Global Veterinaria 18 (1): 20-26, 2017
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2017.20.26

Isolation and Characterization of Avian Influenza Virus (H5N1) in Egypt from Ducks during 2015

[1]R.I. El-Tantawy, [2]H.A. Hussein, [2]A.H. El-Deeb and [1]M.K. Hassan

[1]Reference Laboratory for Veterinary Quality Control on Poultry Production,
Animal Health Research Institute, Dokki, Giza Egypt
[2]Department of Virology Faculty of Veterinary Medicine, Cairo University, Egypt

Abstract: Ducks act as natural reservoir of avian influenza and play important role in transmission and distribution of avian influenza and may be the source of human infection. In the present study, sequence analysis of HA gene of nucleotides and Amino Acids for 5 isolates of H5N1 from duck samples collected from live bird market during 2015 in Egypt (Cairo and Giza governorates) indicated that all isolates are closely related to each other and the identity% of such viruses with A/duck/Egypt/F5/2006 ranged between 96.1%-97%. The phylogenetic analysis of the five isolates indicates their relation to clade 2.2.1.2 and clustered with duck viruses reported in 2014. The study reports the continuous circulation and mutations of H5N1viruses in ducks and their relation to human infection cases reported in 2015.Also, the sequence analysis reports important mutations in sites on the HA gene affecting the receptor binding sites and the increase rate of human infection with such clade in 2015.

Key words: Highly Pathogenic Avian Influenza (HPAI) · H5N1 · HA · Real Time RT-PCR · Conventional RT-PCR · Sequencing · Phylogenetic Analysis

INTRODUCTION

Avian influenza is caused by infection with viruses of the family *orthomyxoviridae*. It is enveloped virus, negative sense, single stranded and contain genome composed of eight separate RNA segments encode for at least 11 viral proteins[1]. Classification of these viruses is based on antigenic properties of the two surface glycoproteins; hemagglutinin (HA) and neuraminidase (NA). At least, there are 18 HA (H1- H18) and 11 NA (N1- N11) subtypes circulating in avian and/or mammalian hosts [2- 3]. The first record of sever outbreak of HPAI H5N1 in Egypt was reported in February[4] and it was the third –highest number of established human infections [5]. Domestic ducks have been associated with the distribution and evolution of HPAI H5N1 [6-7]. They play a role in the epidemiology of HPAI H5N1 viruses as they are the natural reservoir of avian influenza viruses [8-9]. The duck species can shed and spread virus from both the respiratory and intestinal tracts. The nonappearance of disease signs in some duck species has led to the thought that ducks are the "Trojan horses" of H5N1 in their silent spread of virus [10]. The history of increased pathogenicity of HPAI H5N1 viruses for ducks in some countries in Asia, containing Vietnam and Lao PDR such observation has been reported on Egyptian strains in ducks [11-12]. Domestic ducks that are interact with wild waterfowl and also other poultry species can act as important mediators in the spread of avian influenza between birds [13]. The domestic duck has greater chance of disseminating HPAI H5N1 viruses [14]. Reducing the risk of HPAI H5N1 virus infection in ducks is important to control the continuing circulation and spread of HPAI H5N1 [11]. Vaccination is important for protecting ducks against HPAI H5N1 and for controlling the disease [15]. Virus circulation may still occur in clinically healthy vaccinated population, which may result in an endemic condition and in the emergence of antigenic variants [16]. Since March 2006 in Egypt, vaccination was enhanced nationwide based on the national control strategy. While in 2007,recognition of vaccination failure causing epidemics in some poultry farms and variant H5N1 virus

Corresponding Author: Hussein Aly Hussein, Department of Virology Faculty of Veterinary Medicine, Cairo University, Egypt. E-mail: husvirol@cu.edu.eg.

was emerged [17-18]. In July 2009, vaccination in backyard/household locations has been temporarily suspended until a new vaccination strategy is assumed [19]. Continuing outbreaks have been reported in many cases with high mortality in duck farms, backyard ducks, wild ducks, live-market ducks and rooftop ducks [20].

The aim of this work is to molecular characterize and construct the phylogenetic tree of some HPAI H5N1 from duck samples collected from live bird market in Egypt during 2015.

MATERIALS AND METHODS

Sampling: Fifty samples collected from ducks in live bird markets from Cairo and Giza during 2015, each sample represented by 5 tracheal and 5 cloacal swabs, which loaded to 1-2 ml PBS containing antibiotics. OIE [20].

Virus Isolation: Using specific pathogen free (SPF) 9-11 day old Embryonated Chicken Eggs (ECE) the prepared samples were inoculated and propagation was carried out for virus isolation. OIE [20].

