

Zagazig University  
Faculty of Veterinary Medicine  
Department of Avian and Rabbit Medicine



**“Protective Efficacy of Avian Influenza(H9N2)  
Commercial Vaccines in Chickens”**

By

**Hossam Abdel Aleam Abdel Hafez Gado**  
B.V.Sc., Zagazig University, 2009  
M.V.Sc., Zagazig University, 2014

*Under supervision of*

<b>Prof. Dr.</b> <b>Amal A. M. Eid</b> Prof. of Avian and Rabbit Diseases; Faculty of Veterinary Medicine; Zagazig University.	<b>Prof. Dr.</b> <b>Ibrahim A. Ghanem</b> Prof. of Poultry Diseases; Faculty of Veterinary Medicine; Zagazig University.
--	--

**Prof. Dr.**  
**Abdullah A.M. Selim**  
Chief Researcher; RLQP;  
Animal Health Research Institute;  
Dokki; Giza.

**A Thesis**  
**Submitted to Zagazig University**  
**For PhD. degree in Veterinary Medical Sciences**  
**(Avian and Rabbit Diseases)**  
**Avian and Rabbit Medicine Department**

**2021**

## **LIST OF CONTENTS**

<b>Titles</b>	<b>Page</b>
<b>I- INTRODUCTION</b>	1
<b>II-REVIEW OF LITERATURE</b>	3
<b>III- MATERIAL AND METHODS</b>	38
III.1 Materials	38
III.2 Methods	52
<b>IV.RESULTS</b>	61
<b>V. DISCUSSION</b>	133
<b>VI. SUMMARY</b>	147
<b>VI I.REFERENCES</b>	150
<b>VITA</b>	194
<b>ARABIC SUMMARY</b>	----

**LIST OF TABLES**

<b>No.</b>	<b>Titles</b>	<b>Page</b>
<b>1</b>	H9N2 LPAIV co-infections with different pathogens under variable experimental conditions in chickens	<b>27</b>
<b>2</b>	Evaluation of low pathogenic AIV H9N2 Vaccines in experimental studies	<b>36</b>
<b>3</b>	Descriptive data of examined chicken flocks that suspected to be infected with avian influenza virus subtype H9	<b>40</b>
<b>4</b>	The Sequence of primers and probes used in real time PCR	<b>44</b>
<b>5</b>	Primers used in Reverse Transcriptase-Polymerase Chain Reaction (one step RT-PCR) and Sequence reaction of HA gene	<b>45</b>
<b>6</b>	Primers used in Reverse Transcriptase-Polymerase Chain Reaction (one step RT-PCR) and Sequence reaction of NA gene	<b>45</b>
<b>7</b>	The Sequence of primers and probes used in purity test	<b>50</b>
<b>8</b>	Real-Time RT-PCR Reaction Mix Volumes for one reaction for H5, H9, ND, IB virus detection	<b>53</b>
<b>9</b>	Real-Time RT-PCR Reaction Mix Volumes for one reaction for MG and MS detection	<b>54</b>
<b>10</b>	Thermo cycling Conditions for Gene-Specific Probe and Primer Sets	<b>54</b>

<b>11</b>	Reaction components for RT-PCR of the H9 and NA genes of avian influenza	<b>55</b>
<b>12</b>	RT-PCR condition for H9 and NA genes of avian influenza	<b>55</b>
<b>13</b>	Experimental design for evaluation of effectiveness of H9N2 avian influenza inactivated vaccines	<b>60</b>
<b>14</b>	Molecular detection of avian influenza viruses and other suspected co-infections (IBV and NDV) by real-time PCR	<b>63</b>
<b>15</b>	Accession numbers of the H9 AIV field isolates on GenBank.	<b>72</b>
<b>16</b>	Amino acid alteration in the receptor binding site of HA of H9N2 viruses in comparison to A-Quail-HK-G1-97	<b>90</b>
<b>17</b>	Differences in antigenic epitopes of HA gene	<b>91</b>
<b>18</b>	Glycosylation sites of HA in comparison to A-Quail-HK-G1-97 the ancestor of the Egyptian H9N2 viruses	<b>92</b>
<b>19</b>	Amino acid substitution in the hemadsorbing sites and stalk region of NA of H9N2 viruses in comparison to A-Quail-HK-G1-97	<b>107</b>
<b>20</b>	Sequence of amino acids in Glycosylation sites of NA proteins	<b>108</b>
<b>21</b>	Percent amino acid identity comparison between our study isolates with other viruses and vaccinal seeds	<b>110</b>
<b>22</b>	Titration of S6	<b>119</b>
<b>23</b>	Titration of S2	<b>120</b>

