

Cairo University  
Faculty of Veterinary Medicine  
Department of Poultry and Rabbit diseases.

# *Some immunogenic and antigenic studies on avian influenza virus infection in Egypt*

*Submitted by*

*Ahmed Maher Abdel-Hamid Helal*

*(B.V.Sc. 2004, Cairo University)  
(M.V.Sc., poultry diseases, Cairo University, 2010)*

**For the degree of Philosophy of Doctor  
in Veterinary Medical Science**

**Under supervision of**

**Prof. Dr.**

*Manal Afifi Aly Afifi*

Prof. of poultry diseases, Faculty of Veterinary Medicine  
Cairo University.

**Dr.**

*Khaled Madian Ahmed*

Assistant Prof. of poultry diseases  
Faculty of veterinary medicine  
Cairo University

**Dr.**

*Susan Sayed Elmahdy*

Chief Researcher of poultry diseases  
and technical Manager of Central  
Laboratory  
For Control of Veterinary Biologics,  
Abbassia, Cairo.

**{2015}**

جامعة القاهرة  
كلية الطب البيطري  
قسم أمراض الطيور و الأرانب

بعض الدراسات المناعية والأنتيجينية على عدوى  
فيروس انفلونزا الطيور فى مصر

رسالة مقدمة من

ط . ب . / أحمد ماهر عبد الحميد هلال  
بكالوريوس العلوم الطبية البيطرية ٢٠٠٤ - جامعة القاهرة  
ماجستير العلوم الطبية البيطرية ( أمراض الدواجن ) (جامعة القاهرة ٢٠١٠ )

للحصول على  
درجة دكتوراة الفلسفة فى العلوم الطبية البيطرية  
(أمراض الطيور و الأرانب )

تحت إشرافه

الأستاذ الدكتور

**منال عفيفي علي عفيفي**

أستاذ أمراض الطيور و الأرانب  
كلية الطب البيطري - جامعة القاهرة

دكتور

**سوزان سيد المهدي**

رئيس بحوث – و المدير الفني  
المعمل المركزي للرقابة علي المستحضرات  
الحيوية البيطرية  
العباسية - القاهرة

دكتور

**خالد مدين أحمد**

أستاذ مساعد أمراض الطيور و الأرانب  
كلية الطب البيطري – جامعة القاهرة

(٢٠١٥)

## C.V

**University:** Cairo

**Faculty:** Veterinary medicine

**Department:** Poultry and Rabbit diseases.

**Full Name:** Ahmed Maher Abdel-Hamid Helal

**Date of birth:** 9/11/1980

**Last degree:** Master degree of vet. science, Poultry and Rabbit diseases. 2010

**Degree:** PhD of vet. Medicine, Poultry and Rabbit diseases.

**Title of the thesis:** Some immunogenic and antigenic studies on avian influenza in Egypt

**Supervisors:** Prof. Dr. Manal Afifi Aly Afifi

Dr. Khaled Madian Ahmed

Prof. Dr. Susan Sayed Elmahdy

### Abstract

Seventy three isolates of AIV were collected from 11 Egyptian governorates during the period from 2010 to 2014, from different poultry flocks, The clinical signs were cyanosis of comb and wattle, echymosis on the shanks and feet, facial edema and greenish diarrhea and drop in egg production .On necropsy, diffuse subcutaneous hemorrhages on the feet and shanks, the lung showed congestion and hemorrhages. Petechiae were noticed throughout on serosal surfaces especially on the peritoneum. The ovaries were hemorrhagic contains yolk from ruptured ova. RT-PCR revealed 43 positive for M gene while 26 positive using H gene primer whereas cleavage site sequence revealed 17 out 73 positive with percentage 58.9%, 35.6% and 23.2%; respectively.

AI strain (A/chicken/Egypt/M7217B/2013(H5N1) represent the most common clusters of AIV was reassorted at National research center and then adapted on MDCK cell line for preparation of Tissue culture (TC) vaccine in parallel with egg adapted one.

