

# One-Tube RT-PCR for the Simultaneous Detection and Genotyping of Duck Hepatitis A Virus Subtypes 1 and 3

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Running Head: One-Tube RT-PCR for Genotyping DHAV-1 and DHAV-3

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**Abstract:** The co-circulation of duck hepatitis A virus subtypes 1 (DHAV-1) and 3 (DHAV-3) in ducklings has resulted in significant economic losses. Because ducklings infected with DHAV-1 or DHAV-3 show similar clinical signs and gross lesions, it is important to discriminate these subtypes as early as possible for better clinical management. On the basis of multiple alignments of the 5'-noncoding region sequences of strains DHAV-1 and DHAV-3, universal and type-specific primers were designed and synthesized. Using the primers in a one-tube reverse transcription-PCR (RT-PCR) assay, reference strains of DHAV-1 and DHAV-3 (isolated over a span of 60 years and covering many different countries) were successfully amplified, indicating that the primer sequences were completely conserved. The amplicon sequences results and the sizes of amplicons from reference DHAV-1 and DHAV-3 isolates correlated completely with their genotypes. Moreover, with this one-tube RT-PCR system, the amplicon sizes of liver samples of reference DHAV-1- or DHAV-3-infected birds matched perfectly with their respective genotypes, as determined by virus isolation and neutralization tests. No other RNA viruses of duck origin were detected with the synthesized primers. The sensitivity of viral RNA detection was 10 pg. With this system, 20% genotype 1, 45% genotype 3, and 9% co-infection of the two genotypes were detected in 55 clinical samples. This novel approach could be used for the rapid genotyping DHAV-1 and/or DHAV-3 infection in routine clinical surveillance or epidemiologic screening.

**Keywords: DHAV-1; DHAV-3; Phylogenetic analysis; One-tube RT-PCR; Simultaneously genotyping; Serotype**

## 1. Introduction

Duck hepatitis A virus (DHAV), the etiologic agent of viral hepatitis in ducklings, is an acute, highly lethal, and contagious pathogen (1, 2). Three heterologous serotypes of DHAV have been identified: DHAV subtype 1 (DHAV-1, the most common, virulent, and globally prevalent serotype), DHAV subtype 2 (DHAV-2, a Taiwan serotype), and new emerging DHAV subtype 3 (DHAV-3, a South Korea, Vietnam, and China serotype) (3-7).

DHAV, a member of Genus *Avihepatovirus* in Family *Picornaviridae* (8), threatens all duck-growing farms worldwide, causing a greater than 80% mortality rate among ducklings. The DHAV-1 genome is a single-stranded, positive-sense RNA composed of a single, large open reading frame (ORF) that encodes a polyprotein of 2,249 aa and is flanked by a 5'-noncoding region (5'-NCR) and a 3'-noncoding region. As in all members of the *Picornaviridae*, the genome of DHAV-1 is organized as follows: 5'-NCR-VP0-VP3-VP1-2A1-2A2-2B-2C-3A-3B-3C-3D-3'-NCR (8).

The diagnosis of duck hepatitis is usually based on the observations of clinical signs, gross pathologic changes, and reproduction of the disease in susceptible ducklings. However, these methods have been shown to be incapable of detecting or discriminating DHAV-1 from DHAV-3 because of the similar clinical symptoms and pathologies caused by these two serotypes (9). Although the neutralization test (NT) (10), enzyme-linked immunosorbent assay (1), virus isolation (VI) (12), and immunofluorescence assays (11, 13) are reliable for typing DHAV, they are labor intensive and time consuming.

Currently, the most widely preferred diagnostic method is molecular genotyping, where the subtype of an isolate is determined largely by sequencing part of the virus genome and then using phylogenetic analysis to compare it with known subtype reference sequences (14). To date, many RT-PCR has been used widely to simultaneously detect infections of animal and plant viruses (15-17). However, this

method has not been applied to the simultaneous detection of DHAV-1, DHAV-3 or coinfection of DHAV-1 and DHAV-3.

The polyprotein-encoding genome sequence has generally been used to determine the phylogenetic relationships among the picornavirus genotypes, which correlate with the virus serotypes. In this present study, we conducted sequence analysis using information of the DHAV-1 and DHAV-3 sequences available in the GenBank database. The phylogenetic analysis indicated that the 5'-NCR could be used as a target gene for DHAV-1 and DHAV-3 genotyping analysis. With a universal primer and type-specific primers targeted to the 5'-NCR, we developed a one-tube RT-PCR for the simultaneous detection and genotyping of DHAV-1 and DHAV-3, without amplicon sequencing. With this one-tube RT-PCR system, we demonstrated that the RNA amplicon sequences and sizes of reference viruses or of samples from reference virus-infected birds matched perfectly with those obtained using the gold standard method: combined virus isolation and neutralization test (VI/NT). A total of 55 clinical samples were successfully screened with this one-tube RT-PCR assay. Since DHAV-2 strains are unavailable in China, we mainly detected DHAV-1 and DHAV-3 in this study.

## **2. Materials and Methods**

### *2.1. Ethics statement*

All experiments involving animals were approved by the Animal Welfare and Ethical Censor Committee at Harbin Veterinary Research Institute (HVRI) and the Animal Ethics Committee of the HVRI of the Chinese Academy of Agricultural Sciences (License SYXK (Heilongjiang) 2011022).

