Global Veterinaria 16 (6): 557-564, 2016
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2016.16.06.10463

Antibody Response of Commercial Vaccinated Layers Against H9N2 Viruses in Egypt

[1]Amany Adel, [1]Abdelsatar Arafa, [2]Hussein A. Hussein and [2]Ahmed El-Sanousi

[1]National Laboratory for Veterinary Quality Control on Poultry Production,
Animal Health Research Institute, P.O. Box: 264-Dokki, Giza- Egypt, 12618
[2]Virology Department, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt, 12211

Abstract: Low pathogenic avian influenza H9N2 viruses are widely spreading in the world and Middle East resulting in great economic losses in poultry industry especially in combination with secondary infections. In Egypt the control ofH9N2 virus in chickens depends on vaccination, most vaccines used are imported and prepared from heterogonous strains mostly chinese H9N2 viruses. In this study, 80 samples of antisera were collected from 4 farms of vaccinated commercial layers flocks and tested by serological hemagglutination inhibition (HI) assay versus 2 Egyptian antigens represent the genetic groups of Egyptian H9N2 viruses. The study elucidates that the viruses represent the 2 genetic groups of Egyptian H9N2 are highly similar in antigenic response against the vaccinated antisera of studied layers flocks used different vaccines, the high sero-conversion for the vaccinated antisera against both 2 Egyptian antigens indicates the potency of the vaccines in field to give high immunity against circulating H9N2 viruses in Egypt.

Key words: LPAI H9N2 · Hemagglutinin · HI Assay · Vaccinated Layers · H9N2 Vaccines

INTRODUCTION

Low pathogenic avian H9N2 virus is firstly isolated from turkeys in Wisconsin in 1966 (Tu/WS/66) [1] and distributed all over the world countries in Asia, Europ and Africa including the Middle East [2, 3]. All the poultry species were susceptible and infected with LPAI H9N2, the infection leads to severe economic losses with many outbreaks complicated with other pathogens viral infection as Infectious bronchitis and bacterial infection like staphylococcus and mycoplasma [4].

In Egypt, before the first record for the detection of virus in quail flock in 2011 [5] there was serological evidence for the presence of H9N2 infection in chicken population during 2001 [6]. The infection with H9N2 in layers and breeders chicken causes respiratory signs and drop in egg production [7, 8] while, the infected quail and broiler flocks mostly are apparently health with no clinical signs [9] but the prevalence of virus infection in duck is lower than in chicken [10]. The incidence of infection in chicken with H9N2 in Egypt is higher than other species and the infected broilers, layers and breeders are apparently healthy, otherwise the secondary bacterial and viral infection with specific clinical features cause clinical signs on birds [11]. The Egyptian H9N2 virus was related to G1-like lineage and belonged to group B according to hemagglutinin sequence [5, 9].

Application of biosecurity measures, in addition to the vaccination programs are very important to control and prevent the spreading of influenza virus in chickens commercial flocks [12]. Avian Influenza inactivated vaccines either monovalent or polyvalent subtypes, evoke immunological protection and could reduce the morbidity, mortality and clinical signs of influenza virus [13, 14].

Since the emergence of H9N2 in Egypt, many H9N2 vaccines either local or imported have been used for controlling the disease in commercial flocks, a local H9N2 strain is used for immunization as inactivated vaccine, either single or bivalent vaccine with H5 virus and trivalent vaccine with Newcastle disease virus (NDV) and Infectious bronchitis virus (IBV) to control the disease [15]. Recently, a monovalent conventional inactivated H9N2 vaccine was prepared using local Egyptian strain with Montanide 71 ISA VG adjuvant, which is an authorized, potent and safe vaccine and could give protection and reduce virus shedding [16].

Corresponding Author: Amany Adel, National Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, P.O. Box: 264-Dokki, Giza- Egypt, 12618.
Tel: +202 33380121, Fax: +202 33370957, E-mail: a.adel18784@gmail.com.

In this study, we try to find out the level of immunity induced by vaccines against different circulating H9N2 strains. The base of our study depends on measuring the sero-conversion of vaccinated layers flocks, 80 serum samples of 4 vaccinated flocks were tested by hemagglutination inhibition assay (HI) against 2 antigens of H9N2 viruses represent the different circulating viruses in Egypt.

