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Antigenic Drift in H5N1 Avian Influenza Virus in Poultry Is Driven by Mutations in Major Antigenic Sites of the Hemagglutinin Molecule Analogous to Those for Human Influenza Virus▽†

Giovanni Cattoli,[1]* Adelaide Milani,[1] Nigel Temperton,[2] Bianca Zecchin,[1] Alessandra Buratin,[1] Eleonora Molesti,[2] Mona Meherez Aly,[3] Abdel Arafa,[3] and Ilaria Capua[1]

OIE/FAO Reference Laboratory for Avian Influenza and Newcastle Disease, Istituto Zooprofilattico Sperimentale delle Venezie, 35020 Legnaro (PD), Italy[1]; School of Pharmacy, University of Kent at Medway, Chatham Maritime, Kent ME4 4AG, United Kingdom[2]; and National Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, P.O. Box 264, Dokki, Giza 12618, Egypt[3]

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H5N1 highly pathogenic avian influenza virus has been endemic in poultry in Egypt since 2008, notwithstanding the implementation of mass vaccination and culling of infected birds. Extensive circulation of the virus has resulted in a progressive genetic evolution and an antigenic drift. In poultry, the occurrence of antigenic drift in avian influenza viruses is less well documented and the mechanisms remain to be clarified. To test the hypothesis that H5N1 antigenic drift is driven by mechanisms similar to type A influenza viruses in humans, we generated reassortant viruses, by reverse genetics, that harbored molecular changes identified in genetically divergent viruses circulating in the vaccinated population. Parental and reassortant phenotype viruses were antigenically analyzed by hemagglutination inhibition (HI) test and microneutralization (MN) assay. The results of the study indicate that the antigenic drift of H5N1 in poultry is driven by multiple mutations primarily occurring in major antigenic sites at the receptor binding subdomain, similarly to what has been described for human influenza H1 and H3 subtype viruses.

The highly pathogenic avian influenza (HPAI) viruses belonging to the H5N1 subtype emerged for the first time in Southeast China in 1996. After its reemergence in China in 2003, the virus rapidly spread to other Asian countries first and then to Europe and Africa. Its continuous circulation in the animal reservoir has led to a significant genetic diversification represented by multiple phylogenetic lineages, classified as clades 0 to 9 (4, 37).

Egypt officially reported its first outbreak in poultry in February 2006 (http://www.oie.int/eng/info_ev/en_AI_factoids_H5N1_Timeline.htm). Since then, clade 2.2 viruses have spread among domesticated birds throughout the country and HPAI H5N1 virus was declared endemic in Egypt in July 2008 (http://www.oie.int/animal-health-in-the-world/web-portal-on-avian-influenza/about-ai/h5n1-timeline/). The epidemic resulted in disruption of the national poultry industry and in severe economic losses. In addition, the continuous circulation of this virus in the environment has increased the risk of human exposure. Indeed, bird-to-human transmissions with severe public health consequences have been occurring in Egypt since 2006, causing 125 human cases and 41 fatalities to date (28 February 2011; http://www.who.int/csr/disease/avian_influenza/country/cases_table_2011_02_28/en/index.html).

With the aim of reducing the risk for humans and the economic losses to industry, major interventions were taken by the veterinary services to control the epidemic in poultry. In the first period of the epidemic, these interventions were based on the culling of infected birds and movement restrictions (2, 28). Subsequently, mass vaccination in poultry was introduced officially both in the industrial sector and in backyard flocks in March 2006 and May 2007, respectively. As a consequence of the many practical difficulties in vaccinating poultry in the field, particularly household flocks, the vaccine coverage is very low in Egypt with an average of 25 to 30% in some governatorates, dropping to as low as 1% in some villages. Even in commercial flocks, vaccine coverage of only 50 to 60% is a realistic estimate (28).

Despite all the control measures taken, the virus continues to circulate in poultry in both vaccinated commercial and backyard flocks (10). As a result of this persistent circulation, the HPAI H5N1 viruses have evolved and progressively diverged to the extent that they are currently reclassified as a third-order genetic clade, namely, clade 2.2.1 (3, 37).

