We conducted a 3-year longitudinal serologic survey on an open cohort of poultry workers, swine workers, and general population controls to assess avian influenza A virus (AIV) seroprevalence and seroincidence and virologic diversity at live poultry markets (LPMs) in Wuxi City, Jiangsu Province, China. Of 964 poultry workers, 9 (0.93%) were seropositive for subtype H7N9 virus, 18 (1.87%) for H9N2, and 18 (1.87%) for H5N1. Of 468 poultry workers followed longitudinally, 2 (0.43%), 13 (2.78%), and 7 (1.5%) seroconverted, respectively; incidence was 1.27, 8.28, and 4.46/1,000 person-years for H7N9, H9N2, and H5N1 viruses, respectively. Longitudinal surveillance of AIVs at 9 LPMs revealed high co-circulation of H9, H7, and H5 subtypes. We detected AIVs in 726 (23.3%) of 3,121 samples and identified a high diversity (10 subtypes) of new genetic constellations and reassortant viruses. These data suggest that stronger surveillance for AIVs within LPMs and high-risk populations is imperative.

Avian influenza A viruses (AIVs) remain an important threat to human health. With new strains widely circulating in China, an increasing number of human infections with AIVs have been reported since 2013, including subtypes H7N9, H5N6, and H10N8 (1–3). In addition, more human infections with H9N2 have been reported since 2014 (4). Although no sustained human-to-human transmission has been observed for these viral subtypes, serious concern exists that the virus could become more efficient in causing human epidemics (5).

Most human infections with AIVs (e.g., subtypes H7N9, H5N1, and H5N6) have been associated with exposure to poultry and resulted in severe illness (6). However, these severely ill patients could represent the tip of the iceberg because mild and asymptomatic infections with H7N9, H9N2, and H5N1 subtypes have been observed by surveillance (7–11) and serologic studies (12–18). Surveillance might miss persons with mild or asymptomatic infection who do not seek medical care. Cross-sectional serologic studies have limited value for measuring incidence rates of AIV infections, resulting in poor understanding of the prevalence of infection and the proportion of cases that are mild or subclinical in humans.

The southern provinces of China have a high density of poultry and humans and are considered likely hot spots for the emergence of new reassortant influenza viruses (19). China’s Jiangsu Province, one of the hot spots, has reported human infections with H7N9 and H5N1 subtypes. We conducted a 3-year longitudinal serologic study to estimate the seroprevalence and seroincidence of H7N9, H9N2, H5N1, and H5N6 subtypes among animal (poultry and swine) workers and general population controls and to identify the risk factors for seropositivity or seroconversion. We also conducted longitudinal surveillance to measure the diversity and genetic variation of AIVs at live poultry markets (LPMs) in the city of Wuxi, Jiangsu Province, China.

Materials and Methods

Study Population, Sampling, and Data Collection

During July 2013–September 2016, we conducted a longitudinal serologic survey among an open cohort of poultry and swine workers and general population controls in Wuxi. We recruited workers who were ≥18 years of age and were exposed to poultry and pigs or to poultry and pig manure as part of their daily activities (e.g., husbandry, slaughtering, sales). In addition, we recruited control participants from residents at community service centers who reported having no exposure to poultry or pigs or to animal manure as part of their daily activities. After enrolling...
participants in July 2013, we conducted follow-up visits at 1, 2, and 3 years. Because poultry and swine workers in China are often temporarily employed and different workers might be present each year, prospective follow-up of the same persons over the study period was not always feasible. Therefore, we enrolled new participants at each follow-up visit to maintain the number of active cohort participants at ≈2,000.

At participant enrollment, we used a comprehensive questionnaire to collect demographic data, exposure variables, information about any history of chronic medical conditions, influenza vaccination history, self-reported influenza-like illness during the past 12 months, and the extent and nature of exposure to animals or animal manure. At each follow-up visit, we used a shorter questionnaire to collect additional demographic data, recent history of exposure to poultry or pigs, and self-reported recent influenza-like illness. At enrollment and follow-up visits, we asked each participant to provide a 5-mL blood sample.

We obtained written informed consent from all participants before conducting interviews and collecting samples. The institutional review boards of the Beijing Institute of Microbiology and Epidemiology (no number given) approved the study protocol.