MATERIALS AND METHODS

Sampling: 80 samples of sera were collected from 4 layer chicken flocks vaccinated against H9N2, The 2 viruses used in this study as antigens were routinly tested by real time PCR for subtyping as avian influenza subtype H9. All the viruses and antisera used in this study were collected in National laboraory for quality control on poultry production.

Isolation of Viruses: The 2 viruses of H9N2 were isolated on specific pathogen free (SPF) embryonated chicken eggs at 9-11 day old embryo, by intra-allantoic rout, incubated at 37°C for 3-5 days. After isolation, hemagglutination assay was done on collected allantoic fluids for make measure the HA unit for each isolate. As the 2 viruses used in this study were V3413-Quail-2011 with accession no. JN828570 and and S83-Turkey-2013 with accession no. KU296198.

Genetic Characterization of Hemagglutinin Gene: Amplification of hemagglutinin gene: it was implemented by one step RT-PCR using Qiagen kit (QIAGEN, Hilden, Germany) with specific primers for the HA gene of H9 subtype in table no (2), The RT-PCR cycling as follows: one cycle at 50°C for 30 min, one cycle at 95 °C for 15 min and 40 cycles of 94°C for 45 Sec, 56°C for 45 Sec and 72°C for 2 min., a final extension at 72°C for 10 min. in thermo cycler 2720 ABI.

Sequencing for Hemagglutinin (HA) Gene: It was done by using Big dye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer, Foster City, CA). The cycling protocol for sequence reactions is: 1 cycle at 96° C for 1 min, 25 Cycles repeated at 96° C for 10 Sec, 50° C for 5 Sec. and 60°C for 2 min. The sequencing reactions were purified using a spin column Centrisep kit (Applied Biosystems, USA) then loaded in a sequencer plate of ABI (Applied Biosystems 3130 genetic analyzers, USA).

Genetic and Phylogenetic Analysis of HA Gene: The alignment of HA gene was done using Bioedit software (Version 7.1) [18] for analysis of the nucleotide and amino acid sequences. Mega6 software was used for phylogenetic analysis at 1000 Bootstrap [19].

Preparation of H9N2 Inactivated Antigens: The 2 selected isolates were filtered with sterile filter in size 0.20 um and then inactivated by beta-propiolactone (BPL) with concentration 1:1000 [20].

Hemagglutination Inhibition Test (HI): HI test was carried out [20] for serological detection of antibodies of each isolate against 80 samples of sera (20 samples/flock) collected from 4 field flocks of layers, The antigens selected based on the common genetic structure of the Egyptian H9N2 viruses where the first was the original virus of 2011 and the second represent the 2013 virus circulating till now. Each antisera tested against the 2 Egyptian antigens and the test was repeated for 2 times for each sample.

Statistical Analysis for Results of HI Test: By using Microsoft Excel software, we calculated the means and standard deviations for each flock tested against each Egyptian antigen, the One-way ANOVA for Means was applied to find out the significant deviation between the

Table 1: The data of antisera flocks in this study

Code	Age	Type of birds	Time of vaccination	Vaccines other than H9
F1	168 days (24w)	Layers Hylin	133 days (19w)	IB, NDV
F2	203 days (29w)	Layers Hylin	168 days (24w)	H5N3, NDV, IBV
F3	154 days (22w)	Layers Hylin	100 days (14w)	NDV, H5N1
F4	224 days (32w)	Layers Hylin	140 days (20w)	--

Table 2: Primers for HA (H9) RT-PCR amplification

Primer ID	Sequence (site on HA gene)	Reference
F1-6	TAG CAA AAG CAG GGG AAT TTC TT (1-23)	NLQP
H9-Rev	GCC ACC TTT TTC AGT CTG ACA TT (898-920)	[17]
H9-For	GGA AGA ATT AAT TAT TAT TGG TCG GTA C (738-765)	[17]
HT7R	TAA TAC GAC TCA CTA TAA GTA CAA ACA AGG GTG (1731-1763)	SEPREL

different means of HI titers. Also, linear Regression analysis for all the HI values of all vaccinated flocks antisera against each antigen to find out the correlation between the 2 antigens.

RESULTS

Isolation of Viruses: The isolated viruses were collected from inoculated eggs and tested by hemagglutination assay to give the HA activity 2log8 for V3413-Quail-2011 and 2log7 for S83-Turkey-2013.

