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RESEARCH ARTICLE

Antigenic Variation of Clade 2.1 H5N1 Virus Is Determined by a Few Amino Acid Substitutions Immediately Adjacent to the Receptor Binding Site

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ABSTRACT **Highly pathogenic avian influenza (HPAI) viruses of the H5N1 subtype are genetically highly variable and have diversified into multiple phylogenetic clades over the past decade. Antigenic drift is a well-studied phenomenon for seasonal human influenza viruses, but much less is known about the antigenic evolution of HPAI H5N1 viruses that circulate in poultry. In this study, we focused on HPAI H5N1 viruses that are enzootic to Indonesia. We selected representative viruses from genetically distinct lineages that are currently circulating and determined their antigenic properties by hemagglutination inhibition assays. At least six antigenic variants have circulated between 2003, when H5N1 clade 2.1 viruses were first detected in Indonesia, and 2011. During this period, multiple antigenic variants cocirculated in the same geographic regions. Mutant viruses were constructed by site-directed mutagenesis to represent each of the circulating antigenic variants, revealing that antigenic differences between clade 2.1 viruses were due to only one or very few amino acid substitutions immediately adjacent to the receptor binding site. Antigenic variants of H5N1 virus evaded recognition by both ferret and chicken antibodies. The molecular basis for antigenic change in clade 2.1 viruses closely resembled that of seasonal human influenza viruses, indicating that the hemagglutinin of influenza viruses from different hosts and subtypes may be similarly restricted to evade antibody recognition.**

IMPORTANCE **Highly pathogenic avian influenza (HPAI) H5N1 viruses are responsible for severe outbreaks in both commercial and backyard poultry, causing considerable economic losses and regular zoonotic transmissions to humans. Vaccination is used increasingly to reduce the burden of HPAI H5N1 virus in poultry. Influenza viruses can escape from recognition by antibodies induced upon vaccination or infection through genetic changes in the hemagglutinin protein. The evolutionary patterns and molecular basis of antigenic change in HPAI H5N1 viruses are poorly understood, hampering formulation of optimal vaccination strategies. We have shown here that HPAI H5N1 viruses in Indonesia diversified into multiple antigenic variants, that antigenic differences were due to one or a very few substitutions near the receptor binding site, and that the molecular basis for antigenic change was remarkably similar to that for seasonal human influenza viruses. These findings have consequences for future vaccination and surveillance considerations and contribute to the understanding of the antigenic evolution of influenza viruses.**

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Since their first detection in Southeast Asia in 1997, highly pathogenic avian influenza (HPAI) viruses of subtype H5N1 have spread to numerous countries of the Eastern Hemisphere, where they caused outbreaks in poultry and diversified into multiple distinct genetic lineages or clades (1–3). In Indonesia, HPAI H5N1 viruses were first detected in Central Java in August 2003 (1). The viruses were classified as belonging to clade 2.1, and from early 2004 onwards, multiple persistent genetic lineages within this clade have emerged in Indonesia (4). Within the first 2 years after initial detection, the clade 2.1 viruses spread over large parts of the country (5) and frequently caused severe outbreaks in both backyard flocks and commercial poultry farms. Economic losses resulting from HPAI H5N1 virus outbreaks in poultry were estimated at $470 million by 2008 (6). Indonesia's first human case of H5N1 virus infection was identified in 2005 (7). Further zoonotic transmissions resulted in 193 reported human cases of infection, 161 of which were fatal (8).

The Indonesian government launched a mass vaccination program for poultry in February 2004, using A/Chicken/Legok/2003 as the recommended vaccine strain, though imported vaccines,

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often of different clades or subtypes (e.g., clade 1 or subtypes H5N2 and H5N9), were used by the poultry industry initially. Emergence of antigenic variants to which the vaccines did not provide protection was detected in poultry as early as 2005. After a series of HPAI H5N1 virus outbreaks from 2006 to 2008 and as a result of monitoring of avian influenza virus variants by the Indonesian OFFLU project (9), the poultry vaccine recommendation was updated to contain an A/Chicken/West Java/30/07 (A/Chicken/Nagrak/30/07)-like strain. With time, vaccination intensified in commercial poultry farms, with nearly 100% vaccine coverage in breeders and layers, while broilers were vaccinated only during the rainy season. However, implementing a vaccination campaign for backyard poultry appeared to be more challenging, and vaccination coverage by 2008 was estimated to be less than 40% of the standing backyard and commercial poultry population in Indonesia (10). Despite vaccination campaigns, improved hygiene measures, movement controls, and preemptive culling of infected flocks, the virus is still enzootic in large parts of Indonesia.

In 2007, an international consultative group of scientists convened by the World Health Organization (WHO), the World Organization for Animal Health (OIE), and the Food and Agriculture Organization of the United Nations (FAO) reported on the diversification of clade 2.1 into clades 2.1.1, 2.1.2, and 2.1.3. Since 2007, clades 2.1.1 and 2.1.2 seemed to have gone out of circulation, while clade 2.1.3 viruses were further reported to have diversified into fourth-order clades: 2.1.3.1, 2.1.3.2, and 2.1.3.3 (1–3, 11). Given the large genetic diversity of clade 2.1 viruses and continuing outbreaks in vaccinated poultry, we anticipated that clade 2.1 viruses also changed—and possibly diversified—antigenically over time.

Antibodies targeting the hemagglutinin (HA) surface glycoprotein are a critical component of the immune defense against influenza virus infections in both humans and animals (12). In humans, amino acid substitutions in HA have been shown to lead to escape from antibody-mediated neutralization. Studies performed in the 1980s defined five antigenic sites in the globular head of HA that contain the amino acid positions associated with antigenic change in seasonal human influenza viruses (12–14). Similar sites have been identified for HPAI H5N1 viruses (15, 16) and a recent study suggested that antigenic change in HPAI H5N1 viruses in poultry could be attributed to amino acid substitutions in antigenic sites similar to those defined for human influenza viruses (17). However, it was recently shown that antigenic change in seasonal human influenza viruses is primarily caused by amino acid substitutions in a small dominant antigenic domain immediately adjacent to the HA receptor binding site (RBS), rather than across nearly the entire globular head of HA (18). Here, we mapped the antigenic diversity of clade 2.1 viruses and identified the substitutions responsible for antigenic differences, to test the hypothesis that the molecular basis for antigenic change in avian clade 2.1 viruses circulating in poultry is similar to that of seasonal influenza viruses circulating in humans. Analysis of a subset of viruses that represented genetically distinct lineages within clade 2.1 revealed that at least six antigenically diverse variants circulated in Indonesia from 2003 to 2011. Naturally occurring single, double, or quadruple amino acid substitutions were found to determine all major antigenic differences between antigenically distinct viruses. These substitutions occurred at positions immediately adjacent to the RBS, indicating that clade 2.1 viruses evolved to evade neutralization by antibodies directed to the RBS region. These findings imply that avian clade 2.1 H5N1 viruses and seasonal human influenza viruses can escape antibody neutralization in a similar way.

RESULTS

Phylogenetic analysis and selection of representative viruses. The HA sequences of 96 avian clade 2.1 viruses isolated between 2003 and 2011 during HPAI H5N1 virus outbreaks in commercial poultry farms and backyard flocks in Indonesia were determined as part of ongoing surveillance studies (see Table S1 in the supplemental material). Of these 96 HA sequences, 79 were from viruses isolated during the currently underreported period since 2008. To evaluate recent genetic variation of clade 2.1 viruses, we constructed a maximum-likelihood phylogenetic tree using the sequences described in the present study and publically available clade 2.1 sequences (1–3, 19) (Fig. 1; see also Fig. S1).

In general, viruses isolated between 2008 and 2011 were genetically more diverse than those isolated prior to 2008. Almost 85% of the viruses isolated since 2008 belonged to clade 2.1.3.2. Of the 92 clade 2.1.3.2 viruses isolated since 2008, 77 grouped in a single branch that forms the lower part of clade 2.1.3.2 and 94% of the 2010 and 2011 viruses grouped in this part of the tree. Viruses belonging to the other presently circulating clades were much less prevalent in recent years; only seven clade 2.1.3.1 viruses and five clade 2.1.3.3 viruses were isolated since 2008. Two viruses, A/Chicken/West Java/34/2008 and A/Chicken/West Java/6-1/2008, belong to a group of viruses that did not fit in any of the defined clades and are most closely related to clade 2.1.3.

