



Letters to the Editor

Better fit of codon usage of the polymerase and nucleoprotein genes to the chicken host for H7N9 than H9N2 AIVs



Dear Editor,

Recently, the evolution of H7N9 AIVs has been widely studied in this journal.^{1,2} The six internal genes (PB2, PB1, PA, NP, M, and NS) of H7N9 are from chicken H9N2 avian influenza viruses.³ Genotypic analysis of these six internal genes revealed that the most frequent genotypes of H9N2 genotypes did not become the major genotypes in H7N9, thus it appears that H7N9 prefer some genotypes for these genes.⁴ The cause for this preference remains unknown, as is whether this preference helped H7N9 become a successful AIV subtype. Increased replication, i.e. the efficient production of new viruses, is one of the critical factors that influences infection. The translation of viral proteins requires host tRNAs.⁵ Matching viral and host codon usage could enhance the translation of viral proteins.⁶ Codon usage preference was suggested to play a role in the evolution of AIVs,⁷ thus, we compared codon usage of H7N9 and H9N2 to their hosts.

Genomes of H7N9 and H9N2 AIVs were downloaded from the NCBI Influenza Virus Resource at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/genomes/FLU), the Influenza Research Database (IRD) (www.fludb.org/brc/home.spg?decorator=influenza), and the Global Initiative on Sharing Avian Influenza Data (www.gisaid.org). Redundant sequences, laboratory strains and short (<95% of the corresponding gene) sequences were removed. Our final dataset contained 667 chicken-isolated H7N9 genomes, 1051 human-isolated H7N9 genomes, and 704 chicken-isolated H9N2 genomes. Open reading frames (ORFs) for all eight viral genes (PB2, PB1, PA, HA, NP, NS, MP, and NA) were used.

The codon adaptation index (CAI) quantifies the similarity of the codon frequency of a set of test sequences (e.g., viral sequences) with those from a reference set of sequences,⁸ which are typically highly expressed host genes. A greater similarity of codon usage of the viral sequences to the highly expressed host genes predicts adaptation of the viral genes to their hosts, and high expression. CAI values range from 0 to 1, with higher CAIs indicating better adaptation to the hosts. HA, NA, NS, and MP genes from H7N9 do not show better fits to the chicken compared to those from H9N2, but the NP, PA, PB1, and PB2 do have better

fits ($P < 0.05$) (Fig. 1A). Replication and transcription of AIVs are catalyzed by the viral polymerase complex, which is composed of the PB2, PB1, and PA proteins.⁹ Together with the polymerase proteins, the NP encapsulates the viral RNA to form the ribonucleoprotein complex (RNP), which is the minimal functional unit of the viral genome for transcription and replication. The six internal genes of H7N9 are from chicken H9N2 avian influenza viruses, however, the polymerase (PA, PB1, and PB2) and nucleoprotein (NP) genes of H7N9 AIVs show much better fit for chicken than H9N2. This better fit of their codon usage should help the replication of H7N9 within chicken hosts. Genotypic analysis of the six internal genes revealed that H7N9 prefer some genotypes,⁴ with this preference explaining the difference in codon usage between H7N9 and H9N2. A better fit for codon usage by these genotypes should help H7N9 persist in poultry since their emergence in 2013.

The effective numbers of codon (ENC), a measure of codon bias, values range from 49 to 57. Values of ENC greater than 40 indicate that weak bias prevail in all genes from both H7N9 and H9N2 AIVs. An ENC-plot mapping analysis was used to identify factors that influence codon usage bias. ENC values for each gene were plotted against their corresponding GC3 content (Fig. 1B). All plotted values lie considerably below the solid curve, indicating that in addition to mutational pressure, factors such as selection for translational efficiency also influence codon usage patterns in AIVs. We therefore constructed a neutrality plot to identify the effects of natural selection and mutation pressure on the codon usage patterns by plotting P12 (GC12) values of the synonymous codons and P3 (GC3) values. According to this neutrality plot analysis, natural selection accounts for the majority (81–98%) of total selection pressure acting on the evolution of codon usage in the NP, PA, PB1, and PB2 genes.

Unlike many novel AIV subtypes that quickly disappear, H7N9 became the dominant AIV subtype in China soon after its emergence in 2013.¹⁰ H7N9 AIVs frequently reassort with H9N2.³ Our efforts discovered that, although the six internal genes for H7N9 were attained from H9N2, the polymerase (PA, PB1, and PB2) and nucleoprotein (NP) genes of H7N9 have a better fit to codon usage for expression in chicken than those of H9N2. The preference of the internal gene genotypes that have a better fit to codon usage of the host may help H7N9 persist in poultry and become a successful subtype in China.

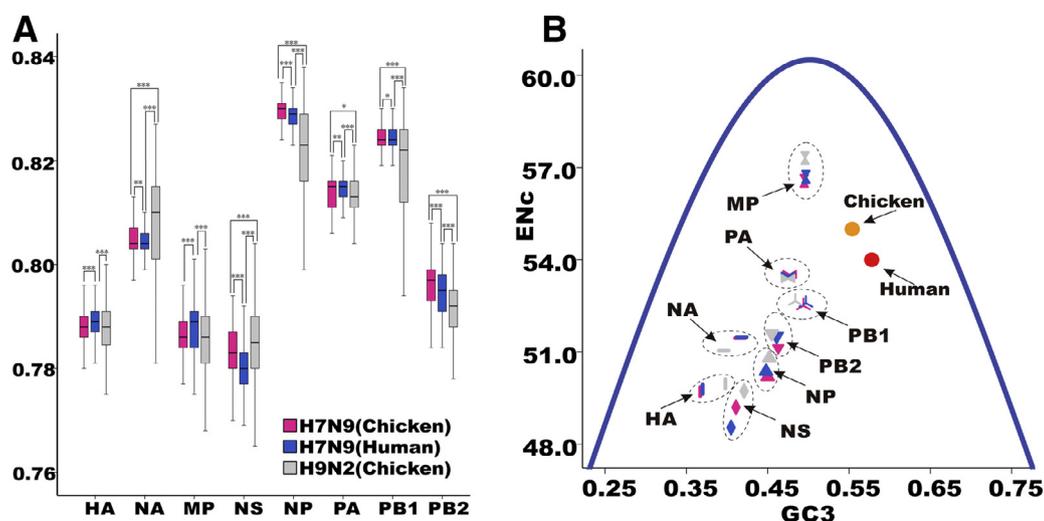


Fig. 1. (A) Codon adaptation index (CAI) analysis of all eight H7N9 and H9N2 AIV genes to their hosts. Chicken-isolated H7N9 AIVs are shown in red, human-isolated H7N9 in blue, and chicken-isolated H9N2 in grey. (B) ENC denotes the effective number of codons, and GC3 denotes the GC content on the third position of synonymous codons. The solid blue line represents the expected curve derived from the positions of strains if codon usage was only determined by GC3 composition (i.e., without selection). Points on or close to the curve means that the bias was caused by mutation pressure, otherwise, the varied positions are due to natural selection or other factors.

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Conflict of interest

The authors declare not conflict of interest.

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The codon usage bias of avian influenza A viruses

Dear Editor,

A recent study in this journal revealed that avian influenza A viruses (AIVs) H5Nx (N1, N6 and N8) showed pathogenicity,¹ and accordingly had different adaptation to the codon usage pattern of its hosts.² AIVs classify into 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes.³ The abundance and distribution of different subtypes varies greatly.⁴ Subtype H5 is the most often isolated AIV, followed by H7 and H9.⁴ It remains unclear which factor(s) associate with this abundance in birds. Considered that codon usage pattern was suggested to influence the adaptation of H5Nx AIVs,² in this study, we calculated the codon adaptation index (CAI) to assess how well AIV HA subtypes H1–H16 and NA subtypes N1–N9 correspond to host codon usage patterns.