Genetic Characterization of Hemagglutinin Gene: The amino acids alignment of mature hemagglutinin in Fig. (1) show 6 mutations at amino acid residues in the S83/Turkey/2013 antigen and the 2 antigen share in the most important amino acid residue Leu (L2106). By phylogenetic analysis, these 2 Egyptian antigens in the study located in 2 different subgroups within the Egyptian viruses clarified in Fig. (2). The sublineage 1 includes the original Egyptian H9N2 strain A/qu/Egy/3413v/11 and enclosed on the viruses isolated during 2011 to 2012, yet the sublineage 2 includes the S83/Turkey/2013 and it represent mainly the circulated viruses isolated during 2013 to 2015 with evidence of presence isolates from 2011 and 2012. The identity % between the isolates of our study is very high and closely similar with identity % = 98.6% and with the local strain candidate for vaccination the identity % = 98.6-99.8%. as shown in Table 3.

Statistical Analysis of Hemagglutination Inhibition Results (HI): The values of HI titers and their means for the antisera of vaccinated layers flocks in this study showed high means of HI titers against the 2 antigens with rang 2log7.24 – 2log9.95 fig.(4). Mostly the flock 3 and flock 4 have relative low HI titers against both of the Egyptian antigens may be due to the time of vaccination is earlier than in both flocks 1 and 2. The results of HI titers and means were briefly clarified in Table no.(4). One-way ANOVA for Means of the HI titers for the 4 flocks against the 2 Egyptian antigens at p-value= 0.05 and confident level 95% showed no significant variations with p-value >0.05 (=0.99), also The linear regression for all the individual titers of all flocks against the 2 Egyptian antigens reveals positive correlation between the V3413-Quail-2011 and S83-Turkey-2013 antigens with high R^2= 0.85 and p-value > 0.05 as shown in Fig. (3).

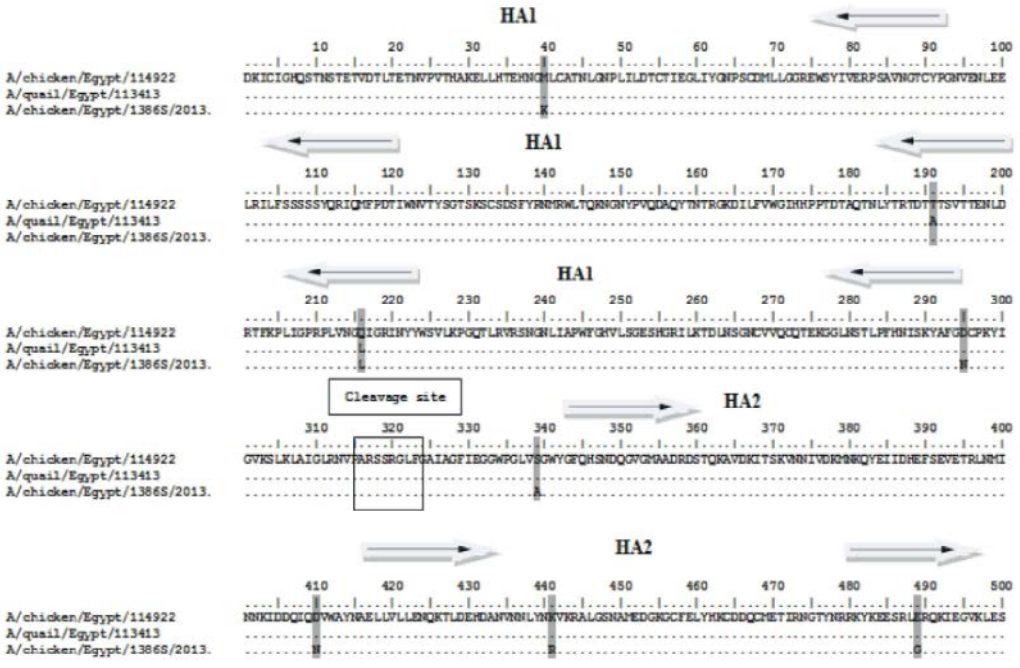

Fig. 1: The amino acids sequence alignment (With H9 numbering for mature hemagglutinin) of the 2 Egyptian isolates used in the study in comparison with the local strain candidate for vaccine preparation (A/ch/Egy/4922v/11). The amino acid residues have substitutions labeled with gray shades and the cleavage site sequence are rounded with black frame