Analysis of antigenic diversity. We next investigated the antigenic diversity of clade 2.1 viruses. We selected viruses from each of the genetically distinct lineages that circulated since 2008, including multiple viruses from the most prominent lineage, to represent the recent genetic diversity of clade 2.1 viruses (Fig. 1 and Table 1). Two additional representative viruses were included: A/Chicken/West Java/30/07, which has been used as poultry vaccine strain since 2010, and A/Indonesia/5/05, which is the current clade 2.1.3.2 candidate vaccine virus for human use (20). The HA genes of the selected viruses were cloned without a multibasic cleavage site and used to make 6:2 recombinant viruses in the context of the A/Puerto Rico/8/1934 reference virus with the neuraminidase gene segment of an H5N1 virus (21). We were unable to construct A/Chicken/West Java/6-1/08.

To select a panel of antisera that covered the antigenic variation between the representative viruses, we initially prepared antisera against a subset of the representative viruses that were genetically most divergent. The antisera were subsequently tested in hemagglutination inhibition (HI) assays using all representative viruses, and additional antisera were generated against the viruses that showed a HI pattern divergent from that of the viruses used for generation of the initial antiserum panel. The antigenic properties of all representative viruses were analyzed in HI assays using a panel of 8 to 16 ferret antisera (see Table S2 in the supplemental material).

The representative viruses differentiated into six distinct antigenic variants (Fig. 2). A/Indonesia/5/05, A/Chicken/Central Java/51/09, and A/Chicken/North Sumatra/27/09, which belong to clade 2.1.3.2, were antigenically similar. Three other antigenic variants were distinguished within clade 2.1.3.2: A/Chicken/West Java/30/07, A/Chicken/West Java/119/10, and A/Chicken/East Java/121/10. The last was antigenically similar to four other repre-

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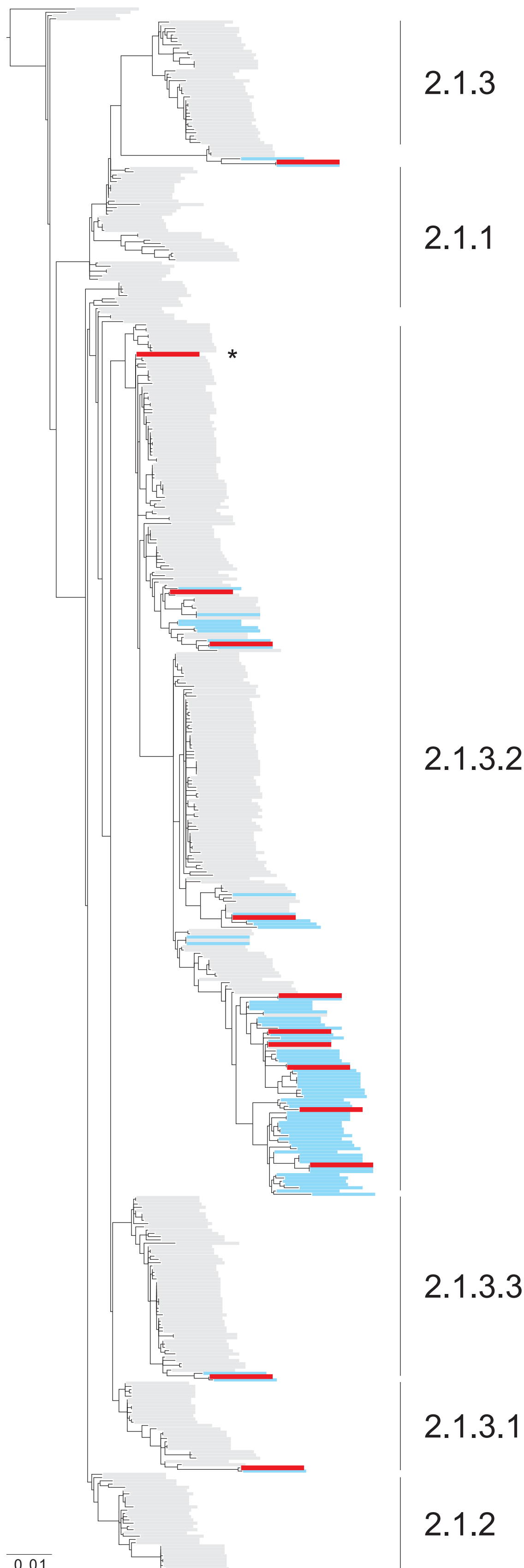

FIG 1 Maximum-likelihood phylogenetic tree for the HA gene of clade 2.1 viruses. HA sequences of 1,600 nucleotides (nt) in length were used, with A/Goose/Guangdong/1/1996 as an outgroup. The clades as defined previously are specified (1–3). The tree contains sequences from the present study from 2008 to 2011 (in blue) and publically available sequences (1–3, 19). Red bars indicate viruses selected for antigenic characterization (Table 1), including the recommended poultry vaccine strain (A/Chicken/West Java/30/07) and the candidate vaccine strain for human use (A/Indonesia/5/05). An asterisk indicates the position of A/Indonesia/5/05. The full tree is available as Fig. S1 in the supplemental material.

TABLE 1 Representative viruses selected for mapping the antigenic diversity of clade 2.1 viruses

Strain	Clade
A/Indonesia/5/05	2.1.3.2
A/Chicken/West Java/30/07	2.1.3.2
A/Chicken/West Java/6-1/08	2.1.3.2
A/Chicken/North Sumatra/27/09	2.1.3.2
A/Chicken/West Java/47/09	2.1.3.2
A/Chicken/Central Java/51/09	2.1.3.2
A/Chicken/Central Java/52/09	2.1.3.2
A/Chicken/West Java/59/09	2.1.3.2
A/Chicken/North Sumatra/72/10	2.1.3.3
A/Chicken/West Java/90/10	2.1.3.2
A/Chicken/West Java/119/10	2.1.3.2
A/Chicken/East Java/121/10	2.1.3.2
A/Chicken/South Sulawesi/157/11	2.1.3.1

sentative viruses of clade 2.1.3.2: A/Chicken/West Java/47/09, A/Chicken/Central Java/52/09, A/Chicken/West Java/59/09, and A/Chicken/West Java/90/10. The representative viruses for clades 2.1.3.1 and 2.1.3.3, A/Chicken/South Sulawesi/157/11 and A/Chicken/North Sumatra/72/10, respectively, were antigenically distinct from each other and all other representative viruses.

Surprisingly, A/Chicken/South Sulawesi/157/11 (clade 2.1.3.1) and A/Chicken/North Sumatra/72/10 (clade 2.1.3.3) were antigenically more closely related to A/Indonesia/5/05 (clade 2.1.3.2) than other antigenic variants of clade 2.1.3.2 (Fig. 2). Vice versa, A/Chicken/West Java/30/07 was genetically very similar to A/Indonesia/5/05 (see Fig. S1 in the supplemental material), yet it was antigenically substantially more distinct from A/Indonesia/5/05 than A/Chicken/Central Java/51/09 and A/Chicken/North Sumatra/27/09, A/Chicken/South Sulawesi/157/11, and A/Chicken/North Sumatra/72/10 (Fig. 2). The correlation between the antigenic distance of the representative viruses from A/Indonesia/5/05 and the number of HA1 amino acid substitutions from A/Indonesia/5/05 was 0.4828, but was not statistically significant (P = 0.1325) (see Table S3, Pearson correlation).