*List of Tables*

---

<b>24</b>	H9N2 virus shedding in vaccinated and non-vaccinated challenged SPF chickens	<b>124</b>
<b>25</b>	Histopathological Lesion score in experimental chickens	<b>127</b>
<b>26</b>	Serological response by ELISA ID-screen H9 kit in experimental chickens	<b>131</b>
<b>27</b>	Serological response by HI test using local and imported antigens in experimental chickens	<b>132</b>

***LIST OF FIGURES***

<b>Fig. No</b>	<b>Title</b>	<b>Page</b>
1	A Broiler chicken 28 day-old naturally infected with H9AIV showing conjunctivitis with facial edema (Farm No. 6).	61
2	Layer flock eggs 305 days naturally infected with H9AIV showing small, thin shell and misshape eggs with 25% drop in egg production (Farm No. 2).	62
3	Amplification curve for AIV Matrix gene rRT-PCR	64
4	Amplification curve for AIV H9 gene rRT-PCR	65
5	Amplification curve for AIV H5 gene rRT-PCR	66
6	Amplification curve for AIV H7 gene rRT-PCR	67
7	Amplification curve for IBV rRT-PCR	68
8	Amplification curve for NDV rRT-PCR	69
9	Agarose gel electrophoresis of the RT-PCR products of H9 gene	70
10	Agarose gel electrophoresis of the RT-PCR products of NA gene	71
11	Alignment of Nucleotide sequences of H9 gene of four Egyptian H9 isolates in comparison to other selected strains	74
12	H9 nucleotide identities and divergence of two Egyptian H9 isolates (S2&S6) in comparison to other selected strains (full gene sequence)	81
13	H9 nucleotide identities and divergence of four Egyptian H9 isolates (S1 &S2&S6&S9) in comparison to other selected strains (Partial gene sequence)	82
14	Alignment of amino acid sequences of H9 protein of	85

*List of Figures*

	four Egyptian H9 isolates in comparison to other selected strains	
15	Alignment of Nucleotide sequences of N2 gene of four Egyptian H9 isolates in comparison to other selected strains	94
16	N2 nucleotide identities and divergence of two Egyptian H9 isolates (S2&S6) in comparison to other selected strains (full gene sequence)	101
17	N2 nucleotide identities and divergence of four Egyptian H9 isolates (S1 &S2&S6&S9) in comparison to other selected strains (Partial gene sequence)	102
18	Alignment of amino acid sequences of N2 protein of four Egyptian H9 isolates in comparison to other selected strains	104
19	Phylogenetic analysis of H9 gene nucleotide sequences of AIV(S2&S6) isolates from Sharkia Province, Egypt and other sequences available in GenBank(Full gene)	112
20	Phylogenetic analysis of H9 gene nucleotide sequences of AIV(S1&S2&&S6 and S9) isolates from Sharkia Province, Egypt and other sequences available in GenBank(Partial gene)	113
21	Phylogenetic analysis of NA gene nucleotide sequences of AIV(S2&S6) isolates from Sharkia Province, Egypt and other sequences available in GenBank(full gene)	114
22	Phylogenetic analysis of NA gene nucleotide sequences of AIV(S1&S2&S6 and S9) isolates from Sharkia Province, Egypt and other sequences available in GenBank (partial gene)	115
23	Amplification curve for AIV H9 gene rRT-	116

*List of Figures*

	PCR(purity test)	
24	Amplification curve for AIV H5 gene rRT-PCR(purity test)	116
25	Amplification curve for MG rRT-PCR(purity test)	117
26	Amplification curve for MS rRT-PCR(purity test)	117
27	Amplification curve for ND (M gene) rRT-PCR(purity test)	118
28	Amplification curve for IBV rRT-PCR(purity test)	118
29	Standard curve for isolate A-Chicken-Egypt-S6ZAG-2018	125
30	Shedding amplification curves for samples (1: 30) 3rd day post challenge	125
31	Shedding amplification curves for samples (31: 60) 5th day post challenge.	126
32	Shedding amplification curves for samples (61: 90) 7th day post challenge	126
33	Representative Photomicrograph of the pulmonary tissue	128
34	Representative Photomicrograph of the tracheal tissue	128
35	Representative Photomicrograph of the bursa tissue	129
36	Representative Photomicrograph of the thymus tissue	129
37	Representative Photomicrograph of the cecal tonsile	130
38	Representative Photomicrograph of the renal tissue	130



## **SUMMARY**

Avian influenza is a contagious disease caused by type A influenza viruses. In spite of the mild nature of H9N2 low pathogenic avian influenza virus, the virus was isolated from frequent disease outbreaks with high mortality in different parts of the world.

