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# Serological and Molecular detection of highly pathogenic avian influenza of Layan Hens circulating in Diyala Governorate

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*AIV (H5N8), ELISA, Commercial layer Hens, RT-PCR*

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## ABSTRACT

This study was done at the college of Veterinary Medicine, University of Diyala, from September 2021 to April 2022 to assess the seroprevalence and molecular detection of avian influenza virus (AIV) H5N8 in layer chickens. All samples came from 8 commercial flocks of layer chickens. Clinical sings included, severe respiratory infection with enlargement of the head, neck, and prominent edema of the face, followed by excessive lacrimation, sinusitis, severe rales, and cyanosis in all parts of unfeather skin predominantly the wattles comb, and the legs. In this regard, ELISA was used as a serological test and RT-PCR as a molecular detection technique. Accordingly, (364) serum samples from 8 probable AIV (H5N8) infected layer farms were collected and subjected to ELISA test using commercial kit that specific for IgG antibodies to AIV H5N8. The results showed a significant increase of IgG antibodies in such serum samples at days 70 and 200 of age. According to the instruction manual of ELISA kit manufacturer and to mean titers of layers of present study, all farms are infected with AIVH5N8 strain. However, at the age of 200 days, 48 postmortem tissue samples including trachea, lung and liver of affected birds were collected from clinically and sub-clinically infected flocks from all farms. These samples were processed, RNA extracted and submitted to RT-PCR using specific primers for H5N8 strain. The results showed that 32 out of the 48 tissue samples (66.6%) tested positive for H5N8.The resulted Amplicon (320bp) was commercially sequenced and analyzed. The sequencing of the local AIV H5N8 revealed a 99% sequence identity with the reference sequences. The detected strain was registered in GenBank data (NCBI) under acc.number ON247929.1. Phylogenetic tree for locally detected virus in comparison to data from of NCBI was created and showed that the investigated S1(ON247929.1, AIV (A/laying hens/Iraq/(H5N8) segment 4 hemagglutinin (HA) gene, partial cds, local sample is closely related to reference isolates from the NCBI acc. no. of MW961428.1, MW961444.1, MW961476.1, MW961436.,1 and MW961484.1. These strains of the Influenza H5 virus have been deposited from Nigeria.

# التحري المصلي والجزيئي عن فايروس انفلونزا الطيور شديد الظراوة في الدجاج البياض في محافظة ديالى

**زينب عبد عون حمه الطالباني و عامر خزعل صالح العزاوي**
**فرع الاحياء المجهريه/ كلية الطب البيطري/ جامعة ديالى**

**الخلاصة**
أجريت هذه الدراسة في كلية الطب البيطري ، جامعة ديالى ، للفترة من أيلول 2021 إلى نيسان 2022 لتقييم الانتشار المصلي وكذلك الكشف الجزيئي عن فيروس أنفلونزا الطيور H5N8 (AIV) في الدجاج البياض . تم جمع جميع العينات من 8 قطعان مرباة تجاريا خاصة بهذا النوع من الدجاج. تضمنت الأعراض السريرية للحقول المصابة, وجود اصابة حادة في الجهاز التنفسي مع تضخم الرأس والرقبة ووذمة بارزة في الوجه ، تليها تدمع غزير في عيون الطائر ، والتهاب الجيوب الأنفية ، وحشرجه شديدة في القصبه الهوائيه ، وازرقاق في جميع أجزاء الجلد غير المغطى بالريش في الغالب مشط الدلايات ، والساقين. في هذا الصدد ، تم استخدام لاختبار ELISA فحص مقايسة الممتز المناعي المرتبط بالانزيم باستخدام الكت الخاص بالفايروس كاختبار مصلي وفحص تفاعل البلمره المتسلسل التقليدي RT-PCR كطريقة للكشف الجزيئي. وفقًا لذلك ، تم جمع (364) عينة مصل من 8 حقول تربيه لدجاج بيض المائده يشك في اصابتها بفيروس AIV (H5N8) وخضعت لاختبار ELISA باستخدام مجموعة تجارية مخصصة للأجسام المضادة لـ IgG لـ .AIV H5N8 أظهرت النتائج زيادة معنوية في الأجسام المضادة IgG في عينات المصل هذه في الفترتين 70 و 200 يوم من العمر. وفقًا لدليل التعليمات الخاصه بالعدة التشخيصيه لمجموعة ELISA في الدراسة الحالية ، فإن جميع المزارع مصابة بسلالة .AIVH5N8. ومع ذلك ولتاكيد الاصابه جزيئيا ، في عمر 200 يوم ، تم جمع 48 عينة من الأنسجة المصابة من الدجاج النافق بما في ذلك القصبة الهوائية والرئة والكبد وكذلك من للطيور المصابة الحيه لقطعان مصابة شخصت سريريا واكلينيكيا على انها مصابه بالمرض من جميع المزارع. تمت معاملة هذه العينات ، واستخلاص الحمض النووي الريبي وفحصها بجهاز RT-PCR باستخدام بادئات محددة لسلالة .H5N8. أظهرت النتائج أن 32 عينة نسيجية من أصل 48 (66.6٪) تم اختبارها إيجابية لفيروسH5N8 . وعلى هلامة الاكاروز اظهرت العينات المفحوصه الموجبه نطاقا محددا من الحامض النووي بمقدار 320 نقطه اساس(bp) باستخدام ازواج من الرايمرات محدده للبروتين الخاص بغلاف الفايروس(الهيمكلوتنين). . تم تسجيل السلالة المكتشفة في بيانات GenBank (NCBI) تحت رقم الحساب. ووجد محرك NCBI BLASTnتشلبها في التسلسل بنسبة 99% بين العينه المحلية المكتشفه لانفلونزا الطيور في محافظة ديالى والعتر المسجلة في بنك الجينات. تم إنشاء شجرة النشوء والتطور للفيروس المكتشف محليًا مقارنة ببيانات من NCBI وأظهرت أن جين S1 (ON247929.1 ) AIV الدجاج البياض الذي تم تسجيله / العراق/ ذا تطابق وصلة قريبه جدا في مناطق محدده من الجين وبنسبة 99% مباشرتا مع سلالات لفايروس الانفلونزا لنفس النوع معزوله في نايجيريا من النمط المصلي H5N8 والتي تم تسجيلها وتاكيدها في بنك الجينات وتحمل الارقام التاليه1.MW961428 و MW961444.1 و MW961476.1 و .MW961436 و 1 و MW961484.1وهذا يدل على ان هذه العتره المسجله محليا ربما تكون واسعة الانتشار عالميا.

