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RESEARCH ARTICLE

Development of Taqman Real-time Fluorescent Quantitative PCR for Rapid Detection and differentiation between DHAV-1 and DHAV-3 in Duck Farming

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ABSTRACT

The duck industry is at high risk from duck viral hepatitis (DVH) and has the potential to cause substantial financial losses because of the high mortality rates observed in duck farming, even with continuous breeder duck flock vaccination. Among the different etiological agents of DVH, duck hepatitis A virus type-1 (DHAV-1) is the most common followed by DHAV-2 and DHAV-3. Although DHAV-I is more common and pathogenic, DHAV-3 has just emerged from duck farms in North Egypt, thus there's a pressing need to find a way to detect both DHAV-1 and DHAV-3 rapidly and simultaneously using real-time qPCR. To assess and compare the sensitivity of the real time reverse transcriptase PCR (rRT-PCR) technique for the detection of DHAV-3 and DHAV-1, dilution range of titrated DHAV-1 and DHAV-3 reference strains from $10^{7.2}$ and 10^{6} EID_{50}/ml to $1EID_{50}$, was implemented, respectively. The results of the current study confirmed that the rRT-PCR assay's had lowest detection limit for DHAV-1 and DHAV-3 was $10^{2.2}$ and 10^{2} EID_{50}/ml, respectively, and it is ten-fold higher than RT-PCR. The rRT-PCR was highly specific to DHAV-1 and DHAV-3, as other avian diseases and nucleic acid isolated from samples that tested negative for DHAV. When examining clinical samples for rRT-PCR, the diagnostic sensitivity was better than the RT-PCR. It detected 25 out of 40 clinical suspected samples but the RT-PCR detected only 15 out of 40 clinical suspected samples. In conclusion, the assay may be used as an efficient, rapid, sensitive, specific, and focused molecular diagnostic technique for detection and epidemiological investigations of DVH caused by both DHAV-1 and DHAV-3.

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INTRODUCTION

Duck virus hepatitis (DVH) is a severe, widely contagious disease that impacts young ducklings and it is a significant danger to the duck industry, resulting in significant financial losses due to the high mortality rates in duck-growing farms despite continuous vaccination (Mohammed *et al*., 2023). Duck hepatitis A virus (DHAV) is member of the *Picornaviridae* family and the *Avihepatovirus* genus. The genome of the DHAV is single-stranded positive-sense RNA. Based on the phylogenetic study, it has been divided into three genotypes: DHAV-1, DHAV-2, and DHAV-3. The DHAV-1 is the most virulent, pathogenic, and distributed

worldwide, while DHAV-2 and DHAV-3 are found only in East and South Asia (Kim *et al.,* 2006; Palya *et al.*, 2006; Hisham *et al.,* 2020).

In 1969, DHAV-1 was first discovered in Egypt (Refaie, 1969). It was spread in Egyptian duck farms causing tremendous financial losses in Egyptian duck farms. Lately, there has been a DHAV-3 epidemic reported in Egypt in vaccinated and non-vaccinated duck farms. Molecular characterization of DHAV-3 in Egypt showed that the DHAV-3 revealed a new subgroup different from China and Korean strains (Erfan *et al.,* 2015; Zanaty *et al.,* 2017; Mansour *et al.,* 2019; Yehia *et al.,* 2021). The DHAV-3 was widely spread together with DHAV-1 causing severe economic losses in duck farms in vaccinated and non-vaccinated farms (Hassan *et al*., 2020; El-Kholy *et al*., 2021; Yehia *et al*., 2021; Lelwa *et al*., 2023).

A polyprotein encoded by the single big open reading frame (ORF) in the DHAV genome splite apart into P1, P2 and P3. The P1 contain three structural proteins VP0, VP1, VP3, and VP0 divided into VP2and VP4 proteins, and P2 and P3 contain 9 non-structural proteins, which are 2A1, 2A2, 2A3, 2B, 2C, 3A, 3B, 3C, and 3D (Wang *et al*., 2008; Gao *et al*., 2012; Xu *et al*., 2012). The VP1 gene among the isolates exhibits the most genetic variability and is responsible for receptor binding, virulence, and immunogenicity (Liu *et al*., 2008), as well as; the genotyping of DHAV depends mainly on the VP1 gene (Wang *et al*., 2008).

DHAV infections can be detected by several methods used, such as enzyme-linked immunosorbent assay (Gabridge and Newman, 1971, Fan *et al*., 1998), microneutralization assay, and immunofluorescent assay but these approaches of low sensitivity and specificity rather than time-consuming (Hwang, 1969; Zhang *et al*., 2014; WU *et al*., 2015).

Many people have used reverse transcriptase polymerase chain reaction (RT-PCR) to simultaneously find viral infections in plants and animals. The method of choice is molecular genotyping of DHAV. By sequencing a segment of the viral genome, a subtype of an isolate is primarily identified and comparing it with reference sequences from known subtypes using phylogenetic analysis (Huang *et al*., 2012). The differentiation between DHAV-1 and DHAV-3 are detected using several RT-PCR methods. However, sequencing is additionally necessary to understand the results because the amplicons are hard to distinguish by size (Fu *et al*., 2008).

Additionally, this method is costly, labor- and time-intensive. A duplex PCR was designed to separate amplicons based on size in order to distinguish between DHAV-1 and DHAV-3; however, Multiple rounds of PCR are required by numerous sets of primers, which are similarly expensive., time-consuming and inconvenient (Kim *et al*., 2008; Hu *et al*., 2016; Chen *et al*., 2013) and one tube PCR for distinguish between DHAV-1 and DHAV-3 were developed, but it has low sensitivity and time-consuming (Chen *et al*., 2019). Therefore, it could be preferable to create a simple, sensitive, rapid, and specific, and economically distinguishable real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assay to distinguish between DHAV-1 and DHAV-3 without the need for any sequencing.

