Assiut Vet. Med. J. Vol. 61 No. 147 October 2015

MOLECULAR CHARACTERIZATION OF A DUCK VIRUS HEPATITIS ISOLATE ISOLATED FROM SHARKIA GOVERNORATE.

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ABSTRACT

Received at: 27/8/2015

Accepted: 17/9/2015

In the present study a Blencher duckling flock with 70% recurrent mortality on each reared batch was examined for the cause of mortality. live duckling were showing nervous signs and opisthotonos, sacrificed samples revealed depressed areas on the liver marking the large blood vessels while Hemorrhagic lesion suggestive for DVH were only noticed on liver of dead duckling carcasses. Liver samples were collected for virus isolation trials and for immunofluorescence. Initial virus isolation in embryonated chicken eggs ECE after ultra-filtration through 400 nm membrane filter revealed small size hemorrhagic edematous embryos this lesion was consistent upon a series of ultra-membrane filtration through 200 and 100 nm membrane filters which indicate a Picorna virus. Immunofluorescence examination revealed positive results for duck virus hepatitis (DVH). Clinical samples examined by generic RT-PCR assays followed by partial sequence analysis of the 3D gene revealed that the isolate was characterized as Duck hepatitis A virus resembling the strain (DHV/Duck / Egypt / Al-Gharbia /2014) in the 3D protein gene whom its gene bank accession number was (KP202874). The current study stresses on the value of ultra-membrane filtration through a 100 nm membrane filters as a rapid diagnostic tool For DVH without the need for extra laboratory diagnostic work. Interpretation of sequence results revealed that. The isolated (DVH/EG. Bayoumieh-Sharkia-2015) is a Duck hepatitis type A virus. With 100% resemblance to the strain (DHV/Duck/ Al-Gharbia /2014) Egypt/ in 3D protein gene with gene bank accession number (KP202874) unpublished data. Sequence and phylogenetic analysis indicated that the (DVH/EG. Bayoumieh-Sharkia-2015) isolate is clustered in the DHAV serotype 1 but was distinguishable from the other isolated Egyptian strains with resemblance ranging between 63.1 - 63.8%.

Key words: *DVH-A, Fluorescence, Ultra membrane filtration, (DVH/EG. Bayoumieh-Sharkia-2015), Picornavirus, Ducklings, Egypt,RT-PCR, sequencing*

INTRODUCTION

Duck virus hepatitis (DVH) is a highly fatal rapidly spreading viral infection of young ducklings, characterized primarily by hepatitis. The disease constitutes a great economic importance to all duck growing farms because of the high potential mortality if not controlled. The disease is caused by at least three different viruses Woolcock and Tsai (2013).

The most pathogenic DVH is a member of the newly proposed Picornavirus genus (*Avihepatovirus*) duck hepatitis A virus 1 (DHAV-1), formerly designated as duck hepatitis virus type 1 or DHV-1 (Levine and Fabricant (1950) followed by many authers {Asplin (1965), Calnek (1993), Kim *et al.* (2006), Ding and Zhang (2007)}.

DHAV-2 isolated in Taiwan by Tseng and Tsai (2007), and DHAV-3 isolated in South Korea by Kim *et al.* (2007), and in China by Fu *et al.* (2009) are two newly described DHV genotypes, belongs to the same proposed *Avihepatovirus* genus; they can also cause high mortality in ducklings.

DHAV-2 and DHAV-3 were first recognized as separate entities because they induced hepatitis in DHAV-1–immune ducklings Asplin (1965), Toth (1969), Haider and Calnek (1979), Calnek (1993), Gough *et al.* (1984) ,Gough *et al.* (1985), Gough and Stuart (1993), Monroe *et al.* (2005), Todd *et al.* (2009), Fu *et al.* (2009). These two viruses are now reclassified and named as Duck Astrovirus types 1 and 2, respectively Fu *et al.* (2009).

MATERIALS

Samples
Twenty thousand 7-day-old Blanchard ducklings reared in Belbees Sharkia., expressing 70% mortality was the subject for our investigation. Affected ducklings were showing nervous signs and opisthotonos (fig - 1).

Embryonated chicken eggs (ECE):
Nine day old ECE were used for DVH isolation trials according to levine and Fabricant (1950), OIE (2010).

Membrane filters
450 nm Thermo scientific Nalgene syringe filter. Cat-no. 190-2545 (8-0404-40493), 200 nm Thermo scientific syringe filter. Nalgene cat. no. 190-2520 (8-0404-391109). 100 nm Sigm Aldrich syringe filter cat. no. (f-7523).

RT-PCR RNA extraction
QIAamp Viral RNA Mini kit (Qiagen, Germany, GmbH®).

Duck virus hepatitis vaccinal strain
Vaccinal strain of {VAC Sera Abbasia kindly obtained from Prof. Dr. Susan Tolba (Head of poult. Vaccine unit)}.

Duck virus hepatitis Antiserum.
Anti-duck virus hepatitis produced in rabbits, against the vaccinal strain of VAC Sera Abbasia.

Preparation of duck virus hepatitis Antiserum.
Anti-duck virus hepatitis serum produced in rabbits using vaccinal strain of DVH was used in immunoflourescent antibody technique (IFAT) according to Hanaa *et al.* (2013).

