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Epidemiological and genetic investigations of human-to-human transmission of zoonotic influenza viruses

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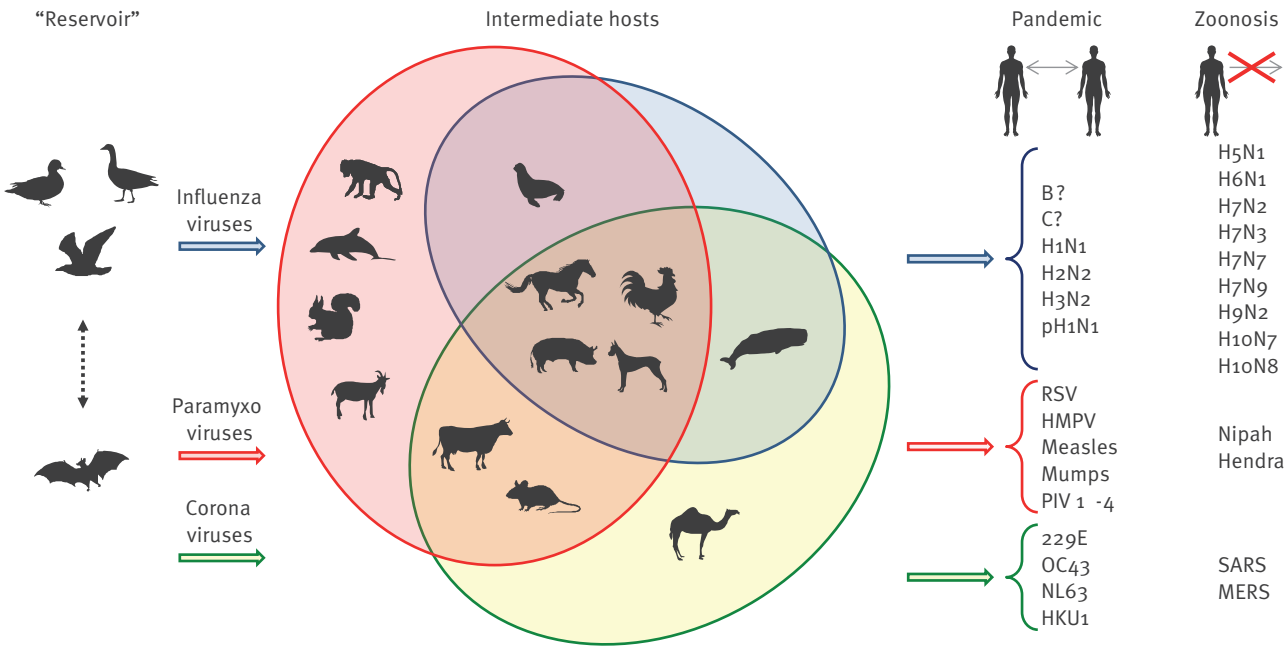
In September 2013, leptospirosis was diagnosed in two Spanish travellers returning from Thailand. The first case walked in floodwater in the Phi Phi Islands in pouring rain: 20 days later he presented with fever and acute hepatitis. The second presented with fever and renal failure 17 days after visiting the islands. These cases remind clinicians to consider leptospirosis in febrile patients with a history of contact with flood or fresh water while travelling to tropical countries.

Of the over 1,400 species of infectious organisms known to be pathogenic to humans, 60% are zoonotic, i.e. transmissible from animals to humans [1]. We therefore continuously face the threat of newly emerging pathogens with a major health impact. In the last decade, viral zoonoses have resulted in numerous human cases of infection with e.g. influenza A viruses, Ebola virus, Nipah virus, Hendra virus, severe acute respiratory syndrome (SARS) coronavirus, and Middle-East

Figure

Wild aquatic birds and bats are thought to be the reservoir for many virus families in nature



HMPV: human metapneumovirus; MERS: Middle-East respiratory syndrome; PIV: parainfluenza virus; RSV: respiratory syncytial virus; SARS: severe acute respiratory syndrome.

Blue: influenza viruses, red: paramyxoviruses, green: coronaviruses. Dotted arrow: The presence of influenza viruses in both species suggests that transmission from birds to bats, or the other way around, may have occurred in the past.

As humans do not frequently come into contact with bats and wild birds, they are more likely to contract zoonotic viruses via intermediate hosts such as domestic birds and mammals. Such zoonotic events almost always result in isolated cases of human infection, as indicated under 'zoonosis'. Rarely, upon mutation or reassortment, these zoonotic viruses adapt to the new human host, acquire human-to-human transmissibility, and may start a pandemic (as depicted under 'pandemic'). In the last century alone, four human influenza pandemics have occurred. Pandemics of paramyxoviruses and coronaviruses are even more rare, yet numerous viruses of animal origin that belong to these families are also endemic in humans

respiratory syndrome (MERS) coronavirus. While these zoonotic viruses often caused severe disease and deaths in humans, they generally did not represent global health threats as the viruses lacked the ability of sustained human-to-human transmission. However, some of these viruses might adapt to replicate in and spread between humans, to cause pandemics. This is true in particular for respiratory viruses associated with recent zoonotic outbreaks that belong to families that also contain viruses that are endemic in humans and thus have been shown to possess pandemic potential in the past (Figure). Monitoring and predicting which of the various zoonotic viruses have the potential to cross the species barrier and emerge in humans globally has become a major topic in infectious disease research.

Wildlife can host an enormous diversity of viruses, which generally do not cause severe disease in these reservoir hosts. Bats have been recognised as reservoir hosts of viruses of the *Filoviridae*, *Paramyxoviridae*, and *Coronaviridae* families [2,3], while wild birds harbour the greatest diversity of influenza A viruses. Recently, close relatives of avian influenza A virus were also found in bats [4]. Occasionally, viruses are transmitted from their reservoir hosts to other animal species, intermediate hosts, from which the viruses may subsequently be more efficiently transmitted to humans, e.g. as a consequence of more frequent contact. Unfortunately, very little is known about the genetic and phenotypic viral and host traits that facilitate interspecies transmission. In addition, knowledge on the mechanisms by which these zoonotic viruses may subsequently adapt to efficient replication and spread in humans is lacking. Recently, progress has been made towards understanding the genetic and phenotypic requirements of avian influenza viruses to become transmissible in ferrets [5-9].

Influenza A viruses are among the most intensively studied viruses when it comes to host range and transmission. The reason is that influenza A virus zoonoses and pandemics occur relatively frequently compared with other virus families. Influenza A viruses have been isolated from many hosts, but wild birds in the orders *Anseriformes* (ducks, geese and swans) and *Charadriiformes* (gulls, terns and waders) as well as bats are thought to form the virus reservoirs in nature (Figure). At least 18 subtypes of the influenza virus surface glycoprotein haemagglutinin (HA or H) and 11 subtypes of neuraminidase (NA or N) have been detected in the reservoirs [4]. In the last century, only influenza A viruses of subtypes H1N1 (in 1918 and 2009), H2N2 (1957), and H3N2 (1968) have spilled over from wild birds to poultry or pigs and subsequently triggered a pandemic, with or without the requirement for prior host adaptation or reassortment (i.e. genetic mixing of gene segments). However, avian influenza viruses circulating in poultry (e.g. of subtypes H5, H6, H7, H9, and H10) have occasionally crossed the species barrier to infect humans, raising concerns of a new pandemic threat. The continuing circulation of highly pathogenic avian influenza (HPAI) H5N1 viruses in poultry in Asia and the Middle East has resulted in hundreds of millions of poultry deaths. Cross-species transmission events of H5N1 virus have been reported for several species of wild birds and mammals, including humans (Figure). However, sustained human-to-human transmission has not yet been described.

A study that appears in the current issue of *Eurosurveillance* by Chea and colleagues investigated two cases of human influenza A(H5N1) virus infection in Cambodia [10]. After the post-mortem diagnosis of influenza A(H5N1) virus infection in a mother and her child, the authors performed a follow-up study of individuals who had been in contact with the two cases. The mother and child were assumed to have been infected from a common poultry source. Although there were no poultry samples available for retrospective laboratory confirmation of H5N1 virus infection, poultry in and around the household had started to die before disease onset of the cases. Respiratory specimens from all contact cases tested negative by RT-PCR for influenza A(H5N1) virus, and no H5N1 antibodies were detected in follow-up sera of contacts. Based on this extensive epidemiological investigation, there was no evidence of human-to-human transmission between the cases or their contacts. The authors conclude that the two cases were most probably exposed to a common source of contaminated environment.

Similar clinical, virological, and epidemiological investigations were performed by Xiao et al. [11] and Hu et al. [12], but for laboratory-confirmed cases of infection with influenza A(H7N9) virus. Since the emergence of the H7N9 virus in February 2013, only one possible human-to-human transmission event had been described [13]. Here, Xiao and colleagues describe the probable transmission of H7N9 virus from a father to his five year-old child after he worked on a wet market contaminated with H7N9 virus [11]. Disease onset in the child was 10 days later than in the father, and virus was detected in the child several days later as well. The child reportedly did not have contact with poultry in the 12 days before onset of the disease. Investigations of 40 close contacts did not result in the detection of additional cases of infection. Hu et al. describe a similar case of human-to-human transmission from a woman who also contracted influenza A(H7N9) virus at a contaminated wet market and probably transmitted the virus to her husband [12]. The man reportedly never visited wet markets and had not purchased or eaten poultry in the two weeks before the onset of his illness. Onset of disease and detection of the virus in the man occurred approximately one week later than in his wife. Again, 27 close contacts were followed up, but they all turned out to be H7N9-negative. These studies thus present two probable cases of human-to-human transmission of H7N9 virus. In both studies, high nucleotide sequence identity between the viruses from the linked patients was in agreement with this assumption. In the first study, the virus could further be linked

genetically to an environmental sample [11]. Although the H7N9 virus has been shown to have some ability of airborne transmission (via aerosol and respiratory droplets) between ferrets [14-18], the human cases are most likely to have occurred through direct contact transmission between family members.

Qi et al. describe the potential origin and genetic diversity of the most recently identified zoonotic influenza virus subtype, H10N8, which was detected in a patient that visited a poultry market a few days before onset of illness [19]. Phylogenetic analysis of the viruses isolated from the patient and the poultry market showed that six genes had the same genetic origin, but the PB1 and PB2 genes (that are part of the viral polymerase complex) were of a different origin. These two genes from the human and chicken virus may be derived from H9N2 chicken and H7 duck influenza viruses, respectively. The genetic differences between the internal genes of the human and chicken viruses suggest that H10N8 viruses continue to undergo reassortment, as reported previously for H7N9 virus. The authors suggest that the H10N8 viruses have become established in poultry and speculate that the diversity of the H10N8 viruses may be much higher than reported so far.

Interestingly, the PB2 gene of the human H10N8 virus contained an E627K mutation, which is a well-known mammalian adaptation mutation associated with virulence and transmission of influenza viruses in mammals, and which was absent in the avian H10N8 strain. This same mutation was also noted in a fifth paper on influenza virus zoonosis in this issue of *Eurosurveillance* by Wang et al. [20]. Based on analyses of large numbers of full virus genome sequences, these authors propose an evolutionary history of H7N9 viruses, involving continuous amino acid substitutions and reassortment events. According to the authors, reassortment events resulted in at least 26 genotypes. Additional host adaptation of H7N9 viruses resulted in the deletion of five amino acid residues from the NA stalk region and changes in the receptor binding site, resulting in increased affinity for human-type receptors. Finally, the well-known mammalian adaptation mutations in the polymerase complex protein PB2 were acquired, which may allow the viruses to replicate more efficiently in the upper respiratory tract of mammalian hosts. The authors conclude that due to this host adaptation process, the pandemic potential of influenza A(H7N9) is higher than that of any other known avian influenza virus. However, it should be noted that the pandemic risk of influenza virus strains may not be equal to their zoonotic risk, and that different influenza virus subtypes or lineages may require different genetic or phenotypic changes to cause zoonoses and pandemics.

Recently, the United States Centers for Disease Control and Prevention developed an inventory of amino acid substitutions in H5N1 viruses linked to critical phenotypic changes. It supports a molecular approach for surveillance and is useful for identifying genetic changes that may affect phenotypic traits of importance such as virulence, (mammalian) host adaptation, polymerase activity, airborne transmissibility etc. [21]. In geographical locations where viruses already have some of the critical mutations associated with mammalian adaptation and transmission, intensified surveillance is key for monitoring the emergence of viruses with potentially high impact. As previously described by Russell et al., deep sequencing of avian, other non-human, and human virus samples, preferably collected at multiple time points, is necessary to better characterise the likelihood of emergence of viruses with an increased public health risk [22]. Additional studies are also needed to better link the genetic changes in influenza viruses of various subtypes with the phenotypic changes that affect within-host fitness and between-host transmissibility. Furthermore, additional experiments are needed to determine the effect of mutations that may affect transmission of newly emerging influenza virus subtypes in humans [22]. Because many substitutions may be functionally equivalent to those described previously, and because the effect of such substitutions may be dependent on the virus backbone, sequence-based virus surveillance alone may be misleading. Therefore, surveillance studies and epidemiological investigations may be improved further by including virus phenotyping assays, using relatively simple methods as described previously for airborne transmissible H5N1 [5].

The five publications in the current issue of *Eurosurveillance* on the potential transmissibility and evolution of influenza viruses highlight the impact of these zoonotic viruses on global health. Surprisingly little is known about the routes and mechanisms of virus transmission. As a consequence of these knowledge gaps, key questions in public health such as "Can newly emerging viruses acquire the ability of human-to-human transmission to trigger a pandemic?" remain unanswered. Increased knowledge is important to predict risks and to adapt pharmaceutical and non-pharmaceutical intervention strategies to prevent outbreaks or pandemics. Currently, we appear to rely on humans as sentinels for virus outbreaks in animals and often apply a reactive rather than pro-active approach to limit the impact of emerging virus infections. Implementation of the One Health concept as an approach to overcome some of the difficulties, including efforts to improve laboratory capacity and surveillance systems in humans and animals, may limit the impact of zoonotic and pandemic threats in the future.

Conflict of interest

None declared

Authors' contributions

Sander Herfst and Ron Fouchier wrote the manuscript.

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Two clustered cases of confirmed influenza A(H5N1) virus infection, Cambodia, 2011

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In February 2011, a mother and her child from Banteay Meanchey Province, Cambodia, were diagnosed, post-mortem, with avian influenza A(H5N1) virus infection. A field investigation was conducted by teams from the Cambodian Ministry of Health, the World Health Organization and the Institut Pasteur in Cambodia. Nasopharyngeal, throat and serum specimens collected from 11 household or three neighbour contacts including two suspect cases tested negative by reverse transcriptase–polymerase chain reaction (RT-PCR) for A(H5N1). Follow-up sera from the 11 household contacts also tested negative for A(H5N1) antibodies. Twenty-six HCW who were exposed to the cases without taking adequate personal protective measures self-monitored and none developed symptoms within the two following weeks. An unknown number of passengers travelling with the cases on a minibus while they were symptomatic could not be traced but no clusters of severe respiratory illnesses were detected through the Cambodian surveillance systems in the two weeks after that. The likely cause of the fatal infection in the mother and the child was common-source exposure in Preah Sdach District, Prey Veng Province. Human-to-human transmission of A(H5N1) virus was unlikely but genetic susceptibility is suspected. Clusters of A(H5N1) virus infection should be systematically investigated to rule out any human-to-human transmission.

Introduction

The first human cases of the ongoing avian influenza A(H5N1) virus epidemic were detected in 2003 in Guangdong, China [1]. Clusters of confirmed human cases have been described in the past in Azerbaijan [2], mainland China [3], Egypt [4], Hong Kong [5], Indonesia [6], Pakistan [7,8] Thailand [9], Turkey [10] and Vietnam [11]. In at least one Indonesian cluster, transmission of A(H5N1) may have occurred through contact with environmentally-contaminated material [12]. However, human-to-human transmission in each of these clusters could not be definitively excluded.

In Cambodia, the first human cases of influenza A(H5N1) were described in 2005 [13]. As of 31 March 2014, there have been 56 confirmed A(H5N1) cases in humans with 37 deaths in 14 provinces and in Phnom Penh, including a mother-and-child cluster described herein [14]. In 2011, two different lineages of influenza A(H5N1) virus co-circulated in Cambodia: lineage 5 which circulated in Cambodia and south Vietnam and lineage 6 believed to be endemic to Cambodia only [15]. Viruses from lineage 6 have also been isolated from other sporadic human and poultry cases (IPC data, not shown).

The event

On 20 February 2011, a fatal influenza A(H5N1) virus infection was laboratory-confirmed by the national influenza center at Institut Pasteur in Cambodia (IPC). The deceased, a child who resided in Banteay Meanchey Province, in western Cambodia, had visited Prey Veng Province in eastern Cambodia, with his parents before the onset of symptoms (Figure 1) [16]. Following the notification, the Rapid Response Team (RRT) of the Ministry of Health (MOH) of Cambodia visited the child's village of residence in western Cambodia and the admitting hospital in Siem Reap Province the following day. His hospital record was reviewed and the child's father, grandmother and healthcare workers (HCW) who had taken care of him were interviewed. Initial investigations revealed that the 19-year-old mother of the child had reportedly died from severe respiratory infection in another district hospital in the same province on 12 February 2011. The RRT subsequently also visited the hospital where the mother had died and obtained clinical information of the mother from hospital staff who provided care before her death. Some stored serum from the mother, retrieved at the hospital and sent to

Figure 1

Map showing the location of the villages in the two provinces targeted by the investigations of two cases of confirmed influenza A(H5N1) virus infection, Cambodia, 2011

IPC, tested positive for influenza A(H5N1) virus antibodies on 22 February 2011.

Additional field investigations were subsequently conducted in Banteay Meanchey and Prey Veng Provinces by staff from the MOH, the World Health Organization (WHO), and IPC to determine the magnitude of the cluster and possible sources of infection, and investigate the possibility of person-to-person transmission. This report summarises the clinical, laboratory, and epidemiological findings of the investigation of this first laboratory-confirmed human A(H5N1) cluster in Cambodia.

Methods

Clinical and epidemiological investigations

Clinical and epidemiological data of the cases were collected by reviewing available medical records at admitting health facilities, and by interviewing household members, relatives, caregivers, other villagers, and HCW in Banteay Meanchey, Siem Reap or Prey Veng Provinces.

Tracing of close contacts was performed by interviewing the household members of the cases and their neighbours in Banteay Meanchey and Prey Veng Provinces, as well as HCW in Banteay Meanchey and Siem Reap Provinces. The purpose was to collect information on types and degrees of exposure to the cases, poultry, and other environmental elements.

Three types of close contacts were defined. Close contacts with the cases referred to those with whom the child had played or those who had taken care of the child and/or the mother for at least 15 minutes and had been within less than one metre distance from the cases while ill. This group included household members (household contacts), neighbours (neighbour contacts) and HCW (HCW contacts). Close contacts with sick or dead poultry referred to those who had touched, carried, defeathered, eviscerated, cleaned or cut any

sick/dead poultry. Close contacts with the cases and with sick or dead poultry were defined as those who met criteria for inclusion in both groups.

Case definition

A suspected influenza A(H5N1) case was defined as a close contact who presented with fever and/or respiratory and digestive signs or symptoms within two weeks after last exposure to the confirmed cases or any sick or dead poultry. A confirmed case was defined as being laboratory-confirmed (see 'Laboratory methods').

Nasopharyngeal, throat, and serum specimens were collected from household contacts and suspect cases in Prey Veng and in Banteay Meanchey Provinces, who agreed to be tested for A(H5N1) infection. Follow-up sera from identified contacts was collected more than three weeks later and tested for A(H5N1) antibody. All identified contacts were monitored for two weeks for development of acute respiratory signs and symptoms after their last exposure.

We defined a cluster of A(H5N1) infections as consisting of at least two symptomatic persons with laboratory-confirmed A(H5N1) infection among household members, relatives or other types of contacts.

As part of urgent public health investigations of human A(H5N1) infections in Cambodia, the national ethics committee was informed by the Ministry of Health and fast-track ethics approval was obtained. All participants in the epidemiological investigations gave their informed consent to be interviewed and tested for A(H5N1) infection, and were informed about their laboratory results and their interpretations.

Laboratory-testing

Laboratory-testing of samples for influenza A(H1N1) collected from field investigations was performed at IPC, the WHO-designated National Influenza Center in Cambodia. Ribonucleic acid (RNA) was extracted from nasopharyngeal, throat and serum specimens and submitted to quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) tests. Serum samples from contacts were tested for A(H5N1) antibodies by hemagglutination-inhibition (HI) assay [17] and microneutralisation (MN) assay in the biosafety level-(BSL) 3 laboratory of the Virology Unit at IPC using the A(H5N1) virus strain isolated from the index case 1. The titre was calculated by the Reed and Muench method [18]. A chemiluminescence-based NA enzyme inhibition assay (NA Star, Applied Biosystems, CA, United States) was used to test the cultured virus for its sensitivity to oseltamivir and zanamivir, as described previously [19].

