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RESEARCH

Serosurveillance and Molecular Characterization of H9N2 Avian Influenza Virus in Lebanon

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ABSTRACT

Background: Since the first report concerning the existence of avian influenza subtype H9N2 (AI H9N2) in 2006 in Lebanon; a lot of pathological changes and mortalities were seen among chicken flocks suggesting the influence of the AI H9N2 virus.
Objective: this study reports the circulation of H9N2 associated with mortalities in broiler sector and decrease egg production in layer hens and breeders in Lebanon.
Methods: A total of 22 poultry flocks have been tested by real-time reverse transcription polymerase chain reaction (rt-RT-PCR) for the presence of H9N2 in different types of chickens including broilers, breeders, laying hens, backyard chickens and ducks. Tracheal swabs were collected from flocks raised in different governorates in Lebanon.
Results: Testing of samples with rt-RT-PCR revealed that 6 flocks reared in 4 Lebanese governorates were positive (CT ranged from 20.42 to 33.86). A Positive sample with CT 20.42 was tested and partially sequenced and submitted to GenBank, acquired accessions number MH266696 and designated A/chicken/Lebanon/21-E03/2018 (H9N2). Phylogenetic analysis of the obtained sequence cluster the characterized strain along with (formerly) G1-like viruses' subtype B like those circulating in the Middle East.
Conclusion: the AI H9N2 avian influenza virus is circulating causing major problem among chickens. More studies need to be addressed to understand the real effect of the H9 virus on chickens and humans in Lebanon.
Keywords: Avian Influenza, H9N2, ELISA, Real-time PCR, chicken, Lebanon.

BACKGROUND

Avian influenza (AI) is a disease of poultry causing high economic losses beside its zoonotic potential. The disease is caused by type A influenza viruses that belong to family *Orthomyxoviridae* (Palese and Shaw., 2007). Avian influenza is further classified into highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI), depending on the severity of the disease in susceptible birds. The silent spread of LP H9N2 has been recorded in the Middle East and the Far East regions for several years indicated additional risk factor to the poultry industry (Zhou *et al.,* 1999). Although H9N2 viruses caused high morbidity and mortality (Naeem *et al.,* 2007), yet the recent emerging H9N2 virus in Egypt was isolated from clinically healthy commercial bobwhite quail flock in May 2011 (El-Zoghby *et al.,* 2012). Birds challenged with H9N2 became more susceptible to the vNDV (Bonfante *et al.,* 2017).

Natural infection with AI-H9 in poultry results in variable clinical signs depending on host species, virus strain, and the presence of secondary respiratory pathogens (Easterday *et al.,* 1997)**.** Clinical signs including swelling of sinuses, nasal and ocular discharge, severe respiratory signs and low mortality rate. Gross lesions included severe congestion in tracheal mucosa and lungs, and caseous material in the tracheal bifurcation and secondary bronchi (Alexander., 1982; Easterday *et al.,* 1997; Nili and Asasi., 2002). Subtain *et al.,* (2011) reported variability in gross lesions of H9 depend on infected birds with AI H9 subtype, some birds showed slight hyperemia

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and congestion in trachea and lungs on 5th and 9th day post infection. However; kidneys were swollen; changes at a rate of 10% and 43%; respectively.

ELISA assays were commonly used for detection of antibodies directed against the conserved nucleoprotein (NP), thereby detecting humoral responses to all AIV subtypes (OIE., 2015). RT-PCR assays of the H9N2 based on primers specific to HA gene have been applied in both conventional and real time format (Monne *et al.,* 2008).

Phylogenetically, AI H9N2 viruses can be grouped into 3 distinct sub-lineages represented by prototype strains: A/Qa/HK/G1/97 (G1-like), A/Ck/Korea/38349-p96323/96 (Korean-like) and A/Dk/HK/ Y280/97 (Y280-like) (Guan *et al.,* 1999). Genetic and antigenic analysis of subtype H9N2 viruses showed that these viruses have been evolved gradually from the Eurasian lineage into several distinct sub-lineages and were established in domestic poultry (Guan *et al.,* 1999; Xu *et al.,* 2007). Further phylogenetic analysis studies on China and the Middle East subtype H9N2 virus isolates shown that these viruses have undergone reassortment with other subtypes to generate multiple novel genotypes consisting of gene segments from different lineages (Guan *et al.,* 1999; Guo *et al.,* 2000; Xu *et al.,* 2003).