METHODS

Sample preparation for ECE inoculation
Livers of necropsied ducklings showing hemorrhages were homogenized and subjected for three successive freeze–thaw cycles, then 1/10 suspension was made of it. The suspension was clarified by centrifugation for 10 min at 3000 rpm OIE (2010). Initial sample was filtered through 450 nm filter for bacterial sterility. Part of allantoic fluid (AF) from the first passage was filtered through 200 nm filter; another part was filtered through 100 nm filter, these were further used for ECE inoculation.

RNA extraction
RNA extraction from the supernatant of liver homo-genates were performed using the QIAamp Viral RNA Mini kit (Qiagen, Germany, GmbH®) according to their manufacturer's recommendations. The RT-PCR assays using oligo-nucleotide primers specific to the sequence coding for the 3D protein. Primers designated DHV-1 ComF (5'-AAG AAG GAG AAA ATY (C or T) AAG GAA GG-3') and DHV-1 ComR (5'- TTG ATG TCA TAG CCC AAs (c or G) ACA GC-3') flank a 467 base pair DNA in the 3D gene as indicated by OIE (2010) was used.

Partial amplification of 3D genes were conducted as performed by Erfan *et al. (2015).* The reactions were performed in a T3 thermo cycler (Biometra®). The amplicons were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) along with 100- bp DNA Ladder (Qiagen, Germany, GmbH®). The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data were analysed by a computer software (Automatic Image Capture Software, Biosciences, USA®).

Sequence and phylogenetic analyses
Partial sequence of the 3D gene of the field isolate was generated using forward and reverse primers of the generic PCRs, Amino acid sequences were deduced from the generated nucleotides using BioEdit software version 7.1.7 Hall (1999). Sequences of query and representative DVH was retrieved from the GenBank database. All sequences were aligned and identity matrices were calculated automatically using BioEdit. Phylogenetic analyses using maximum likelihood, neighbour joining and maximum parsimony in MEGA6 Tamura *et al.* (2013). Consensus unrooted trees were generated with 100 bootstrap replicates and were further edited using Inkscape 0.91 (Free Software Foundation, Inc., USA).

RESULTS

Results of the present study is shown in table (1-2) and figures (1-9).

Table 1: shows the partial sequence analysis of 3 D gene of the (DVH/EG.Bayoumieh-Sharkia-2015).

TGAGATAGTATGCAGCTGATCCAATTGAGTTTAGGACAGTAGTGCATGGTGACCCTGAGCACATAC
CGCCTTCCACCTTCCAAATCTCATCAGTGACATAGTGTGTTGAATAGATGGTTGGCTCATGAATTTT
CTTCACAAGGGCTGGATCGTTATGGAAAAATGACAACACATCAACAGCTTCTTCCAAAATTTGAGC
ACTCAGAGACCCGTCAAAACCAGAAAAGTCCAAACACAAGTTGTAGGGCTGCAGATTTGCTAACA
GATTGTCCCACTCGGCAAAAGGGTTGATTCCAACAGCACAGCCTGATAGAATAAAGCTGTCATCAT
AAATGTTGGAATAAATTTCACCCATAACCATGCGGAACGCAACTGTGTAGTCAAAGTTGCATGCCT
CAATACCTCGAGTCTTTCCTTCCTT

Table 2: Nucleotide (upper right) and amino acid (lower left) of (DVH/EG.Bayoumieh-Sharkia-2015) 3D gene compared to the Egyptian isolates of Erfan *et al.* (2015).

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		
1		96.8	96.8	96.8	96.8	96.8	97.0	96.8	96.8	96.8	96.3	96.8	96.8	96.8	96.3	96.8	63.1	1	KP148263
2	4.5		100.0	100.0	100.0	100.0	99.8	100.0	100.0	100.0	98.8	100.0	100.0	100.0	98.8	100.0	63.8	2	KP148264
3	4.5	0.0		100.0	100.0	100.0	99.8	100.0	100.0	100.0	98.8	100.0	100.0	100.0	98.8	100.0	63.8	3	KP148265
4	4.5	0.0	0.0		100.0	100.0	99.8	100.0	100.0	100.0	98.8	100.0	100.0	100.0	98.8	100.0	63.8	4	KP148266
5	4.5	0.0	0.0	0.0		100.0	99.8	100.0	100.0	100.0	98.8	100.0	100.0	100.0	98.8	100.0	63.8	5	KP148267
6	4.5	0.0	0.0	0.0	0.0		99.8	100.0	100.0	100.0	98.8	100.0	100.0	100.0	98.8	100.0	63.8	6	KP148268
7	4.2	0.2	0.2	0.2	0.2	0.2		99.8	99.8	99.8	99.0	99.8	99.8	99.8	99.0	99.8	63.6	7	KP148269
8	4.5	0.0	0.0	0.0	0.0	0.0	0.2		100.0	100.0	98.8	100.0	100.0	100.0	98.8	100.0	63.8	8	KP148270
9	4.5	0.0	0.0	0.0	0.0	0.0	0.2	0.0		100.0	98.8	100.0	100.0	100.0	98.8	100.0	63.8	9	KP148271
10	4.5	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0		98.8	100.0	100.0	100.0	98.8	100.0	63.8	10	KP148272
11	5.2	1.6	1.6	1.6	1.6	1.6	1.4	1.6	1.6	1.6		98.8	98.8	98.8	100.0	98.8	63.8	11	KP148273
12	4.5	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	1.6		100.0	100.0	98.8	100.0	63.8	12	KP148274
13	4.5	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	1.6	0.0		100.0	98.8	100.0	63.8	13	KP148275
14	4.5	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	1.6	0.0	0.0		98.8	100.0	63.8	14	KP148276
15	5.2	1.6	1.6	1.6	1.6	1.6	1.4	1.6	1.6	1.6	0.0	1.6	1.6	1.6		98.8	63.8	15	KP148277
16	4.5	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	1.6	0.0	0.0	0.0	1.6		63.8	16	KP148278
17	26.9	25.4	25.4	25.4	25.4	25.4	25.8	25.4	25.4	25.4	25.4	25.4	25.4	25.4	25.4	25.4		17	DVH-EG-Bayoumieh-sharkia
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		

