Antigenic analysis of H5N1 highly pathogenic avian influenza viruses circulating in Egypt (2006–2012)

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ABSTRACT

The highly pathogenic avian influenza (HPAI) H5N1 in Egypt circulated continuously after its introduction in February 2006 with substantial economic losses and frequent human infections. Phylogenetic analysis of the available HA sequences revealed the presence of two main sublineages; the classic 2.2.1 and the variant 2.2.1.1. The classic 2.2.1 had subdivided into two clusters of viruses; cluster C1 contained the originally introduced virus and isolates from 2006 to 2009 and cluster C2 emerged in 2007 and continues to circulate. The variant 2.2.1.1 represents the isolates mainly from chickens and subdivided into two clusters; cluster V1 contains isolates from 2007 to 2009 and cluster V2 contains isolates from 2008 to 2011. Sequence analysis revealed 28 amino acid mutations in the previously reported antigenic sites and high evolution rate which may be due to selective pressure from vaccination and/or natural infection. Antigenic analysis of 18 H5N1 isolates from 2006 to 2012 that represent different clusters was conducted using hemagglutination inhibition (HI) and virus neutralization (VN) assays using hyperimmune sera produced by immunizing SPF chickens with inactivated whole-virus. Antigenic relatedness of ancestral Egyptian H5N1 isolate (459–3/06) with other isolates ranged from 30.7% to 79.1% indicating significant antigenic drift of the H5N1 viruses from the ancestral strains. The antigenic relatedness between C2 and V2 clusters ranged from 28.9% to 68% supporting the need for vaccine seed strains from both clusters. Interestingly, A/CK/EG/1709-6/2008 H5N1 strain showed a broad cross reactivity against viruses in different H5N1 clusters (antigenic relatedness ranged from 63.9% to 85.8%) demonstrating a potential candidate as a vaccine strain. Antigenic cartography which facilitates a quantitative interpretation and easy visualization of serological data was constructed based on HI results and further demonstrated the several antigenic groups among Egyptian H5N1 viruses. In conclusion, the cross reactivity between the co-circulating H5N1 strains may not be adequate for protection against each other and it is recommended to test vaccines that contain isolates from different antigenic groups in experimental infection trials for the selection of vaccine seed strain. Furthermore, the continuous monitoring for detecting the emerging variants followed by detailed antigenic analysis for updating vaccines is warranted.

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1. Introduction

The first isolation of the H5N1 highly pathogenic avian influenza (HPAI) virus was from geese in Guangdong Province, China in 1996 which gives the genetic origin of the hemagglutinin (HA) gene of the HPAI H5N1 viruses (Xu et al., 1999). The HPAI H5N1 viruses have evolved into multiple phylogenetically distinct clades, classified from 0 to 9 (WHO/OIE/FAO, 2008). Phylogenetically distinct H5N1 viruses isolated from bar-headed geese at Qinghai Lake in April 2005 in western China was designated as clade 2.2 (Chen et al., 2005), and clade 2.2 viruses rapidly spread west through central Asia and Europe, reaching Africa in 2006 (Salzberg et al., 2007).

The Egyptian poultry industry, commercial and backyard sectors, estimated to be consist of 850 and 250 million birds, respectively (Abdelwhab et al., 2010c). The poultry meat trade in Egypt depends mainly on live bird markets (LBMs) because of insufficient slaughterhouses, lack of marketing infrastructure, and cultural preference for the consumption of freshly slaughtered poultry (Abdelwhab et al., 2010c). LBMs commonly present in Egypt follow minimal food safety standards. In addition, the market infrastructure does not allow for secure disposal of the carcasses (Hosny, 2009; Abdelwhab et al., 2010c).

The first isolation of clade 2.2 (Qinghai Lake-like) virus from poultry in Egypt was in February 2006 (Aly et al., 2008; Saad et al., 2007). The virus spread rapidly throughout the country infecting different poultry species raised in Egypt causing severe economic losses of about 2–3 billion US dollars by mid-2007 with frequent human infections (Meleigy, 2007). Mass vaccination using inactivated H5N1 and H5N2 whole virus vaccines with different seed strains was implemented in 2006 with other control measures including biosecurity, movement control and monitoring (Peyre et al., 2009). The incidence of new outbreaks decreased by the end of 2006 and early 2007. Experimental challenge studies supported that the vaccinated chickens with the commercially available vaccines at that time provided partial protection against challenge with H5N1 field isolates (Kim et al., 2010; Terregino et al., 2010). Nonetheless, the virus continued to circulate in vaccinated commercial and backyard poultry due to vaccine breaks that may be related to lower vaccine quality or improper storage, handling and administration of the vaccine (Hafez et al., 2010) in addition to a shortage of infrastructure (Peyre et al., 2009). Egypt was declared to be H5N1 endemic in 2008.

