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Some Concurrent Bacterial infection with Duck virus Hepatitis in Ducks at EL-Behira Governorate

Gomaa, Y. Elhaddad*;Eid, G.S. Hussein; Nahed, A.E.S. Naem*** and Hanaa, A. EL-Zarkony*****

*Biotechnology, **Avian viral diseases, ***Bacteriology
Reference Laboratory for Veterinary Quality Control on Poultry Production, Damanhour Branch, Animal Health Research Institute, Agricultural Research Center, Egypt.

Research

Corresponding author:
Gomaa Y. Elhaddad
E.mail: gomaaelhaddad@gmail.com

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Abstract

Numerous illnesses have been found in duck farms in EL-Behira Governorate. Some of them were primarily manifested as nervous symptoms, which are associated with or without diarrhea in some flocks and with variable mortalities percent. In order to determine the prevalence of duck viral hepatitis in relation to bacterial infection, 20 duck flocks (12 farms and 8 backyards) were examined in 2023–2024. Mainly *E. coli,* Salmonella spps, Klebsiella spps and *staph aureus* was detected. Molecular detection of duck viral hepatitis DHV was made by RT-PCR followed by subtyping to DHV-1 and DHV-3 and sequencing of 2 viruses. Eleven flocks had concurrent E. coli infections with DHV with occurrence of 4 different serotypes as O55:K59, O125:K70, O26:K60 and O86 a: K61 and 1 isolate were untypeable. Concurrent infection of DVHA with Salmonella occurred in 3 flocks out of 20, which were serotyped as 1 isolate *S. typhimurium* and 2 isolates were S. Montevideo. Four flocks had concurrent K. pneumoniae and DVHA and four flocks also exhibited concurrent DVHA with *staph aureus*.

Conclusion: From the previous results, we recommend conducting more studies, as genetic analysis helps track the development of duck hepatitis virus and develop control measures by following security, biosecurity, and the necessary immunizations to prevent transmission of the disease. To determine whether mixed infection is present and how it is affected.

Keywords*: Duck, bacterial causes, viral causes.*

Introduction

Ducks are significant birds of economic importance for several countries worldwide, **Narhari (2009).** Two predominant duck species are worldwide produce meat, the Muscovy duck (Cairina moschata) and the Moulard duck (a hybrid between the Muscovy and Pekin duck) **Baeza (2006).** One of the most significant illness affecting ducklings globally is duck hepatitis virus (DHV), which resulted in enormous losses throughout the expansion of Egyptian duck farms and is a serious danger to commercial duck farms **Ellakany *et al*. (2002); Erfan *et al* (2015) and Hisham *et al*. (2020).** DHAV is known to induce encephalitis and pancreatitis in Muscovy ducks, but it also infects Mallard and Pekin ducklings **Guerin *et al*. (2007).**

DVH is a highly contagious duck illness that is marked by significant death and serious ill-

ness, particularly among ducklings that are younger than four weeks old **Woolcock (2008).** In the first week of life, there is 100% morbidity and 95–100% death **Mahdy (2005).** Three distinct serotypes (1–3) of the duck hepatitis A virus (DHAV) are responsible for this acute and extremely contagious disease in ducklings; serotype 1 is the most prevalent in poultry, **Rohaim *et al* . (2021).**

Bacterial infections likes, *E.coli* in duck cause a variety of problems. It considered as the one of most dangerous illness usually occurs between the ages of 2 and 6 weeks, when mortality rates can reach 43% **Punnoose *et al.* (2021).** Also Salmonella infection is one of the most serious illnesses affecting ducks, which has a substantial financial cost and a major impact on public health because diseased duck flocks are thought to be the primary source of Salmonella that may be transmitted to humans **Yang *et al*. (2019).**

One of the main zoonotic bacteria in the Enterobacteriaceae family is *Klebsiella pneumonia* **Wang *et al*. (2020).**

Staph aureus in the week-old ducklings result in various clinical manifestations (enlarged abdomen, septicemia, suppurative dermatitis, suppurative arthritis with decreased feed and water intake), which gradually led to a highly dehydrated carcass **Elfeil (2012)**, **Mondal and Sahoo (2014).**

This study aimed to identify the most important causes of death in ducks. Viral infection, primarily duck hepatitis virus (DHV), which can occur concurrently with bacterial infection and affect ducks as early as three weeks of age were examined through genetic analysis of detected pathogens.

