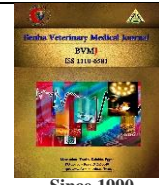




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Molecular characterization of isolated duck viral hepatitis and its pathogenicity in one-day and seven-day old ducklings in Qalyubia Governorate

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ABSTRACT

Duck hepatitis A virus (DHAV) is a cause of duck viral hepatitis (DVH), which is an acute and infectious illness with a high mortality rate. The aim of this study is Molecular characterization of isolated duck viral hepatitis and evaluation of its pathogenicity in one- day and seven-day old ducklings in Qalyubia governorate so thirty samples from several duck farms in Qalyubia Governorate were used in this investigation between 2023 and 2024. The tested birds, which ranged in age from 7 to 12 days had neurotic symptoms including ataxia, opisthotonos, spasmodic kicking, swift death and enlarged liver showing noticeable petechial hemorrhages. Using allantoic sac isolation and RT-PCR, these samples were utilized to identify DVH in SPF-ECEs. After the eggs were harvested, the embryos displayed bleeding, stunting, and greenish necrosis of their livers. A 250 pb sequence of 5'UTR gene fragment was amplified by RT-PCR, and 880 pb VP1 gene was amplified for subtyping, indicating that the isolate is related to DHAV-3. The affirmative subtyped sample was next subjected to sequencing analysis and phylogenetically examined. The examined strain's VP1 nucleotide sequence revealed differences with Chinese and Korean-Vietnamese strains (86.5% to 88.1%) as well as 66.8% and 96.8% to 100% similarity to vaccinal and Egyptian strains, respectively. The pathogenicity of the isolated strain was then assessed using two distinct age groups of pekin duckling. By monitoring liver and kidney functions and doing a histological study, the results verified that our isolate is a highly pathogenic virus.

1. INTRODUCTION

Ducklings can contract duck hepatitis virus (DHV), a highly fatal condition that spreads quickly and is highly contagious (Woolcock, 2003). There are three varieties of heterologous DHV: I, II, and III (Woolcock, 2003). Duck hepatitis A virus (DHAV), which is the current nomenclature for DHV type I, is classified into three genotypes: DHAV-1, DHAV-2, and DHAV-3 (Wang *et al.*, 2008). DHAV-2 is associated with Astrovirus, whereas DHAV-I and DHAV-3 are related to Picornaviruses. It is known that DHAV-1 is the virulent variety that spreads widely (Lefkowitz *et al.*, 2018). Ducklings are particularly vulnerable to DHV infection in the first few weeks of life. Throughout their lives, adult birds develop into carriers with no symptoms (Liu *et al.*, 2011). In white Pekin ducklings, DHAV was initially isolated in the spring of 1949 in New York (Levine and Fabricant, 1950). China and South Korea were the isolation sites for DHAV-3 (Xu *et al.*, 2019). Ducklings from Pekin, Balady and Rowan were the first to be identified as DHAV infected in Egypt (Refaie, 1969). DHAV is a tiny (25–30 nm) non-enveloped virus has sRNA with a genomic size of approximately 7800 nucleotides. It has one open reading frame that is surrounded by two untranslated regions (3'&5' UTRs). The 5' UTR gene is important to promote viral proteins translation and ribonucleic acid (RNA) replication as well as it provides a far more sensitive and specific molecular detection of DHAV using RT-PCR in clinically suspicious samples (Fu *et al.*, 2008). VP1 gene sequences important for virulence, phylogeny, and genotyping of DHAV. Ducklings infected

with DHAV-I experienced ataxia, weakness, dullness, spasmodic movement and kicking of the legs, loss of balance, and ultimately passed away in the opisthotonos position (Woolcock, 2008). According to Hassan *et al.* (2018) dead ducklings with DVH showed enlarged kidneys and spleen as well as an enlarged liver with petechial and ecchymotic hemorrhages. The primary basis for laboratory diagnosis of DVH is the isolation and identification of DHAV (Maiboroda, 1972). Reverse Transcriptase-Polymerase Chain Reaction was successfully used to detect DHAV (Erfan *et al.*, 2015).

