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RESEARCH

PHYLOGENETIC ANALYSIS AND PATHOGENECITY ASSESSMENT OF LOCALLY ISOLATED STRAIN OF HPAI VIRUS IN EGYPT IN 2016

Dalia M. Omar[1], Nermeen A. Marden[1], Lamiaa M. Gaafar[1], Elham A. El-ebiary[1], Khalid El-Dougdoug[2], Badawi Othman[2], Abd Elsattar Arafa[3], Hussein A. Hussein[4]

ABSTRACT

Background*: Avian influenza viruses* (AIV) circulating in Egypt since 2006 causing serious economic losses in poultry sector and human hazard although vaccination programs applied and other control measures.

Objectives: The present study was designed for detection, isolation, identification and characterization of *Avian influenza viruses* (AIV) circulating among poultry in 2016 to determine its suitability to be used in vaccine quality control in Egypt.

Methods: The newly isolated virus was identified and subtyped antigenically by serological test as Haemagglutination inhibition (HI) using standard AI antisera for H5 antigen and genetically by RT-PCR using specific primer for H5 gene and it was confirmed to be H5N1 and grouped according to the year of isolation as 2016 isolate strain.

Results: According to the phylogenetic analysis it falls into the classical group of Egyptian AI viruses subclade 2.2.1.2 which is dominant in Egypt since 2012 till now. Therefore, the virus was identified as HPAI according to the Intravenous Pathogenicity Index (IVPI) score (2.1). On the other hand, the strain identity to some AI vaccine strains was determined for further characterization.

Conclusion*:* our experiments in this recent isolation of avian influenza (AI) viruses denoted clearly that the evolution of H5N1 HPAI viruses in Egypt continues to occur in poultry farms. The subclade 2.2.1.2 of classical HPAI viruses has been the dominant cluster till now. So, continuous monitoring of the circulating viruses is important for better selection of viruses in vaccine studies and to understand the evolution of Avian influenza viruses overtime.

Keywords: HPAI, H5N1, HA test, RT-PCR.

BACKGROUND

Egypt is considered the largest poultry producer in the Arab world that it roughly produces 23% of the total Arab world poultry production (Freiji, 2008). This poultry industry was greatly affected since the 1st outbreak of HPAI virus H5N1 in 2006, moreover it caused major public health threat. Although Egypt used AI vaccination programs as a powerful tool to control and eradicate AI virus, and despite other control efforts like stamping out [Aly, *et al.,* (2008); Abdel-Moneim, *et al.,* (2009) and Abdelwhab, *et al.,* (2016)], the disease became endemic in Egypt since 2008 and nearly every poultry farm was threatened (Abdelwhab, *et al.,* 2011), this mainly due to circulation of the HPAIV H5N1 in different poultry species (chickens, ducks, turkeys, etc.) (Abdelwahab, *et al.,* 2010). Egypt relied primarily on inactivated H5N1 and H5N2 vaccines to limit the spread of H5N1 virus and minimize its socioeconomic impacts (Hafez, *et al.,* 2010), but in the meantime the Egyptian field uses many other types of AI vaccines like live and killed viral vectored vaccines.

The HPAIV H5N1 has been introduced into Egypt in early 2006 (Aly, *et al.,* 2008) as subclade 2.2 probably via infected wild ducks (Saad, *et al.,* 2007), which diversified into two distinct variant and classic groups designated 2.2.1.1 and 2.2.1.2 respectively. Viruses in the variant group (2.2.1.1) circulated in vaccinated poultry from 2007 to 2011. Meanwhile, the classic group (2.2.1.2) viruses were observed mainly in backyard birds and humans, and recently in the commercial birds. (Gupta and Brunak, 2002; and Tamura *et al.,* 2011).

Correspondence: nermeen.marden@gmail.com
Full list of author information is available at the end of the article

The continuous and rapid evolution of the HPAI viruses necessitates reviewing and updating of vaccine development, as well as continuous monitoring of the virus evolution and genetic changes. It is critical to detect genetic drift or shift of H5N1 and other important AI viruses with epizootic potential that may lead to new outbreaks even through the vaccinated chickens.

In this study, the isolation, identification and characterization of a recent AI virus circulating in the poultry farms was carried out to detect the rate of mutation in H5N1 virus in Egypt then subsequently used in AI vaccines evaluation (Marangon *et al.,* 2008) hoping that, the data in this study will help the authorities and provide a comprehensive guidance towards more efficient disease control and prevention for both poultry and humans.