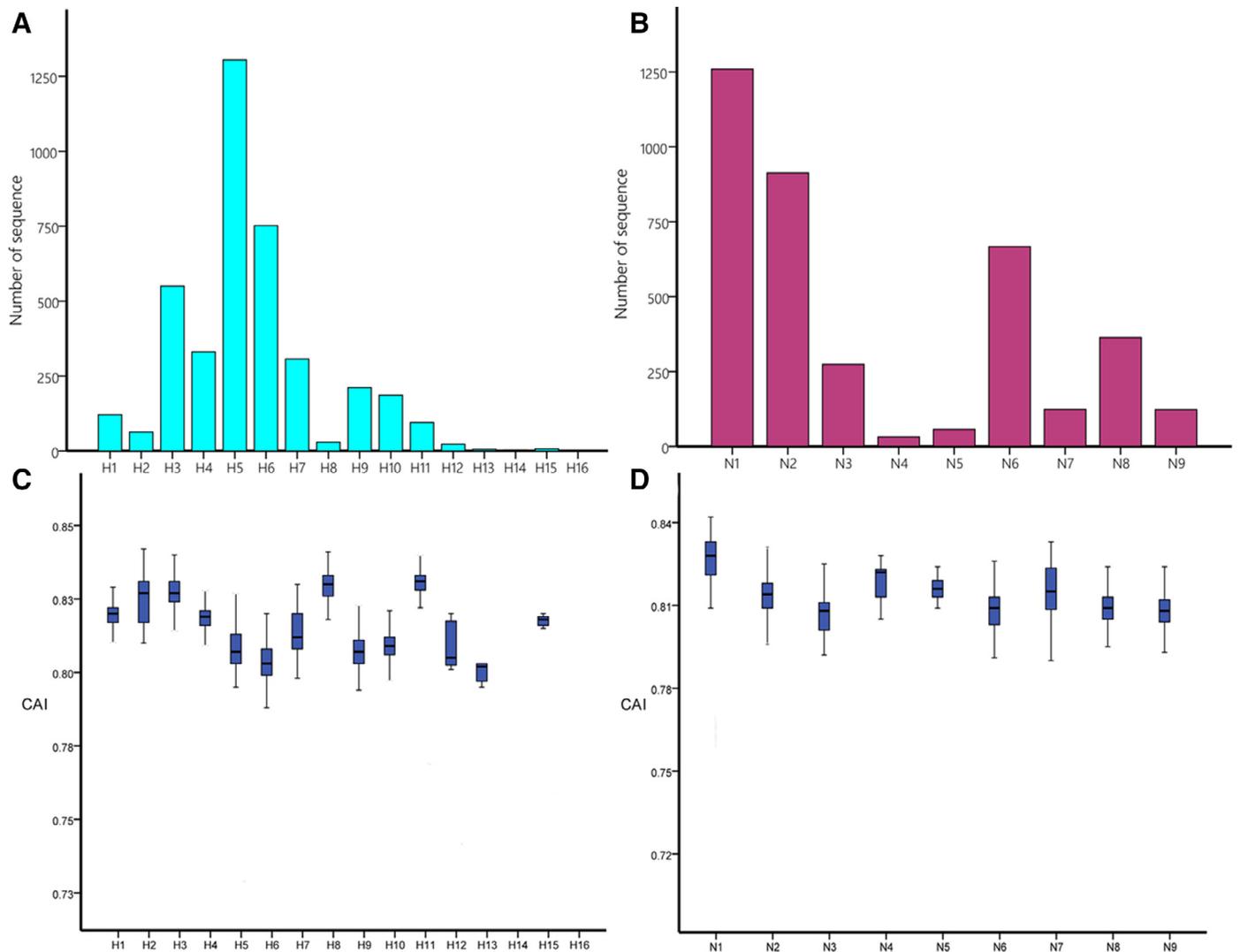


Fig. 1. (A) The abundance of 16 HA subtypes of avian influenza viruses isolated from ducks. (B) The abundance of 9 NA subtypes of avian influenza viruses isolated from ducks. (C) Codon adaptation index (CAI) of 16 HA subtypes. (D) Codon adaptation index (CAI) of 9 NA subtypes. All available sequences of each subtype were collected from NCBI Influenza Virus Resource.

The specific distribution of subtypes varied between different surveillance studies depending on species, time and place. We counted all available sequences of each subtype isolated from ducks to roughly estimate relative abundance. Surveillance data of different subtypes were collected from NCBI Influenza Virus Resource. As shown in Fig. 1(A), HA subtypes H3, H5, and H6 had relatively more sequences than the other subtypes, while H13, H14, H15 and H16 were rare. While, NA subtypes N1 and N2 generally had far more sequences than N4 and N5 (Fig. 1(B)). Subtypes such as H5 and H7, which cause severe illness, have received greater attention than those that cause mild or subclinical disease. Thus, the former had more records and sequences in the databases, and this biased statistics on relative abundance. Although AIVs have been isolated from >100 species, most surveillance studies focused on Galliformes (domestic poultry), Anseriformes (ducks, geese, and swans) and Charadriiformes (shorebirds), which are thought to be the reservoir community for AIVs.⁵ Many limitations in the current surveillance system have also contributed to sampling bias.⁶ Therefore, the available sequence numbers of each subtype (Fig. 1(A) and (B)) only roughly reflected its actual abundance.

Viruses depend on their hosts' cellular structure and metabolism to replicate and assemble. Codon usage patterns of viruses reflect the evolutionary changes that allow them to

optimize their survival and fitness to their hosts.⁷ Thus, we compare the codon usage bias of AIVs to their hosts. Codon adaptation index (CAI) which predicts the level of gene expression and the adaptation of viral genes to their hosts, was performed with the local version of CAIcal server (<http://genomes.urv.cat/CAIcal/>). Codon usage patterns of all subtypes of AIVs were calculated for the common host–duck. HA and NA showed different patterns. For HA subtypes, H5 and H6 had more isolated sequences, while they had lower CAI values than did other subtypes (Fig. 1(A) and (C)). While for NA subtypes, N1 had the most sequence records among NA subtypes (Fig. 1(B)), yet CAI values show that N1 (0.827 ± 0.007) had the highest CAI values (Fig. 1(D)). Because influenza virus replication is based on its host's expression, virus codon usage must coevolve with its host to use host resources efficiently.⁸ It is expected that the common infectious NA subtypes of AIVs are better adapted to host expression system. However, HA which is responsible for binding virus to sialic acid-containing cell-surface receptors and for membrane fusion during virus entry into host cells, is the major antigenic influenza surface glycoprotein for neutralizing antibodies. The deoptimization codon usage of HA may reduce immune stimulation to the host immune system. Deop-

timization of codon usage may reduce competition with host cell translation, reflecting another strategy of adaptation, which was found in Epstein-Barr virus.⁹ Especially, most of the H5 AIVs are highly pathogenic. Reduce their immune stimulation to their hosts would be help for their persistence and expansion in poultry.

The molecular bases for efficient virus replication and transmission are complex and multifactorial.¹⁰ Codon usage patterns of viruses were widely suggested to reflect the evolutionary changes that allow them to optimize their survival and fitness to their hosts,^{2,7,8} little is known about differences in codon usage among AIV subtypes. In conclusion, our analyses of codon usage bias in all subtypes of AIVs discover variation in codon usage bias of different AIV subtypes. The abundance of NA subtypes roughly correlates with their adaptation of codon usage bias to the host. However, HA gene showed a reversed pattern. Therefore, multiple evolutionary forces appear to drive the codon usage of AIVs.

