A Serological Survey of Antibodies to H5, H7 and H9 Avian Influenza Viruses amongst the Duck-Related Workers in Beijing, China

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Abstract

The continued spread of highly pathogenic avian influenza (HPAI) viruses of H5 and H7 subtypes and low pathogenic avian influenza (LPAI) viruses of H5, H7 and H9 subtypes in birds and the subsequent infections in humans pose an ongoing pandemic threat. It has been proposed that poultry workers are at higher risk of exposure to HPAI or LPAI viruses and subsequently infection due to their repeated exposure to chickens or domestic waterfowl. The aim of this study was to examine the seroprevalence of antibodies against H5, H7 and H9 viruses amongst duck-related workers in Beijing, China and the risk factors associated with seropositivity. In March, 2011, 1741 participants were recruited from (1) commercial duck-breeding farms; (2) private duck-breeding farms; and (3) duck-slaughtering farms. Local villagers who bred ducks in their backyards were also recruited. A survey was administered by face-to-face interview, and blood samples were collected from subjects for antibody testing against H5, H7 and H9 viruses. We found that none of the subjects were seropositive for either H5 or H7 viruses, and only 0.7% (12/1741) had antibody against H9. A statistically significant difference in H9 antibody seroprevalence existed between the various categories of workers (P = 0.005), with the highest figures recorded amongst the villagers (1.7%). Independent risk factors associated with seropositivity to infection with H9 virus included less frequent disinfection of worksite (OR, 5.13 [95% CI, 1.07–24.58]; P = 0.041; ≤ twice monthly versus > twice monthly) and handling ducks with wounds on hands (OR, 4.13 [95% CI, 1.26–13.57]; P = 0.019). Whilst the risk of infection with H5, H7 and H9 viruses appears to be low among duck-related workers in Beijing, China, ongoing monitoring of infection with the H9 virus is still warranted, especially amongst villagers who breed backyard ducks to monitor for any changes.

Introduction

The spread of highly pathogenic avian influenza (HPAI) viruses of H5 or H7 subtypes and low pathogenic avian influenza (LPAI) viruses of H5, H7 or H9 subtypes amongst birds and sporadic infection in humans continues to pose a threat to public health [1–10], because of the potential for a strain with pandemic potential to emerge via adaptive mutation or reassortment [11]. The 1918 pandemic began following adaptive mutation of an avian virus, and the pandemics of 1957 and 1968 were the result of genetic reassortment of viruses from human and avian sources [12–14]. While the last pandemic originated from a swine-origin influenza virus (S-OIV) [15], the threat brought by avian influenza viruses continues.

As of September, 2012, a total of 608 human cases of HPAI H5N1 were reported globally, with a case-fatality ratio of 59.0% (359 fatal cases) [16]. In addition, human cases infected with H7 or H9 viruses were also sporadically reported in a couple of countries. In 1996, a woman developed conjunctivitis after a piece of straw had entered her eye while cleaning her duck house, with LPAI H7N7 virus isolated from her conjunctiva specimen [3]. In 2003, 89 people were confirmed as being infected with HPAI H7N7 in the Netherlands with evidence of limited person to person transmission, which was associated with an outbreak of HPAI H7N7 in chickens in over 200 farms [4,5]. In 2007, four cases infected with LPAI H7N2 were identified after an outbreak of LPAI H7N2 in chickens on a small farm in north Wales in the United Kingdom [6]. Eight cases were confirmed as being infected with LPAI H9N2 from mainland and Hong Kong, China in 1998–1999, of which three had contact with live chickens before illness onset [7–9]. In 2003, a five-year-old child infected with LPAI H9N2 was reported in Hong Kong, China. However, the source of infection was unknown [10].

To date most studies, especially those on risk factors of infection, have generally focused on H5 virus rather than H7 or H9 viruses. In 2006, several human infections with H5N1 were reported from China [17], however, the source of infection was unknown. Given the distinct possibility of human infection with H7 or H9 viruses and the minimal human-to-human transmission of avian-adapted viruses, it is important that the research focus shift accordingly. Previous studies have demonstrated that both direct...
and indirect exposure to infected live/dead poultry, including chickens or domestic waterfowl, plays a very important role in the transmission of HPAI or LPAI viruses to human [3,7,17,20,22]. Poultry workers are therefore considered to be at highest risk of infection with HPAI or LPAI viruses because of their frequent exposure to chickens or domestic waterfowl. In addition, as China is considered to be an influenza epicenter [23], ongoing exposure to chickens or domestic waterfowl, plays a very important role in the transmission of HPAI or LPAI viruses to human [3–7,17,20,22]. Poultry workers are therefore considered to be at highest risk of transmission of HPAI or LPAI viruses to human [3–7,17,20,22].

