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Molecular Characterization of H9N2 Avian Influenza Viruses Isolated from Commercial Broiler Chickens in Egypt during 2014-2015

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ABSTRACT
Avian influenza virus H9N2 is a panzootic pathogen that affects poultry causing mild to moderate respiratory distress but has been associated with high morbidity and considerable mortality. In this study, one hundred and twenty cloacal and tracheal swabs and organs (trachea, lung and spleen) were collected from broiler chicken flocks in 8 Egyptian governorates from summer 2014 to spring 2015. Results of RT-PCR revealed that 36 flocks were positive out of 120 flocks for H9N2 (30%) of which 24 infected with H9 only (66.7%) and 12 co-infected with H5N1 (19.4), NDV (11.1%) or IBV (8.3%). Seasonal distribution of positive flocks was as follow: 0.8% in summer of 2014, 5.8% in autumn of 2014, 19.2% in winter of 2014 and 4.1% in spring of 2015. Sequencing of HA gene and phylogenetic analysis of 7 selected H9N2 isolates representative of different governorates indicated that all isolates belong to G1-like lineage and are closely related to other Middle East H9N2 isolates.

Key words:
Avian Influenza, H9N2, Broiler chickens, Sequencing

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1. INRODUCTION

Avian influenza viruses (AIV) are segmented, negative-sense, single-stranded RNA viruses of the family Orthomyxoviridae and are divided into types A, B and C on the basis of the antigenic character of their internal nucleoprotein and matrix proteins. Only type A influenza viruses have been known to cause natural infections in birds (Wood et al., 1993). They can be divided into two distinct groups on the basis of their ability to cause disease in poultry (Capua and Alexander, 2004). Highly pathogenic avian influenza viruses (HPAIV) may cause up to 100% mortality. On the other hand, low pathogenic avian influenza viruses (LPAIV) cause mild to moderate infections in various domestic and wild bird species (Mo et al., 1997; Alexander, 2000).

Recently, LPAIV H9N2 subtype has been isolated worldwide from different types of terrestrial poultry (Cameroon et al., 2000; Li et al., 2003). Outbreaks were initially concentrated in Asia (Capua and Alexander, 2004), and then subsequently spread to Africa, Middle East (Roussan et al., 2009) and America causing significant economic losses related to increased mortality and decreased production (Swayne and Halvorson, 2003). It has also been reported that H9N2 avian influenza virus can cross species barrier and infect humans (Peiris et al., 1999).

The first isolation of H9N2 virus in Egypt was from clinically healthy commercial bobwhite quail flock (A/quail/Egypt/113413v/2011/H9N2) in May 2011 (El-Zoghby et al., 2012) then from commercial broiler, broiler breeder and layer farms (Monne et al., 2012; Shakal et al., 2013; Ahmed et al., 2013; Dabour et al., 2014).

On phylogenetic analysis of the haemagglutinin (HA) gene of the cleavage site, the Egyptian isolates of H9N2 were grouped in the Qa/HK/G1/97 lineage, which is similar to the viruses circulating in the Middle East, with close phylogeny to the Israeli viruses and they were also close to the recently isolated virus in 2011, A/quail/Egypt/113413v/2011/H9N2 (Arafa et al., 2012b). The Egyptian H9N2 strain was found to have a genetic constitution that suggest the ability of the virus to acquire basic amino acids in the HA connecting the peptide sequence needed to become highly pathogenic (Abdel-Moneim et al., 2012).

The disease in poultry is increasing throughout the country and co-infection with other pathogens have resulted in high morbidity and mortality. The situation is not going to improve by itself and the

only intervention strategy being used is vaccination that is based on antigenic and genetic diversity of the circulating virus strains. Continuous surveillance for H9N2 in Egypt and appropriate antigenic analysis could certainly offer better scientific approach to improving control measures of the disease. So, in the present study, the isolation, sequencing and phylogenetic analysis of H9N2 AIV was carried out to provide updated data about the situation of H9N2 in Egypt.

2. MATERIAL AND METHODS

2.1. Sampling

Eighty paired cloacal and tracheal swabs besides 40 tissue samples (trachea, lung and spleen) were collected from 120 broiler flocks suffering from mild to severe respiratory signs with low to moderate mortality. All these flocks were not vaccinated against H9N2 virus. Swab samples were collected in a medium containing 50% glycerol, 50% phosphate-buffered saline (PBS), penicillin (2 $\times$ 10^3 U/ml), streptomycin (200 µg/ml), and amphotericin B (250 µg/ml). Samples were chilled on ice until delivered to the laboratory. All samples were stored at –80°C until used.

