

**Original Paper****Some studies on molecular epidemiology of Duck Hepatitis A virus in Egypt**Ahmed A. Lelwa<sup>1</sup>; Ahmed E. M. Saad<sup>1</sup>; Ibrahim M. Elborae<sup>1</sup>; Abdstar M. Arafa<sup>2</sup><sup>1</sup>Department of Avian and Rabbit Diseases, Faculty of Veterinary Medicine, Benha University, Egypt.<sup>2</sup>Laboratory For Veterinary Quality Control on Poultry production, Animal Health, Dokki, Giza, Egypt.**ARTICLE INFO****ABSTRACT****Keywords**

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Several disease outbreaks were observed in duck farms in Egypt leading to nervous manifestations and high mortality. In this investigation, suspected Duck Viral Hepatitis samples (n=38) were collected from 38 commercial flocks from five Egyptian governorates including (Sharkia, Qalyubia, Menofia, Ismailia and Dakahlia) from December 2019 to May 2022 of age ranged from 3-16 days. The infected flocks were suffered from nervous signs with high mortality ranged from (48%-91%). There is no history of previous vaccination against Duck Hepatitis A Virus (DHAV). Clinical samples were examined by Reverse transcription-PCR depend on molecular identification of UTR and VP1 gene respectively. The overall positive rate was 76.3% (n = 29/38). Out of 29 positive samples, five (17.24%) are subjected to sequencing and phylogenetic analysis. Our findings demonstrated that these novel DHAV strains were more similar and genetically correlated to the newly reported DHAV genotype 3 in Egypt. There were amino acid substitutions detected in VP1 gene in comparison to the first Egyptian genotype 3 (Avihepatovirus A isolate 26 VP).

**1. INTRODUCTION**

Duck viral hepatitis (DVH) is a highly fatal contagious disease of young ducklings that was originally discovered in 1945 in the United States (Wen et al 2018). The disease is widely distributed in Egypt and globally among duck raising countries with a significant economic loss due to high morbidity and mortality (up to 95% in 1-wk-old ducklings) associated with hepatitis and enlarged hemorrhagic livers (Tseng and Tsai, 2007). The disease is caused by three different types of viruses (DHV-1, DHV-2, and DHV-3). No antigenic relationships have been found between the three types of DHVs (Woolcock, 2003). Among the three types of DHVs, DHV-1 is the most prevalent and virulent one which belongs to the genus Avihepatovirus in the family Picornaviridae (Lefkowitz et al. 2018). Recently, DHV-1 has been renamed as duck hepatitis A virus (DHAV) and divided into three serotypes designated serotype 1, 2 and 3: Serotype 1 (DHAV-1) (the classical serotype) is the most widespread and more virulent serotype worldwide (Kamomae et al., 2017), whereas serotype 2 (DHAV-2) was reported in Taiwan and serotype 3 (DHAV-3) in South Korea and China (Kim et al. 2007; Li et al. 2013). DHAV-1 is a nonenveloped, positive-sense, single-stranded RNA virus, encoding a large polyprotein (Kok et al., 2009). Viral polyprotein (VP) coding region is flanked with untranslated region (UTR) at the 5' and 3' ends (Pan et al., 2012). UTR is highly conserved among DHAV-1 strains and usually used as a target for molecular detection of DHAV-1 by using reverse transcription-PCR (RT-PCR) (Fu et al., 2008). The VP1 is the major structural protein (highly variable region) and plays a vital role in receptor binding, neutralizing

antibodies, virulence, pathogenicity, immunogenicity, and protection against DHV (Liu et al. 2008). Phylogenetically, DHAV-1 strains were classified into several groups or clades on the basis of their VP1 sequences (Wang et al. 2008). The Egyptian strains were further categorized genetically based on VP1 gene sequence into three groups (A, B, and C), with two subgroups (B1 and B2) in Group B (Erfan et al., 2015). Recently, DHAV3 is the most prevalent in duck farms in Egypt due to the low cross-protection between DHAV-1 (the classical serotype) and DHAV-3 (the wild type/field strain), which results in vaccination failure (Hassan et al. 2020). The aim of the present work is to survey the incidence of DHAV in duck flocks from five Egyptian governorates including (Sharkia, Qalyubia, Menofia, Ismailia and Dakahlia) by RT-PCR. Thirty-eight collected samples suspected to be infected with DHAV are subjected to molecular characterization targeting UTR and VP1 gene.