In this study, we conducted a serological survey of antibodies against H5, H7 and H9 viruses amongst duck-related workers in Beijing, China to examine previous infection with these viruses and associated risk factors in this population.

### Materials and Methods

#### Ethics Statement

This study was approved by the institutional review board and human research ethics committee of Beijing Center for Disease Prevention and Control (CDC).

#### Subjects and Survey Design

This study was conducted in Beijing, China in March, 2011. In Beijing, 14 of the 18 districts are classified as having some form of duck-related industry. 6/14 districts were randomly selected for inclusion in the study. Workers from commercial and private duck-breeding farms and slaughtering sites were invited to participate in this study. Local villagers involved with breeding ducks in their backyards were also recruited. A duck-slaughtering site refers to a place where ducks are killed, plucked, the heads/viscera removed, and processed ducks are cleaned and packaged. Workers were excluded if they were employed in a position in which exposure to ducks was limited such as those in administrative roles. From the selected six districts, all identified workers who were employed within the sector and households involved with breeding ducks in their backyards were invited to participate in this study. From each household, one family member who was deemed to have the closest contact with ducks was recruited in this study.

After obtaining written informed consent from the participants, a questionnaire was administered via a face-to-face interview conducted by trained staffs, and blood samples were collected for antibody testing against H5, H7 and H9 viruses.

#### Survey Contents

The survey included questions on employment, demographics (sex, age, and education background), presence of any underlying disease, smoking, and alcohol intake. Other items included duration of exposure to ducks, farming practices (e.g. breeding patterns, duck breeds), avian influenza vaccination status of the ducks, exposure of ducks to other species of birds, disinfection of worksite, personal protective equipment use, handling ducks with wounds on hands and contact with sick or dead ducks, etc.

#### Laboratory Testing

Serum samples were pretreated and assayed by hemagglutination-inhibition (HI) assay, as previously described [24]. One volume of serum was treated with four volumes of receptor-destroying enzyme (RDE) at 37°C for 18 hours, and was then incubated at 56°C for 30 minutes, followed by absorption with horse erythrocytes. The titration of 1:10 was first prepared for each pre-treated serum sample to test for specific antibodies against H5, H7 and H9 virus antigens using 1% horse erythrocytes. H5, H7 and H9 virus antigens employed for HI assay were A/Chicken/Anhui/01/2005 (HPAI H5N1), A/Chicken/Hebei/02/2007(LPAI H7N2) and A/Chicken/Shanghai/10/1999 (LPAI H9N2), provided by Qingdao YEBIO Bio-engineering Co., Ltd, China. The serum samples with 1:10 titer that were able to inhibit virus-induced hemagglutination were then diluted into eight titrations (1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280) for the HI assay. The HI titer was calculated as the reciprocal of the highest dilution of serum that inhibited virus-induced hemagglutination of the horse erythrocytes. A titer value of ≥1:40 was regarded as positive, i.e. previous infection [25,26].

#### Statistical Analysis

Data were entered in duplicate using Epitools Software, and was analyzed using SPSS16.0 statistical package (SPSS Inc., Chicago, IL, USA). We estimated the seroprevalence rates of antibodies against H5, H7 and H9 viruses among various types of subjects. Seroprevalence rates were compared between subgroups using Pearson $\chi^2$ test. Seropositive (HI titer≥1:40) and seronegative (HI titer<1:40) groups were compared to identify potential risk factors for past infection with HPAI or LPAI viruses.

| Participant category       | Frequency (% of total) |
|---------------------------|------------------------|
| Commercial duck-breeders  | 313 (18.0)             |
| Private duck-breeders     | 261 (15.0)             |
| Villagers breeding backyard ducks | 605 (34.7) |
| Duck-slaughtering workers | 562 (32.3)             |
| Sex                       |                        |
| Male                      | 759 (43.6)             |
| Female                    | 982 (56.4)             |
| Age group                 |                        |
| <50 years                 | 1191 (68.4)            |
| >50 years                 | 550 (31.6)             |
| Ethnic group              |                        |
| Chinese Han               | 1637 (94.0)            |
| Chinese Minority Groups   | 104 (6.0)              |
| Education background      |                        |
| >Primary school           | 972 (55.8)             |
| ≤Primary school           | 769 (44.2)             |
| Marital status            |                        |
| Not married               | 207 (11.9)             |
| Married                   | 1515 (87.0)            |
| Divorced                  | 6 (0.3)                |
| Widowed                   | 13 (0.7)               |
| Having underlying disease |                        |
| No                        | 1592 (91.4)            |
| Yes                       | 149 (8.6)              |
| Currently smoker          |                        |
| No                        | 1266 (72.7)            |
| Yes                       | 475 (27.3)             |
| Currently drinker         |                        |
| No                        | 1208 (69.4)            |
| Yes                       | 533 (30.6)             |

Table 1. Baseline characteristics of study participants (n=1741).