The evaluation of avian influenza vaccines should be considered on a regular bases to ensure that they are still protective against the circulating virus strains (Maragoni et al., 2008). A previous report on the antigenic drift of Mexican H5N2 viruses showed vaccination failure which may be due to the extensive use of the vaccine (Lee et al., 2004a). Similarly in Egypt, the continuous circulation of the virus under immune pressure in different poultry species may have led to the emergence of genetically distinct variant strains. These variants emerged by late 2007 forming the new third-order clade 2.2.1 (Abdelwhab et al., 2010a,b; Arafa et al., 2010a,b; Balish et al., 2010; Cattoli et al., 2009; WHO/OIE/FAO, 2009). The variant viruses (clade 2.2.1) continued to evolve rapidly in Egyptian poultry forming fourth-order clade 2.2.1.1(WHO/OIE/FAO, 2012). Sequence analysis demonstrated higher mean evolutionary rates for the HA gene of the 2.2.1.1 subclade (7.37 × 10⁻³/year) than the classic group of 2.2.1 subclade (3.69 × 10⁻³/year) (Abdelwhab et al., 2012a). There are up to 31 signature amino acid changes in the HA protein between the variant 2.2.1.1 strains and the ancestral Egyptian H5N1 strain (Arafa et al., 2012a). Moreover, some of the recent variant viruses were not detected by real time RT-PCR assay recommended by the World Organization of Animal Health (OIE) (Abdelwhab et al., 2010a).

The emergence of the genetically diverse H5N1 viruses raised questions about the protection afforded by the commercial vaccines being used. Evidence showed an increased virulence of recent field isolates in poultry causing a drop in egg production and mortality up to 27% even with three vaccinations with commercial H5N1 vaccine (El-Zoghby et al., 2012). Recent studies clearly indicated that H5N2 vaccines did not elicit protection against the variant H5N1 strains in vaccinated chickens and turkey poults (Abdelwhab et al., 2011, 2012b; Grund et al., 2011; Kilany et al., 2011). Another recent study also showed that H5N2 vaccine neither prevented mortality nor virus replication and shedding in vaccinated chickens challenged with one of the classic 2.2.1 strains (Abdelwhab et al., 2012b; Hassan et al., 2012). In contrast, two vaccines containing variant H5N1 strains protected chickens against challenge with the classic 2.2.1 strains (Grund et al., 2011; Hassan et al., 2012).

Antigenic drift of the influenza virus is caused by the accumulation of point mutations in the antigenic epitopes of HA, the major surface glycoprotein targeted by host antibody. Although antigenic epitopes have been identified in other proteins like NA, M1, PA, M2e, and PB1-F2, the majority of them are in HA (Bui et al., 2007; Yen and Peiris, 2009). Epitope mapping of the HA of the H5N9 subtype using a panel of monoclonal antibodies showed that there are five distinct neutralizing epitopes in its H5 molecule (Philpott et al., 1989). In another study it was shown that a single amino acid change in the HA of H5N1 HPAI virus can affect the immune response and protection (Hoffmann et al., 2005). More recently, 21 potential antigenic sites were identified in the HA of HPAI H5N1 including the previously reported sites (Cai et al., 2012).

The antigenic analysis of the earlier H5N1 variant strains in Egypt demonstrated a considerable antigenic variation (Balish et al., 2010), and this antigenic drift is driven by multiple mutations primarily occurring in the major antigenic sites at the globular head of HA (Cattoli et al., 2011b). Other studies showed that the classic 2.2.1 strains are antigenically related and cross-reactive to the ancestral Asian H5N1 strains, but demonstrated weak cross reactivity with the variant 2.2.1.1 strains (Beatu et al., 2013; El-Shesheny et al., 2012; Watanabe et al., 2012). This highlights the importance of continuous monitoring of the circulating H5N1 viruses genetically and antigenically for...
the selection of the best vaccine strains that cross-react with the circulating viruses.

In this study, we conducted phylogenetic analysis of the available HA sequences of HPAI H5N1 strains isolated in Egypt from 2006 to 2012. We also conducted antigenic analysis using HI and VN tests using polyclonal antibodies raised against 18 H5N1 isolates that represent the co-circulating sublineages in Egyptian poultry to determine the degree of cross-reactivity among them and to better understand the antigenic evolution of field viruses that would provide valuable information and enable more efficacious control tools for HPAI H5N1 in Egypt.

