Antigenic analysis of highly pathogenic avian influenza virus H5N1 sublineages co-circulating in Egypt

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Highly pathogenic avian influenza virus H5N1 has spread across Eurasia and Africa, and outbreaks are now endemic in several countries, including Indonesia, Vietnam and Egypt. Continuous circulation of H5N1 virus in Egypt, from a single infected source, has led to significant genetic diversification with phylogenetically separable sublineages, providing an opportunity to study the impact of genetic evolution on viral phenotypic variation. In this study, we analysed the phylogeny of H5 haemagglutinin (HA) genes in influenza viruses isolated in Egypt from 2006 to 2011 and investigated the effect of conserved amino acid mutations in the HA genes in each of the sublineages on their antigenicity. The analysis showed that viruses in at least four sublineages still persisted in poultry in Egypt as of 2011. Using reverse genetics to generate HA-reassortment viruses with specific HA mutations, we found antigenic drift in the HA in two influenza virus sublineages, compared with the other currently co-circulating influenza virus sublineages in Egypt. Moreover, the two sublineages with significant antigenic drift were antigenically distinguishable. Our findings suggested that phylogenetically divergent H5N1 viruses, which were not antigenically cross-reactive, were co-circulating in Egypt, indicating that there was a problem in using a single influenza virus strain as seed virus to produce influenza virus vaccine in Egypt and providing data for designing more efficacious control strategies in H5N1-endemic areas.

INTRODUCTION

Since the emergence of highly pathogenic avian influenza virus (HPAI) subtype H5N1 in China in 1996, the H5N1 virus has evolved to form 10 phylogenetically distinct clades (0–9) (WHO/OIE/FAO H5N1 Evolution Working Group, 2008), spread into South-East and East Asia, and
caused an epidemic in poultry and occasional infections in mammals (WHO, 2012c). In April 2005, HPAI H5N1 viruses caused large outbreaks in wild waterfowl at Qinghai Lake in China (Chen et al., 2005) and one genotype, clade 2.2, unexpectedly spread west to central and southern Asia, Europe and Africa, including Egypt (Salzberg et al., 2007; Wang et al., 2008). Since the initial outbreaks in Egypt, a distinct third-order H5N1 clade, clade 2.2.1, has evolved in Egypt and diverged further into phylogenetically separate branches within clade 2.2.1 (Cattoli et al., 2009). By early 2009, none of these new sublineages had become dominant and all of the sublineages continued to co-circulate in birds in Egypt (Abdel-Moneim et al., 2009; Arafa et al., 2010).

Clade 2.2 H5N1 virus was first isolated in poultry in Egypt in February 2006, possibly after its introduction from infected migrating ducks (Saad et al., 2007). Thereafter, HPAI H5N1 spread swiftly nationwide among birds, including chickens, ducks, turkeys, geese and quail, and was declared endemic in Egypt in July 2008. Other countries with endemic HPAI H5N1 are Indonesia, China and Vietnam (OIE, 2011). To control and attempt to eradicate H5N1 viruses, the Egyptian authorities used a blanket vaccination programme and attempted to heighten biosecurity and quarantine measures in both commercial and household sectors (Peyre et al., 2009). Nevertheless, HPAI H5N1 is still endemic in Egypt and continues to pose a severe threat to the poultry industry (Hafez et al., 2010), causing more than a US$ 1 billion annual loss (Meleigy, 2007). In addition, among countries surveyed by the WHO, Egypt has the second-highest number of human H5N1 infections (WHO, 2012a). As of April 2012, 166 HPAI H5N1 cases, with 59 fatalities, have been reported in Egypt. In particular, the cumulative number since 2009 is notable: of 249 HPAI H5N1 cases worldwide, 123 cases (49% of the total) were in Egypt. Most human infections were linked to close contact with and/or slaughtering of infected birds and no sustained human–human transmission has been documented to date in Egypt (WHO, 2012b, c). However, the long-term endemic status of HPAI H5N1 in Egypt could increase the opportunity for emergence of potential pandemic strains through intra- and interspecies transmission.