Table 3: Identity % of amino acids of the used antigens and the local strain candidate for vaccine preparation

Antigens ID	A/ch/Egy/4922V/11	A/qu/Egy/3413V/11	A/tu/Egy/86S/13
A/ch/Egy/4922V/11		99.8%	98.6%
A/qu/Egy/3413V/11	99.8%		98.6%
A/tu/Egy/86S/13	98.6%	98.6%	

Table 4: The means of HI titer and standard deviations (SD) were calculated for the results of each farm against each antigen

Antigens	V3413-Quail-2011				S83-Turkey-2013			
Flocks	F1	F2	F3	F4	F1	F2	F3	F4
Mean	9.45	9.95	8.05	7.55	9.2	9.85	7.78	7.24
SD	0.81	0.22	1.31	1.36	0.88	0.49	1.15	1.36

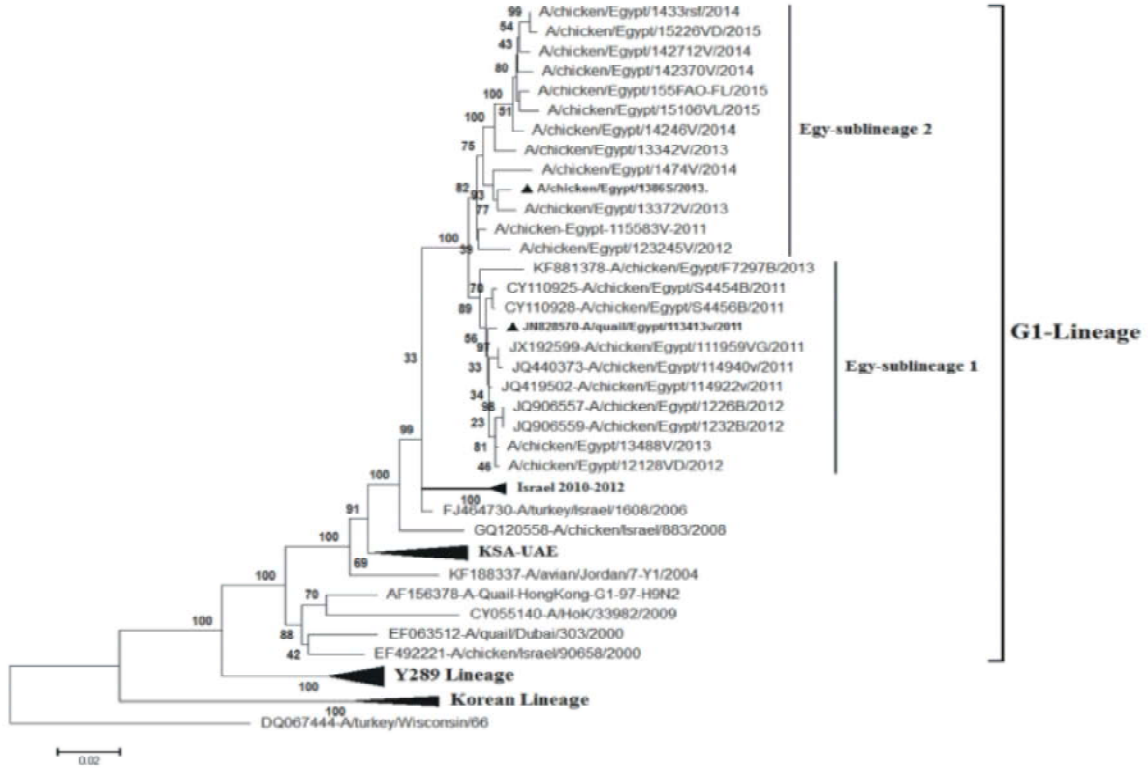

Fig. 2: The phylogenetic tree for nucleotides sequence of HA gene, ▲ selected viruses in our study