### *2.2. Viruses*

Three DHAV-1-related strains (viz., DRL-62 (from ATCC), R85952 (from ATCC), and HP-1 (1,18) and five DHAV-3-related strains (viz., JT and GY (9), and recently isolated strains HLJ-1, ZJ-01309, and SD0517) were used as references in this study. The viruses were propagated in 12-day-old

embryonated duck eggs (free of DHAV-1 and DHAV-3 infections) as described previously (9), and the allantoic fluids of the infected eggs were collected and stored at  $-80^{\circ}\text{C}$ .

### 2.3. *Clinical samples*

Fifty-five clinical liver samples with hemorrhagic lesions were collected and used to screen DHAV-1 and DHAV-3 field infections in bird flocks during 2015–2017 in China.

### 2.4. *Phylogenetic analysis of the 5'-NCR and polyprotein-encoding genes of DHAV-1 and DHAV-3*

Nucleotide sequences of the 5'-NCR and the polyprotein-encoding genes of DHAV-1 and DHAV-3 in GenBank were used to create phylogenetic trees for relationship studies. GenBank information was extracted for nine reference strains of DHAV-1 and seven of DHAV-3, which were isolated from many different countries over a wide time span (>60 years) (Table 1). LASERGENE 7.1 software (DNASTAR 6.0, Madison, WI, USA) was used for the sequence analysis. Phylogenetic trees were generated using the neighbor-joining method in the Molecular Evolutionary Genetics Analysis 4.0 software program (19), where bootstrap probabilities were calculated with 1,000 replicates. The phylogenetic trees were visualized using the TreeView program.

**Table 1.** Duck hepatitis A viruses used in this study.

<b>Virus</b>	<b>Isolate</b>	<b>GenBank No.</b>	<b>Country</b>	<b>Year</b>
DHAV-1	DRL-62	DQ219396	America	1970
	R85952	DQ226541	America	1955
	DHV-HS	DQ812094	South Korea	1994
	C80	DQ864514	China	2011
	HP-1	EF151312	China	1975
	5886	DQ249301	America	2005
	03D	DQ249299	Taiwan	2003
	H	JQ301467	England	1993
	DHV-HSS	DQ812092	South Korea	1995
DHAV-3	AP-03337	DQ256132	South Korea	2003
	JT	JF835025	China	2010
	SD01	GQ485310	China	2008
	AP-04009	DQ256133	South Korea	2011
	B63	EU747874	China	2011
	DN2	JF914944	Viet Nam	2009
	LS	KP233203	China	2014

## 2.5. Primers

The 5'-NCR was selected for primer design on the basis of multiple alignments of the GenBank sequences of DHAV-1 and/or DHAV-3 (Table 1), using the CLUSTAL W program (DNASTAR 6.0). The forward primer pAF was designed for the detection of both DHAV-1 and DHAV-3 on the basis of the conserved region in both serotypes. The type-specific reverse primers pA1R and pA3R were designed on the basis of the type-specific conserved region in DHAV-1 and DHAV-3, respectively. A PCR assay using these three primers in one tube was evaluated for the simultaneous detection and genotyping of DHAV-1 and DHAV-3.

## 2.6. RNA extraction and one-tube/one-step RT-PCR

Two hundred microliters of the reference virus stocks (viz., strains DRL-62, R85952, HP-1, JT, GY, HLJ-1, ZJ-01309, and SD0517) or of the supernatants from clinical sample homogenates were used for RNA extraction with TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The one-tube RT-PCR (Qiagen, Hilden, Germany) was carried out in a 25- $\mu$ l reaction volume, comprising 2  $\mu$ l of RNA and various concentrations of the forward primer pAF and reverse primers pA1R/pA3R. The RT-PCR mixture was subjected to the following thermal cycling conditions: an initial 50°C for 30 min and 95°C for 15 min, followed by 15 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; 30 cycles of 95°C for 30 s, 60 °C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 3 min. The PCR products were detected by 1% agarose gel electrophoresis (Sigma–Aldrich, St. Louis, MO, USA) and stained with ethidium bromide. The PCR products of appropriate size were extracted using an Agarose Gel DNA extraction kit (Watson Biotechnologies, Inc., Shanghai, China) and then submitted for commercial sequencing (DNA Sequence Service, TaKaRa Biotechnology Co. Dalian, China). To exclude laboratory contamination, the RNA extractions and PCR

mixtures were prepared and processed with different sets of pipettes and filter tips. Each RT-PCR was tested for contamination by using reagents as negative controls.

### 2.7. Cloning and sequence analysis

The PCR products of appropriate size were purified and cloned using a pMD18-T cloning kit (TaKaRa Biotechnology Co., Ltd.), as described previously (14). Positive plasmids were purified using a QIAprep spin miniprep kit (Qiagen, Valencia, CA, USA) and submitted to TaKaRa Biotechnology Co. for sequencing. The nucleotide sequences were analyzed with the MegAlign program and deposited in the GenBank database.

