ORIGINAL ARTICLE

# Serological and Virological Surveillance of Avian Influenza A Virus H9N2 Subtype in Humans and Poultry in Shanghai, China, Between 2008 and 2010

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## Impacts

- The study shows evidence of occupational exposure to poultry increases the risk of infection with low-pathogenicity avian influenza virus H9N2.
- Avian H9N2 influenza virus was circulating for 3 years in poultry markets in Shanghai before the novel H7N9 emerged in 2013.
- The long-term maintenance of avian influenza virus genes in live poultry markets represents a major risk for emergence in the occupational poultry-exposed population as well as in the general population.

**Keywords:**
Avian influenza virus; H9N2 subtype; occupational exposure; poultry; serology

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## Summary

We report the serological evidence of low-pathogenic avian influenza (LPAI) H9N2 infection in an occupational poultry-exposed population and a general population. A serological survey of an occupational poultry-exposed population and a general population was conducted using a haemagglutinin-inhibiting (HI) assay in Shanghai, China, from January 2008 to December 2010. Evidence of higher anti-H9 antibodies was found in serum samples collected from poultry workers. During this period, 239 H9N2 avian influenza viruses (AIVs) were isolated from 9297 tracheal and cloacal paired specimens collected from the poultry in live poultry markets. In addition, a total of 733 influenza viruses were isolated from 1569 nasal and throat swabs collected from patients with influenza-like symptoms in a sentinel hospital, which include H3N2, H1N1, pandemic H1N1 and B, but no H9N2 virus was detected. These findings highlight the need for long-term surveillance of avian influenza viruses in occupational poultry-exposed workers.

## Introduction

Influenza A viruses have been categorized into 18 haemagglutinin (HA) and 11 neuraminidase (NA) subtypes (Tong et al., 2013). Avian influenza viruses (AIVs) play a key role in the emergence of pandemic strains. In February 2013, a novel avian influenza H7N9 virus emerged in China and was found to infect humans (Gao et al., 2013). As of 25 October 2013, the novel reassortant avian-origin influenza A/H7N9 subtype has infected 137 population in China, including 45 deaths, all the genes from the H7N9 virus in China were of avian origin, with six internal genes from AIV H9N2 subtype (Gao et al., 2013; World Health Organization, 2013). Meanwhile, genome sequencing data indicated that the 1918 H1N1 virus was likely an avian virus with mutations adapted to its mammalian host

 

(Taubenberger et al., 2005). The 1957 H2N2 and 1968 H3N2 pandemic strains, on the other hand, were originated as avian–human reassortant influenza viruses that had acquired human virus-like receptor-binding properties (Kawaoka et al., 1989). Furthermore, the 2009 pandemic H1N1 influenza virus was shown to possess avian consensus residues at positions 627 (E) and 701 (D) of the PB2 polymerase, indicating a recent acquisition from avian virus (Jagger et al., 2010).

Aquatic avian species are the natural hosts of influenza A viruses, and the host barrier restricts interspecies transmission (Subbarao and Katz, 2000; Perdue and Swayne, 2005). Nevertheless, AIVs can occasionally transmit to mammalian species, including humans. However, there are concerns that mutations and/or reassortment between avian and human/swine viruses may result in potential human influenza infection or even a new pandemic strain. Avian influenza viruses H4, H5 and H9 serotypes have transmitted to pigs in Canada and south-eastern China (Karasin et al., 2000; Peiris et al., 2001; Ninomiya et al., 2002). Highly pathogenic avian influenza (HPAI) viruses H5N1, H7N7 and H7N3 have also caused human illness (Chan, 2002; Fouchier et al., 2004; Tweed et al., 2004; Wang et al., 2006).

The low-pathogenicity avian influenza (LPAI) virus H9N2, first detected from turkeys in the United States in 1966 (Dong et al., 2011), usually cause only mild disease in domestic poultry, such as chickens, ducks, turkeys and quail (Alexander, 2000; Lu et al., 2001; Matrosovich et al., 2001; Miller et al., 2010). In mainland China, avian H9N2 virus was first isolated from Guangdong province in 1994. It has subsequently spread and become the most prevalent subtype of influenza viruses in poultry (Wu et al., 2008; Sun et al., 2010).