Influenza virus haemagglutinin (HA) is a virion-surface glycoprotein and the primary target for neutralizing antibodies (Skehel & Wiley, 2000; Smith & Helenius, 2004). The protein is initially synthesized as precursor HA0 and cleaved to yield HA1, a variable external subunit, and HA2, a conserved transmembrane subunit (Stevens et al., 2006). Most of the HA1 molecule forms a globular head containing the binding pocket for cell-surface sialylglycans (or sialylgangliosides), the primary receptor for influenza viruses (Suzuki, 2005). HA affinity for sialylglycans is one of the determinants of influenza A virus host range (Horimoto & Kawaoka, 2005; Suzuki, 2005). Human and avian influenza viruses differ in their recognition of host-cell receptors: human viruses mainly bind α2,6-linked sialylglycan, while avian viruses have a high affinity for α2,3-linked sialylglycan (Couceiro et al., 1993; Ito et al., 1998). HA is also an antigenically variable protein in which a large number of point mutations accumulate, mainly in HA1 epitope regions (Nelson & Holmes, 2007).

Interestingly, H5N1 was introduced into Egypt from a single infected source (Eladl et al., 2011; Watanabe et al., 2011b). In contrast, in other countries, such as China and Nigeria, co-circulation of different H5 sublineages has allowed antigenic shift due to genetic reassortment among the sublineages (Chen et al., 2006; Fusaro et al., 2010). Continuous replication of H5N1 virus in Egypt during the last 5 years has provided an opportunity to study the relationship between genetic evolution and selection of influenza virus phenotypes, including antigenicity, receptor-binding specificity and pathogenicity. Although previous studies focused on genetic evolution of influenza viruses, there are relatively few reports analysing the effect of genetic evolution on biological characters (Cattoli et al., 2011a).

Several commercial inactivated H5 vaccines, produced using different H5 virus strains, were used during the H5N1 epidemic in Egypt (Peyre et al., 2009). However, mass vaccination has failed to control the continual H5N1 HPAI outbreaks in Egypt (Hafez et al., 2010). The vaccination campaign limited the first wave of 2006 outbreaks. However, antigenic variants were detected in several vaccinated farms in 2007 and are now the dominant strains in vaccinated and non-vaccinated flocks in Egypt (Abdelwhab & Hafez, 2011a). Several studies have suggested that immune pressure due to the vaccines resulted in major antigenic drift of the H5N1 virus, generating phylogenetically distinct clade 2.2.1 variants (denoted here as sublineage C) (Cattoli et al., 2011b; Eladl et al., 2011). There are conflicting data on whether commercially available vaccines provide protection against these new antigenic drift variants. Some studies have reported inadequate protection (Abdelwhab et al., 2011b; Grund et al., 2011; Peyre et al., 2009), while others have reported sufficient protection (Kim et al., 2010; Terregino et al., 2010). These discrepancies were probably due to selection of strains in those studies with phylogenetically discordant or unrepresentative sequences. In addition, polyclonal antibodies in infected chicken and ferret sera, used in conventional analyses, might complicate sensitive determination of the effect of HA amino acid mutations on antigenicity due to cross-reactivity with other viral structural proteins, such as neuraminidase and nucleoprotein (Kaminski & Lee, 2011).

In this study, we performed phylogenetic analyses of HA genes in HPAI H5N1 strains isolated in Egypt from 2006 to 2011, identified the amino acid mutations that were conserved in each of the newly formed H5 sublineages, and investigated the effect of these mutations on the antigenicity and immunogenicity of the HAs with monoclonal and polyclonal antibodies. The results of this study should be useful for understanding antigenic drift among H5N1 viruses in Egypt and for planning more efficacious control strategies for endemic HPAI H5N1.
RESULTS

Phylogenetic analysis of H5N1 HAs and identification of conserved mutations

The phylogeny of H5N1 influenza viruses in Egypt was investigated by analysing 62 HA sequences of representative viruses isolated from birds and humans from 2006 to 2011. Phylogenetic analysis showed that all of the HA genes clustered in clade 2.2.1 with an overall monophyletic topology, indicating that these viruses diverged from a single origin (Fig. 1). In addition, most human H5N1 viruses isolated in 2009–2011 clustered in two sublineages (A and B) and most avian H5N1 viruses isolated in 2009–2011 clustered in two different sublineages (C and D), as described previously (Abdel-Moneim et al., 2009; Balish et al., 2010; Watanabe et al., 2011b). This phylogenetic tree topology was the same as that for a phylogenetic tree reconstructed from 492 HA sequences from H5N1 viruses isolated in Egypt and posted in GenBank (data not shown). Analysis of the 492 H5 HA sequences also identified amino acid mutations that were conserved in each of the sublineages compared with ancestral Egyptian viruses; these conserved mutations are listed in Fig. 1. Most HPAI H5N1 viruses isolated in Egypt at the time of

Fig. 1. Phylogenetic tree of HA genes of H5N1 viruses isolated in Egypt. This tree includes HA sequences of 51 H5N1 influenza A viruses isolated in Egypt, available in GenBank, and 11 HA sequences determined in our study. Strains whose HA sequences were also analysed for antigenicity are marked with ●. Amino acid mutations conserved in each of the sublineages are shown using H5 numbering. Colours are used to highlight human and avian virus strains isolated in 2009–2011. Bar, 0.005 nucleotide substitutions per site.

