Molecular Characterizations of a High Pathogenic Avian Influenza H5N8 in Iraq

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Abstract

High pathogenicity avian influenza subtype H_5N_8 viruses were detected in different areas in Iraq at the last 2017 and early 2018 and 2019 and the disease was detected clinically. This disease is responsible for high economic losses for poultry industry and threat human health, so that, this study was conducted for molecular detection, characterization and phylogenic analysis of avian influenza in Iraq. AI subtype H_5N_8 is an infectious disease primarily in birds and responsible for severe respiratory illness which associated with a high percentage of morbidity and mortality in wild and domestic birds. During this study one hundred fifty different samples including (trachea, larynx and lung) were collected from different areas of broiler chicken from Baghdad and its surrounding regions during winter 2019. Avian influenza virus subtype H_5N_8 was detected by using real time RT-PCR technique, and specific kits (Kylt^R Germany) for AIV subtypes H_5 and N_8 were used respectively. The results revealed that (15) samples out of (150) collected samples were gave positive results for avian influenza H_5 these positive samples were prepared for a second step of detection by RT-PCR test specific for N_8 subtype the results revealed that only (12) out of (15) tested samples were positive for H_5N_8 (8%).Genetic sequencing of isolates and phylogenic analysis of three selected isolates of (H_5N_8) bellowing to different areas indicate that all strains bellowing to H_5 class (2.3.4.4) high pathogenic avian influenza revealed that they are closely related to Egyptian strain (A/ duck/ Egypt/ F446/2017. H_5N_8-MH893737.1) (with 97.6% identity). Analysis of the mono-basic amino acid (PQIEPR / GLF) at the hemagglutinin cleavage site revealed there is no deletion of the stalk region with the neuraminidase indicated that the isolates is a typical HPAI strain (A / duck / Egypt / F446/2017). The similarity of the nucleotide sequence analysis of hemagglutinin gene revealed that there was a high homology (97.6%) to that of A/duck/Egypt/F446/2017 H_5N_8 .

Keywords: *Avian influenza, Bird flu, Fowl plague, H_5N_8 avian flu*

Introduction

Viruses of avian influenza are single-stranded RNA viruses, negative sense, segmented viruses classified by the *Orthomyxoviridae* family into groups A, B, C and D based on variations in their matrix proteins, internal nucleoproteins and antigenic characteristics, Influenza type A are only viruses have been known to have the ability to cause natural infections in birds based on their frequency and potential to cause illness in poultry[1]. Influenza A / H5N1 was initially isolated from a Chinese goose in 1996. Humans infections were first recorded in Hong Kong in 1997 [3].

Informally, the avian flu was known as fowl plague or avian influenza and these viruses triggered a variety of influenza that adapted in birds [4]. Avian flu is related to dog flu, swine flu, horse flu, and human flu, a disease caused by influenza virus strains that can be adapted in a specific species. Of the four influenza virus types (A, B, C and D), the influenza virus type A is a zoonotic disease and has an almost complete natural reservoir in avian[5]. Influenza viruses are typically categorized as surface proteins into a broad range of subtypes based

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on 18 hemagglutinin HA (from H_1 to H_{18}) and 11 neuraminidase NA (from N_1 to N_{11}), some of which pose a threat to public health[6].

Numerous specific subtypes 16 hemagglutinin subtypes from H_1 to H_{16} and Nine neuraminidase subtypes from N_1 to N_9 bellowing to the avian flu viruses, but the $H_{17}N_{10}$ & $H_{18}N_{11}$ subtypes which are found in bats only[2]. Broad distribution of avian influenza viruses in different hosts may result in the exchange of their gene or gene fragments, which in turn contributes to highly antigenic variation and potential development of new strains of avian influenza viruses. This can cause significant epidemics and outbreaks, lead to tremendous economic losses in the poultry industry and may also pose a serious threat to human health[6]**.** The hemagglutinin gene plays a crucial role in the of influenza virus A life cycle, its involvement in recognition of receptor, attachment of virus particles on host cell, e fusion of membrane and entry inside the host cell[7].