Molecular Characterization: Extraction of RNA was carried out from allantoic fluid by using QIAamp viral RNA mini kit (Qiagen, Germany) according to manufacturer's instructions. For RT-PCR amplification of the HA gene, QuantiTect kit was used as described by manufacturer's instruction: Two sets of primers were used including common type A primer& probe to detect type A avian influenza [21] and HA gene specific primers to amplify the full HA gene [22].

Sequencing: Using the Qiagen PCR purification kit, the amplified RT-PCR product was sequenced. Forward and reverse sequences were aligned together using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) in an ABI PRISM® 3100. BioEdit software was used to analyze the sequence of HA gene, BLAST (Basic Local Alignment Search Tool) [23]. Phylogenetic analysis of the obtained sequences was carried out using MEGA. 6.0 software.

GenBank Accession Numbers: Nucleotide sequences of the Egyptian HPAI H5N1 of duck were submitted to GenBank and accession numbers are KY415973 – KY415974 – KY415975 – KY415976 – KY415977.

RESULTS

Detection of type A and H5 viruses in collected samples by rt RT-PCR: Result of rt RT-PCR revealed that out of 50 tested samples 5 were positive (Figure1).

Virus Isolation: Avian influenza viruses in the five positive samples (by rtRT-PCR) were successfully isolated in SPF ECE (9-12) days old chicks via allantoic route. Amplification of full length gene using specific set of primers revealed the correct expected bands.

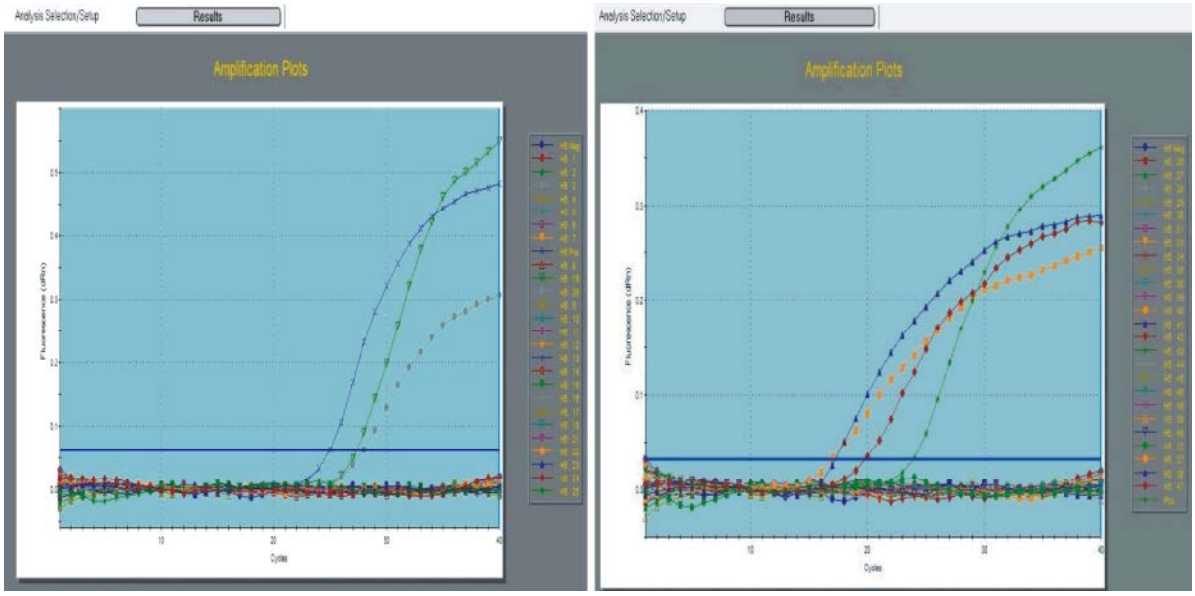

Fig. 1: Amplification curve for AIV H5 gene RRT-PCR Reverse transcriptase PCR for samples from (1- 50) samples.

