Molecular epidemiological surveys of H5 subtype highly pathogenic avian influenza viruses in poultry in China during 2007–2009

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To investigate the prevalence and evolution of the H5 subtype highly pathogenic avian influenza (HPAI) viruses circulating in poultry in China during 2007–2009, five molecular epidemiological surveys were carried out. A total of 21 591 swab samples were collected, and from them 55 H5 HPAI viruses were isolated. None of the 55 viruses carried any known mutations, which can render the virus binding to human SAa2,6Gal receptors. The surveys indicated that live-bird markets, backyard flocks and slaughtering sites were at greater risk of being infected with the viruses during winter, and Clades 2.3.2, 2.3.4 and 7 of the viruses co-circulated in poultry in China during 2007–2009. Viruses within Clades 2.3.2 and 7 have become genetically distinguishable from the viruses isolated before 2007 and antigenically distinguishable from the vaccine strains used in China. Viruses within Clade 2.3.2 have been circulating widely in China and caused a new wave of cross-continental spreading from Asia to Europe.

H5 subtype highly pathogenic avian influenza (HPAI) viruses caused a few outbreaks in poultry in Europe, Asia, Africa and the Oceania during 1959–2000 (Alexander & Brown, 2009; Ma et al., 2007). A novel strain of H5 subtype HPAI viruses was found in China in 1996. UnpreceDentedly, its descendants have been spreading widely in Asia, Europe and Africa for years, and they have killed millions of birds (Chen, 2009; Xu et al., 2005; Smith et al., 2006). Recently, some viruses isolated from wild birds, domestic fowls or human cases during 2006–2008 in China were characterized (Chen, 2009; J. Chen et al., 2009; Guan et al., 2009; Kou et al., 2009; Smith et al., 2009; Xu et al., 2009). However, the evolution of the viruses circulating in China largely remains unclear since 2006. To investigate the prevalence and evolution of the viruses circulating in domestic fowls in China, five molecular epidemiological surveys were performed as shown in Table 1.

Each of the surveys covered 8–13 provinces, autonomous regions or municipalities. They were selected randomly from different parts of China (Fig. 1). Two counties were selected randomly from each province. In each county, usually seven sites (two commercial poultry farms of >2000 fowls, five live-bird markets, slaughtering sites or backyard flocks of <500 fowls) were selected randomly, and 30 fowls (chickens, ducks or geese) from each site were selected randomly for sample collection. In addition, at least 100 ducks or geese were sampled in each county for each survey.

Swab samples were collected by taking smears from the trachea and cloaca of the domestic fowls and placed in a transport medium. The samples were clarified by centrifugation at 1000 g for 5 min, and the supernatants were inoculated in 10-day-old specific-pathogen-free (SPF) chicken embryonated eggs via the allantoic sac route. The eggs were further incubated for 4 days and checked twice each day during the incubation period. The dead ones were picked out and stored in a refrigerator. After the incubation period, the allantoic fluids of the live embryos were collected and tested by using the haemagglutination assay. All the haemagglutination-positive samples and the allantoic fluid of the dead eggs were investigated further by RT-PCR, as described below.

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The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are HM006764–HM006818 and HM583607–HM583609.
Viral RNA was extracted from the allantoic fluid of the dead eggs by using the RNeasy Mini kit (Qiagen). RNA was amplified with the PrimeScript One-step RT-PCR kit (TaKaRa) using the primers 5'-AGCAAAAGCAGGGGT-CCAATCT-3' (forward) and 5'-TCTACCATTCCCTGC-CATC-3' (reverse), specific to a 1122 bp region in the haemagglutinin (HA) gene of H5 subtype avian influenza viruses (AIVs) flanking the HA1 domain. RT-PCR was performed in a 50 μl reaction mixture containing 5 μl template RNA, 25 μl 2× One-step RT-PCR buffer, 1 μl of each primer (20 μM) and 2 μl One-step RT-PCR enzyme mix. The mixture was initially incubated at 50 °C for 30 min and denatured at 94 °C for 2 min, and then 30 cycles were performed with denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 2 min. PCR products were purified and ligated with pGEM-T Easy vector (Promega). Then, both strands of the clones were sequenced using a Perkin-Elmer model 377 XL DNA sequencer.

Phylogenetic relationships of the sequences were analysed using the methods reported previously (J. M. Chen et al., 2009). Briefly, sequences were aligned and edited by using the software CLUSTAL_X. Then, the phylogenetic relationships were calculated by using MEGA 4.1 with the neighbour-joining method and the model of Kimura.

