Amino acid sequences of local isolates of Duck Hepatitis Virus A type 1 (DHAV-1) in Egypt

Hanaa A. El-Samadony¹, Hoda M. Mekky²* and Khaled M. Mahgoub²

¹Animal Health Research Institute, Poultry Diseases and Research Department, Virological Unit, Dokki, Giza, Egypt. ²Department of Poultry Diseases, Veterinary Research Division, National Research Centre, P.O. 12622 Dokki, Giza, Egypt.

*Correspondence: hmekky335@gmail.com  Accepted: 08 Jun. 2018 Published online: 31 July, 2018

Thirty liver samples were collected from commercial duck farms at period started from June 2016 to December 2017 from six different governorates in Egypt. The affected ducks were suffering from nervous manifestations in addition to high mortality rate during the first 3 weeks of life. Specific pathogen free embryonated chicken eggs (SPF ECEs) were used for isolation of suspected virus from processed liver samples. RT-PCR application was done on RNA extracted from allantoic fluids for amplification of 3D gene and VP1 gene of DHAV-1. In case of 3D gene, all examined samples showed negative results. In case of VP1 gene, only one positive sample was appeared. This positive sample was derived from Mallard ducks of age 5-10 days old from Qalyubia governorate. Analysis of the phylogenetic tree according to the VP1 gene sequence showed that the examined strain Kal.1 is distinguishable from the other Egyptian strains, where the identity was 95% to 96% (Nucleotide) and 96 to 98% (Amino acids). The Kal.1 strain showed 100% resemblance to both the Egypt vaccine strain and China DZ strain. Also, The Kal.1 strain had 96% nucleotide homology and 94% amino acid homology to Viet Nam GL08 strain. In conclusion, Phylogenetic analyses indicated the similarity of isolated DHAV-1 strain to the Egypt vaccine strain. Therefore, further study for evaluating efficacy of DHAV-1 vaccine(s) versus recently recovered field isolate should be performed to ensure optimal control of DHAV-1. Also, immunization of breeder ducks before laying in addition to duckling vaccination at the time of hatching may provide early and effective protection against highly lethal DHAV-1 in Egypt.

Keywords: SPF ECEs, DHAV type1, Amino acid sequencing.