Transmission of the H9N2 virus crossing the species barrier to mammals has been reported. For instance, AIV H9N2 was isolated from pigs in southern China in 1998 (Peiris et al., 1999b; Li et al., 2005). A year later, two children in Hong Kong were confirmed with avian influenza H9N2 infections by nasopharyngeal aspirates (a third child case was reported in 2003) (Peiris et al., 1999a; Guo et al., 2000; Wan et al., 2008; Ge et al., 2009; Jia et al., 2009). Between 1998 and 1999, 10 human H9N2 viral infections were reported in China (Khuntirat et al., 2011).

Serological studies have provided strong evidence of H9 avian viral infections in humans. In 2006, 1.7% of the serum specimens from farmers in Xinjiang (north-western China) were H9 positive (Jia et al., 2009). Another serologic surveillance study showed that in Guangzhou from 2007 to 2008, the prevalence of anti-H9 antibodies among all participants was much higher than anti-H5 antibodies (Wang et al., 2009). All these data indicate the existence of asymptomatic AIV H9N2 infections among humans, although the H9N2 seropositivity has not yet been associated with any clinical disease.

To better understand the potential for the LPAI H9N2 spread among humans and domestic poultry in Shanghai, we surveyed the serological H9N2-specific haemagglutinin-inhibiting (HI) antibody in the occupational poultry-exposed workers and the general population in Shanghai. We also investigated the prevalence of H9N2 avian influenza virus in live poultry from wet markets in Shanghai and the positive rate of influenza virus isolation among patients with influenza-like illnesses (ILI).

## Materials and Methods

### Samples collection

After institutional review board approval, a total of 2570 participants were recruited during the period of January 2008–September 2010 in Shanghai, China, and selected at convenience by using an informed consent process. A population consisted of 840 occupational poultry-exposed workers defined as feed poultry workers, slaughters, sellers and transporters. These workers were routinely, heavily exposed to chickens, ducks, geese, pigeons and expected to have the highest level of exposure to avian influenza virus-infected birds. The 840 occupational poultry-exposed workers worked in 35 wet markets in Pudong district and 102 wet markets in Putuo district in Shanghai. There were no exclusion criteria, except for those people who did not want to participate in this study. All the poultry workers reported no serious respiratory illness history and were not vaccinated against avian influenza. The general population (1730 control subjects) consisted of the health medical examination participants and children outpatients who had no occupational exposure to poultry from three districts (Putuo, Pudong and Minhang) in Shanghai. After informed consent was obtained, a standard questionnaire was administered by trained staff to subjects. Information collected in the survey included demographic characteristics (age and gender) and occupational exposure (feed poultry, slaughter poultry, sell poultry and transport poultry) of the study subjects. Each participant permitted serum sample collection. A total of 2570 human serum samples were collected of which 840 were from the occupational poultry-exposed workers and 1730 were collected from the general population as controls.

We defined influenza-like illnesses (ILI) as a sudden onset of fever (a temperature $\geq$ 38°C) and cough or sore throat in the absence of another diagnosis. For monitoring influenza virus activity, sentinel hospitals in Shanghai were required to collect 5–15 nasopharyngeal or throat swabs weekly from ILI outpatients who had a fever (body temperature $\geq$ 38°C) for no longer than 3 days. A total of 1569

  

influenza-like illnesses (ILI) outpatients were enrolled between January 2008 and December 2010, regardless of age from a sentinel hospital (Minhang central hospital) in Minhang district, Shanghai.

There are three wholesale live poultry central markets in Shanghai, most live poultry sold in retail wet markets in Shanghai were purchased from these wholesale markets. We selected two of the three wholesale markets for testing poultry for AIVs: Sanguantang Poultry and Egg Market in Putuo District and Shanghai Agricultural Products Central Wholesale Market in Pudong District. A total of 9297 tracheal and cloacal paired swabs were random collected from live poultry in the two markets we selected between January 2008 and August 2010.

### Laboratory facilities

All experiments were conducted under the BSL-2 laboratory conditions in accordance with the institutional regulation.

### Cells

Madin-Darby canine kidney (MDCK) cells (ATCC, CCL34) were grown in minimal essential medium (Lifetechnologies/Gibco, Chicago, IL, USA) supplemented with 5% foetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Lifetechnologies/Gibco, Chicago, IL, USA).

