International Journal of Infectious Diseases 43 (2016) 85–89

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Is a highly pathogenic avian influenza virus H5N1 fragment recombined in PB1 the key for the epidemic of the novel AIV H7N9 in China, 2013?

Liang Chen [a,b], Liqian Sun [b,c,d], Rui Li [b,c,d], Yue Chen [e], Zhijie Zhang [b,c,d,*], Chenglong Xiong [a,b,*], Genming Zhao [b,c], Qingwu Jiang [b]

[a] Department of Public Health Microbiology, School of Public Health, Fudan University, Bldg 8#, Rd. Dong'an 130, Shanghai 200032, People's Republic of China
[b] Key Laboratory of Public Health Safety, Ministry of Education, Shanghai, People's Republic of China
[c] Department of Epidemiology and Biostatistics, School of Public Health, Fudan University, Bldg 8#, Rd. Dong'an 130, Shanghai 200032, People's Republic of China
[d] Laboratory for Spatial Analysis and Modeling, School of Public Health, Fudan University, Shanghai, People's Republic of China
[e] Department of Epidemiology and Community Medicine, Faculty of Medicine, University of Ottawa, Ontario, Canada

ARTICLE INFO

Article history:
Received 28 October 2015
Received in revised form 4 January 2016
Accepted 5 January 2016

Corresponding Editor: Eskild Petersen, Aarhus, Denmark.

Keywords:
Influenza virus
H7N9
Recombination
China

SUMMARY

Background: A novel avian influenza A H7N9 virus that infects humans was identified in China in 2013. This study is the first to comprehensively investigate the characteristics of genomic recombination, rather than reassortment, which has been the subject of investigation in previously reported studies.
Methods: Novel avian influenza virus (AIV) H7N9 genome sequences were obtained from the NCBI Influenza Virus Sequence Database and the Global Initiative on Sharing Avian Influenza Database (GISAID) and a representative isolate was subjected to homogeneity analysis. A phylogenetic tree was constructed. Eight segments of the isolate were analyzed to identify segments with recombination events, the corresponding recombination fragments, and breakpoints. The evolutionary history of the recombined fragments was tracked by constructing phylogenetic trees of the recombination fragments.
Results: Among the eight segments of the novel AIV H7N9 analyzed, only the PB1 segment showed a marked recombination phenomenon, with 11 recombination events; these included five actual recombination events and six possible misalignment artifact recombination events. The most notable was the recombination of a 291-nucleotide (nt) fragment at the 490–780 nt site that was affiliated to a highly pathogenic avian influenza virus (HPAIV) H5N1 (A/tree sparrow/Thailand/VSMU-16-RBR/2005). The phylogenetic tree of the 291-nt recombination fragment on the PB1 segment showed that the novel AIV H7N9 had a close genetic relationship to H9N2 and H5N1.
Conclusions: The novel AIV H7N9 might have reassorted its PB1 segment from H9N2 circulating in China, and this H9N2 PB1 might have been recombined into a highly pathogenic fragment from HPAIV H5N1, which could be the reason for the high fatality rate among patients with AIV H7N9 influenza.

1. Introduction

An epidemic of human infection with a novel avian influenza virus (AIV) H7N9 first emerged in China in 2013. As of February 23, 2015, a total of 571 laboratory-confirmed cases of human infection with avian influenza A(H7N9) virus, including 212 deaths, had been reported to the World Health Organization (WHO), giving a fatality rate of 37.13%, which is much higher than the rate of <0.25% for patients with AIV H1N1 in 2009–2010. Human cases of H7N9 emerged sporadically in the winter of 2015 in China.[1–3]

The influenza virus contains eight segments of a single-stranded RNA genome with negative polarity, and is more complex than many other single-stranded unsegmented RNA viruses. Previous studies on AIV H7N9 have focused mainly on the features of reassortment among the eight segments of its genome, whereas

* Corresponding authors. Tel./fax: +86 21 5423 7435; Tel./fax: +86 21 5423 7410.
E-mail addresses: epista@gmail.com (Z. Zhang), xiongchenglong@fudan.edu.cn (C. Xiong).

http://dx.doi.org/10.1016/j.ijid.2016.01.002

potential recombination within each segment has not yet been investigated.[4,5] Identifying and excluding the recombinant segments could provide further information on the evolution of this pathogen.[6] In order to investigate the evolutionary origin of the novel H7N9 virus and the reasons for its high virulence in humans, the characteristics of recombination in each of the eight segments of this virus was explored in the present study.

