Novel Reassortant Highly Pathogenic Avian Influenza (H5N5) Viruses in Domestic Ducks, China

Min Gu, Wenbo Liu, Yongzhong Cao, Dixin Peng, Xiaobo Wang, Hongquan Wan, Guo Zhao, Quangang Xu, Wei Zhang, Qingqing Song, Yanfang Li, and Xiufan Liu

Aquatic birds are considered the natural reservoir for influenza A viruses of all known 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes (1). Influenza viruses replicate preferentially in the cells lining the intestinal tracts of wild waterfowl, usually causing no clinical signs. Excretion of substantial amounts of virus in the feces can infect wild and domestic birds by waterborne transmission (1). In the People’s Republic of China, domestic ducks raised in the traditional free-range system often share water with wild aquatic birds. Moreover, domestic ducks are often in close contact with poultry, livestock, and humans in the same village or farm. Therefore, domestic ducks play a major role in the ecology of influenza viruses (2) and can act as potential vessels for genetic reassortment (3). Systematic surveillance of influenza viruses in domestic ducks could provide timely and valuable epidemiologic information and should be continued.

The Study

As part of routine surveillance for avian influenza viruses from December 2008 through January 2009 in eastern China, tracheal and cloacal swab samples from apparently healthy domestic ducks in live poultry markets were collected for virus isolation and identification as described (4). From these samples, 2 influenza (H5N5) viruses—A/duck/eastern China/008/2008 (008 [H5N5]) and A/duck/eastern China/031/2009 (031 [H5N5])—were detected in mallard ducks (Anas platyrhynchos).

These 2 viruses grew efficiently in eggs and in MDCK cells, each with virus titers >8 log10 50% egg infectious dose (EID50)/mL or 8 log10 50% tissue culture infectious dose/mL (Table 1). The intravenous pathogenicity index for chickens and 50% lethal dose for mice were 2.6 and 10^8 EID50 for 008 (H5N5), 2.5 and 10^4 EID50 for 031 (H5N5), respectively (Table 1). Therefore, both novel influenza subtype H5N5 viruses were assumed to be highly pathogenic for chickens and moderately virulent for mice (3).

When mice were inoculated with a sublethal dose of 10^5 50% EID50, each influenza subtype H5N5 virus was able to replicate without prior adaptation. The highest virus titers were detected in the mouse lung. The viruses were able to spread to the brain and heart. Furthermore, influenza virus 008 (H5N5) was isolated from the spleen and liver, and influenza virus 031 (H5N5) was detected in the kidney (Table 1). Microscopic findings in infected mice were interstitial pneumonia with various amounts of erythrocytes in alveolar lumens, hyperemia and lymphocyte infiltration in alveoli, congestion in the renal cortex and glomerulus (data not shown).

Genomic analysis showed that the influenza viruses 008 (H5N5) and 031 (H5N5) were highly homologous with each other, sharing 99.2%–99.7% nt identities among the 8 gene segments except for the polymerase acidic protein (PA) gene (94.1%). Another 3 viruses isolated from the same surveillance study—A/duck/eastern China/108/2008 of H5N1 subtype (108 [H5N1]), A/duck/eastern China/909/2009 of H5N1 subtype (909 [H5N1]), and A/duck/Yangzhou/013/2008 of H6N5 subtype (013 [H6N5])—were more closely related to the novel influenza subtype H5N5 viruses than those in GenBank (Table 2).

As outlined by the World Health Organization/World Organisation for Animal Health/Food and Agriculture Organization unified nomenclature system for subtype H5N1 highly pathogenic avian influenza (HPAI) viruses (6), the HA genes of the 2 subtype H5N5 viruses were classified into clade 2.3.4 (Figure 1, panel A), which has been the prevalent lineage in southern China since 2005 (7–9). In addition to the typical residues Q226 and G228 in HA, conferring receptor preference for SAα2,3Gal, influenza viruses 008 (H5N5) and 031 (H5N5) simultaneously carried an S227R mutation in the receptor-binding pocket. A recent report (10) indicated that the S227N substitution accompanied by deglycosylation at residue 158 could substantially increase the affinity of HA for SAα2,6Gal without reducing its binding affinity for SAα2,3Gal.
Whether this S227R variation with the changed residual polarity affects the receptor-binding property deserves further investigation.

To construct the NA tree, we retrieved 90 complete N5 sequences from GenBank, including the only 2 influenza subtype H5N5 viruses from the United States: A/mallard/MN/105/2000 and A/duck/Massachusetts/sg-00440/2005. The N5 viruses were grouped into 2 lineages—North American and Eurasian—in accordance with their geographic distribution (Figure 1, panel B). The 2 subtype H5N5 isolates from China belonged to the Eurasian lineage, whereas the 2 from the United States clustered within the North American lineage. In addition, 1 aa deletion at residue 42, located in the stalk region of NA, was identified in all Eurasian, but not in the North American, strains.