**2. MATERIAL AND METHODS****2.1. Sample collection and preparation:**

Between December 2019 to May 2022, 38 liver samples were aseptically collected from 3:16-day-old Pekin and Mallard duckling from five Egyptian governorates including (Sharkia, Kalubia, Menofia, Ismailia and Dakahlia). There is no history of previous vaccination against Duck Hepatitis A Virus (DHAV). The liver samples were prepared totally aseptically, according to (OIE, 2017). To generate a 20% suspension (w/v) in sterile PBS (PH: 7.2) solution containing penicillin (1000 IU/ml) and streptomycin (1mg/ml), four pooled livers were manually ground in a sterile mortar and pestle, according to (El-Kholy et al. 2021).

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## 2.2. Detection of DHAV using RT-PCR:

**Extraction of viral RNA:** The viral RNA was extracted from liver supernatants by using Direct QIAamp Viral RNA Mini Kit (QIAGEN, Germany, Cat. no.52904).

**Reverse transcriptase Polymerase Chain Reaction (RT-PCR):**

As shown in table (1), RT-PCR was utilized in a single step to amplify the 5' UTR gene and the VP1 gene. The reaction mixture contained 6 µl of extracted RNA Template, 1 µl of forward primer (20 P MOL), 1 µl of reverse primer (20 P MOL), 12.5µl Patho Gene-spin TM RT-PCR Master mix (2x), and up to 25 µl of nuclease free water. The RT-PCR reactions were carried out in accordance with the parameters in a T3 thermal cyclor (Biometra, Germany). Gel electrophoresis (1.5%) was used to detect the PCR amplicons.

Table 1 Oligonucleotide sequence of forward and reverse primers sets specific to 5'UTR and VP1 genes of DHAV.

Gene	Primer sequence 5'-3'	Amplified Segment(b)	Ref
UTR	F. primer CCTCAGGAACTAGTCTGGA	250	Fu et al. 2008
	R. primer GGAGGTGGTCTGAAA		
Genotyp e1 VP1	F. primer ACACCTGTTTGGGAGGCAAT	609	Mansou r et al. 2019
	R. primer TCCAGATTGAGTTCAAATGCTAGTG		
Genotyp e3 VP1	F. primer ATGCGAGTTGGTAAGGATTTTCAG	880	Doan et al. 2016
	R. primer GATCCTGATTACCAACAACCAT		

## 2.3. VP1 sequencing and Phylogenetic analysis:

The VP1 gene reaction PCR products from Genotype 3 were extracted and purified directly using gel extraction kits (QIAGEN, Germany). The Big dye Terminator TM V3.1 cycle sequencing kit and previously reported forward and reverse primers for the DHAV3 VP1 gene were used to sequence the PCR results. The MEGA X software (Kumar et al., 2018) was used to perform the following tasks: alignment of sequenced nucleotides using the Clustal W method, calculation of identity percentage of nucleotides using the 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

### Clinical picture and postmortem lesions:

The examined flocks had neurological manifestations such as ataxia, head pulled back (opisthotonos), unbalance, and falling on one side with spasmodic kicking till death, whereas the gross pathological alterations of recently deceased ducklings revealed mostly hemorrhagic liver, as shown in Fig. (1).



Figure 1 Three days old Pekin young duckling showed enlarged liver with petechial hemorrhage.

### Molecular detection of DHAV by reverse transcriptase RT-PCR:

Out of the 38 samples tested by RT-PCR, 29 tested sample (76.3%) were positive for 5' UTR gene of DHAV with amplicon size 250 bp. All positive strains were further identified based on VP1 gene amplification reported that all isolates of Duck hepatitis A virus (DHAV) belong to Genotype 3 at 880bp (Table 2), Fig (2 and 3).

Table 2 Detection and genotyping of DHAV using RT-PCR.

Governorate	Number of samples		
	Collected	Positive for UTR gene	Positive for DHAV-1
Menofia	3	3	-
Sharkia	26	17	-
Qalyubia	3	3	-
Ismalia	3	3	-
Dakahlia	3	3	-
Total	38	29	-



Figure 2 The Electrophoretic pattern of the RT-PCR products based on UTR gene of DHAV-1 to three viral strains from Dakhlia governorate on the gel electrophoresis (1.5%). Amplicon size was detected at 250 bp which considered positive results for the DHAV-1. Positive and negative controls are included.