MATERIALS AND METHODS

Development of RNA standard: The Egyptian DHAV-1 (Duck-hepatitis-A-virus- BH3), DHAV-3 (Duck-hepatitis-A-virus- BH1) reference strain, GenBank accession number MN873051, and MN873049 respectively, was used to extract the standard RNA, which was titrated using specific pathogen-free embryonated duck eggs. The Reed and Muench method, as published previously (OIE, 2021), was used to compute the virus titer, which is represented as EID_{50} (50% embryo infective dose)/mL.

Nucleic acid extraction: As per the guidelines provided by the manufacturer, viral RNA was extracted from reference isolates and clinical samples using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). Allantoic fluid or phosphate-buffered saline (PBS) solutions, including tissue homogenate with a total volume of 200 µl, were used as samples. In a final volume of 60 µl, RNA was eluted and then kept at -80 °C. The DNA was extracted using a Genomic DNA Mini kit (Qiagen, Cat. No. GB100).

Primers and probes design: Using the MegAlign tool (DNASTAR 6.0, Madison, WI, USA), based on the DHAV-1 and DHAV-3 VP1 gene sequence alignments in GenBank (Table 1) in specific and conserved region for each DHAV-1 and DHAV-3, we selected the primers and probes. The VP1 nucleotide sequences of 43 of both DHAV -1 and DHAV-3 (Table 1) were aligned to find the maximum amount of sequence conservation. Although different subtypes of DHAV include DHAV-1 and DHAV-3, these strains' VP1 showed certain areas of nucleotide sequence conservation. The standard for type-specific primers should be conserved within a single subtype and maintain the greatest possible sequence divergence among DHAV-1 and DHAV-3. The VP1 sequences of two distinct subtypes were individually aligned to identify the portion of the primers and probes sequence for DHAV-1 (F at 320-339, R at 451-472, and probe at 391-409) and DHAV-3 (F at 130-151, R at 259-288, and probe at 196-215) that, while mostly conserved within a single subtype, significantly differed within subtypes (Table 2). When obtained (blasted) from GenBank (http://www.ncbi.nlm.nih.gov), the primers and probes for DHAV-1, and DHAV-3 strains are entirely conserved but do not resemble DHAV-2 strains, demonstrating that PCR can distinguish DHAV-1 and DHAV-3 but not DHAV-2 using the proper primers and probes.

Optimization of the condition of rRT-PCR for DHAV-1, DHAV-3 virus detection: The rRT-PCR was developed and validated using Stratagene Mx3005p Real-Time PCR System and Quantitect probe RT-PCR kit (Qiagen, Valencia, CA, USA). Following Bora *et al*. (2011), optimizing the TaqMan probe concentration and annealing temperature achieved the maximum fluorescence intensity at the lowest Ct value. The rRT-PCR was optimized by using a Quantitect probe RT-PCR kit (Qiagen, Valencia, CA, USA) in a 20 µL of total mixture including 10 µL 2X RT-PCR Buffer, 0.5 µL 25X RT-PCR Enzyme Mix, 0.5 µL forward primer, 0.5 µL

Table 1: Reference strain used in primers and probes design

Strain name	Accession number	Country
Duck hepatitis A virus strain F355- DHAV1	KP148294	Egypt
Duck hepatitis A virus strain F729- DHAV1	KP148293	Egypt
Duck hepatitis A virus strain F86- DHAV1	KP148290	Egypt
Duck hepatitis A virus strain F340- DHAV1	KP148281	Egypt
Duck hepatitis A virus strain F215- DHAV1	KP148280	Egypt
Avihepatovirus A isolate Du/Eg/A2/140/12- DHAV1	MK510861	Egypt
Avihepatovirus A isolate Du/Eg/B1/HL1/15- DHAV1	MK510860	Egypt
Avihepatovirus A isolate Du/Eg/B1/HL1/15- DHAV1	MK510860	Egypt
Avihepatovirus A isolate Du/Eg/K2/211/14- DHAV1	MK510858	Egypt
Avihepatovirus A isolate FS28- DHAV1	MG992355	Egypt
Avihepatovirus A isolate FS22- DHAV1	MG992349	Egypt
A isolate Du/Eg/K2/211/14- DHAV1	MK510858	Egypt
Avihepatovirus A isolate Du/Eg/Z1/HS1/15- DHAV1	MK510857	Egypt
Duck-hepatitis-A-virus-DU-BH3- DHAV1	MN873051	Egypt
Duck-hepatitis-A-virus-DU-BH7- DHAV1	MN873055	Egypt
Avihepatovirus A isolate FS21- DHAV1	MG992348	Egypt
Duck hepatitis A virus 3 strain NC- DHAV3	JF925121	Vietnam
Duck hepatitis A virus 3 strain DN2- DHAV3	KM361877	Vietnam
Duck hepatitis A virus 3 strain SD1201- DHAV3	KU860089	Vietnam
Duck hepatitis A virus 3 isolate AP-04114- DHAV3	DQ812093	China
Duck hepatitis A virus 3 isolate AP-04203- DHAV3	DQ256134	China
Duck hepatitis A virus 3 strain C-YCW- DHAV3	GU066824	China
Duck hepatitis A virus 3 strain GD- DHAV3	GQ122332	China
Duck hepatitis A virus 3 isolate D11-JW-018- DHAV3	JX312194	China
Duck hepatitis A virus 3 strain C-GY- DHAV3	EU352805	China
Duck hepatitis A virus 3 strain C-YCZ- DHAV3	GU066823	China
Duck hepatitis A virus 3 isolate AP-03337- DHAV3	DQ256132	China
Duck hepatitis A virus 3 strain JT- DHAV3	JF835025	China
Duck hepatitis A virus 3 strain CH-P120- DHAV3	MH752744	China
Duck hepatitis A virus 3 strain CH-P60- DHAV3	MH752742	China
Duck hepatitis A virus 3 strain LS- DHAV3	KP233203	China
Duck hepatitis A virus 3 strain C-BLZ- DHAV3	GU066822	China
Duck-hepatitis-A-virus-DU-BH1- DHAV3	MN873049	Egypt
Duck-hepatitis-A-virus-DU-BH2- DHAV3	MN873050	Egypt
Duck-hepatitis-A-virus-DU-BH4- DHAV3	MN873052	Egypt
Duck-hepatitis-A-virus-DU-BH5- DHAV3	MN873053	Egypt
Duck-hepatitis-A-virus-DU-BH6- DHAV3	MN873054	Egypt
Duck-hepatitis-A-virus-DU-BH8- DHAV3	MN873056	Egypt
Duck-hepatitis-A-virus-DU-BH9- DHAV3	MN873057	Egypt
Avihepatovirus A isolate 26- DHAV3	MK862180	Egypt
Avihepatovirus A isolate 101- DHAV3	MK862182	Egypt
Avihepatovirus A isolate 100- DHAV3	MK862181	Egypt
DVH-Dak-Pk-F36-2022- DHAV3	OP374129	Egypt