Materials and Methods

Collection of samples:

A total of 20 ducklings flocks of different breeds (Muller and Muscovy), during the first 3 weeks of age with mortality rates up to 95% with different nervous symptoms (imbalance, lethargy, and ataxia, falling on their sides and kick spasmodically followed by opisthotonos, with or without diarrhea) as illustrated in table (1) were tested from different localities Behira governorate in 2023–2024, mainly (12 farms and 8 Backyard). The capacity range from (30-2000 duckling), no known history about previous vaccination against DHAV. Samples were examined for detection of DVH as well as bacterial infection.

Table (1). History of collected samples from infected ducks in 2023–2024

Sample No.	Mortality %	Number	Breed	Type ofrearing
1	90	50	Muscovy	Backyard
2	80	50	Muller	Backyard
3	65	500	Muller	Farm
4	70	600	Muller	Farm
5	85	400	Muller	Farm
6	90	100	Muller	Farm
7	75	30	Muscovy	Backyard
8	92	40	Muscovy	Backyard
9	60	300	Muscovy	Farm
10	84	60	Muscovy	Backyard
11	80	80	Muller	Farm
12	77	2000	Muller	Farm
13	95	450	Muller	Farm
14	45	1000	Muller	Farm
15	85	40	Muller	Backyard
16	90	60	Muller	Backyard
17	88	60	Muscovy	Backyard
18	40	1000	Muscovy	Farm
19	90	300	Muscovy	Farm
20	75	400	Muscovy	Farm

For investigation of DVH**:** enlarged congested liver with severe petechial hemorrhages on the surface was collected during the post mortem examination. Samples were homogenized in saline containing 2000 IU/ml penicillin and 200 lg/ml streptomycin. Fifteen minute centrifugation at 3000 rpm to obtain the supernatant which kept at -80 C until investigation.

Samples from internal organs (primarily the liver, heart, pancreas, and lung) were taken as a pooled sample for each flock for bacteriological examination. The samples were kept in ice box then immediately transported to the laboratory.

Detection of DVH by RT-PCR:

Nucleic acid extraction from samples was performed using the QIAamp minielute virus spin kit (Qiagen, Germany, GmbH). Briefly, 200 µl of the sample suspension was incubated with 25 µl of Qiagen protease and 200 µl of AL lysis buffer at 56^{O}C for 15 min. After incubation, 250 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer.

Oligonucleotide Primers. Supplied from (**Metabion Germany)** are listed in table (2).

Table (2). Primers sequences, target genes, amplicon sizes specific to 5'UTR and VP1 genes of DHAV.

Target gene	Primer sequence (5'-3')	amplified product (bp)	Reference
UTR	CCTCAGGAACTAGTCTGGA	250 bp	**Fu *et al.* (2008)**
	GGAGGTGGTGCTGAAA		
genotype 1 VP1	ACACCTGTTTGGGAGGCAAT	609 bp	**Mansour *et al.* (2019)**
	TCCAGATTGAGTTCAAATGCTAGTG		
genotype 3 VP1	ATGCGAGTTGGTAAGGATTTTCAG	880 bp	**Doan *et al.* 92017)**
	GATCCTGATTTACCAACAACCAT		

PCR amplification: Primers were utilized in a 25µl reaction containing 12.5 µl of Quantitect probe rt-PCR buffer **(QIAgen, Gmbh)**, 1 µl of each primer of 20 pmol concentration, 0.25 µl of rt-enzyme 5.25 µl of water, and 5 µl of template. The reaction was performed in a Biometra thermal cycler. Reverse transcription was applied at 50 ^{O}C for 30 min, a primary denaturation step was done at 95 ^{O}C for 5 min, followed by 35 cycles of 94^{O}C for 30 sec, (annealing: 50^{O}C for 5'UTR; 52^{O}C for DVH-1 and 55^{O}C for DVH-3) for 40 sec and 72^{O}C for 45 sec. min. A final extension step was done at 72^{O}C for 10 min.

