DOI:10.1111/j.1750-2659.2007.00032.x
www.blackwellpublishing.com/influenza

Original Article

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

X. Chen,[a,1] G.J.D. Smith,[b,1] B. Zhou,[a] C. Qiu,[c] W.L. Wu,[b] Y. Li,[d] P. Lu,[a] L. Duan,[b] S. Liu,[a] J. Yuan,[a] G. Yang,[a] H. Wang,[a] J. Cheng,[e] H. Jiang,[f] J.S.M. Peiris,[b] H. Chen,[b] K.Y. Yuen,[b] N. Zhong,[d] Y. Guan[b]

[a]Shenzhen Donghu Hospital, Shenzhen, China, [b]State Key Laboratory of Emerging Infectious Diseases, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong, China, [c]Shenzhen Peoples' Hospital, Shenzhen, China,[d]Guangzhou Institute of Respiratory Disease, First Affiliated Hospital, Guangzhou Medical College, Guangzhou, China,[e]Shenzhen Center for Disease Control and Prevention, Shenzhen, China,[f]Shenzhen Health Bureau, Shenzhen, China.

Correspondence: Yi Guan, State Key Laboratory of Emerging Infectious Diseases, Li Ka Shing Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong, China. Email: yguan@hkucc.hku.hk

[1]These authors contributed equally to this manuscript.

Accepted 14 November 2007.

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.

Please cite this paper as: Chen *et al.* (2008) H5N1 infection in China. Influenza and Other Respiratory Viruses 1(5-6), 207–213.

Introduction

Influenza A (H5N1) virus is endemic in poultry in southern China and south-east Asia[1–5] and has repeatedly caused severe human infection with high mortality, giving rise to an unprecedented pandemic threat.[6–8] In the first 7 months of 2007, 27 countries were confirmed as being affected by this highly pathogenic avian influenza (HPAI) virus.[9–11] 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.[4,5,10] Moreover, the long-term endemicity of those viruses in poultry has led to an increase in human infection cases.[11,12]

In 2005, there were 97 confirmed human H5N1 infections from five countries with 42 (43%) fatalities.[9–11] 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.[9–11] A similar situation has also been observed in Indonesia.[8,12]

Systematic influenza surveillance has revealed that one variant of H5N1 virus, genotype Z, had become predominant in most affected regions.[1–5,13] 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.[1,3–5] 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.[3] 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.[3,14]

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 5·2·9 (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 v3·1 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 7·0 (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

Table 1. Antigenic analysis of H5N1 influenza viruses by hemagglutinin inhibition test

Virus	Clade*	MAbs to Ck/HK/YU22/02				Ferret antisera		
		3C8	7C6	8H11	10H4D2	BH goose/QH/65/05	VNM/1203/04	Gs/HK/437·4/99
Shenzhen/406H/06	2·3·4	<**	<	1600	3200	80	80	320
Ck/HK/282/06	2·3·4	<	<	1600	3200	80	80	320
Magpie robin/HK/75/06	2·3·4	<	<	1600	3200	80	80	640
Dk/HN/1204/06	2·3·4	<	<	1600	1600	80	80	320
Ck/HN/999/05	2·3·1	3200	400	6400	6400	320	40	640
VNM/1203/04	1	3200	800	200	12 800	80	320	1280
IDN/5/05	2·1	<	200	3200	6400	320	160	320
BH goose/QH/65/05	2·2	6400	<	12 800	6400	1280	160	640
Ck/HK/YU22/02	8	1600	200	800	6400	160	80	1280
Gs/HK/437·6/99	0	<	1600	12 800	12 800	1280	640	1280

*Clade designations follow World Health Organization guidelines available at: http://www.who.int/csr/disease/avian_influenza/guidelines/nomenclature/en/index.html.
**<, HI titer <1:100 for MAbs or <1:20 for antisera.
BH goose, bar-headed goose; Ck, chicken; Dk, duck; Gs, goose; HK, Hong Kong; HN, Hunan; IDN, Indonesia; QH, Qinghai VNM, Vietnam.

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.[20] 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.[3,21,22]

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).