**Poultry and Environmental Surveillance of AIVs**
During the serologic study period, we also conducted prospective surveillance of AIVs at 9 LPMs in 9 districts of Wuxi (Figure 1). Once each month, we collected ≈54 cloacal swab samples (6 samples from each LPM) from chickens, ducks, or geese and preserved each sample in a tube containing 3 mL of viral transport medium (MT0301; Yocon, Beijing, China). In addition, 18 of each type of environmental swab and fecal/slurry samples were collected (2 samples of each type from each LPM). We collected environmental samples by swabbing surfaces of chicken epilators, chopping boards, cages, and sewage 4–8 times with separate cotton-tipped swabs. We then inserted the swabs into a tube containing 3 mL of viral transport medium (Yocon). Fecal (1 g) or slurry (1 mL) samples were collected at available sites and were diluted in viral transport medium (Yocon).
Sample Processing and Laboratory Analysis
All blood, cloacal, and environmental specimens were kept on frozen cold packs at 2°C–8°C after collection and transported to the local Center for Disease Control and Prevention laboratory. Serum was separated by centrifugation for 5 min at 2,000 rpm. Cloacal and environmental specimens were vortexed, and swabs were discarded. For the fecal/slurry specimens, we conducted an extra centrifugation for 5 min at 2,000 rpm to separate the mixture of virus and viral medium. Each type of specimen was split into 3 aliquots and frozen at −80°C until use.

We first screened all serum samples by hemagglutination inhibition (HI) assay (20), and samples with an HI titer ≥10 were tested by a microneutralization (MN) assay (21). Considering the prevalence of avian-lineage viruses in China and their availability, we used a human H7N9 isolate (A/Jiangsu/Wuxi05/2013), clade 2.3.4.4 H5N6 virus (A/chicken/Jiangsu/WXBING2/2014), clade 2.3.2.1c H5N1 virus (A/chicken/Jiangsu/WX927/2013), and Y280-like H9N2 virus (A/chicken/Jiangsu/WXWA021/2013) for HI and MN assays. We defined a seropositive result as an MN titer ≥80 for all tested viruses. Seroconversion was defined as detection of a 4-fold rise in MN antibody titer between initial serum sample and a paired second serum sample, with the second sample achieving a titer ≥80. Additional details for the HI and MN assays, PCR detection, viral isolation, sequencing of the cloacal and environmental samples, and the phylogenetic analysis of the AIVs we identified are available in the online Technical Appendix (https://wwwnc.cdc.gov/EID/article/24/7/17-2059-Techapp1.pdf). We deposited sequence data in the GISAID database (http://platform.gisaid.org; accession nos. EPI_ISL_277027–277050, 277052–277064, and 277093–277127).

Statistical Analysis
We calculated the proportion (and associated 95% CIs) of poultry workers, swine workers, and control participants who were seropositive or seroconverted. We estimated the incidence of seroconversion per 1,000 person-years (and associated 95% CIs) for participants with multiple longitudinal serum samples using the time between baseline and follow-up as their person-time contribution. We excluded participants who were seropositive at baseline. We analyzed categorical and continuous variables using the χ² or Fisher exact test and the Student t-test where necessary. Risk factors for virus infection (any seropositivity or seroconversion for each individual) were assessed only among participants with paired serum samples using logistic regression models after adjustment for sex and age group or variables with p values <0.05, summarized by odds ratios (ORs) with 95% CIs. Exact Poisson regression model was used to explore the effect of exposure on 1,000 person-year incidence in the cohorts, assessed by incidence rate ratios with 95% CIs. All tests were 2-sided with a 0.05 level of significance. Analyses were performed using SPSS software version 16.0 (SPSS, Chicago, IL, USA).

Results

Demographic Characteristics of Participants
In July 2013, we enrolled 1,995 participants: 511 poultry workers, 569 swine workers, and 915 general population controls. Of these original 1,995 participants, 1,137 were followed up at year 1 (July 2014), 892 at year 2 (July 2015), and 701 at year 3 (July 2016) (Figure 2). To compensate for the number of participants lost to follow-up, we enrolled an additional 866 participants in July 2014, 603 in July 2015, and 124 in July 2016 (Figure 2). New participants enrolled in 2014 were also followed in 2015 (396) and 2016 (339) and new participants enrolled in 2015 were followed in 2016 (479) (Figure 2). Poultry and swine workers tended to be older and less educated than controls (p<0.05), and swine workers comprised a significantly higher proportion of men among the 3 groups (online Technical Appendix Table 1).

Seroconversion
Seroconversion differed by group and over time (Table 1). The overall seroprevalence of H7N9, H9N2, and H5N1 viruses in poultry workers was significantly higher than in swine workers and controls (p<0.05). Of 964 enrolled poultry workers, 9 (0.93% [95% CI 0.43%–1.76%]) were seropositive for H7N9, 18 (1.87% [95% CI 1.11%–2.94%]) for H9N2, and 18 (1.87% [95% CI 1.11%–2.94%]) for H5N1 during the study period. In comparison, of 1,079 enrolled swine workers, only 2 (0.19% [95% CI 0.02%–0.67%]) were seropositive for H7N9 and 3 (0.28% [95% CI 0.06%–0.81%]) for H9N2. Similar seroprevalence was observed among the 1,545 enrolled controls. No poultry workers were found seropositive for H7N9 in the 2016 survey and for H5N1 virus in the 2014 and 2015 surveys. In addition, we observed a significant increase in seroprevalence of 3.46% for H5N1 virus among poultry workers in the 2016 survey, compared with the previous year’s survey. No participants in any group were seropositive for H5N6 throughout the study.

