

Extent of Antigenic Cross-Reactivity among Highly Pathogenic H5N1 Influenza Viruses[∇]

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Highly pathogenic H5N1 avian influenza viruses emerged in 1996 and have since evolved so extensively that a single strain can no longer be used as a pre-pandemic vaccine or diagnostic reagent. We therefore sought to identify the H5N1 strains that may best serve as cross-reactive diagnostic reagents. We compared the cross-reactivity of 27 viruses of clades 0, 1, 2.1, 2.2, 2.3, and 4 and of four computationally designed ancestral H5N1 strains by hemagglutination inhibition (HI) and microneutralization (MN) assays. Antigenic cartography was used to analyze the large quantity of resulting data. Cartographs of HI titers with chicken red blood cells were similar to those of MN titers, but HI with horse red blood cells decreased antigenic distances among the H5N1 strains studied. Thus, HI with horse red blood cells seems to be the assay of choice for H5N1 diagnostics. Whereas clade 2.2 antigens were able to detect antibodies raised to most of the tested H5N1 viruses (and clade 2.2-specific antisera detected most of the H5N1 antigens), ancestral strain A exhibited the widest reactivity pattern and hence was the best candidate diagnostic reagent for broad detection of H5N1 strains.

Since their emergence in China in 1996, highly pathogenic H5N1 influenza viruses have evolved extensively at both genetic and antigenic levels. The World Health Organization (WHO) has classified the strains into 10 phylogenetic clades (0 to 9), most of which contain subclades.

Although they induce some level of cross-protection, H5N1 strains differ at the antigenic level (2). For example, antiserum against A/Indonesia/5/05 (clade 2.1) cross-reacts well with viruses from clades 2.2, 2.3.4, and to a lesser extent, 1, while antiserum against A/whooper swan/Mongolia/244/05 (clade 2.2) cross-reacts well with clade 2.1 strains but poorly with clades 1 and 2.3.4 (2). Two studies used murine monoclonal antibodies to antigenically characterize highly pathogenic avian influenza (HPAI) H5N1 viruses (13, 24). Wu et al. were able to group the 41 viruses they studied into four antigenically distinct clusters (A to D). Group A contained clade 2.1 and 2.4 viruses, as well as A/Hong Kong/213/03 (clade 1); group B contained clades 1, 4, 5, 7, and 9; group C contained clades 2.2, 2.3.2, and 2.3.3; and group D contained clades 2.3.2 and 2.3.4. These findings suggested a link between genetic and antigenic distances, but they also highlighted the antigenic complexity of clade 2 strains. Studies in mice, ferrets, and humans also showed HPAI H5N1 cross-clade reactivity (1, 8–11, 14, 15, 19, 25). Therefore, despite the partial cross-reactivity of certain H5N1 viruses, it has become difficult to predict whether a vaccine strain will protect against a strain of a different clade (or even sometimes of the same clade), and WHO now has 16

H5N1 vaccine seed viruses available and 4 in production or pending (22).

The antigenic diversity of HPAI H5N1 viruses not only increases the difficulty of developing pre-pandemic vaccines but also creates diagnostic problems, since a single antigen (or antiserum) may not detect all H5N1 field specimens. We therefore compared the cross-reactivity of clades 0, 1, 2.1, 2.2, 2.3, 4, and four “ancestral” H5N1 strains to determine that a virus(es) may be a useful diagnostic reagent. The ancestral strains were created from hemagglutinin (HA) and neuraminidase (NA) sequences computationally generated to represent ancestral nodes within the H5 and N1 phylogenetic trees (8).