Laboratory confirmation was defined as a positive PCR and/or virus isolation or a fourfold or greater rise in antibody titre against A(H5N1) in paired sera (acute and follow-up) with the follow-up serum having a titre of 1:80 or higher, or antibody titre of 1:80 or more in a single serum collected at day 14 or later after onset of symptoms, and a titre of 1:160 or greater in HI using horse red blood cells [20,21].

Results

Case descriptions

Case 1

A nine-month-old male infant developed fever and cough in the early morning of 5 February 2011. He and his mother were treated for similar symptoms with over-the-counter paracetamol in a village in Banteay Meanchey Province. Ten days later, on 15 February, he developed dyspnea and was admitted to Jayavarman VII Children Hospital in Siem Reap Province. Clinical evaluation on admission revealed a temperature of 38.2 °C, elevated breathing frequency of 48 breaths/min, oxygen saturation ranging from 93%-99% on ambient air, and bilateral wheezing on lung auscultation. A chest radiograph showed air trapping in both lungs with thickening of bronchi. Complete blood cell count showed normal white blood cells including lymphocytes but low haemoglobin of 7.7 g/dl (norm: 13.5–19.5) and hematocrit of 25% (norm: 44.0–64.0), elevated platelets at 461 x 10^9/L (norm: 150 x 10^9–400 x 10^9) and alanine aminotransferase (ALT) at 95 IU/l (norm: 5–41). Aspartate transaminase (AST) was normal. The child was diagnosed with acute bronchitis, anaemia, malnutrition and possible paracetamol overdose. Despite intravenous amoxicillin/clavulanic acid and nebulised terbutaline and salbutamol, his condition deteriorated rapidly; on 17 February respiratory rate was 68 breaths/min and the child was cyanotic without oxygen supplementation and was transferred to the intensive care unit where he was intubated with handbag ventilation performed by family members. A repeat chest radiograph on the same day showed extensive bilateral infiltrates. Repeat blood tests showed aggravation of anaemia with haemoglobin of 6.6 g/dl and impairment of liver function with ALT at 104 IU/l and normal AST.

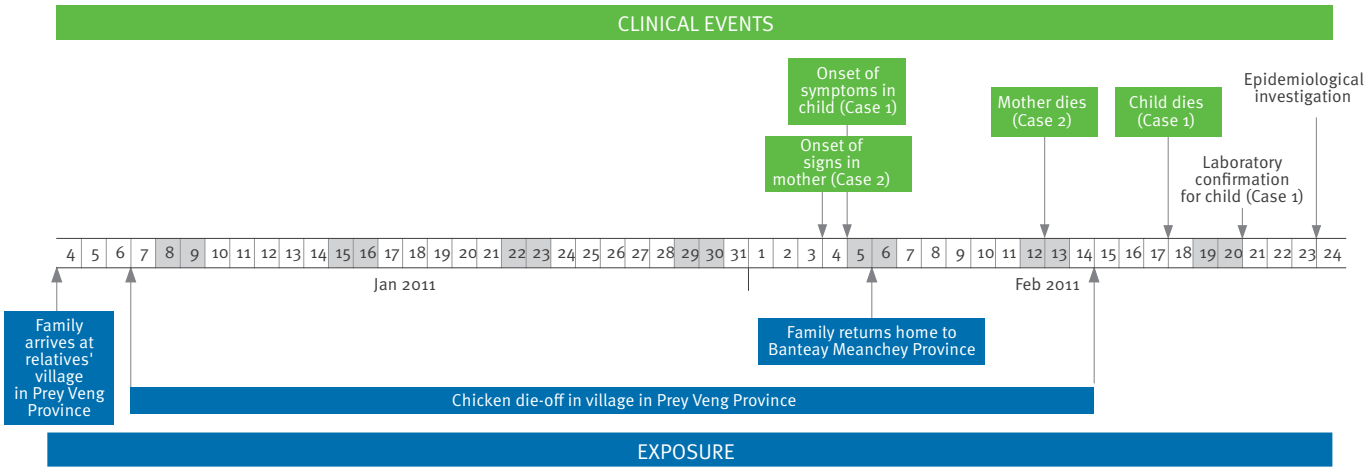
Due to the sudden deterioration of his condition, history of fatal respiratory illness in his mother five days earlier and recent reports of sick and dead poultry in a village in Prey Veng Province where the family visited for a month before onset, A(H5N1) virus infection was suspected. Nasopharyngeal (NP) swabs and serum were collected on 17 February and sent to IPC. Oseltamivir 3mg/kg twice daily was started immediately after specimen collection. However, the child died on the same day only a few hours after transfer to ICU. On 20 February, IPC notified health authorities that nasopharyngeal swabs tested positive for A(H5N1) virus by qRT-PCR.

Case 2

A 19-year-old female, the biological mother of Case 1, developed fever and headache in the evening of 4 February 2011. A day later, she developed cough and mild dyspnea and received over-the-counter

Figure 2

Sequence of exposure and clinical events in two confirmed human cases of influenza A(H5N1) virus infection, Prey Veng cluster, Cambodia, 2011



paracetamol which she took for three days. Still, her dyspnea continued to worsen and on 8 February she was admitted to a private clinic in the capital of Banteay Meanchey Province. On admission, she presented with a temperature of 38.5°C, productive cough, chest pain and shortness of breath. A chest radiograph showed bilateral infiltrates and pleural effusion. She received a third-generation cephalosporin and a chest drain was placed in her right hemithorax. Five days later, on 12 February, she was transferred to a district hospital for severe dyspnea where she died two hours after admission. No respiratory specimens were collected before her death. Stored serum that had been collected on 12 February, was retrieved at the hospital and tested at IPC on 22 February, after samples from Case 1 had tested positive for A(H5N1). The serum of the patient was analysed by qRT-PCR and tested positive for A(H5N1) virus with a viral load of $8.7x10^3$ equivalent cDNA copies/mL.

Exposure histories of cases

On 3 January, the two cases and three other household family members (the child's father, aunt and grandmother) travelled from their home village in Banteay Meanchey Province to spend the Lunar New Year with relatives in a village in Preah Sdach District, Prey Veng Province. From 7 January onwards, poultry in the family and two neighbouring households began to die. By 20 February, all chickens in the three households including the relatives' 20 chickens had died. None of the ducks in any of the households became ill or died (Figure 2).

Case 2 was reportedly present with Case 1 at all times. Case 1 sat, played and crawled on the ground at their relatives' and neigbours' homes. However, none of the cases visited the pond on the homestead or had direct contact with any sick, dead or slaughtered poultry. Both cases reportedly spent time in areas surrounding the three households where chickens had died, including areas contaminated with feathers, feces and discarded waste of sick or dead poultry.

Contact tracing

A total of 48 contacts were identified in Prey Veng, Banteay Meanchey and Siem Reap Provinces (Table), none of them blood relatives of Case 2. Eight were household contacts who had close contact with sick or dead poultry, and two provided home care to Case 2 in the evening of 4 February (for less than 24 hours) after her onset of symptoms. All eight contacts were asymptomatic during the two-week period after their last exposure. A nasopharyngeal swab and acute serum sample were collected from each of the seven household contacts who were present during the investigation on 23 February; samples could not be taken from one contact who was away at school. Follow-up sera were collected on 29 March from all eight household contacts. All tested negative for A(H5N1) virus and anti-A(H5N1) antibodies.

Three other household contacts (father, aunt and grandmother of Case 1) who were also exposed to sick or dead poultry over one month in the village in Prey Veng Province, travelled back to Banteay Mean Chey Province with the cases. These three individuals were the most exposed to the cases after the onset of their symptoms. They cared for them while they were sick, performed hand-bagging ventilation during hospitalisation, and washed Case 2's corpse. However, no acute serum samples or nasopharyngeal swabs were collected from them as they had already left the village in Prey Veng by the time the team started the investigation. Follow-up sera were collected from them on 28 October, as part of another study project. All three said they had been asymptomatic during the period of two weeks following their last exposure and tested negative for anti-A(H5N1) antibodies.

Ten neighbour contacts in Prey Veng Province had repeated direct exposures to sick or dead poultry through touching, carrying, defeathering, eviscerating, cleaning, cutting, or chopping sick or dead chickens. They had no contact with the cases after symptoms onset but had contact with contaminated environments by playing on the ground (for children), dusting or wiping chairs or day beds, clearing trash from the ground contaminated by sick or dead chickens, for more than one month. Eight of them were symptom-free during two weeks after their last exposure to the cases. Two children aged seven and 10 were symptomatic (cough and runny nose) but did not develop severe respiratory disease; both recovered completely after several days. Nasopharyngeal swabs and acute sera were collected from both during the investigation on 23 February, and follow-up sera were collected on 29 March, all of which tested negative for A(H5N1) virus and anti-A(H5N1) antibodies, respectively. No further testing was performed during the cluster investigation in the remaining eight neighbour contacts in Prey Veng as they were not considered exposed to the cases after their symptoms onset.

A total of 26 HCW took care of both confirmed cases in Banteay Mean Chey and Siem Reap Provinces. None of them had any exposure to sick or dead poultry, but had multiple exposures with the cases during their hospitalisations between one to seven days. Of the 26, several had performed high-risk procedures such as endotracheal intubation, nasopharyngeal aspiration or chest drain insertion with no appropriate personal protective equipment (PPE) before A(H5N1) virus infection was suspected. All HCW contacts chose to not get tested and preferred to self-monitor for symptoms for two weeks after their last exposure; none of them developed any symptoms during the observation period.

One neighbour contact in Banteay Mean Chey helped to wash Case 2's corpse; she reported having washed her hands with soap and water after washing the corpse and she was not aware of any exposure to sick or dead poultry. She did not develop symptoms during the two weeks after the last exposure to the case and despite exposure she was not sampled during the initial investigation of the RRT because the investigation team did not return to the village in Banteay Mean Chey for this purpose.

On 5 February, one day after the onset of Case 2's symptoms, the family returned home on a crowded 12-seat minibus with approximately 15 other passengers and both cases developed cough during the 12-hour trip. The fellow passengers were unknown to the family members travelling with the cases. In the absence of passenger lists, contract tracing was not possible.

Table 1

Contact tracing results in cluster of two cases of confirmed influenza A(H5N1) virus infection, Cambodia, 2011

Close contact	Relationship to cases (Province)	Number of contacts		Duration of exposure		Number tested		Results (PCR and/or IgM)
		No symptoms	With symptoms	To sick/dead poultry	To cases after onset	Acute (NP[a] swaps and sera)	Follow-up (Sera only)	
With cases and sick or dead poultry	Household (Prey Veng)	8	0	>1 month	<24 hours	7[b]	8	All negative in first and second testing
	Household (Prey Veng, Banteay Mean Chey)	3[c]	0	1 month	14 days	0	3	
With sick or dead poultry only	Neighbour (Prey Veng)	8	2	>1 month	None	2	0	
With the cases only	HCW (Siem Reap, Banteay Mean Chey)	26[d]	0	None	7 days	n.a.	n.a.	
	Neighbour (Banteay Mean Chey)	1	0	None	Multiple exposure ranging from 1 to 3 days	0	1	
	Passengers on minibus from Prey Veng to Banteay Meanchey	Unknown[e]	Unknown[e]	Unknown[e]	12 hours	n.a.	n.a.	

HCW: healthcare workers; Ig: immunoglobulin; n.a.: not applicable; NP: nasopharyngial; PCR: polymerase chain reaction.

[a] NP swabs tested by PCR.

[b] One contact was at school during the investigation and was not available for sampling.

[c] Father, aunt and grandmother of Case 1 travelled with the cases back to Banteay Meanchey Province, and were exposed for up to 14 days.

[d] HCW chose not to be tested but self-monitor for 14 days instead.

[e] Unknown to cases and, therefore contact tracing was not possible.

Genome analysis of the virus

Whole-genome sequence analysis of the virus isolated from the child (A/Cambodia/Vo219301/20111(H5N1), GenBank accession numbers: JN588806, JN588857, JN 588879, JN588827, JN588926, JN588911, JN588896, JN588863) did not demonstrate any mutation associated with higher virulence or adaptation to human receptors. Phylogenetic analysis of the HA sequence of the virus revealed that the strain isolated from this patient belongs to clade 1.1, lineage 6. This clade had been detected in 19 previous sporadic influenza A(H5N1) cases in Cambodia (IPC, unpublished data). Chemiluminescence-based NA enzyme inhibition assay results demonstrated that the virus was sensitive to both antiviral drugs with IC50 at 0.46 nM and 2.15 nM for oseltamivir and zanamivir, respectively.

Discussion

Since the spread of A(H5N1) avian influenza in Asia beginning in 2003, over 500 confirmed human cases of severe respiratory disease due to A(H5N1) virus infection have been reported [22,23]. The vast majority of these human A(H5N1) infections have been sporadic occurrences involving direct contact with sick or dead poultry through culling, discarding, plucking or preparing food as the likely mode of transmission [2,22,24–26]. However, a small number of confirmed cases reportedly did not have direct poultry contact. In these cases, exposure to environments contaminated by infected poultry, as well as person-to-person transmission in some clusters, could not be ruled out [6,8,9,11,27–29].

Of the 56 laboratory-confirmed cases in Cambodia as of 31 March 2014, the two cases in this report represent the first confirmed human cluster of A(H5N1) infections in this country. Symptoms of respiratory illness in Case 2 began less than 24 hours before onset of illness in Case 1. The incubation period for A(H5N1) virus infection in humans typically ranges from two to five days [24,30] but may be up to 17 days in cases of low-dose exposure such as to live poultry in a market [31]. It was unlikely that Case 2 was the source of infection to Case 1 or vice versa, based on the short interval between symptom onsets of the cases.

Transmission of A(H5N1) virus from patients to HCW or other close contacts, and asymptomatic or subclinical A(H5N1) virus infections are rare but have occurred in the past [32–35]. Despite the presence of symptoms during travel and during home as well as hospital care, there was no clinical or laboratory evidence of person-to-person transmission from Case 1 or Case 2 to neighbours, family members or unprotected HCW. Passengers on the minibus were exposed to both cases for around 12 hours after symptom onset (mild fever and cough). Since they could not be identified follow-up and collection of samples for laboratory-testing was not possible, human-to-human transmission from this cluster to the fellow passengers could not be completely ruled out. However, no cases of severe and acute respiratory infection, suspected or confirmed to be influenza A(H5N1), were reported across Cambodia through the event-based surveillance system during the two weeks after the exposure.

None of the cases had direct contact with sick or dead poultry prior to illness. However, both were present in a village for 33 days during which poultry died. Retrospective laboratory confirmation of A(H5N1) in these poultry could not be performed because poultry remains were not available at the time of the investigation. However, both cases most likely had common exposure to environments contaminated by presumed A(H5N1) virus-infected poultry or their waste. Case 2 likely touched contaminated surfaces, objects placed on the ground, poultry cages or soil, and did not wash hands at all times before eating or touching food, the mouth, nose or eyes. Case 1 probably sat, crawled, or played on the ground shared by the sick or dead poultry and other animals (pigs, dogs and cows). Interviews confirmed that his hands were barely washed before touching his nose, mouth, eyes, or eating. It was also likely that both cases inhaled dust while sitting, playing, crawling, or working on the ground. Direct hand contact with environments or inhalation of dust contaminated by A(H5N1) virus during poultry outbreaks have been previously identified as risk factors for A(H5N1) virus infection in humans in Cambodia [36,37]. However, due to the lack of environmental sampling in this investigation, this route of transmission to the cases could not be confirmed.

Apart from the mother and her child, no other household contacts in Prey Veng Province became infected. Cases and household members were exposed to the same environment; however, different levels of exposure i.e. different amount or concentration of virus in the environment, number of times contacted with the surrounding environment each day could not be verified. As revealed by interviews, Case 2 had much less exposure to sick or dead poultry compared with other household members, and Case 1 had no direct exposure to sick or dead poultry. Yet they were infected while the other household members were not. This may be due to higher levels of infective virus in the environment than that in the sick or dead poultry, which is unlikely. Alternatively, genetic vulnerability could have been a crucial risk factor.

Investigations of earlier clusters have shown that A(H5N1) virus transmission to less exposed individuals may occur, especially to blood relatives of confirmed cases [6–10,13,27–29,38,39] while more intensely exposed individuals remain uninfected or symptom-free [38,40]. This suggests the possibility of genetic susceptibility as a risk factor for infection [41–43].

The virus isolated from Case 1 belongs to clade 1.1, lineage 6, and did not present mutations associated with higher virulence or adaptation to human receptors (SA-α-2,6-Gal).

Conclusion

The carefully conducted epidemiological investigations by the field teams did not bring forward evidence for human-to-human transmission between the cases and their household, HCW or traveling contacts in the Provinces of Prey Veng or Banteay Meanchey. Exposure to contaminated environment, rather than human-to-human transmission or direct contact with sick or dead poultry, was the probable source of infection in this first confirmed mother-and-child cluster of A(H5N1) infections in Cambodia. We are currently conducting contact tracing and seroprevalence studies thanks to funding from the Office of the Assistant Secretary for Preparedness and Response within the US Department of Health and Human Services. A nested genetic study is ongoing to document whether suspected A(H5N1) susceptibility polymorphisms - which could have been a major risk factor of infection in this cluster - are more frequent in cases with confirmed A(H5N1) infection compared to those confirmed negative in Cambodia.

Human cases of A(H5N1) will likely continue to occur in Cambodia. Early suspicion of A(H5N1) virus infection in humans could lead to timely diagnosis and pharmacologic and supportive therapy to prevent transmission and fatal outcomes. Prompt and thorough case investigations are necessary to identify possible routes of infection, including human-to-human transmission of influenza A(H5N1) virus. Close collaboration between clinicians, virologists and epidemiologists, with strong support from national and local health authorities, remains critically important for accomplishing thorough and successful field investigations.

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Conflict of interest

None declared.

Authors' contributions

Nora Chea, Arnaud Tarantola, Nima Asgari: coordinated field investigations of the cluster and wrote the manuscript. Seng Doeurn Yi, Heng Seng, Chuong Penh field investigated the cluster. Sareth Rith, Sek Mardy: tested the specimens from the clusters and contacts. Philippe Buchy: tested the specimens from the clusters and contacts and wrote the manuscript. Touch Sok, Sovann Ly, Paul Kitsutani, Maria Concepcion Roces, Vanra Ieng wrote the manuscript. Denis Laurent, Beat Richner treated the patients and wrote the manuscript.

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Transmission of avian influenza A(H7N9) virus from father to child: a report of limited person-to-person transmission, Guangzhou, China, January 2014

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We investigated a possible person-to-person transmission within a family cluster of two confirmed influenza A(H7N9) patients in Guangzhou, China. The index case, a man in his late twenties, worked in a wet market that was confirmed to be contaminated by the influenza A(H7N9) virus. He developed a consistent fever and severe pneumonia after 4 January 2014. In contrast, the second case, his five-year-old child, who only developed a mild disease 10 days after disease onset of the index case, did not have any contact with poultry and birds but had unprotected and very close contact with the index case. The sequences of the haemagglutinin (HA) genes of the virus stains isolated from the two cases were 100% identical. These findings strongly suggest that the second case might have acquired the infection via transmission of the virus from the sick father. Fortunately, all 40 close contacts, including the other four family members who also had unprotected and very close contact with the cases, did not acquire influenza A(H7N9) virus infection, indicating that the person-to-person transmissibility of the virus remained limited. Our finding underlines the importance of carefully, thoroughly and punctually following-up close contacts of influenza A(H7N9) cases to allow detection of any secondary cases, as these may constitute an early warning signal of the virus's increasing ability to transmit from person-to-person.

Introduction

In February 2013, a novel avian influenza A(H7N9) virus emerged in China [1]. In humans, infection with the virus can result in rapid progressive pneumonia, acute respiratory distress syndrome (ARDS) and even in death [2]. Some young patients (2–4 years-old) nevertheless only develop mild illness or even experience subclinical infections [3,4]. A first epidemic wave of influenza A(H7N9) occurred from February to May 2013, with a total of 132 human infections and 37 deaths reported to the World Health Organization (WHO) [5]. Most cases were sporadically identified in the Yangtze River delta of eastern China [6]. After a period, from June to October 2013 when only four cases were identified [7], the virus re-emerged in the Pearl River delta of southern China in November 2013 resulting in a second epidemic wave. As of 5 April 2014, a total of 98 laboratory-confirmed influenza A(H7N9) cases with 31 deaths, and 411 cases with 145 deaths, have been reported in Guangdong province and mainland China, respectively [8].

