View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Crossref

Article
Chinese Science Bulletin
Molecular Virology
July 2012 Vol.57 No.19: 2419–2424
doi: 10.1007/s11434-012-5215-y

# Increased substitution rate in H5N1 avian influenza viruses during mass vaccination of poultry

WANG ZhaoGuo[1], JIANG WenMing[2], LIU Shuo[2], HOU GuangYu[2], LI JinPing[2], WANG ZhiYu[1*]& CHEN JiMing[2*]

[1] *College of Public Health, Shandong University, Jinan 250012, China;*
[2] *China Animal Health and Epidemiology Center, Qingdao 266032, China*

Received November 29, 2011; accepted March 8, 2012

As a means of heated debate, mass vaccination of poultry has been used in some countries to control H5N1 highly pathogenic avian influenza (HPAI), which remains of global economic and public health significance. Theoretically, mass vaccination can act as an evolutionary selective force facilitating the emergence of vaccine-resistant viruses, similar to that widespread use of antibiotics facilitates the emergence of antibiotic-resistant bacteria. To support the hypothesis, the substitution rates in the two subunits, HA1 and HA2, of the viral hemagglutinin gene, were calculated using a Bayesian Markov Chain Monte Carlo (MCMC) approach. It was found that the rate in the HA1 subunit, but not in the HA2 subunit, increased significantly during periods of mass vaccination (2005–2010 in China and 2003–2009 in Indonesia), in contrast to the periods when no mass vaccination programs took place (1996–2004 in China and 2004–2008 in Thailand). Because substitutions in the HA1 subunit rather than in the HA2 subunit can lead to vaccine-resistant viruses, the results support that mass vaccination programs facilitate the emergence of vaccine-resistant viruses, which, in turn, will render mass vaccination programs less effective. Therefore, caution must be taken when adopting mass vaccination as a long-term strategy to control HPAI.

**highly pathogenic avian influenza, vaccination, virus, substitution rate, disease control, selection, evolution**

**Citation:** Wang Z G, Jiang W M, Liu S, et al. Increased substitution rate in H5N1 avian influenza viruses during mass vaccination of poultry. Chin Sci Bull, 2012, 57: 2419–2424, doi: 10.1007/s11434-012-5215-y

H5N1 subtype highly pathogenic avian influenza (HPAI) viruses have been circulating widely in wild birds and domestic fowls for years in Eurasia and Africa, despite great efforts to control the disease [1–4]. Consequently, the virus has caused huge economic loss in poultry in many affected countries. Furthermore, the virus has also caused hundreds of fatal human cases since the end of the year 2003. It may thus spark a human pandemic influenza, if it has adapted to efficient human-to-human transmission through gene mutation or genetic re-assortment [1,5]. To minimize the direct loss caused by H5N1 HPAI, vaccination of poultry has been used as an important prevention and control means in China, Indonesia, Vietnam, Egypt, and several other countries [6–8].

Circulation of H5N1 HPAI virus in China was firstly identified in 1996, and was confirmed in each of the subsequent years [1–3,9,10]. Gene sequences of some H5N1 HPAI viruses circulating in China in 1996–2010 have been available in GenBank, or reported herein. Vaccination of poultry against HPAI has been used in China since 2004. Initially, only birds in and around areas where outbreaks occurred were vaccinated. In 2005, mass vaccination was compulsorily applied to all domestic poultry. Since then, billions of domestic birds have been vaccinated in China every year [11].

Circulation of H5N1 HPAI virus in Indonesia was firstly identified in 2003, and was confirmed in each of the subsequent years [12]. Gene sequences of some H5N1 HPAI vi-

*Corresponding authors (email: zhiyu.wang@sdu.edu.cn; jmchen66@yahoo.cn)

© The Author(s) 2012. This article is published with open access at Springerlink.com

ruses circulating in Indonesia in 2003–2009 have been available in GenBank. Large commercial poultry producers in Indonesia implemented vaccination against H5N1 HPAI using various vaccines, soon after the disease broke out in the country in 2003. The vaccines were also provided by the government free of charge to the backyard poultry sector from mid-2004 onwards, except that in 2006 and 2007, efforts were focused on twelve high-risk provinces due to limited resources [6].

