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BRIEF REPORT

Reintroduction of highly pathogenic avian influenza A H7N9 virus in southwestern China

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Abstract

Highly pathogenic (HP) avian influenza A H7N9 virus has emerged in China since 2016. In recent years, it has been most prevalent in northern China. However, several strains of HP H7N9 reappeared in southwestern China (Yunnan Province) in 2021. As a result, we are wondering if these viruses have re-emerged in situ or been reintroduced. Here, we present phylogenetic evidence that the HP H7N9 viruses isolated in Yunnan emigrated from northern to southwestern China in 2020. The northern subregion of China has become a novel epicenter in HP H7N9 dissemination. Meanwhile, a cleavage motif re-emerged due to the T341I mutation, implying a parallel evolution. This cross-region transmission, which originated in non-adjacent provinces and traveled a great geographic distance in an unknown way, indicates that HP H7N9 dissemination did not halt in 2020, even under the shadow of the COVID-19 pandemic. Additional surveillance studies in poultry are required to determine the HP H7N9 virus's geographic distribution and spread.

Keywords Avian influenza virus · H7N9 · Highly pathogenic · Epidemiology · Phylogeography

Introduction

Avian influenza viruses (AIVs) are negative-sense and single-stranded RNA viruses commonly found worldwide in their natural reservoir hosts (wild waterfowls or domestic birds). AIVs can occasionally spread from birds to mammals (like humans), leading to zoonoses and sometimes major outbreaks and/or severe diseases. The novel H7N9 AIV subtype, which first emerged in 2013, is convincing evidence that zoonotic AIVs triggered public concern about the potential for AIV transmission in humans. In the middle of 2016, the first detection of a highly pathogenic avian influenza (HPAI) H7N9 variant that possessed the insertion of four amino acids at the cleavage site of the HA protein from its low pathogenic counterpart was in the South of China (Guangdong) [1]. It then spread rapidly westward to adjacent provinces (Guangxi and Hunan), southwestern China (Yunnan) [2, 3], and northern China (Shaanxi) in 2017 [4]. Since the retail poultry market closed once the H7N9 outbreak in certain regions, it may have resulted in market shifts caused by retailers selling live poultry in other regions or neighboring provinces and spreading the virus [5]. Meanwhile, we recently reported that the HP H7N9 virus has a significant spatiotemporal association [6], mainly circulating in North China since 2017. However, recent studies reported that HP H7N9 AIVs had been detected in Anhui [7] and southern (Yunnan and Guangdong) China [8] between 2017 and 2019, indicating that HP H7N9 AIVs are not limited to the North of China. The HP variants spread quickly among the poultry farms from the southern to the North of China, resulting in 100% lethality to the poultry and about 50% mortality in infected humans [9, 10].

To reduce the hazards of H7N9, a national vaccination strategy using the H5/H7 (Re-1) bivalent inactivated vaccine was initiated in China in September 2017. Poultry vaccination successfully eliminated human infection with the H7N9

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virus and significantly decreased poultry's H7N9 virus isolation rate [11, 12]. Although vaccination minimized human and poultry infections [13], the H7N9 increased virulence and expanded the host range to ducks [14], accelerated the evolution rate [8, 15], and increased the genetic and antigenic diversity [15]. Meanwhile, the HP H7N9 virus was endemic, with high levels of local transmission following a northward unidirectional geographic expansion in 2017 [6]. Recently, Chen et al. identified several strains of novel HP H7N9 viruses in Yunnan at the start of 2021, which may have been immune-evading strains resulting from H7N9 Re-3 and rLN79 vaccination [16]. Therefore, we performed additional analysis of these viruses using the phylogenetic tree to determine whether these HP viruses were re-emergent in situ or were reintroduced.

