ANTIGENIC AND GENETIC DIVERSITY OF HIGHLY PATHOGENIC AVIAN INFLUENZA A (H5N1) VIRUSES ISOLATED IN EGYPT DURING 2007AND 2008

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ABSTRACT

Highly pathogenic avian influenza A virus (H5N1) has diverged antigenically and genetically since its initial detection in Asia in 1997. Viruses belonging to clade 2.2 in particular have been reported in numerous countries with the majority occurring in Egypt. Previous reports identified antigenic similarities between viruses belonging to clade 2.2. However, poultry and human viruses isolated in northern Egypt during 2007 and 2008 were found to be antigenically distinct from other clade 2.2 viruses from this country. Genetic analysis of the hemagglutinin revealed a high degree of nucleotide and amino acid divergence. The antigenic changes in Egyptian viruses isolated during 2007–08 necessitated that two of these strains be considered as potential H5N1 pre-pandemic vaccine candidates.

INTRODUCTION:

Highly pathogenic avian influenza (HPAI) has been confined to influenza A subtypes H5 and H7 (7). The outbreaks of HPAI H5N1 in Hong Kong in 1997 were believed to be caused by a reassortant H5N1 virus that derived the hemagglutinin (HA) gene from A/Goose/Guangdong/1/96 (GS/GD) and its internal genes from avian viruses of other subtypes circulating in China (5). GS/GD was isolated from geese on a commercial farm in Guangdong Province, China (25). Since their initial detection in Asia, HPAI viruses originating from GS/GD have undergone extensive evolution resulting in antigenic diversification (5). Because of the extensive genetic divergence of viruses within the GS/GD lineage, H5N1strains are now classified into numerous genetic groups (clades) based on the HA gene (2,23). In 2005 there was an outbreak of HPAI H5N1 in migratory waterfowl in the Qinghai Lake region of western China (4,8). The Qinghai-like viruses have caused severe disease in ducks and migratory birds and rapidly spread throughout Asia and then into Europe, the Middle East, and Africa (1,8,18). H5N1 viruses that emerged from the Qinghai Lake outbreak have been identified as belonging to clade 2.2. Clade 2.2 viruses have been implicated in poultry and wild bird outbreaks in more than 60 countries and appear to be the most geographically diversified of the HPAI H5N1 viruses. As clade 2.2 viruses began to spread westward in 2005 and 2006, Egyptian authorities prepared for their possible introduction into Egypt.

In February 2006 outbreaks of H5N1 in poultry were confirmed in Egypt, and genetic analyses indicated introduction of a Qinghai Lake–like H5N1 strain (10,12). In collaboration with the Egyptian Ministry of Agriculture, initial isolation of H5N1 viruses in poultry was performed at the National Laboratory for Quality Control of Poultry Production (NLQP) and later confirmed by the U.S. Naval Medical Research Unit No. 3 (NAMRU-3). Following the first detection of HPAI H5N1, authorities began a culling and vaccination program to control the spread of the disease in poultry. Several inactivated vaccines derived from low-pathogenicity H5N2 or H5N1 strains were imported, and outbreaks in industrial poultry production facilities were reasonably controlled by late 2006. However, scattered outbreaks in backyard and rooftop flocks that may have been inconsistently vaccinated (10) led to the emergence of many human cases in 2006, 2007, and 2008. As of March 2009, Egypt has had the third highest number of human cases after Indonesia and Vietnam (22).

As a result of the persistent circula-

tion of HPAI H5N1 in Egypt, the viruses have diverged to the extent that they have been reclassified as a third-order clade, termed clade 2.2.1 (23).

In addition to the notable genetic diversification, recent Egyptian viruses from both birds and humans demonstrated significant antigenic drift within clade 2.2. Consequently, the World Health Organization (WHO) has recommended that two strains from the 2007–08 human outbreaks in Egypt be considered as potential H5N1 pre-pandemic vaccine candidates (21). This report describes recent surveillance activities in Egypt that led to the isolation of one of these vaccine candidates as well as molecular and antigenic characterization of this and other Egyptian viruses.