MATERIALS AND METHODS

Sample management and preparation:

Sample collection and processing:

Fresh samples of dead and sick birds from infected flocks in Qaluobia governorate were collected. These samples were stored on ice during transportation that never exceeds one day. The tracheal and cloacal swabs samples were taken on virus transport medium (tryptose phosphate broth with antibiotics). Samples were stored at -80 ºC until processed (Beard, 1989). In the laboratory, samples were clarified by centrifugation at 1000 g for 20 minutes and then supernatants were taken for AIV isolation as described by Lee and Suarez (2004).

Virus isolation:

A volume of 0.1 ml from each specimen supernatant was inoculated into five ,9-11-day-old, specific pathogen free (SPF) embryonated chicken eggs (ECE) via the allantoic sac route according to the methods previously described by (Payungporn *et al.,* 2006). The mortality % of the inoculated ECEs was estimated. The allantoic fluids (AF) of inoculated ECEs were collected and tested for the presence of haemagglutinating (HA) activity using rapid HA test (Swayne *et al.,* 1998).

Identification and Characterization of AIV:

a. Micro plates HA and HI tests (serological identification):

A panel of standard positive sera against Newcastle (ND), Egg drop syndrome (EDS) and avian influenza (AI-H5) for H5N2, H5N1 and H9N2 viruses were used (Animal Health Service Deventer "GD", Netherland). The test was conduct also against negative reference serum. The isolated viral samples were examined for inhibition of the HA activity according to (OIE, 2017). The tested viral samples diluted 2 fold serial dilution and equal amount of 1 % RBCs were added to determine their HA titers. Then, the viral samples were adjusted to a concentration of 4 HA unit/ 50 µl to furtherly be used in HI test. All the standard positive and negative antisera were diluted 2 fold serial dilution and mixed with equal volumes of the adjusted viral samples. After incubation, another equal amounts of 1% RBCs suspension were added and the dilutions were examined for inhibition of the virus HA activity.

b. RT-PCR (genetic identification):

The tested samples were genetically identified by RT-PCR following the same protocol described at (Office International des Epizooties (OIE), 2017). The identification of the AI isolates in swab samples occur using a set of specific primers targeted to the HA gene (H5N1 and H9N2) and F gene of ND as shown in (Table 1). The technique was started with the RNA extraction from the collected samples using RNA extraction kit (QIAamp Viral RNA, RNA

extraction kit, QIAGEN Gmb H, Germany, catalogue No. 52904) and followed by RT-PCR using (QIAGEN One Step RT-PCR Kit Inc, Valencia, CA catalogue No. 210210). The RT-PCR reaction scheme was one cycle at 95 ºC for 30 minutes (initial denaturation), 35 cycle of (94 ºC for 30 seconds "denaturation", 55 ºC for 45 seconds "annealing" and 72 ºC for 1 minute "extension") and final extension at 72 for 10 minutes.

Table (1): Primers of RT-PCR

Gene	Primer name	Primer & probe sequence	Ref.
H5 gene	F-H5	5'- CTC TTC GAG CAA AAG CAG GGT-3'	**El-Zahed *et al.*, (2015)**
	R-H5	5'- TAC CAA CCG TCT ACC ATK CCY TG-3'	
H9 gene	F- H9	5'-TAG CAA AAG CAG GGG AAT TTC TT-3'	**El-Zahed *et al.*, (2015)**
	R- H9	5'-ATC TTG TAT TTG GTC ATC AAT C-3'	
F gene (ND)	F-ND	5'- TCCGGAGGATAC AAGGGTCT-3'	**Wise, *et al.*, (2004)**
	R-ND	5'- AGCTGTTGCAACCCCAAG-3'	

c. DNA Sequencing of H5 gene:

It was performed according to Cattoli *et al.* (2009). The amplified DNA fragments were purified by using (QIAquick PCR purification Kit Qiagen, Valencia, CA). Purified DNA fragments were sequenced from both directions and the complete coding sequences were generated using the (Big Dye Terminator v3.1 cycle sequencing kit, Applied Biosystem, Fostercity, USA) and the same primers that amplify the DNA. For sequence comparison and identification, the sequences were searched against the GenBank by the BLAST program (Altschul *et al.,* 1990) provided by NCBI, USA. The sequence analysis and comparison occurred between sequences derived for this study and sequences posted in GenBank for other Egyptian and standard vaccinal AI strains were shown in Figure (1).

d. Intravenous pathogenicity index (IVPI):

The test was done to classify the H5 subtype of AI virus isolates as HP or LP for chickens based on the OIE definition. The IVPI is defined as the mean score per bird per observation over the 10-day period after intravenous inoculation of the virus to 10 SPF chickens (6 weeks old). The test was done according to Terregino and Capua (2009).

