Molecular and Phylogenetic Study of The H P A I (High Pathogenic Avian Influenza) Virus Sub-type H5N8 in Iraq

Muhammed Abdul-Kadhim[1], Hayder A. Muhammed[2], Ali K. Aljarah[2]

[1]Lecturer, Pathology & Poultry Disease Branch, [2]Assist. Prof., Microbiology Branch, College of Veterinary Medicine, University of Kerbala

Abstract

The Avian influenza (AI) is aspreadableinfection of the birds and humans affected by type A of influenza virus. Highly pathogenic avian influenza (HPAI) caused by the viruses subtype (H5N1) and (H5N8) is consider as a definite risk to the poultry industry and human health and it is started as the occasional revolutionize a satisfactory because of migratory bird which consider as a carrier of the disease. Twenty oropharyngeal swabs were collected from infected flock have suffer from sudden death with high mortality, all these samples were collected from (1-5) days after clinical signs appears and the results summarized by three farms infected with HPAI subtype (H5N8), three viral isolates strain out of 20 samples were identified by RT-PCR assay, two of them have layer origin while one have broiler origin, the three sequencing results in this study have been presented to the gene bank database under accession number MN689674.1 and MN658854.1, phylogenetic analysis of our sequences were used to relatedness of strain obtaining with other avian influenza viruses around the world, the a current sequences of avian H5N8 strains were found to be related as a common ancestor with Iranian registered strain.

Keywords: *Avian influenza H5N8, RT-PCR, degenerate primer, phylogenetic tree.*

Introduction

The Avian influenza disease is a word that used to define influenza viruses type A and it is separate from a wide-ranging of birds species all over the world and mammals[(1)]. Recombination between AIV strains with mammalian derived influenza strains may be take place and that producing a new recombinant influenza viruses have the ability to initiating disease in humane and other species in the same time wild birds, waterfowl and shorebirds consider important source of infection by influenza A viruses and producing important risk for this disease[(2–4)]. AIV can be divided according to inducing pathological lesion in the natural host in to two groups: high pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI), the infection with HPAI strains (H5N1, H5N8) categorized from slight to heavy sickness with high death rate may arrived to 100% in infected flocks [(8)].

AIV was multiplication in the respirational and gastric tract system of sick birds, so the suggesting of isolation of the infections (H5N8) from oropharyngeal swabs, tracheal swabs, cloacal swabs and feces[(5)].

In Egypt HPAI subtype H5N8 was diagnosed since 2016 in both commercial and backyard bird with high mortality and there are six genotypes have been detected in both wild and domestic birds[(5–7)].

During the 2015-2017 HPAI subtype H5N8 widely spread throughout Europe, Asia and Africa and seasonal migration of wild aquatic birds and water birds consider the important source for vector of disease, so that the infected birds were shed the viruses through the rivers, marshes and lakes, eventually reached the poultry flocks[(9–11)]. Interestingly, the H5N8 remained as localized sporadic active infection during the spring, summer and autumn of 2017 in Iran and Italy While, HPAI infection become epidemics in winter and early spring[(9)].

The World Organization for the Animal Health (OIE) in 2018[(22)] was established the HPAI (H5N8) in Iraq infected poultry with high sickness and death rate, In the last years poultry industry in Iraq suffering from high morbidity and mortality with sudden death and that lead to high economic losses in addition, the risk

of this disease on human health, so there are a doubts around this farm infected with HPAI subtype (H5N8), so that this study was designed to diagnosed HPAI subtype (H5N8) by using molecular technic test and phylogenetic analysis with other avian influenza virus sequences from different geographic area due to limited research has been conducted to determine HPAI subtype (H5N8) virus in Iraq.

Materials and Method

Sample Collection: The study was conducted by collection samples from chicken broilers and layers in different unorganized cities of Karbala province,the study was carry on just birds which have suspected clinical respiratory sings and have covered with mucosa congestion in un feathered regions and highly mortality. Within 24 to 48 hours after onset symptoms oropharyngeal swabs were collected from these chickens by sterile ice boxand recently sent to the lab as soon as possible. The course of this study was beginning from February 2018 to January 2019.

RNA extraction procedure: RNA pellet was extracted from oropharyngeal by using (QIAamp Viral RNA Mini Kit/QIAGEN) Germany. The RNA extraction of avian influenza was done by Qia tube connected automated purification system according to manufacturecompany, Interestingly, the extracted RNAwas eluted in 50 µl of DEPEC water and calculated by Nano drop-spectrophotometerdevice,and immediately store in -30C at freezingforRT-PCR analysis[23-32].