2. Materials and methods

2.1. Viruses

Eighteen H5N1 strains used in this study are shown in Table 1. Viruses were isolated from 8 governorates representing different geographic regions in Egypt and from 3 different poultry species (13 chicken, 3 duck and 2 turkey isolates). Four viruses were isolated from diseased poultry showing typical signs of HPAI. Tracheal swab samples were inoculated into embryonated chicken eggs and allantoic fluids containing the propagated virus were collected as previously described (Eladl et al., 2011). Part of the allantoic fluid was inactivated using phenol (Biotechnology grade PH 4.3, GmbH, Germany) for RNA extraction and the other part inactivated by BPL and used for antigenic analysis. Another thirteen beta-probiolactone (BPL) inactivated isolates were provided by the Animal Health Research Institute (AHRI-NLQP, Giza, Egypt). All inactivated viruses were shipped to the Food Animal Health Research Program (FAHRP), The Ohio State University, Wooster Ohio (USDA Shipping permit #110710). A/Chicken/Dakahlia/106/2008 (H5N1) phenol inactivated isolate was obtained from the FAHRP depository and used for preparation of reassortant virus.

2.2. Generation of reassortant viruses

The RNA was extracted with TRIZOL LS Reagent (Life Technologies, Rockville, MD) according to manufacturer’s instructions. The HA genes of A/Chicken/Dakahlia/106/2008 (106/08), and A/Chicken/Egypt/527/2012 (527/12) isolates were amplified using a one-step RT-PCR kit (Qiagen, Valencia, CA) and two reverse genetic primer sets (primers sequences available on request), which removes multiple basic amino acids at the cleavage site as previously described (Liu et al., 2003). Reassortant viruses containing the modified HA of the H5N1 virus in a genetic background of A/WSN/33 (H1N1) were generated using reverse genetics (Lee et al., 2004b; Neumann et al., 1999). Rescued viruses were propagated in 10-day-old SPF embryonated chicken eggs (ECE) and their identity confirmed by RT-PCR and sequencing of the HA1 gene. Viruses were BPL-inactivated and used in the serologic study.

2.3. Polyclonal antibodies preparation

Inoculums were prepared by mixing the BPL-inactivated allantoic fluids of parental or reassortant viruses with Montanide ISA 70 VG oil adjuvant (Seppic, France) according to the manufacturer’s instructions. Three-week-old SPF chicks (n = 3 per isolate) were inoculated subcutaneously with 0.5 ml of inactivated whole virus mixed with oil adjuvant and booster inoculated two weeks later with the same dose. Sera were collected two weeks after the booster inoculation.

2.4. Serologic assays

Sera were heat inactivated at 56 °C for 30 min and tested by HI and VN assays. HI was carried out according to the OIE manual (OIE, 2008) with minor modifications. Briefly, 2-fold serial dilutions of sera were mixed with 8 hemagglutination units of each virus. The HI reactivity was determined using a 1% suspension of turkey red blood cells.