Anitgenic analysis

Antigenic analysis with MAbs 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 MAbs 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

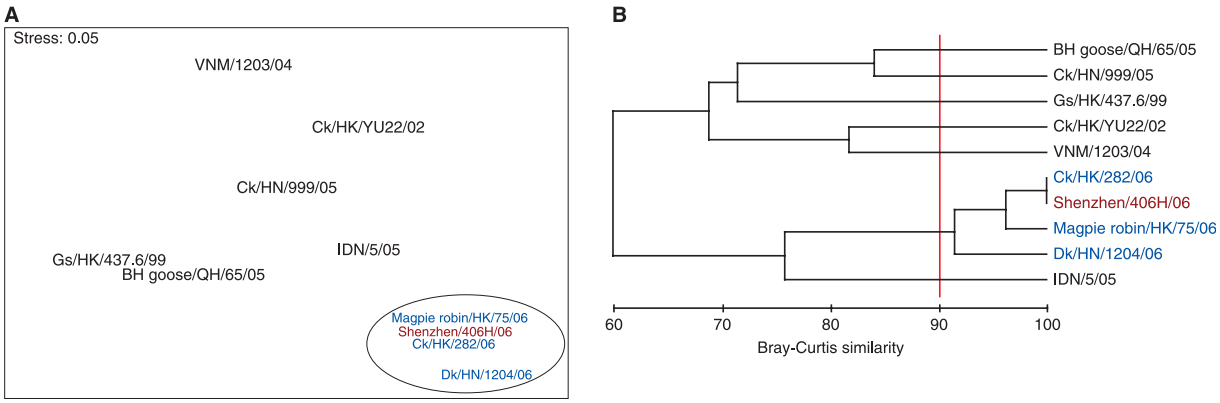

Figure 1. Numerical analysis of hemagglutination inhibition (HI) titers (see Table 1) by using non-metric multidimensional ordination in two dimensions (A) and hierarchical agglomerative clustering (B). BH goose, bar-headed goose; Ck, chicken; Dk, duck; Gs, goose; GX, Guangxi; HK, Hong Kong; HN, Hunan; IDN, Indonesia; QH, Qinghai; VNM, Vietnam.