Methods

We first collected the H7N9 sequences and metadata from the GISAID EpiFlu public database. The HP H7N9 viruses isolated since 2017 and the H7N9 Re-2 vaccine strain (A/chicken/Guangxi/SD098/2017) were selected to formulate the dataset. The signal of the heterochronous sampling date was estimated for Bayes molecular clock correction by Treetime (v0.8.5) [17]. Then, time-scaled tree constructions were executed using the Markov chain Monte Carlo (MCMC) framework applied in Bayesian Evolutionary Analysis Sampling Trees (BEAST, v1.10.4). General time-reversible (GTR) substitution model with four gamma categories, strict molecular clock, and different tree priors (Skygrid coalescent model was chosen for HA) were selected in BEAUti (v1.10.4). After 200 million generations running and sampled every 20,000 steps, Tracer (v1.7.1) inspected the convergence. Following the burn-in of the first 10% of trees, TreeAnnotator (v1.10.4) analyzed the maximum clade credibility (MCC) tree with median node heights. MCC tree with a rectangular layout was used for visualization using ggtree [18, 19]. A light red background was used to highlight HP H7N9 viruses isolated in the Southwest of China in 2021. The non-synonymous substitutions (amino acid mutation) along the tree were analyzed by Treesub (https://github.com/tamuri/treesub).

To analyze the geographic dissemination and spreading routes, China was divided into four geographical subregions: South (Jiangsu, Shanghai, Anhui, Hubei, Hunan, Zhejiang, Fujian, Taiwan, Jiangxi, Guangdong, Guangxi, Hongkong, Macau, and Hainan), North (Heilongjiang, Jilin, Liaoning, Inner Mongolia, Beijing, Tianjin, Hebei, Shanxi, Shaanxi, Henan, and Shandong), Northwest (Gansu, Qinghai, Ningxia, Xinjiang), and Southwest (Chongqing, Sichuan, Guizhou, Yunnan, Tibet). Then, discrete phylogeographic analysis was conducted as in our previous description to depict the migration routes of H7N9 [20]. Briefly, we used a Bayesian stochastic search variable selection procedure (BSSVS) to estimate the transitions between these subregions [21]. A full Markov jump and reward history was recorded separately from the BSSVS run in order to track the expected number of transitions (jumps) and waiting times in given subregions (rewards) using a continuous-time Markov chain (CTMC) model [22]. In addition, we used the Python script (https://github.com/AdmiralenOla/GlobalL4scripts) conducted by Brynildsrud et al. [23] to analyze the direction of migration over time between subregions to investigate the migration history of HP H7N9. Significant migration routes were determined based on the established criteria, which were Bayes factor (BF) ≥ 3 and posterior probability (PP) > 0.5.

Results and discussion

The heatmap of Markov jumps indicates the frequency of transitions between subregions using a discrete trait phylogenetic model. The transition value of HP H7N9 is high from Southern to Northern, whereas the HP H7N9 in the North also is the source to Northwest, Southern, and Southwest (Fig. 1A). The Markov reward bar chart shows the proportion of time the virus spends in each subregion. The Northern has a high reward value, indicating that HP H7N9 has been present in this area for some time (Fig. 1B). The result of the HA MCC tree indicated that the sporadic cases of HP H7N9 in poultry had been generally reported in northern China since 2018 (Fig. 1C), which was consistent with the previous findings [6, 24]. The HP H7N9 has rarely been detected in southern and southwestern China since its fifth epidemic wave (vaccination started at the end of wave 5). We found that the HP H7N9 viruses isolated in Yunnan Province (Southwest) shared an ancestor node with the viruses circulating in northern China (Shanxi and Hebei Province) with a high posterior (0.9991). The median time of most recent common ancestor (tMRCA) of these seven viruses was on October 1, 2020 (2020.7674, 95% HPD: 2020.4984–2020.9654, posterior = 1). The remaining seven segments' MCC tree showed a similar phylogenetic topology with HA about these strains (Figs. S1–S7). As a result, phylogenetic evidence reveals that the HP H7N9 viruses obtained in southwestern China may have originated from northern China around October 2020 (Period 9). After this single dissemination from the North to the Southwest, the HP H7N9 virus in Yunnan experienced local transmission and sporadic detections under independent molecular evolution and adaptation.

