

Origin and diversity of novel avian influenza A H7N9 viruses causing human infection: phylogenetic, structural, and coalescent analyses



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Summary

Background On March 30, 2013, a novel avian influenza A H7N9 virus that infects human beings was identified. This virus had been detected in six provinces and municipal cities in China as of April 18, 2013. We correlated genomic sequences from avian influenza viruses with ecological information and did phylogenetic and coalescent analyses to extrapolate the potential origins of the virus and possible routes of reassortment events.

Methods We downloaded H7N9 virus genome sequences from the Global Initiative on Sharing Avian Influenza Data (GISAID) database and public sequences used from the Influenza Virus Resource. We constructed phylogenetic trees and did 1000 bootstrap replicates for each tree. Two rounds of phylogenetic analyses were done. We used at least 100 closely related sequences for each gene to infer the overall topology, removed suspicious sequences from the trees, and focused on the closest clades to the novel H7N9 viruses. We compared our tree topologies with those from a bayesian evolutionary analysis by sampling trees (BEAST) analysis. We used the bayesian Markov chain Monte Carlo method to jointly estimate phylogenies, divergence times, and other evolutionary parameters for all eight gene fragments. We used sequence alignment and homology-modelling methods to study specific mutations regarding phenotypes, specifically addressing the human receptor binding properties.

Findings The novel avian influenza A H7N9 virus originated from multiple reassortment events. The HA gene might have originated from avian influenza viruses of duck origin, and the NA gene might have transferred from migratory birds infected with avian influenza viruses along the east Asian flyway. The six internal genes of this virus probably originated from two different groups of H9N2 avian influenza viruses, which were isolated from chickens. Detailed analyses also showed that ducks and chickens probably acted as the intermediate hosts leading to the emergence of this virulent H7N9 virus. Genotypic and potential phenotypic differences imply that the isolates causing this outbreak form two separate subclades.

Interpretation The novel avian influenza A H7N9 virus might have evolved from at least four origins. Diversity among isolates implies that the H7N9 virus has evolved into at least two different lineages. Unknown intermediate hosts involved might be implicated, extensive global surveillance is needed, and domestic-poultry-to-person transmission should be closely watched in the future.

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Introduction

On March 30, 2013, a novel avian influenza A H7N9 virus causing human infections was identified in China.¹ As of April 18, 2013, the virus had spread to six provinces and municipal cities—ie, Shanghai, Anhui, Jiangsu, Zhejiang, Beijing, and Henan. To our knowledge, this outbreak represents the first time that the H7N9 subtype has infected people and caused fatal cases (as of April 18, 2013, 87 people have been infected and 17 have died). In modern times, H7 subtype avian influenza viruses, including H7N1, H7N2, H7N3 and H7N7, have caused more than 100 human infections, including a fatal case in the Netherlands.²⁻⁴ In wild birds, H7 and N9 avian influenza viruses have evolved to American, Oceanian, and Eurasian lineages.^{5,6} Preliminary analyses¹ have shown that the H7N9 viruses causing the 2013 outbreak in China are

novel reassortants, the HA gene originated from avian influenza viruses circulating in ducks in Zhejiang Province, the NA gene is related to avian influenza viruses isolated from wild birds, and the internal genes probably originated from an earlier H9N2 lineage. Kageyama and colleagues⁷ have postulated that the internal genes originated from poultry viruses. In this study, we integrated data from phylogenetic analyses, coalescent analyses, and host ecology to infer the potential origins and genetic diversity of the novel avian influenza A H7N9 viruses.

Methods

Sequence alignment

We downloaded the H7N9 virus genome sequences from the Global Initiative on Sharing Avian Influenza Data (GISAID) database—specifically, A/Anhui/1/2013

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(Anhui/1), EPI439503~EPI439510; A/Shanghai/1/2013 (Shanghai/1), EPI439486~EPI439491, EPI439493, EPI439494; A/Shanghai/2/2013 (Shanghai/2), EPI439495~EPI439502; and A/Hangzhou/1/2013 (Hangzhou/1), EPI440095~EPI440097. We downloaded all the public sequences used from the Influenza Virus Resource.⁸ CLC

Main Workbench (CLC Bio, Aarhus, Denmark) was used for alignment and editing of sequences.