**Table 1.** The total numbers and the numbers with positive samples (in parentheses) of sampling places or samples of the five surveys of H5 subtype AIVs

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<tr>
<td>Provinces</td>
<td>13 (7)</td>
<td>12 (4)</td>
<td>8 (7)</td>
<td>11 (4)</td>
<td>11 (6)</td>
</tr>
<tr>
<td>Counties</td>
<td>26 (7)</td>
<td>24 (4)</td>
<td>24 (8)</td>
<td>30 (4)</td>
<td>31 (8)</td>
</tr>
<tr>
<td>Poultry farms</td>
<td>59 (0)</td>
<td>59 (0)</td>
<td>41 (0)</td>
<td>81 (0)</td>
<td>83 (0)</td>
</tr>
<tr>
<td>Live-bird markets</td>
<td>26 (3)</td>
<td>25 (1)</td>
<td>12 (8)</td>
<td>21 (2)</td>
<td>38 (7)</td>
</tr>
<tr>
<td>Backyard flocks</td>
<td>4 (3)</td>
<td>60 (6)</td>
<td>33 (5)</td>
<td>90 (2)</td>
<td>84 (8)</td>
</tr>
<tr>
<td>Slaughtering sites</td>
<td>5 (2)</td>
<td>6 (1)</td>
<td>5 (1)</td>
<td>5 (0)</td>
<td>4 (1)</td>
</tr>
<tr>
<td>Chicken samples</td>
<td>1954 (8)</td>
<td>3729 (2)</td>
<td>1934 (11)</td>
<td>4978 (3)</td>
<td>4660 (19)</td>
</tr>
<tr>
<td>Duck samples</td>
<td>551 (0)</td>
<td>531 (1)</td>
<td>320 (2)</td>
<td>602 (1)</td>
<td>585 (2)</td>
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<tr>
<td>Goose samples</td>
<td>196 (0)</td>
<td>294 (5)</td>
<td>307 (1)</td>
<td>432 (0)</td>
<td>418 (0)</td>
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<td>Clade distribution*</td>
<td>2.3.4 (8)</td>
<td>2.3.4 (7)</td>
<td>2.3.2 (1)</td>
<td>2.3.4 (3)</td>
<td>2.3.2 (9)</td>
</tr>
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</table>

*This is shown by the clade designations with the corresponding numbers of the virus isolates in parentheses.

**Fig. 1.** The provinces, autonomous regions or municipalities involved in the surveys. The relevant ordinal numbers of the surveys were given after or under the provincial designations, and the underlined numbers indicate H5-positive samples were identified in these times of survey. For example, ‘Xinjiang 1, 2, 3’ in the figure indicates that Xinjiang was involved in the first three surveys and H5-positive samples were identified in the first and third surveys.
two-parameter. The substitution rates were set in gamma distribution among sites. Bootstrap values were calculated out of 1000 replicates. Genetic distances were calculated using the same model and parameters set for phylogenetic analysis.

A total of 21,591 swab samples were collected and tested, and from them 55 H5 subtype AIVs were isolated. All 55 viruses were from the embryonated eggs that had died within 3 days after inoculation. Among them, 34 were from live-bird markets, 16 from backyard flocks and five from slaughtering sites (Table 1). The prevalence of the viruses was higher ($P < 0.05$, by $\chi^2$ test) during October–December (0.30–0.55%) than in March–May (0.07–0.17%) over a period of 3 years. None of the samples collected from commercial poultry farms were found to be H5 positive.

The GenBank accession numbers of the HA1 domain sequences of the 55 viruses and three other H5 HPAI viruses, A/chicken/Neimeng/Q4/2007(H5), A/chicken/Tibet/QX2/2009(H5) and A/chicken/Hunan/Q23/2009(H5), which were isolated through other activities, are HM006764–HM006818 and HM583607–HM583609. All the sequences harboured five to seven basic amino acid residues at the cleavage site. This indicated that they were highly pathogenic (Senne, et al., 1996). None of them carried the mutations 129→V, 134→V, 182→K, 192→R, 222→L, 223→N or 224→S (H5 numbering) in the HA gene, which could render the virus binding to human SAα2,6Gal receptors and facilitate the virus replicate efficiently in humans (Auwarakul et al., 2007; Stevens, et al., 2006; Yamada et al., 2006).