2. Materials and methods

2.1. Determination of the reference isolate of the novel H7N9

Genomes of AIV H7N9 were collected from the NCBI Influenza Virus Sequence Database (http://www.ncbi.nlm.nih.gov/genomes/FLU/aboutdatabase.html) and the Global Initiative on Sharing Avian Influenza Data (GISAID) database (http://platform.gisaid.org/epi3/frontend) on May 18, 2015. Phylogenetic trees were constructed using MEGA 5.0 software (http://megasoftware.net) and homogeneous identities were calculated using Lasergene 7 software (http://www.dnastar.com); this was done to determine which isolate of H7N9 should be selected as the reference for further studies.

2.2. Searching for large-scale homogeneous sequences serving as recombination resources

Sequences sharing high pairwise identities with each segment of the genome of the reference were obtained through the Basic Local Alignment Search Tool (BLAST), and the maximum target sequences parameter was set at 1000 (consequently, 8000 sequences in total). Based on the phylogenetic tree and homogeneous identities, sequences were removed if they satisfied the following two conditions: (1) they were isolated from the same area within a period of 2 years, and (2) they showed more than 99.0% identity, since they might be the same strain obtained from different individuals. Sequences released after March 2013 were also removed, because they obviously did not conform to the logical temporal order of recombination.

2.3. Recombination analyses for segments affiliated with the novel H7N9

Homogeneous recombination events were analyzed using the recombination detection program RDP, version 4.16 (http://www.bioinf.manchester.ac.uk/recombination/programs.shtml), as reported by Martin et al. and Boni et al.[7,8]

2.4. Phylogenetic trees of recombinant fragments

To track the evolutionary history of the recombination fragments identified, phylogenetic trees consisting of recombinant fragments were constructed. Sequences corresponding to the segments that had characteristics of recombination and that were established during the period January 2003 to February 2013, regardless of their hosts and subtypes, were downloaded from the NCBI Influenza Virus Sequence Database. After alignment using ClustalW in MEGA 5.0 software, all of the segments were trimmed into the length corresponding to the identified recombination fragments. The jModelTest2 program (http://darwin.uvigo.es) was then applied to estimate the likelihood value of the model to select the best model for tree construction. Maximum likelihood phylogenetic trees were bootstrapped by 1000 replicates for significance testing.

3. Results

An early isolate A/Zhejiang/DTID-ZJU01/2013 (H7N9), which displayed very high homogeneity compared to the other H7N9 isolates established in China in 2013, was selected as the reference H7N9 for this study. Identities between them were as follows: NA, 99.3–99.9%; HA, 99.3–99.9%; M, 98.4–99%; PB1, 99.3–99.9%; NEP/NS1, 97.6–99.9%; NP, 99.2–99.7%; PA, 99.7–99.8%; and PB2, 96.7–99.8%.

After pre-processing the sequences, the numbers of sequences used for recombination analysis corresponding to each segment of the novel H7N9 were 384 PB2, 389 PB1, 423 PA, 247 HA, 414 NP, 289 NA, 411 NS, and 372 M, in accordance with the selection criteria.

Among the eight segments of the reference isolate genome, only the PB1 segment displayed notable evidence of recombination; a total of 11 recombination events were detected, including five possible recombinations and six possible misalignment artifact recombinations. The five fragments were derived from subtypes of influenza virus from different regions or host origins, with length ranging from 41 to 291 nucleotides (nt). Most notable was a 291-nt fragment recombination at the 490–780 nt site. This was affiliated with a highly pathogenic avian influenza virus (HPAIV) isolate of A/tree sparrow/Thailand/VSMU-16-RBR/2005 (H5N1) (accession number EF178509), which was responsible for a regional epidemic of highly pathogenic avian influenza in Southeast Asia in 2005 (Figure 1, Table 1).