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A human infection with a novel reassortant H3N2 swine virus in China



Dear Editor,

We read with interest the recent communication by Guo et al. concerning avian influenza virus pathogenicity.¹ Swine has been considered an intermediate host for avian influenza viruses to adapt to humans. Cross-species transmissions caused by novel reassortant swine-originate influenza A virus (S-OIV) are of particular concern after the 2009 pandemic caused by pdH1N1 virus and epidemic outbreaks caused by H3N2v.^{2,3} The genesis of these viruses shows that reassortant is the major driving force for producing highly infectious variants.^{4,5}

China breeds over 40% of the world's swine (USDA/FAS). A seroprevalence study shows 14.6% and 18.8% percent of swine in southern China are serum positive against H1 and H3 influenza A viruses.⁶ These indicate high viral contamination and the risks of cross-species transmissions in China. In this study, we retrospectively screened the ILI (Influenza Like Illness) samples collected between 2015 and 2017 in Guangdong China and a swine-origin H3N2 influenza A virus was isolated from a clinical case in 2017. The phylogenetic and antigenic analyses found it is a novel reassortant with different antigenicity from seasonal H3N2 viruses.

Retrospective surveillance

Based on the influenza-like illness (ILI) surveillance network of Guangdong, we screened the 7732 H3 positive samples collected in 2015 (2183), 2016 (1336), and 2017 (4231). Virus isolation was prepared, and 764 H3N2 strains were isolated and applied for whole genome sequencing.⁷ The primary phylogenetic analysis showed that one strain (A/GD/277/H3N2/2017, accession number MK117067-MK117074) is comprised of gene segments distinct from other seasonal H3N2 strains but more closely related to the swine-origin influenza viruses (S-OIVs) H3N2.

Epidemiology and antigenicity of the swine-origin H3N2 virus

A/GD/277/H3N2/2017 was sampled from a ten-year-old girl admitted to hospital in March 2017 with influenza symptoms and discharged 2 days later. History of direct swine contact could not be verified. Serum samples were obtained from the child in

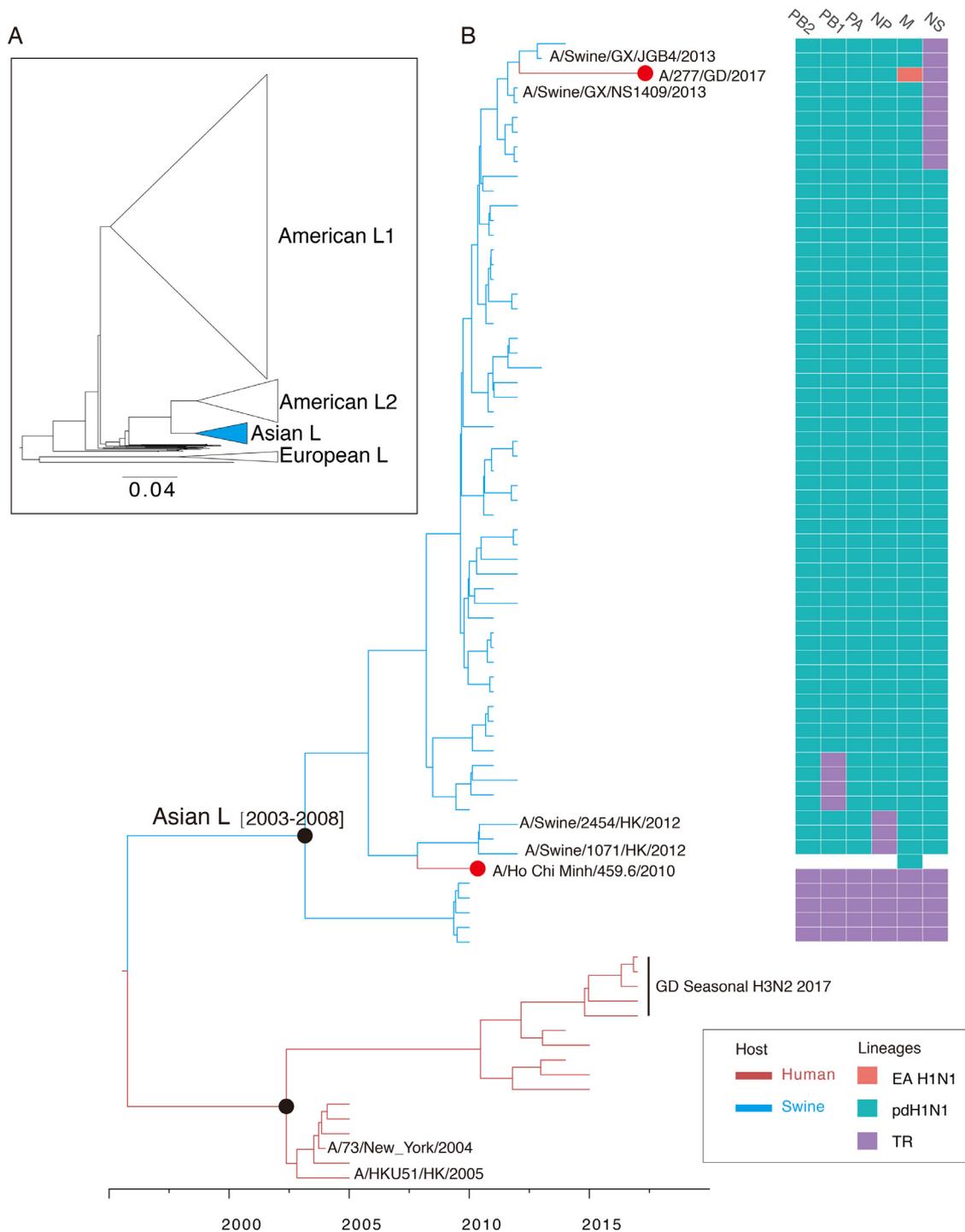


Fig. 1. Phylogenetic analyses of the HA gene segment. (A) Maximum likelihood (ML) tree inferred from swine H3N2 viruses collected in 2012–2017. Phylogenetic lineages were named according to the locations of lineage strains (Fig. S1A). The Asian lineage (Asian L) was highlighted with blue. (B) Bayesian Maximum Clade Credibility (MCC) tree of HA gene inferred from swine H3N2 viruses and closely related human H3N2 viruses. Branch colors represented the most probable ancestral species. A/GD/277/H3N2/2017 and A/Ho Chi Minh/459.6/2010 clinical strains were highlighted with red dots, and their closely related sequences were also marked. The combination of internal genes for A/GD/277/H3N2/2017 and closely related strains were shown on the right of MCC tree. Black circles indicate posterior support >0.95. EA H1N1: Europe-avian like H1N1; pdH1N1: 2009 pandemic H1N1 virus; TR: Triple Reassortant H3N2 virus.

August 2018 with written informed consent. Hemagglutination-inhibition analysis of A/GD/277/H3N2/2017 and four other contemporary seasonal H3N2 viruses revealed the serum titer to A/GD/277/H3N2/2017 was 640 but with no measurable (<10) inhibition by antiserum against other 5 contemporary seasonal H3N2 viruses. The phylogenetic relation between A/GD/277/H3N2/2017

and the other 5 tested seasonal H3N2 viruses was shown in Fig. 1B (Guangdong Seasonal H3N2 2017). The antigenicity test confirmed the S-IOV infection in this child patient and further indicated that A/GD/277/H3N2/2017 virus was antigenically distinct from influenza A(H3N2) viruses currently circulating in the human population.

