Avian influenza A (H5N1) infection in a patient in China, 2006

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Background Highly pathogenic avian influenza H5N1 virus has caused increasing human infection in Eurasia since 2004. So far, H5N1 human infection has been associated with over 50% mortality that is partly because of delay of diagnosis and treatment.

Objectives and methods Here, we report that an H5N1 influenza virus infected a 31-year-old patient in Shenzhen in June 2006. To identify the possible source of the infection, the human isolate and other H5N1 influenza viruses obtained from poultry and wild birds in southern China during the same period of time were characterized.

Results Genetic and antigenic analyses revealed that the human H5N1 influenza virus, Shenzhen/406H/06, is of purely avian origin and is most closely related to viruses detected in poultry and wild birds in Hong Kong in early 2006.

Conclusions The findings of the present study suggest that the continued endemicity of H5N1 influenza virus in the poultry in southern China increases the chance for introduction of the virus to humans. This highlights the importance of continued surveillance of poultry and wild birds for determining the source for human H5N1 infection.

Key words Epidemiology, evolution, human, influenza A virus.

Introduction Influenza A (H5N1) virus is endemic in poultry in southern China and south-east Asia and has repeatedly caused severe human infection with high mortality, giving rise to an unprecedented pandemic threat. In the first 7 months of 2007, 27 countries were confirmed as being affected by this highly pathogenic avian influenza (HPAI) virus. Although H5N1 virus had previously been controlled in most of those countries, repeated outbreaks in poultry indicates that virus has become endemic in the poultry population in those regions. Moreover, the long-term endemicity of those viruses in poultry has led to an increase in human infection cases.

In 2005, there were 97 confirmed human H5N1 infections from five countries with 42 (43%) fatalities. In contrast, 80 of 116 (69%) human cases of H5N1 from nine countries in 2006 alone were fatal. While in China from November 2005 to May 2007, 25 cases from 13 different provinces have been confirmed. Many of those cases in China did not have a history of contact with poultry and no H5N1 influenza activity had been recorded locally. A similar situation has also been observed in Indonesia.

Systematic influenza surveillance has revealed that one variant of H5N1 virus, genotype Z, had become predominant in most affected regions. After circulating continuously for several years in poultry, H5N1 influenza viruses developed into antigenically and genetically distinguishable sublineages in different regions, including sublineages restricted to certain regions within China. However, since October 2005 an H5N1 variant (Fujian-like or Clade 2.3.4), gradually replaced previously dominant variants in different provinces of China and became predominant in the wider southern China region. This variant was also detected from smuggled chicken and wild bird in Hong Kong, and also from duck in Laos, Malaysia and Thailand in early 2006.
In June 2006, an H5N1 influenza virus was isolated from a patient diagnosed with severe pneumonia in Shenzhen, a neighboring city of Hong Kong. Even though there was no reported H5N1 activity locally in poultry at the same time, this case suggested that continuing endemicity of H5N1 influenza viruses in poultry has become a persistent threat of human infection. This study characterizes the H5N1 viruses from this human case along with those isolates from poultry or wild birds in the neighboring regions during the same period to useful epidemiological insights.

Methods

Virus detection and isolation

Influenza A (H5N1) virus was isolated in Madin-Darby canine-kidney cells from a tracheal aspirate specimen obtained on June 12, 2006 using previously described methods.15 The virus subtype was determined by standard hemagglutination inhibition (HI) and neuraminidase inhibition tests, as recommended by World Health Organization (WHO).16 Reverse-transcriptase-polymerase-chain-reaction (RT-PCR) of tracheal aspirates and neutralization assay for antibody detection were also conducted as recommended by the WHO.16,17

Antigenic analysis

To identify the possible source of infection, H5N1 influenza variants belonging to different sublineages, and those recent isolates from different provinces were also included in the antigenic analysis (Table 1). The analysis was performed through HI assay with a panel of monoclonal antibodies (MAbs) and ferret antisera against different WHO H5N1 representative strains, as previously described.16,18 The MAbs started at a 1:100 dilution and ferret antisera started at a 1:20 dilution.

To visualize similarity between the antigenic reaction patterns of different viruses, numerical analysis of HI titers was conducted using PRIMER version 5 (PRIMER-E, Plymouth, UK) as previously described.3,19 Titers <100 for MAbs and <20 for antisera in the HI assay were converted to zero in this analysis.

Phylogenetic and molecular analyses

RNA extraction, cDNA synthesis, and PCR were carried as described previously.18 Sequencing was performed by using BigDye Terminator v3 Cycle Sequencing Kit on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s instructions. Sequences were assembled and edited with Lasergene 7 (DNASTAR, Madison, WI, USA). Multiple sequence alignment and neighbor-joining phylogenetic analyses were carried out as previously described.4

Nucleotide sequence accession numbers

The sequence data generated in this study have been submitted to GenBank under accession numbers EF137706–EF137713.