Table 1. Highly pathogenic H5N1 avian influenza virus variants (groups E and F) characterized for this study.

Strain name	GenBank no.	Source laboratory	Governorate	Date collected	Vaccinated	Sequencing lab
A/chicken/Egypt/9402NAMRU3/2007	EU623467	CLEVB	Sharqiya	Nov. 7	Yes	NAMRU-3
A/chicken/Egypt/9403NAMRU3/2007	EU623468	CLEVB	Sharqiya	Nov. 7	Yes	NAMRU-3
A/chicken/Egypt/07632S-NLQP/2007	EU496395	NLQP	Qalyoubiya	Dec. 7	Yes	NLQP
A/chicken/Egypt/07201-NLQP/2007	EU496388	NLQP	Qena	Dec. 7	Yes	NLQP
A/chicken/Egypt/07202-NLQP/2007	EU496389	NLQP	Sharqiya	Dec. 7	Yes	NLQP
A/turkey/Egypt/07203-NLQP/2007	EU496390	NLQP	Sharqiya	Dec. 7	Yes	NLQP
A/chicken/Egypt/07701S-NLQP/2007	EU496397	NLQP	Behera	Dec. 7	Unknown	NLQP
A/chicken/Egypt/0806Q-NLQP/2008	EU496398	NLQP	Daqahlia	Jan. 8	Unknown	NLQP
A/chicken/Egypt/0808S-NLQP/2008	EU496399	NLQP	Daqahlia	Jan. 8	Unknown	NLQP
A/chicken/Egypt/488N3-CLEVB/2008	GU064350	CLEVB	Sharqiya	Jan. 8	Yes	NAMRU-3
A/chicken/Egypt/489N3-CLEVB/2008	GU064351	CLEVB	Sharqiya	Jan. 8	Yes	NAMRU-3
A/chicken/Egypt/490N3-CLEVB/2008	GU064352	CLEVB	Qalyoubiya	Jan. 8	Unknown	NAMRU-3
A/chicken/Egypt/492N3-CLEVB/2008	GU064353	CLEVB	Sharqiya	Jan. 8	Unknown	NAMRU-3
A/chicken/Egypt/495N3-CLEVB/2008	GU064354	CLEVB	Cairo	Jan. 8	Unknown	NAMRU-3
A/chicken/Egypt/496N3-CLEVB/2008	GU064355	CLEVB	Qalyoubiya	Jan. 8	Yes	NAMRU-3
A/chicken/Israel/1055/2008	EU574927	Israel	N/A	Jan. 8	Unknown	Kimron
A/Egypt/3300-NAMRU3/2008	FJ226061	NAMRU3	Cairo	Apr. 8	No	NAMRU3

MATERIALS & METHODS:-

Surveillance, virus isolation, gene sequencing. In response to reports of vaccinated poultry die-offs at industrial farms in northern Egypt, the NLQP, in collaboration with the Ministry of Agriculture, and the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB) collected cloacal swabs from chickens over a period from April 2007 to January 2008. Using conventional reverse transcription-polymerase chain reaction (RT-PCR) techniques, a total of 32 of these swabs were found to be positive for the presence of HPAI H5N1 HA gene RNA. Following this detection, CLEVB used the remaining sample for virus isolation in embryonated chicken eggs. Twelve isolates collected from eight governorates were then sent to NAMRU-3 for confirmatory testing. Several of these samples were identified as having been collected from poultry previously vaccinated against HPAI H5N1 with inactivated low pathoge-