Incidence of Seroconversion
During the study period, 30 participants seroconverted (Table 2). Among the poultry workers, 2 (0.43%) seroconverted for H7N9, 13 (2.78%) for H9N2, and 7 (1.5%) for H5N1 (Table 3), resulting in incidences of 1.27/1,000 person-years for H7N9, 8.28/1,000 person-years for H9N2, and 4.46/1,000 person-years for H5N1 (Table 4). Among
Avian Influenza A Virus, China

swine workers and controls, only 1 control seroconverted for the H7N9 virus, and 3 (0.58%) swine workers and 4 (0.39%) controls seroconverted for H9N2 (Table 3). Although the incidence among swine workers and controls was low or 0 for H7N9 and H5N1, the incidence of H9N2 was relatively high among swine workers (1.93/1,000 person-years) and controls (1.54/1,000 person-years) (Table 4). Poultry workers were more likely than controls to have infection with H9N2 (incidence rate ratio 5.36 [95% CI 1.65–22.55]) and H5N1, but seroconversion rates between the groups did not differ significantly for H7N9 (Table 4).

**Figure 2.** Flowchart for participant enrollment and follow-up in study of avian influenza A virus infection among workers at live poultry markets, Wuxi, Jiangsu Province, China, 2013–2016.

**Table 1. Seroprevalence of microneutralization titers against influenza A(H7N9), A(H9N2), and A(H5N1) viruses in poultry workers, swine workers, and controls, eastern China, 2013–2016**

<table>
<thead>
<tr>
<th>Antigen/year</th>
<th>Poultry workers</th>
<th>Swine workers</th>
<th>Controls</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>H7N9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2013</td>
<td>3/511 (0.58 [0.12–1.71])</td>
<td>0/569 (0 [0–0.65])</td>
<td>2/915 (0.22 [0.03–0.79])</td>
<td>0.13</td>
</tr>
<tr>
<td>2014</td>
<td>3/533 (0.56 [0.12–1.64])</td>
<td>1/589 (0.17 [0–0.94])</td>
<td>1/881 (0.11 [0–0.63])</td>
<td>0.19</td>
</tr>
<tr>
<td>2015</td>
<td>3/535 (0.56 [0.12–1.63])</td>
<td>0/501 (0 [0–0.73])</td>
<td>0/855 (0 [0–0.43])</td>
<td>0.04</td>
</tr>
<tr>
<td>2016</td>
<td>0/491 (0 [0–0.75])</td>
<td>1/367 (0.27 [0.01–1.51])</td>
<td>1/785 (0.13 [0–0.71])</td>
<td>0.48</td>
</tr>
<tr>
<td>Overall†</td>
<td>9/964 (0.93 [0.43–1.76])</td>
<td>2/1,079 (0.19 [0.02–0.67])</td>
<td>4/1,545 (0.26 [0.07–0.66])</td>
<td>0.03</td>
</tr>
<tr>
<td>H9N2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2013</td>
<td>1/511 (0.20 [0.01–1.09])</td>
<td>0/569 (0 [0–0.65])</td>
<td>2/915 (0.22 [0.03–0.79])</td>
<td>0.61</td>
</tr>
<tr>
<td>2014</td>
<td>2/533 (0.38 [0.05–1.35])</td>
<td>1/589 (0.17 [0–0.94])</td>
<td>1/881 (0.11 [0–0.63])</td>
<td>0.70</td>
</tr>
<tr>
<td>2015</td>
<td>11/535 (2.06 [1.03–3.65])</td>
<td>0/501 (0 [0–0.73])</td>
<td>4/855 (0.47 [0.13–1.19])</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2016</td>
<td>7/491 (1.43 [0.58–2.92])</td>
<td>3/367 (0.82 [0.17–2.37])</td>
<td>2/785 (0.25 [0.03–0.92])</td>
<td>0.05</td>
</tr>
<tr>
<td>Overall†</td>
<td>18/964 (1.87 [1.11–2.94])</td>
<td>3/1,079 (0.28 [0.06–0.81])</td>
<td>9/1,545 (0.58 [0.27–1.10])</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>H5N1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2013</td>
<td>1/511 (0.20 [0–1.09])</td>
<td>0/569 (0 [0–0.65])</td>
<td>0/915 (0 [0–0.40])</td>
<td>0.26</td>
</tr>
<tr>
<td>2014</td>
<td>0/533 (0 [0–0.69])</td>
<td>0/589 (0 [0–0.62])</td>
<td>0/881 (0 [0–0.42])</td>
<td>NA</td>
</tr>
<tr>
<td>2015</td>
<td>0/535 (0 [0–0.69])</td>
<td>0/501 (0 [0–0.73])</td>
<td>0/855 (0 [0–0.43])</td>
<td>NA</td>
</tr>
<tr>
<td>2016</td>
<td>17/491 (3.46 [2.03–5.49])</td>
<td>0/367 (0 [0–1.00])</td>
<td>0/785 (0 [0–0.47])</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Overall†</td>
<td>18/964 (1.87 [1.11–2.94])</td>
<td>0/1,079 (0 [0–0.34])</td>
<td>0/1,545 (0 [0–0.24])</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*N: the statistics were not performed because of 0 in the 2 groups.
†The overall seroprevalence was calculated as the number of seropositive persons divided by the number of all new enrolled persons during the study period.