### 2.8. Sensitivity of the RT-PCR assay

The sensitivity of the one-tube RT-PCR assay for DHAV-1 and DHAV-3 detection was measured by using 10-fold serial dilutions of the allantoic fluids containing strain HP-1 or JT. RNA from each 10-fold dilution ( $10^{-1}$  to  $10^{-7}$ ) of the viruses was extracted as described above, and 2  $\mu$ l ( $10^2$  to  $10^{-4}$  ng) from each dilution was used in the one-tube RT-PCR. The sensitivity of the assay for a mixed virus stock (mixed HP-1 and JT) was also evaluated. In brief, each individual 10-fold serial dilution of HP-1 and JT was mixed prior to RNA extraction, and the sensitivity of the RT-PCR assay was detected in the same way as described above for the single strains.

### 2.9. One-tube RT-PCR for the detection of DHAV-1/DHAV-3 from liver samples of experimentally infected birds

To evaluate the efficiency of the established RT-PCR assay for detecting samples from infected birds, ducklings were infected with the reference DHAV-1 and DHAV-3 strains. In brief, 11 groups of birds (each group consisting of ten 1-day-old specific-pathogen-free (SPF) ducklings) were respectively intramuscularly inoculated with a  $10^{4.5}$  duck embryo lethal dose (ELD<sub>50</sub>) of the individual DHAV-1 (DRL-62, R85952, and HP-1) and DHAV-3 (JT, GY, HLJ-1, ZJ-01309, and SD0517) strains, or mixed



virus stocks A (HP-1 and JT) or B (DRL-62 and GY), or with 0.2 ml of phosphate-buffered saline (PBS) as a negative control. The birds were observed daily for clinical signs, gross lesions, and death. Liver samples from the dead infected birds and non-infected control birds were collected for analyses of the extracted RNA by one-tube RT-PCR and combined VI/NT. The liver samples were pretreated before the RNA extraction or virus isolation, as described previously (9,14). In brief, after three freeze–thaw cycles, the liver samples were homogenized in PBS (1:2, v/v) and pelleted by centrifugation at 5,000 ×g for 15 min. The supernatants were collected for RNA extraction or VI/NT. The extracted RNA was used for one-tube RT-PCR, as described above. Amplicons of 214 and 289 bp in size were submitted for sequencing.

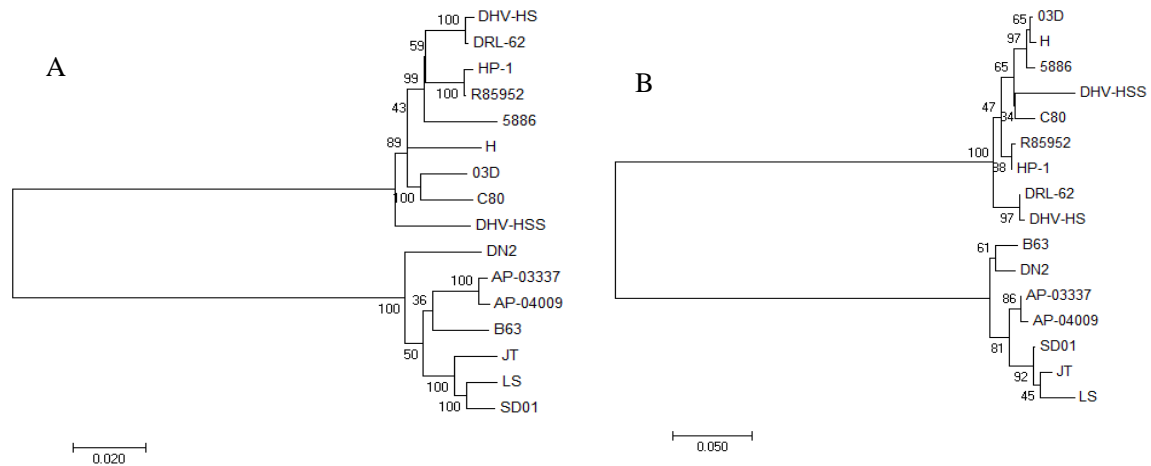
#### *2.10. Virus isolation and neutralization test (VI/NT) for DHAV-1 and DHAV-3 detection*

VI was conducted on the liver homogenate supernatants that had been positively verified by RT-PCR to contain single amplicons of 214 or 289 bp. In brief, 0.2 ml of the positive supernatants was injected into 11-day-old SPF duck embryos. The allantoic fluids were collected from the embryos that had died after 24 h of inoculation and stored at  $-70^{\circ}\text{C}$  until use. The DHAVs in the allantoic fluids were then characterized by NT using anti-DHAV-1 or anti-DHAV-3 sera (1, 9). The virus NTs were performed using the constant-virus variable-serum method, as described previously (20), with SPF duck embryos used as the indicator. The sera against DHAV-1 and DHAV-3 were inactivated at  $56^{\circ}\text{C}$  for 30 min before the test. The endpoint titer of the serum against homologous and heterologous viruses was calculated using the method of Reed and Muench (21). The antigenic relationships (r-values) were calculated as the ratio between the heterologous and homologous serum titers. The supernatant samples from the mixed virus-infected birds were not subjected to VI/NT.