INTRODUCTION
Duck virus hepatitis is an acute, highly lethal infectious disease to ducklings. It is characterized by acute illness, short duration of disease, rapid transmission and high mortality (OIE, 2017). Traditionally, the disease was caused by Duck hepatitis virus types (DHV-1-3). Both DHV-2 and DHV-3 were identified later as astrovirus, known as duck astrovirus type 1 (DAsV-1) and duck astrovirus type 2 (DAsV-2) respectively (Todd et al., 2009). Lately, DHV-1 is rechristened duck hepatitis A virus (DHAV) and represents the most widespread and most virulent type (Fu et al., 2008; Ding and Zhang, 2007; Kim et al., 2006 and Tseng et al., 2007). The disease has appeared and become common for more than half a century. Firstly, it was emerged in 1945 at the United States Long Island (Levine and Fabricant, 1950). In 1954, it was isolated from the British ducks (Asplin and Mclauchlan, 1954), and isolated from Canada in 1957 (Macpherson and Avery, 1957). Subsequently, other countries of the world have reported the spread of the disease (Guerin et al., 2007; Sandhu et al., 1992 and Woolcock, 2008). The disease is ranked as a notifiable disease by The World Organization for Animal
Health requiring mandatory reporting. The disease mainly invades 3 weeks old ducklings, the mortality rate can be as high as 90% to 95% (Jin et al., 2008). Only horizontal transmission is the main source of infection, through the digestive tract and respiratory infections (Woolcock and Tsai, 2013). The disease incubation period about 1~2 d, the clinical manifestations are mainly systemic convulsions, motor disorders, feet spasm, opisthotonos, and other neurological symptoms. Gross lesions mainly in the liver, the performance of swelling, dark color or yellow, with bleeding spots on the surface (Woolcock and Tsai, 2013 and Liu et al., 2008). DHAV is related to the genus Avihepato virus of the Picornaviridae family as reported in the Virus Taxonomy the 10th Report of the International Committee on Taxonomy of Viruses (ICTV, 2017). DHAV consists genetically of three special serotypes, DHAV-1, DHAV-2 and DHAV-3 (Fu et al., 2008). Among the three serotypes, DHAV-1 is the most virulent serotype with significant impact on the worldwide poultry industry (Ding and Zhang, 2007 and Tseng et al., 2007), whereas DHAV-2 and DHAV-3 have been detected in Taiwan China, and Korea (Kim et al., 2007; Xu et al., 2012 and Li et al., 2013). Variant strain of DHAV-1(DHAV-1a) was reported in India (Rao and Gupta, 1967) and Egypt (Shalaby et al., 1978), respectively and serologically distinct of DHAV-1 (Sandhu et al., 1992). The disease was appeared in Egypt at 1970s but with little known disease situation. Application of breeder ducks vaccination using attenuated vaccines is done in the commercial level (Abd-Elhakim et al., 2009). Recently, studies on DHAV epidemiology and molecular characterizaiton in Egypt were done by Erfan et al., 2015; Bayoumie and Abd EL-Samie 2015; El-Samadony et al., 2016 and Zanaty et al., 2017. DHAV is a small, non-enveloped virus, diameter 20~40 nm. Like a typical picornavirus, DHAV genomic sequence analysis showed a single stranded positive sense RNA molecule approximately 7800 nucleotides in length. It consisting of 5’ noncoding region (5’ UTR), open reading frame (ORF), 3’ noncoding region (3’ UTR) and ploy (A) tail (Kim et al., 2006). Open reading frame ORF-encoded polyprotein. This polyprotein is cleaved into nine non-structural proteins, 2A1,2A2,2A3,2B,2C,3A,3B,3C and 3D, and three structural proteins VP0(1AB), VP3(1C), VP1(1D) in addition to a leader protein (L) (Kim et al., 2006). The structural proteins VP0, VP3 and VP1 are located on the surface of the viral capsid and participate in the formation of viral antigenic sites. VP1 protein is the major protein, encoding the main antigenic sites and specific neutralization sites, essential main components to determine the virus antigenicity (Gao et al., 2012 and Wei et al., 2012). The internal ribosome entry site (IRES) element of the DHAV 5’UTR can initiate the synthesis of the downstream protein (Pan et al., 2012). Poly (A) is a template for the synthesis of negative-stranded RNA, whose length is related to the efficiency of synthesis of negative-strand RNA and the infectivity of viral RNA. Several methods were confirmed for detection of DHAV infections, as indirect hemagglutination test, micro neutralization assay, and ELISA (Fan et al., 1998 and Gabridge and Newman, 1971). But these approaches are time consuming in addition to lack sensitivity and specificity. Now, direct reverse transcription polymerase chain reaction (RT-PCR) is widely used for genomic and phylogenetic analysis of DHAVs due to high sensitivity and specificity (Kim et al., 2007; Liu et al., 2007; Anchun et al., 2009 and Wen et al., 2014). Studying the molecular and genetic sequence of 3D gene and VP1 gene of DHAV-1 virus circulating in duck farms in Egypt is the aim of this paper.

MATERIALS AND METHODS

Thirty samples were collected from ducks with clinical disease, from 30 commercial farms of different duck breeds (Mallard, Muscavvy, Pekin and Hurcara) in Egypt from six governorates. The affected ducks were suffering from nervous manifestations in addition to high mortality rate during the first 3 weeks of life. Collected liver samples from commercial duck farms in Egypt from June 2016 to December 2017 were described in Table 1.

Preparation of Tissue Sample Suspension:

Liver samples were collected aseptically from freshly dead or ducks showed clinical sings, homogenized and suspended 20% W/V in sterile saline containing antibiotics (2000 iu/ml Penicillin and 200 mcg/ml Streptomycin). The suspension was clarified by centrifugation at 3000 rpm for 15 minutes. The clear supernatant were harvested and kept at -20°C (Woolcok, 1998).

SPF eggs inoculation:

The prepared liver tissue supernatant was inoculated into the allantoic cavity of 8-10-day-old embryonated specific-pathogen-free chicken eggs (SPF ECEs), five eggs were used for each samples. After incubation for 48-72 hours at 37°C,
allantoic fluid was harvested aseptically from inoculated eggs and stored at -20°C for further passage, embryos from dead or live eggs were observed for typical lesions of DHV. After 4 passages, collected allantoic fluids were used for RNA extracted (OIE, 2017).