Virological research on avian influenza A(H7N9) viruses has demonstrated several characteristic features of mammalian influenza viruses, which are likely to contribute to their infectivity to humans [9]. The amino acid sequence of the receptor-binding site (RBS) of haemagglutinin (HA) determines a preference for human- or avian- type receptors. Mammalian-adapting mutations were found in the RBS of influenza A(H7N9) early strains isolated from Shanghai and Anhui. Furthermore, a T160A substitution in HA of the influenza A(H7N9) virus has been found to result in increased virus binding to human-type receptors [9].

Despite limited person-to-person transmission and few cases of influenza A(H7N9) observed outside China, the further adaptation of influenza A(H7N9) in humans raises serious concerns for a potential pandemic. Previous studies have provided convincing evidence that infected poultry and a contaminated environment might be the key sources of influenza A(H7N9) virus infection in humans [10-13]. However, the risk for a potential pandemic is associated with the person-to-person transmissibility of the virus. Although several family clusters of confirmed influenza A(H7N9) virus

infection were reported previously [2,14,15], only one instance has been strongly suggestive of a possible limited person-to-person transmission of the virus [16]. Clusters of the viral infection may be an early warning sign of the virus adapting to humans, which might result in more efficient person-to-person transmission. In this study, we report the clinical, epidemiological and virological findings of two patients with influenza A(H7N9) in a family cluster identified during the second epidemic wave and provide evidence of a person-to-person transmission of influenza A(H7N9) virus between these two patients.

Methods

Detection and confirmation of influenza A(H7N9) cases

A clinical and laboratory surveillance system for patients with pneumonia of unknown aetiology (PUE), which was designed to identify novel respiratory pathogens like severe acute respiratory syndrome coronavirus (SARS-CoV) and avian influenza A(H5N1) virus, has been in use in China since 2004. All clinical facilities must report any patient lacking a clear diagnosis and meeting the criteria of PUE [17]. Respiratory specimens are collected and sent to the local laboratories of the surveillance network to identify the possible causative pathogens. Since April 2013, these samples are screened for influenza A(H5N1) and A(H7N9), SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) using a reverse transcription-polymerase chain reaction (RT-PCR) assay. The patients reported here were identified using this surveillance system, and the laboratory-confirmed influenza A(H7N9) cases were defined according to the Chinese guidance of diagnosis and treatment for humans infected with avian influenza A(H7N9) [18].

Epidemiological investigation of patients

Since the index case remained in critical condition when the epidemiological investigation was launched and the second case was too young to understand some of the questions, public health staff from the local Centre for Disease Control and Prevention (CDC) interviewed the remaining four family members to obtain basic information, previous health status, daily life schedule, the timeline of disease and possible close contacts of the two patients. We focused on investigating the poultry and animal exposure history prior to the onset of illnesses of the cases to identify a possible source of infection. We also interviewed some of the healthcare workers who provided medical services for these two cases and reviewed all medical records from the hospitals that these two patients visited to clarify the entire course of disease of the two patients.

Infection source tracing

Once the first patient was laboratory-confirmed to be infected with avian influenza A(H7N9) virus, a field investigation was immediately performed to find the possible infection source. To trace possible exposures, we surveyed the household and the residential district where this index case lived and inspected especially the retail wet market, where he was working before the onset of his disease. We gathered basic information on the wet market, such as the location and floor plan, numbers and distribution of poultry stalls, amounts and species of poultry, poultry transportation and unloading procedure, recent diseases in poultry, and how routine disinfection was performed. Twenty-six environmental samples were also collected from all four live poultry stalls of this market on 10 January 2014 to determine whether the market had been contaminated by the avian influenza A(H7N9) virus. These samples included 11 poultry faecal swabs, four chopping block swabs, three scalding machine swabs, two visceral waste swabs, four bloody sewage samples and two poultry drinking water samples.

Medical observation of close contact

All family members who lived with the two cases, relatives and friends who visited these two cases, as well as healthcare workers who had provided medical services to these two cases and been in contact with them within one meter without proper personal protection, from the onset of the patients' illnesses to when the patients were effectively isolated were recognised as close contacts. The investigators interviewed all close contacts to obtain information, including demographic information, previous health status and immunisation, pattern of contact, duration and frequency of contact, and use of personal protective equipment. All close contacts were monitored for respiratory symptoms and fever (≥38°C) for seven days. We also collected throat swabs from all contacts to detect the influenza A(H7N9) viral RNA within 24 hours after the influenza A(H7N9) cases were confirmed. During a seven-day medical observation, throat swab samples were collected repeatedly if any close contact developed influenza-like symptoms.

Identification of causative pathogen

The Universal Transport Medium (Copan Italia) was used to collect the throat swab and environmental samples. The samples were stored at 4°C and transported to the laboratory within two to four hours. Influenza A(H7N9) viral RNA was detected with a real-time reverse-transcriptase polymerase chain reaction (rRT-PCR) assay using pairs of H7- and N9-specific primers provided by the Chinese National Influenza Center as described previously [19]. The detection limit of the rRT-PCR assay was 300 copies/ml, i.e. the viral RNA was considered to be negative when the viral RNA copies in the throat samples were less than 300 copies/ml.

Virus culture, sequencing and phylogenetic analysis

The viruses were isolated using specific pathogen-free (SPF) chicken embryos as described previously [17]. The full length of HA open reading frame (ORF) gene of isolated strains was amplified using two pairs of primers that we designed (15F-5'-AGC AGG GGA TAC AAA

ATG AAC ACT C-3'/753R-5'-TGT ATC ATT GGG ATT TAG CAT TAG CC-3' and 588F-5'-AAC TGC AGA GCA AAC CAA GCT ATA T-3'/1689R-5'-CCA AAC TTA TAT ACA AAT AGT GCA CCG-3') and the OneStep RT-PCR Kit (Invitrogen, USA). The PCR products were purified and sequenced by Life Technologies Inc. using the same primers described above. The obtained HA sequences were sent to the GenBank database whereby the sequences from the two cases were deposited under accession numbers KJ415822 and KJ415823 respectively, while the HA sequence derived from environmental samples collected in the wet market was given accession number KJ415824. We performed multiple sequence alignments and constructed the phylogenetic tree with MEGA 6.0.6 using a neighbour-joining method with 1,000 bootstrap replicates.

Serological test

Paired serum samples were collected from the two cases and the 40 close contacts one week (10–16 January) and four weeks (15–16 February) after they were recruited, respectively. All serum samples were treated with receptor-destroying enzyme (Denka Seiken) for 18 hours at 37°C and then inactivated for 30 min at 56°C. The haemagglutination inhibition (HI) assay was performed using four HA units of inactivated strain A/Guangzhou/1/2014(H7N9) and 1% horse red blood cells to evaluate the H7-specific antibody titres according to the WHO's protocol [20]. The detection limit was 1:10 dilution of the serum samples, i.e. the samples lacking HI activity at 1:10 dilution were considered to be negative.

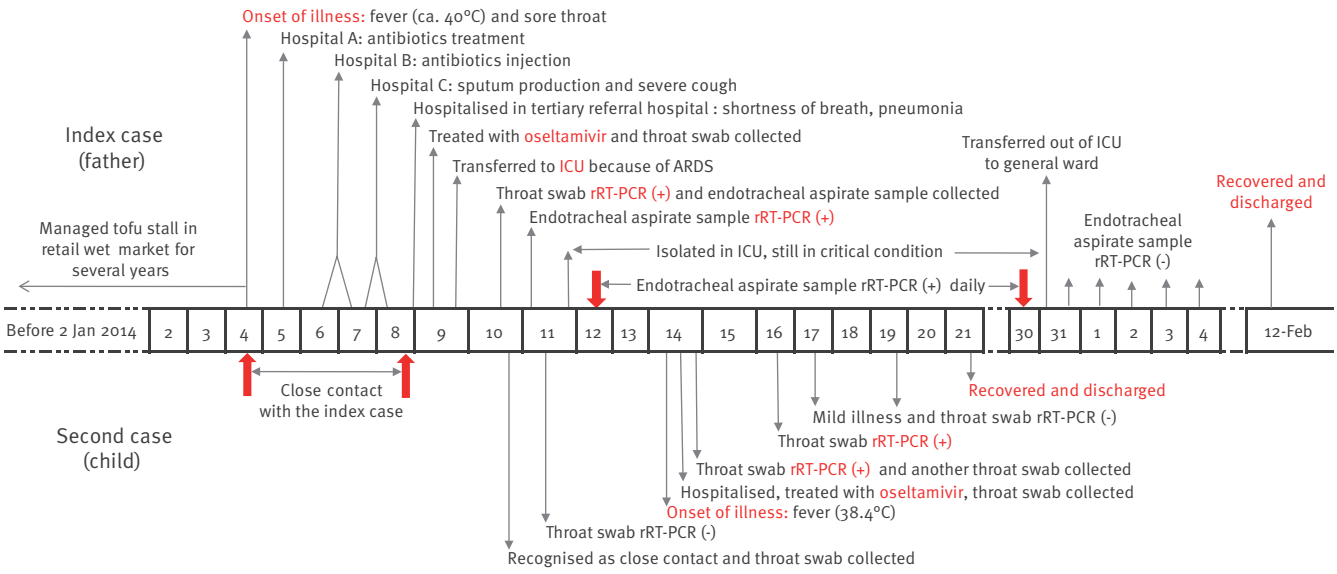
Results

Description of the cases

The family of six members was living in Guangzhou, the capital city of Guangdong province, whose population exceeds 12.7 million (2010 census data). As summarised in Figure 1, the index case, the father in his late 20ies, who had no history of any chronic disease, developed a high fever (approximately 40°C), accompanied by a sore throat and myalgia on 4 January 2014. From 5 to 7 January he visited three local hospitals (hospital A, B and C). At hospital A, he was prescribed oral ciprofloxacin to be taken for two days. He took these on the same day he visited the hospital as well as the next day at home. At hospitals B and C, he was administered ceftriaxone intravenously for two days. The symptoms nevertheless progressed and he experienced a constant high fever, severe cough with heavy sputum and progressive shortness of breath. The patient was therefore admitted to a tertiary referral hospital, and a chest X-ray examination revealed bilateral lower lobe pneumonia on 8 January. Although oral oseltamivir therapy (75 mg twice daily) was started on 9 January, the disease continued to progress, and the patient was transferred to the intensive care unit (ICU) with a diagnosis of viral pneumonia and ARDS. Throat swab and endotracheal aspirate samples collected on 9 and 10 January, respectively, were confirmed to be positive for influenza A(H7N9) viral RNA by rRT-PCR (the Ct values were 30.13 and 28.46, respectively). Thus, the patient was diagnosed with an influenza A(H7N9) virus infection on 10 January. His subsequent specimens were positive for influenza A(H7N9) until

Figure 1

Timeline of disease in two laboratory-confirmed cases of a family cluster of influenza A(H7N9) in Guangzhou, China, January 2014



ARDS: acute respiratory distress syndrome; ICU: intensive care unit; rRT-PCR: real-time reverse-transcriptase polymerase chain reaction.

30 January. On 31 January, the patient was transferred and isolated in an general ward, and his specimens continued to be negative for influenza A(H7N9) until 4 February. On 4 February, the treatment with oseltamivir, which had been continuous since 9 January was stopped. The patient was discharged from the hospital on 12 February after he had fully recovered.

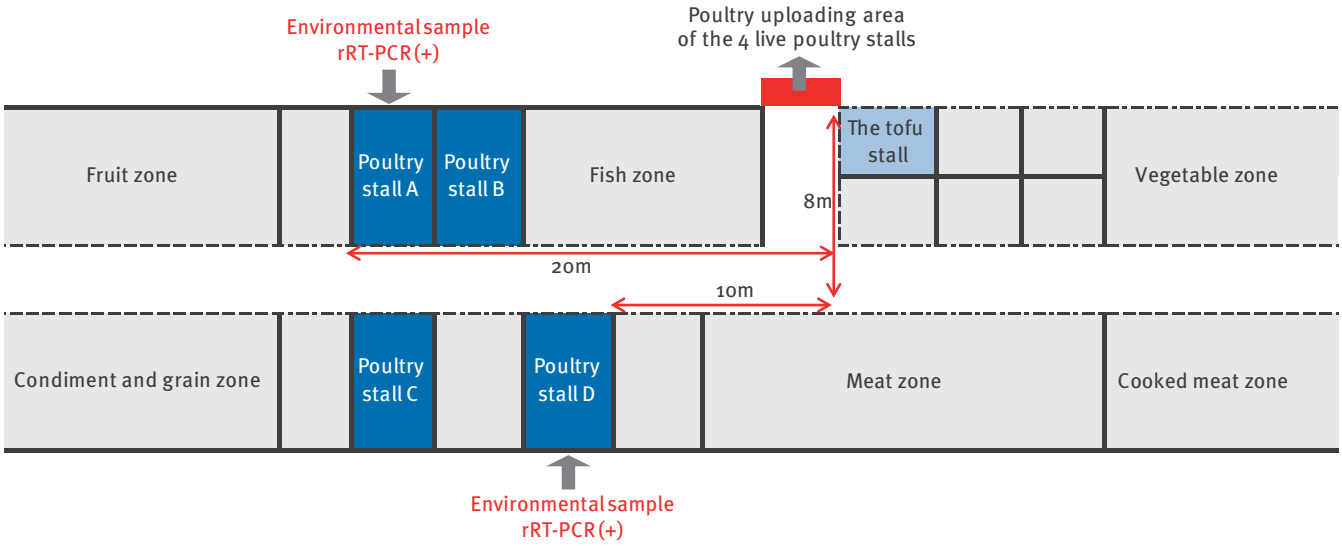
The second patient, the elder child of the index case who was five years-old and healthy without chronic diseases, did not show any influenza symptoms when the father was laboratory-confirmed with influenza A(H7N9). The first throat swab collected on 10 January was negative for influenza A(H7N9) viral RNA, but the child developed a fever (38.4°C) accompanied by cough and fatigue on 14 January. A throat swab sample collected on this day was positive for influenza A(H7N9) viral RNA (Ct value: 31.23). Thus, according to Prevention and Control Protocol for Human Infections with Avian Influenza A(H7N9) (China CDC, 2014), whereby all confirmed cases must be hospitalised in isolation and treated, the child was admitted to an isolated ward of a designated hospital and given oral oseltamivir therapy (45 mg twice daily). A further throat swab sample collected on 16 January was still positive for influenza A(H7N9) viral RNA (Ct value: 32.55) and the patient did not show any other co-morbidities. After 16 January, body temperature returned to normal, and the symptoms subsided. After two throat swab samples collected on 17 and 19 January were negative for influenza A(H7N9) viral RNA, the continuous treatment with oseltamivir from 14 January was stopped and the child was discharged from the hospital on 21 January 2014 with full recovery.

Epidemiological investigations of the two cases

The index case lived with his parents, wife and children, in a seven-story building located in a crowded community. He lacked direct contact with known sick persons, and his family did not buy and cook any poultry in the 12 days prior to the onset of illness. According to our investigation, no poultry, birds or pigs were raised in their residential district within a range of approximately 100 metres. However, he and his wife had been working in a tofu stall located in a retail wet market for several years. He worked at his stall for approximately 11 hours per day (from 7 am to 1 pm and 4 pm to 9 pm) and otherwise remained at home every day before 4 January. As shown in Figure 2, four live poultry stalls (A, B, C, D) are located within a 20-metre distance of the tofu stall in this market. Notably, the distance between the poultry unloading area of these four live poultry stalls and the index case's tofu stall was only approximately two metres. Small trucks transporting live poultry were parked near the tofu stall, and the live poultry were unloaded and moved to live poultry stalls by passing the tofu stall every morning for about an hour when the index case was working. Among the 26 environmental samples collected from these four live

Figure 2

Floor plan of the wet market where the index case of a family cluster of influenza A(H7N9) worked, Guangzhou, China, January 2014



rRT-PCR: real-time reverse-transcriptase polymerase chain reaction.

Locations of four live poultry stalls, unloading area of live poultry in the wet market, as well as the tofu stall, where the index case and his wife worked in the past four years are indicated. Twenty-six environmental samples, including 11 poultry faecal swabs, four chopping block swabs, three scalding machine swabs, two visceral waste swabs, four bloody sewage samples and two poultry drinking water samples, were collected from all four live poultry stalls of this market on 10 January 2014. A chopping block swab of stall A and a bloody sewage sample of stall D were positive for influenza A(H7N9) viral RNA.

poultry stalls, one chopping block swab sample from poultry stall A and one bloody sewage sample from poultry stall D were identified as positive for influenza A(H7N9) viral RNA.

The second case, the index case's five-year-old child, was taken care of by the grandparents, who were well aware of the child's daily activities. They confirmed that the child neither visited any wet market nor had contact with any poultry or birds in the 12 days prior to the child's disease onset. However, from 4 January when the father developed symptoms and had to rest at home, to 8 January, when he developed a severe cough with heavy sputum and progressive shortness of breath that resulted in his admission to hospital, the child had very close and unprotected contact with the sick father every day, such as embracing, kissing, playing, talking face to face, watching television, eating and taking naps together.

Close contacts monitoring

A total of 40 close contacts of the two cases, including the other four family members, three additional relatives, four friends, 24 healthcare workers and five patients at the tertiary referral hospital (Table), were recruited for medical monitoring. The younger sibling of the index case, who visited Guangzhou during the 4 to 8 January period, and the other four family members had very close contact with the two cases. Specifically, the index case's wife provided unprotected care to the index case at home and even bedside care at the hospital until the index case was transferred to the ICU. We collected throat swab samples from the index case's wife daily from 10 to 21 January, but all 12 throat swab samples collected were negative for influenza A(H7N9) viral RNA.

During the seven days of medical observation, fever, conjunctivitis, diarrhoea and respiratory symptoms were not detected in any of the 40 close contacts. Throat swab samples (at least two samples were collected from four family members and the index case's younger sibling who had very close contact with the patients) from these close contacts were also negative for influenza A(H7N9) viral RNA. The results of the HI assay showed that the paired serum samples of all 40 contacts were negative, i.e. the influenza A(H7N9) virus HI titre was lower than 10 (Table).

In addition, we expanded our medical monitoring to 15 occupational poultry workers of the four live poultry stalls at the wet market. None of these workers showed abnormal symptoms during the seven-day medical observation, and all 15 throat swabs collected from these poultry workers were also negative for influenza A(H7N9) viral RNA. We also conducted a serological investigation of these poultry workers and found their paired serum samples to be all negative.

Haemagglutinin gene sequencing and phylogenetic analysis

The influenza A(H7N9) virus was isolated from the throat swab samples collected from the index case (A/Guangzhou/1/2014(H7N9)), the second case (A/Guangzhou/2/2014(H7N9)) and the environmental samples from the wet market (A/environment/Guangzhou/1/2014(H7N9)). The sequencing results of the full length HA ORFs of these three isolated strains showed that the genetic sequences were identical (Figure 3). Moreover, the positions of the cleavage site, RBS and glycosylation motifs did not differ from those of the influenza A(H7N9) virus strains isolated during the first outbreak of avian influenza A(H7N9) virus in eastern China [1-4, 8-14]. However, the similarity of the HA gene was 99.57% between these three isolates and a strain isolated early from chickens in Guangzhou (Figure 3). These virological results strongly supported that the index case acquired the influenza A(H7N9) virus infection in the contaminated environment of the wet market where he worked, and subsequently transmitted the virus to his elder child at home.

Discussion

In the second wave of the influenza A(H7N9) outbreak in China, a family cluster of two influenza A(H7N9) cases was identified. The index case was the first case of influenza A(H7N9) virus infection in Guangzhou because no influenza A(H7N9) case had been previously identified in Guangzhou during the first epidemic wave. He most likely acquired the infection directly from the contaminated environment of the wet market where he worked. This assumption is based on the following findings: (i) he was not exposed to sick persons and had no direct contact with live poultry during the 12 days prior to his disease onset; (ii) the environment of the wet market, where he worked for approximately 11 hours every day prior to his disease onset, was shown to be contaminated by influenza A(H7N9) virus; and (iii) the sequence of the HA gene of the virus strain isolated from the environmental sample completely matched that isolated from this index patient. In fact, 65% (15/23) of influenza A(H7N9) confirmed cases in Guangzhou had a history of wet market exposure, suggesting that contaminated wet markets might be the main source of influenza A(H7N9) virus infection (data not shown). In contrast, the second case, the five-year-old child of the index case, likely acquired the influenza A(H7N9) virus infection from the sick father because of the following facts: (i) the child had not visited any wet market and lacked contact with poultry and birds during the 12 days prior to disease onset; (ii) the child had unprotected and very close contact with the sick father, even after the father's disease onset; (iii) the first throat swab sample collected from the child six days after the father's disease onset was negative for influenza A(H7N9) viral RNA, but the second throat swab sample collected six days after the last exposure to the sick father was positive for the viral RNA; and (iv) the HA gene of the virus strains isolated from father and child were identical.