Fig. 1: A young duckling that is Showing opisthotonos

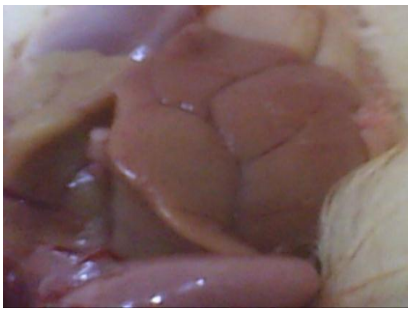


Fig. 2: shows liver of sacrificed ducklings had depressed areas marking large blood vessel

Fig. 3: Young duckling showing typical liver hemorrhagic lesions of duck virus hepatitis

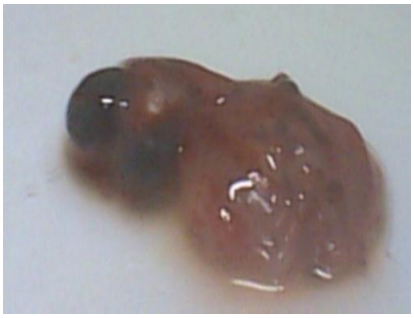


Fig.4: 13 day old chicken embryo was inoculated at 9 days with 100 nm filtrate of the A.S. fluid. Note the small size, hemorrhage, and edema.

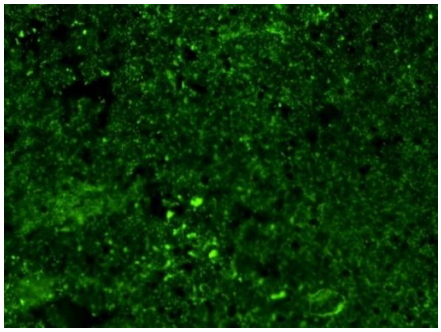


Fig.5: Liver smears from 7 ds old duckling stained with antirabbit IgG conjugated with FITC for DVH. Mark the fluorescence in the infected hepatocytes X 400.

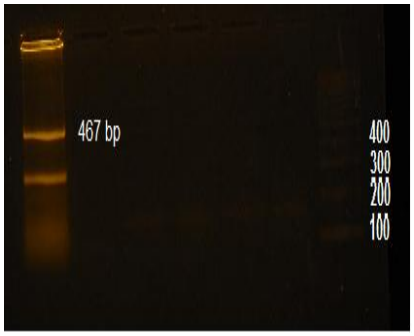


Fig. 6: Shows PCR. Lane 467 bp using a ladder of 100 bp for (DVH/EG.Bayoumieh-Sharkia-2015).

Fig. 7: showing phylogenetic tree of (DVH/EG.Bayoumieh-Sharkia-2015) compared to the Egyptian isolates of Erfan *et al.* (2015).

