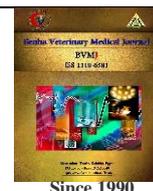




Official Journal Issued by  
Faculty of  
Veterinary Medicine

## Benha Veterinary Medical Journal

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### Original Paper

## Molecular detection and phylogenetic analysis to evaluate the evolutionary pattern of VP1 gene sequence of recent duck hepatitis A virus (DHAV) isolate and assessment of new genotyping emerging in Egypt

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### ARTICLE INFO

#### Keywords

Duck hepatitis A virus  
VP1 gene  
Genotype 3

Received 30/05/2021

Accepted 06/06/2021

Available On-Line  
01/10/2021

### ABSTRACT

Duck hepatitis A virus (DHAV) is an *Avihepatovirus* A family *Picornaviridae* which consider one of the most serious threatening viral pathogens influencing duck rearing in Egypt until now, with disastrous economic effects on duck industries' not only at local level but also worldwide. In this study, suspected DHAV samples (n=10) were collected from commercial flocks and backyard rearing from different localities in Kalubia province from September 2020 to January 2021. The infected flocks were showing high mortality and nervous signs without history of previous vaccination against DHAV. Hemorrhagic liver samples were prepared from recently died (7 to 15) day old ducklings. Trials for isolation of the virus on SPF-ECEs using allantoic route showed stunting, subcutaneous hemorrhage, and yellowish discoloration of liver with necrotic foci in the inoculated embryos. Then, viral isolates were undergone molecular identification using RT-PCR targeting 5' UTR and VP1 gene which revealed amplicon size 250 bp and 880 bp respectively for positive DHAV isolates. The positive isolate was further subjected to sequencing of the partially amplified VP1 gene and phylogenetic analysis was performed in relation to the Egyptian DHAV strains, vaccinal strain and other sequences available on the GenBank. Our results revealed that this new DHAV isolate was more homologous and genetically related to emerging DHAV genotype 3 which recently identified in Egypt. The studied vaccinal strain using in vaccination against the virus was out of grouping. Consequently, further serological studies to update the nature of future vaccine strains for successful preventive strategies would be urgent.

## 1. INTRODUCTION

Duck viral hepatitis (DVH) is viral infection of growing duckling less than 3 weeks' old which distinguished with contiguousness, sudden onset, widely spreading and highly fatal disease with catastrophic economic impacts on the duck rearing industries (OIE, 2018).

The disease caused by 3 different viruses, DVH type I, DVH type II which classified as duck astrovirus-1 (DAstV-1) which only recorded in UK (Koci and Schultz-Cherry, 2002) and DVH type III that classified as duck astrovirus-2 (DAstV-2) recorded only in USA (Todd et al., 2009).

DHAV, formerly known as DVH-1 which belong to genus *Avihepatovirus*, family: *Picornaviridae* (Knowles et al., 2012). DHAV comprise 3 genotypes (DHAV-1, DHAV-2 and DHAV-3) which genetically distinct from each other with no or finite cross neutralization protection (Kim et al., 2009).

DHAV-1 is the widest spreading genotype all over the world (Wang et al., 2008), while DHAV-2 limited spreading in Taiwan (Tseng and Tsai, 2007) and DHAV-3 was detected in Korea (Kim et al., 2007), China (Liu et al., 2011) and Vietnam (Doan et al., 2016a).

DHAV is small size (25-30 nm), non-enveloped, icosahedral capsid and positive polarity single stranded ribonucleic acid (ssRNA) with genomic size about 7800 nucleotides which contain one open reading frame (ORF) encompassed by two untranslated regions (3'&5' UTRs). The single (ORF) encode precursor viral poly protein comprises ~2500 amino acids which subject to post translational cleavage to rise 12 proteins that subdivided into structural proteins (VP1, VP2, VP3 and VP4) and nonstructural proteins (Wen et al., 2015).

The 5' UTR gene play significant role in synthesis of viral proteins and viral ribonucleic acid (RNA), via ribosomal binding to viral RNA occur in of 5' UTR region known as internal ribosome entry segment (IRES). Furthermore, the molecular detection of DHAV in clinically suspected samples by use RT-PCR of 5' UTR gene is much more specific and sensitive than other genes (Fu et al., 2008).

