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Novel H5N1 Highly Pathogenic Avian Influenza Viruses in Egypt: Characterization, Zoonotic Potential and Vaccination Studies

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ABSTRACT

The current study investigated the prevalence and genetic diversity of highly pathogenic avian influenza (HPAI) viruses in non-vaccinated backyard chicken flocks in Qalubia governorate during the period 2012-2014. Results revealed the emergence of two novel H5N1 HPAI viruses, designated A/Chicken/Egypt/D2296E/2012 (CH/E12) and A/Chicken/Egypt/D2296H/2014 (CH/H14). Phylogenetic analysis of the hemagglutinin (HA) genes revealed that CH/E12 belongs to the predominant subclade 2.2.1.2, meanwhile CH/H14 provides an empiric evidence for the ongoing circulation of the 2.2.1.1 viruses, which were thought to have disappeared from Egypt since 2011. In addition, genetic analysis revealed that CH/E12 and CH/H14 retained the amino acid substitutions which characterize the subclade 2.2.1.2 and 2.2.1.1, respectively. However, the HA-receptor binding residues of the two viruses carried numerous substitutions, suggesting a preferable binding to human type receptors. Vaccination-challenge studies were conducted under standard laboratory conditions using three commercially available vaccines. Results revealed that the hemagglutination inhibition (HI) titers as well as the levels of protection conferred by each vaccine were positively correlated with the HA identity between the vaccine and challenge viruses. The antigenically related (90.8–96.2%) A/swan/Hungary/4999/2006 and A/chicken/Egypt/18-H/09 vaccine strains provided the highest (86.6–93.3%) protection, meanwhile the antigenically distinct (78.3–81%) A/chicken/Mexico/232/1994 H5N2 vaccine strain provided the lowest level of protection (80–83.3%) against lethal challenge. These findings highlight the significance of backyard chickens as a constant reservoir of HPAI H5N1 viruses in Egypt and raise the need for monitoring the prevalence of H5N1 viruses in backyard chicken flocks in order to understand the virus evolution and to select the proper vaccination strategy.

1. INTRODUCTION

Avian influenza virus (AIV) belongs to the genus Influenza virus A of the family Orthomyxoviridae. The virus genome consists of eight single-stranded, negative-sense RNA segments encoding ten viral proteins: nucleoprotein (NP), hemagglugtinin (HA), neuraminidase (NA), polymerase acid (PA), polymerase basic 1 (PB1), PB2, matrix 1 (M1), M2 and two non-structural (NS1 and NS2) proteins (Yoon et al., 2014). The HA glycoprotein mediates viral attachment to the cell surface and fusion of the viral envelope with the endosome, while the NA glycoprotein is responsible for detachment of the newly synthesized virions from the infected cell. AIV subtyping is based on the serological reactivity of the HA and NA glycoproteins. To date, eighteen HA (H1-H18) and eleven NA (N1-N11) subtypes have been detected throughout the world (Fouchier et al., 2005; Tong et al., 2012).

On the molecular basis, HA gene contains at least five antigenic sites (A-E) responsible for production of neutralizing antibodies (Wiley et al., 1981). In addition, HA gene contains receptor binding sites responsible for the appropriate attachment

between the virus and host cell (Steven et al., 2006). Point mutations may result in amino acid substitutions in the HA antigenic or receptor binding sites, which could potentially alter the antigenicity and/or pathogenicity and enable the virus to evade the host immune response and preexisting antibodies (Wilson and Cox, 1990).

In poultry, pathogenicity of AIV correlates directly with the deduced amino acid sequence at the HA cleavage site (Bosch et al., 1979; Klenk and Garten, 1994). Low pathogenic (LP) AIV strains possess a trypsin-like monobasic motif at the HA cleavage site and usually confined to the respiratory and gastrointestinal epithelia. Whereas, highly pathogenic (HP) strains with a polybasic HA cleavage site can infect multiple cell types and induce 100% mortality (Alexander, 2007). To date, HPAI infections have been caused by some strains belong to the H5 and H7 subtypes. Since 1997, HPAIV subtype H5N1 caused global economic losses in poultry industry and diversified into ten distinct phylogenetic clades (0–9) and tens of subclades (Smith and Donis, 2015).