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Table 2. Characteristics of poultry workers, swine workers, and controls with seroconversion of influenza A(H7N9), A(H9N2), and A(H5N1) viruses, eastern China, 2013–2016*

<table>
<thead>
<tr>
<th>Virus, participant no.</th>
<th>Age, y/sex</th>
<th>Occupation</th>
<th>Chronic medical condition</th>
<th>MN titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2013</td>
</tr>
<tr>
<td>H7N9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>28/F</td>
<td>Chicken slaughtering</td>
<td>No</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>41/F</td>
<td>Chicken slaughtering</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>63/F</td>
<td>Retired</td>
<td>No</td>
<td>20</td>
</tr>
<tr>
<td>H9N2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>48/F</td>
<td>Chicken backyard grower</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>28/M</td>
<td>Chicken raising</td>
<td>No</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>51/F</td>
<td>Chicken raising</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>47/F</td>
<td>Chicken seller</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>47/M</td>
<td>Chicken seller</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>46/M</td>
<td>Chicken seller</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>51/M</td>
<td>Chicken seller</td>
<td>Chronic bronchitis</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>49/M</td>
<td>Chicken/duck seller</td>
<td>Diabetes</td>
<td>NA</td>
</tr>
<tr>
<td>12</td>
<td>59/F</td>
<td>Chicken/duck seller</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>39/F</td>
<td>Chicken/duck seller</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>14</td>
<td>27/F</td>
<td>Chicken/duck seller</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>57/F</td>
<td>Chicken/pigeon slaughtering</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>16</td>
<td>52/F</td>
<td>Duck/goose seller</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>17</td>
<td>32/M</td>
<td>Pig slaughtering</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>18</td>
<td>52/M</td>
<td>Pig slaughtering</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>19</td>
<td>26/M</td>
<td>Pork seller</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>20</td>
<td>40/M</td>
<td>Grocer, control</td>
<td>Chronic bronchitis</td>
<td>5</td>
</tr>
<tr>
<td>21</td>
<td>48/M</td>
<td>Grocer, control</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>22</td>
<td>38/M</td>
<td>Grocer, control</td>
<td>Diabetes</td>
<td>5</td>
</tr>
<tr>
<td>23</td>
<td>61/M</td>
<td>Retired, control</td>
<td>No</td>
<td>NA</td>
</tr>
<tr>
<td>H5N1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>39/F</td>
<td>Chicken/duck/goose seller</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>25</td>
<td>45/F</td>
<td>Chicken/duck/pigeon raising</td>
<td>No</td>
<td>20</td>
</tr>
<tr>
<td>26</td>
<td>48/M</td>
<td>Pigeon seller</td>
<td>No</td>
<td>10</td>
</tr>
<tr>
<td>27</td>
<td>60/F</td>
<td>Chicken/duck/goose seller</td>
<td>No</td>
<td>10</td>
</tr>
<tr>
<td>28</td>
<td>55/F</td>
<td>Duck/goose seller</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>29</td>
<td>46/F</td>
<td>Chicken slaughtering</td>
<td>No</td>
<td>40</td>
</tr>
<tr>
<td>30</td>
<td>53/F</td>
<td>Chicken slaughtering</td>
<td>No</td>
<td>20</td>
</tr>
</tbody>
</table>

*MN, microneutralization; NA, the participant was not available in this year.

Risk Factors for AIV Infections

Poultry workers who performed selling had 4.25 (95% CI 1.20–25.32) times higher odds of H9N2 virus infection than did poultry workers who performed slaughtering (Table 5). Among poultry workers, female sex (adjusted OR 5.48 [95% CI 2.38–12.62]) and exposure to pigeons (adjusted OR 3.13 [95% CI 1.23–8.00]) were also significant risk factors for H5N1 virus seropositivity or seroconversion. Controls who were male (adjusted OR 8.75 [95% CI 1.09–70.45]) or had chronic respiratory disease (adjusted OR 7.24 [95% CI 1.42–37.00]) were more likely to be seropositive or to seroconvert for H9N2.