Mapping the molecular basis of antigenic change in clade 2.1 viruses. To gain insight into the molecular basis of antigenic change in clade 2.1 viruses, we next performed site-directed mutagenesis of the HA of A/Indonesia/5/05 and generated recombinant viruses that were tested by HI assay. It was shown recently that all major antigenic changes in seasonal human influenza viruses were due to amino acid substitutions immediately adjacent to the RBS (18), and we hypothesized that the same would apply to H5N1 viruses. To test this hypothesis, the amino acid differences between HA of A/Indonesia/5/05 and that of the representative viruses from the remaining five antigenic variants were plotted on an H5N1 HA crystal structure to identify the amino acid substitutions that occurred near the RBS (see Fig. S2 and S3 in the

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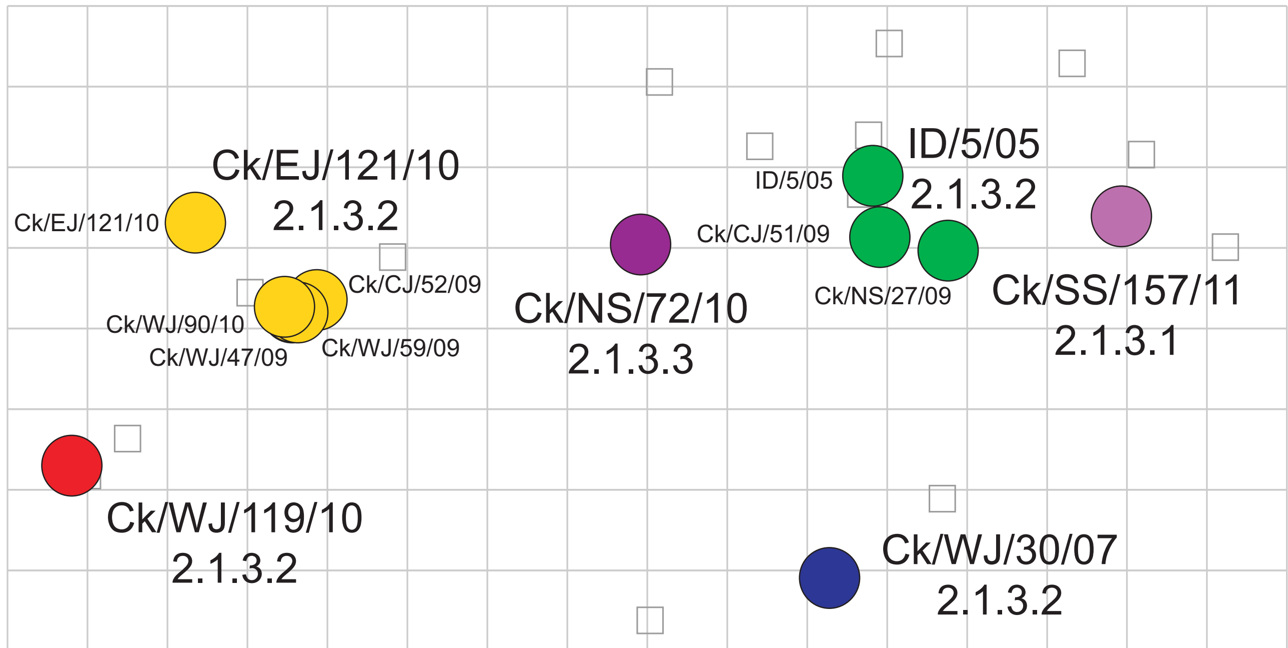

FIG 2 Antigenic map of selected clade 2.1 viruses. Filled circles and open squares indicate the positions of viruses and antisera, respectively. Both axes represent antigenic distance. The spacing between gridlines is 1 antigenic unit of distance, which equals a 2-fold difference in the HI assay. Positions of viruses and antisera in the map were generated using antigenic cartography methods as described previously (25). Briefly, the distance between a virus-antiserum pair is inversely related to the HI titer of the virus to that antiserum. Modified multidimensional scaling methods were used to arrange the relative positions of virus and antiserum points in the map such that they reflect the HI titers with minimal error. The viruses are color coded to indicate the different antigenic variants: A/Indonesia/5/05, green; A/Chicken/West Java/30/07, blue; A/Chicken/North Sumatra/72/10, purple; A/Chicken/East Java/121/10, yellow; A/Chicken/West Java/119/10, red; A/Chicken/South Sulawesi/157/11, pink. We considered viruses to be substantially antigenically different if the distance between them is at least 2 antigenic units. Abbreviations in the virus names are as follows: Ck, Chicken; CJ, Central Java; EJ, East Java; ID, Indonesia; NS, North Sumatra; SS, South Sulawesi; WJ, West Java.

supplemental material). Subsequently, each of these substitutions was introduced independently into the A/Indonesia/5/05 HA gene, and HI assays were performed with the mutant viruses to test if the substitution could fully explain the antigenic difference between A/Indonesia/5/05 and the strain in which the substitution was observed. Combinations of substitutions were tested when single substitutions did not fully explain the antigenic difference.

A/Indonesia/5/05 and A/Chicken/West Java/30/07 differed by 5 amino acids in the HA1 region (see Fig. S2 in the supplemental material), of which substitutions 133SA and 185AE were located adjacent to the RBS. Single mutants of A/Indonesia/5/05 with either 133SA or 185AE in HA did not have the same antigenic properties as A/Chicken/West Java/30/07 (see Fig. S4A). However, introduction of both 133SA and 185AE in A/Indonesia/5/05 resulted in a virus with antigenic properties that were similar to those of A/Chicken/West Java/30/07 (Fig. 3A; see also Fig. S4A). Thus, the antigenic difference between A/Indonesia/5/05 and A/Chicken/West Java/30/07 was due to substitutions 133SA and 185AE.

The five viruses that were antigenically A/Chicken/East Java/121/10-like (Fig. 2) shared 6 amino acids in the HA1 region that were different from those of A/Indonesia/5/05 (see Fig. S2 in the supplemental material), four of which were located adjacent to the RBS: 155SN, 183DN, 184AE, and 189RM. When introduced individually, none of these substitutions changed the antigenic properties of A/Indonesia/5/05 to become A/Chicken/East Java/121/10-like. However, substitutions 183DN and 189RM each had a substantial impact on the antigenic properties of A/Indonesia/5/05 (see Fig. S4B), and we therefore constructed a double mutant virus. A/Indonesia/5/05 with substitutions 183DN and 189RM in HA was antigenically similar to the A/Chicken/East Java/121/10-like viruses, indicating that the combination of substitutions 183DN and 189RM was responsible for the antigenic difference between A/Indonesia/5/05 and A/Chicken/East Java/121/10-like viruses (Fig. 3B; see also Fig. S4B).

A/Chicken/West Java/119/10 displayed 17 amino acid differences in HA1 compared to A/Indonesia/5/05 (see Fig. S2 in the supplemental material), of which 5 occurred at positions adjacent to the RBS: 136, 151, 159, 183, and 189. In addition, A/Chicken/West Java/119/10 HA had a deletion of the amino acid at position 129 compared to the HA of A/Indonesia/5/05. These 6 amino acid changes were introduced individually in the HA of A/Indonesia/5/05, but none resulted in a change in antigenic properties to become A/Chicken/West Java/119/10-like (see Fig. S4C). The Δ129 151IT combination was previously shown to cause conformational changes around the RBS (22), which could affect antibody recognition. Mutations Δ129 151IT in HA of A/Indonesia/5/05 indeed had a substantial effect on the antigenic properties of the virus (see Fig. S4C). Since substitutions 183DN and 189RM were collectively responsible for the antigenic difference between A/Indonesia/5/05 and A/Chicken/East Java/121/10 (Fig. 3B), and both substitutions were also present in A/Chicken/West Java/119/10, we constructed a virus to combine Δ129 151IT with 183DN and 189RM. This quadruple mutant virus was antigenically similar to A/Chicken/West Java/119/10 (Fig. 3B; see also Fig. S4C). Thus, we concluded that the deletion at position 129 and substi-

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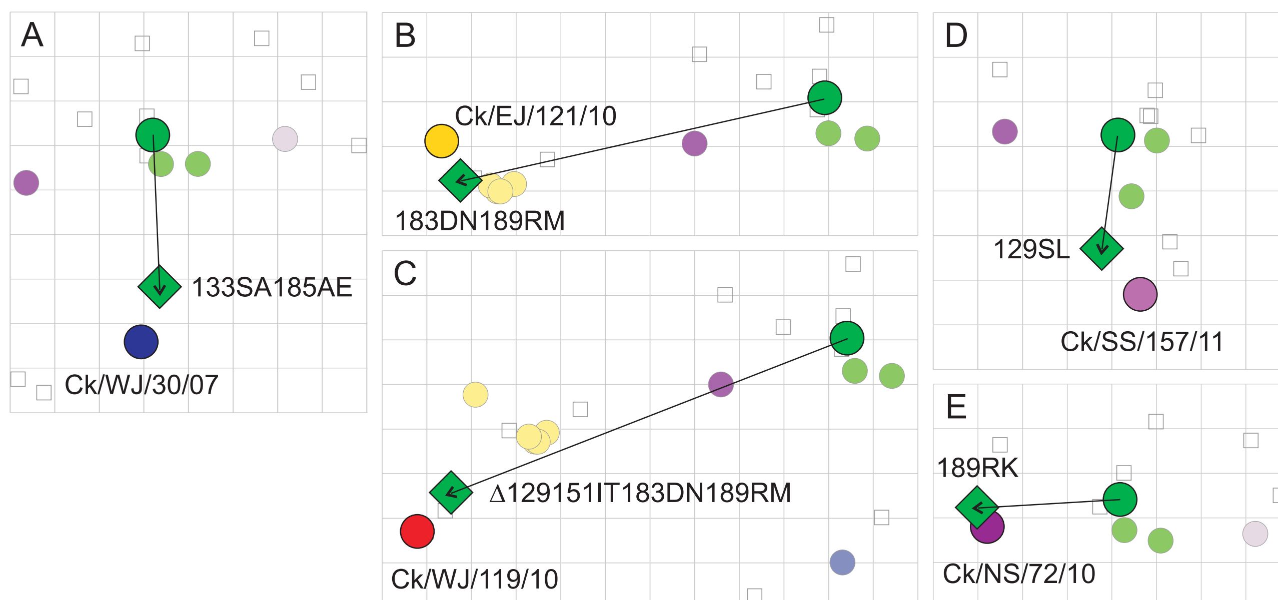