### Viral isolation

From January 2008 to December 2010, a total of 1569 throat swab specimens collected from influenza-like illnesses (ILI) outpatients and were placed into viral transport media tubes and shipped to the laboratory within 48 h in a refrigerated container at 2–8°C then stored at −80°C. The throat swabs should be washed and removed. The supernatant passed through 0.22-μm filters (Millipore, MA, USA) to reduce bacterial contaminants, then incubated onto a monolayer MDCK cells in 24-well plates in a medium containing trypsin and passages three times. During the same period, 9297 tracheal and cloacal paired swab specimens were grown in the allantoic cavity of 10-day-old embryonated chicken eggs.

### RNA extraction, RT-PCR, DNA sequencing and phylogenetic analysis

Viral RNAs were extracted from cell culture supernatant or allantoic using the TRIizol® LS Reagent (Takara, Dalian, China) as suggested by the manufacturer. Reverse transcriptase polymerase chain reaction assay (RT-PCR) was performed using the rTaq PCR Amplification Kit (Takara, Dalian, China). The haemagglutinin (HA) genes of AIV H9N2 and other viruses, including human seasonal influenza viruses (H1N1, H3N2, H1N1pdm09 or B), were amplified with specific primers by means of RT-PCR. All amplified products were visualized after electrophoresis on an ethidium bromide stained 1.5% agarose gel.

We sequenced and analysed the HA genes of seven H9N2 virus isolated in 2008. Subsequently, HA was amplified using pairs forward and reverse primers designed as follows: H9seqF1: 5′- TCGGCTACCAATCAAC- 3′; H9seqR1: 5′- CGAGCAGTTCCCAAAT-3′; H9seqF2: 5′-GAAGAATCCTGAAGACT-3′; H9seqR2: 5′- GAATAAGAAGGCAGCAA-3′. DNA sequencing was carried out by the Life Tecnologies/Invitrogen Corporation, Shanghai, China. The edit and align nucleotide sequences and phylogenetic analysis were performed using MEGA programme and an unrooted phylogenetic tree was generated by the distance-based neighbour-joining method. Bootstrap values were calculated from 1000 replicates. Amino acid residues were numbered according to the HA sequences of H3 (Nobusawa et al., 1991) with GenBank accession number: V01087.

### Haemagglutination inhibition assays

Serum samples collected from 2570 participants were kept in a refrigerated container at 2–8°C immediately after collection and delivered to the laboratory at the end of each working day for storage at −20°C until use.

Serum samples were tested for antibody titres against avian H9 influenza viruses by Haemagglutination inhibition (HI) assays according to the World Health Organization (WHO) Manual for the laboratory diagnosis and virological surveillance of influenza (World Health Organization, 2011). To exclude the cross-reaction of the antibody against human H1 influenza virus, we also detected antibody titres against human H1. The H9 and H1 standard antigen was obtained from Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences.

Prior to the assay, one part of serum was mixed with three parts of receptor-destroying enzyme (RDE) (Denka Seiken, Tokyo, Japan). The mixtures were incubated overnight in a 37°C water bath to remove non-specific HAs. The enzyme was inactivated by a 45-min incubation at 56°C followed by the addition of 1/5 part of chicken erythrocytes and one part of physiological saline (0.85% NaCl). After another 30-min 37°C incubation, the samples were centrifuged at 2000 ***g*** for 5 min. The supernatant was transferred to a new tube with final dilution of 1/5. HI assays were performed in V-bottom 96-well plates with 0.7% chicken red blood cells (RBCs). Positive and negative serum controls were included with sera tested. The HI titre was expressed as the reciprocal of the highest serum dilution that completely inhibited haemagglutination of four

HA units of the virus. As previously reported, HI titres of 40 were correlated with a 50% reduction in the risk of infection or disease with seasonal influenza viruses in human population, and HI titres of 40 or greater were indicative of exposure to virus or vaccine (de Jong et al., 2003; Killian et al., 2013). As cut-off values of HI assays for H9 serosurveillance in humans in published studies often vary (Kayali et al., 2010; Coman et al., 2013; Huang et al., 2013; Zhou et al., 2014), to better understand the subclinical H9N2 infection, we accepted HI antibody titre $\geq 1:40$ of serum samples as positive.