In Lebanon; the first record on the occurrence of H9N2 in meat chicken breeders, commercial layers, free range layers and broilers was reported in 2005, (Barbour *et al.,* 2006). The authors have proved the presence of H9 antibodies by ELISA and HI, where vaccination against H9N2 virus was not practiced at that time. There was little data reported the incidence of H9N2 virus circulation in Lebanon in the years; 2007, 2010 and 2011 (Murtada *et al.,* 2016). They demonstrated the pathogenicity of the virus and the great economic importance to the outbreaks striking the poultry production in Lebanon. Avian influenza H9N2 have been isolated form quail in Lebanon in 2010, the strains were recorded by Influenza Research Database and given the name; A/quail/Lebanon/273/2010 and A/quail/Lebanon/272/2010.

Poultry industry in Lebanon; comprising Chicken, part of the large poultry family (duck, turkey goose), is present in almost every household's or restaurant's kitchen in Lebanon. In fact, consumption /capita is estimated by some at 30 kg/year. The country is home to more than 10 large poultry producers and some 2,000 poultry farms. The Lebanese poultry producers have the capacity to tend to the entirety of local demand (Blominvest Bank., 2016).

According to Lebanese Ministry of Agriculture; all H9N2 vaccines are inactivated and imported from different countries like France, Italy, Mexico and Hungary. The used H9N2 vaccines are monovalent or combined with Newcastle disease virus and/or infectious bursal disease virus. Vaccination programs in most Lebanese poultry farms include H9N2 vaccine, especially in breeders and layer hens. The practice of H9N2 vaccines in broiler is limited to some areas and it is almost absent in backyard chickens.

In recent years; several outbreaks and mortalities in broiler flocks were observed in different regions in Lebanon, mortalities ranged from 20-50% depending on the secondary infection and complex respiratory system. Though the objective of the present study is to measure and evaluate ELISA antibody titers against AI H9 in sera collected of flocks under study as well as molecular detection of H9N2 in tracheal swabs collected from chicken flocks demonstrating mortalities in different regions in Lebanon. Finally; sequence analysis of the detected virus in our study.

MATERIALS AND METHODS

Samples

During March 2014, suspicious IBV infections were found in 14-day-old commercial broiler farm with previous IBV vaccination in Qaluobia province, Egypt. The flock was

vaccinated against IB and Newcastle disease viruses at one day of age using H120 and B1 vaccines respectively. The sick birds presented with respiratory symptoms and the pathological changes in proventriculus (enlarged, filled with fluid, and its mucosa was thickened and exuded a milky fluid when squeezed at postmortem) without renal lesion. Tissue samples of swollen proventriculus were collected and frozen at -70 °C for further analysis.

Chicken flocks:

A total number of 22 chicken flocks located in different Lebanese governorates with different ages, different types of raising (broilers, breeders, layers and backyard chickens have shown clinical sigs including ruffled feather, decrease feed and water intake, respiratory signs and decreased egg production in breeders and layers with variable mortalities. Lesion including general congestion, hemorrhages in tracheal mucosa, airsaculitis congested heart and liver, pericarditis, pneumonia, perihepatitis hemorrhages in coronary fat, enteritis and hemorrhages in intestinal mucosa (Fig1).

Samples:

Blood samples were collected for detection of antibodies in apparent healthy and diseased chicken flocks and tested using ELISA test. Tracheal swabs from life and diseased chicken were collected for molecular detection of AI-H9 subtype using rt-RT-PCR and sequencing of the obtained positive samples.