**Detection of RNA of DHAV-1 by one-step RT-PCR:**

**RNA Extraction Methods:**

Viral RNA extraction from allantoic fluid of inoculated SPF ECEs was applied by using the Patho Gene-spin™ DNA/RNA Extraction Kit (INTRON Biotechnology, Seongnam, Korea) following to the manufacturer’s instructions. RNA concentrations were measured by using the Nano Drop ND-1000 (Nano Drop, Wilmington, DE). The samples were kept at -20°C for one step RT-PCR (OIE, 2017).

**Oligonucleotide Primers Design:**

The one-step RT-PCR is performed using the specific primers DHAV-1 Com F (5'-AAG-GAG-GAG-AAA-ATY-[C or T]- AAG-GAA-GG-3') DHAV-1 Com R (5'-TTG-ATG-TCA-TAG CCC-AAS- [C or G]-ACA-GC-3') Flanked by an expected 467 bp DNA sequence in the 3D gene (OIE, 2017).

The complete sequence of the VP1 gene of all tested samples was amplified with a pair of specific primers (sense primer: 5'-ATC AGG GTGATT CTA ACC AG-3'; reverse primer: 5'-CTT ATT TCT AAT TTG GTCA-G-3'), yielding an expected PCR product of 734 bp (Liu et al., 2008).

**One-Step RT-PCR:**

The one-step RT-PCR is conducted using the 25 μl reaction mixtures for partial amplification of DHAV-1 3D and VP1 gene. T-gradient thermal cycler (Biometra, Gottingen, Germany) was programmed according to the conditions described in Table 2.

**DNA Molecular weight marker:**

The amplified products were read according to 100 bp ladder (NEW ENGLAND BioLabs).

**Agarose gel electrophoreses:**

Electrophoresis for amplicon separation was applied on 1.5% agarose gel that prepared according to (Sambrook et al., 1989). The gel was visualized and photographed by a gel documentation system and the data was analyzed through computer software.

**Sequencing of the amplified part of VP1gene:**

GATC Company by use ABI 3730xl DNA sequencer by using forward and reverse primers combining the traditional Sanger technology with the new 454 used for performing gene sequencing using an Applied Bio systems3130 genetic analyzer (ABI, USA) for sequencing the DHAV-1 (734 bp) PCR product of the VP1 gene. The sequencing data were checked by NCBI Blast search and assembled edited chromatograph using Bio Edit software version 7.1.5 program. Edited sequences of DHAV-1 isolate were characterized using BLAST n for Nucleotide or BLAST p for protein analysis (http://www.ncbi.nlm.nih.gov/BLAST/) (OIE,2017). The amino acid sequences identity matrix was calculated to determine the homology between the isolates and other selected reference isolates. To assess the genetic relatedness among the DHAV-1 and the phylogenetic tree was generated by the distance-based neighbor-joining (NJ) method using MEGA version 6.

**RESULTS**

**Clinical signes and post morture finding:**

Affected ducklings less than 6 weeks of age showed nervous signs as ataxia, imbalance, lateral recumbency, kick spasmodically, opisthotonos followed by death. All deaths in affected flocks were done within 3–4 days. The main gross pathological sign was enlarged liver with haemorrhages. Enlarged spleen and kidneys with congested renal blood vessels were also evident.

**The inoculated SPF eggs:**

By inoculation of the suspected samples in SPF ECEs, inoculated embryos showed stunting with swollen yellowish or reddish liver. Also, greenish allantoic Fluid was more apparent in some cases.

**One-Step RT-PCR:**

**3D gene:**

One-step RT-PCR was applied on RNA extracted from allantoic fluid of inoculated SPF eggs for amplification of a DNA fragment of 467 bp of 3D gene. All examined samples showed negative results with 3D gene.
Table 1: Collected samples from commercial duck farms in Egypt from June 2016 to December 2017