### Microneutralization assay

We performed microneutralization (MN) assay for the detection of functional antibodies with three H9N2 influenza virus isolates. All MN assays were performed with MDCK cells according to the WHO standard protocols (World Health Organization, 2011), with an MN cut-off value of 1 : 20 (Miller et al., 2010; Rizzo et al., 2010). The H9N2 influenza viruses selected for testing were egg-grown as follows: A/Chicken/Shanghai/0734/2007, A/Chicken/Shanghai/0817/2008, and A/Chicken /Shanghai/0867/2008.

### Statistical analysis

Logistic regression was used to screen risk factors associated with positive anti-H9 HI titres $\geq 1:40$. To control for possible interactions between factors, multivariable logistic regression was used to estimate the odds ratio (OR) and 95% confidence interval (95% CI) for factors associated with positive anti-H9 HI titres. We screened a number of possible risk factors, including occupation, age, sex and human influenza H1N1 titre $\geq 1:40$. Independent variables in the original model included occupation, age, sex and human influenza H1N1 titre $\geq 1:40$. All the independent variables remained in the final model after carrying out backwards elimination procedure. In logistic regression analysis, maximum likelihood estimates for the odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using Wald chi-squared test. Agreements between HI and MN assays were calculated by kappa test. All *P*-values were two tailed; *P*-values $< 0.05$ were considered statistically significant. All the statistical analysis was performed using the SPSS 18.0 software (The Predictive Analytics Company, Chicago, IL, USA) and STATA statistical software version 12.0 (Stata Corp, TX, USA).

## Results

### Detection of H9 HI antibodies in serum samples

Serum samples were collected from people who were occupationally exposed to live poultry ($n = 840$) and from the general population ($n = 1730$). Of the 2570 specimens collected for testing, 81 specimens did not meet testing criteria due to insufficient serum amounts or missing demographic data. The median age of the poultry workers was 41 years (range, 15–75 years), and 54.8% were male. The median age of the general participants was 29 years (range, 1–94 years), and 43.2% were male. Regarding age, the majority of younger occupational individuals were in the age between 16 and 39 years, and the older workers were in the age between 40 and 59 years. The minimum age was set at 16 years because of a small number of youth workers in the poultry markets in Shanghai (Table 1). The HI assay was performed to measure the anti-H9 antibody titres with a cut-off value of 1 : 40. HI titres for 2489 (81 missing cases) serum specimens sampled between 2008 and 2010 are provided in Table 2. Among the 16–39 years age group, the positive rate of anti-H9 antibody titres of younger occupational poultry-exposed workers was up to 13.7%, compared with 2.9% in the general population. Among the 40–59 years age group, the positive rate of older workers was 12.2%, compared with 6.3% in the general population. While controlling for age, sex and human influenza H1N1 titre $\geq 1:40$, the adjusted odds of occupational poultry workers (OR: 4.1, 95% CI: 2.7–6.1) were statistically significantly higher than the general controls. The adjusted odds of male (OR: 1.7, 95% CI: 1.2–2.4) was

**Table 1.** Characteristics of seroepidemiologic survey participants ($n = 2570$) in Shanghai

| Characteristics | Occupational poultry-exposed population | General population |
|---|---|---|
| Total | 840 | 1730 |
| Sex, participants, no. (%)[a] | | |
| Male | 460 (54.8) | 745 (43.2) |
| Female | 380 (45.2) | 981 (56.8) |
| Missing data | 0 | 4 |
| Age, years, no. (%)[a] | | |
| 0–15 | 1 (0.1) | 232 (14.0) |
| 16–39 | 328 (39.7) | 848 (51.0) |
| 40–59 | 477 (57.7) | 207 (12.4) |
| ≥60 | 20 (2.4) | 376 (22.6) |
| Missing data | 14 | 67 |
| Age, median, years (range) | 41 (15–75) | 29 (1–94) |
| Occupation, participants, no. (%)[a,b] | | |
| Feed poultry | 339 (40.4) | – |
| Slaughter poultry | 378 (45) | – |
| Sell poultry | 579 (68.9) | – |
| Transport poultry | 8 (1) | – |

[a]Data are no. (%) of subjects.

[b]Most occupational poultry-exposed population had multiple exposure, so the sums of all percentages are >100%.

