Research Article

Improved one-tube RT-PCR method for simultaneous detection and genotyping of duck hepatitis A virus subtypes 1 and 3

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Abstract

Background
The cocirculation of duck hepatitis A virus subtypes 1 (DHAV-1) and 3 (DHAV-3) in ducklings has resulted in significant economic losses. Ducklings with DHAV-1 or DHAV-3 infection show similar clinical signs and gross lesions; hence, it is important to identify the viral subtypes in infected ducklings as early as possible for better clinical management.

Methods and results
Based on multiple 5’ noncoding region (5’-NCR) sequences of DHAV-1 and DHAV-3 strain alignments, universal and type-specific primers were designed and synthesized. With three primers in one-tube reverse transcription-PCR (RT-PCR), reference DHAV-1 and DHAV-3 isolates ranging over 60 years and across many different countries were successfully amplified, indicating that the primer sequences were completely conserved. The sequence results and the sizes of amplicons from reference DHAV-1 and DHAV-3 isolates are completely correlated with their subtypes. Moreover, with this one-tube RT-PCR system, amplicon sizes from liver samples of reference DHAV-1- or DHAV-3-infected birds fit closely with their subtypes, which was determined by virus isolation and neutralization testing. No other duck-origin RNA viruses were detected. The sensitivity of viral RNA detection was 10 pg. With this system, 20% subtype 1, 45% subtype 3, and 9% coinfection of two subtypes were detected in 55 clinical samples.

Conclusions and significance
This novel approach could be used for rapidly typing DHAV-1 or DHAV-3 infection in routine clinical surveillance or epidemiological screening.
Introduction

Duck hepatitis A virus (DHAV) is an acute, highly lethal and contagious etiological agent of viral hepatitis of ducklings [1, 2]. Based on phylogenetic analyses, DHAV has been classified into 3 genotypes: DHAV type 1 (DHAV-1), type 2 (DHAV-2), and type 3 (DHAV-3). DHAV-2 and DHAV-3 are genetically and serologically different from DHAV-1[3–6]. The most common, virulent, and worldwide distribution subtype is DHAV-1. DHAV subtype 2 is only limited in Taiwan. DHAV subtype 3 is often prevalent in South Korea, Vietnam, and China [3–11]. DHAV-1 occurs worldwide and threatens all duck-growing farms, causing more than 80% mortality in ducklings.

DHAV is a member of Avihepatovirus in the family Picornaviridae [4, 12, 13]. The genomes of DHAV-1, DHAV-2 and DHAV-3 are single-stranded, positive-sense RNA containing a single, large open reading frame (ORF), which encodes a polyprotein measuring 2249 aa flanked by the 5'- and 3'-noncoding region (NCR). The genomes of DHAV-1 and DHAV-3 are organized as members of the family Picornaviridae: 5'NCR-VP0–VP3–VP1–2A–2B–2C–3A–3B–3C–3D–3'NCR. There are several approaches for the diagnosis of duck hepatitis disease, including examining clinical signs, determining gross pathological changes, and reproducing the disease in susceptible ducklings. However, these methods have been shown to be incapable of detecting or discriminating DHAV-1 and DHAV-3 because of their similar clinical symptoms and pathology [14]. The neutralization test [15], virus isolation combined with PCR [16], and immunofluorescence assays [11, 17] are reliable for typing DHAV, but they are labor-intensive and time-consuming. Enzyme-linked immunosorbent assays (ELISA) are reliable test for detecting DHAV-1/DHAV-3, but antigen preparation in ELISA is also cumbersome [1,18].

To date, RT-PCR has been used widely to simultaneously detect infections of animal and plant viruses. Due to the emergence of new DHAV-3 subtypes, the widely preferred method is molecular genotyping [8, 10, 19–24], in which the subtype of an isolate is determined largely by sequencing part of the viral genome and then using phylogenetic analysis to compare it with already known subtype reference sequences. Some RT-PCR approaches are employed for the detection and genotyping of DHAV-1 and DHAV-3, but the amplicons are difficult to discriminate by size, and this approach must also employ sequencing results analysis [8]. Moreover, this approach is time-consuming, labor-intensive, and expensive. Duplex PCR differential approach for DHAV-1 and DHAV-3 was successfully developed that enabled easy discrimination of amplicons by size, but multiple sets of primers require multiple rounds of PCR [10, 21, 22], which are also labor-intensive, expensive and inconvenient. The third real-time PCR detection approach [19, 24] is accurate, but this assay is cumbersome, labor-intensive, and expensive and requires special equipment. Therefore, it may be desirable to develop a rapid, simple, sensitive, and economical diagnostic assay for genotyping DHAV-1 and DHAV-3.

