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Identification Of Common Genotypes Of Avian Influenza Virus In Iraq (Najaf And Al-Diwaniyah Governorates)

Sarah Mazin Razoqi
Assistant Prof. Dr. Israa Najm Abdullah Al-Ibadi
University of AL-Qadisiyah College of Veterinary Medicine Department of Pathology and Poultry Diseases
smazin038@gmail.com
Esraa.najm@qu.edu.iq

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Abstract
Avian influenza (AI) is Viral respiratory disease caused by RNA virus type A orthomyxoviruses . Avian influenza was a zoonotic disease, has caused several epidemics in domestic fowl , The different degrees of pathogenicity demonstrated by viruses in diverse avian species, as well as the diversity of viral properties such as antigens and genes, make it difficult to reliably diagnose viral infections in poultry or wild birds. This is because viruses can infection
many bird specie without clinical signs ,The different degrees of pathogenicity demonstrated by viruses in diverse avian species, as well as the diversity of viral properties such as anti gens and genes, make it difficult to reliably diagnose viral infections in poultry or wild birds notably the latest H5,H7 highly virulent avian influenza viruses. Aims of the study are; Identification of AI infection in the broilers , layer, geese and Gallinula in Diwaniyah and Najaf governorates. Fourty suspected birds for each avian species were investigated which are taken from local market, ,veterinary clinics and poultry farms in the area, then rapid test were performed using the AIV Rapid kit. samples were taken randomly from (lung and trachea) , samples kept in Eppendorf tube contain 1.5 ml trizol , from which the RNA was extracted by RNA extraction kit and RNA converted into CDNA , this last product was entered into a real-time PCR with Universal Primer NP to detect the infection, Special primers were designed to identify possible subtypes (H5 primers,H7 primers.H9) .
Results of rapid test kit revealed that only 5 samples shown positive results between others.
Twenty samples were positive in PCR test against NP primer. From these eight samples were positive against H5 and twelve were positive against H7 primers consequently and were negative to H9 primers , all above samples were taken from broilers.
layer, geese and Gallinula birds were negative to all primers despite having upper respiratory signs .Aims of the study Identification of AI infection in the broilers and Gallinula in Diwaniyah and Najaf governorates . by np primer /PCR and Identify the genotype which is diagnosed in each avian species. By H5 H7 H9 primers /PCR.

Introduction
Avian influenza refers to an infection or disease caused by a Type A influenza virus from the Orthomyxoviridae family .It is both infectious and zoonotic, that infects birds andMammals,
The principal reservoirs for influenza A viral genes were Aquatic species wild ducksthe . influenza viral can infect both domestic chickens and a variety of other birds., Avian influenza has a profound impact on both public and veterinary health. sporadic but severe outbreaks of sickness inlate 19th

century with the most severe instances affecting small children and the elderly(**1**)(2) . caused by RNA virus type A orthomyxoviruses These viruses exhibit significant genetic diversity, and new strains arise via both mutation and reassortment. They are characterized by having nucleoprotein and matrix internal proteins that are antigenically similar (3), (4). avian influenza virus subtype H5N1 was first identified in Hong Kong in 1997. Recent outbreaks of a pandemic in Asia, Europe, North America, Oceania, and Africa have prompted concerns about its rapid spread and the virus conversion LPAI to HPAI (6) The potential of H5N1 to vary through mutations and reassortment certainly raises the possibility of viral adaptation to the human species, even though the virus has not been able to sustain human-to-human transmission(7). Planning for a pandemic of influenza requires a high level of awareness that is required to contain the virus's initial outbreaks by using case recognition, sensitive and quick diagnostic techniques, suitable treatment , Antimicrobials may aid in the prevention of Secondary bacterial infection occurs in flocks infected with low-pathogenicity virus strains (8). The viral genome is detected, particular antibodies are used to make the diagnosis, or the virus is isolated. (9). Avian influenza viruses that ex hibit low pathogenicity (LPAI) has the ability to undergo genetic changes and transition into High-pathogenic viruses(HPAI) (15). Poultry-infecting viruses of the influenza type may be categorized Divide into two distinct groups. Virulent viruses generate highly pathogenic avian influenza (HPAI), which has the potential to cause the death of whole flocks. These viruses only target the H5 and H7 strains. Nevertheless, not all H5 and H7 viruses result in the development of highly pathogenic avian influenza (HPAI). Instead, some simply lead to a less severe respiratory infection referred to as low pathogenic avian influenza.(LPAI) (10). Avian influenza may manifest a range of symptoms in birds, ranging from mild illness with little or no observable indications to severe sickness that can rapidly escalate to a lethal condition and result in a significant outbreak. Highly pathogenic avian influenza, caused by viruses of the H5, H7, and H9 subtypes, is marked by severe symptoms and rapid progression leading to death. The mortality rate may exceed 100% within 48 hours. Highly pathogenic strains may cause severe respiratory illness in people. (11). Birds primarily transmit viruses by inhalation or ingestion, as well as direct contact with respiratory secretions, fecal material, contaminated food or water, bird droppings harboring the virus, and infected items (10) (12). Certain avian species, such as ducks, exhibit greater resistance to infection compared to other species. Ducks may be infected by pathogenic strains of influenza A viruses (IAVs) that do not cause apparent clinical indications. As a result, ducks Influenza A virus (IAV) reservoirs are natural hosts that facilitate viral reassortment (13). Quail are a species known for their propensity to act as an amplifying host (14)(15).