Phylogenetic analysis, as shown in Fig. 2 and Table 1, indicated that the H5 subtype viruses circulating during 2007–2009 in China could be divided into three clades, i.e. Clades 2.3.4, 2.3.2 and 7, according to the WHO/the World Organisation for Animal Health/Food and Agriculture Organization of the United Nations nomenclature system (WHO/OIE/FAO H5N1 Evolution Working Group, 2008). Viruses within Clades 2.3.2 and 2.3.4 have been isolated from multiple species of hosts including chickens, ducks and wild birds, while viruses within Clade 7 have never been isolated from ducks.

The results shown in Table 1 indicate that viruses within Clade 2.3.4 were dominant in poultry in China during 2007–2008, and viruses within Clade 2.3.2 also circulated widely in poultry in China during 2009. In addition, viruses within Clade 7 were not found for a period during 2007–2008 and then they re-emerged in 2008.

It was found through analysis of the sequences available in GenBank (WHO/OIE/FAO H5N1 Evolution Working Group, 2008; Liu, et al., 2009) that viruses from at least six clades, i.e. Clades 2.2, 2.3.2, 2.3.4, 4, 7 and 9, co-circulated in China during 2005–2006. Among them, only viruses from three clades (Clades 2.3.4, 2.3.2 and 7) during 2007–2009 in China were found through the surveys reported here. It was assumed that viruses from the other three clades (Clades 2.2, 4 and 9) have largely disappeared from the poultry in China.

Fig. 2 suggested that the viruses in Clades 2.3.2 and 7 have changed significantly compared with the ones isolated before 2007. Rapid evolution of viruses within these clades has also been observed earlier (Boltz et al., 2010; Kim et al., 2006; Nguyen et al., 2009; Smith et al., 2009; Uchida et al., 2008). The genetic distances in the nucleic acid sequences of the HA1 domain between the viruses isolated during 2005–2006 and those isolated during 2008–2009 are $4.3 \pm 0.85$% (mean ± sd) within Clade 2.3.2, and $5.4 \pm 1.45$% within Clade 7. Therefore, the viruses within Clades 7 and 2.3.2 isolated after 2007 have been designated as variants of Clades 7 and 2.3.2, respectively.

Two vaccine strains, Re4 and Re5, generated using reverse genetics, have been widely used in China since 2006 to prevent poultry infections and deaths caused by the HPAI viruses within Clades 7 and 2.3 (including Clades 2.3.2 and 2.3.4), respectively (Chen, 2009). Their HA genes were from A/chicken/Shanxi/2/2006(H5N1) within Clade 7 and A/chicken/Anhui/1/2006(H5N1) within Clade 2.3.4, respectively (Chen, 2009).

Between the variant of Clade 7 and the vaccine strain Re4, the amino acid changes of D43N/K, I71/L, S121Y/E, K140E/N, D183N, K189M/Q, P194L, T195N/L and A238T/S (H5 numbering) in the HA1 domain were observed. Among them, K140E/N, D183N and K189M/Q occurred at antigenic sites, which can change the antigenicity of the viruses (Duuvuri et al., 2009; Kaverin et al., 2007; Wu et al., 2008). Three isolates of the variant of Clade 7 (A/chicken/Jiangsu/Q305/2008, A/chicken/Anhui/QG5/2008 and A/chicken/Tibet/QX2/2009) were randomly selected and characterized antigenically by the haemagglutination inhibition (HI) assay using the SPF chicken serum against the vaccine strain Re4. The serum was made by us and its homologous HI titre was 256. The HI titres of the serum were all ≤8 for the three isolates.

Similarly, between the variant of Clade 2.3.2 and the vaccine strain Re5, the amino acid changes of D45N/K, R53L, S120D, S129L, T140N, P141S, T156A, R162K, A184E, K189R, V200I, A263T and K277R (H5 numbering) in the HA1 domain were observed. Among them, at least R53L, T140N, R162K, K189R, A263T and K277R occurred at antigenic sites, which can change the antigenicity of the viruses (Duuvuri et al., 2009; Kaverin et al., 2007; Wu et al., 2008). Three isolates of the variant of Clade 2.3.2 (A/chicken/Hubei/QG5/2008, A/chicken/Jiangxi/Q1/2008 and A/duck/Guangdong/QH16/2009) were randomly selected and characterized antigenically by the HI assay using the SPF chicken serum against the vaccine strain Re5. The serum was made by us and its homologous HI titre was 256. The HI titres of the serum were all ≤8 for the three isolates.