Previously, the polyprotein genome sequence has usually been used to judge phylogenetic relationships within picornavirus genotypes, which correlated with virus serotypes or subtypes. In this study, we conducted sequence analysis based on the available sequence information of DHAV-1 and DHAV-3 in GenBank. Phylogenetic analysis indicated that the 5′-NCR could be used as a target gene for DHAV-1 and DHAV-3 subtype analysis. Using universal primer and type-specific primers targeted to the 5′-NCR, we developed a one-tube RT-PCR method 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 sequence results and the size of amplicons of reference viruses or samples of reference virus-infected birds fit well with the gold standard method: virus isolation and neutralization test (VI/NT). Fifty-five
clinical samples were successfully screened with this one-tube RT-PCR system. Since DHAV-2 is unavailable in China, we examined DHAV-1 and DHAV-3 detection in this study.

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

Viruses
Three DHAV-1 strains, DRL-62 (from ATCC), R85952 (from ATCC), and HP-1 [1, 25], five DHAV-3 strains, JT and GY [14], and three 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 [14], and the allantoic fluids of infected eggs were collected and stored at -80°C. Avian influenza viruses (AIV) WY11 and WY24 [26], Newcastle disease virus (NDV) vaccine strain Lasota, Muscovy duck reovirus (DRV) S14 strain [27], and duck Tembusu virus (DTMUV)TA strain [28] were used for PCR specificity analysis.

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 from 2015 to 2017 in China.

Phylogenetic analysis of the 5′-NCR and the polyprotein of DHAV-1 and DHAV-3
Sixteen nucleotide sequences from the 5′-NCR and the polyprotein of DHAV-1 and DHAV-3 in GenBank were used to create phylogenetic trees for relationship studies. The strain information from GenBank includes nine reference strains of DHAV-1 and seven of DHAV-3, which covered the greatest variety of countries and ranged most widely in time (across 60 years) (Table 1). LASERGENE 7.1 software (DNASTAR, 6.0, Madison, WI, USA) was used for sequence analysis. Phylogenetic trees were generated by using the Neighbor-Joining method in MEGA 4.0 software [29]; Bootstrap probabilities were calculated with 1000 replicates. The phylogenetic trees were visualized by using the program TreeView.

Primers
The 5′-NCR was selected for primer design based on GenBank sequence alignments of DHAV-1 and/or DHAV-3 (Table 1) by using the CLUSTAL W computer program (DNASTAR 6.0, Madison, WI, USA). The forward primer pAF was designed for the detection of both DHAV-1 and DHAV-3 subtypes based on conserved regions for both DHAV-1 and DHAV-3. The subtype-specific reverse primers pA1R and pA3R were designed based on conserved subtype-specific regions for DHAV-1 or DHAV-3, respectively. A PCR assay using these three primers in one tube was evaluated for detection and genotyping DHAV-1 and DHAV-3.
RNA extractions and one-tube/one-step RT-PCR

For this experiment, 200 μl of reference virus stock (including three DHAV-1 related viruses, DRL-62, R85952, and HP-1; and five DHAV-3 related viruses, JT, GY, HLJ-1, ZJ-01309, and SD0517) or supernatants of clinical sample homogenates were used for RNA extraction with TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. The RNA was eluted in 20 μL of DEPC water. One-Step RT-PCR (Qiagen, Hilden, Germany) was carried out in a 25-μL reaction volume containing 5 μL of 5×PCR buffer, 1 mM dNTP mix, 2 μL of extracted RNA, 2 μL of pAF and 1 μL of each pA1R/pA3R (primer concentration 2.5 pmol/μL), 2 μL of One-Step RT-PCR Enzyme Mix. A thermal cycler (Biometra, Germany) was used for the RT-PCR. The RT-PCR mixture was subjected to the following thermal cycle conditions: a reverse transcription step at 50°C for 30 min, a denaturation step at 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, 72°C for 30 s; and a final extension for 3 min at 72°C. The PCR products were detected by 2% agarose gel electrophoresis (Sigma–Aldrich, St. Louis, MO, USA) stained with ethidium bromide. The appropriate sizes of PCR products were extracted using an Agarose Gel DNA extraction kit (Watson Biotechnologies, Inc. Shanghai, China) and then submitted for sequencing. To exclude laboratory contamination, RNA
extractions and PCR mixture preparations were processed with different sets of pipettes and filter tips. Each RT-PCR was used to screen for contamination using negative reagent controls.

