JOURNAL OF VIROLOGY, Feb. 2008, p. 1798–1807 Vol. 82, No. 4
0022-538X/08/$08.00+0 doi:10.1128/JVI.02256-07

Antigenic Profile of Avian H5N1 Viruses in Asia from 2002 to 2007[▽]

Wai Lan Wu,[1]† Yixin Chen,[3,4]† Pui Wang,[1] Wenjun Song,[1] Siu-Ying Lau,[1] Jane M. Rayner,[1] Gavin J. D. Smith,[1] Robert G. Webster,[5] J. S. Malik Peiris,[1] Tianwei Lin,[1,4] Ningshao Xia,[3,4] Yi Guan,[1,2]* and Honglin Chen[1,2]*

State Key Laboratory for Emerging Infectious Diseases, Department of Microbiology and the Research Center of Infection and Immunology, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong SAR, People's Republic of China[1]; International Institute of Infection and Immunity, Shantou University, Shantou, People's Republic of China[2]; National Institute of Diagnostics and Vaccine Development in Infectious Diseases[3] and School of Life Sciences,[4] Xiamen University, Xiamen, People's Republic of China; and Virology Division, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee 38105[5]

Received 18 October 2007/Accepted 26 November 2007

Antigenic profiles of post-2002 H5N1 viruses representing major genetic clades and various geographic sources were investigated using a panel of 17 monoclonal antibodies raised from five H5N1 strains. Four antigenic groups from seven clades of H5N1 virus were distinguished and characterized based on their cross-reactivity to the monoclonal antibodies in hemagglutination inhibition and cell-based neutralization assays. Genetic polymorphisms associated with the variation of antigenicity of H5N1 strains were identified and further verified in antigenic analysis with recombinant H5N1 viruses carrying specific mutations in the hemagglutinin protein. Modification of some of these genetic variations produced marked improvement to the immunogenicity and cross-reactivity of H5N1 strains in assays utilizing monoclonal antibodies and ferret antisera raised against clade 1 and 2 H5N1 viruses, suggesting that these sites represent antigenically significant amino acids. These results provide a comprehensive antigenic profile for H5N1 virus strains circulating in recent years and will facilitate the recognition of emerging antigenic variants of H5N1 virus and aid in the selection of vaccine strains.

In 2003 human H5N1 infections reemerged in China following the first documented cases in 1997 (11, 32, 43, 57, 58). Subsequently, poultry outbreaks were observed across multiple countries in Southeast Asia (27, 42, 52). Surveillance studies of both chicken and duck in southern China showed that H5N1 virus had persisted in poultry and continued to circulate in waterfowl since 1998 (6, 7, 14), causing repeated outbreaks in chicken, possibly in the process of adapting to land-based poultry (27, 42). Genetic analysis indicates that after 1997 the H5N1 virus evolved into multiple genotypes (14). More recently, these multiple genotypes had been replaced by a single dominant strain, H5N1 genotype Z (27), that has caused outbreaks throughout Asia with sporadic transmission to humans (42, 52). The outbreak in migratory birds at Qinghai Lake, China, in early 2005 led to further expansion of the geographical distribution of H5N1 virus from Asia to Europe, the Middle East, and Africa (9, 12, 29, 36). It is recognized that the long-term endemicity of H5N1 virus in poultry has generated genetically and antigenically diversified viruses in some countries (8, 41). The unprecedented persistence of H5N1 virus in poultry since 1996 and continued human infection observed in multiple affected countries since 2003 have raised the concern that this virus might spark a pandemic.

It is uncertain when another influenza pandemic may occur and even difficult to determine whether the current global spread of H5N1 virus is a false alarm or an indication of another pandemic in the making. In preparation for the worst scenarios, it is believed an effective vaccine will be vital to alleviating the next pandemic. However, it is also anticipated that a vaccine may not be available until several months after a pandemic strain is identified. Several complicating factors differentiate the development of a pandemic H5N1 vaccine from the development of vaccines against seasonal human influenza strains, a program now well established and largely successful (40). First, a pandemic vaccine has never before been developed, meaning that researchers have no prior experience on which to base their efforts. Second, unlike human H1N1, H2N2, and H3N2 strains, the H5N1 virus causing human infections is still of the avian type (8, 27, 41, 54, 55), for which an epidemiological model for antigenic prediction has not been established (39). It is also not clear whether genetic variations in the hemagglutinin (HA) protein are a result of immune escape or associated with host adaptation (54). It would therefore be difficult to predict the likely match of H5N1 vaccines without understanding the antigenic determinants of H5N1 viruses. Two antigenic sites corresponding to sites A and B of the H3 subtype of the HA molecule were recently described by characterizing antibody escape mutants of a recombinant virus containing the HA and neuraminidase (NA) genes of A/Vietnam/1203/04 (A/VNM/1203/04), a clade 1 virus, in the background of A/Puerto Rico/8/34 (H1N1) virus (21). Genetic studies based on phylogenetic analysis of the HA gene have revealed that at least 10 clades of H5N1 viruses have emerged since 2000 (8, 36, 41, 52, 53). However, there has not

* Corresponding author. Mailing address: State Key Laboratory for Emerging Infectious Diseases, Department of Microbiology, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong SAR, People's Republic of China. Phone: 852 28199830. Fax: 852 28551241. E-mail for H. Chen: hlchen@hkucc.hku.hk. E-mail for Y. Guan: yguan@hkucc.hku.hk.
† W.L.W. and Y.C. contributed equally to this study.
▽ Published ahead of print on 12 December 2007.

been a systematic comparison of antigenicity between these genetic variants.

A comprehensive characterization of the antigenicity of H5N1 viruses isolated from the time when human infections reemerged in 2003 until the present is necessary to understanding the antigenic evolution of H5N1 viruses and would provide important information for vaccine selection. In this study, we developed a panel of more than 400 monoclonal antibodies (MAbs) that react with a wide range of H5N1 strains and used these antibodies to investigate the antigenic profiles of H5N1 viruses from different genetic lineages. Genetic polymorphisms on the HA protein were identified that are associated with antigenic variation. Manipulation of these polymorphic sites to improve immunogenicity of H5N1 viruses was explored, with a view to determine antigenically significant amino acids in HA protein.

MATERIALS AND METHODS

Virus propagation and titration. H5N1 viruses were grown in the allantoic cavities of 10-day-old embryonated chicken eggs at 35°C for 48 h. The allantoic fluid was harvested, and aliquots were stored at −80°C until use. Virus titer was determined by calculating the 50% tissue culture infectious dose ($TCID_{50}$) per ml of virus stock, using the method of Reed and Muench (34). Experiments using H5N1 virus were performed inside the biosafety level 3 facility at The University of Hong Kong.

Production of H5 monoclonal antibodies. Five representative H5N1 strains, Chicken/Hong Kong/YU22/2002 (Ck/HK/YU22/2002; clade 8), Duck/VNM/S654/2005 (Dk/VNM/S654/2005; clade 1), Dk/Indonesia/MS/2004 (Dk/IDN/MS/2004; clade 2.1), Barheadded goose/Qinghai/15C/2005 (BH goose/QH/15C/2005; clade 2.2), and Dk/VNM/568/2005 (clade 2.3) were chosen as immunogens for the preparation of anti-H5 MAbs by standard hybridoma technology (45). Based on phylogenetic analysis, these strains were selected from the major H5N1 clades in current circulation (clade 1, 2.1, 2.2, and 2.3) to cover the genetic and antigenic diversities of H5N1 viruses (8, 41). A clade 8 virus, Ck/HK/YU22/02, was included as it represents more ancestral strains isolated during an outbreak in Hong Kong in 2002 (27). Briefly, viruses were inactivated by adding formalin to a final concentration of 0.03%, and allantoic fluids were kept at 4°C for 72 h. Inactivation was confirmed by the absence of detectable infectivity after two blind passages of the treated allantoic fluids in embryonated chicken eggs. We then emulsified 400 μl of inactivated H5N1 virus, adjusted to a titer of 5×10^7 $TCID_{50}$/ml before inactivation, with an equal volume of Freund's complete adjuvant, and injected the virus subcutaneously into six-week-old female BALB/c mice at 2-week intervals. A final dose of 100 μl of inactivated virus was injected intravenously 3 days prior to fusion of spleen cells from the immunized mice with mouse myeloma cells (SP2/0-Ag14). The resulting hybridomas were screened for the secretion of H5-specific MAbs against Ck/HK/YU22/2002 and other non-H5 reference viruses by a hemagglutination inhibition (HI) assay, and positive clones were expanded and cultured in 75-cm^2 flasks. Cells that produced MAbs were then cloned by limiting dilution at least three times. MAbs were prepared by injection of hybridoma culture into the peritoneal cavities of pristine-primed BALB/c mice; the ascitic fluid was collected after 9 to 12 days and stored at −20°C. MAbs were purified using ammonium sulfate precipitation, followed by DE-52 ion exchange chromatography. Immunoglobulin concentrations were determined by spectrophotometry using a Perkin Elmer MBA 2000 spectrometer (Waltham, MA). Isotyping was performed by indirect enzyme-linked immunosorbent assay using mouse MAb isotyping reagent (BD Biosciences, San Jose, CA) as capture antibody and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin as a reporter antibody.

