Research articles

Genesis of the novel human-infecting influenza A(H10N8) virus and potential genetic diversity of the virus in poultry, China

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Human infection with a novel influenza A(H10N8) virus was first described in China in December 2013. However, the origin and genetic diversity of this virus is still poorly understood. We performed a phylogenetic analysis and coalescent analysis of two viruses from the first case of influenza A(H10N8) (A/Jiangxi-Donghu/346-1/2013 and A/Jiangxi-Donghu/346-2/2013 and a novel A(H10N8) virus (A/chicken/Jiangxi/102/2013) isolated from a live poultry market that the patient had visited. The haemagglutinin (HA), neuraminidase (NA), PA subunit of the virus polymerase complex, nucleoprotein (NP), M and non-structural protein (NS) genes of the three virus strains shared the same genetic origins. The origins of their HA and NA genes were similar: originally from wild birds to ducks, and then to chickens. The PA, NP, M, and NS genes were similar to those of chicken influenza A(H9N2) viruses. Coalescent analyses showed that the reassortment of these genes from A(H9N2) to A(H10N8) might have occurred at least twice. However, the PB1 and PB2 genes of the chicken A(H10N8) virus most likely originated from H7-like viruses of ducks, while those of the viruses from the case most likely stemmed from A(H9N2) viruses circulating in chickens. The oseltamivir-resistance mutation, R292K (R291K in A(H10N8) numbering) in the NA protein, occurred after four days of oseltamivir treatment. It seems that A(H10N8) viruses might have become established among poultry and their genetic diversity might be much higher than what we have observed.

Introduction

On 17 December 2013, China formally confirmed the first human infection with an avian influenza A(H10N8) virus in Jiangxi Province [1]. The patient, a woman in her early 70s, was hospitalised on 30 November 2013 due to severe pneumonia and died on 6 December. From 3 December, she had been given oseltamivir as an antiviral treatment. She was also diagnosed with multiple comorbidities. It was suggested that the comorbidities might have accounted for the death of this patient to some degree [1]. Further surveillance did not reveal evidence of inter-human transmission of this virus. Therefore, on the basis of current evidence, it seems that this was most likely a sporadic case [1]. As of 15 February 2014, three cases of human infection with A(H10N8) virus have been confirmed in Jiangxi Province, of whom two died [2]. Notably, these infections in southern China coincided with a second wave of A(H7N9) virus infection in eastern China: as of mid-February, there have been more than 300 human cases [3].

Avian A(H10N8) virus was isolated as early as 1965, among quails in Italy [4]. In China, it has been identified in water samples from Dongting Lake in 2007 [5] and in a duck from Guangdong in 2012 [6]. Preliminary phylogenetic data have shown that the A(H10N8) virus causing the first human infections in China, A/Jiangxi-Donghu/346/2013(H10N8), is a novel reassortant [1]. The H10 haemagglutinin (HA) and N8 neuraminidase (NA) gene segments might have been derived from different influenza viruses from wild birds, while the six internal genes (PB2, PB1, PA, NP, M, NS) most likely originated from A(H9N2) viruses in poultry [1].

To provide further insight into the origin of this A(H10N8) virus that led to a human fatality, we performed an epidemiological study at a live poultry market that the first patient (identified in December

2013) visited a few days before onset of symptoms. An A(H10N8) virus was successfully isolated from a chicken swab sample, which was named A/Chicken/Jiangxi/102/2013(H10N8). Two samples from the patient were collected on 4 and 6 December 2013: the viral isolates were named A/Jiangxi-Donghu/346-1/2013 and A/Jiangxi-Donghu/346-2/2013, respectively. In this study, we performed a phylogenetic analysis and coalescent analysis of full-length genome sequences of these virus stains to infer the potential origin of this novel human-infecting virus and the genetic diversity of the virus in poultry.

Methods

Sample collection and virus isolation

Clinical samples (tracheal aspirates) of the patient were collected on 4 and 6 December 2013. Swab specimens from the live poultry market where the patient had bought a chicken on 23 November 2013 were obtained on 8 December 2013. All these samples were sent to the influenza laboratory of the Nanchang Center for Disease Control and Prevention and were screened by real-time reverse transcription (RT)-PCR. The M gene-positive specimens, which were negative for H5, H7 and H9 subtype viruses, were sent to an animal biosafety level 3 laboratory of South China Agricultural University for virus isolation.

All the specimens were propagated in the allantoic sac of 9–11 day-old specific pathogen-free embryonated chicken eggs for 60 hours at 37 °C. HA assays were performed according to the World Health Organization protocol [7].

RNA extraction, real-time reverse transcription-PCR, reverse transcription-PCR and DNA sequencing

RNA was extracted from the two samples from the patient, the swab specimens of the live poultry and a suspension of the three A(H10N8) virus isolates with the RNeasy Mini Kit (Qiagen). The real-time RT-PCR detections of M gene, H5, H7 and H9 subtype influenza viruses were performed according to national standards of China [8]. Two-step RT-PCR was conducted with universal primers as reported by Hoffmann et al. and each gene segment was amplified under standard conditions [9]. The PCR products were purified with a QIAamp Gel extraction kit (Qiagen) and sequenced with an ABI 3730 DNA Analyzer (Applied Biosystems).

Multiple sequence alignment and phylogenetic analysis

Full-length genome sequences of the A(H10N8) virus strains were combined together, producing eight query datasets that corresponded respectively to the eight gene segments of a type A influenza virus. We performed BLASTn (nucleotide Basic Local Alignment Search Tool) using the eight datasets against GenBank. The BLAST outputs and the inputs (query datasets) were combined together, respectively. Multiple sequence alignment was performed using MUSCLE (multiple sequence comparison by log-expectation) [10]. Phylogenetic analysis was performed using RAxML with the GTRGAMMA model [11]. A total of 1,000 bootstrap replicates were implemented. After the first run, we analysed the phylogenetic trees and selected representative sequences to compose smaller datasets for further phylogenetic analyses.

Sequences from reference strains used in the genetic analysis were obtained from the EpiFlu database of the Global Initiative on Sharing All Influenza Data (GISAID) (Table 1).

Calculation of the estimated time to the most recent common ancestor using BEAST

Sequences with information on month of isolation that were available in the BLAST outputs were selected to compose new datasets to calculate the estimated time to the most recent common ancestor for avian-origin and human-origin A(H10N8) viruses. This was performed using BEAST (Bayesian evolutionary analysis by sampling trees) [12] and the parameters used were the same as those we had implemented in a previous study [13].