MATERIALS AND METHODS

Collection of the samples:

Tissue samples (trachea, pharynx and lung) were collected from suspected birds (Gallinula , Geese ,layer and broilers) Samples were collected from market ,veterinary clinics and local Poultry farms from Najaf and Diwaniyah Governorate, depending on case history, clinical signs , gross lesions , and p.m lesion and rapid examination was performed using the AIV Rapid kit .All samples were collected from October 2023 to January 2024. we collected 40 birds were collected from each species.

Tiny pieces of the organs were cut - approximately 10mg and placed in eppendorf tube containers and Trizol 1ml were added and stored in (- 20 C) and prepared for RNA extraction,Tissue . The samples were then transferred in a coolbox to the laboratory. Upon arrival to the laboratory the samples were centrifuged and the added Trizol was discarded and tissue processing was carried out . Tissue samples undergone grinding and prepared for RNA extraction

The process of creating and increasing the amount of complementary DNA (cDNA) was carried out utilizing the EasyScript one step gDNA removal and DNA amplification method. synthesis supermix as following
RNA Mixing primer with waterThe mixture was incubated for 5 minutes at 65°C and 120 seconds on ice.
. then other components Extracted RNA and all reagent were dissolved on ice ,All solutions were shaken with vortex. 2 µl RNA + 10 µl reaction mix which contains RNase in hibitor, (dNTPs), and balanced concentration for Oligo (dT) and random primers + up to 19 µl Nuclease-Free H2O have been mixed. At 65 ° C, the mixture was heated for 5 minutes, and then it was Subjected to incubation in ice for a minimum duration of one minute. A short centrifugation was conducted to gather the components that may adhere to the inner surface of the tube. A volume of Reverse transcriptase (1 µl) was introduced into a 20 µl solution and gathered through brief centrifugation. The tube was placed inside a 42 °C incubator for a duration of 50 minutes.
Table 4.Kit components for PCR reaction

Component	Volume
2xES reaction mix	10 µl
gDNAremover	1 µl
Random primer (0.1µg/µl)	1 µl
RNA	5 µl
RNase-free water	2 µl
RT/RI enzyme	1 µl

		Product size (bp) Amplification fragment (bp)
NPFa	TGTACGGACTTGCTGTGGCC	106
NPRa	GAGACTGAAGACCTGGCTGTT	
H5F	ACAAAGCTCTATCAAAACCCAAC	499
H5R	TACCCATACCAACCATCTACCAT	
H7F	CAGGCGGAATTGATAAGGAG	409
H7R	TGCCCCATTGAAACTGAAAG	
H9F	ATCGGCTGTTAATGGAATGTGTT	221
H9R	TGGGCGTCTTGAATAGGGTAA	
The NPF and NPR primers bind at nucleotide positions 839–858 and 925–945, respectively, based on GenBank accession no. DQ251452.1.		

Table 5.PCR product

No.	Component	Volume
1	PCR master mix	25µl
2	forward primer	3µl
3	reverse primer	3µl
4	Cdna	10µl
5	Nuclease-free water	8 µL
Total		50 µL

The master mix is form from taqpolymerase ,Ntpa (A,T,G,C) and buffer (mgcl).