Phylogenetic analysis

Phylogenetic trees were inferred on the basis of maximum-likelihood methods, the general time-reversible model⁹ of

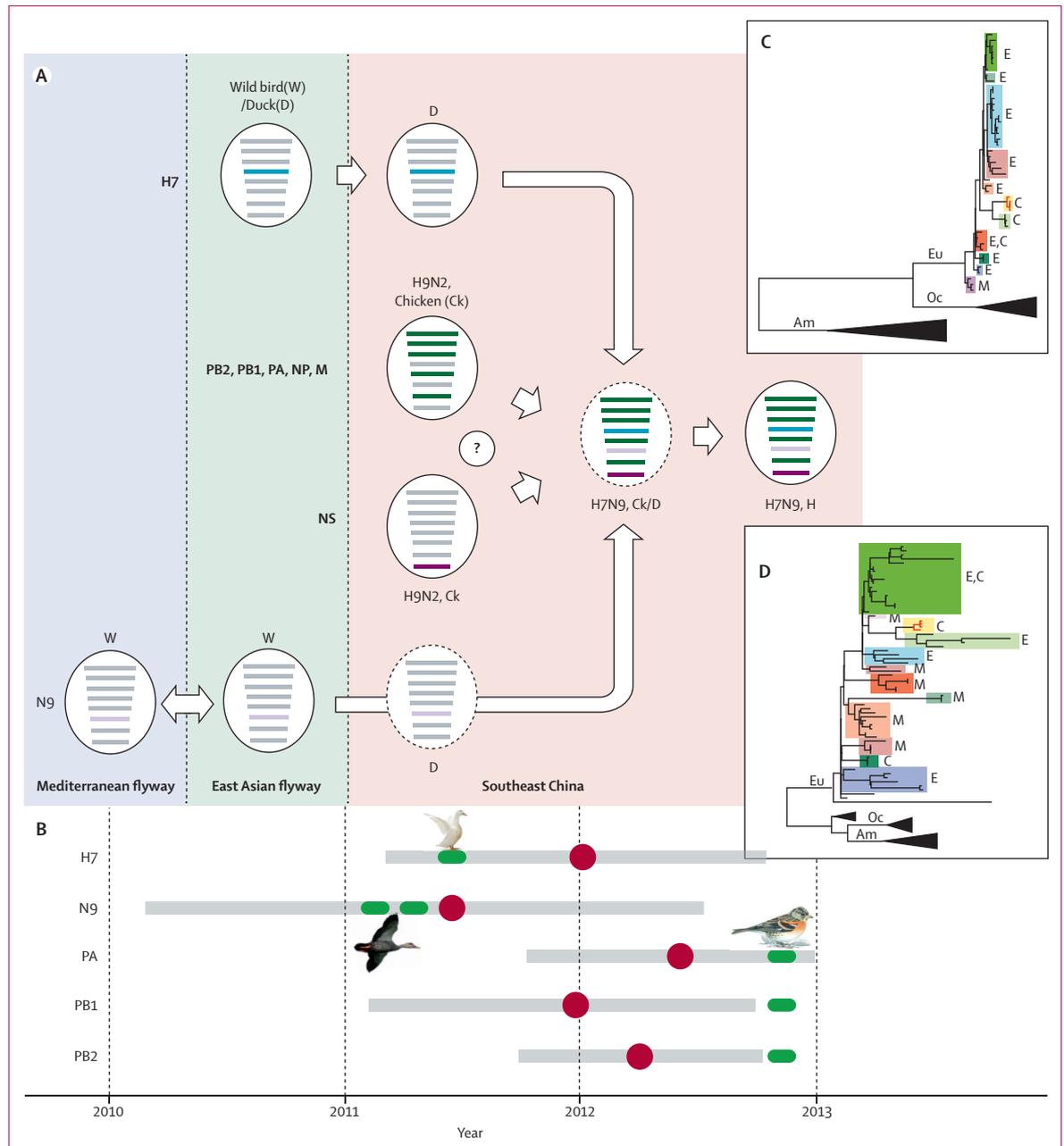


Figure 1: Spatial and temporal model of origin of novel avian influenza A H7N9 virus