The outbreak of this disease is studied among duckling that has no history of previous vaccination from farms in Qalyubia governorate, Egypt between October 2022 and July 2023. RT-PCR was used for detection of DHAV in clinically suspected samples and after isolation in specific pathogen free embryonated chicken eggs (SPF- ECEs). 5'UTR and VP1 genes specific primers were used for identification and typing of isolated DHAV respectively using RT-PCR. Additionally, VP1 gene underwent partial sequencing and phylogenetic analysis to define the genetic characterization. This allowed us to determine whether novel genotypes of DVAH could emerge in Egypt and examined the virulence of our isolated strain by determining its pathogenicity in ducklings of different ages.

2. MATERIAL AND METHODS

2.1. Sampling.

A total of thirty samples were collected from several pekin duck farms in Qalyubia governorate. The ducklings were 7–

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12-day-old and experienced spasmodic movement, ataxia, weakness, and opisthotonos posture when they were lying on one side. Upon postmortem inspection, they revealed an enlarged spleen, kidneys, and liver with petechial and ecchymotic hemorrhages. The samples, which comprise liver, spleen, kidney, and brain, were taken between October 2022 and July 2023 and used for RT-PCR diagnosis. Samples were prepared under complete aseptic condition according to OIE. (2018). Samples were stored at -80 till usage. After that, samples were collected for isolation in SPF-ECEs. Prior to typing and sequencing, the sample exhibiting DVH-specific isolation results was obtained for RT-PCR confirmation.

2.2. Molecular Detection of the virus and typing of DVH

Following the manufacturer's instructions, Viral RNA Mini Kit (QIAGEN, Germany, Cat. no.52904) according to manufacturer's recommendations. 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). 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.

Virus	Primer of gene sequence 5'-3'	Identified genotype	Size of amplicon (bp)	Reference
DVH	5'UTR-F	CCTCAGGAAGTAGTCTGGA	250	Fu et al. (2008)
	5'UTR-R	GGAGGTGGTGCTGAAA		
	VP1-F	ACACCTGTTGGGAGGC AAT	609	Mansour et al. (2019)
	VP1-R	CCAGATTGAGTTCAAA TGCTAGTG		
	VP1-F	ATGCGAGTTGGTAAGGATTTTCAG	880	Doan et al. (2016)
	VP1-R	GATCCTGATTACCAAC -AACCAT		

2.3. Isolation of virus on specific pathogen free embryonated chicken egg (SPF-ECE)

Two hundred microliters of sample homogenate (liver, spleen, and kidney) were injected into the allantoic sac of a 10-day-old SPF-ECE to achieve isolation (OIE, 2010). Every sample received three SPF-ECEs for inoculation. The eggs were then opened and checked for specific DVH replication after the incubation period ended. The inoculated eggs were then incubated for five days at 37°C and monitored daily for the presence of embryonic deaths, apart from one that happened within the first 24 hours after inoculation. (This procedure is repeated three times according to OIE standards). Ultimately, allantoic fluid and embryos were aseptically removed from inoculated SPF-ECEs after each passage to confirm the virus's viability via RT-PCR and to conduct additional molecular analysis.

subcutaneously and the 7-day old group was challenged with 0.5ml of the same titer (Niu et al., 2019) Control groups were injected with 0.2ml and 0.5ml sterile PBS according to their age. Ducklings were observed for signs and mortality. From each group at 3rd and 6th day post infection (dpi), three ducklings from each group were euthanized and slaughtered and their livers, spleens and kidneys were collected for histopathology and Blood samples were collected from each duckling by jugular vein puncture then each blood sample was placed in a plain centrifuge tube for serum separation and determination of biochemical constituents; activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) (Reitman and Frankel, 1957), creatinine (Houot, 1985) and uric acid (Trivedi et al., 1979).