nicity avian influenza vaccines, although the specific vaccines administered were unknown. In addition to poultry isolates, samples of HPAI H5N1 isolated from humans in Egypt were also sent to NAMRU-3 by the Egyptian Ministry of Health for diagnostic confirmation and analysis. Following RNA extrac tion from virus isolates, samples were tested using a real-time RT-PCR method specific for the detection of the influenza A matrix and H5 HA genes according to Spackman et al. (15). N1 neuraminidase gene-specific realtime RT-PCR was performed according to Payungyoung et al. (11). Select human and poultry H5N1 isolates were submitted to the Centers for Disease Control and Prevention, Influenza Division, Atlanta, Georgia, USA (CDC) by NAMRU-3 for comprehensive antigenic and genetic characterization. Viruses were passaged once in 10–11-day-old embryonated chicken eggs under Biosafety Level 3 enhanced conditions. The eggs were inoculated with a 1:10 to 1:1000 virus dilutions in phosphate buffered saline (PBS, pH 7.2) with penicillin/streptomycin and gentamicin added. Eggs were incubated for 24–26 hr at 35 C. After incubation, the allantoic fluid was harvested and a hemagglutination titer was determined using 0.5% turkey red blood cells. Aliquots of each virus were stored at -80 C. Following reisolation of positive samples, PCR amplification of overlapping fragments of the HA gene was performed using H5N1-specific primers (primer sequences available upon request). Sequencing of PCR amplicons was performed using the BigDye Terminator cycle sequencing reaction version 3.1 (Applied Biosystems, Foster City, CA). HA sequences were submited in GenBank (Table 1).

Genetic analysis. HA gene nucleotide sequence alignments were generated using sequences derived for this study, as well as sequences posted in GenBank. Analyses were conducted in MEGA4 using the neighbor-joining method (19). Bootstrap analysis was implemented with 1000 replicates and A/turkey/Turkey/1/2005 (H5N1) was used to root the tree for Fig. 1. Tree groupings were based on significant bootstrap support values (>80), as well as antigenic relatedness of viral isolates as described below. In addition, amino acid sequence alignments were used to infer amino acid substitutions between the isolates analyzed in Fig. 1. The complete open reading frame of the mature HA1 protein was included in the final dataset for the HA translation analysis. Substitutions that were found to be conserved between isolates showing antigenic variation in the Egypt virus hemagglutination inhibition (HI) assay were included at pertinent branches on the HA tree (Fig. 1).

Ferret antisera. Antigenic characterization was performed using postinfection ferret antisera produced at the CDC to selected Egyptian isolates (6). Approximately 1-yr-old ferrets were inoculated via the intranasal route with 1:1000 to 1:10,000 of diluted virus. At day 14, ferrets were boosted with concentrated virus and Titermax. At day 28, ferrets were humanely uthanatized and exsanguinated. Blood was collected in serum separator tubes without additives. Vacutainers were centrifuged, and serum was aliquoted and stored at -20 C. All antisera were treated with DENKA Seiken RDE, according to the manufacturer's recommendation. The HI assay was performed to compare a select number of Egypt clade 2.2.1 viruses with viruses from other clades.

HI assay. Fifty micro liters of treated ferret antiserum was added to the first column of V well micro titer plates. Next 25 µl of PBS, pH 7.2 was added to columns 2 through 10. The antiserum was diluted by twofold serial dilutions of 25 µl each. Then 25 µl of the standardized antigen (4 HA units) was added to columns 1 through 10. The titers were verified by performing a backtitration. The plates were mixed using a plate shaker and incubated at room temperature for 15 min. Then 50 µl of 0.5% Turkey red blood cells were added to columns 1 through 10, and the plates were mixed using a plate shaker and then incubated at room temperature for 30 minutes before reading.

RESULTS:-

Phylogenetic analyses. All strains analyzed from Egypt were identified as clade 2.2.1. The HA gene phylogenetic tree indicated that all Egyptian strains analyzed in this study formed a monophyletic group with a bootstrap support value of 97. Five subgroups within the main Egyptian cluster were identified on the phylogenetic tree with significant bootstrap support (Fig. 1). Groups A–D consisted of viruses collected primarily from humans and poultry between 2006 and 2009 (Table 1; Fig. 1).

