**Cover page**

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**Authors**

**1Ahmed R. Elbestawy**, Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Damanhour University, 22511, Beheira, Egypt

Email: ahmed.elbestawy@vetmed.dmu.edu.eg

**2Mahmoud Gamil Ibrahim\***,Department of Birds and Rabbit Medicine, Faculty of Veterinary Medicine, University of Sadat City, 32958, Menoufiya, Egypt

Email: [mahmoud.jameel@vet.usc.edu.eg](mailto:mahmoud.jameel@vet.usc.edu.eg)

**3Haitham Hammam**,Department Birds and Rabbit Medicine, Faculty of Veterinary Medicine, Aswan University, 81511, Aswan, Egypt

Email: Haithmhmam2020@gmail.com

**5Ahmed Elsayed Noreldin**,Department Histology and Cytology, Faculty of Veterinary Medicine, Damanhour University, 22511, Beheira, Egypt

Email:ahmed.elsayed@damanhour.dmu.eg

**4Amanallah El Bahrawy**, Department of Veterinary Pathology, Faculty of Veterinary Medicine, Sadat City University, 32958, Menoufiya, Egypt

Email: amanallah.elbahrawy@vet.usc.edu.eg

**1Hany F. Ellakany**, Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Damanhour University, 22511, Beheira, Egypt

Email: ellakany\_hany@vetmed.dmu.edu.eg

\*Corresponding author:

Mahmoud Ibrahim, University of Sadat City, 32958, Menoufiya, Egypt

Email: [mahmoud.jameel@vet.usc.edu.eg](mailto:mahmoud.jameel@vet.usc.edu.eg)

**Molecular Characterization of Fowl Adenovirus D Species in Broiler Chickens with Inclusion Body Hepatitis in Egypt**

Ahmed R. Elbestawy1, Mahmoud Ibrahim2\*, Haitham Hammam3, Ahmed Elsayed Noreldin4, Amanallah El Bahrawy5, Hany F. Ellakany1

1Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Damanhour University, 22511, Beheira, Egypt

2Department of Birds and Rabbit Medicine, Faculty of Veterinary Medicine, University of Sadat City, 32958, Menoufiya, Egypt

3Department of Birds and Rabbit Medicine, Faculty of Veterinary Medicine, Aswan University, 81511, Aswan, Egypt.

4Department Histology and Cytology, Faculty of Veterinary Medicine, Damanhour University, 22511, Beheira, Egypt.

5Department Pathology, Department of Veterinary Pathology Faculty of Veterinary Medicine, Sadat City University, 32958, Menoufiya, Egypt.

\*Corresponding author:

Mahmoud Ibrahim, University of Sadat City, 32958, Menoufiya, Egypt

Email: [mahmoud.jameel@vet.usc.edu.eg](mailto:mahmoud.jameel@vet.usc.edu.eg)

**ABSTRACT**

In the current study, fowl adenovirus (FAdV) was detected and characterized from broiler chicken flocks with inclusion body hepatitis (IBH) in Northern Egypt during the period from March 2017 till March 2018. Grossly, the examined flocks showed hydropericardium and enlarged friable livers with ecchymotic hemorrhages. Histopathologically, liver tissues revealed intranuclear inclusion bodies in hepatocytes and hepatitis. Molecular detection by PCR assay using primer specific for the L1 loop region of hexon gene was conducted, the PCR products were sequenced for typing of FAdV. Seventeen out of 37 flocks tested were positive. Sequence analysis revealed that the 17 FAdVs were identical, suggesting a common ancestor for the FAdVs circulating in northern Egypt during the period of study. Phylogenetically, they clustered with fowl adenoviruses D species (FAdV-2 and FAdV-11). Interestingly, mortalities observed in the 17 flocks infected with FAdVs varied from 0.4% to 20%. To our knowledge, this is the first report of FAdV-D from broiler chicken in Egypt. Further studies are needed to examine different poultry species and other geographic areas in Egypt. Moreover, studies on the pathogenicity and potential preventive measures against FAdV infection on poultry farms are necessary.