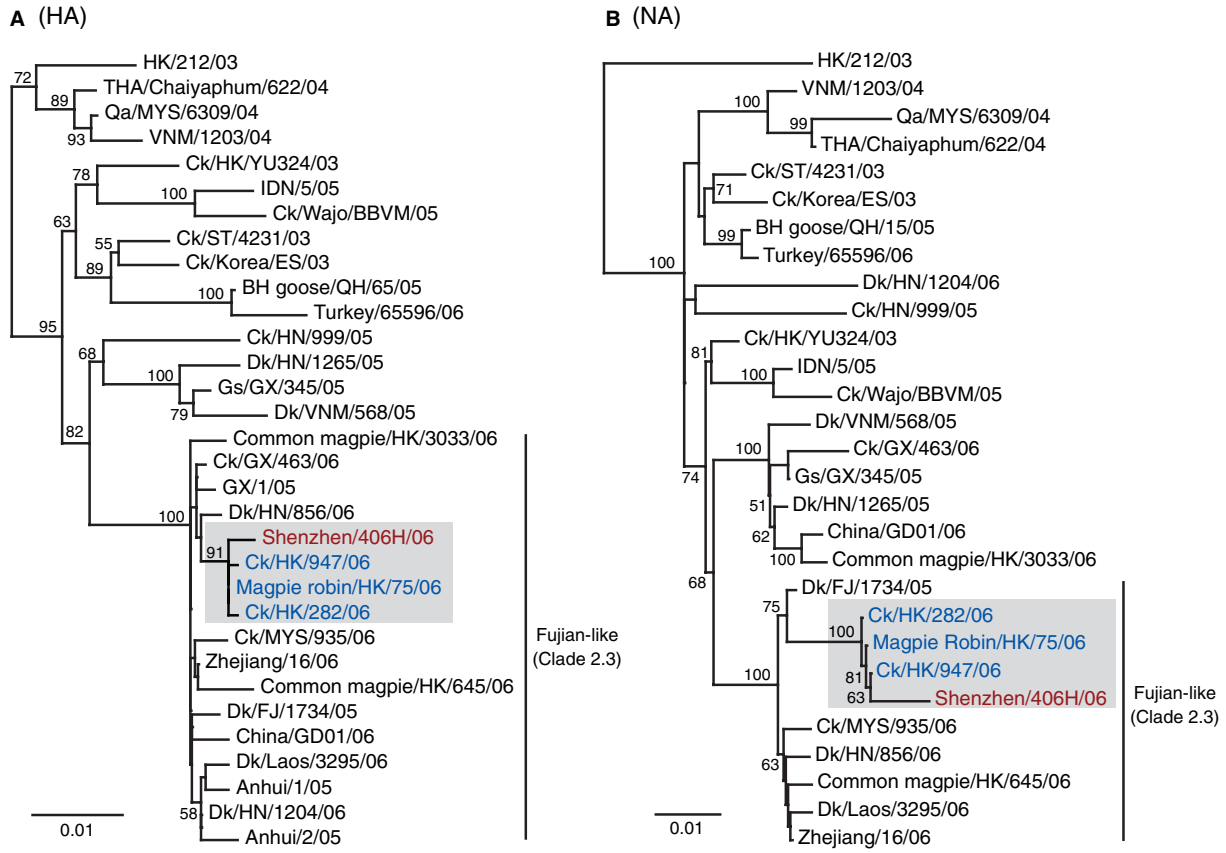

Figure 2. Phylogenetic relationships between representative H5N1 influenza A virus genes. (A) HA, nucleotide positions 1 to 1011. (B) NA, nucleotide positions 1 to 1410 (B). Trees were generated in the program PAUP* by using a general-time-reversible neighbor-joining analysis. Trees were rooted to A/Goose/Guangdong/1/1996. Numbers at branches are bootstrap values from 1000 replicates. Scale bar, 0·01 substitutions per site. The isolate characterized in this study is highlighted in red. BH goose, bar-headed goose; Ck, chicken; Dk, duck; Gs, goose; GX, Guangxi; HK, Hong Kong; HN, Hunan; MYS, Malaysia; Qa, quail; QH, Qinghai; ST, Shantou; THA, Thailand; VNM, Vietnam; YN, Yunnan.

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.

Phylogenic 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.[3,21,22] 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 (LRERRRKR/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 α2,3-NeuAcGal linkages of avian cell-surface receptors.[23] There was an Arg-212-Lys mutation in the receptor-binding domain;[23,24] however, all other amino acid residues relevant to receptor binding were identical to those of Gs/GD-like viruses.[25] The virus Shenzhen/406H/06 did not have any mutations in the M2 or NA proteins associated with resistance to amantadine and oseltamivir, respectively.[26–28] 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.[29]