In panel A, each circle represents an influenza virus. The eight gene segments (horizontal bars) are, from top to bottom, PB2, PB1, PA, HA, NP, NA, MP, and NS. The question mark indicates the uncertainty of internal gene reassortment. In panel B, red circles represent the estimated times of most recent common ancestors (identified from phylogenetic analyses) of the novel H7N9 virus were collected (appendix). Green bars represent the times when the most closely related sequences (identified from phylogenetic analyses) of the novel H7N9 virus were collected (appendix). Panel C shows schematic phylogenetic trees of HA of the H7 subtype, and panel D shows schematic phylogenetic trees of NA of the N9 subtype, constructed on the basis of the maximum likelihood method and with 1000 bootstrap replicates. Figure 2 shows detailed bootstrap values. Coloured boxes and letters show clades in different flyways. E= east Asian flyway. M=Mediterranean flyway. C=China. Am=American clade. Eu=Eurasian clade.

nucleotide substitution, and γ -distributed rates among sites (appendix). We did 1000 bootstrap replicates for each tree. All phylogenetic trees were constructed with RAxML.¹⁰ Two rounds of phylogenetic analyses were done. In the first round, we used at least 100 closely related sequences for each gene to infer the overall topology. In the second round, on the basis of phylogenetic analysis, we removed a few suspicious sequences from the trees and focused on the closest clades to the novel H7N9 viruses. Tree topologies obtained from the two rounds

were compared with those from bayesian evolutionary analysis by sampling trees (BEAST) analysis.¹¹

See Online for appendix

Bayesian Markov chain Monte Carlo evolutionary analyses and homology modelling

We used the bayesian Markov chain Monte Carlo method, implemented in BEAST¹¹ (version 1.7.2) to jointly estimate phylogenies, divergence times, and other evolutionary parameters for all eight gene fragments. For all analyses we used the uncorrelated log-normal-distributed model¹²

