DHAV-3, with 99%–100% bootstrap supportively placed at the basal nodes (Figure 1). The DHAV-1 genotype contained 19 different strains divided into three main subgroups:

Table 3. The variable positions at the C-terminal of the VP1 protein of the DHAV-3 strains with different geographical origins (samples from Vietnam directly used in this study are bolded). The consistent differences from all other DHAV strains are shown in a box.

No	Code of samples	Country	Sites of variation										
			178	182	183	184	186	191	196	200	207	214	219
1	CHR-Y	China	P	T	H	T	L	T	D	Q	K	K	H
2	CLX	China	P	T	H	T	L	T	N	Q	K	K	H
3	C-NXC	China	P	T	H	T	L	T	N	Q	K	K	H
4	C-PJK	China	P	T	H	T	L	T	D	Q	E	K	H
5	C-PSY	China	P	T	H	T	L	T	D	Q	K	K	H
6	CYCW	China	P	T	H	T	L	T	N	Q	K	K	H
7	CYCZ	China	P	T	H	T	L	T	D	Q	K	K	H
8	C-YZC	China	P	T	H	T	L	T	D	Q	E	K	H
9	FS	China	P	T	H	T	L	T	D	Q	E	K	H
10	G	China	P	T	H	T	L	T	D	Q	E	K	H
11	GD1	China	P	T	H	T	L	T	D	Q	E	K	H
12	GD	China	P	T	H	T	L	T	D	Q	K	K	H
13	JN-6	China	P	T	H	T	L	T	N	Q	K	K	H
14	JN-12	China	P	T	H	T	L	T	N	Q	K	K	H
15	SD01	China	P	T	H	T	L	T	N	Q	E	K	H
16	SD1101	China	P	T	H	T	L	T	N	Q	E	K	R
17	VF	China	P	T	H	T	M	T	N	Q	K	K	H
18	YT-63	China	P	T	H	T	L	T	N	Q	E	K	H
19	YT-BX	China	P	T	H	T	L	T	D	Q	E	K	H
20	B-N	China	P	T	H	T	L	T	N	Q	E	K	H
21	B63	China	S	T	**P**	T	L	T	D	Q	E	G	**Y**
22	AP03337	Korea	S	**A**	**P**	T	**S**	**I**	**E**	Q	K	K	**L**
23	AP04009	Korea	S	**A**	**P**	T	**S**	**I**	**E**	Q	K	K	**L**
24	AP04114	Korea	S	**A**	**P**	T	**S**	**I**	**E**	Q	K	K	**L**
25	AP04203	Korea	S	T	**P**	T	**S**	**I**	**E**	Q	K	K	**L**
26	**BM**	**Vietnam**	P	T	H	T	L	T	D	Q	K	K	H
27	**HY**	**Vietnam**	P	T	H	T	L	T	D	Q	K	K	H
28	**DN1**	**Vietnam**	S	T	**P**	**M**	**S**	T	D	**H**	**N**	**R**	**Y**
29	**DN2**	**Vietnam**	S	T	**P**	**M**	**S**	T	D	**H**	**N**	**R**	**Y**
30	**NC**	**Vietnam**	S	T	**P**	**M**	**S**	T	D	**H**	**N**	**R**	**Y**
31	**KH1**	**Vietnam**	S	T	**P**	**M**	**S**	T	D	**H**	**N**	**R**	**Y**
32	**KH2**	**Vietnam**	S	T	**P**	**M**	**S**	T	D	**H**	**N**	**R**	**Y**
33	**LA1**	**Vietnam**	S	T	**P**	**M**	**S**	T	D	**H**	**N**	**R**	**Y**
31	**NT**	**Vietnam**	P	T	H	T	L	T	D	Q	E	K	H
32	**NT2**	**Vietnam**	P	T	H	T	L	T	D	Q	E	K	H

clade 1, clade 2, and clade 3. Two Vietnamese isolates, VXXT and GL08, formed one subgroup, clade 2, which are closely related to the known sequences from Korea, HS-KR (GenBank: DQ812094) and DRL62-ATCC (DQ219396) (Figure 1). The other Vietnamese vaccine strain, VXAC, was clustered together with nine Chinese isolates to form clade 3. Clade 1 contained five DHAV-1 strains isolated in China: AV2111, SY2, SY4, SY5, and ZI07. The average genetic similarities of clades 1, 2, and 3 were 99%, 99%, and 99.2%, respectively.

As shown in Figure 1, the DHAV-3 genotype contained 36 different isolates from China, Korea, and Vietnam, divided into three clades. The average genetic similarities of clades 1, 2, and 3 were 97.4%, 98.2%, and 98%, respectively. Four Vietnamese isolates (BM, HY, NT, and NT2) had close relationships with the 21 known sequences from the Chinese DHAV-3 strains, forming one subgroup, clade 1. Four Korean isolates were closely related to each other and formed one separate branch (clade 2), whereas six Vietnamese isolates (DN1, DN2, NC, LA1, KHO1, and KHO2) belonged to a distinct cluster (clade 3), and had very close relationships with the Chinese B63 strain (GenBank, EU747874). This clade 3 of six Vietnamese DHAV-3 strains and the B63 of China is a newly formed subgroup not previously identified.

The DHAV-2 genotype contained only two identical strains, O4G and 90D, solely forming a distinct genotypic group as reported since their identification from Taiwan.