Table 2: rRT-PCR Primers and probes

Primer and Probes	Sequence
DHAV-1-F	CAGCAATGGGAGGTGTGATG
DHAV-1-R	GACTTCCTGATTGAGTCCACAT
DHAV-1-Probe	CCACTCAGGCCAACTCGAC
DHAV-3-F	ACTGTTCAACACACTAGTGAGG
DHAV-3-R	TGTgCAACCATGCAGGGTGT
DHAV-3-probe	CTGTTGCGCTTCTTTGCCTA

reserve primer (Table 2), 3.35 µL Nuclease-free water, 5 µL DNA template. Three duplicates of each TaqMan probe concentration 20, 40, 60, 80 pmol/µl were employed in each group. Based on the assay's outcomes, the best concentration was picked. Using that concentration, the Stratagene Mx3005p Real-Time PCR System, amplification and detection were done under ideal cycling conditions at 50°C for 30 min, 95°C for 5 min, 40 cycles at 95°C for 10s, 50°C, 52°C, 54°C, 56°C, and 58°C are the annealing temperatures., with three replicates in each group for 10s and at 72°C for 15s to determine the precise annealing temperature.

Sensitivity of the newly developed rRT-PCR reaction to detect of DHAV-1 and DHAV-3: Using a stepwise dilution range of titrated reference strains of DHAV-1 and DHAV-3 from $10^{7.2}$ and 10^{6} EID_{50}/ml to $1EID_{50,}$ respectively, the limit of detection of the rRT-PCR assay using Quantitect probe RT-PCR kit (Qiagen, Valencia, CA, USA) was established and compared it with RT-PCR using EasyScript® One-Step RT-PCR Super Mix as specified by the manufacturer's guidelines and specific primers (Table 3) in three replicates. The rRT-PCR, data collection, and evaluation were conducted using 7300 system software (ABI).

Specificity of the newly developed rRT-PCR reaction: The specificity of the rRT-PCR assay for both 2 sets was evaluated with the extracted DNA and RNA template from seven different avian pathogens including DVAH-1, DVAH-3, the virus of bird flu, duck liver tissue, Marek's disease virus, *Salmonella* and *E. coli* isolates and water served as the negative control.

Table 3: RT-PCR primers

Gene	Primer sequence	Amplicon size	Identification of genotype	Reference
UTR	UTR-F CCTCAGGAACTAGTCTGGA UTR-R GGAGGTGGTGCTGAAA	250	All	Fu *et al*., 2008
VP1	DHAV-1-F ATC AGG GTG ATT CTA ACC AG DHAV-1-R CTT ATT TCT AAT TTG GTC AG	734	DHAV-1	Liu *et al*., 2008
	DHAV-3-F ATGCGAGTTGGTAAGGATTTTCAG DHAV-3-R GATCCTGATTTACCAACAACCAT	800	DHAV-3	Doan *et al*., 2017

Table 4: Epidemiologic information and RT-PCR and rRT-PCR results of the tested samples