Diversity and Reassortment of AIVs at LPMs

During the study period, we collected and screened 3,121 samples from 9 LPMs for IAVs. A total of 466 (23.2%) of 2,010 cloacal swab samples, 145 (24.5%) of 590 environmental swab samples, and 115 (22.0%) of 521 fecal slurry specimens were positive for influenza A (Figure 3, panel A). Single infection with H9, H7, and H5 subtypes was detected in 229 (31.5%), 27 (3.7%), and 25 (3.4%) of 726 AIV-positive specimens, respectively. Sequencing results of 45 isolated strains and 33 original specimens (online Technical Appendix Table 3) showed that 10 AIV subtypes were detected in LPMs (Figure 3, panel B). To further study the origin of these 10 subtypes, we performed a detailed phylogenetic analysis for all available gene segments (online Technical Appendix Figure). The analyses revealed multiple gene segment exchanges among and within subtypes or interspecies among those circulating in domestic and wild birds, resulting in new genetic constellations and reassertive viruses, which we have represented in Table 3.
or both) and internal genes among the different subtypes or lineage and interspecies (Figure 4, panel B). The HA gene of 3 H5N2 viruses originated from clade 2.3.4 H5N2 (A/chicken/Wuhan/HAQL07/2014) or clade 7 H5N1 (A/chicken/Zhejiang/7450/2015)-like viruses and the HA gene of 3 H11N2 viruses from A/duck/Jiangxi/22537/2012-like H11N9 virus, the NA gene in all of them was originated from HxN2-like viruses. The 1 H3N8 virus (A/chicken/Wuxi/4859/2015) also had multireassortments that the HA and NA were respectively generated from the HA of Eurasian (A/duck/Jiangsu/26/2004) and North American lineage (A/pintail/Alberta/232/1992) H3N8-like virus, and the internal genes were reassorted with 6 subtypes circulating in ducks and wild birds. The HA of H1N2-like virus (A/chicken/ Wuxi/4859/2015) had multireassortments that the HA and NA were respectively generated from clade 2.3.4 H5N2 (A/chicken/Wuhan/HAQL07/2014) or clade 7 H5N1 (A/chicken/Zhejiang/7450/2015)-like viruses and the NA gene of Y-280 lineage H9N2-like virus (A/chicken/Wuxi/6082/2015) virus originating from Y280-like H9N2 viruses, the polymerase basic 2 gene of 1 H5N2 virus originated from Y280 lineage H9N2-like virus (A/chicken/Wuxi/6462/2015) virus originating from Y280-like H9N2 viruses, the polymerase basic 2 gene of 1 H5N2 virus originated from clade 2.3.2.1c H7N9-like virus (A/chicken/Wuhan/HAQL07/2014), and 1 H5N2 (A/chicken/Wuxi/5068/2015) and 1 H5N2 (A/chicken/Wuxi/5081/2015) viruses multireasserted from chicken or lineage and interspecies (Figure 4, panel B). The HA gene of 3 H5N2 viruses originated from clade 2.3.4 H5N2 (A/chicken/Wuhan/HAQL07/2014) or clade 7 H5N1 (A/ chicken/Zhejiang/7450/2015)-like viruses and the HA gene of 3 H11N2 viruses from A/duck/Jiangxi/22537/2012-like H11N9 virus, the NA gene in all of them was originated from HxN2-like viruses. The 1 H3N8 virus (A/chicken/ Wuxi/4859/2015) also had multireassortments that the HA and NA were respectively generated from the HA of Eurasian (A/duck/Jiangsu/26/2004) and North American lineage (A/pintail/Alberta/232/1992) H3N8-like virus, and the internal genes were reassorted with 6 subtypes circulating in ducks and wild birds. The HA of H1N2-like virus (A/Anseriformes/Anhui/L6/2014), the NA of clade 2.3.2.1c H5N1-like virus (A/chicken/Wuhan/HAQL07/2014), and the M gene of Y-280 lineage H9N2-like virus (A/chicken/
Shandong/wf0202/2012) reassorted and generated new H1N1 virus (A/chicken/Wuxi/5682/2015).

All H5 subtypes possessed a polybasic amino acid residue at the cleavage site (RERRRKR/GL), indicating they were highly pathogenic in chickens, whereas the other subtypes were low pathogenicity (online Technical Appendix Table 3). We detected the HA Q226L (H3 numbering) mutation in 4 H7N9 and all H9N2 viruses, indicating a binding ability to the human-like receptor. However, all subtypes had no polymerase basic 2 E627K and D701N mutations. All H7, H9, and H5 subtypes had the deletion in NA stalk associated with enhanced virulence in mice, as well as adaptation and transmission in poultry. All H9N2 viruses had oseltamivir resistance mutations of R292K in NA (N2 numbering), and adamantane resistance-associated mutation of S31N of M2 protein in 2 H5N1 and all H5N2, H1N1, H7N9, and H9N2 viruses.