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and statistical significance was defined with \( p \) conducted with a probability of removal set at 0.1, i.e. all variables included in multivariate analysis. Backward logistic regression was conducted to determine risk factors associated with seropositivity for antibodies to H5, H7 or H9 viruses in duck-related workers in the serological survey in Beijing, China.

### Results

#### Characteristics of Subjects

A total of 1741 subjects, with the median of age of 44 years (range: 14–71 years), were involved in this study, including 313/374 (response rate: 83.7%) participants from the commercial duck-breeding farms, 605/710 (response rate: 85.2%) from farms which slaughtered ducks, and 605/710 (response rate: 85.2%) villages who bred backyard ducks. The participant demographic information is summarized in Table 1.

#### Antibody Seroprevalence Against H5, H7 or H9 Viruses

In this study, none of the subjects were seropositive for either H5 or H7 antibodies, and only twelve (0.7%, 12/1741) had antibody against H9, among which four had a titer of 1:80 and eight with a titer of 1:40. Ten of the villagers were seropositive to H9 (1.7%, 10/590), and 605/710 (response rate: 85.2%) villagers who bred backyard ducks. The participant demographic information is summarized in Table 1.

#### Risk Factors Associated with Seropositivity for Antibodies to H9 Virus in Duck-related Workers

In univariate analysis, the following factors were found to be significantly associated with seropositivity for antibodies to H9 virus (Table 3): older age (odds ratio [OR], 4.38 [95% confidence interval [CI], 1.31–14.61]; \( P = 0.016 \); >50 years versus \( \leq 50 \) years), fewer years of education (OR, 3.83 [95% CI, 1.03–14.18]; \( P = 0.045 \); \( \leq \)primary school versus above primary school), exposure to ducks ranging on land and river (OR, 3.91 [95% CI, 1.17–13.04]; \( P = 0.026 \); versus ranging only on land), exposure to layer ducks (OR, 6.36 [95% CI, 1.72–23.59]; \( P = 0.006 \); versus broiler ducks), exposure to ducks in contact with other birds (OR, 3.92 [95% CI, 1.24–12.41]; \( P = 0.020 \), less frequent disinfection of worksite (OR, 7.48 [95% CI, 1.63–34.22]; \( P = 0.010 \); ≤twice monthly versus>twice monthly), noncompliance with mask use (OR, 8.06 [95% CI, 1.04–62.53]; \( P = 0.046 \); and handling ducks with wounds on hands (OR, 6.33 [95% CI, 2.00–20.09]; \( P = 0.002 \).

In multivariate analysis, significant independent risk factors included less frequent disinfection of worksite (OR, 5.13 [95% CI, 1.07–24.58]; \( P = 0.041 \); ≤twice monthly versus>twice monthly) and handling ducks with wounds on hands (OR, 4.13 [95% CI, 1.26–13.57]; \( P = 0.019 \) (Table 4).

#### Discussion

In this study, none of the enrolled workers were seropositive for either H5 or H7 antibodies, but 0.7% had antibody against H9 virus. This finding indicates that the risk of infection with H5, H7 and H9 viruses appears to be low among duck-related workers in Beijing, China, but the risk of infection with H9 virus is slightly elevated in comparison to the other subtypes.

Although HPAI H5N1 virus continues to circulate in chickens and domestic waterfowl in China [27], human infection with HPAI H5N1 virus so far has been rare, with only 43 human cases as of the end of September, 2012 [16]. Low rates of subclinical infection in poultry (chickens of domestic waterfowl) workers have previously been documented in a number of Chinese studies [28,29]. In addition, studies conducted in other countries have also reported low frequency of transmission of this virus to poultry (chickens of domestic waterfowl) workers [30–32]. These reports and our findings indicate that there continues to be a strong host specificity of infection with the H5 virus.