**الكلمات المفتاحية:** الانفلونزا شديدة الظراوة ، دجاج بيض المائده ، جهاز تفاعل البلمره المتسلسل التقليدي

## INTRODUCTION

Due to viral strain and herd immunity, global avian influenza epidemics have an adverse effect on the chicken sector and increase morbidity and mortality (Vigeveno *et al.*, 2020). Negative-sense single-stranded RNA genomes are seen in avian influenza viruses (MacLachlan and Dubovi, 2010). The genome is divided into 8 segaments, totaling 13.5 kilobases. The disease-causing virus belongs to the Orthomyxoviridae family's genus Alphainfluenzavirus. This virus's genome is fragmented by 13.5 kb, includes single-stranded RNA, and is negative sense (Lamb, 2001; Maclachlan and Dubovi, 2010). Differences in the matrix (M1) protein and nucleoprotein (NP) lead to classification into four genera: A, B, C, and recently discovered D (Capua and Alexander, 2007). Influenza A virus only infects birds (Lamb, 2001).

According to several workers, avian influenza can infect humans and other mammals and produce endemic, epidemic, and pandemic diseases in birds and other species ( Capua and Alexander, 2009). Fowl plaque was the first name of AIV that produced severe clinical indications and deaths among poultry globally. The virus caused rapid death in poultry farms within a few days, and the mortality rate skyrocketed (Stegeman et al., 2004). These viruses were divided into four genera—A,

B, C, and recently discovered D—based on variations in their matrix (M1) protein and nucleoprotein (NP) (Capua & Alexander, 2007). Only birds can contract a flu virus (Lamb, 2001). These viruses' neuraminidase (NA) and hemagglutinine (HA) spike structures enabled further subtyping into one of 16 antigenically distinct HA subtypes (H1 to H16) and one of nine NA subtypes (N1 to N9), while two subtypes found in bats (subtypes H17N10 and H18N11) represented the remaining 2 HA (17 to 18) and 2 NA subtypes (Tzarum et al., 2017). Two influenza types A virus has been named based on their capacity to significantly infect a host. The mild to subclinical symptoms of low-pathogenicity (LP) avian influenza virus include minimal respiratory and digestive effects as well as a moderate impact on ovarian follicles, which lower egg output and death in hens (Swayne *et al.*, 2013). The second pathotype, High Pathogenic Avian Influenza Virus (HPAIV), can produce widespread symptoms and have up to 100% mortality rates. Chickens with the condition had neurological symptoms such leg and wing paralysis as well as nasal and ocular discharges (Hemida et al., 2019). The two most dangerous strains of avian influenza are H5 and H7 (Swayne et al., 2013). Due to the possibility of mutation, which could result in the creation of a more virulent virus and render vaccines useless, HPAIVs have resulted in significant financial losses for the poultry sector (Kim, 2017).

Due to its rapid transmission and fatality of domestic poultry, highly PAIV is referred to as the "fowl plague." Due to the Re-assortment of avian and human influenza in the 20th century, epidemics of the H1N1, H2N2, H3N2, H1N1, and H5N1 viruses respectively broke out in Asia, Africa, and Europe in 1918, 1957, 1968, 1977, and 2009. (Capua and Alexander, 2009; Gonzales and Elbers, 2018). The deadly H5N8 strain of HPAI was discovered in wild birds in Asia for the first time in 2010 and spread to farmed birds via migrating aquatic birds. Early in 2014, migratory ducks from China brought the H5N8 HPAIV 2.3.4.4b lineage virus to Southeast Asia, particularly Korea. Migratory aquatic birds were held accountable for the epidemic's global spread (Lee et al., 2015). A global problem is the previous discovery and spread of HPAIV H5N8 influenza viruses by wild birds from several continents (Lee et al., 2015). Iraq first discovered low pathogenicity HIV in flocks of layer hens (Al-Nasrawi, 2002). On March 11, 2018, the first cases of the extremely contagious H5N8 avian influenza were discovered in the Diyala Governorate. There are two strains of this disease, according to a report from the International Office of Zoology (OIE 11/3/26150). Which is part of clade 2.3.4.4.b, that led to a mortality rate of more than 90%. The current study's objectives are to analyze the HPAIV (H5N8) in various flocks of layer chickens in the Diyala Governorate of Iraq by serological, molecular, and phylogenetic methods.

## MATERIALS & METHODS

### BACKGROUND

The current study was conducted at the College of Veterinary Medicine, University of Diyala from September 2021 to April 2022, and sought to assess the seroprevalence and molecular of the major and very harmful viral illness of layer hens termed AIV. All of this study's samples processing and testing were done in the virology laboratory. All samples were collected from 8 suspected commercial layer farms suspected to be infected with AIV(H5N8) that were located in four areas of Diyala Governorate.