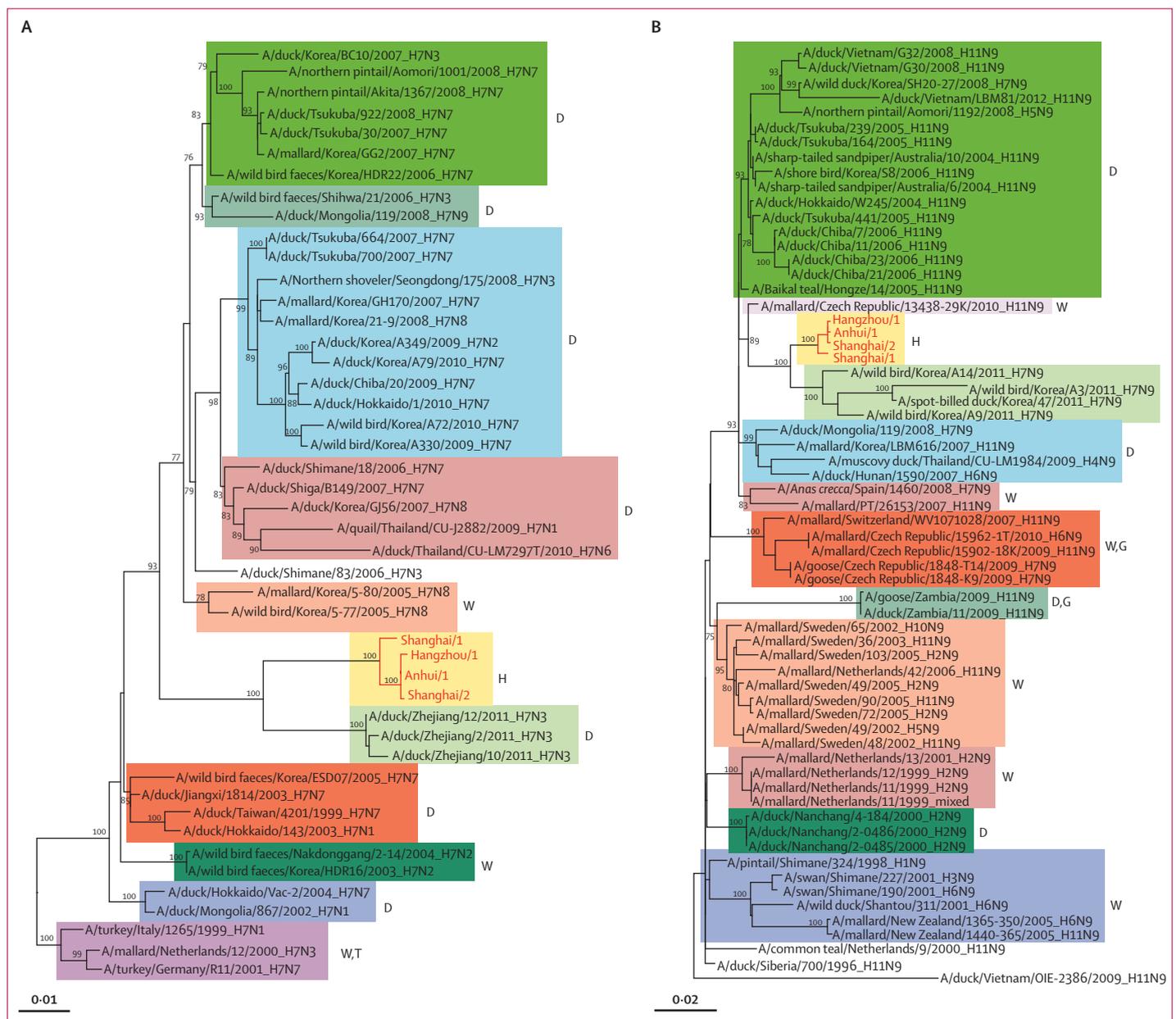


Figure 2: Phylogenetic trees of H7 (A) and H9 (B)

Different lineages are shown by different-coloured boxes (each colour corresponds to that in figure 1C, 1D). H7N9 viruses are shown by red lines and text. D=duck. W=wild bird. H=human being. T=turkey. G=goose.

For more on the **SWISS MODEL** see <http://swissmodel.expasy.org>

and the general time-reversible nucleotide substitution model. To investigate the extent to which dating estimates are affected by the demographic model chosen,¹³ we reanalysed on the basis of the constant size and exponential growth models (appendix). Bayesian Markov chain Monte Carlo analysis was run for different steps, 10% of which were removed as burn-in and sampled every 10 000 steps.

We used the online structure homology-modelling server SWISS MODEL in automated model mode for our homology modelling of the ectodomain protein sequences of the H7 HAs and NAs.

Results

The phylogeny of the H7 gene sequences available showed three main independent lineages—ie, American, Oceanian, and Eurasian lineages. The novel H7N9 virus fell within the Eurasian lineage, and was genetically close to sequences isolated from ducks in Zhejiang Province in 2011 (figures 1, 2). The H7 phylogenetic tree also showed that varied H7 viruses were circulating in wild ducks along the east Asian flyway, which covers eastern China, South Korea, and Japan. Genetic exchange between wild birds and ducks

has also been recorded (figure 2). The estimated time to most recent common ancestor of the novel H7N9 strains was roughly January, 2012 (mean Jan 4, 2012, 95% highest posterior density March 5, 2011–Oct 12, 2012).

Similar to the H7 phylogeny, the N9 phylogenetic tree showed that the NA gene of the novel H7N9 virus also belonged to the Eurasian lineage (figure 1). The most closely related gene sequences were from H7N9 viruses isolated from wild ducks in South Korea in February and April of 2011,¹⁴ implying a similar virus circulating in this area. At that time, these wild ducks colonised for breeding during northward migration from southeast China along the east Asian flyway, and the estimated time to most recent common ancestor of the NA gene was roughly mid-June, 2011 (mean June 18, 2011, 95% highest posterior density March 1, 2010–July 9, 2012; figure 1).

Without exception, the six internal genes of the H7N9 virus were clustered together with H9N2 viruses isolated from China.¹⁷ Nevertheless, phylogenetic analyses suggested at least two different origins of H9N2 avian influenza viruses, one for the NS gene and one for the remaining sets of internal genes (figure 3; appendix).