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Antigenic analysis of H5N1 influenza virus sublineages

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this study were in sublineages B and D, which were recently classified as group 2.2.1/C and subclade 2.2.1.1, respectively, in the WHO classification. In addition, viruses closely related to ancestral viruses were isolated at low frequency. Sublineages A and B were probably formed in early 2008, and contained 96 of the 97 isolates from human infection cases in Egypt since 2008. We previously found that HA mutations Q192H and S129del/I151T (H5 numbering), which were conserved in sublineages A and B, respectively, increased HA binding affinity for α2,6-linked sialylglycan, possibly accounting for the increase in human H5N1 infections in Egypt (Watanabe et al., 2011b). However, sublineages C and D were probably formed in 2007, mainly from isolates from vaccinated birds in commercial farms (Balish et al., 2010). These two sublineages shared several amino acid mutations in the HA globular head and retained the classical binding preference for α2,3-linked sialylglycan (Watanabe et al., 2011b). Previous studies suggested a vaccine-driven emergence of sublineage C viruses (Abdel-Moneim et al., 2011; Cattoli et al., 2011b). Sublineage D diverged from sublineage C in 2007, with additional mutations compared with the ancestral viruses. Viruses in the most recent phylogenetic branches were in sublineage D. Therefore, no single H5N1 sublineage has become dominant, and phylogenetically distinct viruses have persisted in Egypt.

Antigenic analysis using HA recombinant viruses

A panel of five mAbs against the HA of A/crow/Kyoto/53/2004 (H5N1), which had the antigenicity of other contemporary Asian strains, was used for antigenic analysis of H5N1 viruses circulating in Egypt. We have previously mapped the epitopes recognized by these mAbs to conserved regions in the globular head of the HAs of East Asian H5 viruses and shown that they have broad cross-neutralizing activity against Asian H5 lineage strains (Du et al., 2009). The broad cross-reactivity of the mAbs with H5N1, H5N2 and H5N3 HAs was confirmed in this study (Fig. S1, available in JGV Online). The pattern of mAb reactivity with HA of A/duck/Egypt/D2Br21/2007, one of the ancestral H5 viruses in Egypt, was comparable to that of the Asian H5 lineage, indicating an antigenic similarity between the Asian H5 lineage and ancestral Egyptian H5 viruses. In these studies, mAbs C43, which binds influenza nucleoprotein (Okuno et al., 1993), and C179, which binds the HA stalk with cross-reactivity to H1, H2, H5 and H6 viruses (Okuno et al., 1993; Smirnov et al., 1999), were used as controls. The reactivity of these mAbs with 11 HAs that contained sequences representative of sublineage A, B, C and D viruses was analysed by immunofluorescence assays. Both a human- and a bird-derived virus HA were included as representatives of sublineages A and B.

To investigate specific reactivity between the mAbs and HAs, we generated recombinant H5N1 viruses, each containing one of the sublineage HA genes and the other genes from A/duck/D1Br12/2007 (EG/D1), and infected Madin–Darby canine kidney (MDCK) cells with these viruses. Antigenic analysis of the infected cells with the panel of mAbs showed different mAb reactivity patterns against the HAs of recombinant viruses from different sublineages (Fig. 2). All mAbs reacted similarly to cells infected by viruses with sublineage A and B HAs, except for mAb 4G6, which did not react with sublineage B HAs due to the mutation at the epitope (D43N) recognized by mAb 4G6 (Du et al., 2009). However, the mAbs did not react with cells infected with viruses with sublineage C and D HAs. The reactivity of the mAbs with the Egyptian HAs correlated with their neutralizing activity: parental, sublineage A and sublineage B viruses were neutralized by the mAbs with different efficacies, while sublineage C and D viruses were not neutralized by the mAbs (data not shown). These results indicated that ancestral, sublineage A and sublineage B viruses isolated in Egypt shared epitopes in the HA globular head with antigenicity similar to the Asian H5 lineages, but sublineage C and D HAs did not have this antigenicity.