Analysis of the PCR Products: The PCR products were separated by electrophoresis employing gradients of 5V/cm on a 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature. 15 µl of each product was put into a gel slot for gel analysis. To ascertain the fragment sizes, a gene ruler 100 bp ladder (Fermentas, thermofisher, Germany) was employed. A gel documentation system (Biometra) took pictures of the gel, and computer software was used to analyze the data.

Genes sequencing

Two selected positive DHAV viruses (one was DVH-1 and one strain was DVH-3) were taken for gene sequencing, using a QIAquick PCR Product extraction kit, PCR products were purified (Qiagen, Valencia). The sequence reaction was carried out using a Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer), and a Centrisep spin column was used to purify the product. The Applied Biosystems 3500 genetic analyzer (HITACHI, Japan) was used for DNA sequenceing. To confirm the identity of the sequences with GenBank accessions, a BLAST® analysis (Basic Local Alignment Search Tool), **El-Shemy *et al.* (2022)** was initially carried out.

The MegAlign module of Laser gene DNA Star version 12.1 generated the identity percent and maximum likelihood in MEGA6 were used for the phylogenetic studies **Tamura *et al.* (2013).**

Bacterial isolation and identification

E.coli was isolated on MacConkey and Eosin Methylene Blue (EMB) medium, biochemical tests (IMVIC) were performed **Rania and Ahlam (2023).** Rapid antisera sets (DENKA SEIKENCo., Japan) were used for serological determination of the isolates **Heba *et al.* (2012).**

K. pneumoniae was cultured on MacConkey agar and identified by biochemical tests according to **Arya *et al.* (2020).**

Salmonella was detected and serotyped according to **EL-Gaos *et al.* (2019).**

S. aureus was isolated on Baird parker agar and mannitol salt agar then identified by biochemical identification according to **Eid *et al.* (2019).**

Results

Molecular detection of DHAV:

DHAV was detected in all examined flocks (20/20) as all associated with mixed infection which occur with some bacterial isolates as illustrated in table (3).