For assessment of protective: Eighty one 7 day old SPF chicks, were divided into four groups (1-4), 20 each in isolators at CLVEB, were challenged at 28 day old with  $10^6$  / ml pathogenic AIV (A /ch / Egy / 1709-6 /2008 Eu 717857) via intranasal route. All birds of group 3 (Unvaccinated challenged) died at 3<sup>rd</sup> day post- challenge, whereas unvaccinated unchallenged birds (group 4) survived without showing clinical signs. The results revealed that Protection % of vaccinated groups either with prepared tissue culture AI vaccine or egg adapted AI one were 50%.

Key words: avian influenza, vaccine, embryonated chicken egg, tissue cultur



*Dedecated to:*

My great father and mother

My brother

My sisters

My wife

and My son(Samir)(Jailan)



## Acknowledgement

### Acknowledgment

I wish first to thank forever my *God* for helping me to complete this work as he helps all who search for the truth, because he is the way, the truth and the life.

My sincerely thanks to *Prof. Dr. Manal Afifi* , Professor of poultry and rabbit disease, Faculty of Veterinary Medicine, Cairo University, for his skillful supervision of the present work and for kindly offering his experience and time from many preoccupations.

I am deeply grateful to *Dr. Susan el - Mahdy*, Chief Researcher, technical manager; Central Laboratory for Quality Control of Veterinary Biologics, for generously and conscientiously supervising the work and for her distinct kindness in giving me a lot and unfitting help through my study.

I am in indebted to *Dr. Khalid Madian Ahmed* assistant professor of poultry and rabbit disease Faculty of Veterinary Medicine, Cairo University for his help. I warmly thank *Dr. Ibrahim Soliman* professor at VSVRI, Abbasia, and *Dr. Mohammed A. Ali* professor at national research center

I am specially expressed my appreciation and fruitful help to *Dr. Mounir Elsafty*, and *Dr. Lamiaa M. Omar* Central Laboratory for Quality Control of Veterinary Biologics, for all his help to me. I wish to express my thanks to all staff members of Central Laboratory for Quality Control of Veterinary Biologics. Cardiac thanks for my family for their support, great help and sacrifice.



## Acknowledgement

Special gratitude to ***Dr. Mohammed A. Rohaim*** department of virology Faculty of Veterinary Medicine, Cairo University. and ***Dr. Hassanin Abozeid*** department of poultry and rabbit diseases Faculty of Veterinary Medicine, Cairo University for their scientific support.

It is great pleasure to remember the great help and cooperation of all staff member (doctors, technicians and workers) in the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB) Abbasia, Cairo for their help.



## **list of contents**

<b>1. INTRODUCTION.....</b>	<b>1</b>
<b>2. REVIEW OF LITERATURE.....</b>	<b>5</b>
2.1. History of AIV.....	5
2.1. 1. Historical background.....	5
2.1.2. History in the world.....	6
2.1.3. History in Egypt.....	7
2.4.Ecology and epidemiology of AIV.....	12
2.5. Classification.....	17
2.6.Nomenclature.....	18
2.7. Composition.....	20
2.8. Morphology.....	21
2.9. Diagnosis.....	22
2.9.1.Morbidity and mortality.....	22
2.9.2. Incubation Period.....	23
2.9.3. Clinical signs.....	23
2.9.4. Post Mortem Lesions.....	24
2.10. Immunization against AIV H5N1.....	29
2.10.1. History of AI Vaccines.....	30
2.10.2. General consideration of AI Vaccines.....	31
2.10.3. Selection of AI Vaccine Strain .....	32
2.10.4. Immunological Basis for protection of AI Vaccines	34
2.10.5.Assessment and Evaluation of AI Vaccines.....	35
2.10.6. Administration of AI Vaccines.....	40
2.10.7. Types of AI Vaccines	44
2.11.Tissue culture vaccine.....	56