Discussion

Initial studies indicated that duck hepatitis virus (DHV) had numerous clinical and pathological characteristics similar to *Enterovirus* [18]. However, little was known about its molecular characteristics. Recently, the complete genome sequences of many DHV strains were determined in China, Korea, and Taiwan [4–9], which enable us to better understand the genomic organization and precise taxonomic position of DHV, indicating that it is genetically and functionally distinct from enteroviruses. Accordingly, DHV is classified as a member of a novel genus, *Avihepatovirus*, within the family *Picornaviridae*, and was changed to duck hepatitis A virus (DHAV), classified into three genotypes: DHAV-1, 2, and 3 (see ICTV [2013] at http://ictvonline.org/virusTaxonomy.asp). Currently, *Picornaviridae* includes 12 genera: *Enterovirus*, *Cardiovirus*, *Aphthovirus*, *Hepatovirus*, *Parechovirus*, *Erbovirus*, *Kobuvirus*, *Teschovirus*, *Sapelovirus*, *Senecavirus*, *Tremovirus*, and *Avihepatovirus*. Between different DHAV genotypes, the strains cannot be discriminated based on their clinical presentation, microscopic lesions, or the course of the disease. Therefore, PCR/RT-PCR assay is the optimal method for the detection/discrimination of DHAV-1, 2, and 3 infections in ducklings [6,19]. Using this technique, VP1 showed the distinct divergence of the external capsid protein in picornaviruses, including DHAV, which is commonly and reliably used for molecular characterization and phylogenetic analysis [20,21].

DHAV is one of the most important viral infections in ducks worldwide. Genotyping and molecular data are available from numerous endemic countries, but there remains limited information regarding this agent and its molecular characteristics in Vietnam. Indeed, DHAV was detected for the first time during 1983–1984 and continues to occur in many areas of the country. Despite increased knowledge of the duck hepatitis virus worldwide, there is little published information on the genetic characterization of DHAV field isolates, and no international publications about the DHAV endemic/epidemiologic situation from Vietnam.

In the present study, we reported, for the first time, the complete VP1 gene sequences of DHAV from 11 clinical duckling samples collected from seven provinces representing the majority of geographical distribution in Vietnam, including Hanoi, Hung Yen, Dong Nai, Ninh Thuan, Khanh Hoa, Long An, and Sai Gon. We also evaluated two currently used attenuated vaccine strains. Our results showed that both the DHAV-1 and DHAV-3 genotypes exist in Vietnam, with predominant endemicity to DHAV-3 viruses. In particular, DHAV-1 was detected in only one province of north Vietnam (Hanoi), whereas DHAV-3 viruses were detected in all other five provinces of Southern Vietnam (Ninh Thuan, Dong Nai, Long An, Khanh Hoa, and Sai Gon provinces) and one province of North Vietnam (Hung Yen) in this study. Our study revealed that DHAV-3 was more prevalent than DHAV-1 in Vietnam. These results are consistent with a study conducted in China that reported that DHAV-3 is more widely spread than DHAV-1 [19]. However, in another publication, Li *et al*. [22] suggested that DHAV-1 was prevalent in China. Although there was no consistency in the study of prevalence for DHAV genotypes DHAV-1 or DHAV-3 in China, and likewise in Vietnam, the co-existence of both genotypes has been clearly reported and confirmed [4,8,19,20,22].

The criteria for the same serotypes in picornaviruses are VP1 nucleotide identity ≥ 75% and/or amino acid identity ≥ 88% [23]. In this study, the identity between the isolates of genotypes DHAV-1, DHAV-2, and

DHAV-3 did not exceed 72% and 78%, respectively, and the amino acid sequence identities for the five Vietnamese DHAV-3 isolates and two Vietnamese DHAV-1 vaccines were less than 70%–72%. The two Vietnamese attenuated vaccine strains have been clearly classified as DHAV-1. Therefore, the protective effect of the DHAV-1 vaccine with virulent DHAV-3 strains may be restricted [24].

We also performed the genetic characterization of the VP1 genes in DHAV-3. Our study revealed a hypervariable region at the C-terminus of the VP1 gene among the different DHAV-3 strains as previously identified in DHAV-1 viruses [10,22]. Four consistent residues, T182A, T191I, D196E, and H219L for the four Korean isolates (AP03337, AP04009, AP04114, and AP04203) and other T184M, Q200H, K207N, and K214R, respectively, for the six Vietnamese isolates (DN1, DN2, NC, LA1, KHO1, and KHO2), were detected, which are quite distinct from all other DHAV-3 strains (Table 3). This finding indicated that the change in the VP1 gene of DHAV-3 might be related to the geographic location. The consistent amino acids for each group of strains representing geographical localities may be a source for discriminative implementation within the DHAV-3 genotype.

Phylogenetic analysis of the Vietnamese DHAVs confirmed the existence of two genotypes in Vietnam, genotype 1 (DHAV-1) and genotype 3 (DHAV-3), and supported the division of clades within each genotype (clades 1, 2, and 3 for DHAV-1 and DHAV-3). The most remarkable finding of our study is that clade 3 of DHAV-3 comprises six Vietnamese isolates closely related to the B63 strain of China, and this represents a new clade 3 never before reported.

According to a previous study, mixed DHAV-1 and DHAV-3 infections were very serious and common in China (57.7%) [19], and mixed DHAV-1 and DHAV-2 infections were detected in Taiwan [11]. However, in the present study, we did not find any co-infections in the Vietnamese clinical samples via analysis of the VP1 genes. Further molecular characterization of the complete genomic sequences of DHAV in Vietnam must be performed to better understand the genomic reassortments.

Conclusions

We report, for the first time, the genetic characterization of the VP1 gene of the duck hepatitis A virus isolated in Vietnam by examining the nucleotide and amino acid sequences and constructing a phylogenetic tree for genotyping. At least two genotypes were identified, DHAV-1 and DHAV-3, and DHAV-3 seemed to be dominant in the country. Our results will be helpful for understanding the molecular epidemiology of DHAV and genotype-matching vaccine implementation in Vietnam. This study also determined the current status of DHAV infection in ducklings from Vietnam, which has not previously been reported internationally.

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