By late 2007 and early 2008, clade 2.2.1 viruses emerged in Egypt (1, 2, 19) and showed genetically and antigenically distinguishable features. These emerging viruses have not replaced the previous circulating strains but are instead cocirculating with them in poultry. The hemagglutinin (HA) sequence of these viruses is grouped in a separate branch within clade 2.2.1 of the phylogenetic tree (see Fig. S1 in the supplemental material). Accumulation of amino acid (AA) mutations in the major antigen, the HA protein, has raised concerns about the efficacy of "in-field" vaccination of poultry. In addition, in the light of this evolving situation the World Health Organization (WHO) has recently revised the pro-

* Corresponding author. Mailing address: Istituto Zooprofilattico Sperimentale delle Venezie, viale dell'Università 10, 35020 Legnaro (PD), Italy. Phone: 39 0498084384. Fax: 39 0498084360. E-mail: gcattoli@izsvenezie.it.

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TABLE 1. Field and mutant viruses analyzed in this study[a]

Strain no.	Strain description	Titer for serum:									MGT	Titer for cross-HI with:	
		1	2	3	4	5	6	7	8	9		1709-6/08	1709-1/07
1	A/Ck/Eg1709-6/08 stock L.39/08	4	0	4	8	64	2	32	4	4	4	1,024	256
2	A/Ck/Eg1709-1/07 stock L.41/08	256	64	128	256	512	128	512	128	512	362	512	1,024
3	RG PR8+Eg6 HA+NA	4	0	4	8	64	4	32	4	4	4	1,024	256
4	RG PR8+Eg1 HA+NA	128	64	128	512	512	256	512	128	512	256	1,024	2,048
5	RG PR8+Eg6 HA+NA S74P	32	16	64	64	256	32	256	64	64	45		
6	RG PR8+Eg1 HA+NA P74S	128	64	128	128	512	256	512	256	512	256		
7	RG PR8+Eg6 HA+NA G140R	4	4	4	16	128	64	256	16	256	32		
8	RG PR8+Eg1 HA+NA R140G	512	128	128	512	512	128	1,024	128	256	362		
9	RG PR8+Eg6 HA+NA S74P G140R	8	8	8	16	256	64	128	32	128	32		
10	RG PR8+Eg1 HA+NA P74S R140G	256	256	256	128	256	128	512	2,048	256	256		
11	RG PR8+Eg6 HA+NA S74P P141S	32	8	8	16	256	16	128	16	64	45		
12	RG PR8+Eg1 HA+NA P74S S141P	32	32	32	128	256	128	512	128	256	91		
13	RG PR8+Eg6 HA+NA S74P G140R P141S	32	16	32	64	1,024	64	256	64	256	91		
14	RG PR8+Eg1 HA+NA P74S R140G S141P	64	64	64	128	128	64	512	64	64	64		
15	RG PR8+Eg6 HA+NA G140R P141S Y144F	32	16	16	32	512	64	256	64	256	91		
16	RG PR8+Eg1 HA+NA R140G S141P F144Y	64	32	32	64	64	32	256	64	64	64		
17	RG PR8+Eg6 HA+NA S74P G140R P141S Y144F	128	32	128	128	1,024	256	1,024	256	1,024	362		
18	RG PR8+Eg1 HA+NA P74S R140G S141P F144Y	64	64	64	128	128	64	256	64	64	64		
19	RG PR8+Eg6 HA+NA S74P G140R P141S Y144F K162R	64	32	64	128	512	256	512	64	512	181		
20	RG PR8+Eg1 HA+NA P74S R140G S141P F144Y R162K	8	8	16	16	32	8	64	4	8	8		
21	RG PR8+Eg6 HA+NA S74P G140R P141S K162R	32	16	64	64	512	128	256	64	512	128		
22	RG PR8+Eg1 HA+NA P74S R140G S141P R162K	16	16	64	32	256	32	16	16	16	16		
23	RG PR8+Eg6 HA+NA S74P R110H G140R P141S Y144F	64	32	128	128	1,024	256	512	128	1,024	256		
24	RG PR8+Eg1 HA+NA P74S H110R R140G S141P F144Y	64	64	64	128	128	32	256	32	32	45		
25	RG PR8+Eg6 HA+NA S74P S120N G140R P141S Y144F	128	64	256	256	2,048	512	512	128	1,024	362		
26	RG PR8+Eg1 HA+NA P74S N120S R140G S141P F144Y	64	64	64	128	128	64	256	64	64	64		
27	RG PR8+Eg1 HA+NA P74S N120S R140G S141P F144Y R162K A184E	4	4	16	32	128	8	64	8	16	8		
28	RG PR8+Eg1 HA+NA P74S R140G S141P F144Y R162K A184E	16	8	64	64	1,024	32	128	16	16	16		
29	RG PR8+Eg1 HA+NA P74S R140G S141P F144Y A184E	32	16	32	64	128	32	256	32	16	23		
30	RG PR8+Eg1 HA+NA R140G S141P F144Y R162K	32	32	64	32	128	32	128	16	64	45		
31	RG PR8+Eg1 HA+NA N120S R140G S141P F144Y R162K A184E	32	16	32	64	256	32	128	32	64	45		
32	RG PR8+Eg1 HA+NA R140G S141P F144Y A184E	64	64	64	128	128	64	512	64	64	64		
33	RG PR8+Eg1 HA+NA N120S R140G S141P F144Y	128	64	64	128	128	64	256	64	64	91		
34	RG PR8+Eg1 HA+NA N120S R140G S141P F144Y A184E	128	32	64	128	128	64	256	64	64	91		
35	A/Ck/Mex/232/94[b]	2,048	4,096	4,096	4,096	4,096	1,024	2,048	2,048	4,096	3,010		