Reverse Transcriptase Polymerase chain reaction assays and Primer designing: RT-PCRassays were done for diagnosis avian influenza virus, dependedonHemagglutinin glycoprotein gene by using specific degenerate primers. These primers were designed using influenza genomic sequences database available in gene bank, and retrieved by multiple alignment, theses set of primers aremade by (Bioneer/. Korea) as following table (1).

Table (1): Theprimerused in this study with amplicon product size

Primer	Sequence	Amplicon size
Hemagglutinin glycoprotein gene	5-ATCATCCCCARRRRTTCTTG -3	1132bp
	5-GAYTACCCBMARTATTCAG -3	

A total of 5 µl of cDNAs were added to new twenty micro liter of Master mix containTaqPCR Master Mix Qiagen/germany contains Taq DNA Polymerase, the exclusive QIAGEN PCR Buffer that reduces the necessity for optimizing process, and dNTPs, amd 0.5 µm of degenerate primers, The reaction was done in a Thermal cycler system (Mygene Bioneer) using the following protocol : 95ºC for 5 minutes followed by 35 cycles consisted of 94ºC for 35 second, 57ºC for 45 second and 72ºC for 35ºC second as well as final extension 72ºC for 5 minutes, The products were examined by agarosegel electrophoresis on a 1.5% gel under UV illumination[23-32].

DNA sequencing method and Data analysis: Genetic sequencing of Haemagglutinin glycoprotein gene by using analysis of phylogeneticrelationship and study level of alignment by mega multiple sequence software alignment programs a product was purified from thegel by using (QIA quick Gel Extraction/Qiagen). The purified PCR product was sent to Korea for high quality DNA Sequencing service by Macrogen providing techniques (https://dna.macrogen.com/eng/support/ces/guide/ces_sample_submission.jsp). The nucleotides' Sequences were truncated and aligned at both ends using ApE software (A plasmid editor Version 2.0.51); therefore the sequences majority were started and ended at the homologous nucleotide positions. The sequences target was submitted to BLAST (http://www.blast.ncbi.nlm.nih.gov) (Zhang et al., 2000).the nucleotides with diversity index as well as the Euclidean distances calculated were uploaded to the UPGMA (clustering) tree and the Maximum Parsimony phylogenetic tree by using the software MEGA 6 (Molecular Evolutionary Genetics Analysis Version 6.0),

Results and Discussion

Avian influenza is a very contagious viral disease among poultry flocks;the results showed that chicken were infected with highly pathogenic avian influenza by note systemic disease with multiple organ failure, we found lesion of cyanosis and edema of the head, comb wattles (figure 1)and red discoloration of feet and shanks due to subcutaneous ecchymotic hemorrhagic (figure 2). In chicken, medicalmarksreveal the virus duplication and harm to numerous visceral structures and circulatory, nervous systems and integument. Precisemedicalappearanceshang on the level of injury and which structures or organ systems are affected. In the severe stage, the results found clear clinical signs other than listlessness, closer observation of 3 obtaining chicken has discovereda reduced activity; decrease sensitivity to stimuli, reduction in "in-house" noise, dehydration, and decreased feed and water intake that rapidly progressed to severe listlessness and death[12] figure 1.