Table 1

<table>
<thead>
<tr>
<th>No.</th>
<th>Isolate name</th>
<th>Sublineage</th>
<th>Governorate</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A/chicken/Egypt/06459-3-NLQP/2006</td>
<td>2.2.1, C1</td>
<td>Menoufia</td>
<td>459-3/06</td>
</tr>
<tr>
<td>2</td>
<td>A/duck/Egypt/0918-NLQP/2009</td>
<td>2.2.1, C2</td>
<td>Behera</td>
<td>18/09</td>
</tr>
<tr>
<td>3</td>
<td>A/chicken/Egypt/1026/2010</td>
<td>2.2.1, C2</td>
<td>Behera</td>
<td>2d/10</td>
</tr>
<tr>
<td>4</td>
<td>A/turkey/Egypt/112634V/2011</td>
<td>2.2.1, C2</td>
<td>Menoufia</td>
<td>2D/94/11</td>
</tr>
<tr>
<td>5</td>
<td>A/duck/Egypt/111175/2011</td>
<td>2.2.1, C2</td>
<td>Gharbia</td>
<td>1175/11</td>
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<tr>
<td>6</td>
<td>A/chicken/Egypt/111AF/2011</td>
<td>2.2.1, C2</td>
<td>Fayoum</td>
<td>1A/1F/11</td>
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<td>7</td>
<td>A/chicken/Egypt/112AF/2011</td>
<td>2.2.1, C2</td>
<td>Fayoum</td>
<td>2A/1F/11</td>
</tr>
<tr>
<td>8</td>
<td>A/chicken/Egypt/502/2011</td>
<td>2.2.1, C2</td>
<td>Sharkia</td>
<td>502/11</td>
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<tr>
<td>9</td>
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<td>Dakahlia</td>
<td>503/11</td>
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<td>Dakahlia</td>
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</tr>
<tr>
<td>11</td>
<td>A/chicken/Egypt/527/2012</td>
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<td>Dakahlia</td>
<td>527/12</td>
</tr>
<tr>
<td>12</td>
<td>A/chicken/Egypt/1705-6/2008</td>
<td>2.2.1.1, V1</td>
<td>Sharkia</td>
<td>1705-6/08</td>
</tr>
<tr>
<td>13</td>
<td>A/chicken/Dakahlia/106/2008</td>
<td>2.2.1.1, V2</td>
<td>Dakahlia</td>
<td>106/08</td>
</tr>
<tr>
<td>14</td>
<td>A/chicken/Egypt/09519S-NLQP/2009</td>
<td>2.2.1.1, V2</td>
<td>Qalubia</td>
<td>5195S/09</td>
</tr>
<tr>
<td>15</td>
<td>A/chicken/Egypt/1063/2010</td>
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<td>Qalubia</td>
<td>63/10</td>
</tr>
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<td>16</td>
<td>A/turkey/Egypt/101474V/2010</td>
<td>2.2.1.1, V2</td>
<td>Giza</td>
<td>1474V/10</td>
</tr>
<tr>
<td>17</td>
<td>A/chicken/Egypt/10135ss/2010</td>
<td>2.2.1.1, V2</td>
<td>Sharkia</td>
<td>135ss/10</td>
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<tr>
<td>18</td>
<td>A/chicken/Egypt/116AD/2011</td>
<td>2.2.1.1, V2</td>
<td>Behera</td>
<td>6AD/11</td>
</tr>
</tbody>
</table>
The HI antibody titer was determined as the reciprocal of the highest serum dilution that had complete inhibition of hemagglutination. All sera were tested with the homologous and heterologous BPL-inactivated antigens used in this study. The VN was carried out as previously described (Swayne et al., 1998), using the two reassortant viruses, 106/08 and 527/12, for all sera. Two-fold serial dilutions of serum starting from a 1:16 dilution were incubated with an equal volume of virus containing 100 TCID₅₀/25 μl in a 96-well plate for 30 min at 37 °C. The virus-serum mixture was transferred to an MDCK cell monolayer and incubated at 37 °C in 5% CO₂. Plates were observed for cytopathic effect for 4 days post-inoculation to determine the virus neutralization titer. Neutralization titers were expressed as the reciprocal serum dilution giving a 50% reduction of the cytopathic effect. The antigenic relatedness among different isolates was expressed as an R-value based on the Archetti and Horsfall formula (Archetti and Horsfall, 1950), using the cross HI results.

2.5. Genetic analysis

Gel purified RT-PCR products (Gel Extraction Kit, Qiagen) of the HA1 gene for the new isolates (503/11 and 527/12) and the reassortant viruses were sequenced directly using the ABI Prism 3100 automated sequencing machine (Applied Biosystems, Foster City, CA). For phylogenetic analysis, 529 HA sequences of Egyptian H5N1 viruses isolated from birds and humans were retrieved from GenBank and analyzed. The final phylogenetic tree was reconstructed with 55 HA representative sequences from viruses isolated from 2006 to 2012 from different geographic regions. Sequence and phylogenetic analysis were conducted with the Megalign program using the Clustal V alignment algorithm and neighbor-joining method (DNASTAR, Madison, WI).

2.6. Antigenic cartography construction

3D AntigenMap was constructed according to (Barnett et al., 2012) which is based on matrix completion–multiple dimensional scaling (MC-MDS) algorithm described previously (Cai et al., 2010).