Phylogenetic analysis

Maximum likelihood trees were first performed for all available H3N2 gene segments by using Raxml.⁸ A sub-dataset including the closely related sequences of A/GD/277/H3N2/2017 was generated for estimating the molecular clock phylogeny of HA gene with BEAST v2.3.⁹ Due to the population structure, the MSCOT model was applied to investigate transmissions between swine and human populations.¹⁰

The HA phylogeny based on 2012–2017 swine H3N2 sequences showed the endemic circulation of S-OIV viruses (Fig. 1A). In particular, two major established lineages of H3N2 virus (American L1 & American L2) were found in the American swine population; a small lineage (European L) comprised strains from Italy and Spain 2015–2016, and an Asian lineage comprised swine strains circulating in Southeast Asia 2010–2014 (Fig. 1A). A/GD/277/H3N2/2017 fell into the Asian lineage (Fig. 1B). The most closely related sequences of A/GD/277/H3N2/2017 were found on H3N2 swine strains circulating in southern China (Guangdong, Guangxi, Hong Kong) 2012–2014 (Fig. 1B & Fig. S1). Notably, the phylogeny of HA showed there was at least another spillover infection caused by this Asian swine lineage since the H3N2 strain (KJ955515, A/Ho Chi Minh/459.6/2010) isolated from a clinical sample in Vietnam also fell into this clade. Limited by the surveillance data in Asia after 2015 (Fig. S1), the dominant H3N2 lineage in the current Asian swine population was still elusive. However, the strains causing spillover infections in Vietnam in 2010 and in China in 2017 were clustered with Asian S-OIVs in 2010–2014 (Fig. S1B) suggesting this lineage has been firmly established in the Asian swine population. The TMRCA (time to the most common ancestor) was estimated as early as 2004 (95% HPD, 2003–2008) (Fig. 1B). There are a few amino acid differences between A/GD/277/H3N2/2017 and closely related swine strains. Site 138 (H3 numbering) in the 130-loop is of particular concern since the loop plays a determinable role in the interaction of the sialic acid receptor. All closely related swine strains preserve Asp at site 138, which changed to Ser in A/GD/277/H3N2/2017. Fundamental experiments were required to further determine whether N138S was related to this species transmission.

The N2 phylogeny was similar to H3. The phylogenetic analysis on six other internal genes indicated A/GD/277/H3N2/2017 as a reassortant of A/Swine/NS1402/GX/2013-like viruses and Europe-avian-like H1N1 viruses. As shown in Fig. 1B, seven out of eight gene segments could be found in A/Swine/NS1402/GX/2013-like viruses. In contrast, the M gene was separated from these H3N2 viruses but closely related to the Europe-avian-like H1N1 (EA H1N1) viruses (Fig. 1B and Fig. S2). Current data suggested A/GD/277/H3N2/2017 was a novel reassortant H3N2 virus with its M segment adopt from EA H1N1 strains and other segments from endemic H3N2 S-OIVs.

Conclusion

Cross-species transmissions raise public concerns on increasing pathogenicity of avian influenza virus. As an intermediate host, a large gap still exists in the epidemiological and genetic knowledge of influenza viruses in swine populations. In this study, we show that the A/GD/277/H3N2/2017 that caused infection in a child in 2017 and A/Ho Chi Minh/459.6/2010 from clinical samples in Vietnam in 2010 are both from a swine H3N2 lineage which established in Southeast Asia as early as 2004 (Fig. 1B). Moreover, A/GD/277/H3N2/2017 emerged as a novel reassortant and has a distinct antigenicity from contemporary seasonal H3N2 viruses. These results suggest (1) a long-term successive circulation of H3N2 in swine population in Asia; (2) most of human population may naïve to A/GD/277/H3N2/2017-like viruses.

A/GD/277/H3N2/2017 contains internal segments close to swine H3N2 strains circulating in neighboring regions except for M segments from pdH1N1. These data suggest the local circulation of S-OIV H3N2 viruses in Asia contributes to the emergence and spillover infection of the reassortant S-OIV H3N2 virus. Considering the high prevalence of S-OIVs and large swine populations, active molecular surveillance in China, as well as other Asian regions, is required. This will provide us a full picture of S-OIVs' prevalence and genetic diversity rather than waiting for humans to serve as sentinels to detect new reassortant S-OIVs.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2019.04.015.

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No gene communication of HA gene between the human H3N2 and H1N1 pandemic 2009 influenza A viruses



Dear Editor,

Recently, a study in this journal suggested that the 2014 H1N1 pandemic 2009 (H1N1/pdm2009) had gene communication with 2016/2017 H3N2.¹ The influenza A H1N1/pdm2009 virus, a novel swine-derived, triple reassortant virus, was rapidly transmitted between humans and spread to 168 countries, resulting in over 123,000 human deaths globally from March to December 2009.^{2,3} Since then, it has replaced the previous seasonal H1N1 and circulated as a seasonal virus along with the H3N2 virus, posing substantial risks to human populations,⁴ creating an opportunity

for coinfection and therefore recombination or reassortment between them. Occasional case reports have documented heterosubtypic coinfection in humans,^{5,6} however, only one reassortment event was confirmed.⁷ Recombination between their HA genes has not been reported.

HA molecules are categorized into two groups: group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18) and group 2 (H3, H4, H7, H10, H14, and H15).⁸ H1 is in group 1 while H3 is group 2, and these two different phylogenetic groups are separated by a large phylogenetic distance (Fig. 1(A)). Thus, the suggested gene communication between H1N1/pdm2009 and H3N2 reported in the Li et al study is unexpected.¹ To examine this in more detail, we collected all available H1N1/pdm2009 and H3N2

sequences from the NCBI (<https://www.ncbi.nlm.nih.gov>), GISAID (<https://www.gisaid.org>) and FluDB (<https://www.fludb.org>) public databases, and recalculated a phylogenetic network for the HA gene (Fig. 1(B)). Our results show that H1N1/pdm2009 and H3N2 have a very large phylogenetic distance in the phylogenetic network. The link between the 2016/2017 H3N2 and 2014 H1N1/pdm2009 sequences has 824 nucleotide changes (marked in bold in the Fig. 1(B))! This suggests that in the Li et al study, they did not use a weighted genetic distance ratio for their phylogenetic network, and thus reached the incorrect conclusion that 2014 H1N1/pdm2009 had gene communication with 2016/2017 H3N2.¹ Our results show that the HA gene sequences from H1N1/pdm2009 and H3N2 have very large phylogenetic distance, and that gene communication is not detected.

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Conflict of interest

The authors declare not conflict of interest.

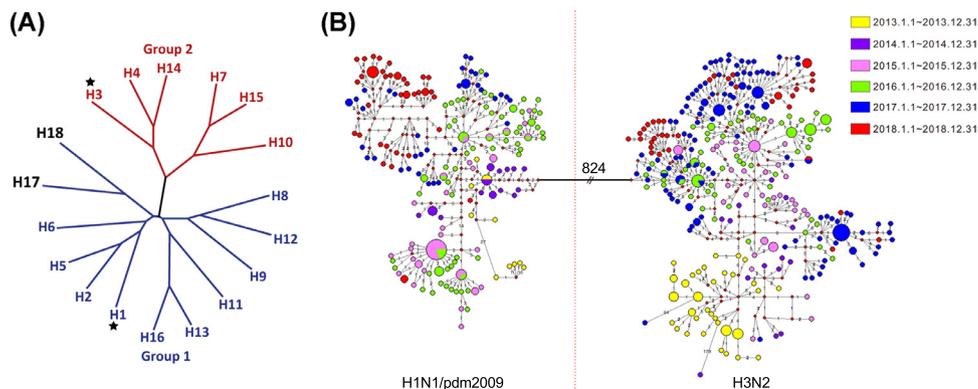


Fig. 1. Phylogenetic relationships of human H3N2 and H1N1/pdm2009 IAVs. (A) Phylogenetic tree of the 18 HA genes. This figure was modified from Wu et al study. (B) Phylogenetic network of HA gene for human H3N2 and H1N1/pdm2009 IAVs. The numbers on the lines represent the numbers of the nucleotide substitutions.

