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BRIEF REPORT

A dominant internal gene cassette of high pathogenicity avian influenza H7N9 virus raised since 2018

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Abstract

The zoonotic H7N9 avian influenza virus emerged with the H9N2-origin internal gene cassette. Previous studies have reported that genetic reassortments with H9N2 were common in the first five human H7N9 epidemic waves. However, our latest work found that the circulating high pathogenicity H7N9 virus has established a dominant internal gene cassette and has decreased the frequency of reassortment with H9N2 since 2018. This dominant cassette of H7N9 was distinct from the cocirculating H9N2, although they shared a common ancestor. As a result, we suppose that this dominant cassette may benefit the viral population fitness and promote its continuous circulation in chickens.

Keywords Avian influenza · H7N9 · High pathogenicity · Internal gene cassette · Viral evolution · H9N2

Introduction

The first case of humans infected with the H10N3 avian influenza virus (AIV), whose internal genes inherited from the H9N2 AIV, has recently been reported in China [1, 2]. The rise of other human-infecting AIVs, such as H5N6, H7N9, and H10N8, was similarly donated by the H9N2-origin gene cassette [3, 4]. Almost all donor H9N2 AIVs were of Genotype S (G57-like), prevalent in China's chicken population since 2010 [5]. These findings show that AIVs with the Genotype S H9N2 internal genes pose a potential threat to human health. Genotype S H9N2 is also of severe concern to the poultry industry and plays a crucial role in novel AIV genesis and evolution in China.

The zoonotic H7N9 virus emerged through reassortment with chicken-origin H9N2 viruses in 2013 [6]. It can cause severe disease in humans and other mammals (e.g., mice, ferrets, guinea pigs, pigs, and nonhuman primates) [7, 8]. It is responsible for at least five human epidemics [8], making it one of the most deadly AIVs, which carries the H9N2 gene cassette. Since February 2013 (http://www.fao.org/ag/againfo/programmes/en/empres/H7N9/situation update.html, last accessed on 19 December 2021), it has caused 1568 human infection cases with 616 fatalities, resulting in about 40% mortality. Reassortment with the dominant H9N2 genotype has played a critical role in the wave 5 human outbreak in 2016–2017, the largest H7N9 zoonotic outbreak [9]. Furthermore, a high pathogenicity (HP) H7N9 variant with four-amino acid insertion in cleavage sites of hemagglutinin (HA) protein emerged from its low pathogenic (LP) counterpart in July 2016 [10]. HP H7N9 has caused significant economic losses in the poultry industry, subsequently promoting a nationwide vaccination program (http://www.moa.gov.cn/govpublic/SYJ/201707/t20170711_5744436.htm, last accessed on 19 December 2021). Following that, the LP H7N9 virus is becoming increasingly rare in the field since 2018, whereas the HP H7N9 viruses are still sporadically detected [1]. The Yangtze River Delta (YRD) and Pearl River Delta lineages have been established [11]. The YRD lineage was responsible for the current circulating HP H7N9 outbreaks under vaccination in China [12–16]. The emerging

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cases of vaccine escape mutation and altered receptor binding characteristics have been reported [12–14, 16, 17].

Previous studies frequently reported genetic reassortments between H9N2 and H7N9 [18–21]. As a result, H7N9 genotypes continue to emerge. However, most of these genotypes were ephemeral, with only a few genotypes enduring for more than two waves [22]. Intensive reassortments with the internal gene cassette from the cocirculate Genotype S H9N2 AIV may significantly affect the adaptation and prevalence of H7N9 in the avian host during the first five waves. [19, 23, 24]. However, the *status quo* relationship between the internal genes of H9N2 and H7N9 remains unknown since wave 5 (2016.10 to 2017.9).

Methods

To elucidate the current relationship of the internal genes between H7N9 and H9N2, we conducted a phylogenetic analysis of the six internal genes. We firstly collected all available internal gene sequences of H7N9 and H9N2 from 2010 to 2021 from the NCBI Influenza Research Database (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi) and Global Initiative on Sharing All Influenza Data (GISAID, https://platform.gisaid.org/). Then, we clean the sequence dataset following our previously described methods [13, 14]. Ultimately, 4643 PB2, 4637 PB1, 4652 PA, 4651 NP, 4718 M, and 4719 NS sequences were obtained. ModelFinder was used to determine the best-fit substitution model using the optimality Bayesian information criterion (BIC) [25]. IQ-tree (v1.6.12) [26] was used to generate a preliminary maximum likelihood (ML) tree for each segment with 10,000 repeats ultra-bootstrap on our High-Performance Computing Cluster.