HI test. The HI test was performed to screen specific H5 MAbs and to assess the reactivity of ferret antiserum against different H5N1 isolates, in accordance with the World Health Organization (WHO) Manual on Animal Influenza Diagnosis and Surveillance (50). Ferret antisera against Dk/HN/101/04 (clade 2.3), A/IDN/5/05 (clade 2.1), BH goose/QH/1A/05 (clade 2.2), A/VNM/1203/04 (clade 2.1), and Swan/Mongolia/244/05 (clade 2.2) were kindly provided by the WHO Collaborating Center on the Ecology of Influenza Viruses in Lower Animals and Birds at St. Jude Children's Research Hospital (41, 56). All polyclonal antisera were treated by receptor-destroying enzyme (Denka Seiken Co., Tokyo, Japan) prior to testing. The HI test was started at a 1:40 dilution for polyclonal antiserum and 1:100 dilution for monoclonal antiserum and adsorbed with a 0.5% suspension of turkey red blood cells (Lampire Biological Laboratories, PA).

Neutralization assay. Determination of endpoint neutralizing antibody titers was performed by microneutralization assay, as previously described (22, 35). Briefly, serial 10-fold dilutions of MAbs were mixed and incubated with virus for 2 h at 37°C, and 35 μl of the mixture, containing 100 $TCID_{50}$ of virus, was then added to Madin-Darby canine kidney (MDCK) cells and allowed to adsorb for 1 h. Virus supernatant was removed and replaced with minimal essential medium plus antibiotics. The H5N1 virus-infected MDCK cells were incubated for 72 h at 37°C in the presence of 5% CO_2, and the neutralizing titer was determined by HA test. For the HA test, 50 μl of 0.5% turkey red blood cells (Lampire Biological Laboratories, Pipersville, PA) was added to 50 μl of cell culture supernatant and incubated at room temperature for 30 min. The neutralization titer was the lowest MAb dilution that was negative for hemagglutination.

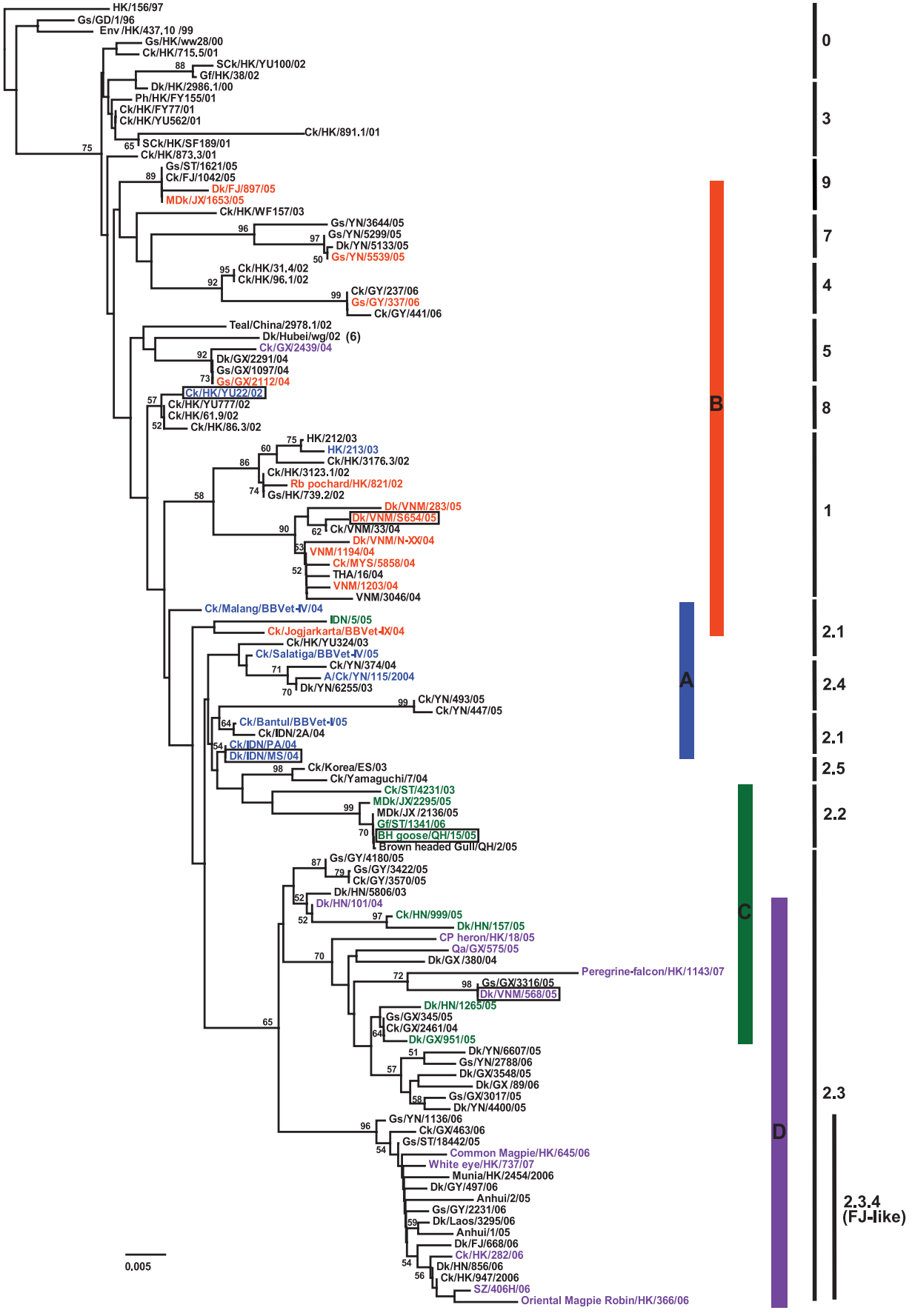
Generation of recombinant viruses. A/VNM/1194/04 and Chinese pond heron/HK/18/05 (CP heron/HK/18/05) viral RNAs were extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Full-length viral RNA segments were amplified using reverse transcription-PCR with segment-specific primers, as described elsewhere (18), and cloned into the pHW2000 vector, kindly provided by Eric Hoffmann of St. Jude Children's Research Hospital (17). Plasmids were sequenced to confirm that no mutations were introduced during the cloning procedure. Specific changes to the HA amino acid sequences of A/VNM/1194/04 and CP heron/HK/18/05 strains, described in Table 3, were introduced by site-specific mutagenesis (Stratagene, La Jolla, CA) and confirmed by sequencing. Recombinant viruses (rc-VNM/1194/04-6a, rc-VNM/1194/04-189, and rc-CP heron/HK/18/05) and their parental prototype viruses were generated by transfecting eight plasmids into mixed MDCK and 293T cells, as described previously (17), using a TransIT LT-1 kit (Mirus, Madison, WI). Recombinant viruses were rescued, and viral sequences were confirmed. HI and neutralization tests with recombinant viruses were carried out as described above.

Phylogenetic analysis and molecular characterization. Sequence assembly, editing, alignment, and residue analysis of the HA1 gene were performed as previously described (8). A neighbor-joining amino acid tree was constructed in MEGA 3.1 using the Jones-Tayler-Thornton amino acid replacement model with 1,000 bootstrap replicates (24).

RESULTS

Grouping of H5 MAbs. Over 400 H5 MAbs were raised against five genetically diverse strains of the H5N1 virus. Forty-one representative strains of H5N1 viruses were selected from 10 different genetic clades (Fig. 1), as defined by the WHO H5N1 virus evolution working group (53), and used to characterize the MAbs by HI testing. A group of 17 MAbs displaying various reactive spectra were selected for neutralization assay in MDCK cells (Table 1). Most of the MAbs showed similar patterns of reactivity in both neutralization assays and HI tests (data not shown), suggesting that they may bind to receptor binding regions. Based on the HI test and neutralization assay results, these MAbs were classified into three classes, as shown in Table 1. Class I antibodies possess broad reactive spectra and react well with almost all tested strains of H5N1 virus in the neutralization assay. Classes II and III have a comparatively more narrow reactive spectra, reacting with some H5N1 virus clades but exhibiting poor or no reactivity with other tested viruses. This panel of MAbs was used for the antigenic characterization of H5N1 viruses described below.

