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Serological and Molecular Detection of H5N8 Influenza Virus in Layer Hens in Diyala Governorate

A Thesis

Submitted to the Council of the College of Veterinary Medicine/ University of Diyala in Partial Fulfillment of the Requirements for the Degree of Master of Science in Veterinary Microbiology

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بسم الله الرحمن الرحيم

أَمَّنْ هُوَ قَانِتٌ آنَاءَ اللَّيْلِ سَاجِدًا وَقَائِمًا يَحْذَرُ

الْآخِرَةَ وَيَرْجُو رَحْمَةَ رَبِّهِ ۗ قُلْ هَلْ يَسْتَوِي الَّذِينَ

يَعْلَمُونَ وَالَّذِينَ لَا يَعْلَمُونَ ۗ إِنَّمَا يَتَذَكَّرُ أُولُو

الْأَلْبَابِ { 9}

صدق الله العلي العظيم

سورة الزمر الاية {9}

Supervisor Certification

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In view of the valuable recommendation, we forward this thesis for debate by the examining committee.

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Examination committee certification

We, the examination committee, certify that the entitled thesis "**Serological and Molecular Detection of H5N8 Influenza Virus in Layer Hens in Diyala Governorate**" by **Zainab Abd Awan Hama** has been examined and read through all of its contents and related topics. The committee recommends that the student passed and awarded the degree of Master of Science in Veterinary Medicine (Veterinary Microbiology).

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Dedication

To the one who encouraged me to persevere all my life, to the most prominent man in my life

*(**Dear father**)*

To the one in whom I rise, and upon whom I rest, to the giving heart

*(**My beloved mother**)*

To the one who made the effort to help me and was the best support

*(**My dear husband**)*

*To the places of my liver, my daughter **Lugain** and my son **Mujtaba***

to my friends and colleagues...

To everyone who contributed even a letter to my academic life…

To all of them: I dedicate this work, which I ask Allah Almighty to accept sincerely...

Zainab Abd Awan

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Praise be to Allah, Lord of the Worlds, and prayers and peace be upon the most honorable prophets and messengers, our master Muhammad, his family and companions, and those who followed them in goodness until the Day of Judgment, and after.

I thank Allah Almighty for His bounty as He allowed me to accomplish this work by His grace, to Him be praised first and foremost.

Then I thank those good people who helped me during this period, foremost among them is my professor supervising the thesis, His Eminence ***Prof. Amer Khazaal Salih Al-Azzawi(Ph.D.)*** *The one who spared no effort to help me, he opened his house for me, as is his habit with all students of knowledge, and I used to sit with him for long hours reading to him and he did not find any embarrassment in that, and he was urging me to search, and he desired me in it and strengthened my resolve to him, so he has the reward from God and from me all appreciation, may Allah preserve him, enjoy health and wellness, and benefit from his knowledge.*

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Finally, I would like to express my sincere thanks to all those who helped me in producing this study to the fullest.

Zainab Abd Awan

Declaration Form

I hereby declare that this thesis entitled titled **(Serological and Molecular Detection of H5N8 Influenza Virus in Layer Hens in Diyala Governorate) presented at the College of Veterinary Medicine-University of Diyala in 2022**, is my original work, except for quotations and citations which have been duly acknowledged. I also declare that it has not been submitted previously or concurrently, for any other degree at the University of Diyala or other Universities.

Zainab Abd Awan Hama

/ /2022

Abstract

The highly virulent strain of influenza virus first appeared in poultry in 1996 and was named the H5N1 avian influenza virus of the clade 2.3.4 H5 HPAIV.

This study is to use serological identification and molecular detection by ELISA, Real-Time PCR, and RT-PCR to locate the H5N8 virus in various commercial layer flock farms in the Governorate of Diyala from four study locations between September 2021 to April 2022. Also, the study aimed to explore the sequence of the suspected AIV and to observe the relatedness of local Iraqi strains and reference strains from the Gene bank by phylogenetic analysis. Blood samples (364) were collected in the current study from four distinct sites of commercial layer flocks located all over Diyala Governorate at the age of 70 and 200 days old for the serological procedure by using the indirect ELISA technique. Furthermore, for molecular detection, according to severe clinical signs and high mortality rate that appeared on the infected commercial layer flocks from each four study regions, six samples from each flock at age of 200 days old were collected to detect the pathogenic virulent strain of AIV by using real-time PCR through using the specific kit for H5 and H8 protein and specific primer and prob (AIH-5 and AIN-8).