Clinical signs included enlargement of the head, neck, and face due to high infestation infection, followed by severe respiratory indications including excessive lacrimation, sinusitis, severe rales, and cyanosis in all sections of unfeather skin notably the wattles, comb, and the legs (Fig 1, A&B). By the third day of the sickness, egg production drops drastically and the shells become deformed and pallid. In severe cases of the suspected disease, HPAIV H5N8's high

morbidity rate is followed by a 65-90 % fatality rate. Postmortem alterations in H5N8-infected hens indicated hemorrhagic pancreatitis, duodenal distension, and cardiac and cecum tonsil hemorrhage. (Fig. 1, C,B and E).


**Figure 1: Layer chickens naturally suspected infected with HPAI of the H5N8 subtype, Showing (A, B) hemorrhages on the shank, congestion and cyanosis of the comb and wattles (arrow), C, hemorrhages on the caecal tonsils,D, g multifocal hemorrhages in the myocardium and In E, hemorrhagic pancreatitis and duodenal distension.**

## COLLECTION OF BLOOD SAMPLES

A total of (364) blood samples were obtained from four laying hen farms in the governorate namely (Baqubah, Kanaan, AL-ghalibiaand, and AL-Khales). The first blood samples were taken at 70 and 200 days of age by a wing veins method and left for a short period of considerable time to clotting at room temperature. Centrifugations of the blood were down at 3000 rpm for 10 minutes. Serum was collected in sterile 2-ml Eppendorf tubes and followed by freezing the collected sera at -20°C until used. Post-mortem tissues for PCR (n = 48) were obtained from clinically and sub-clinically infected layer hens with HPAIVH5N8 from each farm's 70 and 200 days old flocks (trachea, lung, liver). All lab samples were RNA extracted before RT-PCR analysis (Table 1).

**Table 1 Blood (364) for ELISA detection and Postmortem tissues samples (n=48) for PCR was collected from birds according to the farm's location, number, and Age of birds**

| NO | Samples No. for ELISA detection at 70 days old | Samples No. for ELISA detection at 200 days old | Samples No. for molecular at 70 days old | Samples No. for molecular at 200 days old | Place of the farm |
|---|---|---|---|---|---|
| 1 | 46 | 46 | 6 | 6 | Baqubah |
| 2 | 45 | 45 | 6 | 6 | Kanaan |
| 3 | 46 | 46 | 6 | 6 | AL-ghalibia |
| 4 | 45 | 45 | 6 | 6 | AL-Khalis |

## PROCESSING OF SERUM SAMPLES

The ELISA device was used with the commercial kit to examine and evaluate the presence of IgG antibodies against avian influenza in serum samples collected from flocks of laying hens in four areas in Diyala Governorate. The indirect ELISA assay was used to evaluate serum samples using a commercial kit obtained from the company (Sun Long Biotech Co., L, China). ELISA performed at dilution serum of (1:5) for each well. Also, for each microplate, two control wells were negative and two were positive, and only one empty well was included in all assays simultaneously were left. The result was reading by microplate reader (ELx800, BiO-TeK Instruments Inc., USA). The Microplate provided in this kit has been pre-coated with an Antibody specific to influenza H5N8; make it to solid-phase.

## PROCESSING SAMPLES FOR MOLECULAR STUDY

Molecular detection was done by using conventional RT-PCR, performed on tissue samples. The RNA was extracted from 20 gms each tissue of trachea, lung and liver by using an extraction kit for tissue (Kylt® RNA / DNA Purification kit, Germany). The extracted RNA was amplified using the oligonucleotide primer sequences of H5-kha-1F and H5-kha-3R for PCR covering the nucleotide region of partial HA gene as previously described by Slomka et al., (2007). The sequence of the forward primer (5'CCTCCAGARTATGCMTAYAAAATTGTC3') and for the reverse primer (5'TACCAACCGTCTACCATKCCYTG3') produced a DNA fragment of 320 bp. Primers were obtained commercially from (Alpha DNA, Montreal, Canada).

In the current study, PCR was performed in two steps, after converting RNA to cDNA - in the first step by using (Access RT-PCR System Kit, Promega, USA) an improved reverse transcriptase procedure to convert RNA to cDNA, the second step is used to amplify cDNA using (Gradient PCR) programmable thermal cycler (Eppendorf, USA), to detect all samples and determine the necessary temperature in (primary denaturation and annealing), as well as template concentration DNA, set between (1.5 - 2 µl). The program for RT-PCR amplification used a reaction volume of 25 µl as shown in Table (2). The PCR products were analyzed by electrophoresis on a 1.5% agarose gel and visualized using Gel Documentation

**Table 2 Reaction setup and thermal cycler protocol for cDNA synthesis of HPAIV(H5N8)**

| NO | Step | TM °C | Time | No. of cycle |
|---|---|---|---|---|
| 1 | denaturation | 94°C | 30 seconds | 40 cycles |
| 2 | annealing | 60°C | 1 minute | |
| 3 | extension | 68°C | 2 minutes | |
| 4 | final extension | 68°C | 7 minutes | 1 cycle |
| 5 | soak | 4°C | | soak |

## NUCLEIC ACIDS SEQUENCING OF PCR AMPLICONS

The resolved PCR amplicons were commercially sequenced from the forward direction, following to instruction manuals of the sequencing company (AniCon Labor GmbH). By comparing the observed nucleic acid sequences of local viral sample with the retrieved nucleic acid sequences, the virtual positions, and other details of the retrieved PCR fragments were identified.

## COMPREHENSIVE PHYLOGENETIC TREE CONSTRUCTION

Using the NCBI-BLASTn service, a specific comprehensive tree was built in this work, and the detected variants were compared to their nearby homologous reference sequences (Zhang et al. 2000). Then, using the iTOL suit, a full inclusive tree was constructed using the neighbour-joining approach, incorporating the observed variant (Letunic and Bork, 2019). In the comprehensive tree, the sequences of each categorized phylogenetic group were colored accordingly.