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Avian influenza A viruses H5Nx (N1, N2, N6 and N8) show different adaptations of their codon usage patterns to their hosts



Dear Editor,

A recent study in this journal identified three H5 highly pathogenic avian influenza A viruses (HPAIVs) with differing NA subtypes (N1, N6 and N8) and revealed that while the three isolates were all highly pathogenic in chickens and ducks only the H5N1 and H5N6 subtypes showed high pathogenicity in mice, with no mortality observed for H5N8.¹ This suggests differences in their pathogenicity potential. Since the emergence of H5N1 in China in 1996, it has continued to evolve and reassort with other NA subtypes, including N2, N6, and N8.^{2–5} These NA subtypes

of H5Nx HPAIVs show distinct differences in epidemiology and pathogenicity. Viruses depend on their hosts' cellular structure and metabolism for replication and assembly. Most viral genomes do not encode tRNAs, and, consequently, translation of viral proteins requires host tRNAs.⁶ As influenza virus replication is based on host machinery, viral codon usage must coevolve with the host to allow efficient use of host resources.⁷ Here, we compare codon usage among the different NA subtypes (N1, N2, N6, and N8) of H5Nx AIVs.

All available HA and NA sequences of H5Nx (N1, N2, N6, and N8) were downloaded from the Influenza Virus Resource at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/genomes/FLU), the Global Initiative on Sharing Avian Influenza Data (www.gisaid.org) and the Influenza Research Database (IRD) (www.fludb.org/brc/home.sp?decorator=influenza). Redundant sequences were removed. Laboratory strains and short (<95% of the corresponding gene) sequences were also excluded. Our final dataset contained 5504, 899, 1161, and 552 HA sequences and 4064, 672, 905, and 478 NA sequences for H5N1, H5N2, H5N6, and H5N8, respectively.

First, the codon adaptation index (CAI) was employed to predict the level of gene expression and the adaptation of viral genes to their hosts. The CAI analysis of HA and NA from H5Nx AIVs was performed using the CAIcal server (<http://genomes.urv.cat/CAIcal/>). Index values range from 0 to 1, where a score of 1 represents the tendency of a gene to always use the most frequently used synonymous codons in the host.⁸ Higher CAI values for all HA and NA subtypes in ducks indicates that the AIVs were more adapted to the codon usage patterns found in ducks than in humans. For the NA gene, N1 had the highest mean CAI value, followed by N2, N6, and N8 (Fig. 1A), indicating that N1 was significantly ($P < 0.001$) better adapted to the codon usage pattern of its hosts, followed by N2, while N6 and N8 were less adapted. No significant difference in codon usage was seen for the HA genes based on the CAI analysis (Fig. 1B).

The correspondence analysis (CA) was then used to verify the codon usage differences among the N1, N2, N6, and N8 of H5Nx. Relative synonymous codon usage (RSCU) values for the 59 relevant codons were calculated for all NA subtypes using the program CodonW (available at <http://sourceforge.net/projects/codonw/>). CA was then used with the RSCU values for the different NA segments of H5Nx AIVs and its host (duck) sequences. Here, the first three axes from the CA analyses provide a 3-dimensional visualization of the relationships among the sequences. The three principal axes account for 46.66%, 14.99% and 7.21% of the total variation, respectively (Fig. 1C). The different NA segments from H5Nx AIVs are located in different positions in the 3-dimensional graph. N1 and N2 are located closer to each other, compared to N6 and N8. In addition, N1 and N2 are located closer to the duck than N6 and N8, which indicates that the codon usage patterns of the N1 and N2 segments are more similar to duck than N6 and N8. This result is consistent with the CAI analyses of the different NA subtypes (Fig. 1A).

A neutrality plot was used to estimate the effects of natural selection and mutation pressure on the codon usage patterns by plotting P12 (GC12) values of the synonymous codons and P3 (GC3) values.⁹ According to the neutrality plot analysis, natural selection accounts for the majority (98%) of total selection pressure in the evolution of codon usage in the HA genes. In contrast, natural selection accounts for 52%, 69%, 49% and 66% of the total selection pressure for N1, N2, N6, and N8, respectively, indicating that the role of natural selection in the shaping codon usage patterns is much lower for NA than for HA (Fig. 1D). The HA protein is the main target recognized of host immune system, which likely accounts for the intense natural selection pressure acting upon HA gene.

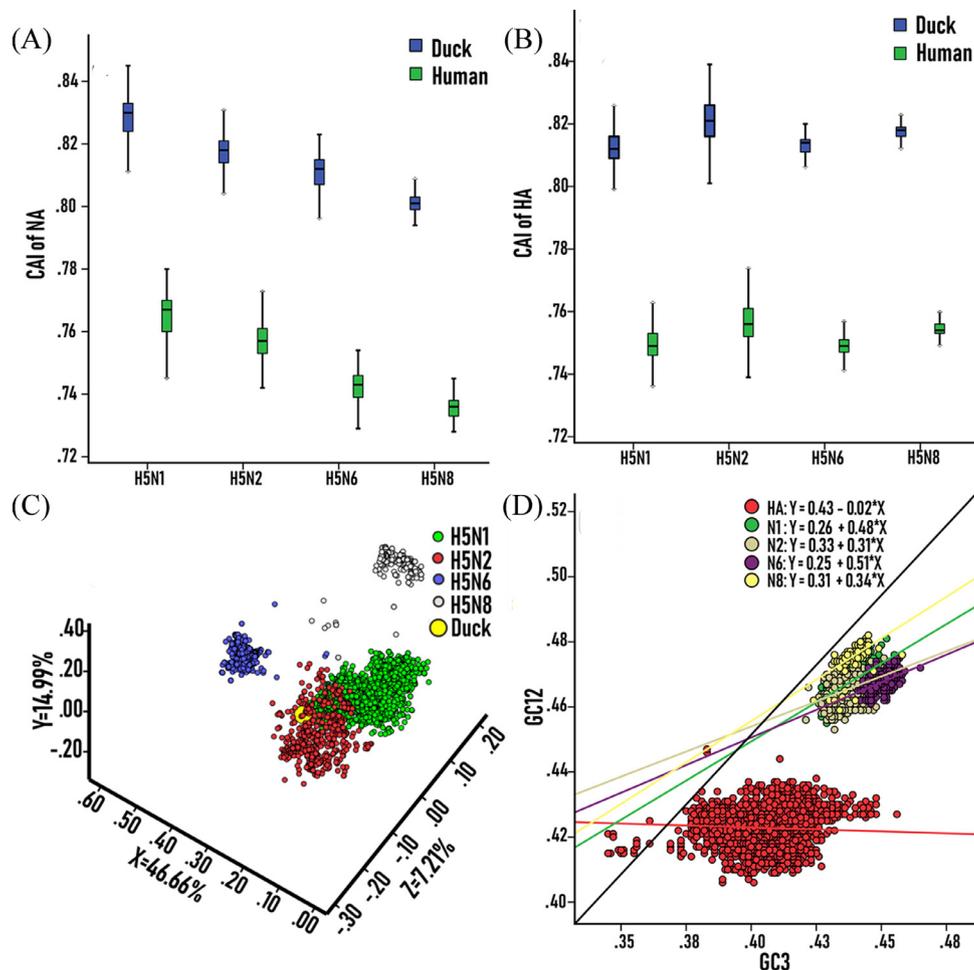


Fig. 1. (A) Codon adaptation index (CAI) analysis of NA subtypes (N1, N2, N6, and N8) to their hosts; (B) Codon adaptation index (CAI) analysis of HA sequences from H5N1, H5N2, H5N6, and H5N8 to their hosts. (C) Correspondence analysis (CA) of host (duck) and the different NA subtypes (N1, N2, N6, and N8) of H5Nx AIV. H5N1, H5N2, H5N6, and H5N8 are shown in green, red, orange, and grey, respectively. The duck is shown in yellow. (D) Neutrality plots for the HA and NA genes from H5Nx AIVs. GC12 stands for the average GC content in the first and second position of codons, while GC3 refers to the GC content in the third position. Dots and regression lines for HA, N1, N2, N6, and N8 are shown in red, green, brown, purple, and yellow, respectively. (Linear regression, HA: $Y = 0.43 - 0.02 \cdot X$; N1: $Y = 0.26 + 0.48 \cdot X$; N2: $Y = 0.33 + 0.31 \cdot X$; N6: $Y = 0.25 + 0.51 \cdot X$; N8: $Y = 0.31 + 0.34 \cdot X$).