The majority of viruses in groups A and B were from southern governorates of Egypt. However, within all of these subgroups, the inclusion of strains from governorates in the Nile Delta region (North) indicated mixing of strains throughout the country. Additionally, 17 strains—10 isolates sequenced by NAMRU-3 and 7 sequenced by the NLQP—formed a subgroup (referred to as Egypt group E and F) within the larger Egyptian cluster of clade 2.2.1 viruses (Table 1; Fig. 1). One of the isolates, A/Egypt/3300 - NAMRU 3/2008, although phylogenetically related to group E, was an outlier to the group and was classified as group F because of antigenic variation to be discussed. All group E and F strains were collected in late 2007 and early 2008, and sequenced.

by independent laboratories (NLQP, NAMRU-3, and CDC). At least five strains from CLEVB and four from NLQP were collected from vaccinated poultry. Groups E and F consisted of 16 poultry viruses and one human virus (6 sequenced for this study, 11 previously posted in GenBank). These groups demonstrated considerable nucleotide distance from the nearest relatives within clade 2.2.1, with a bootstrap support value of 100. Table 1 details the viruses belonging to these groups including GenBank accession numbers, geographic location, and date of collection. Egyptian viruses sent to CDC for antigenic characterization fell primarily into subgroups A–D but also included representative viruses from groups E and F (shown underlined in Fig. 1).

Molecular characterization of groups E and F.

HA gene nucleotide sequences of the group E strains were nearly identical to each other (99.9% on average) but only 97%–98% identical to the remaining Egyptian strains. The group E strains also possessed 11 conserved amino acid substitutions in the HA1 protein in comparison to other Egyptian viruses (Fig. 1). Four of the 11 amino acid changes (S123P, R140G, S141P, and A184E) were identified in previously described putative antigenic sites (17). The N165H mutation indicates a predicted loss of glycosylation at this site. Additionally, the glycosylation motif (N-X-S/T) at amino acids 154–156 recorded in the majority of HPAI H5N1 was absent in the HA genes of these isolates (7). In addition to these 11 conserved amino acid substitutions, the group F strain, A/Egypt/3300-NAMRU3/2008, possessed seven other substitutions. Five of the seven substitutions were found in other putative influenza A antigenic sites (E126G, A129L, A156T, L190I, Q192K), and the D154N substitution is predicted to add a glycosylation site to the HA1 protein. Furthermore, three of the amino acid substitutions identified were located at residues previously shown to be in or near receptor-binding sites (E126G, A129L, and L190I) (17).

ANTIGENIC ANALYSIS:

Table 2: presents data on HI analysis of H5N1 viruses from different clades. In addition to antisera raised against viruses from Egypt, Table 2 includes ferret sera obtained with use of clade 2.2 viruses that have been chosen by the WHO as potential vaccine candidates:
A/bar-headed-goose/Qinghai/ 1A / 2005, A / turkey /Turkey/1/2005, A/ whooper swan/ Mongolia/244/2005, A/chicken /India /NIV33487/2006, A/Egypt/ 321/2007, and A/Egypt/ 3300-NAMRU3/2008. Table 2 demonstrates slight cross-reactivity between the clade 2.1 and majority of clade 2.2 viruses. The majority of clade 2.2.1 Egyptian viruses col-

lected from 2006 to 2007 were antigenically similar to each other, while group E (represented by A chicken/Egypt/9403-NAMRU3/2007) and F isolates (represented by A / Egypt /3300-NAMRU3/2008; EG/3300) formed antigenically unique clusters within the clade 2.2.1 viruses isolated in Egypt.