MATERIALS AND METHODS

Viruses and antisera. Twenty-seven H5N1 strains were used in the present study: the ancestral strains A, B, C, and D (8) and 23 of their theoretical descendants. Thirteen were generated by cloning gene segments into the dual-promoter plasmid pHW2000 and then creating reverse genetics (rg) 6+2 viruses by combining the HA and NA genes of HPAI viruses with the six internal genes of A/Puerto Rico/8/1934 (PR8) by DNA transfection, as described previously (12). The HA connecting peptide was modified to match that of low-pathogenicity viruses to allow study of the rg strains in biosafety level (BSL) 2+ laboratories. These 13 rg 6+2 viruses were: the ancestral strains A, B, C, and D; A/Vietnam/1203/04 (04-VNM, clade 1 [accession number for the rg HA sequence, CY077101]), A/whooper swan/Mongolia/244/05 (05-MNG, clade 2.2, wild-type [wt] HA [EU723707]), A/duck/Hunan/795/02 (02-CHN, clade 2.1, wt HA [CY028963]), A/duck/Laos/3295/06 (06-LAO, clade 2.3.4, rg HA [FJ147207]), A/Japanese white-eye/Hong Kong/1038/06 (06-HKG, clade 2.3.4, wt HA [ISDN184028]), A/goose/Guizhou/337/06 (06-CHN, clade 4, wt HA [DQ992765]), A/Hong Kong/213/03 (03-HKG, clade 1, wt HA [AY575870]), A/Cambodia/R0405050/07 (07-KHM, clade 1, wt HA [FJ225472]), and A/turkey/Egypt/7/07 (07-EGY, clade 2.2.1, wt HA [CY055191]). Fourteen wt HPAI H5N1 viruses were studied in BSL3+ laboratories: A/Hong Kong/156/97 (97-HKG, clade 0 [AF046088]), A/chicken/Hong Kong/AP156/08 (08-HKG, clade 2.3.4, rg HA [CY095707]), A/common magpie/Hong Kong/5052/07 (07-HKG, clade 2.3.2 [CY036173]), A/gray heron/Hong Kong/1046/08 (08-HKG, clade 2.3.2

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[CY036245]), A/falcon/Saudi Arabia/D1795/05 (05-SAU, clade 2.2 [EU748903]), A/falcon/Saudi Arabia/D1936/07 (07-SAU, clade 2.2 [CY035241]), A/chicken/Nigeria/42/06 (06-NGA, clade 2.2), A/chicken/Egypt/1/08 (08-EGY, clade 2.2.1 [CY061552]), A/Vietnam/1194/04 (04-VNM2, clade 1 [AY651333]), A/Muscovy duck/Vietnam/33/07 (07-VNM, clade 1 [CY029639]), A/duck/Laos/A0301/07 (07-LAO, clade 2.3.4 [CY040934]), A/chicken/Cambodia/13LC1/05 (05-KHM, clade 1 [EF473073]), A/duck/Hunan/101/04 (04-CHN, clade 2.3.1 [AY651365]), and A/chicken/Guiyang/3570/05 (05-CHN, clade 2.3.3 [DQ992758]).

With the exception of the cleavage site (PQIETRGLF replacing the polybasic cleavage site as described by Subbarao et al. in 2003 [21]), the rg viruses were identical to the wt viruses in HA sequence.

Ferret antisera (four ferrets per virus) were raised against the four ancestral strains and against 04-VNM (clade 1), 05-MNG (clade 2.2), 02-CHN (clade 2.1), 06-LAO and 06-HKG (clade 2.3.4), and 06-CHN (clade 4) as previously described (8).

Non-H5 antisera tested included (i) chicken antiserum to A/aquatic bird/Hong Kong/D125/03(H1), A/wild duck/Shantou/992/00(H2), A/chicken/Nanching/3-120/01(H3), A/duck/Shantou/461/00(H4), and A/duck/Hong Kong/M603/98(H11), (ii) goat antiserum to A/turkey/MA/65(H6), A/Netherlands/219/03(H7), A/turkey/Ontario/6118/68(H8), A/chicken/Germany/N/49(H10), and A/duck/Alberta/60/76(H12), (iii) ferret antiserum to A/chicken/Pakistan/NARC-2434/00(H9) and A/shorebird/DE/172/06(H16), and (iv) rabbit antiserum to A/gull/MD/707/77(H113) and A/mallard/Astrakhan/263/82(H14).

HI and MN assays. Ferret antisera were treated with receptor-destroying enzyme (Denka Seiken Co., Tokyo, Japan) overnight at 37°C, heat inactivated at 56°C for 30 min, diluted 1:6 with phosphate-buffered saline (for a final dilution of 1:10), and tested by hemagglutination inhibition (HI) assay with either 0.5% packed chicken red blood cells (cRBCs) or 1% packed horse red blood cells (hRBCs) complemented with 0.5% bovine serum albumin (Sigma, St. Louis, MO) as described in the WHO manual on animal influenza diagnosis and surveillance (23).

Filtered antisera were tested by MN assay in MDCK cells as previously described (23). Neutralizing titers were expressed as the reciprocal of the serum dilution that inhibited 50% of the growth of 100 50% tissue culture infectious doses (TCID₅₀) of virus. The four ferret antisera obtained per virus were tested individually before the mean HI and MN titers were calculated.

Antigenic cartography. Antigenic cartographs were constructed by using the integrative matrix completion-multidimensional scaling (MC-MDS) method as recently described (4). Matrix completion was used to remove the data noise in HI and MN experiments. Multidimensional scaling projected the antigens onto a two-dimensional grid. We constructed three antigenic maps (MN, HI with cRBCs, and HI with hRBCs). Before the antigenic cartographs were constructed, the HI data were normalized to a reference HI value (the HI titer of the respective antigen and homologous ferret antiserum). The normalized value was the ratio between an experimental HI titer and the reference HI titer. In case the experimental HI titer was higher than the reference HI titer, a normalized value of 1 was attributed. An outlier antigen was defined as distant from its counterparts by more than two boxes, i.e., by more than 2 log₂.