❖ Pathogenicity test of the challenge virus:

- **Virus propagation and titration:**

It was done according to Woolcock *et al*. (2001), the AF of isolated virus was diluted 10 fold serial dilution using PBS up to 10-4 for virus propagation and 10-12 for virus titration. SPF ECE 9 day old was inoculated via allantoic sac route with each dilution. AFs from haemagglutination positive eggs were pooled and aliquoted for long term storage at -70OC in propagation step. For titration, dead and live egg per dilution were examined by slide HA test at the end of incubation period and the titer was calculated according to Reed and Muench (1938).

- **Pathogenicity test:**

It was done in 4 weeks old SPF chickens according to Kim et al., (2010) and Spickler et al., (2008) that tenfold Serial dilution of the virus was prepared. Viral titer ranged from 104.5 -108.5 EID50/0.1 ml was inoculated into 50 susceptible SPF chickens intranasally (10 chickens per titer). Chickens were observed daily with record of AI clinical signs and mortality for 10 days. Uninoculated control SPF chickens were also observed for the same period of observation. All dead and diseased birds were counted on day of termination. The infective dose of the challenge virus that kills 100% of chickens through 72 hours was calculated.

RESULTS:

Virus isolation:

The inoculated ECE were examined and embryo mortality was recorded. Embryo deaths occurred 24-48 hours post inoculation as 10/25 after 24 hours post inoculation and ended to be 18/25 at 48 hours post inoculation. All samples showed complete positive haemagglutination.

Identification and characterization of AIV:

The data from Table (2) showed that all isolate samples were H5-AIV positive while they were serologically negative for H9N2, ND and EDS by HI test. Also, the results of RT-PCR test confirmed that isolate samples were H5 AI positive where they yielded strong positive reaction. While the same samples were ND and H9 negative.

Table (2): Serological and genetic identification of AIV isolate.

Isolation year	Serological identification					RT-PCR		
	H5N1	H5N2	H9N2	ND	EDS	AI H5	AI H9	ND
2016	10.3	8.8	3 (-ve)	-ve	-ve	+ve	-ve	-ve

The data depicted in **Table (3)** showed that the HA titer for newly AI isolate was 7 $\log_2$. While, the IVPI value was 2.1 and so, it is considered to be a highly pathogenic AIV for chicken. On the other hand, the AIV titer was $10^{11.5}EID_{50}$/ml.

Table (3): Characterization of AI isolate.

Isolation year	HA titer ($\log_2$)	Titer in ECE (EID_{50}/ml)	IVPI*
2016	7	$10^{11.5}$	2.1

*** IVPI** = intravenous pathogenicity index

Beside that, it was found that a titer of $10^{7.5}$ EID_{50} in 0.1 ml saline / bird was sufficient to induce 100% mortality to the infected chickens within 72 hours **(Table 4).**

Table (4): Results of pathogenicity of the local classical HPAI 2016 H5N1 challenge virus (A/chicken/Egypt/Qal-3/2016).

Virus Titer (EID_{50}/0.1ml)	No. of inoculated birds	Clinical signs	Daily observation (dpi)										No. of dead / total	Mortality	
			1	2	3	4	5	6	7	8	9	10		Time (dpi)**	%
$10^{4.5}$	10	Dead					1*	1					2/10	5th 6th	10 20
		Diseased													
$10^{5.5}$	10	Dead					1	3					4/10	5th 6th	10 40
		Diseased					3								
$10^{6.5}$	10	Dead			3	3	1						7/10	3rd 4th 5th	30 60 70
		Diseased			1	1									
$10^{7.5}$	10	Dead		3	7								10/10	2nd 3rd	30 100
		Diseased		1											
$10^{8.5}$	10	Dead		6	4								10/10	2nd 3rd	60 100
		Diseased		2											