Despite some antigenic similarity observed between members of group E and F, sera generated against viruses from each group showed low levels of cross-reactivity between heterologous viruses. The addition of seven amino acid substitutions between / chicken / Egypt / 9403-NAMRU3 /2007 and A/Egypt/3300-NAMRU3/2008 is likely to have influenced these results. Importantly, these recent virus isolates were antigenically dissimilar to the currently proposed clade 2.2.1 vaccine candidate, A/Egypt/321-NAMRU3 /2007. These data indicate the need for the production of a new clade 2.2.1 H5N1 vaccine candidate, in addition to the previously recommended A / Egypt / 321-NAMRU3 /2007 virus (group B, Fig. 1). The majority of viruses from ge
netic groups A–D were antigenically similar to each other although viruses within each discrete group did show on average higher cross-reactivity to one another. Amino acid differences between strains included in the HI assay (Table 2) are shown in Fig. 1 at relevant branches of the phylogenetic tree. Notable amino acid substitutions were observed between isolates A/chicken /Egypt/9403-NAMRU3-CLEVB214 / 2007 and A / Egypt/ 3300-NAMRU3/2008 (as indicated in Fig. 1) and the majority of clade 2.2.1 isolates included in the test. Other amino acid substitutions shared by isolates belonging to subgroups A, B, C, and D are also indicated in Fig. 1 and may play a role in the minor antigenic differences observed in the HI assay (Table 2). One or more unique substitutions were found to be conserved among members of the same groups as indicated in Fig. 1, and these changes were observed to correlate with higher cross-reactivity between isolates belonging to the same group. For example, isolates in group C showed high titers to each other in comparison to isolates from groups A, B, and D. Several amino acid substitutions conserved amongst members of groups A–D were identified as residing in or around putative influenza A antigenic sites (D43N, A127T, A129del, I151T), while others were identified in or near previously identified receptor-binding sites (I151T, V219I) (16). Together, these data suggest that the higher cross-reactivity seen between isolates in the same phylogenetic/antigenic group results from shared amino acid residues in antigenically significant regions of the HA1 protein

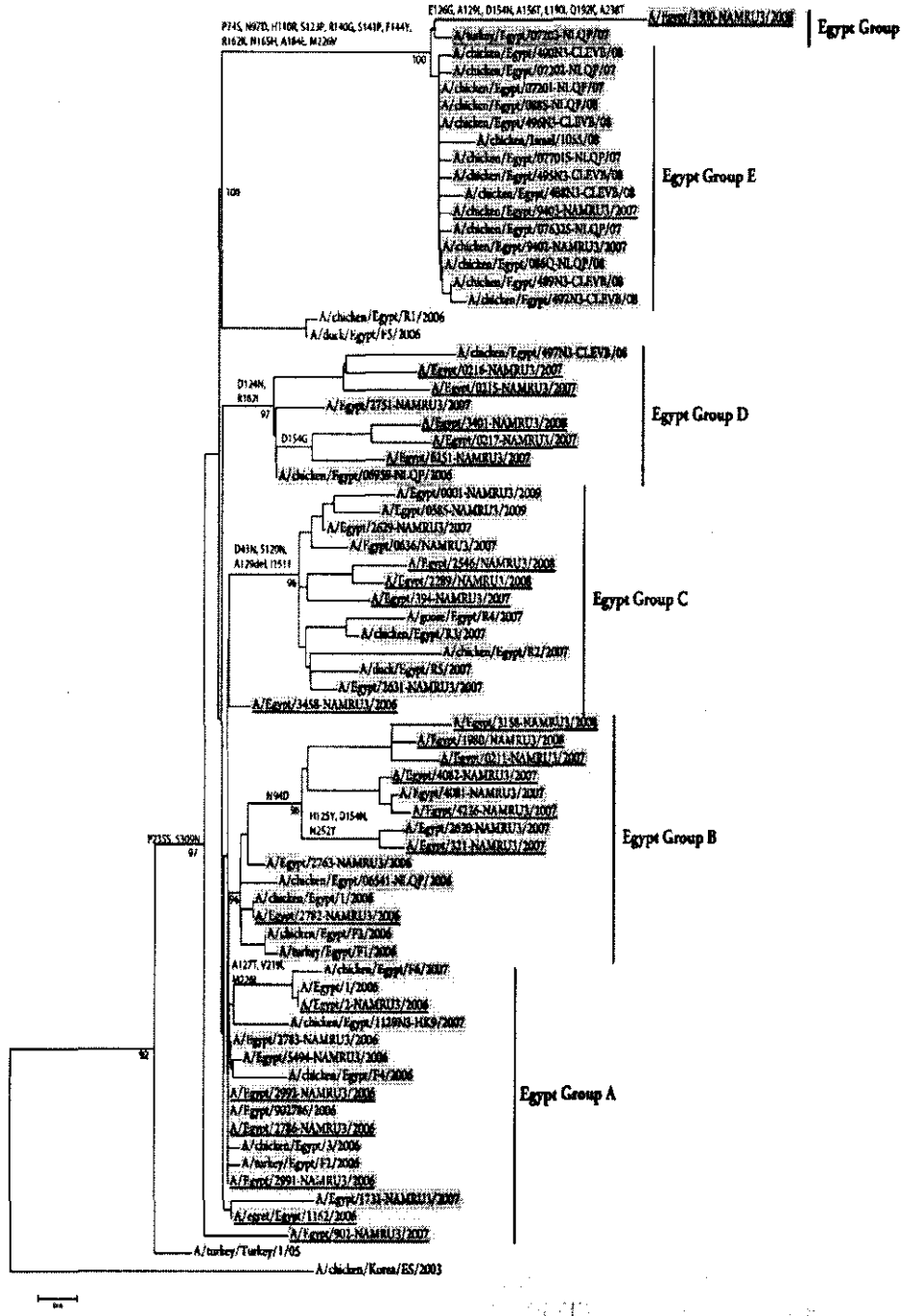
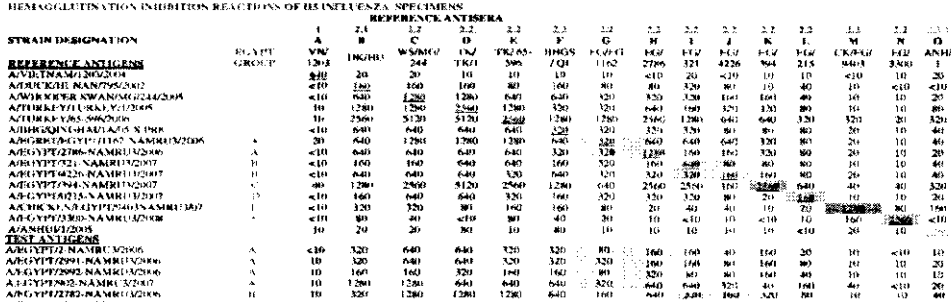


