Genetic tuning of the novel avian influenza A(H7N9) virus during interspecies transmission, China, 2013

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A novel avian influenza A(H7N9) virus causing human infection emerged in February 2013 in China. To elucidate the mechanism of interspecies transmission, we compared the signature amino acids of avian influenza A(H7N9) viruses from human and non-human hosts and analysed the reassortants of 146 influenza A(H7N9) viruses with full genome sequences. We propose a genetic tuning procedure with continuous amino acid substitutions and reassorting that mediates host adaptation and interspecies transmission. When the early influenza A(H7N9) virus, containing ancestor haemagglutinin (HA) and neuraminidase (NA) genes similar to A/Shanghai/05 virus, circulated in waterfowl and transmitted to terrestrial poultry, it acquired an NA stalk deletion at amino acid positions 69 to 73. Then, receptor binding preference was tuned to increase the affinity to human-like receptors through HA G186V and Q226L mutations in terrestrial poultry. Additional mammalian adaptations such as PB2 E627K were selected in humans. The continual reassortation between H7N9 and H9N2 viruses resulted in multiple genotypes for further host adaptation. When we analysed a potential association of mutations and reassortants with clinical outcome, only the PB2 E627K mutation slightly increased the case fatality rate. Genetic tuning may create opportunities for further adaptation of influenza A(H7N9) and its potential to cause a pandemic.

Introduction

Human infections with a novel avian influenza A(H7N9) virus were reported in Yangtze river delta region in March, 2013 [1], the virus further spread to northern and southern China within one month and human cases decreased during summer time [2]. Human infection with influenza A(H7N9) virus re-emerged in late 2013 and became the most prevalent avian influenza affecting humans in China. The virus caused 379 human cases with 135 deaths in mainland China by 10 March 2014 [3]. Most of the affected regions were areas with a high density of poultry and humans and were considered as the most likely hotspots for the generation of novel reassortant influenza viruses [4]. In addition, these areas are on the East Asia/Australian migratory flyway, indicating potential contributions of wild birds to the emergence of H7N9 viruses [1]. The complicated circumstances make it difficult to fully determine the origin of the novel emerging virus or to predict its further epidemic tendency. Previous studies suggested that the H7N9 virus contained several mammalian-adaptive mutations such as HA 226L and PB2 627K, and that the internal genes were diverse [5,6]. However, the genetic evolution pathway of the H7N9 viruses during interspecies transmission was not comprehensively illustrated. Here, we conducted a molecular epidemiology investigation of 173 influenza A(H7N9) viruses in China to extrapolate the potential interspecies transmission mechanism.

Methods

Virus sampling and isolation

Specimens as well as clinical and epidemiological information were collected from human cases. Environmental samples and avian samples were collected in the area where human cases identified. Virus isolation was conducted by Chinese National Influenza Center (CNIC) in a biosafety level 3 facility using nine-day-old specific pathogen-free (SPF) embryonated chicken eggs and incubated at 37 °C for 48–72 hours. The allantoic fluid was harvested, aliquoted and stored at -80 ºC until use.

RNA extraction and genome sequencing

Viral RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). Gene segments were amplified using the Qiagen OneStep RT-PCR Kit. A total of 48 primer pairs were used to generate PCR amplicons between 378 and 1,123 bp in length for full genome sequencing. Primer sequences are available from the authors on request. Amplified PCR products were purified using ExoSAP-IT reagent (USB, Cleveland, US). Complete genome sequencing was performed with an ABI 3730XL automatic DNA analyser (Applied Biosystems, Foster City, US) using the ABI BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems; Foster City, US).

Genomic signature and phylogenetic analysis

All sequences were submitted to the Global Initiative on Sharing All Influenza Data (GISAID) database. Genomic signature and phylogenetic analyses were performed with sequences retrieved from GenBank and GISAID databases. The novel influenza A(H7N9) virus sequences used in this study were from samples collected between 26 February 2013 and 5 February 2014 in China. A maximum likelihood phylogenetic tree for the nucleotide sequences of each gene of selected influenza viruses was constructed using MEGA5.1 [7]. In the phylogenetic analysis, six internal genes of these viruses were compared with sequences of influenza A(H9N2) viruses. All datasets for the phylogenetic analyses had been tested in jModelTest to determine the best substitution model for each segment [8]. The GTR+I+Γ_4 model was used as substitution model for the polymerase genes PB2, PB1, PA genes and the nucleoprotein (NP) genes; GTR+Γ_4 was used as substitution model for haemagglutinin (HA) and neuraminidase (NA) genes; K80+Γ_4 was used for the matrix (M) and the genes for the non-structural proteins (NS). The robustness of each node of the tree was assessed using a bootstrap resampling analysis (1,000 replicates, with topologies inferred using the maximum likelihood method). The clade definition of each internal gene was based on the following criteria: each clade shared a common ancestral node with a bootstrap value of ≥60, and percentage pairwise nucleotide distances between clades of >1.5%.

Selective pressure calculating

We estimated the number of non-synonymous (dN) to synonymous (dS) nucleotide changes for codons 627 and 701 on the PB2 genes of both human-infecting H7N9 virus and recent avian H9N2 virus with the method of the fixed effects likelihood method available in the hypothesis testing using phylogenies (HyPhy) package accessed through the Datamonkey website [9]; dN>dS was an indication of positive selection.

Results

To analyse the molecular evolution of the novel avian influenza A(H7N9) virus during interspecies transmission, we compared the signature amino acids between 103 human and 70 non-human (animal and environmental) H7N9 viruses from available databases including 53 viruses isolated by the Chinese National Influenza Center (CNIC) between 26 February 2013 and 5 February 2014 (51 from human, one from environment, one from chicken; Tables 1 and 2). The substitutions G186V and Q226L/I (H3 numbering) in the HA gene have been reported to increase the affinity of avian influenza virus to human-like receptors [10,11]. Only A/Shanghai/1/2013 and A/Shanghai/05/2013 had 186G and 226Q; two human and 11 non-human H7N9 viruses had 186V and 226Q; 95 human and 58 non-human viruses had G186V and G226L/I mutations, one human virus had G186V and G226P mutations (Table 2).

A deletion in the NA stalk region in avian influenza A(H5N1) viruses has been suggested to be associated with adaptation and transmission in domestic poultry [12,13]. All H7N9 viruses analysed in this study had a deletion of five amino acids (positions 69–73) in the stalk region, except the A/Shanghai/05/2013 virus (Figure 1).

E627K and/or D701N mutations in the PB2 protein are considered critical for mammalian adaptation of avian influenza viruses [14,15]. All 59 PB2 segments from non-human viruses had PB2 627E and 701D except A/Tree sparrow/Shanghai/01/2013(H7N9) with 627K. Among the 87 H7N9 viruses from humans, 63 possessed PB2 627K and 701D, 18 possessed 627E and 701D, and six had the 627E and 701N mutation. Dual mutations of E627K and D701N were not detected. We identified significantly higher *dN* than *dS* in PB2 position 627 (dN=20.17, dS=1.68×10^{-5}, p=0.0023), indicating positive selection pressure. Full length PB1-F2 protein (87–90 amino acids) has been connected with increased virulence in mice [16]. A PB1-F2 protein with 90 amino acids was detected in 70 of 88 human and 42 of 59 non-human H7N9 viruses (Table 2). The potential human-like signatures in the PA gene have been reported previously [17]. In this study, the PA genes from 88 human and 60 non-human viruses were analysed, with 100A found in 74 human and 47 non-human viruses, 356R in 84 human and 59 non-human viruses, 409N in 84 human and 53 non-human viruses (Table 2). In addition, all H7N9 viruses had a truncated NS gene

Table 1a

Novel avian influenza A(H7N9) viruses isolated by the Chinese National Influenza Center, 26 February 2013–5 February 2014 (n=53)