Table

Information on close contacts of two cases of a family cluster of influenza A(H7N9), Guangzhou, China, January 2014

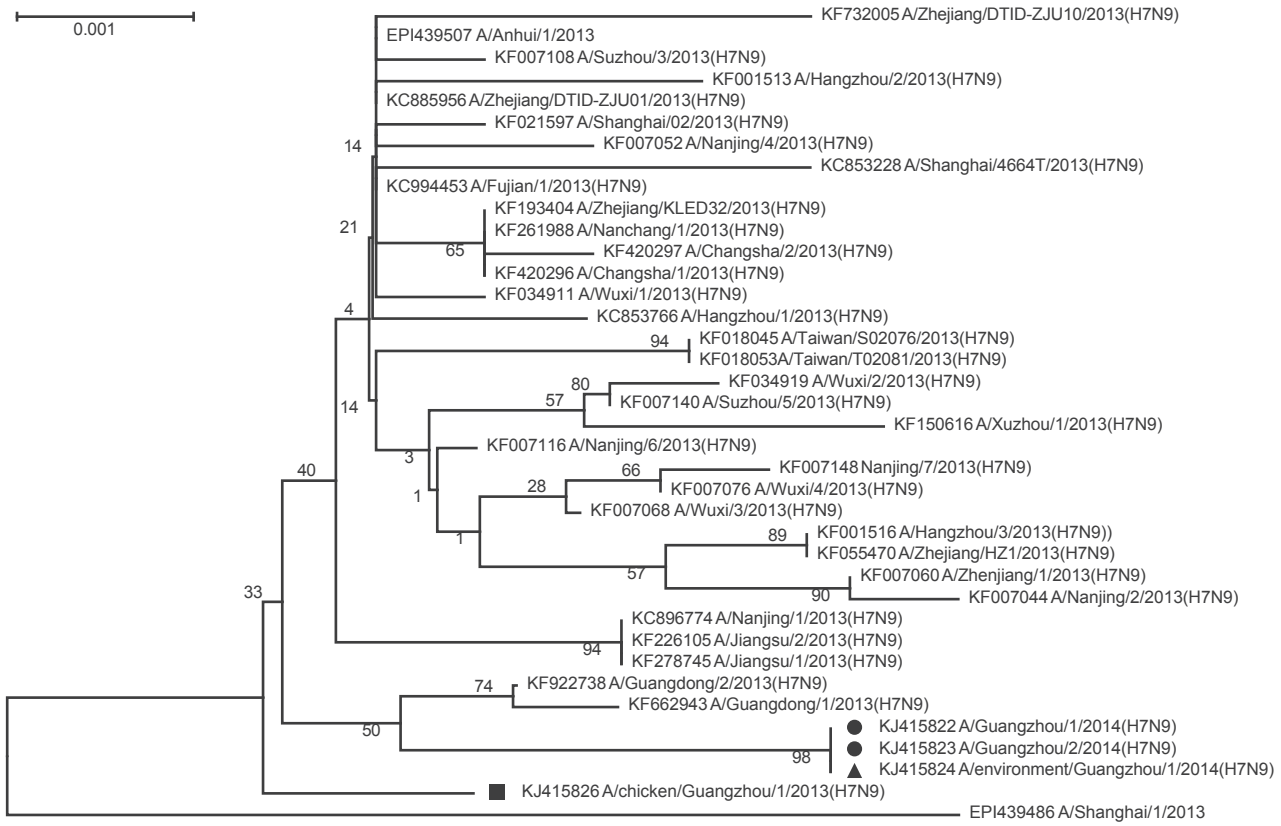
Classification of close contacts	Relations with the index case/ approximate age (years)	Contact with which case	Description of contact	Result of HI assay[a]	
				Date of sampling /titre	Date of Sampling /titre
Five family members of the index case	Wife/late 20s	Both	Provided close care at home and unprotected bedside care at the hospital to the index case; Provided conventional care at home to the second case	10 Jan / <10	15 Feb / <10
	Mother/early 60s	Both	Provided conventional care at home the both cases	10 Jan / <10	15 Feb / <10
	Father/late 60s	Both	Family daily life contact with both cases	10 Jan / <10	15 Feb / < 10
	Child/5	Index case	Family daily life contact with the index cases	10 Jan / <10	15 Feb / 20
	Child/ <5	Both	Family daily life contact with both cases	10 Jan / <10	15 Feb / < 10
Three relatives of the index case	Cousin/early 20s	Index case	Worked with the index case and accompanied him to seek medical care	10 Jan / <10	15 Feb / < 10
	Younger sibling/early 20s	Both	Visited the index case after his hospitalisation; Provided conventional care at home to the second case	10 Jan / <10	15 Feb / < 10
	Uncle/late 50s	Second case	Family daily life contact with the second case	16 Jan / <10	15 Feb / < 10
Four friends of the index case	One friend/late 40s	Second case	Talking and playing with the second case	16 Jan / <10	16 Feb / < 10
	Three other friends/mid to early 30s	Index case	Visited the index case after his hospitalisation	11 Jan / <10	16 Feb / < 10
Twenty-four healthcare workers	Ten staff of hospital A/mid-40s to mid-70s	Index case	Provided various medical services without effective personal protective equipment	11 Jan /<10	16 Feb / < 10
	Two staff of hospital B/early 40s to early 60s			11 Jan / <10	16 Feb / < 10
	Two staff of hospital C/early 20s			11 Jan / <10	16 Feb / < 10
	Ten staff of tertiary referral hospital/ early 20s to mid-50s			10 Jan / <10	16 Feb / < 10
Five patients of tertiary referral hospital	Roommates/early 40s to early 80s	Index case	Shared the same room with the index case before his isolation	10 Jan / <10	16 Feb / < 10

HI: haemagglutination inhibition.

[a] Paired serum samples were collected on 10–16 Jan (week 1) and 15–16 Feb (week 4), respectively.

Figure 3

Phylogenetic analysis of haemagglutinin (HA) genetic sequences obtained in the investigation of a family cluster of avian influenza A(H7N9), Guangzhou, China, January 2014 (n=38 sequences in total in the phylogenetic tree)



● HA genes of two influenza A(H7N9) virus strains isolated from two patients reported in this manuscript
▲ HA gene of the virus strain isolated in this study from environmental samples collected in the wet market (Figure 2)
■ Virus strain isolated from chicken earlier at a wet wholesale poultry market of Guangzhou

Bootstrap values are indicated on the tree nodes.

Several family clusters of confirmed avian influenza A(H7N9) virus infection have been reported previously [2,14-16]. Compared to these reports, our investigation of this family cluster has provided clearer and more solid evidence that the second case acquired the infection of influenza A(H7N9) virus from the sick father. The influenza A(H7N9) virus can reportedly bind to both avian-type (α 2, 3-linked sialic acid) and human-type (α2, 6-linked sialic acid) receptors [21] and replicate efficiently in mammals and human airway cells [22,23], which suggest that the virus possesses the potential for person-to-person transmission. In fact, limited person-to-person transmission of other subtypes of avian influenza, such as influenza A(H7N7) [24] and A(H5N1) [25-28], has been reported previously. Moreover, more than 90% of secondary person-to-person transmission of influenza A(H5N1) virus occurred in blood-related persons, especially in first-degree blood-related persons [29], suggesting a genetic basis for the susceptibility to influenza A(H5N1) virus infection. Similarly, our study and those previously published [2,14-16] have also shown that the person-to-person transmission of influenza A(H7N9) virus occurred in closely related persons. Cases of influenza A(H7N9) transmission between genetically unrelated persons have not yet been reported. Fortunately, the person-to-person transmission of influenza A(H7N9) has been very limited thus far. In this study, the other four family members, i.e. the parents, wife and younger child of the index case, did not acquire the infection with influenza A(H7N9) virus, although they had unprotected and very close contact with him. Moreover, among these family members, the index case's parents and other child, who were genetically closely related to him, were not infected by the virus.

Notably, the symptoms were much milder in the second case than in the index case, and the throat swab samples of the second patient were positive for influenza A(H7N9) viral RNA for only three days after this child's disease onset, while those of the index case were positive for approximately 22 days after his disease onset. In light of the fact that milder disease has

been reported for some young children, the outcome of influenza A(H7N9), in function of age warrants further observation. The symptom duration and virus persistence in patients who acquired the infection from other avian influenza patients may moreover be an index to monitor the adaptation of the virus for infection and growth in humans. In the event reported here, the genetic sequencing data of the virus isolates from the two cases showed no adaptive changes in the RBS and glycosylation motifs of HA. Although we did not investigate internal genes, previous studies on influenza A(H7N9) viral sequences causing human infections only point to the E627K mutation of the polymerase subunit PB2, as potentially contributing to transmission among humans [9]. Thus, the influenza A(H7N9) virus might not yet be well adapted for human infection, and the risk for community-level spread of this virus is still considered to be very low.

In summary, our clinical, epidemiological and virological findings strongly implicate that the contaminated live poultry market might be the most possible source of influenza A(H7N9) virus infection for the index case. As a tofu seller, the index case had a good habit of hand hygiene. However if sufficient contaminated aerosolised materials were inhaled by this case, airborne transmission of influenza A(H7N9) virus could have occurred. Remarkably, our study indicated that the second case most likely acquired the virus from the index case. Fortunately, the investigation also suggested that the person-to-person transmission of influenza A(H7N9) was still highly limited. However, the influenza A(H7N9) virus reportedly continues to undergo reassortment [30], the strict surveillance of the influenza A(H7N9) virus infection, including monitoring the symptoms and viral load in patients who acquired the infection from person-to-person transmission and genetically unrelated persons who have had close contact with patients, should be continued.

Acknowledgments

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Conflict of interest

None declared.

Authors' contributions

Ming Wang and Zhicong Yang conceptualised the study design and supervised the study. Ming Wang was responsible for epidemiological survey, Xincai Xiao, Zongqiu Chen, Jun Yuan, Shuanglang Ye, Hui Liu and Jianyun Lu participated in field investigation and sample collection. Hongbing Luo, Zhi Nie and Xiaoping Tang acquired clinical data, Zhicong Yang,Kuibiao Li and Biao Di performed the laboratory testing, including rRT-PCR, genome sequence and phylogenetic tree analysis. Xincai Xiao, Zongqiu Chen and Bojian Zheng drafted the manuscript and other co-authors contributed to review and approved the final version.

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Limited human-to-human transmission of avian influenza A(H7N9) virus, Shanghai, China, March to April 2013

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In April 2013, two members of one family were successively confirmed as cases of avian influenza A(H7N9) virus infection in Shanghai, China. Respiratory specimens from the two cases and their close contacts were tested using real-time reverse-transcription (RT)-PCR. Paired serum specimens from contacts were tested by haemagglutination inhibition assay and microneutralisation test. The index patient developed severe pneumonia. Her husband presented with pneumonia shortly thereafter. Both cases had highly similar clinical features and infection with A(H7N9) virus was confirmed in both cases by genetic analysis. Phylogenetic analysis revealed a high level of similarity between the sequences from the two patients and environmental samples collected from wet markets in Minhang and Changning districts. Six samples from the Changning wet market were confirmed as A(H7N9) positive. Of 27 close contacts, one developed mild respiratory symptoms and another tested positive for A(H7N9) antibodies, but both were negative by real-time RT-PCR. The other 25 close contacts of both cases were A(H7N9) negative. Limited human-to-human transmission of the virus most likely occurred in the family cluster. However, other close contacts did not test positive for the virus, suggesting limited potential for extensive human-to-human transmission of the virus.

Introduction

A novel influenza virus was isolated from two fatal human cases in Shanghai, China in March 2013 [1]. Genomic sequencing indicated that the recombinant virus belonged to the influenza A subtype H7N9. Low pathogenic avian influenza A(H7N9) virus infection occurs in animals and is mostly asymptomatic [2] but had not been previously reported in humans [3]. Illness caused by this novel virus in humans is characterised by a sudden onset of high fever (≥39 °C) and respiratory symptoms, followed by a progressive decline in lung function. The global impact of influenza A(H7N9) is not yet well understood and a pandemic was feared as the number of cases continued to increase in China [4].

Past epidemiological evidence has demonstrated that infection with avian influenza A(H5N1) virus may occur by handling or coming into contact with infected poultry or birds [5]. Unprotected exposure to critically ill patients may have led to human-to-human transmission of A(H5N1) virus in Thailand in 2004 [6,7]. Familial clustering of A(H5N1) virus infection was also observed in Jiangsu Province, China in 2007 [8]. As for A(H5N1), human-to-human transmission of A(H7N9) virus merits further study.

Three family clusters of A(H7N9) virus infection were reported in 2013: all cases were blood relatives [9,10]. Due to the limited number of cases, possible human-to-human transmission could not be ruled out. More and more cases have been reported in 2014 in Zhejiang, Guangdong, Jiangsu and Shanghai provinces of China, indicating that A(H7N9) viruses were circulating asymptomatically among natural hosts and that the transmission route of this virus is complex [4,11]. As of 17 June 2014, 247cases have been reported in 2014 [12]. Evidence of human-to-human transmission is important for making recommendations on vaccination and treatment, as how readily the virus moves between people determines what kind of preventive measures and policies should be enacted.

We report the results of an investigation into a family cluster of cases with A(H7N9) virus infection in Shanghai, China. A couple who presented with severe pneumonia were hospitalised in April 2013: both were confirmed as A(H7N9) positive. Epidemiological, clinical and laboratory findings are described and provide

clues to the possibility of human-to-human transmission – data vital to the prevention and control of re-emerging human infection with A(H7N9) virus.

Methods

Epidemiological investigation

The Shanghai Municipal Center for Disease Control and Prevention (SCDC) was informed about the index case, on 3 April. Investigative staff from the SCDC interviewed the couple the same day, during Case A's hospitalisation to verify the reported exposure history starting one week before the onset of symptoms and to establish event timelines. A standardised structured questionnaire was used to collect demographic information, information on syndromes and symptoms, epidemiological history (including exposure to poultry and livestock, extent of outdoor activity and poultry-market visits) and information on close contacts. An epidemiological investigation, identification and active surveillance of close contacts were initiated on 3 April immediately once the SCDC had been informed about the index case.

Medical records were also reviewed for the time of symptom onset and progression of the illness. Nasal swabs and pharyngeal swabs from the two patients were collected during their hospitalisation.

Close contacts were defined as individuals known to have been within 1 metre or to have had contact with the serum, respiratory or faecal secretions of a case of A(H7N9) infection without having used effective protective equipment at any time from one day before the symptoms onset of the case to the day the case was placed in isolation or died [13]. The close contacts were monitored twice daily for fever and respiratory symptoms via repeated interviews over the 10 days after their last exposure to the cases. Nasopharyngeal swabs were collected if febrile respiratory symptoms appeared during observation. Paired sera were collected from close contacts during both acute and follow-up phases (≤1 week and 3–4 weeks, respectively, after their last exposure to the cases).

Households and places known to have been visited by the cases in the seven days before the onset of symptoms were surveyed to assess the source of possible environmental exposure. These included the Taiyang (TY) wet market (an open market selling fish, poultry, pork, etc.) in Changning district, which the index case often visited before the onset of symptoms. Because poultry at the TY wet market came from a wholesale market that also supplied Ruili (RL) wet market (in Minhang district), we also collected environmental samples from this wet market.

Faeces samples and tracheal and cloacal swabs were collected from pigeons raised in the community where the patients resided. Sera samples were also collected from some of these pigeons to test serologically for A(H7N9) antibodies.

Environmental samples (including chicken faeces, wastewater from a slaughterhouse, swabs from a chicken cage surface and poultry chopping boards) from the TY and RL wet markets were collected according to the standard operation procedures (SOPs) of the China Center for Disease Control and Prevention (CDC). All samples were placed in sterile viral transport medium at 4 °C and transferred to an SCDC laboratory for A(H7N9) virus detection within two hours.

All investigations were conducted in accordance with the policy approved by the SCDC ethics committee. Informed consent was obtained from patients and close contacts.

Laboratory testing

Total nucleic acid was isolated from each sample (200 µl) using MagNA Pure LC 2.0 (Roche, Switzerland) with the MagNA Pure LC DNA Isolation Kit (Roche, Germany). Real-time reverse transcription (RT)-PCR, to detect influenza A, H7 and N9, was performed according to the SOPs of the World Health Organization (WHO) [14].

In order to explore the genetic relationship between the two cases, total genomic segments were amplified using RNA isolated directly from original specimens as described previously [15]. The A(H7N9)-positive environmental samples collected from the TY wet market in Changning were sequenced simultaneously. On the basis of the threshold cycle value, we also chose one A(H7N9) virus-positive environmental sample, collected from the RL wet market for sequencing. To facilitate the phylogenetic analysis, sequences of novel A(H7N9) viruses from the two members of this family and environmental samples collected in Shanghai in 2013 were downloaded from the Global Initiative on Sharing All Influenza Data (GISAID) EpiFlu database (Table 1). In addition, the standard A(H7N9) strain A/Anhui/01/2013 [1] was also analysed. Sequence similarity analyses were performed with MegAlign method [16] in the Lasergene 7.01 software package. We used MEGA software (version 5.05) [17], multiple alignments by MUSCLE algorithm (a multiple sequence alignment method with reduced time and space complexity) and phylogenetic trees constructed by neighbour-joining method to estimate the viral gene relationship with selected influenza A virus strains obtained from GISAID.

The A(H7N9) antibody titre was determined in paired sera from close contacts using the haemagglutination inhibition (HI) assay from the China CDC [18]. A microneutralisation (MN) test was performed for serum samples with titres ≥40. Two assays were conducted according to WHO SOPs [19,20]; the standard antigen used in the two assays was produced from A(H7N9) isolates (A/AH/1/2013(H7N9)).

Table 1

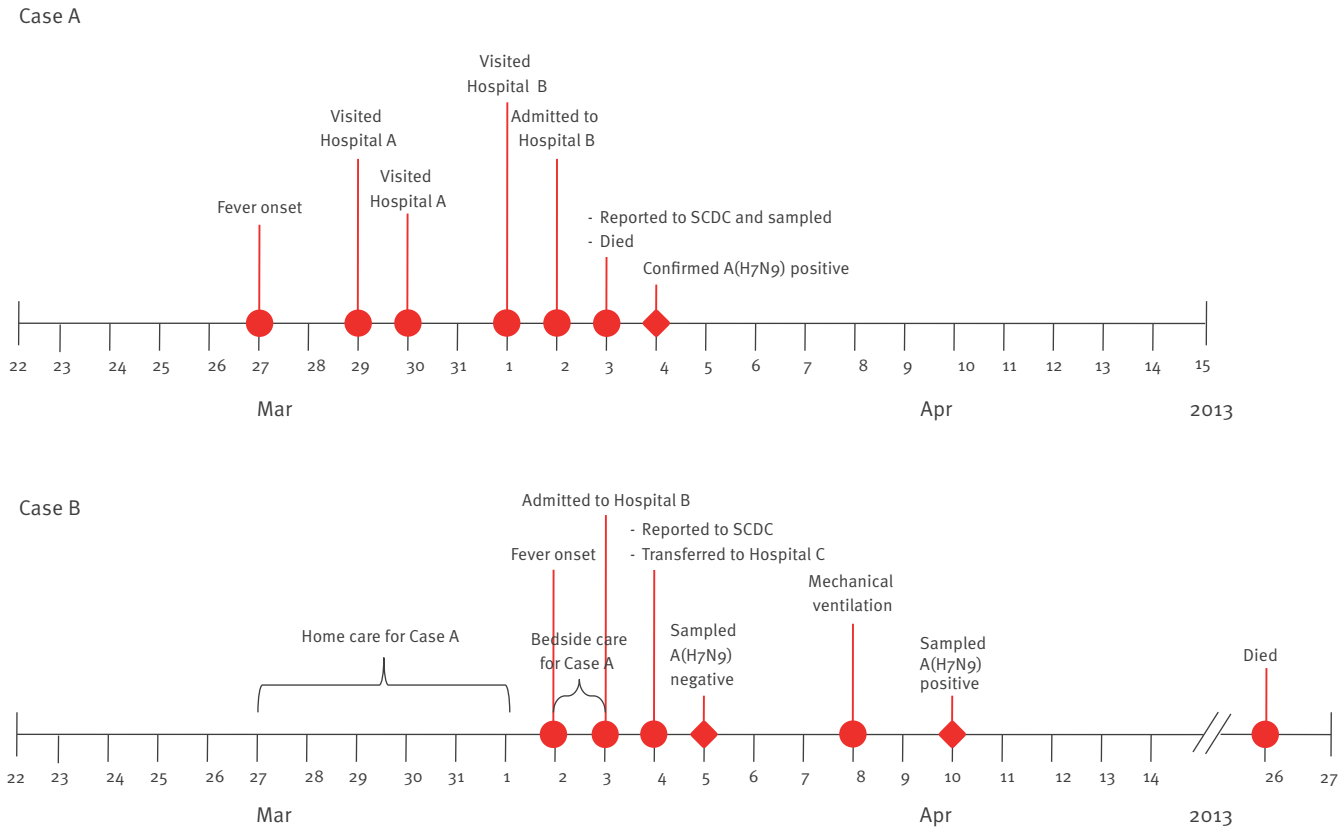
Origin of the haemagglutinin and neuramidinase sequences of influenza A(H7N9) isolates used for the phylogenetic analysis