Prevalence of mutations characteristic of sublineages C and D

To investigate the effect of vaccination on antigenic drift of sublineage C and D viruses, the prevalence of conserved amino acid mutations in these sublineages was compared between HAs from H5N1 viruses isolated from 28 vaccinated and 10 non-vaccinated geographically distant poultry flocks in northern Egypt during 2007–2009, which was the putative time when these sublineages arose (Arafa et al., 2010; Balish et al., 2010). In H5N1 viruses isolated from vaccinated flocks, mutations in HA characteristic of sublineage C were identified in 30–80 % of viruses isolated in 2007, 54–77 % of viruses isolated in 2008, and 80–100 % of viruses isolated in 2009, although the number of virus strains from vaccinated flocks was small (Table 1). In H5N1 viruses isolated from vaccinated flocks, mutations in HA characteristic of sublineage D were identified in 10–30 % of viruses isolated in 2007, 8–23 % of viruses isolated in 2008, and 0–80 % of viruses isolated in 2009 (Table 1). In contrast, in H5N1 viruses isolated from non-vaccinated flocks, the prevalence of HA mutations characteristic of sublineage C was essentially zero in 2007 and 2008 and 100 % in 2009, and was essentially zero for HA mutations characteristic of sublineage D in 2007, 2008 and 2009, although the number of virus strains from non-vaccinated flocks was small. For H5N1 viruses isolated in Asia, the prevalence of HA mutations characteristic of sublineages C and D was low in 2007, 2008 and 2009, with a few exceptions (at HA residues 120, 141, 154, 156 and 162). These results indicated that sublineage C and D viruses spread preferentially among vaccinated flocks in northern Egypt, implying vaccine-driven evolution of these viruses.

Antigenic analysis of recombinant viruses with specific mutations

To compare antigenic variation among sublineage HAs, the effect of strain-specific amino acids had to be excluded. Therefore, mutations characteristic of each sublineage were introduced in the HA gene of EG/D1, and recombinant
viruses with the mutated HA gene and the unmodified other seven protein genes were generated. For sublineage D, the 11 mutations found in both sublineages C and D were also introduced. The antigenicity of these viruses was investigated by using them to infect MDCK cells and analysing the reactivity patterns of the infected cells with the panel of mAbs described above. With one exception, HA mutations characteristic of sublineages A and B had little effect on the reactivity patterns of the mAbs (Fig. 3). In contrast, no reactivity was seen in cells infected by viruses with HA mutations characteristic of sublineages C and D. These results confirmed that sublineage C and D HAs generally had different antigenicity from ancestral Asian and Egyptian H5 viruses.

**Glycosylation of recombinant HAs with specific mutations**

The HA1 proteins of Egyptian H5 lineage viruses contain different combinations of three N-linked glycosylation sites (NGS) at residues 72, 154 and 165. To determine the attachment of N-glycans to the globular head of sublineage HAs, recombinant HAs with specific mutations were prepared as described in Methods. Each protein carried the mutations conserved in one of the sublineages. In addition, variant forms of sublineage D HA were prepared in which amino acid residues were introduced in the NGS of sublineage D HA. Electrophoretic analysis of the proteins showed different patterns of N-glycosylation among the sublineage HAs (Fig. 4). The mobility shift for sublineage D HA1 with mutated NGS showed that the slower mobility of HA1 with mutations characteristic of sublineage D was due to glycosylation of residue 154N as a result of amino acid changes D154N and A156T in sublineage D HA1. Amino acid changes P74S and N165H, which were conserved in both sublineage C and D HAs, resulted in the generation and loss of glycosylation sites, respectively, which explained the lack of a mobility shift for sublineage C HA1. The N-glycosylation patterns identified in sublineage HAs are summarized in Table 2. The effect of N-glycosylation changes in sublineage D HA on antigenicity was analysed by immunofluorescence assays. The results suggested that

![Antigenic variation in H5N1 viruses isolated in Egypt. MDCK cells were infected with recombinant viruses with different HAs at an m.o.i. of 0.5 and the reactivity of the HAs against a panel of mAbs against influenza A viruses was determined by immunofluorescence assays. mAbs 3C11, 3H4, 3H12, 4C12 and 4G6 were cross-reactive to HA1 of the Asian H5 lineage, and mAbs C43 and C179 were controls. The recombinant virus designations are on the left and the phylogenetic sublineages are on the right. The mAb designations are at the top, with their antigens in parentheses.](http://vir.sgmjournals.org/2219)
Table 1. Prevalence of HA mutations characteristic of H5 sublineages C and D in viruses isolated in Egypt and Asia