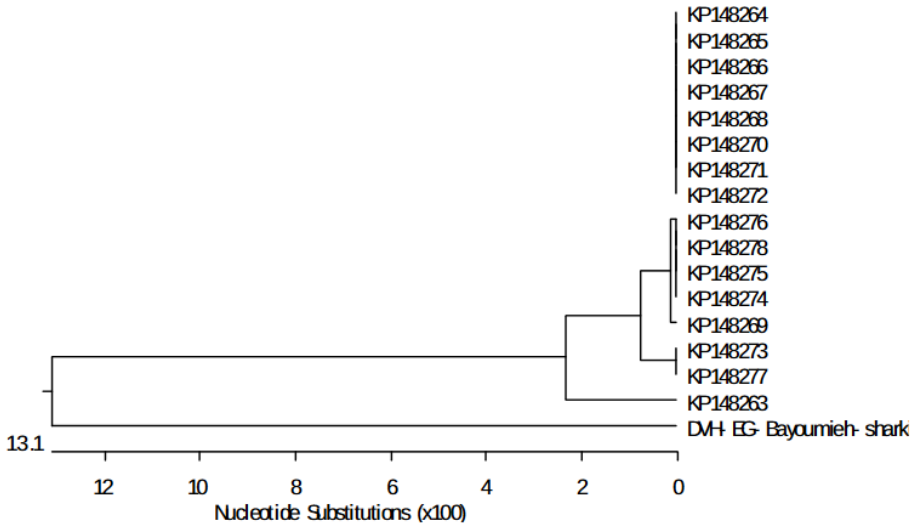
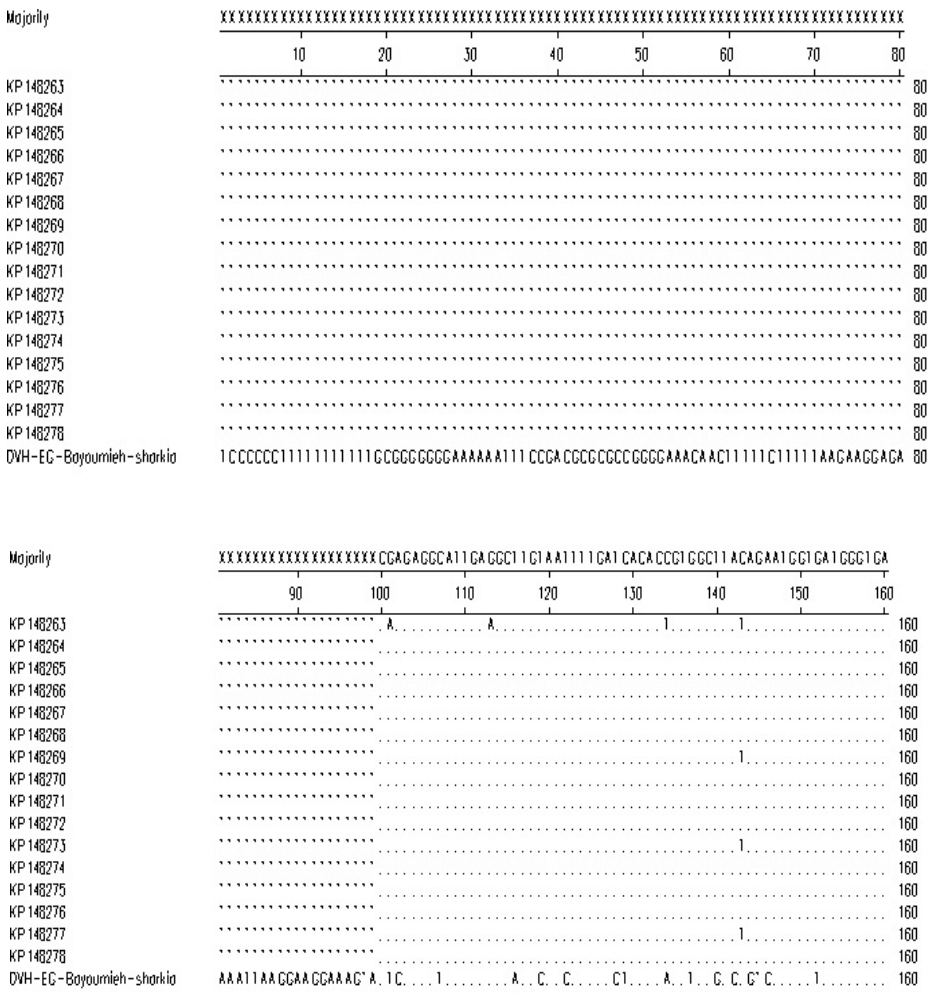
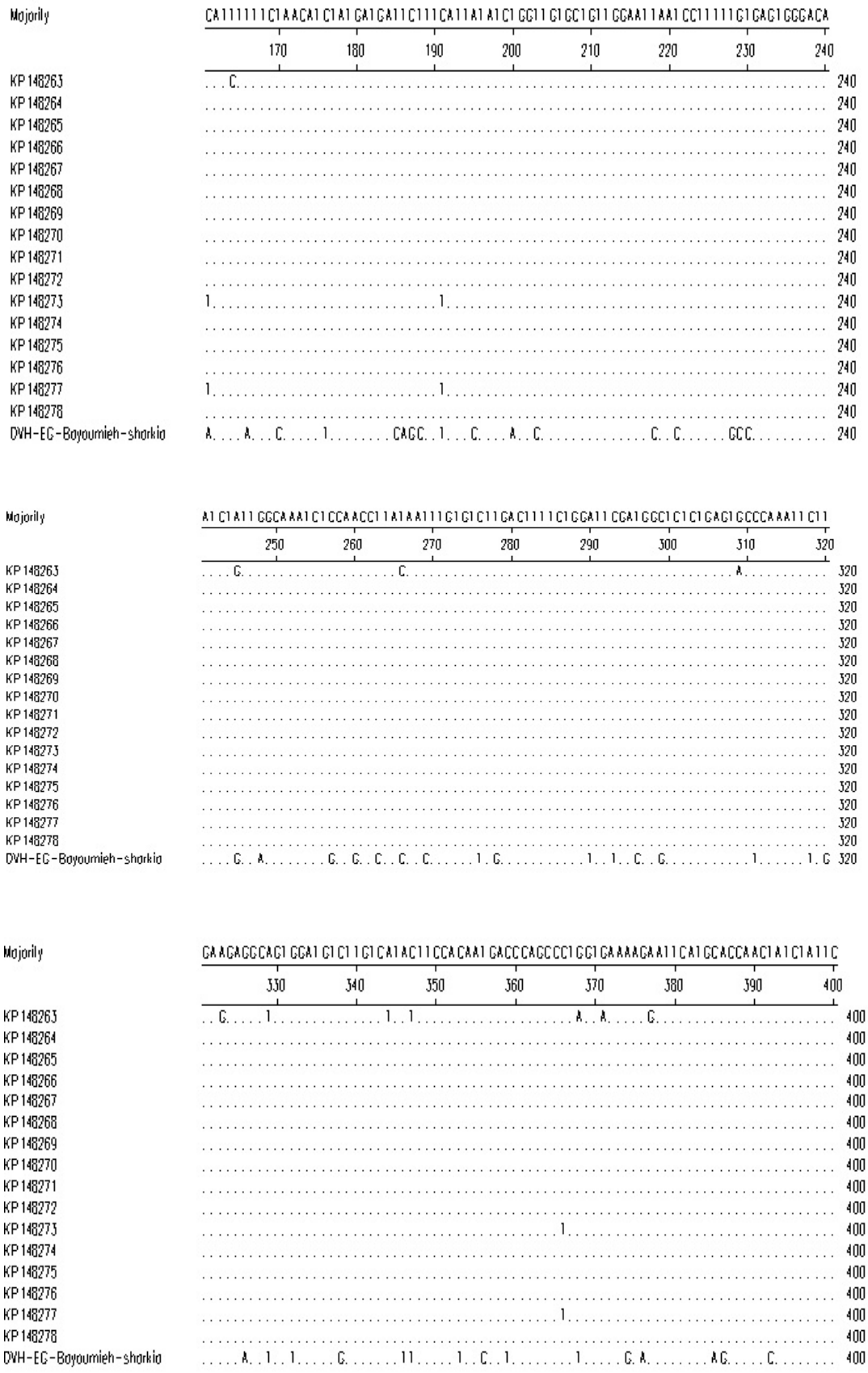