ELISA:

Commercial ELISA kits "ID Screen Influenza A Nucleoprotein Indirect"; for the detection of antibodies against the Influenza A virus Nucleoprotein in chicken or turkey serum or plasma was used according to the manufacturer instructions, (IDvet, 310, rue Louis Pasteur - Grabels – FRANCE). The provided reagents with the ELISA Kit were microplates coated with purified NP antigen, positive control, negative control, concentrated conjugate (10X), dilution buffer 14, dilution buffer 3, wash concentrate (20X), substrate solution and stop solution (0.5 M). The kit has been kindly supplied by JOVAC Company from Hashemite Kingdom of Jordan for free.

The required materials not provided with the kit; Mono and multi-channel pipettes capable of delivering volumes of 5 ul,10 ul, 100 ul and 200 ul, disposable tips, 96 well microplate reader, distilled water, and 96 well pre-dilution plate. These materials and the working space (laboratory) were kindly supplied by LEBANVET company/Lebanon.

The CV% is the standard deviation divided by the mean, multiplied by 100, whether it was relating to antibody titers. Interpretation of CV values in vaccinated birds can be done as: > 30%: Excellent; 30-50%: Good; 51-80%: Fair and >80%: poor.

Extraction of H9 RNA:

RNA was extracted from collected swabs using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, Calif., USA, Cat. no. 52904). The kit possesses the selective binding properties of a silica- gel-based membrane with the speed of micro-spin technology.

Real-time RT-PCR:

Quantitect probe RT-PCR kit (Qiagen, Inc. Valencia CA, cat no 204443) was used and the reactions were employed using Stratagen MX3005P machine (Stratagene, USA). Sequences of the primer and probe used in rt-RT-PCR were shown in (Table 1); PCR Master Mix was composed of 12.5 µl 2x QuantiTect Probe RT-PCR Master Mix, 0.25 µl forward primer (50 pmol), 0.25 µl reverse primer (50 pmol), 0.125 µl probe (30 pmol), 0.25 µl QuantiTect RT Mix,

8.625 µl RNase Free water and 3 µl template RNA. Thermal profile for amplification of HA gene of H9 subtypes was as follows: 50° C for 30 min, 95 °C for 15 min, cycling steps of 94 °C for 10 s, 54 °C for 30 s and 72 °C for 10 sec repeated for 40 cycles. rt-RT-PCR was performed at Reference Lab for Veterinary Quality Control on Poultry Production. Animal Health Research Institute, Dokki, Giza, Egypt.

Conventional RT-PCR for amplification of HA gene of H9N2 viruses The extracted RNAs were used to amplify the HA gene by means of one step RT-PCR with Qiagen®kit (QIAGEN, Hilden, Germany) with primers specific for the HA gene of H9 subtype. The RT-PCR was conducted as follows: one cycle of RT step at 50 °C for 30 min, one cycle at 95 °C for 15 min for initial denaturation followed by 40 cycles of 94 °C for 45 sec, 56 °C for 45 s and 72 °C for 2 min and final extension at 72 °C for 10 min. The test was achieved on thermocycler 2720 ABI (Applied Biosystems, USA). The electrophoresis of PCR products was done on Ethidium bromide 1% stained agarose gel 1.5% and the amplified products were visualized by gel documentation system – Image capture (Biometra, Germany).

Sequencing of hemagglutinin gene:

Only one positive sample could be partially sequenced and submitted to GenBank, acquired accessions MH266696 and designated (A/chicken/Lebanon/21-E03/2018 (H9N2)). The sequencing of HA gene was carried out using of 2 µl of Big dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster City, CA), 1 µl of each primer specific for HA gene The cycling protocol for sequence reactions was done as follows: one cycle at 96 °C for 1 min, 25 repeated cycles of 96 °C for 10 Sec, 50 °C for 5 Sec and 60 °C for 2 min. Sequencing reactions were purified using a spin column Centrisep® kit (Applied Biosystems, USA) to remove the extra free dNTPs bases, and followed by loading the purified reactions in a sequencer plate of ABI (Applied Biosystems 3130 genetic analyzers, USA).