<table>
<thead>
<tr>
<th>Sublineage/mutation in HA*</th>
<th>Strains (%) with mutations isolated in:†</th>
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<tbody>
<tr>
<td></td>
<td>From vaccinated flocks</td>
</tr>
<tr>
<td></td>
<td>(n=38)</td>
</tr>
<tr>
<td></td>
<td>2007 (n=10)</td>
</tr>
<tr>
<td>C</td>
<td></td>
</tr>
<tr>
<td>P74S</td>
<td>50.0</td>
</tr>
<tr>
<td>D97N</td>
<td>50.0</td>
</tr>
<tr>
<td>H110R</td>
<td>40.0</td>
</tr>
<tr>
<td>S123P</td>
<td>40.0</td>
</tr>
<tr>
<td>R140G</td>
<td>30.0</td>
</tr>
<tr>
<td>S141P</td>
<td>80.0</td>
</tr>
<tr>
<td>F144Y</td>
<td>70.0</td>
</tr>
<tr>
<td>R162K</td>
<td>60.0</td>
</tr>
<tr>
<td>N165H</td>
<td>40.0</td>
</tr>
<tr>
<td>A184E</td>
<td>60.0</td>
</tr>
<tr>
<td>M226V</td>
<td>40.0</td>
</tr>
<tr>
<td>D</td>
<td></td>
</tr>
<tr>
<td>S120L</td>
<td>20.0</td>
</tr>
<tr>
<td>D154N</td>
<td>30.0</td>
</tr>
<tr>
<td>A156T</td>
<td>10.0</td>
</tr>
<tr>
<td>L190I</td>
<td>10.0</td>
</tr>
<tr>
<td>A238T</td>
<td>10.0</td>
</tr>
</tbody>
</table>

*Mutations are shown according to H5 numbering.
†Percentage of H5N1 viruses that have mutation(s) characteristic of sublineages C and D for each geographical region, type of flock and year of virus isolation. Sequence information is from GenBank and from sequences analysed in this study.

Fig. 3. Effect of HA conserved mutations in different sublineages on antigenicity. MDCK cells were infected with recombinant viruses with HAs containing the mutations conserved in one of the sublineages and the other genes from virus EG/D1, and the reactivity of the HAs against a panel of mAbs was determined by immunofluorescence assays as described in the legend to Fig. 2. The mutations introduced into each EG/D1 HA are on the left and the HA sublineages are on the right. Mutations specific to sublineage D are underlined. The mAb designations are at the top, with their antigens in parentheses.
and B HAs, and that mouse polyclonal antibodies induced by divergence of these phylogenetic branches from sublineage A was significant antigenic drift in sublineage C and D HAs after sublineages A–D (Table 3). These results indicated that the patterns of HAI and neutralizing antibody titres for HA1s (18–57) (Table 3). There was good correlation between much lower titres against viruses with sublineage A and B (508 and 127), lower titres against each other (18 and 50), and significantly different HAI titres against homologous virus from mice immunized with sublineage C and D HA1s had viruses with sublineage C and D HA1s (7–36) (Table 3). Sera from mice immunized with sublineage A and B HA1s had similar haemagglutination (HAI) titres against homologous virus and heterologous HAs were determined post-immunization. Sera from mice immunized with sublineage A and B HA1s had similar haemagglutination inhibition (HAI) titres against homologous virus (453 and 570) and against each other (403 and 570), and much lower HAI titres against viruses with sublineage C and D HA1s (7–36) (Table 3). These results indicated that there was significant antigenic drift in sublineage C and D HAs after divergence of these phylogenetic branches from sublineage A and B HAs, and that mouse polyclonal antibodies induced by the mutations at NGS and the other sites (non-NGS) in sublineage D HA impacted antigenic variation synergistically (Fig. S2).

Variations in immunogenicity of recombinant viruses with specific mutations

To evaluate the immunogenicity of sublineage HAs, recombinant HA proteins with specific mutations were prepared and purified as described in Methods. Electrophoretic analysis of the purified proteins showed the specificity and purity of the HA1 preparations (Fig. S3). We examined whether the amino acid changes in each sublineage affected induction of serum antibody responses to HA1. Groups of mice were vaccinated intra-peritoneally with purified HA1s, and serum HAI and neutralizing antibody titres against viruses with homologous and heterologous HAs were determined post-immunization. Sera from mice immunized with sublineage A and B HA1s had similar haemagglutination inhibition (HAI) titres against homologous virus (453 and 570) and against each other (403 and 570), and much lower HAI titres against viruses with sublineage C and D HA1s (7–36) (Table 3). These results indicated that there was significant antigenic drift in sublineage C and D HAs after divergence of these phylogenetic branches from sublineage A and B HAs, and that mouse polyclonal antibodies induced by sublineage C or D HAs are not cross-reactive with sublineage A and B HAs.