**KEYWORDS:** Fowl aviadenovirus (FAdV); inclusion body hepatitis (IBH); FAdV-D; FAdV-2; FAdV-11; Egypt.

**1. INTRODUCTION**

Adenoviruses are ubiquitous in chickens and have been isolated from either sick or apparently healthy birds. Adenoviruses isolated from chickens are called fowl adenoviruses (FAdVs) (Harrach et al., 2011). FAdVs are non-enveloped, dsDNA viruses belonging to the genus Aviadenovirus within the family Adenoviridae. FAdVs based on restriction fragment length polymorphism (RFLP) were grouped into five species FAdV-A to FAdV-E (Hess, 2000), and based on serum cross- neutralization tests were divided into 12 serotypes (FAdV-1 to FAdV-8a and FAdV-8b to FAdV-11) (Meulemans et al., 2004).

The most notable diseases associated with FAdV infection in chickens are the inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS) and gizzard erosions (GE) (McFerran & Smyth, 2000). IBH is an acute disease usually caused by FAdV of groups D and E including FAdV-2, -3, -9, -11 and FAdV-6, -7, -8a,-8b serotypes, respectively (Ojkic et al., 2008; Schachner et al., 2016; Steer et al., 2011). IBH outbreaks were distributed worldwide and the disease has been detected in different countries resulting in considerable economic losses. The majority of detected outbreaks caused by serotypes 2, 4, 8a, 8b and 11 as reported in New Zealand (Christensen & Saifuddin, 1989), Canada (Grgic et al., 2011), Japan (Nakamura et al., 2011), Australia (Steer et al., 2011), Korea (Choi et al., 2012), Hungary (Kajan et al., 2013), South Africa (Joubert et al., 2014), China (Zhao et al., 2015), and recently Saudia Arabia (Mohamed et al., 2018) and Egypt (Radwan et al., 2018).

IBH characterized by a sudden increase in mortality, usually ranges between 5% and 10% (McFerran & Smyth, 2000), but occasionally can reach to 30% (Barr and Scott, 1998). Diagnosis of IBH can be carried out through the observation of gross lesions along with histological lesions in examined birds. Grossly, IBH-infected birds usually show pale, friable and swollen livers, also petechial or ecchymotic haemorrhages may be present in liver and skeletal muscles (Macpherson et al., 1974; McFerran et al., 1976). Intranuclear inclusion bodies (INIB) are often observed in degenerated hepatocytes which can be eosinophilic, large, round, or irregularly shaped with a clear pale halo or in some cases can be basophilic inclusions (Grimes et al., 1977; Itakura et al., 1974).

Molecular characterization of FAdVs performed by polymerase chain reaction (PCR) using gene specific primers. The most common gene used for FAdVs detection is the hexon gene (Raue & Hess, 1998; Xie et al., 1999). Also primer specific for DNA polymerase (Kajan et al., 2011), and the 52K gene (Meulemans et al., 2001; Günes et al., 2012) have been used. Further DNA sequencing and/or restriction enzyme analysis has been used for FAdV typing (Raue & Hess, 1998; Meulemans et al., 2001). However, hexon is the major protein of the adenovirus that possess the neutralizing epitope, and known to be serotype specific (Russell, 2009; Liu et al., 2016), which mean that serotyping of FAdV is mainly related to the hexon gene sequence (Niczyporuk, 2016).

In this study, 37 broiler chicken farms showing liver lesions with variable mortalities in Northern Egypt during 2017-2018 were examined for FAdV infection. History, signs and gross lesions reported then liver samples were collected for histopathology and molecular detection by PCR. Further typing of FAdV positive samples were performed by hexon gene sequencing.

**2. MATERIALS AND METHODS**

**2.1. Clinical cases and samples**

Thirty-seven broiler chicken flocks showed liver lesions accompanied with varying mortalities in northern Egypt (Alexandria, Beheira, and Kafr El Sheikh governorates) were examined during the period from March 2017 till March 2018. The age of examined chickens varied from 3-31 days-old. Affected birds showed depression, decreased body weight, also watery diarrhea observed. The main gross lesions observed in the examined flocks were pale, swollen livers with subcapsular ecchymotic hemorrhages and hydropericardium (Fig. 1).