Genetic and phylogenetic analysis of HA gene:

Bioedit software (version 7.1) was used for alignment of the nucleotide and amino acid sequences, (Hall., 1999). The phylogenetic analysis was done by using maximum likelihood (ML) tree method by Mega 7 software (Tamura *et al.,* 2013), used the general time-reversible (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites and estimated proportion of invariant sites (I) (with 8 rate categories, Γ4), with 1000 replicates of bootstrap analysis.The determination of glycosylation sites on hemagglutinin gene was accomplished by NetNGlyc 1.0 Server (Gupta *et al.,* 2004).

RESULTS

Respiratory signs, sneezing, coughing, ruffled feather, green or white or bloody or undigested feed diarrhea, gasping and bad husbandry situation. The observed clinical sings lead to the suspicion of respiratory pathogens affect egg production, (figures, 1 and 2).

Lesion including general congestion, hemorrhages in tracheal mucosa, Airsaculitis congested heart and liver, pericarditis, pneumonia, perihepatitis hemorrhages in coronary fat, enteritis and hemorrhages in intestinal mucosa. The recorded lesions are suggestive to septicemic infections including H9N2, (figure 3).The results of the tested samples by ELISA and rt-RT-PCR are summarized in table (2).

The obtained results of ELISA and rt-RT-PCR have shown 7 and 6 flocks were positive to infection with AIV H9 by ELISA and rt-RT-PCR respectively. These flocks were distributed

as follows: in North Lebanon governorate 3 samples were positive by ELISA while all were negative by rt-RT-PCR. In Akkar; the 3 tested flocks by ELISA were negative for AI H9, one of them was positive when tested by rt-RT-PCR. The results of Beqaa's flocks 3 samples were positive to the presence of AI H9 antibodies (Abs) by ELISA and 3 samples were positive to AI H9 virus by rt-RT-PCR, 2 of them were positive to AI H9 by both tests. From the Baalbek/Hermel flocks; only one sample was tested positive by rt-RT-PCR. The two samples from South Lebanon came with positive results by ELISA whereas negative by rt-RT-PCR. One sample from Nabatea have conferred positivity to both ELISA and rt-RT-PCR. The result of the only sample acquired from Mount Lebanon was positive by ELISA and negative by rt-RT-PCR.

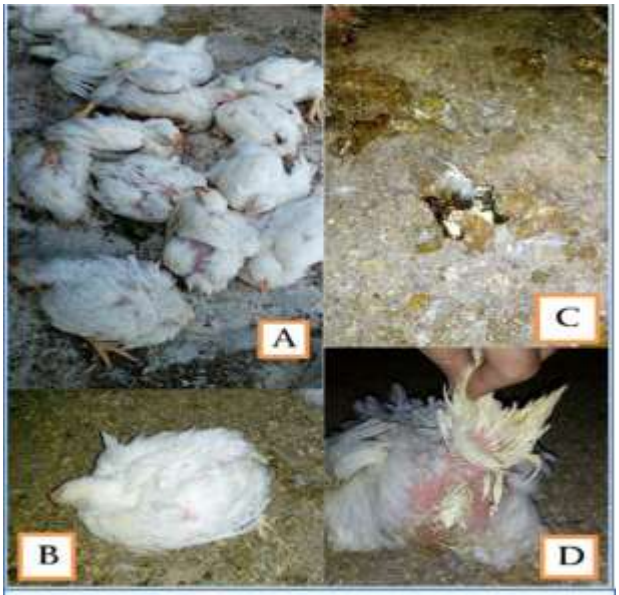


Fig. 1- clinical signs in broilers

A, B: ruffled feather, huddling, lethargy, growth retard, C, D: whitish or bloody diarrhia