ID	Region	Virus name	Isolate ID in GISAID	Patient's sex	Patient's age group (years)	Date of illness onset (Y/M/D)	Clinical outcome	Sample type	Sample collecting date (Y/M/D)
1	Shanghai	A/Shanghai/1/2013(H7N9)	EPI_ISL_138737	Male	≥71	2013/2/24	Fatal	Throat swab	2013/2/26
2		A/Shanghai/2/2013(H7N9)	EPI_ISL_138738	Male	21–30	2013/2/28	Fatal	Throat swab	2013/3/5
3		A/Shanghai/3/2013(H7N9)	EPI_ISL_139649	Male	≥71	2013/2/21	Fatal	Throat swab	2013/2/27
4		A/Shanghai/4/2013(H7N9)	EPI_ISL_139650	Male	61–70	2013/3/5	Fatal	Sputum	2013/3/5
5		A/Shanghai/05/2013(H7N9)	EPI_ISL_141159	Male	0–10	2013/3/31	Recovered	Throat swab	2013/4/2
6		A/Shanghai/06-A/2013(H7N9)	EPI_ISL_141166	Male	51–60	2013/4/3	Fatal	Throat swab	2013/4/10
7[a]		A/Shanghai/07/2013(H7N9)[a]	EPI_ISL_141160	Male	0–10	2013/3/17	Recovered	Throat swab	2013/3/18
8		A/Shanghai/8/2013(H7N9)	EPI_ISL_141173	Male	61–70	2013/4/1	Fatal	Throat swab	2013/4/7
9		A/Shanghai/9/2013(H7N9)	EPI_ISL_141174	Male	61–70	2013/4/1	Recovered	Throat swab	2013/4/8
10		A/Shanghai/10/2013(H7N9)	EPI_ISL_141167	Female	≥71	2013/4/4	Recovered	Throat swab	2013/4/9
11		A/Shanghai/11/2013(H7N9)	EPI_ISL_141168	Male	≥71	2013/4/6	Fatal	Throat swab	2013/4/10
12		A/Shanghai/12/2013(H7N9)	EPI_ISL_141169	Female	≥71	2013/4/4	Recovered	Throat swab	2013/4/10
13		A/Shanghai/13/2013(H7N9)	EPI_ISL_141170	Male	61–70	2013/4/6	Recovered	Throat swab	2013/4/10
14		A/Shanghai/14/2013(H7N9)	EPI_ISL_141171	Male	51–60	2013/4/4	Fatal	Throat swab	2013/4/10
15		A/Shanghai/15/2013(H7N9)	EPI_ISL_141176	Female	≥71	2013/4/1	Recovered	Throat swab	2013/4/9
16		A/Shanghai/16/2013(H7N9)	EPI_ISL_141177	Male	≥71	2013/4/2	Fatal	Throat swab	2013/4/10
17		A/Shanghai/17/2013(H7N9)	EPI_ISL_141172	Female	51–60	2013/3/30	Fatal	Nasopharyngeal swab	2013/4/3
18	Anhui	A/Anhui/1/2013(H7N9)	EPI_ISL_138739	Female	31–40	2013/3/15	Fatal	Throat swab	2012/3/20
19		A/Anhui/02/2013(H7N9)	EPI_ISL_141190	Male	51–60	2013/4/10	Recover ed	Throat swab	2013/4/14
20		A/Anhui/03/2013(H7N9)	EPI_ISL_141191	Male	≥71	2013/4/14	Fatal	Throat swab	2013/4/21
21[a]		A/Anhui/04/2013(H7N9)a	EPI_ISL_157287	Male	51–60	2013/3/26	Recovered	Throat swab	2013/4/1
22	Jiangsu	A/Jiangsu/01/2013(H7N9)	EPI_ISL_141158	Female	41–50	2013/3/19	Fatal	Tracheal aspirate	2013/3/30
23		A/Jiangsu/02/2013(H7N9)	EPI_ISL_141162	Female	31–40	2013/3/21	Fatal	Throat swab	2013/3/31
24		A/Jiangsu/03/2013(H7N9)	EPI_ISL_141163	Female	21–30	2013/3/30	Recovered	Throat swab	2013/4/6
25		A/Jiangsu/04/2013(H7N9)	EPI_ISL_141164	Female	61–70	2013/3/21	Recovered	Tracheal aspirate	2013/4/5
26		A/Jiangsu/05/2013(H7N9)	EPI_ISL_141184	Male	31–40	2013/3/31	Recovered	Throat swab	2013/4/8
27		A/Jiangsu/06/2013(H7N9)	EPI_ISL_141185	Male	51–60	2013/4/3	Recovered	Throat swab	2013/4/10
28		A/Jiangsu/07/2013(H7N9)	EPI_ISL_141186	Male	≥71	2013/4/1	Fatal	Throat swab	2013/4/3
29		A/Jiangsu/08/2013(H7N9)	EPI_ISL_141187	Male	41–50	2013/4/1	Recovered	Throat swab	2013/4/11
30		A/Jiangsu/09/2013(H7N9)	EPI_ISL_141188	Female	≥71	2013/4/5	Fatal	Throat swab	2013/4/9
31	Zhejiang	A/Zhejiang/01/2013(H7N9)	EPI_ISL_139651	Male	31–40	2013/3/8	Fatal	Tracheal aspirate	2013/3/25
32		A/Zhejiang/02/2013(H7N9)	EPI_ISL_141175	Male	61–70	2013/3/29	Fatal	Throat swab	2013/4/3

NA: Not applicable.

[a] A/Shanghai/07/2013(H7N9) and A/Anhui/04/2013(H7N9) were isolated by the local Center for Disease Control using MDCK cells.

Table 1b

Novel avian influenza A(H7N9) viruses isolated by the Chinese National Influenza Center, 26 February 2013–5 February 2014 (n=53)

ID	Region	Virus name	Isolate ID in GISAID	Patient's sex	Patient's age group (years)	Date of illness onset (Y/M/D)	Clinical outcome	Sample type	Sample collecting date (Y/M/D)
33	Hunan	A/Hunan/01/2013(H7N9)	EPI_ISL_141182	Female	61–70	2013/4/14	Recovered	Throat swab	2013/4/24
34		A/Hunan/02/2013(H7N9)	EPI_ISL_141183	Male	51–60	2013/4/15	Fatal	Tracheal aspirate	2013/4/25
35		A/Hunan/0070/2013(H7N9)	EPI_ISL_142859	Male	61–70	2013/4/23	Recovered	Throat swab	2013/4/29
36	Beijing	A/Beijing/01-A/2013(H7N9)	EPI_ISL_141165	Female	0–10	2013/4/11	Recovered	Throat swab	2013/4/12
37		A/Beijing/02/2013(H7N9)	EPI_ISL_157286	Male	0–10	2013/5/22	Recovered	Throat swab	2013/5/28
38	Henan	A/Henan/01/2013(H7N9)	EPI_ISL_141178	Male	31–40	2013/4/7	Recovered	Throat swab	2013/4/7
39		A/Henan/02/2013(H7N9)	EPI_ISL_157284	Male	51–60	2013/4/17	Fatal	Throat swab	2013/4/23
40	Shandong	A/Shandong/01/2013(H7N9)	EPI_ISL_141179	Male	31–40	2013/4/19	Recovered	Throat swab	2013/4/21
41		A/Shandong/0068A/2013(H7N9)	EPI_ISL_142858	Male	0–10	2013/4/27	Recovered	Throat swab	2013/4/28
42	Fujian	A/Fujian/01/2013(H7N9)	EPI_ISL_141180	Male	61–70	2013/4/16	Recovered	Throat swab	2013/4/23
43		A/Fujian/02/2013(H7N9)	EPI_ISL_157288	Male	≥71	2013/4/17	Fatal	Throat swab	2013/4/27
44		A/Fujian/03/2013(H7N9)	EPI_ISL_157289	Male	51–60	2013/4/20	Recovered	Throat swab	2013/4/29
45		A/Fujian/04/2013(H7N9)	EPI_ISL_157290	Male	61–70	2013/5/1	Recovered	Throat swab	2013/5/2
46		A/Fujian/05/2013(H7N9)	EPI_ISL_157292	Male	0–10	2013/4/26	Recovered	Throat swab	2013/4/27
47	Jiangxi	A/Jiangxi/01/2013(H7N9)	EPI_ISL_141181	Male	61–70	2013/4/21	Recovered	Tracheal aspirate	2013/4/24
48	Hebei	A/Hebei/01/2013(H7N9)	EPI_ISL_157293	Female	61–70	2013/7/12	Fatal	Tracheal aspirate	2013/7/19
49	Guangxi	A/Guangxi/08970/2014(H7N9)	EPI_ISL_157294	Female	41–50	2014/1/27	Recovered	Tracheal aspirate	2014/2/3
50		A/Guangxi/08971/2014(H7N9)	EPI_ISL_157295	Male	0–10	2014/2/4	Recovered	Throat swab	2014/2/5
51		A/Guangxi/08309/2014(H7N9)	EPI_ISL_157296	Female	51–60	2014/1/20	Fatal	Throat swab	2014/1/27
52	Shandong	A/Environment/Shandong/1/2013(H7N9)	EPI_ISL_141189	NA	NA	NA	NA	Swab on chopping board (environmental sample)	2013/4/24
53	Anhui	A/Chicken/Anhui-Chuzhou/01/2013	EPI_ISL_141161	NA	NA	NA	NA	Chicken throat swab	2013/3/29

NA: Not applicable.