Discussion
We estimated the seroprevalence and seroincidence of H7N9, H9N2, H5N1, and H5N6 viruses in an open cohort of poultry workers, swine workers, and the general population in Wuxi, Jiangsu Province, China. Poultry workers had relatively higher seroprevalence and seroincidence of H7N9, H9N2, and H5N1 than swine workers and the general population, although the overall seroprevalence and seroincidence was low. Active surveillance for AIVs revealed that 10 subtypes were circulating at LPMs, and
extensive gene segment reassorts occurred among and within subtype or interspecies that circulate in domestic poultry and wild birds.

Serologic evidence of human infection with H7N9 has previously been reported (14–18,22–24). In those studies, the seroprevalence ranged from 0% to 17.1%. In our study, a much lower seroprevalence of anti-H7N9 virus ranged from 0% to 0.56% during the enrollment and follow-up times in poultry workers. Our findings are similar to the 0.11% seroprevalence of MN titers >20 found in poultry workers in 6 provinces in China (18). In comparison with studies that did not perform MN testing, the proportion of elevated HI titers >20 in our study was also much lower (0%–2.83% in poultry workers). For example, 7.2%–14.9% of poultry workers in Shenzhen had HI titers >160 (14). Another study found that 1.6% of poultry workers with HI...
Human infections with H9N2 virus have been reported since 1998, and concern about its pandemic potential has increased, especially in recent years. Because this virus always causes mild upper respiratory tract illness that is clinically indistinguishable from the symptoms of common influenza caused by seasonal human H1N1 and H3N2 viruses, the incidence of H9N2 infections might be underestimated. Previous studies in China (25–28) and other countries (29–35) estimated that seroprevalence ranged from 0.5% to 4.6% in poultry workers. Our results showed that poultry workers had an overall H9N2 seroprevalence of 1.87% and a seroincidence of 8.78/1,000 person-years, which is significantly higher than those of H7N9 and H5N1. We detected no significant serologic response at baseline, which is significantly higher than those of H7N9 and H5N1.

Our active surveillance data revealed a high diversity of AIVs at LPMs. We observed genetic evidence of extensive reassortment of viral genes among and within subtype, and the new viral genes were introduced from the wild bird gene pool to domestic poultry, which further enriched such diversity. Additional co-detections of H9N2 with H7N9, H5N1, or H5N6 might provide the potential conditions for intersubtype reassortment. Our data also showed that H9N2 was the dominant circulating subtype, showing a high prevalence of 31.5%. Furthermore, all or some of the internal genes of the viruses we identified were from the Y280-like H9N2 virus, such as H7N9, H5N1, H5N2, and H1N1. All H5 subtype viruses showed a polybasic cleavage site, indicating its high pathogenicity in poultry. Although no outbreaks of H5 subtype viruses were reported in Wuxi, the outbreaks of H5N1, H5N2, and H5N6 in poultry were reported in several cities of Jiangsu Province surrounding Wuxi (39). Because H9N2 is not highly pathogenic, the extent of infection in poultry and humans is likely to remain underappreciated. Interaction or reassortment between the prevailing human and avian influenza viruses is considered the most probable scenario for generating new pandemic strains. We also argue that almost anywhere in the world where LPMs exist, especially large LPMs with different poultry, disparate viruses could be mixed yielding new AIVs. These viruses can move quickly across large geographic areas and change rapidly. Hence, our findings support the conclusion that LPMs play a critical role in the continual emergence of new reassortant AIVs that can spread through poultry populations. Thus, influenza surveillance among wild bird and domestic poultry at LPMs should be strengthened.

Our study had several limitations. First, although our study provides serologic evidence of virus infection, we did not conduct surveillance for influenza-like illness among participants, which prevents us from identifying laboratory-confirmed human disease and obtaining evidence of direct transmission from poultry to humans. Second, because of possible waning of antibodies or lack of antibody response to AIVs during the 1-year follow-up period, we were unlikely to have detected all seroconversions during the study period; thus, our study might underestimate the seroincidence.

In conclusion, conducting surveillance for new influenza virus surveillance at LPMs, especially when the LPMs are large and can sustain virus transmission, and monitoring the poultry and poultry workers for the new AIV infections are critical. Despite overall low seroprevalence or seroincidence, poultry workers had a higher risk for infection than swine workers and controls. Thus, it seems prudent to encourage poultry workers to use personal protective equipment (e.g., masks and gloves) and to undergo educational programs to help them understand and prevent AIV transmission between humans and poultry.
Acknowledgments
We thank the staff of Wuxi Center for Disease Control and Prevention for sampling and all participants for their cooperation.