Liver samples were collected from affected birds; part kept in 10% neutral buffered formalin (NBF) for histopathological investigation, and another part kept at - 80oC to be further processed for molecular diagnosis by PCR. Also, Bursa of fabricious collected and kept at - 80oC for detection of IBDV.

**2.2. Histopathology**

Liver tissues were taken and fixed in 10% neutral buffered formalin (NBF) for histopathological investigation. After 72 h of fixation, samples were dehydrated, embedded in paraffin wax and sectioned (3µm) for hematoxylin and eosin (HE) staining. Histological photos were taken by using Leica EC3 digital camera.

**2.3. PCR assay**

Liver samples were homogenized with saline containing antibiotics then clarified by centrifugation. The supernatants were collected and kept at -80 °C until used for DNA extraction. DNA was extracted from the supernatant of the liver homogenate using a Viral Gene-spin viral DNA/RNA extraction Kit (iNtRON, South Korea) according to the manufacturer’s instructions.

PCR was conducted using the PCR Master Mix (2X) (Thermo Scientific, Lithuania) in 25 μl reaction volume and 5 μl DNA template with hexon gene specific primers according to (Raue et al., 2005). The PCR reaction heated at 95 ◦C for 5 min as initial denaturation step. Then 40 cycles of; denaturation at 94 ◦C for 30 sec, primer annealing at 56 ◦C for 30 sec and elongation at 72 ◦C for 45 sec, followed by a final elongation step of 5 min at 72 ◦C. The PCR product was analyzed by agarose gel electrophoresis to visualize the specific band by UV Transilluminator.

Moreover, molecular detection of chicken infectious anemia virus (CIAV) and infectious bursal disease virus (IBDV) were performed using the extracted viral DNA/RNA from liver and bursa of fabricious tissues by PCR and RT-PCR assays, respectively (Todd et al., 1992; Islam et al., 2012).

**2.4. Partial hexone gene sequence**

Specific DNA bands with 590 bp size were excised from the gel and the PCR product was extracted using GeneJET Gel Extraction kit (Thermo scientific, Lithuania) according to the manufacturer instructions. Direct Sanger sequencing was performed using the forward and reverse primers in two reactions with the purified DNA fragment.

**2.5. Genetic analysis**

A BLAST analyses (<http://www.ncbi.nlm.nih.gov/BLAST>) were conducted on each sequence to identify related reference viruses. The nucleotide sequences were analyzed with the BIOEDIT program using the Clustal W alignment algorithm. The obtained sequences in this study were aligned with adenovirus field and reference strain sequences from different countries that are accessible in the NCBI GenBank database. Phylogenetic trees were constructed by the maximum likelihood method using MEGA 6 software.

**3. RESULTS**

**3.1. Gross and Pathological results**

Grossly, the heart of dead chickens had accumulated serous fluid in the pericardium (hydropericardium). In addition, the liver was pale yellow in colour and had pin point ecchymotic hemorrhage on the surface, swollen edges, and friable in consistency.

Microscopically, the hepatic parenchyma had two shapes of intranuclear inclusion bodies; the first was dense basophilic inclusion which occupied most of the nucleus, the second was eosinophilic inclusion which surrounded by a halo. Moreover, hepatic parenchyma showed congestion of the central veins and hepatic sinusoids, vacuolation of hepatocytes and severe necrosis with or without inflammatory cells infiltration. Inflammatory cells were mainly few heterophils and plasma cells (Fig. 2).

**3.2. Molecular detection**

The presence of FAdV was confirmed by PCR amplification of a 590bp fragment from samples of 17 flocks out of 37 total flocks tested (45.9%). Fifteen IBH outbreaks detected during 2017 from Beheira, Alexandria, and Kafr El Sheikh governorates and 2 cases reported during 2018 from Beheira governorate. Mortality rates varied from 0.4% to 16.6%, and only one flock (EG.12) revealed 20% mortalities, this flock showed very virulent infectious bursal disease (vvIBD) typical PM lesions including swelled and hemorrhagic bursa, also hemorrhage in thigh muscles and hemorrhage between gizzard and proventriculus. Mortalities observed in EG.12 flock supposed to be due to very virulent IBDV infection while FADV may have a minor role. All 17 FADV positive flocks were negative for CIAV and IBDV detection by PCR and RT-PCR assays, respectively, except EG.12 flock was tested positive for IBDV by RT-PCR. The history of the PCR positive flocks were listed in Table 1.