1. Agarose gel produced by dissolve the agarose 1.5 g in 100 ml of 1XTBE and then allow mixture to cool to 45–50°C.
2. Next, 0.5µl of Ethidium bromide stain was added to the solution of agarose gel. 3. After adding the agarose gel solution to the tray and positioning the comb correctly, it was allowed to harden for half an hour at room temperature. After that, the comb was carefully taken out of the tray.
4. After The gel tray in the electrophoresis chamber was filled with 1X TBE buffer.

5. Each comb well received 10 µl of the PCR product and 10 ul of the 100 bp ladder.

Results

Trachea, pharynx and lung samples were collected from suspected Gallinula , goose and broilers chicken Samples were collected from market ,veterinary clinics and local Poultry farms collected randomly from different areas from Najaf and Diwaniyah Governorate, depending on case history, clinical signs , gross lesions , and p.m lesion , All samples were collected from October 2023 to January 2024 In this study a total of 40 chickens broiler and /or layers were clinically examined have respiratory signs swollen head , Symptoms include ocular discharge, fatigue and despondency, wing drooping, and leg dragging. The symptoms include edema and cyanosis of the comb and wattles, hemorrhages and erythema on the legs, and subcutaneous bleeding on the neck. . we collected suspected 40 Gallinula birds from market Najaf and Diwaniyah Governorate and we collected suspected 40Geese .

The birds are healthy there are no apparent clinical signs and Postmortem no pathological lesion , PCR diagnosis were negative .

AIV Rapid test diagnostic results

The AIV rapid kit field examination revealed that only 5 samples showed positive results, while the negative result was only found at the C line.

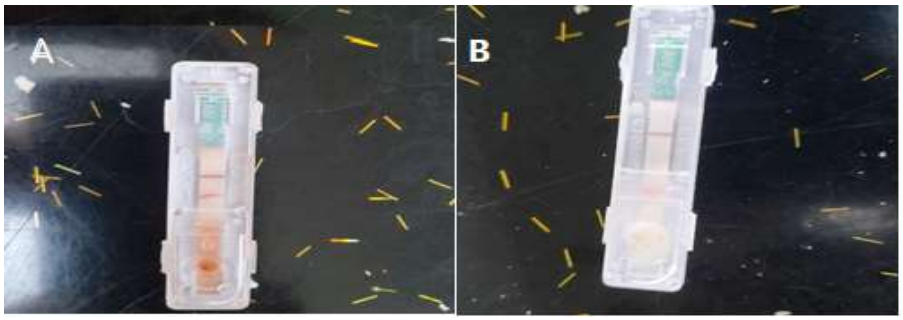

Figure 5. Rapid kit result.

a- positive, two lines at C (control) and T (test) , b- negative, one line at C (control)

4.2.Results of Molecular examinations

Results of PCR

Twenty sample PCR product were loaded in agarose gel 1.5%with hypo ladder then electrophoresed bands were visuals under UU light, 12 positive sample at 106 bp gene NP (**Figure 13)** were taken from broilers birds while layer, geese and Gallinula birds were negative to all primers despite having upper respiratory signs

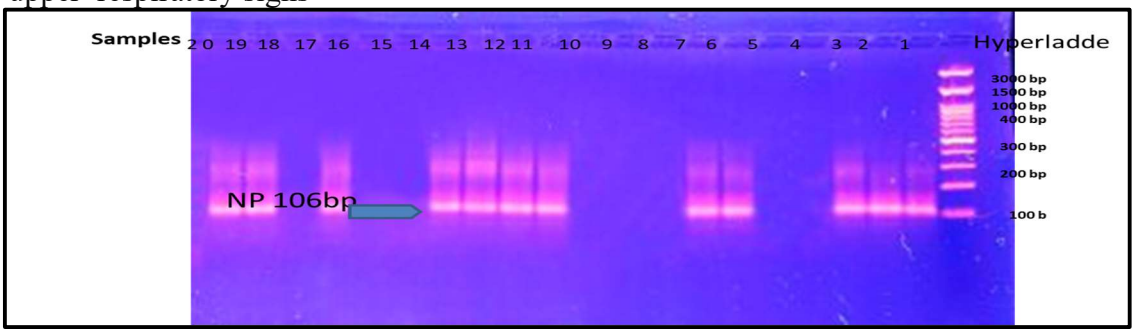

Figure 6. Agarose gel electrophoresis image that show Analysis of PCR reaction products by electrophoresis of AIV NP band 1,2,3,6,7,11,12,13,17,19,20 represent AIV positive sample at size (106bp).