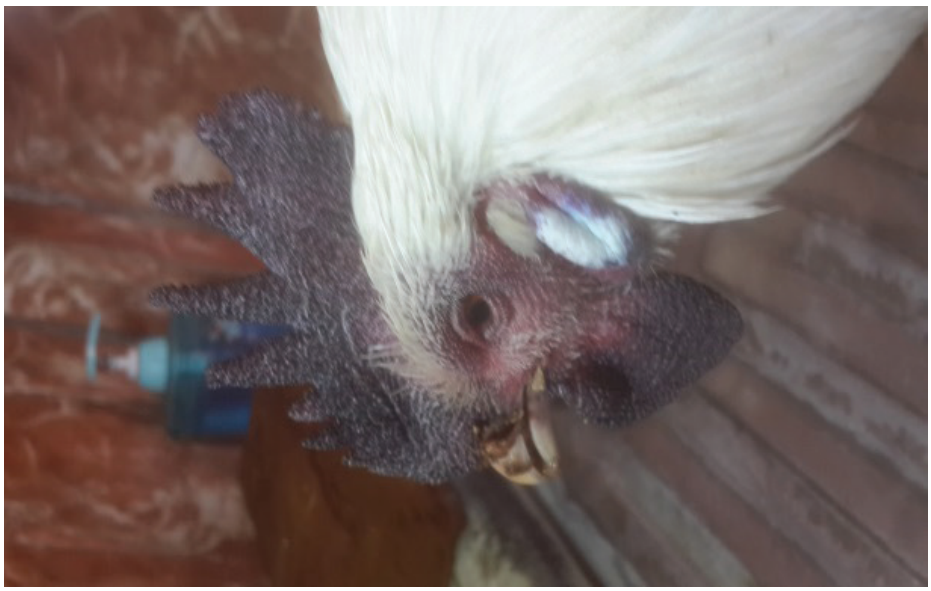

Figure 1: head of infected bird suffer from conjunctivitis with congested comb and wattles (cyanosis)

Figure 2: subcutaneous ecchymotic hemorrhagic of legs

The medicalsigns of disease are particularly flexible and depend on other aspects including host species, age, sex, concurrent infections, acquired immunity and environmental factors[8]. the prevalence of H5N8 virus were revealed as 15% (3 out f 20) suspected viral samples in oropharyngeal swabs in different unorganized chicken farm field by using RT-PCR assays through amplification and visualization on agarose gel electrophoresis 1132 bp in length of HA glycoprotein gene figure 3.

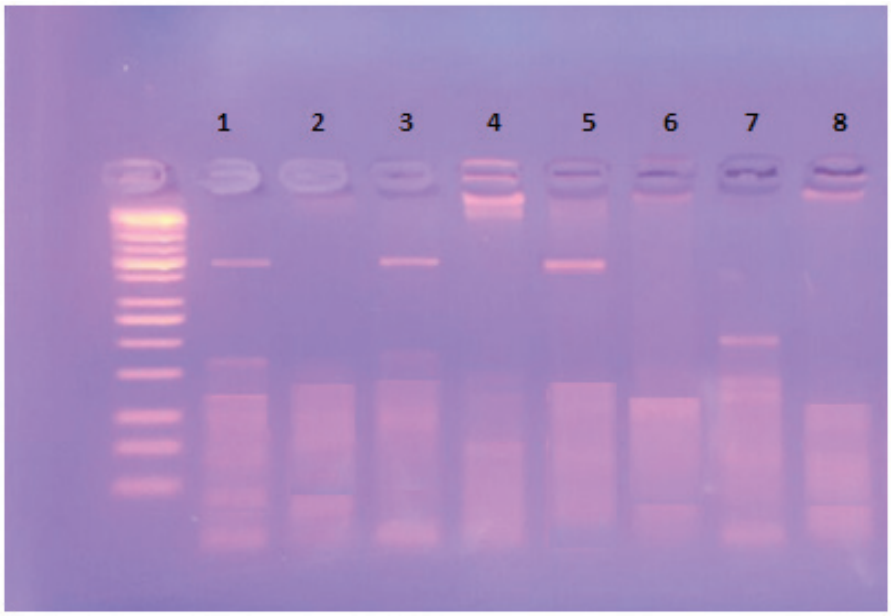

Figure (3): Agarose gel under UV light thatshows the RT-PCR product analysis of hemagglutinin gene in avian influenza virus H5N8 isolates. M: marker (range between 100 to 2000bp), lane (1,3 and 5)wells showed positive results of avian influenza virus strain.

The Iraqi viral isolates shared features characteristic for recent HPAI virus H5N8 isolates,the genomic sequences of 3 isolates avian influenza strains were verified by