Results

In total, 86 samples, including 64 swabs of poultry (42 chickens, 12 ducks and 10 pigeons), 10 fresh poultry faeces, 8 swabs of 8 poultry cages and 4 sewage samples, were collected from the live poultry market visited by the first influenza A(H10N8) patient. The M gene-positive specimens that were negative for H5, H7 and H9 subtype influenza virus were used for virus isolation. A/Chicken/Jiangxi/102/2013(H10N8) was obtained from the chicken specimens. A/Jiangxi-Donghu/346-1/2013 and A/Jiangxi-Donghu/346-2/2013 were isolated from the patient's samples on 4 and 6 December, 2013, respectively. No other influenza viruses were isolated in these specimens.

A comparison of sequences showed that A/Jiangxi-Donghu/346-1/2013 (GISAID accession numbers: EPI530523–EPI530530) was 100% identical to A/Jiangxi-Donghu/346-2/2013 (GISAID accession numbers: EPI530531–EPI530538) in six genes, with the only differences lying in the NA and nonstructural protein (NS) genes, whose sequence identities were 99.8%. Both A(H10N8) virus isolates from the case were highly similar (>99.3%) to the A/chicken/Jiangxi/102/2013 (GISAID accession numbers: EPI530539–EPI530546) in six genes, except for the PB1 (89.4%) and PB2 (93.3%) genes. In contrast, the PB1 and PB2 genes of A/Jiangxi-Donghu/346-1/2013 were highly similar to the counterparts of two A(H9N2) viruses, A/environment/Chongqing/00516/2013 and A/environment/Jiangxi/00449/2013.

In the human and chicken isolates, only one basic amino acid (arginine, R) was noted at the HA cleavage site. In addition, the amino acid residues 226Q and

Table 1

Origin of sequences used for the phylogenetic analysis of the novel influenza A(H10N8) sequences

Segment ID	Segment	Country	Collection date	Isolate name	Submitting laboratory	Authors
EPI497517	PB2	China	2013-Feb-17	A/environment/ Jiangxi/00449/2013	WHO Chinese National Influenza Center	Rongbao, Gao; Xiaodan, Li; Ye, Zhang; Shumei, Zou; Xiang, Zhao; Xiyan, Li; Dayan, Wang; Yuelong, Shu
EPI497519	PB1	China	2013-Feb-17	A/environment/ Jiangxi/00449/2013	WHO Chinese National Influenza Center	Rongbao, Gao; Xiaodan, Li; Ye, Zhang; Shumei, Zou; Xiang, Zhao; Xiyan, Li; Dayan, Wang; Yuelong, Shu
EPI497516	PA	China	2013-Feb-17	A/environment/ Jiangxi/00449/2013	WHO Chinese National Influenza Center	Rongbao, Gao;X iaodan, Li; Ye, Zhang; Shumei, Zou; Xiang, Zhao; Xiyan, Li; Dayan, Wang; Yuelong, Shu
EPI497512	HA	China	2013-Feb-17	A/environment/ Jiangxi/00449/2013	WHO Chinese National Influenza Center	Rongbao, Gao; Xiaodan, Li; Ye, Zhang; Shumei, Zou; Xiang, Zhao; Xiyan, Li; Dayan, Wang; Yuelong, Shu
EPI497515	NP	China	2013-Feb-17	A/environment/ Jiangxi/00449/2013	WHO Chinese National Influenza Center	Rongbao, Gao; Xiaodan, Li; Ye, Zhang; Shumei, Zou; Xiang, Zhao; Xiyan, Li; Dayan, Wang; Yuelong, Shu
EPI497513	NA	China	2013-Feb-17	A/environment/ Jiangxi/00449/2013	WHO Chinese National Influenza Center	Rongbao, Gao; Xiaodan, Li; Ye, Zhang; Shumei, Zou; Xiang, Zhao; Xiyan, Li; Dayan, Wang; Yuelong, Shu
EPI497514	MP	China	2013-Feb-17	A/environment/ Jiangxi/00449/2013	WHO Chinese National Influenza Center	Rongbao, Gao; Xiaodan, Li; Ye, Zhang; Shumei, Zou; Xiang, Zhao; Xiyan, Li; Dayan, Wang; Yuelong, Shu
EPI497518	NS	China	2013-Feb-17	A/environment/ Jiangxi/00449/2013	WHO Chinese National Influenza Center	Rongbao, Gao; Xiaodan, Li; Ye, Zhang; Shumei, Zou; Xiang, Zhao; Xiyan, Li; Dayan, Wang; Yuelong, Shu
EPI497497	PB2	China	2013-Mar-13	A/environment/ Chongqing/00516/2013	WHO Chinese National Influenza Center	Rongbao, Gao; Xiaodan, Li; Ye, Zhang; Shumei, Zou; Xiang, Zhao; Xiyan, Li; Dayan, Wang; Yuelong, Shu
EPI497498	PB1	China	2013-Mar-13	A/environment/ Chongqing/00516/2013	WHO Chinese National Influenza Center	Rongbao, Gao; Xiaodan, Li; Ye, Zhang; Shumei, Zou; Xiang, Zhao; Xiyan, Li; Dayan, Wang; Yuelong, Shu
EPI497499	PA	China	2013-Mar-13	A/environment/ Chongqing/00516/2013	WHO Chinese National Influenza Center	Rongbao, Gao; Xiaodan, Li; Ye, Zhang; Shumei, Zou; Xiang, Zhao; Xiyan, Li; Dayan, Wang; Yuelong, Shu
EPI497495	HA	China	2013-Mar-13	A/environment/ Chongqing/00516/2013	WHO Chinese National Influenza Center	Rongbao, Gao; Xiaodan, Li; Ye, Zhang; Shumei, Zou; Xiang, Zhao; Xiyan, Li; Dayan, Wang; Yuelong, Shu
EPI497501	NP	China	2013-Mar-13	A/environment/ Chongqing/00516/2013	WHO Chinese National Influenza Center	Rongbao, Gao; Xiaodan, Li; Ye, Zhang; Shumei, Zou; Xiang, Zhao; Xiyan, Li; Dayan, Wang; Yuelong, Shu
EPI497496	NA	China	2013-Mar-13	A/environment/ Chongqing/00516/2013	WHO Chinese National Influenza Center	Rongbao, Gao; Xiaodan, Li; Ye, Zhang; Shumei, Zou; Xiang, Zhao; Xiyan, Li; Dayan, Wang; Yuelong, Shu
EPI497502	MP	China	2013-Mar-13	A/environment/ Chongqing/00516/2013	WHO Chinese National Influenza Center	Rongbao, Gao; Xiaodan, Li; Ye, Zhang; Shumei, Zou; Xiang, Zhao; Xiyan, Li; Dayan, Wang; Yuelong, Shu
EPI497500	NS	China	2013-Mar-13	A/environment/ Chongqing/00516/2013	WHO Chinese National Influenza Center	Rongbao, Gao; Xiaodan, Li; Ye, Zhang; Shumei, Zou; Xiang, Zhao; Xiyan, Li; Dayan, Wang; Yuelong, Shu

HA: haemagglutinin; NA: neuraminidase; NP: nucleoprotein; NS: nonstructural protein.

