Serological Surveillance of Influenza A Virus Infection in Swine Populations in Fujian Province, China: No Evidence of Naturally Occurring H5N1 Infection in Pigs

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Impacts

• H5N1 highly pathogenic avian influenza virus (HPAIV) was isolated from several pigs in Fujian Province, China, since 2001 and hence caused concern in the research field and the public.
• We performed serological surveillance of the antibodies against H5 over 2 years as well those against H1, H3 and H9, in the pig populations in Fujian Province, China, to test if these isolates were sporadic event or H5N1 virus has adapted to infect pig populations.
• We found no sera positive for H5 out of 1407 samples in the 2-year period and conclude that there is no evidence that H5N1 has spread in the pig population, at least in this region studied, although antibodies were detected in different sera against H1, H3 and H9. The case reported in 2003 appears to have been sporadic.

Keywords:
Serological surveillance; swine influenza virus; H5N1

Summary

Several highly pathogenic H5N1 avian influenza viruses were isolated from swine populations in Fujian Province, China, since 2001. Because it is thought that H5N1 infection in pigs might result in virus adaptation to humans, we surveyed swine populations in Fujian Province in 2004 and 2007 for serological evidence of the infection. Twenty-five pig farms covering all nine administrative districts of Fujian Province were sampled and a total of 1407 serum specimens were collected. The haemagglutination inhibition (HI) tests revealed no evidence of H5 infection and only a few cases of H9 infection. The negative results for H5 infection were further verified by micro-neutralization tests. By contrast, H1 influenza virus infections were prevalent in swine in both surveys according to the results of enzyme-linked immunosorbent assay (ELISA). The H3 infection rate was reduced dramatically in 2007 compared with 2004, when examined by HI and ELISA. In summary, the results imply that the swine populations in Fujian Province had not been affected greatly by the H5N1 avian influenza virus, given that there is no serological evidence that H5N1 influenza virus has infected the pig populations. The reported isolates represent only sporadic cases.
Introduction

Although aquatic birds serve as the reservoir of influenza A viruses (IAVs), pigs are considered to be leading candidates for the intermediate hosts and reassortment vessels for IAVs from avian and human sources (Brown, 2000, 2001; Peiris et al., 2001b). Studies have demonstrated that pigs possess both avian (α-2,3Gal) and human (α-2,6Gal) receptors and therefore are infected by both kinds of IAVs (Kida et al., 1994; Ito et al., 1998). Moreover, pigs are domesticated and have frequent contact with both humans and birds. A series of IAV infections and reassortment events in pigs has been reported, from either human or bird sources or both (Kida et al., 1994; Guan et al., 1996; Brown, 2001; Peiris et al., 2001b; Landolt et al., 2003; Yu et al., 2007). Southeast Asia (including southern China) has long been regarded as an influenza epicenter, where all subtypes of IAVs are found to be prevalent in ducks and aquatic birds and where close contacts between domestic ducks, wild aquatic birds, pigs, humans and other mammals are frequently observed (Shortridge and Stuart-Harris, 1982; Shortridge, 1997; Brown, 2001). Therefore the roles of pigs as an important potential intermediary should be paid more attention and the surveillance should be continued around these areas. It has been reported that subtypes H1N1, H3N2 and H1N2 are mainly circulating in the swine populations (Guan et al., 1996; Brown, 2000, 2001; Peiris et al., 2001a,b; Ma et al., 2006; Qi and Lu, 2006; Yu et al., 2007). Since 1999, the H9N2 subtype of IAV has emerged in southern China and has co-circulated with other subtypes of IAV in pigs (Guan et al., 2000; Peiris et al., 2001a,b; Ninomiya et al., 2002; Choi et al., 2004; Cong et al., 2007; Xu et al., 2007).