Table 2

Characterisation of selected molecular markers of novel avian influenza A(H7N9) viruses[a] China, 26 February 2013–5 February 2014

Gene	Significance	Mutation[b]	Amino acid	Human	Non-Human	Fatal[c]	Recovered[c]
HA	Favours mammalian adaptation	G186V	G	2	0	1	1
			V	98	70	22	26
	Receptor-binding site	Q226L/I	Q	4	11	2	1
			L	92	58	20	25
			I	3	0	1	1
			P	1	0	0	0
NA	Related to drug resistance	E119V	E	96	67	22	27
			V	1	0	1	0
		R152K	R	95	67	23	26
			K	2	0	0	1
		R292K	R	92	67	20	27
			K	5	0	3	0
PB2	Increased virulence in mice	E627K	E	24	59	4	10
			K	63	1	19	17
	Enhanced transmission in guinea pigs	D701N	D	81	60	22	24
			N	6	0	1	3
PB1	Increased transmission in ferrets	I368V	I	10	1	4	3
			V	78	58	19	24
	Increased replication in mammalian cells	L598P	L	87	59	22	27
			M	1	0	1	0
PB1-F2	Increased pathogenicity in mice	87–90 amino acids in length	11AA	1	0	0	0
			25AA	14	17	3	7
			76AA	3	0	1	1
			90AA	70	42	19	19
PA	Species-associated signature positions	V100A	V	12	13	2	3
			I	2	0	0	1
			A	74	47	21	23
		K356R	K	4	1	0	0
			R	84	59	23	27
		S409N	S	4	7	1	1
			N	84	53	22	26

HA: haemagglutinin; NA: neuraminidase; PA, PB1, PB2: polymerase subunits.

[a] Identical molecular markers that exist in all influenza A(H7N9) virus isolates and have been reported to have functions in other influenza virus subtypes are not listed in the Table. They include the following: HA (a single R at the link peptide; 160A, without N-glycosylation at this position and indicating increased virus binding to human-type receptors), PB2 (89V and 357H, indicating increased pathology in mice), PB1 (99H, indicating increased transmission in ferrets; 473V, indicating increased replication in mammalian cells), PA (36A, reported to be related to increased replication), M1 (30D and 215A, related to increased virulence in mice), M2 (31N, indicating reduced sensitivity to Amantadine), NS1 (42S, indicating increased virulence in mice; PDZ motif deletion, indicating decreased virulence in mice).

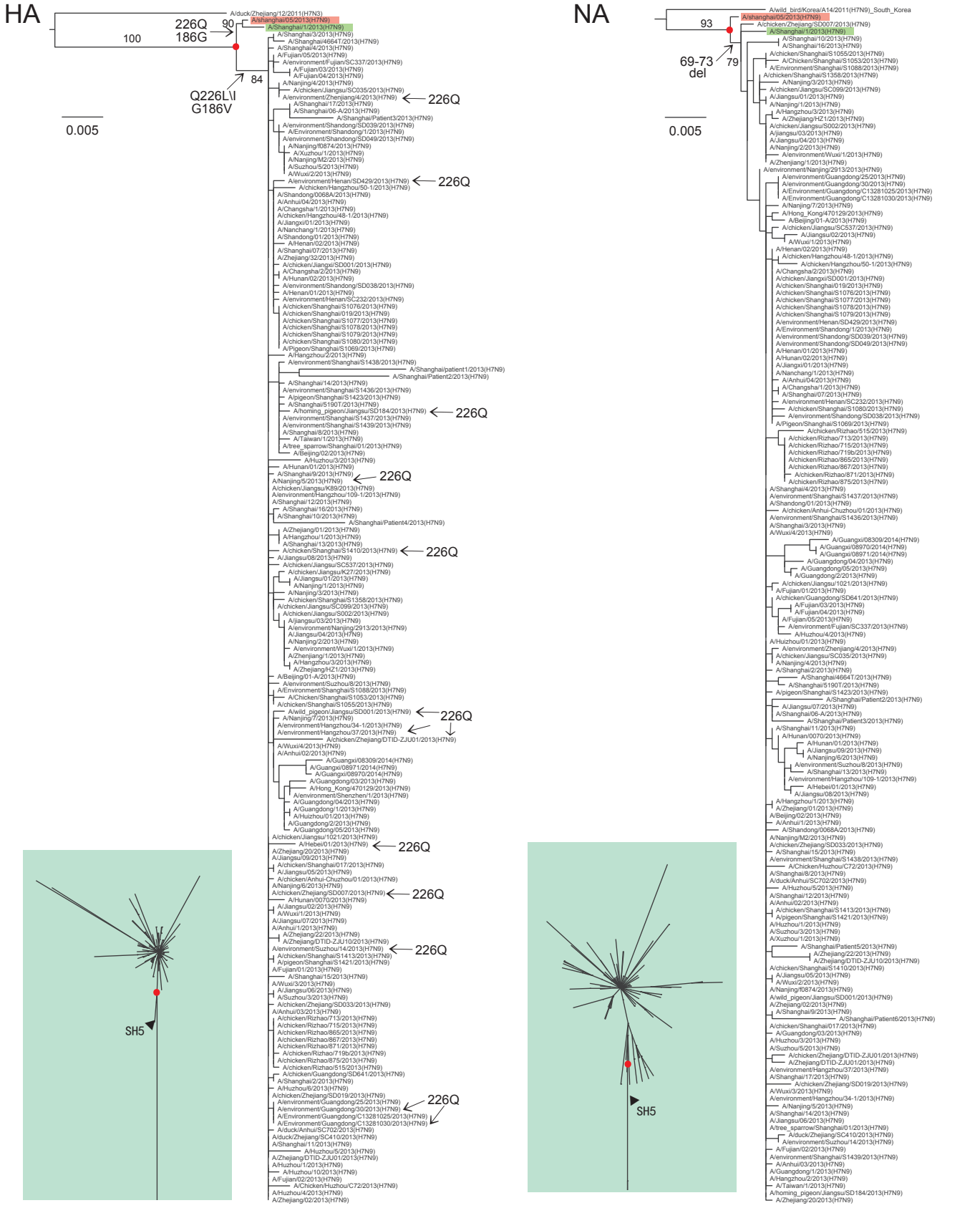
[b] The HA gene was under H3 numbering system. The NA gene was under N2 numbering system. Other internal genes were numbered from start codon (M).

[c] Only viruses with available data on clinical outcome were included.

53 viruses were from the Chinese National Influenza Center.

Figure 1

Phylogenetic trees of haemagglutinin (HA) and neuraminisdase (NA) genes of influenza A(H7N9) viruses, China, 26 February 2013–5 February 2014 (n=170 for HA, n=164 for NA)



HA: haemagglutinin; NA: neuraminidase.

Red dots represent the common ancestor of the novel H7N9 virus. A/Shanghai/5/2013 and A/Shanghai/1/2013 are highlighted in pink and green, respectively. Schematic unrooted trees of HA and NA genes are shown in lower left boxes.

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53 HA and NA sequences were sequenced by the Chinese National Influenza Center.

with a PDZ motif deletion (Table 2). The PDZ motif deletion in H5N1 viruses has been shown to determine the virulence of the virus in mice, without affecting the replication of the virus [18]. PDZ deletion might influence the pathogenicity of H7N9 viruses in human or avian hosts.