| Characteristic                  | Subject number | H5 virus | H7 virus | H9 virus | \( P \text{ value}\)* |
|---------------------------------|----------------|----------|----------|----------|----------------------|
| Participant category            |                |          |          |          |                      |
| Commercial duck-breeder         | 313            | 0 (0)    | 0 (0)    | 0 (0)    | 0.005\( \)            |
| Private duck-breeder            | 261            | 0 (0)    | 0 (0)    | 1 (0.4)  |                      |
| Villagers breeding backyard ducks| 605            | 0 (0)    | 0 (0)    | 10 (1.7) |                      |
| Duck-slaughtering workers       | 562            | 0 (0)    | 0 (0)    | 1 (0.2)  |                      |
| Sex                             |                |          |          |          |                      |
| Male                            | 759            | 0 (0)    | 0 (0)    | 5 (0.7)  | 0.892\( b\)           |
| Female                          | 982            | 0 (0)    | 0 (0)    | 7 (0.7)  |                      |
| Age group                       |                |          |          |          |                      |
| \( \leq 50 \) years             | 1191           | 0 (0)    | 0 (0)    | 4 (0.3)  | 0.021\( c\)           |
| >50 years                       | 550            | 0 (0)    | 0 (0)    | 8 (1.5)  |                      |
| Total                           | 1741           | 0 (0)    | 0 (0)    | 12 (0.7) |                      |

NOTE. Data are seropositive no (%), unless otherwise indicated.

\( a \)Comparison of H9 antibody status.

\( b \)Compared by Pearson \( \chi^2 \) test.

\( c \)Compared by Pearson \( \chi^2 \) test with continuity correction.

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Table 3. Univariate analysis for risk factors associated with seropositivity for antibodies to H9 virus amongst duck-related workers, Beijing, China.

| Factors                              | Seropositivity (n = 12) | Seronegativity (n = 1729) | OR (95% CI)          | P value*       |
|--------------------------------------|-------------------------|---------------------------|----------------------|----------------|
| Sex                                  |                         |                           |                      |                |
| Male                                 | 5 (41.7)                | 754 (43.6)                | Reference            |                |
| Female                               | 7 (58.3)                | 975 (56.4)                | 1.08 (0.34–3.43)     | 0.892          |
| Age group                            |                         |                           |                      |                |
| ≤50 years                            | 4 (33.3)                | 1187 (68.7)               | Reference            | 0.016          |
| >50 years                            | 8 (66.7)                | 542 (31.3)                | 4.38 (1.31–14.61)    |                |
| Education background                 |                         |                           |                      |                |
| >Primary school                      | 3 (25.0)                | 969 (56.0)                | Reference            |                |
| ≤Primary school                      | 9 (75.0)                | 760 (44.0)                | 3.83 (1.03–14.18)    | 0.045          |
| Having underlying disease            |                         |                           |                      |                |
| No                                   | 10 (83.3)               | 1582 (91.5)               | Reference            |                |
| Yes                                  | 2 (16.7)                | 147 (8.5)                 | 2.15 (0.47–9.92)     | 0.325          |
| Currently smoker                     |                         |                           |                      |                |
| No                                   | 9 (75.0)                | 1257 (72.7)               | Reference            |                |
| Yes                                  | 3 (25.0)                | 472 (27.3)                | 0.89 (0.24–3.29)     | 0.859          |
| Currently drinker                    |                         |                           |                      |                |
| No                                   | 7 (58.3)                | 1201 (69.5)               | Reference            |                |
| Yes                                  | 5 (41.7)                | 528 (30.5)                | 1.63 (0.51–5.14)     | 0.409          |
| Years of exposure                    |                         |                           |                      |                |
| ≤5 years                             | 9 (75.0)                | 1259 (72.8)               | Reference            |                |
| >5 years                             | 3 (25.0)                | 470 (27.2)                | 0.89 (0.24–3.31)     | 0.866          |
| Breeding pattern of ducks            |                         |                           |                      |                |
| Ranging only on land                 | 4 (33.3)                | 1144 (66.2)               | Reference            | 0.026          |
| Ranging on land and river            | 8 (66.7)                | 585 (33.8)                | 3.91 (1.17–13.04)    |                |
| Type of ducks                        |                         |                           |                      |                |
| Broiler ducks                        | 3 (25.0)                | 1175 (68.0)               | Reference            | 0.006          |
| Layer ducks                          | 9 (75.0)                | 554 (32.0)                | 6.36 (1.72–23.59)    |                |
| Avian influenza vaccination in ducks |                         |                           |                      |                |
| Yes                                  | 11 (91.7)               | 1314 (76.0)               | Reference            |                |
| No                                   | 1 (8.3)                 | 415 (24.0)                | 0.29 (0.04–2.24)     | 0.234          |
| Ducks in contact with other birds    |                         |                           |                      |                |
| No                                   | 5 (41.7)                | 1274 (73.7)               | Reference            |                |
| Yes                                  | 7 (58.3)                | 455 (26.3)                | 3.92 (1.24–12.41)    | 0.020          |
| Frequency of disinfection of worksite|                         |                           |                      |                |
| >twice monthly                       | 2 (16.7)                | 1036 (59.9)               | Reference            | 0.010          |
| ≤twice monthly                       | 10 (83.3)               | 693 (40.1)                | 7.48 (1.63–34.22)    |                |
| Mask use                             |                         |                           |                      |                |
| Yes                                  | 1 (8.3)                 | 731 (42.3)                | Reference            |                |
| No                                   | 11 (91.7)               | 998 (57.7)                | 8.06 (1.04–62.55)    | 0.046          |
| Glove use                            |                         |                           |                      |                |
| Yes                                  | 3 (25.0)                | 815 (47.1)                | Reference            |                |
| No                                   | 9 (75.0)                | 914 (52.9)                | 2.68 (0.72–9.92)     | 0.141          |
| Handling ducks with wounds on hands  |                         |                           |                      |                |
| No                                   | 5 (41.7)                | 1416 (81.9)               | Reference            |                |
| Yes                                  | 7 (58.3)                | 313 (18.1)                | 6.33 (2.00–20.09)    | 0.002          |
| Occurrence of sick/dead ducks at worksite |                 |                           |                      |                |
| No                                   | 10 (83.3)               | 1454 (84.1)               | Reference            |                |
| Yes                                  | 2 (16.7)                | 275 (15.9)                | 1.06 (0.23–4.85)     | 0.943          |
In agreement with a previous study from Northern China, we did not find any subjects who were seropositive to the H7 virus [33]. In comparison, previous European studies have documented higher frequencies of human infection with the H7 viruses [3–6]. This correlates well to the surveillance studies of H7 viruses in birds which have documented persistent epidemics of the virus in birds in many European countries [1], but not in China.