In conclusion, we found that for the HA gene, which encodes the main target recognized by the host immune system, natural selection accounts for 98% of the total selection pressure. Codon usage in the N1 subtype is best adapted to its host, consistent with their prevalence and circulation in poultry since 1996. The current epidemic NA subtypes (N6 and N8) are less adapted to the codon usage of their host compared with N1. H5N6 has become the dominant H5Nx in China, and has a series of genotypic markers that are associated with cross-species transmission from avian to humans.¹⁰ The poorer adaptation of the codon usage in N6 and N8 to its hosts is consistent with the much fewer number of cases of human infection.

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Conflict of interest

The authors declare not conflict of interest.

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Genetic characterization of H7N4 avian influenza virus in China in 2018



Dear Editor,

Recent study in this journal reported that the first case of human infection with H7N4 avian influenza virus (AIV) was confirmed in China.¹ Subsequently, 11 H7N4 AIVs were isolated from poultries, 4 viruses isolated from chickens and 7 viruses isolated from ducks. Presently, H7N4 AIVs were only identified from USA, Australia, Italy, Canada, Netherlands, Germany, Keara, Thailand, and China.² Most of the H7N4 AIVs were low pathogenicity, except those isolated from Australia in 1997. In the present study, we performed a genetic analysis of these H7N4 AIVs in China during 2018.

The human-origin H7N4 AIV, A/Jiangsu/1/2018, and the other 11 poultry-origin H7N4 AIVs, that identified from China in 2018, as well as the reference sequences downloaded from the National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov>), and the Global Initiative on Sharing Avian Influenza Data (GISAID) database (<https://www.gisaid.org>). The phylogenetic trees of all eight genes were reconstructed, respectively, using the neighbor-joining method with 1000 bootstrap tests in MEGA 6.06. As shown in Fig. 1, the HA and NA genes of A/Jiangsu/2018 and the other 11 poultry-origin H7N4 AIVs were clustered with Eurasian lineage, closely relating to the viruses isolated from Thailand in 2010. All internal genes are also grouped into Eurasian lineage (Supplementary Fig. S1A–F). The HA, NA, PB2, PB1, PA, NP, M, and NS genes of A/Jiangsu/2018 shared 99.2–99.5%, 99.4–99.8%, 99.7–99.9%, 100%, 99.8%, 100%, 99.6–100%, and 100% nucleotide identity with all 11 poultry-origin H7N4 AIVs. By Blast in GISAID and NCBI, the HA, NA, PB2, PB1, PA, NP, M, and NS genes of all 12 H7N4 AIVs isolated from China in 2018 are most closely related to A/duck/Ibaraki/1/2015 (H7N2) isolated

from Japan, A/mallard/Chany/126K-2/2014 (H8N4) isolated from Russia, A/duck/Miyazaki/450,307/2016 (H1N1) isolated from Japan, A/pintail/Russia_Primorje/222/2015 (H11N9) isolated from Russia, A/duck/Bangladesh/30,827/2016 (H3N8) isolated from Bangladesh, A/duck/Dongting/D76-1/2016 (H5N7) isolated from China, A/black-necked crane/Zhaotong/ZT-12/2013 (H1N2) isolated from China, A/teal/Russi_Primorje/390/2016 (H1N1) isolated from Russia, respectively. Previous study has demonstrated that wild birds act an important role in the unexpected emergence of novel AIVs in poultry, and mammals.³ China locates in the middle of the Central Asian Flyway and East Asian–Australasian migratory flyway. Thus, the results of the homology analysis indicating that the migratory birds might cause these multi-reassortant viruses in China.

We also investigate the amino acid mutation sites that reported to be associated with host adaptation, pathogenesis, receptor specificity, and antiviral resistance in all 12 H7N4 AIVs. No mutations E119V, I222L, and R294K in the NA protein, as well as S31N in the M2 protein were not found in these viruses, indicating that they remained sensitive to amantadine and oseltamivir (Table 1). Based on the deduced amino acid sequence of the HA gene, the HA cleavage site pattern PELPKGR/G, a typical motif, observed in low pathogenicity AIVs. The mutations Q226L, and G228S were not identified in the HA proteins of all 12 H7N4 AIVs, indicating that they preferred to bind to α -2,3-linked sialic acid AIV receptor.⁴ Virulence-related signatures, such as the 90 amino acid PB1-F2 protein and the P42S substitutions in the NS1 protein, were identified in all 12 H7N4 AIVs isolated from China in 2018. Previous research has demonstrated that the substitutions A588V, E627K, and D701N in the PB2 protein associated with increased virulence in mammals.^{5,6} The substitution E627K was observed in the PB2 protein of the A/Jiangsu/1/2018, but not the other 11 poultry-origin H7N4 AIVs. This is maybe the reason that A/Jiangsu/1/2018 triggered a case of human infection.

Beside H7N4 AIV, human infection with H5N1, H5N6, H7N9, H10N8 and H9N2 AIVs have been reported in China, especially H7N9 resulting in over 1500 laboratory-confirmed human cases.⁷ Several studies have confirmed that exposure to live poultry significantly increases the risk of human infection with AIV.^{8,9} The bivalent H5/H7 vaccine successfully prevents chickens from H7N9 AIV infections, and thus preventing human infections.¹⁰ To prevent human infection with AIV, we should strengthen surveillance, control these viruses in poultry, and avoid exposure to live poultry.

Competing interests

The authors declare that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2019.04.012.