In 2014 the influenza/goose /Guangdong (1/1996) lineage clade (2.3.4.4) H_5N_8 avian influenza viruses with a high pathogenicity were originated in poultry and wild birds throughout Europe, Asia, and North America. At this time, the wild birds in the Netherlands were extensively investigated for HPAI H5N8 virus (real-time PCR targeting the M and H5 genes) and antibody detection (inhibition of hemagglutination and neutral virus) [11]**.** The rapid detection by RT-PCR of AIV subtypes H_5 has a crucial role for control of avian influenzas disease infection, Pathogenicity of avian influenza infection was varies greatly depending several factor as a host species, virulence of strain, infective doses, and infection routs[12].

In Iraq, according to OIE ($H_5N_{8)}$ struck again on a commercial farm near the Baghdad metropolitan area and it were killed 13,240 susceptible birds out of 29,000. The survivors were pulled out to control the virus spread, this detection makes Iraq's sixth ($H_5N_{8)}$ outbreak during 2018. avian influenza viruses with a high pathogenicity was identified during the last few years in several governorates and the disease was established at 2018 by the organization for animal health in the infected poultry with high sickness and high mortality rate, this reason lead to high economic losses for poultry industry with possible risk for human health[13]. Continuous surveillance of HPAI H_5 in Iraq is necessary to avoid economic losses in poultry industry and detection of outbreak points, so this study is the first to identified high pathogenic avian influenza H_5N_8. And it is aimed for genetic characterization and sequencing and phylogenic analyses of AI subtype H5N8 and study the genetic variation between isolates, for these reasons this study was done.

Materials and Methods

Samples collection and screening:-

Between December 2018 and October 2019 , 150 samples including (trachea, larynx and lung) were collected from different areas from(Baghdad 75 samples, Dialla 42 samples, and Hilla 33 samples) from suspected poultry farm which given symptoms of avian influenza illness. These farms show a different symptom in poultry as ocular and nasal discharge, sneezing, enlarged infra-orbital sinuses. The mortality rate varies from 10% to 65% as well as subcutaneous hemorrhages, petechial hemorrhages on visceral organs and muscles, extreme inflammation, edema and red discoloration of the shanks and feet. Greenish diarrhea was common in badly affected birds, paralysis, and drooping of wings. These samples were collected in sterile container and send to specific diagnostic laboratory for confirmative RT-PCR test.

Samples preparation and RNA extraction:

A part of collected samples were pooled in a sufficient volume of sterile buffer (1 ml of normal saline), another parts were preserved for molecular and histopathological tests. Samples were soak for an adequate period of time and finally wash out the sample by plus vortexing, RNA from samples were extracted from tissues by using kit of the total RNA extraction (Kylt^R RNA extraction Kit) after extraction the RNA was elute in 60μL of RNase-free D.D water, then store after adding 20 U of RN as inhibitor at (-80 ºC) until using for Real-Time TR-PCR.

Subtyping of isolates by using Real-Time PCR:

To differentiate influenza isolates from other non-influenza. real-time RT PCR specific for influenza A virus that amplifies the cleavage site of hemagglutinin gene coding sequence which performed using total of 800 bp RNA according the WHO guidelines[8]. Then the

positive isolates for influenza A test were further tested to detect the **H_5** by using Real-Time PCR, then using a positive samples for detect the type of **N_8** gene according to instruction of manufacturer company (AniCon Labor GmbH)^R Kit (Germany).

Sequencing and phylogenetic analysis (incl. comparison to NCBI database):

A 800 bp fragment containing the cleavage site of the HA gene of Influenza A type H_5N_8 has been sequenced and was phylogenetically analyzed based on comparison to known reference strains by(AniCon Labor. GmbH Emstek, Germany). Influenza viruses were known via a database that was created by downloading influenza virus sequences from the (NCBI) information. Then, tested in the influenza virus database by Basic Local Alignment Analysis Tool for nucleotides (BLASTn).