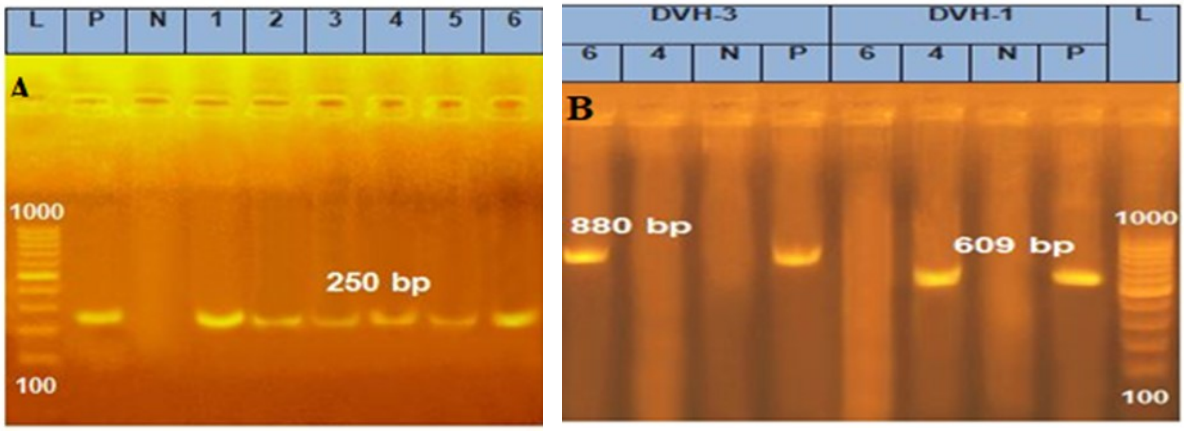

Fig. (1). Electrophoretic patterns of RT-PCR products:
A: For the DHAV UTR gene at 250 bp: Positive lanes of 6 viral strains on the gel electrophoresis (1.5%).
B: Detection of DHAV-1 in Lane (4) at 609 bp and lane (6): Positive DVH-3 at 880 bp.
Lane (P): Control positive, lane (N): Control negative.

Sequence and phylogenetic analysis:

One sample of DVH-1 and DVH-3 was subjected to partial gene sequencing, This sequence was uploaded to the NCBI platform with accession No. PQ261040 -2024 DHAV-1 Bah1 and PQ261041-2024 DHAV-3 Bah2 respectively .

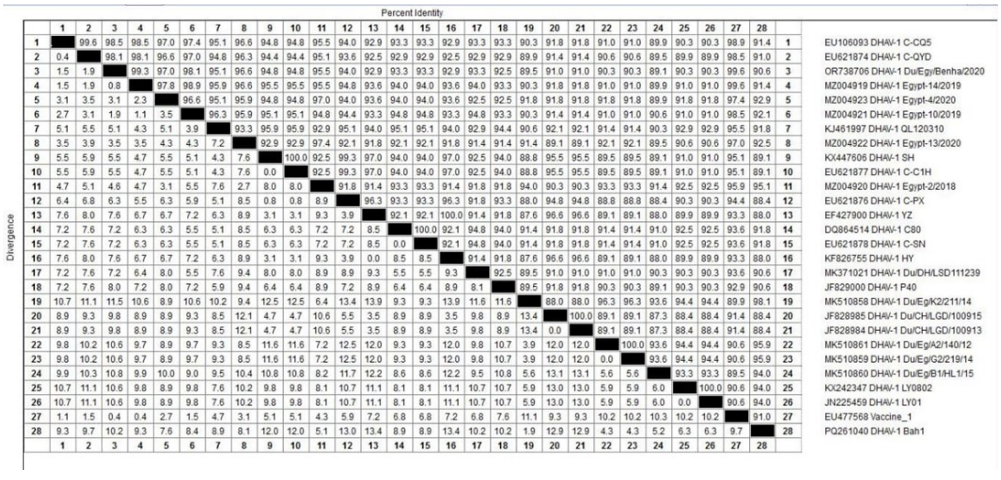

Figure (2). Identity percentage for nucleotide sequences of PQ261040 DHAV-1-2024 Bah1 and other related strains.

