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Outbreak of Inclusion Body Hepatitis-Causing Adenovirus in Lebanese Broiler Flocks

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Abstract

During the summer of 2017, broiler flocks in three farms of northern Lebanon were affected by a disease that resulted in poor birds' performance, and raised mortality up to 53.3% in farm 1. Broilers of farms 1, 2 and 3 were 16, 21 and 30 days of age when brought to the Animal Pathology and Physiology Laboratory at the American University of Beirut for diagnosis. Blood chemistry analysis demonstrated very high levels of alkaline phosphatase (> 3000 U/L) while gross lesions were limited to liver congestion and kidney tanning. Histopathological observations showed the formation of intranuclear inclusion bodies in the broilers hepatocytes while PCR analysis of liver specimens, targeting the amplification of the hexon gene of the fowl adenovirus (FAdV), was positive for all of the diagnosed broilers (three/farm) but not for the breeders, thus refuting the possibility of vertical transmission. Sequencing of the amplified 590 bp DNA fragment showed that the virus is of the strain D, serotype 11. The phylogenetic analysis demonstrated a 100% similarity of the Lebanese isolate with the Iranian isolate IR-HM-1/2011 and high similarity to other Iranian strains, namely IR/H1303.9/15 and IR/H653/13 suggesting the circulation of the same clade of viruses regionally.

Keywords: *Broilers; Fowl Adenovirus; Histopathology; PCR; Phylogenetic Analysis*

Introduction

Avian adenoviruses isolated from healthy or diseased birds, uninfected cell cultures and uninoculated eggs are found to be widespread throughout avian species in all parts of the globe demonstrating economic losses in commercial poultry farming with incidence of infection in flocks reaching nearly 100% in most countries [1,2]. Avian adenovirus is known to be transmitted vertically through the egg in addition to being associated with disease conditions as quail bronchitis (QB), egg drop syndrome (EDS), haemorrhagic enteritis (HE) and inclusion body hepatitis (IBH) [3,4]. Moreover, the virus can be present as a secondary infection associated with respiratory, nervous, reproductive and skeletal system diseases as signs may be mild or asymptomatic [5].

Group I describe adenovirus infection of chickens and turkeys that is generally sub-clinical or accompanied by mild symptoms [6]. Virulence is contingent upon the strain of virus, infectivity titer [7] and the age of the bird where the virus is observed to be most severe in birds under 21 days of age [2,3]. Primary disease signs include increased mortality, decreased feed intake, ruffled feathers, rales, coughing, lacrimation, and depression [8,9]. IBH cases illustrate the liver as the main affected lesion becoming enlarged, brittle and yellowish-brown pale in color [5]. In addition, haemorrhages can occur on liver surface, and may appear on leg and breast muscle. Liver histopathology reveals dispersed hepatic necrosis along intra-nuclear inclusions within hepatocytes [5]. Isolation of group I adenovirus virus is achieved through feces, pharynx and affected organs as the liver [10].

Group II pathogenicity describes HE in turkeys displaying dark red/black distended intestines full of bloody contents affecting ages 6-11 weeks old [11,12]. Spleen enlargement is a factor of group II adenovirus which may be mottled and brittle [13]. Spleen histopathology demonstrates hyperplasia of white pulp, with great quantity of inclusion bodies in lympho-reticular cells [2,14], accompanied by necrosis and lymphoid depletion in thymus and bursa of Fabricius [11,15]. Liver enlargement and congestion of lungs may also occur. Gut lesions are also affected by severe blockage of intestinal mucosa, deterioration and hemorrhage of villi [14,16]. HE is known to be transmissible via direct contact with infected feces [17] and unlike group I, there is no evidence shown for vertical transmission [15].

Group III affects laying hens and is depicted by abnormal egg production known as egg drop syndrome (EDS) virus. The virus occurs in hens between the onset of laying stage and 36 weeks of age, and is able to remain for up to 10 weeks [18]. EDS primary symptoms are illustrated after 7 - 9 days along the loss of eggshell pigment; thinning of egg shell or formation of shell-less eggs [2,19]. Following infection, oedema of the uterine mucosa and exudate in the lumen are indicated. Layers may show transient diarrhea and decreased feed intake [20]. Inclusion bodies are observed in all regions of the oviduct, though they stand prevalent in epithelial cells of pouch shell gland of uterus [21,22]. EDS is transmissible in three ways through vertical, lateral or inoculation transmission (practiced for vaccination or bleeding of viraemic birds) [23].