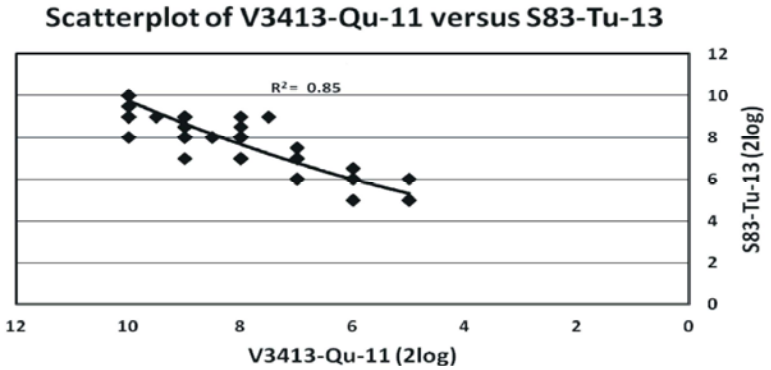

Fig. 3: Linear regression for all HI titers against the 2 Egyptian antigens, Y-Axis is the S83-Tu-13 antigen and X-Axis is the V3413-Qu-11 antigen

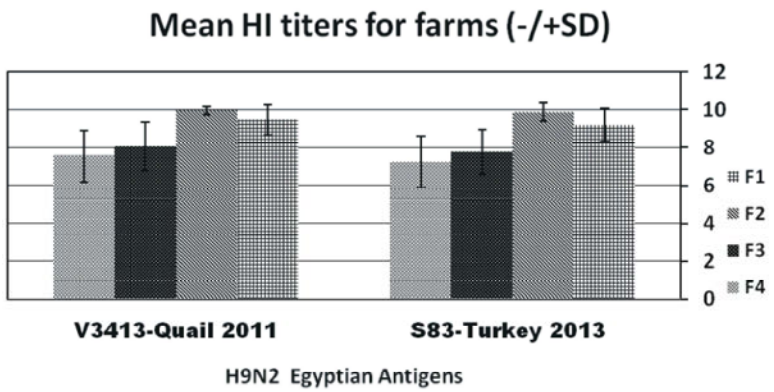

Fig. 4: Statistical chart of mean HI titer for each flocks against each antigens with standard deviation bars (± SD) for each mean

DISCUSSION

Since the H9N2 showed wide spreading in different countries with economic losses in poultry flocks [4]. In spite of application of inactivated vaccines against H9N2, the virus still circulates especially in broiler chickens and commercial flocks [21]. Our study is to prove that the genetic evolution of hemagglutinin gene of circulated H9N2 viruses in Egypt could affect the efficacy of vaccination in field.

The genetic classification for hemagglutinin gene of Egyptian H9N2 in recent studies determine 2 subgroups [9, 22] so we select 2 different antigens to represent the both subgroups. The antigen V3413-Qu-2011 is the original Egyptian H9N2 virus isolated in 2011 [5] and this isolate is genetically closed to v4922-ch-2011 virus which is the candidate strain for preparation of H9N2 local vaccine [16] with high identity 99.8% as table (3). Both of v4922-ch-2011 and V3413-Qu-2011 are belonging to the Egyptian phylogenetic sub-lineage 1 fig.2 which includes mainly the viruses isolated during 2011-2012. Otherwise, the second antigen used in this study is S83-Tu-2013 which is the virus isolated from turkey in 2013, this virus is phylogenetically belonging to the Egyptian sub-lineage 2 that contains mainly the viruses isolated during 2013 to 2015 fig.2 , the S83-Tu-2013 virus has high genetic similarity to V3413-Qu-2011 the virus of subgroup1 with identity 98.6% . The genetic constitutions of the 2 antigens are nearly the same, but the S83-Tu-2013 virus has minor different alterations as shown in Fig. (1). The most characteristic amino acid residue 216 in mature hemagglutinin protein is one of the receptor binding sites and belonged to epitopes of antigenic sites on H9 [23, 24]. Although the H9N2 viruses isolated from avian species characterized with Gln (Q216) [23] but the 2 viruses in this study shared with Leu(L216) similar to most of Egyptian avian H9N2 isolates [9, 11, 22] amino acid residue Leu (L216) is specific for human receptor α2, 6 sialic acid linkages which is specific for human influenza viruses subtypes H2 and H3 [25, 26].

In this study, we purposed to collect the vaccinated serum samples from layer flocks because layers and breeders have longer life span than broilers, thus the vaccination in layers evoke prolonged immunization time by booster doses [27] which give opportunities for our study depending on the sero-conversion of vaccinated flocks. Unfortunately, the data about types or strains of vaccines used in each flocks is undetermined, but the available data for each flock told that they were vaccinated with imported H9 vaccines. Also, we excluded the antisera of flocks vaccinated with H5N2 or any other N2 subtype to avoid the partial cross reactivity that may be induced against H9N2 viruses [28, 29].