The VP1 protein is external surface capsid protein and major responsible for host cellular receptor attachment. The immune system has been modulated to elicit neutralizing antibodies, also VP1 responsible for virulence, phylogeny, and genotyping of DHAV.

The first detection of DHAV was in 1950 in the United State of America (Wen et al., 2014), while the first report of DHAV in Egypt was in 1970 (Refaie, 1969).

The protection of breeder ducks sector in Egypt usually provided with DHAV-1 egg-adapted live attenuated vaccine produced from E52 Rispen strain (FAO, 2009).

In the current work, we study the prevalence of DHAV in clinically suspected samples collected from ducklings without history of previous vaccination by virus isolation on specific pathogen free embryonated chicken eggs (SPF-ECEs). The isolated viruses were molecularly characterized by reverse transcriptase polymerase chain reaction (RT-PCR) for 5'UTR and VP1 genes. Furthermore, the VP1 gene was partially sequenced, and the genetic characterization was described by phylogenetic analysis to find out the possibility of new genotyping emerging of DVAV in Egypt.

## 2. MATERIAL AND METHODS

### 2.1. Sample collection and preparation

Ten liver samples were aseptically collected from 1:2-week-old commercial and backyard duckling from different localities of Kalubia governorate between September 2020: January 2021. Diseased duckling were showing typical clinical signs of duck viral hepatitis as described by (Mansour., 2019). Liver samples were prepared under complete aseptic condition according to (OIE.,2018). Each sample consisted from (4 pooled liver) were manually grinded in sterile mortar and pestle to prepare 20% suspension (w/v) in sterile PBS (PH:7.2) solution contain penicillin (1000 IN/ml) & streptomycin (100mg/ml). The suspension subjected to 3 successive cycles of freezing and thawing then sample is clarified by centrifugation at 3000 rpm for 15 minutes at 4°C. the clarified supernatants were transferred into sterile tubes, then filtrated by 250 nm syringe filter.

### 2.2. Virus isolation on specific pathogen free embryonated chicken eggs (SPF-ECEs)

Trails for virus isolation were performed by allantoic route inoculation of 9-day old age SPF-ECEs (Nile SPF, Koom Oshiem, Fayoum, Egypt) (OIE.,2018). About (100 µL) of each sample was inoculated in 3 SPF-ECEs, after that incubated at 37°C for 3-5 days and daily examined for detection of embryonic deaths with exclusion of nonspecific one occurred in first 24-hour post incubation and determination effect of virus replication on the embryo (3 successive passage were done). Finally, allantoic fluid and embryos were aseptically harvested from inoculated SPF-ECEs for molecular identification.

### 2.3. RNA Extraction

Viral RNAs were extracted from allantoic fluid harvest by using Viral RNA Mini Kit (QIAGEN, Germany, Cat. no.52904) according to manufacturer's recommendations.

### 2.4. Reverse transcriptase Polymerase Chain Reaction (RT-PCR)

One step RT-PCR was used for amplification of 5' UTR gene and VP1 gene as mentioned in table (1). The reaction mixture was composed from 6 µl of extracted RNA Template, 1 µl of forward primer (20 P MOL), 1 µl of reverse primer (20 P MOL), 12.5 µl Patho Gene-spin™ RT-PCR Master mix (2x) and nuclease free water added up to 25 µl. The RT- PCR reactions were run in T3 thermal cycler (Biometra, Germany) according to conditions shown in table (3). The PCR amplicons were detected by gel electrophoresis (1.5%).

**Table (1):** Oligonucleotide sequence of primer sets specific to 5'UTR and VP1 genes of DHAV

Gene	Primer sequence 5'-3'	product (bp)	Reference
5'UTR	CCTCAGGAACTAGTCTG	250	(Fu et al., 2008)
	-GA		
	GGAGGTGGTGCTGAAA		
Genotype1 VP1	ACACCTGTTGGGAGGC	609	(Mansouret al., 2019)
	-AAT		
	TCCAGATTGAGTTCAAA		
Genotype3 VP1	-TGCTAGTG	880	(Doan et al., 2015)
	ATGCGAGTTGGTAAGG-		
	ATTTTCAG		
	GATCCTGATTACCAAC		
	-AACCAT		

### 2.5. VP1 sequencing and Phylogenetic analysis

The PCR products of Genotype 3 VP1 gene reaction were directly extracted and purified by use gel extraction kits (QIAGEN, Germany). The sequencing of PCR products was done by using Bigdye Terminator TM V3.1 cycle sequencing kit using the same previously forward and reverse primer of DHAV3 VP1 gene. The obtained nucleotide sequences were analyzed by MEGA X software (Kumar et al., 2018) which used for: alignment study of sequenced nucleotides by using Clustal W method, Calculation of identity percentage of nucleotides through utilize pairwise distance method and construction of phylogenetic tree using maximum likelihood method with moderate strength and 1000 bootstrap replicates (Tamura et al., 2013).