Antigenic classification of H5N1 virus. Forty-one H5N1 viruses selected from current genetic clades and tested in this study (Fig. 1) were, in turn, grouped according to their reactivity to the three classes of MAbs in the cell-based neutralization assay (Table 1). Group A viruses, comprised mainly of clade 2.1 viruses isolated from Indonesia, react to most members of the MAb classes. A clade 2.4 virus, Ck/Yunan/115/04 (Ck/YN/115/04), which is phylogenetically close to clade 2.1, showed a similar antigenic profile to other viruses in this group (Fig. 1). HK/213/03, a clade 1 virus in this antigenic group, has


HK/156/97
Gs/GD/1/96
Env /HK/437.10 /99
Gs/HK/ww28/00
Ck/HK/715.5/01
SCk/HK/YU100/02
Gf/HK/38/02
Dk/HK/2986.1/00
Ph/HK/FY155/01
Ck/HK/FY77/01
Ck/HK/YU562/01
Ck/HK/891.1/01
SCk/HK/SF189/01
Ck/HK/873.3/01
Gs/ST/1621/05
Ck/FJ/1042/05
Dk/FJ/897/05
MDk/JX/1653/05
Ck/HK/WF157/03
Gs/YN/3644/05
Gs/YN/5299/05
Dk/YN/5133/05
Gs/YN/5539/05
Ck/HK/31.4/02
Ck/HK/96.1/02
Ck/GY/237/06
Gs/GY/337/06
Ck/GY/441/06
Teal/China/2978.1/02
Dk/Hubei/wg/02 (6)
Ck/GX/2439/04
Dk/GX/2291/04
Gs/GX/1097/04
Gs/GX/2112/04
Ck/HK/YU22/02
Ck/HK/YU777/02
Ck/HK/61.9/02
Ck/HK/86.3/02
HK/212/03
HK/213/03
Ck/HK/3176.3/02
Ck/HK/3123.1/02
Rb pochard/HK/821/02
Gs/HK/739.2/02
Dk/VNM/283/05
Dk/VNM/S654/05
Ck/VNM/33/04
Dk/VNM/N-XX/04
VNM/1194/04
Ck/MYS/5858/04
THA/16/04
VNM/1203/04
VNM/3046/04
Ck/Malang/BBVet-IV/04
IDN/5/05
Ck/Jogjarkarta/BBVet-IX/04
Ck/HK/YU324/03
Ck/Salatiga/BBVet-IV/05
Ck/YN/374/04
A/Ck/YN/115/2004
Dk/YN/6255/03
Ck/YN/493/05
Ck/YN/447/05
Ck/Bantul/BBVet-I/05
Ck/IDN/2A/04
Ck/IDN/PA/04
Dk/IDN/MS/04
Ck/Korea/ES/03
Ck/Yamaguchi/7/04
Ck/ST/4231/03
MDk/JX/2295/05
MDk/JX /2136/05
Gf/ST/1341/06
BH goose/QH/15/05
Brown headed Gull/QH/2/05
Gs/GY/4180/05
Gs/GY/3422/05
Ck/GY/3570/05
Dk/HN/5806/03
Dk/HN/101/04
Ck/HN/999/05
Dk/HN/157/05
CP heron/HK/18/05
Qa/GX/575/05
Dk/GX /380/04
Peregrine-falcon/HK/1143/07
Gs/GX/3316/05
Dk/VNM/568/05
Dk/HN/1265/05
Gs/GX/345/05
Ck/GX/2461/04
Dk/GX/951/05
Dk/YN/6607/05
Gs/YN/2788/06
Dk/GX/3548/05
Dk/GX /89/06
Gs/GX/3017/05
Dk/YN/4400/05
Gs/YN/1136/06
Ck/GX/463/06
Gs/ST/18442/05
Common Magpie/HK/645/06
White eye/HK/737/07
Munia/HK/2454/2006
Dk/GY/497/06
Anhui/2/05
Gs/GY/2231/06
Dk/Laos/3295/06
Anhui/1/05
Dk/FJ/668/06
Ck/HK/282/06
Dk/HN/856/06
Ck/HK/947/2006
SZ/406H/06
Oriental Magpie Robin/HK/366/06
0.005
0
3
9
7
4
5
8
1
2.1
2.4
2.1
2.5
2.2
2.3
2.3.4
(FJ-like)
A
B
C
D

an HA position 223 amino acid substitution compared to other clade 1 viruses and was reported to display a unique antigenic feature (16, 27, 52). Group B primarily consists of clade 1 viruses, together with viruses from clades 4, 5, 7, and 9, which share similar polymorphism patterns in the HA protein (Table 2). This group of viruses reacts with class I and II but not class III MAbs. The antigenic similarity in viruses from different phylogenetic clades may be the result of sharing similar characteristics in the antigenic sites while maintaining their clade-specific sequences. Group C contains clade 2.2 Qinghai-like viruses and also viruses from clade 2.3. This group of viruses reacts with class I and III MAbs, similar to group A viruses, but not with class II MAbs, with the exception of 3C8 and, to a lesser extent, 9H12B (Table 1). One Qinghai-like virus isolated in 2006, Guinea fowl/Shantou/1341/06 (Gf/ST/1341/06) (41), showed no obvious difference in antigenicity to the Qinghai virus, BH Goose/QH/15/05, which was first isolated in early 2005 during the initial outbreak (9). Group D is composed almost exclusively of clade 2.3.4 Fujian-like viruses from the highly diverse clade 2.3 (Table 1 and Fig. 1) (41). A striking feature of group D viruses is that they react only with class I MAbs, displaying little or no reactivity with class II and the majority of III MAbs except 1G2 and 16F8. As previously reported, clade 2.3.4 viruses became prevalent in China from late 2005 onwards and were subsequently detected in Hong Kong, Laos, Malaysia, Thailand, and Vietnam (1, 10, 41). These viruses represent one of the four distinct groups of H5N1 viruses that are currently circulating, in addition to the Qinghai-like viruses (clade 2.2) (9, 12, 29, 36) and those in Indonesia (clade 2.1) and Vietnam (clade 1) (42, 52). Two clade 2.3 group D viruses, CP heron/HK/18/05 and Dk/Hunan/101/04 (Dk/HN/101/04) (8), showed very poor reactivity to most of the three classes of MAbs tested. The four antigenic groups defined here by MAbs are largely related to their phylogenetic classification (Fig. 1), with four major currently circulating clades falling into distinct antigenic groups (Table 1). It appears that the currently circulating clade 2 viruses are more antigenically diverse than other clades. The distinct antigenic features of group D viruses indicate that a major drift may have occurred.

Genetic polymorphisms and antigenic variation. To understand the molecular basis of the antigenic variation of H5N1 viruses, genetic mutations on the HA protein were analyzed. Table 2 shows the clusters of genetic polymorphisms identified among groups A, B, C, and D viruses. These genetic polymorphisms reflect the antigenic patterns revealed by MAb analysis (Table 1). Nine HA polymorphisms (amino acid residues 86, 94, 124, 129, 138, 140, 189, 212, and 263) were identified in the majority of group B H5N1 viruses (Table 2). Polymorphisms in group C were differentially distributed between clades 2.2 and 2.3; furthermore, the clade 2.3 viruses in group C carry mutations that are also prevalent in group D viruses. However, consistent with their distinct antigenic pattern, group D H5N1 viruses had 11 (amino acid residues 140, 155, 156, 174, 181, 189, 227, 269, 282, 310, and 322) genetic polymorphisms (Table 2). Based on the HA structure of H5N1 virus (15, 38), the majority of the polymorphic sites identified in Table 2 are exterior residues in the HA protein, except for 94, 174, and 263 that are in interior positions and not shown (Fig. 2). It remains to be investigated if those interior residues may contribute to antibody binding.

Taken together, genetic data demonstrate that each of the four antigenic groups of H5N1 virus carry distinct patterns of polymorphism in the HA molecule. Interestingly, many of the mutations listed in Table 2 are potential antigenic sites similar to the mapping of H3N2 virus antigenic structure (22, 49). Detailed characterization with MAbs is necessary to confirm the antigenic function of these sites.

Genetic polymorphisms associated with antigenicity. Two antigenic epitopes, corresponding to sites A and B of the H3 HA molecule, have recently been described in a recombinant virus containing the HA and neuraminidase of A/VNM/1203/04, but other antigenic sites are less clear (21). Similar genetic polymorphisms were observed within the group B viruses characterized in this study (Tables 1 and 2). To investigate if the polymorphic regions/sites identified in Table 2 are in fact associated with the altered immunogenicity of H5N1 viruses, we first selected group-specific mutations for site-directed mutagenesis. A neutralization assay showed that both group B and D viruses poorly reacted with class III MAbs (Table 1). One of the group B H5N1 viruses, VNM/1194/04, which has been well studied and recommended by WHO in the first group of prepandemic vaccine strains (2), was used to address this question. There are seven amino acid polymorphisms in VNM/1194/04 virus HA, and the majority of these positions correspond to antigenic epitopes as described in H3N2 human influenza virus (49). Most group B and D viruses, which demonstrated poor reactivity with class III MAbs, have a 189-Lys polymorphism compared to group A and C viruses.