No	Date of collection	Governorates	UTR-RT-PCR	RT-PCR	rRT-PCR
1	2-2022	Qalyubia	Positive	DHAV-1	DHAV-1
2	3-2022	Kafr-El Sheikh	Negative		
3	5-2022	Monufia	Positive	DHAV-3	DHAV-3
4	1-2023	Qalyubia	Negative		DHAV-1
5	4-2022	Monufia	Negative		
6	1-2023	Cairo	Positive	DHAV-3	DHAV-3
7	4-2022	Monufia	Positive	DHAV-3	DHAV-3
8	6-2022	Giza	Positive		DHAV-3
9	7-2022	Monufia	Negative		
10	8-2022	Cairo	Positive	DHAV-1	DHAV-1
11	3-2023	Giza	Negative		DHAV-3
12	3-2022	Kafr-El Sheikh	Positive	DHAV-3	DHAV-3
13	5-2022	Qalyubia	Negative		
14	4-2022	Kafr-El Sheikh	Negative		DHAV-3
15	11-2022	Cairo	Positive	DHAV-3	DHAV-3
16	2-2022	Cairo	Negative		
17	5-2022	Kafr-El Sheikh	Positive	DHAV-3	DHAV-3
18	9-2022	Giza	Negative		DHAV-1
19	10-2022	Kafr-El Sheikh	Negative		DHAV-3
20	3-3023	Qalyubia	Positive	DHAV-3	DHAV-3
21	12-2022	Kafr-El Sheikh	Negative		DHAV-3
22	5-2022	Cairo	Negative		
23	2-2022	Cairo	Negative		
24	3-2022	Kafr-El Sheikh	Negative		DHAV-3
25	5-2022	Giza	Negative		
26	2-2022	Kafr-El Sheikh	Negative		
27	3-2022	Qalyubia	Positive	DHAV-3	DHAV-3
28	2-2022	Qalyubia	Negative		
29	3-2023	Giza	Positive	DHAV-3	DHAV-3
30	6-2022	Giza	Positive	DHAV-3	DHAV-3
31	4-2022	Giza	Negative		
32	2-2023	Kafr-El Sheikh	Negative		
33	5-2022	Cairo	Positive	DHAV-3	DHAV-3
34	2-2023	Qalyubia	Negative		
35	12-2022	Kafr-El Sheikh	Positive	DHAV-1	DHAV-1
36	11-2022	Cairo	Negative		DHAV-3
37	1-2023	Kafr-El Sheikh	Negative		
38	5-2022	Qalyubia	Negative		DHAV-3
39	9-2022	Qalyubia	Negative		
40	11-2022	Qalyubia	Negative	DHAV-3	DHAV-3

Clinical validation of rRT-PCR: Forty liver samples were collected from recently deceased suspicious ducks from five governorates (Qalyubia, Kafr-El Sheikh, Monufia, Cairo, Giza) from 2022-2023. They showed symptoms of illness, including nervous signs and sudden mortality, and ten negative liver samples from evidently wholesome ducks were utilized to evaluate the clinical performance of DHAV-1 and DHAV-3. This rRT-PCR test was compared to the traditional RT-PCR method. The samples were collected from duck farms during 2022 and 2023 from different governorates in Egypt, including Qalyubia, Monufia, Kafr-El Sheikh, Cairo, and Giza (Table 4). All samples were tested for DHAV common gene (UTR) and subtyping DHAV-1and DHAV-3 by VP1 gene by RT-PCR and rRT-PCR as previously described.

Virus isolation: An amount of 0.2 ml of the homogenized liver suspension of positive samples was injected into the allantoic sac of 10 days old specific-pathogen-free (SPF) embryonated duck eggs. The injected eggs were inoculated for seven days at 37ºC, and every day they were checked for abnormal growth alterations and embryonic demise. The allantoic fluid was then collected and confirmed by rRT-PCR.

RESULTS

Optimization of the rRT-PCR assay conditions: The final concentrations for each primer and probe were selected after rRT-PCR optimization and were 20 pmol/µL and 40 pmol/µL respectively. The rRT-PCR reaction was optimized to be 20 µL in the volume containing 10 µL 2X RT-PCR Buffer, 0.5 µL 25X RT-PCR Enzyme Mix, 0.5 µL forward primer, 0.5 µL reserve primer, 3.35 µL Nuclease-free water, 0.15 µL probe, and 5 µL DNA template at 50°C for 30 min proceeded by 95°C for 5 min then 40 cycles at 95°C for 10s and at 56°C for 10s and at 72°C for 15s for specific detection of both DHAV-1 and DHAV-3.

Sensitivity of the newly developed rRT-PCR reaction to detect DHAV-1 and DHAV-3: By testing 10-fold serial dilutions of the DNA standards from $10^{7.2}$ and 10^{6} EID50/ml for DHAV-1 and DHAV-3, respectively, to 1 EID50/ml, the sensitivity of the rRT-PCR assay was assessed and compared with RT-PCR. The outcomes demonstrated that the assay's lowest limit of detection was $10^{2.2}$ and $10^{2}EID_{50}$/ml for rRT-PCR assay Fig. 1 (A, B), higher than RT-PCR $10^{3.2}$ and $10^{3}EID_{50}$/ml for DHAV-1 and DHAV-3, respectively in three times. Compared to the RT-PCR, rRT- PCR had ten times higher sensitivity than RT-PCR.

Specificity of the rRT-PCR reaction: Seven distinct avian pathogens, including isolates of the bird flu virus, *Salmonella*, *E. coli*, Marek's disease virus, and duck liver tissue, were used to test the specificity of the rRT-PCR for the two sets. Water served as the negative control. Reactions with DHAV-1 in the set defined for DHAV-1 and DHAV-3 from the set specified for DHAV-3