In Egypt, clade 2.2.1 H5N1 viruses first emerged in 2006 and rapidly evolved into subclade 2.2.1.1 and 2.2.1.2 viruses. The 2.2.1.1 viruses have emerged in commercial poultry from 2007 to 2009 as a result of the ineffective vaccination strategies (Grund et al., 2011). In 2008, the 2.2.1 viruses evolved into the subclade 2.2.1.2, which represented the dominant cluster in commercial poultry and backyard birds since 2011 (Younan et al., 2013; El-Shesheny et al., 2014).

Backyard production system is primarily based on rearing multiple species of birds in close contact and is usually associated with minimal biosecurity measures. Thus, backyard flocks represent a constant reservoir of AIV and pose an increasing threat to commercial poultry and public health (Cristalli and Capua, 2007; ElMasry et al., 2017). Therefore, the present study aimed to assess the prevalence and genetic diversity of HPAI H5N1 viruses in backyard chicken flocks and to investigate the protective efficacy of the currently used AI vaccines against the emerging strains.

2. MATERIALS AND METHODS

2.1. Flock history and field samples

During the period between December 2012 and November 2014, tracheal and cloacal swabs were collected from five birds from each of 110 backyard chickens flocks raised in Qalubia governorate. The flocks exhibited AI-suspected signs including ruffled feathers, depression, tremors and incoordination, dyspnea and diarrhea. None of the flocks had a history of vaccination against AI. The tracheal and cloacal swabs collected form each flock were pooled separately and held at 4°C until processed.

2.2. Virus isolation and serological identification

For each pooled sample, the swab fluid was centrifuged at 1500 xg for 15 min, and a 0.2ml volume of the clarified supernatant was inoculated into five embryonated (10-day-old) specific-pathogen-free chicken eggs via the allantoic sac route. After incubation at 37°C for 72 h, eggs containing dead and surviving embryos were chilled at 4°C for 2 h and then the allantoic fluids were harvested and tested for HA activity. The HA-positive allantoic fluids were subsequently screened by hemagglutination inhibition (HI) test for antigenic specificity with H5N1 antisera (kindly supplied by Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo, Egypt) according to the Office International Des Epizooties (OIE) guidelines (OIE, 2001).

2.3. Virus titration and pathotyping

The 50% embryo infective dose (EID_{50}) of the obtained virus isolates was calculated using 10-day-old SPF emberyonated chicken eggs as previously described (Reed and Muench, 1938). In addition, virulence of these virus isolates to chicken was assessed by the intravenous pathogenicity index (IVPI) test. Briefly, the allantoic fluid of each virus isolate was diluted 1:10 with sterile saline and a volume of 0.1ml was intravenously inoculated into each of ten 6-week-old SPF chickens. The birds were housed in HEPA-filtered isolation cabinets and observed at 24-intervals for 10 days. Each bird at each observation was assigned a score of 3 if dead, 2 if severely sick, 1 if sick and 0 if normal. The IVPI was calculated as the mean score per bird per the 10 observations. Viruses with IVPI exceeding 1.2 were considered to be highly pathogenic (OIE, 2001).