Isolate ID	HA Segment ID	NA Segment ID	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	Authors
EPI_ISL_142903	EPI457637	EPI457636	China	2013-Apr-03	A/pigeon/Shanghai/S1421/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang, Q., Shi, J., Chen, H, et al.
EPI_ISL_142902	EPI457629	EPI457628	China	2013-Apr-03	A/pigeon/Shanghai/S1423/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang, Q., Shi, J., Chen, H, et al.
EPI_ISL_138985	EPI440701	EPI440700	China	2013-Apr-02	A/Pigeon/Shanghai/S1069/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI_ISL_142908	EPI457677	EPI457676	China	2013-Apr-03	A/environment/Shanghai/S1436/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang, Q., Shi, J., Chen, H, et al.
EPI_ISL_138984	EPI440693	EPI440692	China	2013-Apr-03	A/Environment/Shanghai/S1088/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI_ISL_141177	EPI447616	EPI447730	China	2013-Apr-10	A/Shanghai/16/2013	–	WHO Chinese National Influenza Center	Wang, Dayan; Gao, Rongbao; Shu,Yuelong, et al.
EPI_ISL_141176	EPI447615	EPI447737	China	2013-Apr-09	A/Shanghai/15/2013	–	WHO Chinese National Influenza Center	Wang, Dayan; Gao, Rongbao; Shu, Yuelong, et al.
EPI_ISL_141174	EPI447613	EPI447751	China	2013-Apr-08	A/Shanghai/9/2013	–	WHO Chinese National Influenza Center	Wang, Dayan; Gao, Rongbao; Shu, Yuelong, et al.
EPI_ISL_141173	EPI447612	EPI447758	China	2013-Apr-07	A/Shanghai/8/2013	–	WHO Chinese National Influenza Center	Wang, Dayan; Gao, Rongbao; Shu, Yuelong, et al.
EPI_ISL_141172	EPI447611	EPI447765	China	2013-Apr-03	A/Shanghai/17/2013	–	WHO Chinese National Influenza Center	Wang, Dayan; Gao, Rongbao; Shu, Yuelong, et al.
EPI_ISL_141171	EPI447610	EPI447772	China	2013-Apr-10	A/Shanghai/14/2013	–	WHO Chinese National Influenza Center	Wang, Dayan; Gao, Rongbao; Shu, Yuelong, et al.
EPI_ISL_141170	EPI447609	EPI447780	China	2013-Apr-10	A/Shanghai/13/2013	–	WHO Chinese National Influenza Center	Wang, Dayan; Gao, Rongbao; Shu, Yuelong, et al.
EPI_ISL_141169	EPI447608	EPI447787	China	2013-Apr-10	A/Shanghai/12/2013	–	WHO Chinese National Influenza Center	Wang, Dayan; Gao, Rongbao; Shu, Yuelong, et al.
EPI_ISL_141168	EPI447607	EPI447797	China	2013-Apr-10	A/Shanghai/11/2013	–	WHO Chinese National Influenza Center	Wang, Dayan; Gao, Rongbao; Shu, Yuelong, et al.
EPI_ISL_141167	EPI447606	EPI447804	China	2013-Apr-09	A/Shanghai/10/2013	–	WHO Chinese National Influenza Center	Wang, Dayan; Gao, Rongbao; Shu, Yuelong, et al.
EPI_ISL_141160	EPI447599	EPI447893	China	2013-Mar-18	A/Shanghai/07/2013	–	WHO Chinese National Influenza Center	Wang, Dayan; Gao, Rongbao; Shu, Yuelong, et al.
EPI_ISL_139650	EPI443025	EPI443026	China	2013-Mar-09	A/Shanghai/4/2013	–	WHO Chinese National Influenza Center	Wang, Dayan; Gao, Rongbao; Shu, Yuelong, et al.
EPI_ISL_139649	EPI443022	EPI443023	China	2013-Feb-27	A/Shanghai/3/2013	–	WHO Chinese National Influenza Center	Wang, Dayan; Gao, Rongbao; Shu, Yuelong, et al.
EPI_ISL_138739	EPI439507	EPI439509	China	2013-Mar-20	A/Anhui/1/2013	–	WHO Chinese National Influenza Center	–
EPI_ISL_138738	EPI439502	EPI439500	China	2013-Mar-05	A/Shanghai/2/2013	–	WHO Chinese National Influenza Center	–
EPI_ISL_138737	EPI439486	EPI439487	China	2013-Feb-26	A/Shanghai/1/2013	–	WHO Chinese National Influenza Center	–
EPI_ISL_146876	EPI471834	EPI471838	China	2013-Apr	A/chicken/Shanghai/017/2013	–	Other Database Import	Yang ,D.-Q., Ju, H.-B., Zhou, J, et al.
EPI_ISL_142927	EPI457829	EPI457828	China	2013-Apr-03	A/chicken/Shanghai/S1076/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang, Q., Shi, J., Chen, H, et al.
EPI_ISL_142923	EPI457797	EPI457796	China	2013-Apr-03	A/chicken/Shanghai/S1080/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang, Q., Shi, J., Chen, H, et al.
EPI_ISL_142922	EPI457789	EPI457788	China	2013-Apr-03	A/chicken/Shanghai/S1358/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang, Q., Shi, J., Chen, H, et al.
EPI_ISL_142920	EPI457773	EPI457772	China	2013-Apr-03	A/chicken/Shanghai/S1413/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Zhang, Q., Shi, J., Chen, H, et al.

HA: haemagglutinin; NA: neuramidinase.

Figure 1

Timeline of key events during illness of two cases of influenza A(H7N9) virus infection, Shanghai, China, March–June 2013



SCDC: Shanghai Municipal Center for Disease Control and Prevention.

Results

Cases A and B

Case A, the index case, in this family cluster was a woman in her early 50s who lived in the Changning district of Shanghai with her husband (Case B). The patient was very weak and developed a low-grade fever, chills and arthralgia on 27 March 2013. She took acetaminophen and aminophenazone at home the following day and visited the local hospital (Hospital A) on 29 and 30March. Chest X-ray showed right-lower-lobe pneumonia. She was given a three-day prescription of ceftazidime and levofloxacin for symptomatic and anti-infection treatment. However, she visited a second hospital (Hospital B) with a temperature of 39.5 °C on 1 April. Initial testing showed that her white blood cell count was normal; she received further antibiotic therapy with ceftriaxone and ofloxacin. She was admitted the following day with a diagnosis of severe pneumonia and acute respiratory distress syndrome (ARDS). Tests upon admission revealed leucopenia and hypoxaemia. A lung computerised tomography scan showed diffuse bilateral lung infiltrates associated with right pleural effusion. Despite endotracheal intubation and administration of oseltamivir (75 mg twice a day via a nasogastric feeding tube, a total of two doses of 75 mg before her death) and corticosteroids, the patient died on 3 April.

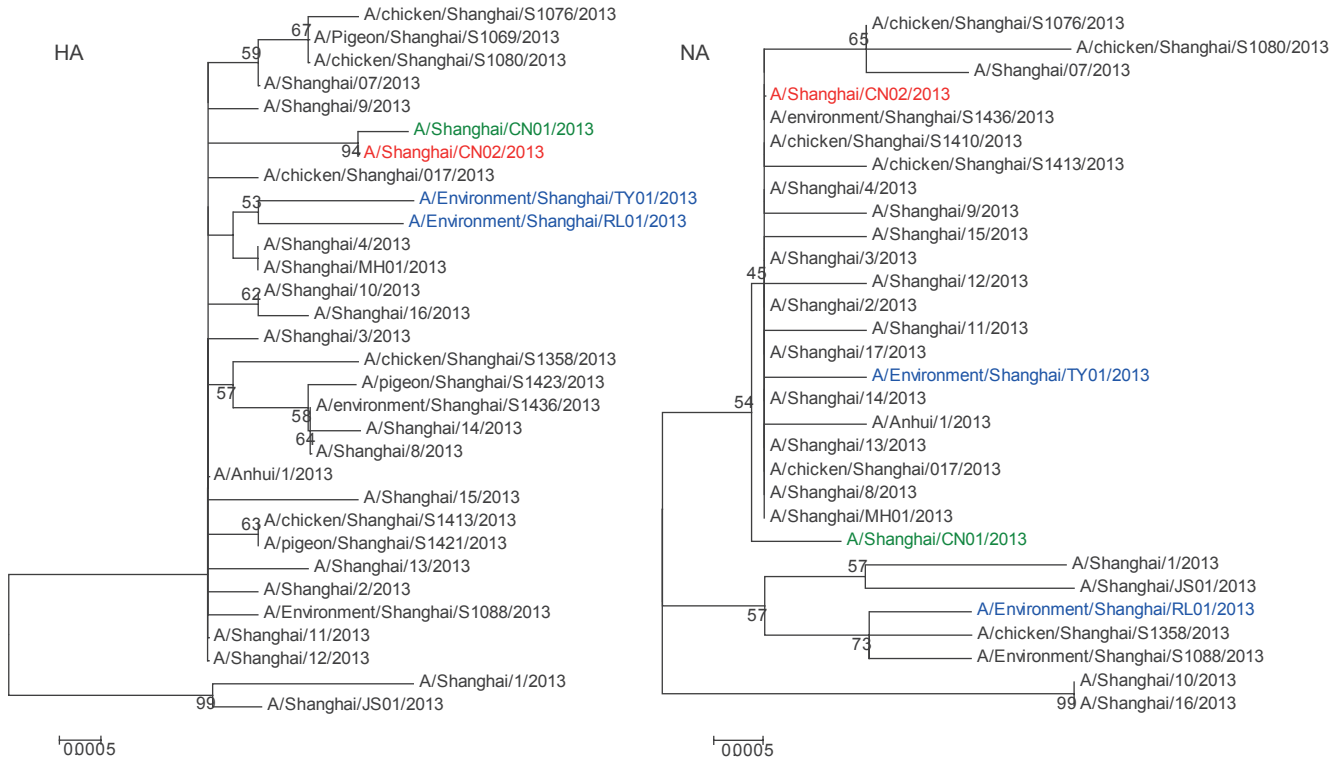
Case B, Case A's husband who was in his mid-50s, developed a fever on 2 April. He was admitted to Hospital Bon 3 April with a temperature of 39.5 °C, rhinorrhoea, dry cough and sore throat. A chest X-ray showed bilateral lower-lobe infiltrates. On 4 April, he was transferred to another hospital (Hospital C, where suspected or confirmed A(H7N9) cases were treated). He began treatment with oseltamivir (75 mg orally twice daily from 3 to 10 April). Although he was mechanically ventilated on 8 April, he also developed ARDS, on 11 April. He progressively deteriorated in the hospital until his death on 26 June.

A timeline of key events is shown in Figure 1.

On 4 April, A(H7N9) virus was detected by real-time RT-PCR of RNA extracts from respiratory specimens taken from Case A during the acute phase of her illness. Real-time RT-PCR of RNA extracts from pharyngeal swabs from Case B were negative for A(H7N9) on

Figure 2

Phyloygenetic tree of avian influenza A (H7N9) virus haemagglutinin and neuraminidase sequences for two cases in a family cluster, Shanghai, China, March–June 2013



HA: haemagglutinin; NA: neuraminidase.

Multiple alignments were constructed by using the MUSCLE algorithm (a multiple sequence alignment method with reduced time and space complexity) of MEGA software version 5.05. Phylogenetic trees were constructed by using the neighbour-joining method with bootstrap analyses of 1,000 replications. Bootstrap values are shown in the nodes. Sequences of CN01 are shown in green, sequences of CN02 are shown in red, and sequences of TY01 and RL01 are shown in blue. The scale bar indicates base substitutions per site.

5 April, whereas testing of pharyngeal swabs taken on 10 April confirmed the presence of the A(H7N9) virus.

The index case had had surgery several years before this infection, but had otherwise been in good physical condition before symptom onset. She had not eaten poultry or come into direct contact with live poultry or individuals with febrile respiratory symptoms during the two weeks before symptom onset. The pork and vegetables that made up her daily diet were purchased in a supermarket and the TY market near her home. Live poultry were sold and slaughtered at this wet market that Case A visited daily. The area where live poultry were available was approximately 2 metres from the vegetable stands she often visited. Although homing pigeons were raised on roofs adjacent to the couple's residence, neither had come into contact with pigeons or their faeces. No other birds or poultry were raised in her residential quarter and no live poultry was ever brought home.

Case B had not visited any wet market 14 days before symptom onset. He reported no history of live poultry exposure and had not been in contact with symptomatic individuals except for Case A. He too had not eaten or purchased live poultry or poultry products during the two weeks before the onset of his symptoms. He had participated in the care of Case A, providing unprotected care, including bedside care day and night either at home or in the hospitals between 27 March and 3 April.

Environmental samples

A total of 12 faeces samples and 51 tracheal or cloacal swabs from 27 pigeons in the couple's community collected on 13 April were negative for A(H7N9) by real-time RT-PCR. A total of 27 serum samples from these pigeons were negative for A(H7N9) using the HI assay.

Environmental sampling of markets was carried out on 4 April. As all poultry markets in Shanghai were closed on 5 April on order from the Shanghai Municipal Government in response to the A(H7N9) outbreak [21], samples from chickens in the TY market were unavailable at the time of sampling (the live poultry stand in the market closed on 4 April). However, six samples

collected from a poultry chopping board, two chicken faeces samples and two water samples from the live poultry trading area on the day the TY market closed were positive for influenza A and five samples from the poultry chopping board and one chicken faeces sample were positive for A(H7N9) by real-time RT-PCR.

Five samples from RL wet market, including one chicken faeces, one swab from a pigeon cage surface, one swab from duck cage surface, one swab from a pork chopping board and one waste-water sample from a slaughterhouse, were tested for influenza A and A(H7N9) by real-time RT-PCR. All five samples were positive for influenza A. Four samples were also positive for A(H7N9): the swab from the pork chopping board was negative.

The viruses detected in the patients and the chicken faeces samples from the TY and RL wet markets were designated as follows:

- A/Shanghai/patient CN02/2013 (H7N9), from Case A (referred to hereafter as CNO2);
- A/Shanghai/patient CN01/2013 (H7N9), from Case B (CN01);
- A/Environment/Shanghai/TY01/2013 (H7N9), from Changning TY market (TY01);
- A/Environment/Shanghai/RL01/2013 (H7N9) (from Minhang RL market (RL01).

Sequences of eight RNA segments of CN02, CN01 and TY01 viruses were submitted to GenBank under accession numbers KF918656–KF918663, KF609516–KF609523 and KF918664–KF918671, respectively. Sequences of the five RNA segments – polymerase (PA), haemagglutinin (HA), neuraminidase (NA), matrix protein (MP), non-structural protein (NS) – of RL01 virus were submitted to GenBank under accession numbers KJ572519–KJ572523. Eight gene segments from Case A (CN02) in this study were most similar to those of Case B isolates (CN01) (≥99.9%). Phylogenetic analysis of the HA genes revealed that CN02 and CN01 strains were in one branch, distinctly separate from other human A(H7N9) strains isolated in Shanghai (Figure 2). Both homology and phylogenetic analysis indicated that CN02 possessed a similar genetic constellation as CN01. The HA genes of the TY01 and RL01 strains were 99.6% similar by MegAlign method, which suggests the possibility that the A(H7N9)-positive chickens in these two markets could have been from the same source. With regard to the NA gene, CN02, CN01 and TY01 strains were located in the same large branch whereas RL01 was in another branch, which indicates that the NA gene of this novel avian influenza virus may come from different strains.

Similar to most human A(H7N9) viruses, CN02 and CN01 acquired the ability to bind to human-like receptors with the substitutions G186V and Q226L in the HA protein (H3 numbering) [22,23]. The 69–73 amino acid deletion was also presented in the NA gene (N2 numbering). Base pairs 1–429 of CN01 were not sequenced, so full data are not available on the sequence or any deletions in the NA gene of this isolate.

The presence of lysine at amino acid 294 of the NA gene in the CN01 strain indicates possible resistance to oseltamivir [24], whereas arginine was maintained at this position in the CN02 NA gene, indicating its sensitivity to neuraminidase inhibitors [25]. However, both CN01 and CN02 had an S31N mutation in the M2 protein, which confers resistance to adamantine. No truncated PB1-F2, associated with increased virulence [26,27], was observed in CN01 and CN02. Aspartic acid at PB2 residue 701 (present in CN01 and CN02) is associated with reduced transmissibility [28]. Amino acid 627K was present in PB2 of CN01 and CN02, but amino acid 627E was detected in TY01 (E627K is associated with increased virulence [29,30]).

Close contacts

An additional 27 persons, including 22 medical personnel, four relatives and a friend, were identified as close contacts. All but one – a sibling of Case A – underwent 10 days of medical observation and did not develop acute respiratory symptoms. The sibling, in their early 50s, escorted Case A to an outpatient appointment on 1 April, cleaned her corpse without protection and attended the funeral of Case A on 3 April. This patient subsequently developed a fever of 37.6 °C, headache and sore throat on 4 April and was admitted on 5 April, but recovered and was discharged on 9 April. The patient was administered oseltamivir from 5 to 9 April (75 mg twice daily). The patient reported no history of exposure to live poultry in a wet market or other symptomatic individuals two weeks before the onset of symptoms. Throat swabs collected from the patient on the first day of medical observation were all negative for A(H7N9) by real-time RT-PCR.

Of the 27 close contacts, 14 were sampled at both acute phase and follow-up phase (four weeks after last contact); the other 13 close contacts chose not to be sampled for paired sera.

Nine of the 14 close contacts' acute serum samples were positive in the HI assay, with titres ≥40. The MN assay was used for further confirmation of the nine HI-positive serum samples. Only one acute serum was positive, with a titre of 20: the other eight had an MN titre<10. Follow-up sera of all 14 were negative for A(H7N9) antibodies in the HI assay.

Details of the type of exposure of the close contacts and sera collection are shown in Table 2.

Discussion

The epidemiological and clinical features described here are most consistent with another family cluster of avian influenza A(H7N9) in Shanghai, China. A similar clinical picture was seen in both cases, which provides

Table 2

Type of exposure and sera collection of 27 close contacts of two cases of influenza A(H7N9) infection, Shanghai, China, March–June 2013

Characteristic	Close contacts[a,b]				
	Only exposed to Case A		Only exposed to Case B		Exposed to both cases
	Household (n=1)	Healthcare workers (n=22)	Household (n=1)	Social (n=1)	Household (n=2)
Age range in years	50s[c]	28–52	50s[c]	50s[c]	50–56
Sex (male)	0	8	0	1	0
Possible exposure					
Contact with poultry	0	0	0	0	0
Visited wet market	0	3	0	0	0
Type of contact with A(H7N9) cases					
Provided direct care[d]	1	2	1	0	2
Close physical contact[e]	1	21	1	0	2
Exposed to case (<1 metre)	1	22	1	0	2
Contact with serum, respiratory or faecal secretions of case	0	8	0	0	1
Duration of exposure to casein hours (range)	1[c]	1 (0.2–8)	13[c]	1[c]	10.5 (6.5–15)
Personal protection equipment used					
N95 respirator	0	0	0	0	0
Surgical mask	0	22	0	0	0
Goggles	0	0	0	0	0
Face shield	0	0	0	0	0
Gloves	0	10	0	0	0
Gowns	0	22	0	0	0
Symptoms					
Febrile respiratory symptoms	0	0	0	0	1
Serum collection					
Paired sera samples collected	0	13	0	1	0
Time in days from last exposure to acute serum collection for paired sera in days (range)	NA	1 (1–2)	NA	0	NA
Time in days from last exposure to convalescent serum collection for paired sera in days (range)	NA	21 (21–25)	NA	13[c]	NA

NA: not applicable.

[a] Close contacts were defined as individuals known to have been within 1 metre or to have had contact with the serum, respiratory or faecal secretions of a case of influenza A(H7N9) infection without having used effective protective equipment at any time from one day before the symptoms onset of the case to the day the case was placed in isolation or died [13].

[b] Data are numbers of close contacts unless otherwise indicated.

[c] No range given as only one close contact.

[d] Provided care for the patient and had the possibility of coming into contact with respiratory secretions or faecal material.

[e] Within 1 metre and in contact with the patient without personal protection.

support to the hypothesis that the same pathogen caused both illnesses.