**Cloning and sequence analysis**

Appropriate sizes of PCR products were purified and cloned into a pMD18-T cloning kit (TaKaRa Biotechnology Co., Ltd.) as described previously (27). Positive plasmids were purified using a QIAprep spin miniprep kit (Qiagen, Valencia, CA, USA) and submitted for sequencing (DNA Sequence Service, TaKaRa Biotech Co). The nucleotide sequences were analyzed with the MegAlign program and deposited in GenBank.

**Sensitivity of the RT-PCR assay**

The sensitivity of the one-tube RT-PCR for detecting DHAV-1 and DHAV-3 was measured by using 10-fold serial dilutions of the allantoic fluids of the HP-1 or JT strains. RNA from each dilution (10-fold, \(10^{-1}\) to \(10^{-7}\)) of viruses was extracted as described above. Two microliters of RNA (approximately \(10^2\) to \(10^{-4}\) ng) from each dilution in a 25-μL reaction volume was employed with one-tube RT-PCR. Sensitivity for mixed stock (mixed HP-1 and JT) was also evaluated. Briefly, each individual 10-fold serial dilution of HP-1 and JT was mixed prior to RNA extraction. The sensitivity of the RT-PCR process was evaluated as described above.

**One-tube RT-PCR for DHAV-1/-3 detection samples from experimentally infected birds**

To evaluate the efficiency of the established RT-PCR for detection samples from infected birds, ducklings were infected with reference DHAV-1/-3. Briefly, the eleven groups of birds (each group consisted of 10 1-day-old SPF ducklings) were intramuscularly inoculated with \(10^{4.5}\) duck embryo lethal dose (ELD\(_{50}\)) of single DHAV-1 (DRL-62, R85952, and HP-1), single DHAV-3 (JT, GY, HLJ-1, ZJ-01309, and SD0517), mixed stocks A (HP-1 and JT) (with equal dose) and B (DRL-62 and GY), and 0.2 ml PBS as a negative control. The birds were observed daily for clinical signs, gross lesions, and death. Liver samples from dead infected or noninfected control birds were collected for one-tube RT-PCR analysis and virus isolation combined with the neutralization test (VI/NT). Liver samples were pretreated before being subjected to RNA extraction or virus isolation as described previously (13, 29). Briefly, after three freeze–thaw cycles, liver samples were homogenized at 1:2 (v/v) in phosphate-buffered saline (PBS) and pelleted by centrifugation at 5,000×g for 15 min. The supernatants were collected for RNA extraction or VI/NT. Extracted RNA was then used for one-tube RT-PCR as described above. Amplicons of 214 bp and/or 289 bp were submitted for sequencing.

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

When supernatants of liver samples were determined with single amplicons of 214 bp or 289 bp by RT-PCR, we next conducted virus isolation from these positive supernatants. Briefly, 0.2 mL selected positive homogenized supernatants were injected into 11-day-old SPF duck embryos. Allantoic fluids were collected after the embryos died over 24 h of inoculation and stored at -70°C until use. DHAV in the allantoic fluids was then characterized by a neutralization test using anti-DHAV-1 or anti-DHAV-3 sera [1, 14]. Virus neutralization tests were performed using the constant virus variable serum method as described previously [1]. Sera against DHAV-1 and DHAV-3 were inactivated at 56°C for 30 min before the neutralization test. SPF duck embryos were used as the indicator in the virus neutralization test. The end-point titer of the serum against homologous and heterologous viruses was calculated by the
method of Reed and Muench [30]. Antigenic relationships (r-values) were calculated as the ratio between heterologous/homologous serum titer. Samples of DHAV-1 and DHAV-3 mixed-infection birds were not subjected to VI/NT.

Results
Phylogenetic analysis of the 5'-NCR and the polyprotein genome of DHAV-1 and DHAV-3