2.4. Sequencing and phylogenetic analysis of the HA gene

The HI-positive samples were subjected to RNA extraction using an EZ-10 spin column total RNA mini-prep kit (Biobasic INC, Canada) following the manufacture's protocol. Subsequently, the extracted RNA samples were subjected to reverse transcriptase polymerase chain reaction (RT-PCR) assay targeting the HA gene using a one-step RT-PCR kit (Qiagen, CA) and specific H5 primers as previously described (Spackman et al., 2002). The PCR products were analyzed by agarose gel

electrophoresis and ethidium bromide staining. Amplicons of appropriate size (456 bp) were purified using a QIAquick kit (Qiagen) following the manufacture's protocol. Sequencing reaction was carried out using the ABI PRISM 3130xl genetic analyzer (Applied Biosystems, USA). The obtained HA gene sequences were deposited in the GenBank under the accession numbers MK782974 and MK782975. Sequence alignments were carried out using BioEdit software version 7.0 and phylogenetic analysis was conducted using the Phylogeny.fr tool (Dereeper et al., 2008).

2.5. Vaccination-challenge studies in SPF chickens

Two hundred and seventy, one-day-old SPF chicks (Kom Oshim farm, Fayoum, Egypt) were randomly divided into nine groups (I-IX) of 30 birds each. In addition, three commercial H5 vaccines were used: (i) live recombinant HVT-H5 (rHVT-H5) vaccine (Vectormune-AI; CEVA) containing the HPAI H5N1 strain (A/swan/Hungary/4999/2006); (ii) inactivated EGY FLU AI vaccine (Harbin Veterinary Research Institute, China) containing the H5N1 strain (A/chicken/Egypt/18-H/09); and (iii) inactivated Mexican H5N2 vaccine (Boehringer Ingelheim, Mexico) containing the LPAI H5N2 strain (A/chicken/Mexico/232/1994). At 1-day-old, groups I and II were vaccinated subcutaneously with rHVT-H5 vaccine (0.1ml /chick). At 4 weeks of age, groups III and IV were vaccinated subcutaneously with EGY FLU vaccine (0.5ml /chick), meanwhile groups V and VI were vaccinated subcutaneously with Mexican H5N2 vaccine (0.5ml /chick) according to the manufacturer's instructions. Groups VII, VIII and IX were kept unvaccinated.

Four weeks post-vaccination, sera of five randomly selected birds in each group were subjected to HI testing against the challenge virus according to standard procedures (OIE, 2001). In addition, chickens in groups I, III, V and VII were intranasally challenged with $10^{9.8}$ EID_{50} of the strain A/Chicken/Egypt/D2296E/2012 (CH/E12) in 0.1ml. Meanwhile, chickens in groups II, IV, VI and VIII received $10^{6.8}$ EID_{50} of the strain A/Chicken/Egypt/D2296H/2014 (CH/H14). The challenge dose of both isolates was previously shown to induce 100% mortality within three days post infection. Group IX was kept as a negative control. Three days post-challenge, birds within each group were monitored for mortality. The percentage protection from mortality within each vaccine group was evaluated using the following formula: (% mortality in the unvaccinated challenged group – % mortality in the corresponding vaccinated challenged group / % mortality in the unvaccinated challenged group) × 100 (Swayne et al., 1999).

2.6. Statistical analysis

One-way analysis of variance (ANOVA) model was used to determine means and standard deviations of HI titers within each group. In addition, ANOVA and post hoc t-tests were used to identify significant differences in the HI titers and levels of protection between groups. A probability (P) value of less than 0.05 indicates statistical significance.

3. RESULTS

3.1. Virus identification, titration and pathotyping

The first egg passage revealed the ability of the inoculated samples to induce death of embryos at 48 hr post-inoculation. In addition, the harvested allantoic fluids showed positive HA activity (HA titers in the range between 5 and 8 log2) and subsequently confirmed for the presence of H5N1 virus (HI titers in the range between 6 and 9 log2). The virus titers were recorded in the range between 10.8 and 13.2 log 10 EID_{50}. Moreover, on the basis of the IVPI test, the isolates were characterized as HPAI H5N1 viruses (indices in the range between 2.32 and 2.94).