Circulating strains of avian adenovirus in the middle east include presence of EDS in Israel [24]; reported IBH in Turkey [25]; Egypt [26,27] adenovirus-like IBH and IBH of strains D and E with two high prevalence Fowl Adenovirus (FAdV) serotypes 11 and 8b in Iran [28,29]; as well as Hydropericardium syndrome of fowl adenovirus serotype 4 (FAdV-4) strain recognized in Kuwait and Iraq [4,30]. This study reports, for the first time, the occurrence of adenovirus infection of broiler flocks in the north of Lebanon and characterizes the newly isolated virus.

Materials and Methods

Case History

During the summer of 2017, three broiler flocks of the Ross 308 strain showed mediocre performance and/or high mortality in the North of Lebanon. Around 11000 broilers were reared in an open system in Farm 1; their performance was very poor with a feed conversion ratio (FCR) value of 2.66 and mortality of 53%. Signs of illness and morality started at five days of age and the birds were administered fosfomycin, oxytetracycline, amprolium, amoxicillin and colistin. Farm 2 was the largest, with 26000 broilers being reared in a closed system. Mortality in this farm was around 1.1% and the birds showed poor growth with an average live body weight of 2 kg at 40 days of age, and were treated with tetracycline at day one and amprolium at 21 days of age. In Farm 3, 12300 broilers were reared in an open system. Mortality in the third farm didn't exceed 1.1% yet the birds demonstrated poor growth with an average live body weight of 2 kg at marketing age of 45 days and an FCR of 1.82. Birds in farm 3 were treated with amoxicillin at day 1 and florfenicol at 21 days of age at which birds start showing morbidity signs. Three birds per farm were brought to the Animal Pathology and Physiology lab at the American University of Beirut for diagnosis. Birds from farms 1, 2 and 3 were 30, 16 and 21 days of age respectively, when brought to the lab. In addition, six breeders were also brought to assess the probability of vertical transmission of any potential pathogen.

Blood chemistry analysis and Gross pathology

A volume of 2 mL of blood was collected from the wing vein of three broilers/farm. Blood was centrifuged at 2000 rpm for 10 minutes and the sera were collected in a 1.5 mL capacity Eppendorf tube. Levels of gamma glutamyl transferase (GGT), alanine aminotransferase (ALT) and alkaline phosphatase (ALKP) were determined in order to assess the liver enzymatic functions. Broilers were then humanely euthanized and observed for the determination of gross lesions in the liver, kidneys, respiratory and immune organs.

Microbiological analysis

Intestinal *Clostridium perfringens* analysis

An amount of 1.5 grams of duodenum, jejunum, ileum and cecum, along with their contents were homogenized together in saline. A vol-

ume of 0.1 mL of the homogenate was cultured into Trypticase Sulfite Neomycin Agar (BioMérieux sa, 69280 Marcy l'Etoile, France) using the pour plate method. The plates were then incubated for 2 days at 37^{0}C in anaerobic jars, in the presence of Palladium catalyst. Samples from each bird were observed in duplicates for *C. perfringens* counts.

Eimeria sp. Analysis

Floatation test was applied to count *Eimeria* sp oocysts in the intestines of the birds. An amount of 1.5 grams of ceca and its contents were pooled from three birds/farm and homogenized in tubes containing four mL of 30% saline. The tubes were left to stand for 30 minutes, then 20 µl of the supernatant were observed under the microscope at 400X for counting using the improved Neubauer counting chamber (Marienfeld GmbH and Co, Germany). The same protocol was applied for *Eimeria* sp oocysts count in the duodenum, jejunum and ileum pooled specimens.