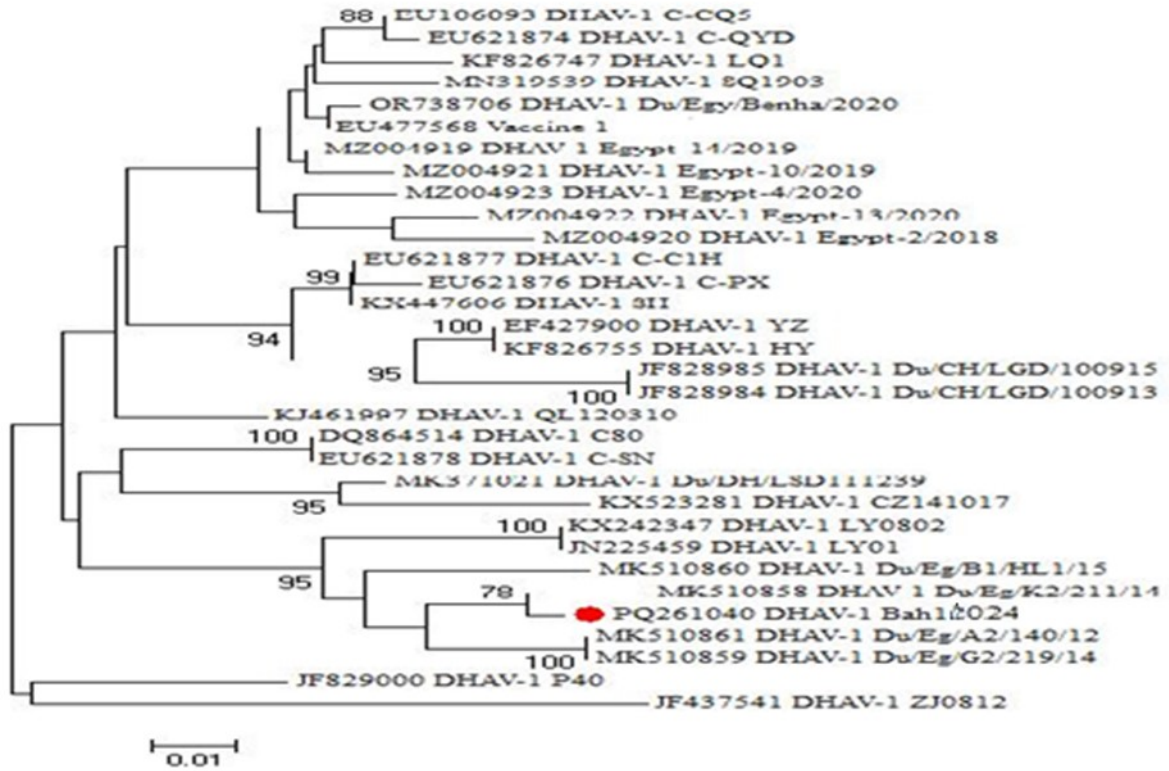

Figure (3). Phylogenetic tree for the nucleotide sequence of of PQ261040 -2024 DHAV-1 Bah1 along with other related strains.