<b>3. MATERIAL AND METHODS.....</b>	<b>61</b>
<b>3.1. Material .....</b>	<b>61</b>
3.1.1. Samples collection of suspected AI (H5N1) from different governorates.....	64
3.1.2. Transport and storage of specimens ...	65
3.1.3. Avian Influenza Viruses .....	65
3.1.4. Materials used for virus detection and isolation .....	66
3.1.5. Reagents and buffers for extraction of viral RNA .....	68
3.1.6. Reagents and buffers for real-time reverse transcriptase rt-PCR	69
3.1.7. Reagents and buffers for conventional RT-PCR .....	70
3.1.8. Buffers and reagents used for agarose gel electrophoresis .....	71
3.1.9. QIAquick® PCR Purification kit.....	72
3.1.10. BigDye® Terminator v1.1 Cycle Sequencing kit.....	72
3.1.11. DyeEx 2.0® Spin kit.....	72
3.1.12 Buffers and solutions used for virus isolation.....	73
3.1.13. Materials used for virus identification	74
3.1.14. Materials used for viral titration.....	74
3.1.15. Reagents used in preparation of inactivated vaccine .....	75
3.1.16. Media used for testing the prepared vaccine .....	75
3.1.17. Solutions and buffers used agar gel precipitation test (AGPT)	76
<b>3.2. Methods .....</b>	<b>76</b>
3.2.1. Virus isolation and detection.....	76
3.2.2. Virus identification by slide HA .....	77
3.2.3. Infectivity titration in SPF ECEs .....	78
3.2.4. Real time-Reverse Transcription-Polymerase Chain Reaction	78
3.2.5. Identification of virus by reverse transcriptase polymerase chain reaction.....	82





	3.2.6. Analysis of amplified RNA by agarose gel Electrophoresis	83
	3.2.7. Purification of the PCR Products.....	84
	3.2.8. Sequence PCR a part of H gene from AI isolate by using BigDye Terminator	86
	3.2.9. Sequence analysis	86
	3.2.10. Sequence submission to the GenBank	87
	3.2.11. Preparation of media and reagent formulas	87
	3.2.12. Some steps of reassortment	88
	3.2.13. Inoculation of reassortant avian influenza H5N1 virus	88
	3.2.14. Inactivation by Binary ethylenimine (BEI) for the reassortant H5N1 virus	89
	3.2.15. Infectivity test for testing complete inactivation	89
	3.2.16. Vaccines preparation using oil adjuvant	89
	3.2.17. Physical evaluation of the prepared vaccines( Emulsion type)	90
	3.2.18. Quality control of the prepared vaccines	90
	3.2.19. Statistical analysis	92
	<b>4. Experiments and Results.....</b>	<b>94</b>
	<b>5. DISCUSSION.....</b>	<b>130</b>
	<b>6. SUMMARY.....</b>	<b>151</b>
	<b>7. REFERENCES.....</b>	<b>155</b>
	<b>8. ARABIC SUMMARY.....</b>	<b>1</b>



## LIST OF TABLES

<i>Table no.</i>	<i>Title</i>	<i>page</i>
<b>1</b>	Clades of Asian-lineage H5N1 HPAI viruses (H5N1 Evolution Working Group, (WHO, OIE and FAO) 2007).	<b>19</b>
<b>2</b>	Some epidemiological data on AI during 2010 – 2014 in Egypt	<b>61</b>
<b>3</b>	The oligonucleotide for amplification of part of H5 gene for AIV-H5	<b>68</b>
<b>4</b>	The oligonucleotide for amplification of cleavage site region of H5 gene for AIV	<b>70</b>
<b>5</b>	Probe and primer sets phase step	<b>80</b>
<b>6</b>	Thermal cycles	<b>81</b>
<b>7</b>	The cycling protocol of RT-PCR for amplification of internal sequence of HA gene of H5N1 AIV	<b>83</b>
<b>8</b>	some historical and field data of some poultry flocks suspected to be infected with AI	<b>96</b>
<b>9</b>	Results of seventeen cleavages H5 in ECE	<b>105</b>
<b>10</b>	Percentage identity of partial HA sequences	<b>108</b>
<b>11</b>	Results of propagation of AIV in ECE	<b>114</b>
<b>12</b>	Results of propagation of AIV in MDCK	<b>115</b>
<b>13</b>	Titration of AIV in ECE	<b>117</b>
<b>14</b>	Inactivation rate of the reasserted AIVs	<b>119</b>
<b>15</b>	Sterility of vaccine	<b>122</b>
<b>16</b>	HI titer of chick of serial diluted seed AIVs	<b>124</b>
<b>17</b>	Shelf life time of the prepared vaccines	<b>126</b>
<b>18</b>	The protection percentage post-HPAI challenge of vaccinated SPF chicks compared with control birds.	<b>128</b>
<b>19</b>	Shedding results of AIV from experimental chickens	<b>129</b>