2.4. VP1 sequencing and Phylogenetic analysis

The gel extraction kits (QIAGEN, Germany) were utilized to directly extract and purify the PCR results of the Genotype 3 VP1 gene reaction. Using the same DHAV3 VP1 gene forward and reverse primers as before, the Bigdye Terminator TM V3.1 cycle sequencing kit was used to sequence the PCR results. The obtained nucleotide sequences were subjected to analysis using MEGA X software (Kumar et al., 2018). The software was utilized for three purposes: the construction of a phylogenetic tree using the maximum likelihood method with moderate strength and 1000 bootstrap replicates (Tamura et al., 2013), identification percentage calculation of nucleotides through the use of pairwise distance method, and alignment study of sequenced nucleotides.

2.7. Histopathological Analysis

This study first preserved tissue samples in buffered formalin to prevent autolysis. Subsequently, these tissue samples underwent a standardized histological preparation process, including embedding in paraffin wax, sectioning to a thickness conducive to microscopic examination, and staining. The Hematoxylin and Eosin staining protocols by Bancroft were applied (Suvarna et al., 2019). An ordinal semiquantitative scoring system was employed for microscopic evaluation of the tissue sections. This system, rigorously developed to assess the extent of tissue damage, allowed for classifying observed pathological changes into discrete categories. Specifically, the system quantified degenerative transformations, inflammatory processes, and cellular damage on a scale from normal (-) to very severe (++++), encompassing mild (+), moderate (++) and severe (+++) lesions as intermediate stages. This scoring paradigm aligns with the validated histopathologic scoring principles advocated by (Gibson-Corley et al., 2013).

2.5. Ethics Declarations.

The authors of this study followed the guidelines established by the animal welfare committee. The protocols used in this research were approved by the Research Ethics Committee at the Faculty of Veterinary Medicine, Benha University (BUFVTM 01-02-24).

The severity of histopathological changes in liver and kidney samples was quantitatively assessed using a scoring system. Samples exhibiting no discernible lesions received a score of 0. Samples characterized by minor, intermediate, and extensive renal damage were assigned scores of 1, 2, and 3, respectively, reflecting the escalating severity of kidney lesions (Xu et al., 2021).

2.6. Pathogenicity test

Sixty Pekin ducklings of 1-day and 7-day old were purchased from a Pekin duck hatchery plant in Qaluybia. Each age group was equally divided into 2 groups. One of each was control -ve and the other group was challenged with the virus according to their age. The 1-day old ducklings were challenged with 0.2ml 105 EID50

2.8. Statistical Analysis

The statistical analysis was carried out using Two-Way ANOVA using SPSS, ver. 27 (IBM Corp. Released 2013). Data were treated as a complete randomization design according to (Steel et al., 1997). Multiple comparisons were

carried out applying Duncun test the significance level was set at ≤ 0.05 .

3. RESULTS

3.1. DHAV detection by RT-PCR

Thirty samples were tested using RT-PCR, and only one isolate tested positive for the Duck Hepatitis Virus type 1 (DHV-1) 5' UTR gene with an amplified segment size of 250 bp (Fig. 1). VP1 gene amplification was used to genotype the positive isolate. In the positive sample, DHAV Genotype 3 was detected at 880 bp (Fig. 2).

3.2. Isolation of virus in embryonated chicken egg

Following the harvest of the inoculated embryonated chicken eggs. The dead embryos displayed stunting, subcutaneous bleeding, and hepatic hemorrhage with greenish necrosis on the fifth day after injection; these signs appeared after the third passage (Fig. 3).

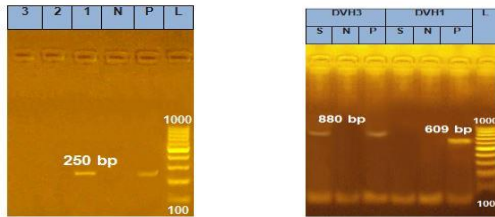


Figure 1 RT-PCR of sample 1 showing positive result amplicon at 250 bp, samples 2 & 3 showing negative results.