## RESULTS AND DISCISSION

All samples were from commercial layer flocks with characteristic HPAI H5N8 symptoms and postmortem findings (Figure 1). The serological methods employing the ELISA technique was evaluated for the presence of **Anti –AIV (H5N8**) IgG according to the ELISA kit's instruction manual (SunLong Biotech Co., L, China). The 364 blood samples were obtained from eight commercial layer farms in four districts of Diyala Governorate. All samples were ELISA-tested for avian influenza antibodies (Table1). According to CUT OFF, the ELISA test result was down (Cut-OFF value computed as mean of Negative Control + Positive Control+0.15). If the optic density value is less than cut-off grade, the serum samples are computed negative, and the result is negative for AIVH5N8-IgG. If the value is larger than cut-off grade, the result is positive for AIVH5N8-IgG. The overall positive result rate of anti AIVH5N8 IgG across all serum samples in the Baqubah region was 43.4(20/46) with a mean titer of IgG (0.34433± 0.019451) units at 70 days old, while the positivity rate was 41.3(19/46) with a mean titer of (0.36257± 0.21811) at 200 days old ,there was no statistically significant difference between 70 and 200 days old.

In Kannan was 37.7 (17/45) and slightly higher in age of 200 days, with a rate of 44.4 (20/45) and mean IgG titer (0.33880± 0.19184) at 70 days old and 0.36762 ±0.26392) units at 200 days old. The difference between 70- and 200-day-olds was statistically significant. In Al-Ghalibia at 70 days old, the anti-AIV H5N8 IgG positivity rate in all blood samples was 45.6(21/46) with a mean titer of IgG (0.38007 ±0.0267530) and 80.4(37/46) with a mean titer of 0.53774 ±0.026767). At 70 and 200, the variation between 70 and 200-day-old fish in the same region was statistically significant. In the Al-Khales region, the number of infected layer chickens grew somewhat at 70 days, with a rate of 60(27/45) and a mean titer of IgG (0.58764 ± 0.028425), and again at 200 days, with a rate of 91.1(41/45) , but the changes failed to extend the thresholds of significance. In the same site, the statistical difference between 70 and 200 day olds was substantial. The overall anti-AIV (H5N8) IgG positive rate in infected layer flocks that were clinically suspected to have AIV was 46.7 (85/182) at 70 days old and 64.2 (117/182) at 200 days old, respectively. At 70 days old, the mean anti-AIV H5N8 titer rate in AL-Khales flocks was (0.58764 ±0.028425), which was considerably higher than the three other locations (P0.05), followed by those layers infected with H5N8 in AL-Ghalibia flocks with a mean titer of (0.38007 ±0.026753). The variations in positive serum between the current research flocks in two areas (AL-Khales and AL-Ghalibia) were statistically significant.

The mean ± SE IgG positive rate in Baqubah and Kanaan was (0.34433±0.019451) and (0.33880± 0.019184) correspondingly, according to the results in table (2). When compared to the AL-Khales region, the differences between the current research flocks at three regions with positive serum were statistically significant. According to the findings of this study, antiAIV H5N8 titer rates were substantially higher in AL-Khales flocks at 200 days old (0.74236±0.023195), compared to AL-Ghalibia areas (P0.05). As shown in table (3), it was substantially higher (P≤0.05) than all other two regions, namely (Baqubah and Kanaan), with positive rates of (0.36762±0.026392) and (0.36257±0.021811), respectively. It was also very significant (P≤0.05) when comparing the positive

rates of the flocks from Al-Ghalibia to those from Baqubah and Kanaan. The flocks of a commercial layer in two places (AL-Ghalibia and AL-Khales) at the ages of 70 and 200 days old had the highest positive rate, which was statistically significant (P≤0.05) as showed in table 3 and figure 2.

**Table 3 Showed anti-AIV H5N8 IgG positive rates in commercial layer flocks by age in all four areas**

| Region | NO | Age 70 days | Age 200 days |
|---|---|---|---|
| Baqubah | 46 | 34433 ± .019451 B a | .36257 ± .021811 C a |
| Kanaan | 45 | .33880 ± .019184 B a | .36762 ± .026392 C a |
| AL_ghalibia | 46 | .38007 ± .026753 B b | .53774 ± .026767 B a |
| AL_Khales | 45 | .58764 ± .028425 A b | .74236 ± .023195 A a |

**Legend: The presence of various capital letters indicates statistical significance (P0.05) vertically, whereas the presence of different small letters shows statistical significance (P≤0.05) horizontally.**