[a] The stock and mutant viruses created (1 to 34) were tested by HI assay against avian sera of chickens vaccinated with the Mexican-derived H5N2 strain (sera 1 to 9). The HI titers of sera 1 to 9 were used to calculate the mean geometric titers (MGTs). Strains 1 to 4 were tested by cross-HI against avian sera homologous to the field strains (sera 1709-1/07 and 1709-6/08).

[b] Homologous vaccine strain A/chicken/Mexico/232/94.

posed 2.2.1 strains as candidates for the development of human vaccines (39).

In order to control the infection in poultry, different types of inactivated avian influenza (AI) virus vaccines have been imported and applied in Egypt. Vaccine strains are represented by "homologous" H5N1 subtypes (e.g., mostly H5N1 Re-1 Chinese vaccines) and are predominantly used in household poultry. Mexican-derived A/H5N2 vaccines are most commonly applied in commercial poultry farms (29). Before being released on the market, all imported vaccines are evaluated according to international standards for vaccine quality assurance. For potency testing, vaccines are challenged against Egyptian H5N1 isolates (29). At the time of writing, the strain used for official challenge testing in Egypt is an early 2008 isolate belonging to a genetically distinguishable group of viruses that emerged in the country (in this work, this challenge strain is named 1709-6/2008).

Compared to the previously circulating strains (2006 to 2007), a lower level of antigenic cross-reactivity was revealed by HI testing between the selected 2008 challenge strain and the Mexican-derived H5N2 vaccine strain, indicating a significant vaccine drift (35). Interestingly, this lower cross-reactivity did not adequately predict the clinical protection induced by the vaccine in challenged chickens. In fact, 80% protection was

TABLE 2. Microneutralization test results of chicken sera containing antibodies raised against the Mexican-derived H5N2 strain (sera 1 to 9), A/Ck/Eg/1709-1/2007 (Ck Eg1) strain, A/Ck/Eg/1709-6/2008 (Ck Eg6) strain, and A/Avian/It/2000 H7N1 (Av H7N1) strain

Test antigen	Titer for serum:												
	1	2	3	4	5	6	7	8	9	Ck Eg1	Ck Eg6	Av H7N1	Negative
A/Ck/Eg/1709-1/2007	1,280	640	640	1,280	5,120	2,560	5,120	1,280	5,120	10,240	5,120	<80	<80
A/Ck/Eg/1709-6/2008	<80	<80	<80	<80	160	<80	160	<80	<80	2,560	10,240	<80	<80
RG PR8+Eg1 HA+NA	1,280	640	640	1,280	10,240	2,560	10,240	1,280	10,240	20,480	10,240	<80	<80
RG PR8+Eg6 HA+NA	<80	<80	<80	<80	320	<80	160	<80	<80	5,120	20,480	<80	<80
A/Avian/It/2000 H7N1	<80	<80	<80	<80	<80	<80	<80	<80	<80	<80	<80	2,560	<80

achieved after challenge with 1709-6/2008, and viral shedding was significantly reduced (35). Similar results were obtained in chickens immunized with the same vaccine and challenged with a distinct 2008 Egyptian strain (19).

There is scientific evidence indicating that evolution and antigenic drift of human influenza (H1 and H3 influenza A) viruses are driven by multiple mutations within major antigenic sites of HA located in the receptor binding subdomain (RBD) (11, 32). In poultry, the occurrence of antigenic drift in avian influenza viruses is less well documented and the mechanisms remain to be clarified, particularly with reference to the use of vaccines as control measures for HPAI epidemics. Antigenic drift was demonstrated in Mexico for H5N2 viruses causing a low-pathogenic AI epidemic; however, its association with vaccination in poultry was not clearly established (22). Although antigenic diversity has been described for H5N1 HPAI viruses in Asia and in Egypt (2, 40), its driving mechanisms and the impact on control measures have not yet been established.

