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Epidemiological Study on the Impact of Vaccination Programs on Antigenic Relatedness, Genetic Characterization of Highly pathogenic Avian Influenza H5N1

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Key words

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ABSTRACT:

Two HPAI H5N1 viruses were isolated from vaccinated layer and broiler commercial poultry farms in Egypt at years 2011 and 2013; respectively. By phylogenetic analysis, the viruses fall into two genetically diverse clades: (i) A/chicken/Egypt/VRLCU67/2011 classified as a variant virus, clade 2.2.1.1; and (ii) A/chicken/Egypt/13VIR3729-4/2013 classified as a classic virus, clade 2.2.1.

Cross HI-test confirmed that the reaction between the two viruses is weak; furthermore, it showed the antigenic diversity between viruses belong to different clades and antigenic groups.

Antigenic relatedness was calculated between six AI antigens and their antisera representing the different clades and antigenic groups circulated in Egyptian field; including the A/chicken/Egypt/VRLCU67/2011 strain which showed very low R-values with the other viruses' groups; ranging from 17 % to zero. Results demonstrated the genetic and antigenic diversity of the variant viruses and how can the vaccine seed be a weak point in the vaccination program that could be broken by the drifted viruses antigenically distant from the vaccine strain.

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1. INTRODUCTION

Vaccines are used to prevent disease and death, reduce virus replication and shedding, and subsequently diminish environmental load and transmission of the virus. However, there is no "Sterilizing immunity" could be achieved in the field due of several factors; the most important one is the degree of antigenic relatedness of vaccine seed strain with the circulating field virus (Swayne, 2006).

Genes of influenza virus, as the genes of other RNA viruses, mutate with high frequency. The survival of influenza A viruses in nature seems to depend on the continuing evolution to new antigenic phenotypes. Epidemiologically significant inter-pandemic antigenic drift reflects successive point mutations, principally in the HA gene. Not surprisingly, the frequency of amino acid changes in the HA exceeds that of genes coding for internal proteins which are less subject to immune-pressure (Kilbourne et al., 1990).

Egypt implemented large-scale vaccination of domestic poultry to fight H5N1 highly-pathogenic avian influenza (HPAI) epidemics (Peyre et al. 2009). The blanket vaccination strategy was successful to control the first wave of outbreaks, then the immune pressure promoted antigenic drift of the virus, which resulted in the emergence of a newly differentiated cluster of HPAIV H5N1 in 2007, described as 2.2.1 variant (classified recently as a separate clade 2.2.1.1 according to the World Health Organization 'WHO' classification) (Abdelwhab et al. 2012a).

The variant strain appeared mainly in vaccinated commercial farms which showed vaccination failure, while the old classic group was present widely in backyard raised poultry at the same time period. In season 2009/2010, the classic group 2.2.1 started to spread to commercial farms (Arafa et al. 2012a).

The major surface proteins of avian influenza (AI) virus are the hemagglutinin (HA) and neuraminidase (NA) glycoproteins which are

inserted into the viral envelope as spike-like projections in a ratio (HA: NA) of about 4:1 but varying in proportion in some mutant or reassortant viruses. Moreover, they are considered the principal immunizing antigens of the virus. Neutralization of the virus is mediated mainly through the HA, therefore it is subjected to major selective pressure by antibody, leading to mutant viruses to emerge and producing new epidemics (Kilbourne et al., 1990).

Examination of antigenic relatedness between the Eurasian or American H5N2 vaccines and the variant viruses in Egypt by HI assay revealed huge distance (13% and 6% respectively) (Terregino et al., 2010). Moreover, challenge studies proved that these variant strains escaped immunity induced by those antigenically distant LPAI H5 vaccines. Distantly related vaccine strains could provide clinical protection against circulating virus but do not prevent shedding of the variant field strains (Grund et al., 2011). So, antigenic characterization should be kept in pace of the changing field virus in order to update vaccination programs implemented in Egypt.

The goal of this study was to evaluate the genetic and antigenic relatedness of two recent Egyptian H5N1 HPAI viruses by cross-HI test, putting in mind the vaccination status of poultry flocks which affect both the disease epidemiology and the biosecurity controlling program.