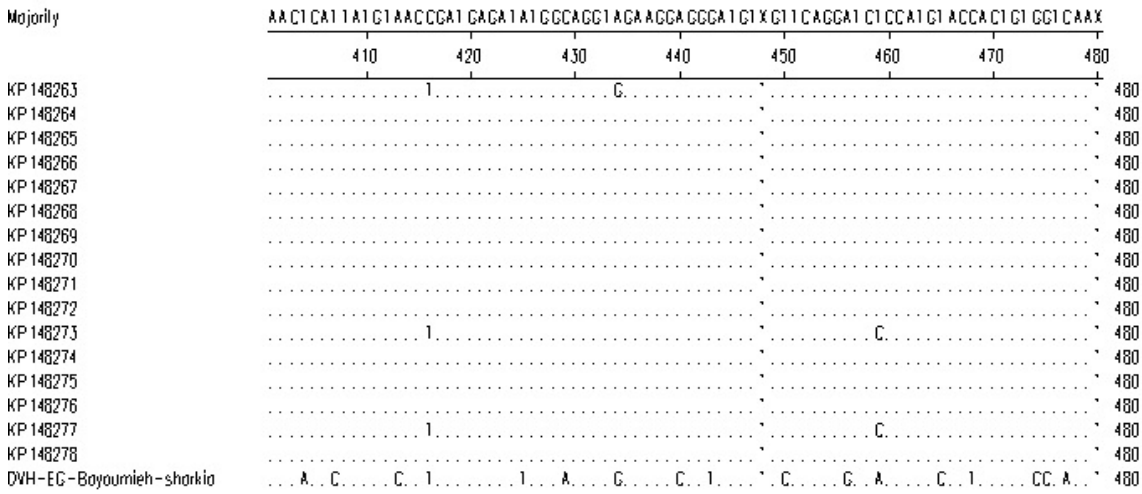
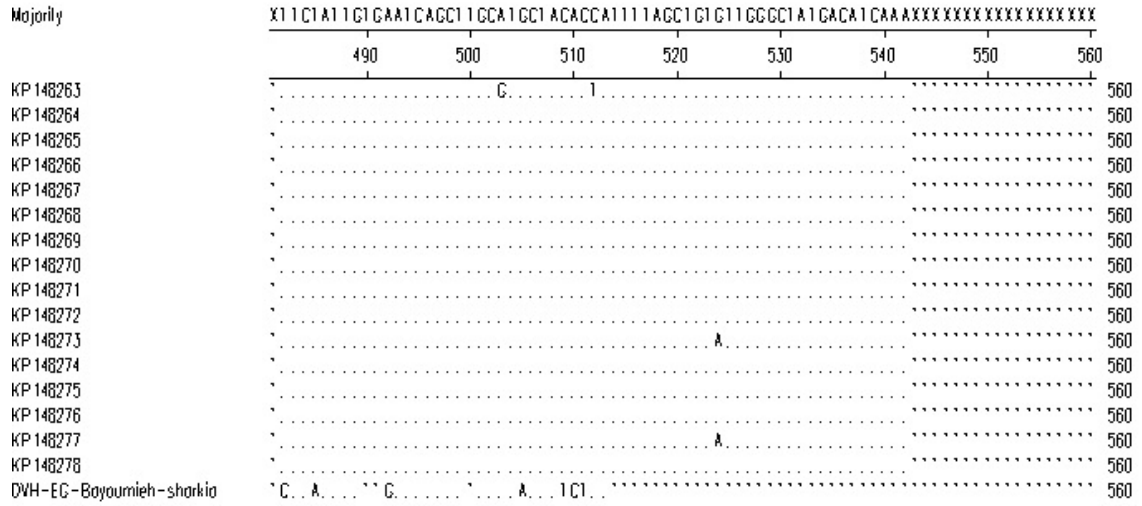