2.2. Virus isolation

Tissue samples were homogenized in a sterile mortar and pestle to give approximately 20% suspension in PBS supplemented with antibiotics. Both tissue suspensions and swab samples were centrifuged at 500 × g for 10 min. and supernatant fluids were collected. About, 0.2ml from each sample was inoculated aseptically into the allantoic cavity of five 9-11 day-old embryonated chicken egg (ECG). Eggs were incubated at 37°C for 5 days, candled daily and dead embryos were collected. The collected allantoic fluids were centrifuged 3000 rpm/ 5 minutes to remove blood and cells. If there is no HA activity, another blind passage was done before identifying the specimen as a negative sample.

2.3. Assessing the haemagglutinating activity of the collected isolates

The allantoic fluid of pooled infected embryos was subjected to slide haemagglutination (HA) test and positive ones were titrated with plate HA in a 25-µl volume in 96-well-haemagglutionation-plate (Beard, 1989).

2.4. Detection of viral RNA using RT-PCR

RNA was extracted from virus-containing allantoic fluids using GeneJET viral DNA and RNA Purification kit (Thermo Scientific, USA) Cat. No. # K0821. The procedure was performed according to the manufacturer's instruction. Amplification process was performed using Verso one step RT-PCR ready mix Cat. No AB-1454/LD/A (Thermo Scientific, USA) in a 25 µl reaction mixture containing 5 µl RNA template and 10 pmol of each primer. For H9 gene amplification, the reaction mixture was subjected to 50°C for 30 min and 95° C for 15 min then 35 cycles of 94°C, 53°C and 72°C each for 1 min, followed by a final extension at 72°C for 10 min (Tajmanesh et al., 2006). The PCR products were separated in 1% agarose gel and visualized under ultraviolet light (Fig. 2). To detect mixed infection of H9N2 positive samples, RT-PCR assays for H5N1, Newcastle disease virus (NDV) and infectious bronchitis virus (IBV) were also performed.

Table (1): Oligonucleotide primers used for detection of influenza viruses (H5 and H9), NDV and IBV by RT-PCR assay

Target gene fragment	Primer	Sequence (5'-3')	Amplicon size	Reference
H5	H5-f	CT CCA GAR TAT GCM TAY AAA ATT GTC-3'	311	Slomka et al., 2007
	H5-R	AC CAA CCG TCT ACC ATK CCY TG -3'		
H9	H9-f	5´-CTY CAC ACA GAR CAC AAT GG-3´	486	Tajmanesh et al., 2006
	H9-R	5´-GTC ACA CTT GTT GTT GTR TC-3´		
NDV	ND-f	5′-TGGAGCCAAACCGCGCACCTGCGG-3′	766	Mazumder et al., 2012
	ND-R	(5′-GAGGATGTTGGCAGCAT-3′)		
IB	XCE3r	5′-CAGATTGCTTACAACCACC -3´		Adzhar et al., 1997
	BCE1f	5′-AGTAGTTTTGTGTATAAACCA-3´	154	
	DCE1f	5′-ATACAATTATATCAAACCAGC-3´	217	
	MCE1f	5′-AATACTACTTTTACGTTACAC -3´	295	

2.5. Sequencing of HA gene and phylogenetic analysis

Purified RT-PCR products of seven representative samples were sequenced directly in both forward and reverse directions (Macrogen, Korea). Different gene sequences were assembled by trimming primer-linker. Phylogenetic analysis based on the HA gene was performed using MEGA version 6.0 (www.megasoftware.net) (Tamura et al., 2007).

For comparison, the multisequence and phylogenetic analysis of the isolated H9N2 strains in the current study and the recently isolated Egyptian strain from quail as well as different H9N2 virus lineages circulated in the Middle East together with the ancestor Asian strains were included. All gene sequence data were collected from the National Center for Biotechnology Information (NCBI) flu database.

3. RESULTS AND DISCUSSION

Since the first emergence of H9N2 AIV in 1966 (Homme and Easterday, 1970) and although H9N2 viruses did not satisfy the criteria for highly pathogenic avian influenza, they were unique among this category, infecting a wide variety of species, including chickens (Wu et al., 2008), quails (Xu et al., 2007) pigeons (Nagarajan et al., 2009), turkeys, ducks (Perk et al., 2009), geese, pigs (Yu et al., 2008) and humans (Li et al., 2003).