* = No signs ** dpi = day post infection Viral titer = 11.5 $\log_{10}$ EID_{50}

The genetic analysis of the isolated virus:

It was shown from phylogenetic tree (Figure 1) and Table (5) that the AIV samples isolated at 2016 were identical with each other and fell into a group of the Egyptian AI viruses clade 2.2.1.2 reference strains that related to classical group isolated and circulated from 2012 and till now. Also, the isolated AIV at 2016 was named as A/Ch/Egypt/Qal-3/2016 (H5N1) with accession number MF664437 on gene bank. When the newly isolated 2016 AIV compared with the other ex-isolated challenge viruses isolated at 2008, 2010, 2012 and 2015, the % of identity were 94, 92, 98 and 99% with them respectively.

As regarded from Table (5), the vaccinal strain of rHVT-AI was identical to classical 2016 challenge virus with 97% and to variant 2008 challenge virus with 95%. While the degrees of identity between classical 2016 isolate to vaccinal strains of rFP-AI/Scot and rFP-AI/Ire vaccines were 85.5% and 84.5%, respectively. But percentage % of similarity between them and the variant 2008 challenge virus were 83.2% and 83%, respectively.

Also, the degree of identity between classical 2016 and variant 2008 challenge viruses and vaccinal strain of krND-AI vaccine were 92% and 97%, respectively. Meanwhile, the vaccinal strain of krBacu-AI+ND vaccine was identical to classical 2016 and variant 2008 challenge viruses with 92% and 93%, respectively.

Also, the degree of identity between vaccinal strain of kH5N1 vaccine and the classical 2016 and variant 2008 challenge viruses were 92% and 96%, respectively. On the other hand, the vacccinal strain of kH5N2 and kAI+ND vaccines was identical to classical 2016 with 70% and to variant 2008 challenge viruses with 69%. But, the degree of identity between classical 2016 and variant 2008 challenge viruses and vaccinal strain of kH5N2 (Pot) vaccine were 84% and 82%, respectively.

Table (5): Sequence identity matrix of Egyptian AIV challenge viruses and some vaccine strains

AI viruses (isolates or vaccinal strains)	Strains	Sequence identities / challenge viruses				
		2008	2010	2012	2015	2016
2008	**A/cicken/Egypt/1709-6/2008**	ID	0.97	0.94	0.95	0.94
2010	**A/chicken/Egypt/1063/2010**	0.97	ID	0.94	0.92	0.92
2012	**A/chicken/Egypt/128s/2012**	0.94	0.94	ID	0.98	0.98
2015	**A/duck/Egypt/AR236-A3NLQP/2015**	0.95	0.92	0.98	ID	0.99
2016	**A/chicken/Egypt/Qal-3/2016**	0.94	0.92	0.98	0.99	ID
rHVT-AI	**A/mute swan/Hungary/4999/2006**	0.95	0.94	0.96	0.97	0.97
rFP-AI/Scot	**A/chicken/Scotland/1959**	0.832	0.83	0.87	0.85	0.855
rFP-AI/Ire	**A/chicken/Ireland/1983**	0.83	0.824	0.86	0.84	0.845
krND-AI	**A/chicken/Egypt/1063/2010**	0.97	1	0.94	0.92	0.92
krBacu-AI+ND	**A/duck/China/E319-2/2003**	0.92	0.92	0.93	0.93	0.93
kH5N1	**A/chicken/Egypt/18-H/2009**	0.96	0.97	0.94	0.92	0.92
kH5N1 Re-1	**A/goose/Guandong/1/1996**	0.91	0.90	0.88	0.89	0.89
Local kH5N1	**A/chicken/Egypt/Q1995D/2010**	0.97	0.99	0.94	0.92	0.91
	A/duck/Egypt/M2583D/2010	0.96	0.94	0.97	0,98	0.98
kH5N2 (Mex)	**A/chicken/Mexico/232/1994**	0.69	0.64	0.70	0.70	0.70
KAI+ND	**A/chicken/Mexico/232/1994**	0.69	0.64	0.70	0.70	0.70
kH5N2 (Pot)	**A/duck/Potsdam1402-6/1986**	0.82	0.82	0.83	0.84	0.84
kH5N3	**A/chicken/Vietnam/C58/2004**	0.93	0.93	0.90	0.91	0.91

Also, the degree of identity between classical 2016 and variant 2008 challenge viruses and vaccinal strain of kH5N1 Re-1vaccine were 89% and 91%, respectively. Meanwhile, the vaccinal strains of local kH5N1 vaccine were identical to classical 2016 and variant 2008 challenge viruses with 91%, and 97% (Q strain) and 98% and 96% (M strains) respectively.