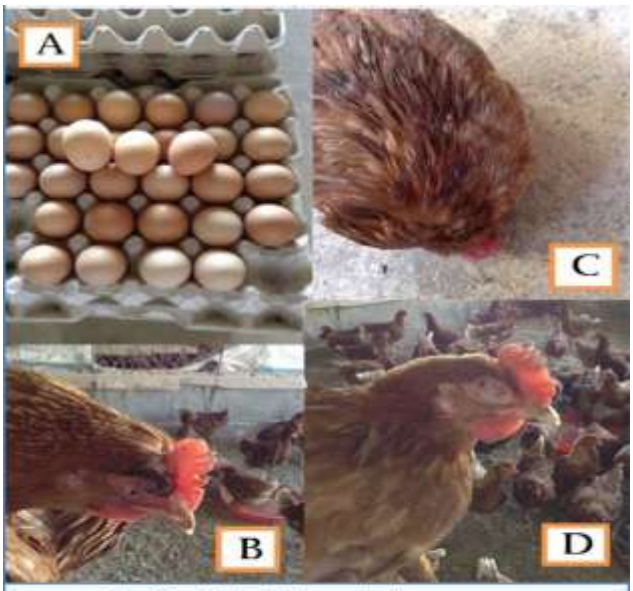


Fig. 2- clinical signs in layers

A: change in egg quality, B: torticollis; nervous signs, C: blisters on comb and wattle D: excessive lacrimation and conjunctivitis

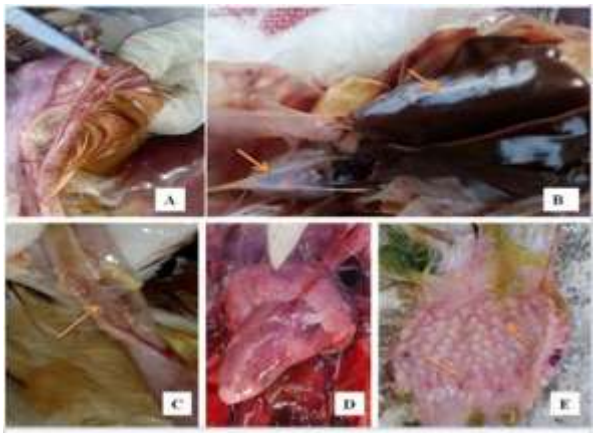
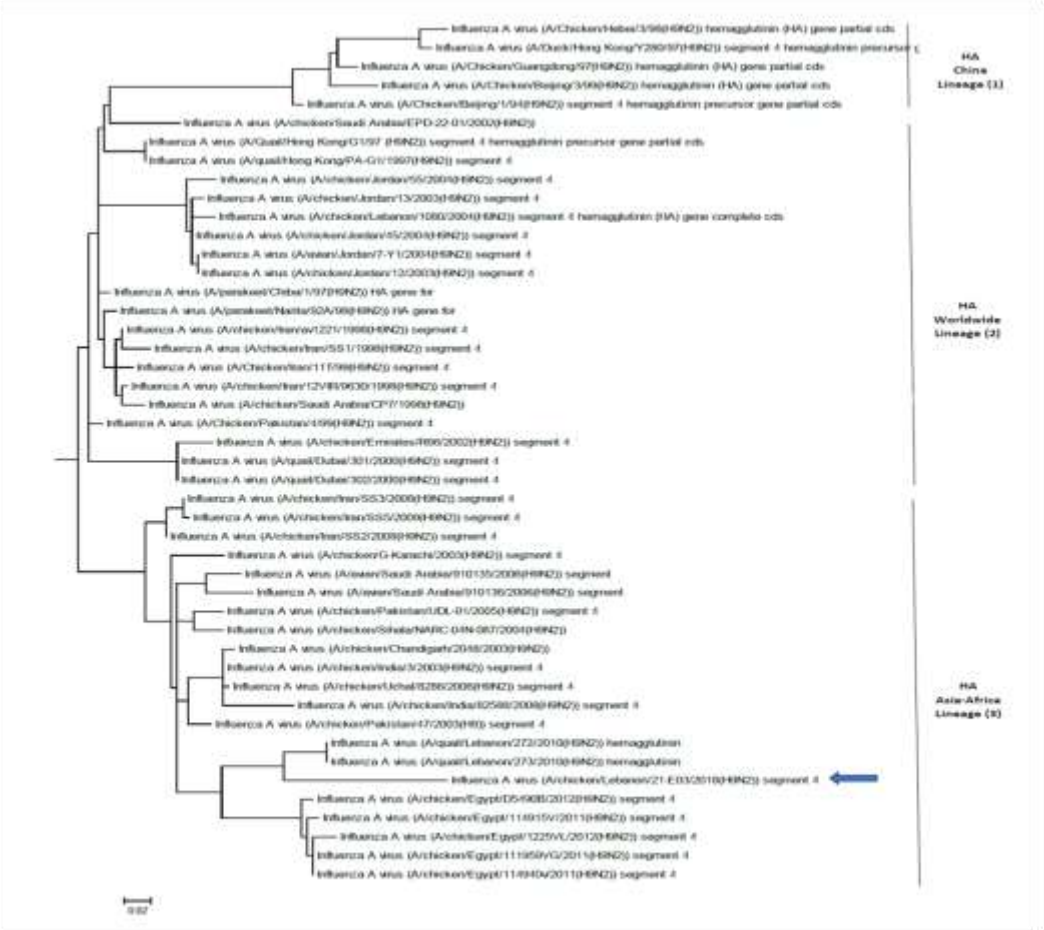