HA and NA genes each formed a single phylogenetic group including a subgroup consisting of A/Shanghai/05/2013 (SH5) and A/Shanghai/1/2013 (SH1). SH5 located most closely to the common ancestor (Figure 1). The topologies of the maximum likelihood phylogenetic trees exhibited higher diversity of internal genes than of surface HA and NA genes, consistent with previous studies [1,5,6] (Figure 2). PB2, PB1, PA, NP, M and NS genes were divided into four, six, eight, six, three and five clades, respectively. Combination of the clades of the individual internal genes in an isolate defined a genotype.

Among 146 H7N9 viruses with full genome sequences, we detected at least 26 genotypes. The abbreviation of one selected virus was used to name each genotype (Figure 3A). Among all the genotypes, 20 were detected only once or twice in this study; this might suggest transient circulation of these genotypes in avian species. Genotype AnH1, represented by A/Anhui/1/2013 virus, was detected in nine Chinese provinces and applied to 45% (66/146) of all H7N9 viruses throughout the study period (Figure 3). Genotype SH7, represented by A/Shanghai/7/2013, was distributed in eight provinces but only comprised 17% (25/146) of the H7N9 viruses. Genotype JS1, represented by A/Jiangsu/1/2013, occurred in only three provinces and contained 15% (22/146) of the H7N9 viruses. Most genotypes (20/26) could be detected in the Yangtze river delta region.

We further analysed the potential association of mammalian-adaptative mutations and genotypes with clinical outcome of human infection. Most of the mutations did not affect the case fatality rate, only the PB2 E627K mutation slightly increased the case fatality rate (29% for E, 53% for K) without statistical significance (corrected chi-squared test, p=0.22019) (Table 2). The case fatality rate of patients infected with AH1, SH7, JS1 genotype viruses was 46% (12/26), 43% (3/7) and 50% (4/8), respectively.

Discussion

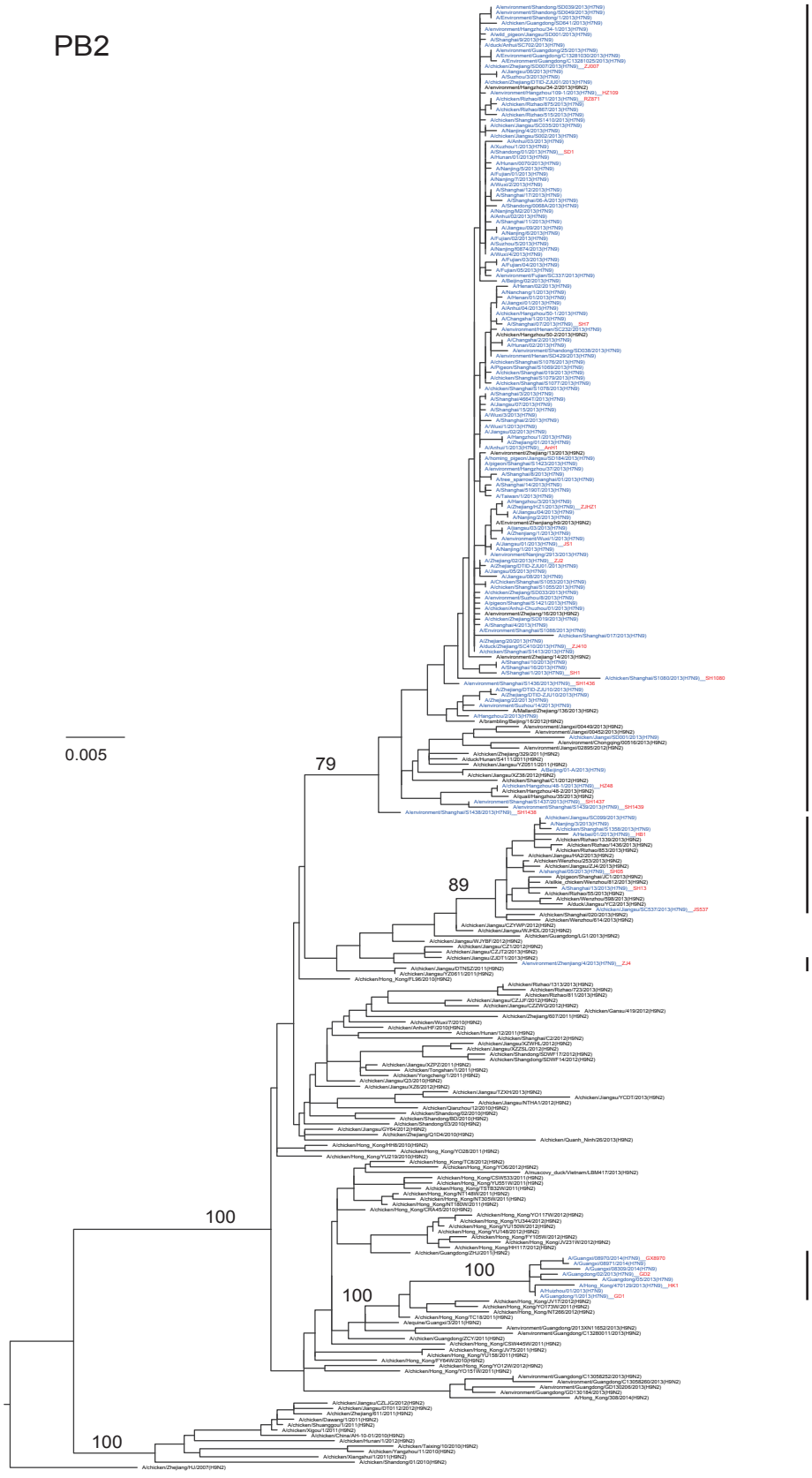
Since the emergence of the novel avian influenza A(H7N9) viruses in China, its unusual features indicated a potential pandemic risk that may persist over a long time [19,20]. Previous studies have demonstrated that the HA of this virus could be of duck origin, the NA was most likely to be related to N9 viruses detected in migratory birds, and the six internal genes could be from avian influenza H9N2 viruses [1,5,21]. Further studies suggested that domestic ducks may act as key intermediate hosts for the generation of H7N9 viruses, and may, after transmission to chickens and reassortment with distinct H9N2 donor viruses in at least two sequential steps have generated the currently circulating H7N9 virus with their high diversity of internal genes, using BEAST analysis [6,21]. However, the procedure of interspecies transmission of the avian influenza A(H7N9) virus has not been fully characterised. Here, we propose that a genetic tuning procedure with continuous amino acid substitutions and reassortations, mediates the host adaptation and interspecies transmission of H7N9 viruses (Figure 4).

The SH5 virus contained the most likely ancestral HA and NA genes of the H7N9 virus, as both located most closely to the common ancestor. The NA of SH5 virus had a long stalk, similar to the influenza viruses in wild birds such as A/Wild birds/Korea/A14/2011 (H7N9), while all the other novel H7N9 viruses had a deletion of five amino acids (position 69–73) in the stalk region. NA deletion is considered a hallmark of aquatic bird viruses adapted to terrestrial poultry [12,13]. SH5 virus also contained HA 186G and 226Q, which are recognised as typical molecular markers of avian-origin influenza viruses. Thus, when the early H7N9 virus that contained similar HA and NA genes as A/Shanghai/05 virus circulated in waterfowl, it transmitted to terrestrial poultry and acquired the NA stalk deletion at position 69–73, creating SH1-like viruses, which were better adapted to terrestrial poultry. In terrestrial poultry, the receptor-binding preference was tuned to increase the affinity to human-like receptors through G186V and Q226L mutations in the receptor-binding site of HA. Through this tuning procedure, the virus acquired dual receptor-binding capacity. The previous 858 H7 subtype viruses before 2013 for which sequences were available in GenBank, rarely had the G186V (n=61) or Q226L (n=0) substitutions. The novel H7N9 viruses may have acquired the mutations either in chickens, as observed for avian influenza A(H9N2) virus which acquired the HA Q226L mutation in the year 2000 [22], or in other potential hosts containing human-like receptors such as quail or pigeon [23,24]. Finally, additional mammalian adaptation mutations such as PB2 E627K were positively selected for in humans (Figure 4). That *dN* was significantly higher than *dS* in PB2 627 due to a burst of adaptive evolution in a new host, indicated that the H7N9 virus was at the early stage of interspecies transmission from avian to human. The 627K and/or 701N mutations in the PB2 protein, which confer enhanced replication at the temperature of the upper airway of mammalian and possibly human hosts, are considered critical for mammalian adaptation of avian influenza viruses [14,15].