Fig. 1. Phylogenetic tree of viral HA sequences of Egyptian H5N1 viruses generated by neighbor-joining analysis. Bootstrap values at relevant nodes represent 1,000 replicates. Scale bar represents 0.002 nucleotide substitutions. Amino acid substitutions at group-defining branches are shown and represent substitutions in the HA1 relative to A/turkey/Turkey/1/05. Viruses included in the HI test are underlined. Del indicates a deletion of an amino acid.

Table 2:- HI assay of Egyptian H5N1 antigenic variants. The underlined titers represent the homologous titers of the reference antigens to reference antisera. Group A viruses are highlighted in yellow, group B in blue, group C in rose, group D in gray, group E in orange, and group F in green.



DISCUSSION:-

Since the initial outbreaks of HPAI H5N1 in 1997 (3), the virus has diverged both antigenically and genetically with novel genotypes and variant viruses continuing to emerge (20). Similar to that of seasonal influenza viruses, their continuous and rapid evolution necessitates reviewing and updating, if necessary, strains used for vaccine development. Continuous monitoring of virus evolution and genetic changes of H5N1 and other important avian influenza viruses with epizootic potential is critical to detect genetic drift or a genetic shift that may lead to new outbreaks or a future influenza pandemic. In order to slow the rate of mutation and reassortment of viruses circulating in poultry populations, complete eradication by culling or culling with vaccination are commonly used (9). Initially, most HPAI H5N1 affected countries followed the eradication policy for controlling the spread of the disease. Vaccination of poultry, however, has become more common and is considered essential in many countries for economic reasons (9).