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228G (H3 numbering) of the HA protein indicated an avian-like receptor-binding preference. The substitution E627K in the PB2 gene was found in both A/Jiangxi-Donghu/346-1/2013 and A/Jiangxi-Donghu/346-2/2013 viruses, but not in A/Chicken/Jiangxi/102/2013. In particular, both 292R and 292K were detected in the NA protein in all nine NA gene clones from the original trachea aspirate on 6 December 2013, among which four clones had Rs and five clones had Ks. However, only R was detected in the isolate from the sample taken from the patient on 4 December 2013.

Phylogenetic trees constructed using the associated HA gene sequences showed that the three Nanchang A(H10N8) isolates were clustered together and fell within a cluster that includes two H10 isolates from China and Vietnam and an earlier strain from Sweden, A/mallard/Sweden/105522/2009 (Figure 1A). However, two previous Chinese A(H10N8) isolates, A/duck/Guangdong/E1/2012 and A/environment/DongtingLake/Hunan/3-9/2007, fell within a different cluster (Figure 1A). Similarly, the three recent Chinese A(H10N8) isolates were also clustered together in the NA phylogenetic tree and were most closely related to A/duck/Vietnam/OIE-2747/2012 (Figure 1B). However, there was a long branch length between the Chinese A(H10N8) and the Vietnamese A(H3N8) strains. In addition, there were also a few A(H3N8) strains from Japan and South Korea isolated in 2008 and 2010 in this cluster (Figure 1B). It is noteworthy that the recent Chinese A(H10N8) strains, as well as several N8-containing strains circulating in eastern Asia and south-east Asia, fell within a North American N8 lineage (Figure 2).
For the PA, NP, M and NS genes, A/chicken/Jiangxi/102/2013 and the two isolates from the influenza A(H10N8) case were always clustered together. All three fell within the H9N2 lineages that were circulating in Chinese chickens in 2013 (Figures 3–6). Notably, in the PA phylogenetic tree, they were also grouped together with a few A(H7N9) strains, besides the A(H9N2) viruses (Figure 3).

To investigate further the potential time of occurrence of the reassortment events, we calculated the most recent time to the common ancestor for A/chicken/Jiangxi/102/2013 and A/Jiangxi-Donghu/346-1/2013. The most recent time to the common ancestor, calculated using the HA data, was in late October 2013, approximately a month before laboratory confirmation of the patient's infection, while that calculated using the NA data was dated in late June 2013 (Table 2). For internal genes PA, NP, M and NS, the most recent time to the common ancestor was dated from approximately 3 (for PA) to 11 (for NP) months before the human infection with the A(H10N8) virus (Table 2). Given that the 95% confidence intervals of the most recent time to the common ancestor calculated using the NP and PA gene sequences were not overlapping (Table 2), we conclude that at least two temporarily separated reassortment events of internal genes from A(H9N2) to A(H10N8) might have occurred (Table 2, Figures 1–7).

In the PB1 and PB2 phylogenetic trees, the human-infecting H10N8 were still clustered together with the H9N2 influenza viruses that were circulating in chickens in 2013 (Figure 7). However, the chicken H10N8 isolate (A/chicken/Jiangxi/102/2013) fell within a different lineage. In detail, the PB1 gene of A/chicken/Jiangxi/102/2013 was more closely related to the PB1 genes from strains isolated from ducks in Zhejiang Province in 2011–13 (Figure 7A), while the PB2 gene was most similar to the PB2 genes from ducks from Jiangxi Province in 2009 (Figure 7B). It should be noted that the majority of strains in the chicken A(H10N8) lineages belonged to the subtype H7. Similarly, the gap of branch length between A/chicken/Jiangxi/102/2013 and its H7 relatives was very long (Figure 7).

Discussion

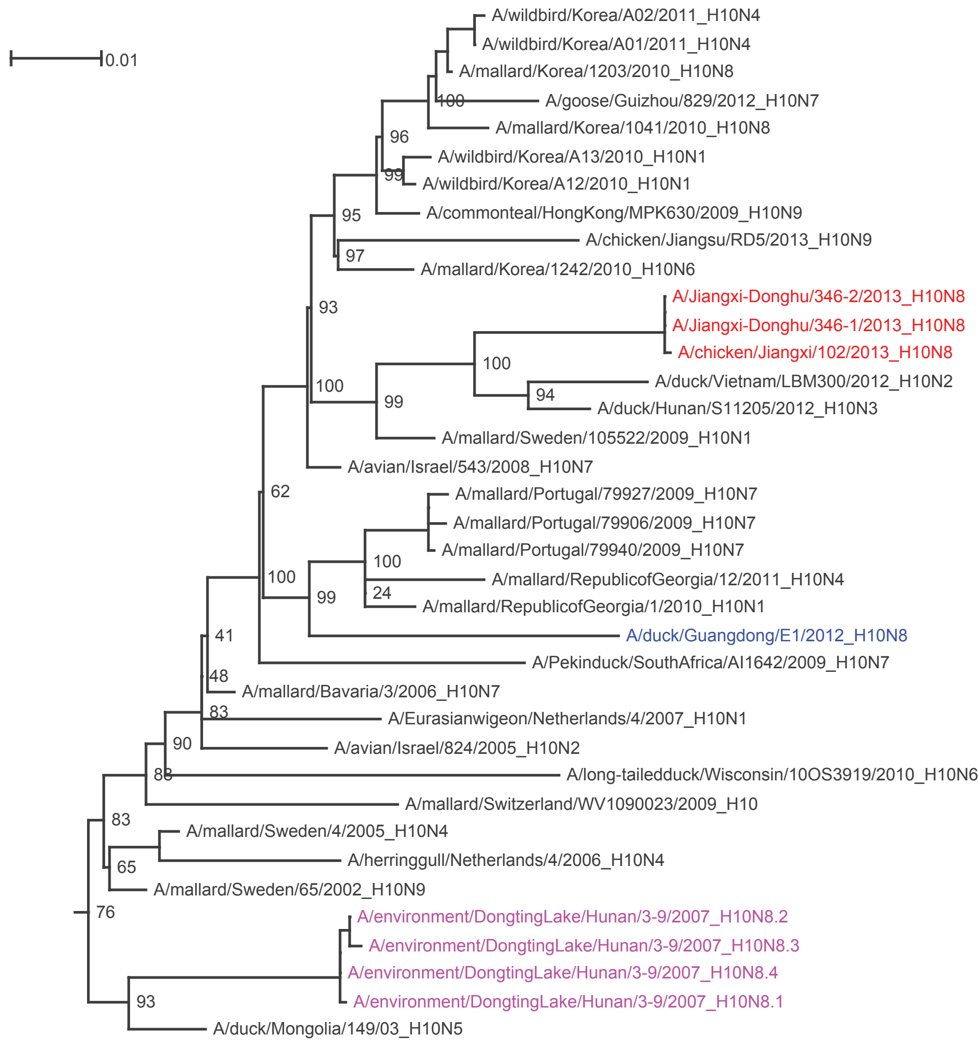
Phylogenetic analysis and coalescent analysis of the A(H10N8) viruses from a human case and an A(H10N8) virus isolated from a chicken from the live poultry market that the patient visited a few days before symptom onset show that both A/chicken/Jiangxi/102/2013 and A/Jiangxi-Donghu/346-1/2013 were multiple reassortants (Figure 8). They shared the same genetic origins in six gene segments, although they had different PB1 and PB2 genes. The PB1 and PB2 genes of A/chicken/Jiangxi/102/2013 most likely originated from the counterparts of the subtype H7 from ducks, whereas those of A/Jiangxi-Donghu/346-1/2013 might be derived from contemporarily circulating chicken A(H9N2) influenza viruses.