Since the first fatal human case occurred in Hong Kong SAR of China in 1997 (Subbarao et al., 1998), the H5N1 subtype of IAV has been regarded as the next potential pandemic threat. A number of authors reported the isolation and genesis of H5N1 from domestic or wild waterfowl (Li et al., 2005; Liu et al., 2005; Normile, 2006; Smith et al., 2006; Wang et al., 2008) as well as sporadic seropositivity of pigs to H5 (Ninomiya et al., 2002; Choi et al., 2005; Jung et al., 2007). Li and colleagues isolated H5N1 IAV from infected pigs in southern China (Li et al., 2004) and this raised public health concerns. Briefly, the researchers tested serum samples collected from pig herds in 14 Provinces in China and found H5N1-positives in serum samples from Fujian via haemagglutination inhibition (HI) and neuraminidase inhibition experiments, following the method in the Textbook of Influenza (Nicholson, 1998). Moreover, they isolated H5N1 viruses from swabs samples taken from dead or sick pigs in Fujian Province. Recently, Zhu et al. reported further characterization of the H5N1 virus isolate (A/swine/Fujian/1/03) obtained from pigs in Fujian province in 2003 (Zhu et al., 2008). The serious effects that potentially may be caused by H5N1 infection in pigs have not yet been observed. To determine the prevalence of H5 subtype IAV in the pig populations of southern China, we investigated pig farms in Fujian Province southern China and performed serological surveillance, which has been widely used for this purpose elsewhere (Guan et al., 1996; Peiris et al., 2001b; Ninomiya et al., 2002; Choi et al., 2004; Jung et al., 2007). We surveyed the pig farms in 2004 and 2007 respectively, to investigate the seroprevalence of avian influenza virus in the pig populations of Fujian Province, following the reported H5N1 infection of pigs in 2003 (Li et al., 2004; Zhu et al., 2008) and its high prevalence in poultry markets from 2005 to 2006 (Chen et al., 2006; Smith et al., 2006). In total, over 1400 porcine serum samples from 25 pig farms in the nine administrative districts of Fujian Province were collected and analysed and the serological surveillance presented no evidence of H5 infection in the swine populations, although infections with H1, H3 and H9 IAV are still found. We surmise that the reported cases (Li et al., 2004; Zhu et al., 2008) were sporadic and rare and that avian H5N1 IAV has not yet adapted to the porcine host.

Materials and Methods

Surveillance regions and background

In 2004 and 2007 respectively, we performed prospective surveillance for influenza viruses in the swine population of Fujian Province, China. Specifically, 25 pig farms in all nine administrative districts of Fujian Province, Fuzhou, Longyan, Nanping, Ningde, Putian, Quanzhou, Sanming, Xiamen and Zhangzhou were under our surveillance (Fig. 1). The pig farms surveyed were all large- or medium-scale enterprises, producing 100–3000 slaughter pigs per year. These farms contribute the majority of pig production in Fujian Province and thus can be considered to be representative of the pig farms in Fujian Province. The local official veterinary department inspects these farms. It should be noted that pigs from small farms and backyard production were sometimes gathered on those farms. These were then slaughtered together. No vaccination programme for influenza virus has been executed in this pig population. Our surveillance lacks data from small farms, which may have lower bio-security standards, but it covered some small farms whose pigs were grouped on the large-scale farms. The date of the survey in each year is from April/May to November and was begun after the...
H5N1 outbreak in Fujian Province was reported and H5N1 viruses were isolated from sick pigs (Li et al., 2004).

Collection of porcine sera

Most of the serum samples obtained in 2004 were collected from adult sows, except that 95 were from pigs of 1 day to 4-month-old pigs. All the serum samples in 2007 were collected from adult sows.

Antigens, positive sera and virus

For the HI tests for H3, we used antigen produced from virus strain A/Fujian/411/2002 (H3N2) (FJ/411/02) and positive sera were prepared in chickens. The antigen was bought from the Institute for Viral Disease Control and Prevention, China Center for Disease Control and Prevention (China CDC). It should be noted that we failed to obtain H3N2 virus from pig in Fujian in that year and therefore we used the human strain FJ/411/02, as recommended by China CDC. FJ/411/02 was a prevalent strain in 2002 and was very similar to viruses prevailing in 2004. For the HI test for H5 and H9, we used antigens produced from the A/Guangdong/Goose/1/96 (H5N1) (GS/GD/1/96) and A/Chicken/Shanghai/10/2001 (H9N2) (CK/SH/10/01) virus stains and positive sera were prepared in chicken. These antigens were bought from Harbin Veterinary Research Institute, Chinese Academy of Agriculture Sciences. The antigens and positive sera for H5 and H9 are widely used for serological surveillance in China by the National Reference Laboratory in the above institute. For the neutralization tests for H5, we used the virus A/Bar-headed goose/Qinghai/1/2005 (H5N1) (BhG/QH/1/05), in which the HA sequence is similar to that of the prevalent virus in southern China.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA tests were applied for H1 (year 2004 and 2007) and H3 (year 2007 only) detection. HerdChek® antibody test kits for swine influenza viruses H1N1 and H3N2 were purchased from IDEXX Laboratories, Inc. The H1N1 and H3N2 ELISA tests were performed according to the manufacturer’s instructions and the resulting optical densities were converted into sample-to-positive (S/P) ratios using the formula provided by the manufacturer. Samples with an S/P ratio greater than or equal to 0.4 were considered to be positive for antibody against H1N1 and those with S/P ratio greater than or equal to 0.3 were considered to be positive for antibody against H3N2. All procedures were completed at ambient temperature (20–25°C).