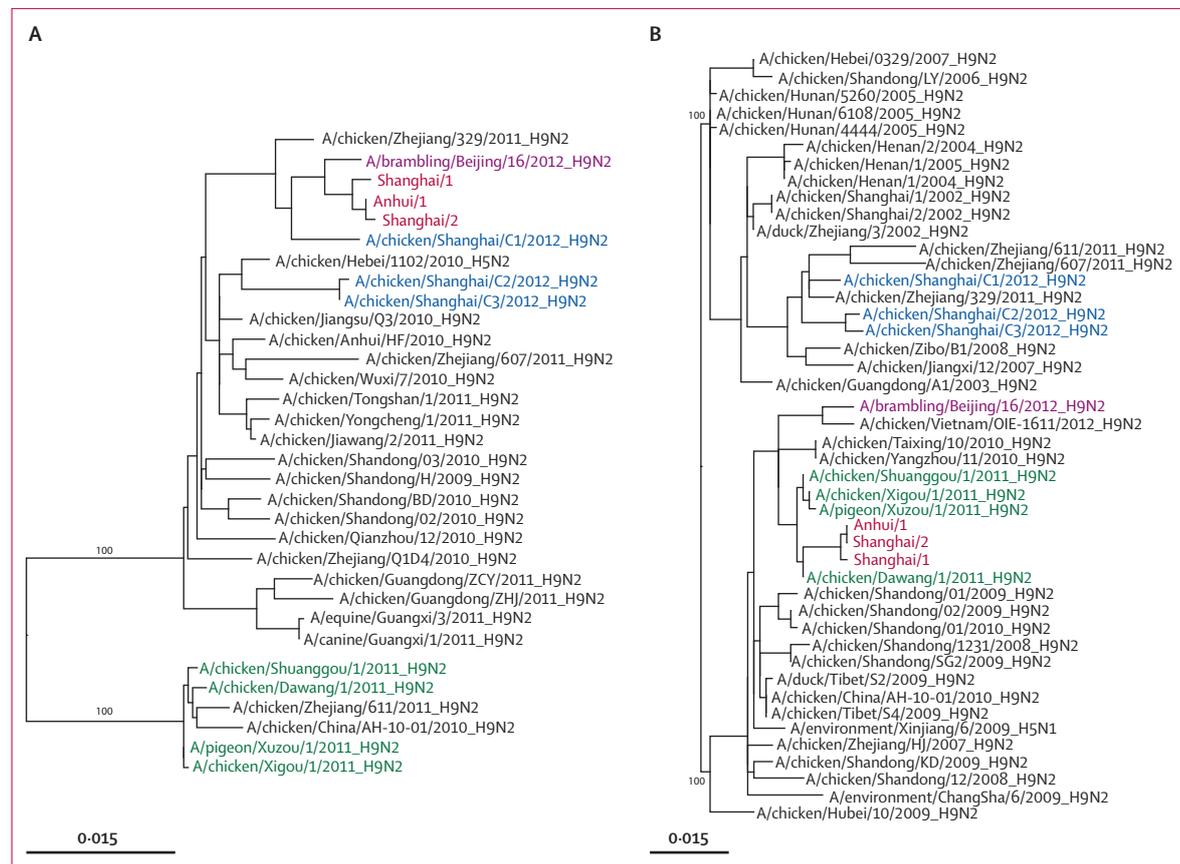


Figure 3: Phylogenetic trees of PB2 (A) and NS (B)

Avian influenza A H7N9 viruses are shown in red, brambling viruses in pink, viruses isolated from Shanghai in 2012 in blue, and viruses isolated from Jiangsu province in green.

The most closely related genes—PB2, PB1, and PA—were all from one strain, A/brambling/Beijing/16/2012(H9N2), which was collected on Nov 7, 2012. The brambling is a migratory bird and might fly south for wintering. The phylogenetic trees also showed that the polymerase genes of this virus fell within a clade of chicken H9N2 viruses in the vicinity of Shanghai in 2012 and Zhejiang in 2011; viruses from Beijing or other northern provinces were not identified in this clade (figure 3; appendix). The long branch between the brambling virus and the novel H7N9 viruses shows that few surveillance data are available. Furthermore, the estimated time to most recent common ancestor to the

polymerase genes was before June, 2012 (figure 1), suggesting that the brambling H9N2 virus is a more recent strain than is the ancestor of the novel H7N9 avian influenza viruses.