The polyprotein genome sequence is usually used to judge phylogenetic relationships within picornavirus genotypes, which correlate with virus serotypes. To prove that the 5'-NCR sequence could be used for virus genotyping, we compared phylogenetic trees constructed by polyprotein and the 5'-NCR. The phylogenetic tree constructed by the polyprotein genome clearly demonstrated that DHAV-1 or DHAV-3 constitute two monophyletic clades, DHAV-1 clade (9 strains) and DHAV-3 clade (7 strains) (Fig 1A). Similar to the phylogenetic tree of the polyprotein, the phylogenetic tree built by the 5'-NCR also forms two groups: 9 strains of DHAV-1 form group A and 7 strains of DHAV-3 form group B (Fig 1B). Sequence analysis indicated that the polyprotein genome of DHAV-1 and DHAV-3 strains showed 93.8–100% homology within the same genotype (clade) and 66.3–70.2% identity between two genotypes (clades). Similar to that of the polyprotein genome, the 5’-NCR of DHAV-1 or DHAV-3 strains from different locales and years were closely related within the same genotype (clade), showing 94.2–99.8% homology within genotypes and 51.4–66.7% identity between different genotypes (clades). The similar pattern of phylogenetic trees and the similar genetic distance of the 5’-NCR and the polyprotein genome of DHAV-1 and DHAV-3 suggested that the 5’-NCR might also be used to study genotyping or phylogenetic relationships.

Primer sequences

Since the 5’-NCR could be used for genotyping analysis, we selected the 5’-NCR for primer design in this study. To locate maximum sequence conservation, the 5’-NCR nucleotide sequences of 16 DHAV (listed in Table 1) from two different genotypes were aligned. Though DHAV-1 and DHAV-3 are different subtypes, the 5’-NCRs of these strains exhibited some regions of conservation in their nucleotide sequences. After careful analysis of the sequences of the 5’-NCR, one absolutely conserved region for both DHAV-1 (nucleotide from 271 to 289) and DHAV-3 (nucleotide from 298 to 316) was selected as PCR common forward primer pAF: 5’-GGAGGTGGTGCTGAAATAT-3’ (Fig 2A). The pAF sequence is completely conserved when it is retrieved (blasted) for similarity to other DHAV-1 and DHAV-3 strains in GenBank (http://www.ncbi.nlm.nih.gov) but without any similarities to DHAV-2 strains, suggesting that pAF is suitable for PCR detection of DHAV-1 and DHAV-3 but not for DHAV-2. To allow the specific detection of DHAV-1 or DHAV-3, reverse primers were designed separately. The criterion of type-specific primers should not only maintain maximum sequence differences between DHAV-1 and DHAV-3 but should also be completely conserved within the same subtype. To find the primer sequence region highly conserved within the same subtype but highly divergent between different subtypes, the 5’-NCR sequences of two different subtypes were aligned separately. The highly conserved region from 468 to 484 was selected as the DHAV-1-specific PCR reverse primer (pA1R) region (Fig 2B) and 570 to 586 was selected as the DHAV-3-specific PCR reverse primer (pA3R) region (Fig 2C). Since DHAV-2 is unavailable in China, the primers designed in this study were mainly intended for DHAV-1 and DHAV-3 detection. Primer information is listed in Table 2. The pAF, pA1R, and pA3R sequences are completely conserved when retrieved for similarity to corresponding DHAV-1
and DHAV-3 strains in GenBank (http://www.ncbi.nlm.nih.gov) but without any similarities to DHAV-2 or other organism, suggesting that pAF, pA1R, and pA3R should be valid for PCR detection of DHAV-1 and DHAV-3 but not for DHAV-2.

Fig 1. Phylogenetic analysis of the polyprotein genome (A) and the 5’-NCR (B) of the 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.

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Fig 2. Primers designed by using three alignments of 5’non-coding region sequences from DHAV-1 and DHAV-3 viruses. Identical nucleotides to the top sequences are indicated by dashes. Nucleotide positions are written to the right of sequences. Three conserved regions (in shadow box) 271–289 for DHAV-1 (A) and 298–316 for DHAV-3(A), 468–484 (B) and 570–586 (C) were used for the design of primers for pAF, pA1R, and pA3R, respectively.

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Detection of reference DHAV-1 and DHAV-3

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

Sensitivity of the one-tube RT-PCR

Sensitivities of the one-tube RT-PCR for detection representative DHAV-1 (HP-1) and DHAV-3 (JT) were measured by using 10-fold serial dilutions (from $10^{-1}$ to $10^{-7}$) of the virus stocks as described previously [27], representing $10^3$ to $10^{-3}$ ng virion RNA in the reaction at each dilution. In this assay, $10^{-6}$ dilutions of viruses, equivalent to approximately 10 pg of viral RNA per reaction, were detected (Fig 4). Similar sensitivity was obtained from mixed DHAV-1 and DHAV-3 stock (data not shown).

Specificity of one-tube RT-PCR

To test the specificity of the designed primers for DHAV-1 and DHAV-3, RNA was extracted from other avian viral isolates corresponding to a number of distinct virus families, including avian influenza viruses (AIV), Newcastle disease virus (NDV), Muscovy duck reovirus (DRV), and duck Tembusu virus (DTMUV). The amplification process was performed with RNA as described above. In no case did the three primers amplify sequence fragments of the appropriate sizes of 214 bp or/and 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.