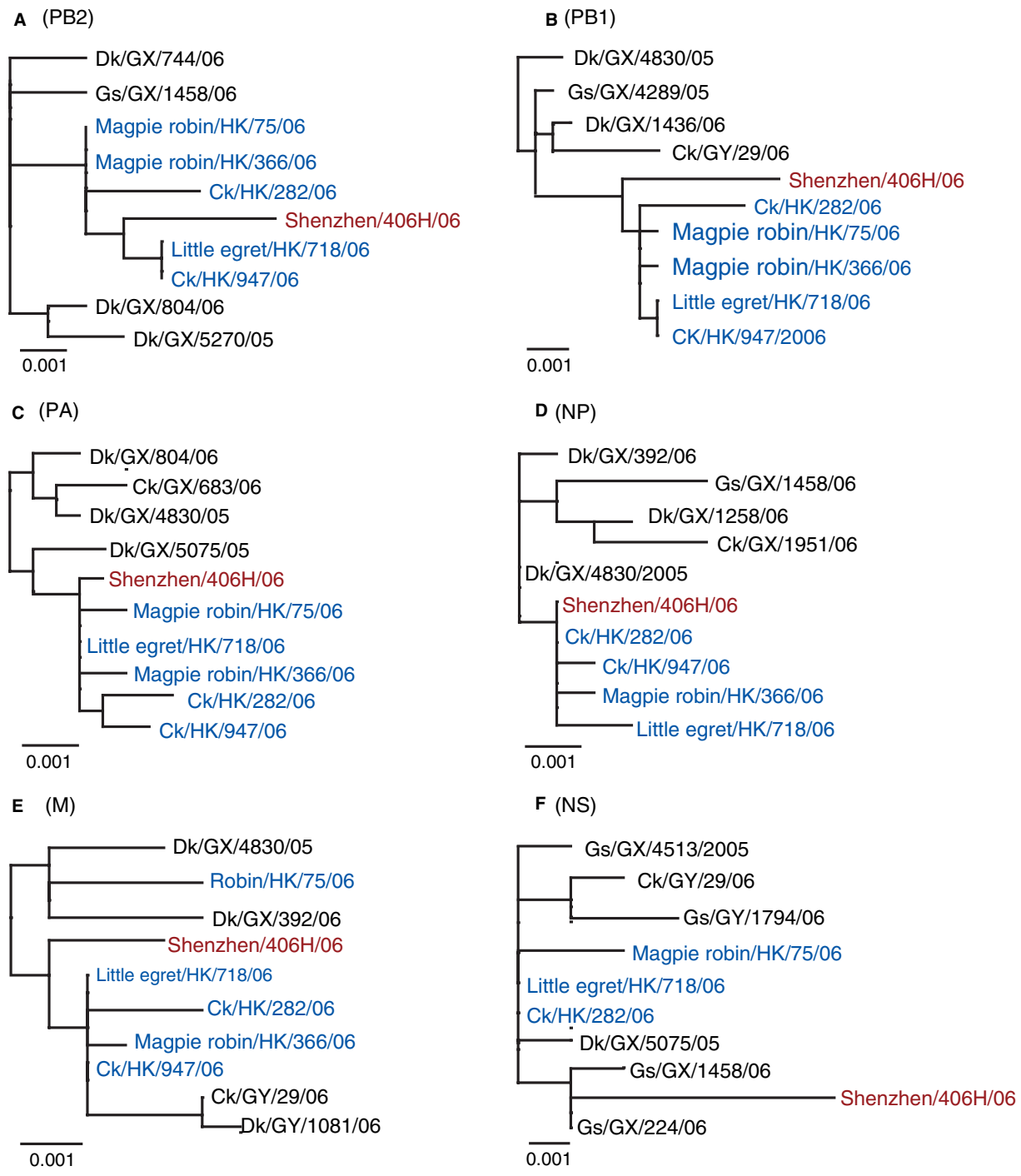

Figure 3. Phylogenetic relationships of Shenzhen/406H/06 within Fujian-like (Clade 2·3·4) H5N1 influenza A virus genes. (A) PB2 (polymerase basic 2), nucleotide positions 1–2280. (B) PB1 (polymerase basic 1), nucleotide positions 1–2274. (C) PA (polymerase acidic), nucleotide positions 1–2151. (D) NP (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.[8,12]

From 2004 onwards, H5N1 virus has continued to expand its territory, transmitting primarily through poultry and poultry product movement, along with bird migration [1,13] 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.

Acknowledgements

This work was supported by the Shenzhen Health Bureau and Guangdong Provincial Government and the Li Ka Shing Foundation. GJDS is supported by a career development award from the National Institutes of Health under NIAID contract HHSN266200700005C.

References

1 Chen H, Smith GJD, Li KS *et al.* Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. Proc Natl Acad Sci USA 2006;103:2845–2850.
2 Li KS, Guan Y, Wang J *et al.* Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature 2004;430:209–213.
3 Smith GJD, Fan XH, Wang J *et al.* Emergence and predominance of an H5N1 influenza variant in China. Proc Natl Acad Sci USA 2006;103:16936–16941.
4 Smith GJD, Naipospos TSP, Nguyen TD *et al.* Evolution and adaptation of H5N1 influenza virus in avian and human hosts in Indonesia and Vietnam. Virology 2006;350:258–268.
5 World Health Organization Global Influenza Program Surveillance Network. Evolution of H5N1 avian influenza viruses in Asia. Emerg Inf Dis 2005b;11:1515–1521.
6 Tran TH, Nguyen TL, Nguyen TD *et al.* Avian influenza A (H5N1) in 10 patients in Vietnam. N Engl J Med 2004;350:1179–1188.
7 Chotpitayasunondh T, Ungchusak K, Hanshaoworakul W *et al.* Human disease from influenza A (H5N1). Thailand, 2004. Emerg Infect Dis 2005;11:201–209.
8 World Health Organization Consultation on Human Influenza A/H5. Avian influenza A (H5N1) infection in humans. N Engl J Med 2005a;353:1374–1385.
9 Centre for Health Protection, Department of Health. Global Statistics of Avian Influenza. Hong Kong, China: Government of the Hong Kong SAR, 2007. http://www.info.gov.hk/info/flu/eng/global.htm.
10 Food and Agricultural Organization of the United Nations. Avian Influenza Disease Emergency Bulletin, Issue 42. Rome, Italy, 2006. http://www.fao.org/avianflu/en/AIDEnews.html.
11 World Health Organization. Situation updates – Avian influenza. 2005 to 2007. Geneva, Switzerland: World Health Organization, 2007. http://who.int/csr/disease/avian_influenza/updates/en/index.html.