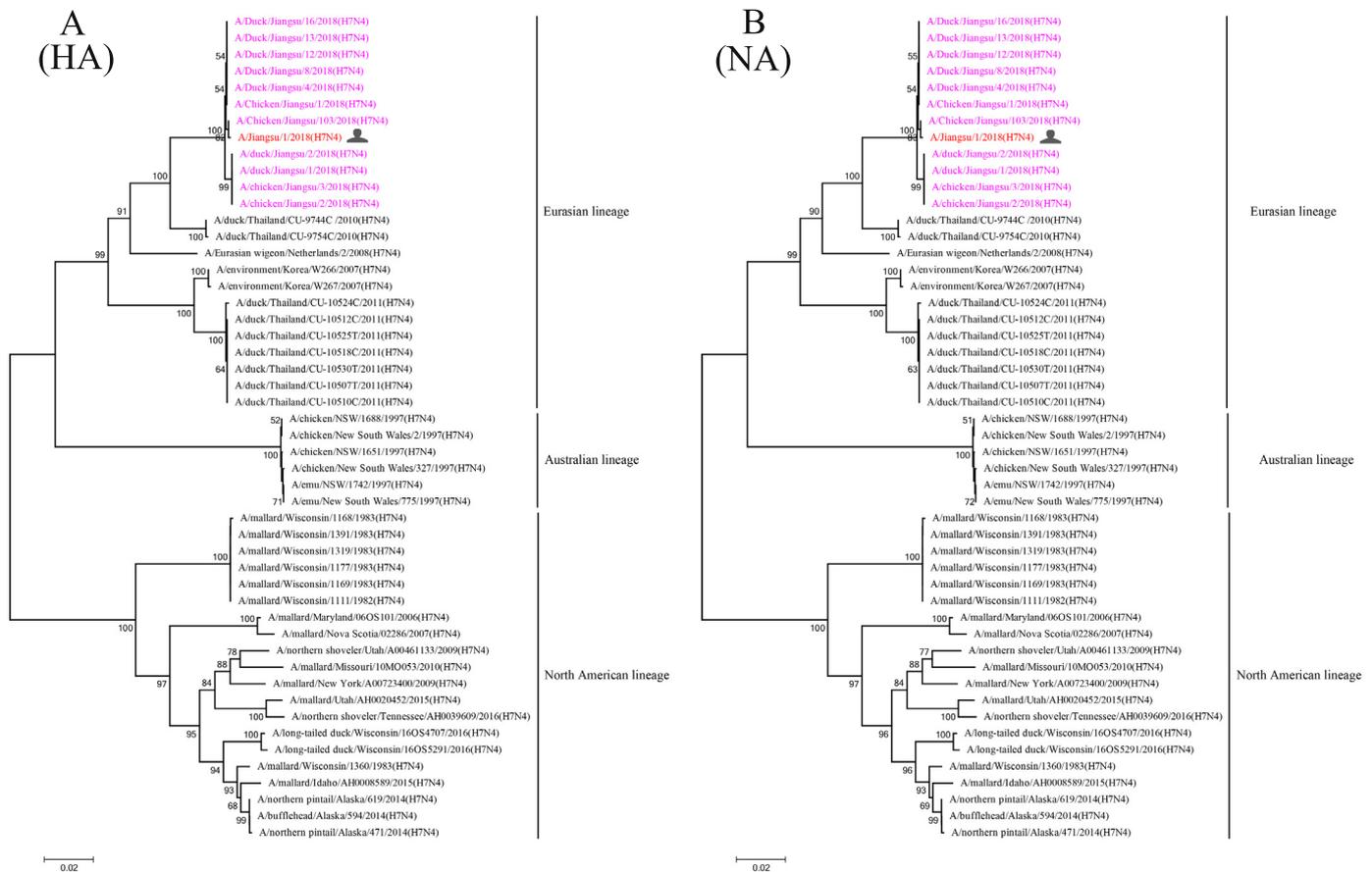


Fig. 1. Phylogenetic trees of the open reading frame of HA (A) and NA (B) genes of H7N4 AIVs isolated from China in 2018. The trees were constructed by the neighbor-joining method with 1000 bootstrap replicates in MEGA 6.06. For each node, only the bootstrap values ($>50\%$) are shown above the branches. The human-origin H7N4 AIV was indicated with red font.

Table 1

Molecular analysis of the human-original and the other avian-original H7N4 AIVs isolated from China in 2018.

Protein	Molecular feature or amino acid substitution	Function	Human-original H7N4 AIV strain (A/Jiangsu/1/2018)	Avian-original H7N4 AIVs in China ^a
HA	Multibasic cleavage site H160A	Expand viral tropism	PELPKGR/G	PELPKGR/G
	G186V	Increased binding to human-type influenza receptor	A	A
	Q226L	Increased binding to human-type influenza receptor	G	G
	G228S	Increased binding to human-type influenza receptor	G	G
NA	E119V	Oseltamivir resistance	E	E
	I222L	Oseltamivir resistance	I	I
PB2	R294K	Oseltamivir and zanamivir resistance	R	R
	A588V	Increased virulence in mice	A	A
PB1	E627K	Increased virulence in mice	K	E
	D701N	Increased virulence in mice	D	D
PB1-F2	I368V	Increased transmission in ferrets	I	I
	87–90 aa in length	Increased pathogenicity in mice	90 aa	90 aa
PA	T97I	Increased virulence in mice	T	T
	L336M	Increased polymerase activity in mice	L	L
M2	S31N	Amantadine resistance	S	S
	P42S	Increased virulence in mice	S	S
NS1	D92E	Increased virulence in mice	D	D
	N205S	Altered antiviral response in host	S	S
	G210R	Altered antiviral response in host	G	G

^a All the other 11 avian-original H7N4 AIVs were isolated from China in 2018.

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Phylogeographic patterns of the African swine fever virus



Dear Editor,

The emergence of novel avian influenza A viruses is frequently reported in this journal,^{1,2} while other emergent diseases, e.g., African swine fever (ASF), which also cause great losses in recent years. African swine fever is caused by the African swine fever virus (ASFV). ASFV is the only member of the Asfarviridae family, and the genus Asfivirus.³ Infections of swine with ASFV show high

morbidity and mortality (up to 100%). At present, there is no effective vaccine to prevent ASF and thus it is a great threat to global pig production. Since its first described in Kenya in the 1920s, ASF has been recorded in most sub-Saharan African countries, and has spread outside Africa to Europe (Spain, Portugal, Italy, and France) in the 1950s, although it was subsequently eradicated from those countries. More recently, ASF was export to Georgia in 2007, and then spread throughout Eastern Europe, including Russia, Belarus, Ukraine, Estonia, Lithuania, Latvia, Romania, Moldova, Czech Republic, and Poland.⁴ The virus has continued to spread worldwide, and has now been reported in 37 countries or regions. On August 3, 2018, China – the world's largest pork producer, reported its first ASF outbreak. ASF has now been reported across all provinces of China, leading to the deaths of more than 1 million pigs. At least three other Asian countries, Cambodia, Mongolia and Vietnam, have reported ASF outbreaks to the OIE (<http://www.oie.int/>). However, how ASF was able to quickly spread across the world remains a mystery. Here, to address this question, we studied the phylogeographic patterns of this virus.

The ASFV genome is 170–193 kilobase pairs in length.⁵ A pair of genes (p72 and p54) has been widely used to examine ASFV phylogenetic relationships.^{6–8} Genotypes for p72 have been classified for the identification of circulating ASFVs. Therefore, we collected all 716 p72 and 680 p54 available gene sequences from NCBI (<http://www.ncbi.nlm.nih.gov/>) to investigate the phylogeographic patterns of ASFVs. A Median-joint network of the p72 sequences was constructed using Network 5.0 (<http://www.fluxus-engineering.com/sharenet.htm>). The phylogenetic network could be divided into the 24 previously described genotypes (I–XXIV, Fig. 1(A)). Genotypes I and II are the most widely distributed genotypes, with 31.4% of the sequences belonging to genotype I (225 of the 716 sequences) and 15.1% belonging to genotype II (108 of the 716 sequences). East Africa and South Africa have their own unique ASFV lineages, suggesting independent evolution of this virus in these two regions. As shown in Table 1, for the p72 gene, ASFVs from East Africa and South Africa have the most genotypes (15 and 14, respectively), the largest numbers of unique genotypes (8 and 8, respectively), the greatest haplotype diversity (0.78 and 0.951, respectively), and highest nucleotide diversity (0.03022 and 0.02188, respectively). Similarly, for p54, ASFVs from East Africa and South Africa have the greatest haplotype diversity (0.835 and 0.974, respectively) and highest nucleotide diversity (0.15120 and 0.16511, respectively). Since the largest genetic diversity in ASFVs is found in East Africa and South Africa, this supports the conclusion that East Africa and South Africa are the two main origin regions for ASFVs.