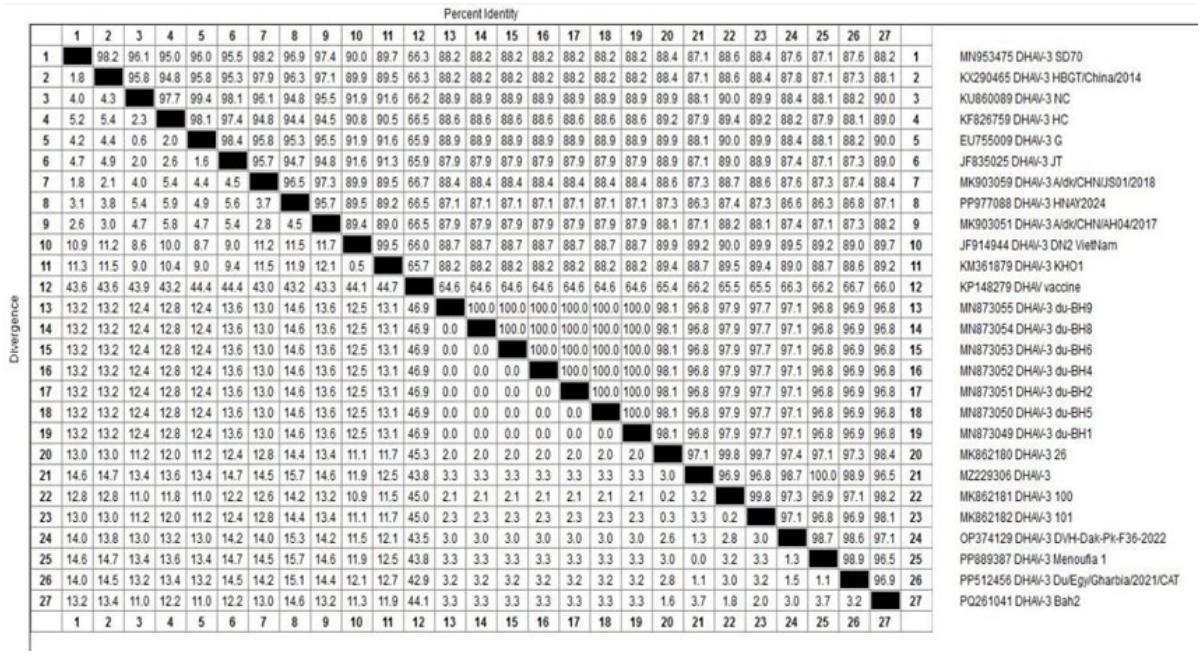

Figure (4). Identity percentage for nucleotide sequences of PQ261041-2024 DHAV-3 Bah 2 and other related strains.

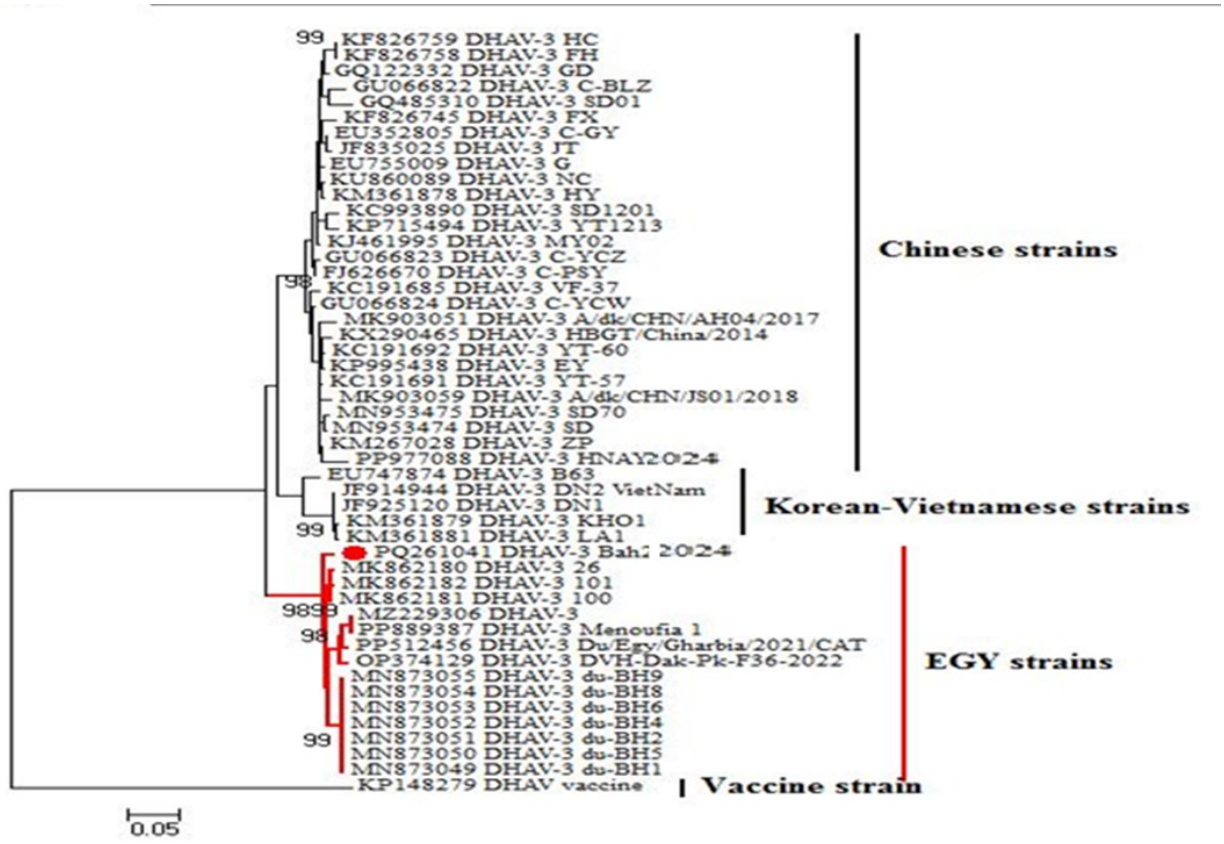

Figure (5). Phylogenetic tree for the nucleotide sequence of PQ261041 -2024 DHAV-3 Bah2 clustered with DHAV-3 Egyptian, Chinese and Korean-Vietnamese strains