Table 1: Amino acids differences in HA protein of the five isolated viruses in relation to A/duck/Egypt/F5/2006 strain

Isolate ID	Amino acids differences	total
A/duck/Egypt/VRLCU-Giza1/2015(H5N1)	D43N -S120D - Δ129 - I151T - D154N -N155D - R162K - Y252N - G272S - K373R - R325K - I 396V - F463L - F537S - **D54N - S357I - D439S - R453Q**	18
A/duck/Egypt/VRLCU-Helwan1/2015(H5N1)	D43N -S120D - Δ129 - I151T - D154N -N155D - R162K - Y252N - G272S - K373R - R325K - I 396V - F463L - F537S	14
A/duck/Egypt/VRLCU-Helwan2/2015(H5N1)	D43N -S120D - Δ129 - I151T - D154N -N155D - R162K - Y252N - G272S - K373R - R325K - I 396V - F463L - F537S - **K152R**	15
A/duck/Egypt/VRLCU-Giza2/2015(H5N1)	D43N -S120D - Δ129 - I151T - D154N -N155D - R162K - Y252N - G272S - K373R - R325K - I 396V - F463L - F537S - **K265R**	15
A/duck/Egypt/VRLCU-Giza3/2015(H5N1)	D43N -S120D - Δ129 - I151T - D154N -N155D - R162K - Y252N - G272S - K373R - R325K - I 396V - F463L - F537S - **F95V - L171I -P321H**	17

Table 2: Identity % of Amino Acids and diversions of Egyptian H5N1 strains (2015) in comparison with A/duck/Egypt/F5/2006 strain

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
1		97.2	96.7	98.9	98.3	97.0	97.8	96.7	96.7	97.0	97.0	98.5	98.2	97.6	96.9	96.9	96.5	96.5	96.7	96.5	97.0	96.9	96.9	96.1	95.0	1
2	2.4		98.9	97.2	98.9	99.3	99.1	98.9	98.9	98.9	99.3	96.9	96.5	98.9	99.1	99.1	98.2	98.7	98.9	98.7	99.3	99.1	99.1	98.3	93.7	2
3	3.0	1.1		96.7	98.2	98.9	98.3	98.9	98.9	98.9	98.9	96.3	95.9	98.2	98.7	99.1	98.3	98.9	98.9	98.7	99.3	99.1	99.1	98.3	93.7	3
4	1.1	2.4	3.0		98.3	97.0	97.8	96.7	96.7	97.0	97.0	98.5	98.2	97.6	96.9	96.9	96.1	96.5	96.7	96.5	97.0	96.9	96.9	96.1	95.0	4
5	1.3	1.1	1.9	1.3		98.5	99.1	98.2	98.2	98.2	98.5	98.0	97.6	98.9	98.3	98.3	97.6	98.0	98.2	98.0	98.5	98.3	98.3	97.6	94.1	5
6	2.6	0.7	1.1	2.6	1.5		98.7	98.9	98.9	98.9	99.3	96.7	96.3	98.5	99.1	99.1	98.2	98.7	98.9	98.7	99.3	99.1	99.1	98.3	93.4	6
7	1.9	0.9	1.7	1.9	0.9	1.3		98.3	98.3	98.7	98.7	97.4	97.0	99.1	98.5	98.5	97.8	98.2	98.3	98.2	98.7	98.5	98.5	97.8	93.9	7
8	3.0	1.1	1.1	3.0	1.9	1.1	1.7		100.0	98.5	98.9	96.3	95.9	98.5	98.7	99.1	98.2	98.7	98.9	98.7	99.3	99.1	99.1	98.3	93.7	8
9	3.0	1.1	1.1	3.0	1.9	1.1	1.7	0.0		98.5	98.9	96.3	95.9	98.5	98.7	99.1	98.2	98.7	98.9	98.7	99.3	99.1	99.1	98.3	93.7	9
10	2.6	1.1	1.1	2.6	1.9	1.1	1.3	1.5	1.5		98.9	96.7	96.3	98.5	98.7	98.7	97.8	98.3	98.5	98.3	98.9	98.7	98.7	98.0	93.7	10
11	2.6	0.7	1.1	2.6	1.5	0.7	1.3	1.1	1.1	1.1		96.7	96.3	98.5	99.1	99.1	98.2	98.7	98.9	98.7	99.3	99.1	99.1	98.3	93.4	11
12	1.5	2.8	3.4	1.5	1.7	3.0	2.3	3.4	3.4	3.0	3.0		98.5	97.2	96.5	96.7	95.8	96.1	96.3	96.1	96.7	96.5	96.5	95.8	94.8	12
13	1.9	3.2	3.8	1.9	2.1	3.4	2.6	3.8	3.8	3.4	3.4	1.5		96.9	96.5	96.3	95.8	96.1	95.9	95.8	96.3	96.1	96.1	95.4	94.8	13
14	2.1	1.1	1.9	2.1	1.1	1.5	0.9	1.5	1.5	1.5	1.5	2.4	2.8		98.3	98.3	97.6	98.0	98.2	98.0	98.5	98.3	98.3	97.6	93.7	14
15	2.8	0.9	1.3	2.8	1.7	0.9	1.5	1.3	1.3	1.3	0.9	3.2	3.2	1.7		98.9	98.3	98.9	98.7	98.5	99.1	98.9	98.9	98.2	93.9	15
16	2.8	0.9	0.9	2.8	1.7	0.9	1.5	0.9	0.9	1.3	0.9	3.0	3.4	1.7	1.1		98.7	99.3	99.4	99.3	99.8	99.6	99.6	98.9	93.7	16
17	3.2	1.9	1.7	3.6	2.4	1.9	2.3	1.9	1.9	2.3	1.9	4.0	4.0	2.4	1.7	1.3		99.4	98.9	98.3	98.9	98.7	98.7	98.0	93.5	17
18	3.2	1.3	1.1	3.2	2.1	1.3	1.9	1.3	1.3	1.7	1.3	3.6	3.6	2.1	1.1	0.7	0.6		99.4	98.9	99.4	99.3	99.3	98.5	93.5	18
19	3.0	1.1	1.1	3.0	1.9	1.1	1.7	1.1	1.1	1.5	1.1	3.4	3.8	1.9	1.3	0.6	1.1	0.6		99.1	99.6	99.4	99.4	98.7	93.4	19
20	3.2	1.3	1.3	3.2	2.1	1.3	1.9	1.3	1.3	1.7	1.3	3.6	4.0	2.1	1.5	0.7	1.7	1.1	0.9		99.4	99.3	99.3	98.5	93.2	20
21	2.6	0.7	0.7	2.6	1.5	0.7	1.3	0.7	0.7	1.1	0.7	3.0	3.4	1.5	0.9	0.2	1.1	0.6	0.4	0.6		99.8	99.8	99.1	93.7	21
22	2.8	0.9	0.9	2.8	1.7	0.9	1.5	0.9	0.9	1.3	0.9	3.2	3.6	1.7	1.1	0.4	1.3	0.7	0.6	0.7	0.2		99.6	98.9	93.5	22
23	2.8	0.9	0.9	2.8	1.7	0.9	1.5	0.9	0.9	1.3	0.9	3.2	3.6	1.7	1.1	0.4	1.3	0.7	0.6	0.7	0.2	0.4		98.9	93.5	23
24	3.6	1.7	1.7	3.6	2.4	1.7	2.3	1.7	1.7	2.1	1.7	4.0	4.4	2.4	1.9	1.1	2.1	1.5	1.3	1.5	0.9	1.1	1.1		92.8	24
25	5.2	6.2	6.2	5.2	5.8	6.6	6.0	6.2	6.2	6.2	6.6	5.4	5.4	6.2	6.0	6.2	6.4	6.4	6.6	6.8	6.2	6.4	6.4	7.2		25
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	