In addition to the fine tuning of amino acid substitutions, the dynamic reassortment of H7N9 virus with H9N2 virus resulted in at least 26 genotypes based on the internal genes, indicating another way of genetic tuning for further host adaptation. The genotype diversity of the novel H7N9 viruses indicated that the internal genes of H7N9 and H9N2 viruses were genetically compatible and that, because of the phylogenetic

Figure 2

Phylogenetic trees of PB2 (n=153), PB1 (n=152), PA (n=153), NP (n=154), M (n=163) and NS (n=162) genes of the novel avian influenza A(H7N9) viruses, China, 26 February 2013–5 February 2014



HA: haemagglutinin; M: matrix protein; NA: neuraminidase; NP: nucleoprotein; PA, PB1, PB2: polymerase subunits.

The H7N9 viruses are highlighted in blue and the clades of each gene are labelled with capital letters. The abbreviations of selected viruses to name each genotype are highlighted in red.

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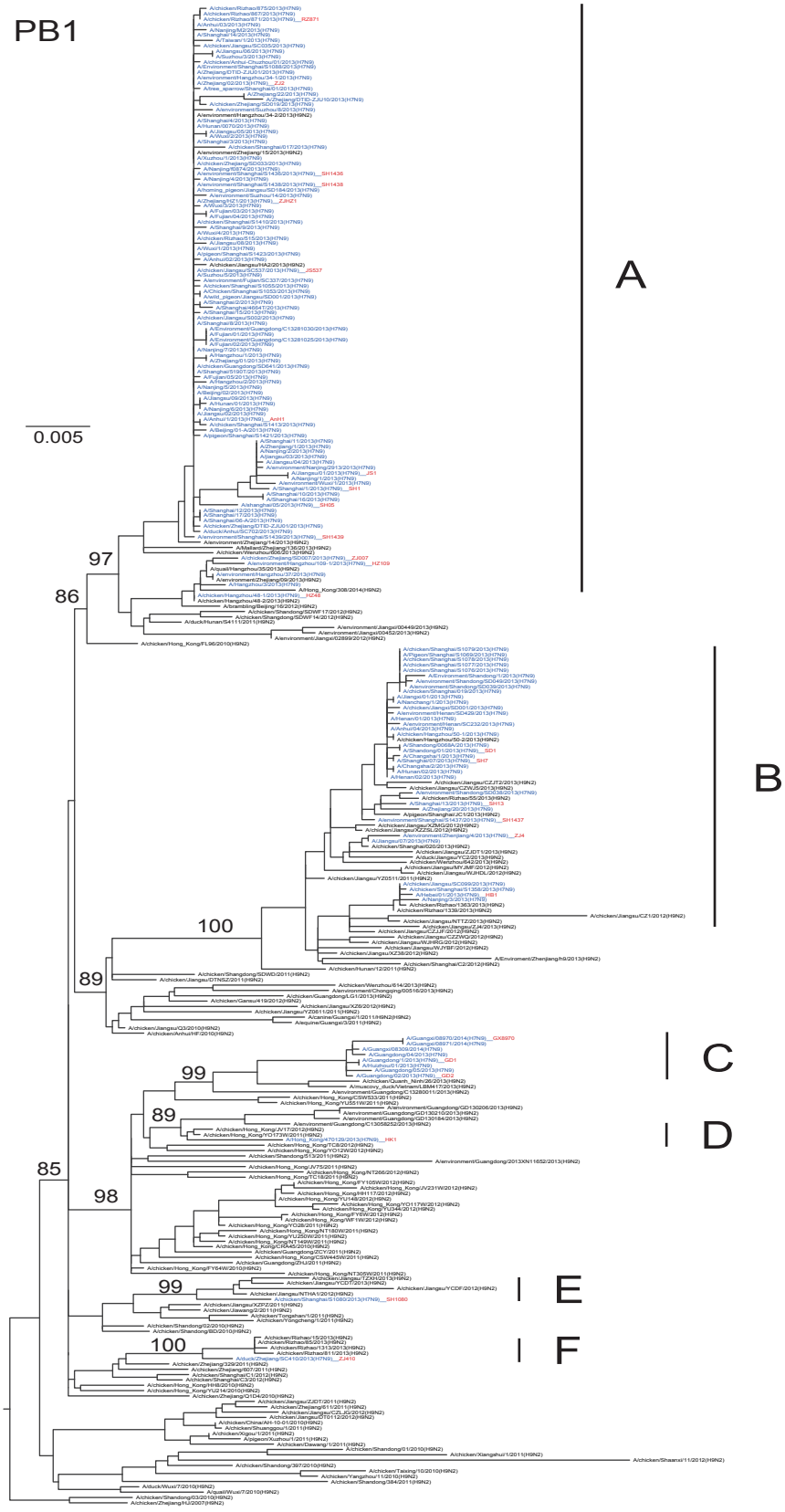

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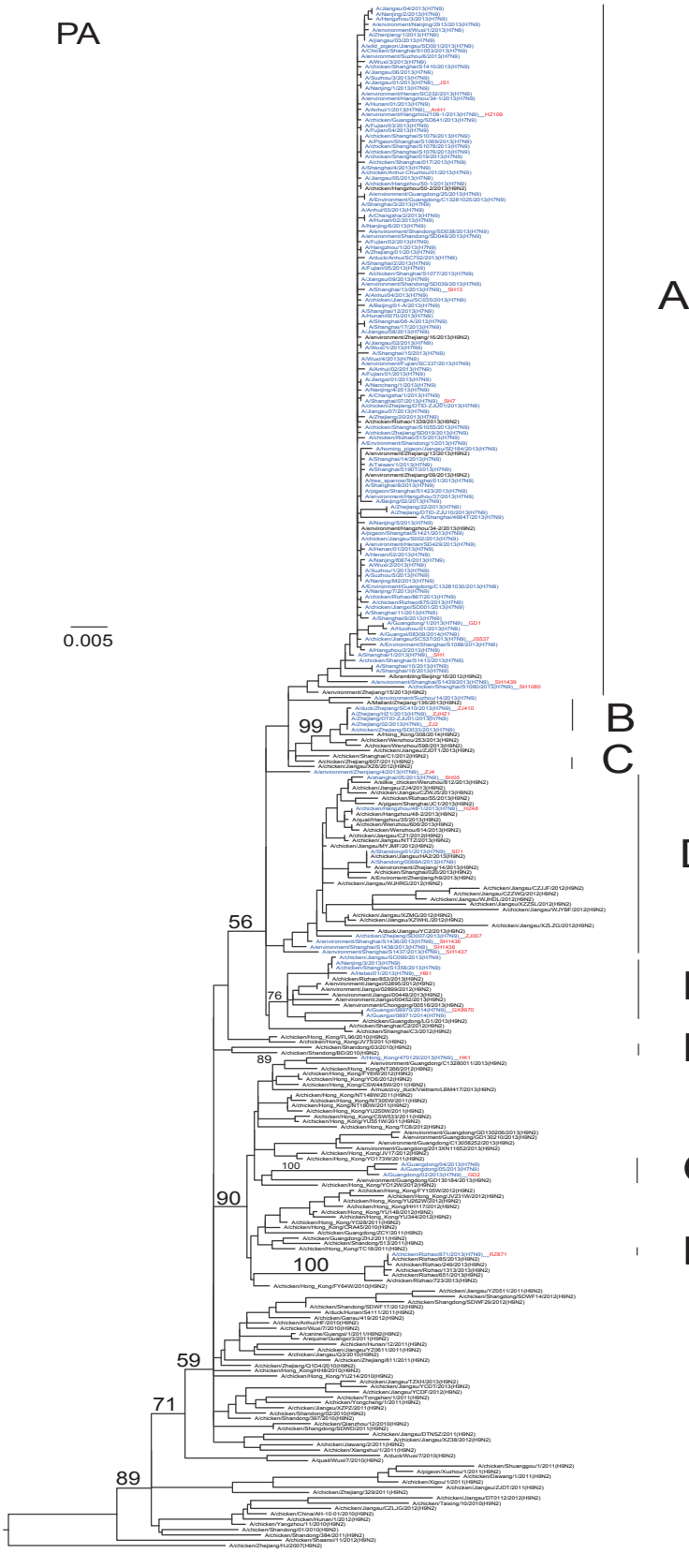