Circulation of H5N1 HPAI virus in Thailand was firstly identified in 2004, and was confirmed in each of the subsequent years till 2008 [13]. Gene sequences of some H5N1 HPAI viruses circulating in Thailand in 2004–2008 have been available in GenBank. Vaccination against HPAI has never been approved in Thailand.

Circulation of H5N1 HPAI virus in Vietnam was firstly identified in 2001, and was almost confirmed in each of the subsequent years [14,15]. The sequence data have revealed several times of introduction of exotic H5N1 viruses into Vietnam [14–16]. A vaccination campaign targeting flocks and regions of a higher risk to be infected with the deadly viruses started at the end of 2005 in Vietnam, and resulted in decline of poultry and human cases [17].

Circulation of H5N1 HPAI virus in Egypt was firstly identified in 2005, and was confirmed in each of the subsequent years [18]. However, the impact on disease control of the vaccination in Egypt has been very limited, and a recent assessment study highlighted substantial weaknesses in the vaccination programs in Egypt [19].

Except in Egypt, the vaccination strategy has proved to be effective in protecting poultry from H5N1 virus infection, reducing the virus load in the environment, and preventing the transmission of H5N1 viruses from poultry to humans, at least from the experience in China and Vietnam [6,11,17]. However, the use of mass vaccination has been the subject of heated debate in recent years due to its obvious negative effects [20–24]. Firstly, vaccination is a logistically demanding and costly method [6,22,24]. Secondly, vaccination facilitates "silent" spread of the virus, because vaccinated birds usually show no severe clinical signs if they are infected with the virus, and thus farmers may relax their vigilance and relevant biosecurity measures [24]. Furthermore, vaccine-resistant influenza viruses, which reduce the efficacy of mass vaccination programs, have been reported to have emerged in China, Egypt, and Indonesia [6,18,25].

Theoretically, in a similar manner as the widespread use of antibiotics facilitates the emergence of antibiotic-resistant bacteria strains [26], effective mass vaccination should facilitate the emergence of vaccine-resistant viruses, which can spread among vaccinated hosts, and thus have an advantage over the non-resistant viruses [27]. With this consideration, mass vaccination programs against HPAI may act as an evolutionary selective force driving the substitution rate in the gene of the virus involved in vaccine resistance. So far, however, no direct data have been reported to support this idea.

This study aims to investigate whether the substitution rates in the two subunits, HA1 and HA2, of the viral hemagglutinin (HA) increased during periods of mass vaccination. HA is a glycoprotein present in the viral membrane as a single polypeptide (HA0), which is cleaved by the host's proteases into HA1 and HA2. The HA1 subunit is the major surface antigen, a target of neutralizing antibodies produced during infection or vaccination, and substitutions in the HA1 subunit can result in vaccine-resistant influenza viruses [28,29]. In effect, as far as we know, substitutions in the HA1 subunit play a much more important role than those in the HA2 subunit in vaccine escape [18,25,30–32]. In this sense, the HA2 subunit serves as a control in this study when analyzing the substitution rate of the HA1 subunit.

In this study, we compared the nucleotide and amino acid substitution rates in the HA1 and HA2 subunits in four periods: 1996–2004 in China, 2005–2010 in China, 2003–2009 in Indonesia, and 2004–2008 in Thailand. We selected these four periods because mass vaccination was not implemented in China until 2005, and HPAI vaccination has never been approved in Thailand, but was implemented in Indonesia in 2003–2009. We did not analyze the data of Egypt and Vietnam, because the vaccination strategy were not well implemented in Egypt, and some exotic H5N1 viruses have been introduced into Vietnam in the past several years for multiple times [14–16,19]. The substitution rate can be overestimated if these exotic viruses were not well identified and excluded from the calculation. In addition, many H5N1 HPAI viruses circulating in Vietnam might be just under a slight vaccination pressure as the vaccination strategy in Vietnam targeted only flocks and regions of a higher risk to be infected with the deadly virus [17].