3. Results

3.1. Phylogenetic analyses

All 529 Egyptian H5N1 strains analyzed in this study were clustered in clade 2.2.1 forming a monophyletic group, indicating a single introduction of the virus which then spread widely in the country with significant evolution forming multiple clusters or sublineages (Fig. 1). The Egyptian H5N1 strains formed two main

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**Fig. 1.** Phylogenetic tree based on nucleotide sequences of the HA gene. The black squares indicate the strains used in the antigenic analysis in this study. The tree was generated using the neighbor-joining method and phylogenetic relationships were estimated through a bootstrap trial of 1000.
sublineages; the classic 2.2.1 and the variant 2.2.1.1. The classic 2.2.1 represent strains isolated mainly from different poultry species and all human isolates except one isolate (A/Egypt/3300-NAMRU3/2008), which belongs to different sublineage and was recommended as a pre-pandemic vaccine candidate (Balish et al., 2010). Poultry isolates in the classic 2.2.1 lineage were mainly isolated from non-vaccinated backyard and household poultry including chickens, ducks, geese and turkeys from all governorates. It subdivided into two clusters of strains; cluster C1 contains the ancestor strains from 2006 and disappeared in 2009 and cluster C2 emerged in 2007 and became the predominant H5N1 lineage in Egypt containing all 2011 and 2012 isolates except three chicken isolates (5AD/11, 6AD/11 and 3Q/11) which belong to the C2 cluster. The variant 2.2.1.1 lineage represents strains that escaped from the selection pressures imposed by immune response and the majority of viruses were isolated from the Nile Delta region which is intensively populated with commercial chicken farms that use AI vaccines. The 2.2.1.1 lineage formed two clusters that include strains isolated mainly from vaccinated flocks (Arafa et al., 2012a). Cluster V1 contains early variant strains that emerged in 2007 and disappeared by 2009. All viruses in this cluster were isolated from chickens except for one turkey isolate (A/turkey/Egypt/07203-NLQP/2007). Cluster V2 emerged in 2008 and continues to circulate. The majority of the viruses were isolated from chickens and three isolates were from ducks. The V2 cluster includes only one human isolate that mentioned above.

3.2. Antigenic analysis of H5N1 virus isolates

The cross HI titers are shown in Fig. 2 and the antigenic and genetic relatedness are shown in Table 2. The cross reactivity of the antisera against the 459-3/06 strain that represents the original H5N1 viruses introduced in Egypt showed lower reactivity with both C2 and V2 strains than with the homologous virus in HI assay (more than 4 fold). The antigenic relatedness of the 459-3/06 isolate with other isolates ranged from 30.7% to 79.1% indicating the significant antigenic drift of the H5N1 viruses from the ancestral strains. The C2 cluster strains were antigenically similar to each other with antigenic relatedness ranging from 85% to 100% which decreased with both the C1 cluster strain (64.5–79.1%) and the V1 cluster strain (69.4–84.4%). In contrast, C2 antisera showed a dramatic decrease in cross HI titers with V2 cluster strains, more than a 6 fold difference was observed with some isolates. Their antigenic relatedness with V2 strains ranged from 28.9% to 68% with cross HI titer differences indicating the presence of a major antigenic variation between C2 cluster strains and V2 cluster strains that co-circulate in Egypt. The 1709-6/08 strain that belongs to the variant V1 cluster showed broader reactivity against representative strains from different Egyptian H5N1 clusters tested in this study.

**Fig. 2.** Cross-reactivity of antisera produced in this study against different Egyptian H5N1 viruses determined by the HI test. (A) HI titers against classical 2.2.1 sublineage viruses. (B) HI titers against variant 2.2.1.1 sublineage viruses. Homologous titers are shown in light gray. The values indicate mean ± standard deviation from three chicken sera.

Table 2: Antigenic and genetic relatedness between H5N1 isolates used in this study.

<table>
<thead>
<tr>
<th>Sublineage</th>
<th>Variant</th>
<th>Isolate</th>
<th>Relatedness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classic 2.2.1</td>
<td>C1</td>
<td>459-3/06</td>
<td>96.9</td>
</tr>
<tr>
<td>Classic 2.2.1</td>
<td>C2</td>
<td>18/09</td>
<td>87.6</td>
</tr>
<tr>
<td>Cluster C1</td>
<td>V1</td>
<td>2d/10</td>
<td>77.8</td>
</tr>
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<td>Cluster C1</td>
<td>V2</td>
<td>2694V/11</td>
<td>82.5</td>
</tr>
<tr>
<td>Cluster C1</td>
<td>V1</td>
<td>117S/11</td>
<td>75.1</td>
</tr>
<tr>
<td>Cluster C1</td>
<td>V2</td>
<td>1AF/11</td>
<td>73.8</td>
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<td>Cluster C1</td>
<td>V1</td>
<td>2AF/11</td>
<td>72.6</td>
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<td>Cluster C1</td>
<td>V2</td>
<td>503/11</td>
<td>70.3</td>
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<td>V1</td>
<td>502/11</td>
<td>67.5</td>
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<td>V2</td>
<td>504/11</td>
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<td>V1</td>
<td>527/12</td>
<td>61.1</td>
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<td>V2</td>
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<td>60.7</td>
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<td>V2</td>
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<td>Cluster C1</td>
<td>V2</td>
<td>6AD/11</td>
<td>57.1</td>
</tr>
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</table>

a Antigenic relatedness (bold numbers) was calculated using the Archetti and Horsfall formula to calculate Rn values based on the cross HI titers.

b The percentage similarities (italic numbers) based on the HA gene sequence and were determined using the MegAlign-DNASTAR program; NA: not available.
showing antigenic relatedness ranging from 63.9% to 85.8%. The cross reactivity between V2 cluster strains was variable and antigenic relatedness ranged from 52.7% to 94.7% indicating within-cluster antigenic variation and drift.