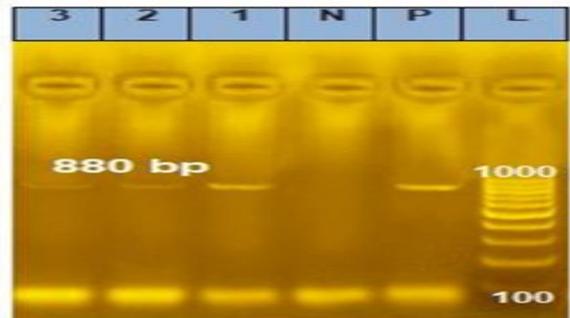


Figure 3 The Electrophoretic pattern of the RT-PCR products based on VP1 gene of both DHAV-1 (609 bp) and DHAV-3 (880 bp) to three viral strains from Dakhlia governorate on the gel electrophoresis (1.5%). Amplicon size was detected at 880 bp which considered positive results for the DHAV (Genotype 3). Positive and negative controls are included.

### VP1 gene sequencing and phylogenetic analysis:

The multiple sequence alignment for VP1 gene partial sequence of our new DHAV isolate has showed 96% nucleotide identity with recently identified DHAV-3 Egyptian strains.

Sequencing was performed to five viral strains (only strains recovered from flocks with high mortalities (86%:91%) of different breeds from five governorates and recovered through the four years of investigation). These five isolates named DVH-Manf-Md-F1-2019, DVH-Sh-Pk-F6-2020, DVH-Kal-Pk-F11-2021, DVH-Is-Pk-F27-2022, DVH-Dak-Pk-F36-2022 and submitted on Gen Bank with accession numbers OP374125, OP374126, OP374127, OP374128 and OP374129, respectively.

There were four amino acid substitutions detected in VP1 gene in comparison to the first Egyptian genotype 3 (Avihepatovirus A isolate 26 VP ) which were (L187P, L188P, D198N, X238H), (L187P, L188P, D198N, X238H), (L187T, D198N, K209R, X238H) and (L187T, D198N, K209R, X238H) in case of (DVH-Menf-Md-F1-2019, DVH-Sh-Pk-F6-2020, DVH-Kal-Pk-F11-2021, DVH-Is-Pk-F27-2022), respectively. There were six amino acid substitutions (L187P, L188P, D198N, F235X, E236X, L237X) in case of (DVH-Dak-Pk-F36-2022) strain (Fig. 4).

Phylogenetic tree was constructed using nucleotide sequence of the partial VP1 gene showing that five selected Egyptian DHAV strains (DVH-Manf-Md-F1-2019, DVH-Sh-Pk-F6-2020, DVH-Kal-Pk-F11-2021, DVH-Is-Pk-F27-2022, DVH-Dak-Pk-F36-2022) were present in the same group of Egyptian genotype3 strains as (Avihepatovirus A isolate 26, Avihepatovirus A isolate BH) and foreign strain as (Duck hepatitis A virus strain B-N) which characterized also as DHAV-3 like strains.

The similarity of the first four strains (DVH-Manf-Md-F1-2019, DVH-Sh-Pk-F6-2020, DVH-Kal-Pk-F11-2021, DVH-Is-Pk-F27-2022) among each other in this study was ranged from 95%:100%. On the other hand, the similarity of these strains was reduced in comparison to the fifth strain (DVH-Dak-Pk-F36-2022) and it was ranged from 88:93%. The multiple sequence alignment for VP1 gene partial sequence of our new DHAV-3 strains showed 91% - 94% nucleotide identity with first Egyptian DHAV-3 (Avihepatovirus A isolate 26), while nucleotide identity with Vietnamese strains, Korean strains, and Chinese strains as 77%:87%, 75%:84%, 79%:89% respectively. The lowest similarity was observed with DHAV-1 vaccine used in Egypt which ranged from 47%:52% (Fig. 5).

