

## Phylogenetic analysis and pathological characterization of fowl adenovirus isolated during inclusion body hepatitis outbreak in Tubas, Palestine

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### Abstract

Fowl adenoviruses (FAdVs) associated with inclusion body hepatitis (IBH) was identified in commercial broiler chickens in Palestine. Investigated birds showed primary clinical signs and lesions of IBH including growth retardation, congested and enlarged liver with necrosis, petechial hemorrhage and basophilic intra-nuclear inclusion bodies. The mortality rate was from 15.00%. The FAdV was detected and sequenced in the liver samples of infected chicken by polymerase chain reaction using hexon gene-specific primers. Phylogenetic analysis revealed that FAdVs belong to FAdV-D serotype 10, clustered within the European highly pathogenic isolates. The highest nucleotide sequence similarity was 99.48% with highly pathogenic FAdV-D serotype 10 detected from infected chicken in Poland (GenBank: LN907532.1) and FAdV-D from infected chicken in Sweden (GenBank: HE961828.1). The lowest similarity was 93.46% with Canadian FAdV-D (GenBank: EF685576.1). In conclusion, this is the first report describing the presence of IBH revealing that the causative virus is closely similar to the highly pathogenic FAdV-D serotype 10 of IBH in broiler chickens in Palestine.

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### Introduction

*Adenoviridae* virus is a non-enveloped, double-stranded DNA genome, approximately 43.7 kbp to 44.08 kbp in size. This family contains five genera including *Atadenovirus*, *Aviadenovirus*, *Ichtadenovirus*, *Mastadenovirus* and *Siadenovirus*.<sup>1</sup> The viral genome encodes approximately 40 structural and non-structural proteins. Structural proteins include those making up the hexons, pentons, penton fibers and others associated with the virion core.<sup>2</sup> The hexon loop 1 (Hex L1) region represents the most variable region related to virus neutralization and serotype specificity.<sup>3</sup> Fowl Adenoviruses (FAdVs) belong to the genus *Aviadenovirus*, associated with many economically important diseases in different species of birds including inclusion body hepatitis (IBH).<sup>4</sup> Since the IBH was first identified in 1963 in the USA, the disease became a significant threat to the poultry industry worldwide causing tremendous economic losses in many countries in the world.<sup>5,6</sup>

The current classification system recognizes five species of FAdVs; A to E (FAdV-A to -E) based on genomic differences, and 12 serotypes (FAdV-1–8a and 8b–11)

determined by cross-neutralization tests.<sup>1</sup> Serological methods and polymerase chain reaction (PCR) coupled with DNA sequencing proved identifying and differentiating of FAdV serotypes. Recently, serological methods have been increasingly replaced by molecular typing based on the nucleotide sequence of the Hex L1.<sup>7</sup> The Hex L1 gene region has adequate variability for reliable identification and distinction of at least 12 genotypes within the currently acknowledged species.<sup>8</sup>

In Palestine, the occurrence and characterization of FAdV in broilers have never been reported. The present study included a Palestinian FAdV isolate obtained during 2018 with documented clinical signs and gross and histopathological findings of IBH to determine the serotype and the species as well as the orthologues of the circulating pathogenic FAdV strain.

### Materials and Methods

**Clinical samples.** Samples were collected from a 17 days-old unvaccinated broiler flock. Liver samples were collected during necropsy of dead birds for histo-

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pathological and molecular analyses. Samples were kept at the Central Diagnostic Laboratory of the Department of Veterinary Medicine at An-Najah National University, Nablus, Palestine, for further analyses. Samples were obtained from cases sent to the Veterinary Clinic at the Department of Veterinary Medicine, An-Najah National University, Nablus, Palestine, for the diagnosis of the disease under the usual veterinary service work in Palestine (No. ANNU-1819-Sc018, September 2018). Also, tissue samples were collected from dead birds only.

**Histopathology.** After necropsy, obtained samples were fixed in 10.00% neutral-buffered formalin, routinely embedded in paraffin wax and stained with Hematoxylin and Eosin (H & E) for histopathological examination following standard procedures.<sup>9</sup>

**DNA extraction, PCR amplification and sequencing.** The DNA was extracted from 25.00 mg of the liver, using ISOLATE II Genomic DNA Kit (Bioline, Alexandria, USA) according to the manufacturer's instruction. A primer pair (Forward 5'- CAARTTCAGR CAGACGGT-3' nucleotide positions 144-161; Reverse, 5'- TAGTGATGMC GSGACATCAT-3' nucleotide positions 1041-1021) was used to amplify 897 bp of the hexon gene including the L1 loop region as described previously.<sup>7</sup> The PCR was performed in the Veriti® Thermal Cycler (Applied Biosystem, Bedford, USA) with the following reaction conditions: An initial denaturation step at 94.00 °C for 5 min, followed by 30 cycles of 94.00 °C for 1 min, 55.00 °C for 45 sec and 72.00 °C for 1 min with a final extension at 72.00 °C for 10 min. The amplified PCR products were analyzed by electrophoresis on a 1.00% agarose (Sigma-Aldrich) with 0.50 µg mL<sup>-1</sup> ethidium bromide. The PCR products of the Hex L1 gene with the specific size were submitted to Sanger sequencing (Syntezza Bioscience, Jerusalem, Palestine).