3.2. Phylogenetic and molecular analysis of the HA gene

Phylogenetic analysis of the obtained HA gene sequences revealed the existence of two genetic groups (Fig. 1). The CH/E12 isolate was grouped in the subclade 2.2.1.2 H5N1 viruses and close to A/goose/Egypt/M2794A/2011 and A/duck/Egypt/Q4596A/2012 viruses with 99% and 99.6% nucleotide identities, respectively. Meanwhile, the CH/H14 isolate was grouped in the subclade 2.2.1.1 viruses and exhibited 99.3% and 99.5% nucleotide identities to A/chicken/Qalubia/CAI12/2008 and A/chicken/Qalubia/CAI20/2008, respectively. In addition, CH/E12 and CH/H14 viruses shared 96.7% nucleotide identity to each other and 97.5–98.2% identities to A/chicken/Qalubia/1/2006, which is the earliest recorded strain in the same governorate. Alignment analysis of the two isolates in comparison with the ancestral strain (A goose/Guangdong/1/96), revealed the presence of amino acid substitutions in the HA cleavage, receptor-binding, antigenic and N-linked glycosylation sites (Table 1).

Table 1**: Amino acid characteristics of the HA proteins of CH/E12 and CH/H14 viruses**

HA amino acid residues			Virus (accession number)	
			CH/E12 (MK782974)	CH/H14 (MK782975)
Cleavage site (321–330)[A]			PQG***E****K*RRKKR	PQGEGRRKKR
Antigenic sites				
	A	138	***Q***[B]	***Q***
		140	R	***G***
		141	S	***P***
		162	***K***	***K***
	B	151	***T***	I
		154	N	***D***
		155	***N***	***N***
		184	A	***E***
		189	***R***	***R***
	C	282	***I***	***I***
	D	212	***K***	***K***
		226	M	***V***
	E	263	***T***	***T***
Receptor binding sites		189	***R***	***R***
		222	Q	Q
		224	G	G
N-linked glycosylation sites		165	NNT	***H***NT
		286	NSS	NSS

[A]The H5 numbering was according to the amino acid sequence of the ancestral strain (A goose/Guangdong/1/96) without the signal peptide.
[B]Substitutions are indicated in italic and bold fonts.

Table 2: Mean log_2 HI titers and levels of protection against lethal challenge with CH/E12 and CH/H14 viruses.

Group	Vaccine strain	Challenge strain	[a]HA antigenic identity	Mean HI titers four weeks post vaccination	Protection %
I	A/swan/Hungary/4999/2006	CH/E12	96.2	6.04±0.36	90
II	A/swan/Hungary/4999/2006	CH/H14	94.5	5.84±0.37	86.6
III	A/chicken/Egypt/18-H/09	CH/E12	90.8	5.14±0.24	90
IV	A/chicken/Egypt/18-H/09	CH/H14	92.9	5.5±0.36	93.3
V	A/chicken/Mexico/232/1994	CH/E12	81	1.92±0.34	83.3
VI	A/chicken/Mexico/232/1994	CH/H14	78.3	1.18±0.24	80
VII	None	CH/E12		0.0±0.0	0.0
VIII	None	CH/H14		0.0±0.0	0.0
IX	None	None		0.0±0.0	100

[a] HA identity between the vaccine and challenge viruses.

3.3. Serological monitoring and post-challenge protection

The unvaccinated groups (VII-IX) remained serologically negative during the entire period of the experiment. Meanwhile, vaccinated groups (I-VI) had significantly different ($p<0.05$) HI titers. Based on mortality, the unvaccinated challenged groups (VII and VIII) showed 100% mortality within three days post-challenge, meanwhile the unvaccinated unchallenged group (IX) remained normal throughout the observation period. The vaccinated challenged groups (I-VI) exhibited significantly different levels of protection against mortality. In addition, the HI titers as well as the levels of protection conferred by each vaccine seemed to be positively correlated with the HA identity between the vaccine and challenge viruses (Table 2).