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

One-day-old SPF ducklings with single DHAV-1 (DRL-62, R85952, and HP-1) or single DHAV-3 (JT, GY, HLJ-1, ZJ-01309, and SD0517) or mixed DHAV-1 and DHAV-3 stocks A (HP-1 and JT) and B (DRL-62 and GY) infections showed 100% (infection with single DRL-62 or R85952 or HP-1), 70% (infection with single JT or GY), 80% (infection with single HLJ-1 or ZJ-01309 or SD0517), 100% (with mixed HP-1 and JT infection) and 90% (with mixed DRL-62 and GY infection) mortalities within two weeks, respectively. All birds, regardless of whether they were infected with single DHAV-1 or DHAV-3 or mixed stocks, showed similar clinical symptoms and pathological changes, including typical hepatitis lesions and enlarged liver with hemorrhages. No signs of disease or death occurred in PBS-negative control birds.

Table 2. Primer information.

| Viruses | Primers | Primer sequences 5’ NCR region | Amplicon size (bp) |
|---------|---------|---------------------------------|-------------------|
| DHAV-1  | pAF     | 5-GGAGGTGGTGCTGAAATAT-3         | 271–289           | 214               |
|         | pA1R    | 5-CATGTGCCCTGGAACAGAT-3         | 468–484           |                   |
| DHAV-3  | pAF     | 5-GGAGGTGGTGCTGAAATAT-3         | 298–316           | 289               |
|         | pA3R    | 5-GGATCAAAGGGGTTTTC-3           | 570–586           |                   |

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RNA from liver homogenates of each group was subjected to one-tube/one-step RT-PCR for detection DHAV-1 and DHAV-3 or for VI/NT. The PCR results showed that single 214-bp and 289-bp fragments were amplified correspondingly from birds infected with single DHAV-1- and DHAV-3-related viruses, respectively (Fig 5, lanes 3, 4, and 9 for DHAV-1; lanes 1, 5, 6, 8, and 10 for DHAV-3). The mixed 214-bp and 289-bp fragments were simultaneously amplified from samples of birds infected with mixed DHAV-1 and DHAV-3 stock A (HP-1 and JT) and stock B (DRL-62 and GY). As demonstrated in Fig 5, amplicons of 214 bp and 289 bp were simultaneously detected from samples of coinfected birds (Fig 5, lanes 2 and 7), which are in accordance with stock A or B in the test tube (data not shown). The sequences of amplicons are consistent with GenBank sequences of corresponding reference viruses, suggesting...
that this one-tube/one-step RT-PCR could simultaneously detect DHAV-1 and DHAV-3 coinfections in liver samples. No amplicons were detected in bird samples from the PBS control group. Virus isolation combined with neutralization test (VI/NT) are considered "gold standard" methods to test single DHAV-1 and DHAV-3 infection. Neutralization test results showed that allantoic fluids corresponding to reference single DHAV-1 and DHAV-3 infected birds were both neutralized homologous antisera (with r ≥ 1) and only, to some extent, in the presence of undiluted heterologous antisera. These data demonstrated that single amplicons of 214 bp or 289 bp from liver samples are correlated with virus isolation and neutralization tests, suggesting that a one-tube RT-PCR system is also suitable for DHAV-1 and DHAV-3 detection in liver samples of experimentally infected birds. The VI/NT is not applicable for birds coinfected with DHAV-1 and DHAV-3, but the sequence results of 214-bp and 289-bp coamplified products were consistent with those of mixed reference viruses in this study, suggesting that this one-tube/one-step RT-PCR system is also suitable for clinical screening with coinfected birds. To help discriminate between two amplicons, a reference 250 bp line should be included in DNA molecular weight marker (amplicon size 214bp < 250 bp and 289 bp > 250 bp) and 1.5–2.0% agarose gel is recommended in electrophoresis.