RESULTS

Antigenic cartography. Because a large amount of data was generated to compare the antigenic cross-reactivity of 10 groups of sera with 27 viruses by MN, by HI with cRBCs, and by HI with hRBCs (Table 1), a visualization method for the titers was needed. Influenza antigenic cartography is analogous to geographic cartography; it projects influenza antigens onto a two-dimensional map on the basis of their titers. The distances between the antigens can then be measured just as geographic distances are measured on a geographic map. Influenza antigenic cartography can thus be used to identify antigenic variants and is useful for influenza vaccine strain selection. Maps representing our HI cRBCs, MN, and HI hRBCs data are shown in Fig. 1, 2, and 3, respectively.

Intraclade cross-reactivity. In the MN assay (Fig. 2), all clade 1 viruses grouped together with the exception of 04-VNM and 03-HKG. In clade 2.2, 05-MNG was the only outlier. Interestingly, clade 2.3.2 and 2.3.4 strains remained within 2

log₂ of one another, although 08-HKG (clade 2.3.4) was somewhat distant from its counterparts. Ancestral viruses A, B, C, and D were distant from all isolates except the clade 1 03-HKG, (1 to 2 log₂ away). When all 27 viruses were considered, ancestral A strain was positioned centrally and therefore was putatively the best candidate diagnostic reagent for detection of anti-H5N1 antibodies by MN. However, when clear outliers (04-VNM, 03-HKG, and the ancestral viruses), were excluded, a 2.2 strain (e.g., 05-SAU, 06-NGA, 07-EGY, or 08-EGY) appeared to be a better candidate diagnostic reagent.

Cartography of HI with cRBCs (Fig. 1) showed a pattern very similar to that of MN cartography. The same outlier strains were detected in clades 1 and 2.2. In clade 2.3.4, in contrast, 08-HKG appeared near its counterparts while 06-HKG was 1 log₂ away. Ancestral strains A, B, and D were not as tightly clustered in the cartograph of HI with cRBCs (Fig. 1) than in the cartography of MN (Fig. 2). Ancestral strain A and 06-HKG were located in the center of the overall map, but 2.2 strains again appeared to be better candidate diagnostic reagents for most strains when the outliers (the clade 0 97-HKG, clade 1 03-HKG and 04-VNM, and clade 4 06-CHN viruses) were excluded.

Cartography of HI with hRBCs was much less dispersed; all points on the map fit within eight squares (<3 to 4 log₂ apart, Fig. 3). Differences between strains were therefore less distinct. 07-KHM and 04-VNM (clade 1) were very closely related. Clade 2.2 strains were within 2 log₂ of each other, and 05-MNG was no longer a clade outlier. In clade 2.3.4, the Lao strains still clustered closely, while strains from Hong Kong were distant from the Lao viruses and even more distant from each other. A central strain was harder to identify in Fig. 3, but clade 2.2 strains still remain in the middle of the map. The smaller antigenic distances may suggest broader cross-reactivity in HI assays with hRBCs.

Interclade cross-reactivity and subtype specificity. Clades 0, 1, 2, and 4 were not spatially distinct in any of the three antigenic cartographs. For example, the clade 2 viruses were not closer to one another than to clade 1 or 4 strains. Interestingly, 04-VNM (clade 1), 02-CHN (clade 4), 05-MNG (clade 2.2), and 06-CHN (clade 2.1) clustered together in the MN assay (<1 log₂ apart, Fig. 2). The four ancestral strains and 03-HKG (clade 1) were consistently separate from the other tested viruses in the HI cRBCs and MN assays (Fig. 1 and 2).

In HI assays with cRBCs and with hRBCs, the ancestral viruses (A to D), 04-VNM, and 02-CHN did not cross-react with any non-H5 antisera (HI titers < 10) except H13 in the HI-hRBC assay (HI titers 10 to 40; homologous HI titers, 200 to 720 [data not shown]).