2. MATERIALS AND METHODS

2.1. Farms description

Farm 1*:* On May 2011, the farm (1) which consisted of three flocks (A, B and C) of Fourty-week-old chicken (Layer) in the Ismailia Governorate, Egypt suffered from respiratory distress with low mortality rate 0.13% (3/2400), 0.07% (3/4500) and 0.25% (15/6000) in the three flocks, respectively. The general health signs of the flocks were apparently healthy with observed drop in egg production, but the third flock (c) showed blood in the tracheal swaps, congestion of comp and wattles, red eyes and redness in shanks due to subcutaneous haemorrhage. The post-mortem (P.M) findings revealed severe tracheitis, cyanosis of the head and patches of congestion in the shanks particularly under the hock joint.

After 3-4 days the mortality rates increased and the owner get rid of the flocks.

The farm was located in the region called Elsalhya Elgadida. There were no neighboring farms; however, farm biosecurity was poor: The farm was not fenced, two pigeon towers were present inside the farm, there were lots of wild birds, dogs and cats, snacks and rodents inhabited the place, a cultivated field was inside the farm area, garbage was noticed everywhere. Hygienic management (cleaning, disinfection application, ventilation,…etc.) was unsatisfactory.

The three flocks received 3 injections of AI-inactivated vaccine (H5N1 Re-1 strain) Sub-cutaneously (S/C) at 10, 60 and 115 days old with 0.5, 0.7 and 0.7 cc dose; respectively.

Farm 2: Farm (2) was located in Beni-Suif governorate- Egypt, where Arber-acre broiler chickens of 28 days of age on April 2013 were kept in semi-closed housing system with automatic ventilation, on deep litter. The farm consisted of three houses (floors).

Biosecurity program was not well developed. Two days ago; the farm was subjected to accidental cut off electric current in a hot day which accompanied with bad ventilation, and led to heat stress of birds. Subsequently, mortality rate was 5-6% and the AI infection started from the upper (3^{rd}) floor, then to the 2^{nd} and the 1^{st} floors. At the end of the rearing cycle, the mortality rate increased on three successive days to 20%, 40% and reached 50% at 42 days of age.

The three flocks received one injection of AI-inactivated vaccine (H5N2 strain) Sub-cutaneously (S/C) at 8 days old with 0.5 cc dose.

On April 2013, the birds showed low food intake, high water consumption, respiratory rales and greenish diarrhea. P.M examination revealed petechiae in gizzard, signs of fever and kidney involvements.

2.2. Sample collection and preparation

Tracheal and cloacal swabs were collected. Tracheas of the dead birds were excised and kept in ice box till sent to the lab., where they were stored at -20 ºC till further examination.

2.3. Viruses

The virus isolates; A/chicken/Egypt/VRLCU67/2011 (farm 1) and A/chicken/Egypt/13VIR3729-4/2013 (farm 2), analyzed in this study were isolated from the birds of those two infected premises in Egypt. Viruses were detected by virus isolation in 9–11 day-old embryonating chicken eggs (ECEs) after incubation at 37 °C for 3–5 days ***(OIE, 2012)***. All activities involving infectious allantoic fluids were carried out under BSL3 (biocontainment) conditions.

2.4. Sequencing and phylogenetic analysis of influenza virus HA gene

RNA extraction, cDNA synthesis and PCR were carried out as previously described (Cattoli and Monne, 2009). The phylogenetic tree was constructed with 167 HA sequences of mostly Egyptian H5N1 viruses isolated from birds and were retrieved from GenBank and analyzed, and were representative sequences from viruses isolated from 2006 to 2012. Sequence and phylogenetic analysis were conducted with the MEGA4 software using neighbor-joining method.

2.5. GenBank accession numbers

The nucleotide sequences of the two isolates presented in this article have been deposited in the GenBank database under accession numbers JX024234 and KF715072. **2.6. Production of hyperimmune antisera**

The original allantoic fluid (containing H5N1 HPAI strain) was diluted 1:10 in PBS containing antibiotics and inoculated into the allantoic cavity of each SPF egg (0.1-0.2 ml/egg) and incubated at 37°C (range 35–39°C) for 7 days. A pool of HA and RRT-PCR positive allantoic fluid (HA titre 5 log2) was inactivated with 0.05% (v/v) of beta-propiolactone (BPL, Sigma®, St. Louis, Mo.) for 3 hours at +37°C water bath. Virus inactivation was confirmed by 3 blind passages in embryonated eggs (OIE, 2012). the freeze-dried antigen was resuspended with 1 ml of sterile PBS and mixed in 30/70 water-in-oil emulsion adjuvant (Montanide ISA 763 VG, Seppic®) as recommended by the manufacturer. The emulsion was used to immunise ten 6-week-old Specific Pathogen Free (SPF) White Leghorn chickens by administering 1 ml doses of the preparation intramuscularly twice, three weeks apart. Hyper-immune sera were collected by bleeding the birds two weeks after the second administration.