Although the PA genes of influenza viruses 008 (H5N5) and 031 (H5N5) diverged to assemble respectively with influenza viruses 013 (H6N5) and 108 (H5N1), the novel influenza subtype H5N5 viruses aggregated closely with recent Eurasian subtype H5N1 viruses, especially influenza viruses 108 (H5N1) and 909 (H5N1) in the trees of HA (Figure 1, panel A), polymerase basic protein (PB) 2, PB1, nucleocapsid protein, matrix protein, and nonstructural protein genes (online Technical Appendix, www.cdc.gov/EID/content/17/6/1060-Techapp.pdf). For NA, spatiotemporal correlation indicates that influenza

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**Table 1. Characteristics of 2 novel avian influenza (H5N5) viruses isolated from domestic ducks, China, December 2008–January 2009***

<table>
<thead>
<tr>
<th>Virus</th>
<th>Characteristics</th>
<th>Virus replication in experimentally infected mice, no. virus-positive mice/no. tested mice (mean titer + SD)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tissue dpi 2 dpi 4 dpi 6 dpi 8 dpi 10</td>
</tr>
<tr>
<td>008</td>
<td></td>
<td>Lung 1/2 (3.2 ± 0.2) 2/2 (3.8 ± 0.4) 2/2 (4.7 ± 0.4) 2/2 (4.4 ± 0.5) 0/2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brain 0/2 0/2 0/2 0/2 1/2 (3.4 ± 0.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heart 1/2 (2.0 ± 0.2) 2/2 (2.6 ± 0.5) 2/2 (3.1 ± 0.1) 0/2 0/2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spleen 0/2 0/2 0/2 0/2 1/2 (2.3 ± 0.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver 0/2 0/2 0/2 0/2 1/2 (2.2 ± 0.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney 0/2 0/2 0/2 0/2 0/2</td>
</tr>
<tr>
<td>301</td>
<td></td>
<td>Lung 1/2 (3.0 ± 0.0) 1/2 (3.3 ± 0.0) 2/2 (4.1 ± 0.2) 2/2 (4.5 ± 0.3) 1/2 (2.6 ± 0.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brain 0/2 0/2 0/2 0/2 1/2 (3.4 ± 0.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heart 0/2 1/2 (2.5 ± 0.0) 1/2 (3.0 ± 0.0) 0/2 0/2</td>
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<td>Spleen 0/2 0/2 0/2 0/2 0/2</td>
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<td>Liver 0/2 0/2 0/2 0/2 0/2</td>
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<tr>
<td></td>
<td></td>
<td>Kidney 0/2 0/2 1/2 (2.1 ± 0.0) 0/2 0/2</td>
</tr>
</tbody>
</table>

†EID50, 50% egg infectious dose; IVPI, intravenous pathogenicity index (determined in chickens); TCID50, 50% tissue culture infectious dose (determined in MDCK cells); MLD50, 50% lethal dose in mice (expressed as the EID50 value corresponding to 1 LD50); dpi, day postinoculation; 008, A/duck/eastern China/008/2008; 301, A/duck/eastern China/031/2009.

**Table 2. Influenza viruses with highest nucleotide identity to each gene of 008, and 031***

<table>
<thead>
<tr>
<th>Gene segment</th>
<th>Closest viruses in GenBank</th>
<th>Closest viruses isolated during surveillance, China, December 2008–January 2009</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Strain Nucleotide identity, %</td>
<td>Strain Nucleotide identity, %</td>
</tr>
<tr>
<td>PB2 008</td>
<td>A/duck/Guangxi/xa/2001 (H5N1) 96.0</td>
<td>108 100</td>
</tr>
<tr>
<td>301</td>
<td>A/duck/Guangxi/xFZ/2001 (H5N1) 95.9</td>
<td>909 99.8</td>
</tr>
<tr>
<td>PB1 008</td>
<td>A/duck/Hokkaido/Vac-1/04 (H5N1) 97.2</td>
<td>031 99.5</td>
</tr>
<tr>
<td>301</td>
<td>A/duck/Hokkaido/Vac-1/04 (H5N1) 97.1</td>
<td>909 99.7</td>
</tr>
<tr>
<td>PA 008</td>
<td>A/chicken/Hunan/8/2008 (H5N1) 98.1</td>
<td>013 99.7</td>
</tr>
<tr>
<td>301</td>
<td>A/wild duck/Hunan/021/2005 (H5N1) 98.0</td>
<td>108 99.5</td>
</tr>
<tr>
<td>HA 008</td>
<td>A/wild duck/Hunan/211/2005 (H5N1) 97.7</td>
<td>108 99.9</td>
</tr>
<tr>
<td>301</td>
<td>A/wild duck/Hunan/211/2005 (H5N1) 97.8</td>
<td>008 and 108 99.4</td>
</tr>
<tr>
<td>NP 008</td>
<td>A/chicken/Jilin/hk/2004 (H5N1) 96.1</td>
<td>108 99.8</td>
</tr>
<tr>
<td>301</td>
<td>A/chicken/Jilin/hk/2004 (H5N1) 96.5</td>
<td>108 99.6</td>
</tr>
<tr>
<td>NA 008</td>
<td>A/mallard/Switzerland/WV4060167/2006 (H3N5) 95.8</td>
<td>031 99.2</td>
</tr>
<tr>
<td>301</td>
<td>A/mallard/Switzerland/WV4060167/2006 (H3N5) 95.8</td>
<td>008 99.2</td>
</tr>
<tr>
<td>M 008</td>
<td>A/china/GD01/2006 (H5N1) 99.1</td>
<td>031 and 909 99.8</td>
</tr>
<tr>
<td>301</td>
<td>A/china/GD01/2006 (H5N1) 99.1</td>
<td>909 100</td>
</tr>
<tr>
<td>NS 008</td>
<td>A/wild duck/Hunan/211/2005 (H5N1) 97.9</td>
<td>108 99.5</td>
</tr>
<tr>
<td>301</td>
<td>A/wild duck/Hunan/211/2005 (H5N1) 97.9</td>
<td>909 99.9</td>
</tr>
</tbody>
</table>