Based on genetic polymorphism in the group B H5N1 viruses, two recombinant viruses were derived from VNM/1194/04 virus by site-directed mutagenesis: rc-VNM/1194/04-6a with six mutations at amino acid residues 86 (site E), 124 (site B), 129 (receptor binding site [RBS]), 189 (site B), 212 (site D), 263 (site E); and rc-VNM/1194/04-189 with a single mutation at residue 189 (Table 3). These site-directed mutations changed the residues to the consensus sequences of isolates shown in Tables 2 and 3. The recombinant HA genes were reconstituted into viruses together with the seven other parental segments using reverse genetic tech-

FIG. 1. Phylogenetic tree built with HA amino acid sequences illustrating the phylogenetic clades of viruses used in this study. Clades of H5N1 virus were classified according to recommended nomenclature of the WHO evolution working group, e.g., 2.1 indicates 2003 to 2007 from Indonesia (mixed avian/human). A detailed description of each clade is provided at the WHO website (53). A, B, C, and D represent four antigenic groups of H5N1 viruses characterized in this study with MAbs (Table 1) and are highlighted in blue, orange, green, and purple, respectively. Clade 2.3.4 was specified as it was also called "FJ-like" virus previously (41). Isolates of H5N1 virus used for generating monoclonal antibodies are labeled in a blank box. Abbreviations: Env, environment; FJ, Fujian; GD, Guangdong; Gs, goose; GX, Guangxi; GY, Guiyang; JX, Jiangxi; MDk, migratory duck; MYS, Malaysia; Ph, pheasant; Qa, quail; Rb pochard, rosybilled pochard; SCk, silky chicken; SZ, Shenzhen; THA, Thailand.

TABLE 1. Neutralization assay of H5N1 virus with monoclonal antibodies

Group	H5N1 virus[a]	Clade	Neutralization titer of the indicated MAb by class[b]																
			I						II				III						
			20A11	16F13	13D4	16G3	14E4	20H2	3C8	9H12B	16A12	14D4	1G2	3F1B8	16F8	4GE1	1D8	6CF3	10DD2
A	Ck/HKYU22/02	8	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200
	Ck/IDN/2A/04	2.1	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	800	≥3200	≥3200	≥3200	≥3200	≥3200	1600	≥3200	≥3200	≥3200
	Dk/IDN/MS/04	2.1	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	≥3200	≥3200	≥3200	800	≥3200	≥3200	≥3200
	Ck/Salatiga/BBVet1/05	2.1	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200
	Ck/Malang/BBVet4/04	2.1	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	<	≥3200	≥3200	≥3200	≥3200	<	≥3200	≥3200	≥3200
	HK213/03	1	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	1600	<	≥3200	≥3200	≥3200	<	≥3200	≥3200	≥3200	<
	Ck/Bantul/BBVet1/05	2.1	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200
	Ck/YN/115/04	2.4	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	1600	<	≥3200	≥3200	≥3200	800	≥3200	≥3200	≥3200
B	Gs/GX/2112/04	5	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	≥3200	≥3200	≥3200	400	<	≥3200	<	<	<	<
	Ck/Jogjakarta/BBVet-IX/04	2.1	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	≥3200	<	≥3200	≥3200	≥3200	<	<	<	<
	Dk/VNM/283/05	1	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	≥3200	≥3200	<	<	≥3200	<	400	<
	Rb pochard/HK/821/02	1	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	1600	≥3200	<	<	≥3200	<	<	<
	Ck/MYS/5858/04	1	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	1600	≥3200	800	<	<	<	<	<	<	<
	VNM/1194/04	1	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	1600	1600	1600	<	<	<	<	<	<	<	<
	VNM/1203/04	1	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	<	<	<	<	<	<	<
	Dk/VNM/N-XX/04	1	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	<	<	<	<	<	<	<
	Dk/VNM/S654/05	1	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	<	<	<	<	<	<
	MDk/JX/1653/05	9	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	≥3200	≥3200	≥3200	400	<	≥3200	<	<	<	<
	Dk/FJ/897/05	9	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	≥3200	≥3200	≥3200	<	<	≥3200	<	<	<	<
	Gs/GY/337/06	4	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	≥3200	≥3200	≥3200	<	<	<	<	<	<	<
	Gs/YN/5539/05	7	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	≥3200	≥3200	≥3200	<	<	<	<	<	<	<
C	IDN/5/05	2.1	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	≥3200	≥3200	<	≥3200	≥3200	≥3200	800	<	800	1600
	BH goose/QH15/05	2.2	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	≥3200	<	≥3200	≥3200	≥3200	800	≥3200	≥3200	≥3200
	Gf/ST/1341/06	2.2	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	<	<	≥3200	≥3200	≥3200	<	≥3200	≥3200	≥3200
	MDk/JX/2295/05	2.2	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	<	800	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200
	Ck/HN/999/05	2.3	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	<	<	≥3200	≥3200	≥3200	<	≥3200	<	≥3200
	Dk/HN/157/05	2.3	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	<	≥3200	≥3200	≥3200	800	≥3200	≥3200	≥3200
	Ck/ST/4231/03	2.2	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	400	<	≥3200	≥3200	≥3200	400	≥3200	≥3200	≥3200
	Dk/GX/951/05	2.3	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	800	<	400	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	800
	Dk/HN/1265/05	2.3	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	400	≥3200	≥3200	≥3200	≥3200	800	≥3200	≥3200
D	Ck/GX/2439/04	5	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	<	<	<	≥3200	<	≥3200	<	<	<	<
	Qa/Gx/575/05	2.3	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	400	<	<	<	≥3200	<	<	<	<	≥3200	<
	Common Magpie/HK/645/2006	2.3	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	ND	<	<	<	800	<	≥3200	<	<	<	<
	Oriental Magpie Robin/HK/366/2006	2.3	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	<	<	<	800	<	≥3200	<	<	<	<
	Ck/HK/282/2006	2.3	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	<	<	<	400	<	≥3200	<	<	<	<
	White eye/HK/737/07	2.3	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	<	<	<	≥3200	<	≥3200	<	<	<	<
	SZ/406H/2006	2.3	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	<	<	1600	1600	<	≥3200	<	<	<	<
	Dk/VNM/568/05	2.3	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	<	<	<	<	<	<	<	<	<	<
	Peregrine-falcon/HK/1143/07	2.3	≥3200	≥3200	≥3200	≥3200	≥3200	≥3200	<	800	<	<	<	<	<	≥3200	<	<	<
	Dk/HN/101/04	2.3	1600	800	1600	800	800	400	≥3200	<	<	400	≥3200	≥3200	<	<	<	<	<
	CP heron/HK/18/05	2.3	<	<	<	<	<	<	<	800	<	≥3200	<	1600	<	<	<	<	1600

[a] Abbreviations: FJ, Fujian; GD, Guangdong; Gs, goose; GX, Guangxi; GY, Guanyang; JX, Jiangxi; MDk, migratory duck; MYS, Malaysia; Qa, quail; Rb pochard, rosybilled pochard; SCk, silky chicken; SZ, Shenzhen (see also text).

[b] Virus neutralization titers of ≥3200, 400 to 1600, and <400 (<) were viewed as indicating good, moderate, and poor reactivity, respectively. Clade classification of H5N1 viruses was based on phylogenetic genetic analysis of amino acids, as shown in Fig. 1. MAbs were grouped into three classes (I, II, and III) based on their reaction patterns in the cell-based neutralizing assay. ND, not done.