The origin of infection for the index case was plausibly live poultry in her local wet market, acquiring A(H7N9) from the environment during the course of her daily visit to the market. Visiting a live poultry market, where avian influenza A viruses can be maintained and amplified [31], is a known risk factor for A(H5N1) infection in mainland China [32] and Hong Kong [33]. The Ministry of Agriculture in China announced that A(H7N9) virus detected from poultry specimens from markets in Shanghai had a high degree of similarity with the A(H7N9) virus isolated from patients on 4 April [34]. After live poultry markets were shut down, the number of reports of fatal cases of A(H7N9) virus infection dramatically diminished in Shanghai [21]. Before the poultry markets were disinfected, environmental samples collected in the market that Case A visited daily tested positive for avian influenza A(H7N9), which supports the hypothesis that the local wet market was the source of the infection in this case.

Several pieces of epidemiological evidence gathered in the family cluster investigations support the origin of infection for Case B being the virus transmitted from the index patient (his wife). First, he developed symptoms six days after the onset of his wife's symptoms and had provided care for her on each of these six days. Influenza virus incubation period is generally two to five days [35]. Second, during the course of his wife's illness, Case B was in close and regular contact with her, including eating and living together and providing unprotected bedside care. Prolonged and direct exposure to his critically ill wife made person-to-person spread of the virus possible. Third, Case B never visited the market and had not purchased or eaten poultry two weeks before the onset of his symptoms. Pigeons found in the community where the couple resided were negative for A(H7N9). Therefore it is highly unlikely that the novel virus was transmitted from pigeons or poultry to the secondary case in this family cluster.

The sequence comparison showed high similarity between the virus strains from samples collected from the two cases. Gene similarity analysis and phylogenetic analysis showed that Case B was likely to be the secondary case in this household infection. Genetic analysis supports the hypothesis that this was another family cluster of avian influenza infection [8].

One close contact developed mild symptoms during the medical observation phase, but real-time RT-PCR and HI assay ruled out the possibility of A(H7N9) virus infection. An acute serum sample from another close contact tested positive at a 1:20 dilution in the MN assay, but no fourfold elevation was detected in the follow-up serum. Other close contacts did not develop acute respiratory symptoms during the 10 days of medical observation, with all other paired sera testing negative for A(H7N9) antibodies.

We previously found that only two of 440 close contacts of 32 confirmed cases of A(H7N9) virus infection developed fever, cough or other respiratory symptoms in Shanghai in 2013 (data not shown). Given this finding and the fact that no further transmission was observed in the family cluster studied here, we speculate that human-to-human transmission of this novel avian A(H7N9) virus is limited. Even if, as seems likely, Case B was infected by the same virus that infected his wife, transmission arose following very close, frequent and unprotected contact. In this instance, limited person-to-person transmission of avian influenza A(H7N9) virus possibly occurred in this couple, as documented for subtype H7 transmission in the Netherlands in 2003 [36]. Similar to A(H5N1) virus, A(H7N9) virus mainly affects the lower respiratory tract, sputum and endotracheal aspirates [37], which may lead to reduced virus shedding in the upper respiratory tract.

Whether susceptibility to severe influenza in humans is heritable or not is still controversial [38]. More than 90% of A(H5N1) case clusters have occurred in blood relatives [39]. Previous reports of family clusters of A(H7N9) virus infection imply genetic susceptibility to this virus in a family. The cases in these family clusters were blood relatives. In contrast, however, the couple in our study were not blood relatives, which implies that prolonged, intense exposure to a sick person without any personal protective equipment can lead to human-to-human transmission of the virus to someone who is no more likely than anyone else in the population to share genes that make them more susceptible to infection. Thus, further in-depth study on genetic susceptibility to A(H7N9) virus infection is needed [40].

Taken together, our results indicate that human-to-human transmission of A(H7N9) virus most likely occurred between these two family members. Our investigation of their close contacts does not suggest that the virus has developed the ability to transmit readily and widely between humans. More urgent research is required to better understand the characteristics of the virus and determine its transmissibility between humans.

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Conflicts of interest

None declared.

Authors' contributions

Jiayu Hu, Yiyi Zhu, Jian Li, Zijian Feng and Fan Wu designed the protocol of investigation, set up the field epidemiology and clinical investigation, contacted all investigators; Baihui Zhao, Liguo Liu, Haoxiang Su, Zhen Teng were responsible for real-time RT-PCR testing, sequence analysis and serological assay, including the experimental design and data analysis; KaikaiGu, Wenhong Zhang and Songzhe Tang provided technical assistance for the epidemiological investigations and clinical data and helped to review the data. All other co-authors participated in collection and management of data.

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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).

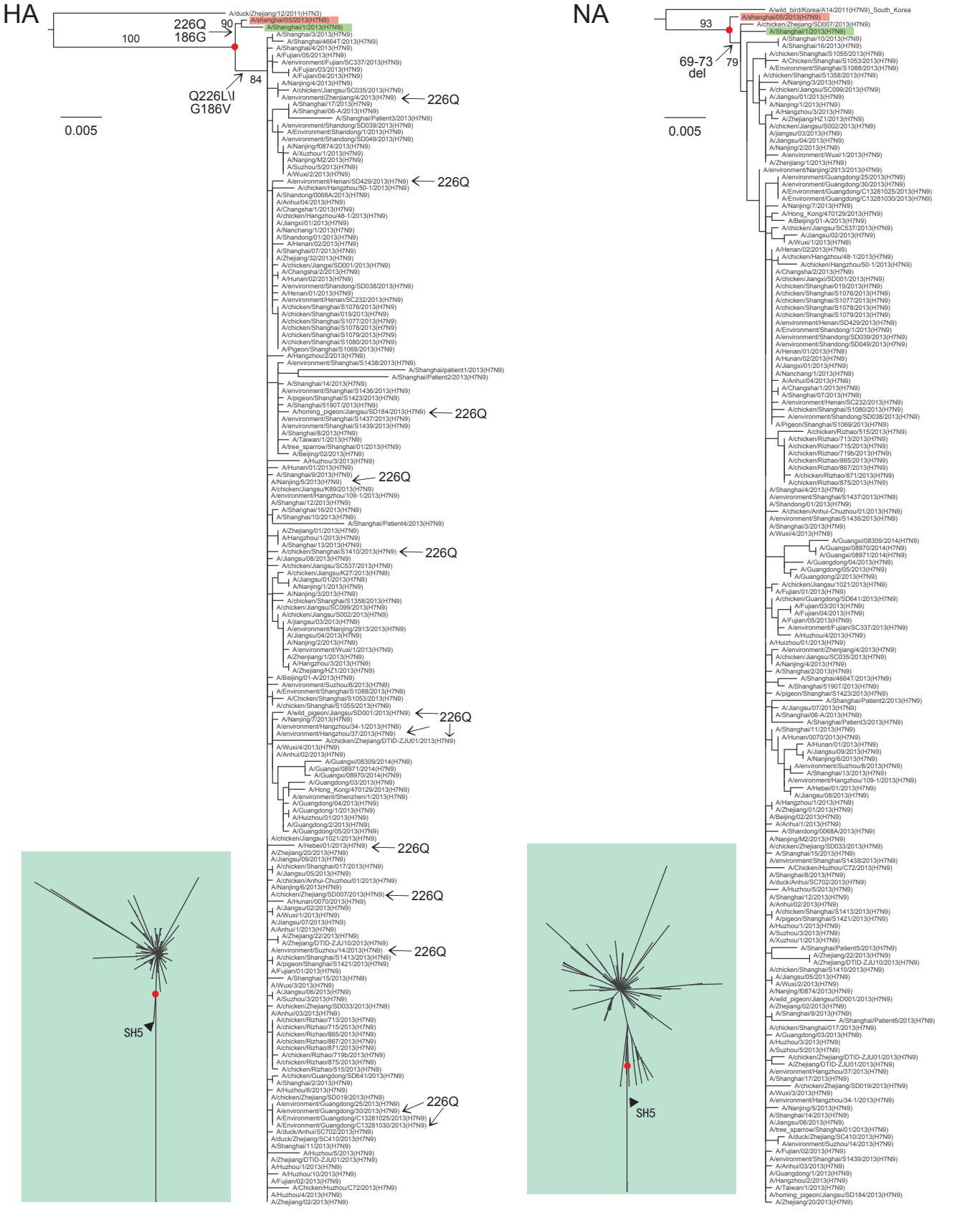
[b] The HA gene was under H3 numbering system. The NA gene was under N2 numbering system. Other internal genes were numbered from start codon (M).

[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.

The authors gratefully acknowledge the originating and submitting laboratories who contributed sequences used in the phylogenetic analysis to GISAID, and recognise in particular the laboratories who contributed A(H7N9) sequences in our phylogenetic analysis.

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

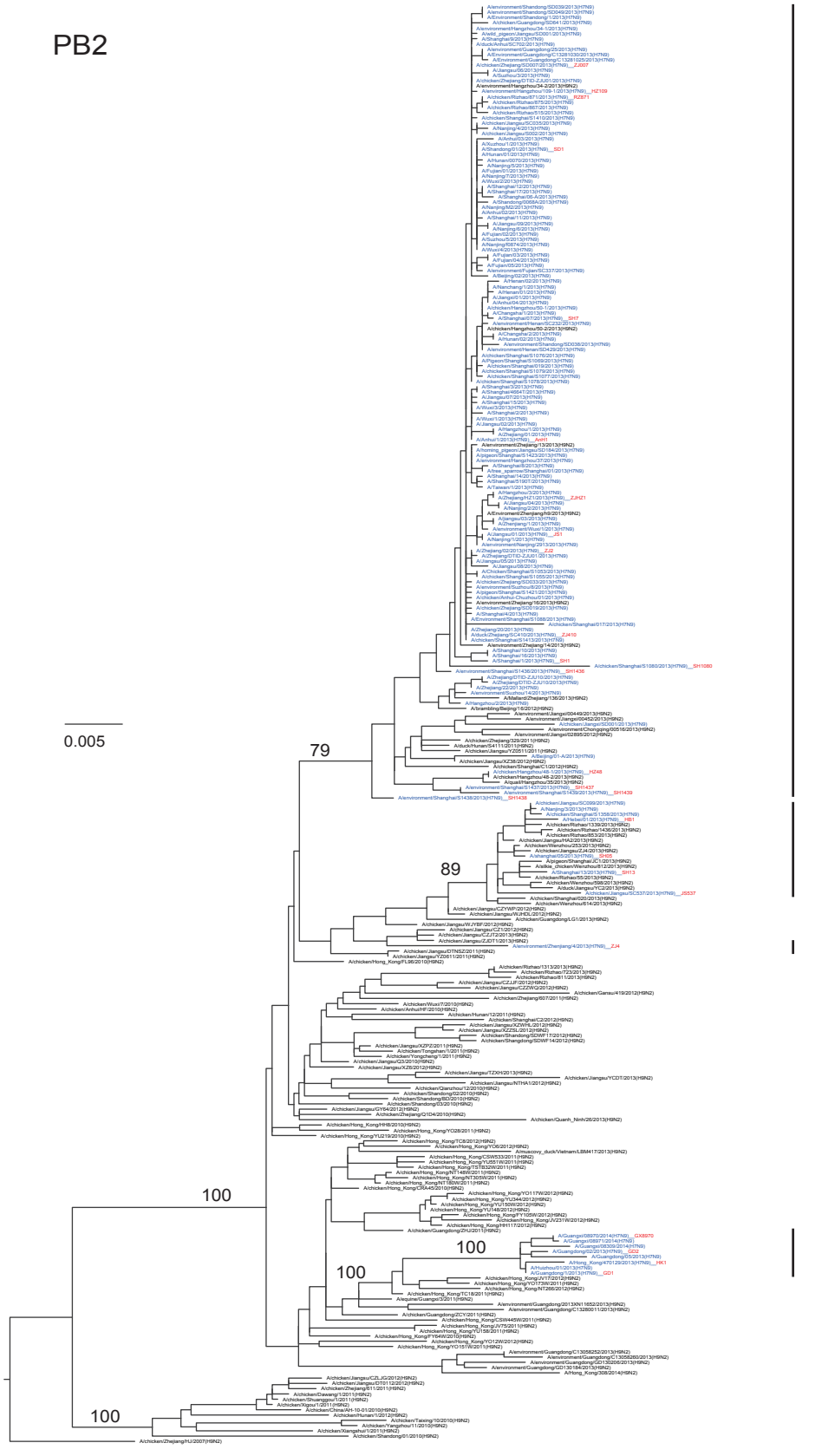
Since the emergence of the novel avian influenza A(H7N9) viruses in China, its unusual features indicated a potential pandemic risk that may persist over a long time [19,20]. Previous studies have demonstrated that the HA of this virus could be of duck origin, the NA was most likely to be related to 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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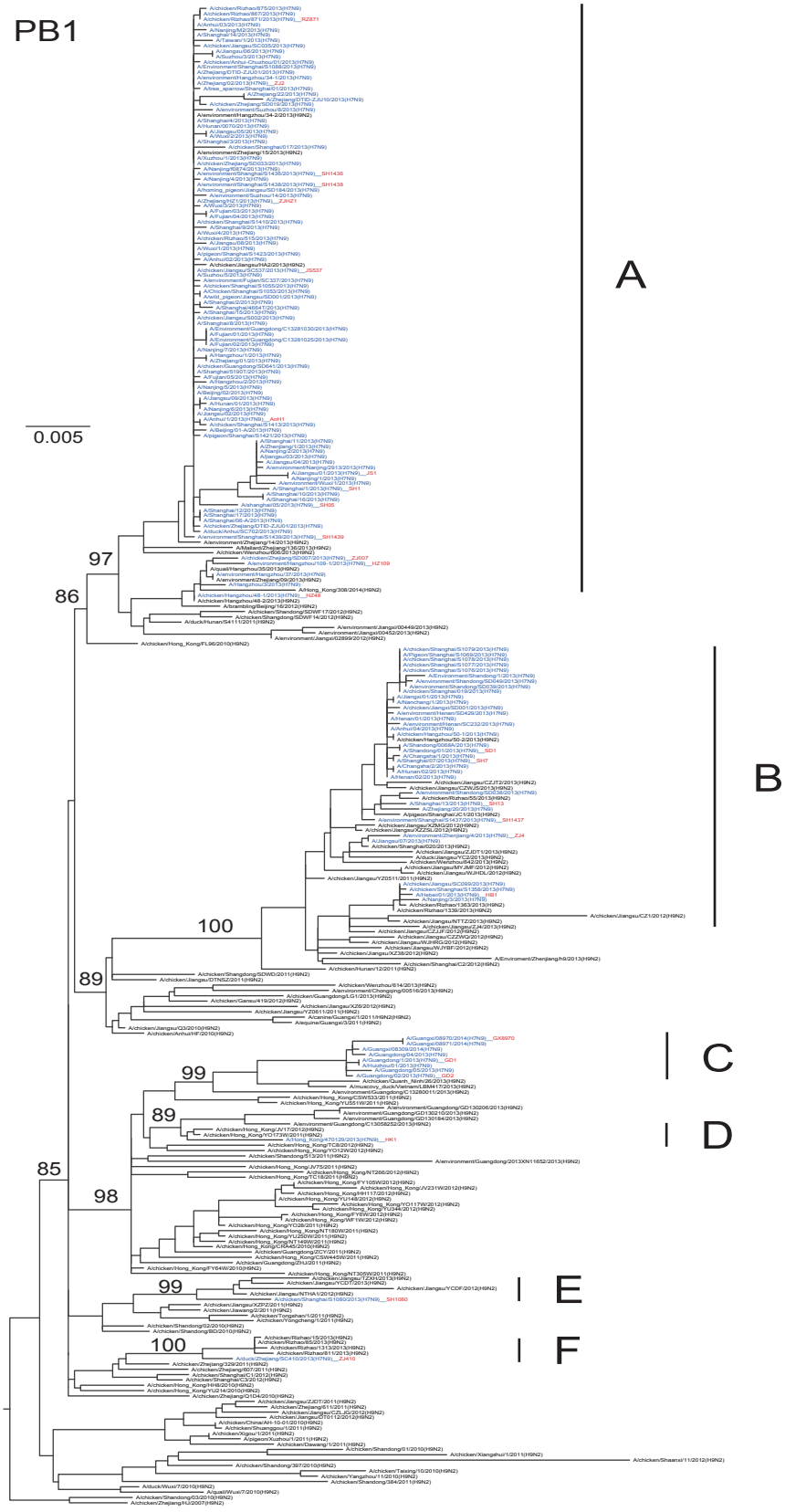

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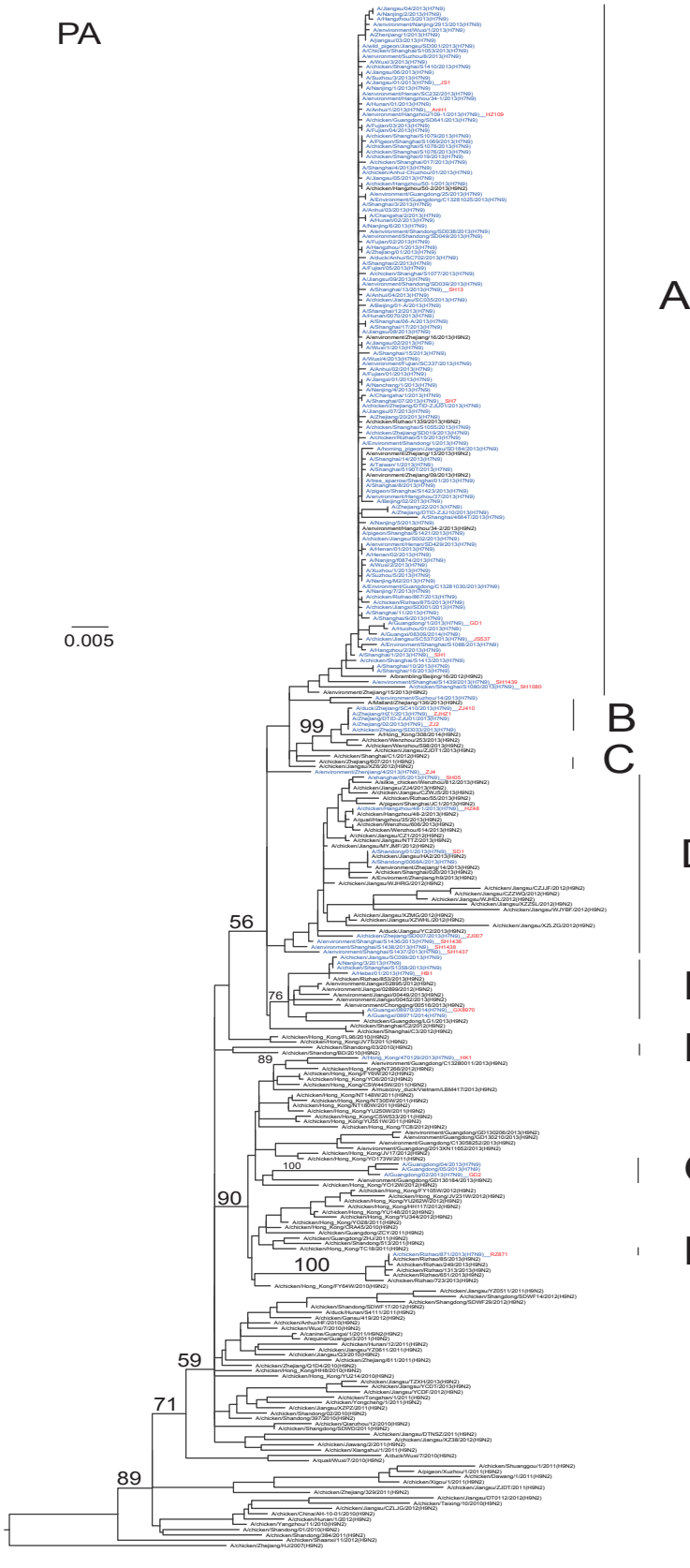