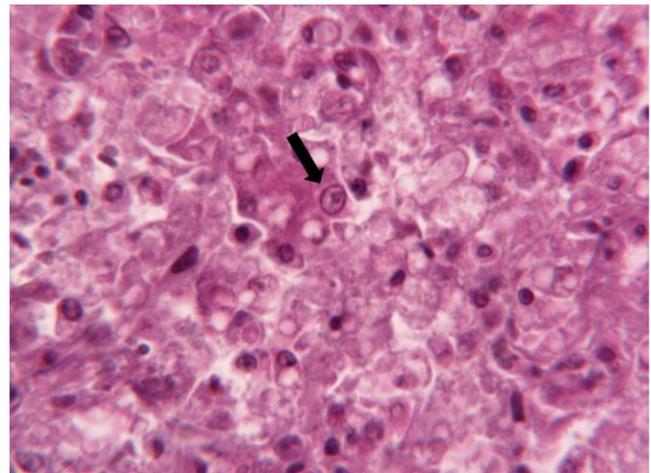
**Sequence analysis.** The nucleotide sequences results were retrieved by Finch TV 1.4 (<https://finchtv.software.informer.com/1.4/>) software, aligned and edited with Vector NTI 9.1.0® (Invitrogen, Carlsbad, USA), compared with reference FAdVs strains. The phylogenetic tree was generated by neighbor-joining method using the MEGA software (version 10.0.5; Biodesign Institute, Tempe, USA)<sup>10</sup> with 1,000 bootstrap replication.

## Results

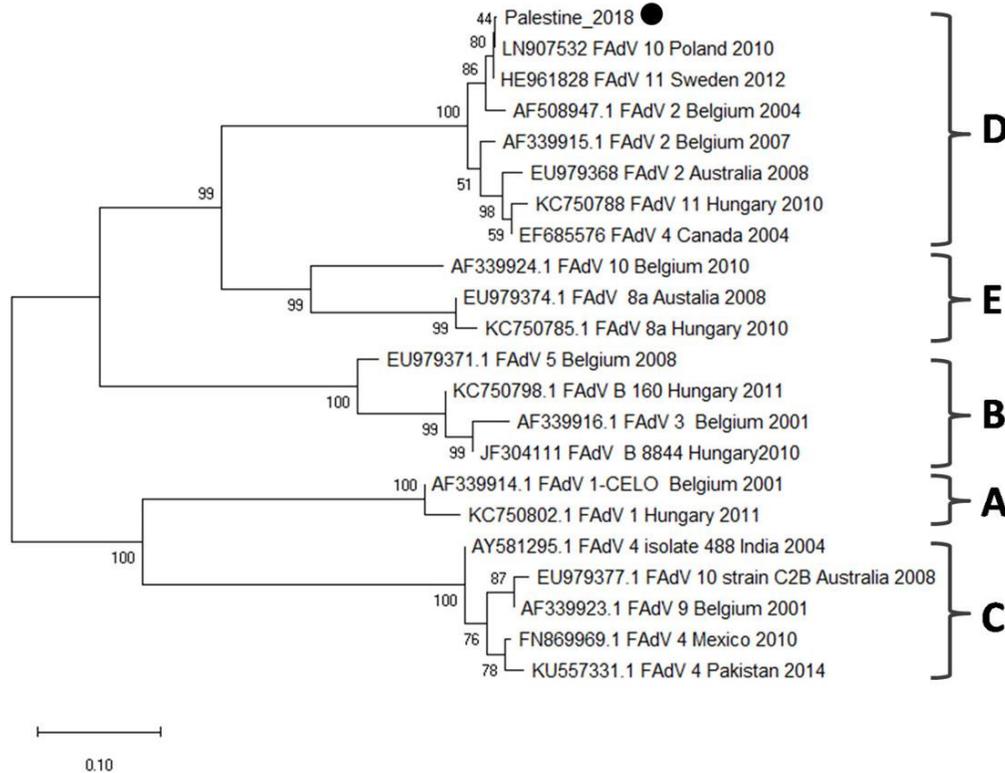
**Clinical signs and gross and histopathological lesions.** Affected birds showed signs of anemia including weakness, depression, pale comb and wattles, growth retardation and 15.00% mortality rate. All necropsied birds from infected flock showed lesions of mild to severe hepatitis including hepatomegaly with diffusely yellowish-pale discoloration and multi-focal pale areas of hepatic necrosis. Additional lesions included markedly atrophied thymus glands and the bursa of Fabricius and

splenomegaly. Subcutaneous hemorrhages were also noticed. Histopathological examination showed 90.00% of the liver section with multi-focal to coalescing randomly distributed areas of coagulative necrosis, characterized by preservation of normal architecture of necrotic tissue with hyper-eosinophilic cytoplasm of necrotic hepatocytes with pyknosis, karyorrhexis, and karyolysis of nuclei and moderate infiltration of inflammatory cells consisting of lymphocytes and few macrophages. Also, degenerated hepatocytes showed intra-nuclear inclusion bodies with margined chromatin (Fig. 1).