TABLE 2. Genetic polymorphism of HA gene of H5N1 virus

Group	Virus[a]	Clade	Residue at the indicated antigenic site and position[b]:																			
			Site E		Site B	RBS	Site A		Site B	Glyco				Site B	Site D		Site E			Site C		
			86	94	124	129	138	140	155	156	174	181	183	189	212	227	263	269	277	282	310	322
A	Ck/HK/YU22/02	8	A	**D**	**N**	S	Q	K	S	S	V	P	D	R	K	E	A	L	K	M	R	Q
	Ck/IDN/2A/04	2.1	A	N	D	S	Q	K	S	T	V	P	D	R	K	E	A	L	K	M	R	Q
	Dk/IDN/MS/04	2.1	A	N	D	S	Q	K	S	A	V	P	D	R	K	E	A	L	K	M	R	Q
	Ck/Salatiga/BBVet-IV/05	2.1	A	N	D	S	**L**	**R**	S	S	V	P	D	R	K	E	A	L	K	M	R	Q
	Ck/Malang/BBVet-IV/04	2.1	A	N	**N**	S	Q	K	S	A	V	P	D	R	K	E	A	L	K	M	R	Q
	HK213/03	1	A	**D**	**S**	**L**	Q	K	**N**	A	V	P	D	R	K	E	A	L	K	M	R	Q
	Ck/Bantul/BBVet-I/05	2.1	A	N	D	S	Q	K	S	T	V	P	D	R	K	E	A	L	K	M	R	Q
	Ck/YN/115/04	2.4	A	N	D	S	**L**	**R**	S	S	V	P	D	R	K	**D**	A	L	K	**V**	R	Q
B	Gs/GX/2112/04	5	A	**D**	**N**	S	Q	**R**	S	A	V	P	D	**K**	K	E	A	L	K	M	R	Q
	Ck/Jogjakarta/BBVet-IX/04	2.1	A	**S**	D	S	**L**	**R**	S	T	V	P	D	**K**	K	E	A	L	K	M	R	Q
	Dk/Vietnam/283/05	1	**V**	**D**	**S**	**L**	Q	**R**	S	T	**I**	P	D	R	**R**	E	**T**	L	K	M	R	Q
	Dk/HK/821/02	1	A	**D**	**S**	**L**	Q	K	S	A	V	P	D	R	K	E	A	L	K	M	R	Q
	Ck/Malaysia/5858/04	1	**V**	**D**	**S**	**L**	Q	K	S	T	V	P	D	**K**	**R**	E	**T**	L	K	M	R	Q
	VNM/1194/04	1	**V**	**D**	**S**	**L**	Q	K	S	T	V	P	D	**K**	**R**	E	**T**	L	K	M	R	Q
	VNM/1203/04	1	**V**	**D**	**S**	**L**	Q	K	S	T	V	P	D	**K**	**R**	E	**T**	L	K	M	R	Q
	Dk/VNM/N-XX/04	1	**V**	**D**	**S**	**L**	Q	K	S	T	V	P	D	**K**	**R**	E	**T**	L	K	M	R	Q
	Dk/VNM/S654/05	1	**V**	**D**	**S**	**L**	Q	K	S	T	V	P	D	**K**	**R**	E	**T**	L	K	M	R	Q
	MDk/JX/1653/05	9	A	**D**	**N**	S	**L**	K	S	A	V	P	D	**K**	K	E	A	L	K	M	R	Q
	Dk/Fujian/897/05	9	A	**D**	**N**	S	**L**	K	S	A	V	P	D	**K**	K	E	A	L	K	M	R	Q
	Gs/GY/337/06	4	A	**D**	**N**	**L**	**L**	E	S	S	V	P	D	**K**	**T**	E	A	L	K	M	R	Q
	Gs/YN/5539/05	7	A	**D**	**N**	**L**	**L**	K	**N**	T	V	P	D	**K**	K	E	A	L	K	M	R	Q
C	IDN/5/05	2.1	**T**	**S**	D	S	**L**	**S**	S	T	V	P	D	R	K	E	A	L	K	M	R	Q
	BH goose/QH15/05	2.2	A	N	D	S	Q	**R**	**N**	A	V	P	D	R	K	E	**T**	L	K	**I**	R	Q
	Gf/ST/1341/06	2.2	A	N	D	S	Q	**R**	**N**	A	V	P	D	R	K	E	**T**	L	K	**I**	R	Q
	MDk/JX/2295/05	2.2	A	N	D	S	Q	**R**	**N**	A	V	P	D	R	K	E	**T**	L	K	**I**	R	Q
	Ck/HN/999/05	2.3	**V**	N	D	S	Q	K	**N**	A	V	P	D	R	K	**D**	A	**V**	K	M	**K**	Q
	Dk/HN/157/05	2.3	**V**	N	D	S	Q	K	**N**	A	V	P	D	R	K	**D**	A	**V**	K	M	**K**	Q
	Ck/ST/4231/03	2.2	A	N	D	S	Q	**R**	S	T	V	P	D	R	K	E	A	L	K	**I**	R	Q
	Dk/GX/951/05	2.3	A	N	D	**L**	Q	**S**	**N**	A	V	P	D	R	K	**D**	A	**V**	K	M	**K**	Q
	Dk/HN/1265/05	2.3	A	N	D	**L**	Q	**S**	**N**	A	V	P	D	R	K	**D**	A	**V**	K	M	**K**	Q
D	Ck/GX/2439/04	5	A	**D**	**N**	S	Q	**R**	S	A	V	P	D	**K**	**R**	**X**	A	L	K	M	R	Q
	Qa/Gx/575/05	2.3	A	N	D	**L**	Q	**S**	**N**	A	V	P	D	R	K	**D**	A	**V**	K	M	**K**	Q
	Common Magpie/HK/645/2006	2.3	A	N	D	S	Q	**T**	**N**	T	**I**	**S**	D	**K**	K	**D**	A	**V**	K	**I**	**K**	**L**
	Oriental Magpie Robin/HK/366/2006	2.3	A	N	D	S	Q	**T**	**N**	T	**I**	**S**	D	**K**	K	**D**	A	**V**	K	**I**	**K**	**L**
	Ck/HK/282/2006	2.3	A	N	D	S	Q	**T**	**N**	T	**I**	**S**	D	**K**	K	**D**	A	**V**	K	**I**	**K**	**L**
	White eye/HK/737/07	2.3	A	N	D	S	Q	**T**	**N**	T	**I**	**S**	D	**K**	K	**D**	A	**V**	K	**I**	**K**	**L**
	SZ/406H/2006	2.3	A	N	D	S	Q	**T**	**N**	T	**I**	**S**	D	**K**	K	**D**	A	**V**	K	**I**	**K**	**L**
	Dk/VNM/568/05	2.3	A	N	D	L	Q	**N**	**D**	A	V	P	D	R	K	**D**	A	**V**	K	M	**K**	Q
	Peregrine-falcon/HK/1143/07	2.3	A	**A**	**N**	**L**	Q	**N**	S	A	V	P	D	R	K	**D**	**T**	**V**	R	M	R	Q
	Dk/HN/101/04	2.3	A	N	D	S	Q	K	**N**	A	V	P	D	R	K	**D**	A	**V**	K	M	**K**	Q
	CP heron/HK/18/05	2.3	A	N	**N**	**V**	Q	**S**	**N**	A	V	P	**N**	**M**	K	**D**	A	**V**	**R**	M	**K**	Q
	Consensus		A	N	D	S	Q	K	S		V	P	D	R	K	E	A	L	K	M	R	Q

[a] Abbreviations: FJ, Fujian; GD, Guangdong; Gs, goose; GX, Guangxi; GY, Guanyang JX, Jiangxi; MDk, migratory duck; MYS, Malaysia; Qa, quail; Rb pochard, rosybilled pochard; SCk, silky chicken; SZ, Shenzhen (see also text); Glyco, glycosylation.

[b] The amino acid residue positions are derived from mature H5 sequence. Residues in boldface indicate polymorphisms in the HA sequence of the H5N1 viruses compared with the consensus sequence.

nology (17). The neutralization assay demonstrated that reactivity with MAbs was improved in rc-VNM/1194/04-6a (Table 4). Virus rc-VNM/1194/04-6a showed higher reactivity with all class III MAbs and most class II MAbs in comparison to the group B prototype virus VNM/1194/04. In contrast, rc-VNM/1194/04-189 showed no significant improvement in reactivity to the prototype virus in any of three MAb classes. This suggests that 189-Lys alone has little effect on antigenic variation in group B viruses. However, it remains to be investigated if interaction between HA resi-

TABLE 3. Amino acid residue positions in the HA gene selected for site mutagenesis

Virus	Residue at amino acid position[a]:							
	86	124	129	183	189	212	263	277
VNM/1194/04	V	S	L	D	K	R	T	K
rc-VNM/1194/04-6a	**A**	**D**	**S**	D	**R**	**K**	**A**	K
rc-VNM/1194/04-189	V	S	L	D	**R**	R	T	K
CP heron/HK/18/05	A	N	V	N	M	K	A	R
rc-CP heron/HK/18/05	A	N	**S**	**D**	**R**	K	A	**K**

[a] Mutated amino acid residues are in boldface.

due 189 and the other five amino acids examined in this study may be important for antibody binding (5, 22, 49).

HI (data not shown) and neutralization assays (Table 1) showed that CP heron/HK/18/05 (group D) reacted poorly with virtually all MAbs used in this study, which provides a good prototype for investigating the association of genetic mutations and antigenic variation in H5N1 virus. Comparison of the HA sequence of this virus with consensus sequences of other H5N1 viruses revealed that CP heron/HK/18/05 carried 10 mutations against the consensus (Table 2). Among them, 129-Val, 183-Asn, and 189-Met are unique mutations and found only in CP heron/HK/18/05, while 277-Arg was shared with just a single virus (Table 2). Mutations at positions 129 and 189, but with different amino acid residues, were also seen in VNM/1194/04 (Table 2).

We then examined if these four polymorphisms at positions 129, 183, 189, and 277 might contribute to the loss of reactivity of CP heron/HK/18/05 to class I, II, and III MAbs (Table 1). Antigenic tests with recombinant virus (rc-CP heron/HK/18/05) in both the HI test (data not shown) and neutralization assay (Table 4) showed marked improvement in reactivity with all class I and most class III MAbs but no obvious improvement against class II MAbs, suggesting that the targeted genetic variations are associated with the binding sites of class I and III MAbs.

Ferret antiserum has been commonly used for the evaluation of influenza virus antigenicity (40, 51, 52). The HA-modified recombinant derivatives of rc-VNM/1194/04 and rc-CP heron/HK/18/05 were tested for reactivity to antisera raised against different H5N1 strains (Table 5). Both HA-modified rc-VNM/1194/04 and rc-CP heron/HK/18/05 exhibited improved reactivity to a panel of ferret antisera over that of their parental strains in the HI test. More importantly, both HA-modified recombinant H5N1 strains cross-react with reference ferret serum raised against clade 1 and 2 H5N1 viruses. The broad cross-reactivity of the HA-modified strains to antiserum raised against antigenically different strains demonstrates the contribution of these genetic mutations to the lack of cross-clade antigenic reactivity among antigenically diverse H5N1 viruses. Taken together, these results demonstrate that the HA polymorphisms identified in this study are associated with H5N1 virus antigenicity and that manipulation of these sites may improve viral immunogenicity.