DISCUSSION

Our cartographic comparison of the antigenic cross-reactivity of 27 HPAI H5N1 viruses of nine subclades, using three different assays, has shed light on both the assays and the antigenic similarities of the strains tested. The HI-cRBC and MN data sets were very similar (Fig. 1 and 2), suggesting limited effect of H5N1 antigens binding to cRBCs in the serum neutralization. Although the viruses we tested are not representative of all circulating H5N1 strains, 05-MNG was anti-

TABLE 1. Arithmetic mean normalized HI and MN titers

Antigen	Titers obtained with various ferret antisera ^a									
	A	B	C	D	1-04-VNM	2.2-05-MNG	2.1-02-CHN	2.3.4-06-LAO	2.3.4-06-CHN	4-06-CHN
A	1 (45) <i>1 (720)</i> 1 (600)	0.31 3.25 0.93	0.38 <i>1.46</i> 0.21	0.28 <i>0.79</i> 0.72	1 <i>0.60</i> 0.44	0.33 <i>0.45</i> 0.86	0.38 <i>0.81</i> 0.21	0 <i>1.19</i> 4	0.10 <i>0.75</i> 0.31	0.14 <i>0.30</i> 0.03
B	1.50 <i>0.49</i> 1.19	1 (95) <i>1 (220)</i> 1 (760)	0.69 <i>0.71</i> 0.22	0.50 <i>0.31</i> 0.46	0.69 <i>0.16</i> 0.02	0.56 <i>0.15</i> 0.86	0.94 <i>0.54</i> 0.96	0.13 <i>0.44</i> 1.63	0.15 <i>0.69</i> 0.19	0.09 <i>0.07</i> 0.02
C	1.63 <i>1.10</i> 2.04	0.91 <i>1.69</i> 1.71	1 (60) <i>1 (200)</i> 1 (880)	0.35 <i>1.21</i> 1.21	1.83 <i>0.23</i> 0.44	0.50 <i>0.52</i> 1.53	1 <i>1.02</i> 0.81	0.13 <i>1</i> 2.38	0.32 <i>1</i> 0.63	0.04 <i>0.16</i> 0.03
D	1.50 <i>0.67</i> 0.81	0.88 <i>1.50</i> 0.40	0.63 <i>1.08</i> 0.23	1 (150) <i>1 (720)</i> 1 (380)	0.58 <i>0.28</i> 0.08	0.76 <i>0.48</i> 2.05	2.13 <i>1.02</i> 1.63	0.29 <i>1.06</i> 2.63	0.23 <i>0.88</i> 0.33	0 <i>0.08</i> 0.01
1-04-VNM	0.28 <i>0.31</i> 0.16	0.02 <i>0.63</i> 0.10	0.25 <i>0.83</i> 0.11	0.02 <i>0.21</i> 0.13	1 (33) <i>1 (440)</i> 1 (150)	0 <i>0.38</i> 0.53	0.16 <i>0.96</i> 0.16	0 <i>1.78</i> 2.75	0 <i>0.50</i> 0.10	0.12 <i>0.47</i> 0.13
2.2-05-MNG	0.53 <i>0.24</i> 0.21	0.11 <i>0.69</i> 0.19	0.23 <i>0.83</i> 0.11	0.35 <i>0.35</i> 1.18	0.29 <i>0.19</i> 0.50	1 (85) <i>1 (380)</i> 1 (333)	0.88 <i>0.96</i> 0.90	0.29 <i>2.25</i> 2.88	0.06 <i>0.47</i> 0.29	0 <i>0.11</i> 0.11
2.1-02-CHN	0.03 <i>0.12</i> 0.12	0.09 <i>0.41</i> 0.11	0.22 <i>0.42</i> 0.08	0.21 <i>0.27</i> 0.21	0.33 <i>0.32</i> 0.13	0.24 <i>0.33</i> 0.94	1 (180) <i>1 (840)</i> 1 (920)	0.21 <i>2.88</i> 3	0.13 <i>0.44</i> 0.12	0 <i>0.11</i> 0.02