Low pathogenic AI H9N2 was first reported in Egypt in November 2011 from bobwhite quails; the isolated virus was closely related to viruses of the G1-like lineage isolated from neighboring countries, indicating possible epidemiological link

s (El-Zoghby et al., 2012). Subsequent isolation and characterization of H9N2 AIV from chickens in Egypt were reported (Abdel- Moneim et al., 2012; Arafa et al., 2012b; Shakal et al., 2013; Monne et al., 2013; Kayali et al., 2014; Dabour et al., 2014).

Although H9N2 viruses were characterized as low pathogenic avian influenza (LPAI) viruses, they caused high morbidity and mortality especially in cases of complications with other viruses as NDV, IBV, IBD or their live vaccines and other bacteria as E.coli and ORT as well as mycoplasma (Naeem et al., 2007).

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In this study, one hundred and twenty poultry flocks in 8 Egyptian governorates (El-behira, Alexandria, El-Gharbia, Marsa Matrouh, Kafr El-Sheikh, El-Fayoum, Assiut and El-menia) were randomly sampled and investigated for presence of H9N2 AIV during 2014 and 2015.

Clinical signs of examined flocks ranged from very mild disease to high morbidity and mortality with generalized clinical signs as huddling, ruffled feathers, depression, decreased feed and water consumption, decrease in weight gain and edema of face and head in most flocks. Greenish diarrhea and rare nervous signs were also observed.

The most frequent post mortem lesions were in the respiratory tract, especially sinuses, and characterized as catarrhal, fibrinous, serofibrinous, mucopurulent, or fibrinopurulent inflammation. Mild congestion of trachea and lungs besides intestinal ballooning were also observed. There are many other lesions have been reported including firm pancreas with pale mottling and hemorrhage (pancreatitis). Also in some cases there were petechial haemorrhages in abdominal fats , serosal surfaces and peritoneum.

Fifty samples were HA positive after first passage in ECE with mean log_2 6-8 HAU. Negative samples were inoculated for a second passage and of which 9 samples were HA positive with mean log_2 4-6 HAU.

The results of RT-PCR revealed 36 positive samples out of 120 examined broiler flocks (30%) of which 24 infected with H9 only (66.7%) and 12 co-infected with H5N1 (19.4), NDV (11.1%) or IBV (8.3%).

Regarding the distribution of positive cases among different seasons, we found that 1 out of 36 (2.8%) were positive during summer of 2014, 7 out of 36 (19.5%) during autumn of 2014, 23 out of 36 (63.9%) during winter of 2014 and 5 out of 36 (13.9%) during spring of 2015 were H9 positive.

The incidence of LPAI H9N2 in Egypt was previously reported by Monne et al., (2012) who described identification and characterization of the H9N2 subtype in Egyptian commercial broiler and broiler breeder farms for the first time.

Within the same year, by application of multiplex RT – PCR, Shakal et al. 2013 detected 2 positive cases out of 76 broiler farms (2.63%) from 13 Egyptian governorates. Also, Kaoud et al., 2014 reported 1 positive flock out of 50 samples collected from commercial and backyard broiler chickens at

2012 from different Egyptian governorates. Furthermore, Kayali et al., 2014 performed active surveillance for AIV from 2010-2012 and detected 18.7% positive cases for H9N2 and 21.7% H9N2/H5N1 coinfected flocks from December 2011 till December 2012.

On the other hand, Dabour et al., 2014 detected 80 H9 positive broiler flocks out of 142 (56.3%) examined flocks by RT-PCR from 6 Egyptian governorates during 2012-2014.

Increased number of positive cases during winter months more than other seasons supports the theory of increasing the activity of H9N2 AIVs by low temperature as well as importance of cold stress and bad ventilation with increased ammonia in poultry houses. The results mentioned here were agreed with Naeem et al. 1999 and 2003 who found that AIV H9N2 caused lesions and mortalities during winter in northern Pakistan more than that occurred in summer season in southern Pakistan.

Detection of H5N1 AIV from H9N2 positive cases indicated continuous co-circulation of the two subtypes in commercial chicken flocks. These results agreed with previous studies reported by (Monne et al., 2012; Kayali et al., 2014) and provide possible explanation for increased infectivity of H9N2 AIV overtime due to genetic reassortment with H5N1 subtype.