1 A/duck/Egypt/F5/2006(H5N1)
2 A/duck/Egypt/141AI/2014 1707 bp
3 A/duck/Egypt/14688/2014 1707 bp
4 A/duck/Egypt/07322S-NLQP/2007(H5N1
5 A/duck/Egypt/08218-NLQP/2008(H5N1
6 A/duck/Egypt/10118/2010(H5N1))
7 A/duck/Egypt/1011d/2010(H5N1)
8 A/duck/Egypt/11106SF/2011(H5N1)
9 (A/duck/Egypt/11106SF/2011(H5N1)
10 A/duck/Egypt/121068/2012(H5N1)
11 A/duck/Egypt/121338/2012(H5N1)
12 A/duck/Egypt/072643-NLQP/2007(H5N1)
13 (A/duck/Egypt/083558-NLQP/2008(H5N1))
14 A/duck/Egypt/1310SD/2013 1707 bp
15 A/turkey/Egypt/14125S/2014 1707 bp
16 A/Duck/Egypt/15328G/2015(H5N1)
17 A/EGYPT/1525/'2015
18 A/Egypt/1524/2015
19 A/Egypt/1578ca/2015
20 A/duck/Egypt/VRLCU-Giza1/2015(H5N1)
21 A/duck/Egypt/VRLCU-Helwan1/2015(H5N1)
22 A/duck/Egypt/VRLCU-Helwan2/2015(H5N1)
23 A/duck/Egypt/VRLCU-Giza2/2015(H5N1)
24 A/duck/Egypt/VRLCU-Giza3/2015(H5N1)
25 guangdong 1996 H5N1

Sequence Analysis: Similarities between the five isolates were ranged from 98.5% to 99.8%and the similarities with the firstly isolated duck strain in Egypt (A/duck/Egypt/F5/2006) were ranged from 96.1% to 97%indicating continuous mutation of these viruses (Table 2). There are several mutations in amino acids reported in the isolated viruses compared to the duck virus isolated in 2006 in Egypt strain A/duck/Egypt/F5/2006 (Table 1).