In this study, ten villagers and two farm workers were seropositive to H9 antibody. Previously, a study from Guangzhou in southern China also found that the seroprevalence of antibody against H9 virus (4.5%) in poultry workers was much higher than that against H5 virus (0.2%) [29]. In addition, a study conducted in northern China found that 1.5% (12/783) of villagers breeding backyard poultry (chickens or domestic waterfowl) were seropositive to H9 virus, but identified no persons seropositive to H7 virus [33]. Our findings, along with those from previous studies support the notion that there is relatively higher risk from H9 virus in China, in comparison to H5 and H7 subtypes.

In the present study, the seroprevalence of antibodies against H9 virus amongst villagers breeding ducks in their backyard was significantly greater than participants from other categories, which was supported by a previous work [33]. This difference may be attributed to two reasons: firstly, people raising ducks in their backyards have closer and longer periods of exposure to ducks, and this may put them at increased risk for exposure to viruses; secondly, people who work with ducks in their job are more likely to use personal protective equipment like masks and gloves than people raising ducks in their backyards.

In this study, the seroprevalence of antibodies to H9 virus amongst participants above 50 years was significantly higher than those less than 50 years. As there is no report about the change of susceptibility in human of the H9 viruses that have circulated in China in recent years, we consider that this difference by age may be attributed to a longer period of exposure to ducks in older participants.

Participants who reported that the worksite was infrequently disinfected were more likely to be identified as seropositive for antibodies to H9 virus in our study. In corroboration are the results from a Korean study that found an increased risk of seropositivity against H9 virus in chickens associated with less frequent cleaning with disinfectants [34]. We also found that contact with ducks while having a hand wound was also a risk factor for previous infection with H9 virus amongst the duck workers. Given this finding, glove should be recommended for workers with wounds on hands to use.

It has been reported that handling healthy, sick and dead chickens or domestic waterfowl was the predominant means of human infection with HPAI H5N1, HPAI/LPAI H7 and LPAI H9N2 viruses [2–7]. In addition, for patients infected with HPAI H5N1, contact with virus-contaminated fomites followed by self-inoculation of the respiratory tract or inhalation of aerosolized infectious excreta was also plausible transmission route [2]. Therefore, mask or glove use may theoretically provide protection against H5, H7 and H9 viruses for persons having frequent contact with chickens or domestic waterfowl. In the present study, no mask use was a significant risk factor for previous infection with H9 virus in univariate analysis, but not in multivariate analysis; no glove use was not a significant risk factor either in univariate analysis or in multivariate analysis. Due to the small number of persons seropositive to H5, H7 and H9 viruses, this study may be underpowered to assess the effect of mask or glove use.