Certainly, what is to be avoided is to favor virus spread and antigenic evolution by applying uncontrolled live viruses vaccination. Continuous virological and serological monitoring have to be implemented to assess the extent of H9N2 circulation in the country and to guarantee the early detection of H9N2/H5N1 reassortants (Monne et al., 2012).

All cases which proved positive for H9N2 AIV without coinfection with other pathogens suffered from mild respiratory signs with low morbidity and mortality supports the idea that silent spread of LP H9N2 recorded in the Middle East and the Far East regions for several years and subsequent increased risk to the poultry industry. These results agree with Arafa et al., 2012 who reported 136 H9 positive cases from apparently healthy commercial broiler flocks and 14 from diseased ones and the recorded mortalities were 5-10% and 20-30% respectively.

Moreover, increased vaccination against H9N2 in Egypt as a control strategy does not prevent infection but can reduce or prevent clinical disease signs, mortality, and reduce virus shedding.

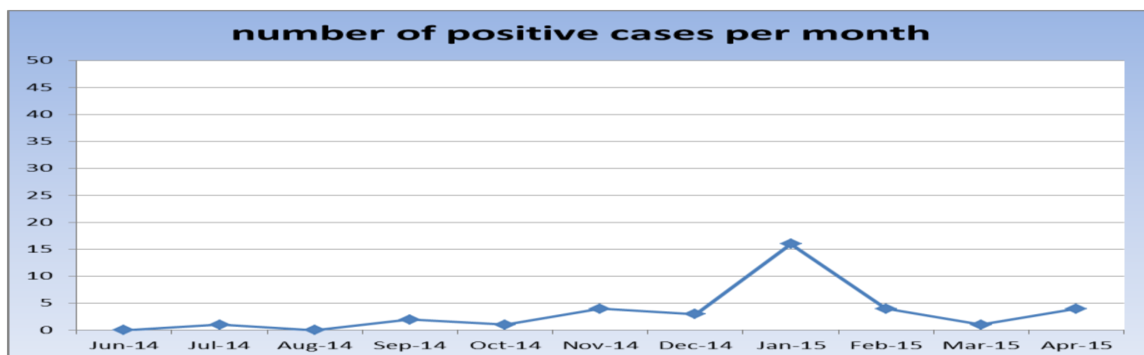

Fig. (1): Seasonal distribution of the 36 field isolates of H9N2.

Table (2) Geographical distribution of the collected Egyptian isolates.

No.	Governorate	No. of collected samples	No. of positive samples
1	Alexandria	29	16
2	El-Behira	42	9
3	El-Gharbia	14	1
4	Kafr El-Sheikh	4	1
5	Fayoum	3	1
6	El-Menia	14	4
7	Assuit	11	3
8	Marsa Matrouh	3	1
	Total	120	36

Table (3) History of positive H9N2 samples and RT-PCR results for H9, H5, NDV and IBV.

Sample Code	Age	Capacity	Severity of Resp. signs	Mortality last 3 days (%)	RT-PCR			
					H9	H5	NDV	IB
18	28	11000	++	0.85%	+	-	-	-
26	33	9200	++	1.3%	+	-	-	-
37	38	11500	+++	5.1%	+	-	+	-
44	32	6000	++	1.5%	+	-	+	-
57	29	3500	++	8%	+	-	+	-
61	36	5000	+++	11.8%	+	-	+	-
63	32	45000	++	9.3%	+	-	+	-
66	24	7000	+++	17.2%	+	+	+	-
69	35	8000	+++	38.8%	+	+	+	-
79	30	9000	+++	35.8%	+	+	+	-
80	28	7500	+++	20.3%	+	+	-	-
81	35	8000	++	1.2%	+	-	-	-
84	34	9500	+++	4.9%	+	-	-	-
85	35	11500	+++	1.8%	+	-	-	-
87	29	12000	++	6.9%	+	+	-	-
89	35	11000	++	1.7%	+	-	-	-
90	28	6000	+++	1.6%	+	-	-	-
91	31	10000	+++	7%	+	-	-	-
92	27	8000	++	4%	+	-	-	-
93	34	7000	++	8.5%	+	+	-	-
94	29	3000	+++	6%	+	-	-	-
95	29	5500	++	3.3%	+	-	-	-
96	27	12000	+++	7.8%	+	-	-	-
97	25	9000	++	3.3%	+	+	-	-
98	28	8000	++	3%	+	-	-	-
99	31	11000	++	3.3%	+	-	-	-
100	28	19000	+++	4.4%	+	-	-	-
101	28	4700	+++	2.1%	+	-	-	-
104	29	10000	++	4.5%	+	-	-	-
106	28	12000		2%	+	-	-	-
109	30	5000	+++	6%	+	-	-	-
115	31	8000	++	3.3%	+	-	-	-
116	30	9000	++	2.7%	+	-	-	-
117	35	14000	++	2.6%	+	-	-	-
118	26	7000	+++	3.1%	+	-	-	-
119	32	5000	++	2.4%	+	-	-	-