2.3.4-06-LAO	0 <i>0.09</i> 0.02	0 <i>0.20</i> 0.02	0.03 <i>0.06</i> 0	0 <i>0.10</i> 0	0.06 <i>0.14</i> 0	0.05 <i>0.11</i> 0	0.06 <i>0.23</i> 0.02	1 (43) <i>1 (80)</i> 1 (35)	0 <i>0.09</i> 0.01	0.05 <i>0.07</i> 0
2.3.4-06-CHN	1.28 <i>0.32</i> 0.12	0.70 <i>2.06</i> 0.08	0.34 <i>0.76</i> 0.05	0.63 <i>0.83</i> 0.17	0.09 <i>0.10</i> 0.02	0.02 <i>0.11</i> 0.17	0.08 <i>0.20</i> 0.03	0.25 <i>1.50</i> 1.13	1 (40) <i>1 (60)</i> 1 (310)	0 <i>0.08</i> 0.01
4-06-CHN	0.31 <i>0.22</i> 0.19	0.05 <i>0.63</i> 0.11	0.03 <i>0.52</i> 0.08	0 <i>0.18</i> 0.17	0.79 <i>0.71</i> 0.63	0.05 <i>0.23</i> 0.43	0.06 <i>0.46</i> 0.10	0.19 <i>2.06</i> 2.75	0 <i>0.44</i> 0.26	1 (260) <i>1 (960)</i> 1 (1720)
1-03-CHN	5 <i>1.71</i> 1.08	7 <i>5</i> 0.92	3 <i>5.67</i> 0.90	5.42 <i>2.08</i> 2.31	2.17 <i>0.67</i> 0.41	0.79 <i>0.89</i> 2.87	0.94 <i>1.29</i> 0.20	0.13 <i>1.38</i> 0.63	0.81 <i>24</i> 1.04	0.13 <i>0.17</i> 0.02
0-97-CHN	2.75 <i>0.79</i> 0.02	1.56 <i>1.75</i> 0.01	0.75 <i>0.71</i> 0	0.44 <i>0.63</i> 0	2.33 <i>0.28</i> 0.04	0.40 <i>0.36</i> 0.10	0.13 <i>0.19</i> 0	0 <i>0.88</i> 0	0.21 <i>1.13</i> 0	0.27 <i>0.31</i> 0
2.3.4-08-CHN	0 <i>0</i> 0	0 <i>0.02</i> 0	0.03 <i>0.03</i> 0	0 <i>0.03</i> 0	0.06 <i>0.03</i> 0	0 <i>0.03</i> 0	0.03 <i>0.06</i> 0	0 <i>0.31</i> 0	0 <i>0.34</i> 0	0 <i>0.03</i> 0
2.3.2-07-CHN	0 <i>0.15</i> 0	0 <i>0.50</i> 0	0 <i>0.54</i> 0	0 <i>0.19</i> 0.01	0 <i>0.09</i> 0	0 <i>0.20</i> 0	0.02 <i>0.29</i> 0.01	0 <i>0.81</i> 0	0 <i>0.63</i> 0	0 <i>0.06</i> 0
2.3.2-08-CHN	0 <i>0.11</i> 0	0 <i>0.31</i> 0	0 <i>0.40</i> 0	0 <i>0.16</i> 0	0 <i>0.09</i> 0	0.03 <i>0.40</i> 0.31	0.03 <i>0.29</i> 0	0 <i>0.53</i> 0	0 <i>0.63</i> 0	0 <i>0.05</i> 0
2.2-05-SAU	0 <i>0.15</i> 0.03	0 <i>0.50</i> 0.03	0 <i>0.48</i> 0.01	0 <i>0.27</i> 0.18	0 <i>0.14</i> 0.04	0.18 <i>0.54</i> 2.40	0.17 <i>0.71</i> 0.16	0 <i>1.38</i> 0.16	0 <i>0.63</i> 0	0 <i>0.06</i> 0
2.2-07-SAU	0 <i>0.40</i> 0.31	0.05 <i>1.50</i> 0.18	0 <i>0.73</i> 0.05	0 <i>0.75</i> 0.84	0 <i>0.10</i> 0.04	0.14 <i>0.42</i> 1.88	0.19 <i>0.48</i> 0.17	0 <i>1.06</i> 0.13	0.10 <i>1.38</i> 0.12	0 <i>0.06</i> 0