Table 1: List of viruses used in the cross-HI test:

Viruses	Abbreviations	Antigenic group	Genetic clade
A/Turkey/Turkey/1/2005	Tk/Tk/05		2.2
A/ chicken/Bangaladesh/11RS1984-26/2011 (H5N1 1915-26/V11)	1915-26/V11		2.2
A/chicken/Egypt/1709-1/2007	1709-1/2007	A1	2.2.1
A/ chicken/Egypt/1709-2/2008	1709-2/2008	A1	2.2.1
A/ chicken/Egypt/1709-4/2008	1709-4/2008	A1	2.2.1
A/Duck/Egypt/1709-10/2008	1709-10/2008	A1	2.2.1
A/ chicken/Nigeria/4337-343/2008	4337-343/2008	A1	2.2.1
A/ chicken/Egypt/1553-1/2010	1553-1/2010	A2	2.2.1
A/chicken/Egypt/1553-15/2010	1553-15/2010	A2	2.2.1
A/chicken/Egypt/3982-5/2010	3982-5/2010	A2	2.2.1
A/Broiler/Egypt/3982-8/2010	3982-8/2010	A2	2.2.1
A/ Broiler/Egypt/3982-9/2010	3982-9/2010	A2	2.2.1
A/Chicken/Egypt/13VIR3729-4/2013**	13VIR3729-4/2013		2.2.1
A/Egypt/2962-195/2013	2962-195/2013		
A/Egypt/2962-196/2013	2962-196/2013		
A/ chicken/Egypt/1709-5/2008	1709-5/2008	B1	2.2.1.1
A/chicken/Egypt/1709-6/2008	1709-6/2008	B1	2.2.1.1
A/chicken/Egypt/3982-19/2010	3982-19/2010	B1	2.2.1.1
A/chicken/Egypt/1553-2/2010	1553-2/2010	B2	2.2.1.1
A/ chicken/Egypt/1553-6/2010	1553-6/2010	B2	2.2.1.1
A/chicken/Egypt/1553-13/2010	1553-13/2010	B2	2.2.1.1
A/ chicken/Egypt/1553-26/2010	1553-26/2010	B2	2.2.1.1
A/ chicken/Egypt/1553-28/2010	1553-28/2010	B2	2.2.1.1
A/ chicken/Egypt/2095-75/2010	2095-75/2010	B2	2.2.1.1
A/Broiler/Egypt/3982-3/2010	3982-3/2010	B2	2.2.1.1
A/Broiler/Egypt/3982-21/2010	3982-21/2010	B2	2.2.1.1
A/chicken/Egypt/3982-50/2010	3982-50/2010	B2	2.2.1.1
A/chicken/Egypt/3982-52/2010	3982-52/2010	B2	2.2.1.1
A/ chicken/Egypt/3982-78/2010	3982-78/2010	B2	2.2.1.1
A/ chicken/Egypt/VRLCU67/2011*	VRLCU67/2011		2.2.1.1

* Inactivated antigen from the isolate of Farm (1).

** Inactivated antigen from the isolate of Farm (2).

Table 2: List of antisera used in the cross-HI test:

Antisera	Abbreviations	Antigenic group	Genetic clade
A/mallard/It/3401/05(H5N1) (Italian)	It/3401/05		
A/Turkey/Turkey/1/2005	Tk/Tk/05		2.2
A/chicken/Egypt/1709-1/2007	1709-1/2007	A1	2.2.1
A/chicken/Egypt/1553-1/2010	1553-1/2010	A2	2.2.1
A/chicken/Egypt/1709-6/2008	1709-6/2008	B1	2.2.1.1
A/chicken/Egypt/1553-2/2010	1553-2/2010	B2	2.2.1.1
A/chicken/Egypt/VRLCU67/2011*	VRLCU67/2011		2.2.1.1

* Antiserum produced from virus isolate of Farm (FA-2).

2.7. Cross-HI test

The HI test was performed as previously described (OIE, 2012), by using serial two-fold dilutions of antisera, 4 HA units of homologous and heterologous antigens, and a 1% suspension of chicken erythrocytes per test well. The isolates and the antisera used in the HI test are listed in Tables 1 and 2.