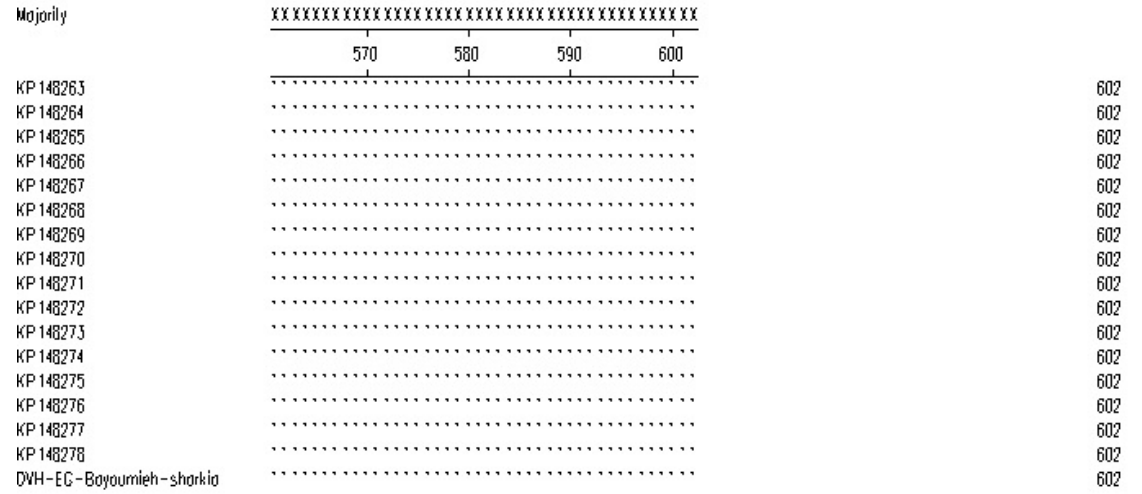
Fig. 8: Nucleotide Alignment (DVH/EG. Bayoumieh-Sharkia-2015) compared to the Egyptian isolates of Erfan *et al.* (2015).