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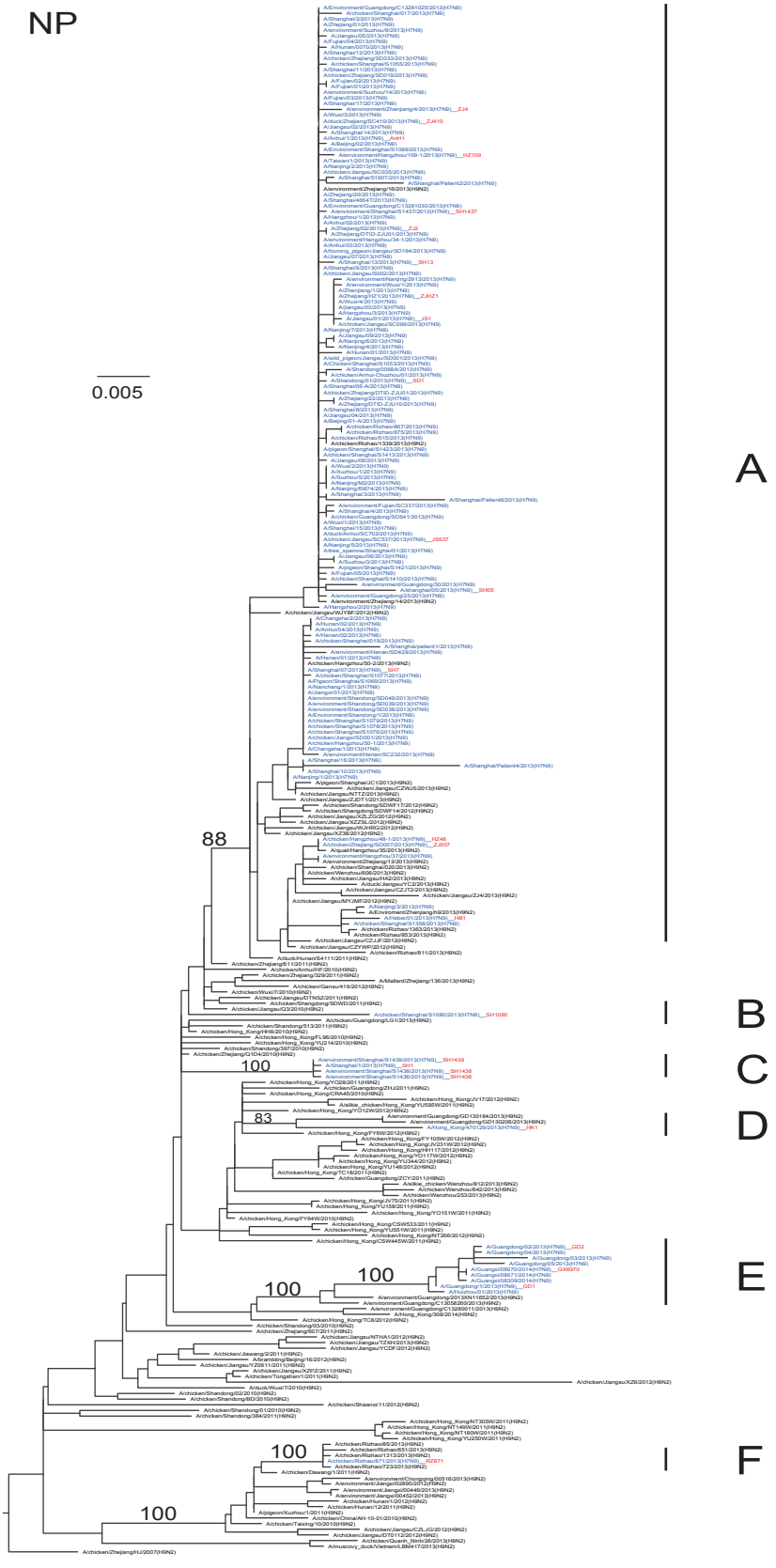

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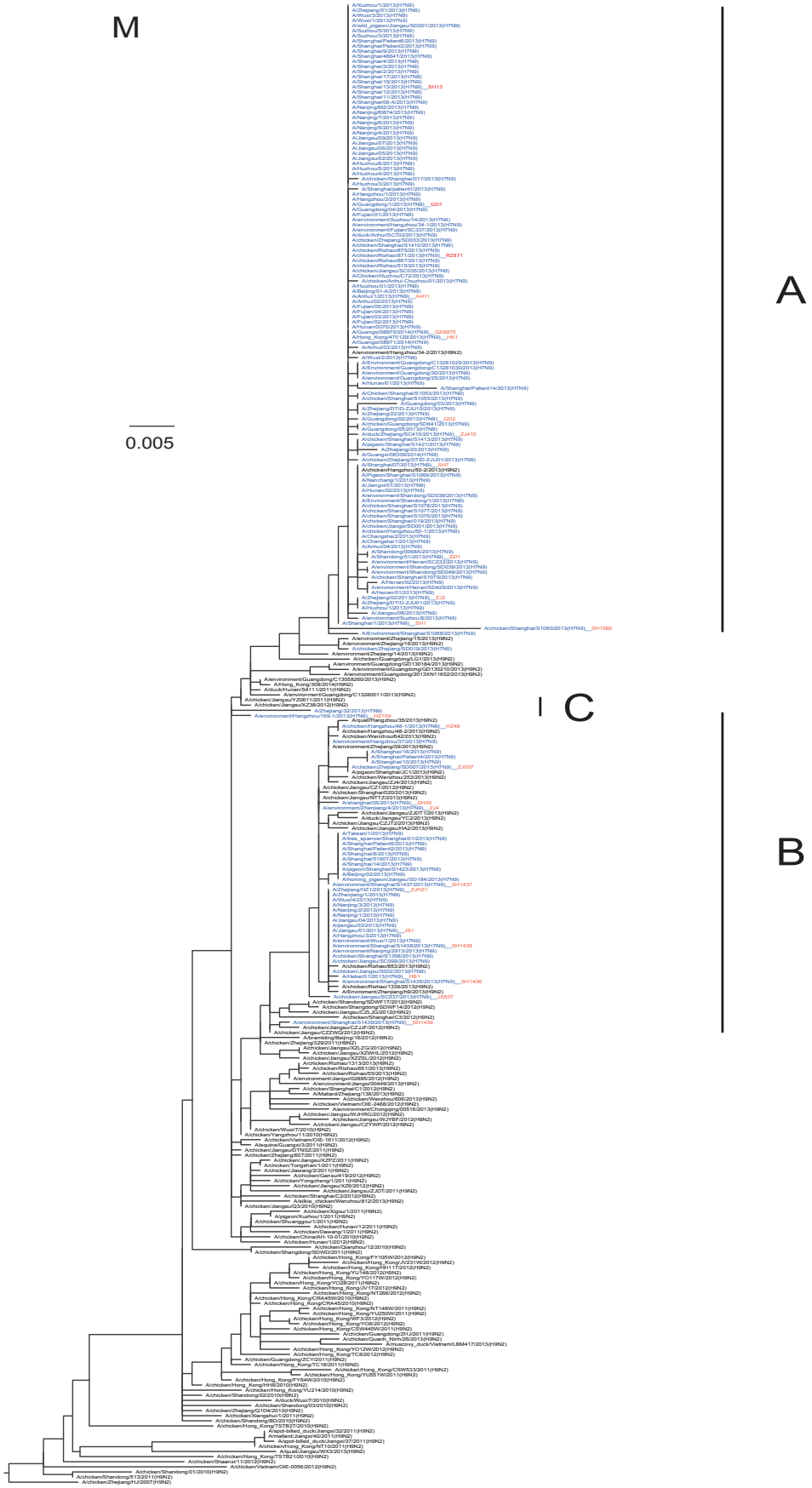