Fig. 3- Lesions of natural field cases suspected to infection with H9N2

A: Hemorrhages in tracheal mucosa. B: Congested heart & liver with pericarditis and perihepatitis. C: Hemorrhages in intestinal mucosa and enteritis. D: Hemorrhages in coronary fat. E: Hemorrhages in proventriculus.

Fig. 4- Bayesian phylogenetic tree of the hemagglutinin (HA) gene of avian influenza A H9N2



The sequences in HA lineages shown in the above tree are grouped according to Mingda Hu *et al*., 2017. The HA sequences from China and Hong Kong were given the Name China Lineage (1), while HA sequences from other Asian Countries were named World Lineage (2). The sequences in HA lineage 3 were from Asia and Africa, so we named Asia-Africa Lineage (3).

DISCUSSION

AI H9 antibodies have been detected in sera samples of layers and broiler breeders in Lebanon by ELISA test even though the vaccination was not practiced at that time, *(Barbour et al., 2006).* Similarly, we could detect the existence of AI H9 Abs in chicken sera of unvaccinated backyards and certain broiler flocks by ELISA test. We have also detected by rt-RT-PCR 6 positive samples of AI H9N2 (27.2% of the flocks- from four governorates; Akkar, Beqaa, Baalbek/Hermel and Nabatea). This is almost similar to *Barbour et al., (2007)* when examined oropharyngeal swabs collected from wild and domestic birds in Lebanon by RT-PCR, the percentage of the positive samples they have tested was 14.3%. Interestingly; they have detected H7 positive samples confined to sparrows and backyard chicken in the southern province of Lebanon. Although in our study; not all ELISA results did match the rt-RT-PCR results when tracheal swabs were tested, but it might be due to the absence of virus shedding, since the AI H9 virus is shed from the tracheal mucosa at day 3-5 after infection, *(Chaharaeina, et a.l, 2007).*

AI H9N2 virus is low pathogenic and the disease may pass unnoticed, or cause mild respiratory infection in affected poultry, even though when AI H9 is accompanied with other organisms -such as E-coli or mycoplasma or other respiratory viruses-, the H9 virus causes

enormous loss, and mortalities may range from 20-65%, *(Jaleel et al., 2017, Hassan et al., 2016).* In our study we have observed the presence of E-coli in many cases in which severe infection was observed, *(Tonu et al., 2011).* IBV and NDV were also detected but the results came negative by rt-RT-PCR. *Hassan et al, (2016)* have demonstrated that early infection in non-vaccinated chickens with AI H9N2 virus may result in immunosuppression to other viral diseases, and the interference between co-infection with other viruses such as IBV or NDV will alter the severity of clinical signs and lesions, and it might generate a novel reassortants within each virus including AI H9N2.