These reintroduced viruses shared three amino acid substitutions (Q71R, Q231K, and T341I, H7 full-length numbering) on the HA tree. Notably, the novel Q231K mutation,

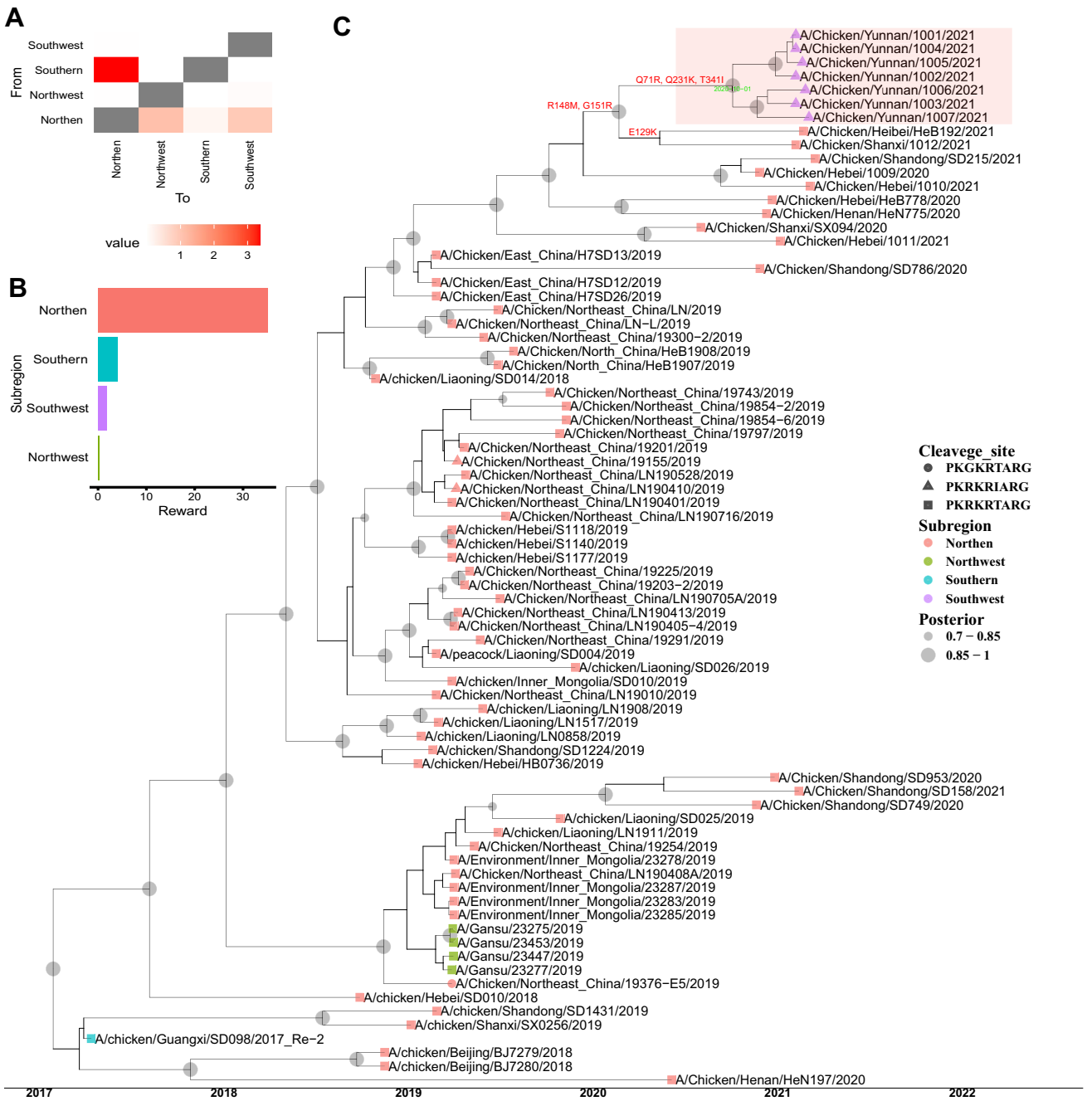

Fig. 1 Time-resolved maximum clade credibility (MCC) tree of HA genes and subregion jumps and rewards since 2018. **A** Markov jumps' heatmap indicates the frequency of transitions between subregions using a discrete trait phylogenetic model. The transition value of HP H7N9 is high from Southern to Northern, whereas the HP H7N9 in the North also is the source to Northwest, Southern, and Southwest. **B** The Markov reward bar chart shows the proportion of time the virus spends in each subregion. The Northern has a high reward value, indicating that HP H7N9 has been present in this area for some time. **C** The MCC tree of HP H7N9 HA genes has been collected since 2018. Viruses isolated in the Southwest are highlighted with a light red background and purple triangle tip points. They cluster with the viruses isolated in the Northern, indicating that viruses in the Southwest originated from the North around October 2020

which is part of the 220 loop of the receptor-binding site (RBS) of HA, possibly affects the tissue tropism and/or viral virulence since the 231K (222 in H3 numbering) mutation in H5N1 may allow efficient infection of cells in the central nervous