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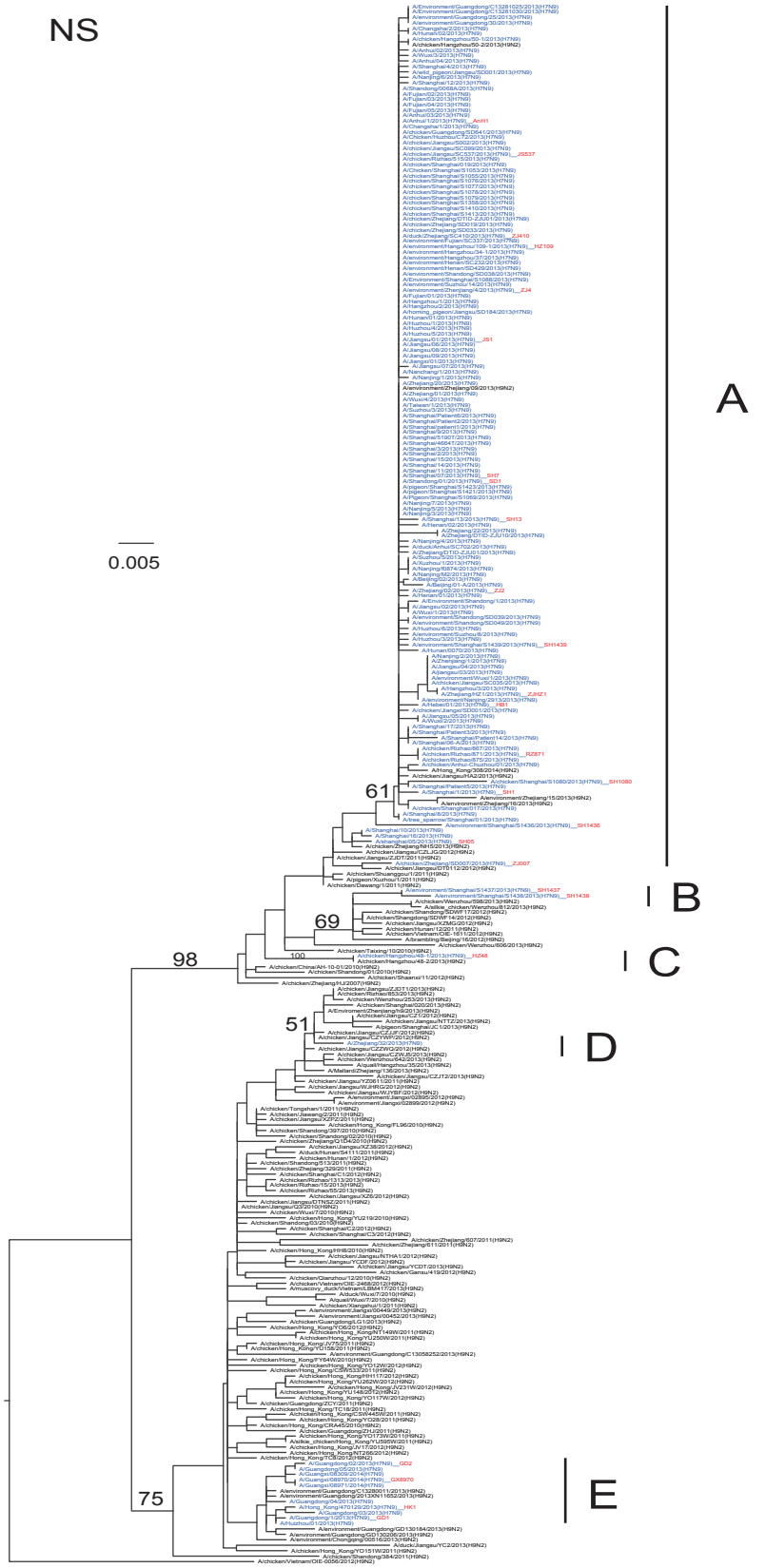

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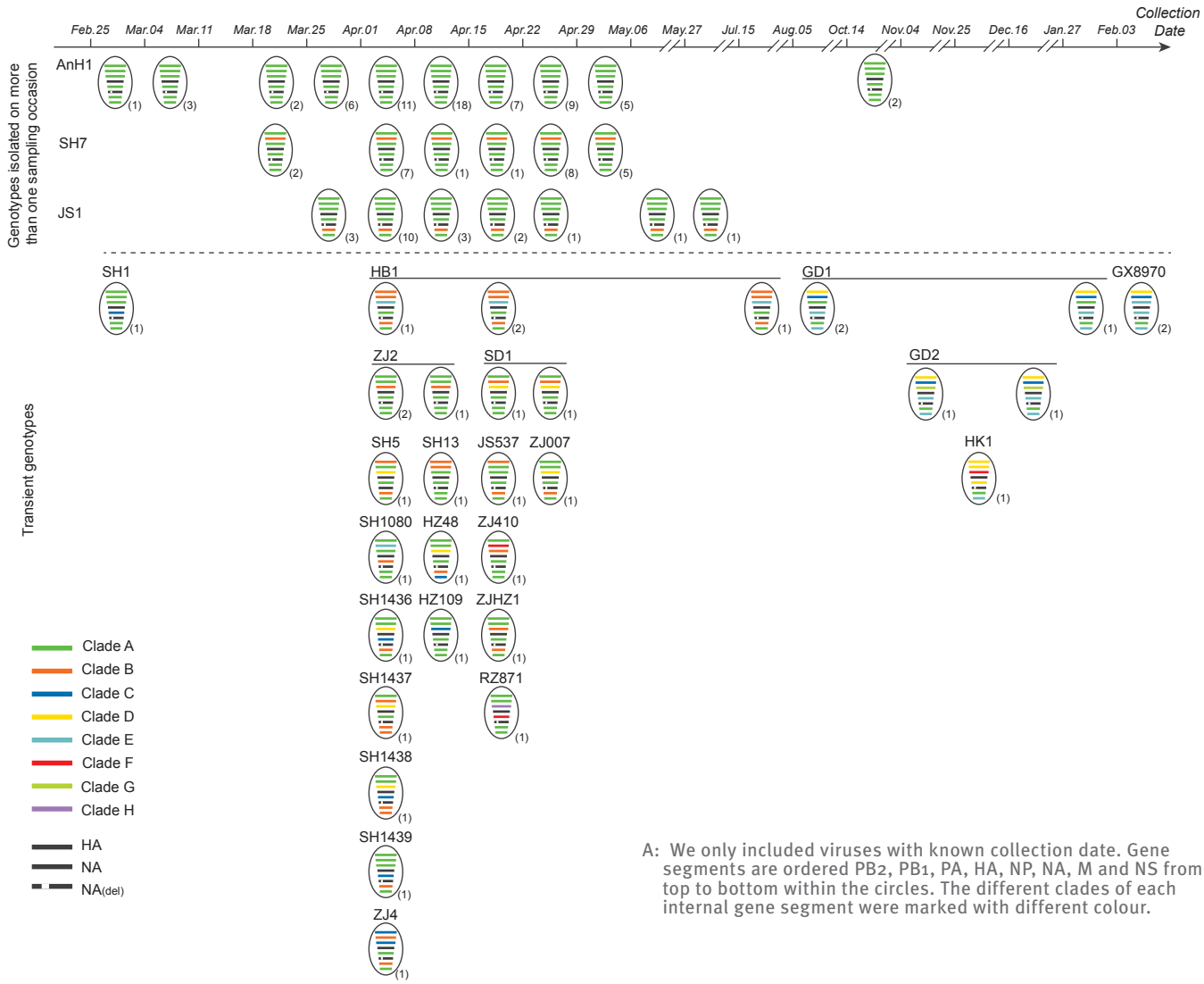
Figure 3