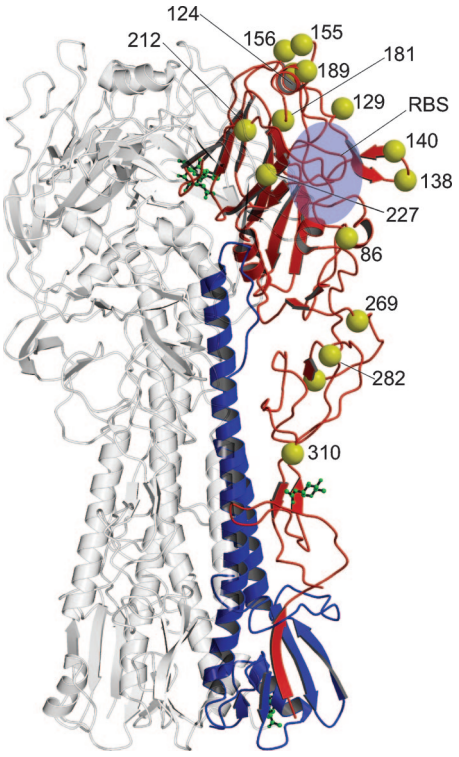


FIG. 2. Ribbon diagram of the trimeric HA molecule with the mutations shown as yellow spheres. The majority of the polymorphic sites (Table 2) are exterior residues, with the exception of residues 94, 174, and 263 (not shown). The RBS is indicated with a blue oval. The reference monomer is drawn in color, and the other two monomers are shown in gray. In the reference monomer, polypeptide HA1 is in red, polypeptide HA2 is in blue, and the polysaccharides are drawn in ball-and-stick form in green. The diagram was prepared with the program MOLSCRIPT (23) and Raster3D (30).

TABLE 4. Comparison of antigenicity of recombinant viruses in neutralization assay using MAbs

Virus	Neutralization titer of the indicated MAb by class[a]																
	I						II				III						
	20A11	16F13	13D4	16G3	14E4	20H2	3C8	9H12B	16A12	14D4	1G2	3F1B8	16F8	4GE1	1D8	6CF3	10DD2
VNM/1194/04	12,800	3,200	12,800	12,800	6,400	12,800	1,600	1,600	1,600	<	<	<	<	<	<	<	<
rc-VNM/1194/04-6a	12,800	12,800	12,800	12,800	12,800	12,800	12,800	1,600	12,800	<	12,800	12,800	12,800	<	3,200	12,800	12,800
rc-VNM/1194/04-189	12,800	12,800	12,800	12,800	12,800	12,800	12,800	400	<	200	200	<	<	<	<	<	<
CP heron/HK/18/05	<	<	<	<	<	<	100	800	<	12,800	12,800	1,600	100	<	<	<	1600
rc-CP heron/HK/18/05	12,800	12,800	12,800	12,800	12,800	12,800	3,200	200	<	12,800	12,800	12,800	12,800	400	12,800	12,800	12,800

[a] Same panel of MAbs as in Table 1. rc, recombinant; <, <100.

TABLE 5. Comparison of antigenicity of recombinant viruses in HI test using ferret antiserum[a]

Virus	HI titer of the indicated virus in ferret antiserum:				
	Dk/HN/101/04 (clade 2.3)	BH goose/ QH/1A/05 (clade 2.2)	IDN/5/05 (clade 2.1)	VNM/1203/04 (clade 1)	Swan/Mongolia/244/05 (clade 2.1)
Dk/HN/101/04	*640*	2,560	1,280	80	320
BH goose/QH/1A/05	320	*640*	640	40	*320*
IDN/5/05	80	320	*1,280*	40	40
VNM/1203/04	80	80	160	*160*	<40
CP heron/HK/18/05	80	80	80	40	<40
rc-CP heron/HK/18/05	**320**	**1,280**	**1,280**	**80**	**160**
VNM/1194/04	<40	<40	80	80	<40
rc-VNM/1194/04-6a	**320**	**640**	**1,280**	**80**	**80**
rc-VNM/1194/04-189	**80**	**160**	**640**	**80**	**40**

[a] Values in italics are HI titers with homologous H5N1 strains which were used to generate ferret antiserum. Viruses and values in boldface are genetically modified H5N1 viruses described in this study and their respective HI titers.

DISCUSSION

Antigenic drift of influenza virus is caused by an accumulation of mutations in the epitope regions of the HA to which neutralizing antibodies bind (38, 46, 49). Drift on viral surface proteins, mainly the HA, allows the virus to evade antibody binding within the host (40, 46). Prediction of antigenic drift is a factor in determining if a vaccine will match the circulating strain (3, 40). Though highly pathogenic avian influenza H5N1 virus has been continuously circulating for more than a decade in Asia, antigenic drift in H5N1 viruses has not been fully evaluated. The current understanding of influenza virus antigenic drift is based solely on the analysis of the cartography of human H3N2 and H1N1 influenza viruses, which are under the selection of population-level immunity (4, 31, 39, 47, 49). Unlike human influenza virus subtypes, H5N1 viruses have circulated only in domestic and wild birds to date (48). Avian influenza viruses are thought to maintain a stable antigenic and nonpathogenic status in their natural hosts, wild aquatic birds (46). Poultry has a relatively short life span, and massive seasonal vaccinations of poultry against avian influenza virus are a recent development (25, 33). In unvaccinated aberrant hosts, such as chicken, infection with highly pathogenic H5N1 virus is lethal (13, 20, 37). Thus, accumulation of mutations on the HA of avian influenza virus may not be caused by continuous antibody selection, as has occurred with human influenza viruses. Alteration of receptor binding specificity by introducing mutations onto avian H5 influenza HA also caused changes in virus immunogenicity (55), making it unclear if the model for H3N2 would apply for H5N1 viruses (40). Therefore, a working model for predicting the antigenicity of H5N1 virus is necessary in preparation for a potential pandemic (39).

The traditional approach using antisera raised from immunized ferrets provided a gold standard for evaluating antigenic changes to human influenza viruses (40), and a similar methodology was adopted for H5N1 virus, as recommended by the WHO (8, 12, 41, 52). Antiserum must be updated with newly circulating strains and then used to characterize emerging antigenic variations. Cocirculation of multiple antigenic variants of H5N1 virus hampers the timely production of standard antiserum for antigenic analysis. The highly pathogenic and avian features, including an avian-type receptor binding preference, of H5N1 virus also increase the difficulty of generating H5N1-specific ferret antisera (56). MAbs have been used for identifying antigenic epitopes on the HA of influenza virus but less commonly for antigenic profiling because each antibody binds only to a single epitope (5, 19, 21). This study evaluated the use of a group of 17 MAbs selected from a large panel of 400 clones that may provide an alternative for antigenic profiling of H5N1 viruses, in addition to the traditional practice of using subtype-specific ferret antiserum. With this approach, a newly emerged antigenic variant can be characterized before a ferret antiserum is available. More importantly, as each MAb maps to a specific epitope, this approach can also provide information on genetic variations that may be associated with antigenic change.

Consistent with our previous genetic analysis, antigenic profiling with MAbs demonstrated that H5N1 virus has undergone rapid antigenic changes since 2003 (8, 27, 41). Three antigenically distinct groups (A, C, and D) of H5N1 viruses are composed mainly of clade 2 viruses, which are currently cocirculating in broad regions and have evolved into several genetically distinct subclades (53). H5N1 viruses in the same phylogenetic group generally share similar antigenic properties in cell-based neutralization experiments, with some exceptions. While the majority of clade 2.1 viruses are antigenically classified as group A (Table 1), Ck/Jogiarkarta/BBVet-lX/04 and IDN/5/05 were classified in groups B and C, respectively (Table 1). The different antigenic features of viruses in the same genetic clade may be the result of limited mutations in the antigenically important amino acids. This may also explain the classification of some clade 2.3 viruses in group C with clade 2.2 viruses (Fig. 1). Virus phylogeny also does not correlate with antigenic properties in group B, which includes viruses from five different genetic clades (Table 1). It is interesting that viruses in group D, which represent a dominant genetic variant (clade 2.3.4) in southern China, showed a distinctive antigenic profile that may imply a major antigenic drift has occurred. It remains to be seen if the recent development of large-scale vaccination of poultry in some Asian countries has contributed to this phenomenon (33).