### *3. Results*

### 3.1. Phylogenetic analysis of the 5'-NCR and polyprotein-encoding gene of DHAV-1 and DHAV-3

The polyprotein-encoding gene sequence is usually used to ascertain the phylogenetic relationships among picornavirus genotypes, which correlate with the virus serotypes. To prove that the 5'-NCR sequence could be used for virus genotyping, we compared the phylogenetic trees constructed on the basis of the polyprotein-encoding gene and the 5'-NCR sequences. The phylogenetic tree based on the polyprotein-encoding gene sequences clearly demonstrated that DHAV-1 and DHAV-3 constitute two monophyletic clades, with nine strains in the DHAV-1 clade and seven strains in the DHAV-3 clade (Figure 1A). Similarly, the phylogenetic tree based on the 5'-NCR also formed two groups, with nine DHAV-1 strains forming one group and seven DHAV-3 strains forming another group (Figure 1B). Sequence analysis indicated that the polyprotein-encoding gene of the DHAV-1 and DHAV-3 strains showed 93.8–100% homology within the same genotype (clade) and 66.3–70.2% identity between the two genotypes (clades). Similarly, the 5'-NCR of the DHAV-1 or DHAV-3 strains isolated in different locales and years were closely related, showing 94.2–99.8% homology within genotypes (clade), and 51.4–66.7% identity between different genotypes (clades). The similar phylogenetic patterns and genetic distances of the 5'-NCR and polyprotein-encoding gene of DHAV-1 and DHAV-3 suggested that the 5'-NCR could also be used for genotyping or phylogenetic relationship studies of these viruses.

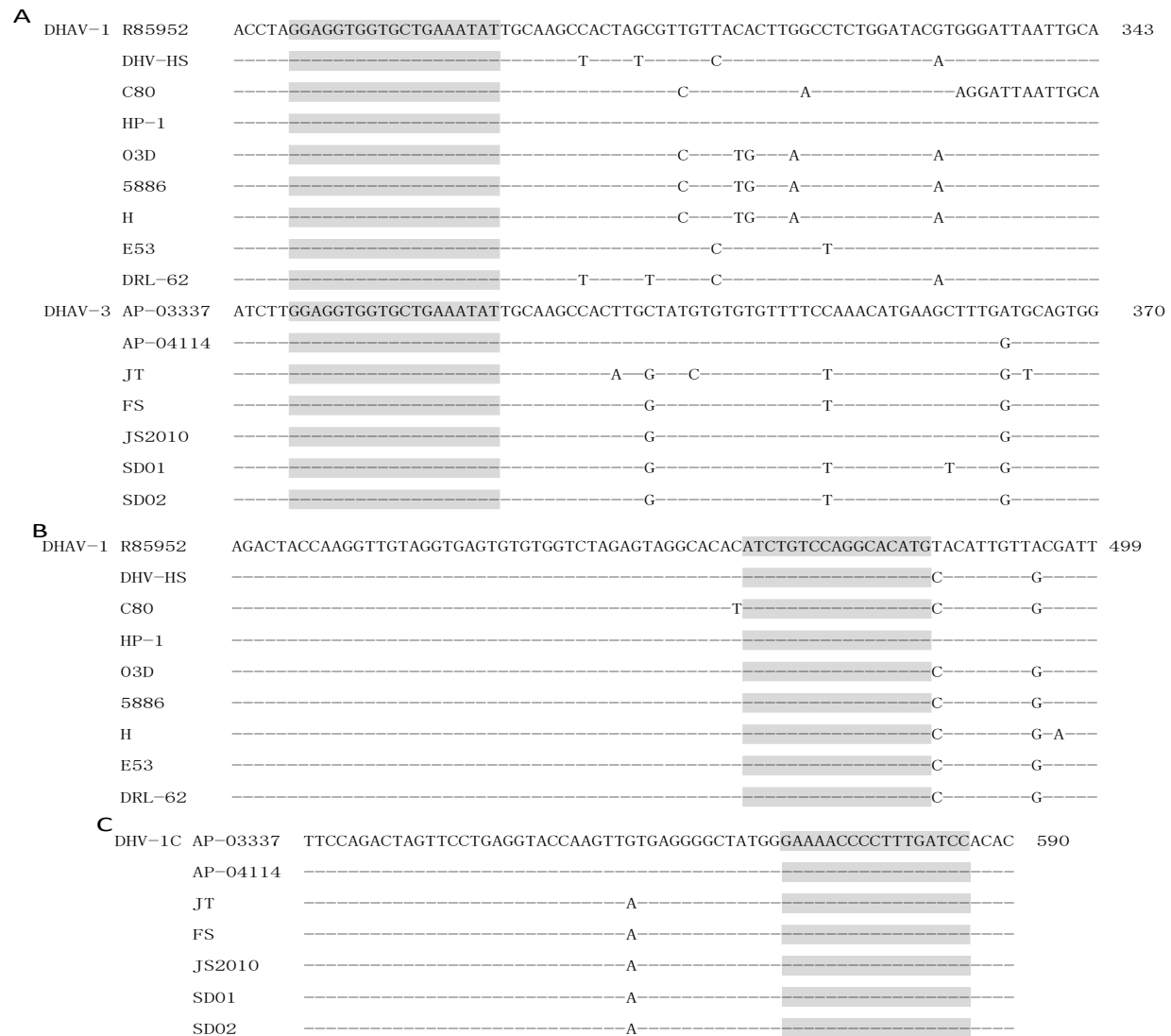


**Figure 1.** Phylogenetic analysis of the polyprotein-encoding gene and the 5'-noncoding region of DHAV-1 and DHAV-3 strains from GenBank. The numbers above and below the branches indicate bootstrap values. The scale bar represents the nucleotide substitutions per site.