Nephrotropic Infectious Bronchitis (IB) analysis

Kidneys of three birds/farm were pooled and homogenized in 5 mL of viral transport medium [31]. The homogenates were subjected to freezing and thawing three times and centrifuged at 2000 rpm for 10 minutes. The presumptive IB RNA was extracted from the supernatants using the QIAamp Viral RNA Mini Kit (Qiagen, Germany) and amplified by reverse transcription Polymerase Chain Reaction (RT-PCR) using One-Step RT-PCR kit (Qiagen, Germany) targeting a segment of 706 bp of the S1 gene of IB [32]. PCR products were subjected to electrophoresis and visualized in 2% agarose gel using Ethidium bromide for wet staining.

Adenovirus analysis

Histopathology

Broilers liver specimens were fixed in 10% formalin in phosphate buffered saline (PBS). Tissue sections of 4µm of thickness were fixed on slides and stained with H and E according to Cardiff., *et al*. (2014) [33]. The tissue sections were observed at 400x magnification for the presence of intranuclear basophilic inclusion bodies that characterize the adenovirus infection in broilers.

PCR analysis for Avian Adenovirus

Liver specimens from broilers and breeders were homogenized, individually, in transport medium [31] then the homogenates were subjected to freezing and thawing, three times. The DNA was extracted using the Qiagen DNA minikit (Qiagen GmBH, Hilden, Germany) and subjected to PCR amplification using the RedTaq Ready Mix (Sigma-Aldriche, 3050 Spruce Street, St Louis, MO, USA) and the primers Hex L1-s (5′- ATGGGAGCSA CCTAYTTCGACAT-3′) and Hex L1-as (5′- AAATTG TCCCKRAANCCGATGTA-3′) that corresponded to nucleotides 301 - 323 and 868 - 890 of the hexon gene, respectively [34]. The cycling conditions were as follows: 15 minutes at 95^{0}C followed by 32 cycles of: 95^{0}C for 1 min, 54^{0}C for 2 minutes, and 72^{0}C for 3 minutes. A final extension was performed at 72^{0}C for 10 minutes.

Sequencing and phylogenetic analysis of the Avian Adenovirus

The FAdV DNA amplicon of 590 bp was excised from the gel and purified using the QIAquick Gel Extraction kit (Qiagen GmBH, Hilden, Germany). The nucleotide sequence was determined using 3100 Avant Genetic Analyzer- ABI PRISM (Applied Biosystems, Hitachi).

Nucleotide sequences were compared with internationally reported sequences of avian adenovirus strains using the National Center for Biotechnology Information Website (www.ncbi.nlm.nih.gov). A cladogram was constructed based on the 590 bp Hexon gene fragment sequence alignment.

Results

Blood chemistry analysis and gross lesions

Blood chemistry analysis revealed normal levels of gamma glutamyl transferase and alanine aminotransferase in birds of flocks 1, 2 and 3 as shown in Table 1.

Farm	Gamma glutamyl transferase (U/L)	Alanine aminotransferase (U/L)	Alkaline phosphatase (U/L)
1	52	10	> 3000
2	56	10	> 3000
3	37	10	2000

Table 1: *Blood chemistry parameters**

**Average of three birds/farm*

However, the level of alkaline phosphatase in birds of two flocks was greater than 3000 U/L, exceeding the normal levels and indicating an abnormal function of the liver [35,36].

The abnormal function of the broilers liver was also reflected through gross lesions observation which revealed congested livers in all of the diagnosed birds (Figure 1).