PCR products of all virulence genes were analyzed by loading in 1.5% Agarose, gel electrophoresis results as follows; total positive samples by PCR were only five positive samples against H7 primer out of 40 (figure 14) at 409 bp

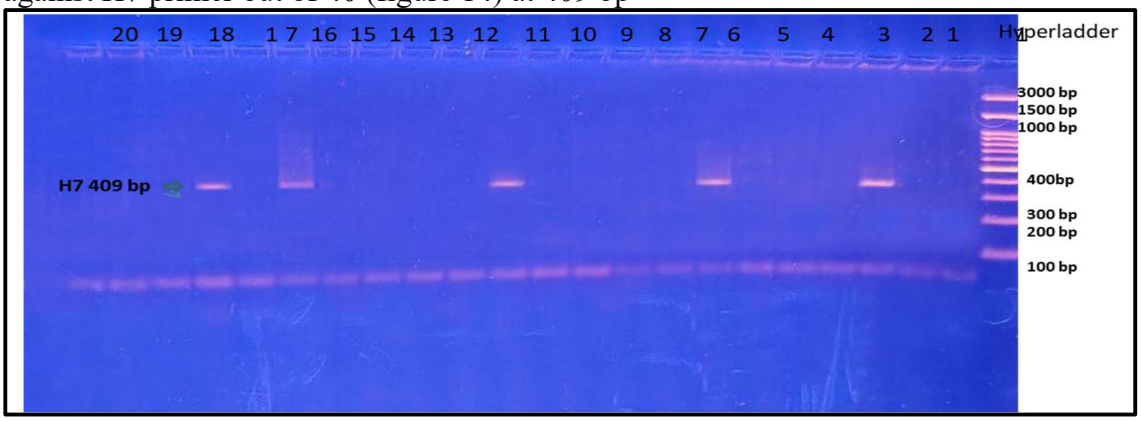

Figure 7. Agarose gel electrophoresis image that show Analysis of pcr reaction products by electrophoresis of AIV H7 band Lane 3,7,12,17,18 represent AIV H7 positive sample at size

(409bp) ز20sample pcr product were loaded in agarose gel 1.5%with hypo ladder then electrophoresed bands were visuals under UU light , all positive sample at 409bp gene H7 were taken from broilers birds while layer, geese and Gallinula birds were negative to all primers despite having upper respiratory signs

PCR products of all virulence genes were analyzed by loading in 1.5% Agarose , gel electrophoresis results as follows; twenty samples were positive against H5 primers as shown in figure 15 and the bands were visualised at 499 bp under UV light.

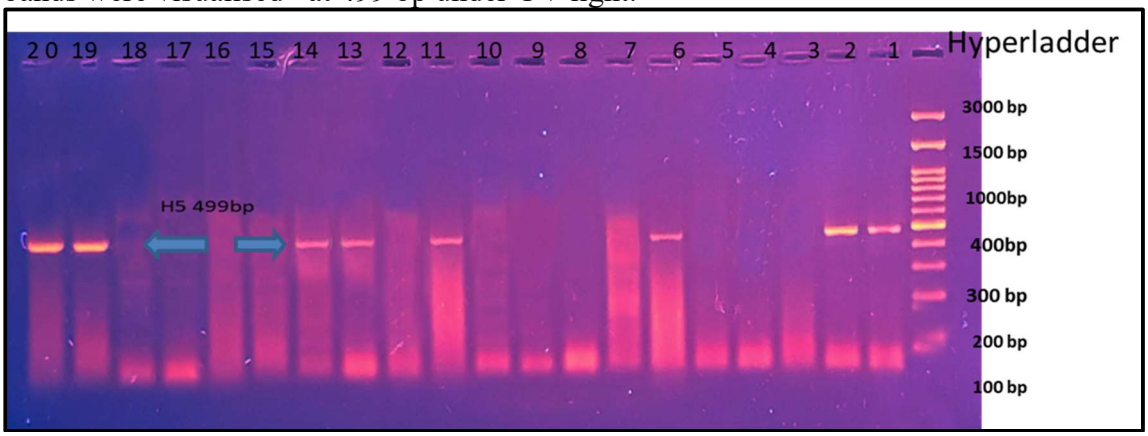

Figure 8. Agarose gel electrophoresis image that show Analysis of pcr reaction products by electrophoresis of AIV H5 band Lane 1,2,6,11,13,14,19,20 represent AIV positive sample control at size 499bp). 20sample PCR product were loaded in agarose gel 1.5%with hypo ladder then electrophoresed bands were visuals under UU light these eight samples were positive against H5.