Further examination of the H7 phylogeny showed a long branch separating Shanghai/1 from Anhui/1, Shanghai/2, and Hangzhou/1 in the the H7N9 viruses that infected people (figure 2). Sequence alignment of the HA protein sequences identified nine aminoacid mutations that distinguished Shanghai/1 from the other three strains (figure 4). The diversification between Shanghai/1 and other human isolates (shown by the long branch) was also noted in the NA and internal genes (figures 2, 3;

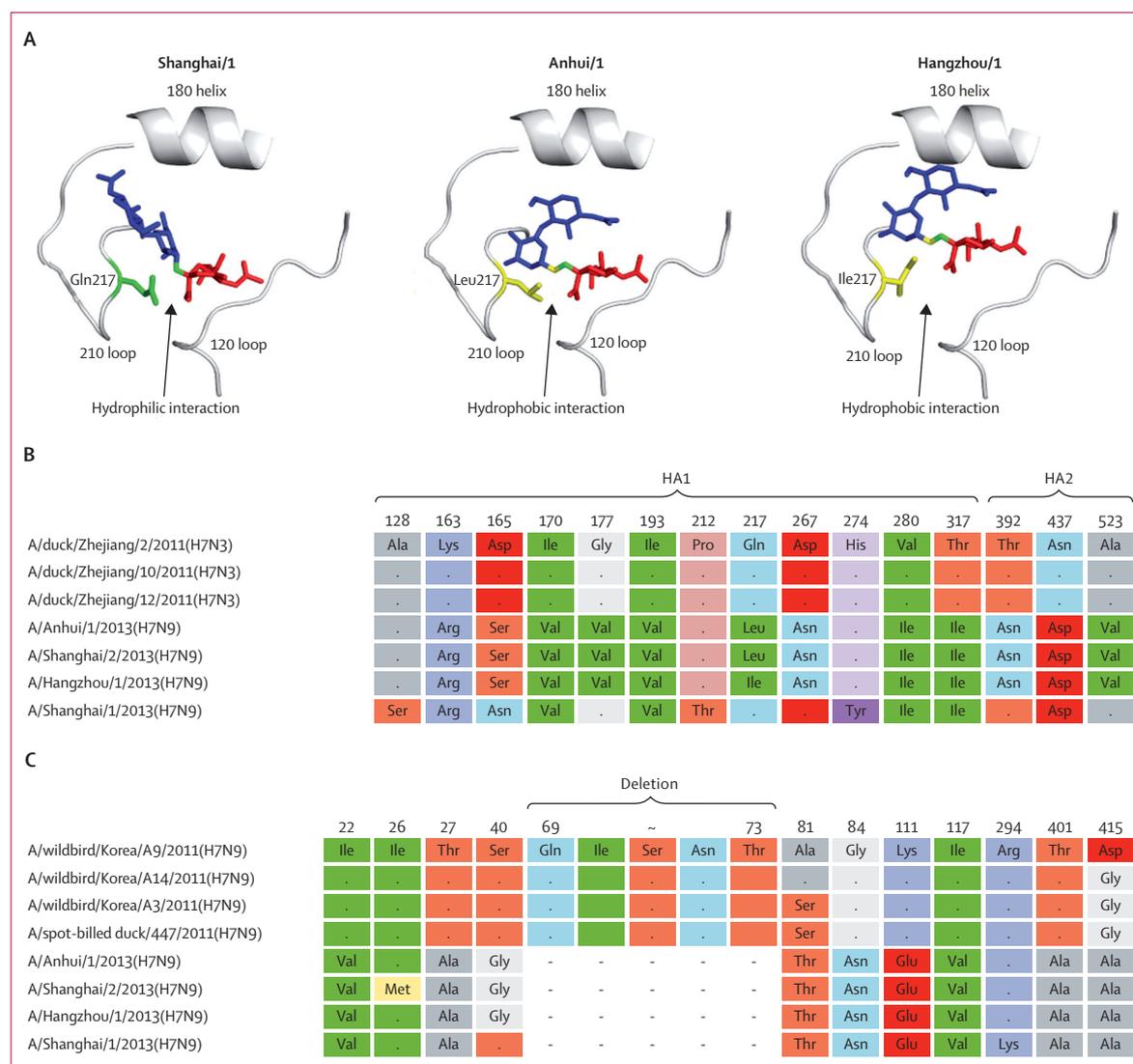


Figure 4: Homology-modelling structural analysis of haemagglutinins derived from human avian influenza A H7N9 virus isolates (A); and alignment of aminoacids of both haemagglutinins and neuraminidases between H7N9 and viruses in adjacent clade (B, C)

At position 217 (corresponding to position 226 of H3 numbering), the haemagglutinin of Shanghai/1 has a hydrophilic Gln, that of Anhui/1 and Shanghai/2 a hydrophobic Leu, and that of Hangzhou/1 a hydrophobic Ile. Gln is compatible with the avian receptor (which is hydrophilic), whereas Leu and Ile are compatible with the human receptor (which is hydrophobic). Numbering in (B) starts from the mature haemagglutinin polypeptide, whereas that in (C) starts from the first Met of the alignments. The Rasmol colouring scheme in CLC Main Workbench was used; it shows different properties of aminoacids.