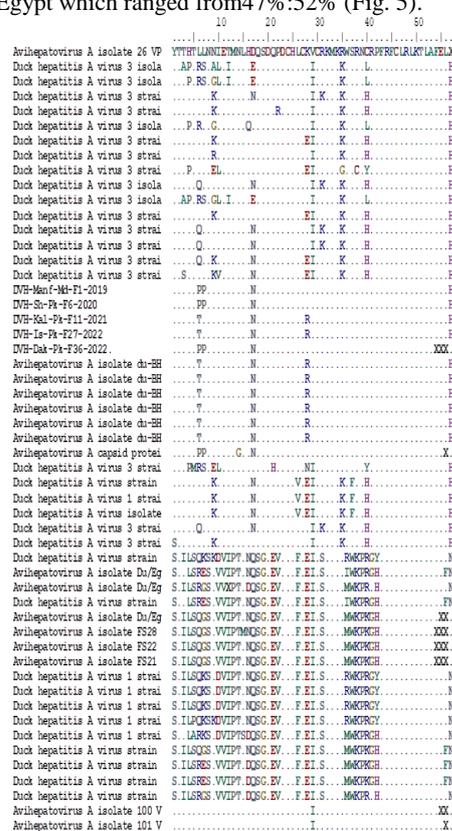


Figure 4 Amino acid Sequence alignment of the 5detected DHAV-3 strains in comparison with other published DHAV strains. The figure was generated by Meg Align module of Laser gene DNA Star software.



Figure 5 Phylogenetic tree pattern of the nucleotide sequence alignment of DHAV strains (DVH-Manf-Md-F1-2019, DVH-Sh-Pk-F6-2020, DVH-Kal-Pk-F11-2021, DVH-Is-Pk-F27-2022, DVH-Dak-Pk-F36-2022). The black dots refer to our field strains. The figure was generated by MEGA5 software.

4. DISCUSSION

DHAV-3 is highly contagious viral disease causes economic losses among duck raising farms in Egypt (Hassan et al., 2018 and El-Kholy et al., 2021) and worldwide (Song et al., 2019).

Most outbreaks of duckling farms in Egypt are due to DHAV Yahia et al. (2020), so that molecular characterization of VP1 gene is essential to identify major circulating DHAV strain. In our study we make surveillance of DHAV in five governorates in Egypt from December 2019 to May2022 including (Sharkia, Kalubia, Menofia, Ismalia and Dakahlia). The clinical signs of all investigated ducklings were nervous manifestation including imbalance, lethargy, and ataxia, falling on their sides and kick spasmodically followed by opisthotonos have been occurred in ducklings within 1-2 hours prior to death. The obtained results were similar to previously recorded clinical signs by Erfan et al. (2015), Zanaty et al. (2017), Hassaan et al. (2018), Mohamed et al. (2018) and El-Kholy et al. (2021). In our study mortality rate was ranged 2021) who-91%) and this was nearly Similar to El-Kholy et al. (2021) who recorded high mortalities ranged from (50-90%). The high variation between mortality (48-91%) may be due to the bad management which may exacerbated the disease. The postmortem examination of recently dead and sacrificed ducklings showed hemorrhagic liver. Similar results were reported by Hisham et al. (2020) and Yahia et al. (2020). For viral detection, RT-PCR which targeting 5'UTR gene was performed to 38 viral isolates which showed amplicon size at 250 bp in 29/38 isolates (76,3%) that considered a positive RT-PCR. Similar results detected by Fu et al. (2008) who

performed RT-PCR for 28 liver samples which amplify 250-bp genomic region in the 5'UTR and reported that all samples were positive for UTR gene. Recently, similar results reported by Hisham et al. (2020) who revealed that 10 out of 30 (33%) were positive for DHAV based on 5' UTR RT-PCR. VP1 is responsible for DHAV pathogenicity, virulence, evolution, and vaccination so that the genetic analysis of the VP1 is the major tool in the determination of genotype features. To record the circulating genotypes, another RT-PCR targeting VP1 of both DHAV genotype 1 and 3 was approved to 29 viral strains and our results reported that all strains showed amplification at 880bp targeting VP1 of genotype 3. Similar result agreed with those of other studies by Hassan et al. (2020) who first reported DHAV3 in Egypt and revealed that 5 out of 15 isolates (33%) were positive for DHAV3 targeting primers amplified VP1 of genotype 3 (880bp). Additionally, El-Kholy et al. (2021) who revealed that 9 out of 10 isolates (90%) were positive for DHAV targeting UTR gene. Out of these 9 isolates, two samples (3 and 10) were further genotyped based on VP1 gene amplification. Duck hepatitis A virus (DHAV) Genotype 3 were detected in both samples at 880 bp. In our study, the genetic analysis of five selected strains (DVH-Manf-Md-F1-2019, DVH-Sh-Pk-F6-2020, DVH-Kal-Pk-F11-2021, DVH-Is-Pk-F27-2022, DVH-Dak-Pk-F36-2022) were clustered in the same group of Egyptian genotype 3 strain with nucleotide similarity ranged from (88%:100%) and this agreed with El-Kholy et al. (2021) who reported nucleotide similarity reached 96%. The genetic analysis revealed that the first four viral strains were like each other (95%:100% identity). Additionally, they showed (88%:93%) identity to the fifth selected strain DVH-Dak-Pk-F36-2022. This lower in similarity may be due to host adaptation which resulted from genetic diversity of the same genotype (Kim et al., 2008). The lowest similarity was observed with the Egyptian DHAV-1 vaccine (47%:52%). Similar results were recorded by El-kholy et al. (2021) who reported 50% identity. These results confirm that our viral strains are antigenically different from vaccinal strain supporting the break of vaccination because the DHAV-1 and DHAV-3 are genetically variant. The vaccine cannot protect against heterologous virus infections, and this is probably the main cause of vaccination failure against DHAV in Egypt. The DHAV diversity resulted by point mutations and/or genomic recombination (Wei et al., 2012) so that continuous substitutions in amino acid were observed. This confirm our obtained results that there were four amino acids substitutions in first four viral strains which collected in period between December 2019 to January 2022 and six amino acid substitutions in fifth strain collected in May 2022.