**Table 2.** Haemagglutination inhibition (HI) antibody titres against avian influenza virus H9 of occupationally poultry-exposed and general population in Shanghai

| Age (years) | No. of samples tested ($n$ = 2489) | HI titres <1 : 10 | 1 : 10 | 1 : 20 | 1 : 40 | 1 : 80 | 1 : 160 | No. (%) of samples with HI titres ≥ 1 : 40 |
|---|---|---|---|---|---|---|---|---|
| Occupationally poultry-exposed population | | | | | | | | |
| 0–15 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 (0) |
| 16–39 | 328 | 177 | 54 | 52 | 32 | 12 | 1 | 45 (13.7) |
| 40–59 | 477 | 246 | 93 | 80 | 38 | 18 | 2 | 58 (12.2) |
| ≥60 | 20 | 9 | 4 | 7 | 0 | 0 | 0 | 0 (0) |
| General population | | | | | | | | |
| 0–15 | 232 | 213 | 16 | 2 | 1 | 0 | 0 | 1 (0.4) |
| 16–39 | 848 | 698 | 81 | 44 | 20 | 5 | 0 | 25 (2.9) |
| 40–59 | 207 | 165 | 17 | 12 | 10 | 2 | 1 | 13 (6.3) |
| ≥60 | 376 | 311 | 39 | 18 | 7 | 1 | 0 | 8 (2.1) |

**Table 3.** Odds ratio for a positive Haemagglutination inhibition (HI) antibody titre against avian influenza virus H9N2 using logistical regression[a]

| Risk factors | Unadjusted OR | 95% CI | Adjusted OR | 95% CI |
|---|---|---|---|---|
| Occupation | | | | |
| Occupational poultry workers | 5.2 | 3.6–7.4 | 4.1 | 2.7–6.1 |
| General controls | Ref | Ref | Ref | Ref |
| Age | | | | |
| 0–15 | Ref | Ref | Ref | Ref |
| 16–39 | 0.2 | 0.0–1.7 | 0.2 | 0.0–1.4 |
| 40–59 | 3.1 | 1.5–6.4 | 1.4 | 0.7–3.1 |
| ≥60 | 5.6 | 2.7–11.8 | 1.7 | 0.8–3.9 |
| Sex | | | | |
| Male | 1.5 | 1.1–2.1 | 1.7 | 1.2–2.4 |
| Female | Ref | Ref | Ref | Ref |
| Human influenza H1 titre ≥ 1 : 40 | | | | |
| Positive | 2.3 | 1.6–3.3 | 2.7 | 1.8–3.8 |
| Negative | Ref | Ref | Ref | Ref |

OR, odds ratio; CI, confidence interval.
[a]Titre ≥ 1 : 40 were considered to be positive; all potential factors were included in final model.

statistically higher than female. The adjusted odds of positive human influenza H1 titre ≥ 1 : 40 (OR: 2.7, 95% CI: 1.8–3.8) were statistically higher than the negative (Table 3).

### Agreement between serological tests

Twenty serum samples with HI titres of 0 ($n$ = 3), 1 : 20 ($n$ = 4), 1 : 40 ($n$ = 7) and 1 : 80 ($n$ = 6) were tested in an MN assay. The positive results obtained with HI (≥1 : 40) and MN (≥1 : 20) assays were in good correlation with a kappa value of 0.781 ($P < 0.05$), indicating a strong concordance between these two serological assays.

### Virological surveillance for influenza viruses in humans

We isolated 733 influenza viruses from the 1569 nasal and throat swabs collected of ILIs in Minhang District, Shanghai, from January 2008 to December 2010. The positive rate was 46.7% (733/1569). The majority of the isolates were of H3N2, H1N1 subtypes and B type. No H9N2 virus was detected.

### The prevalence of avian influenza virus in poultry

During the same period of time, we collected 9297 tracheal and cloacal paired swabs from live poultry in the wet markets in Shanghai. We successfully isolated 239 avian influenza viruses (Table 4). The positive rate was 2.5% (239/9297). Of note, all the 239 isolates were of H9N2 subtype.