The sero-conversion of the antisera from the different flocks in this study showed nearly with the same values against both 2 antigens (V3413-Qu-2011 and S83-Tu-2013 Egyptian viruses), it is clear in table(4) that the HI titers and means of flocks 3 and 4 (F3 & F4) against both the 2 antigens are relatively lower than the values of F1 and F2 and this reduction is due to the time of vaccination in each flock, as F3 and F4 were last time vaccinated with H9N2 earlier than the last time vaccination of F1 and F2 as shown in table no(1). Through our study, the F3 and F4 have post vaccine duration 8 and 12 weeks respectively, in the same point some studies on inactivated vaccine discussed that the antibody curve reach peak at 4 weeks post vaccination and recommended for booster doses within 12 - 20 weeks post vaccination to evoke prolonged high immunity [27].

The statistical analysis for HI titers values and means showed the same relative sero-conversion of the antisera against the 2 antigens used in this study. The one-way ANOVA analysis for means showed non-significant variations between the response of the 2 antigens against the antisera of the 4 vaccinated flocks with p-value >0.05 (=0.99) at confident level 95%. Also, the linear regression analysis for the individual HI titers against the 2 antigens showed strong correlation between both of them with R2 = 0.85 at P-value = 0.05 , statistically, R2 has an irreversible relationship with the variation in the populations and that means the value of R2 < 1 , > 0 is indicate that the populations are relative to each other.

The Immune response due to vaccination against the 2 strains used in this study indicate satisfactory results that support the protection of vaccinated birds against the current circulated viruses of H9N2. However partial immunity can lead to immune pressure that lead to alteration of amino acids within and around and the receptor binding domains and antigenic sites, which cause change in antigenicity of viruses and raising new escape mutants. Like previous studies on Korean LPAI H9N2 virus demonstrated antigenic drifts and raised antigenic escape groups [30, 31]. Therefore, the immunity release by previously vaccination with other different strains cannot neutralize the escape mutants [32]. So, it is recommended for vaccine efficacy to use strains with antigenicity matches the circulating and escape mutant viruses [33, 34].

In conclusion, the Egyptian H9N2 viruses are continuously in genetic evolution, So, periodical and continuous surveillance is recommended to follow up the evolution of Egyptian viruses, also, the serological surveillance and evaluation of the seroconversion of vaccinated flocks against different strains of circulating H9N2 are recommended as a tool to evaluate vaccines.