DISCUSSION

In this study, we elucidated the antigenic drift among the H5N1 viruses currently co-circulating in Egypt. Our analyses indicated that the HAs in sublineage C and D viruses were antigenically different from those in ancestral Asian and Egyptian H5 viruses and had undergone significant antigenic drift since their divergence from the sublineage A and B phylogenetic branches. To our knowledge, this is the first report that sublineage D HAs were generally not cross-immunogenic with other sublineage HAs, including even sublineage C HAs, although sublineages C and D form a single phylogenetic branch and have a number of amino acid mutations in common.

Viruses in the sublineage C phylogenetic branch were first isolated in 2007. Our survey of vaccination records found that sublineage C viruses preferentially circulated in vaccinated poultry flocks during this time. These data supported previous suggestions that emergence of sublineage C may have resulted from suboptimal vaccinations in Egypt (Abdel-Moneim et al., 2011; Cattoli et al., 2011b), although there is no direct evidence of this. Indeed, seven of the 11 mutations conserved in sublineage C HAs were in residues corresponding to the A and B antigenic sites in H3 HAs, which contain epitopes with high neutralizing efficiency (Kaverin et al., 2007; Wiley et al., 1981) (Fig. 5). H5N1 variants with some of the mutations characteristic of sublineage C HAs have been isolated in Egypt since April 2007, and viruses with all of the mutations characteristic of sublineage C HAs have been isolated since December 2007. The first date, April 2007, was about 13 months after the launch of mass vaccination in industrial poultry sectors in Egypt, and about 5 months before the reported emergence of sublineage C (Arafa et al.,

Table 2. Glycosylation patterns on HAs investigated in this study

Glycosylation at the indicated residue in the globular head of HAs was determined by the mobility shift of HAs with mutated NGS as shown in Fig. 4. Residues are shown according to H5 numbering. +, Glycosylated; −, non-glycosylated.

<table>
<thead>
<tr>
<th>Sublineage</th>
<th>N-Glycosylation at residue:</th>
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<tbody>
<tr>
<td></td>
<td>72</td>
</tr>
<tr>
<td>Parental</td>
<td>−</td>
</tr>
<tr>
<td>A</td>
<td>−</td>
</tr>
<tr>
<td>B</td>
<td>−</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
</tr>
<tr>
<td>D_{S74P}</td>
<td>−</td>
</tr>
<tr>
<td>D_{N154D,T156A}</td>
<td>+</td>
</tr>
<tr>
<td>D_{H165N}</td>
<td>+</td>
</tr>
<tr>
<td>D_{S74P,N154D,T156A}</td>
<td>−</td>
</tr>
</tbody>
</table>

http://vir.sgmjournals.org 2221
Table 3. Serum antibody response in mice immunized with H5N1 viruses isolated in Egypt with HA mutations characteristic of each sublineage

Values are geometric mean antibody titres (GMT) in serum from six mice, obtained 2 weeks after the second administration of virus with HA mutations characteristic of the indicated viral sublineage. Homologous titres are shown in bold.

<table>
<thead>
<tr>
<th>Sublineage</th>
<th>HAI GMT* (95 % confidence interval)</th>
<th>NT GMT† (95 % confidence interval)</th>
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<tbody>
<tr>
<td></td>
<td>Parental A B C D</td>
<td>Parental A B C D</td>
</tr>
<tr>
<td>Parental</td>
<td>359 (208–621)</td>
<td>254 (140–460)</td>
</tr>
<tr>
<td>A</td>
<td>403 (277–587)</td>
<td>453 (304–674)</td>
</tr>
<tr>
<td>B</td>
<td>403 (277–587)</td>
<td>453 (304–674)</td>
</tr>
<tr>
<td>C</td>
<td>22 (10–53)</td>
<td>18 (9–36)</td>
</tr>
<tr>
<td>D</td>
<td>7 (5–11)</td>
<td>7 (4–13)</td>
</tr>
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</table>

*Each HAI titre against the challenge virus is the reciprocal of the highest serum dilution that inhibited haemagglutination by 8 virus haemagglutination units.
†Each neutralizing titre (NT) against the challenge virus is the reciprocal of the highest serum dilution that reduced infection of 100 virus f.f.u. by >50 %.
in the field. Aquatic poultry probably plays a substantial role in H5N1 evolution in Egypt, along with terrestrial birds. Therefore, the ecology of sublineage A and B viruses in aquatic birds needs to be studied further.