To test the hypothesis that HPAI H5N1 virus antigenic drift is driven by mechanisms similar to type A influenza viruses in humans, the molecular changes in genetically divergent viruses circulating among vaccinated poultry in Egypt have been mapped and antigenically analyzed in this study. In addition, this study aims to clarify the correlation of hemagglutination inhibition (HI) and microneutralization (MN) test results with vaccine efficacy prediction in poultry. This type of data will provide information for a better selection of vaccine strains and vaccine strategies.

MATERIALS AND METHODS

Virus strains used in this study and generation of recombinant viruses. Viral strains included in this study, namely, 1709-1/2007 (GenBank accession number EU717849) and 1709-6/2008 (GenBank accession number EU717857), were isolated by the Animal Health Research Institute in Egypt (AHRI-NLQP, Giza, Egypt). Viruses were obtained from samples collected in commercial poultry flocks on 25 February 2007 and 3 January 2008, respectively. Strain 1709-6/2008 is the challenge virus applied in Egypt at the time of writing and also used in a previous challenge study (35). Based on its position in the phylogenetic tree (see Fig. S1 in the supplemental material), this strain could be considered one of the earliest genetically distinct viruses that emerged in Egypt by late 2007 to early 2008. Compared to the Mexican-derived H5N2 vaccine strain, the analyzed AA sequence of the HA protein of 1709-1/2007 was considered representative of the H5N1 viruses circulating in Egypt before the emergence of the distinct group of viruses in late 2007. In fact, the AA differences between 1709-1/2007 and the vaccine strain were common to the vast majority of the 157 H5N1 Egyptian HA sequences (2006 to 2007) downloaded from the public database (GenBank) and analyzed for this study (data not shown).

Reassortant viruses containing the HA and NA of the H5N1 strains in a genetic background of A/PR/8/34 (H1N1) were generated by reverse genetics (RG) as described previously (13). Mutations of interest in the HA gene were introduced by PCR using the QuikChange Site-Directed Mutagenesis kit (Stratagene). The whole-genome sequence of the parental viruses and the full-length HA gene sequences of the RG reassortants were confirmed by sequence analysis of the cDNA amplified from viral RNA by reverse transcription-PCR (RT-PCR).

Serological tests. HI and MN assays were applied to evaluate the immune response to the vaccine. Sera containing antibodies to the H5N2 vaccine strain (A/chicken/Mexico/232/94) were obtained from specific-pathogen-free (SPF) chickens vaccinated at 21 days of age and boosted after 3 weeks with a commercially available vaccine, which had been used in previous studies (19, 35). The HI assay was performed according to the international standard procedure (27) for testing avian sera using parental and reassortant viruses as antigens (4 hemagglutination units [HAU]).

The serum neutralizing activity was assessed by a standard MN assay performed as previously described (31). Avian sera were treated with a receptor-destroying enzyme (RDE) before use and heat inactivated at 56°C for 30 min. Sera raised against homologous antigens (i.e., 1709-1/2007 and 1709-6/2008), one unrelated avian influenza virus antigen (H7N1/HPAI), and one negative chicken serum were included in the assay as controls.

RESULTS

Serology on field (parental) strains. Table 1 and Table 2 report serological results obtained using the HI and MN assays performed on field strains 1709-1/2007 and 1709-6/2008. A significant reduction, in some sera as great as 6 to 7 $\log_2$, evident in the HI titer for field strain 1709-6/2008, indicates major antigenic differences between the H5N2 vaccine strain and the 2008 Egyptian strain. This is in agreement with previous findings using the same or genetically related 2007 and 2008 Egyptian strains demonstrating within-clade antigenic variations and drift among circulating H5N1 viruses (2, 7, 19, 30).

The results obtained with the MN assay revealed a 3- to 6-fold reduction of the neutralizing titer in the sera obtained from vaccinated birds for field strain 1709-6/2008 compared to 1709-1/2007, consistent with the HI test results.