### 3.2. Primer sequences

Since the 5'-NCR could be used for genotyping analysis, we selected it for the primer design in this study. To locate the most highly conserved sequences, the 5'-NCR nucleotide sequences of 16 DHAVs (listed in Table 1) from the two different genotypes were aligned. Despite being different species, the 5'-NCRs of the DHAV-1 and DHAV-3 strains revealed some conserved regions upon careful analysis of their nucleotide sequences. One absolutely conserved sequence (5'-GGAGGTGGTGCTGAAATAT-3') in both DHAV-1 (nucleotides 271–289) and DHAV-3 (nucleotides 298–316) was selected for design of the common forward primer pAF for PCR (Figure 2A). To allow the specific detection of DHAV-1 or DHAV-3, the reverse primers were designed separately. The design criterion of type-specific primers is to not only maintain maximum sequence differences between two genotypes but also to keep completely

sequence conservation within the same genotype. To find sequences that are highly conserved within the same genotype but highly divergent between different genotypes, the 5'-NCR sequences of various strains from the two different genotypes were aligned separately. A highly conserved region from nucleotides 468 to 484 was selected as the DHAV-1-specific reverse primer (pA1R) region (Figure 2B) and that from nucleotides 570 to 586 as the DHAV-3-specific reverse primer (pA3R) region (Figure 2C). Since DHAV-2 strains are unavailable in China, the primers designed in this study were mainly intended for DHAV-1 and DHAV-3 detection only. The primer information is listed in [Table 2](#).



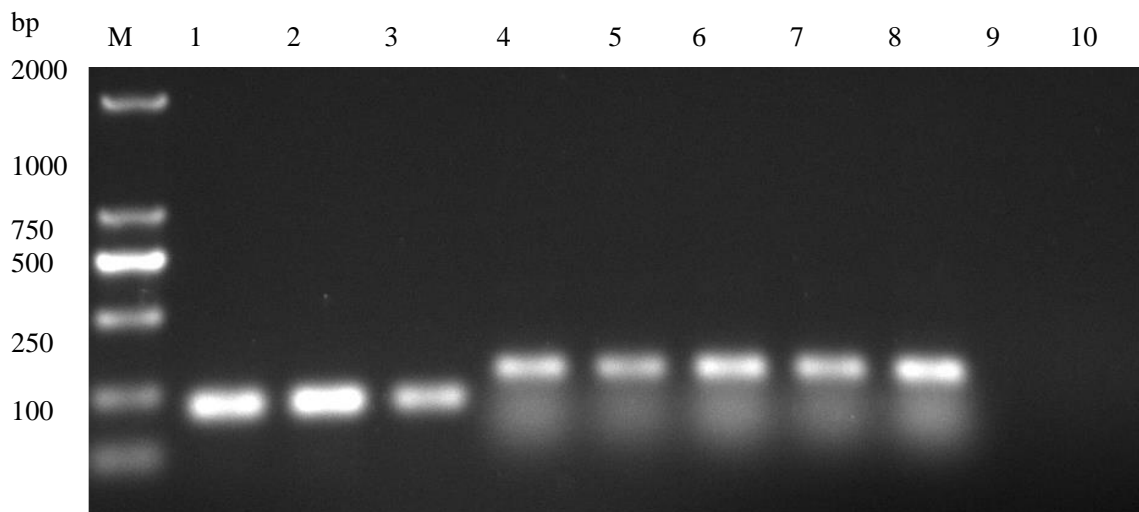
**Figure 2.** Primers designed using three alignments of the 5'-noncoding region sequences from DHAV-1 and DHAV-3 strains. Nucleotides identical to the top sequences are indicated by dashes. Nucleotide positions are numbered to the right of the sequences. Three conserved regions, namely (in shaded boxes) 271–289 for DHAV-1 and 298–316 for DHAV-3 (A), 468–484 (B) and 570–586 (C), were used for the design of primers pAF, pA1R, and pA3R, respectively.