REFERENCES

1. Homme, P.J. and B.C. Easterday, 1970. Avian influenza virus infections: I. Characteristics of influenza A-turkey-Wisconsin-1966. Virus Avian Dis., 14: 66-74.
2. Alexander, D.J., 2000 A review of avian influenza in different bird species. Vet. Microbiol., 74: 3-13.
3. Fusaro, A., I. Monne, A. Salviato, V. Valastro, A. Schivo, N.M. Amarin, C. Gonzalez, M.M. Ismail, A.R. Al-Ankari, M.H. Al-Blowi, O.A. Khan, A.S. Maken Ali, A. Hedayati, G.J. Garcia, G.M. Ziay, A. Shoushtari, K.N. Al Qahtani, I. Capua, E.C. Holmes and G. Cattoli, 2011 Phylogeography and evolutionary history of reassortant H9N2 viruses with potential human health implications. J. Virol., 85: 8413-8421.
4. Naeem, K., A. Ullah, R.J. Manvell and D.J. Alexander, 1999 Avian influenza A subtype H9N2 in poultry in Pakistan. Vet. Rec., 145: 560.
5. El-Zoghby, E.F., A.S. Arafa, M.K. Hassan, M.M. Aly, A. Selim, W.H. Kilany, U. Selim, S. Nasef, M.G. Aggor, E.M. Abdelwhab and H.M. Hafez, 2012 Isolation of H9N2 avian influenza virus from bobwhite quail (Colinus virginianus) in Egypt. Arch Virol, (2012) 157: 1167-1172 DOI 10.1007/s00705-012-1269-z
6. Hussein, H.A. and M. ElAzab, 2002. Evidence for the presence of H6 and H9 among broiler and layer breeders in Egypt.Proce. XII. Int. Cong. World. Vet. Poultry Assoc. Cairo-Egypt, pp: 227-235.
7. Afifi, M.A., M.F. El-Kady, S.A. Zoelfakar and A.S Abdel-Moneim, 2013. Serological surveillance reveals widespread influenza A H7 and H9 subtypes among chicken flocks in Egypt. Trop Anim Health Prod., 45: 687-90.
8. Monne, I., H.A. Hussein, A. Fusaro, V. Valastro M.M. Hamoud, R.A. Khalefa S.N. Dardir, M.I. Radwan, I. Capua and G. Cattoli, 2013. H9N2 influenza A virus circulates in H5N1 endemically infected poultry population in Egypt. Influenza Other Respir Viruses, 7: 240-243.
9. Kandeil, A., R. El-Shesheny, A.M. Maatouq, Y. Moatasim, M.M. Shehata, O. Bagato, A. Rubrum, K. Shanmuganatham, R.J. Webby, M.A. Ali and G. Kayali, 2014. Genetic and antigenic evolution of H9N2 avian influenza viruses circulating in Egypt between, 2011 and 2013, Arch Virol DOI 10.1007/s00705-014-2118-z
10. Kayali, G., A. Kandeil, R. El-Shesheny, A.S. Kayed, M.M. Gomaa, A.M. Maatouq, M.M. Shehata, Y. Moatasim, O. Bagato, Z. Cai, A. Rubrum, M.A. Kutkat, P.P. McKenzie, R.G. Webster, R.J. Webby and M.A. Ali , 2014. Active surveillance for avian influenza virus, Egypt, 2010–2012. Emerg Infect Dis., 20: 542-51.
11. Arafa, A., N.M. Hagag, N. Yehia, A.M. Zanaty, M.M. Naguib and S.A. Nasef, 2012. Effect of Cocirculation of Highly Pathogenic Avian Influenza H5N1 Subtype with Low Pathogenic H9N2 Subtype on the Spread of Infections. Avian Diseases, 56(4s1):849-857.