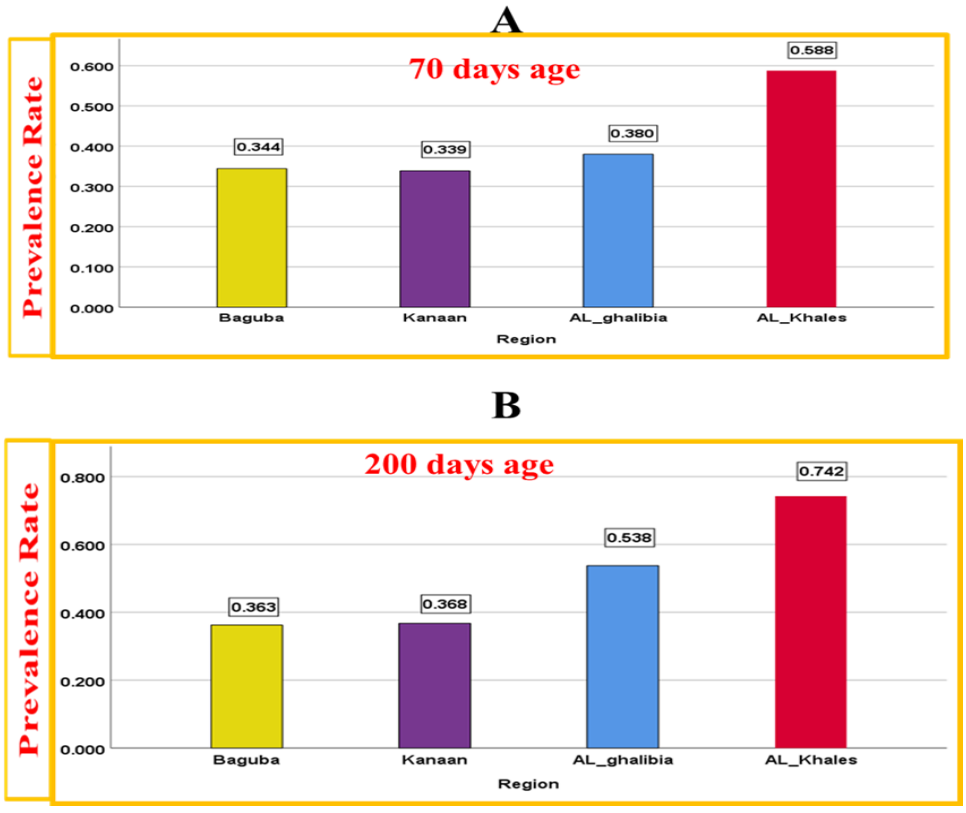


**Figure 2 (A&B) Anti-AIVH5N8 rate by age group in infected commercial layer flocks in all four areas at the same time (70 and 200 days old).**

**DETECTION OF AIV (H5N8) FROM TISSUE SAMPLES USING RT- PCR**

At the ages of 10 and 29 weeks, six tissue samples from commercial layers exhibiting overt signs of influenza virus infection were collected from each farm. For the AIVH5N8 strain, 32 out of the 48 tissue samples (66.6%) tested positive. All of the amplified cDNA (AIV) samples had the same mobility on a 1.5 % agarose gel. Positive RT-PCR template samples produced a distinctive 320 bp DNA band, as seen in figure 3.

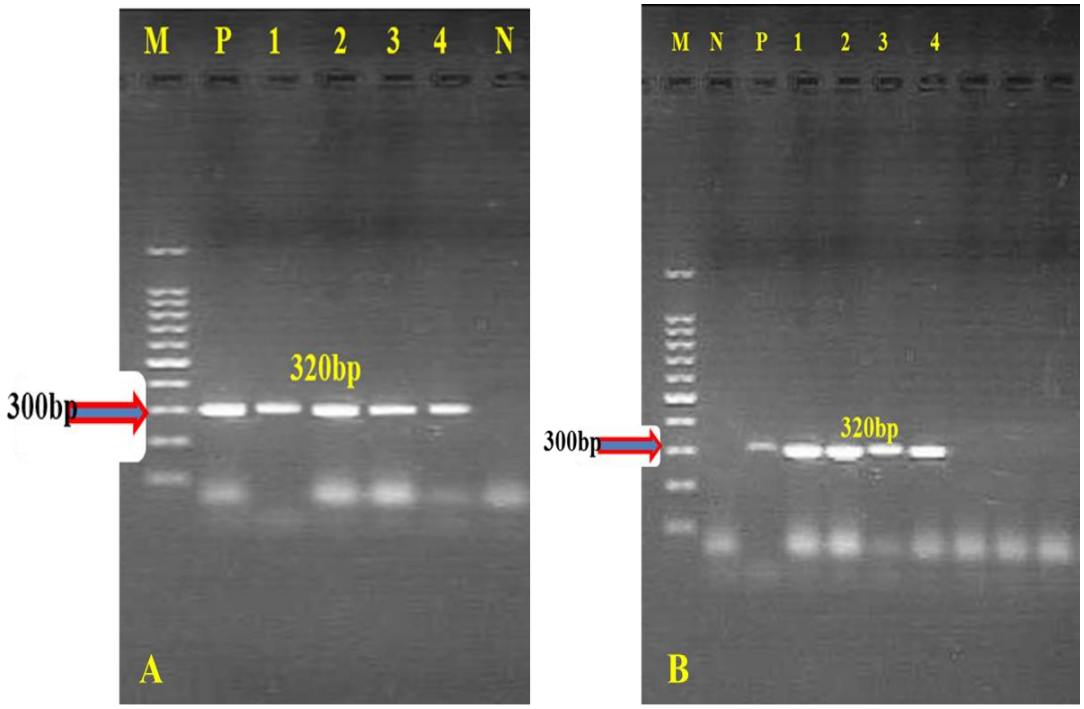


**Figure 3 Conventional RT-PCR test of the HA region used to screen AIV H5N8 tissue samples. The Kha-1f/Kha-3r primer sets detect the HA gene's outer region, which has a product size of 320 bp. The 1.5 % agarose gel was electrophoresed, and 0.5 g/ml Red safe was used to stain it.**

**SEQUENCING RESULTS**

The current analysis inside this locus only contained one sample result from a positive PCR. The HA (hemagglutinin) gene sequences of the avian influenza A virus were examined in this sample. The HA gene variation can be used for Influenza A virus serotyping due to its likely ability to adapt to diverse genetic diversity, as proven in different viral serotypes of the avian influenza virus. According to, the sequencing procedures for these PCR amplicons after NCBI blastn revealed their precise identification (Ye et al., 2012). The sequencing of the local H5N8 avian influenza virus revealed a 99% sequence identity with the reference sequences, according to the NCBI BLASTn engine. By comparing the observed nucleic acid sequences of the analyzed local sample with reference sequences, the correct positions and other properties of the produced PCR fragments were discovered (GenBank acc. MW961484.1). The overall length of the targeted site as well as the beginning and ending positions of the targeted locus inside the viral target with the highest similarity were determined using the NCBI server (Fig, 4).