## **LIST OF FIGURES**

<i>table</i>	<i>Title</i>	<i>page</i>
<b>1</b>	Highly pathogenic Avian Influenza H5 confirmed outbreak World health organization (W.H.O., 2012).	<b>13</b>
<b>2</b>	Diagrammatic representation of the source and movement of influenza A viruses or their genes within avian and mammalian ecological and epidemiological situations	<b>14</b>
<b>3</b>	Schematic diagram for influenza A virus gene segment shape length corresponds to original segment length, function to the right	<b>21</b>
<b>4</b>	Clinical signs of suspected avian influenza from the examined flocks	<b>99</b>
<b>5</b>	Post mortem of suspected avian influenza from the examined flocks	<b>100</b>
<b>6</b>	The amplification blot of the AI isolates of HPAI H5N1 showed variation in the Ct value	<b>102</b>
<b>7</b>	Ethidium bromide stained agarose gel electrophoresis containing the RT-PCR products of; lane 1, 2, 3, 4 and 5: internal part of partial HA gene of AIV (320 bp); lane 6: represents positive control along with 100 bp plus DNA ladder (lane M).	<b>103</b>
<b>8</b>	Multiple nucleotide sequences alignment of partial HA gene of our recently isolated strains during 2014 in comparison with other representative strains of H5N1 AIVs circulating among chicken population	<b>107</b>
<b>9</b>	Amino acid alignment of partial HA gene of our recently isolated strains during 2014 in comparison with other representative strains of H5N1 AIVs circulating among Egyptian chicken population. The	<b>109</b>



	Dot (.) represents identity whereas single alphabet represents difference in the amino acid sequence	
<b>10</b>	Neighbor joining Phylogenetic rooted tree	<b>113</b>
<b>11</b>	Curve of infectivity titration of AIV in MDCK	<b>115</b>
<b>12</b>	Ethidium bromide stained agarose gel electrophoresis containing the RT-PCR products of; lane 1: internal part of partial HA gene of AIV (320 bp) along with 100 bp plus DNA ladder (lane M).	<b>116</b>
<b>13</b>	Embryos of ECE with different AIV inoculation	<b>118</b>
<b>14</b>	Geometric Mean HI titer	<b>124</b>
<b>15</b>	Mean Antibodies titer HI test / log2	<b>126</b>
<b>16</b>	Curve of protection percentage of prepared vaccines	<b>128</b>
<b>17</b>	Curve of shedding percentage of the virus	<b>129</b>



## **1- Introduction**

Avian Influenza (AI) is an important poultry pathogen and a massive menace to the poultry industry. Since the late 1990s, highly pathogenic avian influenza (HPAI) viruses of the H5N1 subtype have devastated the poultry industry of numerous countries. After 2004, H5N1 has spread from Asia to Europe, Africa, and the Middle East, resulting in the killing or culling of hundreds of millions of domestic birds (*Li et al., 2004*). Furthermore, highly pathogenic AIVs (HPAIVs) H5N1 infections in poultry constitute a threat to humans and resulted, till the 2<sup>nd</sup> of October 2014, in 676 human cases with 398 deaths being reported worldwide and in Egypt alone, a total of 185 cases with 68 deaths have been reported (*WHO, 2014*). Occasionally, HPAI H5N1 viruses cross the species barrier and infect humans, sometimes with a severe clinical outcome. This direct transmission of HPAI H5N1 virus to humans was first detected in 1997 (*De Jong et al.1997*).

According to the nucleoprotein (NP) and matrix (M) proteins, influenza viruses are classified into 3 types; A, B and C. AIVs belong to type A which is further diversified based on the surface glycoproteins; hemagglutinin (HA) and neuraminidase (NA), into 18 HA and 11 NA subtypes (*Tong et al., 2013*).

Based on their virulence, two main AI pathotypes do exist; HPAIVs and low pathogenic ones (LPAIVs). Infection of poultry with HPAIVs usually results in multiorgan systemic disease. Such infections, especially whenever H5 subtype is incriminated, constitute a serious impact on both poultry production, including high rates of morbidity and mortality in susceptible flocks and human health (*Koopmans et al., 2004; Fauci, 2006*).