On the basis of current evidence, we propose a model to illustrate the potential origin of the A(H10N8) strain that can infect humans (Figure 8). However, due to the limited surveillance data, the possibility that viruses possessing this HA gene might have been circulating in China over a prolonged time cannot be fully ruled out. The origin and spread of the NA gene was similar, but its original source was North America, not Europe. Then, the H10 and N8 gene segments underwent reassortment in wild birds or most likely in ducks, because viruses possessing such H10 and N8 genes have been isolated from ducks, respectively. This gave rise to a hypothetical A(H10N8) influenza virus in ducks, which continued to reassort with chicken A(H9N2) influenza viruses and obtained the A(H9N2) internal genes through a series of reassortment events. Based on current evidence, the most possible region where these reassortments might have occurred is China, because the internal genes of the human-infecting A(H10N8) influenza viruses are H9N2-like and these A(H9N2) viruses were isolated from China. Some A(H10N8) virus strains, such as A/chicken/Jiangxi/102/2013, obtained part of the internal gene cassette from A(H9N2), while others (e.g. A/Jiangxi-Donghu/346-1/2013) obtained the whole internal gene cassette from A(H9N2). Since avian influenza viruses from wild birds have seldom infected humans directly, reassortment with poultry influenza A(H9N2) viruses might endow them with

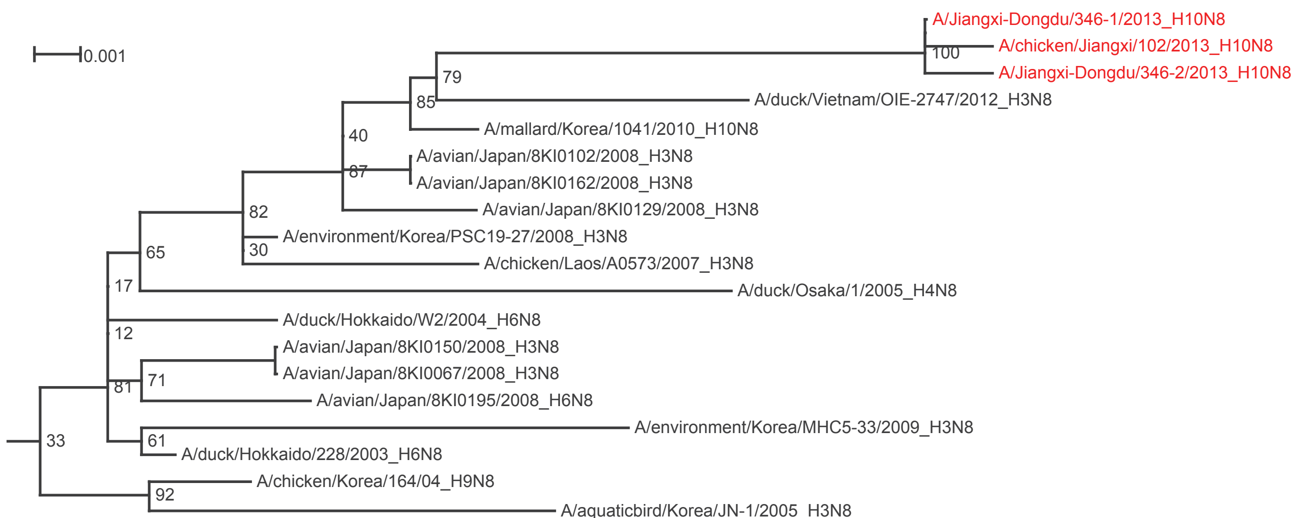
Figure 1

Phylogenetic trees constructed using haemagglutinin and neuraminidase gene sequences from the novel influenza A(H10N8) viruses and closely related sequences from public databases

A. Haemagglutinin gene sequences



B. Neuraminidase gene sequences



Multiple sequence alignment was performed using MUSCLE (multiple sequence comparison by log-expectation). Phylogenetic analysis was performed using RAxML with the GTRGAMMA model. A total of 1,000 bootstrap replicates were implemented. After the first run, we analysed the phylogenetic trees and selected representative sequences to compose smaller datasets for further phylogenetic analyses. The scale bar shows the length of branch that represents an amount of genetic change of 0.01. The unit of the bar is nucleotide substitutions per site. The three Nanchang influenza A(H10N8) viruses are shown in red. Other Chinese A(H10N8) isolates are shown in other colours.

We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu Database on which this research is based.

Figure 2

Large phylogenetic tree constructed using neuraminidase gene sequences from the novel influenza A(H10N8) viruses and their closely related sequences searched from public databases

Multiple sequence alignment was performed using MUSCLE (multiple sequence comparison by log-expectation). Phylogenetic analysis was performed using RAxML with the GTRGAMMA model. A total of 1,000 bootstrap replicates were implemented. After the first run, we analysed the phylogenetic trees and selected representative sequences to compose smaller datasets for further phylogenetic analyses. The scale bar shows the length of branch that represents an amount of genetic change. The unit of the bar is nucleotide substitutions per site. The three Nanchang influenza A(H10N8)viruses are shown in red.