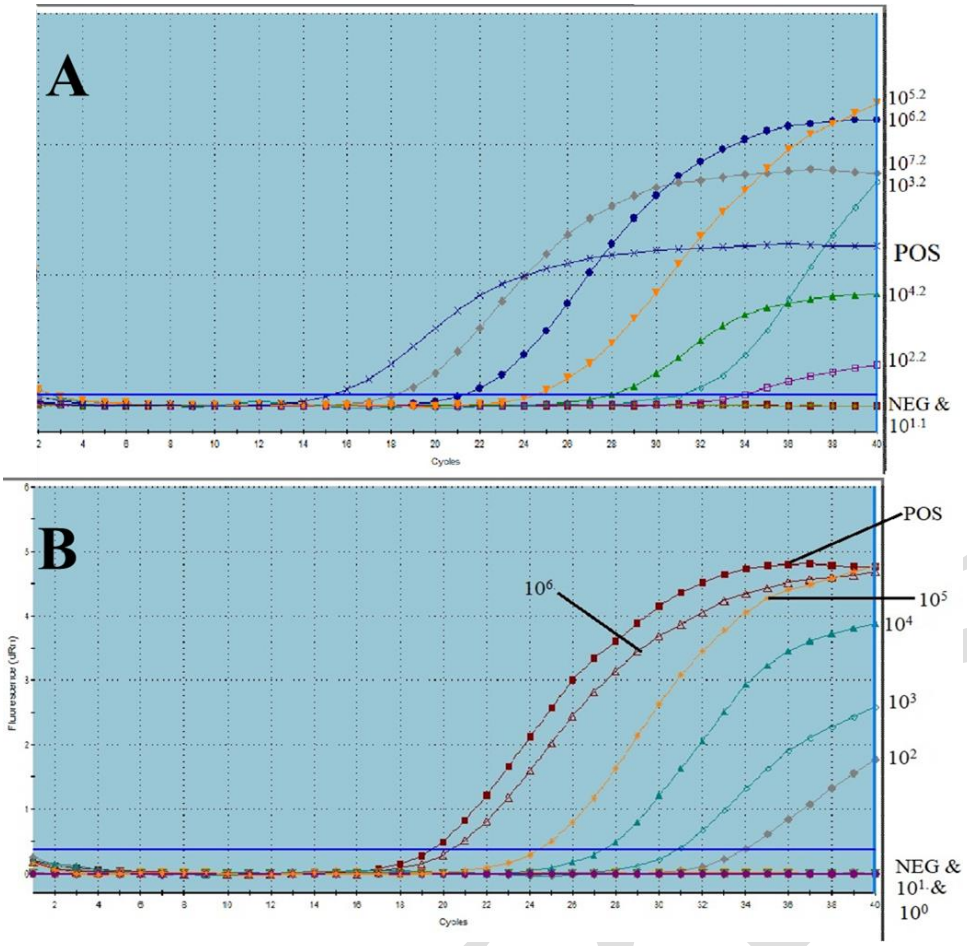

Fig. 1: The analytical sensitivity of the rRT-PCR reaction using a tenfold serial dilution of standard virus from titer $10^{7.2}$ to 1 EID50 and 10^{6} to 1EID50 for DHAV-1 and 3 (A, B). The results showed that the lowest limit of detection of the assay was $10^{2.2}$ and 10^{2} EID_{50}/ml for DHAV-1 and DHAV-3 (A, B), respectively.

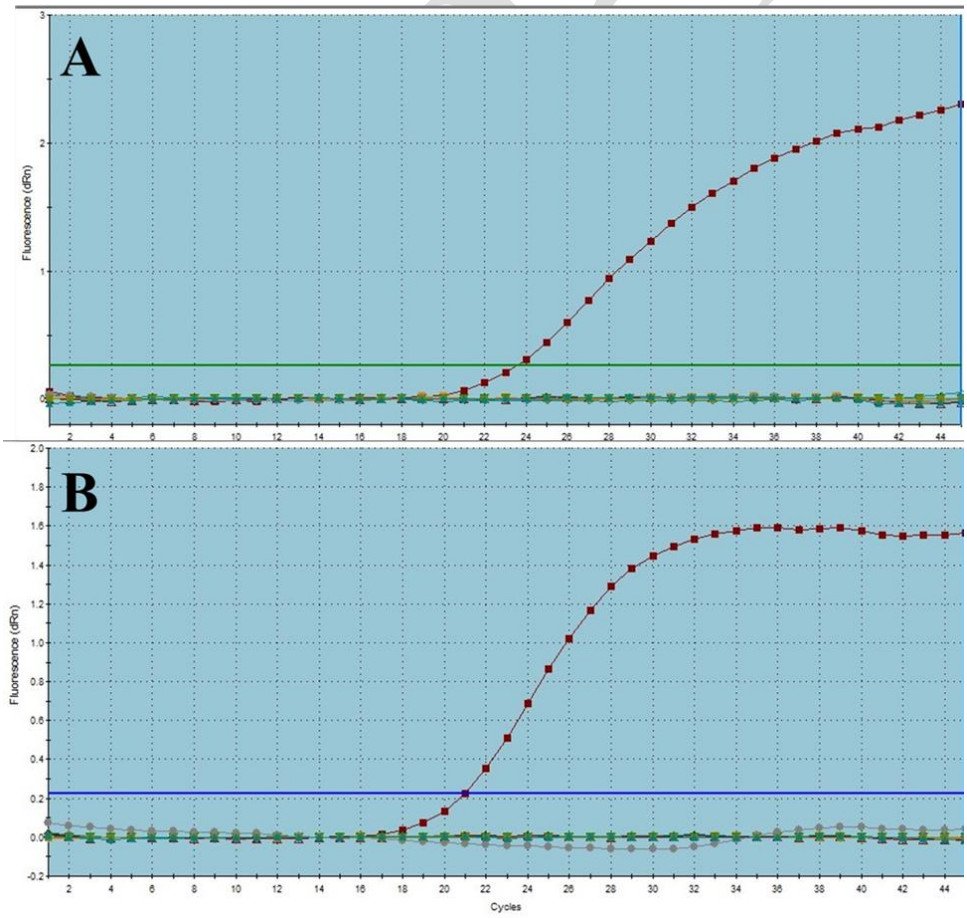

Fig. 2: The specificity of the rRT-PCR reaction for DHAV-1(A) and DHAV- 3 (B). Only RNA of DHAV-1 was amplified, and DHAV-3, avian influenza virus, duck liver tissue, Marek's disease virus, *Salmonella,* and *E. coli* isolates; water were not detected (A). Only RNA of DHAV-3 was amplified, and duck hepatitis virus-1, avian influenza virus, duck liver tissue, Marek's disease virus, *Salmonella,* and *E. coli* isolates; water were not detected (B).