The variant of Clade 2.3.2 was firstly isolated in Hong Kong SAR and mainland China in 2007 (J. Chen et al., 2007; Stevens, et al., 2007; Wu et al., 2008).
Fig. 2. Phylogenetic relationship of H5 subtype AIVs isolated in China in recent years based on their HA1 domain sequences. The sequences marked with triangles are reported by this study, and the other sequences were accessed from GenBank. Clade designations and bootstrap values are given at the right-hand side and the relevant nodes, respectively.
2009; Smith et al., 2009). It was further isolated in Russia, South Korea, Japan and Laos in 2008 (Boltz et al., 2010; J. Chen et al., 2009; Kim et al., 2010; Nguyen et al., 2009; Uchida et al., 2008), and in Mongolia, Nepal, Israel and Romania during 2009–2010 (http://www.defra.gov.uk/foodfarm/farmanimal/diseases/monitoring/documents/h5n1-romania-100315.pdf; accessed on 21 June 2010). Therefore, this novel variant has caused a new wave of cross-continental spreading of the HPAI viruses during 2007–2010 from Asia to Europe. It would be interesting to examine in future whether the novel variant will replace or co-circulate with Clade 2.2 of H5 HPAI viruses, which spread from Asia to Europe and Africa during 2005–2006 and has been circulating widely in South Asia, the Middle East, Europe and Africa for years.

China began mass vaccination in poultry against the H5 subtype viruses at the end of 2005 (Chen, 2009). The surveys indicated that commercial farms have been protected by the vaccines, but the viruses are still circulating in China, especially during the winter period, and live-bird markets and backyard flocks are at greater risk of infection. The wide existence of the viruses in wild birds in East Asia, as shown by recent reports (Kou et al., 2009; Smith et al., 2009; Uchida et al., 2008), may play an important role in the circulation of the viruses in poultry in this region, because many domestic fowls (especially ducks) share the same niches as wild birds, although it cannot be excluded that wild bird populations are continuously infected by poultry sources.

All the 55 H5 HPAI viruses were isolated from clinically healthy domestic fowls (43 from chickens, six from ducks and six from geese, Table 1). Infection with HPAI viruses seems to cause no clinical signs in some species of unvaccinated birds, like ducks and geese, but usually, unvaccinated chickens will show some obvious signs after infection with the viruses. In this report, 43 H5 subtype HPAI viruses were isolated from clinically healthy chickens. This may be because the chickens obtained some immunity through vaccination (Domenech et al., 2009). It may also be possible that some of the chickens were in the incubation period when the samples were collected. Nevertheless, the surveys indicated that the vaccination against the HPAI viruses may also cause some new problems for the control of the disease. Firstly, infections with the HPAI viruses in vaccinated birds may become ‘silent’ without clinical signs. Secondly, vaccination may drive the viral evolution because novel viral strains should have a selective advantage in spreading in vaccinated flocks, if they have become antigenically different from the vaccine strains (Lee et al., 2004).

Three other kinds of pathogens, H7 and H9 subtype AIVs plus Newcastle disease viruses (NDVs), have also been detected in the same 21,591 swab samples. Among the samples, none of the samples were positive for H7 subtype AIVs, 301 samples were positive for H9 subtype AIVs, 54 samples were positive for low pathogenic NDVs and 15 samples were positive for highly pathogenic NDVs. Some of the data about H9 subtype AIVs have been published previously (Ji et al., 2010).

In conclusion, the five molecular surveys reported herein identified 55 H5 subtype AIVs from 21,591 swab samples. None of them carried any known mutations which can render the virus binding to natural human SAa2,6Gal receptors. Distribution of the 55 positive samples indicated that live-bird markets, backyard flocks, slaughtering sites and the cold season were all risk factors of the viral infection. Phylogenetic analysis indicated that three clades (2.3.4, 2.3.2 and 7) co-circulated in poultry in China during 2007–2009. Clade 2.3.2 has circulated widely in poultry in China, and caused a new wave of cross-continental spreading from Asia to Europe. Clades 2.3.2 and 7 have become genetically distinguishable from the viruses isolated before 2007 and antigenically distinguishable from the vaccine strains widely used in China. Evolution of the viral clades, impact of the vaccination on the circulation and control of the HPAI viruses, and detection of other pathogens from the samples have also been discussed in this report.

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References