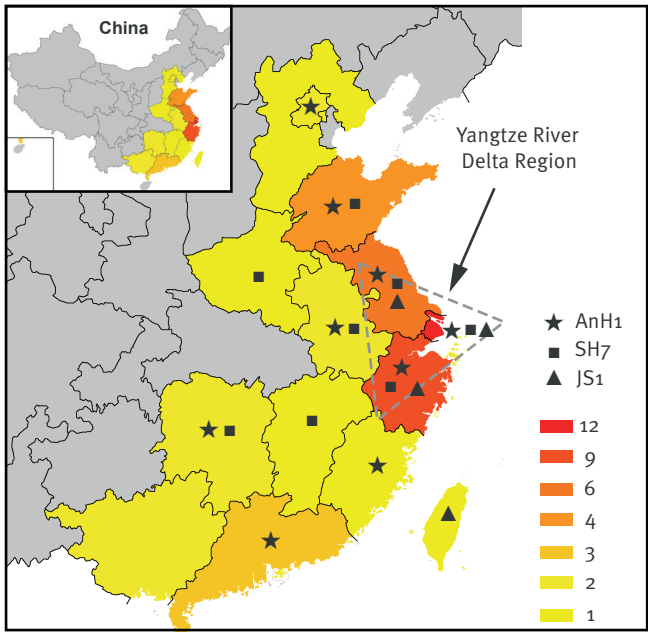
Multiple genotypes of influenza A(H7N9) viruses (A) and geographic distribution of different genotypes (B), China, 26 February 2013–5 February 2014 (n=142)

A



A: We only included viruses with known collection date. Gene segments are ordered PB2, PB1, PA, HA, NP, NA, M and NS from top to bottom within the circles. The different clades of each internal gene segment were marked with different colour.

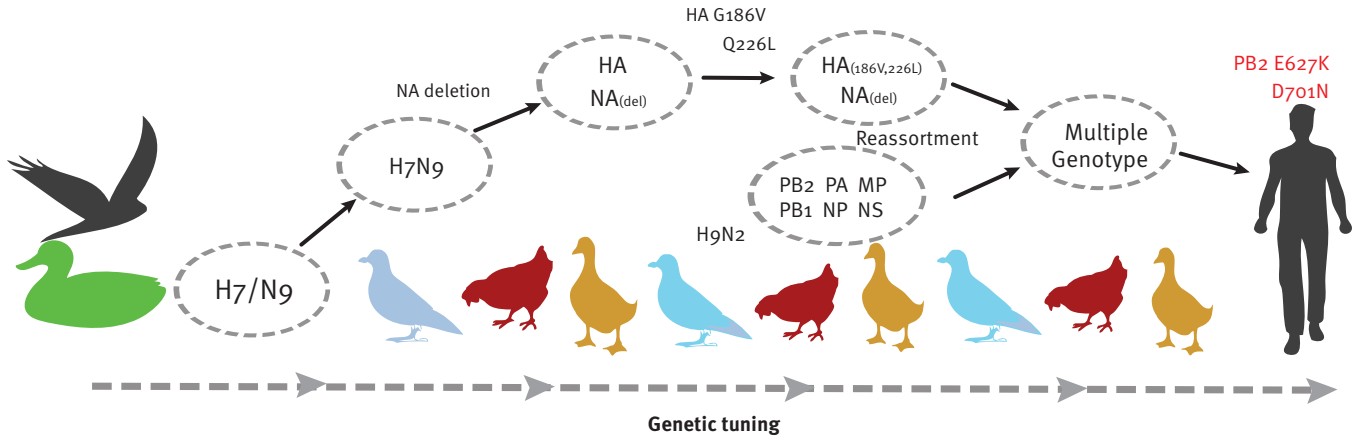
B



B: The colours represent the number of genotypes in each province.

Figure 4

Proposed genetic tuning mechanism of avian influenza A(H7N9) viruses during interspecies transmission



The arrow indicates the proposed schematic procedure of genetic tuning, including amino acid mutations and internal gene reassortment during interspecies transmission.

diversity and genotypic complexity existing in H9N2 viruses, there could have been multiple introductions of internal genes from H9N2 viruses [25]. Based on the comparison with different subtypes in publicly available databases, all six internal genes of the H7N9 virus shared the highest similarity with H9N2 viruses. The gene pool of H7N9 viruses was the result of multiple reassortment events with H9N2 viruses only; no other avian influenza virus subtype was identified that might have provided the internal genes to the H7N9 virus,

Our results showed that most genotypes were detected in the region of the Yangtze river delta (Figure 3B), further supporting the hypothesis that H7N9 viruses originated from this area [5]. Only six genotypes were detected outside this region, namely GD1, GD2, GX8970 and HK1detected in southern China, and RZ871 and SD1 in northern China. All these genotypes contained at least one difference in an internal gene compared with the genotypes detected in the Yangtze river delta, indicating that those genotypes may have been generated through inter-provincial poultry trade and further reassortment with local H9N2 viruses. Genotypes AH1, SH7 and JS1 were detected more frequently than others, which may imply that these three genotypes possess better fitness in poultry and/or were more prone to infect humans [26]. However, the infectivity, transmissibility and pathogenicity of different genotypes have not been characterised yet. New genotypes with better fitness may be generated by genetic tuning in the future.

Our study had several limitations. Firstly, biases may have been introduced through the sampling of potential influenza A(H7N9) cases as well as virus isolation and Sanger sequencing, because some genotypes may have been be missed or not yet identified. Secondly, the association of genotypes with clinical outcomes could not be statistically analysed owing to the limited number of cases for each genotype. Thirdly, the number and proportion of genotypes may change when more data become available.

In conclusion, we proposed that a genetic tuning procedure mediated host adaptation during interspecies transmission. However, the mechanism driving genetic tuning could not be fully elucidated yet. One possibility is that the favoured molecular evolution and compatible reassortment occurred to a large extent in poultry because of the backyard poultry breeding system and the ubiquitous live poultry markets in China where chickens and ducks are kept together [27].The frequent contact among different avian species enabled reassortment between different avian influenza viruses, and some poultry species that contain both human and avian like receptors [23,24], such as quail and pigeon, may facilitate the mammalian-adaptative mutations. Avian-to-human host transmission may be positively influenced by the diversity of the avian host species infected by influenza A(H7N9), while human-to-human transmission may be restricted by the single human species infected by influenza A(H7N9). Hence, it is important to change the breeding system and to close live poultry markets in order to control the spread of avian influenza A(H7N9) virus and to reduce the chance for emergence of avian influenza viruses with pandemic potential.

Our well-developed national influenza surveillance network made it possible to discover the host adaptation

sequence of the novel influenza A(H7N9) virus at an early stage when the virus infected both birds and humans. Genetic tuning not only mediated species switching, but may also allow the virus to adapt so that it infects humans more easily and transmits among people more efficiently. Recently, Malaysia reported its first human case of influenza A(H7N9), imported from Guangdong province, China [28]. Rapid transportation and frequent travelling have made it possible to transfer the virus from China to other regions. Overall, due to the genetic tuning procedure, the potential pandemic risk posed by the novel avian influenza A(H7N9) viruses is greater than that of any other known avian influenza viruses. A response to this threat requires the combined effort of different sectors related to human health, poultry and wild birds, as well as vigilance and co-operation of the world.

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Conflict of interest

None declared.

Authors' contributions

D.Y.W., L.Y., R.B.G, X.Z., Y.T and A.P.W. contributed equally to this work. Y.L.S. designed the work; Y.L.S., D.Y.W., W.F.Z., J.F.Z and L.Y. performed analyses and wrote the paper. R.B.G, S.M.Z., J.F.G., J.D., Y. Zhang, L.B.D., and Y.L. isolated viruses. H.C.Z., Y.G., A.P.W. and T.J.J commented on the paper. X.Z., X.Q., Y.S., Y.J.Z., Y.Z.L., T.L., Y.X., J.X., L.J.C., and Y.W.W. collected and transferred samples. X.Y.L., W.J.H., X.Z., and X.D.L. conducted sequencing. L.Q.L., J.L., H.J.W., L.X., Y.K.C., C.L.X., T.C., Y. Zhu, Z.J.F., W.Z.Y., Y.W., D.X.L., J.H., S.W.W., and G.Z.W. conducted surveillance.

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