FIG 3 Summary of substitutions responsible for antigenic differences between representative viruses and A/Indonesia/5/05. The mutants were analyzed in the context of the antigenic map shown in Fig. 2. Panels A to E display a detail of the antigenic map and show the mutants with the substitutions responsible for the antigenic change from A/Indonesia/5/05 to A/Chicken/West Java/30/07 (A), A/Chicken East Java/121/10 (B), A/Chicken/West Java/119/10 (C), A/Chicken/South Sulawesi/157/11 (D), or A/Chicken/North Sumatra/72/10 (E). A representative virus for each antigenic variant is shown as a large colored circle; the remaining representative viruses are shown as smaller, faint colored circles. Mutants are indicated by green diamond shapes, A/Indonesia/5/05 as large green circles. Grid lines and antigenic map construction are as in Fig. 1. Antigenic maps with all mutants made to investigate the molecular basis of antigenic change from A/Indonesia/5/05 are shown in Fig. S4 in the supplemental material. The antigenic distances between A/Indonesia/5/05, representative viruses, and mutants are listed in Table S4.

tutions 151IT, 183DN, and 189RM were responsible for the antigenic difference between A/Indonesia/5/05 and A/Chicken/West Java/119/10.

A/Chicken/South Sulawesi/157/11 displayed 15 amino acid differences from HA1 of A/Indonesia/5/05 (see Fig. S2 in the supplemental material). Positions 129, 155, and 184 were located close to the RBS, and the corresponding substitutions were introduced individually into the HA of A/Indonesia/5/05. Substitutions 155SN and 184AE had no antigenic effect (see Fig. S4D). Introduction of substitution 129SL into A/Indonesia/5/05 changed it antigenically to A/Chicken/South Sulawesi/157/11 (Fig. 3D; see also Fig. S4D)

There were 10 amino acid differences between HA1 of A/Indonesia/5/05 and that of A/Chicken/North Sumatra/72/10 (see Fig. S2 in the supplemental material). We constructed point mutants of the two substitutions that were located close to the RBS: 155SN and 189RK. Substitution 155SN did not have an antigenic effect (see Fig. S4E). Introduction of 189RK was sufficient to change the antigenic properties of A/Indonesia/5/05 to become A/Chicken/North Sumatra/72/10-like (Fig. 3E; see also Fig. S4E).

To summarize these results, the amino acid changes responsible for antigenic change among all selected representative clade 2.1 viruses occurred at six key positions; 129, 133, 151, 183, 185, and 189. Five positions form a nearly continuous ridge located on the periphery of the RBS. The sixth, position 133, is located in the 130 loop that includes positions that are part of the RBS (Fig. 4).

Seven of the eight amino acid changes responsible for the antigenic differences from A/Indonesia/5/05 involved a large change in the biophysical properties of the amino acids (see Table S5 in the supplemental material). Seven changes resulted in a modification of the hydrophilicity of the amino acids and five resulted in a charge change, and a single substitution involved a substantial increase in the volume of the amino acid side chain. The A/Indonesia/5/05 HA amino acid sequence contains six predicted N-linked glycosylation sites (N-X-S/T-X), but these sites were not affected by the substitutions responsible for the antigenic changes, nor were any new glycosylation sites introduced by these substitutions.

We next marked the amino acid substitutions that were responsible for antigenic change in clade 2.1 viruses in the phylogenetic tree shown in Fig. 1 and analyzed in which years and geographic regions the antigenic variants identified in this study may have circulated in (Fig. 5). The single substitutions responsible for the antigenic change to A/Chicken/North Sumatra/72/10 or A/Chicken/South Sulawesi/157/11 were detected in viruses of all third- and fourth-order clades of clade 2.1. These viruses were isolated intermittently between 2004 and 2011. Viruses that contained the 2 to 4 substitutions responsible for the antigenic change to A/Chicken/West Java/30/07, A/Chicken/East Java/121/10, and A/Chicken/West Java/119/10 were more confined to specific regions of the tree. The quadruple change that was responsible for the antigenic change to A/Chicken/West Java/119/10 was found in two unique sequences only, both of which were isolated in 2010.

Viruses with the substitutions typical for the A/Chicken/South Sulawesi/157/11, A/Chicken/East Java/121/10, A/Chicken/North Sumatra/72/10, and A/Indonesia/5/05 antigenic variants were isolated from multiple geographic regions of Indonesia (Fig. 5). The A/Chicken/West Java/119/10-like and A/Chicken/West Java/30/07-like viruses from our data set were isolated exclusively on the island of Java. Cocirculation in the same geographic region of two

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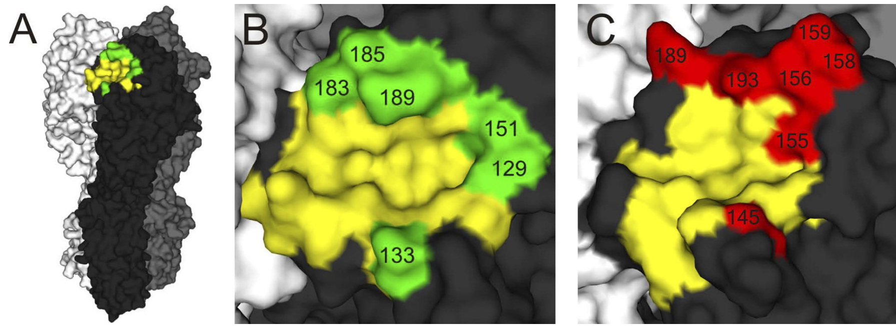

FIG 4 Amino acid positions responsible for antigenic change in clade 2.1 viruses plotted on an A/Indonesia/5/05 HA crystal structure. (A) Monomers are shown in black, gray, and white, the RBS in yellow, and amino acid positions responsible for antigenic change in green. (B) Enlarged view of the area around the RBS. The amino acid positions are based on H5 numbering. (C) Amino acid positions responsible for antigenic change in seasonal human H3N2 viruses are shown for comparison (18). Key positions are plotted on an A/Aichi/2/68 HA trimer and are indicated in red. The amino acid positions are based on H3 numbering. H5 amino acid positions 133, 151, 183, 185, and 189 correspond to H3 positions 137, 155, 187, 189, and 193, respectively. There is no equivalent of H5 amino acid position 129 in H3N2 viruses.

or more viruses that had the substitutions typical for the antigenic variants identified here was noticed from 2005 onwards.

Antigenic effect of substitutions tested using chicken sera. Ferret antisera obtained after inoculation with influenza viruses under laboratory-controlled conditions are the most sensitive reagents for detection of antigenic variation of influenza viruses. We next examined if substitutions leading to antigenic variation as tested by ferret antisera also lead to evasion of recognition by antibodies in antisera of vaccinated chickens. Ten chickens were vaccinated with a vaccine prepared from A/Indonesia/5/05. Three weeks postvaccination, the antisera were harvested and tested in HI assays using the same protocol as for HI tests using ferret antisera.