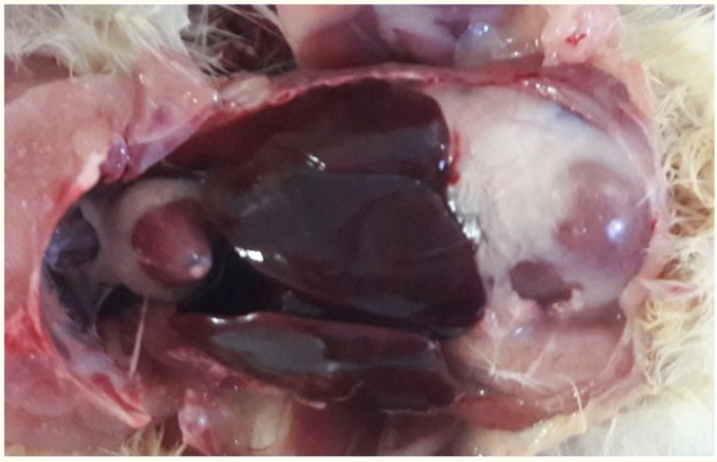

Figure 1: *Congested liver of an affected broiler (Farm 2).*

Birds also showed tanned kidneys and absence of urates accumulation in the ureter, with no lesions in the respiratory and digestive tract organs. In addition, the spleen and thymus were normal and the Bursa of Fabricius didn't show any congestion or atrophy indicating that the immune system was not targeted by the causative pathogen.

Histopathological observations of microscopic lesions

H and E staining of the liver of birds of the three flocks revealed the presence of basophilic intranuclear inclusion bodies (BIIB) that characterize inclusion body hepatitis-causing adenovirus infection (IBH), as shown in figure 2.

All of the examined birds showed the same BIIB microscopic lesion but no one demonstrated the presence of fibrosis, tissue degeneration, presence of apoptotic cells or heterophils infiltration.

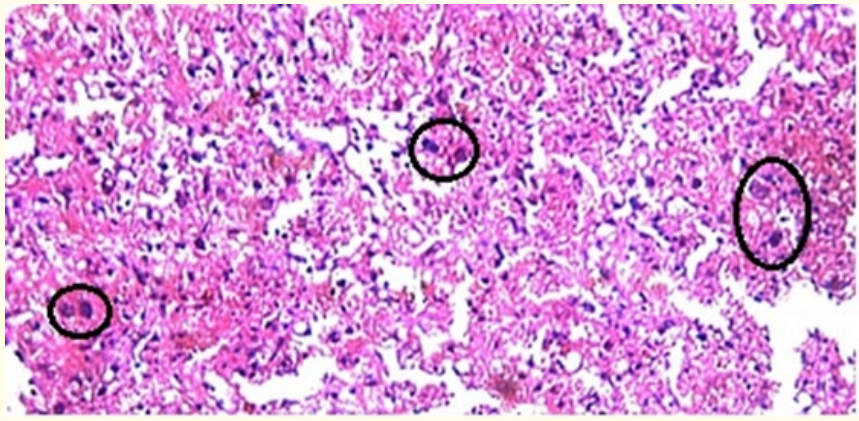

Figure 2: *Intranuclear inclusion bodies in the hepatocytes of the FAdV-infected broilers.*

Microbiological analysis of the intestines and kidneys

The count of *Eimeria* species and *Clostridium perfringens* in the intestines were within the acceptable range with less than 1000 *Eimeria* oocysts/g of intestinal organ and contents, and less than 1000 CFU of *C. perfringens*/g of intestines. In addition, PCR analysis of the kidneys was negative for nephrotopic infectious bronchitis infection as indicated by the absence of the 706 bp amplicon following gel electrophoresis and staining with ethidium bromide. Results of the microbiological analysis of the intestines and kidneys are summarized in Table 2.

Flock	*Eimeria* sp. count* (/g of tissue)			*Clostridium perfringens* count in intestines	Nephrotropic IB virus presence in the kidneys
	Duodenum	Jejunum	Ileum		
1	< 1000	< 1000	< 1000	< 1000	-
2	< 1000	< 1000	< 1000	< 1000	-
3	< 1000	< 1000	< 1000	< 1000	-

Table 2: *Microbiological analysis of the intestines and kidneys of affected broilers.*

**Average of three birds/farm*

Avian adenovirus PCR analysis

The PCR amplification of the FAdV Hexon gene in the livers was positive for all of the individual nine samples (three broilers/farm x 3 farms) showing a 590 bp amplicon (Figure 3).

These results confirm the adenovirus infection as demonstrated in the histopathological analysis of the liver that showed typical adenovirus-microscopic lesions with intranuclear inclusion bodies formation in the hepatocytes. PCR results were negative for the liver specimens collected from the six breeders indicating that the transmission of the pathogen to the progeny was not vertical.