Fig. 3: The infected duck shows ducklings had Depression and Fall on the side, paddling of legs, arching of back, rapid deterioration, and death, often in opisthotonus**.**

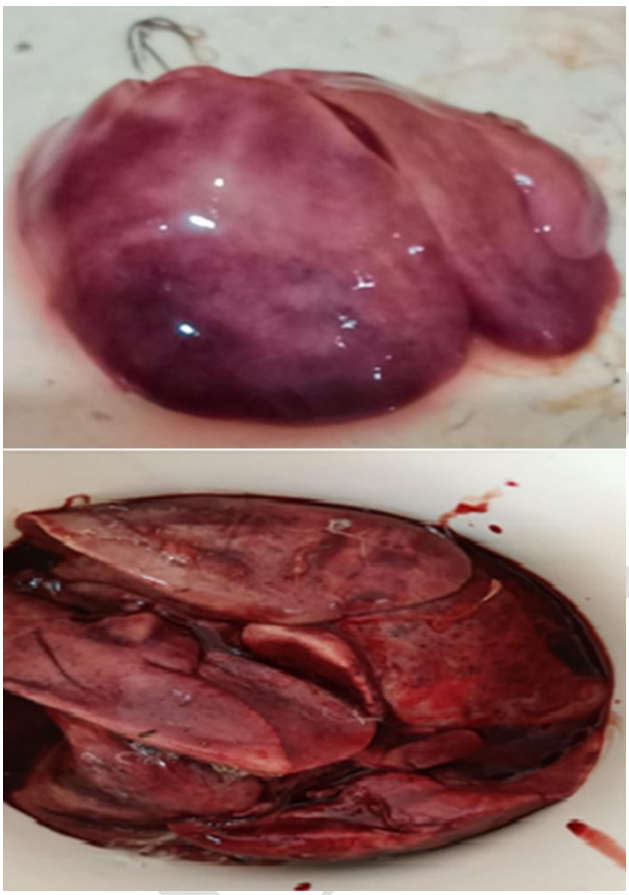

Fig. 4: The liver of the infected duck was enlarged and covered with hemorrhagic foci.

produced strongly positive results. In contrast, the other pathogensgave negative results similar to the negative control, equal to baseline values when the reaction is optimized. Thus, both sets were specified and distinguished between DHAV-1 and DHAV-3 Fig. 2 (A and B).

Clinical validation of rRT-PCR

Clinical symptoms and gross pathology: Mortality rates for young, infected ducklings from five governorates ranged from 50% to 80%. These symptoms included lethargy, numerous neurological indications (movement difficulties, instability, and opisthotonos), and abrupt death Fig. 3. The postmortem showed an enlarged liver featuring 1 cm-diameter hemorrhagic foci (Fig. 4) the spleen was speckled and enlarged, and the kidneys were swollen and clogged.

Virus detection by rRT-PCR: Fifteen samples out of the 40 examined samples were confirmed to be positive by RT-PCR for the DHAV depending on the UTR gene at the expected weight of 250 bp. DHAV-3 was found depending on the VP1 gene in 12 out of the 15 positive samples, representing 80%, at the weight of 800 bp, while three samples showed DHAV-1 positive amplification at 734 bp. Clinical validation of rRT-PCR was carried out for forty samples. The rRT-PCR detected ten samples not detected by RT-PCR in the 30-35 Cycle threshold (Ct) value. The result was positive for twenty-five samples. DHAV-3 was detected in 20 samples Fig. 5, and DHAV-1 in five samples (Fig. 6). In clinical validation, the rRT-PCR provides better sensitivity than the RT-PCR (Table 4). In addition, the ten negative samples collected from apparently healthy ducks were negative for both RT-PCR and rRT-PCR.

Virus isolation: Duck embryonated eggs were used to isolate the positive rRT-PCR samples. DHAV-1 and DHAV-3 caused embryonic mortality, ranging from 35% to 50%. Stunting, liver hemorrhages, and edema were all visible in the inoculated embryos. 20% of the embryos were still alive 7 days following injection; however, their livers were mottled due to regional hepatic necrosis. The embryo had engorged visceral organs and were positive for five DHAV-1 and twenty DHAV-3 using rRT-PCR.

DISCUSSION

Duck hepatitis A is an acute viral infection that spreads widely and has a significant fatality rate among ducklings (Levine and Fabricant, 2019). Three genotypes of DHAV have been identified based on phylogenetic analyses: DHAV-1, DHAV-2, and DHAV-3. DHAV-2 and DHAV-3 differ from DHAV-1 genetically and serologically (Kim *et al*., 2007a; Wang *et al*., 2008). DHAV-1 is the most prevalent, hazardous, and widely dispersed subtype. There is a constrained availability of DHAV subtype 2 in just Taiwan. DHAV subtype 3 is often found in Vietnam, South Korea, and China (Kim *et al*., 2007a; Zhang *et al*., 2014).

The duck farming sector in Egypt suffers significant losses due to the highly contagious, deadly, and quickly spreading DHAV disease. In addition to the widespread distribution of DHAV-1, DHAV-3 was discovered in Pekin duck farms in North Egypt recently, and it was genetically distinct from DHAV-3 isolated from China and Korea (Yehia *et al*., 2021). Since the recent advent of the novel DHAV-3 and the clinical signs and pathological alterations are so similar to those of DHAV-1 infection. (Yehia *et al*., 2021), molecular biological techniques are required to distinguish DHAV-3 infection from DHAV-1 infection. The RT-PCR and rRT-PCR have higher specificity and sensitivity and recently emerged as the preferred accurate method used for efficient genotyping of different DHAVs in Korea and China (Fu *et al*., 2008, Kim *et al*., 2008, Yang *et al*., 2008, Huang *et al*., 2012).