The HI antibody titers against A/Chicken/West Java/30/07 were at least 4-fold lower than the HI antibody titers against A/Indonesia/5/05 with all 10 A/Indonesia/5/05 antisera (Fig. 6A). Of the 10 A/Indonesia/5/05 antisera, 4 also had at least 4-fold-lower HI titers against the 133SA 185AE mutant than against A/Indonesia/5/05, 5 had 2-fold-or-less-lower HI titers, and the remaining antiserum had the same HI titers against the wild-type and mutant

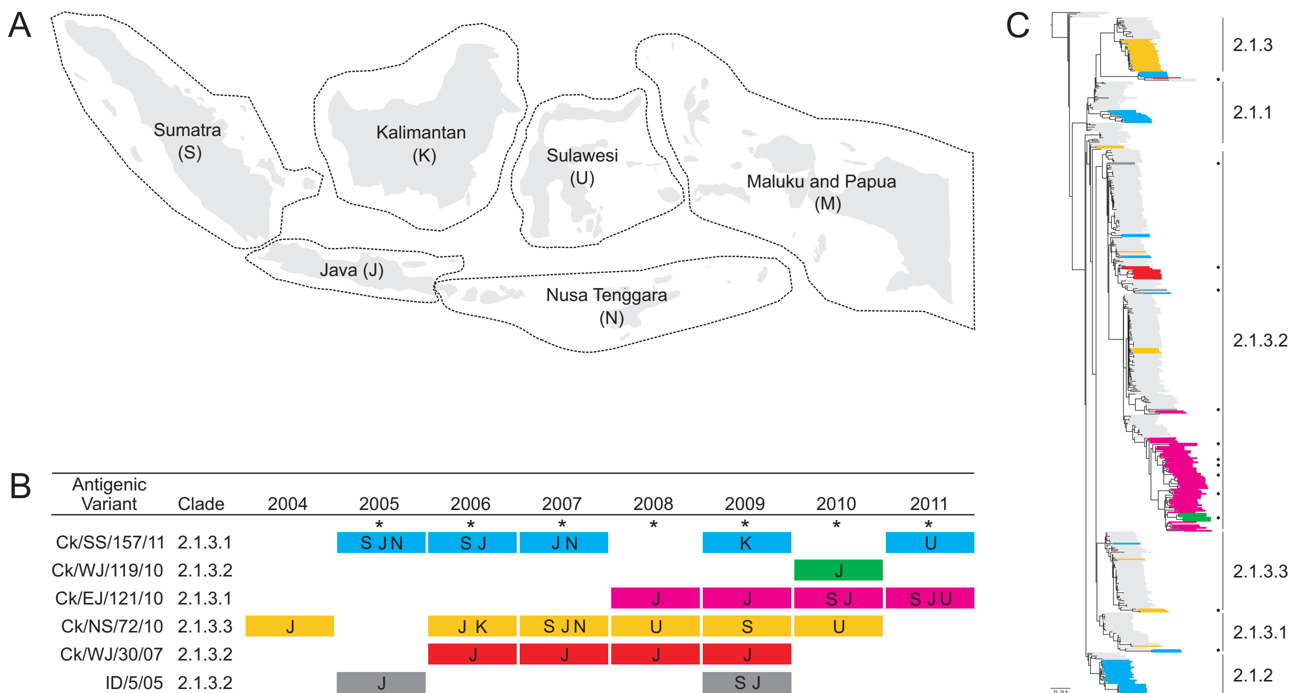

FIG 5 Identification of years and geographic region of isolation of clade 2.1 virus antigenic variants. (A) Geographical regions of Indonesia defined for this study. (B) Colored bars specify the years in which viruses with the substitutions that define the antigenic variants were isolated. The letters S, J, K, U, and N each correspond to the geographical region from which isolates were obtained in a given year and are as defined in panel A. An asterisk indicates the years in which multiple antigenic variants circulated in the same geographical region. Abbreviations in the virus names are explained in the legend to Fig. 2. (C) The phylogenetic tree of Fig. 1, color coded for the substitutions that define the antigenic variants (Fig. 3). Color coding corresponds to the antigenic variants specified in panel B. The positions of the representative viruses in the tree are indicated by black dots. Sequences that did not contain the identified (combinations of) substitutions responsible for antigenic variation are shown in light gray.

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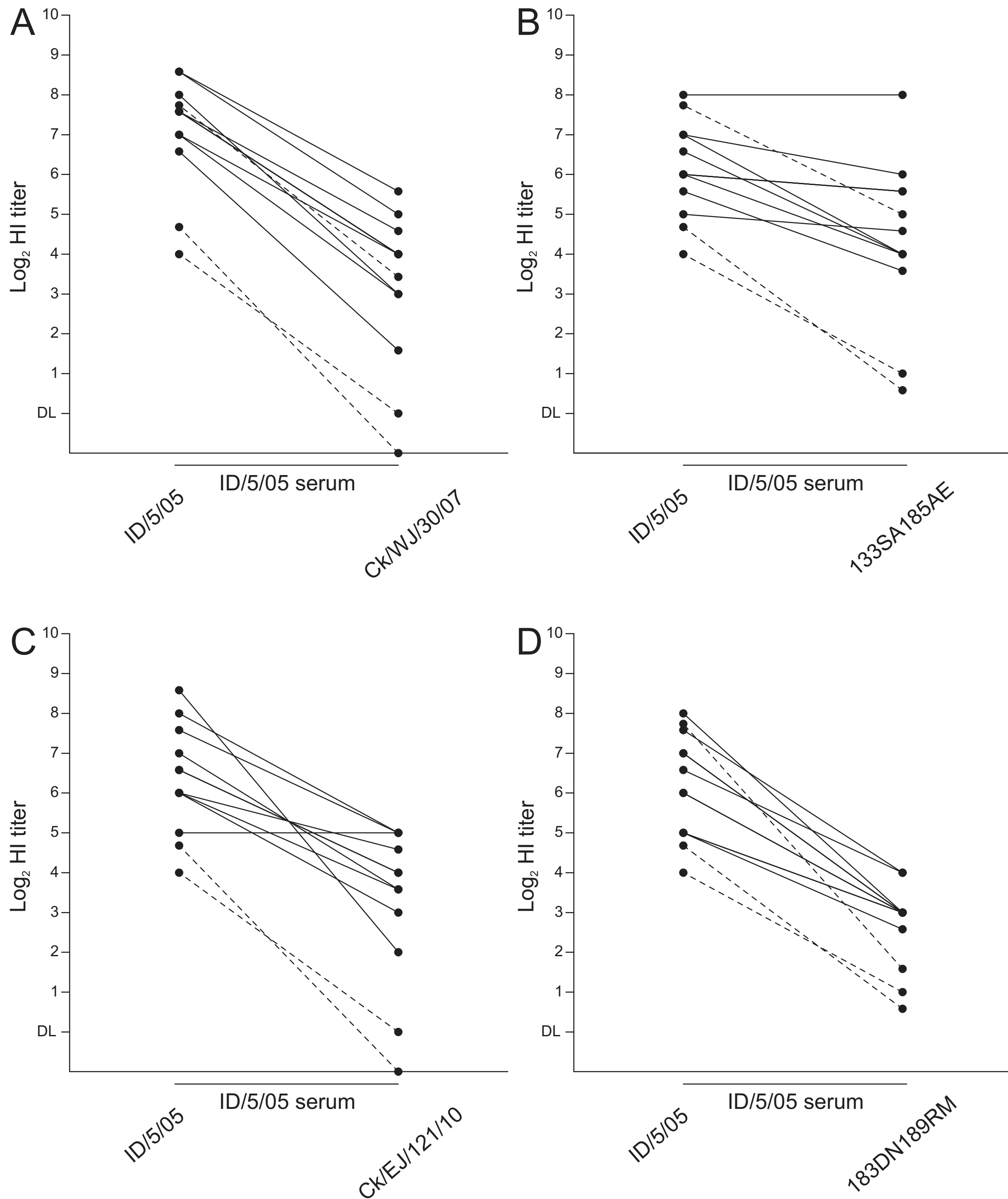

FIG 6 Reactivities of viruses with wild-type or mutant HAs to A/Indonesia/5/05 chicken antisera. Antisera obtained from chickens vaccinated with A/Indonesia/5/05 were tested in HI assays with A/Indonesia/5/05 and Ck/West Java/30/07 (A), A/Indonesia/5/05 and A/Indonesia/5/05 133SA 185AE (B), A/Indonesia/5/05 and Ck/East Java/121/10 (C), or A/Indonesia/5/05 and A/Indonesia/5/05 183DN 189RM (D). Solid lines connect the $\log_2$ HI titers obtained for viruses tested with the same chicken antiserum. Dashed lines connect $\log_2$ HI titers obtained from HI assays using ferret antisera. "DL" indicates the detection limit of the HI assay at the 1:20 starting dilution that was used.

viruses (Fig. 6B). The HI antibody titers against A/Chicken/East Java/121/10 were also at least 4-fold lower than the HI antibody titers to A/Indonesia/5/05 for 8 of the 10 A/Indonesia/5/05 antisera. The remaining two antisera had either a 2.7-fold-lower HI titer against A/Chicken/East Java/121/10 than against A/Indonesia/5/05 or had the same HI titers (Fig. 6C). The HI antibody titers against the 183DN 189RM mutant virus were at least 4-fold lower than those against A/Indonesia/5/05 with all 10 chicken antisera (Fig. 6D). These data thus show that 2 amino acid substitutions near the RBS can also be sufficient to substantially decrease recognition by chicken antibodies.