Figure 2 Based on VP1 gene of both DHAV-1 (609 bp) and DHAV-3 (880 bp), positive sample showed amplification at 880 bp that means the isolate belongs to DHAV-3.

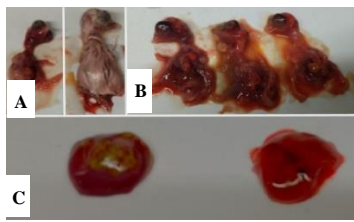


Figure 3 Typical picture of DVH isolation on SPF-ECE, stunting of the embryo (A) compared to negative control (B) with subcutaneous hemorrhage and hemorrhagic liver with greenish necrotic foci (C).

3.3. Sequencing and phylogenetic analysis.

Our isolate's VP1 gene sequence (accession no. OR543968) was compared to the gene sequences of 26 strains of Chinese, Korean Vietnamese, and Egyptians (Figure 4).

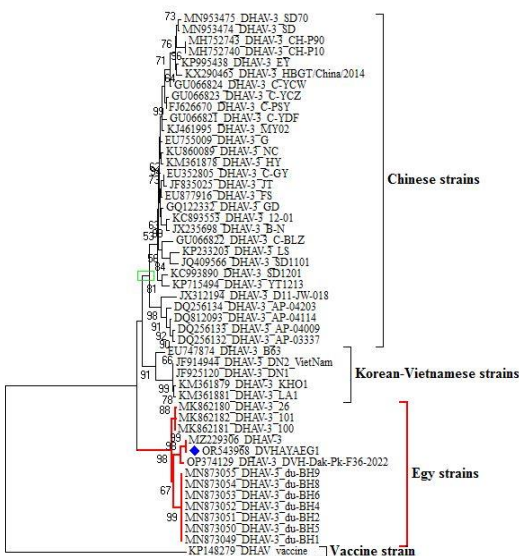


Figure 4 Phylogenetic tree showing Four separate phyloclusters have been identified in the evolutionary tree of the DHAV-3 clusters: Chinese strains, Korean strains, Vietnamese strains, and Egyptian strains subgroups. As an outgroup to DHAV-3, the Egyptian vaccination strain is found in a different cluster.

3.4. The pathogenicity results.

3.4.1. Clinical signs, mortality rate and gross lesions

Main descriptive data for the difference in signs, mortality and lesions between the age groups as mentioned in table (3) and figure (5).

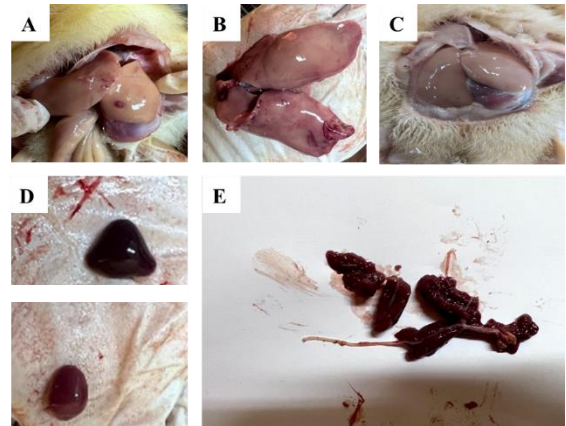


Figure 5 Showing enlarged liver with petechial hemorrhage (A , B). Compared with (C) which shows no changes (normal liver) also showing the difference between infected (D) & normal (E) which show swelling and necrosis in spleen. Infected kidney (F) show hemorrhage and swelling.

Table 2 The strain under study's VP1 nucleotide sequence revealed 66.8% similarity to a vaccine strain and 96.8% to 100% similarity to Egyptian strains; in contrast, it displayed notable similarities with Chinese strains (86.5% to 88.1%) and Korean-Vietnamese strains (88.6% to 89.2%). Remarkably, it has revealed a negligible degree of nucleotide similarity (66.8%) with the Egyptian DHAV-1 vaccination strain.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
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3.4.2. Liver enzymes testing

When comparing the challenged groups (1-day & 7-day) to the blank control groups, liver enzyme levels significantly rose. Compared to the 7-day old group, it was notably higher in the 1-day old group on the third day following the challenge as shown in table (4).