A total of 1368 sequences from the period January 2003 to February 2013 were downloaded to track the evolutionary history of this 291-nt recombination fragment in PB1 of the novel H7N9. After evaluation of the likelihood value, GTR+G was finally chosen [AIC (Akaike information criterion) = 58 725.19] as the optimum model to construct the maximum likelihood phylogenetic tree. It was confirmed that the novel AIV H7N9 might have a close genetic relationship to the H9N2 viruses isolated from Eastern China in 2009–2012, since they were located within the same lineage on the phylogenetic tree. Another phylogenetic lineage, composed of 2007–2009 H9N2 and 2007–2010 H5N1 strains, was closely related to the aforementioned lineage. This indicates that the procedure of recombination into the PB1 might already have been accomplished around 2007; this recombinant PB1 prevailed in

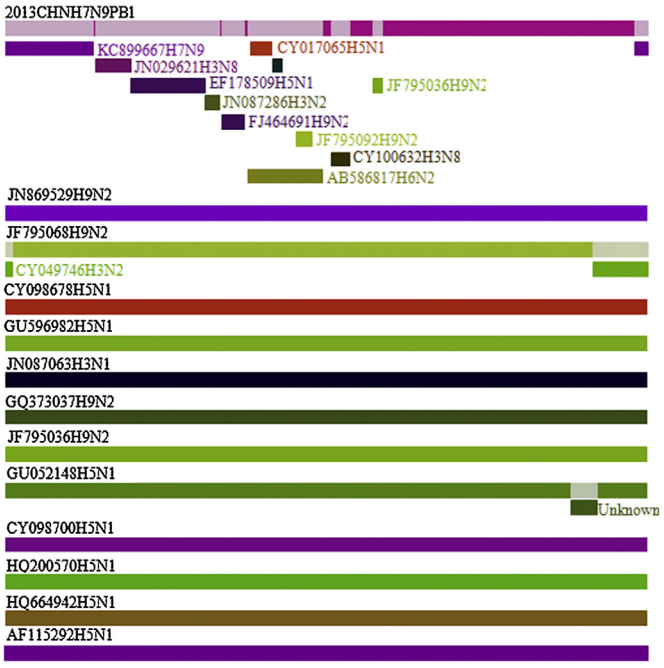

Figure 1. Patterns of recombination of the PB1 segment of the 2013 novel avian influenza virus H7N9 from China (partial segment). Series of recombinant fragments within the PB1 segment of avian influenza virus H7N9 isolated in China in 2013 are displayed.

Table 1
Possible recombinations, and their donors, of the PB1 segment affiliated with the novel avian influenza virus H7N9

Event	Related sequences	Sub-genotype	Original area	Host	Years	KA *p*-value	Global KA *p*-value	Beginning breakpoint	Ending breakpoint
1	CY036821	H2N2	Korea	Human	1968	1.296 E−73	9.185 E−68	490	780
	EF178509[a]	H5N1[b]	Thailand	Sparrow	2005				
2	JX175251	H3N2	CHN (Guangdong)	Duck	2011	9.971 E−22	7.065 E−16	780	838
	JN087286[a]	H3N2	Korea	Duck	2007				
3	CY115542	H3N2	CHN (Hong Kong)	Human	2009	6.646 E−34	4.709 E−28	839	935
	FJ464691[a]	H9N2	Israel	Turkey	2007				
4	FJ913004	H3N2	Thailand	Human	2008	3.914 E−36	2.773 E−30	947	1035
	CY017065[a]	H5N1[b]	Viet Nam	Chicken	2005				
5	FJ912932	H1N1	Thailand	Human	2006	3.610 E−17	2.558 E−11	1042	1082
	AB586849[a]	H6N2	CHN (Hong Kong)	Guinea fowl	2002				

PB1, polymerase basic 1; AIV, avian influenza virus; KA, chi-square.
[a] Accession numbers are the actual recombinations recommended by RDP 4.16 software.
[b] The donors of the main recombination fragments. There were two fragments derived from different highly pathogenic avian influenza virus H5N1 strains, which had high identity and were isolated from two neighboring Southeast Asian countries during the same epidemic period.