## 1 Materials and methods

### 1.1 Sample collection

We have reported the *HA* gene sequences of 55 H5N1 HPAI viruses isolated in 2007–2009 with GenBank accession numbers HM006764–HM006818 and HM583607–HM583609 [3]. Here, we report sequences of 17 additional H5N1 HPAI viruses isolated in 2010 (the relevant infection information has been released online by Ministry of Agriculture of China, http://www.moa.gov.cn/zwllm/tzgg/gb/sygb/). Swab samples were collected by taking smears from the trachea and cloacae of the domestic fowls in randomly selected live bird markets. The samples were clarified by centrifugation, and the supernatants were inoculated in specific-pathogen-free (SPF) chicken embryonated eggs via the allantoic sac route. The eggs were further incubated for 4 d. Thereafter, the allantoic fluids of the embryos were collected and tested using the hemagglutination assay. All the hemagglutination-positive samples and the allantoic fluid of the embryos died during the incubation, were investigated further using

the universal RT-PCR, which targets the whole length of the *HA* gene of all subtypes of influenza viruses [33]. PCR products were purified and ligated with pGEM-T Easy vector (Promega, Beijing, China). Then, both strands of the clones were sequenced using a Perkin-Elmer model 377 XL DNA sequencer. The GenBank accession numbers of the *HA* gene sequences reported herein are JF789566–JF789582.

### 1.2 Representative sequence selection

A total of 1288 nucleotide sequences (>800 bp) of the *HA* gene of H5 subtype HPAI viruses from China, Indonesia, and Thailand available in GenBank, or reported herein, were compiled using the web server of Influenza Virus Resource [34]. If available from these compiled sequences, eight full-length representative sequences were selected for each year in each of the countries. They were selected approximately according to phylogenetic distribution of the sequences, namely that more representative sequences were randomly selected from a bigger branch (i.e. the one covering more viruses) than from a smaller branch (i.e. the one covering less viruses).

### 1.3 Phylogenetic analysis

Sequences were aligned using the software MUSCLE [35]. Genetic distances and phylogenetic relationships under the GTR model were calculated with the maximum-likelihood method using the software MEGA 5.0 (http://www.megasoftware.net/) [36]. Bootstrap values were calculated from 1000 replicates. Clades of H5N1 HPAI viruses were designated according to the WHO/OIE/FAO nomenclature system [31].

### 1.4 Calculation of the substitution rate

The substitution rate in the gene was estimated using the software BEAST 1.6.1 (http://evolve.zoo.ox.ac.uk/Beast/), which employs a sophisticated Bayesian Markov Chain Monte Carlo (MCMC) approach that allows for rate variation among lineages and a range of demographic histories [37]. Nucleotide substitution and amino acid substitution were set to the General Time Reversible (GTR) model and the Jones-Taylor-Thornton (JTT) model, respectively [38]. The software was run using the Bayesian skyline plot under the uncorrelated exponential relaxed-clock model. A previous analysis of human influenza A virus evolution found that the uncorrelated exponential relaxed clock model provided a better fit to the data than the uncorrelated lognormal model [37], and this was further confirmed by Chen and Holmes in analyses of the data of avian influenza viruses [39], although the parameter values estimated were similar under both models. Each analysis was run at least four times (10 million chain lengths for each time) to check convergence of the Markov chain to the posterior distribution.