Haemagglutination inhibition test

Prior to the HI test, serum samples were treated with the “Trypsin-Heat-Periodate” method (WHO, 2002) to inactivate non-specific haemagglutination inhibitors. Briefly, 30 μl of swine serum was added to 15 μl of trypsin (0.8% W/V) and then incubated at 56°C for 30 min to inactivate...
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the trypsin. Then, 90 µl of metapotassium periodate (0.01 M), 90 µl of 1% glycerin phosphate-buffered saline and 75 µl of NaCl (0.85%) containing 10 µl of chicken red blood cells (cRBCs) were added to the serum in turn and the mixture was incubated for 1 h at 4°C. Finally, the treated serum was incubated with cRBCs to eliminate non-specific haemagglutinators. The HI tests were subsequently performed according to the World Organization for Animal Health (OIE) recommendation (http://www.oie.int/eng/normes/monnual/A_00037.htm). In brief, 25 µl of serial twofold dilutions of the treated serum samples were mixed with four haemagglutinin units (HAU) of virus in V-shaped microtiter plates and incubated at room temperature for 30 min and then 50 µl of 1% cRBCs were added to each well and incubated at room temperature for 30 min. The HI titre was calculated as the reciprocal of the highest serum dilution that completely inhibited haemagglutination of four HAU of virus.

In each test conducted, the sera against GS/GD/1/96 and CK/SH/10/01 respectively were used as positive controls. The HI titre for the H5 positive control was >9log2 and the HI titre for the H9 positive control was >10log2. The H5 and H9 standard negative sera were used for negative controls and the HI titre was <2log2. It should be noted that the methods, antigens and positive sera for the HI tests for H5 and H9 are the same as those reported in the studies of Li et al. (2004), and the tests were performed shortly after the serum samples were collected.

Neutralization test

We randomly selected sera in each year and each county for examination in the neutralization tests (NT) for H5.

For most pig farms, one-third to one-half of the serum samples were selected. Given that all the farms we surveyed used uniform management procedures for feeding and medicine, the number of the serum samples used for the NT was sufficient. Serum samples were tested for neutralizing antibodies against BhG/QH/1/05 by using a traditional micro-neutralization method. In brief, first, swine and positive sera were twofold diluted in serum-free DMEM starting at a dilution of 1:10. Second, a dilution of virus containing 100 TCID₅₀ of virus in 0.1 ml was prepared in serum-free DMEM. Third, 100 TCID₅₀ of virus were added to the serially diluted serum at a 1:1 ratio (V/V) and incubated at 37°C for 1 h. Finally, 0.2 ml of the virus-serum mixtures was transferred to 96-well monolayer plates and incubated in 5% CO₂ at 37°C for 96 h. Three wells were run for each dilution of each serum sample. Pathogenic effects were observed every day and cell supernatants were tested with 1% cRBCs for their haemagglutination activity to confirm the virus infection.

Results

Serological analyses of influenza virus infection

From the pig population surveyed, we collected 499 and 908 serum samples in 2004 and 2007 respectively and performed serological detection of influenza virus infection. The results are shown in Tables 1 and 2. In 2004, influenza virus H1 infections were detected using the ELISA method and infections with H3, H5 and H9 were detected using HI, because only ELISA kits for detection of H1 were available at that time in China. In 2007, H1 and H3 were detected by ELISA and H5 and H9 by HI tests, because kits for H3 had become available.