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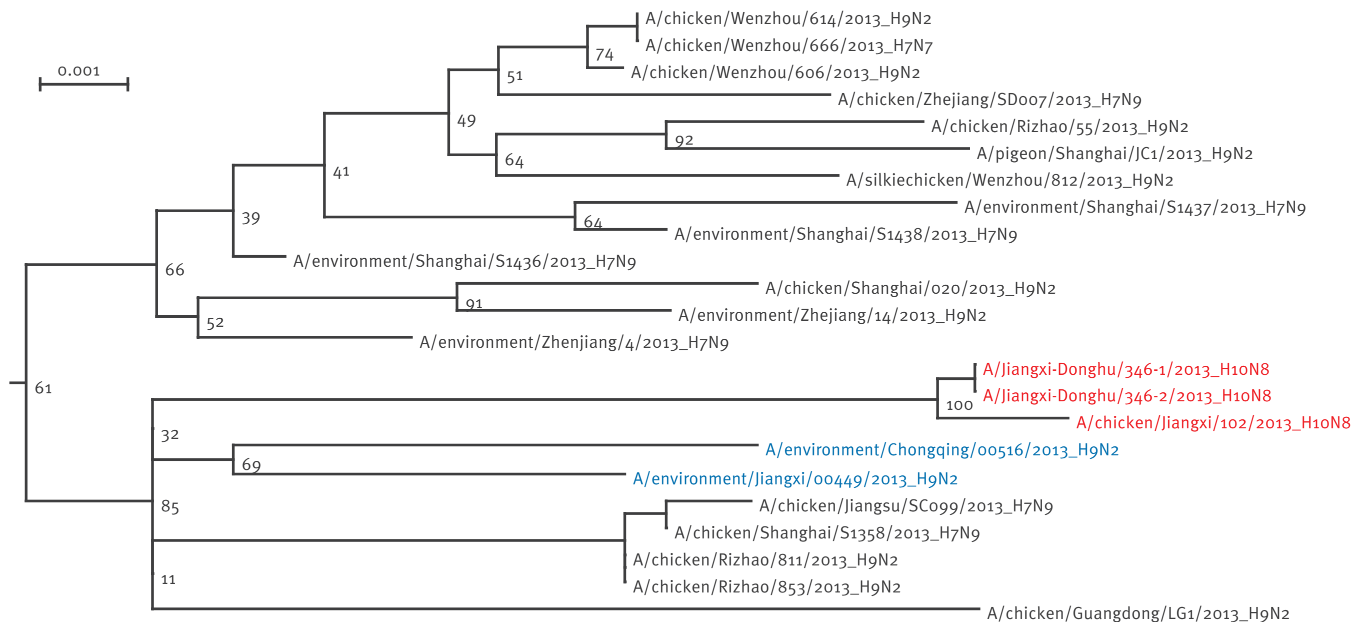
Figure 3

Phylogenetic tree constructed using gene sequences of the PA subunit of the virus polymerase complex from the novel influenza A(H10N8) viruses and their closely related sequences searched from public databases



Multiple sequence alignment was performed using MUSCLE (multiple sequence comparison by log-expectation). Phylogenetic analysis was performed using RAxML with the GTRGAMMA model. A total of 1,000 bootstrap replicates were implemented. After the first run, we analysed the phylogenetic trees and selected representative sequences to compose smaller datasets for further phylogenetic analyses. The scale bar shows the length of branch that represents an amount of genetic change. The unit of the bar is nucleotide substitutions per site. The three Nanchang influenza A(H10N8) viruses are shown in red. The viruses shown in blue are important for the reassortment and origin of the three Nanchang influenza A(H10N8) viruses.

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certain genetic characteristics that would enable them to infect humans. This pattern has also been observed in human-infecting influenza A(H7N9) viruses, whose internal genes were also derived from poultry-derived A(H9N2) viruses [13].

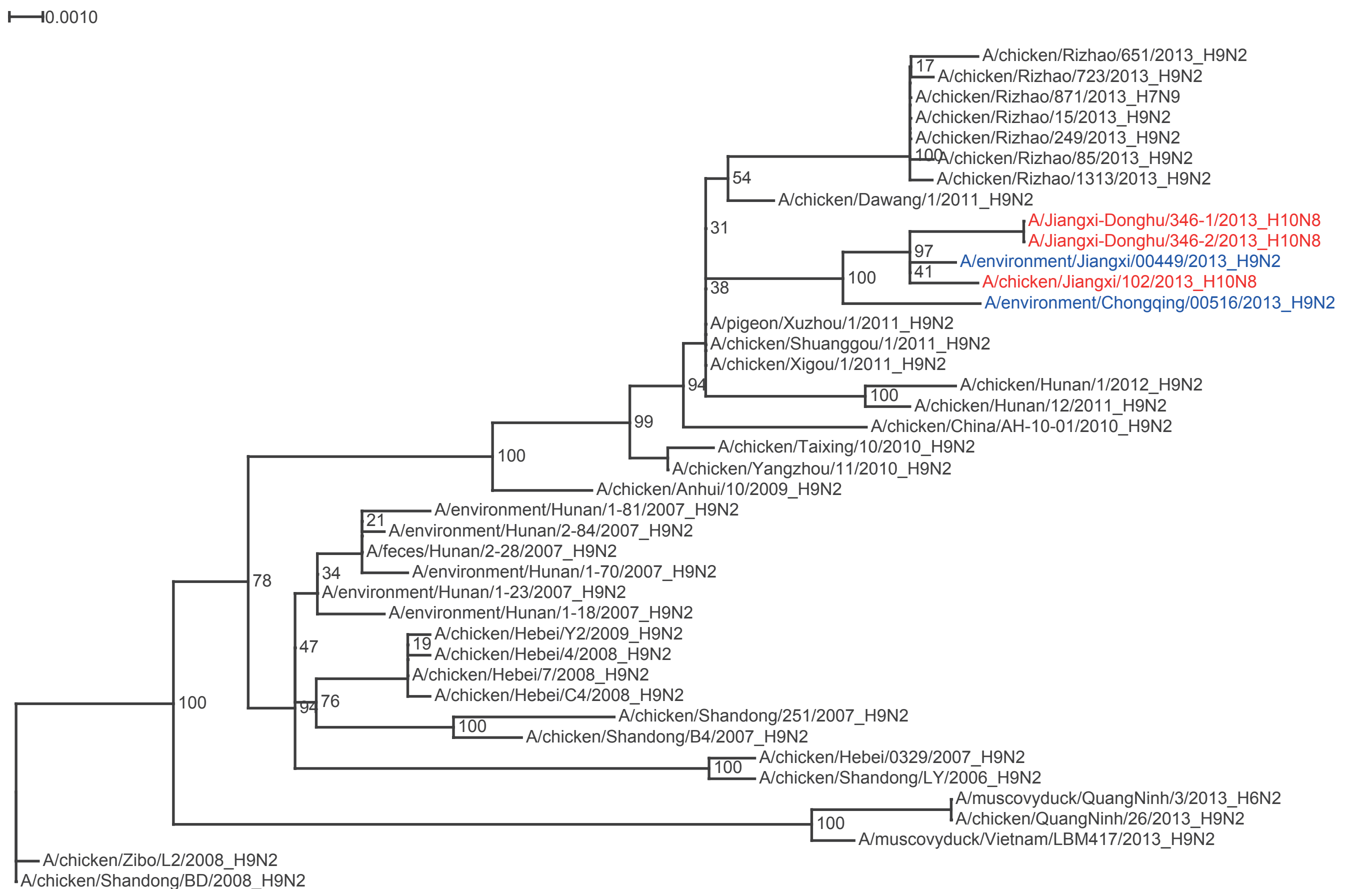
Our analyses showed that the HA and NA genes of the human-infecting influenza A(H10N8)-like viruses might have formed a stable lineage in Jiangxi Province. In addition, the genetic difference in the internal genes between the human-infecting and chicken isolates suggests that they might also undergo a dynamic reassortment process that A(H7N9) viruses have [14,15], so we speculate that the genetic diversity of these influenza A(H10N8) viruses might be much higher than we have observed thus far.