## 2 Results

### 2.1 Representative sequence selection

Nucleotide sequences (>800 bp) of the *HA* gene of a total of 1288 (844 from China, 171 from Indonesia, 273 from Thailand) H5 subtype HPAI viruses were available in GenBank on March 20, 2011, or reported herein. From them, 168 HA1 representative sequences (44 for 1996–2004 in China, 48 for 2005–2010 in China, 36 for 2003–2009 in Indonesia, 40 for 2004–2008 in Thailand), and 163 HA2 representative sequences (42 for1996–2004 in China, 47 for 2005–2010 in China, 36 for 2003–2009 in Indonesia, 38 for 2004–2008 in Thailand) were selected. Their designations and phylogenetic relationships are given in Figures S1 and S2, which were largely consistent with each other, and consistent with the global phylogenetic distribution of H5N1 HPAI viruses in recent years [31]. All the representative sequences were involved in the subsequent substitution rate analysis, except three from Thailand and one from Indonesia, which, as shown in Figure S1, were not indigenous in these two countries. Similar exotic sequences were excluded as representatives for HA2, and thus they were not covered in Figure S2. The selected representatives covered all known major clades circulating during the relevant periods in the three countries [1–3,9,10,13,31]. It should be noted that full-length sequences of some H5N1 HPAI viruses were not available for both HA1 and HA2 in GenBank, and the HA1 and HA2 representative sequences were selected at random, respectively. Therefore, the HA1 and HA2 representative sequences selected in this study are not always from the same viruses. In addition, not as much as eight representative sequences were selected for each of the years in each of the countries, because inadequate candidates of relevant years in relevant countries were available in GenBank.

### 2.2 Changes in substitution rates in HA1 and HA2

Table 1 summarizes the substitution rates in the HA1 and HA2 subunits calculated in this study using the aforementioned representative sequences. For the two data sets where mass vaccination was implemented, China 2005–2010 and Indonesia 2003–2009, the substitution rates in both nucleotide sequences and amino acid sequences of the HA1 subunit, were substantially higher than their counterparts for China 1996–2004 and Thailand 2004–2008, where no mass vaccination took place. The difference in substitution rates of the HA1 subunit between the vaccinated versus the non-vaccinated data sets is statistically significant, because their 95% highest posterior density (HPD) intervals do not overlap (Table 1). In contrast, Table 1 also shows that the substitution rates in both nucleotide sequences and amino acid sequences of the HA2 subunit did not increase significantly during periods of mass vaccination (China 2005–2010 and Indonesia 2003–2009), as compared with their counterparts

**Table 1** Substitution rates (in $10^{-3}$ substitutions site$^{-1}$ year$^{-1}$) in the HA1 and HA2 subunits of the viral *HA* gene

| Country | Period | Mass vaccination | HA1 nucleotide substitution rate | | HA1 amino acid substitution rate | | HA2 nucleotide substitution rate | | HA2 amino acid substitution rate | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean | 95% HPD | Mean | 95% HPD | Mean | 95% HPD | Mean | 95% HPD |
| China | 2005–2010 | Yes | 7.28 | 5.11–9.41 | 10.31 | 6.85–13.88 | 3.97 | 2.66–5.25 | 2.55 | 1.26–3.95 |
| Indonesia | 2003–2009 | Yes | 7.75 | 6.01–9.58 | 8.67 | 5.92–11.50 | 4.37 | 3.01–5.71 | 2.03 | 0.80–3.40 |
| China | 1996–2004 | No | 3.37 | 2.37–4.32 | 3.52 | 1.98–5.17 | 2.34 | 1.37–3.34 | 2.31 | 1.16–3.52 |
| Thailand | 2004–2008 | No | 2.69 | 1.78–3.62 | 3.65 | 1.95–5.60 | 2.44 | 1.46–3.47 | 1.23 | 0.22–2.25 |

during periods when no mass vaccination programs took place (China 1996–2004 and Thailand 2004–2008). The difference in substitution rates of the HA2 subunit between the vaccinated versus the non-vaccinated data sets is of no marked difference as their intervals of 95% HPD overlapped.

### 2.3 Evaluation of sampling error

The substitution rates in HA1 and HA2 subunits were calculated using another set of representative sequences which were selected according to the same criteria stated above, and the results were quite similar to the data given above for the same gene during the same period in the same country (data not shown). Therefore, the sampling error can be excluded as the possible main reason for the significant increase in the substitution rate of the HA1 subunit observed in this study, which is consistent with a previous report also targeting the substitution rate of genes of avian influenza viruses [39].