PCR products of all virulence genes were analyzed by loading in 1.5% Agarose as follows. no positive samples were detected by PCR were against H9 primers **Figure 16**.

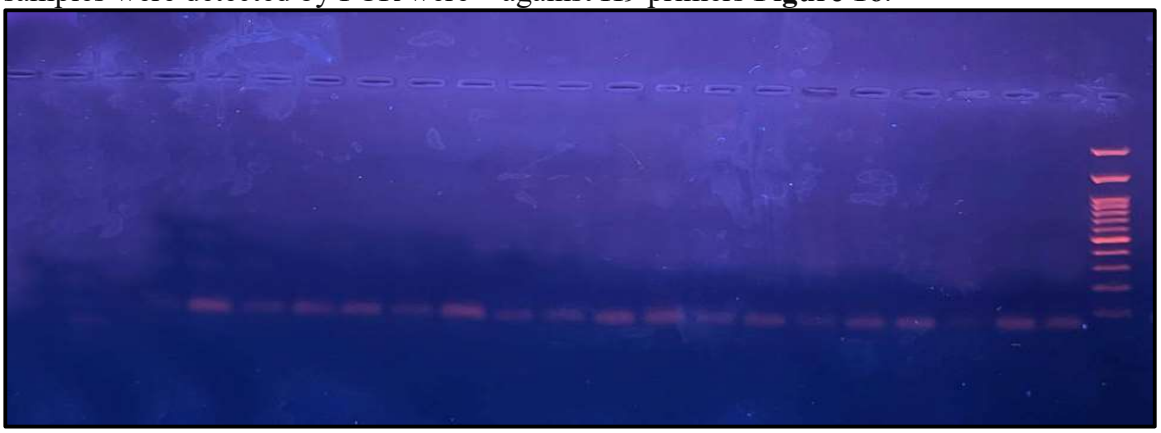

Figure 9. Agarose gel electrophoresis image that show Analysis of PCR reaction products by electrophoresis of AIV of H9 gene all sample were results negative

Statistical Analysis

Data were analyesd using Microsoft excel Version 2020. The mean and SE were used to present quantitative data. Frequency and percentage were used to display qualitative data. We employed the least significant difference, analysis of variance test, and T-test to compare between the groups. P was less than or equal to 0.05 as the significance level value (Daniel, 2009).

Discussion :

Avian influenza viruses are highly contagious and pathogenic viruses that Induced a very pathological condition in domesticated birds (Van et al., 2005).(Channa et al., 2022) The avian influenza subtype

H5N1 has resulted in several outbreaks in poultry globally and hundreds of human cases, most of which have been deadly, since its identification in Hong Kong in the late 1990s (Bridges CB, et al., 2002; WHO, 2019).The H5N1 subtype of avian influenza (AI) is of great concern due to its ability to infect both animals and humans, posing serious risks to the chicken business and the general public's health. This has been confirmed by Rehman et al. (2020) and Rathore et al. (2022), who highlight the zoonotic relevance of H5N1 and its impact on global chicken production, leading to substantial economic losses. The HPAI virus undergoes replication inside the endothelial cells of certain organs within the chicken's vascular system (Brown CC, et al., 1992). The trachea showed significant bleeding upon microscopic inspection, which is consistent with Kim et al's findings (Kim HR, et al.,2015) Systemic infection caused by AIV-H5 infection results in severe morphological and histological alterations, as demonstrated by the fast chromatographic strips. excellent sensitivity and specificity as a field diagnostic instrument. For the identification of avian influenza virus subtypes with highly pathogenic qualities, the RRT-PCR method proved extremely sensitive and specific (HPAI)this in agreement with Isihak, et all (Isihak,et al.,2020) and agreement with chacharaein et all (chacharaein et al.,2009) .In both geese and ducks, the H5 subtype was substantially more prevalent than the H7 subtype. (Al-Mubarak,et,al.,2024), (Channa AA **et,al.,20210)**

Conclusions:

1- Genotyping H5 and H7 Identified in broiler
2- layers, geese and galinula brides were negative to avian influenza varies
3- Phylogenetic analysis revealed similarity with AIV genotypes in nearby countries.

Recommendations

1- Similar studies should be repeated frequently

2- Develope new vaccines depending on current genotypes

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