Antigenic cartography using the cross HI data showed that Egyptian H5N1 isolates formed two distinct antigenic clusters (Fig. 3). Isolates from the classic 2.2.1 sublineage clustered with each other and were distant from the variant V2 cluster strains which represent how different Egyptian H5N1 viruses are related to each other. The 1709-6/08 strain placed in the middle between the classic and variant V2 strains supporting its partial cross reactivity with strains in different clusters. The cross reactivity pattern of 502/11 and 504/11 with different antisera was similar to the C2 cluster strains and were antigenically distant from V2 cluster strains. To confirm the cross HI results, two reasortant viruses, 106/08 which belongs to the variant V2 cluster and 527/12 which belongs to the classic C2 cluster, were used in the VN test (Table 3). The VN titers were consistent with cross HI titers with up to 5 log2 variations between the two H5N1 viruses.

### 3.3. Molecular characterization of the H5N1 strains tested

There were 47 amino acid (aa) substitutions in the HA1 protein and 8 aa substitutions in the HA2 protein, respectively, among the H5N1 strains tested (Fig. 4). Twenty eight aa mutations were present in the previously reported antigenic sites (Cai et al., 2012; Duvvuri et al., 2009). The majority of these mutations with other 19 aa mutations were located within or adjacent to the receptor binding domain (RBD) in the HA1 that may affect the virus replication and transmission. There were 6 conserved mutations in previously reported antigenic sites (D43N, S120N/D, S129del, I151T, D154N and R162K) between the

### Table 3

<table>
<thead>
<tr>
<th>Sublineage</th>
<th>Antisera</th>
<th>A(CK/EG)/527/11 C2 cluster strain</th>
<th>A(CK/EG)/106/08 V2 cluster strain</th>
</tr>
</thead>
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<tr>
<td>Classic 2.2.1</td>
<td>C1</td>
<td>459-3/06 7.0 ± 0.8*</td>
<td>9.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>18/09    10.7 ± 0.5</td>
<td>9.0 ± 0.0</td>
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<td></td>
<td>2d/10    10.7 ± 0.5</td>
<td>8.0 ± 0.8</td>
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<td></td>
<td>2694/11  10.3 ± 0.5</td>
<td>9.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1175/11  10.7 ± 0.5</td>
<td>9.0 ± 0.8</td>
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<tr>
<td></td>
<td></td>
<td>1AF/11   11.0 ± 0.7</td>
<td>9.0 ± 0.7</td>
</tr>
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<td></td>
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<td>2AF/11   11.0 ± 0.5</td>
<td>9.0 ± 0.7</td>
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<tr>
<td></td>
<td></td>
<td>503/11   10.0 ± 0.8</td>
<td>8.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>502/11   11.0 ± 0.0</td>
<td>9.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>504/11   10.5 ± 0.5</td>
<td>8.0 ± 0.0</td>
</tr>
<tr>
<td>Variant 2.2.1.1</td>
<td>V1</td>
<td>1709-6/08 8.0 ± 0.7</td>
<td>9.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>V2</td>
<td>106/08   6.0 ± 0.7</td>
<td>11.0 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>519S/09  6.0 ± 0.8</td>
<td>9.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>63/10    7.25 ± 1.1</td>
<td>9.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1474/10  6.0 ± 0.7</td>
<td>10.0 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>135S/10  6.0 ± 0.0</td>
<td>10.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6AD/11   5.0 ± 0.0</td>
<td>9.0 ± 0.8</td>
</tr>
</tbody>
</table>

* Mean VN titer (log2) ± standard deviation based on data obtained from three chicken sera.