Phylogenetic Analysis: Phylogenetic analysis of five isolates revealed that these viruses were related to viruses isolated from Egypt in 2015 which belonging to clade 2.2.1.2. The viruses were clustered with the 2014 strain of HPAI isolated from A/duck/1468s/2014.

Clustering of the five isolates with these viruses within clade 2.2.1.2 originated from the duck strains isolated in year 2014 in Egypt which indicating the continuous evolution of these viruses (Figure 2).

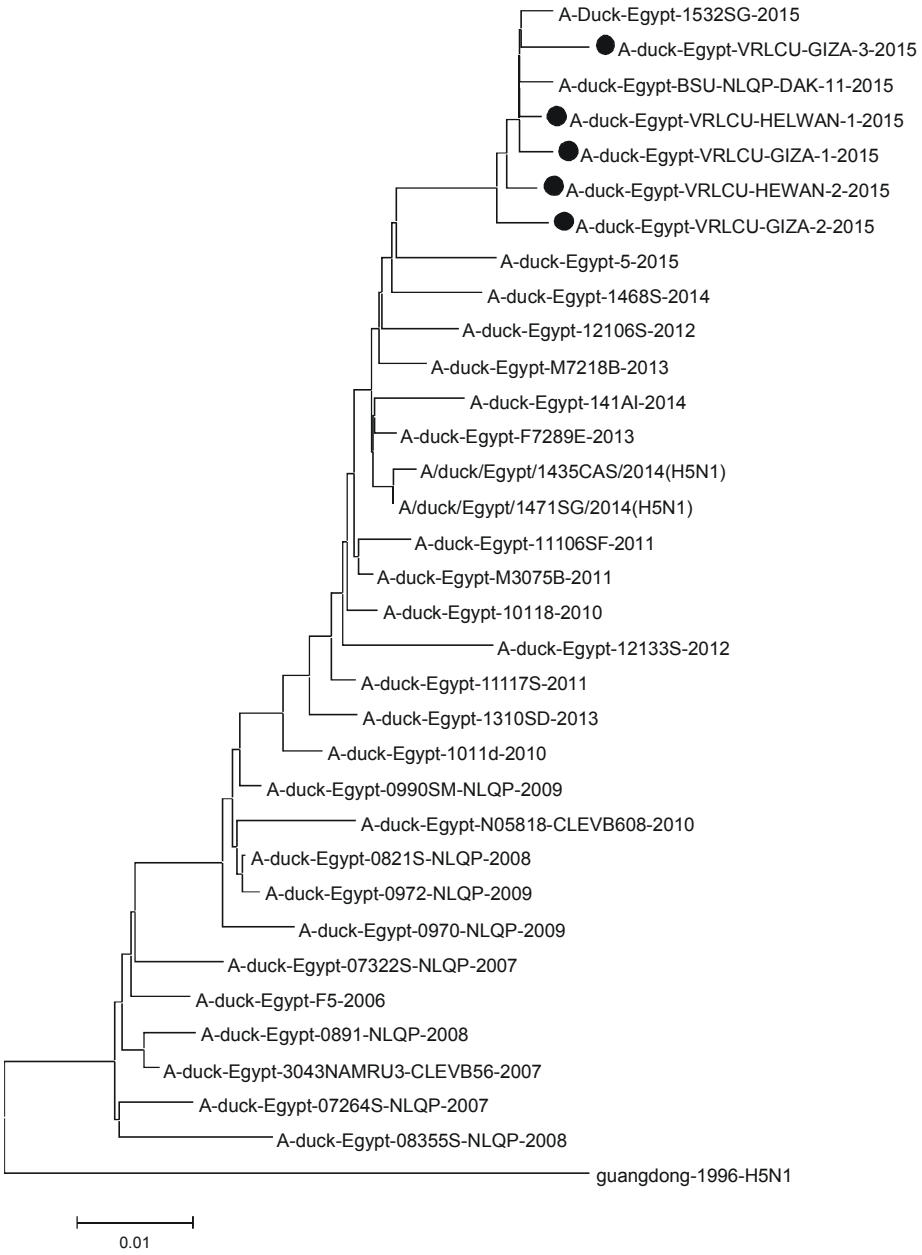

Fig. 2: Phylogenetic tree of HA nucleotide sequences of Egyptian isolates compared with other representative H5N1 strains. The viruses were related to 2.2.1.2 sub clade H5N1