On the other hand, the vaccinal strain of kH5N3 vaccine was identical to classical 2016 and variant 2008 challenge viruses with 91% and 93%, respectively.

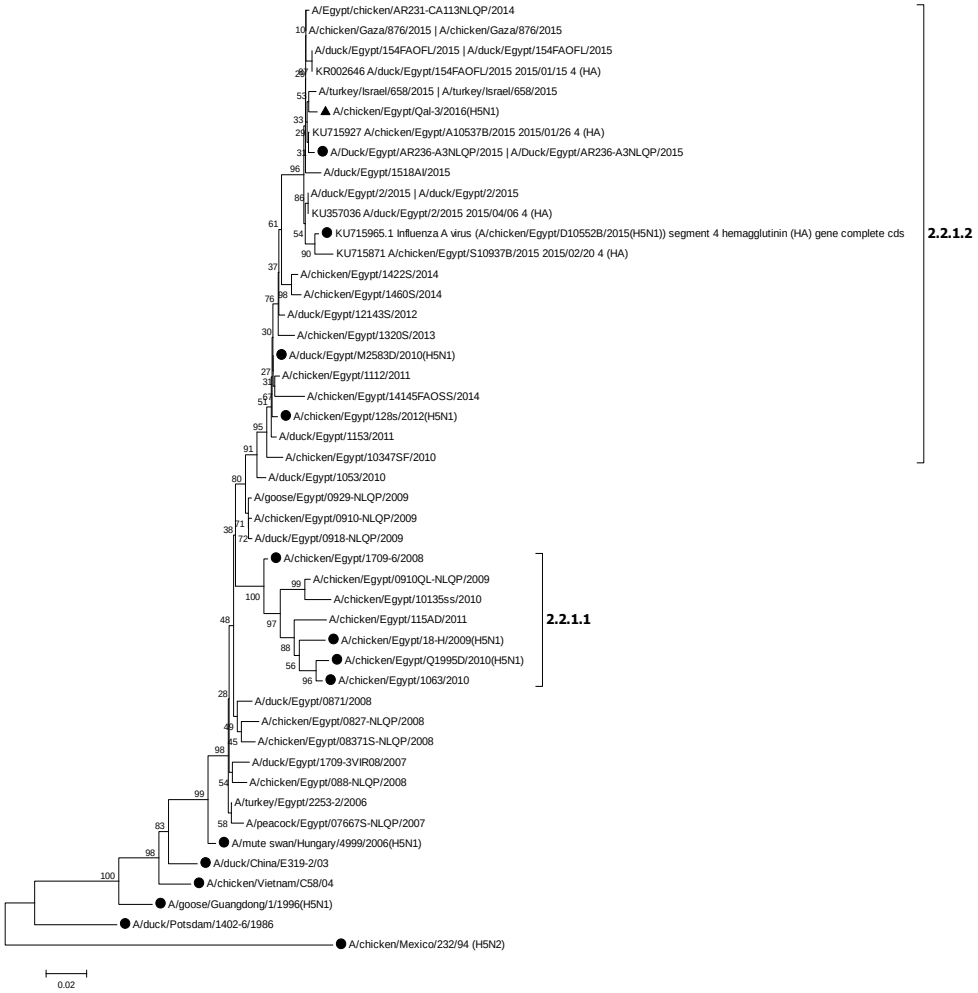

Fig. (1): Phylogenetic analysis of the AI isolate and some standard vaccinal strains

Virus name: A/chicken/Egypt/Qal-3/2016 (H5N1)

Accession number: MF664437

DISCUSSION

Since the initial outbreaks of HPAI H5N1 in 1997 (Claas *et al.*, 1998), the virus has diverged both antigenically and genetically with continuous emerging of new genotypes into classical and variant strains (Wan *et al.*, 2007). Similar to that of seasonal influenza viruses, the continuous and rapid evolution of those HPAI viruses necessitates reviewing and updating of vaccine development. Also, continuous monitoring of virus evolution and genetic changes of the circulating H5N1 and other important avian influenza viruses with epizootic potential. It is critical to detect genetic drift or a genetic shift that may lead to new outbreaks or a future influenza pandemic. In order to slow the rate of mutation and reassortment of viruses circulating in poultry populations, complete eradication by culling or culling with vaccination strategies are commonly used (Marangon *et al.*, 2008).