Nine chicken flocks of different breeds and ages during 2018 were examined in Sharkia Governorate. Samples were collected from farms that experienced respiratory distresses or drop in egg production.

The flocks were tested by real time PCR for determination of the H9N2 subtype in each sample, also they were tested for the presence of other viruses (H5, H7, NDV & IBV), which revealed four flocks were positive for H9AIV, negative for other avian pathogens; two were positive for IBV and three flocks were negative.

The examined broiler flocks showed mild respiratory signs: sneezing, swelling of periorbital tissues with conjunctivitis, nasal and ocular discharges. Whitish diarrhea also noticed. While, examined layer flocks showed depression, decrease in feed consumption with drop in egg production with thin-shelled, rough and misshapen eggs.

The postmortem findings of the examined broiler flocks revealed tracheitis, congestion of lungs and air-sacculitis. Swollen Kidney was also detected.

## *Summary*

---

The molecular characterization was carried out for HA and NA genes of the H9N2 isolates, at first the conventional PCR amplification for the HA and NA genes into 2 fragments was applied, the positive PCR products were sequenced for the nucleotides of the HA and NA genes, All of these sequences have been submitted to the GenBank database.

The HA identity % among our isolates shows high percent (96.9 – 99.8 %), also with other Egyptian isolates in gene bank (2011-2019) (94.3-98.4%). Egyptian viruses were closely related to the Israeli viruses with high identity% (94-96.2%). In the other hand, The NA identity % among our isolates shows (97.5 – 99.9 %), also with other Egyptian isolates in gene bank shows very high percent (96-97.9%) and, were closely related to the Israeli viruses with high identity% (94.1-96.1%).

Phylogenetic analysis revealed that the HA and NA of our Egyptian H9N2 isolates are related to the Middle Eastern H9N2 isolates and maintained a direct out-group relationship to the prototype G1-like viruses, forming a distinct cluster.

The four samples were isolated and propagated in the SPF embryonated chicken eggs then the collected allantoic fluids were tested by HA assay for the detection of the virus titers which produce a titers ranged from  $2^4$  to  $2^9$ . Then (S2 & S6) titrated in 10-day-old SPF embryonating eggs to determine the 50% embryo infectious dose titer (EID<sub>50</sub>) calculated by the Reed and Muench. Virus (A/chicken/Egypt/S6ZAG/2018) was diluted with sterile phosphate buffered saline (PBS, pH 7.4) to adjust the

## *Summary*

---

amount of inoculum to  $1 \times 10^6$  EID<sub>50</sub> per bird. 100µl of diluted virus per bird was given.

Protection efficacy of commercial H9N2 vaccines were evaluated by two field available vaccines (**vaccine 1 A/chicken/Egypt/ME543V/2016 and vaccine 2 A/chicken/Iran/Av1221/ 1998**) through groups each (15 birds) of SPF one day old chicks named (G1/G2/G3/G4/G5/G5/G6) respectively then challenged them with field isolate A/chicken/ Egypt/ S6ZAG/ 2018.

Protection was evaluated by clinical signs, histopathology, immune response weekly by ELISA and HI tests and tracheal swabs were used to monitor virus shedding at 3, 5 and 7 days post challenge by qRT-PCR test.

Our findings indicated that only some depression was obvious in positive control group. Also, there was not any mortality in all groups during the experiment, which confirmed the low pathogenic nature of H9N2 AIV.

It has been seemed that H9N2 vaccine 1 stopped shedding of the field virus at the 3rd day post challenge and the vaccine 2 succeeded to prevent any field virus shedding.

From this study, it concluded that H9N2 vaccines are efficient to decrease and also prevent AI shedding in specific pathogen free (SPF) chickens and give very good antibodies response against H9N2 after single vaccination with oil emulsion inactivated vaccines.