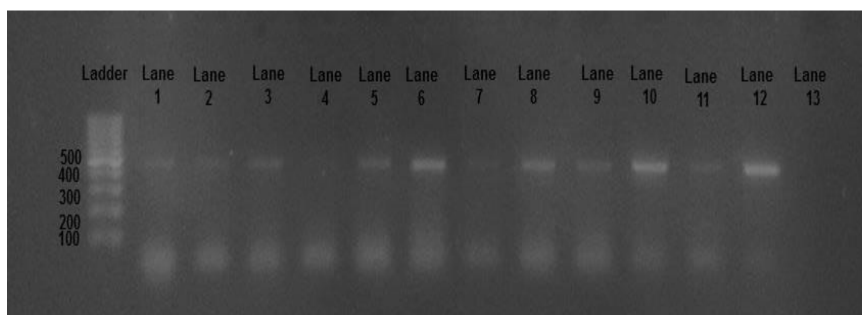

Fig (2): Agarose gel electrophoresis of RT-PCR products (486 bp) of H9N2 samples. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 (positive samples), lane 12 (positive control) and lane 13 (control negative).

The low pathogenicity nature of H9N2 influenza viruses enables them to become widespread since they did not raise concern and permitted their hosts to survive unhindered. Thus, they were free to continue to reassort and were more likely to have the opportunity to find the best gene constellation (Webster et al., 1992).

The presence of H9N2 in commercial Egyptian farms may indicate some defect in applying the biosecurity measures that will threaten the poultry

industry especially with the frequent presence of mortalities associated with other pathogen infection.
The phylogenetic relationship between the H9 HA gene of the tested isolates and those of selected H9N2 strains isolated in several other countries were analyzed. All the Egyptian isolates were closer to the viruses isolated in the Middle East which according to Banks et al. (2000) belonging to viruses from G1 lineage with more close relationship to the Israeli strains (A-Chicken-Israeli-1163-2011-H9N2) (average amino acid identity 88.7%-93.3%) that together formed a characteristic group among G1 like viruses.
The H9 isolates was clustered with recent Egyptian and the samples was G1-like group of HA gene sequencing (Figure 3). The isolates were similar to the Egyptian strain of 2011(A/Quail/Egypt/113413v/2011) with about 92.3%-97.1%.
In the last few years, the H9N2 AIV seems to regain importance as novel genotypes continue to arise. The recently isolated genotypes from poultry in Pakistan were found to contain NS genes similar to highly pathogenic H7N3 and H5N1 viruses (Iqbal et al., 2009). Moreover, the Egyptian H9N2 strain was found to have a genetic constitution that suggest the ability of the virus to acquire basic amino acids in the HA connecting the peptide sequence needed to become highly pathogenic (Abdel-Moneim et al., 2012).
By the end of year 2013, molecular characterization of avian influenza viruses (H9N2) isolated from freshly dead birds suffered from respiratory distress including rhinitis, gasping, coughing, conjunctivitis and ocular discharge, revealed that Egyptian virus, A/chicken/Egypt/VRLCU-ZK2/2012, grouped in the G1/97-like lineage in one group with Israeli strains and other related strains that circulating in the Middle East (Ahmed et al., 2013). Also, phylogenetic analysis of LPAI H9N2 isolated from backyard birds as well a s LBMs bird species (ducks, geese, turkeys, chickens) during 2012 - 2013 from some Egyptian provinces showed that, the virus isolated (Egypt/VRLCUR33/2012/H9N2), was approximately similar to the other Middle East H9N2 strains (Kaoud et al., 2014).
Recently there was dramatic increase in the incidence of H9N2 virus in Egypt and the phylogenetic analysis of the recent isolates confirmed that only H9N2 viruses with a G1-like lineage circulated among poultry in Egypt (Kayali et al., 2014), and all the tested viruses had conserved cleavage site motif RSSR/GLF suggested that they were low pathogenic viruses (Soliman, 2014). While recent study indicated that H9N2 isolated from broiler sectors showed 93.9 - 97.4% homology with the original Egyptian strains 2011 – 2013 (Dabour, 2015).