12 World Health Organization. Influenza Research at the Human and Animal Interface. Geneva, Switzerland: World Health Organization, 2006. http://www.who.int/csr/resources/publications/influenza/WHO_CDS_EPR_GIP_2006_3C.pdf.

13 Chen H, Smith GJD, Zhang SY *et al.* H5N1 virus outbreak in migratory waterfowl. Nature 2005;436:191–192.

14 Boltz DA, Douangngeun D, Sinthasak S *et al.* H5N1 influenza viruses in Lao People's Democratic Republic. Emerg Infect Dis 2006;12:1593–1595.

15 Peiris JSM, Yu WC, Leung CW *et al.* Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet 2004;363:617–619.

16 World Health Organization. WHO Manual on Animal Influenza Diagnosis and Surveillance. Geneva, Switzerland: World Health Organization, 2002. http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf.

17 World Health Organization. Recommendations and Laboratory Procedures for Detection of Avian Influenza A (H5N1) Virus Specimens from Suspected Human Cases. Geneva, Switzerland: World Health Organization, 2007. http://www.who.int/csr/disease/avian_influenza/guidelines/RecAllabtestsAug07.pdf.

18 Guan Y, Poon LLM, Cheung CY *et al.* H5N1 influenza: a protean pandemic threat. Proc Natl Acad Sci USA 2004;101:8156–8161.

19 Xu KM, Li KS, Smith GJD *et al.* Evolution and molecular epidemiology of H9N2 influenza A viruses from quail in southern China, 2000 to 2005. J Virol 2007;81:2635–2645.

20 Zhou B, Zhong N, Guan Y. Treatment with convalescent plasma for influenza A (H5N1) infection. New Engl J Med 2007;357:1450–1451.

21 Shu Y, Yu H, Li D. Lethal avian influenza A (H5N1) infection in a pregnant woman in Anhui Province, China. N Engl J Med 2006;354:1421–1422.

22 Yu H, Shu Y, Hu S *et al.* The first confirmed human case of avian influenza A (H5N1) in Mainland China. Lancet 2006;367:84.

23 Ha Y, Stevens DJ, Skehel JJ, Wiley DC. X-ray structures of H5 avian and H9 swine influenza virus hemagglutinins bound to avian and human receptor analogs. Proc Natl Acad Sci USA 2001;98:11181–11186.

24 Stevens J, Blixt O, Tumpey TM, Taubenberger JK, Paulson JC, Wilson IA. Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. Science 2006;312:404–410.

25 Claas EC, Osterhaus ADME, van Beek R *et al.* Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 1998;351:472–477.

26 Scholtissek C, Quack G, Klenk HD, Webster RG. How to overcome resistance of influenza A viruses against adamantane derivatives. Antiviral Res 1998;37:83–95.

27 Treanor JJ, Hayden FG, Vrooman PS *et al.* Efficacy and safety of the oral neuraminidase inhibitor oseltamivir in treating acute influenza: a randomized controlled trial. JAMA 2000;283:1016–1024.

28 Le QM, Kiso M, Someya K *et al.* Avian flu: isolation of drug-resistant H5N1 virus. Nature 2005;437:1108.

29 Hatta M, Gao P, Halfmann P, Kawaoka Y. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 2001;293:1840–1842.

30 Olsen SJ, Kumnuan U, Ly S *et al.* Family clustering of avian influenza A (H5N1). Emerg Infect Dis 2005;11:1799–1801.

31 Ungchusak K, Auewarakul P, Dowell SF *et al.* Probable person-to-person transmission of avian influenza A (H5N1). N Engl J Med 2005;352:333–340.

32 Kandun IN, Wibisono H, Sedyaningsih ER *et al.* Three Indonesian clusters of H5N1 virus infection in 2005. N Engl J Med 2006;355:2186–2194.

33 Shortridge KF, Zhou NN, Guan Y *et al.* Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology 1998;252:331–342.

34 Kung NY, Guan Y, Perkins NR *et al.* The impact of a monthly rest day on avian influenza virus isolation rates in retail live poultry markets in Hong Kong. Avian Dis 2003;47(Suppl. 3):1037–1041.