Continued on following page

TABLE 1—Continued

Antigen	Titers obtained with various ferret antisera ^a									
	A	B	C	D	1-04-VNM	2.2-05-MNG	2.1-02-CHN	2.3.4-06-LAO	2.3.4-06-CHN	4-06-CHN
1-07-KHM	0 <i>0.93</i> 0	0 <i>0.69</i> 0	0 <i>0.48</i> 0	0 <i>0.19</i> 0.01	0 <i>1.11</i> 0.02	0 <i>2.90</i> 0	0 <i>0.71</i> 0	0 <i>5.75</i> 0	0 <i>2</i> 0	0 <i>0.50</i> 0.01
2.2-07-EGY	0.06 <i>0.42</i> 0.07	0.07 <i>1.13</i> 0.06	0 <i>1.29</i> 0.01	0.02 <i>0.63</i> 0.22	0 <i>0.29</i> 0	0.08 <i>0.48</i> 0.37	0.25 <i>0.65</i> 0.07	0 <i>0.75</i> 0.09	0 <i>1.50</i> 0.05	0 <i>0.06</i> 0
2.2-06-NGA	0 <i>0.15</i> 0.02	0 <i>0.26</i> 0	0.03 <i>0.23</i> 0	0.09 <i>0.24</i> 0.10	0 <i>0.08</i> 0.01	0.18 <i>0.40</i> 3.74	0.17 <i>0.48</i> 0.12	0 <i>0.63</i> 0.06	0 <i>0.50</i> 0.01	0 <i>0.05</i> 0
2.2-08-EGY	0 <i>0.01</i> 0	0 <i>0.03</i> 0	0 <i>0</i> 0	0 <i>0.03</i> 0	0 <i>0.01</i> 0	0.03 <i>0.27</i> 1.08	0 <i>0.07</i> 0.01	0 <i>0.44</i> 0.16	0 <i>0.50</i> 0.05	0 <i>0.01</i> 0
1-04-VNM2	0 <i>0.18</i> 0.01	0 <i>0.19</i> 0	0.13 <i>0.54</i> 0.01	0 <i>0.14</i> 0	0.29 <i>0.36</i> 0.25	0 <i>0.18</i> 0	0.03 <i>0.35</i> 0.02	0 <i>0.81</i> 0.03	0 <i>0.25</i> 0	0.08 <i>0.38</i> 0.01
1-07-VNM	0 <i>0.15</i> 0	0 <i>0.27</i> 0	0.03 <i>0.38</i> 0.01	0 <i>0.13</i> 0	0 <i>0.35</i> 0.12	0 <i>0.10</i> 0	0.03 <i>0.24</i> 0.02	0 <i>0.75</i> 0	0 <i>0.38</i> 0	0.03 <i>0.25</i> 0.02
2.3.4-07-LAO	0 <i>0.09</i> 0	0 <i>0.13</i> 0	0 <i>0.11</i> 0	0 <i>0.09</i> 0	0 <i>0.12</i> 0	0 <i>0.10</i> 0.17	0 <i>0.19</i> 0.03	0.13 <i>2.50</i> 0.81	0 <i>0.75</i> 0	0 <i>0.11</i> 0
1-05-KHM	0 <i>0.18</i> 0.01	0 <i>0.20</i> 0	0 <i>0.24</i> 0	0 <i>0.16</i> 0	0 <i>0.36</i> 0.16	0 <i>0.24</i> 0	0.05 <i>0.29</i> 0.02	0 <i>1</i> 0.03	0 <i>0.44</i> 0	0.02 <i>0.22</i> 0.01
2.3.1-04-CHN	0.56 <i>0.67</i> 0.24	1.22 <i>1.25</i> 0.24	0.28 <i>0.92</i> 0.03	0.35 <i>0.75</i> 0.65	0 <i>0.12</i> 0.01	0.06 <i>0.27</i> 0.53	0.23 <i>0.65</i> 0.26	0 <i>0.81</i> 0.09	0.15 <i>1.50</i> 0.07	0 <i>0.06</i> 0
2.3.3-05-CHN	0 <i>0.18</i> 0.01	0 <i>0.47</i> 0	0 <i>0.54</i> 0	0.02 <i>0.21</i> 0.02	0.10 <i>0.27</i> 0.13	0 <i>0.27</i> 0.13	0.09 <i>0.39</i> 0.06	0.03 <i>2.25</i> 0.31	0 <i>1.25</i> 0	0.02 <i>0.22</i> 0

^a Homologous normalized titers (in parentheses) were assigned a value of "1." Mean homologous titers are indicated in parentheses. Arithmetic mean normalized HI titers are indicated in either a regular (cRBCs) or an italic (hRBCs) typeface. Arithmetic mean MN titers are given in boldface. Antisera from four animals were tested against each antigen.

genically different in the present study from its clade 2.2 counterparts in the MN and HI-cRBC assays; the HA K328R amino acid mutation may be responsible for this finding, although the N154D and/or F537S mutations (shared by 08-EGY and 07-SAU, respectively) may be involved. In our small-scale study, 04-VNM appeared as a clade 1 outlier. The only HA amino acid that distinguishes 04-VNM from the other clade-1 strains tested is a lysine at position 36 (T36K). In contrast, there are nine amino acids that distinguish 03-HKG (another clade 1 outlier in the MN and HI-cRBC assays): S12W, V86A, S120N, T156A, K189R, R212K, S223N, T263A, and I513T. HA positions 86, 189, 212, and 263 were previously identified by Wu et al. as antigenic group B signature amino acids (24). Kaverin et al. reported that HA positions 131 and 156 were highly conserved among HPAI H5N1 strains and appeared to promote antigenic cross-reactivity among viruses of different clades (13).