**Screen DHAV-1/DHAV-3 field infection**

To screen clinical DHAV-1 and DHAV-3 field infections in China, 55 clinical liver samples were analyzed by one-tube RT-PCR assay and VI/NT. Amplicons with sizes of 214 bp or 289 bp were cloned into the pMD18-T vector, and positive clones were submitted for sequencing. Of the 55 samples, 11 amplicons (approximately 20%) with a single size of 214 bp, 25 amplicons (approximately 45%) with a single size of 289 bp, and 5 amplicons (approximately 9%) with both sizes of 214 bp and 289 bp were obtained. Sequence results showed that 11 clinical samples (isolates) are DHAV-1, 25 are DHAV-3, and 5 are both DHAV-1 and DHAV-3. Because some sequences of amplicons showed 100% homology to each other, we selected and submitted 5 different sequences among DHAV-1 or DHAV-3 or coinfected DHAV-1/-3 to GenBank, and GenBank accession numbers are listed in Table 3.
The similar phylogenetic topology of the 5′-NCR and polyprotein-encoding gene of reference DHAVs confirmed that the 5′-NCR might be suitable for genetic subtype analysis. The criterion for routine primer design is that a set of primers should accommodate both the similarities and differences exhibited by most of the virus sequences. The completely conserved 5′-GGAGGTGGTGCTGAAATAT-3′ sequence [retrieved from GenBank (http://www.ncbi.nlm.nih.gov)] between DHAV-1 and DHAV-3 might be functionally related and suitable as a universal primer to enable the detection of both serotypes. The 34.3–48.6% diversities in the 5′-NCR sequences suggest that DHAV-1 and DHAV-3 have been evolving separately for a long time, resulting in two distinct genogroups, making this sequence suitable for the design of type-specific primers. Only the main 214-bp or 289-bp product was obtained when nucleic acids of the specified DHAV-1 or DHAV-3 strains were used as the template. With this one-tube RT-PCR, we successfully detected and genotyped samples from dead birds with a single virus infection or a coinfection, validating the approach used in this study. Compared to other molecular genotype methods [8, 10, 19–24], which require amplicon purification, sequencing, and sequencing results analysis, our one-tube system will save at least 6 to 24 h. Similar to other methods, the 10 pg of viral RNA detection was sensitive enough for virus detection and genotyping. The specificity and sensitivity of the one-tube RT-PCR assay were confirmed by VI/NT, and the lack of cross-reaction between DHAV-1 and DHAV-3 also supports the utility of the assay for the detection of DHAV-1 and DHAV-3 coinfection.

The applicability of one-tube RT-PCR to detecting and genotyping clinical samples was also tested. Of the 55 clinical samples tested, 20% were detected as a DHAV-1 infection, 45% as a DHAV-3 infection, and 9% as coinfections, indicating that the DHAV-3 subtype was the main problem facing duck industries in China. The development of a DHAV-3-specific vaccine is therefore highly important, given the current high prevalence of this subtype in duck flocks and the unavailability of measures to control the virus.

This one-tube RT-PCR approach has the following advantages: the size difference of the amplicons is large enough to allow easy interpretation of the results by untrained staff without requiring amplicon sequencing. Moreover, the technique uses fewer costly PCR reagents, and

| Isolate/Sample | Serotype | Size(bp) | Subtype | 5′-NCR identity to (%) | GenBank No |
|---------------|----------|---------|---------|------------------------|------------|
| HRB01507      | 1        | 214     | DHAV-1  | 95–99                  | MK292314   |
| GZ01506       | 3        | 289     | DHAV-3  | 66–68                  | MK292305   |
| LY01602A/B    | 1/3      | 214/289 | DHAV-1/-3 | 96-100/65-67 | MK292317/MK292308 |
| FJ01501       | 3        | 289     | DHAV-3  | 67–68                  | MK292304   |
| BJ01704       | 3        | 289     | DHAV-3  | 66–68                  | MK292302   |
| JNO1706A/B    | 1/3      | 214/289 | DHAV-1/-3 | 95-100/66-68 | MK292316/MK292307 |
| SH01609       | 3        | 289     | DHAV-3  | 66–67                  | MK292309   |
| CC01508       | 1        | 214     | DHAV-1  | 96-100                 | MK292312   |
| CF01701A/B    | 1/3      | 214/289 | DHAV-1/-3 | 95-100/67-69 | MK292314   |
| SY01701       | 1        | 214     | DHAV-1  | 95–99                  | MK292319   |
| JNO153A/B     | 1/3      | 214/289 | DHAV-1/-3 | 95-100/66-67 | MK292315/MK292306 |
| ZZ01507A/B    | 1/3      | 214/289 | DHAV-1/-3 | 96-100/66-68 | MK292321/MK292311 |
| ZH01605       | 3        | 289     | DHAV-3  | 66–68                  | MK292310   |
| YY01607       | 1        | 214     | DHAV-1  | 96–100                 | MK292320   |

Table 3. Virus detection results by one-tube RT-PCR and virus isolation/neutralization test.