Results

Clinical findings

A previously healthy 31-year-old male van driver presented to a local clinic in Shenzhen on June 7, 2006 with a 4-day
history of high fever (39.9°C), chills, and a cough with clear sputum. On June 8 he maintained a high fever (40°C) and developed frequent and severe coughing, shortness of breath, and watery diarrhea. After a chest radiograph revealed large opacities in his left lung, the patient was admitted to the hospital with a diagnosis of pulmonary infection and possible respiratory failure on the evening of June 9. After admission, the patient received high-dose oxygen, antibiotics, and intubated mechanical ventilation. RT-PCR assay of tracheal aspirate obtained on June 12 were positive for influenza A (H5N1) virus. The virus Shenzhen/406H/06 was successfully isolated from this tracheal specimen. From June 12 to 16 he was given oseltamivir treatment and from June 15 to 16 the patient was treated with convalescent plasma obtained from a patient who recovered from H5N1 infection in February 2006. RT-PCR test on tracheal aspirates were performed daily, and were persistently negative for H5N1 influenza virus from June 16 onwards. On June 16, a pure growth of Pseudomonas aeruginosa was obtained from a tracheal aspirate and the possibility of hospital-acquired secondary infection was considered. Drug sensitivity tests showed the isolate to be resistant to all available antibiotics except polymyxin. Following antibiotic treatment the patient had recovered by early August.

Although the patient was a van driver, he did not have a history of travel outside of Shenzhen in the recent past and had no evidence of occupational exposure to poultry. However, he had visited a local live-poultry market on two occasions between 5 and 10 days before onset of symptoms, but denied purchasing any poultry. None of his close contacts had recently developed any symptoms of respiratory disease. Also, there was no influenza A (H5N1) outbreak locally in poultry before or after the case was identified, in common with several recent cases of human infection China in 2006.

Neutralizing antibody titration
The patient’s serum neutralization antibody titer was determined to A/chicken/Hong Kong/282/2006, a virus most closely related to A/Shenzhen/406H/2006 (Figures 2 and 3). It was negative on June 14 and 15, and rose steadily to between 1:40 and 1:80 by June 20 (data not shown). This may be the result of both the convalescent plasma therapy and the patient’s own humoral immune response as the neutralization antibody titers were maintained at this level subsequently (data not shown).

Antigenic analysis
Antigenic analysis with MAb against Ck/HK/YU22/03 and H5N1 reference antisera with representative viruses from different sublineages demonstrated a diversity of reaction patterns that generally corresponded to their phylogenetic relationships (Table 1, and see below). Shenzhen/406H/06 together with Ck/HK/282/06, Magpie robin/HK/75/06 and Dk/HN/1204/06 showed the same reaction pattern to both MAb and antisera, confirming their antigenic similarity. Ck/HN/999/05 and all representative viruses had significant antigenic differences (Table 1). These relationships were confirmed by numerical analysis of the HI titers, particularly the 100% antigenic similarity of Shenzhen/406H/06 with Ck/HK/282/06 (Figure 1).

Phylogenetic analysis
To determine the evolutionary relationship of the human isolate Shenzhen/406H/06, other H5N1 viruses isolated from wild bird, poultry and previous human isolates were genetically analyzed. Phylogenetic analysis showed that the HA and NA genes of the virus Shenzhen/406H/06 fell into the Fujian-like (Clade 2.3.4) sublineage (Figure 2). The virus was most closely related to those viruses isolated from wild birds and smuggled chicken in Hong Kong in February 2006 (Figure 2). These viruses formed a distinguishable
subgroup within the Fujian-like sublineage. This Fujian-like sublineage was in turn most closely related to other viruses from throughout southern China (Figure 2). It was noted that in the HA gene tree, the H5N1 influenza viruses isolated from several other human cases in different provinces since November 2005, including Anhui/1/05, Zhejiang/16/06 and Guangdong/1/06, all clustered into the Fujian-like sublineage.

Phylogenetic analysis of the internal genes showed the same relationships as the surface genes (Figure 3). Five of six internal genes directly clustered with those H5N1 viruses isolated from smuggled chicken or wild birds in Hong Kong in early 2006, and formed a distinguishable subgroup within the Fujian-like sublineage (Figure 3). All other recent human H5N1 isolates from China were also most closely related to those viruses isolated from poultry. Analysis of gene constellation also confirmed that the virus belonged to H5N1 genotype Z that is present throughout Southeast Asia and southern China (Figure 3).

These findings suggest that the patient was infected from the local environment.