**Table 2.** Primer information.

Virus	Primer	Primer sequence	Sequence region	Amplicon size (bp)
DHAV-1	pAF	5'-GGAGGTGGTGCTGAAATAT-3'	271–289	214
	pA1R	5'-CATGTGCCTGGACAGAT-3'	468–484	
DHAV-3	pAF	5'-GGAGGTGGTGCTGAAATAT-3'	298–316	289
	pA3R	5'-GGATCAAAGGGGTTTTTC-3'	570–586	

### 3.3. Detection of reference DHAV-1 and DHAV-3 strains

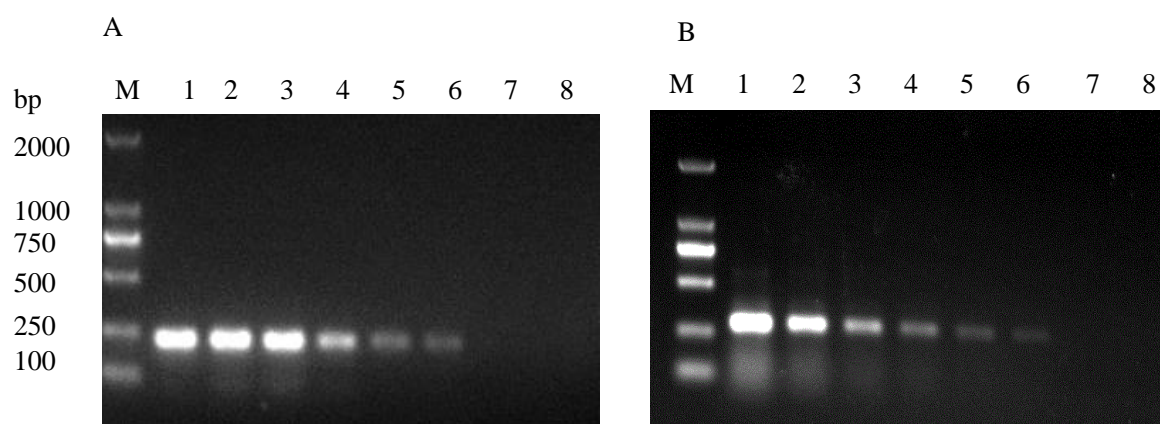
One-tube/one-step RT-PCR was carried out using the RNA from the different strains (i.e., DHAV-1-related strains DRL-62, R85952, and HP-1; and DHAV-3-related strains JT, GY, HLJ-1, ZJ-01309, and SD0517) as templates. By using all three primers (pAF, pA1R, and pA3R) in one tube, the one-step RT-PCR detected only single products of either 214 or 289 bp (Figure 3). The amplified PCR products were then cloned into the pMD18-T vector, and positive clones were purified and again submitted for sequencing. The nucleotide sequence of each clone (corresponding to the viral RNA in the test tube) was exactly the same as the 5'-NCR sequence of the corresponding reference strain in GenBank (data not shown). The cloned amplicon sizes of 214 or 289 bp were completely in accord with the corresponding DHAV subtypes.



**Figure 3.** One-tube PCR for differentiating DHAV-1 and DHAV-3. The one-tube PCR was performed using mixed primers. Lanes 1, 2, and 3, DHAV-1 (strains DRL-62, R85952, and HP-1) PCR products (214 bp), respectively; lanes 4, 5, 6, 7, and 8, DHAV-3 (strains JT, GY, HLJ-1, ZJ-01309, and SD0517) PCR products (289 bp); lanes 9 and 10, PBS-injected allantoic fluid as negative control; lane M, DNA molecular marker.

### 3.4. Sensitivity of the one-tube RT-PCR

The sensitivity of the one-tube RT-PCR for detecting representative DHAV-1 (HP-1) and DHAV-3 (JT) strains was measured using 10-fold serial dilutions (from  $10^{-1}$  to  $10^{-7}$ ) of the virus stocks, as described previously (14), representing  $10^3$  to  $10^{-3}$  ng of viral RNA in the reaction at each dilution. The RT-PCR system detected up to the  $10^{-6}$  dilution of the viruses, which was equivalent to approximately 10 pg of viral RNA per reaction (Figure 4). A similar sensitivity was obtained for the mixed DHAV-1 and DHAV-3 stocks (data not shown).



**Figure 4.** Determination of one-tube RT-PCR detection sensitivity for DHAV-1 (A) and DHAV-3 (B). Lanes 1 to 7, one-tube RT-PCR products obtained from  $10^{-1}$  to  $10^{-7}$  DHAV-1 (A) or DHAV-3 (B) stock dilutions, respectively; lane 8, PBS-injected allantoic fluids as negative controls; lane M, DNA molecular marker. (A) and (B) represent the amplicons of RNA templates extracted from serial 10-fold dilutions of DHAV-1 strain HP-1 and DHAV-3 strain JT virus stocks, respectively. The lowest dilution of DHAV-1 or DHAV-3 virus stocks detected was  $10^{-6}$ .



### 3.5. Specificity of the one-tube RT-PCR

To test the specificity of the designed primers for DHAV-1 and DHAV-3, RNAs were extracted from other avian virus isolates corresponding to a number of distinct virus families; namely, avian influenza viruses (H9N2 and H5N1), Newcastle disease virus, Muscovy duck reovirus, and duck Tembusu virus. The amplification processes were performed with RNA, as described above. None of the three primers amplified sequence fragments of the appropriate sizes (i.e., 214 and/or 289 bp; data not shown). Therefore, the primers designed in this study possess a high degree of specificity for the detection and genotyping of DHAV-1 and DHAV-3.