2.8. Antigenic relatedness of Egyptian H5N1 viruses

Five AI isolates besides the A/chicken/Egypt/VRLCU67/2011 isolate and their antisera were selected on the basis of phylogenetic and one-way serologic data as made by ***Beato et al. (2013)***. Finally, we determined the percentage of antigenic relatedness (R-value) with the ***Archetti and Horsfall (1950)*** equation by calculating homologous and heterologous titer ratios (R % = $\sqrt{R1xR2}$), where r1 is the ratio of heterologous titer obtained with virus 2 to homologous titer obtained with virus 1; r2 is the ratio of the heterologous titer obtained with virus 1 to homologous titer obtained with virus 2. In general, serologically related isolates have R values of more than 50%.

3. RESULTS

3.1. Genetic characterization of the two Egyptian isolates:

phylogenetic analysis of the haemagglutinin (HA) identified that virus isolates A/chicken/Egypt/VRLCU67/2011(H5N1) and A/chicken/Egypt/13VIR3729-4/2013(H5N1) fall in the clades 2.2.1.1/variant and 2.2.1/classic; respectively (Fig. 1) as recently identified by the WHO **(WHO/OIE/FAO H5N1 Evolution Working Group, 2012)**.

3.2. Antigenic characterization of Egyptian H5N1 viruses:

3.2.1. One-way serologic test of vaccine antisera to the field isolates.

To determine the cross-reactivity of A/chicken/Egypt/VRLCU67/2011 antisera to the viruses from different clades and antigenic groups, the HI test was conducted with a panel of AI antigens previously isolated and characterized by *OIE/FAO Reference Laboratory for Avian Influenza and Newcastle Disease in Italy (IZSVe, Legnaro–Padova)* (Table 3). Results were compared with an Italian H5N1 antiserum A/mallard/It/3401/05(H5N1). The results of the HI assay demonstrated that there is high cross-reactivity of VRLCU67/2011 antiserum to the viruses belonging to the variant 2.2.1.1 clade, especially the viruses of the antigenic group B2, which was the same clade of the VRLCU67/2011 strain. In contrast, the Italian It/3401/05 antiserum showed high cross-reactivity with the classic 2.2.1 clade.

3.2.3. Cross-HI and antigenic relatedness (R %)

Comparing the results of the HI assay of both VRLCU67/2011 (2.2.1.1) and 13VIR3729-4/2013 (2.2.1) antigens demonstrated that the variant one reacted with the antisera belonging to clade 2.2.1.1, while the classic strain showed higher titers with antisera belonging to the 2.2.1 clade (table 4 and fig. 2). The variant VRLCU67/2011 strain shared 17% relatedness only with the A/chicken/Egypt/1553-2/2010 isolate belonging to clade 2.2.1.1- antigenic group B2. With other clades, VRLCU67/2011 strain showed R-value ranged from 0 to 2 %.

4. DISCUSSION

This study showed the genetic and antigenic diversity of two Egyptian AI virus isolates which may be the main cause in the failure of vaccination strategy implemented in Egypt. VRLCU67/2011 virus was isolated on May 2011 from a chicken layer production commercial farm, vaccinated with “Re-1 H5N1” and located in Ismailia governorate. The virus was classified as a variant strain 2.2.1.1 by phylogenetic analysis (Fig. 1). The birds were vaccinated with AI vaccine but

this didn't protect them from infection with the variant virus, mortalities or shedding of the virus and its spread between flocks. The same was observed by Abdelwhab et al. (2011), Abdelwhab et al. (2012b), Grund et al. (2011) and Kilany et al. (2011). H5 viruses have become endemic & drift variants with resistance to H5 AI vaccines had emerged in Egypt as reported by Swayne and Kapczynski (2008-a).

13VIR3729-4/2013 virus was isolated from Upper Egypt (Beni-Suif governorate) from broiler production commercial farm. This virus was classified as a classic strain 2.2.1 by phylogenetic analysis (Fig. 1). The flock was vaccinated with H5N2 vaccine but this didn't protect the bird from infection and mortalities and shedding of the virus and this agreed with Ibrahim et al. (2013) who mentioned that H5N2 vaccine neither prevented mortality nor virus replication and shedding in vaccinated chickens challenged with one of the classic 2.2.1 strains according to recent studies by Abdelwhab et al. (2012b) and Hassan et al. (2012). Also, this result agreed with Arafa et al. (2012a) who reported that during the 2010/2011 season, the classic sublineage appeared to be dominant in commercial farms with a marked decrease in variant viruses.