**Polymerase chain reaction and sequencing analyses.** The PCR was performed on the extracted DNA from the liver of diseased chicken and 881 bp of the Hex L1 gene was used in the phylogenetic analyses. The nucleotide sequence utilized in this study was deposited in GenBank with the accession number of MT274428. Using the Blast program of the NCBI,<sup>11</sup> the obtained nucleotide sequences showed a 99.48% nucleotide sequence identity with FAdV-D isolate 10-10761 detected in a highly pathogenic IBH from chicken in Poland in 2010 (GenBank: LN907532.1)<sup>3</sup> and FAdV-D isolate 11-15941 from chicken in Sweden (GenBank: HE961828.1). Both of these isolates belong to the European highly pathogenic FAdV-D. The lowest similarity was 93.46% with Canadian isolates of FAdV-D 04-40372 (GenBank: EF685576.1).<sup>12</sup> For the phylogenetic tree construction; the Palestinian Hex L1 gene nucleotide sequences and most related FAdVs isolates were retrieved from the GenBank database. Nucleotide sequences were aligned with other isolates in the Clustal W method.<sup>13</sup> Phylogenetic analysis of nucleic acid showed branching pattern of Hex L1 gene of the Palestinian IBH isolates closely related to the IBH isolates of European FAdV-D, having close relation to isolates from Poland and Sweden (Fig. 2).



**Fig. 1.** Liver cross-section of broiler chicken. Areas of coagulative necrosis are scattered throughout the liver. Within areas of necrosis, degenerating hepatocytes contain intra-nuclear inclusion bodies (arrow), (H & E, 1000×).



**Fig. 2.** Phylogenetic analysis of the selected fowl adenoviruses (FAdVs) using nucleotide sequences encoding for the hexon L1 gene. The data included the Palestinian isolate (indicated with a black dot) and other already published FAdV strains retrieved from GenBank. The FAdV strains are classified at species level e.g., species A, B, C, D and E. The evolutionary history was inferred using the neighbor-joining method. Numbers indicate the bootstrap values (1000 replicates).

## Discussion

The IBH is caused by several serotypes of FAdVs. Field outbreaks are increasingly reported worldwide, related to economic losses in poultry industry. The characterization of the prevalent serotype in Palestine is not detected yet. Therefore, the present study provides an investigation of IBH caused by FAdV-D serotype 10 in broiler farm in Palestine. This finding extends the knowledge about the characterization of this serotype and enables a wider context with phylogenetic comparison to reports from other countries. The IBH was observed as a result of concurrent infections by FAdVs and immunosuppressive agents such as infectious bursal disease virus and chicken anemia virus. In this case, FAdVs were considered opportunistic agents.<sup>14</sup> However, recent reports have indicated that IBH can cause disease in the absence of predisposing co-infections or immunosuppressants.<sup>15</sup>

In the current study, FAdV serotype 10 was characterized from non-vaccinated chickens showing clinical signs related to IBH. The reported clinical signs and post-mortem lesions were similar to highly pathogenic IBH clinical signs lesions described by Chandra *et al.*<sup>16</sup> Histopathological lesions, particularly the lymphocytic aggregation in the liver, is probably due to the fact that the

lack of lymph nodes in most poultry species increases the importance of liver and lymphoid organs in resistance to various diseases.<sup>17</sup> The characteristic basophilic intranuclear inclusion bodies in the hepatocytes confirmed the disease like IBH.<sup>14</sup>

In this study, phylogenetic analysis based on the Hex L1 gene from different geographic areas worldwide showed that the Palestinian strain was clustered into FAdV-D related to serotypes 10. Similar viruses were described previously from Europe particularly Austria, Germany and Poland,<sup>18</sup> and from Canada.<sup>12</sup> Our findings are in agreement with these reports describing the high pathogenicity of this serotype.<sup>19</sup> The introduction route of the causative FAdV-D virus is not known. It is proposed that the virus might spread from Europe to Palestine through imported eggs and chicks. Besides, previous reports have also indicated the role of wild and migratory birds in the transmission of the FAdV.<sup>20</sup> Chicks from vaccinated breeders have a protecting maternal antibody until approximately three weeks of age.<sup>19</sup> The occurrence of IBH in this study can be due to the lack of maternal antibodies. There are no vaccines against FAV in Palestine, and the assumption is that the breeder may not have been infected with FAV recently. Vaccination of breeder flocks against FAV may be necessary to protect commercial broilers from IBH in the future.

Identification of FAdV serotypes is important in epidemiological studies of disease outbreaks, development of preventative measures and adoption of vaccination strategies. Nucleotide sequence diversity analysis is a reliable molecular epidemiology method for the characterization of FAdV viruses. In conclusion, we report for the first time the presence along with phylogenetic and pathological characterizations of FAdVs in Palestine. It appears that IBH has been recently increasing in broilers in Palestine that can result in large economic losses in the poultry industry.

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### Conflict of interest

The authors declare that they have no competing interests.

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