Table 1. Serological analyses of influenza virus infections in the swine population of Fujian Province, China, 2004

<table>
<thead>
<tr>
<th>Administrative District</th>
<th>No. Pig Farms Surveyed</th>
<th>Date of Survey (mm/yy)</th>
<th>H1 (ELISA)</th>
<th>H3 (HI) (FJ/411/02)</th>
<th>H5 (HI) (GS/GD/1/96)</th>
<th>H5 (NT) (BhG/QH/1/05)</th>
<th>H9 (HI) (CK/Sh/10/01)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fuzhou</td>
<td>3</td>
<td>05/04</td>
<td></td>
<td>14/60 (23.3%)</td>
<td>44/60 (73.3%)</td>
<td>0/60 (0)</td>
<td>0/60 (0)</td>
</tr>
<tr>
<td>Longyan</td>
<td>3</td>
<td>09/04</td>
<td></td>
<td>33/42 (78.6%)</td>
<td>26/40 (65.0%)</td>
<td>0/46 (0)</td>
<td>0/46 (0)</td>
</tr>
<tr>
<td>Nanping</td>
<td>7</td>
<td>04/04</td>
<td></td>
<td>52/134 (38.8%)</td>
<td>115/133 (86.5%)</td>
<td>0/153 (0)</td>
<td>0/35 (0)</td>
</tr>
<tr>
<td>Ningde</td>
<td>2</td>
<td>11/04</td>
<td></td>
<td>32/38 (84.2%)</td>
<td>28/37 (75.7%)</td>
<td>0/38 (0)</td>
<td>0/24 (0)</td>
</tr>
<tr>
<td>Putian</td>
<td>3</td>
<td>09/04</td>
<td></td>
<td>58/65 (89.2%)</td>
<td>42/61 (68.8%)</td>
<td>0/65 (0)</td>
<td>0/24 (0)</td>
</tr>
<tr>
<td>Quanzhou</td>
<td>2</td>
<td>09/04</td>
<td></td>
<td>12/43 (27.9%)</td>
<td>36/41 (87.8%)</td>
<td>0/43 (0)</td>
<td>0/43 (0)</td>
</tr>
<tr>
<td>Sanming</td>
<td>2</td>
<td>11/04</td>
<td></td>
<td>25/49 (51.0%)</td>
<td>44/49 (89.8%)</td>
<td>0/49 (0)</td>
<td>0/13 (0)</td>
</tr>
<tr>
<td>Xiamen</td>
<td>1</td>
<td>09/04</td>
<td></td>
<td>4/26 (15.4%)</td>
<td>24/26 (92.3%)</td>
<td>0/26 (0)</td>
<td>0/16 (0)</td>
</tr>
<tr>
<td>Zhangzhou</td>
<td>2</td>
<td>09/04</td>
<td></td>
<td>9/17 (52.9%)</td>
<td>13/16 (81.3%)</td>
<td>0/26 (0)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>Total²</td>
<td>25</td>
<td>–</td>
<td></td>
<td>239/474 (50.4%)</td>
<td>372/463 (80.3%)</td>
<td>0/499 (0)</td>
<td>0/208 (0)</td>
</tr>
</tbody>
</table>

*ELISA: Sample positive ratio (SPR) ≥ 0.4 for H1N1; HI: titre ≥ 4 log2; NT: titre ≥ 1:16.

²The serum samples were tested by HI assay for the presence of antibodies, recognizing the selected viruses. The titres of positive control sera to FJ/411/02, BhG/QH/1/05 and CK/Sh/10/01 strains are >7log2, >9log2 and >10log2 respectively.