We next tested the effect of the same amino acid substitutions,

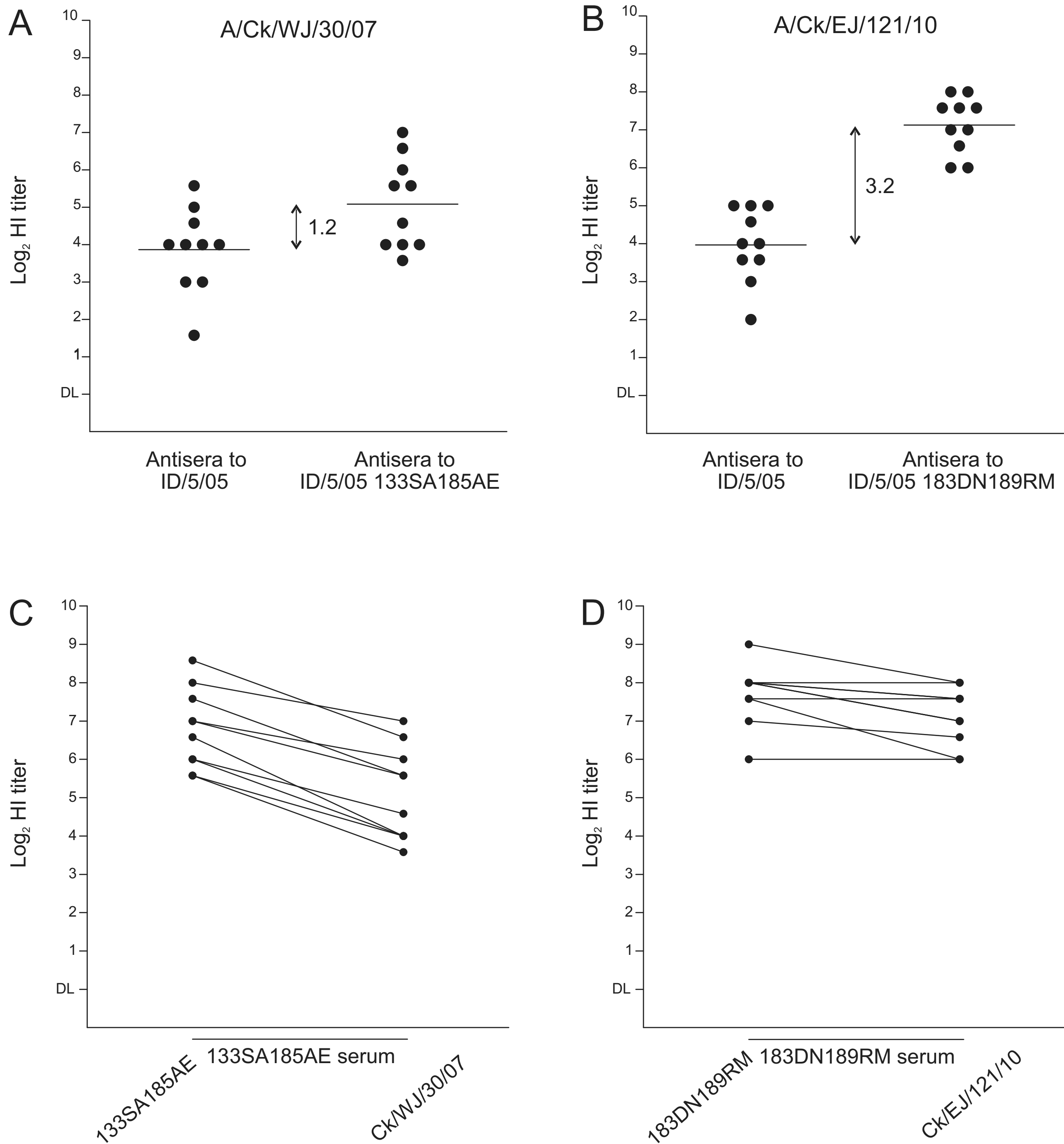

FIG 7 Effects of substitutions responsible for antigenic change on the antibody response of chickens. Antisera obtained from chickens vaccinated with A/Indonesia/5/05, A/Indonesia/5/05 with substitutions 133SA and 185AE, or A/Indonesia/5/05 with substitutions 183DN and 189RM were titrated in HI assays with A/Indonesia/5/05 and A/Chicken/West Java/30/07 (A) or A/Indonesia/5/05 and A/Chicken/East Java/121/10 (B). Each point represents the $\log_2$ HI titer for an individual antiserum. Horizontal lines show the mean $\log_2$ HI titer. The mean $\log_2$ HI titer difference is indicated between the groups. Antisera obtained from chickens vaccinated with A/Indonesia/5/05 with substitutions 133SA and 185AE or A/Indonesia/5/05 with substitutions 183DN and 189RM were titrated in HI assays with homologous virus and A/Chicken/West Java/30/07 (C) or homologous virus and A/Chicken/East Java/121/10 (D). Solid lines connect the $\log_2$ HI titers obtained for viruses tested with the same chicken antiserum. "DL" indicates the detection limit of the HI assay at the 1:20 starting dilution that was used.

133SA 185AE and 183DN 189RM, on the antibody response of chickens upon vaccination. To this end, we vaccinated chickens with A/Indonesia/5/05 with substitutions 133SA 185AE or A/Indonesia/5/05 with substitutions 183DN 189RM. These postvaccination antisera were compared with antisera raised against the wild-type A/Indonesia/5/05 vaccine upon titration in HI assays against A/Indonesia/5/05 and A/Chicken/West Java/30/07 or A/Chicken/East Java/121/10. Antisera prepared by vaccination with the 133SA 185AE mutant had on average 2.5-fold (1.2-$\log_2$)-higher HI antibody titers against A/Chicken/West Java/30/07 than did antisera prepared with the A/Indonesia/5/05 vaccine (Fig. 7A). Antisera prepared by vaccination with the 183DN 189RM mutant

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had on average 8.3-fold (3.2-$\log_2$)-higher titers to A/Chicken/East Java/121/10 than did antisera prepared with the A/Indonesia/5/05 vaccine (Fig. 7B). We then tested if antisera prepared against the 133SA 185AE vaccine inhibited the binding of A/Chicken/West Java/30/07 in HI assays and did the same for 183DN 189RM antisera and A/Chicken/East Java/121/10. Using the antisera raised against the 133SA 185AE vaccine, HI antibody titers against A/Chicken/West Java/30/07 were 2- to 4-fold lower than those to the homologous virus for 5 of 10 antisera, 4-fold lower for 4 antisera, and 6-fold lower for 1 antiserum (Fig. 7C). Using the antisera raised against the 183DN 189RM vaccine, the HI antibody titers against A/Chicken/East Java/121/10 and homologous virus were the same for 3 of the 10 antisera, less than 2-fold lower than that to the homologous virus for 6 of the 10 antisera, and 3-fold lower for 1 antiserum (Fig. 7D). Thus, amino acid substitutions responsible for antigenic change as defined with ferret antisera substantially affected the reactivity of chicken antisera upon vaccination.

DISCUSSION

Following the initial detection of H5N1 clade 2.1 viruses in 2003 in Central Java, the viruses diversified into three distinct genetic lineages (clades 2.1.1, 2.1.2, and 2.1.3) from 2004 onwards and became enzootic in most parts of Indonesia (1–3, 5, 19). Since 2004, the ongoing genetic diversification of clade 2.1.3 viruses necessitated further classification into fourth-order clades (2.1.3.1, 2.1.3.2, and 2.1.3.3), while viruses of clades 2.1.1 and 2.1.2 have apparently disappeared. However, data have been extremely sparse since 2008, which has hampered further analysis of clade 2.1 virus evolution. The present study shows that viruses from clades 2.1.3.1, 2.1.3.2, and 2.1.3.3 have circulated between 2008 and 2011, while viruses that belong to clade 2.1.1 or 2.1.2 were not detected. This finding further substantiates the suggestion that viruses of the latter clades are no longer circulating in poultry. Although viruses from clades 2.1.3.1 and 2.1.3.3 were isolated, the majority of viruses belonged to clade 2.1.3.2, indicating that this was the dominant genotype in recent years. The data set used in this study contains sequences from viruses isolated throughout Indonesia, but the majority of samples originate from outbreaks on the island of Java. This geographical bias is (at least in part) explained by the poultry population densities within Indonesia; 70% of the commercial poultry population and poultry production is on the island of Java.