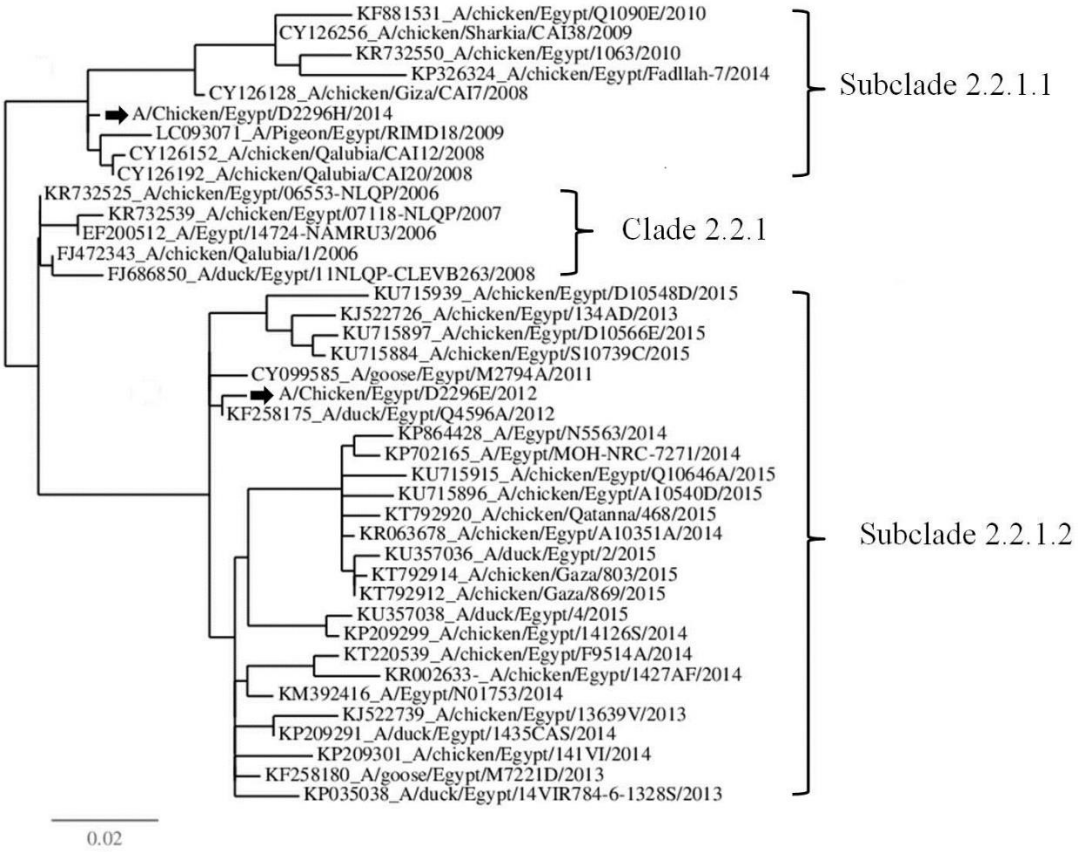

Fig. 1. Phylogenetic analysis of the HA nucleotide sequences of CH/E12, CH/H14 and other H5N1 viruses isolated from poultry and humans. CH/E12 and CH/H14 viruses are marked with black filled arrows.

4. DISCUSSION

Since 2006, HPAI H5N1 viruses have been regularly reported in Egypt despite intensive control strategies (Peyre et al., 2009). Many studies have reported a relatively higher prevalence of H5N1 viruses in backyard flocks in comparison to commercial poultry (Cristalli and Capua, 2007). The higher vulnerability of backyard chickens to AIV infection is mainly attributed to implantation of low biosecurity measures and reluctance of many backyard farmers to vaccinate their chickens. The present study aimed to investigate the characteristics of H5N1 viruses circulating in backyard chicken flocks in Qalubia governorate, which represents an important location for transition of H5N1 viruses between Egyptian governorates in Delta region (Scotch et al., 2013).