Moreover, the human-origin H10 viruses had the E627K substitution in the PB2 gene, which has been associated with the increased virulence of avian influenza viruses in mammals, such as A(H5N1), A(H9N2) and A(H7N9) [16-18]. A previous study has shown that an oseltamivir-resistance mutation emerged after four days of oseltamivir treatment in a patient with the novel influenza A(H7N9) virus infection [19]. Similarly, the oseltamivir-resistance mutation, R292K (R291K in A(H10N8) numbering) in the NA protein, was also found to have occurred after four days of oseltamivir treatment in a patient with the novel A(H10N8) virus infection. The N8 NA belongs to the group 1 NA whose mechanism of resistance to oseltamivir is much more frequently associated with H275Y substitution [20]. The report of R292K in group 1 NA is extremely rare. The R292K of NA gene has been confirmed by sequencing material amplified from the patient's specimen and the isolated virus. This R292K mutation perhaps affected the inhibitor-binding site of the novel influenza A(H10N8) virus and posed a threat to the anti-influenza virus treatment strategy currently implemented. The phenotype of the susceptibility of the virus should be characterised to confirm the resistance profiles. Unlike H5 and H7, some subtypes of which are highly pathogenic, the H10 viruses can have variable pathogenicity in chickens [21,22]. More seriously, some H10 viruses that do not have multiple basic amino acids at the cleavage site in the HA protein could also be highly pathogenic to chickens [22].

As our results suggest that the influenza A(H10N8) viruses might have been established among poultry and that their genetic diversity might be much higher

Figure 4

Phylogenetic tree constructed using the nucleoprotein gene sequences from the novel influenza A(H10N8) viruses and their closely related sequences searched from public databases



Multiple sequence alignment was performed using MUSCLE (multiple sequence comparison by log-expectation). Phylogenetic analysis was performed using RAxML with the GTRGAMMA model. A total of 1,000 bootstrap replicates were implemented. After the first run, we analysed the phylogenetic trees and selected representative sequences to compose smaller datasets for further phylogenetic analyses. The scale bar shows the length of branch that represents an amount of genetic change. The unit of the bar is nucleotide substitutions per site. The three Nanchang influenza A(H10N8) viruses are shown in red. The viruses shown in blue are important for the reassortment and origin of the three Nanchang influenza A(H10N8) viruses.

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than what we have observed, we call for intensive surveillance of avian influenza viruses circulating in poultry. In addition, as A(H9N2) influenza viruses have acted as gene donors for A(H7N9) and A(H10N8) viruses [1,10], the role of A(H9N2) virus in enabling wild bird influenza viruses to infect humans deserves further study.

Acknowledgements

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Conflict of interest

None declared.

Figure 5

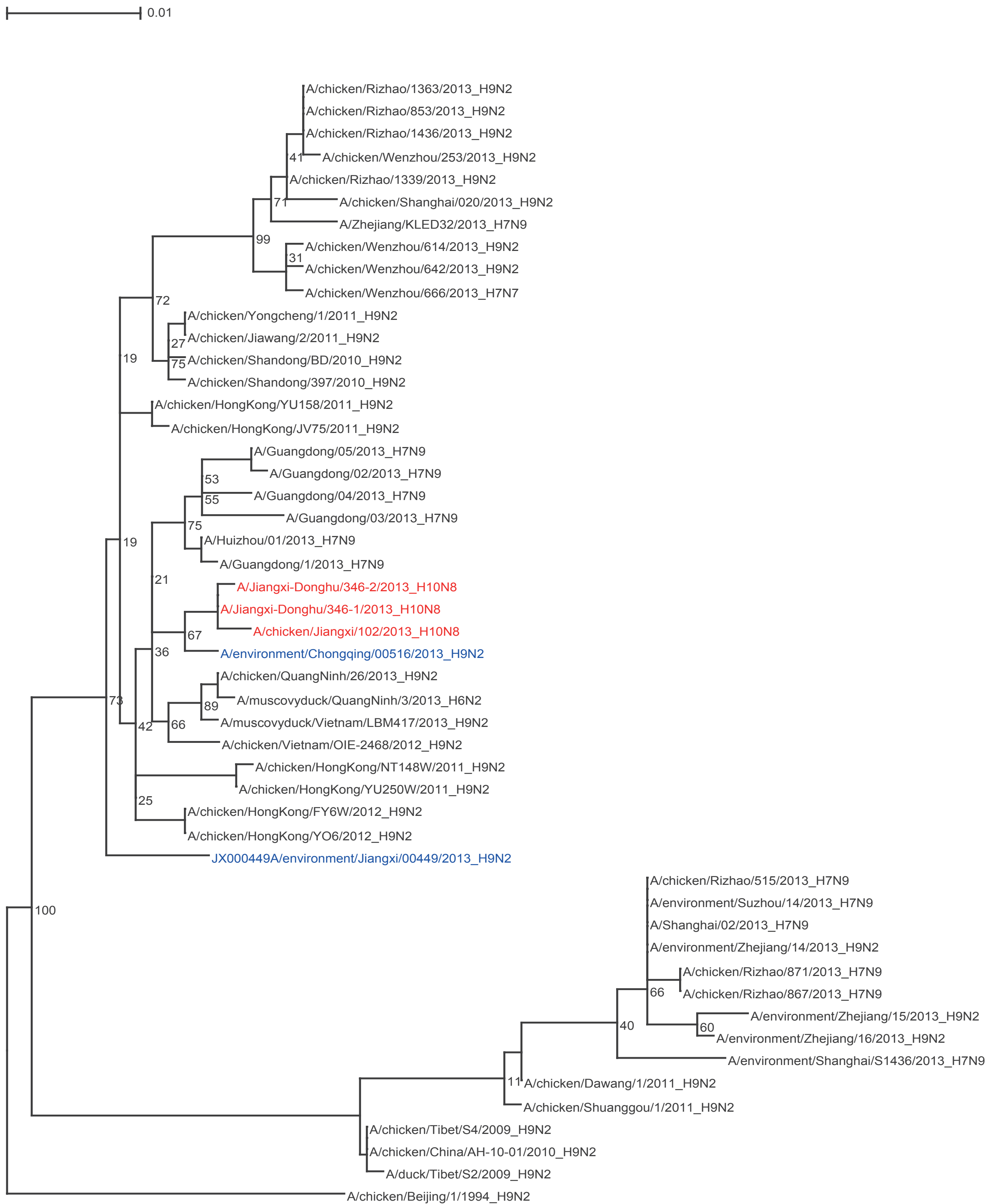
Phylogenetic tree constructed using the M gene sequences from the novel influenza A(H10N8) viruses and their closely related sequences searched from public databases