DISCUSSION

Free-ranging ducks and local abundance of ducks are the main risk factor of the presence of HPAI H5N1 viruses in countries like Thailand and Vietnam [24-25] and can be extended to other countries including Egypt, where duck may be the main source of persistence HPAI H5N1 virus[26]. Out breaks of HPAI H5N1 in Egypt have been reported in ducks from farms, backyards, live bird markets and roof-tops in addition to wild ducks even in vaccinated ducks[20]. Due to continuous mutation and evolution of H5N1, the virus demonstrating an increase in the pathogenicity in ducks [12]. From 2006 to 2014, the phylogenetic analysis of the H5 gene of Egyptian viruses showed the presence of two main subgroups: classic group 2.2.1 and variant group 2.2.1.1 [27]. The classic group of clade 2.2.1 that was introduced into Egypt in 2006 continued to be stable through 2009 and

characterized the original viruses. The variant group 2.2.1.1, was emerged in late 2007 from vaccinated commercial poultry and was divided into 2 clusters from 2008 to 2011 (2.2.1.1 and 2.2.1.1a). The first cluster emerged in late 2007 (2.2.1.1) and continued until 2009, although the second cluster (2.2.1.1a) emerged in 2008 and continued until 2011. Since then, these variant clusters have not been detected [28]. Due to increase of genetic mutations in the HA protein in 2008, the classic viruses was evolved into a new clade 2.2.1.2 and was the main cluster through 2009 and 2014amongboth the household and commercial poultry sectors regardless of their vaccination status [28]. The first identification of the endemic 2.2.1.2 cluster was proposed in 2008 and continues to circulate and distributed in greatest governorates in Egypt [28].

In this study, five isolates of HPAI H5N1 viruses of duck during 2015 found to be belonging to clade 2.2.1.2. These viruses are related to viruses isolated during 2014 and constitute an endemic cluster in Egypt [29]. Mutation reported in the present study were similar to those detected in viruses infected humans in 2015 and categorized to the same phylogenetic group, indicating the importance of transmission of HPAI H5N1 rom duck to human [28]. Also, sequencing results showed common mutations in HA gene (Table1) and other characteristic mutations of clade 2.2.1.2 (K373R and F537S) [29]. In addition, three mutations (S120D, Δ129, I151T) were detected in the isolated viruses which known to be associated with increased binding affinity to human receptor [28]. Thus confirming that these viruses might be the main source of human infections and subsequently implication in the fatal cases reported in 2015 in Egypt [28]. The deletion Δ129 in receptor binding domain of hemagglutinin detected in the isolated viruses could facilitate the interspecies and other species transmission [30-31]. This deletion Δ129, with the reported 2 amino acid mutations (N155D and I151T) are known to be the cause of increasing binding affinity to α2,6 sialic acid receptor and infectivity in human lower respiratory tract [15-16]. On the other hand, there were mutations in antigenic sites (S120D, I151T,R162K) which may cause evolving of escape mutant viruses affecting immune response to vaccine [32]. Indeed, the persistence of virus in the host reservoir, playing important role in virus maintenance especially in duck, which don't show any clinical signs and this allows the virus transmission to poultry population and human. Although the vaccination is the important factor in the control of HPAI H5N1 in Egypt [15], vaccination failure led to antigenic drift of H5N1 viruses and virus mutations [16]. This study reflects the importance of role of duck as natural reservoir of H5N1 and reports the continuous mutations of HPAI H5N1 viruses circulating in the live bird market in Egypt.

CONCLUSION

Isolation and characterization of HPAI H5N1 virus from duck during 2015 indicate the role of duck in transmission of HPAI H5N1. The phylogenetic tree indicates that five isolates related to viruses of 2014 which indicates continuous circulation and mutation of virus H5N1. The isolated viruses is belonging to clade 2.2.1.2 which have the ability to affect human and reported in many human cases in 2015.There is a need to have an effective control strategy for such viruses to reduce the risk of transmission of these viruses from duck to human and domestic poultry populations.

REFERENCES

1. Palese, P. and M. Shaw, 2007. Orthomyxoviridae: the viruses and their replication. In: Knipe DM, Howley PM, editors. Fields Virology. Philadelphia, PA: Lippincott Williams & Wilkins., pp: 1647-1689.
2. Tong, S., Y. Li, P. Rivailler, C. Conrardy, D.A.A. Castillo, L.M. Chen and R.O. Donis, 2012. A distinct lineage of influenza A virus from bats. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(11), 4269-4274. doi:10.1073/pnas.1116200109
3. Tong, S., X. Zhu, Y. Li, M.Shi, J. Zhang, M. Bourgeois and R. O.Donis, 2013. New World Bats Harbor Diverse Influenza A Viruses. *PLoS Pathogens*, *9*(10). doi:10.1371/journal.ppat.1003657
4. Aly, M.M., A. Arafa, M.K. Hassan, M.M. Aly and A. Arafa, 2008. Epidemiological Findings of Outbreaks of Disease Caused by Highly Pathogenic H5N1 Avian Influenza Virus in Poultry in Egypt during 2006 Published by?: American Association of Avian Pathologists content in a trusted digital archive. We use information tec, 52(2): 269-277.
5. WHO., 2009. H5N1 avian influenza: timeline of major events. Retrieved from http:// www.who.int/ topics/avian_influenza/en/
6. Chen, H., G. Deng, Z. Li, G.Tian, Y. Li, P. Jiao and K. Yu, 2004. The evolution of H5N1 influenza viruses in ducks in southern China. Proceedings of the National Academy of Sciences of the United States of America, *101*(28), 10452–7. doi:10.1073/ pnas.0403212101