4. CONCLUSION

From this study we concluded that there is increased rate of isolation of H9N2 infection during 2014-2015 compared with previous three years. Moreover, continuous co-circulation of H9N2 with H5N1 increasing chance of genetic reassortment with each other and subsequent increased pathogenicity of H9N2 in poultry. So, Continuous surveillance with viral sequence comparison and phylogenetic analysis of current LPAI H9N2 are necessary to recognize newly emerging influenza variants and to monitor the global spread of these viruses. Lower incidence of H5N1 cases in comparison with previous seasons was linked to widespread prevalence of H9N2 LPAI subtype.

5. REFERENCES

Abdel-Moneim, A.S., Afifi, M.A., El-Kady, M.F. 2012. Isolation and mutation trend analysis of influenza A virus subtype H9N2 in Egypt. Virol J. 9: 173-182.

Adzhar , A., Gough, R.E., Haydon, D., Shaw, K., Britton, P., Cavanagh, D. 1997. Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain. Avian Pathology 26 : 625-640.

Ahmed, Z.A., Hussein, H.A., Rohaim, M.A. 2013. Efficacy of Composting Poultry Mortality and Farms Wastes with Mixed Respiratory Infection Viruses H9N2 and NDV in Egypt. Global Veterinaria 11(2): 177 – 185.

Alexander, D.J. 2000. A review of avian influenza in different bird species. Vet Micro 74 : 3–13.

Arafa, A., Hagag, N., Yehia, N., Zantary, A.M., Naguib, M.M., Nasef, S.A. 2012. Effect of cocirculation of highly pathogenic avian influenza H5N1subtype with low pathogenic H9N2 subtype on the spread of infections. Avian Dis 56:849-857.

Banks, J., Speidel, E.C., Harris, P.A., Alexander, D.J. 2000. Phylogenetic analysis of influenza A viruses of H9 haemagglutinin subtype. Avian Path 29:353–60.

Beard, C.W. 1989. Serological procedures. In A laboratory manual for the isolation and identification of avian pathogens. 3rd edition. Edited by Purchase HG, Arp LH, Domermuth CH, Pearson JE. Dubuque, Iowa: Kendall/Hunt Publishing Company, 192–200.

Cameron, K.R., Gregory, V., Banks, J., Brown, I.H., Alexander, D.J., Hay, A.J. and Lin, Y.P. 2000. H9N2 subtype influenza A viruses in poultry in Pakistan are closely related to the H9N2 viruses responsible for human infection in Hong Kong. Virology 278 : 36–41.