Majority
170 180 190 200 210 220 230 240
KP148263
KP148264
KP148265
KP148266
KP148267
KP148268
KP148269
KP148270
KP148271
KP148272
KP148273
KP148274
KP148275
KP148276
KP148277
KP148278
DVH-EG-Boyoumieh-sharkia
250 260 270 280 290 300 310 320
330 340 350 360 370 380 390 400

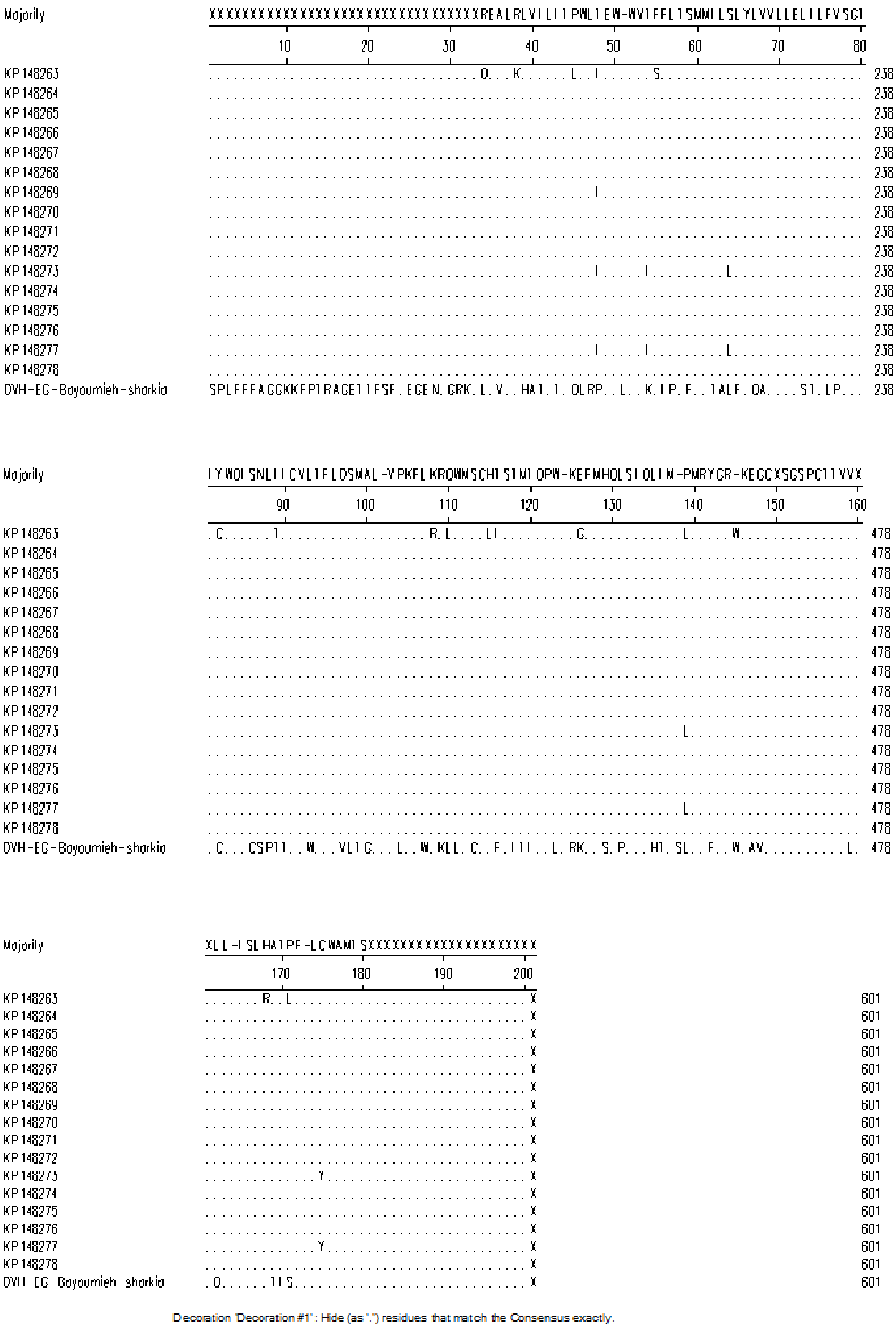






Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

Fig 9: Shows Amino acid sequence of (DVH/EG. Bayoumieh-Sharkia-2015) compared to the Egyptian isolates of Erfan *et al.* (2015).



DISCUSSION

Duck virus hepatitis was first described in 1950, causing severe losses in ducklings in Long Island, New York, then spread to the major duck-growing area of the USA, and ever since It has been described in most important duck-growing areas of the world. The disease has usually become endemic in these regions. The disease is an acute, highly infectious viral disease of ducklings aged from 2 days to 3 weeks. Older ducklings may be diseased, particularly if affected by toxic substances or suboptimal nutrition, but adult stock are resistant. Age resistance to disease is essentially complete from 7 weeks of age Woolcock (2003).

Gough and McNutly (2008) mentioned that Signs of DVH- I infection are per acute., death usually follows within an hour of their onset. Affected birds are often in good condition but start to lose contact with the main flock. Soon they fall over on their sides and, after a short struggle, with paddling movements of the legs, the birds die. The head is usually stretched upwards and backwards (opisthotonos).The mortality rate may be over 90% of the flock. In the present study examined ducklings were showing nervous signs and opisthonous as seen in (fig-1) with a history of 70% mortality until they were delivered to the lab.

The main lesions appear in the liver as mentioned by Gough and McNutly (2008) is liver enlargement with a number of petechial and ecchymotic hemorrhages. In addition, fatty kidneys described as duck fatty kidney syndrome may be caused by DVHV. In the present study PM of the sacrificed samples revealed depressed areas on the liver surface marking the large blood (fig-2). Hemorrhagic lesion of DVH was only noticed in dead duckling carcasses (fig-3).

The sudden onset of a disease., the high mortality in young ducklings, the opisthotonos of the bird and, the characteristic liver hemorrhages are together pathognomonic and sufficient to justify the diagnosis of DVH But the Occurrence of similar disease outbreaks caused by serologic variants of DHAV-1, -2 and -3 are the main problem in differential diagnosis and the need for laboratory diagnosis is raised for legislative purposes.

Woolcock (2008) and Woolcock (2010). Mentioned that the presence of DVH may be confirmed by many Laboratory procedures. Diagnosis of type I virus infection is based on virus isolation following the inoculation of organ suspension from affected ducklings into the allantoic sac of 9-day-old ECE or 10 to 14 day EDE. Levine and Fabricant (1950) were the first to propagate the virus in the allantoic sac of 9-day-old chicken embryos. 10 - 60% of the embryos died by the 5^{th} or 6^{th} day and were stunted or edematous. In the present study virus isolation was attempted by inoculation of 1/10 dilution of liver suspension filtered through a 450 nm filter into 9 day old ECE through the allantoic cavity according to the standard protocol of OIE (2010). Eggs were candled daily for up to 6 days. Inoculated embryos showed stunting and subcutaneous hemorrhages over the whole body with edema, particularly of the abdominal and hind limb regions (fig. 4). The embryo livers may be swollen, red, in color, and show necrotic foci. The liver lesions and embryo stunting become more apparent in embryos that took longer time to die. The Collected allantoic fluid was filtered through 200 nm and 100 nm membrane filter and used again for another inoculation ECE. Which revealed hemorrhagic edematous embryos (fig-4). Ultrastructure, Size, Density, Symmetry of DHAV-1 is naked virus with an icosahedral capsid and has been estimated to be 20–40 nm in size Reus (1959), Richter *et al.* (1964), Tauraso *et al.* (1969) which is a typical picornavirus morphology.