Fig. 3. The antigenic cartography of Egyptian H5N1 HPAI viruses made by using 3D AntigenMap (http://sysbio.cvm.msstate.edu/AntigenMap3D) (Barnett et al., 2012). Cartography is based on the cross HI results. The cartography includes 18 influenza viruses, which represent different H5N1 clusters co-circulating in Egypt.
459-3/06 strain and all classic C2 cluster strains. It was shown that the D43N mutation resulted in antigenic drift between classic C1 and C2 clusters (Watanabe et al., 2012). The variant V2 cluster strains showed 18 conserved mutations while the V1 1709-6/08 strain had 10 aa mutations in relation to the originally introduced 459-3/06 strain. This indicates that the differences in the HI and VN titers may be related to these mutations.

All isolates examined in this study had three potential glycosylation sites (PGS) at 10, 23 and 286. Substitutions P74S and A156T generate additional PGS in the variant 2.2.1.1 strains. The classic 2.2.1 strains has another PGS at position 165 that is absent in the variant 2.2.1.1 sublineage strains caused by the N165H substitution. There are three additional PGSs at 193, 236 and 273 in some of the V2 cluster strains which may warrant further investigation.

The 2012 isolate (527/12) that belongs to the C2 cluster has three specific aa mutations (K152R, K218E and G334R) in the HA gene and the 6AD/11 isolate (V2 cluster) has four specific aa mutations (T167I, Q169K, T188I and V533M) in comparison to different isolates. These mutations may be related to their reduced reactivity with different antisera and indicating the continuous evolution of Egyptian H5N1 viruses of C2 and V2 clusters.

4. Discussion

In the present study, we evaluated the degree of antigenic relatedness between 18 H5N1 strains isolated between 2006 and 2012, representative of different co-circulating sublineages in Egyptian poultry using cross HI and VN tests. We observed antigenic variation between different H5N1 clusters, especially between the variant V2 and the classic C2 cluster strains showing a significant antigenic drift. Moreover, HPAI H5N1 viruses antigenically evolved from the ancestral strains forming two distinct antigenic clusters that facilitated its establishment in Egyptian poultry and complicated AI control and eradication. This is the first time 2012 and 2011 isolates from classic and variant clusters respectively are included in the antigenic analysis of Egyptian HPAI H5N1 viruses which further demonstrates the continuous genetic and antigenic evolution of these viruses.

We observed antigenic variations between the classic C2 cluster strains and the 459-3/06 strain (classic C1 cluster) as shown in Table 2. However in previous studies using monoclonal antibodies (MAB) it was shown that strains from both clusters are antigenically related (El-Shesheny et al., 2012; Watanabe et al., 2012). The majority
of the classic C2 strains in our study are recent isolates (2011 and 2012 isolates) that may have evolved further than strains used in previous studies. The reactivity of the 459-3/06 strain with the V1 strain (1709-6/08) was higher than with the V2 cluster strains (Fig. 2). We found that these reactivity patterns follow the mutations in the major antigenic sites where the 459-3/06 strain shared with the 1709-6/08 strain some residues in antigenic sites A (1335), B (154D and 156A), D (190L and 192Q) and E (71L). Interestingly, the V1 strain (1709-6/08) showed a broader reactivity to all the strains that represent different H5N1 clusters circulating in Egypt, as it shared residues with all the strains in the major antigenic sites (Fig. 4). This is consistent with the previous observation that the antigenic sites of the V2 cluster strains have had good cross-reactivity against the classic and the variant strains (Beato et al., 2013). However, a previous study also revealed antigenic variation between the V1 strain (1709-6/08) and another C1 strain (1709-1/07) (Cattoli et al., 2011b). Our results further validate the previous observation that aa residues in the HA of H5N1 viruses in areas corresponding to the antigenic epitopes represent the major determinant of their antigenicity (Kaverin et al., 2007).

The variant V2 cluster strains have 20, 28 and 17 aa substitutions in previously reported antigenic sites in relation to the 459-3/06 strain, C2 cluster strains and the 1709-6/08 strain, respectively (Fig. 4). These substitutions support the significant antigenic difference of the variant V2 viruses from strains of other clusters. Moreover, we observed within-cluster antigenic drift between the V2 cluster strains with up to a 4 fold difference in HI titers between some isolates (Fig. 2). There are 27 aa substitutions in the HA1 among the V2 cluster strains under study, fifteen of them in previously reported antigenic sites. Previous study revealed that the 2.2.1.1 variant V2 cluster has higher mean evolution rate than other H5N1 clusters that estimated to be 9.76 × 10⁻³ for HA1 (Abdelwhab et al., 2012a).