*PB, polybasic protein; 008, A/duck/eastern China/008/2008/H5N5; 031, A/duck/eastern China/031/2009/H5N5; 013, A/duck/Yangzhou/013/2008/H6N5; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleocapsid protein; NA, neuraminidase; M, matrix protein; NS, nonstructural protein.
virus 013 (H6N5), rather than its phylogenetically
equidistant counterpart A/mallard/Switzerland/WV4060167/
2006(H3N5), might be the N5 donor (Figure 1, panel B).
However, because of the relatively low (95%) sequence
similarity, it is also possible that Eurasian viruses of an
unidentified HA subtype (H?N5), not detected in our
epidemiologic survey, could provide the NA genes.
Therefore, we speculate that in
fluenza viruses 008 (H5N5)
and 031 (H5N5) may be reassortants between contemporary
Eurasian subtype H5N1 and some subtype H?N5 and/or
H6N5 avian influenza viruses with distant evolutionary
relationship with the 2 subtype H5N5 viruses from the
United States (Figure 1; online Technical Appendix). In
addition, regarding the especially high sequence identities
of PB1, HA, nucleocapside protein, NA, and matrix protein
genes exclusively between influenza viruses 008 (H5N5)
and 031 (H5N5), the possibility that the 2 subtype H5N5
viruses donated some gene segments to each other cannot
be excluded (Figure 2).

Conclusions
The 2 novel HPAI (H5N5) viruses isolated and
characterized in this study are most likely reassortants of
recent Eurasian viruses sharing approximate spatiotemporal
distribution. It is less likely that they were introduced
through intercontinental transmission of subtype H5N5
strains from North America. Considering the endemicity
that clade 2.3.4 subtype H5N1 viruses have gained in China
since 2005 (7–9), it is plausible that subtype H5N1 viruses
have provided the backbone for generating the novel
subtype H5N5 viruses instead of the opposite gene
flow.

Ducks have been considered “Trojan horses”
for influenza (H5N1) because of their pivotal role in virus
propagation and evolution (11–13). In our study, the 2
reassortant influenza viruses (008 [H5N5] and 031 [H5N5])
and their 3 possible parent viruses (108 [H5N1], 909
[H5N1], and 013 [H6N5]) were all isolated from apparently
healthy domestic ducks. We speculate that domestic ducks
may serve as reassortant vessels for creating new subtypes
of influenza viruses. In view of the practice of raising
ducks in a free-range system, these novel strains could be
transmitted to other domestic poultry and even humans.
There is evidence that these subtype H5N5 viruses have
been transmitted to terrestrial poultry (Zhao et al., unpub.
data). Thus, the role of domestic ducks in the influenza
virus ecosystem should not be neglected. Systematic
surveillance should be instituted to identify emerging HPAI (H5N5) viruses and to reduce their potential threat to animal and human health.

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References


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Technical Appendix

Shown on the following pages are phylogenetic trees of the polymerase basic protein (PB) 2, PB1, polymerase acidic protein (PA), nucleocapsid protein (NP), matrix protein (M), and nonstructural protein (NS) genes of the novel avian influenza (H5N5) viruses isolated from domestic ducks in the People’s Republic of China, December 2008–January 2009, with reference sequences. Green, 2 subtype H5N5 viruses (A/duck/eastern China/008/2008 and A/duck/eastern China/031/2009); purple, 2 subtype H5N1 viruses (A/duck/eastern China/108/2008 and A/duck/eastern China/909/2009); blue, 1 subtype H6N5 virus (A/duck/Yangzhou/013/2008); **boldface**, other subtype H5N5 viruses available from GenBank. Trees were generated by applying the neighbor-joining method in MEGA 4.0 (www.megasoftware.net) on the basis of full-length coding sequences. Numbers above or below the branch nodes indicate bootstrap values. Scale bars indicate branch length based on the number of nucleotide substitutions per site.