Genetic characterization of field (parental) viruses. The AA sequence of the HA protein of the field strains showed that 12 AA substitutions occurred in the 2008 strain, 11 of which were located in the mature HA1 subunit (Table 3). In 1709-6/2008, all but one of the 11 AA substitutions were located in the receptor binding subdomain (RBD) (9) in positions proximal to the functional receptor binding site represented by the 190 α-helix, the 130 loop, and 220 loop and a number of conserved residues, such as Tyr98, Trp153, and His183 (6, 9, 26, 33, 42) (Fig. 1 and Fig. 2). Out of the 11 AA substitutions detected, 4 (positions 140, 141, 162, and 184) were mapped to the corresponding H3 antigenic sites A and B (Fig. 3) (14, 16, 23). Interestingly, strain 1709-6/2008 exhibited AA mutations almost exclusively at positions where 1709-1/2007 and the H5N2 vaccine strain possessed the same AA (Table 3). Substitution P74S, positioned outside the RBD, generates a potential additional glycosylation site in 1709-6/2008, while substitution

TABLE 3. Amino acid differences in the HA1 protein between the vaccine strain A/ck/Mex/232/94 and the strains A/ck/Egypt/1709-1/07 and A/ck/Egypt/1709-6/08[a]

Amino acid position	Residue in strain:		
	A/ck/Mex/232/94	A/ck/Egypt/1709-1/07	A/ck/Egypt/1709-6/08
14	K	E	E
36	E	T	T
40	R	K	K
43	S	D	D
45	K	D	D
53	K	R	R
74	**P**	**P**	**S**
83	D	I	I
88	G	D	D
97	D	N	N
105	M	L	L
107	S	R	R
108	T	I	I
110	**H**	**H**	**R**
117	F	I	I
119	R	K	K
120	**S**	**N**	**S**
123	**S**	**S**	**P**
124	N	D	D
126	D	E	E
138	N	Q	Q
140	**R**	**R**	**G**
141	**S**	**S**	**P**
144	**F**	**F**	**Y**
154	N	D	D
156	V	A	A
158	Q	P	P
162	**R**	**R**	**K**
163	T	S	S
165	**N**	**N**	**H**
169	I	I	Q
174	I	V	V
184	**A**	**A**	**E**
188	I	T	T
189	K	R	R
195	N	T	T
198	V	I	I
209	S	L	L
210	I	V	V
212	E	K	K
217	P	S	S
226	**M**	**M**	**V**
234	R	K	K
235	P	S	S
238	S	A	A
240	T	N	N
244	T	N	N
252	Y	N	N
257	I	V	V
263	A	T	T
270	N	E	E
275	D	N	N
276	A	T	T
282	V	I	I
294	V	I	I
297	F	L	L
309	K	N	N
310	K	R	R
320	V	S	S
323	R	G	G
325	T	R	R

[a] In bold are shown the 11 amino acid differences between the strains A/ck/Egypt/1709-1/07 and A/ck/Egypt/1709-6/08. The amino acid numbering used for H3 in references 26 and 38 was adopted.

N165H in the same virus deletes a site for potential glycosylation (Fig. 1).

Generation and antigenic characterization of recombinant viruses. RG reassortants included in this study are listed in Table 1. These include the RG reassortants containing the unmodified HA and NA of the 1709-1/2007 and 1709-6/2008 field strains (Table 1; mutants 4 and 3, respectively). In the test system adopted in this study, they reacted very similarly to the respective parental field strains, as demonstrated by the single HI titers and by the mean geometric titer (MGT) in Table 1 as well as by the results obtained using these RG reassortants as antigens in the MN assay (Table 2).

To test their influence on immunogenicity and drift, the AA substitutions located in proximity to the receptor binding site and in major antigenic sites (mapped by genetic analysis or predicted by computational analysis using the Kolaskar-Tongaonkar method [5, 21; data not shown]) were selected for the generation of RG reassortant mutants and subsequent testing (Table 1). Substitution P74S, generating one potential additional glycosylation site in 1709-6/2008, did not produce significant variation when introduced in the HA protein of 1709-1/2007 (mutant 6, Table 1). However, its deletion in the 1709-6/2008 HA altered the antigenicity as highlighted by the moderate to relevant increase in the HI titer (mutant 5, Table 1).

Introduction of substitutions at positions 140 and 141, singly or in combination with P74S, produced an effect on antigenicity (mutants 7 to 14, Table 1). The effects became progressively more evident when two other AA substitutions were added, particularly at positions 144 and 162 (mutants 15 to 22, Table 1). The antigenicity of mutant 4 and the respective field strain 1709-1/2007 was restored in mutant 17, harboring mutations at positions 74, 140, 141, and 144 in the HA genetic backbone of mutant 3 (Table 1). Mutants 20 and 22, with AA substitutions at positions 74, 140, 141, 144, and 162 in the HA genetic backbone of mutant 4, showed HI titers and MGTs similar to mutant 3 and the respective field strain 1709-6/2008, indicating that this minimum constellation of multiple substitutions produced major alterations in HA antigenicity and was responsible for the drift. Introduction of substitution A184E (e.g., mutant 29; Table 1), located in close proximity to the 190 α-helix in 1709-6/2008, in the HA genetic backbone of mutant 4 also altered the antigenicity, although variation was less pronounced than in mutant 20. Introductions of other AA substitutions (e.g., in positions 110 and 120) did not produce major effects.