For detection and sequencing, RT-PCR was used for this purpose, 15 gm of tissue samples (trachea, lung, and liver) from clinically commercial layers flocks showing obvious influenza virus infection were taken at 70 and 200 days of age, were exposed to molecular detection by using RT- a polymerase chain reaction. From all samples collected RNA was extracted and screened by RT-PCR by using two Paris of publishing selective primers . Extracted samples were screened

by RT-PCR to amplify the HA- gene of the avian influenza virus. Sequencing present study was followed to determine the biological diversity of the predominant serotype in Diyala Governorate. Only one sample (S1) result from positive PCR covering the coding regions of the HA gene in poultry infected with AIV was amplified. the pattern of observed nucleic acid sequences of infected samples with references to nucleic acid sequencing from the NCBI database.

After that, a thorough tree was constructed using the neighbor-joining method, to evaluate the precise serotyping of the detected variations and their phylogenetic spread by the NCBI BLASTn.The results of the current study showed that the positivity rate of the mean anti-AIV H5N8 rate in AL-Khales flocks which was considerably higher than the three other locations , followed by those layers of AL-Ghalibia flocks . The variations in positive serum between the current research flocks in two areas (AL-Khales and AL-Ghalibia) were statistically significant. Whereas the positive rate in Baqubah and Kanaan, when compared to the AL-Khales region, the differences between the current research flocks at three regions with positive serum were not significant but statistically significant .

The positivity of antiAIV H5N8 titer rates was substantially higher in AL-Khales flocks at 200 days old compared to AL-Ghalibia areas) and also it was substantially higher than all other two regions, namely (Baqubah and Kanaan . Using qRT- PCR in molecular detection of HPAIVH5N8, results showed that 6 out of 24 tracheal tissue samples from suspected flocks were positive with prevalence results of 25%.

. Using conventional Reverse Transcriptase -PCR for H5N8HPAIV detection, the results showed that, out of 48 samples from four regions flock, 32 samples (66.6%) were positive for H5N8. One strain was recorded in NCBI and got an accession number of

(ON247929). The NCBI BLASTn engine found a 99 percent sequence similarity between the sequencing of the local avian influenza virus H5N8 sample and the targeted reference for the 320 bp amplicons. Analyzing the sample detection from layer hens infected with HPAIVH5N8, the present work indicated two variants in the nucleic acid of studied samples C>T70 and G>A106.

These variants showed silent effects (p. Gly288= and p. Ala300=) on the investigated HA-encoded protein. It was inferred from the tree that our investigated sample was suited in the immediate vicinity to Nigerian strains of the serotype H5N8 which were recorded and confirmed in GenBank with acc.

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List of Abbreviations

Abbreviation	Description
a. a	amino acid
AGID	Agar Gel immunodiffusion
AIV	Avian Influenza Virus
Arg	Arginine
Ct	Cycle threshold
ELISA	Enzyme-Linked Immuno Sorbent Assay
EtBr	Ethidium Bromide
HA	Haem Agglutinin
HI	Hemagglutination Inhibition
HPAI	Highly Pathogenic Avian Influenza
HRP	Horseradish Peroxidase
IgG	Immunoglobulin G
IVPI	I/V Pathogenicity Index
LPAI	Low Pathogenic Avian Influenza
M	Matrix
mRNA	Messenger Ribo Nucleic Acids
NA	Neuraminidase
NCBI	National Central for Biotechnology Information
NPAIV	Non-Pathogenic Avian Influenza Virus
NS	Nuclear Signals
O. D	Optical Density
OIE	World Organization for Animal Health
PCR	Polymerase Chain Reaction
PPE	Personal Protective Equipment

RRT-PCR	Quantitative Real-Time Reverse Transcription-PCR
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
vRNA	viral RNA
vRNPs	viral Ribo Nucleoproteins

Chapter One
Introduction

Introduction

The avian influenza virus epidemics that are sweeping the globe have a significant impact on the poultry sector. Because of the virus strain involved and the immunity of the flocks that are being raised, these outbreaks are linked to increased rates of morbidity and death (Vigeveno *et al*., 2020).

Avian influenza is a highly pathogenic disease caused by infection with type A viruses within the family *Orthomyxoviridae*. The causative agent is one of the most catastrophic diseases affecting the poultry industry and is spreading all over the world.

Etiologically, the virus is a single-stranded RNA (ssRNA), negative sense, segmented genome (8 segments) with a total length of 13.5 kb (Lamb, 2001; Maclachlan and Dubovi, 2010).