12. Tavakkoli, H., K. Asasi and A. Mohammadi, 2011. Effectiveness of two H9N2 low pathogenic avian influenza conventional inactivated oil emulsion vaccines on H9N2 viral replication and shedding in broiler chickens. Iranian Journal of Veterinary Research, Shiraz University, 12(3), Ser. No. 36, 2011.
13. Butterfield, W.K. and C.H. Campbell, 1979. Vaccination of chickens with influenza A/turkey/Oregon/71 virus and immunity challenge exposure to five strains of fowl plague virus. Veterinary Microbiology, 4: 101-107.
14. Swayne, D.E., J.R. Beck, M.L. Perdue and C.W. Beard, 2001. Efficacy of vaccines in chickens against highly pathogenic Hong Kong H5N1 avian influenza. Avian Diseases, 45: 355-365.
15. Abdelwhab, E.M. and A.S. Abdel-Moneim, 2014. Epidemiology, ecology and gene pool of influenza A virus in Egypt: Will Egypt be the epicentre of the next influenza pandemic? Virulence, 0: 1-13.
16. Khalil, A.A., H.A. Hussein, S.K. Tolba and A.A. El-Sanousi, 2015. Preparation and Evaluation of H9n2 Vaccine Adjuvated with Montanide ISA71 VG. Global Veterinaria, 14(5): 670-674, 2015 ISSN 1992-6197.
17. Ben Shabat, M., R. Meir, R. Haddas, E. Lapin, I. Shkoda, I. Raibstein, S. Perk and I. Davidson, 2010. Development of a real-time TaqMan RT-PCR assay for the detection of H9N2 avian influenza viruses Journal of Virological Methods, Volume 168, Issues 1-2, September, 2010, pp: 72-77.1
18. Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser., 41: 95-98.
19. Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar, 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution, 30: 2725-2729.
20. SOIE World Organisation for Animal Health, 2015. Terrestrial Animal Health Code. OIE, Paris.
21. Swayne, D.E. and D.A. Halvorson, 2008. Influenza. In: Saif, YM; Barnes, HJ; Fadl, AM; Glisson, JR; McDougald, LR and Swayne, DE (Eds.), Disease of poultry. (12th Edn.), Ames, Iowa, Iowa State University Press, pp: 153-184
22. Soliman, M.M., A. Arafa, S.M. Tammam, M.M. Aly and H.M. Madbouly, 2014. Molecular Characterization of Avian Influenza Virus Subtype H9N2 in Poultry in Egypt During 2011-2013 with Emerging of a New Variant in Quails. Global Veterinaria, 13(1): 117-126, 2014. ISSN 1992-6197. DOI: 10.5829/idosi.gv.2014.13.01.8426.
23. Neumann, G. and Y. Kawaoka, 2006. Host Range Restriction and Pathogenicity in the Context of Influenza Pandemic. Emerging Infectious Diseases ● www.cdc.gov/eid ● 12(6), June 2006.
24. Kaverin, N.V., I.A. Rudneva, N.A. Ilyushina, A.S. Lipatov, S. Krauss and R.G. Webster, 2004. Structural Differences among Hemagglutinins of Influenza A Virus Subtypes Are Reflected in Their Antigenic Architecture: Analysis of H9 Escape Mutants. JOURNAL OFVIROLOGY, Jan. 2004, pp: 240–249 Vol. 78, No. 10022-538X/04/$08.000 DOI: 10.1128/JVI.78.1.240–249.2004.
25. Rogers, G.N., J.C. Paulson, R.S. Daniels, J.J. Skehel, I.A. Wilson and D.C. Wiley, 1983. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. Nature., 304: 76-8.
26. Matrosovich, M., A. Tuzikov, N. Bovin, A. Gambaryan, A. Klimov, M.R. Castrucci, I. Donatelli, Y. Kawaoka , 2000. Early alterations of the receptor-binding proper-ties of H1, H2 and H3 avian influenza virus hemagglutinins after their introduction into mammals. J Virol., 74: 8502-12.
27. Vasfi Marandi, M., M.H. Bozorgmehri Fard and M. Hashemzadeh, 2002. Efficacy of Inactivated H9N2 Avian Influenza Vaccine against Non-highly Pathogenic A/Chicken/Iran/ZMT-173/1999 Infection.
28. Khalenkov, A., S. Perk, A. Panshin, N. Golender and R.G. Webster, 2009. Modulation of the severity of highly pathogenic H5N1 influenza in chickens previously inoculated with Israeli H9N2 influenza viruses. Virology, 383: 32-38.
29. Imai, K., K. Nakamura, M. Mase, K. Tsukamoto, T. Imada and S. Yamaguchi, 2007. Partial protection against challenge with the highly pathogenic H5N1 influenza virus isolated in Japan in chickens infected with the H9N2 influenza virus. Arch Virol., 152: 1395-1400
30. Lee, C.H., S.H. Byun, Y.J. Lee and I.P. Mo, 2012. Genetic evolution of the H9N2 avian influenza virus in Korean poultry farms. Virus Genes, 45: 38-47.
31. Park, K.J., H.I. Kwon, M.S. Song, P.N. Pascua, Y.H. Baek, J.H. Lee, H.L. Jang, J.Y. Lim, I.P. Mo, H.J. Moon, C.J. Kim and Y.K. Choi, 2011. Rapid evolution of low-pathogenic H9N2 avian influenza viruses following poultry vaccination programmes. J. Gen. Virol., 92(Pt 1): 36-50.
32. Sun, Y., J. Pu, L. Fan, H. Sun, J. Wang, Y. Zhang, L. Liu and J. Liu, 2012. Evaluation of the protective efficacy of a commercial vaccine against different antigenic groups of H9N2 influenza viruses in chickens. Vet. Micro-biol., 156: 1939.

33. Choi, J.G., Y.J. Lee, Y.J. Kim, E.K. Lee, O. M. Jeong, H. W. Sung, J. H. Kim and J.H. Kwon, 2008. An inactivated vaccine to control the current H9N2 low pathogenic avian influenza in Korea. J. Vet. Sci., 9(1): 67-74.

34. Lee, D. and C. Song, 2012. H9N2 avian influenza virus in Korea: evolution and vaccination. Clin Exp Vaccine Res, 2013, 2:26-33 http://dx.doi.org/ 10.7774 /cevr.2013.2.1.26 pISSN 2287-3651 ● eISSN 2287-366X.