## 3 Discussion

The data of this study suggest that the substitution rate of the H5N1 HPAI virus in the HA1 subunit, but not in the HA2 subunit, increased significantly during periods of mass vaccination, in contrast to the periods when no mass vaccination programs took place. Because substitutions in the HA1 subunit play a much more important role than those in the HA2 subunit in vaccine escape, the results support the idea that mass vaccination, as an evolutionary selective force, facilitates the emergence of vaccine-resistant viruses. This is also consistent with previous reports that many amino acid substitutions in the HA1 subunit of H5N1 HPAI viruses occurred at antigenic sites [3,25,40]. In addition, it has also been found by others that the substitution rate of viruses may be elevated by selection of immune escape [41,42].

As mass vaccination programs should lose efficacy with the emergence of vaccine-resistant strains, caution must be taken when adopting mass vaccination as a long-term strategy to control HPAI. Other control measures, such as enhancing biosecurity of poultry systems, should be strengthened to combat the disease.

The ratio of the synonymous substitution rate (dS) versus the non-synonymous substitution rate (dN) is widely used as an indicator of selective pressure acting on a protein-coding gene [43,44]. Homologous genes of the dN/dS ratio =1, >1 or <1 are usually assumed to be evolving under neutral evolution, negative selection or positive selection, respectively. We have estimated the dN/dS ratio of the two subunits using the representative sequences selected in this study (data not shown). However, we did not find that the dN/dS ratio increased significantly in either of the subunits during the periods of mass vaccination. This may result from that the ratio is of a very high background of negative selection (i.e. dS is usually much larger than dN), and dS may be also elevated through hitch-hiking when dN is driven faster by a positive selection pressure [45]. Therefore, the dN/dS ratio may be not sensitive to a positive selection pressure, which has also been identified by our previous study on the HA1 subunit of human H3N2 subtype influenza viruses [46]. Chen and Holmes [39] also found that the dN/dS ratios of the *HA* gene of mammalian influenza A viruses and avian influenza A viruses are not significantly higher than their counterparts of the neuraminidase (*NA*) gene, though the *HA* gene is widely thought to be under more positive selection pressure than the *NA* gene.

It would be interesting to know whether the substitution rate in another gene of H5N1 HPAI viruses also increased during the mass vaccination periods. However, such work was hindered either by inadequate sequences available in GenBank, or by the frequent gene re-assortment between H5N1 HPAI viruses and other avian influenza viruses [10,12,47–51]. Some re-assortment activities involving similar sequences cannot be easily identified through phylogenetic analysis, and the substitution rate may be overestimated if some re-assortment activities are ignored in the estimation. Nevertheless, more data in the future are favored to support the suggestion of this report that H5N1 mass vaccination programs in poultry drive the substitution rate in the HA1 subunit of the viral *HA* gene, and facilitate the emergence of vaccine-resistant viruses, which, in turn, will render mass vaccination programs less effective.

*We sincerely thank Dr. Mario dos Reis in Department of Genetics, Evolution and Environment, University College London, for his precious contribution to the design, data analysis, and report writing of this study. This work was supported by the Sci-tech Basic Work Project of Ministry of Science and Technology (SQ2012FY3260033).*