Results

Molecular detection of avian influenza H_5N_8 and Real-Time PCR:-

From about 150 collected samples 15 positive samples that mean an avian influenza virus was present in many area of country in this collection period. In real-Time PCR 15 positive samples of avian influenza A virus and only 12 samples were give a positive result for H_5N_8 (about 8%) by using H_5 kit and N_8 respectively from(Kylt^R) company for veterinary diagnostic kits.

Based on the amplified sequence of nucleotide coding for the hemagglutinin glycoprotein of Influenza A Virus (type H_5), the RNA extracted from sample numbers A1910987.001, A1910987.002 and A1910987.003 belongs to HPAI-H_5 Clade (2.3.4.4) and is most related to strain A/duck/Egypt/F446/2017(H5N8) (MH893737.1) (each 97.6%).

Table 1: Results of Real-Time RT- PCR specific for influenza A-H_5.

Site ID	Sample ID	Assay Result	Sample Type	Ct	Endpt
A1	1T	Positive	SPEC	21.5	756
A2	2T	Positive	SPEC	29.6	486
A3	3T	Positive	SPEC	29.4	420
A4	4T	Positive	SPEC	32.4	320
A5	5T	Positive	SPEC	26.6	661
A6	6T	Negative	SPEC	0	1
A7	7T	Positive	SPEC	35.1	225
A8	8T	Positive	SPEC	38.1	69
A9	9T	Positive	SPEC	37.4	116
A10	10T	Negative	SPEC	0	1
A11	11T	Negative	SPEC	0	2
A12	12 Lung	Positive	SPEC	36.2	143
A13	13 Larynx	Positive	SPEC	38.1	85
A14	14 larynx	Positive	SPEC	21.7	88
A15	15 lung	Positive	SPEC	18.4	82

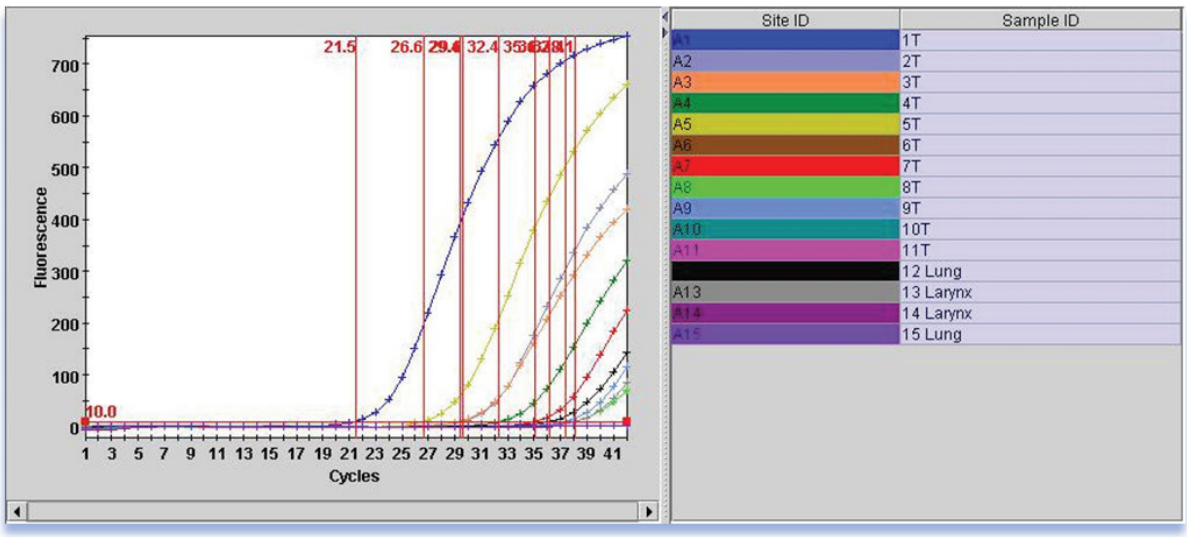

Figure 1:- Real Time PCR amplification of RNA from samples infected with avian influenza H_5.