Panel: Research in context**Systematic review**

We did a basic local alignment search tool (BLAST) search of each H7N9 gene segment against the Influenza Virus Resource on April 6, 2013. The most 100 similar sequences were then extracted for the phylogenetic and coalescent analyses. Recent reports have described clinical information about the first patients infected with avian influenza A H7N9 virus and provided preliminary information about the origins of the virus.¹⁷ The authors of a previous report proposed three origins of the H7N9 virus.¹

Interpretation

This study is the first (to our knowledge) in which comprehensive methods were applied to infer the evolution of the H7N9 virus. We proposed that the virus might have evolved from at least four origins and discussed the potential roles of migratory birds and poultry in the H7N9 outbreak. Both the phylogenetic analysis and the phenotypic inference on some key aminoacid sites have emphasised that extensive surveillance of avian influenza A H7N9 is necessary in humans, poultry, and wild birds.

appendix). 11 aminoacid mutations and a five aminoacid deletion in the NA protein distinguished the novel H7N9 viruses from those noted in wild birds; two of these mutations were noted in Shanghai/1 only (figure 4).

One glutamine (Gln) to leucine (Leu) mutation (position 226 in H3 numbering and 217 in H7 numbering) at the receptor-binding site might cause the H7N9 virus to bind with high affinity to the human receptor.¹⁵ This finding could be explained by the hydrophilic characteristics of the Gln that interacts with the avian receptor and the hydrophobic characteristics of the Leu that favours the human receptor (figure 4).¹⁶ The substitution Gln226Leu, which was noted in Anhui/1 and Shanghai/2, suggests that the novel H7N9 virus might have changed receptor-binding properties. Additionally, the substitution Gln226Ile (isoleucine) was noted in Hangzhou/1, which probably increases the potential for high-affinity binding to human receptors because isoleucine is hydrophobic. This Gln226Ile substitution has not been noted previously for any HA subtypes (figure 4).

Although we integrated data from various sources and proposed the potential origins and reassortment routes of the novel avian origin H7N9 influenza virus, we emphasise that our analysis did not have extensive surveillance data—a bottleneck for all researchers—partly because, compared with highly pathogenic H5N1 avian influenza viruses, viruses of the H7 subtype have low pathogenicity for avian hosts. Hence, avian hosts could harbour viruses for a long time without showing symptoms. However, we have analysed most publicly available, genetically related sequences.

Discussion

Tracing the origin of the novel H7N9 virus is of vital importance for formulation of effective prevention and surveillance policies. We propose at least four possible gene-segment origins are proposed for the novel H7N9 virus (panel).

The estimated time to most recent common ancestor of the novel H7N9 strain was during the wintering period for wild birds. Thus, corresponding H7 strains might have been circulating in poultry (most likely in ducks) for at least 1 year.

An earlier H11N9 strain of mallard (*Anas platyrhynchos*) origin from the Czech Republic in 2010, and an H7N9 strain of common teal (*Anas crecca*) origin from Spain in 2008, also had similar N9 genes to the H7N9 viruses, suggesting that the earlier N9 genes might have been introduced from Europe through bird migration (figure 2). Therefore, the NA gene fragment of the novel H7N9 virus possibly originated from avian influenza viruses carried by wild birds. However, wild ducks are unlikely to transfer avian influenza viruses directly to chickens. Most probably, the wild ducks first transferred the viruses to domesticated ducks (wild and domesticated ducks have similar behaviours and share habitats in eastern China). Additionally, the long-branch between the novel H7N9 and avian influenza viruses from wild birds in the phylogenetic tree raised the possibility of the existence of an intermediate host (figure 2).