1 Alexander D J, Brown I H. History of highly pathogenic avian influenza. Rev Sci Tech, 2009, 28: 19–38
2 Chen H, Deng G, Li Z, et al. The evolution of H5N1 influenza viruses in ducks in southern China. Proc Natl Acad Sci USA, 2004, 101: 10452–10457
3 Jiang W M, Liu S, Chen J, et al. Molecular epidemiological surveys of H5 subtype highly pathogenic avian influenza viruses in poultry in China during 2007–2009. J Gen Virol, 2010, 91: 2491–2496
4 Liu J, Xiao H, Lei F, et al. Highly pathogenic H5N1 influenza virus infection in migratory birds. Science, 2005, 309: 1206
5 Webster R G, Peiris M, Chen H, et al. H5N1 outbreaks and enzootic influenza. Emerg Infect Dis, 2006, 12: 3–8
6 Domenech J, Dauphin G, Rushton J, et al. Experiences with vaccination in countries endemically infected with highly pathogenic avian influenza: The Food and Agriculture Organization perspective. Rev Sci Tech, 2009, 28: 293–305
7 Marangon S, Busani L. The use of vaccination of poultry production. Rev Sci Tech, 2007, 26: 265–274
8 Sims L D. Experience in control of avian influenza in Asia. Dev Biol (Basel), 2007, 130: 39–43
9 Shortridge K F. Poultry and the influenza H5N1 outbreak in Hong Kong, 1997: Abridged chronology and virus isolation. Vaccine, 1999, 17: S26–S29
10 Li K S, 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
11 Chen H. Avian influenza vaccination: The experience in China. Rev Sci Tech, 2009, 28: 267–274
12 Lam T T, Hon C C, Pybus O G, et al. Evolutionary and transmission dynamics of reassortant H5N1 influenza virus in Indonesia. PLoS Pathog, 2008, 4: e1000130
13 Suwannakarn K, Amonsin A, Sasipreeyajan J, et al. Molecular evolution of H5N1 in Thailand between 2004 and 2008. Infect Genet Evol, 2009, 9: 896–902
14 Wan X F, Nguyen T, Davis C T, et al. Evolution of highly pathogenic H5N1 avian influenza viruses in Vietnam between 2001 and 2007. PLoS ONE, 2008, 3: e3462
15 Nguyen T, Davis C T, Stembridge W, et al. Characterization of a highly pathogenic avian influenza H5N1 virus sublineage in poultry seized at ports of entry into Vietnam. Virology, 2009, 387: 250–256
16 Wang J, Vijaykrishna D, Duan L, et al. Identification of the progenitors of Indonesian and Vietnamese avian influenza A (H5N1) viruses from southern China. J Virol, 2008, 82: 3405–3414
17 Soares Magalhães R J, Pfeiffer D U, Otte J. Evaluating the control of HPAIV H5N1 in Vietnam: Virus transmission within infected flocks reported before and after vaccination. BMC Vet Res, 2010, 6: 31
18 Balish A L, Davis T, Saad M D, et al. Antigenic and genetic diversity of highly pathogenic avian influenza A (H5N1) viruses isolated in Egypt. Avian Dis, 2010, 54: 329–334
19 Peyre M, Samaha H, Makonnen Y J, et al. Avian influenza vaccination in Egypt: Limitations of the current strategy. J Mol Genet Med, 2009, 3: 198–204
20 Lee C W, Senne D A, Suarez D L. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. J Virol, 2004, 78: 8372–8381
21 Capua I, Marangon S. Control and prevention of avian influenza in an evolving scenario. Vaccine, 2007, 25: 5645–5652
22 Iwami S, Suzuki T, Takeuchi Y. Paradox of vaccination: Is vaccination really effective against avian flu epidemics? PLoS ONE, 2009, 4: e4915
23 Peyre M, Fusheng G, Desvaux S, et al. Avian influenza vaccines: A practical review in relation to their application in the field with a specifically examine the Asian experience. Epidemiol Infect, 2008, 14: 1–21
24 Savill N J, St Rose S G, Keeling M J, et al. Silent spread of H5N1 in vaccinated poultry. Nature, 2006, 442: 757
25 Tian G, Zeng X, Li Y, et al. Protective efficacy of the H5 inactivated vaccine against different highly pathogenic H5N1 avian influenza viruses isolated in China and Vietnam. Avian Dis, 2010, 54: S287–S289
26 Levy S B, Marshall B. Antibacterial resistance worldwide: Causes, challenges and responses. Nat Med, 2004, 10: S122–S129