### 3.6. Detection and genotyping of DHAV-1/DHAV-3 from infected birds

In the 1-day-old SPF ducklings, the mortality rates within 2 weeks of virus inoculation were 100% in the groups infected with DHAV-1-related strains DRL-62, R85952, or HP-1; 70% in the groups infected with DHAV-3-related strains JT or GY; 80% in the groups infected with DHAV-3-related strains HLJ-1, ZJ-01309, or SD0517; 100% in the group infected with mixed strains HP-1 and JT; and 90% in the group infected with mixed strains DRL-62 and GY. All birds, no matter the type of infection, showed similar clinical symptoms and pathologic changes, including typical hepatitis lesions and enlarged livers with hemorrhages. No signs of disease or death occurred in the PBS-injected negative control birds. RNA from the liver homogenates of each group was subjected to one-tube/one-step RT-PCR for DHAV-1 and DHAV-3 detection or for VI/NT.

The PCR results showed that single 214- and 289-bp fragments were correspondingly amplified from the birds infected with the single DHAV-1- and DHAV-3-related strains, respectively (Figure 5, lanes 3, 4, and 9 for DHAV-1; lanes 1, 5, 6, 8, and 10 for DHAV-3). Mixed 214- and 289-bp fragments were simultaneously amplified from the liver samples of birds infected with mixed virus stocks A (HP-1 and JT) and B (DRL-62 and GY). Those 214- and 289-bp amplicons were simultaneously detected on

the electrophoresis gel from samples of co-infected birds (Figure 5, lanes 2 and 7), which are in accord with the stock A or B solutions (data not shown). The amplicon sequences were consistent with the corresponding GenBank sequences of reference viruses, suggesting that this one-tube/one-step RT-PCR could simultaneously detect DHAV-1 and DHAV-3 co-infections in liver samples. No amplicons were detected in the liver samples from the PBS-injected control group.

Combined VI/NT is considered a “gold-standard” method for testing single DHAV-1 and DHAV-3 infections. The NT results showed that the allantoic fluids from the birds infected with the single reference DHAV-1 and DHAV-3 strains were neutralized by the homologous antisera (with  $r \geq 1$ ), and only, to some extent in the presence of undiluted heterologous antisera. These data demonstrated that the single 214- or 289-bp amplicons from the liver samples correlated with the VI/NT results, suggesting that the one-tube RT-PCR system is also suitable for DHAV-1 and DHAV-3 detection in liver samples of experimentally infected birds. Whereas the VI/NT is not applicable for detecting DHAV-1 and DHAV-3 co-infection in birds, the consistent correlation of the 214 and 289-bp co-amplified products with the mixed reference virus sequences in this study suggests that this one-tube/one-step RT-PCR system is also suitable for the clinical screening of co-infected birds.



**Figure 5.** One-tube RT-PCR for genotyping DHAV-1 and DHAV-3 in experimentally infected birds. Liver samples were collected from dead birds after single DHAV-1 or DHAV-3 infection or DHAV-1 and DHAV-3 co-infection. Lane M, 100-bp DNA marker. Lanes 1, 5, 6, and 10, amplicons (289 bp) from birds with single DHAV-3 infection (represented by strains JT, GY, ZJ-01309, SD0517, and HLJ-1, respectively). Lanes 3, 4, and 9, amplicons (214 bp) from birds with single DHAV-1 infection (represented by strains DRL-62, R85952, and HP-1, respectively). Lanes 2 and 7, amplicons (214 and 289 bp) from birds with DHAV-1 and DHAV-3 co-infections (represented by stocks A (mixed HP-1 and JT) and B (mixed DRL-62 and YG), respectively). No PCR products were detected from PBS-injected control birds (data not shown).

### 3.7. Screening of DHAV-1/DHAV-3 field infections

To further compare the one-tube RT-PCR and VI/NT assays, both methods were used to screen 55 liver samples from birds with natural field infections of DHAV-1 and DHAV-3 in China. Amplicons of 214 or 289 bp were cloned into the pMD18-T vector, and the positive clones were submitted for sequencing. Of the 55 samples, 11 amplicons (about 20%) with a single size of 214 bp, 25 amplicons (about 45%) with a single size of 289 bp, and 5 amplicons (about 9%) with both 214- and 289-bp sizes were obtained. The sequencing results showed that 11 clinical samples (isolates) were of DHAV-1, 25 were of DHAV-3, and 5 were of both DHAV-1 and DHAV-3. Because some amplicon sequences showed 100% homology to one another, we selected five different sequences in each of the DHAV-1, DHAV-3, and co-infected DHAV-1/-3 groups for submission to GenBank (Accession Nos. listed in [Table 3](#)).

1 **Table 3.** Results of virus detection by the one-tube RT-PCR and the virus isolation/neutralization test.