<table>
<thead>
<tr>
<th>Governorate</th>
<th>No. of samples</th>
<th>Age</th>
<th>Breed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sharkia</td>
<td>5</td>
<td>3,7,10, 12, 14-days</td>
<td>Mallard and Muscovy</td>
</tr>
<tr>
<td>Gharbia</td>
<td>6</td>
<td>5,7,10,14,17, 20-days</td>
<td>Pekin and Muscovy</td>
</tr>
<tr>
<td>Menoufiya</td>
<td>4</td>
<td>4,13,17 and 21-days</td>
<td>Muscovy</td>
</tr>
<tr>
<td>Assiut</td>
<td>5</td>
<td>5,7,10,18,20-days</td>
<td>Pekin</td>
</tr>
<tr>
<td>Qalyubia</td>
<td>7</td>
<td>4,5,10,14,16,18, 20-days</td>
<td>Mallard</td>
</tr>
<tr>
<td>Beni Suef</td>
<td>3</td>
<td>5,10, 15-days</td>
<td>Hurcar and Mallard</td>
</tr>
<tr>
<td>Total No. of samples</td>
<td>30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: RT-PCR cycling conditions of Primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reverse transcription</th>
<th>Primary Denaturation</th>
<th>Secondary Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final Extension</th>
<th>No. of cycles</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D gene</td>
<td>45 °C 30 min.</td>
<td>94 ºC 5 min.</td>
<td>94 ºC 20 sec.</td>
<td>52 ºC 30 sec.</td>
<td>72 ºC 30 sec.</td>
<td>72 ºC 5 min.</td>
<td>40</td>
<td>OIE, 2017</td>
</tr>
<tr>
<td>VP1 gene</td>
<td>50 °C 30 min.</td>
<td>95 ºC 5 min.</td>
<td>94 ºC 30 sec.</td>
<td>48 ºC 60 sec.</td>
<td>72 ºC 60 sec.</td>
<td>72 ºC 10 min.</td>
<td>35</td>
<td>Liu et al., 2008</td>
</tr>
</tbody>
</table>

Table 3: Nucleotide and amino acid identities of MH084664 with selected references, Egyptian strains and vaccinal DHAV sequence.
Figure 1: Deduced amino acid sequences of the isolated DHAV in comparison to other DHAV strains circulating in Egypt and related countries.

Partial amino acid alignment of the amplified fragment of VP1 of the identified DHAV-1 isolated which related to some DHAV-1 strains circulating in Egypt and other countries using Bio-edit program. The dot (.) represents identity while single alphabet represents the difference in the nucleotide sequence.
Figure 2: Phylogenetic relationships among DHAV-1 strain based on the alignment of the amplified VP1 sequences. The tree was constructed by the neighbor-joining method in the MEGA 6. The VP1 sequence of DHAV-1 on Gen Bank with accession number MH084664.

**VP1gene:**

One-step RT-PCR was applied on RNA extracted from allantoic fluid of inoculated SPF eggs for amplification of a DNA fragment of 734 bp of VP1 gene. There is one positive sample with VP1 gene isolated from Mallard ducks of age 5-days old from Qalyubia governorate.

**Gen Bank accession numbers:**

GenBank accession number of the generated nucleotide sequence of DHAV isolate reported in this study is (MH084664) for the VP1 gene fragments.

**Sequence analysis of the VP1 gene**

The generated phylogenetic tree in addition to Nucleotide and amino acid identity table showed the homology with different percentages between the MH084664 isolates and other selected reference isolates circulating in Egypt and related countries (Table 3, Figures 1, 2).

**Discussion**

DHAV causes one of the most economically important diseases in the duck growing farms due to its high potential mortality results in great economic losses. Initial appearance of the disease in Egypt at 1970s was reported by Shalaby et al., 1978. DHAV diagnosis is based initially on clinical signs and liver lesion in ducklings followed by viral isolation and serological tests. Now, different rapid and specific RT-PCR techniques were applied for detection of the DHAV RNA (Kim et al., 2007; Liu et al., 2007; Anchun et al., 2009, Wen et al., 2014 and OIE, 2017). DHAV is the only member of a novel genus Avihepatovirus in the family Picornaviridae. DHAV occurs worldwide and threatens all duck-growing farms. DHAV-1, DHAV-2, and DHAV-3 are three serotypes of DHAV as a result of phylogenetic analysis and cross neutralization tests. But the most virulent serotype is DHAV-1 (Tseng et al., 2007, Fu et al., 2008 and ICTV, 2017). The DHAV genome contains a single, long ORF encoding a polyprotein, which is cleaved into a leader protein, three structural proteins (VP0,VP1 and VP3) and nine nonstructural proteins (2A1,2A2,2A3,2B,2C,3A,3B,3C and 3D). The 3D protein plays an important role in the viral RNA synthesis as it is RNA-dependent RNA-polymerase (Kok and McMinn, 2009). VP1 is the most external capsid protein containing the primary neutralization epitope. Genetic diversity at VP1 of DHAV can be used as a target for genotyping of DHAV (Wang et al., 2008).