A broadly cross-protective vaccine for antigenic variants of H5N1 virus will be a very important component in strategizing prepandemic-strain vaccine development. Several vaccine strains derived from clade 1 viruses, which are currently under

different stages of clinical trials, showed limited cross-clade reactivity (2, 28, 44). Application of adjuvant was shown to improve immunogenicity and induce cross-reactive immunity by A/VNM/1194/04 H5N1 (clade 1) pandemic-virus vaccine (26). On the other hand, additional H5N1 vaccine viruses selected from clade 2 have been recommended by WHO for the development of prepandemic-strain vaccines in response to the rapid evolution of H5N1 virus and the cocirculation of multiple antigenic variants in multiple regions (51). However, there is a lack of data showing that these strains will provide desirable cross-protection between antigenically distinctive clade 1 and increasingly diversified clade 2 H5N1 viruses. This study characterized the clusters of genetic polymorphisms associated with the antigenic variations of H5N1 viruses to in vitro neutralization reactivity of MAbs. Modification of some of the genetic variations brought about drastic improvements to antigenic reactivity of two H5N1 strains in assays utilizing both MAbs and ferret antiserum, suggesting that these amino acids may associate with antigenic sites. Further mapping of these sites with those MAbs is necessary. It is important to investigate whether these modified HAs may serve as better immunogens in inducing broad cross-neutralizing antibodies in animal models. As H5N1 viruses are still of avian type, it would also be necessary to evaluate if these sites are recognized by human antibodies. Recent studies using recombinant H5N1 virus with altered receptor binding specificity to human-type receptors elicited more effective neutralizing MAbs that inhibit such variants (16, 54, 55). Therefore, it may be worthwhile to explore the vaccine potential of H5N1 HA with alterations of both receptor binding affinity and antigenically significant amino acids in a virus-like particle system.

ACKNOWLEDGMENTS

We thank Vijaykrishna Dhanasekaran and Justin Bahl for assistance in phylogenetic analysis and Hung Tai and Chung-Lam Cheung for technical assistance in DNA sequencing.

This study was supported by the Area of Excellence Scheme of the University Grants Committee (Grant AoE/M-12/06), the Research Grants Council of the Hong Kong SAR Government (RGC7619/07 M), the National Institutes of Health (NIAID contract HHSN2662007 00005C), the Research Fund for the Control of Infectious Diseases of the Health, Welfare and Food Bureau of the Hong Kong SAR Government, the National Key Technology R&D Program of China (grants 2004BA519A73 and 2006BAI01B06), National Natural Science Foundation of China (30640017), and the Li Ka Shing Foundation.

REFERENCES

1. **Boltz, D. A., B. Douangngeun, S. Sinthasak, P. Phommachanh, S. Rolston, H. Chen, Y. Guan, J. S. Peiris, G. J. D. Smith, and R. G. Webster.** 2006. H5N1 influenza viruses in Lao People's Democratic Republic. Emerg. Infect. Dis. **12:**1593–1595.
2. **Bresson, J. L., C. Perronne, O. Launay, C. Gerdil, M. Saville, J. Wood, K. Hoschler, and M. C. Zambon.** 2006. Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomised trial. Lancet **367:**1657–1664.
3. **Bush, R. M., C. A. Bender, K. Subbarao, N. J. Cox, and W. M. Fitch.** 1999. Predicting the evolution of human influenza A. Science **286:**1921–1925.
4. **Bush, R. M., W. M. Fitch, C. A. Bender, and N. J. Cox.** 1999. Positive selection on the H3 hemagglutinin gene of human influenza virus A. Mol. Biol. Evol. **16:**1457–1465.
5. **Caton, A. J., G. G. Brownlee, J. W. Yewdell, and W. Gerhard.** 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell **31:**417–427.
6. **Cauthen, A. N., D. E. Swayne, S. Schultz-Cherry, M. L. Perdue, and D. L. Suarez.** 2000. Continued circulation in China of highly pathogenic avian influenza viruses encoding the hemagglutinin gene associated with the 1997 H5N1 outbreak in poultry and humans. J. Virol. **74:**6592–6599.
7. **Chen, H., G. Deng, Z. Li, G. Tian, Y. Li, P. Jiao, L. Zhang, Z. Liu, R. G. Webster, and K. Yu.** 2004. The evolution of H5N1 influenza viruses in ducks in southern China. Proc. Natl. Acad. Sci. USA **101:**10452–10457.
8. **Chen, H., G. J. D. Smith, K. S. Li, J. Wang, X. H. Fan, J. M. Rayner, D. Vijaykrishna, J. X. Zhang, L. J. Zhang, C. T. Guo, C. L. Cheung, K. M. Xu, L. Duan, K. Huang, K. Qin, Y. H. Leung, W. L. Wu, H. R. Lu, Y. Chen, N. S. Xia, T. S. Naipospos, K. Y. Yuen, S. S. Hassan, S. Bahri, T. D. Nguyen, R. G. Webster, J. S. Peiris, and Y. Guan.** 2006. Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. Proc. Natl. Acad. Sci. USA **103:**2845–2850.
9. **Chen, H., G. J. D. Smith, S. Y. Zhang, K. Qin, J. Wang, K. S. Li, R. G. Webster, J. S. Peiris, and Y. Guan.** 2005. Avian flu: H5N1 virus outbreak in migratory waterfowl. Nature **436:**191–192.
10. **Chutinimitkul, S., T. Songserm, A. Amonsin, S. Payungporn, K. Suwannakarn, S. Damrongwatanapokin, A. Chaisingh, B. Nuansrichay, T. Chieochansin, A. Theamboonlers, and Y. Poovorawan.** 2007. New strain of influenza A virus (H5N1), Thailand. Emerg. Infect. Dis. **13:**506–507.
11. **Claas, E. C., A. D. Osterhaus, R. van Beek, J. C. De Jong, G. F. Rimmelzwaan, D. A. Senne, S. Krauss, K. F. Shortridge, and R. G. Webster.** 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet **351:**472–477.
12. **Ducatez, M. F., C. M. Olinger, A. A. Owoade, S. De Landtsheer, W. Ammerlaan, H. G. Niesters, A. D. Osterhaus, R. A. Fouchier, and C. P. Muller.** 2006. Avian flu: multiple introductions of H5N1 in Nigeria. Nature **442:**37.
13. **Ellis, T. M., C. Y. Leung, M. K. Chow, L. A. Bissett, W. Wong, Y. Guan, and J. S. Malik Peiris.** 2004. Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission. Avian Pathol. **33:**405–412.
14. **Guan, Y., J. S. Peiris, A. S. Lipatov, T. M. Ellis, K. C. Dyrting, S. Krauss, L. J. Zhang, R. G. Webster, and K. F. Shortridge.** 2002. Emergence of multiple genotypes of H5N1 avian influenza viruses in Hong Kong SAR. Proc. Natl. Acad. Sci. USA **99:**8950–8955.
15. **Ha, Y., D. J. Stevens, J. J. Skehel, and D. C. Wiley.** 2002. H5 avian and H9 swine influenza virus haemagglutinin structures: possible origin of influenza subtypes. EMBO J. **21:**865–875.
16. **Hoffmann, E., A. S. Lipatov, R. J. Webby, E. A. Govorkova, and R. G. Webster.** 2005. Role of specific hemagglutinin amino acids in the immunogenicity and protection of H5N1 influenza virus vaccines. Proc. Natl. Acad. Sci. USA **102:**12915–12920.
17. **Hoffmann, E., G. Neumann, Y. Kawaoka, G. Hobom, and R. G. Webster.** 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl. Acad. Sci. USA **97:**6108–6113.
18. **Hoffmann, E., J. Stech, Y. Guan, R. G. Webster, and D. R. Perez.** 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. **146:**2275–2289.
19. **Horimoto, T., N. Fukuda, K. Iwatsuki-Horimoto, Y. Guan, W. Lim, M. Peiris, S. Sugii, T. Odagiri, M. Tashiro, and Y. Kawaoka.** 2004. Antigenic differences between H5N1 human influenza viruses isolated in 1997 and 2003. J. Vet. Med. Sci. **66:**303–305.
20. **Hulse-Post, D. J., K. M. Sturm-Ramirez, J. Humberd, P. Seiler, E. A. Govorkova, S. Krauss, C. Scholtissek, P. Puthavathana, C. Buranathai, T. D. Nguyen, H. T. Long, T. S. Naipospos, H. Chen, T. M. Ellis, Y. Guan, J. S. Peiris, and R. G. Webster.** 2005. Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proc. Natl. Acad. Sci. USA **102:**10682–10687.
21. **Kaverin, N. V., I. A. Rudneva, E. A. Govorkova, T. A. Timofeeva, A. A. Shilov, K. S. Kochergin-Nikitsky, P. S. Krylov, and R. G. Webster.** 2007. Epitope mapping of the hemagglutinin molecule of a highly pathogenic H5N1 influenza virus by using monoclonal antibodies. J. Virol. **81:**12911–12917.
22. **Kaverin, N. V., I. A. Rudneva, N. A. Ilyushina, N. L. Varich, A. S. Lipatov, Y. A. Smirnov, E. A. Govorkova, A. K. Gitelman, D. K. Lvov, and R. G. Webster.** 2002. Structure of antigenic sites on the haemagglutinin molecule of H5 avian influenza virus and phenotypic variation of escape mutants. J. Gen. Virol. **83:**2497–2505.
23. **Kraulis, P. J.** 1991. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J. App. Crystallogr. **24:**946–950.
24. **Kumar, S., K. Tamura, and N. M.** 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinformatics **5:**150–163.
25. **Lee, C. W., D. A. Senne, and D. L. Suarez.** 2004. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. J. Virol. **78:**8372–8381.
26. **Leroux-Roels, I., A. Borkowski, T. Vanwolleghem, M. Drame, F. Clement, E. Hons, J. M. Devaster, and G. Leroux-Roels.** 2007. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. Lancet **370:**580–589.
27. **Li, K. S., Y. Guan, J. Wang, G. J. D. Smith, K. M. Xu, L. Duan, A. P. Rahardjo, P. Puthavathana, C. Buranathai, T. D. Nguyen, A. T. Estoepangestie, A. Chaisingh, P. Auewarakul, H. T. Long, N. T. Hanh, R. J. Webby, L. L. Poon, H. Chen, K. F. Shortridge, K. Y. Yuen, R. G. Webster, and J. S. Peiris.** 2004. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature **430:**209–213.