**Molecular characterization**

The virus Shenzhen/406H/06 maintained the motif of multiple basic amino acids (LRERRRRK/G) at the HA cleavage site characteristic of HPAI. The receptor-binding pocket of HA1 retains amino acid residues Gln-222 and Gly-224 (H5 numbering used throughout) that preferentially bind to a 2,3-NeuAcGal linkages of avian cell-surface receptors. There was an Arg-212-Lys mutation in the receptor-binding domain; however, all other amino acid residues relevant to receptor binding were identical to those of Gs/GD-like viruses. The virus Shenzhen/406H/06 did not have any mutations in the M2 or NA proteins associated with resistance to amantadine and oseltamivir, respectively. Also, the virus did not have the Lys-627 mutation in the PB2 protein that is associated with increased virulence of avian influenza viruses in mammals.
Figure 3. Phylogenetic relationships of Shenzhen/406H/06 within Fujian-like (Clade 2.3.4) H5N1 influenza A virus genes. (A) PB2 (polymerase basic 2), nucleotide positions 1–2280. (B) PB1 (polymerase basic 1), nucleotide positions 1–2274. (C) PA (polymerase acidic), nucleotide positions 1–2151. (D) NP (nucleoprotein), nucleotide positions 1–1494. (E) M (matrix), nucleotide positions 1–981. (F) NS (non-structural), nucleotide positions 1–816. Trees were generated in the program PAUP* by using a general-time-reversible neighbor-joining analysis. The PB1 and PA tree are rooted to A/duck/Hong Kong/Y280/97. The PB2, NP, M, and NS trees are rooted to A/equine/Prague/1/56. Scale bar, 0.001 substitutions per site. The isolate characterized in this study is underlined. Ck, chicken; Dk, duck; Gs, goose; GX, Guangxi; GY, Guiyang; HK, Hong Kong.
Discussion

In the present study, genetic and antigenic analyses of the H5N1 influenza virus isolated from a patient in Shenzhen in June 2006 revealed that it was most closely related to those H5N1 viruses isolated from smuggled chicken and wild birds in early 2006 in Hong Kong.3 These findings suggest that the patient was very likely infected from affected poultry locally at live-poultry markets. This situation is similar to that observed in most human H5N1 infection cases in other south-east Asian countries.1,3,18

From 2004 onwards, H5N1 virus has continued to expand its territory, transmitting primarily through poultry and poultry product movement, along with bird migration1,13 and over 60 countries and regions have now reported H5N1 outbreaks in poultry or detected H5N1 positive dead birds.10 Among H5N1 influenza affected countries, most human infection cases have resulted from direct contact with infected birds during local outbreaks, including cases of workers that have sera converted following involvement in culling operations of infected bird populations.12 Despite reports of family cluster cases and evidence of limited human-to-human transmission, epidemiological and virological studies concur that current H5N1 viruses still lack the ability for efficient human-to-human transmission.8,12,30–32

In China, human infection with H5N1 virus appears to occur via a different mechanism. In 2006, only few poultry outbreaks were reported from China. However, of the 22 confirmed human cases, the infectious source could only be identified in 10 cases as there were no reports of poultry outbreaks or H5N1 positive birds detected locally. Of those recent cases in China, five patients came from highly urbanized metropolitan areas in southern China. Therefore, the human case in Shenzhen may represent the dynamic of infection from live-poultry markets to humans, where people are infected from silently transmitting virus in the absence of recorded H5N1 activity in poultry.1–3,33,34 However, it must be noted that without effective surveillance, active or passive, it is unlikely that H5N1 virus would be detected. For instance, the accumulated findings of our systematic surveillance in poultry markets during the past 6 years clearly demonstrated that H5N1 influenza virus has circulated continuously in apparently healthy poultry in southern China.1–3,18 Moreover, since the emergence of the Fujian-like (Clade 2.3.4) virus the prevalence of H5N1 virus in southern China has increased dramatically compared with previous years.3 This was also reflected by the increase in human infection cases in southern China during 2006.9,11

In Vietnam, large-scale vaccination of poultry appeared to prevent poultry outbreaks and human infection cases in the past year; however, H5N1 outbreaks have reoccurred in poultry in the winter of 2006/07.9,11 This situation challenges whether poultry vaccination can effectively disconnect the H5N1 transmission chain among birds, or from bird to human. After 11 years of repeated outbreaks and human infection, there are still many H5N1 affected countries that do not have effective surveillance of their poultry. Moreover, as the virus is endemic in these areas surveillance only of outbreaks events is not sufficient to provide a comprehensive view of virus transmission. The present study again demonstrates the importance of both systematic virological and serological surveillance of poultry and wild birds, along with surveillance of humans occupationally exposed to poultry, in understanding the dynamic behavior of this virus.

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