## 3. RESULTS

### 3.1. Clinical Samples and postmortem lesions

The examined flocks showed neurological symptoms such as ataxia, head drawn back (opisthotonos), imbalance and fall on one side with spasmodically kicking till death. The gross pathological changes of recently dead appeared mainly in liver which suffering from AI.

### 3.2. Virus isolation

The inoculated (SPF-ECEs) were consistent in their response and usually embryo died at (3-5) days PI. There were 9 dead embryos of 10 inoculated SPF-ECE showing dwarfism, congestion, and hemorrhagic with abdominal edema. Livers of the inoculated embryos become reddish or yellowish discoloration with scattered points of necrosis (Fig.2)

### 3.3. Molecular identification of viral isolates by reverse transcriptase RT-PCR

Out of the 10 isolates tested by RT-PCR, 90 % isolates were positive for 5' UTR gene of DHAV with amplicon size 250 bp as shown in Fig. (3). Out of these 9 isolates, two samples (3 &10) were further genotyped based on VP1 gene amplification. Duck hepatitis A virus (DHAV) Genotype 3 were detected in both samples at 880 bp as shown in Fig. (4). As expected, the negative control sample did not show any amplicon products for each reaction

### 3.7. VP1 gene sequencing and phylogenetic analysis:

VP1 gene sequence of the new isolate in the current study which have gene bank accession number (MZ229306) was compared with VP1 gene sequences of 29 different DHAV strains from Egypt, vaccinal strain and other sequences available on GenBank database.

The multiple sequence alignment for VP1 gene partial sequence of our new DHAV isolate has showed 96.73% - 96.87% nucleotide identity with recently identified DHAV-3 Egyptian strains, while nucleotide identity with

Vietnamese strains, Korean strains and Chinese strains was 87.83 % - 88.40 %, 86.96% - 88.11 % and 87.61% - 88.64% respectively (Fig. 5). Surprisingly, it has shown insignificant level of nucleotide similarity (50.22%) with the DHAV-1 vaccinal strain used in Egypt. The phylogenetic tree of the DHAV-3 clusters has shown 4 distinct phylocusters as follows: Chinese strains, Korean strains, Vietnamese strains, and Egyptian strains subgroups. While vaccinal strain in Egypt present in separate cluster from DHAV-3 as outgroup (Fig. 6).

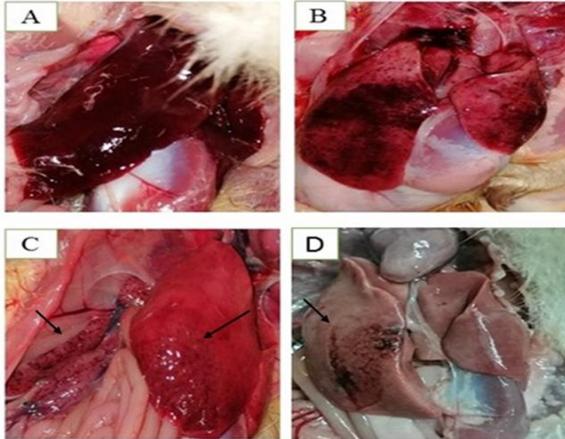


Fig. (1): Panel A; liver with mottled appearance (Hepatitis). Panel B; Diffuse echymotic hemorrhages on the infected liver. Panel C; Hepatic congestion & swollen hemorrhagic kidney. Panel D; Hepatic fissure due to sever degree of hepatitis