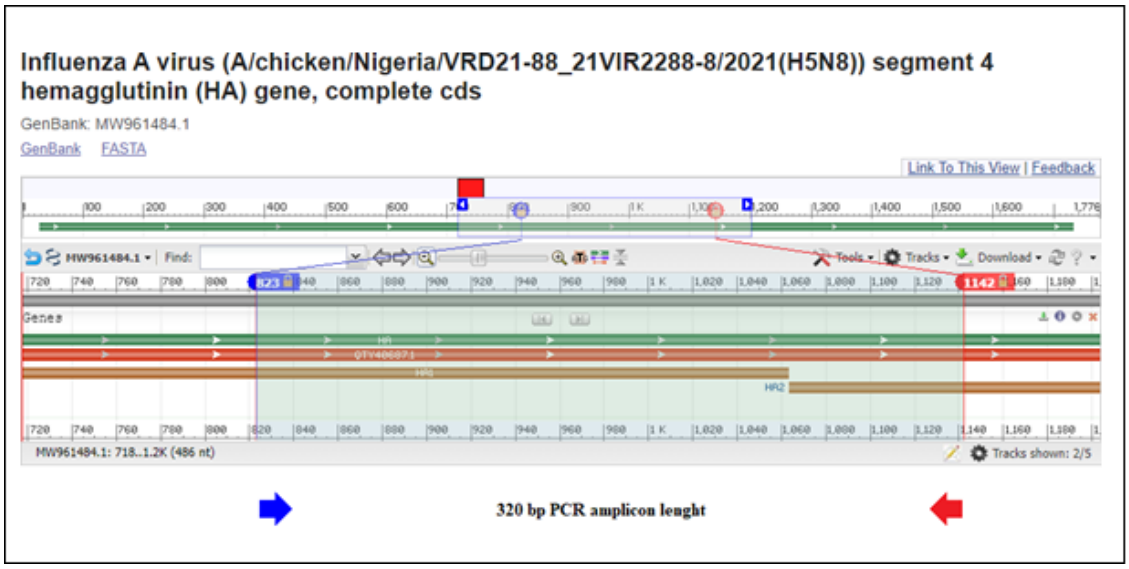


Figure 4 The specific site of the obtained 320 bp amplicon partially encompassed a section of the HA gene (GenBank acc. no. MW961484.1). The blue arrow in the diagram denoted the start point, while the red arrow denoted the finish position.

## PHYLOGENETIC ANALYSIS

Based on amplified 320 bp of the HA gene, the current study created a complete phylogenetic tree to provide a phylogenetic understanding of the actual distances between our examined local AIV sample and the other NCBI reference serotypes. The majority of the integrated sequences in the cladogram were created using relative reference sequences from this phylogenetic tree and the current, studied local sample.

There were a total of 75 aligned nucleic acid sequences in this entire tree. The cladogram separated the combined sequences of the influenza A virus into five strain groups. The association between our sample and its nearby sequences being classified into five different serotypes of the Influenza A virus is the most exciting discovery in the current local viral isolates investigation. The serotypes implicated are H5N1, H5N2, H5N5, H5N6, and H5N8. Sample S1, the viral strain under examination, belongs to the major lineage of the H5N8 serotype. The H5N8 serotype was present in all of the integrated samples in this clade, and the largest number of incorporated Influenza A virus sequences—20 different strains—were inserted at various phylogenetic distances (Fig. 5). However, the examined S1 local sample was suited in closely related to the reference samples from Nigeria which were recorded and confirmed in GenBank with acc. Number of MW961428.1, MW961444.1, MW961476.1, and MW961436.1 and MW961484.1. The second cluster includes samples of African isolates namely OL366043.1, OL354982.1, OL353696.1, OL362018.1, OL362016.1, OL362015.1, OK160062.1, OK160062.1, and MN658766.1, which were placed according to the present tree in locations close to the examined local sample of AIV H5N8 S1. Thus, the African origins of our investigated sample could not be ignored.