DISCUSSION

In the present study, we have mapped and identified the mutations of the HA protein involved in the antigenic drift observable in the HPAI H5N1 variant virus which appeared in Egypt in 2008 (strain 1709-6/2008). In addition, we have gathered information suggesting that H5N1 virus evolution and drift in vaccinated poultry are driven by mechanisms previously described for human influenza viruses.

The sequence of the mature HA protein of strain 1709-6/2008 differs from the HA of the selected 2007 virus (strain 1709-1/2007) by 11 amino acids, exclusively located in the HA1 protein. This is in line with previous findings which indicate 11 amino acid substitutions in the HA1 protein of these newly emerging variants (1, 2). In 1709-6/2008, 10 out of 11 AA

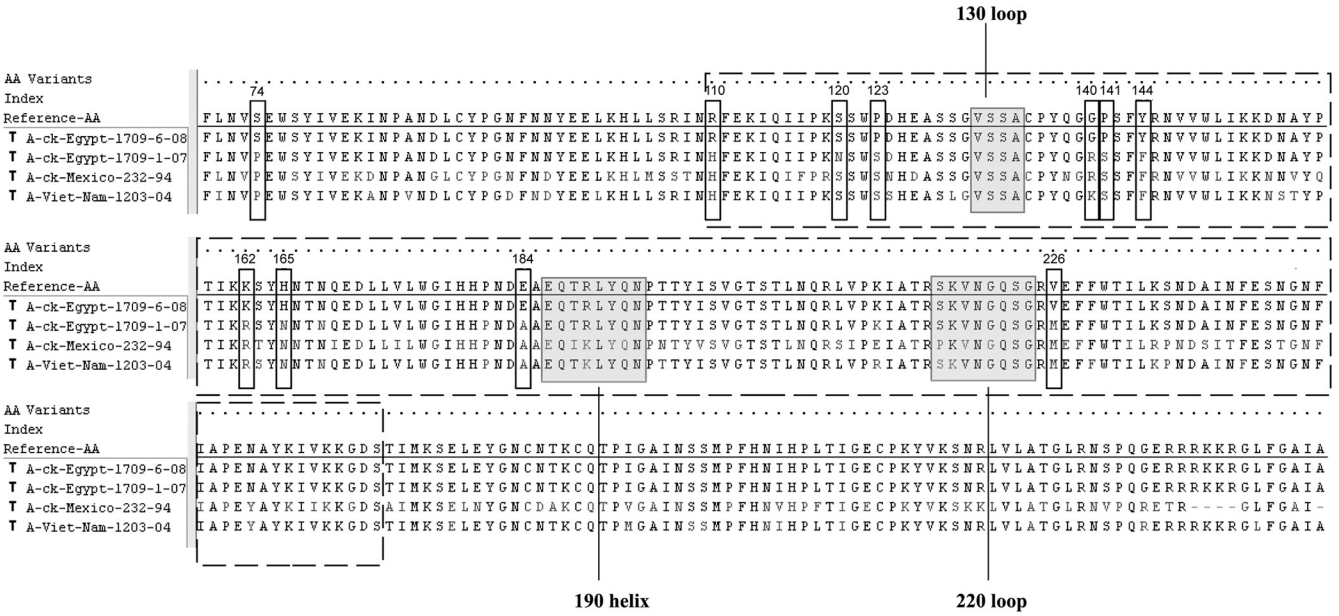

FIG. 1. Amino acid sequence alignment between HA proteins of A/chicken/Egypt/1709-6/2008, A/chicken/Egypt/1709-1/2007, A/chicken/Mexico/232/94, and A/Viet-Nam/1203/04 strains. The figure shows the receptor binding subdomain (9) as a dashed line, the major 130 and 220 loops and 190 α-helix as shaded squares, and amino acid differences between A/chicken/Egypt/1709-6/2008 and A/chicken/Egypt/1709-1/2007 as smaller, unshaded squares.