The antigenic sites against the 63/10 strain showed increased cross reactivity to all H5N1 isolates tested. This is the only variant strain possessing 141S which was previously shown to increase the reactivity of A/CK/EG/1709-6/08 to antigenic raised against Mexican H5N2 (Cattoli et al., 2011b). Moreover, the 63/10 strain has a mutation in antigenic epitope E (183T) and epitope C (128V) and mutations in these epitopes are predicted to increase the neutralization titers of influenza A/H3N2 due to steric interference between antibodies (Ndifon et al., 2009).

Phylogenetic analysis revealed the genetic diversification of H5N1 viruses with the presence of multiple clusters (Abdel-Moneim et al., 2009; Abdelwhab et al., 2010b,a; Arafa et al., 2012a, 2010a; Cattoli et al., 2011a; Eladl et al., 2011). Two clusters of them are still co-circulating in Egypt. Sequence analysis revealed the presence of conserved mutations located mainly within or near the major antigenic sites in the HA gene of the variant 2.2.1.1 sublineage strains (Balish et al., 2010; Watanabe et al., 2012). These mutations may have occurred as a result of selective pressure to escape the immune response from multiple vaccinations with inactivated H5N1 and H5N2 AI vaccines (Abdelwhab et al., 2012a; Grund et al., 2011) as previously reported in Mexico (Lee et al., 2004a). The inefficiency of the commercially available H5N2 and H5N1 vaccines have been reported confirming the antigenic evolution of the Egyptian H5N1 viruses (Abdelwhab et al., 2011; Hassan et al., 2012; Kilany et al., 2011). This highlights the need for updating the currently available AI vaccines in Egypt.

The newly isolated 527/12 strain has K152R and K218E mutations that were located in epitope A and RBS of the H5N1 HA, respectively (Duvvuri et al., 2009). The evolution of the variant 2.2.1.1 strains also led to addition of new N-linked PGS in the HA as shown in Fig. 4. Influenza A viruses have been shown to use this mechanism to mask the antigenic epitopes (Kaverin et al., 2007) or to affect the receptor binding preferences (Wang et al., 2009). The P74S substitution creates a new PGS at residue 72, which was shown to decrease the reactivity of the variant 1709-6/08 strain when tested against the H5N2/Mexican strain antisera (Cattoli et al., 2011b). Another PGS at 154 was introduced by the A156T mutation in all V2 cluster strains which have been shown to decrease the immunogenicity of the A/Vietnam/1203/2004 H5N1 virus (Wang et al., 2010). The loss of PGS at 154 in the classic 2.2.1 strains contributed to increased affinity to human type receptors (Gao et al., 2009). Moreover, the glycosylation at position 165 in the classic 2.2.1 strains was shown to interfere with the binding of SA-α-2,3-Gal analogs (Chen et al., 2012).

The prevalence of the variant V2 cluster decreased in 2010 and only three isolates were identified in 2011 while the classic C2 strains were widespread in all poultry sectors. This may be caused by some mutations in the antigenic epitopes due to selective pressures related to a decrease in the replication efficiency of variant v2 cluster viruses, as previously reported for the H5N9/66 virus (Philpott et al., 1989) and H3N2 viruses (Ndifon et al., 2009). Also, 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. In addition, decreased surveillance activity in the commercial sector should be considered (Arafa et al., 2012a). The widespread prevalence of the low pathogenic H9N2 AI virus in Egypt was shown to decrease the incidence of HPAI H5N1 cases (Arafa et al., 2012b). The variant group may be still circulating in Egyptian poultry at a low level and the high rate of evolution makes its detection unsuccessful, as previously reported (Abdelwhab et al., 2010a). Moreover, the R325G aa substitution in the recent variant V2 strains was shown to decrease the pathogenicity in chickens and this may lead to unnoticed circulation of these viruses (Yoon et al., 2013). However, consistent isolation of the H5N1 viruses belonging to the classic C2 cluster indicates that this cluster is the predominant one in Egypt.

In conclusion, the HPAI H5N1 virus in Egypt continues to circulate and new genetic and antigenic clusters have emerged. The antigenic drift of HPAI H5N1 viruses in Egypt is very likely an important cause of failure to control HPAI and creating the endemic situation. Our data suggest that strains from the C2 and V2 clusters should be included in AI vaccines and seed strains should be updated regularly to
match the circulating strains. We observed broad cross-reactivity of the 1709-6/08 strain against viruses in different H5N1 clusters and testing the potential of 1709-6/08 strain as a vaccine seed strain is warranted. Continuous surveillance of the circulating HPAI H5N1 viruses in Egypt and their antigenic characterization for the selection of the most efficient seed strains is a must and this should be paralleled by well-organized efforts for efficient HPAI control and eradication.

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References