The LPAI H9N2 virus strain used in our HI assay, belonging to the BJ94-like lineage, was isolated in 1999, and it was the diagnostic antigen for detecting antibodies to H9 virus recommended by the agricultural authorities in China. Although H9N2 viruses circulating in China had experienced genetic and antigenic changes since 1999, it was found that similar sequence and obvious

### Table 3. Cont.

| Factors                              | Seropositivity (n = 12) | Seronegativity (n = 1729) | OR (95% CI) | P value* |
|--------------------------------------|-------------------------|---------------------------|-------------|----------|
| Close contact with sick/dead ducks   |                         |                           |             |          |
| No                                   | 11 (91.7)               | 1498 (86.6)               | Reference   |          |
| Yes                                  | 1 (8.3)                 | 231 (13.4)               | 0.59 (0.08–4.59) | 0.614   |
| Close contact with other animals     |                         |                           |             |          |
| No                                   | 9 (75.0)                | 917 (53.0)               | Reference   |          |
| Yes                                  | 3 (25.0)                | 812 (47.0)               | 0.38 (0.10–1.40) | 0.144   |
| Exposure to birds outside of work setting* |                       |                           |             |          |
| No                                   | 12 (100)                | 1700 (98.3)              | Reference   | 1.000    |
| Yes                                  | 0 (0)                   | 29 (1.7)                 | NA          |          |

**NOTE.** Data are frequency (%) of subjects, unless otherwise indicated. Univariate unconditional logistic regression was employed to compare frequencies of exposure between seropositive group and seronegative group. OR, odd ratio; CI, confidence interval; NA, not available. Boldface indicates P<0.1.

*Fisher’s exact test was used because data distribution could not be analyzed by logistic regression.

In this study, the seroprevalence of antibodies to H9 virus amongst villagers breeding ducks in their backyard was significantly higher than participants from other categories, which was supported by a previous work [33]. This difference may be attributed to two reasons as follows: firstly, people raising ducks in their backyard have closer and longer periods of exposure to ducks, and this may put them at increased risk for exposure to H9 virus amongst the duck workers. Given this finding, glove should be recommended for workers with wounds on hands to use.

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In this study, the seroprevalence of antibodies to H9 virus amongst participants above 50 years was significantly higher than those less than 50 years. As there is no report about the change of susceptibility in human of the H9 viruses that have circulated in China in recent years, we consider that this difference by age may be attributed to a longer period of exposure to ducks in older participants.

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cross-reactivity in HI assay existed between H9N2 viruses isolated around 1999 and those isolated in the recent years, and all these viruses still belonged to BJ94-like lineage [35,36]. In addition, as most of patients infected with LPAI H9N2 virus in China were also found around 1999 [7–10], using the virus isolated at that time for HI assay may have higher efficiency to detect human infection with H9 virus crossing the species barrier. In addition, although Beijing and Shanghai are located in different regions of China, there were no distinct regional differences found in LPAI H9N2 viruses circulating in China previously [35,36]. Based on the above-mentioned reasons, we selected A/Chicken/Shanghai/10/1999 (LPAI H9N2) strain used in the HI assay for our study.

In this study, we applied HI assay using horse erythrocytes to detect human sera for antibodies to H5, H7 and H9 viruses. HI assay using horse erythrocytes has high sensitivity and specificity in detecting human antibodies against avian-specific influenza viruses [24,26]. In comparison with HI assay using chicken or turkey erythrocytes, HI assay using horse erythrocytes has increased sensitivity, which may be explained by the fact that horse erythrocytes express a higher proportion of sialic acid containing N-acetyllactosamine acid 2,3-galactose (SA2,3Gal) linkages which avian-specific influenza viruses preferentially bind [37–39].

The study population in this study was the people related to domestic ducks. However, in fact the people frequently exposed to wild ducks should be concerned about as well because wild birds are thought to form the reservoir of influenza A viruses in nature [40]. It was ever reported by Gill et al that of 39 duck hunters and 68 wildlife professionals in the US, three had previous infection with H11N9 virus [41]. In addition, as the worksites of the participants in our study were mainly located in rural areas, they also had the opportunity to contact wild birds and be exposed to wild bird-origin virus strains. The antigens used in this study are the diagnostic antigens recommended by agricultural authorities in China for testing antibodies against viruses in poultry (chickens or domestic waterfowl), thus we are not sure if these antigens could be used for detecting antibodies elicited by wild bird-origin strains.

However, considering the similarity between wild bird-origin strains and poultry-origin strains [42–44], we think antigenic cross-reactivity in HI assay may exist between antigens used in this study and wild bird-origin strains.

This study has several limitations. Firstly, information regarding the exposures of participants to ducks was all based on self-report, and this study is therefore subject to recall bias. Secondly, we only detected twelve subjects with antibody against H9 virus; therefore, this study was probably underpowered as the result of the small sample size to detect other potentially significant risk factors for previous infection. Thirdly, there could be some risk factors that were not taken into account in this study. It was a limitation that we could not assess the effect of visiting live poultry markets because selling live poultry in food markets had been banned in Beijing since 2005.