²Fewer serum samples were examined for H1 and H3 than for H5 and H9. This is because some serum samples were contaminated.
Table 2. Serological analyses of influenza virus infections in swine populations in Fujian Province, China, 2007

<table>
<thead>
<tr>
<th>Administrative District</th>
<th>No. Pig Farms Surveyed</th>
<th>Date of Survey (mm/yy)</th>
<th>Positive results for influenza virus infection*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>H1 (ELISA) H3 (ELISA) H5 (HI) (G5/GD/1/96) H5 (NT) (BhlQ/GH1/05) H9 (HI) (CK/SH10/01)</td>
</tr>
<tr>
<td>Fuzhou</td>
<td>3</td>
<td>05/07</td>
<td>51/104 (49.0%) 3/104 (2.9%) 0/104 (0) 0/48 (0) 2/104 (1.9%)</td>
</tr>
<tr>
<td>Longyan</td>
<td>3</td>
<td>09/07</td>
<td>59/127 (46.4%) 2/127 (1.57%) 0/127 (0) 0/60 (0) 5/127 (3.9%)</td>
</tr>
<tr>
<td>Nanping</td>
<td>7</td>
<td>07/07</td>
<td>55/94 (58.5%) 1/94 (1.0%) 0/94 (0) 0/36 (0) 1/94 (1.1%)</td>
</tr>
<tr>
<td>Ningde</td>
<td>2</td>
<td>11/07</td>
<td>29/100 (29.0%) 2/100 (2.0%) 0/100 (0) 0/48 (0) 5/100 (5.0%)</td>
</tr>
<tr>
<td>Putian</td>
<td>3</td>
<td>09/07</td>
<td>52/116 (44.8%) 4/116 (3.4%) 0/116 (0) 0/48 (0) 3/116 (2.6%)</td>
</tr>
<tr>
<td>Quanzhou</td>
<td>2</td>
<td>09/07</td>
<td>35/100 (35.0%) 6/100 (6.0%) 0/100 (0) 0/48 (0) 0/100 (0)</td>
</tr>
<tr>
<td>Sanming</td>
<td>2</td>
<td>11/07</td>
<td>46/100 (46.0%) 8/100 (8.0%) 0/100 (0) 0/48 (0) 3/100 (3.0%)</td>
</tr>
<tr>
<td>Xiamen</td>
<td>1</td>
<td>09/07</td>
<td>47/96 (48.9%) 1/96 (1.0%) 0/96 (0) 0/36 (0) 2/96 (2.1%)</td>
</tr>
<tr>
<td>Zhangzhou</td>
<td>2</td>
<td>09/07</td>
<td>39/71 (54.9%) 0/71 (0) 0/71 (0) 0/36 (0) 3/71 (4.2%)</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>–</td>
<td>413/908 (45.5%) 27/908 (2.97%) 0/908 (0) 0/408 (0) 24/908 (2.6%)</td>
</tr>
</tbody>
</table>

*ELISA: S/P ≥ 0.4 for H1N1, S/P ≥ 0.3 for H3N2; HI: titre ≥ 4 log2; NT: titre ≥ 1 : 16.

The serological results from 2004 (Table 1) show that the rate of H1 infection in the Fujian pig population was relatively high, because 239 out of 474 serum samples (50.4%) were found positive using ELISA. Samples from three of the nine counties exhibited H1 infection rates >75%. The highest positive rate (89.2%) was in samples from Putian District. The total H3 infection rate of this survey reached 80.3%, with sera from every district showing a proportion positive of >65%; the highest rate was above 92%. However, the numbers of infection with the H5 and H9 subtypes of avian influenza virus were very different from those of H1 and H3 in this survey. Specifically, no H5 positive serum was detected by H1 in all 499 samples and only five serum samples, two from Nanning, H3 positive samples were involved in antibody coexistence. The H9 antibodies in a given individual in 2004 were all detected with other serotypes. One out of the five multiple antibody positive cases was with H1, H3 and H9 positive samples were detected in eight districts.

Verification of H5N1 infection by neutralization tests

A total of 616 serum samples (208 from 2004 and 408 from 2007) were used to test for neutralizing antibody against H5 IAV infection. The NT may provide more accurate results for the detection of avian IAV infection in pigs (Ninomiya et al., 2002; Lipatov et al., 2008). All the sera tested showed an NT titre of <16, suggesting a negative result for H5N1 infection (Tables 1 and 2). This result agrees with that obtained using the HI test.