The clade classification was established on the basis of phylogenetic analysis. Strains of a given clade or subclade are expected to be antigenically related to some extent, but very

few studies have thoroughly investigated the antigenic cross-reactivity among HPAI H5N1 strains, especially from the perspective of diagnostics (rather than of vaccine design). Three articles have described broadly reactive monoclonal antibodies generated for the treatment of H5N1 infection (6, 7, 26). Their use as HI and MN diagnostic reagents may also be worth investigating, although they would serve only for antigen detection. Recent efforts have been made to develop new reagents and assays in response to the extensive antigenic diversity of HPAI H5N1 and the difficulty of automating and standardizing HI and MN assays (5, 16–18). However, to date no single standard test can identify the full range of H5N1 strains.

To generate robust antigenic cartographs, we prospectively compared the normalization method described above with three other normalization methods available on the Antigen-Map web server (<http://sysbio.cvm.msstate.edu/AntigenMap>) (3, 4). We found that normalization based on reference HI was superior. For example, the distances in the cartographs were too small when normalization was based on the maximum HI

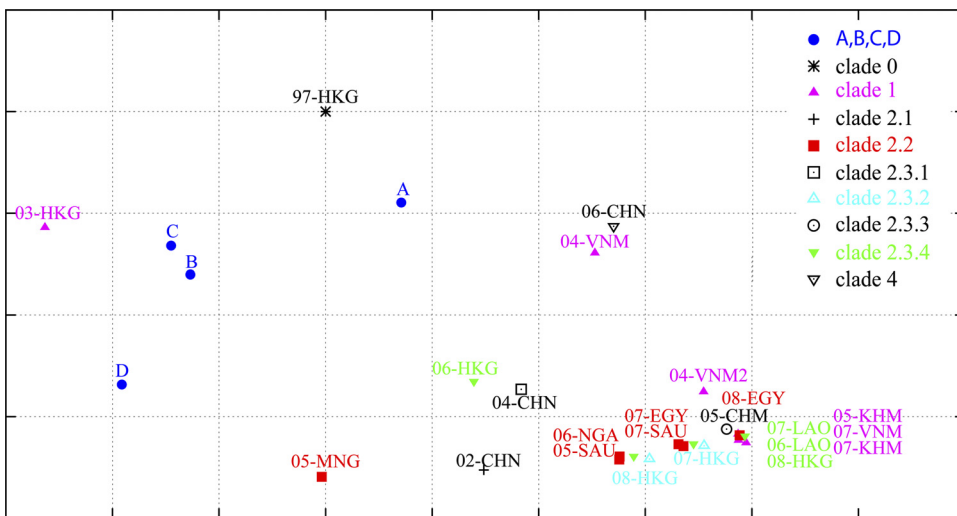


FIG. 1. Antigenic cartograph of H5N1 HPAI constructed by using AntigenMap (<http://sysbio.cvm.msstate.edu/AntigenMap>). Placement is based on hemagglutination inhibition titers with chicken red blood cells (4). Two groups of H5N1 viruses are shown: 14 reassortants (HPAI H5 and N1 plus 6 PR8 internal gene segments) and 13 H5N1 wild-type strains. Reassortants included ancestral strains A, B, C, and D (8), 03-HKG (clade 1), 07-KHM (clade 1), 04-VNM (clade 1), 02-CHN (clade 2.1), 07-EGY (clade 2.2), 08-EGY (clade 2.2), 05-MNG (clade 2.2), 06-LAO (clade 2.3.4), 06-HKG (clade 2.3.4), and 06-CHN (clade 4). The H5N1 wt strains were 08-HKG (clade 2.3.4), 07-HKG (clade 2.3.2), 08-HKG (clade 2.3.2), 05-SAU (clade 2.2), 07-SAU (clade 2.2), 06-NGA (clade 2.2), 04-VNM2 (clade 1), 07-VNM (clade 1), 07-LAO (clade 2.3.4), 05-KHM (clade 1), 04-CHN (clade 2.3.1), and 05-CHN (clade 2.3.3). One grid unit corresponds to a 2-fold difference in HI titer.

titer. One possible reason is the large number of low reactors and low HI titers (only 20 to 80, versus a maximum HI titer of >1,280). Normalization based on only the highest value markedly reduced the distances between the majority of antigens, causing them to form a close cluster in which they could not be distinguished (data not shown).