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About the Author
Dr. Ma is a scientist at the Beijing Institute of Microbiology and Epidemiology. His primary research interests include molecular epidemiology and seroepidemiology of zoonotic influenza viruses and zoonotic transmission of influenza between species.

References

Technical Appendix

Methods

Serologic Assays

Hemagglutination-inhibition (HI) assay was performed as previously described (1). Briefly, after treatment by 1:4 solution of receptor-destroying enzyme (Denka Seiken Co Ltd, Tokyo, Japan) at 37°C for 18 h and then heat-inactivated at 56°C for 30 min to eliminate nonspecific inhibitors, 2-fold serial dilutions of serum (from 1:10 to 1:1,280) were tested against 8 hemagglutinin (HA) units of antigen using 1% horse red blood cells. For the H9N2 virus, 0.5% turkey red cells were used as the receptor of H9N2 virus circulation in China has changed to the human-like receptor. The HI titers were defined as the reciprocal of the highest serum dilution that completely inhibited hemagglutination.

Serum with HI titer ≥1:10 were further confirmed by microneutralization (MN) assay as previously described (2) using a culture of MDCK cells as with minor adaption. In brief, 2-fold serial dilutions of serum from 1:10 to 1:1,280 were incubated with 100 median 50% tissue culture infective dose of the H7N9 virus. 100 µL MDCK cells suspension with 2.0 × 10⁵ cells/mL to each well and the plate incubated at 37°C with 5% CO₂ for 24 h, followed by ELISA to determine virus titer. The MN titer was expressed as the reciprocal of the highest dilution of serum with optical density (OD) <X, where X = [(average OD of virus control wells) + (average OD of cell control wells)]/2. The MN titer as the reciprocal of the highest serum dilution that yielded >50% neutralization. For final titers <1:10 we assigned a value of 1:5 (seronegative).

Molecular Detection, Viral Isolation, and Sequencing

The viral RNA of each sample was extracted (Cat. No. 9766, TaKaRa, Dalian, China) and was subsequently screened for influenza virus A virus (IAV) by real-time reverse
transcription PCR (rRT-PCR) (cat. no. 56046, TaKaRa, Dalian, China) targeting the influenza matrix genome segment (3). These IAV-positive specimens were further subtyped for avian influenza H5, H7, and H9 as previously described (3,4). Meanwhile, IAV-positive specimens were inoculated into 9–11-day specific pathogen–free embryonated chicken eggs for virus isolation.

The full genome of cultured isolates was amplified (cat: 055A, TaKaRa, Dalian, China) using a pair of universal primers that amplify full-length viral genome sequences (5). PCR amplicons were purified (cat. no. 28004, QIAGEN) and then sequenced on Ion Torrent Personal Genome Machine (PGM, Life Technologies, South San Francisco, CA, USA). For samples that failed for virus isolation, HA, neuraminidase (NA), and matrix (M) genes were tried to amplify using universal primers (6) for sequencing. The sequence data were deposited in Global Initiative on Sharing All Influenza Data (GISAID) (accession no. EPI_ISL_277027–277050, EPI_ISL_277052–277064, and EPI_ISL_277093–277127).

**Phylogenetic Analysis**

To understand the molecular epidemiology of identified viruses in the study, we first examined ≥100 closely related sequences for each gene in GenBank and GISAID to infer the overall topology, and then we removed a few extreme outlying sequences from the trees. Maximum-likelihood phylogenetic trees were inferred for available gene segments by using MEGA software, version 6.06 (http://www.megasoftware.net). To assess the robustness of individual nodes on phylogenetic trees, we used a bootstrap resampling process (1,000 replications), the neighbor-joining method, and a best-fit, general time-reversible model of nucleotide substitution.

**Results**

During July 2013–June 2016, active surveillance of IAVs at 9 LPMs was conducted in Wuxi City, Jiangsu Province, China. A total of 3,121 samples (including 2,010 cloacal swabs, 590 environment swabs, and 521 fecal/slurry samples) were collected. A total of 726 (23.3%) samples, including 466 (23.2%) cloacal swabs, 145 (24.5%) environment swabs, and 115 (22.0%) fecal/slurry samples were rRT-PCR positive for IAVs. Of these 726 IAV-positive specimens, 229 (31.5%), 27 (3.7%), and 25 (3.4%) were single infection with H9, H7, and H5
subtype virus, respectively. co-infections of H7 and H9; H5 and H9; and H5, H7, and H9 were detected in 11 (1.5%), 2 (0.3%), and 1 (0.1%) specimens, respectively. A total of 45 samples were successfully isolated, and the whole-genome sequence was sequenced. The HA, NA, and M genes of 33 original specimens that failed for virus isolation were sequenced. The sequencing data revealed 10 subtypes of AIVs in live poultry markets, including H7N9 (n = 5), H9N2 (n = 45), H5N6 (n = 5), H5N1 (n = 6), H5N2 (n = 6), H11N2 (n = 3), H3N8 (n = 3), H1N1 (n = 1), H2N2 (n = 1), and H5N8 (n = 3).