### Genetic and phylogenetic analysis

DNA sequencing of seven H9N2 isolates revealed that all had an RSSR↓GLF motif at the cleavage site of HA,

**Table 4.** Prevalence of avian influenza virus H9N2 in live poultry in Shanghai (2008–2010)

| Year | No. of samples tested | No. of H9N2 isolates | Positive rate (%) |
|---|---|---|---|
| 2008 | 4370 | 58 | 1.3 |
| 2009 | 1646 | 134 | 8.1 |
| 2010 | 3281 | 47 | 1.4 |
| Totals | 9297 | 239 | 2.5 |

representing low pathogenicity in chicken. In addition, the avian-to-mammalian mutation at amino acid 226 (Q226L) at the receptor-binding site (RBS) was also detected (H3 numbering), which is consistent with our previously published results (Ge et al., 2009). To understand the evolution of AIV H9N2 in Shanghai, seven H9 HA genes (CK/SH/Y1/08, CK/SH/Q0801-1/08, Q0801-2/08, Q0808-1/08, Q0808-2/08, Q0812-1/08 and Q0812-2/08) were phylogenetically analysed. All of the H9N2 isolates belonged to Chicken/Beijing/1/94-like lineage. The results have been published previously (Ge et al., 2009; Zhou et al., 2012). The NCBI accession numbers for the haemagglutinin (HA) sequences are GQ335490, JF519770, JF519771, JF519772, JF519773, JF519774 and JF519775.

## Discussion

Avian influenza viruses (AIVs) played a key role in the emergence of all the pandemic strains. So far, there is little evidence of H9N2 human-to-human transmission. But repeated interspecies transmission of H9N2 influenza viruses from poultry to humans raises concerns of a pandemic. The present study was undertaken to investigate the epidemiology of H9N2 subtype in human and live poultry populations in Shanghai.

Our serological results provided strong evidence that avian-to-human transmission of H9N2 subtype influenza virus exists. Serum H9 HI antibody positive rate in occupational poultry-exposed workers was significantly higher than that in the general population in Shanghai. Multivariate logistic regression revealed that the odds of positive anti-H9 antibody titre were significantly higher in the poultry workers compared with the general controls. These results are consistent with previous studies showing that the seroprevalence of H9 antibodies in China was higher among the occupational poultry-exposed population (Jia et al., 2009; Wang et al., 2009). Moreover, it has been reported that direct contact with live poultry was common in southern China, and the interspecies transmission of H9N2 influenza virus into humans was not a rare event (Peiris et al., 1999a,b; Guan et al., 2000; Butt et al., 2005). In accordance with previous studies, the present study indicates the occupational exposure to infected poultry as a risk factor and contributes to human H9 HI antibody seropositivity and/or human H9N2 asymptomatic infection. Multivariate logistic regression revealed that male had significantly higher odds of positive anti-H9 antibody titre. It is possible that the differences in frequency or intensity of avian influenza virus exposures could account for the increased risks of male. Men engaged more frequently than women in feeding and selling poultry-related occupation. It is interesting to note that the H9 HI antibody positive rate in the general population among 16–39 was 2.9% and among 40–69 was 6.3%, suggesting the possible acquisition of H9N2 asymptomatic infection through routine contact to birds in the general population (e.g. purchasing live or fresh killed poultry such as chicken, duck, pigeon, quail from wet markets, touching unclean eggs with poultry faeces, preparing poultry for cooking, etc.). The specific environmental exposure could be a risk factor for AIV infection. A previous survey report suggested that contaminated environmental exposure (e.g. contaminated water) as a risk factor for seropositivity (Vong et al., 2008).

On the other hand, human-to-human transmission of H9N2 influenza viruses is very unlikely. In our virological surveillance in ILI patients, none of the 938 throat swabs isolations were found to be the H9 subtype (Xu et al., 2007). The HA sequence analyses of H9N2 influenza viruses isolated from poultry revealed a leucine (L) at the amino acid 226 of RBS. Although the presence of L226 in the HA appears to be critical for transmissibility of the H9N2 virus to mammals (Wan et al., 2008), the low pathogenicity of the H9N2 virus with L226 at the cleavage site limits viral proliferation in human airway epithelial cells and may merely cause a mild or latent infection. Host range restriction of viral nucleoprotein and RNA polymerase (PB2, PB1 and PA) may also contribute (Moncorge et al., 2010; Mok et al., 2011; Li et al., 2012). This may explain why H9N2 was not isolated from throat swabs of ILI patients. It was notable that the information of influenza based on the sampling of ILI patients from sentinel hospitals may be representative of subjects with more severe illness since they sought medical attention. It appeared that those individuals with influenza may be different than those have H9N2 with either mild disease or were asymptomatic.