In Egypt, in February of 2006, severe outbreaks of HPAI, H5N1; have emerged in several governorates and were associated with drastic mortality up to 100% in infected chickens (*Aly et al., 2006*).

Presumptive diagnosis of HPAI in poultry may include the clinical signs and postmortem lesions but none of these are pathognomonic, and the etiology must be

confirmed by diagnostic tests (*Swayne and Suarez, 2000*). These tests include virus isolation, antigen detection, nucleic acid detection and sero-diagnosis. Reverse transcriptase polymerase chain reaction (RT-PCR) for viral nucleic acid detection is considered a valuable and rapid tool for accurate diagnosis followed by sequencing of the full length haemagglutinating gene (*Spackman et al., 2002; Dybkaer et al., 2004; Chua et al., 2007*).

Control of AI based on biosecurity level, surveillance and vaccination strategy which consider a supportive tool in AIV control strategies was implemented to limit the spread of H5N1 infection and to reduce the losses (*Lee and Suarez, 2005; EFSA, 2008*). Control policy of H5N1AI in poultry in Egypt was initially based on stamping out and surveillance and, in late 2006; vaccination was implemented as an additional control measure using inactivated whole virion H5N1 or H5N2 vaccines. Mass vaccination using commercial vaccines led to decrease in the incidence of new outbreaks by the end of 2006 and early 2007(*Peyre et al., 2009*). The government at that time was providing vaccination of household birds free of charge. However, since July 2009, vaccination in household settings has been provisionally suspended until a new vaccination strategy is adopted (*FAO, 2008*).

There are different types of AI vaccines widely used in Egypt. Reverse genetically H5N1 Chinese strain A/goose/ Guangdong /1/1996 (H5N1) and H5N2 low pathogenic killed Mexican strain vaccine A/chicken/Mexico/232/94 (H5N2) are vaccines ‘types widely used in Egypt. These vaccines must decrease shedding of the virus, morbidity, mortality, and transmissibility; reduce field virus replication; and decrease egg production (*Van den Berg et al., 2008; Swayne, 2009*) as well as minimize its socioeconomic impacts. The poultry industry has received large amount of mass vaccination policy to reduce circulation of the H5N1 virus, but with limited success (*Hafez, 2010*).

Despite the nationwide vaccination strategy of poultry in Egypt to combat H5N1 AI, continuous circulation of the virus in vaccinated and non-vaccinated commercial and backyard poultry was reported (*Hafez et al., 2010*).

One of the main causes of vaccination failure as a control measure is the variation in AIV antigenicity which develops gradually by point mutation (antigenic drift) occurring in the major antigenic sites at the globular head of HA (*Cattoli et al., 2011*) or drastically by genetic reassortment (antigenic shift) (*Bouvier and Palese, 2008*). Antigenic analysis of earlier H5N1 strains in Egypt demonstrated considerable variations (*Balish et al., 2010*) with circulation of stable lineages of H5N1 viruses since late 2007. Presence of the virus under immune pressure in vaccinated birds accelerated the mutation rate of the virus to escape from the repertoire of immune response (*Hafez et al., 2010*).

Increase in incidence of H5N1 outbreaks in vaccinated poultry in Egypt highlighted the significance of continuous monitoring of circulating viruses both genetically and antigenically to define the virus cluster prevalent in the field as well as to select the best vaccine strain which is able to cross-react with circulating viruses from different lineages. The ideal seed virus for vaccine production is, typically, a strain of low pathogenicity that is well matched antigenically with the prevailing virus (*Chen et al., 2003*). All H5N1 viruses isolated all over the world since 1996 have multiple basic amino acids in the cleavage site of HA and, therefore, all are highly pathogenic and so they are unsuitable for vaccine production. The main problem is the inability to obtain high yields of virus in embryonated chickens eggs (ECE) (*Subbarao and Katz, 2004*). New cell line substrates for the manufacture of inactivated influenza vaccine may alleviate the limitations of egg-based vaccines (*Mabrouk and Ellis 2002*).

Recent research has focused on the development of cell culture– derived vaccine production technology using either mammalian cell lines for viruses or insect cells for recombinant antigens. Production in mammalian cell lines offers significant advantages in the manufacturing process over egg-based production, because it allows more flexibility, faster production, and adequate availability of substrate for virus growth (*Genzel et al., 2004*).