substitutions were located in the receptor binding subdomain close to the functional receptor binding sites at the globular head of the protein (6, 9, 33, 42). RG-generated mutants of 1709-1/2007 demonstrated that 5 AA substitutions, at positions 74, 140, 141, 144, and 162, are primarily involved in the antigenic drift and that these mutations alone can reproduce the antigenic vaccine drift observed for the 2008 field strain. The opposite mutations introduced into the same sites in the HA genetic backbone of 1709-6/2008 resulted in HI titers similar to the 2007 strain, thus confirming the important role of these AAs for the antigenic variation and drift (e.g., compare mutants 17 and 18, 19 and 20, and 21 and 22; Table 1). Besides, these results indicate that progressive accumulation of mutations at multiple sites within the H5N1 HA1 molecule enhances antigenic drift and is necessary to increase the mutant ability to escape polyclonal antibody response as revealed by HI testing. This is in agreement with a previous study on H3N2 human influenza virus (32). Substitution P74S generates a potential additional glycosylation site in 1709-6/2008, while substitution N165H in the same virus deletes a site for potential glycosylation. HA glycosylation can affect receptor binding and immune response and can modulate virulence of influenza

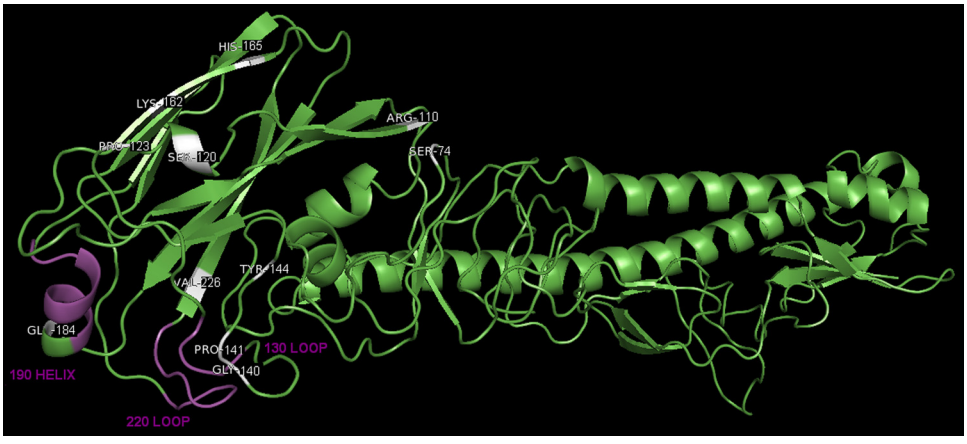

FIG. 2. A/chicken/Egypt/1709-6/2008 HA monomer. Amino acid differences in the HA monomer between A/chicken/Egypt/1709-6/2008 and A/chicken/Egypt/1709-1/2007 are shown in white; the major 130 and 220 loops and 190 helix are shown in magenta (image drawn with Pymol software). The template protein was obtained by submitting the amino acid sequence of the HA protein to the structure homology modeling server http://swissmodel.expasy.org. The Protein Data Bank accession codes obtained refer to influenza A virus (24) for the full monomer (Protein Data Bank identification 2wr1A) and to strain A/Vietnam/1194/04 (41) for the portion from amino acid residue 1 to 340 (Protein Data Bank identification 2ibX).

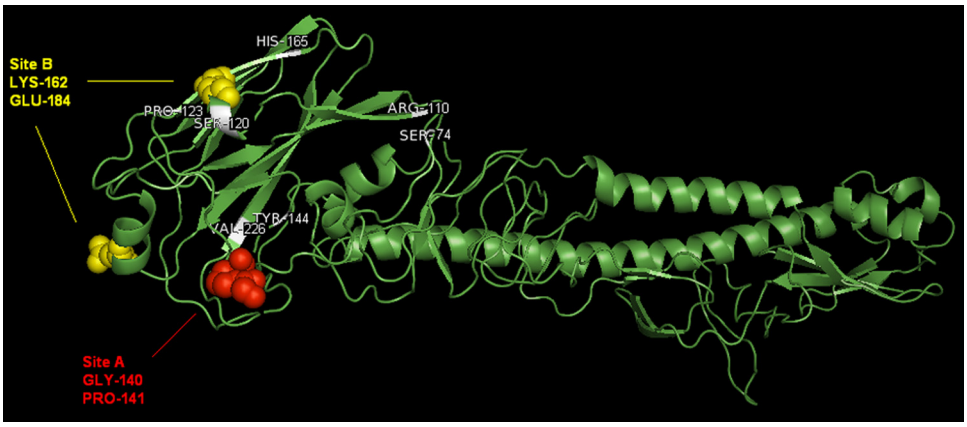

FIG. 3. A/chicken/Egypt/1709-6/2008 HA monomer. Amino acid differences in the HA monomer between A/chicken/Egypt/1709-6/2008 and A/chicken/Egypt/1709-1/2007. In red are amino acid substitutions in antigenic site A (positions 140 and 141); in yellow are amino acid substitutions in antigenic site B (positions 162 and 184). All the positions in white are the differences not included in the antigenic sites.