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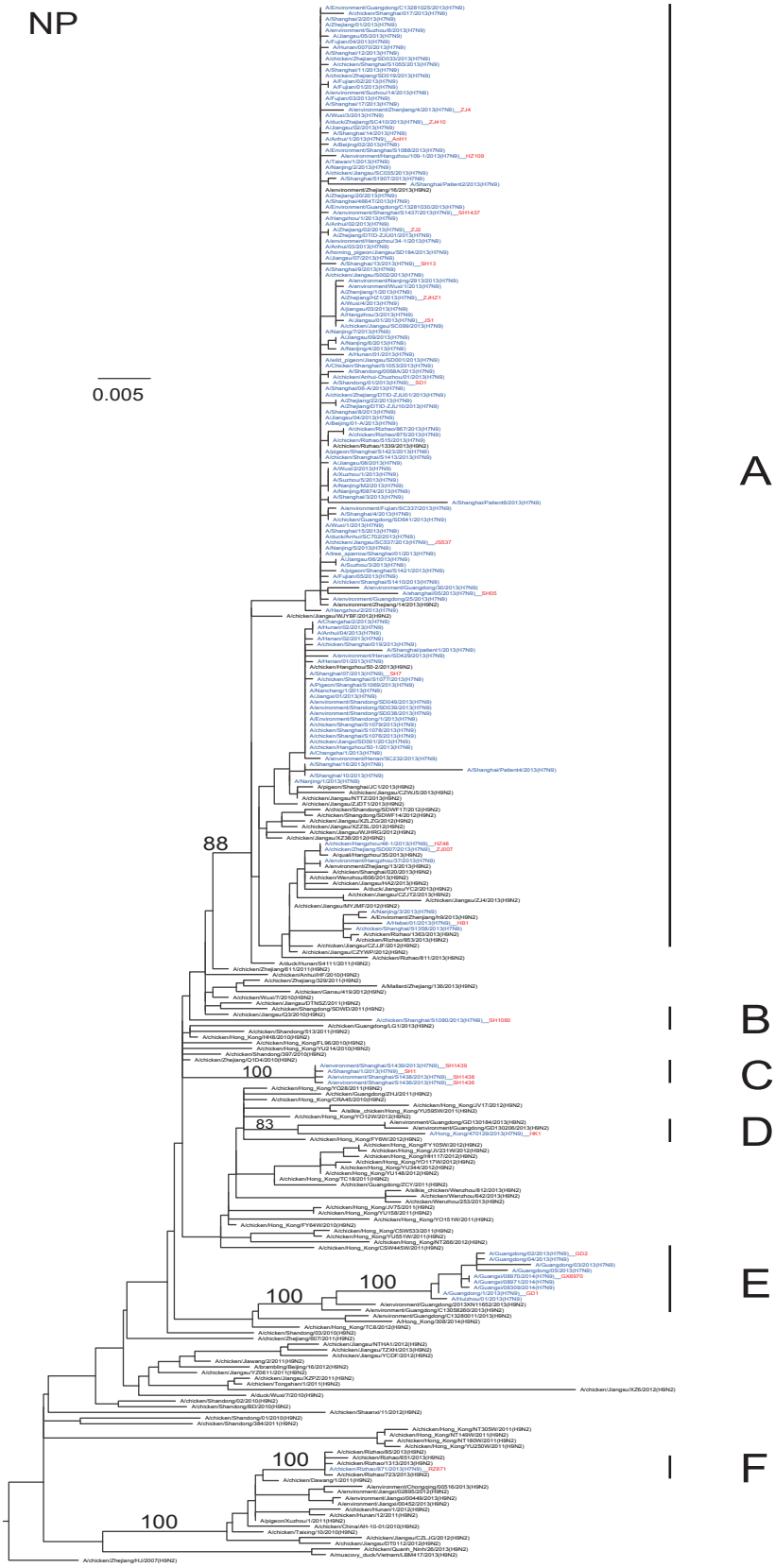
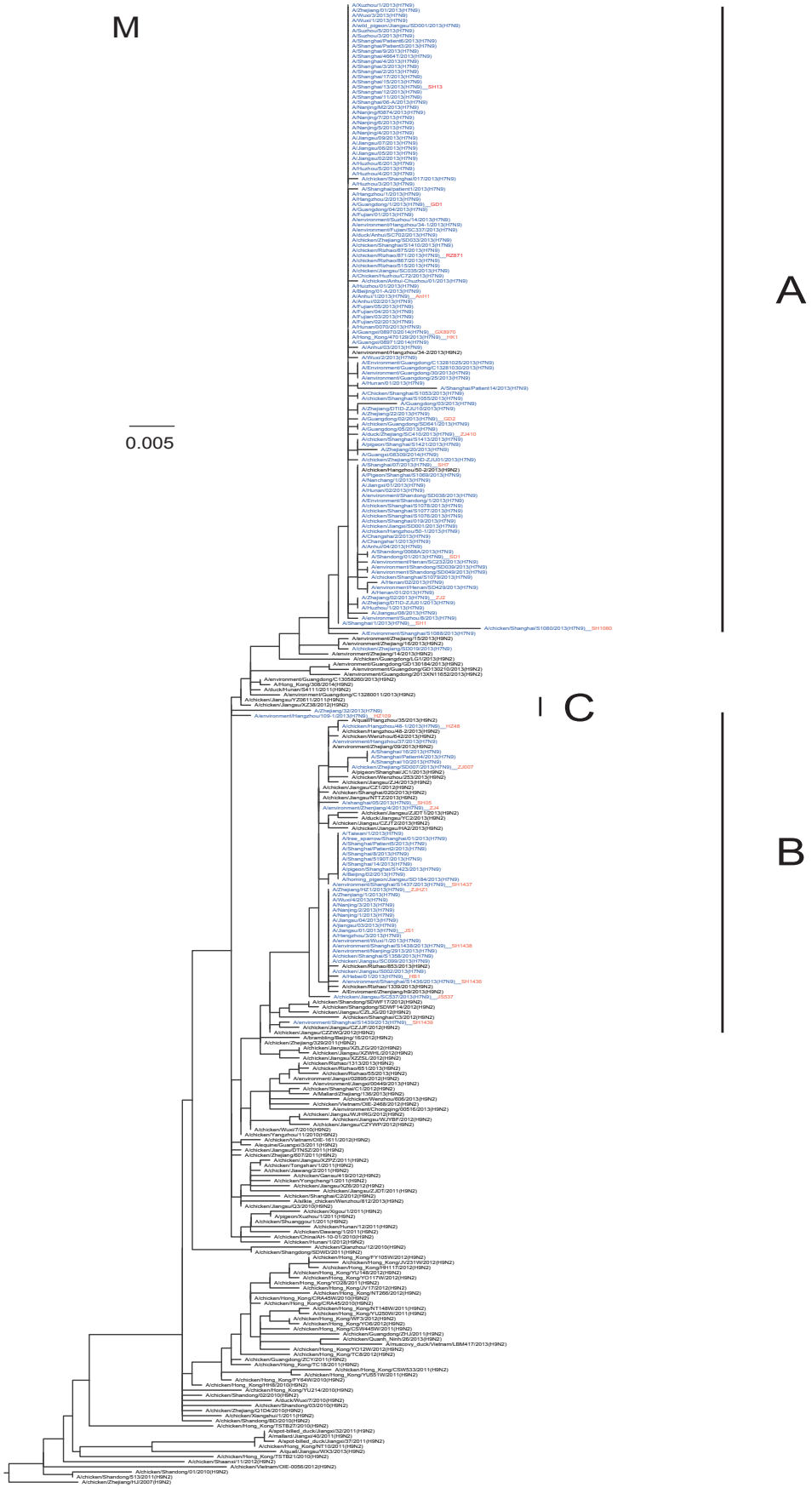


HA: haemagglutinin; M: matrix protein; NA: neuraminidase; NP: nucleoprotein; PA, PB1, PB2: polymerase subunits.

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.

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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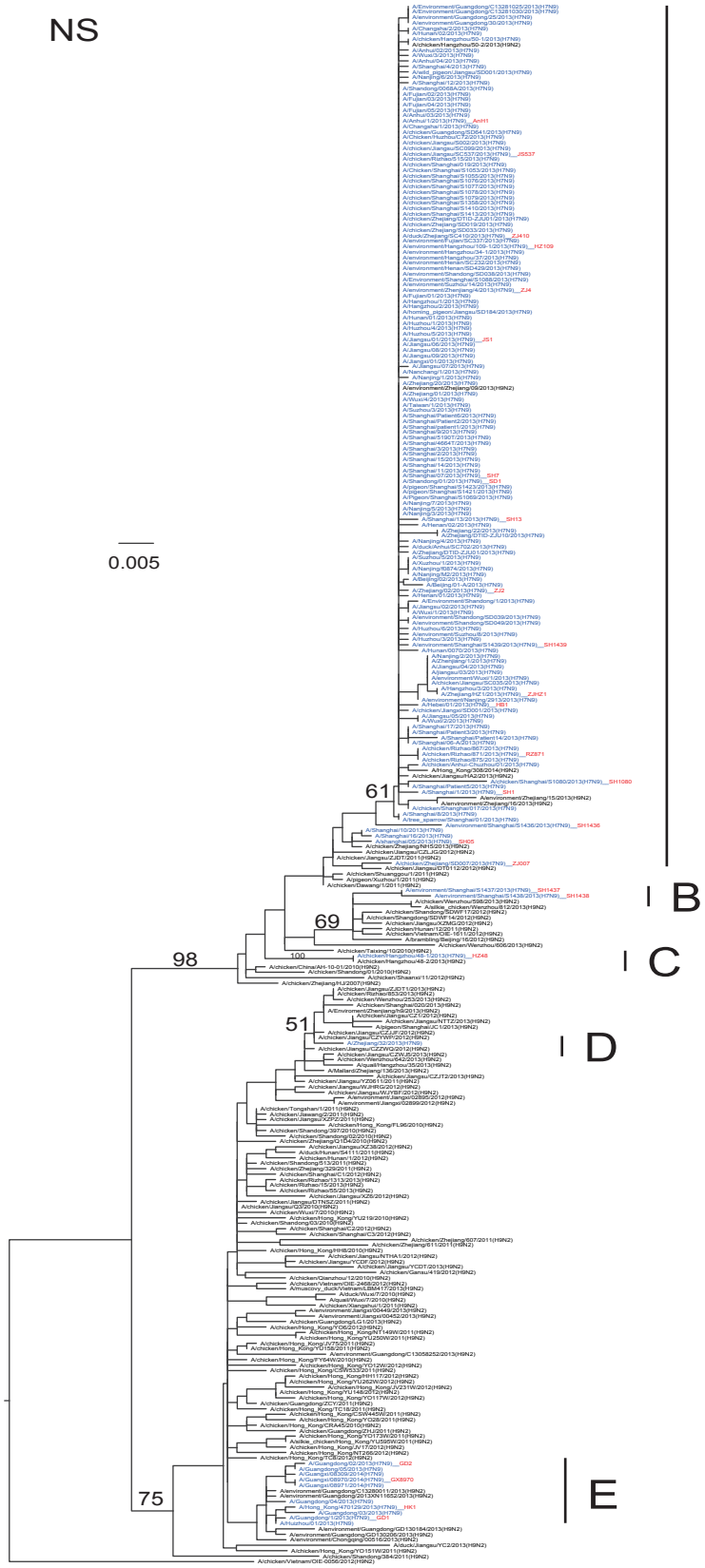


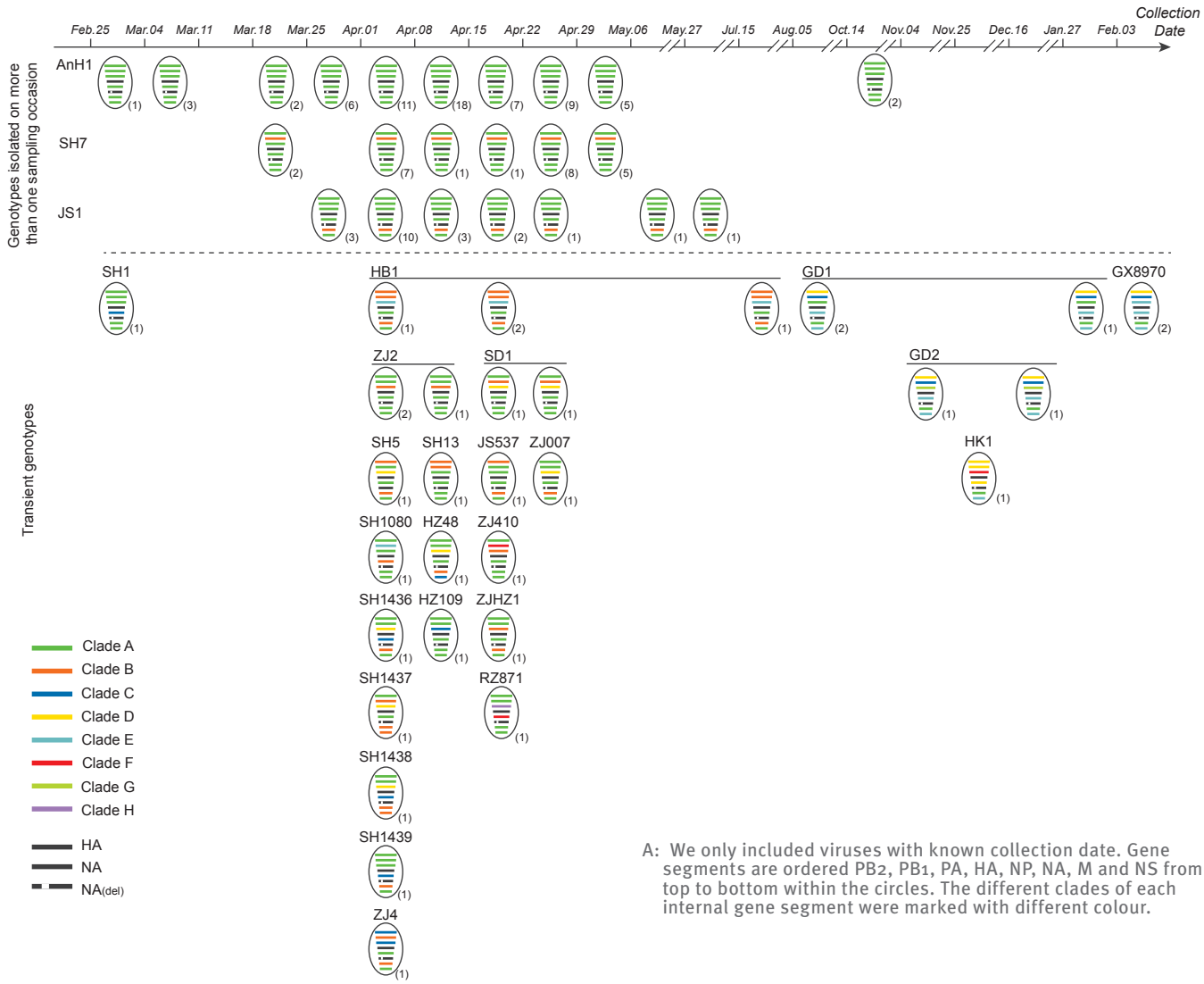
HA: haemagglutinin; M: matrix protein; NA: neuraminidase; NP: nucleoprotein; PA, PB1, PB2: polymerase subunits.

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.

Figure 3

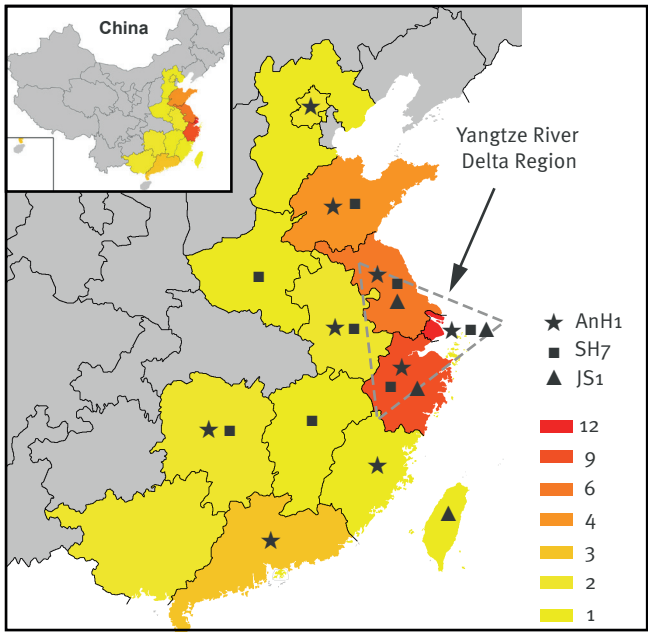
Multiple genotypes of influenza A(H7N9) viruses (A) and geographic distribution of different genotypes (B), China, 26 February 2013–5 February 2014 (n=142)

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.

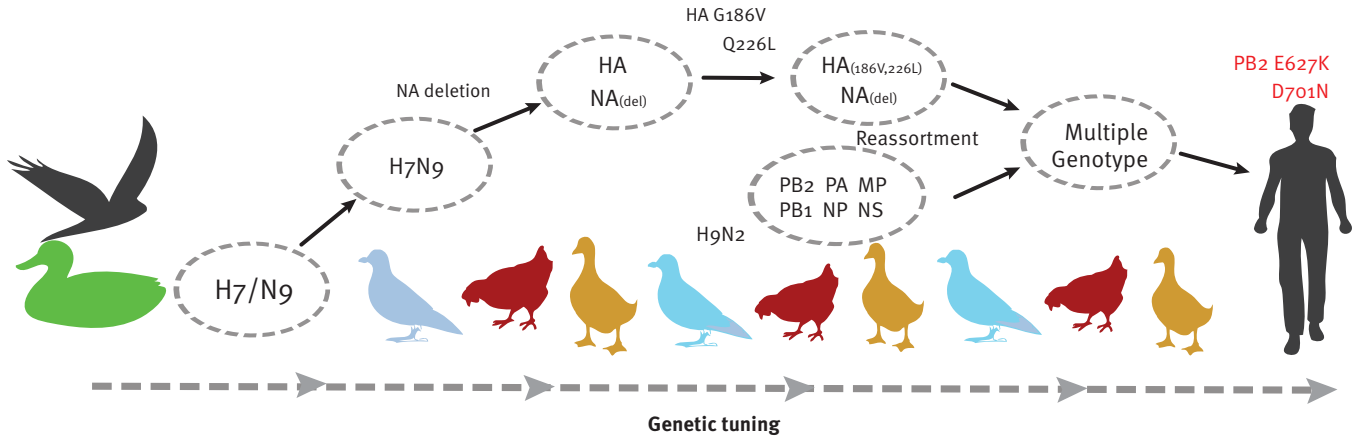
B



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.

Acknowledgements

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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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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

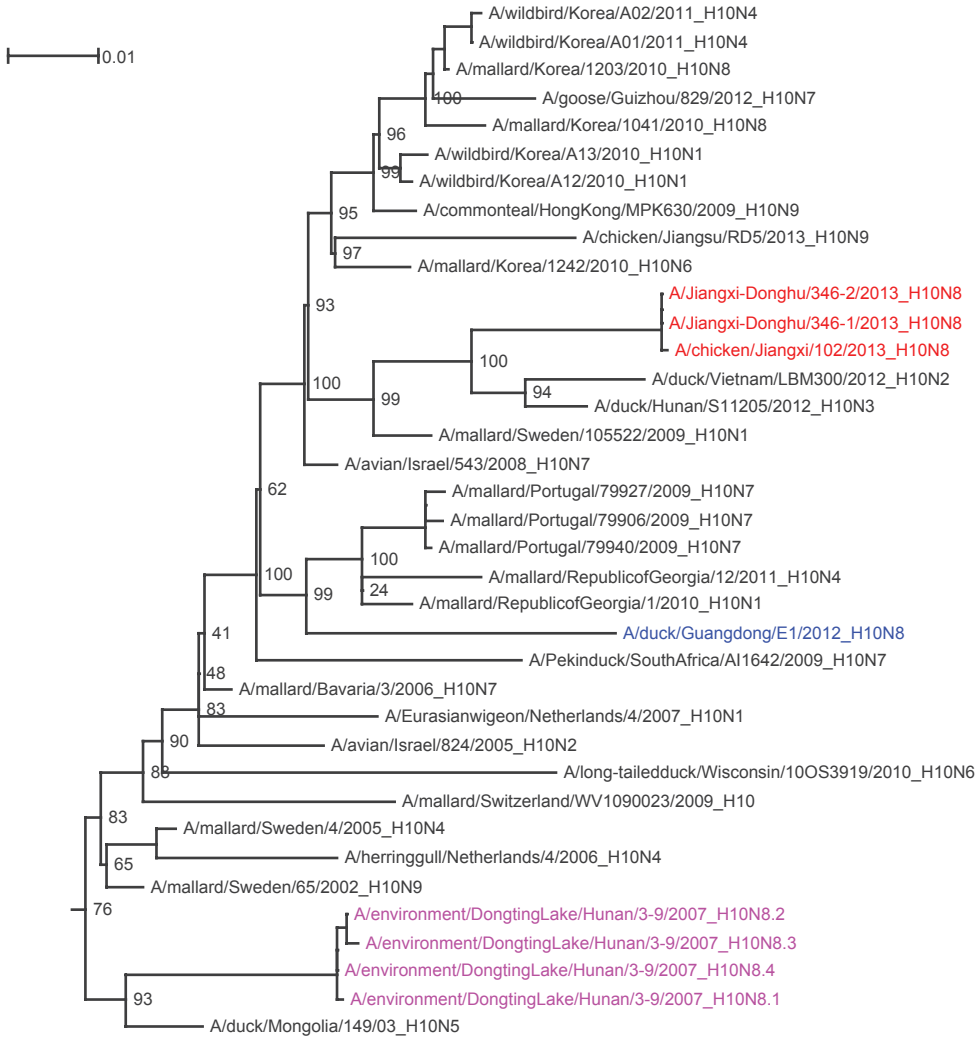
Phylogenetic analysis and coalescent analysis of the A(H10N8) viruses from a human case and an A(H10N8) virus isolated from a chicken from the live poultry market that the patient visited a few days before symptom onset show that both A/chicken/Jiangxi/102/2013 and A/Jiangxi-Donghu/346-1/2013 were multiple 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