dual sets of degenerate primers, two of them have shared 100% no identity with linear genomic product 1130 bp in length figure 1, except one of strain have different in some nucleotides from two previous strain, the three sequencing results in this study have been transduced to the gene bank database under the accession number MN689674.1 and MN658854.1, a neighbor-joining phylogenetic tree based on alignment of the deduced amino acid sequences of the H5 Haemoagglutingene showed that 3 isolates iniraq country has the same multiple basic amino acids at the connecting peptides of cleavage site between HA1 and HA2 (PLREKRRKR▲GLF) with other some recent strain isolates of different geographical distribution cities in Asian, European and American group, on this basis, it was considered that those strain high pathogenic avian influenza virus[13] figure 4.Interestingly, Phylogenetic analyses of HA gene fragments from three strain indicated that most of the sequences obtaining from distinctgeographic regions of world were closely related and cluster in to distinct branches based on geographic origins, the current study noted that two sequence obtaining are separated by a distinct strain originated from Iran have accession number KY701529.1figure (5)[14], the current study was limited to used reverse transcriptase Real time PCR and sequences analysis of Avian Influenza virus, otherwise, there are many method should be consider to confirm this strain by epidemiologic, Immunological results by hemagglutination-inhibition (HI) test, where the amount of antibody titers can be detected by using inhibition activity overthe agar gel immunodiffusion (AGID) and enzyme-linked immunosorbent assays (antibody detection ELISA tests)[15,16]. Otherwise, the

incidence of AI virus can also be definite by the use of reverse –transcription polymerase chain reaction (RT – PCR) or by the examination of a commercially offered immunoassay kit particular for type A influenza, e.g. Directigen TM Flu A[17] or Flu Detect®[18, 23].

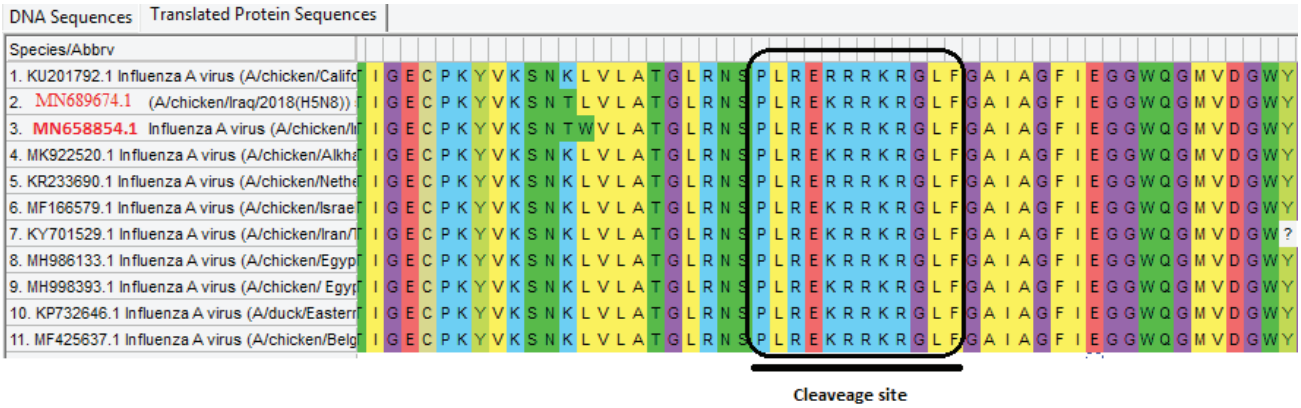

Figure 4: Multiple sequence alignment of two stain isolates with other sequences around the world showed the position of cleavage site

The phylogenic of *H5N8 has* been making Homology sequence identity by userRNA gene according to NCBI-BLAST site show table (2) highly sensitivity to accuracy of molecular detection. Analysis of Phylogenetic tree has donedepend on the clone Hemagglutininglycoprotein, a that used for final detection of H5N8draw a tree by Phylogenetic analysis of Hemagglutin in glycoprotein gene sequences has become the principal method for knowing avian influenzaphylogeny**.**Ourresultshowthe phylogenetic tree has done according to these strains isolates figure (4), the evolution histories of *H5 N8* were contingent using the Neighbor-Joining method [20].

Figure (5): Phylogenetic tree wascreated by using method named (UPG) Unweighted Pair Group with in (MEGA software 6.0 version), the red arrow showed the present of our sequences.

Our results were found that Avian influenza H5 N8 in the polygenetic tree diagram with anaccession number (MN658854.1), a species identified in layer,was the next-highest match at 100% homology with our sequence[21, 23].

Finally, The host species; the geographical factors and environmental conditions are main causes to formed contract values pheromones. We also found Avian influenza H5 N8 genomic sequences showed identical with other species sequences that isolates according to the geographical regions, the present study was found identical nucleotides of Avian influenza H5[21].

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Conflict of Interest: None to declare.

Ethical Clearance: All experimental protocols were approved under the College of Veterinary Medicine and all experiments were carried out in accordance with approved guidelines.

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