The reason of the widespread of classic strains inspite of the variant ones was suggested by Ibrahim et al. (2013) to be due to decrease in the replication efficiency of variant viruses which may be caused by some mutations in the antigenic epitopes in response to selective pressures, as previously reported for the H5N9/66 virus by Philpott et al. (1989) and H3N2 viruses by Ndifon et al. (2009). Another theory suggested that it may be related to some adaptive mutations in the receptor binding domain of the classic viruses providing a competitive advantage over the variant cluster viruses. Arafa et al. (2012a) pointed that decreased surveillance activity in the commercial sector should be considered. On the other hand, Arafa et al. (2012b) added that the widespread prevalence of the low pathogenic H9N2 AI virus in Egypt decreased the incidence of HPAI H5N1 cases.

It was concluded from these results that new strains have emerged although implementing mass vaccination program which agrees with ZhaoGuo et al. (2012) who studied the effect of mass vaccination on the emergence of vaccine-resistant AI viruses and found that during mass vaccination periods, the substitution rate of HA1 gene increased in a noticeable manner in contrast to the periods when no mass vaccination programs took place in China and Indonesia. In turn, that makes long-term mass vaccination programs to control HPAI less effective and caution should be taken when designing a vaccination plan.

Results show significant degree of antigenic variation between the tested Egyptian antigens which have been isolated from different outbreaks. The HI titer of the VRLCU67 antisera recorded 10 log 2 with the homologus antigen (VRLCU67/2011). Viruses differed in their cross-HI reaction according to the genetic and antigenic groups they belong. Viruses belong to the same clade and antigenic group showed higher HI titers in cross-HI test (table 4). Recent studies (Cattoli et al., 2011 and Arafa et al., 2012a) stated that the mutations occured in the antigenic sites of haemagglutinin gene led to changes in immunogenic epitopes which may increase their ability to escape antibody response that could be revealed by HI testing and consequently compromise validation of the vaccination process. The same was observed with Escorcia et al. (2010) who stated that the rapid mutation rate of AI viruses and the fast buildup of antigenic drift need a periodic update of reference antigens and antisera for effective surveillance. They found that early isolates of Mexican LPAI H5N2 (e.g. 2002) gave higher HI titers with antisera of early isolates (e.g. 1994). On the other hand, latest isolates (i.e. 2007 and 2008) gave higher HI titers with antisera of recent LPAI isolated strains (i.e. 2008). Finally, they concluded that those results explained the high variability of HI titers produced by A/Ck/México/CPA-232/1994 (H5N2) (isolated in 1994) reference antigen when used in testing the sera of commercial flocks.

Results in table (5) show that the degree of antigenic relatedness between VRLCU67/2011- clade 2.2.1.1/variant isolate with A/chicken/Egypt/1553-1/2010- clade 2.2.1-A2/classic antigen is zero (R=Zero). A recent study reported antigenic relatedness percentages ranged from 28.9% to 68% between 2.2.1-A2 and 2.2.1.1-B2 clusters, and supported the need for vaccine seed strains from both clusters (Ibrahim et al., 2013).

Other cross-HI antigens; A/turkey/Turkey/1/2005- clade 2.2 (near to the ancestral Egyptian strain), A/chicken/Egypt/1709-1/2007- clade 2.2.1-A1/classic and A/chicken/Egypt/1709-6/2008- clade 2.2.1.1-B1/variant, showed a very weak antigenic relation between them and VRLCU67/2011 (R=1%, 1% and 2% respectively). This agree with Archetti and Horsfall (1950) who concluded that AI strains

obtained from a single epidemic did not differ strikingly but that strains recovered from epidemics occurring in different years may show major antigenic differences.

On the other hand, only A/chicken/Egypt/1553-2/2010 virus (clade 2.2.1.1-B2/variant) recorded a relatively higher antigenic relatedness (but still low) toward VRLCU67/2011 (R=17%). This isolate (A/chicken/Egypt/1553-2/2010) represents the most nearest group to VRLCU67/2011 isolate. This result agree with Grund et al. (2011) who noted that even the viruses belonged to the same genetic grouping as 2.2.1var lineage, may show different antigenic reaction and lower titers.

Low R-values (R<50%) indicate that VRLCU67/2011 is antigenically different from other Egyptian H5N1 AI viruses, which agree with Lee et al. (2004) who reported that by cross-HI and cross-Virus Neutralization (VN) tests, the antigenic relatedness of Mexican sublineage A and B viruses to the vaccine strain was less than 50%, which indicated their antigenic drift away from the vaccine strain.