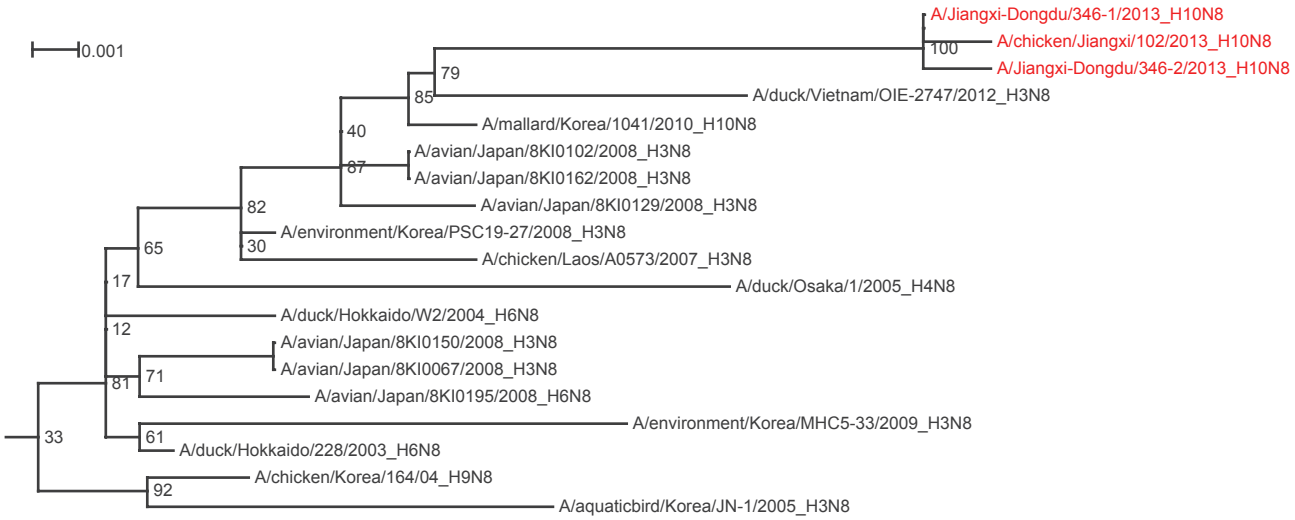
Figure 1

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

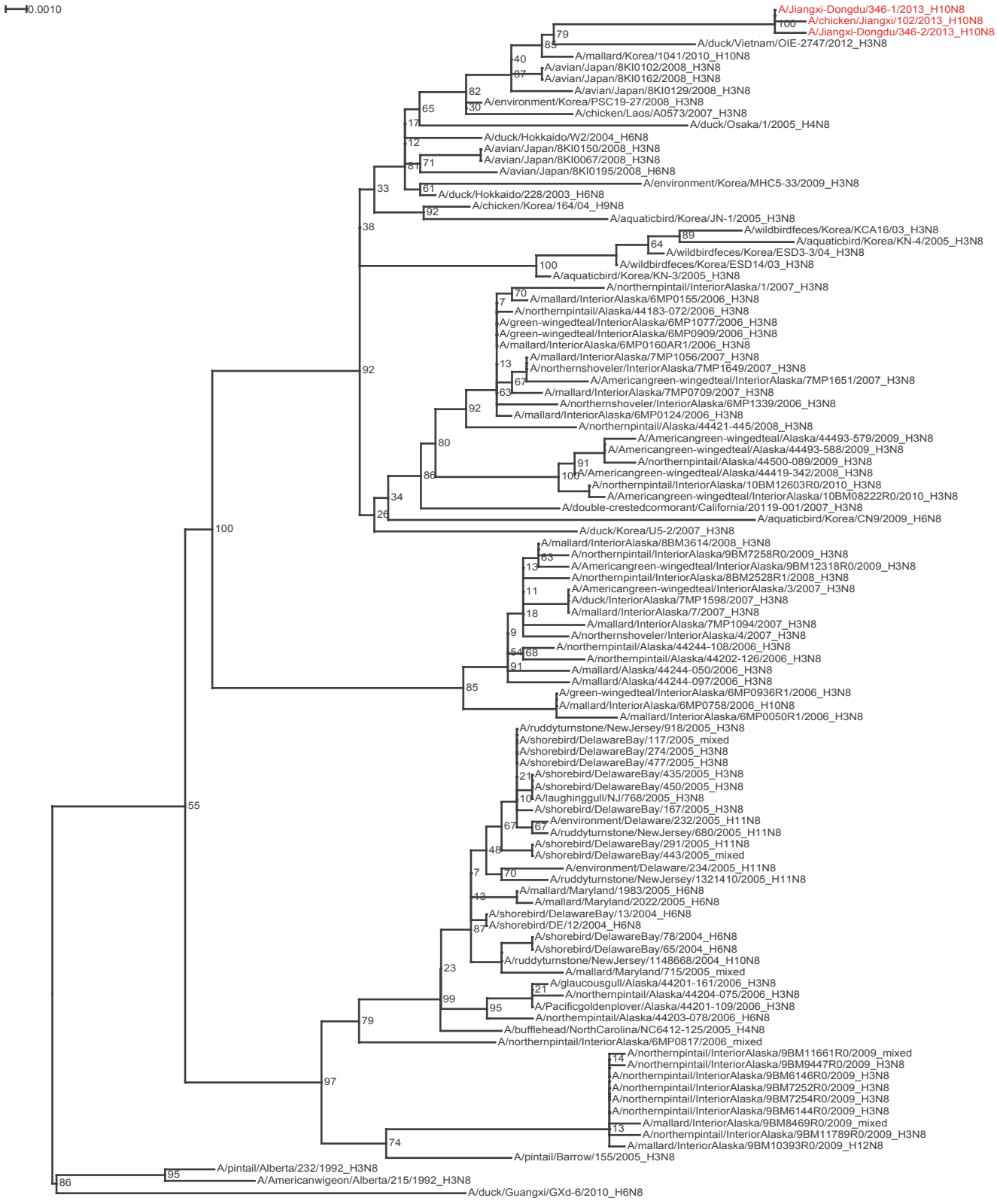


Multiple sequence alignment was performed using MUSCLE (multiple sequence comparison by log-expectation). Phylogenetic analysis was performed using RAxML with the GTRGAMMA model. A total of 1,000 bootstrap replicates were implemented. After the first run, we analysed the phylogenetic trees and selected 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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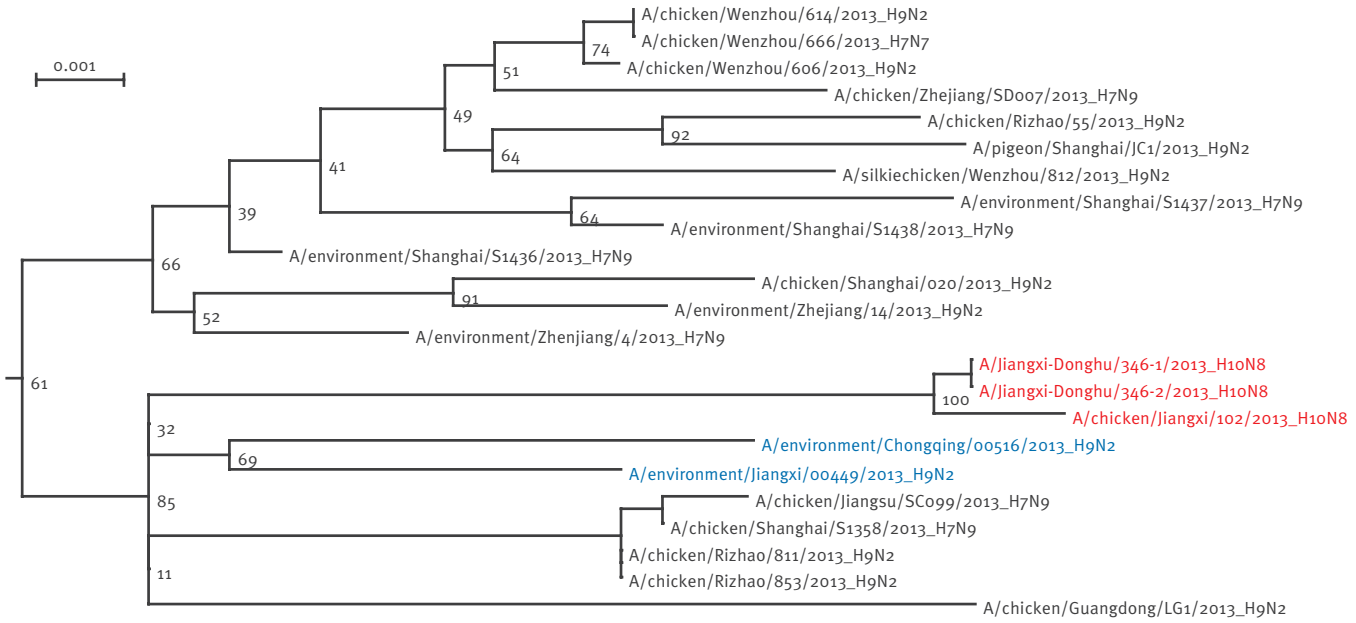
Figure 3

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].

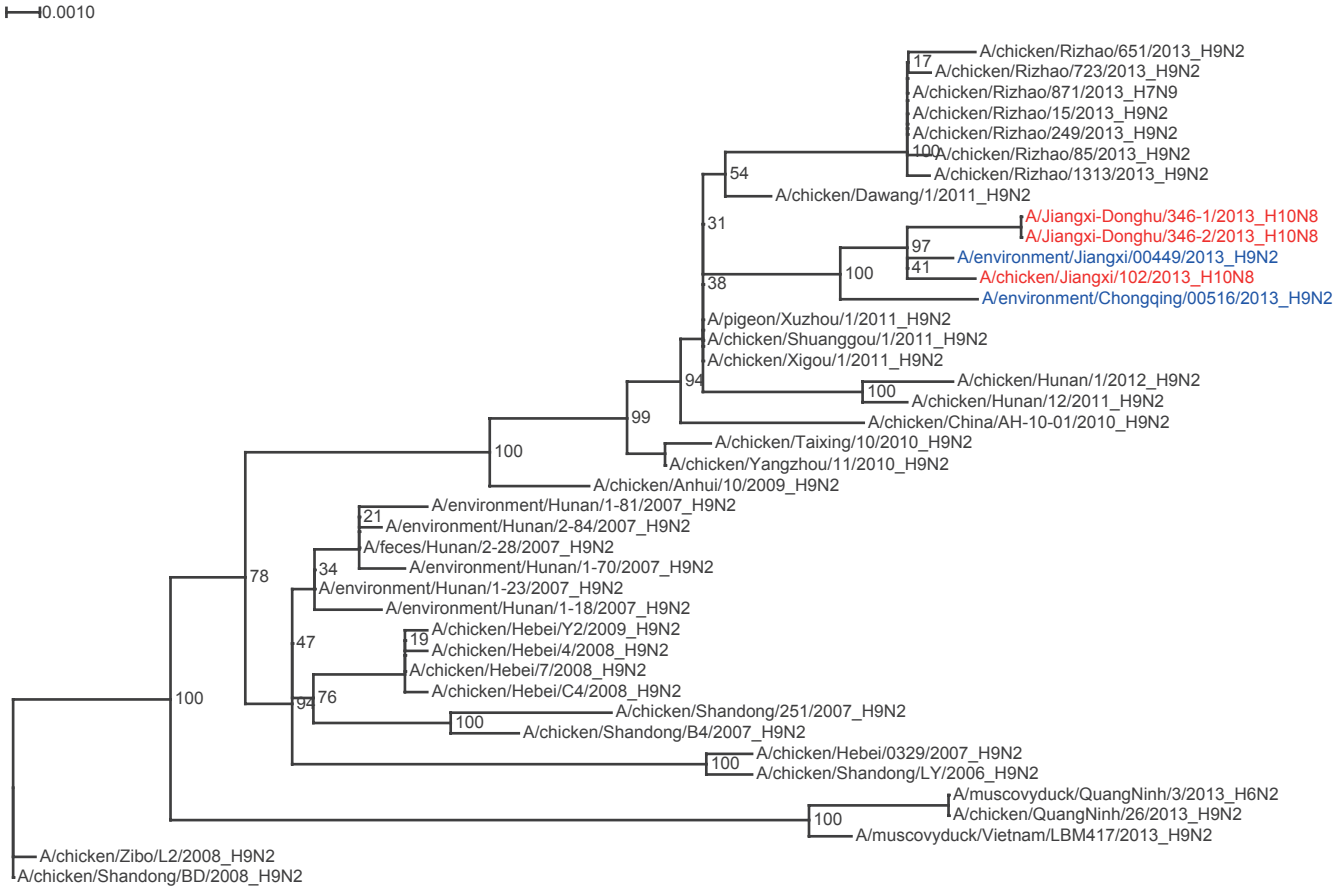
Our analyses showed that the HA and NA genes of the human-infecting influenza A(H10N8)-like viruses might have formed a stable lineage in Jiangxi Province. In addition, the genetic difference in the internal genes between the human-infecting and chicken isolates suggests that they might also undergo 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

We acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu Database on which this research is based (see Table 1). All submitters of data may be contacted directly via the GISAID website www.gisaid.org.

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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.

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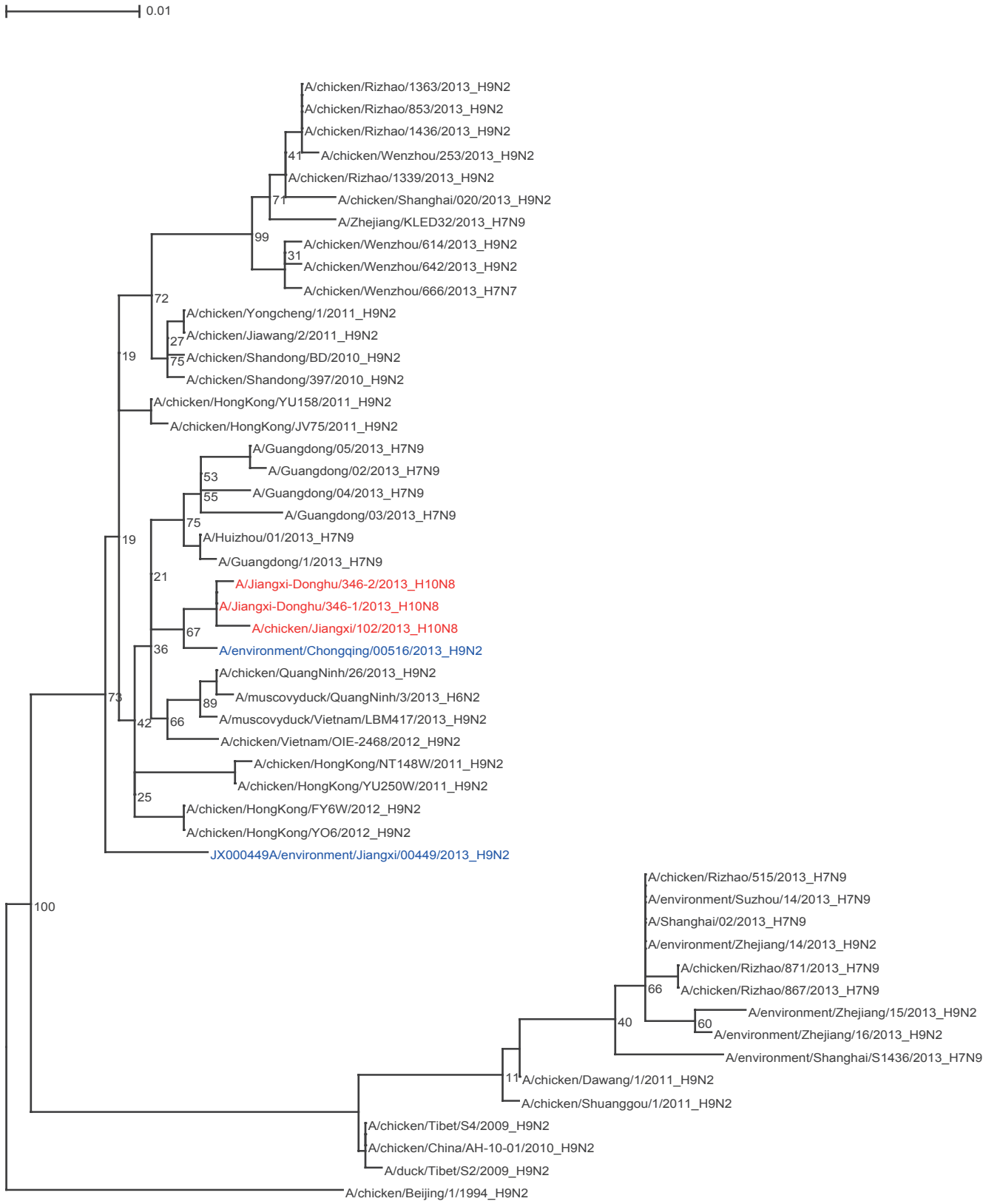
Figure 6

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.

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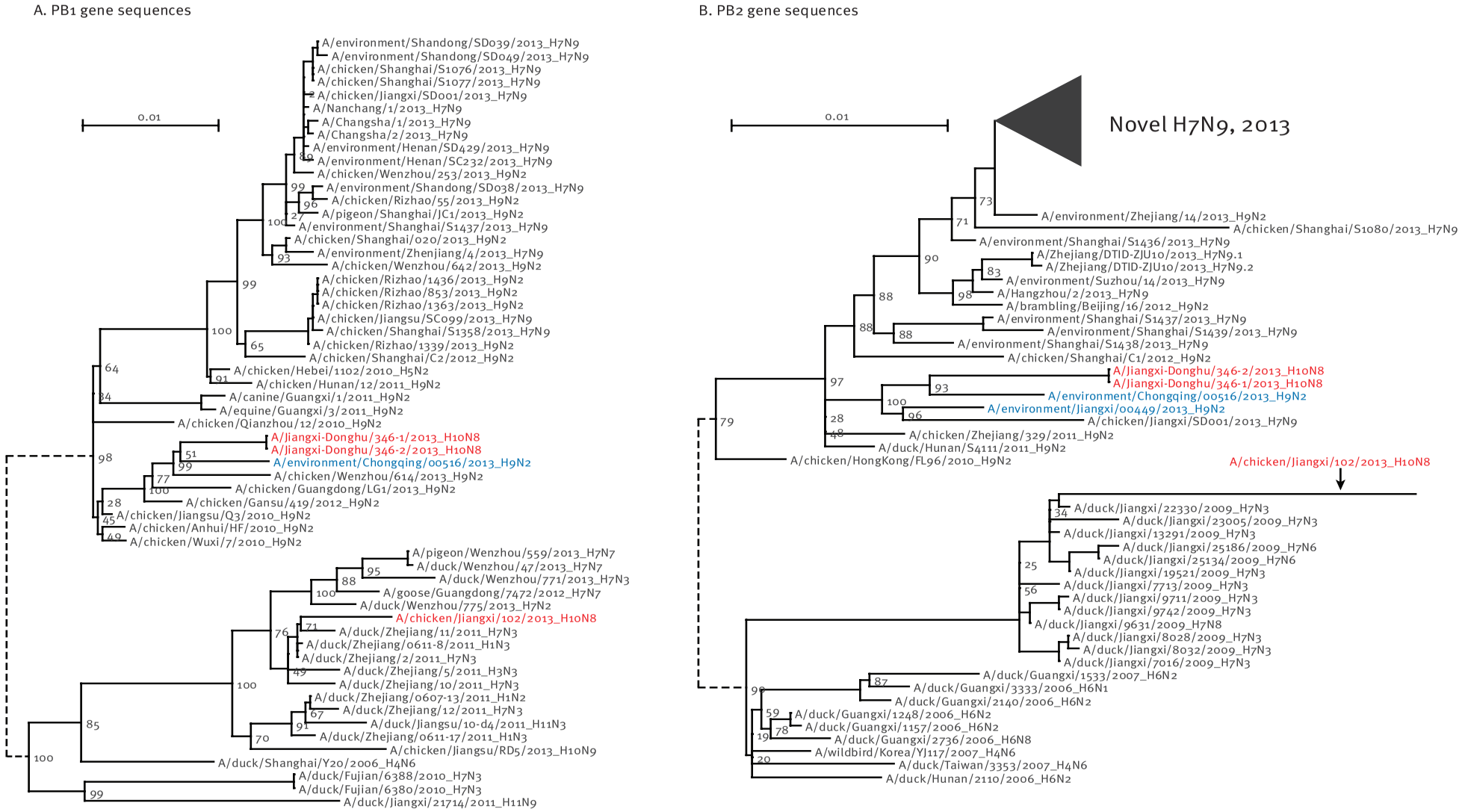
Figure 7

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



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