**3.3. Sequence analysis**

Blast analysis revealed that the nucleotide identity percent of the detected FAdVs sequences with the available FAdV-D and FAdV-2&11 strains on the NCBI GenBank database ranged from 93% to 99%. Majority of FAdV-D strains showing 99% nucleotide identity with FAdV-D Egyptian strains sequenced in this study were isolated from Europe. Sequence alignment of the obtained sequences revealed that the 17 FAdVs strains detected in this study were identical, suggesting a common ancestor for the FAdVs circulating in Egypt during 2017/2018. The partial hexon gene sequences of four representative FAdV-D strains (EG.33, EG.12, EG.14, and EG.17) detected in this study were submitted to the NCBI GenBank (https://www.ncbi.nlm.nih.gov/WebSub/) under accession numbers (MH782423, MH782424, MH782425, MH782426), respectively.

Phylogenetic analysis indicated that these strains clustered with fowl adenoviruses species D in the FAdV 2 & 11 serotypes cluster (Fig. 3). Moreover, FAdVs strains detected in this study viruses clustered with those isolated from Europe, Australia, India and Saudia Arabia. To our knowledge, this is the first report of FAdV-D (serotype 2 and 11) circulating in broiler chickens in Egypt.

**4. DISCUSSION**

With the higher frequency of viral diseases affecting poultry flocks during the last 15 years, some vertically transmitted viral infections remain highly important as it causes immunosuppression in the affected flocks specially during 1st 3-4 weeks of life. IBH is one of these diseases that has been emerged as an economically important disease in many different countries over the world (Schachner et al., 2017). FAdV may be a possible primary pathogen specially in broilers exacerbating other diseases, increasing losses in the poultry industry in Egypt.

In our study we noticed high mortalities in broiler flocks aged from 3-31 days-old associated with macroscopic lesions in the liver of infected broilers including pale, friable, swollen livers with pin point ecchymotic hemorrhages and hydropericardium which were similar to IBH lesions described previously (Grimes et al., 1978). Moreover, Histopathology of livers revealed the presence of intranuclear inclusion bodies in hepatocytes which indicates infection with IBH. Two shapes of intranuclear inclusion were observed; dense basophilic inclusions which occupy most of the nucleus, and eosinophilic inclusion that has a halo around it. These findings were in agreement with previous studies which reported basophilic and eosinophilic INIB in hepatocytes in IBH infections (Grimes et al., 1977; Itakura et al., 1974). Interestingly, virus particles detected in the basophilic inclusions while the eosinophilic inclusions contain a fibrillar, granular material (Itakura et al., 1977).

Mortalities observed in the broilers with IBH varied from negligible to high mortality as it can reach to over 30% and mostly observed in broilers of 3 to 5 weeks of age (Barr and Scott, 1998). Although the FAdV-D strains detected in this study are genetically similar, the mortalities varies from 0.3% to 20%. This is in agreement with recent study showing only 0.8% differences in the genomic sequences between two FAdV-11 strains, one non-pathogenic strain and the other virulent strain (Absalón et al., 2017). IBH reported as a primary cause of mortalities in broilers in Canada with 4 serotypes detected; FAdV-7, FAdV-8a, FAdV-8b, and FAdV-11 (Gomis et al., 2006). Moreover, FAdV-2 serotype was detected from an outbreak in 9-14 days old broilers with 17% mortalities in Japan in 2010 (Nakamura et al., 2011). In another outbreak in Iran during 2012, 2- day old broiler chickens showed increased mortalities which reached 14% by day 21 of age (Rahimi & Minoosh, 2015). Neither CIAV nor IBDV detected in these outbreaks and FADVs considered the cause of mortalities.

In a recent study, mortalities caused by a FAdV-11 strain was 8.6% while FAdV-4 strain killed 28.6% of SPF infected chickens (Zhao et al., 2015). However, another experimental infection study in one-day-old chickens showed mild clinical signs and mortality after infection with FAdV-8b by the ocular route (Steer et al., 2015). The variation in mortalities could depends on the virus pathogenicity, age, chicken’s susceptibility, and concurrent immunosuppressive infections.