viruses (36, 43). In the present study, the single substitution P74S in the HA backbone of 1709-1/2007 did not alter the antigenicity of this mutant significantly. In contrast, the deletion of the potential glycosylation site (substitution S74P) introduced in the HA genetic backbone of 1709-6/2008 increased the MGT and HI titer, in some cases by a factor of 4 (Table 1). This is consistent with previous findings indicating that single selected substitutions in the HA protein may increase the HI titer significantly (12). Additionally, substitution P74S introduced together with R140G, S141P, F144Y, and R162K contributed to the further reduction of the HI titer (e.g., compare mutants 30 and 20, Table 1).

It is interesting that 3 out of 5 key amino acid substitutions detected in this study (positions 140, 141, and 162) are located in corresponding H3 antigenic sites A and B (16, 23). Mutations in 6 positions of 1709-6/2008, namely, 110, 120, 123, 165, 184, and 226, do not appear critical for escape from antibody binding. This could be explained by the fact that some of these positions are either located outside major antigenic epitopes (positions 110, 123, and 165) or not exposed to the surface of the HA antigen (position 226) as predicted by the computational analysis (5, 8, 21).

The results obtained in this study indicate that antigenic drift of H5N1 viruses in Egypt was initiated and driven by mutations primarily occurring in the RBD and decreasing HI antibody activities, similarly to what has been described for the antigenic drift observed in human H1N1 and H3N2 influenza viruses under experimental and field conditions (11, 15, 20). This is also consistent with structural similarities in the antigenic sites shared by H1, H3, and H5 subtypes (17, 18). The AA mutations are located at the globular head of the HA1 protein in proximity to functional binding sites, and some of the key mutations are in the H3-corresponding A and B sites. These sites contain functional epitopes with high neutralizing efficiency (25). The HI and MN test results obtained in this study are consistent with this observation. For H3N2 viruses, AA changes of epitopes in antigenic sites A, B, and D could be highly favored by natural selection in promoting the selection of escape mutants, whereas changes in other epitopes might not be advantageous for the virus (25). The immune pressure on H5N1 viruses circulating in the vaccinated population likely favored the generation of mutants harboring substitutions at the globular head of the HA1 protein that alter the antigenicity, resulting in lower HI antibody activities (i.e., lower HI titer). Interestingly, our analysis demonstrated that the strain which appeared in early 2008 did possess AA mutations exclusively at positions shared between the previously circulating strain (1709-1/2007) and the vaccine strain contained in one of the vaccines most commonly used in Egypt (Table 3).

The major antigenic differences revealed by the HI assay in viruses 1709-1/2007 and 1709-6/2008 were confirmed by the MN assay, indicating that some of the key mutations identified in this study are located in neutralizing epitopes. The apparently conflicting data between *in vitro* (i.e., serological assays) and *in vivo* (i.e., vaccine challenge) studies reported in previous publications (12, 14, 35) deserve further investigations. Although serological tests are extremely useful tools to screen and identify the occurrence of antigenically drifted viruses, it should also be considered that prediction of vaccine efficacy for poultry based on *in vitro* assays may not correlate well with *in vivo* challenge studies (30, 34). In this regard, factors related to vaccine formulation, such as antigen content, vaccine dose, and type of adjuvant, should be taken into account.

In regions of endemicity, reducing H5N1 virus circulation through the administration of appropriate vaccines coupled with the implementation of vaccination strategies in the animal reservoir is essential to reduce the risk of human exposure and the occurrence of viral mutations, which may have an unpredictable impact on human health. The use of strongly adjuvanted vaccines in poultry could partially overcome the need for the accurate matching of vaccine strain in order to confer satisfactory cross-protection and to increase vaccine efficacy (19, 35). Previous publications suggested that extensive vaccination of poultry for avian influenza can favor the emergence of viruses antigenically drifted from the vaccines applied in the field. This could potentially reduce vaccine efficacy and be responsible for vaccination failures (2, 7, 22, 30, 34). Furthermore, factors other than the type of vaccine used (e.g., heterologous versus homologous vaccine strains) can contribute to vaccination failures, such as quality of the vaccines and their

proper storage and administration, the potential interference of maternal antibodies, and vaccine coverage, as suggested or demonstrated in previous publications (7, 11, 19, 28). All these factors should be taken into account to better understand the evolution of the virus and the mechanisms favoring its endemic circulation in the poultry population.

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