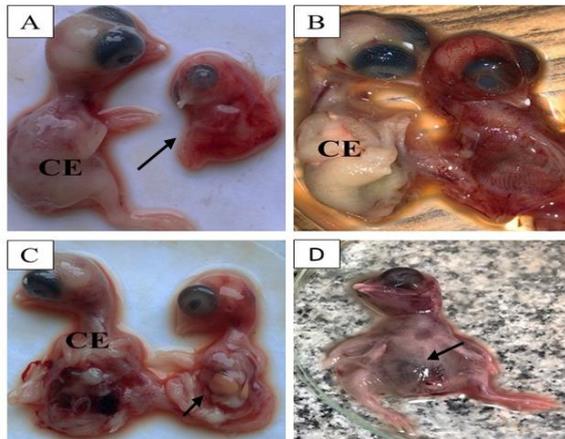


Fig. (2) Panel A; Inoculated embryo showing dwarfism and congestion. Panel B; Subcutaneous hemorrhage over whole body. Panel C; Yellowish discoloration of embryo liver. Panel D; edema in abdominal cavity and hind limb of embryo

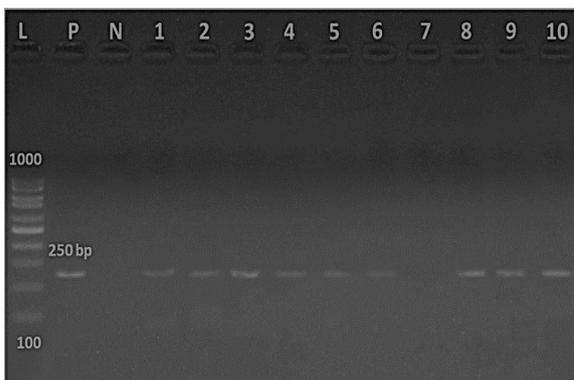


Fig. (3): Panel A; liver with mottled appearance (Hepatitis). Panel B; Diffuse echymotic hemorrhages on the infected liver. Panel C; Hepatic congestion & swollen hemorrhagic kidney. Panel D; Hepatic fissure due to sever degree of hepatitis

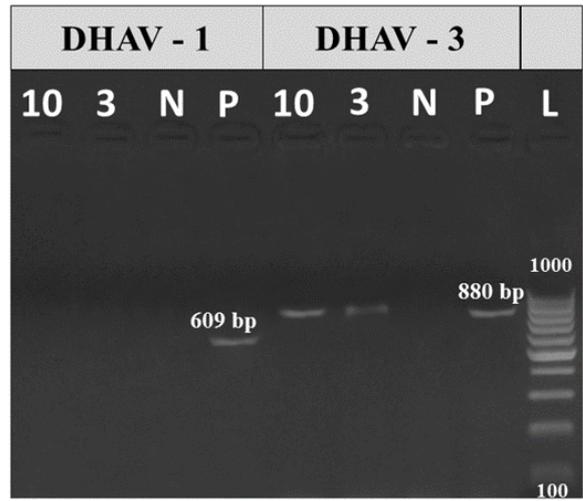


Fig. (4): The Electrophoretic pattern of the RT-PCR products based on VP1 gene of both DHAV-1 (609 bp) and DHAV-3 (880 bp) to the viral isolates (3&10) on the gel electrophoresis (1.5%). Amplicon size was detected at 880 bp which considered positive results for the DHAV (Genotype 3). Positive and negative controls are included

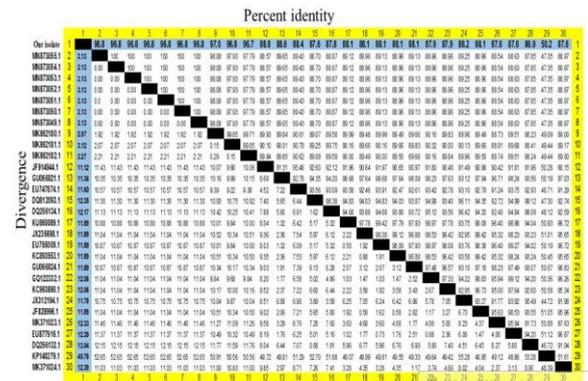


Fig.(5):Nucleotide identities and divergence of VP1 gene-sequenced isolate compared to other selected strains retrieved from the GenBank database that showed 96.73% - 96.87% nucleotide identity with DHAV -3 Egyptian strains