Furthermore, VP1 of DHAV may play important roles in animal vaccination, evolution, and virulence (Jin et al., 2008 and Liu et al., 2008, 2010) and can be used to monitor of DHAV to follow local trends of DHAV infection (Li et al., 2008).
In this study, thirty liver samples were collected from duck farms represented different breeds at ages ranged from 3 to 21 days and located in 6 different governorates (Sharkia, Gharbia, Menoufiya, Assiut, Qaliubiya and Beni Suef), at period started from June 2016 to December 2017. Affected ducks suffered from high mortality and nervous manifestations like ataxia, lateral recumbency and opisthotonos. Gross lesions were found only in the liver; which was swollen with hemorrhages. Processing of liver samples was done for isolation of suspected virus in SPF ECEs. In the trail for isolation of DHAV, four blind passages were performed to 30 samples in SPF ECEs. Embryos were stunted with yellow or reddish embryonic liver (OIE, 2017). Application of one step RT-PCR on RNA extracted from allantoic fluids was done for 3D gene and VP1 gene of DHAV-1. All examined samples showed negative results in case of 3D gene. In case of VP1 gene, only one positive sample was appeared. This positive sample was derived from Mallard ducks of age 5-days-old from Qalyubia governare. DHAV-1 negative flocks require further investigation for other potential causes responsible for acute mortality in ducklings associated with nervous signs such as Pasteurella, Salmonella or E. Coli infection (Nasef et al., 2016). Aflatoxicosis also may cause nervous signs as convulsions, and opisthotonos in addition to bile duct hyperplasia as a microscopic liver lesion (Woolcock and Tsai, 2013). Immunization of breeder ducks in Egypt is usually via the intramuscular administration of live attenuated DHAV vaccines produced from E52 Rispens strain 2-3 weeks before laying. Breeder ducks immunization provide the new hatched duckling with high titers of passively transferred maternal antibody in order to protect them up to 15 days of age (Ellakany et al., 2002 and Abd-Elhakim et al., 2009). Sometimes, shedding of vaccines from vaccinated individuals could occur causing appearance of the infection in unvaccinated individuals with low immunity. The infection of vaccinated ducklings was reported (Jin et al., 2008 and Li et al., 2013). Also, outbreaks of DHAV disease were reappeared in some duck farms that stopped vaccination (Mahdy, 2005).

Unfortunately, no reports are available about the vaccination status of the breeder duck flocks. According to the obtained result, it is not known if stopped DHAV-1 vaccination or low maternal antibody titre is the cause for disease appearance. Analysis of the phylogenetic tree according to the VP1 gene sequence showed that the examined strain Kal.1 with gene bank accession number MH084664 is distinguishable from the Egyptian strains of Erfan et al., (2015), where the identity was 95% to 96% (Nucleotide) and 96 to 98% (Amino acids). The Kal.1 strain showed 100% resemblance to both the Egypt vaccinal strain with gene bank accession number (KP148279) Erfan et al., (2015) and China DZ strain with gene bank accession number (KF826754) Ma et al., (2015). Also, The Kal.1 strain had 96% nucleotide homology and 94% amino acid homology to Viet Nam GL08 strain with gene bank accession number (JF925122) Doan et al., (2011) unpublished data (Table 3, Figures 1, 2).

**CONCLUSION**

DHAV-1 was detected in one out of 30 commercial duck farms. Phylogenetic analyses indicated the similarity of isolated DHAV-1 strain to the Egypt vaccine strain. Therefore, further study for evaluating efficacy of DHAV vaccine(s) versus recently recovered field isolate should be performed to ensure optimal control of DHAV. Also, immunization of breeder ducks before laying in addition to duckling vaccination at the time of hatching may provide early and effective protection against highly lethal DHAV-1 in Egypt.

**CONFLICT OF INTEREST**
The present study was performed in absence of any conflict of interest.

**ACKNOWLEDGEMENT**
The authors are thankful to Prof. Dr. Eid El Said Abd Elaziz Soliman and Dr. Aalaa S.A. Saad, Animal Health Research Institute for helping our study. No fund was received for this study.

**AUTHOR CONTRIBUTIONS**
All authors contributed equally in all parts of this study.

**Copyrights:** © 2017 @ author(s).

This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author(s) and source are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.
REFERENCES


Mahdy SA, 2005. Clinicopathological studies on the effect of duck viral hepatitis in ducks. M.V.Sc Thesis (Clinical Pathology), Faculty of Veterinary Medicine, Zagazig University.