We selected viruses to represent the most prominent recently circulating lineages and used these viruses to map the antigenic diversity of clade 2.1 viruses. Using antigenic cartography methods, we show that at least six antigenically distinct variants had circulated in Indonesia since 2003. Representative viruses that belonged to different phylogenetic clades or to distinct lineages within the same clade were generally also antigenically distinct. However, antigenic distances between the variants correlated poorly with distances in the phylogenetic tree or with the number of amino acid differences in HA1. As was suggested previously (16), antigenic differences between clade 2.1 viruses are therefore not necessarily predictable from (phylo)genetic information alone. Antigenic analysis of the representative viruses also indicated that new antigenic variants emerged with time but that these new variants did not replace previously circulating viruses *per se*, and existing antigenic variants continued to circulate alongside newly emerged, antigenically distinct viruses. However, the predominance of isolates with the 183DN 189RM substitutions that define the A/Chicken/East Java/121/10 antigenic variant in recent years may also signal ongoing directional selection toward viruses of this antigenic phenotype or genotype.

Previously, we reported that major antigenic changes during the evolution of seasonal human H3N2, H1N1, and influenza B viruses were caused by a very few amino acid substitutions that occurred exclusively at positions immediately adjacent to the RBS (18). Although amino acid substitutions that affect the antigenic properties of H3N2 and H1N1 influenza viruses can occur on positions distributed over large parts of the HA globular head (12–14), nature has selected for major antigenic change in these subtypes caused by substitutions at only a few positions located adjacent to the RBS. In the present study, we tested the hypothesis that the molecular basis for antigenic change in clade 2.1 viruses resembles that of seasonal human influenza viruses. Indeed, antigenic differences from A/Indonesia/5/05 were due to single, double, or, in a single case quadruple amino acid substitutions only. Each of these substitutions occurred at a small set of amino acid positions located immediately adjacent to the RBS. Five key positions are located on the membrane-distal periphery of the RBS and form a nearly continuous antigenic ridge, while the sixth position is located on the membrane-proximal part of the RBS and is located in the 130 loop that is involved in receptor binding.

The location of substitutions responsible for antigenic change in clade 2.1 viruses is thus remarkably similar to that of seasonal human influenza viruses. The key substitutions structurally map to positions comparable to those responsible for antigenic change in human H3N2 virus (Fig. 4) (18), and a single or very few substitutions near the RBS were responsible for the antigenic differences. Interestingly, antigenic change in a clade 2.2.1 virus from Egypt was also due to substitutions near the RBS (17). The combination of Δ129 and 151IT involved in the antigenic change from A/Indonesia/5/05 to A/Chicken/West Java/119/10 was observed in clade 2.2.1 viruses isolated between 2007 and 2009 (22). Consistent with the finding that Δ129 151IT results in a conformational change in the area around the RBS, we found that the combination of these amino acid changes resulted in reduced antibody recognition of clade 2.1 viruses.

We previously found that large changes in biophysical properties were involved in all major antigenic changes in H3N2 viruses (18). The antigenic changes in clade 2.1 viruses similarly involved substitutions with large changes in the biophysical properties of the amino acids responsible for the antigenic difference from A/Indonesia/5/05 to four of the five antigenic variants. Changes in charges of the amino acids seemed to play an important role in antigenic change in clade 2.1 viruses, as well as human H3N2 viruses. Interestingly, substitutions that involved hydrophilicity changes were common in clade 2.1 virus but were almost completely absent among substitutions that led to antigenic change in H3N2 viruses. Variation in the number of carbohydrate side chains on HA has been associated with changes in the antigenic properties of influenza A viruses because they can mask antibody epitopes (23, 24), but changes in glycosylation sites were not involved in the major antigenic changes in clade 2.1 viruses. The location of the amino acid positions involved in antigenic change in clade 2.1 viruses exclusively in the periphery of the RBS implies that clade 2.1 viruses, like seasonal human influenza viruses, evolved to specifically evade the antibodies directed at the RBS region. Follow-up studies looking into the effect of substitutions involved in antigenic change on receptor binding could help to

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understand potential limitations of influenza virus antigenic change.

Evolutionary patterns that become noticeable after extensive periods of virus circulation may not yet be clear in the relatively short period of HPAI H5N1 virus circulation in Indonesia. However, we observed some distinct differences in the antigenic evolution of clade 2.1 H5N1 and seasonal human influenza viruses. Human influenza viruses accumulate antigenically important substitutions over time. To avoid neutralization by antibodies raised to an earlier strain, antigenic evolution is typically away from previously circulating antigenic variants and shows a somewhat linear pattern (25). Moreover, antigenic variants are periodically replaced by newly emerging variants that can better evade population immunity. In contrast, the substitutions responsible for antigenic change in clade 2.1 viruses typically did not accumulate over time, and antigenic evolution did not follow a clear pattern away from earlier viruses. Additionally, multiple antigenic variants cocirculated in the same geographical region during multiple consecutive years. A plausible explanation for the different evolutionary patterns of seasonal human influenza viruses and clade 2.1 viruses is the difference in selective pressures acting on these viruses. The short life span of chickens, approximately 28 to 35 days for broilers and 18 months for breeders and layers in commercial poultry farms, prevents buildup of immunity to multiple antigenic variants in the poultry population. Similar to what was described by de Jong et al. for H3N2 viruses in pigs (26), the necessity of evading population immunity that was acquired through infection or vaccination with consecutive antigenic variants is absent in poultry. Population immunity is therefore unlikely to be a major driving force of clade 2.1 virus antigenic evolution, in contrast to the case with seasonal human influenza virus. Moreover, whereas antigenic variants of seasonal human influenza viruses can spread around the globe in a matter of months (27), the limited spatial and temporal mixing of chicken populations limits competition among antigenic variants and therefore may allow coexistence of multiple antigenically different viruses in the same geographic region of Indonesia.

Vaccination can be an efficient method to reduce the burden of HPAI H5N1 viruses in poultry (28–30). The current poultry vaccine strain recommendation for Indonesia is A/Chicken/West Java/30/07 or an antigenically similar strain. Viruses of this antigenic phenotype circulated until at least 2009. However, our analyses indicate that A/Chicken/East Java/121/10-like viruses have become the major antigenic variant in more recent years. Jadhao et al. (29) showed that chickens vaccinated with an antigenically poorly matched vaccine are fully protected from morbidity and mortality but shed virus at much higher levels than those vaccinated with an antigenically matched strain. Reduced virus shedding decreases the probability of further spread between poultry flocks and zoonotic events. It is therefore advantageous to pursue the use of vaccine strains that match the antigenic variants in circulation if vaccination is chosen as one of the approaches to stop or limit the spread of HPAI viruses.

Antigenic analysis of clade 2.1.3.2 viruses isolated from humans in 2011 and 2012 indicated that these viruses were antigenically different from A/Indonesia/5/05, which is the current candidate vaccine strain for human use. An updated vaccine based on an A/Indonesia/NIHRD11771/2011-like virus, which has the 183DN and 189RM substitutions typical for the A/Chicken/East Java/121/10-like viruses, is currently in preparation (31).

Updating the vaccines for poultry or human use to a contemporary strain that matches the major antigenic variant is an important measure that helps to ensure optimal vaccine efficacy. Given that one or two amino acid substitutions near the RBS can already substantially decrease recognition by ferret and chicken antibodies, the long-term efficacy of vaccines that target a single antigenic variant is questionable. We here have shown that vaccination of chickens with a recombinant virus that contains only two key substitutions for antigenic change can be sufficient to substantially change the reactivity of chicken antisera to antigenic variants. Recombinant viruses can be designed to contain the key amino acids necessary to match an antigenic variant while maintaining the growth characteristics endowed by other parts of the virus genome and can be constructed without multibasic cleavage site. Although the efficacy of such vaccines should be evaluated in *in vivo* models, antigenically engineered viruses may provide a means to quickly adapt the vaccine to emerging antigenic variants. However, antigenic variants have cocirculated in the same geographic region of Indonesia from 2005 onwards. These variants were isolated from chickens, thus excluding that they circulated exclusively in different host species. Therefore, any vaccine that is designed to neutralize a single antigenic variant may be of only limited use. The identification of persistent cocirculation of antigenic variants stresses the need for more potent, broadly reactive, next-generation vaccines. At the same time, this study underscores the need for continued monitoring of circulating influenza viruses in poultry, certainly when vaccines are employed to contribute to control of outbreaks.