Multiple sequence alignment was performed using MUSCLE (multiple sequence comparison by log-expectation). Phylogenetic analysis was performed using RAxML with the GTRGAMMA model. A total of 1,000 bootstrap replicates were implemented. After the first run, we analysed the phylogenetic trees and selected representative sequences to compose smaller datasets for further phylogenetic analyses. The scale bar shows the length of branch that represents an amount of genetic change. The unit of the bar is nucleotide substitutions per site. The three Nanchang influenza A(H10N8) viruses are shown in red. The viruses shown in blue are important for the reassortment and origin of the three Nanchang influenza A(H10N8) viruses.

We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu Database on which this research is based.

Figure 6

Phylogenetic tree constructed using the nonstructural protein gene sequences from the novel influenza A(H10N8) viruses and their closely related sequences searched from public databases



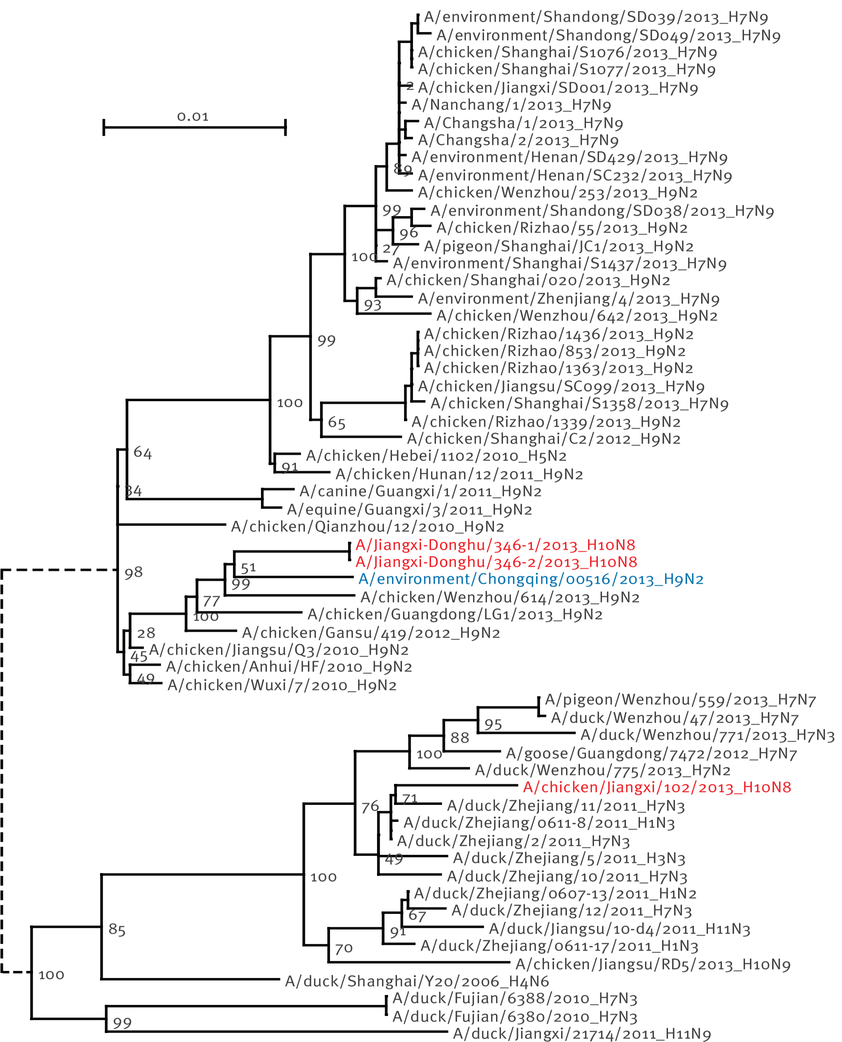
Multiple sequence alignment was performed using MUSCLE (multiple sequence comparison by log-expectation). Phylogenetic analysis was performed using RAxML with the GTRGAMMA model. A total of 1,000 bootstrap replicates were implemented. After the first run, we analysed the phylogenetic trees and selected representative sequences to compose smaller datasets for further phylogenetic analyses. The scale bar shows the length of branch that represents an amount of genetic change. The unit of the bar is nucleotide substitutions per site. The three Nanchang influenza A(H10N8) viruses are shown in red. The viruses shown in blue are important for the reassortment and origin of the three Nanchang influenza A(H10N8) viruses.

We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu Database on which this research is based.