Between 2008 and 2010, the overall isolation rate of H9N2 was relatively high in live poultry and all of the 239 isolates were H9N2 subtype. Compared with our previous study surveying live poultry, the isolation rate of AIV H9N2 increased (Ge et al., 2009). The prevalence of H9N2 during 2009 in live poultry was higher than the other 2 years. The commercial vaccines for H9N2 have been used in chicken in China since 1998. The effectiveness of AIV vaccine was concern with the antigenic relationship between vaccine and prevailing strains (Lee et al., 2004; Zhang et al., 2012). However, one explanation for the

  

prevalence variation of H9 may be that the commercial vaccines commonly made of an earlier H9N2 isolate such as Ck/Shandong/6/96 or Ck/Shanghai/F/98 that was not able to provide sufficient protection. In addition, it should be considered that the H9N2 virus was circulating in live poultry markets in Shanghai for at last 3 years before the novel H7N9 virus emerged in 2013 (Kageyama et al., 2013). It is important to realize that diverse subtypes of AIV isolation in domestic poultry (such as H6N2, H6N6, H1N2, H1N3 and H5N1 etc.) cocirculated in eastern China and southern China, the neighbour regions to Shanghai (Li et al., 2010; Zhao et al., 2011; Wu et al., 2012a,b). We can think of the following reasons that could explain only the H9N2 subtype in our outcome: Avian influenza virus H9 was the most common subtype in domestic poultry in Shanghai in recent years. The prevalence of other subtypes was much lower and mainly isolated from domestic ducks while the majority of our strains were isolated from chickens.

All of the H9N2 isolates in 2008 that we sequenced were derived from Chicken/Beijing/1/94-like lineage. Shanghai is an important transit point for the migration routes of birds in the Asia-Pacific region. Recent studies propose that the assortment events of AIV H9N2 circulating in chickens and AIV H7N9 in ducks probably took place in Shanghai or the adjacent provinces (Feng et al., 2013; Liu et al., 2013). All six internal genes of the novel H7N9 virus share highest identities with the H9N2 viral strains which were circulating in the mainland China in the past 2 years (Chen et al., 2013). Meanwhile, the live poultry wholesale market is a gathering place for birds where the spread and sharing of avian influenza virus can speed the emergence of novel recombinant strains. Unfortunately, there is little surveillance data available about H9N2 isolates in Shanghai from 2011 to 2012. It is therefore difficult to trace the evolutionary pathway of H9N2. Given the great genetic diversity in H9N2 variants and potential role as pandemic influenza agent, the long-term maintenance of avian influenza virus genes in live poultry markets represents a major risk for emergence in the occupational poultry-exposed population as well as in the general population.

There are several limitations of this study. The study design did not allow for evaluation of the prevalence of H9N2 virus in the general population. Samples were only collected from ILI patients in a sentinel hospital, which could bias prevalence estimates. Additionally, cross-reactions from previous infection with human viruses might confound AIV serological results. H1N1 was the most prevalent subtype in humans in Shanghai during the sampling period. Our estimates of the sera-positive rate would be biased if infection with circulating H1N1 influenza viruses led to potential cross-reactive antibody responses resulting in antibody titre $\geq 1:40$. We sought to control such potential confounding by adding H1N1 serology covariates to the multivariate models. Furthermore, another limitation was the specificity of the HI assay. HI assay has been reported to be less sensitive for detecting antibody responses to avian viruses in mammalian sera (Profeta and Palladino, 1986; Rowe et al., 1999). As the microneutralization (MN) assay has the advantage of confirming the specificity of the HI results, samples with HI titre $\geq 1:40$ need to be further confirmed by MN assay to accurately detect anti-H9 antibody titres in humans.

To make predictions for the emergence risk of novel strains of influenza A, this study highlights the necessity for more surveillance of influenza viruses in the occupational poultry-exposed population. Increased focus should be placed on the natural evolution of avian influenza virus.

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## Conflicts of Interest

The authors have not declared any potential conflicts.

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