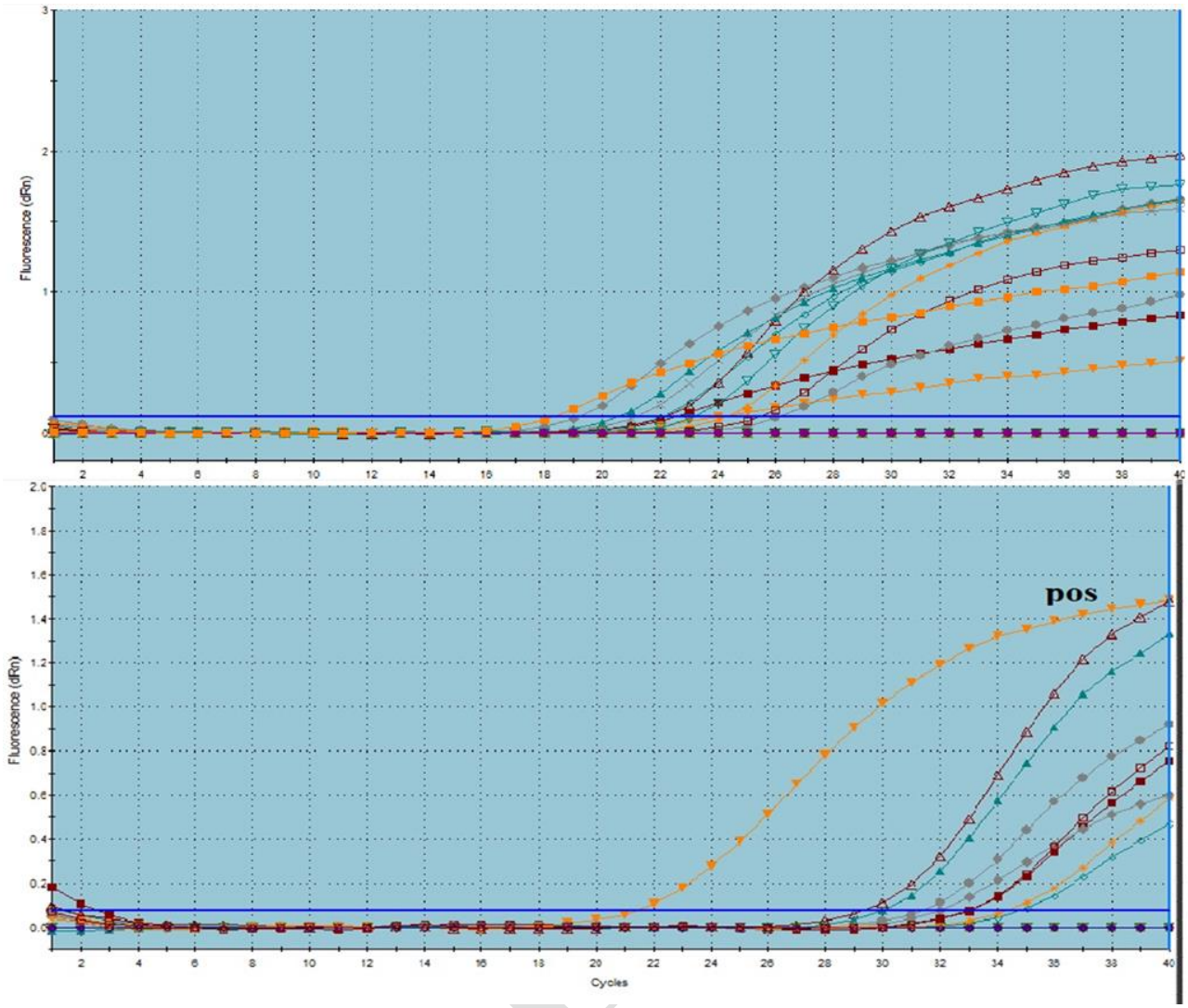

Fig. 5: The clinical validation of rRT-PCR for DHAV-3. The result showed twenty positive samples for DHAV-3.