Existence of multiple serotype antibodies in some single individual pigs

Among the serum samples collected and tested, we found that some pigs had antibodies against different serotypes of influenza viruses simultaneously (Table 3). Because of the serological methods used, the detection of multiple antibodies in a given individual does not necessarily suggest co-infection, but cover the so-called co-infection. When multiple antibodies are detected, we cannot tell whether the viruses infected the host simultaneously or sequentially. In 2004, the main multiple antibodies in a given individual comprised H1 and H3. Of the 499 samples, 121 showed antibody positive for both H1 and H3 and such cases were discovered in seven out of the nine counties surveyed. Given that 239 H1 positive samples and 372 H3 positive samples were detected among all the sera collected, over 50% of the H1 and over 30% of the H3 positive samples were involved in antibody co-existence. The H9 antibodies in a given individual in 2004 were all detected with other serotypes. One out of the five multiple antibody positive cases was with H1, H3 and
H9; two involved H1 and H9 only; two involved H3 and H9 only. In 2007, there were some differences in the antibody co-existence. All 27 H3 positive cases were also positive for H1, but no H3 and H9 co-existence positives were observed. Of all 24 H9 positive cases, only four were detected also with H1 antibody.

Discussion

Southern China and Southeast Asia are thought to be the epicentre for human influenza epidemics (pandemics) throughout the history. Fujian Province of China is in the frontline of this centre. The province is one of the major pig and duck breeding regions, especially backyard feeding is popularized here, which might provide more chance for wild aquatic birds, domestic poultry, pigs and humans to contact closely, creating the opportunity for inter-species transmission and generation of new reassortants of influenza virus, including H5N1. Breakage and expansion of host range of H5N1 influenza virus is worrisome. Our serological surveillance of influenza virus infection performed in 2004 and 2007 covered the swine population in all nine administrative counties of Fujian Province, where a few H5N1 viruses were isolated from sick pigs since 2001 (Li et al., 2003). Furthermore, we also found simultaneous presence of antibodies against two serotypes, H1 and H3, and in some cases against three serotypes, H1, H3, and H9. As no vaccination programme for swine influenza virus has been used in China, such complicated situations of concurrent or consecutive infections might be brought by high circulation of pig production in this area. Pigs are mixed frequently from different sources in the area or even the neighbouring provinces and once infected pigs are imported the infections of such a virus then it will be distributed. Moreover, incomplete management, such as poor hygiene, overcrowded living conditions etc. also made the virus hard to clear out.

Table 3. Existence of multiple serotype antibodies in single individual pigs

<table>
<thead>
<tr>
<th>Administrative District</th>
<th>2004</th>
<th>2007</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H1 + H3</td>
<td>H1 + H9</td>
</tr>
<tr>
<td>Fuzhou</td>
<td>10/60</td>
<td>0/60</td>
</tr>
<tr>
<td>Longyan</td>
<td>18/46</td>
<td>0/46</td>
</tr>
<tr>
<td>Nanping</td>
<td>30/153</td>
<td>2/153</td>
</tr>
<tr>
<td>Ningde</td>
<td>0/38</td>
<td>0/38</td>
</tr>
<tr>
<td>Putian</td>
<td>32/65</td>
<td>1/65</td>
</tr>
<tr>
<td>Quanzhou</td>
<td>9/43</td>
<td>0/43</td>
</tr>
<tr>
<td>Sanming</td>
<td>15/49</td>
<td>0/49</td>
</tr>
<tr>
<td>Xiamen</td>
<td>0/53</td>
<td>0/26</td>
</tr>
<tr>
<td>Zhangzhou</td>
<td>7/19</td>
<td>0/19</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>121/499</td>
<td>3/499</td>
</tr>
</tbody>
</table>

No. of multiply infected positive sera/no. of total sera tested.
H9N2 avian influenza viruses have been identified from avian species in many provinces in China and we found positive sera for H9 in both 2004 and 2007 in this study. As pigs can serve as mixing vessels for the reassortment of human and avian influenza viruses, pig infection of H9N2 has been the focus of increasing attention. In 2004, Xu et al. (2004) reported that H9N2 caused pig disease and death in clinics and deduced that it probably originated from a reassortment of chicken influenza virus serotype H5N1 and duck of chicken H9N2. Combined with results from other reports in China, serological positive for H9N2 in pig farms was found in many provinces, including Guangdong, Heilongjiang, Shandong, Zhejiang, Henan and Beijing (Yu, 2008). The situation of H9N2 influenza virus infection in China needs to be paid more attentions and serological surveillance should be continuously carried out, which might provide necessary data for swine influenza control and possibly also some useful information for the prediction and preparedness of future human influenza pandemics. Meanwhile the situation of H5N1 in swine populations should also be surveyed.