The standard method for control of an HPAI outbreak is testing and culling of all poultry in a farm (Suarez, 2010). However, it has been suggested by the OIE that, when outbreaks spread to a broad area and become uncontrollable, vaccination would be an additional method to reduce nascent virus production and, thereby, suppress further virus infection (OIE, 2003). Vaccination of poultry is now considered a preventive or auxiliary control approach in several H5N1-endemic countries, including Egypt (Peiris et al., 2007; Swayne & Kapczynski, 2008). The problem is that antigenically divergent groups of viruses, which are not cross-reactive, are co-circulating in Egypt. A similar situation was also found in Indonesia even though major antigenic variation had not been detected there as of 2008 (Wibawa et al., 2011). Only several of the recently isolated H5N1 strains circulating in Indonesia had lost reactivity to mAb 4G6 (Mieko Kosaka, personal communication). This makes selection of one influenza virus strain as the vaccine seed virus especially problematic. Furthermore, random rearing of many bird species and their hybrid breeds with uncontrolled confinement is common in rural areas (Suarez, 2010), leaving ducks and geese free to fly away. Therefore, circulation of viruses in each sublineage in Egypt was not restricted in terms of geography or host species, complicating efforts to use a vaccine produced against antigens from a single virus strain. In the future, when vaccination is implemented as part of a comprehensive control strategy for endemic HPAI in Egypt, along with terrestrial birds, the ecology of sublineage A and B viruses, which are now the dominant strains co-circulating in Egypt, would need to be revised periodically (Swayne, 2009), although multivalent or universal vaccines. Broad-spectrum efficacy of these vaccines, especially human–human transmission. Large-scale surveillance of avian influenza viruses in endemic areas should be expanded to better understand evolution of the virus and enable more efficacious control strategies in these regions.

**METHODS**

*Generation of recombinant viruses.* Recombinant H5N1 viruses were generated using a plasmid-based reverse-genetics system as described previously (Fodor et al., 1999; Watanabe et al., 2011b). Each virus generated by reverse genetics (denoted here as rEG(X)) carried the HA gene of the virus being studied, with the other genes coming from A/duck/D1Br12/2007 (EG/D1), one of the ancestral influenza virus strains isolated in Egypt. The 11 recombinant viruses, each containing an HA from an Egyptian virus, were EG/D1, A/chicken/Egypt/RIMD/1-5/2008 (EG/1), A/chicken/Egypt/RIMD4-3/2008 (EG/4), A/chicken/Egypt/RIMD5-3/2008 (EG/5), A/chicken/Egypt/RIMD11-1/2008 (EG/11), A/chicken/Egypt/RIMD12-3/2008 (EG/12), A/chicken/Egypt/RIMD28-1/2009 (EG/28), A/chicken/Egypt/RIMD29-3/2008 (EG/29), A/goose/Egypt/0929-NLQP/2009 (EG/0929), A/Egypt/N04822/2009 (EG/4822) and A/Egypt/N02039/2009 (EG/2039). The HA genes of EG/0929, EG/4822 and EG/2039 were synthesized using the sequences registered in GenBank (http://www.ncbi.nlm.nih.gov/nucleotide) and site-directed mutagenesis PCR. Mutant HA genes were generated by PCR-based site-directed mutagenesis of the HA genes of EG/D1, EG/11, EG/12 and EG/29. The HA genes of the virus stocks were sequenced to detect the possible emergence of revertants or unwanted mutations during amplification as described previously (Watanabe et al., 2007).

*Cells.* MDCK and 293T cells were purchased from the RIKEN BioResource Center Cell Bank (http://www.brc.riken.jp/lab/cell/english/). Chicken embryo fibroblasts (CEF) cells were prepared from 11-day-old embryonated eggs. These cell lines were maintained as described previously (Watanabe et al., 2009).
Virus propagation. The recombinant viruses generated in this study were propagated by single passage in the allantoic cavity of 11-day-old embryonated chicken eggs. The allantoic fluids were harvested 3 days post-infection and stored at −80 °C. Virus titres were assayed as f.f.u. by focus-forming assays (Di Lonardo et al., 2002) on MDCK cells. All experiments with live H5N1 viruses were performed in Biosafety Level 3+ (BSL 3+) conditions at Osaka University, as approved for work with these viruses by the Ministry of Agriculture, Forestry and Fisheries, Japan.