DHAV and bacterial infections in examined duckling:

DHAV was combined with *E. coli* infection in 12 flocks, Serotyping of 5 random selected *E. coli* isolates revealed occurrence of 4 serotypes as O55:K59, O125:K70, O26:K60, O86 a:K61 and 1 isolate was untypeable. While *K. pneumoniae* and *S. aureus* were isolated and identified in 4 /20 for each one in combination with DHAV.

DHAV was combined with Salmonella infection only at 3 farms which were serotyped as one isolate was *S .typhimurium* and 2 isolates were *S. Montevideo* .

Table (3). Mortality rates of investigated flocks:

Age	No. of investigated flocks	PCR and Bacterial isolation	No. of flocks	Mortality rates
1 week	10	DHAV+*E. coli* DHAV+*E. coli*+ *K. pneumoniae* DHAV+ Salmonella	6 1 3	80 82 95
2 weeks	7	DHAV+*E. coli*+ *K. pneumoniae* DHAV+*E. coli* DHAV+ *S. aureus*	2 3 2	75 70 60
3 weeks	3	DHAV+ *S. aureus* DHAV+ *K. pneumoniae*	2 1	45 40

Discussion

DHAV is a highly contagious, quickly spreading, and fatal disease which induces significant losses for the duck farming sector **Doan *et al*. (2016).** DHAV was detected in all flocks under investigation in EL-Behira Governorate with variable mortality rates associated with some bacterial infection.

During our study we reported presence of 2 subtypes of DHAV mainly DHAV-1 and DHAV-3 during random analysis of 2 isolates, Despite the occurrence of maternal protection, epidemiologic and genetic investigations in Egypt show that DHAV-1 has produced significant outbreaks with high mortality rates and significant losses for duck farms in various governorates **Erfan *et al*. (2015), Zanaty *et al*. (2017) and Mansour *et al*. (2019).**

Phylogenetic analysis of The of PQ261040 - 2024 DHAV-1 Bah1 revealed presence of amino acid identities at high percent with GenBank accession no KX242347 DHAV-1 LY0802 and JF828985 (Chinese strains) while sharing percent of 95.1 % of Egyptian strains with GenBank accession no MZ004920 - 2018 and sharing percent of 98.1% with MK510858-2019 while identity percent was 91% to EU477568- vaccine 1strain (attenuated vaccine strain).

- The phylogenetic tree of the DHAV-3 VP1 clusters into three subgroups using the bootstrap method. The nucleotide sequence of PQ261041 DHAV-3 Bah2 was clustered with Egyptian strains with identity percent 96.8% to published strains of **Yehia *et al*. (2021)** which was investigated from 6 Egyptian governorates (Dakahlia, Monufia, Gharbia, Beheira, Alexandria and Qalyubia), named as Duck-hepatitis-A-virus- BH1, BH2, BH4, BH5, BH6 and BH9 with accession no MN873049, MN873050, MN873052, MN873053, MN873054 and MN873057, respectively while identity percent was 89.7% when compared to VietNam strain with GenBank accession no JF914944 DHAV-3 DN2.

The molecular data indicated that the DHAV-3 Bah2 isolated in our study was similar to other Egyptian strains from other governorates but clearly differentiated from the vaccine strain shared low nucleotide similarity (66%) with the DHAV-1 vaccine strain used in Egypt.