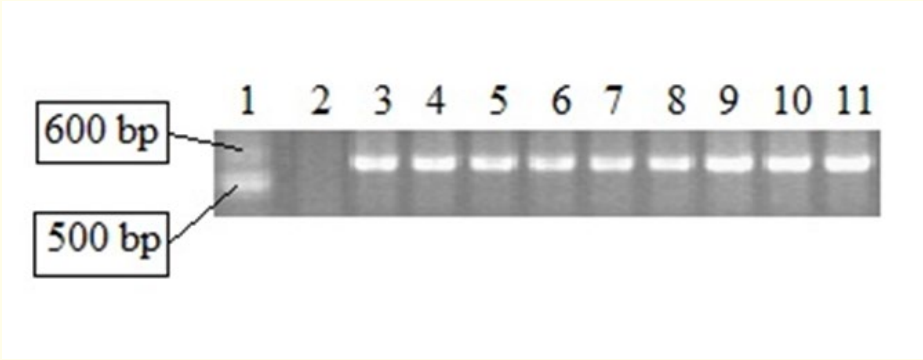

Figure 3: *PCR amplicons of hexon gene fragment of FAdV isolated from birds of farm 1 (Lanes 3, 4 and 5), farm 2 (lanes 6, 7 and 8) and farm 3 (lanes 9, 10 and 11). Lane 1: 100 bp molecular ladder; lane 2: negative control.*

Sequencing and phylogenetic analysis of the Avian Adenovirus

All of the amplicons showed the same nucleotide sequence of the FAdv Hexon gene indicating a common source of infection for the infected flocks. The alignment of these sequences with internationally reported ones revealed a 100% similarity with the Iranian isolate IR-HM-1/2011 which is of the strain D, serotype 11. Fowl adenoviruses of this serotype include mainly the clade of viruses that cause inclusion body hepatitis in broilers. Other Iranian isolates namely IR/H1303.9/15 and IR/H653/13 and Asian isolates, except for the Pakistani ones, showed high similarity to the Lebanese strain as well. The cladogram or tree of genetic similarity is presented in figure 4.

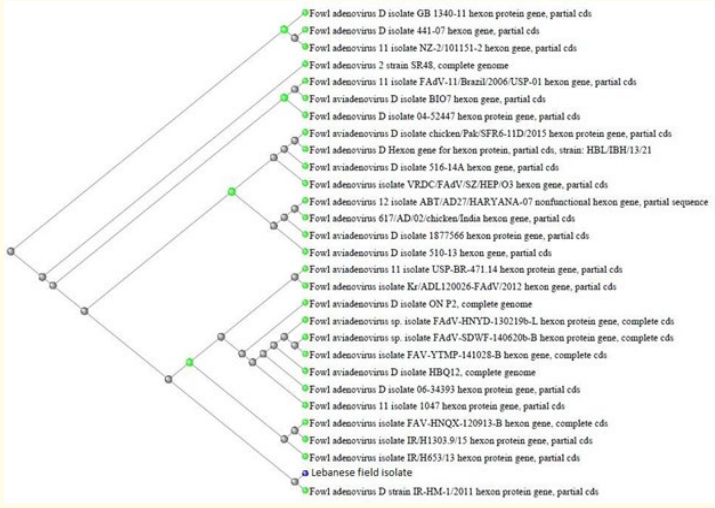

Figure 4: *Cladogram showing multiple alignment of the nucleotide sequence of the 590 bp hexon gene fragment of the isolated FAdV.*

Discussion

This is the first study reporting an outbreak of inclusion body hepatitis-causing adenovirus in broilers of Lebanon. The absence of any concurrent microbiological infection might have resulted in the low mortality rate observed in two out of three flocks, as mentioned by McFerran and Smith (2000) [6] who reported that infections with adenoviruses of Group I are generally sub-clinical or accompanied by mild symptoms. Nevertheless, the infection with adenovirus alone resulted in high economic losses due to the poor performance of the

broilers of farms 2 and 3 namely, live body weight and feed conversion. These results are in accordance with those of Niu., *et al*. (2017) [37] who reported that infection with FAdV-D field strains significantly affected growth, enzymatic systems, and metabolite concentrations of chickens, yet the mortality rate remained low.