2	Isolate/Sample	Serotype	Size (bp)	Genotype	Homology to (%)		GenBank No.
					DHAV-1	DHAV-3	
4	HRB01507	1	214	DHAV-1	95–99	66–69	MK292314
5	GZ01506	3	289	DHAV-3	66–68	98–100	MK292305
6	LY01602A/B	1/3	214/289	DHAV-1/3	96–100/65–67	66–69/97–99	MK292317/MK292308
7	FJ01501	3	289	DHAV-3	67–68	97–100	MK292304
8	BJ01704	3	289	DHAV-3	66–68	97–100	MK292302
9	JN01706A/B	1/3	214/289	DHAV-1/3	95–100/66–68	66–69/97–100	MK292316/MK292307
10	SH01609	3	289	DHAV-3	66–68	97–100	MK292309
11	CC01508	1	214	DHAV-1	96–100	67–69	MK292312
12	CF01701A/B	1/3	214/289	DHAV-1/3	95–100/67–69	66–68/97–100	MK292313/MK292303
13	SY01701	1	214	DHAV-1	95–99	65–68	MK292319
14	JL0153A/B	1/3	214/289	DHAV-1/3	95–100/66–67	66–68/96–100	MK292315/MK292306
15	ZZ01507A/B	1/3	214/289	DHAV-1/3	96–100/66–68	66–69/98–100	MK292321/MK292311
16	RZ015010	1	214	DHAV-1	95–100	66–68	MK292318
17	ZH01605	3	289	DHAV-3	66–68	98–100	MK292310
18	YY01607	1	214	DHAV-3	96–100	66–69	MK292320

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#### 21 4. Discussion

22 The polyprotein-encoding gene sequences have been widely used to evaluate the phylogenetic  
23 relationships among picornavirus genotypes, which also correlate with the virus serotypes. The similar  
24 phylogenetic topology of the 5'-NCR and polyprotein-encoding gene of reference DHAVs confirmed  
25 that 5'-NCR might be suitable for genetic subtype analysis. The criterion for routine primer design is that  
26 a set of primers should accommodate both the similarities and differences exhibited by most of the virus  
27 sequences. The homologies at the 5'-NCR nucleotide level indicated that DHAV-1 and DHAV-3 had  
28 adapted to their hosts over a long time. The completely conserved 5'-GGAGGTGGTGCTGAAATAT-3'  
29 sequence between DHAV-1 and DHAV-3 might be functionally related and suitable as a universal  
30 primer to allow the detection of both serotypes. The 34.3–48.6% diversities in the 5'-NCR sequences  
31 suggest that DHAV-1 and DHAV-3 have been evolving separately for a long time, resulting in two  
32 distinct genogroups, making this sequence suitable for the design of type-specific primers. After careful  
33 examination of the 5'-NCR sequences of each subtype, we designed completely conserved type-specific  
34 primers that allowed us to amplify type-specific RNAs from DHAV-1 and DHAV-3 strains. In the  
35 RT-PCR with the three primers in one tube, only the main DHAV-1-specific 214-bp or  
36 DHAV-3-specific 289-bp product was obtained for samples with the respective virus subtype, and no  
37 other products were obtained when nucleic acids other than the genomic RNA of the specified DHAV-1  
38 or DHAV-3 strains were used as the template. With this one-tube RT-PCR, we successfully detected and  
39 genotyped samples from dead birds with a single virus infection or a co-infection, validating the  
40 approach used in this study. When the one-tube RT-PCR method was tested for its sensitivity, as little as  
41 10 pg of viral RNA was detected, suggesting that the assay is sensitive enough for virus detection and  
42 genotyping. The specificity and sensitivity of the one-tube RT-PCR assay were confirmed by the  
43 sequencing results and combined VI/NT, which verified the amplified products to be of DHAV-1 (214

44 bp) and DHAV-3 (289 bp), respectively. The easily discriminating sizes of amplicons could  
45 simultaneously differentiate DHAV-1 and DHAV-3 infection without sequencing results. The lack of  
46 cross-reaction between DHAV-1 and DHAV-3 also strengthens the assay for the detection of DHAV-1  
47 and DHAV-3 co-infection.

48 The applicability of the one-tube RT-PCR to detecting and genotyping clinical samples was also  
49 tested. Of the 55 clinical samples tested, 20% were detected as a DHAV-1 infection, 45% as a DHAV-3  
50 infection, and 9% as co-infections, indicating that the DHAV-3 subtype was the main problem for duck  
51 industries in China. The development of a DHAV-3-specific vaccine is therefore of utmost importance,  
52 given the current high prevalence of this subtype in duck flocks and the unavailability of measures to  
53 control the virus.

54 This one-tube RT-PCR approach has the following advantages: the size difference of the amplicons  
55 is big enough to allow easy interpretation of the results by untrained staff, without requiring amplicon  
56 sequencing. Moreover, the technique uses fewer costly PCR reagents, and combining the RT and PCR  
57 processes into a single step greatly reduces the time, labor, and contamination potential. In addition, this  
58 one-step RT-PCR protocol is easy to carry out, even for untrained staff, and it does not yield a separate  
59 volume of cDNA as in the two-step and real-time protocols that need to be used in multiple PCRs for  
60 different targets. Considering the disease severity and emergence of new serotype DHAV-3, this novel  
61 approach would be most helpful to specifically and accurately diagnose the closely related DHAV-1 and  
62 DHAV-3 strains and enable their early detection in clinical samples during routine examination. This  
63 simple RT-PCR system could be applied in resource-limited settings or farms, for surveillance or for  
64 routine epidemiologic screening.

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69 **Author Contributions**

70 Y.Z. designed the experiments. X.C., Y.C. and C.L. performed the experiments. X.L., M.L., X.B. and  
71 H.L. analyzed the data.

72 **Conflicts of Interest**

73 The authors declare no conflict of interest.



74 **References and Notes**

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