In summary, the risk of infection with H5, H7 and H9 viruses appears to be low among duck-related workers in Beijing, China, but closer monitoring of infection with the H9 virus should be warranted, especially amongst villagers breeding ducks in their backyards. Less frequent disinfection of the worksites and having contact with ducks while having hand wounds were both independent risk factors for previous infection with H9 virus amongst the duck workers.

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Author Contributions

Conceived and designed the experiments: QW PY. Performed the experiments: QW PY CM WS SC GL XP HL. Analyzed the data: PY YL. Wrote the paper: QW PY. Critical revision of the manuscript for important intellectual content: HS.

References

1. Alexander DJ (2007) Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002–2006. Avian Dis 51: 161–166.
2. Abdel-Ghafar AN, Chotpitayasunondh T, Gao Z, Hayden FG, Nguyen DH, et al. (2008) Update on avian influenza A (H5N1) virus infection in humans. N Engl J Med 359: 261–272.
3. Kurtz J, Manwell RJ, Banks J (1996) Avian influenza virus isolated from a woman with conjunctivitis. Lancet 348: 901–902.
4. Koopmans M, Wilfflem B, Coryn M, Natrop G, van der Nat H, et al. (2004) Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. Lancet 363: 587–593.
5. Fouquier RA, Schneeberger PM, Rouzehad FW, Broekman JM, Kemink SA, et al. (2004) Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci U S A 101: 1356–1361.
6. Editorial team (2007) Avian influenza A/(H7N2) outbreak in the United Kingdom. Euro Surveill 12: 3206.
7. Guo Y, Li J, Cheng X (1999) Discovery of men infected by avian influenza A (H9N2) virus. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi 13: 105–108.
8. Guo Y, Xie J, Wang M (2000) A strain of influenza A H9N2 virus repeatedly isolated from human population in China. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi 14: 209–213.
9. Peiris M, Yuan KY, Leung CW, Chan KH, Ip PH, et al. (1999) Human infection with H9N2 virus. Lancet 354: 916–917.
10. Butt KM, Smith GJ, Chen H, Zhang LJ, Leung YH, et al. (2005) Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. J Clin Microbiol 43: 5760–5767.
11. Lin YP, Shaw M, Gregory V, Cameron K, Lim W, et al. (2000) Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H9N1 human isolates. Proc Natl Acad Sci U S A 97: 9634–9635.
12. Reid AH, Fanning TG, Hultin JV, Taubenberger JK (1999) Origin and evolution of the 1918 “Spanish” influenza virus hemagglutinin gene. Proc Natl Acad Sci U S A 96: 1631–1636.
13. Schäfer JR, Kawaoka Y, Bean WJ, Suss J, Senne D, et al. (1990) Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir. Virology 194: 781–788.
14. Scholtissek C, Rohde W, Von Hoyningen V, Rott R (1978) On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology 87: 13–20.
15. Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, et al. (2009) Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med 360: 2007–2015.
16. World Health Organization (2012) Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003–2012.
17. Zhou L, Liao Q, Dong L, Hua Y, Bai T, et al. (2009) Risk factors for human illness with avian influenza A (H5N1) virus infection in China. J Infect Dis 199: 1726–1734.
18. Cavailler P, Chu S, Ly S, Garcia JM, Ha do Q, et al. (2010) Seroprevalence of anti-H5 antibody in rural Cambodia, 2007. J Clin Virol 48: 123–126.
19. Van Kerkhove MD, Ly S, Holl D, Guitian J, Mangtani P, et al. (2008) Frequency and patterns of contact with domestic poultry and potential risk of H5N1 transmission to humans living in rural Cambodia. Influenza Other Respir Viruses 2: 155–163.
20. Dinh PN, Long HT, Tien NT, Hien NT, Mai le TQ, et al. (2006) Risk factors for human infection with avian influenza A H5N1, Vietnam, 2004. Emerg Infect Dis 12: 1841–1847.
21. Song V, Ly S, Van Kerkhove MD, Achenbach J, Holl D, et al. (2009) Risk factors associated with subclinical human infection with avian influenza A (H5N1) virus–Camodia, 2006. J Infect Dis 199: 1744–1752.
22. Mounis AW, Kswong H, Izurieta HS, Ho Y, Au T, et al. (1999) Case-control study of risk factors for avian influenza A (H5N1) disease, Hong Kong, 1997. J Infect Dis 180: 505–508.
23. ShortridgeKF,Stuart-HarrisCH(1982)Aninfluenzaepidemic?Lancet:812–813.
24. Katzih G, Netzeuzist SF, Capuano AW, Myers KP, Gill JS, et al. (2008) Testing human sera for antibodies against avian influenza viruses: horse RBC
hemagglutination inhibition vs. microneutralization assays. J Clin Virol 43: 73–78.