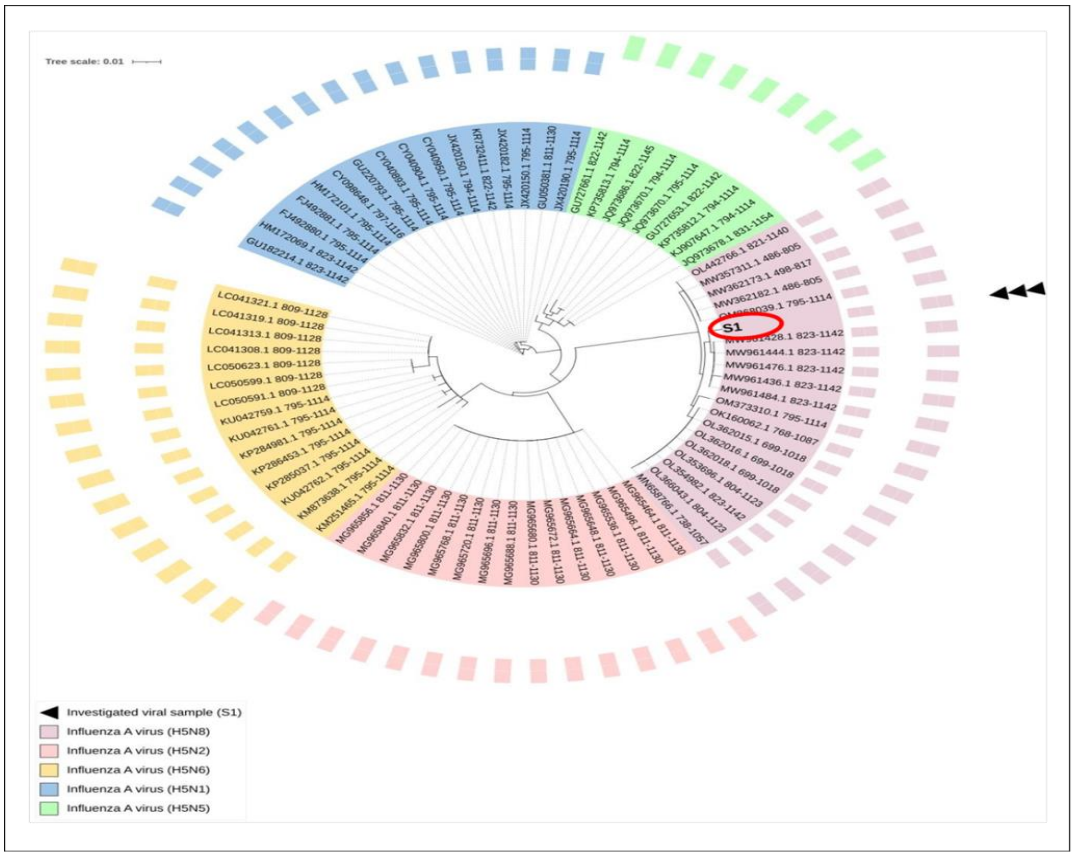


Figure 5 The evolutionary tree of genetic variations of the HA gene segment of chicken-infecting Influenza is represented by a circular cladogram. A sample of a virus. The investigated viral variations are shown by the black-colored triangle. All of the numbers related to each referencing species' GenBank entry number. The number "0.01" at the top of the tree denotes the degree of scale range among the creatures classified by the comprehensive tree. The letter "S" stands for the code of the AIV H5N8 local sample under investigation.

**DISCUSSION**

Worldwide, highly pathogenic avian influenza (HPAI) viruses have emerged in poultry and other animals, causing infrequent but devastating outbreaks. The (AIV) H5N8 subtype among these produces a serious respiratory disease in chicken that has a high morbidity and mortality rate. Most bird species have been affected by highly pathogenic avian influenza (HPAI)(H5N8) epidemics over the past 20 years (Chen et al., 2005; Kapczynski et al., 2013).

**CLINICAL SIGNS OF INFECTED LAYER**

Clinical signs are vague and depend on the severity of the virus strain. Chickens with HPAI may perish suddenly. In all four sites, birds suspected to ا reduced feed intake, cyanosis of the unfeathers skin, severe respiratory illness, and edema of the head and wattle with severe diarrhea followed by quick mortality. Infected flocks displayed significant drops in or cessations of egg production as well as abnormal egg quality (deformed, thin-shelled, soft-shelled). The results of the present study are in agreement with those of (Spickler et al., 2008; Stoute et al., 2016; Stephens et al., 2020), who also noticed that the initial symptoms of HPAIV infection in layer chickens were tremors, incoordination, depression, and anorexia. Some infected layer chickens displayed hock petechial hemorrhages or comb and wattle cyanosis. Within 48 to 72 hours of the onset of clinical signs, all birds died.

**SEROPREVALENCE OF AIV H5N8 OF LAYER CHICKENS ACCORDING TO AGE**

To date, only a few experimental or commercial ELISAs which detect H5 antibodies exist, and they were mainly developed for human use (Postel et al., 2011; Desvaux et al., 2012; Moreno et al., 2013). The results showed that the level of AIV H5N8 antibody increased significantly ($P \leq 0.05$) in all four groups of layer chicken flocks clinically suspected to have AIV at 70 days of age with antibody titers (0.344 0.0194, 0.338 ±0.0194, 0.380 ±0.0267, and 0.587 ±0.0284) for Baqubah, Kanaan, AL-Ghalibia, and AL-Khales respectively, and a total positive rate of anti-AIV (Table,2 and Fig 2). Other nations' chicken populations showed similar trends (Henning et al., 2011; Ghafouri et al., 2017). According to Gupta et al. (2021), the increase in H5 antibodies in chicken flocks, especially layer flocks, is due to multiple exposures to an endemic virus at the same age of the birds and a decline in the birds' immunological condition. All 4 regions of the current study showed highly elevated in IgG antibody titer, the mean titer at 200 days old (0.36762±0.026392 for Baqubah, 0.36257±0.021811 for Kanaan, 0.38007±0.026753 for AL-Ghalibia and 0.74236±0.023195 for AL-Khales) and this may be attributed to infection with the highly pathogenic H5N8 avian influenza virus according to the instructions of the manual kit of ELISA in four different regions of Diyala Governorate in spite of all these flocks vaccinated or not vaccinated. AL-Khales and AL-Ghalibia exhibited a higher IgG titer at 200 days old ($p < 0.05$). These differences in HPAIVH5N8 antibody levels among the 4 research groups may be attributable to geographical dispersion, changes in breeding plans, or both. AL- Khales and AL-Ghalibia is a corridor for shipping poultry products (meat and eggs) from outside Iraq and the Kurdistan region to Baghdad. Researchers and the current study have found evidence that the spread of infection in most flocks of laying hens in various regions is due to a lack of biosecurity in the field of clearing dead birds, where stray dogs play a prominent and important role in the transmission of infection by eating dead birds and transferring their waste to disease-free fields. Scholarly literature shows similar outcomes from earlier studies (Stone, 1993; Capua et al., 2002). However, differences in the levels of elevated antibodies between the four regions may be attributed to epidemiological factors. In addition to the severity of virus strains that infected flocks, poorly controlled bird movement between these regions and the Kurdistan region, north Iraq, and lack of essential biosecurity information caused the virus to become endemic in the poultry population (Swayne and Halvorson, 2003). It concluded that most layer farms of Diyala Governorate are endemic with HPAIV H5N8 as four locations of layer flocks with wide geographical distributions were infected with this virulent virus.