Early infection by immunosuppressive viruses like chicken anemia virus (CAV) or infectious bursal disease virus (IBDV) may play a role in IBH lateral transmission (Fadly et al., 1976). However, It has been reported that IBH could cause mortalities independently without presence of immunosuppression (Christensen & Saifuddin, 1989; Gomis et al., 2006). Interestingly, we observed increased mortalities in 4 IBH outbreaks (EG.1, EG.2, EG.4, and EG.14). Only one IBH outbreak (EG.12), showed typical lesions of vvIBD in which 20% mortality was recorded. This highlighting the importance of these circulating FAdV-D strains which can cause primary infection with increased losses. So considering the FAdVs in the prevention programs in poultry farms in Egypt should be reevaluated.

Molecular detection of FAdVs in the tested flocks was performed through PCR assay using FAdV hexon L1-s and L1-as primers for amplification of the expected 590bp DNA fragment between nucleotides 301 and 890 that is type-specific domains in loop 1 of the hexon of fowl adenoviruses (Toogood et al., 1992). Hexon is the major protein of the adenovirus, and is known to contain the neutralizing epitope (Russell, 2009; Liu et al., 2016), so hexon gene sequencing routinely used for FAdVs serotyping. Phylogenetic analysis indicated that the 17 strains detected in this study belonged to fowl adenoviruses D species and serotyped as FAdV-2 and FAdV-11. Moreover, they are clustered with FAdVs strains isolated from Europe, Australia, India and Saudia Arabia, indicating the worldwide spread of FAdVs. FAdV-D have been detected from different countries all over the world, like Canada (Ojkic et al., 2008), South Africa (Joubert et al., 2014), China (Zhao et al., 2015), Poland (Niczyporuk, 2016), Lebanon (Shaib et al., 2017), and recently Saudia Arabia (Mohamed et al., 2018).

To our knowledge, this is the first report of FAdV-D from broiler chicken in Egypt. Two strains of serotype FAdV-8a (FAdV-E) previously detected in Egyptian chickens during 2015 (Radwan et al., 2018). Surveillance studies for FAdVs in different poultry species and geographic regions in Egypt is needed. Moreover, the pathogenicity of the detected FAdV strains need further studies and the potential risks should be evaluated.

**5. CONCLUSIONS**

We characterized FAdV from broiler chicken flocks with IBH in Northern Egypt in 2017 and 2018. Seventeen out of 37 flocks tested were positive by PCR assay using primer specific for the L1 loop region of hexon gene. Sequence analysis revealed that the 17 FAdVs were identical and phylogenetically they clustered with fowl adenoviruses D species (FAdV-2 and FAdV-11). Studies on the virus pathogenicity and surveillance in different species and geographic areas are important. Finally, preventive measures against FAdV infection on the commercial poultry farms should be considered.

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**Figure Legend**

**Figure 1.** The gross lesions observed in the examined broiler chickens. (a) Heart of dead chicken showing accumulation of serous fluid in the pericardium (arrow). (b and c) Liver of dead chickens is swollen, pale yellow, friable and containing subcapsular ecchymotic hemorrhages.

**Figure 2.** Histopathological lesions observed in Livers. (a) Liver of dead chicken has intranuclear inclusion bodies in the hepatocytes. Two shapes of intranuclear inclusions are observed, the first is dense basophilic inclusions which occupies most of the nucleus (white arrows), the second is eosinophilic inclusion that has a halo around it (black arrows). Inset showing higher magnification of inclusion bodies. (b) The hepatic parenchyma showing basophilic intranuclear inclusion body (white arrow), congestion of central veins and hepatic sinusoids, vacuolation of hepatocytes and severe necrosis with few heterophils and plasma cells (dashed arrows). HE stain, a ×20; inset ×40 and b ×40.

**Figure 3.** Phylogenetic tree based on the nucleotide sequences of the hexon gene of the FAdVs viruses. The black triangle indicate the 17 FAdVs strains detected in this study which clustered with strains from serotypes FAdV-2 and FAdV-11. The alignment was generated using the Clustal W alignment algorithm and the phylogenetic tree was generated by using MEGA 6 program.