## 5. CONCLUSION

In conclusion, DHAV still circulating in the Egyptian duckling farms causing high mortalities. Antigenic diversity between DHAV-1 and DHAV-3 lead to vaccination failure due to no heterologous protection. Therefore, further studies are required to select the appropriate vaccine strains.

## 6. REFERENCES

- Doan H. T., Le X. T., Do R. T., Hoang C. T., Nguyen K.T. and Le T. H. (2016): Molecular genotyping 271 of duck hepatitis A viruses (DHAV) in Vietnam. *J. Infect. Dev. Ctries.* 10: 988-995.
- El-Kholy A.W., Sharawi S.S., Khodier M.H. and El-Nahas E.M. (2021): 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. *Benha Veterinary Medical Journal* 41: 29-33.
- Erfan A. M., Selim A.A., Moursi M.K., Nasef S.A. and Abdelwhab E.M. (2015): Epidemiology and molecular characterization of duck hepatitis A virus from different duck breeds in Egypt. *Vet. Microbiol.* 177:347-352.
- Fu Y., Pan M., Wang X., Xu Y., Yang H. and Zhang D. (2008): Molecular detection and typing of duck hepatitis A virus directly from clinical specimens. *Vet. Microbiol.* 131, 247-257.
- Hassan T.I.R., Eid A.A.M., Ghanem I.A.I., Shahin A.M., Adael S.A.A. and Mohamed F.F. (2020): First Report of Duck Hepatitis A Virus 3 from Duckling Flocks of Egypt. *Avian Dis.* 64, 269-276.
- Hassan M.N., Shahin A.M. and Eid A.A. (2018): Isolation and Molecular Identification of Duck Hepatitis A Virus in Sharkia Governorate, Zagazig Veterinary Journal, Volume 46, 88-95.
- Hisham I., Ellakany H.F., Selim A.A., Abdalla M.A.M., Zain El-Abideen M.A., Kilany W.H., Ali A. and Elbestawy A.R. (2020): Comparative Pathogenicity of Duck Hepatitis A Virus-1 Isolates in Experimentally Infected Pekin and Muscovy Ducklings. *Front. Vet. Sci.* 7, 1-12.
- Kamomae M., Kameyama M., Ishii J., Nabe M., Ogura Y., Iseki H., and Yamamoto Y. (2017): An outbreak of duck hepatitis A virus type 1 infection in Japan. *J. Vet. Med. Sci.* 79:917-920.
- Kim M.C., Kwon Y.K., Joh S.J., Kwon J.H., Kim J.H. and Kim S.J. (2007): Development of one-step reverse transcriptase-polymerase chain reaction to detect duck hepatitis virus type 1. *Avian Dis.*, 51: 540-545.
- Kim M.C., Kwon, Y.K., Joh, S.J., Kwon, J.H. and Lindberg, A.M. (2008): Differential diagnosis between type-specific duck hepatitis virus type 1 (DHV-1) and recent Korean DHV-1-like isolates using a multiplex polymerase chain reaction. *Avian Pathol.*, 37: 171-177.
- Kok C. C. and McMinn P.C. (2009): Picornavirus RNA-dependent RNA polymerase. *Int. J. Biochem. Cell Biol.* 41:498-502.
- Kumar S., Stecher G., Li M., Knyaz C. and Tamura K. (2018): MEGA X. Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35, 1547-1549.
- Lefkowitz E.J., Dempsey D.M., Hendrickson R.C., Orton R.J., Siddell S.G and Smith B.D. (2018): The database of the international committee on taxonomy of viruses (ICTV). *Virus taxonomy. Nucleic Acids Res* 46: D708-D717.
- Li J., Bi Y., Chen C., Yang L., Ding C. and Liu W. (2013): Genetic characterization of duck hepatitis A viruses isolated in China. *Virus Res.* 178:211-216.
- Liu G., Wang F., Ni Z., Yun T., Yu B., Huang J. and Chen J. (2008): Genetic diversity of the VP1 gene of duck hepatitis virus type I (DHV-I) isolates from Southeast China are related to isolate attenuation. *Virus Res.* 137, 137-141.
- Mansour S.M., Mohamed F.F., Fakry F., ElBakrey R.M., Eid A.M., Mor S.K. and Goyal S.M. (2019): Outbreaks of Duck Hepatitis A Virus in Egyptian Duckling Flocks. *Avian Diseases* 74-63:68.
- Mohamed F.F., Mansour S.M., Fakry F., ElBakrey R.M., Eid A.M., Mor S.K. and Goyal S.M. (2018): Outbreaks of Duck Hepatitis A Virus in Egyptian Duckling Flocks. *Avian Diseases* 74-63:68.
- OIE, (2017): Duck Virus Hepatitis. OIE Terrestrial Manual 2017. (Chapter 2.3.8), available online at: [http://www.oie.int/fileadmin/Home/eng/Health\\_standards/tahm/2.03.08\\_DVH.pdf](http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.08_DVH.pdf).
- Pan M., Yang X., Zhou L., Ge X., Guo X., Liu J., Zhang D. and Yang H. (2012): Duck Hepatitis A virus possesses a distinct type IV internal ribosome entry site element of picornavirus. *J. Virol.* 86, 1129-1144.