28. **Lin, J., J. Zhang, X. Dong, H. Fang, J. Chen, N. Su, Q. Gao, Z. Zhang, Y. Liu, Z. Wang, M. Yang, R. Sun, C. Li, S. Lin, M. Ji, X. Wang, J. Wood, Z. Feng, Y. Wang, and W. Yin.** 2006. Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. Lancet **368:**991–997.
29. **Liu, J., H. Xiao, F. Lei, Q. Zhu, K. Qin, X. W. Zhang, X. L. Zhang, D. Zhao, G. Wang, Y. Feng, J. Ma, W. Liu, J. Wang, and G. F. Gao.** 2005. Highly pathogenic H5N1 influenza virus infection in migratory birds. Science **309:** 1206.
30. **Merritt, E. A., and D. J. Bacon.** 1997. Raster3D photorealistic molecular graphics. Methods Enzymol. **277:**505–524.
31. **Munoz, E. T., and M. W. Deem.** 2005. Epitope analysis for influenza vaccine design. Vaccine **23:**1144–1148.
32. **Peiris, J. S., W. C. Yu, C. W. Leung, C. Y. Cheung, W. F. Ng, J. M. Nicholls, T. K. Ng, K. H. Chan, S. T. Lai, W. L. Lim, K. Y. Yuen, and Y. Guan.** 2004. Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet **363:**617–619.
33. **Qiao, C., G. Tian, Y. Jiang, Y. Li, J. Shi, K. Yu, and H. Chen.** 2006. Vaccines developed for H5 highly pathogenic avian influenza in China. Ann. N. Y. Acad. Sci. **1081:**182–192.
34. **Reed, L. J., and H. Muench.** 1938. A simple method for estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
35. **Rowe, T., R. A. Abernathy, J. Hu-Primmer, W. W. Thompson, X. Lu, W. Lim, K. Fukuda, N. J. Cox, and J. M. Katz.** 1999. Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. J. Clin. Microbiol. **37:**937–943.
36. **Salzberg, S. L.** 2007. Genome analysis linking recent European and African influenza (H5N1) viruses. Emerg. Infect. Dis. **13:**713–718.
37. **Shortridge, K. F.** 1999. Poultry and the influenza H5N1 outbreak in Hong Kong, 1997: abridged chronology and virus isolation. Vaccine **17**(Suppl 1)**:** S26–S29.
38. **Skehel, J. J., and D. C. Wiley.** 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu. Rev. Biochem. **69:**531–569.
39. **Smith, D. J.** 2006. Predictability and preparedness in influenza control. Science **312:**392–394.
40. **Smith, D. J., A. S. Lapedes, J. C. de Jong, T. M. Bestebroer, G. F. Rimmelzwaan, A. D. Osterhaus, and R. A. Fouchier.** 2004. Mapping the antigenic and genetic evolution of influenza virus. Science **305:**371–376.
41. **Smith, G. J. D., X. H. Fan, J. Wang, K. S. Li, K. Qin, J. X. Zhang, D. Vijaykrishna, C. L. Cheung, K. Huang, J. M. Rayner, J. S. Peiris, H. Chen, R. G. Webster, and Y. Guan.** 2006. Emergence and predominance of an H5N1 influenza variant in China. Proc. Natl. Acad. Sci. USA **103:**16936–16941.
42. **Smith, G. J. D., T. S. Naipospos, T. D. Nguyen, M. D. de Jong, D. Vijaykrishna, T. B. Usman, S. S. Hassan, T. V. Nguyen, T. V. Dao, N. A. Bui, Y. H. Leung, C. L. Cheung, J. M. Rayner, J. X. Zhang, L. J. Zhang, L. L. Poon, K. S. Li, V. C. Nguyen, T. T. Hien, J. Farrar, R. G. Webster, H. Chen, J. S. Peiris, and Y. Guan.** 2006. Evolution and adaptation of H5N1 influenza virus in avian and human hosts in Indonesia and Vietnam. Virology **350:** 258–268.
43. **Subbarao, K., A. Klimov, J. Katz, H. Regnery, W. Lim, H. Hall, M. Perdue, D. Swayne, C. Bender, J. Huang, M. Hemphill, T. Rowe, M. Shaw, X. Xu, K. Fukuda, and N. Cox.** 1998. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science **279:**393–396.
44. **Treanor, J. J., J. D. Campbell, K. M. Zangwill, T. Rowe, and M. Wolff.** 2006. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. N. Engl. J. Med. **354:**1343–1351.
45. **Vareckova, E., T. Betakova, V. Mucha, L. Solarikova, F. Kostolansky, M. Waris, and G. Russ.** 1995. Preparation of monoclonal antibodies for the diagnosis of influenza A infection using different immunization protocols. J. Immunol. Methods **180:**107–116.
46. **Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka.** 1992. Evolution and ecology of influenza A viruses. Microbiol. Rev. **56:**152–179.
47. **Webster, R. G., and W. G. Laver.** 1980. Determination of the number of nonoverlapping antigenic areas on Hong Kong (H3N2) influenza virus hemagglutinin with monoclonal antibodies and the selection of variants with potential epidemiological significance. Virology **104:**139–148.
48. **Webster, R. G., M. Peiris, H. Chen, and Y. Guan.** 2006. H5N1 outbreaks and enzootic influenza. Emerg. Infect. Dis. **12:**3–8.
49. **Wiley, D. C., I. A. Wilson, and J. J. Skehel.** 1981. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature **289:**373–378.
50. **World Health Organization.** 2002. Manual on animal influenza diagnosis and surveillance, 2nd ed. World Health Organization. Geneva, Switzerland. http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf.
51. **World Health Organization.** 2007. Antigenic and genetic characteristics of H5N1 viruses and candidate H5N1 vaccine viruses developed for potential us as prepandemic vaccines. World Health Organization. Geneva, Switzerland. http://www.who.int/csr/disease/avian_influenza/guidelines/h5n1virus/en/index.html.
52. **World Health Organization Global Influenza Program Surveillance Network.** 2005. Evolution of H5N1 avian influenza viruses in Asia. Emerg Infect. Dis. **11:**1515–1521.
53. **World Health Organization H5N1 Evolution Working Group.** 2007. H5N1 nomenclature. World Health Organization. Geneva, Switzerland. http://www.who.int/csr/disease/avian_influenza/guidelines/nomenclature/en/index.html.
54. **Yamada, S., Y. Suzuki, T. Suzuki, M. Q. Le, C. A. Nidom, Y. Sakai-Tagawa, Y. Muramoto, M. Ito, M. Kiso, T. Horimoto, K. Shinya, T. Sawada, T. Usui, T. Murata, Y. Lin, A. Hay, L. F. Haire, D. J. Stevens, R. J. Russell, S. J. Gamblin, J. J. Skehel, and Y. Kawaoka.** 2006. Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. Nature **444:**378–382.
55. **Yang, Z. Y., C. J. Wei, W. P. Kong, L. Wu, L. Xu, D. F. Smith, and G. J. Nabel.** 2007. Immunization by avian H5 influenza hemagglutinin mutants with altered receptor binding specificity. Science **317:**825–828.
56. **Yen, H. L., A. S. Lipatov, N. A. Ilyushina, E. A. Govorkova, J. Franks, N. Yilmaz, A. Douglas, A. Hay, S. Krauss, J. E. Rehg, E. Hoffmann, and R. G. Webster.** 2007. Inefficient transmission of H5N1 influenza viruses in a ferret contact model. J. Virol. **81:**6890–6898.
57. **Yuen, K. Y., P. K. Chan, M. Peiris, D. N. Tsang, T. L. Que, K. F. Shortridge, P. T. Cheung, W. K. To, E. T. Ho, R. Sung, and A. F. Cheng.** 1998. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. Lancet **351:**467–471.
58. **Zhu, Q. Y., E. D. Qin, W. Wang, J. Yu, B. H. Liu, Y. Hu, J. F. Hu, and W. C. Cao.** 2006. Fatal infection with influenza A (H5N1) virus in China. N. Engl. J. Med. **354:**2731–2732.