Analysis of the amino acid sequence variation immediately adjacent to the RBS region of HA can be applied as a proxy to identify the most prominent antigenic phenotypes and could provide an early indication of emerging antigenic variants. Such genetic data should be complemented with antigenic data and vaccination challenge experiments with the circulating viruses to ensure that the vaccines continue to match the circulating viruses associated with outbreaks.

MATERIALS AND METHODS

Sample collection and virus isolation. Samples were collected during the investigations of outbreaks in both commercial and backyard poultry flocks from 2003 onwards. From 2008 onwards, samples were collected during outbreaks in commercial farms and backyard poultry throughout Indonesia as part of ongoing surveillance studies. Tracheal and oropharyngeal swabs and organ samples, typically the brain, trachea, lung, spleen, pancreas, and intestines, were used to inoculate specific-pathogen-free (SPF) embryonated chicken eggs, and allantoic fluids were tested for viral presence by hemagglutination assay. Subsequently, virus isolates were subject to H5N1 virus specific reverse-transcription PCR followed by sequencing of the full HA gene.

Construction of phylogenetic trees. The HA sequences of 96 H5N1 viruses isolated in Indonesia that were obtained as described above were combined with the publically available clade 2.1 HA sequences (1–3, 19). Phylogenetic tree construction of HA sequences was performed using the maximum-likelihood method available from the PAUP* software package (version 4.0b10) (32) under the GTR + I + Γ_4 model (the general time-reversible model with the proportion of invariant sites and the gamma distribution of among-site rate variation with four categories estimated from the empirical data) as determined by the ModelTest program (33). Global optimization of the tree topology was performed by tree bisection-reconnection branch swapping. The tree was rooted to A/Goose/Guangdong/1/1996.

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Cells. 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Cambrex, Heerhugowaard, The Netherlands) supplemented with 10% fetal calf serum (FCS), 100 μg/ml streptomycin, 100 IU/ml penicillin, 2 mM glutamine, 1 mM sodium pyruvate, and nonessential amino acids (MP Biomedicals, Europe, Illkirch, France). Madin-Darby canine kidney (MDCK) cells were cultured in Eagle minimal essential medium (EMEM) (Cambrex) supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodium bicarbonate (Cambrex), 10 mM HEPES (Cambrex), and nonessential amino acids (MP Biomedicals).

Plasmid construction and site-directed mutagenesis. A/Indonesia/5/05 was isolated from a human case of HPAI virus infection (7) and passaged once in embryonated chicken eggs, followed by a single passage in MDCK cells before amplification of the HA segment by reverse transcription-PCR. The HA gene of A/Chicken/North Sumatra/27/09 was synthesized by Life Technologies Corporation (Carlsbad, CA, USA), while full HA genes of other representative viruses (see Table S1 in the supplemental material) were synthesized by BaseClear (Leiden, The Netherlands). All HA genes were synthesized without a multibasic cleavage site and were cloned into the modified pHW2000 expression plasmid as described previously (34, 35). We were unable to construct the HA of A/Chicken/West Java/6-1/08. Mutations were introduced into the HA gene of A/Indonesia/5/05 using the QuikChange multisite-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions.

Generation of recombinant viruses. Plasmids containing wild-type or modified HA genes were used to generate recombinant viruses by reverse genetics as described elsewhere (34). Briefly, 1 day prior to transfection, 293T cells were seeded in 100-mm gelatinized culture dishes. Cells were transfected overnight with 40 μg of plasmid DNA. Subsequently, transfection medium was replaced by fresh medium supplemented with 2% FCS. After incubation for 72 h at 37°C/5% CO_2, the supernatant was harvested. Virus stocks were propagated by inoculation of MDCK cells with 2 ml 293T transfection supernatant. Inoculum was removed after 2 h and replaced by MDCK infection medium, consisting of EMEM (Cambrex), 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodium bicarbonate, 10 mM HEPES, nonessential amino acids, and 25 μg/ml trypsin. Viruses were harvested after incubation at 37°C/5% CO_2 for 72 h. All viruses were rescued as 6:2 viruses and contained six segments of A/Puerto Rico/8/1934 (A/H1N1), the neuraminidase (NA) gene of A/Hong Kong/156/1997 (H5N1), and the HA gene of the appropriate clade 2.1 viruses, except for A/Indonesia/5/05 and A/Chicken/West Java/30/07 that contained the NA from A/Indonesia/5/05. Absence of undesired mutations and presence of introduced substitutions was confirmed by sequence analysis.

Antisera. Ferret antisera were prepared by intranasal inoculation with 500 μl recombinant virus stock. Fourteen days postinoculation, ferrets were boosted using 500 μl of a 1:1 mix of concentrated virus ($>$2,048 hemagglutinating units [HAU]) and Titermax Gold adjuvant (Sigma-Aldrich, St. Louis, MO, USA). Antisera were collected 4 weeks later. Chicken antisera were prepared by subcutaneous vaccination of 4-week-old SPF White Leghorn chickens (Charles River Laboratories, Wilmington, MA) with 0.5 ml of a 1:1 mix of 512 HAU of virus and Montanide ISA50V (Seppic, France). Antisera were collected 3 weeks postvaccination. All antisera were pretreated overnight at 37°C with receptor-destroying enzyme (*Vibrio cholerae* neuraminidase), followed by inactivation for 1 h at 56°C before use in hemagglutination inhibition (HI) assays.

Serological assays. HI assays were performed following standard procedures (36). Briefly, 2-fold serial dilutions of the antisera, starting at a 1:20 dilution, were mixed with 25 μl of a virus stock containing 4 hemagglutinating units and were incubated at 37°C for 30 min. Subsequently, 25 μl of 1% turkey erythrocytes was added, and the mixture was incubated at 4°C for 1 h. The HI titer is expressed as the reciprocal value of the highest serum dilution that completely inhibited hemagglutination.

Antigenic cartography. Analysis of antigenic properties was performed using antigenic cartography methods as described previously (25). Briefly, antigenic cartography is a method to increase the resolution of, solve paradoxes in, and visualize HI assay data or other binding assay data. In an antigenic map, the distance between antigen point A and antiserum point S corresponds to the difference between the $\log_2$ value of the maximum observed titer to antiserum S from any antigen and the titer of antigen A to antiserum S. The titers in an HI table can be thought of as specifying target distances between the antigens and the antisera. Modified multidimensional scaling methods are then used to arrange the antiserum and antigen points in an antigenic map to best satisfy the target distances specified by the HI data. The distances between the points in an antigenic map represent antigenic distance as measured by the HI assay, in which the distances between antigens and antisera are inversely related to the $\log_2$ HI titer. Because antigens are tested against multiple antisera, and antisera are tested against multiple antigens, many measurements can be used to determine the position of the antigens and antisera in an antigenic map, thus improving the resolution of HI data.

Structural analysis. The amino acid positions responsible for antigenic change were plotted on the crystal structure of the A/Indonesia/5/2005 virus HA (PDB accession code 4K62 [37]). MacPyMOL (The PyMOL Molecular Graphics System, version 1.3; Schrödinger, LLC) was used to visualize the trimer.

Ethics statement. The surveillance program from which virus samples were derived was initiated following the recommendations of the Government of Indonesia to monitor vaccinated flocks and the environment. No special permits were required for this program since it included only flocks and the environment of farms associated with the authors' organizations. Ferrets were housed and experiments were conducted in strict compliance with European guidelines (EU directive on animal testing 86/609/EEC) and Dutch legislation (Experiments on Animal Act, 1997). The protocol was approved by the independent animal experimentation ethical review committee Stichting DEC Consult (Erasmus MC permit number EMC 2114) and was performed under animal biosafety level 3 (ABSL-3) conditions. Animal welfare was monitored daily, and all animal handling was performed under light anesthesia (ketamine) to minimize animal discomfort. Studies that involved the use of chickens were conducted under ABSL-3 conditions approved by USDA and performed according to R-12-51 protocols ("Inactivated vaccines against antigenic variants of highly pathogenic H5N1 influenza viruses") approved by the Institutional Animal Care and Use Committee of the University of Maryland. Chicken studies adhered strictly to U.S. Animal Welfare Act (AWA) laws and regulations.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01070-14/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.3 MB.
Figure S3, PDF file, 1.9 MB.
Figure S4, PDF file, 0.2 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOCX file, 0.1 MB.
Table S4, DOCX file, 0.1 MB.
Table S5, DOCX file, 0.1 MB.

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