Table 2: A positive samples that give high titer of H_5 (and low CT value) were selected and used for detection of N_8 by using specific kit for neuraminidase :-

Site ID	Sample ID	Assay result	Sample type	Ct	EndPt
A9	1T	Positive	SPEC	18.3	482
A10	2T	Positive	SPEC	26.5	294
A11	3T	Positive	SPEC	29.2	145
A12	4T	Positive	SPEC	32.2	76
A13	5T	Positive	SPEC	28.2	181

Table 3: Sequencing and phylogenic analysis (incl. comparison to NCBI database):-

Method: H- & N-specific Real-Time RT-PCR (Kylt® Influenza A - H5 / N1) (a)

Sample No	Sample description	CT.H5	CT.N1	Result
A1910987.001	FTA-card(spot 1)	24.3	-	H5 positive
A1910987.002	FTA-card(spot 2)	31.8	-	H5 positive
A1910987.003	FTA-card(spot 3)	31.8	-	H5 positive

H- & N-specific Real-Time RT-PCR (Kylt® Influenza A - H5 / N1) (a).

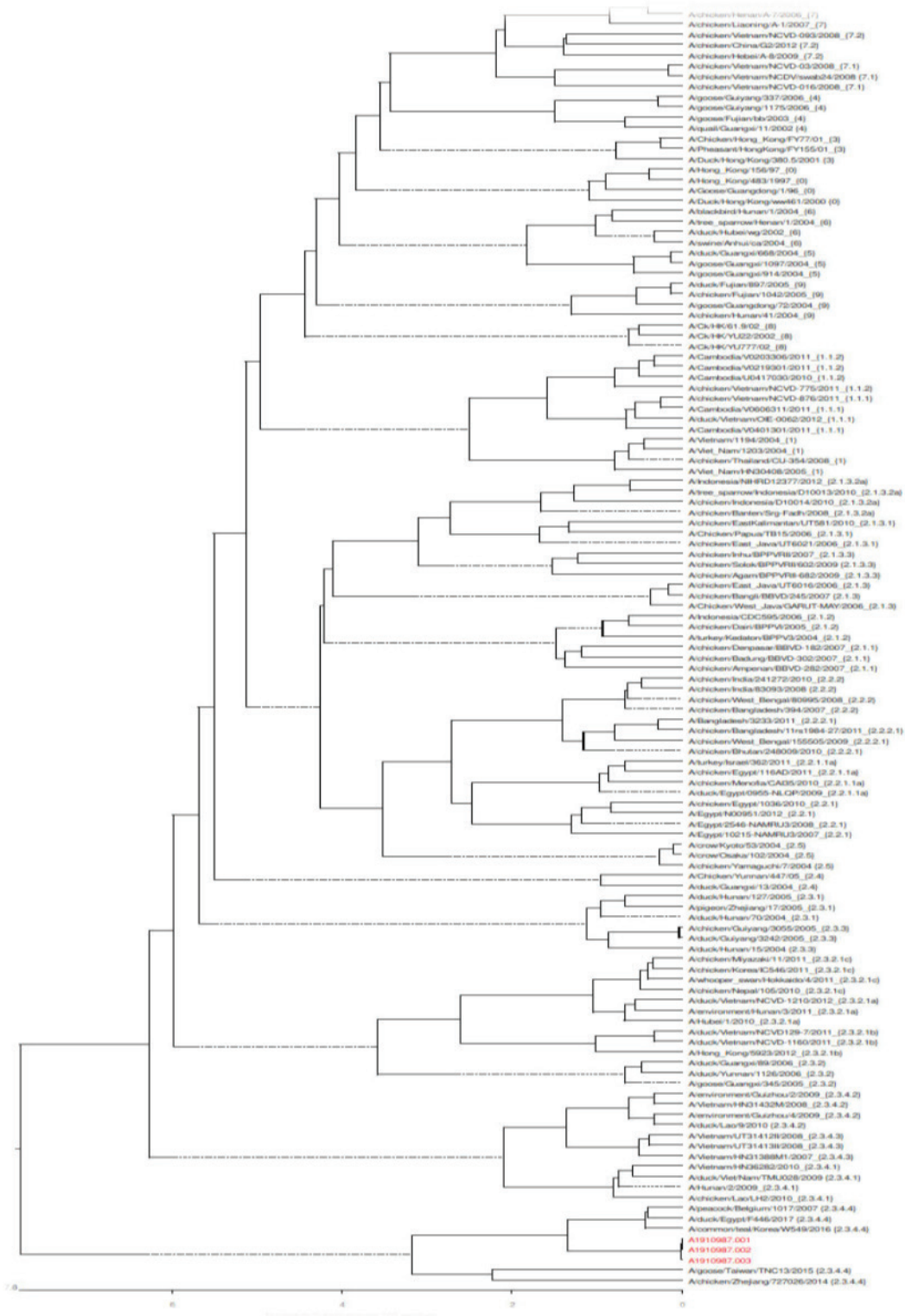

Figure 2: explain the phylogenetic analysis and sequences hemagglutinin of influenza A virus subtype H5N8 of the detected tissues samples from birds in Iraq. The phylogenetic tree was built using the neighbor-joining method. Within the research was included the representative of viral sequences which were quite close to those recorded in this review. These labels the H5N8 viruses identified in this study. viral sequences are provided. Scale bar indicates estimated genetic distance belongs to HPAI H5 Clade 2.3.4.4 and is most related to strain A/duck/Egypt/F446/2017(H5N8) (MH893737.1) (each 97,6 %).

Discussion

Because of lack the continuously surveillance of avian influenza subtype H_5 in some countries, therefore H_5 avian influenza viruses are still circulating in different areas of the world[9]. So that, continuous surveillance of the domestic and wild birds and annually genomic analysis of high and low pathogenic avian influenza viruses by different methods such as next-generation sequencing is recommended due to the high mutation rate of influenza viruses which has been determined by the sequencing[10]. Real time RT-PCR technique was used to detect avian influenza from collected samples by amplification of hemagglutinin gene. The results of sequencing of three selected positive local isolates (A1910987.001, A1910987.002, A1910987.003) for hemagglutinin gene by obtaining A 800 bp fragment containing