25. Pawar SD, Tandale BV, Raut CG, Parkhi SS, Barde TD, et al. (2012) Avian influenza H9N2 seroprevalence among poultry workers in Pune, India, 2010. PLoS One 7: e36374.

26. Meijer A, Bouman A, van de Kamp EE, Wilbrink B, Da Ry van Beest Holle M, et al. (2006) Measurement of antibodies to avian influenza virus A(H7N7) in humans by hemagglutination inhibition test. J Virol Methods 132: 113–120.

27. Martin V, Pfeiffer DU, Zhou X, Xiao X, Prosser DJ, et al. (2011) Spatial distribution and risk factors of highly pathogenic avian influenza HPAI H5N1 in China. PLoS Pathog 7: e1001308.

28. Huo X, Zu R, Qi X, Qin Y, Li L, et al. (2012) Seroprevalence of avian influenza A (H5N1) virus among poultry workers in Jiangsu Province, China: an observational study. BMC Infect Dis 12: 93.

29. Wang M, Fu CX, Zheng BJ (2009) Antibodies against H5 and H9 avian influenza among poultry workers in China. N Engl J Med 360: 2583–2584.

30. Schulz C, Nguyen VD, Hai le T, Do QH, Peiris JS, et al. (2009) Prevalence of antibodies against avian influenza A (H5N1) virus among Cullers and poultry workers in Ho Chi Minh City, 2003. PLoS ONE 4: e7948.

31. Hinjey S, Puthavathana P, Lamsakarnjanarat K, Pooruk P, et al. (2008) Low frequency of infection with avian influenza virus (H5N1) among poultry farmers, Thailand, 2004. Emerg Infect Dis 14: 499–501.

32. Yong S, Gogliani B, Marily S, Holf D, Seng H, et al. (2006) Low frequency of poultry-to-human H5N1 virus transmission, southern Cambodia, 2005. Emerg Infect Dis 12: 1542–1547.

33. Jia N, de Vlas SJ, Liu YX, Zhang JS, Zhan L, et al. (2009) Serological reports of human infections of H7 and H9 avian influenza viruses in northern China. J Clin Virol 44: 225–229.

34. Woo JT, Park BK (2008) Seroprevalence of low pathogenic avian influenza (H9N2) and associated risk factors in the Gyeonggi-do of Korea during 2005–2006. J Vet Sci 9: 161–168.

35. Ji K, Jiang WM, Liu S, Chen JM, Chen J, et al. (2010) Characterization of the haemagglutinin gene of subtype H9 avian influenza viruses isolated in 2007–2009 in China. J Virol Methods 163: 186–189.

36. Zhang Y, Yin Y, Li Y, Wang S, Xu S, et al. (2012) Molecular and antigenic characterization of H9N2 avian influenza virus isolates from chicken flocks between 1998 and 2007 in China. Vet Microbiol 156: 283–293.

37. Jia N, Wang SX, Liu YX, Zhang PH, Zuo SQ, et al. (2008) Increased sensitivity for detecting avian influenza-specific antibodies by a modified hemagglutination inhibition assay using horse erythrocytes. J Virol Methods 153: 43–48.

38. Ito T, Suzuki Y, Mitnaul L, Vines A, Kida H, et al. (1997) Receptor specificity of influenza A viruses correlates with the agglutination of erythrocytes from different animal species. Virology 227: 493–499.

39. Stephenson I, Wood JM, Nicholson KG, Zambon MC (2003) Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. J Med Virol 76: 391–398.

40. Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, et al. (2006) Global patterns of influenza A virus in wild birds. Science 312: 384–388.

41. Gill JS, Wobbe R, Gilchrist MJ, Gray GC (2006) Avian influenza among waterfowl hunters and wildlife professionals. Emerg Infect Dis 12: 1284–1286.

42. Li Y, Shi J, Zhong G, Deng G, Tian G, et al. (2010) Continued evolution of H5N1 influenza viruses in wild birds, domestic poultry, and humans in China from 2004 to 2009. J Virol 84: 8389–8397.

43. Kim HR, Lee YJ, Park CK, Oem JK, Lee OS, et al. (2012) Highly pathogenic avian influenza (H5N1) outbreaks in wild birds and poultry, South Korea. Emerg Infect Dis 18: 480–483.

44. Kou Z, Li Y, Yin Z, Guo S, Wang M, et al. (2009) The survey of H5N1 flu virus in wild birds in 14 Provinces of China from 2004 to 2007. PLoS ONE 4: e6926.