20. Song S., Li P., Zhang R., Chen J., Lan J., Lin S., Guo G., Xie Z. and Jiang S. (2019): Oral vaccine of recombinant *Lactococcus lactis* expressing the VP1 protein of duck hepatitis A virus type 3 induces mucosal and systemic immune responses. *Vaccine*.37, 4364-4369.
21. Tamura K., Stecher G., Peterson D. and Kumar S. (2013): MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* 30:2725–2729.
22. Tseng C.H. and Tsai H.J. (2007): Molecular characterization of a new serotype of duck hepatitis virus. *Virus Res.*, 126: 19-31.
23. Wang L.; Pan M.; Fu Y. and Zhang D. (2008): Classification of duck hepatitis virus into three genotypes based on molecular evolutionary analysis. *Virus Genes*, 37: 52-59.
24. Wen X., Zhu D., Cheng A., Wang M., Chen S., Jia R., Liu M., Sun K., Zhao X., Yang Q., and Wu Y. (2018): Molecular epidemiology of duck hepatitis a virus types 1 and 3 in China, 2010–2015. *Transbound. Emerg. Dis.* 65:10–15.
25. Wei C. Y., Su S., Huang Z., Zhu W.J., Chen J.D., Zhao F.R., Wang Y.J., Xie J.X., Wang H. and G. Zhang G. (2012): Complete genome sequence of a novel duck hepatitis A virus discovered in southern China. *J. Virol.* 86:10247–10247.
26. Woolcock P.R. (2003): Duck hepatitis. In *Diseases of Poultry*: Saif, Y.M., Barnes, H.J., Glisson J.R., Fadly, A.M., McDougald, L.R., Swayne D.E. (Eds.), Eleventh ed. Iowa State University Press, Ames, IA, pp. 343-354.
27. Yehia N., Erfan A.M., Omar S.E. and Soliman M.A. (2020): Dual Circulation of Duck Hepatitis A Virus Genotypes 1 and 3 in Egypt. *Avian Dis.* 65, 1–9 .
28. Zanaty A., Hagag N., M. Samy M., Abdel-Halim A., Soliman M.A., Arafa A. and Nasif S. (2017): Molecular and pathological studies of duck hepatitis virus in Egypt. *J. Vet. Med. Res.* 24:374–384.