3.4.3. Kidney function testing

Challenged groups show highly increased values in comparison to blank groups that indicate a noticeable deterioration in kidney functions as shown in table (4).

3.4.4. Histopathological findings.

In the liver, significant histopathological changes were observed one-day post-infection with Duck Hepatitis, including marked dilation and congestion in central veins and hepatic sinusoids, hepatocellular vacuolar degeneration, necrosis, and apoptosis. Additionally, hyperplasia of the biliary epithelium, portal infiltration by inflammatory cells, and hepatic bleeding were noted. However, one-week post-infection, the severity of these changes decreased, with reduced hepatocellular degeneration, necrosis, and

inflammatory response. Similarly, significant pathological changes in the kidneys were observed one-day post-infection, including severe degeneration and necrosis of

renal tubular epithelial cells, exfoliation into tubular lumens, and infiltration by heterophilic granulocytes.

Table 3 Clinical signs, P.M and mortality occurred in challenged groups.

Day after challenge	One – day old group		Seven – day old group	
	Clinical signs	Mortality, PM EX.	Clinical signs	Mortality, PM EX.
1 st	Greenish diarrhea – depression – anorexia	3 ducklings died. Enlarged liver with hemorrhage. Nephritis	depression – anorexia	No mortality
2 nd	Greenish diarrhea – Ataxia – paddling	4 ducklings died in opisthotonos position. Petechial and ecchymotic hemorrhage in liver – cyanosed enlarged kidneys – enlarged spleen with necrosis.	Greenish diarrhea – Paddling	2 ducklings dead in opisthotonos position. Petechial and ecchymotic hemorrhage in liver
3 rd	Ataxia, paddling, depression	1 duckling died in opisthotonos position. Petechial and ecchymotic hemorrhage in liver with swollen gall bladder – cyanosed enlarged kidneys – enlarged spleen with necrosis.	Ataxia, paddling, Depression	3 ducklings died in opisthotonos position. Petechial and ecchymotic hemorrhage in liver – cyanosed enlarged kidneys – enlarged spleen with necrosis
4 th	Depression	No mortality	Depression, paddling	1 duckling died. Petechial and ecchymotic hemorrhage in liver
5 th	-	1 duckling died in opisthotonos position. Petechial and ecchymotic hemorrhage in liver – cyanosed enlarged kidneys – enlarged spleen with necrosis.	No signs	No mortality
6 th	-	-	No signs	No mortality

Table 4 Liver, Kidney enzymes testing results (mean± SE).

Enzyme (U/L)	Day post infection (dpi)	One- day		Seven-day	
		Control group	Infected group	Control group	Infected group
GPT	3	27.33±0.88 ^{bb}	182.67±1.76 ^{aa}	34.33±0.88 ^{bb}	138.00±1.53 ^{aa}
	6	31.33±0.88 ^{ab}	151.67±1.76 ^{ba}	38.67±0.88 ^{ab}	102.00±1.53 ^{ba}
GOT	3	28.33±0.33 ^{bb}	178.67±1.20 ^{aa}	33.67±0.88 ^{bb}	129.33±0.88 ^{aa}
	6	35.67±1.20 ^{ab}	146.00±1.73 ^{ba}	42.67±1.45 ^{ab}	100.67±1.20 ^{ba}

Enzyme (mg/dl)	Day post infection (dpi)	One- day		Seven-day	
		Control group	Control group	Control group	Infected group
Creatinine	3	1.22±0.01 ^{ab}	3.80±0.06 ^{aa}	1.25±0.05 ^{aa}	3.21±0.11 ^{aa}
	6	1.16±0.02 ^{ab}	3.47±0.03 ^{ba}	3.99±2.90 ^{aa}	2.68±0.17 ^{aa}
Uric acid	3	2.56±0.03 ^{bb}	18.33±0.15 ^{aa}	2.29±0.08 ^{bb}	17.18±0.19 ^{aa}
	6	3.19±0.02 ^{ab}	14.09±0.11 ^{ba}	2.65±0.17 ^{ab}	11.99±0.36 ^{ba}

a & b: There is no significant difference (P>0.05) between any two means for each parameter, within the same column have the same superscript letter. A, B & C: There is no significant difference (P>0.05) between any two means, within the same row have the same superscript letter.