Extensive vaccination of poultry has been demonstrated to result in the emergence of vaccine escape mutants in studies of H5N2 viruses in Mexico (7), and despite the differences in duration and number of doses administered (13 years; two

billion doses in Mexico), vaccine use may impart immune pressure selection in H5N1 viruses as well. Following the detection and spread of Fujian-like (clade 2.3.4) viruses in Southeast Asia, it was hypothesized that the emergence of this sublineage resulted from the selection of possible vaccine escape mutants (14). In Egypt, culling with vaccination has been implemented since March 2006. While there was some initial success in large-scale poultry operations, it was observed that household poultry vaccination was either incomplete or inefficient because of failures in vaccine efficacy (10).

Viruses collected in Egypt during 2006 and 2007 were found to be similar to the viruses from Qinghai Lake and viruses from neighboring Middle Eastern and European countries (clade 2.2). However, as indicated by these and other phylogenetic studies (13), the persistent circulation of H5N1 in Egypt has resulted in the emergence of a distinct sublineage of H5N1 viruses within clade 2.2, termed clade 2.2.1, that consists of viruses almost exclusively from Egypt (24). This study has also identified a subset of poultry and human viruses isolated in northern Egypt during 2007 and 2008 that were found to be antigenically distinct from early clade 2.2 viruses, as well as other clade 2.2.1 viruses from Egypt. As illustrated in Table 2, poultry viruses belonging to this variant subgroup (Egypt group E) were found to be antigenically distinct from related clade 2.2.1 viruses. In addition to the poultry isolates collected in 2008, an isolate from a human case in 2008 from Cairo, A/Egypt/3300-NAMRU3/2008, was identified as being genetically and antigenically distinct from viruses collected in earlier years. This isolate was found to be genetically related to several poultry isolates collected from similar time periods in nearby governates. Subsequent characterization described herein confirmed that these variants were antigenically related.

Phylogenetic analysis of the HA gene of strains analyzed for this study showed six distinct subgroups with high bootstrap support values (Fig. 1). In late 2007 and early 2008, a new variant appeared and was clearly distinct from other Egyptian subgroups (classified here as Egypt groups E and F; Fig. 1) with a bootstrap support value of 100%. The HA gene of the strains in these sub

groups had at least 11 different amino acid mutations from the rest of the Egyptian H5N1 strains sequenced from human or poultry cases. To our knowledge, at least nine of these poultry strains (Table

1) were from vaccinated farms. Similar findings of the emergence of possible vaccine escape variants were reported in China one year after the implementation of vaccination in poultry (14). The detection of these variant strains occurred approximately 18 months after the beginning of vaccination in poultry, and because surveillance of poultry in Egypt has been extremely active, it is less likely that this variant represents a gap in Egyptian surveillance activates but rather a unique event leading to the rapid emergence of this variant. The detection of this variant strain in seven governorates indicated wide geographical spread of the variant strain in Egyptian poultry. Furthermore, a very closely related strain (99.9% identical at the HA nucleotide level) was also reported in Israel in early 2008 and posted in GenBank under accession number EU574927, indicating even further geographic spread. Antigenic analysis of the strains within these variant subgroups indicated significant diversity from previously isolated Egyptian viruses. While there is antigenic diversity commonly seen between distinct clades, this report illustrates within-clade variation, as shown by Table 2. Data presented in this study suggest the emergence of a new variant of HPAI influenza A(H5N1) viruses circulating in northern Egypt that appears to have spread widely. Since the detection of these isolates, review and evaluation of vaccines by WHO and other organizations led to the recommendation to include strain A/Egypt/3300-NAMRU3/2008 as a pre-pandemic vaccine candidate, which is currently under development (22). The emergence of this variant strain, its spread in a short period of time to at least seven governorates in Egypt as well as Israel, and its considerable antigenic variation from previous Egyptian isolates emphasizes the need for continuous monitoring of genetic and antigenic changes in HPAI H5N1 as an early warning system of the detection of new variants and faster response to contain disease spread in the future.

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