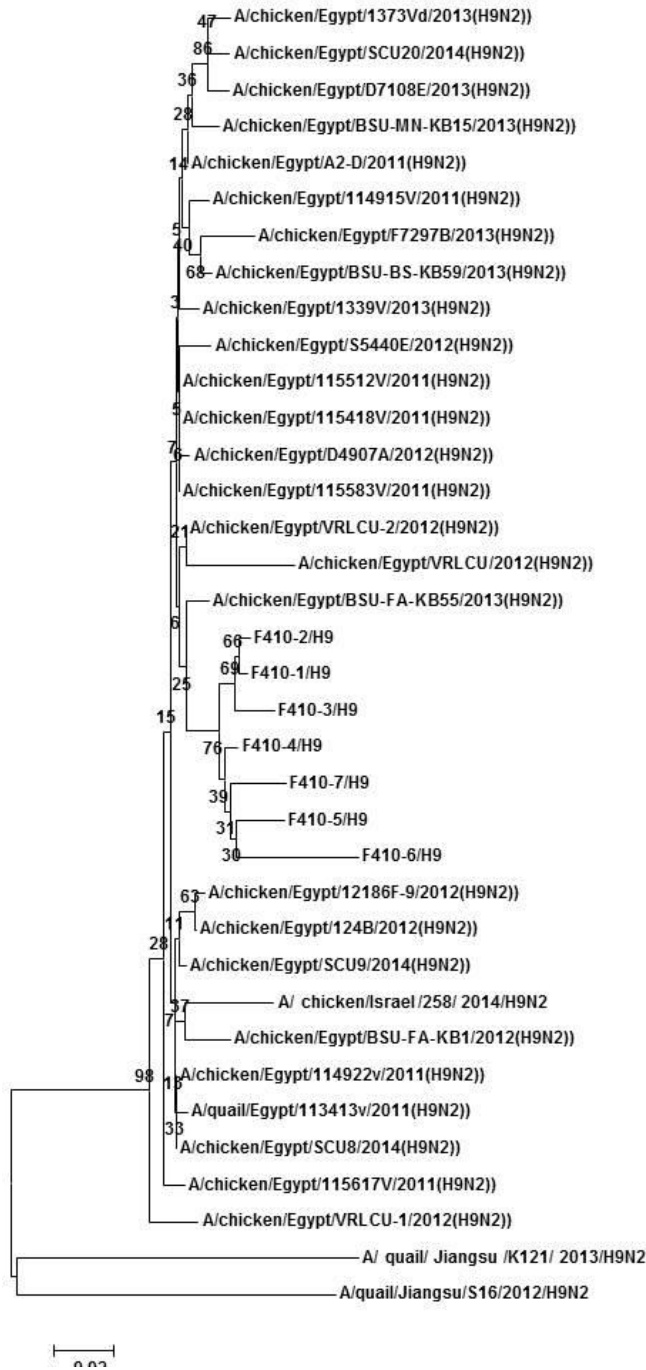

Fig. (3): Phylogenetic tree of 7 selected H9N2 isolates compared with other Egyptian, Middle East (Israeli) and Asian (Hong Kong) isolates.

El-Zoghby, E.F., Arafa, A.S., Hassan, M.K., Aly, M.M., Selim, A., Kilany, W.H., Selim, U., Nasef, S., Aggor, M.G., Abdelwhab E.M., Hafez, H.M. 2012. Isolation of H9N2 avian influenza virus from bobwhite quail (Colinus virginianus) in Egypt. Arch. Virol. 157: 1167–1172.

Capua, I., Alexander, DJ. 2004. Avian influenza: recent developments. Avian Path 33:393–404.

Dabour, S.A. 2015. Studies on avian influenza subtype H9 in west delta governments in Egypt. M.V.Sc (poultry diseases). Faculty of veterinary medicine. Alexandria university.

Shakal, M.A., Youssef, Y.I., El Zeedy, S.A., Ibrahim, S.M., Al Baroudi, B.M. 2013. Surveillance on Avian Influenza H5N1 and H9N2 Subtypes In Egypt 2012-2013. Poult. Fish Wild Science 2: 111.

Shakal, M.A., Youssef, Y.I., El Zeedy, S.A., Ibrahim, S.M., Al Baroudi, B.M. 2013 Surveillance on Avian Influenza H5N1 and H9N2 Subtypes In Egypt 2012-2013. Poult. Fish Wild Science 2: 111.

Slomka, M.J., Pavlidis, T., Banks, J., Shell, W., Mcnally, A., Essen, S., Brown, I.H. 2007. Validated H5 Eurasian real-time reverse transcriptase–polymerase chain reaction and its application in H5N1 outbreaks in 2005–2006. Avian Dis 51: 373–377.

Soliman, M.M. 2014. Molecular characterization of recent isolates of influenza virus strains in Egypt. M.V.Sc (Virology). Faculty of Veterinary Medicine, Beni sueif Univ.

Swayne, D.E., Halvorson, D.A. 2003. Influenza. In: Disease of Poultry, Ames Iowa State Press, 11:135-160.

Tajmanesh, S., Toroghi, R., Momayez, R., Pourbakhsh, S.A. 2006. Establishment of RT-PCR for detection of avian influenza virus (H9N2) in field cases compared to virus isolation method. Arch. Razi. Inst. 61: 111-116.

Tamura, K., Dudley, J., Nei, M., Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596–1599.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., Kawaoka, Y. 1992. Evolution and Ecology of Influenza Viruses. Microbiol. Rev. 56:152-179.

Wood, G.W., Mccauly, J.W., Bashiruddin, J. B. and Aalexander, D.J., 1993 Deduced amino acid sequences of the haemagglutinin cleavage site of avian influenza A viruses of the H5 and H7 subtypes. Arch. Virol. 130: 209 -217.

Wu, R., Sui, Z.W., Zhang, H.B., Chen, Q.J., Liang, W.W., Yang, K.L., Xiong, Z., Liu, Z.W., Chen, Z., Xu, D.P. 2008 Characterization of a pathogenic H9N2 influenza A virus isolated from central China in 2007. Arch. Virol. 153:1549–1555.