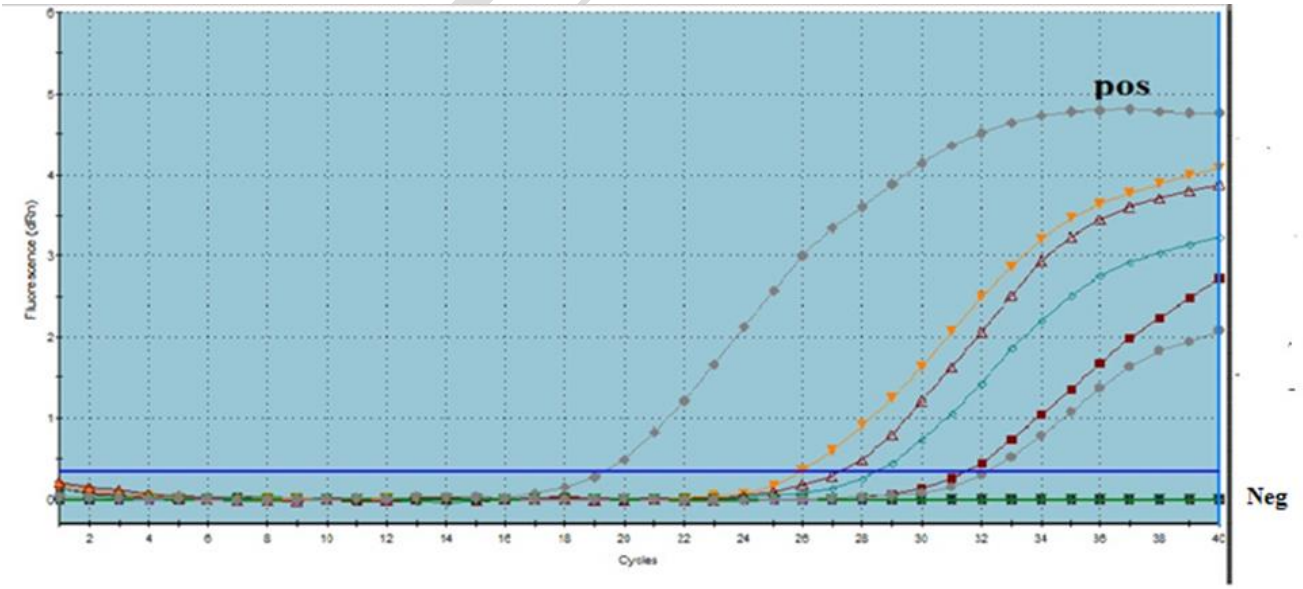

Fig. 6: Shown clinical validation of rRT-PCR for DHAV-1. The result showed five positive samples for DHAV -1.

So, this work intended to develop a sensitive, rapid, and specific Real-time RT-PCR technique for simultaneous detection of DHAV-1 and DHAV-3.

Since the VP1 gene is primarily used in the phylogenetic and genotyping of picornaviruses (Wang *et al*., 2008), the VP1 gene was targeted for the primers and probes designed

to distinguish between DHAV-1 and DHAV-3.

To check the conserved area used for detection of DHAV-1, and DHAV-3, we studied 43 genomic sequences of DHAV-1, DHAV-3, 16 DHAV-1, and 27 DHAV-3 sequences were published in GenBank in Egypt, China, and Vietnam. Based on bioinformatics analysis, the primers and probe were designed to distinguish between DHAV-1 and DHAV-3 in a very conserved and specific region for both subtypes. The results showed that the optimized rRT-PCR in the current study successfully identified both DHAV-1 and DHAV-3 and could differentiate between them. Our established rRT-PCR was extremely specific for each one. It did not amplify the DNA from the other five similar frequently isolated avian viruses and bacteria, including avian influenza virus, duck liver tissue, Marek's disease virus, *Salmonella*, and *E. coli* isolates (Pinheiro *et al*. 2020), as well as reactions with DHAV-1 with the set specified for DHAV -1 show strong positive for DHAV-1 and negative for DHAV-3. DHAV-3 with the set specified for DHAV-3 yielded strongly positive results for DHAV-3 and negative for DHAV-1. As a result, the two sets were specified and distinguished into both DHAV -1 and DHAV-3; these results were similar to previous studies (Kim *et al*., 2008; Saad et al., 2015; Niu *et al*., 2016).

Regarding the sensitivity of the rRT-PCR optimized in our study, by analyzing 10-fold serial dilutions of the reference virus ($10^{7.2}$ and 10^6 EID_{50}/ml for DHAV-1 and DHAV-3, respectively), the sensitivity of rRT-PCR was assessed and compared to RT-PCR and the results revealed that the rRT-PCR assay's lowest limit of detection for DHAV-1 and DHAV-3 was $10^{2.2}$ and 10^2 EID50/ml for the rRT-PCR assay, respectively. It was tenfold higher than RT-PCR. That detects at $10^{3.2}$ and 10^3 EID50/ml for DHAV-1 and DHAV-3, respectively. This was similar to previous studies, showing a calculated detection limit of $3.36x10^3$ and $3.36x10^4$ copies of the RNA template of DHAV-C by rRT-PCR and RT-PCR, respectively (Huang *et al*., 2012). Real-time RT-PCR's enhanced sensitivity in comparison to RT-PCR may result from the ability to detect the fluorescent signal given off by particular amplification products (Acevedo *et al*., 2013).

The rRT-PCR's suitability for detecting clinical samples for both subtypes of DHAV was also investigated. Forty duck farm samples from five Egyptian governorates were examined; sick birds displayed symptoms. In this investigation, DHAV was discovered in 15 of the 40 samples from farms of Pekin duck in 5 governorates of Egypt that were analyzed. Infected animals displayed disease symptoms as detected by RT-PCR (Kim *et al*., 2007b; Kozdru *et al*., 2014). A total of three samples were positive for DHAV-1 and twelve samples were positive for DHAV-3, according to subtyping by VP1 RT-PCR, and this is according to (Yehia *et al*., 2021), who revealed that DHAV-3 was exhibiting a relatively high incidence throughout the governorates under investigation. According to several earlier research, DHAV-3 is more prevalent than DHAV-1 in Vietnam, Korea, and China (Soliman *et al*., 2015; Doan *et al*., 2016; OIE, 2021).

The rRT-PCR detected all positive samples detected by RT-PCR. The ten samples detected with Cycle threshold (CT) range 30-35 were positive by rRT-CR and not detected by RT-PCR, and it was verified by virus isolation and retest of rRT-PCR. It indicates that rRT-PCR was more sensitive than RT-PCR in the detection and differentiation of clinical samples, as previously mentioned (Acevedo *et al*., 2013, Niu *et al*., 2016). Real-time PCR is regarded as the most effective diagnostic technique since it is faster, more sensitive, and more repeatable than PCR, and the possibility of carryover contamination is reduced (Mackay, 2004). Because the real-time RT-PCR assay is quick, easy, effective, extremely specific, and sensitive, it has become a crucial tool for inspecting samples in suspected DHAV cases.

Conclusion: This study established an optimized real-time reverse transcriptase polymerase chain reaction (rRT-PR) test for DHAV-1 and DHAV-3 detection and differentiation. The assay could be used as a quick, accurate, and focused molecular diagnostic for DHAV-1 and DHAV-3 infection and for epidemiological studies.

Competing interests: The authors declare that they have no competing interests.

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