Although ASFVs have invaded other parts of the world from Africa several times, and have disseminated to many countries and regions, all of the sequences that have been isolated outside Africa belong to genotypes I and II. Genotype II is now endemic in east Europe and Russia, and constitutes the 2018 outbreak in China. It is unknown whether these two particular genotypes are better adapted to these regions, or whether their presence is simply due to founder effects (random export events). ASFVs are DNA viruses, thus have much lower mutation rates than RNA viruses. The nucleotide diversity of p72 is much lower than p54 gene (Table 1), suggesting that p72 is a better conserved gene. This further explains the lack of genetic diversity seen in p72 in regions outside of Africa (Table 1).

In the current and previously affected areas, domestic pigs, wild boar, wild African suids (warthogs, bush pigs, and giant forest hogs), and Ornithodoros ticks have been infected through different transmission models. Ornithodoros ticks and African suids act as the biological vectors and reservoirs for ASFVs.⁹ In South and East Africa, the complex transmission cycle of involving African suids, domestic pigs, and ticks results in most of the ASFV genotypes be-

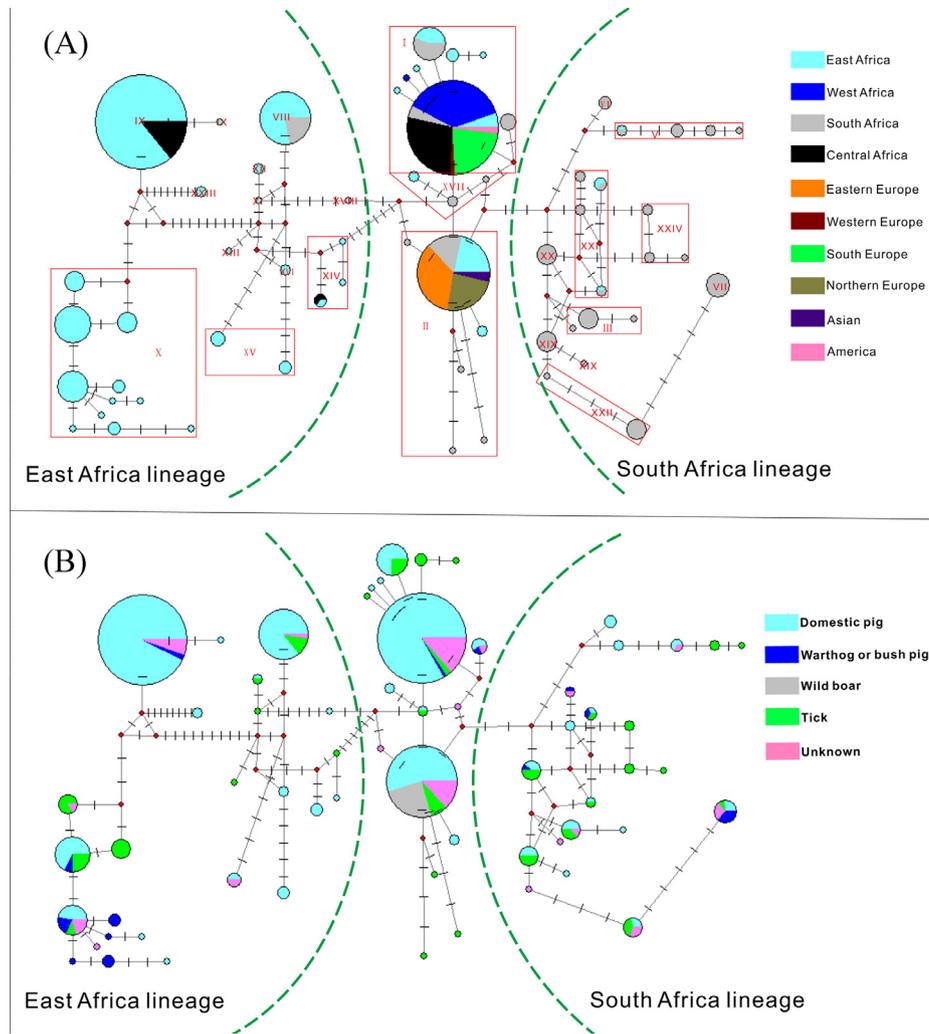


Fig. 1. Phylogenetic network for the p72 gene from ASFV. (A) Phylogenetic network displaying the geographical distribution of where the viruses were isolated. (B) Phylogenetic network marked for host information.

Table 1
Genetic diversity of p72 and p54 genes in ASFV.

Gene	Region	N ^a	nHT ^b	HTdiv ^c	pdiv ^d	Genotypes	Unique genotypes
P72	East Africa	325	38	0.78	0.03022	I, II, IX, V, VIII, X, XI, XII, XIII, XIV, XV, XVI, XVII, XXI, XXIII	X, XI, XII, XIII, XIV, XV, XVI, XXIII
	West Africa	73	3	0.054	0.00014	I	
	South Africa	126	40	0.951	0.02188	I, II, III, V, VI, VII, VIII, XIX, XVII, XVIII, XX, XXI, XXII, XXIV	III, VI, VII, XIX, XVIII, XX, XXII, XXIV
	Central Africa	79	3	0.453	0.02402	I, IX	
	Eastern Europe	36	1	0	0	II	
	Western Europe	3	1	0	0	I	
	South Europe	42	1	0	0	I	
	Northern Europe	24	1	0	0	II	
	America	4	1	0	0	I	
	Asian	4	1	0	0	II	
P54	East Africa	320	62	0.835	0.15120		
	West Africa	75	7	0.488	0.10150		
	South Africa	91	48	0.974	0.16511		
	Central Africa	62	7	0.650	0.07178		
	Eastern Europe	30	1	0	0		
	Western Europe	2	1	0	0		
	South Europe	89	27	0.547	0.0446		
	Northern Europe	2	1	0	0		
	America	4	1	0	0		
	Asian	5	1	0	0		

Notes:

- ^a Number of sequences.
- ^b Number of haplotypes.
- ^c Haplotype diversity.
- ^d Nucleotide diversity.

ing shared (Fig. 1(B)). In other regions, transmission cycles seem to without the involvement of ticks.⁹ Considering the rapid expansion and the sporadic, long-distance outbreaks, direct contact with infected pigs may play a limited role in the spread of ASF. ASFVs can survive long periods in frozen meat and in undercooked pork products, thus swill-feeding and pig/pork transport might play primary roles in transmission. Infections are more likely to occur in small-scale and backyard farms with low biosecurity, with farmers then often attempting to limit their economic losses by an emergency sale of affected pigs to traders, markets, and meat producers, when ASF clinical symptoms are found in some animals. Thus, these sites could act as “amplifying spots”, and play important roles in the rapid transmission of ASF. In addition, infections in wild boars were reported in European and Asian countries, including China. Due to the maintenance of ASFVs in wild boars, ASF is now endemic in Eastern Europe and is far from being eliminated. Wild boars are distributed across Eurasia, thus the establishment of ASFVs in wildlife could intensify the spread of this disease, and make control more difficult.

Improving biosecurity on farms, forbidding swill-feeding, strict animal movement control, and increased disease awareness by pig farmers should greatly help in disease control. Vaccination is one of the best control measures for infectious diseases. Inactivated vaccines to date have been unsuccessful for ASFVs. Live attenuated vaccines often produce a stronger and longer-lasting immune response than inactive vaccines. However, a traditional live attenuated ASFV vaccine caused a chronic disease outbreak.¹⁰ Generating an attenuated vaccine through the sequential deletion of virulence genes is the most promising approach, however, extensive investigation is required to test the safety and efficacy of any attenuated vaccine before it can be used in the field.

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Conflict of interest

The authors declare no conflict of interest.

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