So, the aim of this study is isolation and identification of a new AI (if present) that circulate in the poultry farms during a year of 2016 to diagnose the rate of mutation or reassortment in the AI virus that used in AI vaccines evaluation.

Sample selection and handling are important in the detection of the AI virus. The type and quality of samples collected will affect the success of diagnosis. In addition, storage conditions from the time of collection until the specimen is processed in the laboratory are very important for correct diagnosis of AI, this agreed with Johnson (1990). Swabs collected for AI diagnosis and isolation should be taken from clinically ill or freshly dead animals where the virus is present in the highest quantity at this time.

Virus isolation (VI) technique is considered the reference and standard method for the diagnosis of AI virus in the collected samples. This was agreed with Swayne et al. (1998) who told that virus isolation is the best test method to accurately identify the presence of AIV from tracheal and cloacal swab samples. In this study, embryo deaths due to newly isolated virus infection occurred as early as 24-48 hours post inoculation where 10/25 after 24 hours post and 18/25 after 48 hours post inoculation. All the inoculated samples showed complete positive haemagglutination by HA assay. This agreed with Swayne and Halvorson (2003), who said that the high sensitivity of virus isolation (VI) makes this method, can be used to detect AI virus during any stage of an active infection. So, AI can be detected within 24 hours of infection in an individual bird and for several weeks post exposure in a flock.

Additional tests as HI assay for the positive HA samples was applied to differentiate AI virus from other haemagglutinating viruses like H9N2, ND and EDS (Beard, 1989). Once avian paramyxoviruses and adenoviruses had been ruled out, the presence of AI virus can be confirmed by type specific HI test using AI virus-specific subtyping serums for H5 antigens (reference H5N1 and H5N2 antisera). It was found from Table (2) that all the viral samples serologically H5-AIV positive while they were H9, ND and EDS negative. This agreed with Webster et al. (2002) who said that VI is very sensitive, but not highly specific or selective because other viruses that maybe present in poultry samples will grow in chicken embryos. So, it must be followed by a confirmatory test as HI to differentiate AI virus from other haemagglutinating viruses.

The isolated Egyptian strain of AIV in 2016 was tested for determination of its virulence in chicken. IVPI is necessary to be done for further characterization and confirmation of the high pathogenicity of the newly isolated virus. The IVPI of that AI isolate was 2.1 indicating that it was classified as HP for chicken. This agreed with Terregino and Capua, (2009) who mentioned that any influenza A virus, regardless of subtype, yielding a value > 1.2 in an IVPI test is considered to be highly pathogenic avian influenza virus.

Also, it was found that, the newly Egyptian isolated virus had a titer of $10^{11.5}$ EID_{50}/ml and HA titer 7 $\log_2$ (Table 3). On the meantime, the most suitable challenge dose of that virus according to the pathogenicity test in chickens was $10^{7.5}$ EID_{50}/0.1ml (Table 4). This agreed with (Capua and Marangon, 2003 and Swayne and Slemons 2008) who explain that the sufficient dose to be used for challenge of vaccinated chicken against HPAIV should be able to kill 100% of control SPF chicken within 72 hrs.

In recent years, the application of molecular methods for the detection of viral nucleic acid has become an important tool for the detection of AI virus (M gene) and identification of HA and NA subtypes. RT-PCR based tests are the most widely used molecular method either quantitative

real time reverse transcriptase polymerase chain reaction (rRT-PCR) (Iqbal *et al.*, 2013) or conventional RT-PCR (Fouchier *et al.*, 2000 and Poddar 2002).

The RT-PCR technique with specific set of primers was successful in analyzing of viral samples and confirming the presence of H5N1 AI virus. While RT-PCR using primers for F gene of NDV and HA gene of H9N2 proved that all viral samples were ND and H9N2 negative (Table 5). This agreed with Lee et al. (2001) who said that the application of molecular methods for the detection of viral nucleic acid has become an important, high speed and low cost tool for detection of AI virus type A and directly targeted to the different HA and NA subtypes.