Table 5 Semi-Quantitative Scoring for Pathogenicity Test.

Organ	Control Group Scores	Field	1 Day Post-Infection Scores	Field	1 Week Post-Infection Scores	Field	Average Score (1 Day Post-Infection)	Average Score (1 Week Post-Infection)
Liver	- , + , - , + , +	++++	++++	++++	+++	+++	Very Severe	Severe
Kidney	- , + , - , - , -	++	++	++	+	+	Moderate to severe	Mild to moderate
Spleen	- , + , - , + , -	+++	+++	+++	+++	+++	Severe	Moderate

(-) no lesion, (+) mild lesion, (++) moderate lesion, (+++) severe lesion, (++++) very severe lesion.

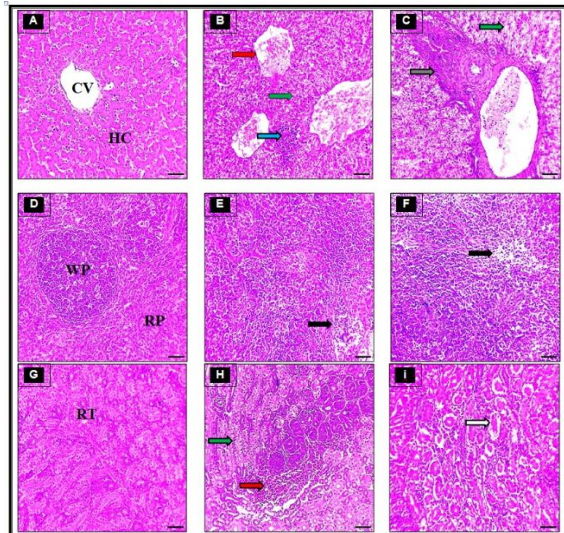


Figure 6 The histological effects of Duck Hepatitis Virus (DHV) infection on the liver, spleen, and kidney tissues of ducklings are shown in Figure7 , along with a comparison with the normal histological appearances of each tissue over a period of one week. Panel A shows the typical histological organization of the hepatocytes without any pathological abnormalities. Panel B illustrates the liver's immediate reaction to DHV infection. It shows notable mononuclear cell infiltration (blue arrow), hepatocytic vacuolar degeneration and necrosis (green arrow), and dilatation and congestion (red arrow). The typical lymphoid structure of the spleen is depicted in Panel D. In contrast, Panel E shows significant bleeding, immediate heterophilic granulocyte infiltration (black arrow), and lymphocyte depletion following infection, all of which point to a strong inflammatory response. The histology of renal tubular epithelial cells is shown in Panel G, as it is. Panel H clearly illustrates the post-infection renal alterations, which include significant heterophilic granulocyte infiltration, glandular tubule atrophy, bleeding, and severe tubular epithelial cell degeneration and necrosis (green and red arrows, respectively). Panels C (liver), F (spleen), and I (kidneys) show less histological changes and a fibrous tissue reaction in the portal region (gray arrow) after one week after infection, indicating a continuing recuperation or adaptation process. Hepatocytic and renal vacuolar degeneration, hemorrhage, necrosis, and inflammatory infiltration are reduced during this phase, and the splenic immune response is modulated, indicating a reduced response to DHV infection. Scale bar = 50 µm, Magnification 20x.

One-week post-infection, although renal tissue damage persisted, the severity was reduced, with diminished cellular degeneration, necrosis, and inflammatory response. In the spleen, significant pathological changes were noted one day post-infection, including lymphocyte depletion, hemorrhage, and infiltration by heterophilic granulocytes. However, one-week post-infection, while evidence of lymphocyte depletion remained, the severity of hemorrhage and granulocyte infiltration decreased, suggesting a reduction in the intensity of the immune response.