During our study we detected occurrence of DHAV-1 and DHAV-3 in investigated flocks to which agree with finding of **Yehia *et al*. (2021)** when reported occurrences of Duck Hepatitis A Virus Genotypes 1 and 3 in Egypt. Some earlier research, demonstrated DHAV-3 as more prevalent in China, Korea, and Vietnam than DHAV-1 **Soliman *et al*. (2015); Doan *et al*. (2016) and OIE (2021).**

The primary factor influencing the severity of infection in young ducklings with DHAV is their immature immune systems, which cannot defend them against viral infection and replication, which render them susceptible to other infections **Song *et al*. (2014),** which may explain presence of other infections with bacteria in our study as concurrent infection which also explain the marked increase in mortality rates reached up to 90% in some investigated flocks at the first weeks of age as in case of concurrent Salmonella infection Additionally, no more study was conducted to examine the concurrent infection.

Salmonella was detected in 3/20 our investigation, This is higher than the 4.6% isolated from diarrhoeal ducks published by **Tsai and Hsiang (2005),** but it approach to isolation rate of **Abd El Tawab *et al*. (2020),** which was approximately 66 isolates from 72 dead ducklings and 33 diseased ducklings' internal organs represented 14.1% and 15.3% of all cases examined. Additionally, this agree with the findings of **Rania and Ahlam (2023),** who found that 16% of the bacterial isolates linked to increased duckling mortality in Behira province caused by Salmonella.

Two serotypes of Salmonella were found in our study, *S. Typhimurium* and *S. Montevideo*. This is in contrast to findings from **Martelli *et al*. (2016) and Eid *et al*. (2019),** who reported the presence of *S. Typhimurium* in ducks but believe it to be the most prevalent serotype that all of the isolates of Salmonella in this experiment were serologically identified as being, while **Badr and Nasef (2016)** recognized *S. Typhimurium* as the primary causes of the 95% mortality rate among ducklings at Pekin duck farms. **Punnoose *et al*. (2021)** consid-

ered *S. Typhimurium* as main cause of morbidity and mortality in ducklings, especially when they are two weeks old.

In our investigation, *E. coli* was shown to be a common concurrent infection in 12/20 flocks, indicating its significant contribution to duckling death. This rate is higher than the 36% reported by **Rania and Ahlam (2023)** in a study on the causes of duckling mortality. As reported by **Islam *et al.* (2004)** with a mortality rate of 11%, **Bariha *et al.* (2019)** with a mortality rate of 55%, and **Roshdy *et al.* (2012)** with a mortality rate of 30.8%, numerous studies have connected duckling mortality to *E. coli* infection with varied mortality percent.

- *Klebsiella pneumonae was* detected in 4 flocks which considered lower than rate reported by **Rania and Ahlam (2023)** as the isolation rate was 36% of examined duck farms. **Khelfa and Morsy (2015)** reported occurrence of *E. coli* and *Klebsiella pneumonae* as main cause for variety of illnesses that have a 20–30% fatality rate.

According to the study's results, Staph aureus was detected in 4 of the 20 flock samples that were analyzed. This is higher than the 12.2% percentage reported by **Eid *et al.* (2019)** but lower than the 28% isolation rate noted by **Rania and Ahlam (2023).**

Conclusion

This study examines the coexisting DHAV and bacterial illnesses that affect ducklings in Behira, Egypt. Genetic analysis revealed presence of 2 subtypes of DHAV as DHAV-1 and DHAV-3 which was accompanied with bacterial infections as *E. coli* in 12 flocks with 4 different serotypes: O55:K59, O125:K70, O26:K60 and O86 a: K61. It associated with Salmonella in 3 flocks, also dual infections with *staph aureus* in 3 flocks which explain the increase in mortality rates in these flocks. Genetic analysis assists in tracking the evolution of DHAV and putting control measures in place to prevent disease transmission. To determine whether mixed infection exists and how it affects duck illness, more research is necessary.

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