As shown in Figure (1), phylogenetic analysis of HA gene of AI strains in this study showed 2 distinct subgroups (2.2.1.1 and 2.2.1.2). In late 2007 and early 2008 a new variant strains (2.2.1.1) appeared until 2011 (El-Shesheny, *et al.*, 2014) and was clearly distinct from the original classical group appeared from 2006 and early 2007 in Egypt (2.2.1) (Arafa *et al.*, 2016). A new classical cluster (2.2.1.2) became predominant in Egypt since 2012 with disappearance of the variant group in the field (Kayali, *et al.*, 2016). Consequently, AI sample isolated at 2016 showed the closest relation to Egyptian group 2.2.1.2 as shown in Figure (1).

On comparing between the newly isolated subtype at 2016 to other reference Egyptian AI strains, it was found that 2016 isolate was identical to variant 2.2.1.1 group (2008 and 2010) with range of 92-94% while to classical 2.2.1.2 group (2012 – 2015) with a range of 98-99%, as in Table (5).

The newly isolated AI virus at 2016 was named A/Ch/Egypt/Qal-3/2016 (H5N1) and its sequence was put in gene bank with accession number MF664437. It was very similar to the Egyptian 2015 reference strain of the same claster 2.2.1.2 (99%) with minimum divergence (1%). The previous data indicating that the 2.2.1.2 claster viruses is still till now the predominant circulating subtypes causing AI outbreaks in Egypt.

According to the other previous studies, analysis of virus population dynamics of the entire data set of the Egyptian H5N1 viruses showed a rise in genetic diversity in the 2.2.1.1 cluster from early 2008 and from 2009 exhibiting a constant progressive adaptation to poultry to be an endemic cluster (WHO, 2012). The viruses from classical and variant groups (2.2.1.2 and 2.2.1.1) had the highest record of mutations in the positive selection sites which may be attributed to vaccination pressure due to long standing application of vaccines with high virus load in the endemic environment (El-Zoghaby et al. 2012). Also, Cattoli et al. (2009) indicated that evolutionary dynamics and positive selection significantly increased in virus population in countries applying the avian influenza vaccination for H5N1, compared to viruses in countries that had never used vaccination. They also indicated that the rapid evolution of H5N1 viruses in Egypt was possibly linked to vaccination pressure due to sub-optimal use of vaccines.

In the meantime, the comparison between the Egyptian locally isolated variant AI strain group 2.2.1.1 and the classical AI strain group 2.2.1.2 with the most vaccinal AI strains forming the most common AI vaccines applied in the field was carried out. It was found that the variant 2.2.1.1 (2008) strain was identical to the tested vaccinal strains of rHVT-AI, rFP-AI, KrND-AI, KrBac-AI+ND, kH5N1, kH5N2 (Mex), kAI+ND, kH5N1 Re-1, local kH5N1 (Q), local kH5N1 (M), kH5N2 (Pots) and kH5N3 vaccines with percentages of 95, 83, 97, 93, 96, 69, 69, 91, 97, 96, 82 and 93 respectively. While, the identity of classical 2.2.1.2 (2016) virus and the sane vaccinal strains were 97%, 85%, 92%, 93%, 92%, 70%, 70%, 89%, 91%, 98%, 84% and 91% respectively as shown in Table (5).

CONCLUSION

Our experiments in this recent isolation of avian influenza (AI) viruses cleared that the evolution of H5N1 HPAI viruses in Egypt continuous to occur in poultry farms. The clade 2.2.1.2 of classical HPAI has been the dominant cluster with a wide circulation since 2011 and till now and this agreed with Arafa *et al.,* (2015). So, continuous monitoring of the circulating viruses and sequencing of haemagglutinin (HA) and other genes as Neuraminidase (NA) gene, is important to better select viruses for vaccine studies and to understand the evolution of viruses overtime.

AUTHOR DETAILS

[1]The Central Laboratory for Evaluation of Veterinary Biologics, El Abassia, Cairo.
[2]Dept. of Agric. Microbiology (Virology Lab.), Faculty of Agriculture, Ain Shams University, Cairo, Egypt.
[3]National Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute.
[4]Department of Virology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt

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