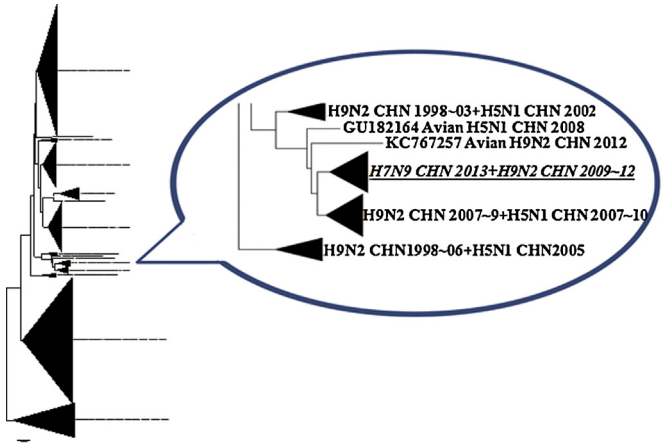

Figure 2. Phylogenetic tree of the 291-nt recombination fragment of 1369 PB1 segments. The novel avian influenza virus H7N9 has a close genetic relationship with the H9N2 viruses isolated from Eastern China (shown in italic). Another phylogenetic lineage composed of 2007–2009 H9N2 and 2007–2010 H5N1 strains is also closely related to the lineage of the novel avian influenza virus H7N9.

Eastern China in the forms of H9N2 or H5N1 subtype influenza virus, and was then reassorted into the novel H7N9 (Figure 2).

4. Discussion

There is general agreement concerning the donor subtypes that provide the eight segments for the novel AIV H7N9. All of its genomic segments are of avian origin. The H7 subtype of hemagglutinin is closest to that of the H7N3 virus from domestic ducks in Zhejiang Province, whereas the N9 subtype of neuraminidase is closest to that of the wild bird isolate A/wild bird/Korea/A14/2011 (H7N9) virus in South Korea or A/Baikal teal/Hongze/14/2005 (H11N9) virus in Jiangsu Province. The six internal genes are derived from a clade of chicken H9N2 viruses.[3–5,9] The current study confirmed that the PB1 segment of the novel H7N9 might have been characterized by recombination. At the 490–780 nt site, a fragment derived from an HPAIV strain, A/tree sparrow/Thailand/VSMU-16-RBR/2005 (H5N1), has been recombined into its PB1 segment.

Genetic recombination is one of the primary processes that produces the genetic diversity upon which natural selection acts.[6] Although intra-segment homologous recombination has only rarely been reported in RNA viruses, some studies have described the sequence patterns that appear compatible with homologous recombination events in the influenza virus.[10,11] Recombination contributing to the generation of genetic diversity can only occur among viruses that replicate within the same cells. The prerequisite for recombination is that an individual host is simultaneously infected with multiple divergent viral strains and then a quasi-species pool is formed consisting of relatively closely related members.[12,13]

For a long time, three genetic lineages of H9 virus (G1, G9, and Y439) circulated in Eastern and Southern China, and poultry were thought to be a stable and lasting maintenance reservoir.[14,15] The perennial positive rate of antibody against H9N2, a typical low pathogenic avian influenza virus (LPAIV) and a major contributor to the novel H7N9, fluctuated between 5.3% and 12.8%; however the rate of virus isolation reached as high as 9% in poultry, although this did not cause any obvious epidemic with mass poultry death.[16,17] Such a high carriage rate of AIV H9N2 in poultry resulted in an extremely high opportunity for co-infection with other influenza viruses, which consequently increased the risk of virus reassortment and recombination. In fact, reassortment between H5N1 and H9N2 has frequently occurred in China. For example, Gu et al. reported that the PB1, PB2, PA, and M segments of A/duck/Shandong/009/2008 (H5N1) from Eastern China were reassorted from an H9N2 isolate.[18] Zhang et al. and Cong et al. also found that many H9N2 AIV strains in China possessed at least two internal genome segments inherited from H5N1.[19,20] Recombination has also been observed previously. Guan et al. reported that the H9N2 influenza virus possesses heterozygous features of H5N1 in its PB1 segment, and Dong et al. found reassortant H9N2 influenza viruses containing H5N1-like PB1 genes in isolates from black-billed magpies in Southern China.[21,22] Many other studies have also provided evidence of a genetic basis for the recombination phenomenon.[10,11,23,24] It is likely that the recombinant PB1 segment appeared earlier, in about 2007, in the form of H9N2 or H5N1, before being reassorted into the novel H7N9 that caused an epidemic in 2013.