AI H9N2 have been endemic among poultry flocks in the Middle East, North Africa and Asia. AIV H9N2 is responsible of severe economic losses presented by moderate to severe mortality in broilers and decline in egg production in layers and breeders, *(Chrzastek et al., 2018). Nagy et al., (2017)* also have summarized the epidemiological distribution of AI H9 in several countries of the Middle East and South Africa, such as: Lebanon, Syria, Palestine, Egypt, Tunisia and Sudan…. etc. This study has demonstrated that the AIV H9N2 subtype is circulating long time ago and it became endemic in the region. The first isolation of the AIV H9N2 was from Iran in 1998 and then in 1999 from the United Arabs of Emirates, then, in the year 2000 the virus was introduced to chicken flocks in occupied Palestine, onward to the whole region.

Genetic and phylogenetic analysis of the H9 hemagglutinin of the AI virus in different Asian, and Middle Eastern countries have shown a genetic evolution of the H9 subtype and formed a distinct subgroup from the previous H9 subtype, *(Ghorbani1, et al., 2016).* The virus has evolved through point mutation and several amino acids substitution at or adjacent to the cleavage site, *(Ghorbani et al., 2016, Adel et al., 2017).*

One sample could be sequenced and submitted to GenBank and acquired accession MH266696. Previously; Hanna et al., (2004) were the first to sequence the full gene of AI H9 subtype presented in Lebanese territories *(*GenBank, *(Slomka et al., 2010).* This leads to the conclusion that AI H9N2 virus have been circulating in Lebanon before the year 2006. In 2010; the AI H9N2 was isolated from two quails of Lebanon and a whole genome was sequenced for these isolates by *Webby et al., (2010).* Phylogenetic analysis has revealed that the AI H9 Lebanese isolate belongs to the Asian- Africa lineage, *(Hu et al., 2017).*

Recent sequence analysis for the whole genome of AI H9N2 has revealed substantial genetic diversity and frequent reassortment events in internal genes of PB2, PA and NP among circulating H9N2 strains with other viruses; (H5N1 and H7N9). A genetic analysis has been conducted for AIV H9 isolated from domestic pigeons in Egypt; the study designated that this isolate acquired five internal genes from Eurasian AIVs circulating in wild birds. While these reassortments between HP AIV and H9N2 might be virulent, such event is of public health concern in some countries like Egypt and China, *(Xu et al., 2007, Gunalan et al., 2015, Hassan et al., 2016, Nagy et al; 2017, Chrzastek et al; 2018).*

Many reports have confirmed the presence of AIV H9N2 Abs in human sera; *(Kang & Jang., 2017).* Infection of kids caused fever and hospitalization, *(Peiris et al., 1996). Barbour et al., 2006* have collected 34 sera from farmers in the eastern region of Lebanon, the seroprevalence has detected 19 cases with positive results for the presence of H9 Antibodies in their blood by HI test. Also, in Lebanon *Murtada et al., 2016* have revealed that 32.3% of individuals exposed to H9 infected poultry had elevated antibody titer against viruses of the same subtypes among Lebanese chicken growers. Thus, full genome sequence of the detected virus is needed to be conducted and essential to study the effect of evolutionary mutations on the biological behavior of H9 virus in Lebanon as well as the zoonotic importance of the circulating H9 viruses.

CONCLUSION

In conclusion: the AI H9N2 avian influenza virus is circulating and causing major problem among chickens in Lebanon. Its economic importance among poultry flocks, epidemiology, genetic variation and the real impact of the virus on humans especially children are still a Mistry. More researches should be conducted to understand the evolution of the H9 virus in Lebanon. The Lebanese authorities should raise the awareness of the impact of the AI H9 virus on humans and take serious actions to prepare considerable national plan for the control of the disease in poultry including surveillance programs, vaccine monitoring and crucial control measures.

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