The structure of neuraminidase (NA) and hemagglutinin (HA) spikes on the surface of these viruses allowed for further subtyping into one of 16 antigenically different HA subtypes ranging from H1 to the H16, and one of nine NA subtypes ranging from N1 to the N9, while two subtypes which have been detected in bats (subtype H17N10 and subtype H18N11) represented the remaining 2 HA (17 to 18) and 2 NA sub (Tong *et al*., 2013; Tzarum *et al*., 2017).

These viruses can attach to the sialic acid of host cells' surface via the HA protein that presents on their surface, but the (NA) protein helped the virus escapes from infected cells once it had completed its reproduction cycle (Tzarum *et al*., 2017; Wang *et al*., 2018).

Besides birds, avian influenza A commonly infects other species and has been isolated from humans and other various animals like pigs, sea mammals, and horses (Ineson et al., 2022).

In contrast to influenza type B, which can exclusively infect humans, viruses of subtype A viruses have evolved to infect different animal species, and humans (Capua and Munoz, 2013; Tong et al., 2013; Dou *et al*., 2018; Shrestha *et al*., 2021).

Based on their capacity to cause this serious infection, two different types of these viruses have been detected and identified. Low pathogenicity (LP) avian influenza virus causes mild to sub-clinical symptoms in poultry (Webster and Rott, 1987; Swayne et al., 2013).

Whereas the second pathotype is very virulent namely (HPAIV) which may induce systemic effects and result in significantly high mortality rates that can occasionally exceed 100% (Hemida *et al.*,2019). Due to its rapid spread and extensive destruction of domestic poultry, HPAIV is sometimes referred to as the "fowl plague." This pandemic that occurred in the 20th century as a result of reassortment between influenza from avians and humans (genetic shift) caused numerous outbreaks in Asia, Africa, and Europe in five time periods during the years 1918, 1957, 1968, 1977, and 2009 by the types of H1N1, H2N2, H3N2, H1N1, and H5N1 respectively (Capua and Alexander, 2007; Dou et al., 2018; Gonzales *et al*., 2018).

A crucial component of the identification and control of infection is the quick and accurate diagnosis and identification of infections caused by these viruses in avians. A conventional approach that is still widely used and recognized for the diagnosis of AIV is viral isolation in embryonated eggs from specified pathogen-free eggs

(SPF), followed by serological detection of HA and NA subtyping (Lee *et al*., 2004; Golabi *et al*., 2021).

To identify the AIV and HA subtypes, some researchers have employed molecular biology techniques such as polymerase chain reaction. Furthermore, another PCR technique was used which is quantitative, fluorescence-based real-time PCR which is able in attaining sensitivity of exceptional nature, specificity, and stability (Elizalde *et al*., 2014; Yang *et al*., 2020).

The highly deadly H5N8 strain of avian influenza that was known as HPAI was initially discovered in wild birds in Asia in 2010 and subsequently spread to domesticated birds via migrating aquatic birds (Lee and Saif, 2009; Lee et al., 2015; Putri et al., 2018).

There are many reports on HPAIV H5N8 that spread in laying hens in many countries of the Middle East, including Iraq and its neighboring countries Iran, Saudi Arabia, and Lebanon (Al-Ghadir *et al.*, 2018). The surface HA receptor protein of H5 viruses belonged to various clades, whereas the 2.3.4.4.4 H5 clade was classified to be 8 sub-clades from (2.3.4.4a) to (2.3.4.4.h) as reported by Gu *et al*., (2022). The first isolation of low pathogenic AIV from flocks of layers occurred in Iraq (Al-Nasrawi *et al.*, 2002). The first cases of the highly contagious avian influenza H5N8 strain were discovered in numerous flocks of laying hens in Diyala Province on March 11, 2018. The disease was discovered through a report from the International Office of Zoological Epidemiology with the report number (26150/11/3 OIE), which officially stated that there are two types of this strain. Its app in the Diyala herds, which are part of clade 2.3.4.4.b, led to a mortality rate of more than 90%.

Aim of The Study

This study aims to identify:

1- Serological identification of HPAIV (H5N8) in some flocks of layer chickens in the Governorate of Diyala.

2- Molecular detection of HPAIV (H5N8) in some flocks of layer chickens in the Governorate of Diyala.

3- Phylogenetic analysis of the HPAIV (H5N8) to observe the relatedness of local Iraqi strains and reference strains from GenBank (NCBI).