4. DISCUSSION

The highly contagious and deadly disease caused by DHAV generates significant losses for the duck business. According to former study (Wen *et al.*, 2017), DHAV is still losing duck farms. Since DHAV-3 was first identified in Egypt (Hassan *et al.*, 2020) our research indicates that the virus is still widespread. In this study, the death rate at the investigated farms was 75%, and neurological symptoms e.g., ataxia, opisthotonos, etc. were noted.

An autopsy that was recorded similar to those reported by (Woolcock, 2008). Due to fewer samples in this study, DHAV was not conclusively diagnosed based on postmortem examination and clinical signs, RT-PCR was used to target the 5'UTR gene. One sample had an amplified segment at 250 bp, which was regarded as a positive result as was detected by Fu *et al.* (2008). On the positive RNA sample, RT-PCR targeting the VP1 gene of DHAV genotypes 1 and 3 was performed once more to identify the subtype of DHAV. As a result of amplification occurred at a size of 880 bp, the virus was shown to belong to DHAV-3,

as previously described by Gao *et al.* (2012). The VP1 amplicon of the positive sample underwent nucleotide sequencing and alignment, and its identity percentage was compared with sequences that were recovered from the Gene Bank. Nucleotide similarity between our isolate (accession no. OR543968) and the Egyptian vaccine strain (KP148279.1) was found to be 66.8% while 96.8% to 100% similarity to DHAV-3 identified in Egypt (Hassan *et al.*, 2020). This virus spreads through the allantoic sac and was isolated on SPF-ECE. The inoculated suspicious sample exhibited greenish discolored allantoic fluid, stunting, subcutaneous hemorrhage, enlarged embryonic liver with bleeding, and greenish necrotic foci. Within five days of the inoculation, the embryo died. The same results reported by (OIE, 2010; Hassan *et al.*, 2020). These outcomes were shown after three passages in certain circumstances. The pathogenicity test's objective was to describe and contrast the clinical symptoms, macroscopic lesions, histopathological lesions and enzyme systems of two age groups of DHAV-3-infected ducklings one-day and seven-day ducklings. Comparing our findings to those of Zhang *et al.* (2018), the mortality occurred at 40 hpi in his study, whereas in ours it occurred at 24 hpi in the group of 1-day old group and 48 hpi in 7-day-old group. The ducklings died on their sides, paddling their legs (opisthotonos), with no difference in the neurological symptoms. The PM ex. showed that the infected ducklings had the usual visible lesions in the liver, which is typically enlarged with petechial hemorrhage with a swollen gall bladder filled with dark green bile. The kidney was extremely swollen, and the spleen, which is brown in hue, had yellowish necrosis. The histopathological alterations noted in the liver, spleen, and kidney were compared to those reported by Zhang *et al.* (2018), who similarly reported that DHAV-3 caused necrosis and heterophil infiltration of kidney tubules, beside loss of numerous lymphocytes and round vacuoles in the spleen parenchyma and infiltration of neutrophils in the parenchyma and liver cells. Liver and renal enzymes were noted here. Liver enzyme levels (AST and ALT) increased significantly in both the 1-day and 7-day groups, potentially due to the deleterious impact of DHV on hepatocytes and biliary canaliculi in the liver. This outcome resembled that of Ellakany *et al.* (2002) and Mahdy (2005). Uric acid and creatinine levels significantly increased in both infected groups. The detrimental effects of DHV on the kidneys may be the cause of the increase in uric acid (Ellakany *et al.*, 2002; Mahdy, 2005).

5. CONCLUSIONS

In this study DHAV strain is genotype (3), and the availability of a vaccination only had no effect to low the disease's incidence. To prevent the emergence of new strains locally, drastic measures must be implemented to restrict the disease's transmission and severity by using plant extracts with vaccination programs.

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