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combining the RT and PCR processes into a single step considerably reduces the time, labor, and contamination potential. In addition, this one-step RT-PCR protocol is easy to carry out, even for untrained staff, and it does not yield a separate volume of cDNA as in the two-step and real-time protocols that need to be used in multiple PCRs for different targets. Considering the disease severity and emergence of new serotype DHAV-3, this novel approach would be most helpful to specifically and accurately diagnose the closely related DHAV-1 and DHAV-3 strains and enable their early detection in clinical samples during routine examination. This simple RT-PCR system could be applied in resource-limited settings or on farms for surveillance or for routine epidemiological screening.

Author Contributions

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Visualization: Ming Liu.

References

1. Liu M, Zhang TT, Zhang Y, Meng FY, Li XJ, Hou ZZ, et al. Development and evaluation of a VP1-ELISA for detection of antibodies to duck hepatitis type 1 virus. J Virol Meth. 2010; 169: 66–69

2. Woolcock PR. Duck hepatitis. In: Diseases of Poultry, 11th ed. Iowa State Press, 2003.

3. Kim MC, Kwon YK, Joh SJ, Kim SJ, Tolf C, Kim JH, et al. Recent Korean isolates of duck hepatitis virus revealed the presence of a new geno- and serotype when compared to duck hepatitis virus type 1 type strains. Arch Virol. 2007; 152: 2059–2072. https://doi.org/10.1007/s00705-007-1023-0 PMID: 17701025

4. Tseng CH, Knowles NJ, Tsai HJ. Molecular analysis of duck hepatitis virus type 1 indicates that it should be assigned to a new genus. Virus Res. 2007; 123:190–203. https://doi.org/10.1016/j.virusres.2006.09.007 PMID: 17067712

5. Tseng CH, Tsai HJ. Molecular characterization of a new serotype of duck hepatitis virus. Virus Res. 2007; 126:19–31. https://doi.org/10.1016/j.virusres.2007.01.012 PMID: 17292992

6. Wang L, Pan M, Fu Y, Zhang D. Classification of duck hepatitis virus into three genotypes based on molecular evolutionary analysis, Virus Genes. 2008; 37:52–59. https://doi.org/10.1007/s11262-008-0233-1 PMID: 18437547

7. Doan HT, Le XT, Do RT, Hoang CT, Nguyen KT, Le TH. Molecular genotyping of duck hepatitis A viruses (DHAV) in Vietnam. J Infect Dev Ctries. 2006; 10(9): 988–995.

8. 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. 2008; 131: 247–257. https://doi.org/10.1016/j.vetmic.2008.03.011 PMID: 18462894

9. Kim MC, Kwon YK, Joh SJ, Kwon JH, Kim JH, Kim SJ. Development of one-step reverse transcriptase polymerase chain reaction to detect duck hepatitis virus type 1. Avian Dis. 2007; 51:540–545. https://doi.org/10.1637/0005-2086(2007)51[540:DOORT]2.0.CO;2 PMID: 17626480

10. Kim MC, Kwon YK, Joh SJ, Kwon JH, Lindberg AM. Differential diagnosis between type-specific duck hepatitis virus type 1 (DHV-1) and recent Korean DHV-1-like isolates using a multiplex polymerase chain reaction. Avian Pathol. 2008; 37:171–177. https://doi.org/10.1080/03079450801918670 PMID: 18393095
11. Zhang TT, Li XJ, Wu XY, Shaozhou WL, Bai XF, Liu SG et al. Characterization of monoclonal antibodies against duck hepatitis type1 virus VP1 protein. J Virol Meth. 2014; 208: 166–170.

12. Kim MC, Kwon YK, Joh SJ, Lindberg AM, Kwon JH, Kim JH, et al. Molecular analysis of duck hepatitis virus type 1 reveals a novel lineage close to the genus Parechovirus in the family Picornaviridae. J Gen Virol. 2006; 87: 3307–3316. https://doi.org/10.1099/vir.0.81804-0 PMID: 17030865

13. Wu XY, Zhang TT, Meng FY, Guo DC, Yin XC, Wulin SZ, et al. Mapping a Type-specific Epitope by Monoclonal Antibody against VP3 Protein of Duck Hepatitis A Type 1 Virus. Scientific Reports. 2017; 7: 10820. https://doi.org/10.1038/s41598-017-10909-7 PMID: 28883462

14. Liu M, Meng F, Li XJ, Zhang Z, Liu S, Zhang Y. Goose haemorrhagic hepatitis caused by a new subtype duck hepatitis type 1 virus. Vet Microbiol. 2011; 152: 280–283. https://doi.org/10.1016/j.vetmic.2011.05.015 PMID: 21641125

15. Hwang J. Duck hepatitis virus-neutralization test in chicken embryos. Am J Vet Res. 1969; 30: 861–864. PMID: 5813677

16. Saha K, Firdaus R, Chakrabarti S, Sadhukhan PS. Development of rapid, sensitive one-tube duplex RT-PCR assay for Specific and differential diagnosis of Chikungunya and dengue. J Virol Meth. 2013; 193: 521–524.