From a pathogenic perspective, the novel AIV H7N9 in China would be characterized as an LPAIV on the site of H7A1/H7A2 cleavage of the HA segment. However, the human fatality rate has been very high (37.13%), which is an important feature of HPAIV. As the donor for PB1 and other internal segments, H9N2 infections in humans have been reported repeatedly since 1980.[25] A large-scale survey revealed that the hemagglutination inhibition test (HI) titer of antibody against H9N2 virus in human sera ranged from 1.8% to 14.7%, and could reach as high as 17.4% in those with particular occupations such as the breeding and slaughtering of poultry.[17,26] A number of studies have reported humans to be susceptible to H9N2.[27] Due to its low pathogenicity, H9N2 infections are usually asymptomatic in both birds and humans.

In the present study, HPAIV H5N1 served as another form of donor, a recombination donor rather than a reassortment donor, for the PB1 segment. Since the first recorded direct bird-to-human

transmission of HPAIV H5N1 in Hong Kong in 1997, these viruses have spread widely across the world. They have caused widespread morbidity and mortality in domestic and wild birds, as well as humans.[28,29] Since low pathogenicity features are displayed in the key proteins of HA and NA of the novel H7N9, it is suspected that the PB1 segment recombination with the 291-nt fragment of HPAIV H5N1 possibly increased its virulence to a large extent, since the fatality rate for the novel H7N9 is between that of LPAIV H9N2 and HPAIV H5N1.

It is well known that the RNA-dependent polymerase of influenza viruses is highly host- and cell type-specific, depending on the identity of a few key amino acid positions in its three subunits. The PB1 protein is one of the three subunits constituting the RNA polymerase, and plays an important role in viral infection and pathogenicity. Avian influenza A viruses, such as the HPAIV H5N1, can sporadically affect human populations, but they are not able to transmit effectively and persistently; RNA polymerase plays a key role in this. There is compelling evidence that these viruses can acquire adaptive mutations in the polymerase subunits such as PB1, PB2, and PA, and nucleoprotein NP, as well as novel polymerase co-factor NEP, to obtain the ability to replicate rapidly in species, and as a result, the avian influenza virus might be able to break through the species barrier to transfer from animal reservoirs to humans.[30,31] A recent study examining the combinations of avian and human influenza polymerases showed that the most efficient influenza transcriptional activity in vitro occurred in an avian-derived PB1 segment, even if the PB2, PA, and NP proteins were from a human virus.[32]

Some efforts have also been made to map the PB1 regions that might modulate the polymerase activity; an adaptive PB1 might provide an advantage to the virus, allowing it to replicate in new hosts.[33] A recent study suggested that some mutations in the polymerase genes, including L13P and S678N in the PB1 subunit, increased the polymerase activity in mammalian cells, resulting in the adaptation of highly pathogenic avian AIV SC35 (H7N7) to mice.[34] Other studies have suggested that the PB1 segment of AIV can reassort into human H3N2 viruses and then increase its virulence in human and other mammal hosts such as mice.[33] Furthermore, PB1 has been shown to be the only segment that was reassorted with avian fragments in two previous influenza pandemics of H2N2 in 1957 and H3N2 in 1968.[35,36]

So far, no studies have reported that the recombination of PB1 segments with certain gene fragments of avian origin influenza virus can affect host tropism and pathogenicity, although it has been reported that the 177aa and 187–211aa sites of the PB1 protein have a significant influence on host adaptation. Coincidentally, the gene sequences encoding these amino acid residues in the PB1 gene segment are located at the 490–780 nt site of the PB1 gene segment of this new AIV H7N9, where the recombination events were found to have occurred. As a recombination donor, the HPAIV H5N1 strain A/tree sparrow/Thailand/VSMU-16-RBR/2005 (H5N1) (or the same strain isolated from different individual hosts in the same region) caused a serious epidemic in Southeast Asia and infected various hosts including wild birds, poultry, and humans, and the new AIV H7N9 subtype is comparable, to a certain degree, to the HPAIV H5N1 virus in host tropism and pathogenicity.[37] Reported clinical characteristics of H7N9-infected human cases and its fatality rate further support this inference.