Phylogenetic analysis (Fig. 1) of CH/H14 revealed the ongoing circulation of the 2.2.1.1 viruses, which were thought to have disappeared from Egypt since 2011 (El-Shesheny et al., 2014). In a previous study (Rohaim et al., 2017), the isolate A/chicken/Egypt/Fadllah-7/2014, which shared 96.1% nucleotide identity with CH/H14, was emerged in a commercial layer flock in El-Menoufia governorate. Moreover, CH/E12 and those isolated from other avian species (A/goose/Egypt/M2794A/2011 and A/duck/Egypt/Q4596A/2012) were closely related (99-99.6% nucleotide identity). These findings strongly raise the significance of backyard poultry as a constant reservoir of HPAI H5N1 viruses in Egypt and highlight the probability of interspecies transmission.

Many studies have reported that presence of multibasic amino acid sequence at the HA cleavage site aggravates the virulence of H5N1 viruses (Senne et al., 1996). Amino acid sequence analysis revealed that CH/E12 contains the HA cleavage motif PQGEKRRKKR/G in addition to the amino acid substitutions I151T and R162K, which characterize the

2.2.1.2 viruses since 2011 (Ibrahim et al., 2013). Meanwhile CH/H14 retains the HA cleavage motif PQGERRRKKR/G, which was thought to have disappeared since 2012 (Arafa et al., 2016). In addition, CH/H14 carries the amino acid substitutions R140G, S141P, N154D and R162K, which characterizes the 2.2.1.1 viruses (Table 1). In comparison with CH/E12, prevalence of the substitution N165H in CH/H14 results in loss of glycosylation at the residue 165, which may alters the antigenicity of the isolate (Schulze, 1997).

Most of the reported cases of human infection in Egypt have arisen during handling or slaughtering infected backyard birds with H5N1 virus (Fasina et al., 2010). Mutations in the HA-receptor binding residues are responsible for switching of the binding specificity from avian type receptors (Sia2-3Gal) to human type receptors (Sia2- 6Gal). Although the two isolates retain the conserved receptor binding residues Q222 and G224 which represent preferential binding to avian type receptors, they contain the substitutions K189R, R162K and P235S, which are thought to be associated with increased binding preference to human type receptors (Maines et al., 2011; Watanabe et al., 2011). Also, prevalence of the substitution N154D in CH/H14 can alter virus transmission (Imai et al., 2012; Hu, 2013). Furthermore, prevalence of the substitutions I151T, and G272S in CH/E12 may significantly increase the affinity to human type receptors (Schmier et al., 2015).

Vaccination represents the main strategy to control AIV infection in Egypt. However, the continuous circulation of HPAI H5N1 viruses is the ongoing result of applying inefficient vaccination strategies, which may further enhance the emergence of vaccine-escape variants (Lee et al., 2004; Kim et al., 2010). Studies have shown that vaccine efficacy is positively correlated with the degree of HA antigenic identity between the vaccine strain and the currently circulating H5N1 viruses (Smith et al., 1999). The present study investigated the efficacy of three commercially used vaccines to protect chickens against the lethal challenge with CH/E12 and CH/H14 viruses (Table 2). As expected, the HI titers were positively correlated with the levels of protection conferred by each vaccine (Swayne, 2009). In addition, in is not surprising for the antigenically distinct A/chicken/Mexico/232/1994 H5N2 vaccine strain to provide the lowest HI titers and levels of protection (Kim et al., 2010). However, the unexpectedly higher efficacy of the EGY FLU vaccine, despite the relatively lower HI titers, may be attributed to the generation of antibodies against the conserved matrix proteins which have been shown to induce effective protection in mice and ferrets (Tompkins et al., 2007; Price et al., 2009).

5. CONCLUSION

The present study provides an empiric evidence for the continued circulation of HPAI H5N1 subclade 2.2.1.1 viruses in Egypt and highlights the need for monitoring the prevalence of H5N1 viruses in backyard chicken flocks in order to understand the virus evolution and to select the proper vaccination strategy.

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