The phylogeny of the H7N9 viruses showed that they all were derived from the Yangtze River Delta lineage associated with the 2013 outbreak of H7N9 in southern China (technical Appendix Figure, panel A). For the H9N2 viruses, their HA and NA genes were fell into the Y280-like lineage, while internal genes were closely related with those of H9N2 viruses in China except for the polymerase basic 2 gene of A/chicken/Wuxi/6082/2015 that was closely related to H5N2 and H5N8 viruses identified in poultry and wild birds, suggestive of reassortment (Technical Appendix Figure, panel B). The 3 H5N6 viruses fell into Jiangxi lineage, which belonged to clade 2.3.4.4, and all gene segments of the H5N6 viruses were closely related to those H5N6 viruses circulating in China and Japan but differed from our previously reported 2 Sichuan lineage–like H5N6 viruses (7) (Technical Appendix Figure, panel C). While the 4 H5N1 viruses were similar to our previously reported (7) and those of H5N1 viruses circulating in China and Vietnam, the M gene was closely related to those H5N1, and H5N1 containing M gene of Y280-like H9N2 viruses identified in China (Technical Appendix Figure, panel D). The HA genes of 5 newly identified H5N2 viruses fell into clade 2.3.4.4 and clade 7. The NA gene of these viruses was closely related to HxN2 viruses circulating in China and Vietnam, while the M gene was closely related to viruses of Y280-like H9N2 and H5 subtypes circulating in China, suggesting multiple reassortment occurred (Technical Appendix Figure, panel E). The HA gene of the 3 H11N2 viruses was closely related to H11Ny subtype viruses circulating in China and Thailand. Whereas the NA and 6 internal genes were derived from the HxN2 subtypes and wild bird origin subtypes circulating in Asian, respectively, indicating that it was in fact derived from multiple and interspecies reassortment events (Technical Appendix Figure, panel F). The HA gene of all three H3N8 viruses was Eurasian lineage and was closely related to those H3Ny subtypes in China, whereas the NA gene fell into Eurasian and North American lineage. The internal genes of the H3N8 viruses were most likely derived from HxNy subtypes in wild birds,
H3N2 and 2.3.2.1c H5N1 viruses in poultry, suggesting multiple and interspecies reassortment (Technical Appendix Figure, panel G). The HA gene of the H1N1 virus was closely related to H1 from multiple subtypes circulating in wild bird and belonged to the Eurasian avian lineage. However, the NA gene was closely related to clade 2.3.2.1c H5N1 viruses, while the M gene was closely related to H5N1 viruses containing M segment of Y280-like H9N2 viruses circulating in China, suggesting an interspecies reassortment occurred among H1Ny subtypes, H5N1, and H9N2 viruses (Technical Appendix Figure, panel H).

References


## Technical Appendix Table 1. Demographic characteristics of study participants, eastern China, 2013–2016*

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<td>168 (29.7)</td>
<td>216 (23.7)</td>
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<td>ILI of family members, no. (%)†</td>
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*ILI, influenza-like illness; NA, not available.
†Variable has missing data; The unit of cost is Chinese RMB.
**Technical Appendix Table 2.** Characteristic of participants with seropositive for influenza A(H7N9), A(H9N2), A(H5N1), and A(H5N6) viruses, eastern China, 2013–2016*

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*F, female; M, male; ILI, influenza-like illness; MN, microneutralization; NA, the participant was not available in this year.*
### Technical Appendix

**Table 3.** Characterization of selected molecular markers associated with infectivity, pathogenicity and antiviral susceptibility of viruses identified in the present study, eastern China, 2013–2016

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*Viral isolation was successful. HA, hemagglutinin; NA, neuraminidase; M, matrix; PB2, polymerase basic 2; NSD, no sequence data.
null
Technical Appendix Figure. Maximum-likelihood phylogenetic tree of influenza A viruses identified in the present study, Wuxi City, Jiangsu Province, eastern China, 2013–2016. The phylogenetic trees of the H7N9 (A), H9N2 (B), H5N6 (C), H5N1 (D), H5N2 (E), H11N2 (F), H3N8 (G), and H1N1 (H) subtypes are shown in panels A–H successively. Bootstrap values (n = 1,000) at key nodes are indicated. Values <75 were not shown. Scale bar indicates evolutionary distance (nucleotide substitutions per site). Black dots indicate the viruses reported in this study. Black triangles indicate the viruses had been previously reported. HA, hemagglutinin; NA, neuraminidase; NS, nonstructural; M, matrix; NP, nucleoprotein; PA, polymerase acidic; PB1, polymerase basic 1; PB2, polymerase basic 2.