Acknowledgements

We thank all of the scientists who contributed the genomes of AIV H7N9 to the NCBI Influenza Virus Sequence Database and the Global Initiative on Sharing Avian Influenza Data (GISAID) database. This research was supported by grants from the Talent Programs for Fostering Outstanding Youth of Shanghai (grant number XYQ2013071), the National Science Fund for Distinguished Young Scholars (grant number 81325017), Chang Jiang Scholars Program (grant number T2014089), and the Key Discipline Construction of Public Health of Shanghai of Shanghai Municipal Health and Family Planning Commission (grant number 12GWZX0101).

Conflict of interest: The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

References

1. Li Q, Zhou L, Zhou M, Chen Z, Li F, Wu H, et al. Preliminary report: epidemiology of the avian influenza A (H7N9) outbreak in China. *N Engl J Med* 2013. http://dx.doi.org/10.1056/NEJMoa1304617. http://www.nejm.org/doi/pdf/10.1056/NEJMoa1304617.
2. Chen Y, Liang W, Yang S, Wu N, Gao H, Sheng J, et al. Human infections with the emerging avian influenza A H7N9 virus from wet market poultry: clinical analysis and characterisation of viral genome. *Lancet* 2013;**381**:1916–25.
3. World Health Organization. WHO risk assessment as of 23 February 2015. - Geneva: WHO; 2015. Available at: http://www.who.int/influenza/human_animal_interface/influenza_h7n9/RiskAssessment_H7N9_23Feb20115.pdf?ua=1 (accessed January 4, 2016).
4. Xiong C, Zhang Z, Jiang Q, Chen Y. Evolutionary characteristics of A/Hangzhou/1/2013 and source of avian influenza virus H7N9 subtype in China. *Clin Infect Dis* 2013;**57**:622–4.
5. Liu D, Shi W, Shi Y, Wang D, Xiao H, Li W, et al. Origin and diversity of novel avian influenza A H7N9 viruses causing human infection: phylogenetic, structural, and coalescent analyses. *Lancet* 2013;**381**:1926–32.
6. Heath L, van der Walt E, Varsani A, Martin DP. Recombination patterns in aphthoviruses mirror those found in other picornaviruses. *J Virol* 2006;**80**:11827–32.
7. Boni MF, de Jong MD, van Doorn HR, Holmes EC. Guidelines for identifying homologous recombination events in influenza A virus. *PLoS One* 2010;**5**:e10434.
8. Martin DP, Lemey P, Lott M, Moulton V, Posada D, Lefeuvre P. RDP3: a flexible and fast computer program for analyzing recombination. *Bioinformatics* 2010;**26**:2462–3.
9. Van Ranst M, Lemey P. Genesis of avian-origin H7N9 influenza A viruses. *Lancet* 2013;**381**:1883–5.
10. Gibbs M, Armstrong J, Gibbs A. Recombination in the hemagglutinin gene of the 1918 "Spanish flu". *Science* 2001;**293**:1842–5.
11. He C, Han G, Wang D, Liu W, Li G, Liu X, Ding N. Homologous recombination evidence in human and swine influenza A viruses. *Virol* 2008;**380**:12–20.
12. Dugan VG, Chen R, Spiro DJ, Sengamalay N, Zaborsky J, Ghedin E, et al. The evolutionary genetics and emergence of avian influenza viruses in wild birds. *PLoS Pathog* 2008;**4**:e1000076.
13. Ghedin E, Fitch A, Boyne A, Griesemer S, DePasse J, Bera J, et al. Mixed infection and the genesis of influenza virus diversity. *J Virol* 2009;**83**:8832–41.
14. Guo YJ, Krauss S, Senne DA, Mo IP, Lo KS, Xiong XP, et al. Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. *Virol* 2000;**267**:279–88.
15. Peiris M, Yuen KY, Leung CW, Chan KH, Ip PL, Lai RW, et al. Human infection with influenza H9N2. *Lancet* 1999;**354**:916–7.
16. Lin YP, Shaw M, Gregory V, Cameron K, Lim W, Klimov A, et al. Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. *Proc Natl Acad Sci U S A* 2002;**97**:9654–8.
17. Cheng X, Liu J, He J, Shan F. Virological and serological surveys for H9N2 subtype of influenza A virus in chickens and men in Shenzhen city. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi* 2002;**16**:319–23.
18. Gu M, Liu WB, Cao JP, Cao YZ, Zhang XR, Peng DX, et al. Genome sequencing and genetic analysis of a natural reassortant H5N1 subtype avian influenza virus possessing H9N2 internal genes. *Bing Du Xue Bao* 2010;**26**:298–304.
19. Zhang P, Tang Y, Liu X, Liu W, Zhang X, Liu H, et al. A novel genotype H9N2 influenza virus possessing human H5N1 internal genomes has been circulating in poultry in eastern China since 1998. *J Virol* 2009;**83**:8428–38.
20. Cong YL, Pu J, Liu QF, Wang S, Zhang GZ, Zhang XL, et al. Antigenic and genetic characterization of H9N2 swine influenza viruses in China. *J Gen Virol* 2007;**88**:2035–41.
21. Guan Y, Shortridge KF, Krauss S, Webster RG. Molecular characterization of H9N2 influenza viruses: were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? *Proc Natl Acad Sci U S A* 1999;**96**:9363–7.
22. Dong G, Xu C, Wang C, Wu B, Luo J, Zhang H, et al. Reassortant H9N2 influenza viruses containing H5N1-like PB1 genes isolated from black-billed magpies in Southern China. *PLoS One* 2011;**6**:e25808.
23. He C, Xie Z, Han G, Dong J, Wang D, Liu J, et al. Homologous recombination as an evolutionary force in the avian influenza A virus. *Mol Biol Evol* 2009;**26**:177–87.
24. Gibbs M, Armstrong J, Gibbs A. The haemagglutinin gene, but not the neuraminidase gene, of 'Spanish flu' was a recombinant. *Philos Trans R Soc Lond B Biol Sci* 2001;**356**:1845–55.
25. Laver WG. The origin of pandemic influenza viruses. New York: Amsterdam-Oxford; 1983: 211–20.