the cleavage site of the HA gene of Influenza A type H_5 was disagree with[14] which was found that H5H8 isolated in this year was bellowing to clade 2.3.4.4 group B and clustered with isolates from Iran and Belgium. The genetic sequences of isolates indicated that there were a similarity between the local isolates and isolate from Egypt and Saudi Arabia. Genetic analysis of AIV H_5N_8 virus was indicates that the virus probably reach to Iraq by wild birds migration due to the position of Iraq as a road of migratory pathway of birds, other several outbreaks of AIV (H_5N_8) virus infections was reported in Egypt, Iran and Saudi Arabia at the end of 2017 and 2018 and it cause exponentially increasing of mortality in flocks of birds with high economic losses. The location of isolates at the end of phylogenetic tree is fits with the hypothesis route of (H_5N_8) virus was introduce into Iraq via the migratory bird but not through the direct contact with infected birds, because the viruses when detected there is no active direct trade and contact with other infected countries and before this time the virus was never been detected in the country. In conclusion the Iraqi HPAI H_5N_8 isolated detected in this study were belonged to (A/duck/Egypt/F446/2017(H5N8) with (97.6%) identity and it may contain virulence-enhancing properties to infect the mammalian host as a result of its continuous reassortment of it is genes, for this reasons the identification and monitoring of both low and high pathogenic viral strains is important for the early management and control of avian influenza viruses. Although the AI H5N8 virus was not detected so far in humans, we should remain aware of the potential of this virus to be transmitted from the avian host to the human population.

Ethics Approval and Consent to Participate:-

The study was conducted following the ethical guidelines of the Animal Care and Use Committee at the College of Veterinary Medicine, University of Baghdad, and consent to collect samples from the infected animals was provided by the owner of the farm. After confirmation that influenza infection has spread in the farm.

Source of Funding – Self

Conflict of Interest – Nil

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