17. Wu XY, Li XJ, Zhang QS, Wulin SZ, Bai XF, Zhang TT, et al. Development of a Conserved B-Cell Epitope on Duck Hepatitis A Type 1 Virus VP1 Protein. PLoS ONE. 2015; 10(2): e0118041. https://doi.org/10.1371/journal.pone.0118041 PMID: 25706372

18. Shen Y, Cheng A, Wang M, Chen S, Jia R, Zhu D, et al. Development of an indirect ELISA method based on the VP3 protein of duck hepatitis A virus type 1 (DHAV-1) for dual detection of DHAV-1 and DHAV-3 antibodies. J Virol Methods. 2015; 225:30–4. https://doi.org/10.1016/j.jviromet.2015.08.016 PMID: 26341062

19. Yang M, Cheng A, Wang M, Xing H. Development and application of a one-step real-time Taqman RT-PCR assay for detection of duck hepatitis virus type1. J Virol Meth. 2008; 153:55–60.

20. Wen XJ, Cheng AC, Wang MS, Jia RY, Zhu DK, Chen S, et al. Detection, differentiation, and VP1 sequencing of duck hepatitis A virus type 1 and type 3 by a 1-step duplex reverse transcription-PCR assay. Poult Sci. 2014; 93(9): 2184–2192. https://doi.org/10.3382/ps.2014-04024 PMID: 25012848

21. Hu Q, Zhu D, Ma G, Cheng A, Wang M, Chen S, et al. A one-step duplex rRT-PCR assay for the simultaneous detection of duck hepatitis A virus genotypes 1 and 3. J Virol Meth. 2016; 236: 207–214.

22. Chen LL, Xu Q, Zhang RH, Yang L, Li JX, Xie ZJ, et al. 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 Meth. 2013; 192(1–2):12–17.

23. Li C, Chen Z, Meng C, Liu G. Rapid detection of duck hepatitis A virus genotype C using reverse transcription loop-mediated isothermal amplification. J Virol Methods. 2014; 196:193–198. https://doi.org/10.1016/j.jviromet.2013.11.009 PMID: 24291148

24. Huang OX, Yue H, Zhang B, Nie PT, Tang C. Development of a Real-Time Quantitative PCR for Detecting Duck Hepatitis A Virus Genotype C. J Clin Microbiol. 2012; 50(10): 3318–3323. https://doi.org/10.1128/JCM.01080-11 PMID: 22855514

25. Li XJ, Zhao R, Lin W, Li CX, Zhang TT, Meng FY, et al. Evidence of VP1 of duck hepatitis A type 1 virus as a target of neutralizing antibodies and involving receptor-binding activity. Virus Res. 2017; 227: 240–244. https://doi.org/10.1016/j.viruses.2016.10.018 PMID: 27816429

26. Liu CG, Liu M, Liu F, Lv R, Liu DF, Qu LD, et al. Emerging multiple reassortant H5N5 avian influenza viruses in ducks, China, 2008. Vet Microbiol. 2013; 167: 296–306. https://doi.org/10.1016/j.vetmic.2013.09.004 PMID: 24080350

27. Zhang Y, Liu M, Ouyang SD, Hu QL, Guo DC, Chen HY, et al. Detection and identification of avian, duck, and goose reoviruses by RT-PCR: goose and duck reoviruses are part of the same genogroup in the genus Orthoreovirus. ArchVirol. 2006; 151: 1525–1538.

28. Liu M, Liu CG, Li G, Li XJ, Yin XC, Chen YH, et al. Complete Genomic Sequence of Duck Flavivirus from China. J Virol. 2012; 86(6):3398. https://doi.org/10.1128/JVI.07086-11 PMID: 22354941

29. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol. 2007; 24: 1596–1599. https://doi.org/10.1093/molbev/msm092 PMID: 17488738

30. Reed LJ, Muench H. A simple method for estimating fifty percent endpoints. Am J Hyg. 1938; 27: 493–497.