Do ducks obtain the HA and NA genes from migratory birds sequentially or simultaneously? We think that the two alternative possibilities coexist. Wild ducks, especially mallards, were frequently infected with or carried H7 viruses (according to sequence information available at the Influenza Virus Resource), and mallards and spot-billed ducks (*Anas poecilorhyncha*) often mix together in a very large colony for molting and wintering.¹⁷ Furthermore, mallards and spot-billed ducks, along with the common teal, are the dominant wintering ducks in southeast China.¹⁷ These large mixed colonies of wild ducks that share habitats with domestic ducks might increase opportunities for genetic reassortment of viruses.

The NS gene was clearly closely related to a group of H9N2 avian influenza viruses in chickens in Jiangsu, China, whereas the remaining internal genes were closely related to those noted in avian influenza viruses isolated from chickens in Shanghai and the vicinity. The distance between these locations where different groups of H9N2 avian influenza viruses were collected is roughly 200 km, and hence virus transmission could have occurred via chicken transportation. Although the NP genes of all three H7N9 isolates were clustered together with avian influenza viruses from Shanghai, they fell within two separate subclades, which implies that the genetic diversity of H9N2 avian influenza viruses in chickens in this area is high (appendix).

We believe that, similar to the other three internal genes, PB2, PB2 and PA might have arisen from H9N2

virus within chicken populations, although wild birds could have brought earlier lineages of viruses to southern China. Increased surveillance data are needed in this region to further elucidate the origin of this virus and trace the evolution of the polymerase genes.

We hypothesise that the H7N9 viruses that infected human beings resulted from a reassortment of avian influenza viruses of at least four origins—duck origin for HA, duck (probably also wild bird) origin for NA, and at least two H9N2 chicken viruses for the internal genes (figure 1). Regarding the potential intermediate hosts and the place where the reassortment events could have taken place, we extrapolated, on the basis of available evidence, that the HA genes were circulating in the east Asian flyway in both wild birds and ducks, the NA genes were introduced from European lineages at an early time and transferred to ducks in China by wild birds through migration along the east Asian flyway, and H9N2 avian influenza viruses were circulating in chicken and duck populations in eastern China and possibly reassorted with the H7 and N9 avian influenza viruses in ducks, which led to the emergence of the new H7N9 lineage. After these reassortment events, the new viruses started to circulate in chickens, with low pathogenicity. Furthermore, we propose that these reassortment events most probably took place in Shanghai or the adjacent provinces, such as Zhejiang or Anhui.

Position 292 of the NA gene (position 294 in N9) has been previously reported to confer resistance to oseltamivir.^{18,19} The strain Shanghai/1 had a lysine at this position and was thus deemed resistant to oseltamivir, whereas all the other strains had an arginine at this site, and were thus supposed to be sensitive to oseltamivir.¹⁹ These genotypic and postulated phenotypic differences imply that the H7N9 lineage has diversified since its emergence and emphasise the necessity of extensive surveillance of this virus in human beings, poultry, and wild birds.

Several long branches existed between the novel H7N9 clade and closely related sequences, which could be explained as unknown intermediate hosts or an absence of genetic diversity because few sequence data are available. Because few surveillance data are available, to establish whether the noted aminoacid substitutions that increased receptor binding in people resulted from fixation of genetic drifts or under host-specific selective pressures is difficult. So far, no mammals other than human beings have been reported to be infected with H7 or N9 influenza viruses in China, but H9N2 viruses have been isolated from pigs.

In conclusion, on the basis of available evidence, we believe that the novel avian influenza A H7N9 virus was a multiple reassortant. The HA and NA genes might originate from duck avian influenza viruses, which might have obtained the viral genes from migratory birds a year previously, whereas the internal genes might come from chicken avian influenza viruses. We believe that the estimated times to most recent common

ancestor for the eight genomic fragments and the frequent poultry transportation in China account for the increased number of confirmed sporadic cases of human infection. In particular, this novel H7N9 virus has diversified into different lineages since its emergence several months ago.

Contributors

GFG, FL, DL, WS, and YShu designed the study. DL, WS, YShi, HX, DW, WLi, YB, YWu, XL, JY, WLi, and FL analysed the data. DL, WS, YShi, GZ, WY, YWa, JM, YShu, FL, and GFG interpreted the data. DL, WS, YShi, FL, and GFG prepared the figures. DL, WS, YShi, YShu, FL, and GFG wrote the paper.

Conflicts of interest

We declare that we have no conflicts of interest.

Acknowledgments

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