27 Lee C W, Senne D A, Suarez D L. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. J Virol, 2004, 78: 8372–8381
28 Stevens J, Blixt O, Tumpey T M, et al. Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. Science, 2006, 312: 404–410
29 Wiley D C, Wilson I A, Skehel J J. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature, 1981, 289: 373–378
30 Bush R M, Fitch W M, Bender C A, et al. Positive selection on the H3 hemagglutinin gene of human influenza virus A. Mol Biol Evol, 1999, 16: 1457–1465
31 WHO/OIE/FAO H5N1 Evolution Working Group. Toward a unified nomenclature system for highly pathogenic avian influenza virus (H5N1). Emerg Infect Dis, 2008, 14: e1
32 Wang Q, Tao Y J. Influenza: Molecular Virology. Norwich: Caister Academic Press, 2010
33 Hoffmann E, Stech J, Guan Y, et al. Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol, 2001, 146: 2275–2289
34 Bao Y, Bolotov P, Dernovoy D, et al. The influenza virus resource at the National Center for Biotechnology Information. J Virol, 2008, 82: 596–601
35 Edgar R C. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res, 2004, 32: 1792–1797
36 Tamura K, Dudley J, Nei M, et al. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol, 2007, 24: 1596–1599
37 Drummond A J, Ho S Y W, Phillips M J, et al. Relaxed phylogenetics and dating with confidence. PLoS Biol, 2006, 4: e88
38 Jones D T, Taylor W R, Thornton J M. The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci, 1992, 8: 275–282
39 Chen R, Holmes E C. Avian influenza virus exhibits rapid evolutionary dynamics. Mol Biol Evol, 2006, 23: 2336–2341
40 Kaverin N V, Rudneva I A, Govorkova E A, et al. Epitope mapping of the hemagglutinin molecule of a highly pathogenic H5N1 influenza virus by using monoclonal antibodies. J Virol, 2007, 81: 12911–12917
41 Duffy S, Shackelton L A, Holmes E C. Rates of evolutionary changes in viruses: Patterns and determinants. Nat Rev Genet, 2008, 9: 267–276
42 Crandall K A, Kelsey C R, Imamichi H, et al. Parallel evolution of drug resistance in HIV: Failure of nonsynonymous/synonymous substitution rate ratio to detect selection. Mol Biol Evol, 1999, 16: 372–382
43 Yang Z. Computational Molecular Evolution. New York: Oxford University Press, 2006
44 Kryazhimskiy S, Plotkin J B. The population genetics of dN/dS. PLoS Genet, 2008, 4: e1000304
45 Rocha E P, Smith J M, Hurst L D, et al. Comparisons of dN/dS are time dependent for closely related bacterial genomes. J Theor Biol, 2006, 239: 226–235
46 Chen J, Sun Y. Variation in the analysis of positively selected sites using nonsynonymous/synonymous rate ratios: An example using influenza virus. PLoS ONE, 2011, 6: e19996
47 Amonsin A, Lapkuntod J, Suwannakarn K, et al. Genetic characterization of 2008 reassortant influenza A virus (H5N1), Thailand. Virol J, 2010, 7: 233
48 Chen J, Fang F, Yang Z, et al. Characterization of highly pathogenic

H5N1 avian influenza viruses isolated from poultry markets in central China. Virus Res, 2009, 146: 19–28
49 Guan Y, Smith G J, Webby R, et al. Molecular epidemiology of H5N1 avian influenza. Rev Sci Tech, 2009, 28: 39–47
50 Dung Nguyen T, Vinh Nguyen T, Vijaykrishna D, et al. Multiple sublineages of influenza A virus (H5N1), Vietnam, 2005–2007. Emerg Infect Dis, 2008, 14: 632–636
51 Usui T, Yamaguchi T, Ito H, et al. Evolutionary genetics of highly pathogenic H5N1 avian influenza viruses isolated from whooper swans in northern Japan in 2008. Virus Genes, 2009, 39: 319–323

**Open Access** This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

## Supporting Information

**Figure S1** Phylogenetic relationships of the selected HA1 representative sequences.
**Figure S2** Phylogenetic relationships of the selected HA2 representative sequences.

The supporting information is available online at csb.scichina.com and www.springerlink.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.