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References


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<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>AUTHOR: A running head short title was not supplied; please check if this one is suitable and, if not, please supply a short title that can be used instead.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>AUTHOR: Please provide department name for affiliation 3 if applicable.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>AUTHOR: Please provide telephone and fax number for corresponding author.</td>
<td></td>
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<tr>
<td>5</td>
<td>AUTHOR: Please check this website address and confirm that it is correct. (Please note that it is the responsibility of the author(s) to ensure that all URLs given in this article are correct and useable.)</td>
<td></td>
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<td>6</td>
<td>AUTHOR: Please provide city and country name for IDEXX Laboratories</td>
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<tr>
<td>7</td>
<td>AUTHOR: Please check this website address and confirm that it is correct. (Please note that it is the responsibility of the author(s) to ensure that all URLs given in this article are correct and useable.)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>AUTHOR: “Furthermore...... three serotypes, H1, H3 and H9”. This sentence has been reworded for clarity. Please check and confirm it is correct.</td>
<td></td>
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<tr>
<td>10</td>
<td>AUTHOR: Please provide the name of the publisher for reference WHO (2002).</td>
<td></td>
</tr>
<tr>
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<td>AUTHOR: Figure 1 has been saved at a low resolution of 285.115 dpi. Please resupply at 600 dpi. Check required artwork specifications at <a href="http://www.blackwellpublishing.com/authors/digill.asp">http://www.blackwellpublishing.com/authors/digill.asp</a></td>
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<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
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</tbody>
</table>

# MARKED PROOF

Please correct and return this set

Please use the proof correction marks shown below for all alterations and corrections. If you wish to return your proof by fax you should ensure that all amendments are written clearly in dark ink and are made well within the page margins.

<table>
<thead>
<tr>
<th>Instruction to printer</th>
<th>Textual mark</th>
<th>Marginal mark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leave unchanged</td>
<td>• • • under matter to remain</td>
<td>1</td>
</tr>
<tr>
<td>Insert in text the matter indicated in the margin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delete</td>
<td>/ through single character, rule or underline</td>
<td></td>
</tr>
<tr>
<td></td>
<td>or</td>
<td></td>
</tr>
<tr>
<td></td>
<td>──── through all characters to be deleted</td>
<td></td>
</tr>
<tr>
<td>Substitute character or</td>
<td>/ through letter or</td>
<td></td>
</tr>
<tr>
<td>substitute part of one or more word(s)</td>
<td>──── through characters</td>
<td></td>
</tr>
<tr>
<td>Change to italics</td>
<td>— — under matter to be changed</td>
<td></td>
</tr>
<tr>
<td>Change to capitals</td>
<td>— — under matter to be changed</td>
<td></td>
</tr>
<tr>
<td>Change to small capitals</td>
<td>— — under matter to be changed</td>
<td></td>
</tr>
<tr>
<td>Change to bold type</td>
<td>—— under matter to be changed</td>
<td></td>
</tr>
<tr>
<td>Change to bold italic</td>
<td>Encircle matter to be changed</td>
<td></td>
</tr>
<tr>
<td>Change to lower case</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change italic to upright type</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>Change bold to non-bold type</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>Insert ‘superior’ character</td>
<td>/ through character or</td>
<td></td>
</tr>
<tr>
<td></td>
<td>\ where required</td>
<td></td>
</tr>
<tr>
<td>Insert ‘inferior’ character</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>Insert full stop</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>Insert comma</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>Insert single quotation marks</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>Insert double quotation marks</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>Insert hyphen</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>Start new paragraph</td>
<td>(As above)</td>
<td></td>
</tr>
<tr>
<td>No new paragraph</td>
<td></td>
<td></td>
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<tr>
<td>Transpose</td>
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<tr>
<td>Close up</td>
<td>linking characters</td>
<td></td>
</tr>
<tr>
<td>Insert or substitute space</td>
<td>/ through character or</td>
<td></td>
</tr>
<tr>
<td>between characters or words</td>
<td>\ where required</td>
<td></td>
</tr>
<tr>
<td>Reduce space between characters or words</td>
<td>between characters or words affected</td>
<td></td>
</tr>
</tbody>
</table>