Mixed infection with FAdV, or environmental stress usually results in high mortality and morbidity among affected birds [38] which explains the high mortality rate of broilers, in farm 3, raised in an open system with little or no control of temperature especially during hot seasons. Due to economic reasons, most of the farmers in the North of Lebanon find themselves obliged to adopt the open system for broiler production despite the risks of pathogenic or environmental challenges as in the case of farm 3.

Microscopic lesions were limited to liver congestion and kidney tanning indicating that the infection, as such, was not pronounced. In case of severe FAdV infection, birds normally show enlarged, pale and friable liver and sometimes ecchymotic hepatic hemorrhages [39], necrotic pancreatitis and gizzard erosions [6,40]. Literature also reports inflammation of the lungs as well as multifocal, pale lesions in the liver and spleen [2,9] and affected kidneys, heart and thymus; nevertheless, none of these gross lesions was observed in this study.

The liver showed intranuclear inclusion bodies that are typical of FAdV infection, yet the lesions were not prominent, as it was reflected by the absence of fibrosis, cellular apoptosis or heterophil infiltration [41]. This was further reflected by the normal levels of two out of three tested hepatic enzymes namely GGT and ALT. Therefore, the ALKP can be used as one of the hepatic damage indicators when testing for FAdV infection in broilers even at earlier stages of infection. Apparently the infection of birds with FAdV raises drastically the levels of ALKP that exceeded 3000 U/L of serum [42,43].

FAdV PCR analysis was positive for birds of the three flocks. Nevertheless, the absence of adenovirus in the breeders flock rejects the possibility of a vertical transmission to the diagnosed progeny. Consequently, proper epidemiological investigation should be carried out to depict the common source of the disease horizontal transmission to the affected flocks. Literature reporting horizontal transmission of fowl adenovirus is abundant; for instance, adenoviruses are heavily present in feces of infected birds and can be also transmitted through fomites, egg trays, crates, transport vehicles and personnel [44,45].

The circulating virus is of strain D, serotype-11, as demonstrated by sequencing of the Hexon gene and alignment with international FAdV DNA sequences [46]. This strain was responsible for different inclusion body hepatitis outbreaks in poultry of Canada in 2003 [47], Korea in 2007 [48] and Hungary in 2010 [49]. In Iran, the same adenovirus strain affected two broiler breeder farms where the birds were less than three weeks of age, and showed depression and mortality of up to 30%. The histopathological analysis of the Iranian flocks that were affected with the serotype 11-FAdV revealed the formation of intranuclear inclusion bodies, similarly to those reported in this study [46]. It is worth noting that the FAdV outbreak reported in this study resulted in different patterns of mortality as the birds were raised in different rearing systems. This variability in the mortality percentage among broilers, caused or accentuated by the presence of FAdV infection, emphasizes the need to apply strict biosecurity measures in poultry husbandry, avoiding the adoption of open systems in which the birds are exposed to pathogens and extreme weather conditions.

The phylogenetic analysis demonstrated a 100% similarity of the Lebanese isolate with the Iranian isolate IR-HM-1/2011 and high similarity to other Iranian strains IR/H1303.9/15 and IR/H653/13. All of these isolates are of strain D, serotype 11 that affects broilers at different ages causing inclusion body hepatitis of the affected birds [29]. Apparently the Chinese strains that were circulating between 2007 and 2014 [50] were even more similar to the Lebanese isolate than the Pakistani, Indian and Korean strains. Geographically distant European and American isolates showed higher differences with the Lebanese isolate. In general, and although adenoviruses of different strains can affect a farm at the same time [38], mutations within the same clade are not likely to occur in a short period of time due to the fact that adenoviruses are double-stranded DNA pathogens, and resist DNA deletions or insertions. Consequently, the high similarity of the Lebanese isolate to some Asian strains, namely Iranian and Chinese ones that are circulating for more than a decade, might indicate the transmission of this clade among different Asian countries and emphasizes the need for the implementation of proper biosecurity and control measures to contain this virus.

Conflict of Interest statement

Authors declare no conflict of interest.

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