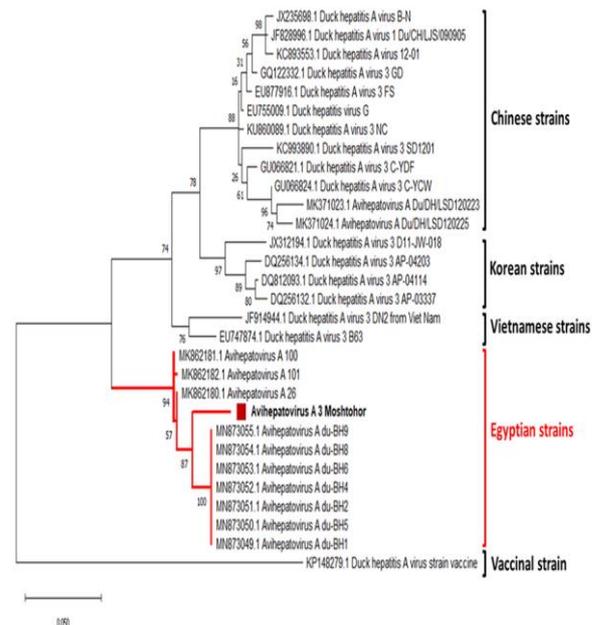


Fig. (6): Phylogenetic tree using maximum likelihood method based on VP1 gene nucleotide sequences of our new DHAV-3 isolate (indicated by red quadrate) with other sequences of DHAV-3 retrieved from the GenBank database. Numbers at the internal nodes represent the bootstrap probabilities (1000 replicates). The scale bar (0.05) means nucleotide changes or substitutions per site.

#### 4. DISCUSSION

DHAV is the main cause of DVH disease, that has negative feedback on Egyptian duckling flocks. In our study, we recorded high mortalities (50-90%) in duckling and variable grades of nervous signs including ataxia, opisthotonos and spasmodic muscular convulsion in examined suspected flocks. After postmortem examination, diseased ducklings showed hepatic hemorrhage (diffuse petechial or ecchymotic) with hepatomegaly, in addition to splenomegaly and congestion of renal vessels with kidney enlargement which are similar to the lesions recorded by Hassaan et al., (2015).

After samples isolation, most of the inoculated suspected samples showed embryonic deaths within 5<sup>th</sup> day post inoculation in SPF-ECE and the embryo showed dwarfism, congestion, hemorrhage, and abdominal edema accompanied with slightly greenish discoloration of allantoic fluid. Embryonic liver also showed enlargement with yellowish discoloration that associated with necrotic foci. These findings came in accordance with (Hassan et al., 2020; Mansour et al., 2019; Yehia et al., 2020).

For viral detection, RT-PCR which targeting 5'UTR gene was performed to 10 viral isolates which showed amplicon size at 250 bp in nine isolates that considered a positive RT-PCR result also detected by (Fu et al., 2008; Hisham et al., 2020).

To reveal the circulating genotypes in Kalubia governorate, another RT-PCR targeting VP1 of both DHAV genotype 1 and 3 was repeated on positive RNA samples number 3 and 10. Both samples did not show any amplification products in RT-PCR targeting VP1 of genotype 1. On the other hand, the same samples showed amplicons at size of 880 bp that indicate the formerly samples are genotype 3 of DHAV (Doan et al., 2016). It is well established that DHAV-3 is circulating in Egypt since 2016 (Hassan et al., 2020; Yehia et al., 2020) and our results came consistent with these recent records.

Nucleotide sequencing and alignment were performed for VP1 amplicon of sample (10) and examined for its identity percent when compared with retrieved sequences from the GenBank. The analysis of nucleotide sequence of our new isolate (MZ229306) has shown 96.73% - 96.87% nucleotide similarity with recently identified DHAV-3 in Egypt (Hassan et al., 2020; Yehia et al., 2020) while nucleotide similarity with Egyptian vaccinal strain (KP1482791) is 50.22%. Based on our finding, we draw attention about new circulating DHAV-3 which genetically variant from vaccinal strain used in vaccine, so it is necessary to determine if the vaccine has ability to protect against heterologous strains or not (Hisham et al., 2020).

#### 5. CONCLUSION

Our results revealed that this new DHAV isolate is more homologous and genetically related to emerging DHAV genotype 3 circulating in Egypt. Consequently, further serological studies to update the nature of future vaccine strains for successful preventive strategies would be urgent.

#### CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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