**MOLECULAR DETECTION OF HPAIV H5N8 FROM TISSUE SAMPLES**

Iraq has endemic H5N8 avian flu. The World Organization for Animal Health (OIE) found H5N8 in Iraqi poultry with substantial morbidity and mortality (Adlhoch et al., 2018). Utilizing RT-PCR, the HA region gene was amplified. With this technique, sick chickens are located before any clinical symptoms manifest. For the purpose of identifying AIV H5N8 Positive samples, Slomka et al. (2007) developed selective RT-PCR primer sequences from a highly conserved portion of the HA gene that resulted 320bp DNA amplicon. By RT-PCR, 66.6% of samples in the current investigation had the AIV H5N8 HA glycoprotein gene, compared to 2.7% in Shehata et al. (2019). 11 commercial layers from three Egyptian farms died of respiratory problems between January 2016 and December 2017. RT-PCR in the current investigation confirmed Abdul-Kadhim et al. (2020). From February 2018 through January 2019, the authors researched unstructured cities in Karbala, Iraq. Twenty tracheal samples were taken from a heavily infected herd. Only birds with cyanosis and edema of the head, comb wattles, and red feet and shanks with considerable mortality were studied. Infected avian tracheal samples showed a 1132 bp HA glycoprotein gene target band on agarose gel electrophoresis. The latest study found 15% H5N8 prevalence (3 out of 20). Abdul-Sada (2020) and Mahmoud and

Allawi (2021) found a lower AIV positive rate in their investigations than in the current study. Each flock's virus intensity and rearing conditions affect the results.

**SEQUENCE AND PHYLOGENETIC ANALYSIS**

This is the first study in Diyala Governorate to use molecular and phylogenetic approaches to determine AIVH5N8 prevalence in layer chicken flocks. A particular PCR fragment spanned the HA gene coding areas in HPAIV-infected layer hens. Direct sequenced amplified fragment (320bp) was used to investigate AIV genetic diversity. All positive PCR results contained the influenza A virus (A/laying hens/Iraq/ZAINAB-S1/2021(H5N8)) segment 4 hemagglutinin (HA) gene (acc.No.ON247929.1). Local layer chicken isolates (ON247929.1) and the NCBI reference target sequence exhibited 99% similarity. Similar research found 99% sequence match with HA Egyptian isolates from HPAIVH5N8-infected layer hens and NCBI references (Hegazy et al., 2019). In the current work, complete phylogenetic tree was created depending on nucleic acid changes observed in the amplified 320 bp of the HA gene amplicons to provide a phylogenetic comprehension of the true distances between our studied sample and the other 75 serotypes from GenBank. S1 was a local isolate included with other influenza A virus genomes in this phylogenetic tree. Local (ON247929.1) sequences were grouped into five phylogenetic clades within the influenza HA virus in the current study. Five S1 serotypes in our virus isolates are noteworthy (H5N1, H5N2, H5N5, H5N6, and H5N8) and S1, a local virus isolate, belonged to the H5N8 lineage. This branch yielded 20 strain sequences. The current study showed that the investigated S1(ON247929.1, Influenza A virus segment 4 hemagglutinin (HA) gene, partial cds, local sample is closely related to reference isolates from the GenBank acc. no. of MW961428.1, MW961444.1, MW961476.1, MW961436.,1 and MW961484.1. These strains of the Influenza H5 virus have been deposited from Nigeria. Laleye et al. (2022) found that 20 HPAI H5N8 viruses collected in Nigerian layer hen flocks between 2016 and 2019 cluster into four genetic groups. The HPAI H5N8 Iraqi strain (ON247929.1) was isolated in Diyala Governorate and resembled Nigerian Influenza H5 virus strains best (MW961428.1, MW961444.1, MW961476.1, MW961436.1, and MW961484.1). The HPAI H5 clade (2.3.4.4b) is connected to Iraqi virus strains in Diyala. Other neighboring sequences in the same lineage, including OL366043.1, OL354982.1, OL353696.1, OL362018.1, OL362016.1, OL362015.1, OK160062.1, and MN658766.1, also supported the sample's African origins. Most Egyptian reference sequences were found. Our sample's African background was therefore unavoidable. Within H5N8, we combined 20 S1 samples. Despite the little biological variety found in our H5N8 samples, the H5N8 and H5N2 serotypes had distinct phylogenetic distances. The H5N2 clade has 15 identical viral sequence samples. Same goes for serotype-H5N8 and serotype-H5N5. Serotype-H5N5 has 9 samples. Next to H5N5, H1N1 samples had a unique evolutionary position. 16 samples comprised this clade. The final clade included 15 serotypeH5N6 samples. In Europe, Africa, and North America, H5N8 viruses have reassorted with other avian influenza viruses to generate H5N1, H5N2, H5N3, H5N6, and H5N5 viruses. The viral sample is close to the cladogram's main clade. H5N8 virus sequences were comparable. Using this HA gene-based tree, viral serotypes can be identified. This reveals that HA gene-specific primers may accurately characterize an influenza A virus's phylogenetic position. HA gene fragments showed genetic fragments' ability to detect viral sequences. HA gene-specific primers may accurately identify Influenza A viruses and their evolutionary positions.

## CONCLUSION

It concluded that most layer farms of Diyala Governorate are endemic with HPAIV H5N8 as four locations of layer flocks with wide geographical distributions were infected with this virulent virus.

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