Genetic analysis. For phylogenetic analysis of HA genes, we used HA sequences of 51 representative H5N1 influenza A viruses isolated in Egypt from 2006 to 2011 and obtained from GenBank, and 11 HA sequences representative of the 21 HA s sequenced in our studies. Phylogenetic analysis of the 62 HA nucleotide sequences was done by the neighbour-joining method using MEGA4 software (Tamura et al., 2007), with the nucleotide sequences covering most of the HA genes. Estimates of the variability of the reconstructed phylogenetic trees were calculated for 1000 bootstrap replicates. For reconstruction of a detailed phylogenetic tree, 398 HA sequences from avian viruses and 98 HA sequences from human viruses isolated in Egypt from 2006 to 2011 were obtained from a GenBank search and analysed. For comparison, 366 published HA sequences of H5N1 influenza A viruses recently isolated in Asia were obtained from GenBank. The prevalence of mutations characteristic of H5N1 sublineages C and D was calculated for 17 HA sequences in previous studies and 21 HA sequences in our studies, all isolated from different poultry sectors in northern Egypt (detailed data are available on request). These sequences were aligned by the MAFFT program (Katoh et al., 2002) and the HA1 regions were compared with each other.

Antigenic analysis. The antigenic specificity of HAs in Egypt was assessed by immunofluorescence assays with a panel of five mouse mAbs against A/crow/Kyoto/53/2004 (H5N1), which we produced and characterized previously (Du et al., 2009). MDCK cells were infected with recombinant viruses at a m.o.i. of 0.5 and analysed for reactivity with each of the mAbs by immunofluorescence using an FITC-conjugated secondary antibody.

Purification of recombinant HA1s. Each HA1 expression plasmid was produced by fusing an HA1 gene with the C terminus of a Flag tag and inserting it into a pcXN2 vector (Watanabe et al., 2009). Insertion of the correct sequences for wild-type and mutant HA1s was confirmed by DNA sequencing and Western blot analysis of the expressed proteins. Recombinant HA1s were produced by transfecting 293T cells with plasmid DNAs using TransIT-LT1 (Mirus Bio), according to the manufacturer’s instructions. At 5 days post-transfection, culture supernatants containing released HA1s were collected and centrifuged twice at 8400 g for 20 min. The supernatants were incubated with pre-equilibrated anti-Flag M2 agarose (Sigma-Aldrich) for 5 h at 4 °C with gentle rotation. The beads were then collected by centrifugation at 4700 g for 40 s and washed four times with PBS containing 0.1% Tween 20 (PBST). The proteins immunoprecipitated by anti-Flag agarose were eluted with 3 × Flag peptide (Sigma-Aldrich) in PBST and dialysed against PBS for 40 h at 4 °C. Western blotting was performed as described previously (Watanabe et al., 2009).

Immunization of mice. Groups of six mice (Japan SLC Inc.) were immunized with two intraperitoneal injections of 100 μg purified HA1 protein with Freund’s complete adjuvant, with 2 weeks between injections. Two weeks after the second administration, sera were collected and specific antibody responses were examined by HA1 and microneutralization assays as described below. Control mice were immunized with PBS with Freund’s adjuvant. All animal studies were conducted under the applicable laws and guidelines for the care and use of laboratory animals in the Research Institute for Microbial Diseases, Osaka University.

Serological analysis. Antibody levels against homologous and heterologous viruses in post-immunization mouse sera were determined by HAI and microneutralization assays as follows. Sera were treated overnight with receptor-destroying enzyme (Denka-Seiken) at 37 °C for 18 h and heat-inactivated at 56 °C for 45 min. HAI assays were performed as described previously (Okuno et al., 1993), with serial twofold dilutions of mouse serum starting from a 1:10 dilution. The HAI antibody titre was defined as the reciprocal of the highest serum dilution that inhibited haemagglutination. For HAI calculations, it was useful to assign HAI titres <10 HAI a value of 5 HAI. For neutralization assays, serial twofold dilutions of serum starting from a 1:10 dilution were incubated with an equal volume of virus containing 100 f.f.u. in a 96-well U-bottom plate for 60 min at 37 °C. The virus–serum mixture was transferred to an MDCK cell monolayer and incubated at 37 °C for 8 h. The neutralization antibody titre was defined as the reciprocal of the highest serum dilution that neutralized ≥50% of the viruses in a focus-forming assay.

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