In general, in vitro serologic tests assessing similarity were more discriminating than in vivo cross-protection studies as stated by lee et al. (2004) who conducted a cross-protection study and reported that the tested commercial AI vaccine could not prevent virus shedding when they challenged the chickens with antigenically different field isolates, and said that these results just confirmed the serologic data obtained by cross-HI and antigenic relatedness testing.

From these results it could be concluded that antigenic drift of HPAI H5N1 viruses in Egypt is very likely an important cause of failure to control HPAI virus and creating the endemic situation as suggested by Ibrahim et al., 2013. And so, monitoring of vaccine-induced evolution of HPAI H5N1 should be done through surveillance, antigenic characterization, and challenge studies for assessing AI vaccination efficacy, as recommended by Grund et al. (2011). Similar recommendations were advised by Marangon et al. (2008), Yassine et al. (2008) and Ibrahim et al. (2013) about the importance of continuous monitoring and evaluation of AI vaccination efficiency toward the circulating H5N1 viruses on regular bases, through genetic and antigenic testing, to ensure that vaccines are still protective against the circulating AI viruses, otherwise selection of the best vaccine strains that cross-react with the circulating viruses.

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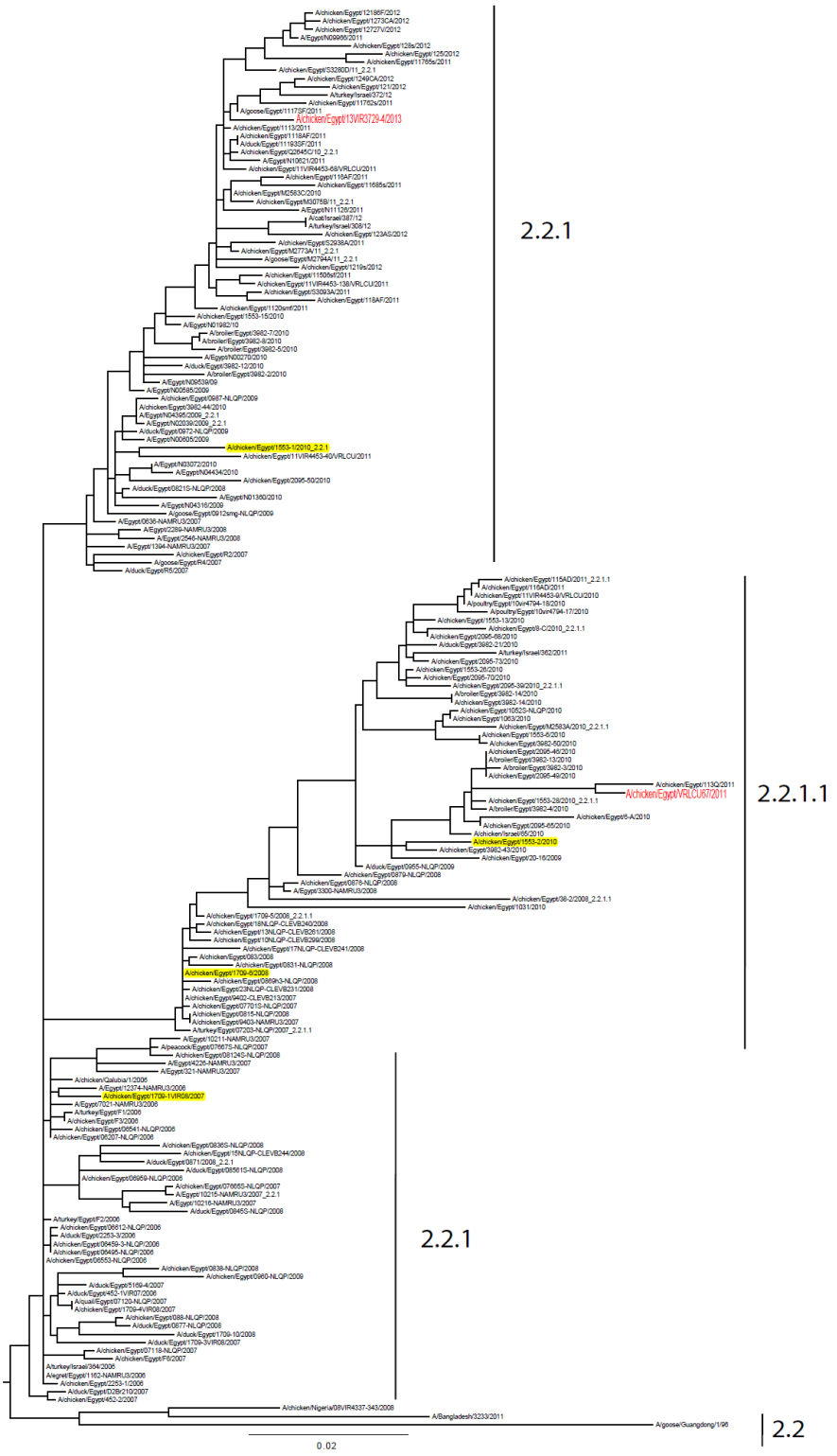