7. Hulse-Post, D.J., K.M. Sturm-Ramirez, J. Humberd, P. Seiler, E.A. Govorkova, S. Krauss and R.G. Webster, 2005. Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proceedings of the National Academy of Sciences of the United States of America, 102(30): 10682-7. doi:10.1073/pnas.0504662102
8. Webster, R.G., W.J. Bean, O.T. Gorman, T.M. Chambers and Y. Kawaoka, 1992. Evolution and ecology of influenza A viruses. Microbiological reviews, *56*(1), 152–79. doi:10.1111/j.1541-0420.2008.01180.x
9. Songserm, T., R. Jam-On, N. Sae-Heng, N. Meemak, D.J. Hulse-Post, K.M. Sturm-Ramirez and R.G. Webster, 2006. Domestic ducks and H5N1 influenza epidemic, Thailand. Emerging Infectious Diseases, *12*(4), 575–581. doi:10.3201/eid1204.051614
10. Truszczynski, M. and E. SamorekSalamonowicz, 2008. The role of wild migrating birds in the spread of avian influenza viruses. Medycyna Weterynaryjna, *64*, 853–857. Retrieved from http:// www.ingentaconnect.com/ content/ docdel/art1083073393
11. Kim, J.K., P. Seiler, H.L. Forrest, A.M. Khalenkov, J. Franks, M. Kumar and R.G. Webster, 2008. Pathogenicity and vaccine efficacy of different clades of Asian H5N1 avian influenza A viruses in domestic ducks. Journal of virology, 82(22): 11374-82. doi:10.1128/JVI.01176-08
12. Pfeiffer, J., M. Pantin-Jackwood, T.L. To, T. Nguyen and D.L. Suarez, 2009. Phylogenetic and biological characterization of highly pathogenic H5N1 avian influenza viruses (Vietnam 2005) in chickens and ducks. Virus Research, 142(1-2), 108-120. doi:10.1016/j.virusres.2009.01.019
13. Li, K.S., Y. Guan, J. Wang, G.J.D. Smith, K.M. Xu, L. Duan and J.S.M. Peiris, 2004. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature*, *430*(6996), 209-13. doi:10.1038/nature02746
14. Kim, J.K., N.J. Negovetich, H.L. Forrest and R. G.Webster, 2009. Ducks: The "Trojan Horses" of H5N1 influenza. Influenza and other Respiratory Viruses. doi:10.1111/j.1750-2659.2009.00084.x
15. Sims, L.D., 2007. Experience in control of avian influenza in Asia. Developments in biologicals, *130*, 39–43. Retrieved from http:// www.ncbi.nlm.nih.gov/ pubmed/18411934
16. Capua, I. and D.J. Alexander, 2008. Avian influenza vaccines and vaccination in birds. Vaccine, *26*(SUPPL. 4). doi:10.1016/j.vaccine.2008.07.044
17. Abdel-Moneim, A.S., S.A.S. Shany, S.R. Fereidouni, B.T.M. Eid, M.F. el-Kady, E. Starick and G.M. Keil, 2009. Sequence diversity of the haemagglutinin open reading frame of recent highly pathogenic avian influenza H5N1 isolates from Egypt. Archives of virology, *154*(9), 1559–1562. doi:10.1007/s00705-009-0461-2
18. Arafa, A., D.L. Suarez, M.K. Hassan and M.M. Aly, 2010. Phylogenetic analysis of hemagglutinin and neuraminidase genes of highly pathogenic avian influenza H5N1 Egyptian strains isolated from 2006 to 2008 indicates heterogeneity with multiple distinct lineages. *Avian Diseases*, *54*(1suppl), 345–349. doi:10.1637/8927-051509-ResNote.1
19. FAO., 2009. H5N1 HPAI Global Overview. Issue No.14. Retrieved from http:// www.fao.org/ avianflu/en/index.html
20. OIE., 2008. OIE Report Ref: 7012, Report Date: 07/07/2008, Country: Egypt. Retrieved from http:// www.oie.int/ dwnld/AVIAN INFLUENZA/Egypt_followup7_final report.pdf
21. Spackman, E., D.A. Senne, T.J. Myers, L.L. Bulaga, L.P. Garber, M.L. Perdue and D.L. Suarez, 2002. Development of a Real-Time Reverse Transcriptase PCR Assay for Type A Influenza Virus and the Avian H5 and H7 Hemagglutinin Subtypes Development of a Real-Time Reverse Transcriptase PCR Assay for Type A Influenza Virus and the Avian H5 and H7 Hemagglutini. Journal of Clinical Microbiology, *40*(9), 3256–3260. doi:10.1128/JCM.40.9.3256
22. Slomka, M.J., T. Pavlidis, J. Banks, W. Shell, A. McNally, S. Essen and I.H. Brown, 2007. Validated H5 Eurasian real-time reverse transcriptase-polymerase chain reaction and its application in H5N1 outbreaks in 2005-2006. *Avian Diseases*, *51*(1), 373-377. doi:10.1637/7664-060906R1.1
23. Altschul, S.F., W. Gish, W. Miller, E.W. Myers, D.J. Lipman, I. Korf and Bedell, 1990. Basic local alignment search tool. Journal of Molecular Biology, 215(3): 403-410. doi:10.1016/S0022-2836(05)80360-2
24. Gilbert, M., P. Chaitaweesub, T. Parakamawongsa, S. Premashthira, T. Tiensin, W. Kalpravidh and J. Slingenbergh, 2006. Free-grazing ducks and highly pathogenic avian influenza, Thailand. Emerging Infectious Diseases, 12(2): 227-234. doi:10.3201/eid1202.050640