Rapid diagnosis using direct immunofluorescence may be made on the livers of affected ducklings Woolcock and Tsai (2013), Maiboroda (1972), Vertinskii *et al.* (1968). In the present study an impression smears were prepared from tissues taken from field samples ., these smears were stained using rabbit anti-duck hepatitis poly clonal serum and an anti-rabbit IF conjugate {Vertinskii *et al.* (1968), Maiboroda (1972), and Hanaa *et al.* (2013)}. Positive results were recorded as showen (fig-5).

Woolcock and Tsai (2013) mentioned that during Differential Diagnosis The sudden onset, rapid spread, and acute course of the disease caused by DHAV-1 are characteristic, and the hemorrhagic lesions in liver of ducklings up to 3 weeks of age are pathognomonic. Occurrence of similar disease outbreaks caused by serologic variants of (DVH) -1, -2 and -3 are the main problem in differential diagnosis. Gough and Wallis (1986) reported the association of DVH -1 with influenza virus in 2- to 5-week-old mallard ducks reared on a game farm. The DVH -1 isolated was of low virulence, and it is suggested that the influenza virus may have exacerbated the hepatitis infection. Other potential causes of acute mortality in ducklings include salmonellosis and aflatoxicosis. The latter disease may cause ataxia, convulsions, and opisthotonos as well as microscopic lesions of bile duct hyperplasia suggestive of DVH, but does not cause the same characteristic liver hemorrhages. None of the other common lethal diseases of ducks occur frequently in this young age group.

Several reverse transcriptase polymerase chain reactions (RT-PCR) have been developed and are useful for identifying DHAV -1 infection such as Kim *et al.* (2007), Cheng *et al.* (2009). Fu *et al.* (2008) reported the detection limit of RT-PCR for

DHAV -1 RNA as 3 pg/10 µl. They also demonstrated that RT-PCR was the most sensitive when the detection rates were compared on 185 clinically suspected DHAV -1-infected liver tissues by RT-PCR, ELISA, and virus isolation methods. Rapid and specific detection of the DHAV RNA using different RT-PCR assays were described Fu *et al.* (2008), Liu *et al.* (2008), OIE (2010) and Wei *et al.* (2012).

Molecular techniques have been described for the detection of DHAVH -1 in clinical material. Sequence results for our examined sample are shown in (fig-7). Interpretation of sequence revealed that The investigated Sample (DVH/EG. Bayoumieh-Sharkia-2015) is a Duck hepatitis A virus. With 100% resemblance to the strain (DHV/Duck/Egypt/Al-Gharbia/2014) in 3D protein gene with gene bank accession number (KP202874) Sultan, and Talaat, (2014) unpublished data. Sequence and phylogenetic analyses also indicated that the (DVH/EG. Bayoumieh-Sharkia-2015) isolate cluster in the DHAV - 1 and was distinguishable from the Egyptian strains of Erfan *et al.* (2015) with a resemblance % ranging between (63.1-63.8%) (table -2 , fig - 8).

ACKNOWLEDGMENTS

The author is grateful to his colleagues at NLQP and Poult. Dis. Dept. AHRI

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الوصف الجزيئي لمعزولة التهاب كبدي فيروسي من البط بمحافظة الشرقية

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في هذه الدراسة تم فحص عينات من قطيع بلنشار ٢٠٠٠٠ بطة عمر سبعة أيام يظهر عليها اعراض عصبية مع ارتفاع نفوق وصل الي ٧٠% يوم جمع العينة. عمر البط والاعراض الظاهرة والافات التشريحية جعلتنا نشتبه في الإصابة بمرض التهاب الكبد الفيروسي للبط. كان الهدف الاول للدراسة هو إظهار القيمة التشخيصية السريعة للفلترة المرحلية بالفلاتر الفيروسية الغشائية كطريقة سريعة فعالة لتشخيص مرض التهاب الكبد الفيروسي لخصائص الفيروس الذي ينتمي الي عائلة Picornaviridae وتم مقارنة نتائج هذا الاجراء مع نتائج بصمة الانطباع الفلورسينتي ونتائج اختبار تفاعل البلمرة المتسلسل. وبدراسة نتائج التتابع النيوكلوتيدي لهذة المعزولة المسماه (DVH/EG.Bayoumieh-Sharkia-2015) أظهرت النتائج ان التتابع النيوكلوتيدي لهذه المعزولة يتطابق بنسبة ١٠٠% مع المعزولة (DHV/Duck/Egypt/Al-Gharbia/2014) من محافظة الغربية ذات رقم الدخول الي بنك الجينات (KP202874) والتي تم عزلها دونما نشربمعرفة (قسم امراض الطيور والارانب بكلية الطب البيطري جامعة السادات – المنوفية) وذلك عند فحص (3D protein gene fragment) Sultan, H.A. and Talaat, S.M (2014) وان هذه المعزولة كانت تتشابه بنسبة تتراوح من ٦٣.١ الي ٦٣.٨ % عند المقارنة بالمعزولات المصرية من مختلف المحافظات (2015) Erfan *et al.* والتي تبدء ارقام الدخول الي بنك الجينات الخاص بها (KP148263 to KP148278) لنفس الجين المفحوص 3D protein gene fragment.