Fig. (1): Phylogenetic tree for H5 influenza A viruses HA genes: Red color —New isolates sequences: A/chicken/Egypt/VRLCU67/2011 (accession number JX024234) and A/chicken/Egypt/13VIR3729-4/2013 (accession number KF715072). The viruses studied in antigenic relatedness (R-value) experiment are highlighted by yellow.

Table 3: A panel of AI antigens used to measure HI titer of VRLCU67/2011 and It/3401/05 antisera:

Clade	Viruses (H5N1)	Serum HI titer (log 2)	
		VRLCU67/2011	Italian H5N1
2.2	A/Turkey/Turkey/1/05	3	---
	A/ chicken/Bangaladesh/11RS1984-26/2011	5	7
2.2.1	A/Chicken/Egypt/13VIR3729-4/2013**	4	---
	A/Egypt/2962-195/2013	3	---
	A/Egypt/2962-196/2013	3	---
2.2.1– A1	A/chicken/Egypt/1709-1/2007	4	6
	A/ chicken/Egypt/1709-2/2008	5	7
	A/ chicken/Egypt/1709-4/2008	5	7
	A/Duck/Egypt/1709-10/2008	4	6
	A/ chicken/Nigeria/4337-343/2008	5	7
2.2.1– A2	A/ chicken/Egypt/1553-1/2010	5	6
	A/chicken/Egypt/1553-1/2010 (Another lot)	3	4
	A/chicken/Egypt/1553-15/2010	Zero	Zero
	A/chicken/Egypt/3982-5/2010	5	5
	A/Broiler/Egypt/3982-8/2010	4	5
	A/ Broiler/Egypt/3982-9/2010	4	7
2.2.1. 1– B1	A/ chicken/Egypt/1709-5/2008	5	5
	A/chicken/Egypt/1709-6/2008	4	4
	A/chicken/Egypt/3982-19/2010	6	2
2.2.1.1– B2	A/chicken/Egypt/1553-2/2010	5	1
	A/chicken/Egypt/1553-2/2010 (Another lot)	7	1
	A/ chicken/Egypt/1553-6/2010	7	3
	A/chicken/Egypt/1553-13/2010	4	1
	A/ chicken/Egypt/1553-26/2010	7	2
	A/ chicken/Egypt/1553-28/2010	7	2
	A/ chicken/Egypt/2095-75/2010	7	2
	A/Broiler/Egypt/3982-3/2010	9	2
	A/Broiler/Egypt/3982-21/2010	7	2
	A/chicken/Egypt/3982-50/2010	8	3
	A/chicken/Egypt/3982-52/2010	7	1
	A/ chicken/Egypt/3982-78/2010	7	2
2.2.1.1	A/ chicken/Egypt/VRLCU67/2011*	10*	---

* Homologus antigen VRLCU67/2011 (Farm 1).

** Farm (2) Antigen

Table 4: Cross-HI test between sera and antigens that represent different AI clades:

Clade	Ag group	Antigen	Antisera (Log 2)					
			2.2	2.2.1– A1	2.2.1– A2	2.2.1.1– B1	2.2.1.1– B2	2.2.1.1
			Tk/Tk	1709-1	1553-1	1709-6	1553-2	VRLCU67
2.2		Tk/Tk/05	9	10	5	6	3	3
		1915-26/V11	8	10	3	4	4	4
2.2.1	A1	1709-1/2007	8	9	5	5	3	3
		1709-4/2008	9	11	4	6	5	3
		1709-10/2008	8	8	5	5	5	2
		4337-343/2008	6	9	3	5	3	3
	A2	1553-1/2010	8	8	6	3	5	4
		3982-8/2010	7	8	6	5	3	3
		3982-9/2010	7	9	4	4	4	4
	Un-identified	13VIR3729-4/2013	8	10	6	4	4	4
		2962-195/2013	7	8	5	4	3	3
		2962-196/2013	8	9	5	4	3	3
2.2.1.1	B1	1709-5/2008	7	7	2	7	4	3
		1709-6/2008	4	4	1	4	3	2
		3982-19/2010	4	4	2	2	4	5
	B2	1553-2/2010	4	3	Ø	6	7	6
		1553-13/2010	4	3	Ø	4	5	5
		2095-75/2010	4	4	Ø	6	7	7
		3982-52/2010	4	3	Ø	6	8	6
		3982-78/2010	5	4	Ø	6	6	6
		VRLCU67/2011	3	3	Ø	1	6	10

Ø: Negative result (zero log 2).

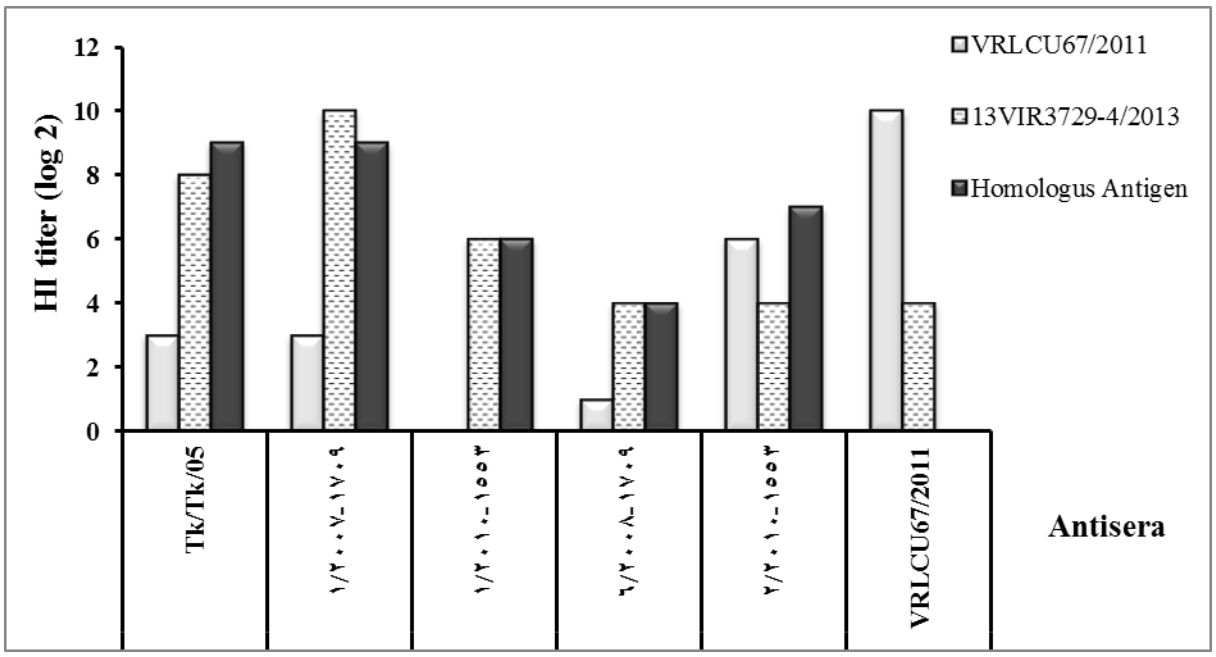

Fig. (2): HI readings of six antisera using the 2 AI isolates: VRLCU67/2011 represents the clade 2.2.1.1 (Variant), while 13VIR3729-4/2013 represents the clade 2.2.1 (classic). They were compared to the homologous antigen for each tested sera.

Table 5: Antigenic relatedness (R-value) (%):

	Antisera	1	2	3	4	5	6
Antigen		1553-1/2010	1553-2/2010	1709-1/2007	1709-6/2008	VRLCU67/2011	Tk/TK/05
1	A/chicken/Egypt/1553-1/2010	100	0	50	12	0	50
2	A/chicken/Egypt/1553-2/2010		100	3	50	17	4
3	A/chicken/Egypt/1709-1/2007			100	25	1	100
4	A/chicken/Egypt/1709-6/2008				100	2	35
5	A/chicken/Egypt/VRLCU67/2011					100	1
6	A/turkey/Turkey/1/2005						100

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