26. Liu Y, Lu EJ, Wang YL, Di B, Li TG, Zhou Y, et al. Avian influenza virus infection in people occupied in poultry fields in Guangzhou city. *Chinese Journal of Epidemiology* 2009;**30**:1111–5.
27. Guo Y, Li J, Cheng X. Discovery of men infected by avian influenza A (H9N2) virus. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi* 1999;**13**:1–6.
28. World Health Organization. Cumulative number of confirmed human cases of avian influenza A (H5N1) reported to WHO. Geneva: WHO; Available at: http://www.who.int/entity/influenza/human_animal_interface/EN_GIP_20151214cumulativeNumberH5N1cases.pdf?ua=1 (accessed December 14, 2015).
29. Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, et al. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* 1998;**279**:393–6.
30. Mänz B, Schwemmle M, Brunotte L. Adaptation of avian influenza A virus polymerase in mammals to overcome the host species barrier. *J Virol* 2013;**87**:7200–9.
31. Mehle A, Dugan VG, Taubenberger JK, Doudna JA. Reassortment and mutation of the avian influenza virus polymerase PA subunit overcome species barriers. *J Virol* 2012;**86**:1750–7.
32. Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG. Characterization of the 1918 influenza virus polymerase genes. *Nature* 2005;**437**:889–93.
33. Li OT, Chan MC, Leung CS, Chan RW, Guan Y, Nicholls JM, et al. Full factorial analysis of mammalian and avian influenza polymerase subunits suggests a role of an efficient polymerase for virus adaptation. *PLoS One* 2009;**4**:e5658.
34. Gabriel G, Dauber B, Wolff T, Planz O, Klenk HD, Stech J. The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. *Proc Natl Acad Sci U S A* 2005;**102**:18590–5.
35. Kawaoka Y, Krauss S, Webster RG. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. *J Virol* 1989;**63**:4603–8.
36. Scholtissek C, Rohde W, Von Hoyningen V, Rott R. On the origin of the human influenza virus subtypes H2N2 and H3N2. *Virology* 1978;**87**:13–20.
37. Puthavathana P, Auewarakul P, Charoenying PC, Sangsiriwut K, Pooruk P, Boonnak K, et al. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. *J Gen Virol* 2005;**86**:423–33.