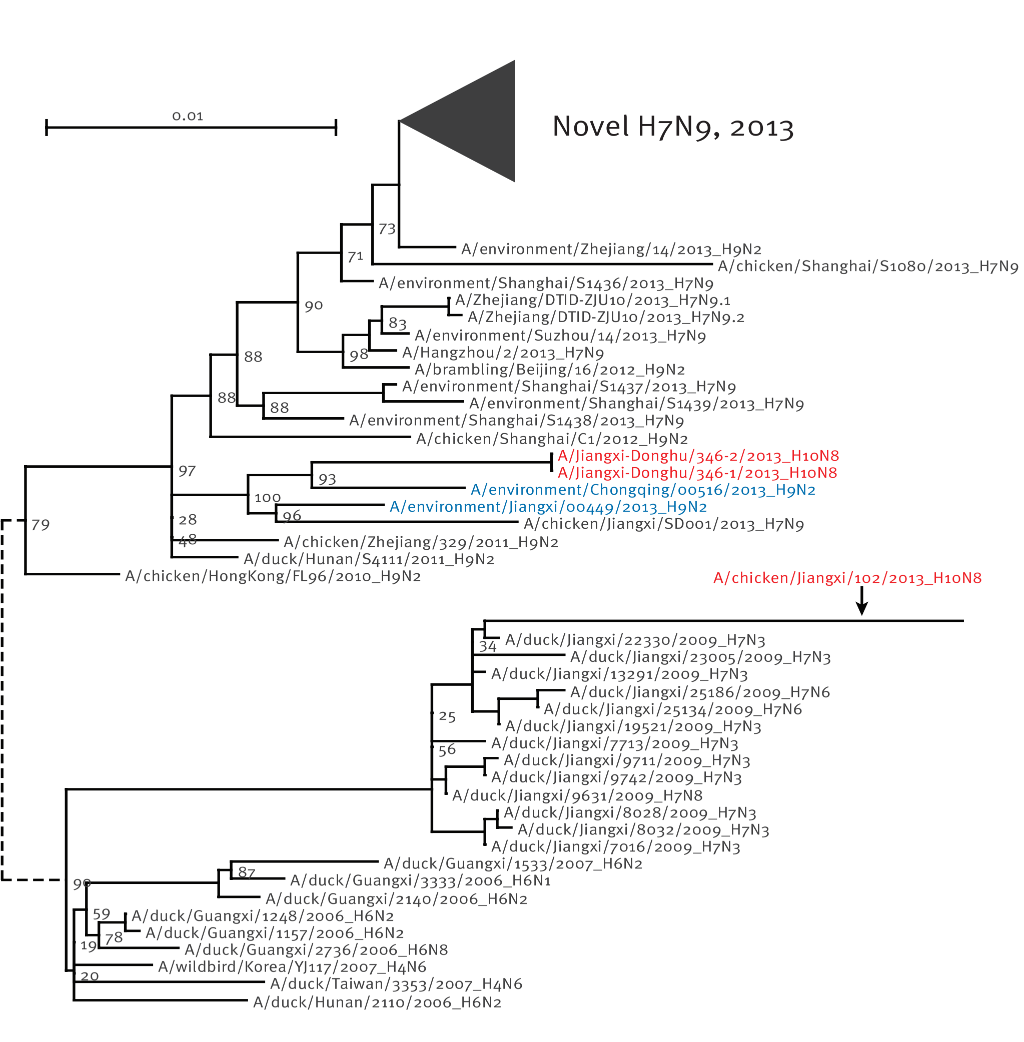
Figure 7

Schematic phylogenetic trees constructed using the PB1 and PB2 gene sequences from the novel influenza A(H10N8) viruses and their closely related sequences searched from public databases

A. PB1 gene sequences



B. PB2 gene sequences



Multiple sequence alignment was performed using MUSCLE (multiple sequence comparison by log-expectation). Phylogenetic analysis was performed using RAxML with the GTRGAMMA model. A total of 1,000 bootstrap replicates were implemented. After the first run, we analysed the phylogenetic trees and selected representative sequences to compose smaller datasets for further phylogenetic analyses. The scale bar shows the length of branch that represents an amount of genetic change. The unit of the bar is nucleotide substitutions per site. The three Nanchang influenza A(H10N8) viruses are shown in red. The viruses shown in blue are important for the reassortment and origination of the three Nanchang influenza A(H10N8) viruses.

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Table 2

Calculated times to the most recent common ancestor for two novel influenza A(H10N8 isolates: chicken isolate (A/chicken/Jiangxi/102/2013) and human isolate (A/Jiangxi-Donghu/346-1/2013)

Gene name	Mean calculated time to the most recent common ancestor (95% confidence intervals)		
	Constant size[a]	Exponential growth[a]	Log-normal growth[a]
HA	23 Oct 2013 (4 Sep 2013–29 Nov 2013)	23 Oct 2013 (2 Sep 2013–29 Nov 2013)	23 Oct 2013 (31 Aug 2013–28 Nov 2013)
NA	28 Jun 2013 (11 Jan 2013–11 Nov 2013)	26 Jun 2013 (11 Jan 2013–12 Nov 2013)	25 Jun 2013 (1 Jan 2013–12 Nov 2013)
PA	6 Sep 2013 (27 Jun 2013–9 Nov 2013)	6 Sep2013 (27 Jun 2013–13 Nov 2013)	5 Sep2013 (21 Jun 2013–7 Nov 2013)
NP	13 Dec 2012 (30 Jun 2012–9 Jun 2013)	19 Dec 2012 (22 Jun 2012–29 May 2013)	13 Dec 2012 (27 Jun 2012–25 May 2013)
M	21 May 2013 (12 Jan 2013–29 Sep 2013)	19 May 2013 (12 Jan 2013–29 Sep 2013)	19 May 2013 (16 Jan 2013–7 Oct 2013)
NS	28 Jun 2013 (18 Jan 2013–29 Oct 2013)	9 Jun 2013 (25 Dec 2012–10 Nov 2013)	11 Jun 2013 (23 Dec 2012–8 Nov 2013)

HA: haemagglutinin; NA: neuraminidase; NP: nucleoprotein; NS: nonstructural protein.

[a] Three different models used to calculate the time to the most recent common ancestor are shown.

Authors' contributions

Conceived and designed the experiments: Wenbao Qi, Xianfeng Zhou, Weifeng Shi, Ming Liao and Mingbin Liu. Epidemiological investigation: Shengen Chen, Lan Cao, Jingwen Wu, Fenglan He, Wentao Song, Hui Li. Performed the experiments: Wenbao Qi, Lihong Huang, Wen Xia, Huanan Li and Qian Li. Contributed analysis: Wenbao Qi, Weifeng Shi, Di Liu and Fumin Lei. Drafted the manuscript: Wenbao Qi and Weifeng Shi. All authors reviewed and revised the first and final drafts of this manuscript. M Liao and M Liu are co-corresponding authors who contributed equally to this article.

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Figure 8

Model of the origin of the novel human-infecting influenza A(H10N8) virus