To construct the time-scaled tree, we first estimated the temporal signal of the collected sequences through root-to-tip regression of genetic divergence and sampling times based on ML trees using Treetime [27]. Then, we removed the sequences without time signal. Sequences acquired before 2013 (H7N9 was first detected in February 2013) were removed. Sequences collected between 2013 and 2017 with a similarity of greater than 99% (highly similar) and sequences collected after 2018 with a similarity of greater than 100% (identical) were deleted using Bioaider [28]. Eventually, 843 (PB2), 889 (PB1), 757 (PA), 705 (NP), 604 (M), and 691 (NS) were retained. Then, the time-scaled tree construction was performed using BEAST (v1.10.4). BEAST running was set following our previous study [13, 29]. Generally, the substitution model of GTR + G4 was adopted under an uncorrelated relaxed molecular clock model and different tree priors (Constant size and GMRF Bayesian Skyride plot). The MCMC chain length was set to 200 million generations, and trees were collected every 20,000 steps. Tracer (v1.7.1) was used to analyze the log file convergence (effective sample size > 200). Following the burn-in of the first 10% of trees, TreeAnnotator prepared the maximum clade credibility (MCC) tree with median heights. The phylogenetic trees were visualized using ggtree [30] and ggtreeExtra [31].

Results and discussion

Phylogenetic analyses revealed that the HP H7N9 virus isolated since 2018 was separately clustered together on each time-resolved tree. Before 2018, the internal gene of the H7N9 virus was intensively associated with the distinct clusters of the H9N2 AIVs on each phylogenetic tree (Figure S1-S6), indicating that their reassortments were extensive. However, the majority of HP H7N9 AIVs isolated since 2018 were clustered in the same region within the trees, suggesting that reassortment events have decreased and the HP H7N9 virus has evolved independently since 2018. To further elucidate these findings, we first divided the tree into HP H7N9 cluster and non-HP H7N9 cluster based on the MCC tree (Figure S1-S6). Then, the segment genotype heatmap was drawn and demonstrated our finding (Fig. 1, right panel), showing reduced reassortment since 2018 compared to before 2018. Although the internal genes present reassortment between a few HP H7N9 AIVs and cocirculate H9N2 AIVs, most HP H7N9 AIVs contain a dominant internal gene cassette without reassortment with H9N2. According to the phylogenetic incongruence tree analysis, the internal segments of H7N9 show a dominant internal gene cassette across the six MCC trees (Figure S7). To conclude, our investigation found that a dominant internal gene cassette of the HP avian influenza H7N9 virus was raised since 2018, and the six internal segments of most HP H7N9 AIVs evolved independently after 2018. This dominant internal gene cassette emerged at or before wave 5.

In addition, the PB2 K526R mutation was identified in the dominant cluster, whereas the PB2 627 site was kept as glutamate (E). The K526R mutation enhances viral replication when combined with E627K [32]. The strains after 2018 mainly had alanine at a position 100 of PA, which is a human relevant amino acid (usually V in avian, A in human) [33, 34]. However, some strains had threonine at this position, implying host adaptation to avian species around 2018. T357S substitution of PA were also found in the strains in the dominant cluster. This position was suggested to be involved in viral polymerase activity and pathogenicity in duck [35]. The role of the mutation should be further explored.

Our findings suggest that most HP H7N9 AIVs have a stable internal gene cassette and have evolved independently since 2018. In other words, the frequency of reassortment