Xu, K.M., Li, K.S., Smith, G.J., Li, J.W., Tai, H., Zhang, J.X., Webster, R.G., Peiris, J.S., Chen, H., Guan, Y. 2007. Evolution and molecular epidemiology of H9N2 influenza A viruses from quail insouthern China, 2000 to 2005. J. Virol. 81: 2635-2645.

Yu, H., Hua, R.H., Wei, T.C., Zhou, Y.J., Tian, Z.J., Li, G.X., Liu, T.Q., Tong, G.Z. 2008. Isolation and genetic characterization of avian origin H9N2 influenza viruses from pigs in China. Vet. Microbiol. 131, 82–92.

Homme, P.J., Easterday, B.C. 1970. Avian influenza virus infections. I. Characteristics of influenza A-turkey-Wisconsin-1966 virus. Avian Dis 14(1): 66-74.

Kaoud, H.A., Hussein, H.A., El-Dahshan, A.R., Kaliefa, H.S., Rohaim, M.A. 2014. Co-circulation of avian influenza viruses in commercial farms, backyards and live market birds in Egypt. International J. of Vet Sci and Med 2: 114 – 121.

Kayali, G., Kandeil, A., El-Shesheny, R., Kayed, A.S., Gomaa, M.M., Maatouq, A.M., Shehata, M.M., Kutkat, M.A., McKenzie, P.P., Webster, R.G., Webby, R.J., Ali, M.A. 2014. Active Surveillance for Avian Influenza Virus, Egypt, 2010 – 2012. Emerg Infect Dis 20(4).

Li, K.S., Xu, K.M.; Peiris, J.S. ; Poon, L.L.; Yu, K.Z.; Yuen, K.Y. et al. 2003. Characterization of H9 subtype influenza viruses from the ducks of southern China: a candidate for the next influenza pandemic in humans?.J. Virol. 77 (12): 6988–6994.

Mazumder, A.C., Khatun, S., Nooruzzaman, M., Chowdhury, E.H., Das, P.M., Islam, M.R. 2012. Isolation and identification of Newcastle disease viruses from field outbreaks in chickens and pigeons. Bangladesh Vet 29,: 41–48.

Mo, I.P., Song, C.S., Kim, K.S., Rhee J.C. 1997. An occurrence of non-highly pathogenic avian influenza in Korea. Proceedings of the Forth International Symposium on Avian Influenza, United States, Animal Health Association, Tallahassee, FL pp. 379-383 .

Monne, I., Hussein, A.H., Fusaro, A., Valastro, V., Hamoud, M.M., Khalefa, R.A., Dardir, S.N., Radwan, M.I., Capua, I., Cattolia, G. 2013. H9N2 influenza A virus circulates in H5N1 endemically infected poultry population in Egypt. Influenza and Other Respiratory Viruses 7(3), 240–243.

Naeem, K., Naurin, M., Rashid, S., Bano, S. 2003. Seroprevalence of avian influenza virus and its relationship with increased mortality and decreased egg production. Avian Path 32(3) 285: 289.

Naeem, K., Siddique, N., Ayaz, M., Jalalee, M.A. 2007. Avian influenza in Pakistan:outbreaks of low- and high-pathogenicity avian influenza in Pakistan during 2003-2006. Avian Dis 51(1 Suppl), 189-193.

Naeem, K., Ullah, A., Manvell, R.J., Alexander, D.J. 1999. Avian influenza A subtype H9N2 in poultry in Pakistan .Vet Rec 145 (19) p. 560.

Nagarajan, S., Rajukumar, K., Tosh, C., Ramaswamy, V., Purohit, K., Saxena, G., Behera, P., Pattnaik, B., Pradhan, H.K., Dubey, S.C. 2009. Isolation and pathotyping of H9N2 avian influenza viruses in Indian poultry. Vet Micro 133: 154 – 163.

Peiris, M., Yuen, K.Y., Leung, C.W., Chan, K.H., Lai, R.W. 1999. Human infection with influenza H9N2. Lancet 354: 916 – 917.

Roussan, D.A., Khawaldeh, G.Y., Al Rifai, R.H., Totanji, W.S.. Shaheen, I.A. 2009. Avian influenza virus H9 subtype in poultry flocks in Jordan. Prev Vet Med 88:77–81.