25. Ha, Y., D.J. Stevens, J.J. Skehel and D.C. Wiley, 2001. X-ray structures of H5 avian and H9 swine influenza virus hemagglutinins bound to avian and human receptor analogs. Proceedings of the National Academy of Sciences of the United States of America,98(20):11181–6.doi:10.1073/pnas.201401198
26. Gilbert, M., X. Xiao, D.U. Pfeiffer, M. Epprecht, S. Boles, C. Czarnecki and J. Slingenbergh, 2008. Mapping H5N1 highly pathogenic avian influenza risk in Southeast Asia. Proceedings of the National Academy of Sciences of the United States of America, 105(12): 4769-4774. doi:10.1073/ pnas.0710581105
27. WHO/ OIE/ FAO H5N1. (2014). Revised and updated nomenclature for highly pathogenic avian influenza A (H5N1) viruses. Evolution Working Group. Influenza and other respiratory viruses, 8(3): 384-8. doi:10.1111/ irv.12230
28. Arafa, A., I. El-Masry, S. Kholosy, M.K. Hassan, G. Dauphin, J. Lubroth and Y.J. Makonnen, 2016. Phylodynamics of avian influenza clade 2.2.1 H5N1 viruses in Egypt. Virology Journal, 13(1): 49. doi:10.1186/s12985-016-0477-7
29. Arafa, A.S., M.M. Naguib, C. Luttermann, A.A. Selim, W.H. Kilany, N. Hagag and T.C. Harder, 2015. Emergence of a novel cluster of influenza A(H5N1) virus clade 2.2.1.2 with putative human health impact in Egypt, 2014/15. Euro surveillance?: bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin, *20*(13), 2–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/25860390
30. Auewarakul, P., O. Suptawiwat, C. Sangma, Y. Suzuki, S. Louisirirotchanakul, H. Lerdsamran and P. Puthavathana, 2007. An Avian Influenza H5N1 Virus That Binds to a Human-Type Receptor An Avian Influenza H5N1 Virus That Binds to a Human-Type Receptor. Journal of virology, *81*(18), 9950–9955. doi:10.1128/JVI.00468-07
31. Kaverin, N.V., R.G. Webster, E.A. Govorkova, N.L. Varich, A.K. Gitelman, A.S. Lipatov and N.A. Ilyushina, 2002. Structure of antigenic sites on the haemagglutinin molecule of H5 avian influenza virus and phenotypic variation of escape mutants. Journal of General Virology, 83(10): 2497-2505. doi:10.1099/0022-1317-83-10-2497
32. Kim, J.K., G. Kayali, D. Walker, H.L. Forrest, A.H. Ellebedy, Y.S. Griffin and R.G. Webster, 2010. Puzzling Inefficiency of H5N1 Influenza Vaccines in Egyptian Poultry. Proceedings of the National Academy of Sciences, 107(24): 11044–11049. doi:10.1073/pnas.1006419107