Our aim was to broaden the capabilities of the existing “gold standard” diagnostic HI and MN assays for HPAI H5N1 by identifying H5N1 strain(s) that may offer superior cross-reactivity. Although our HI-cRBC and MN data sets were very similar (Fig. 1 and 2), the HI-hRBC assay reduced antigenic distances between the HPAI H5N1 strains (Fig. 3). The results

of the present study would therefore lead to recommending the use of HI with hRBCs when a qualitative (positive/negative) answer is expected. In the latter case, using a random reference strain should allow the detection of most HPAI H5N1 (antibodies or antigens). We can link the differences observed between HI with cRBCs and hRBCs and their sialic acids linkage differences (horse erythrocytes contain almost exclusively α 2-3 while chicken contain both α 2-3- and α 2-6-linked sialic acids [20]). The lack of antigenic distinction in HI with hRBCs remains unclear, however. Ancestral strain A appears to be the most broadly reactive reagent for detection of HPAI H5N1 strains, and a clade 2.2 serum/antigen appears to be optimal for

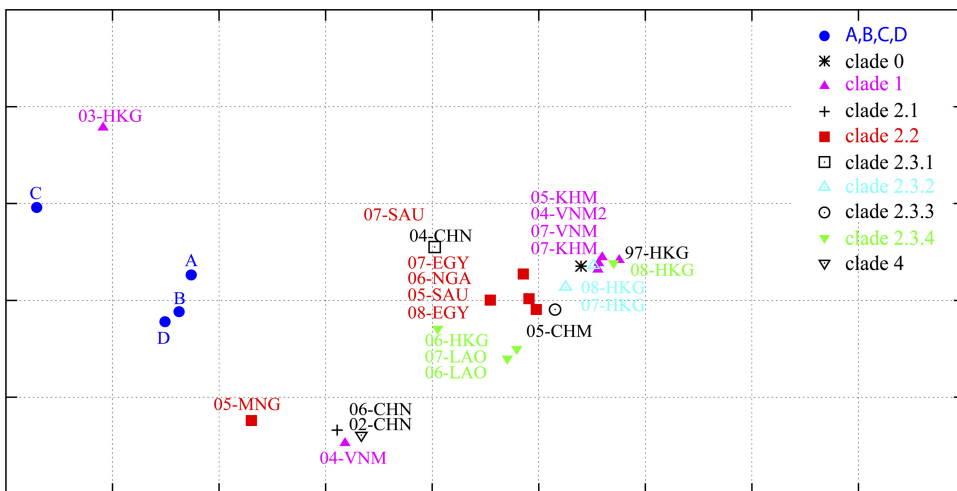


FIG. 2. Antigenic cartograph of H5N1 highly pathogenic avian influenza viruses constructed by using AntigenMap (<http://sysbio.cvm.msstate.edu/AntigenMap>). The positions are based on the results of MN assays (4). The viruses are listed in the Fig. 1 legend. One grid unit corresponds to a 2-fold difference in microneutralization titer.

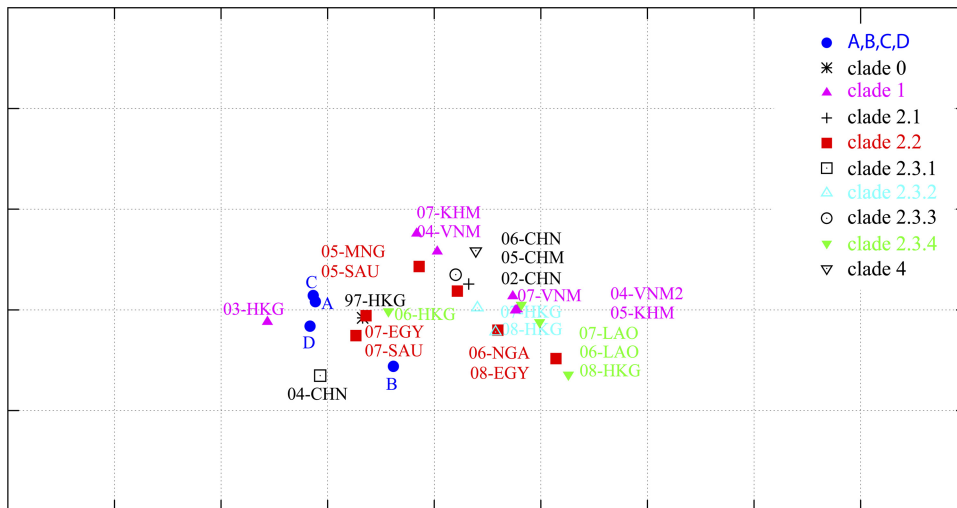


FIG. 3. Antigenic cartograph of H5N1 highly pathogenic avian influenza viruses constructed by using AntigenMap (<http://sysbio.cvm.msstate.edu/AntigenMap>). The positions are based on HI titers using hRBCs (4). Viruses are listed in the Fig. 1 legend. One grid unit corresponds to a 2-fold difference in HI titer.

detecting most HPAI H5N1 viruses/sera by HI-cRBC or MN assays.

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