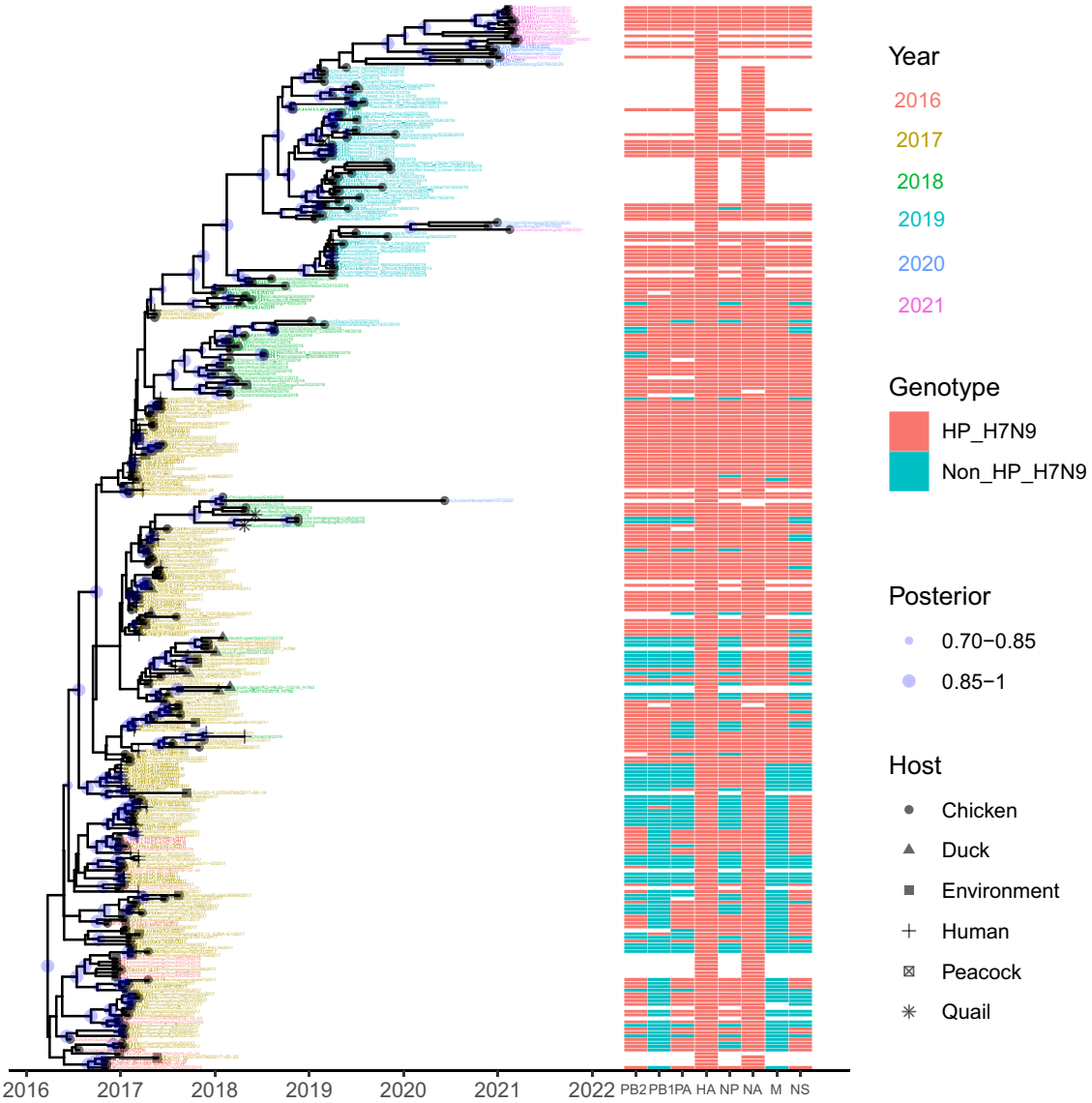

Fig. 1 Time-resolved maximum clade credibility (MCC) tree and segment cluster heatmap. The HA tree of the HP H7N9 influenza virus (left panel) with a genotype table presented as a heatmap (right panel). Tips' colors are labeled by isolation year, and the host determines the tips' shape of the different symbols. The blanks represent the missing data

between HP H7N9 and H9N2 has evidently decreased compared to earlier waves. Despite significant reductions in H7N9 population size [14] and positive rates among poultry [36] since the H7N9 Re-1 vaccine administration, the circulating HP H7N9 AIVs still have a high risk of coinfection with the H9N2 virus in the field. Since the H9N2 virus is basically ubiquitous in Chinese poultry farms, vaccination cannot prevent viral shedding and transmission in chickens after infection [37–39]. Coinfection may result in the shuffling of individual viral genes and generation of H7N9 reassortants whose phylogenetic topology would closely cluster with H9N2 AIVs and resemble the phylogenetic relationship of the first 5 waves. However, only a few reassortants were identified among the six phylogenetic trees after 2018. Our data imply that the HP H7N9 virus has experienced a considerable reduction in reassortment with the H9N2 virus in recent years and evolved independently in chickens. However, the isolation of H7N9 has significantly dropped in recent years due to the shrinking size of the H7N9 under vaccination. Meanwhile, the "sampling strategy" in the H7N9 surveillance may be affected by the declining human infection and the shadow of COVID-19. As a result, sample bias cannot be ruled out entirely.

Our findings show that circulating HP H7N9 viruses currently have a dominant genetic cassette, resulting in a stable genetic background. HP H7N9 AIVs are less likely to be

reassortants than their LP counterparts. Our findings also show the adaptative mutations of the HP H7N9 internal gene cassette, which evolved separately from H9N2 even though they shared a common ancestor. The genesis and convergent evolution of stable gene cassettes warrant further investigation. It is also vital to monitor the further evolution of the HP H7N9 viruses to provide timely prevention and control. Other influenza viruses with zoonotic potential also deserve to be closely monitored. It is also advised that better control of the H9N2 virus is essential to avoid introducing novel zoonotic influenza viruses.

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Author contributions D.H performed research and wrote the paper; XL, MG and LH designed study; DH, HW, and XW analyzed data; TZ, XW and SH contributed new methods. All authors reviewed the manuscript.

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Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval Not applicable since no sample collection or questionnaires from animals or humans were involved in this study.

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