system and result in enhanced pathogenicity in mice model [25]. Meanwhile, the G151R may involve antigenic drift/escape mutation [26]. Additionally, the T341I mutation resulted in a new mutation at the HA cleavage

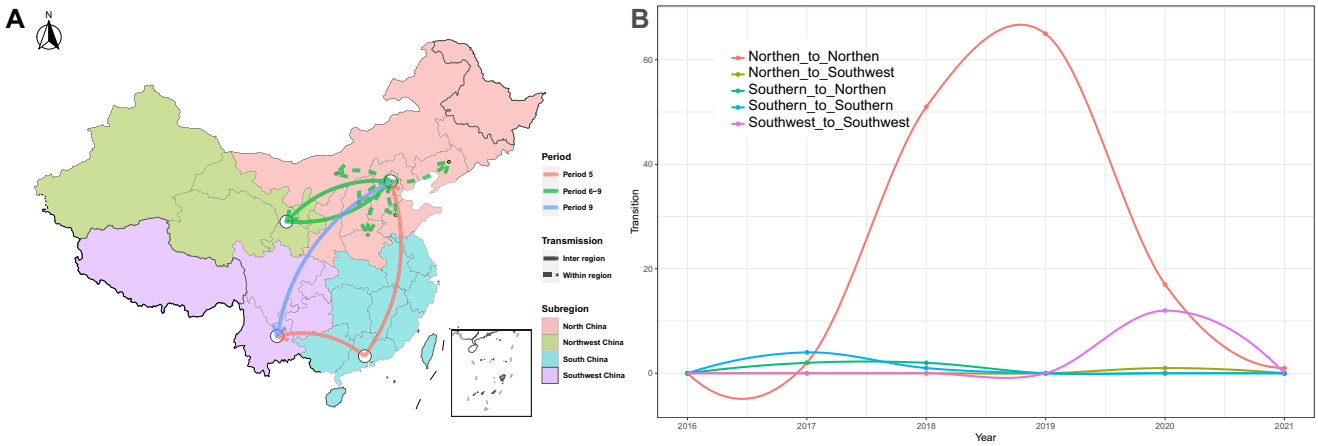

Fig. 2 Spatiotemporal dissemination of highly pathogenic avian influenza H7N9 in China. **A** Migration pathway among subregions. Solid-line depicts diffusion and spread throughout subregions, whereas the dashed line represents dissemination and spread within subregions. The HP H7N9 in Southwest China was first introduced from adjacent Southern in Period 5. Nonetheless, the virus was reintroduced in Period 9 from Northern. **B** The plots for migration among subregions. The *x*-axis indicates the years, and the *y*-axis indicates the migration events on $\log_{10}$ scale. The HP H7N9 viruses have had an elevated level of intra-subregion transmission in the Northern since 2017 and in the Southwest since 2020

site, changing the cleavage motif from PKRKR**T**AR↓G to PKRKR**I**AR↓G. Since 2018, the former motif pattern has become overwhelming in H7N9 (Fig. 1). PKRKRIAR↓G was first reported as the seventh novel cleavage motif and defined as V7 [27]. However, this novel motif was independently evolved rather than inherited from the circulating HP H7N9 virus in northern China, suggesting the site of 341 may be under a parallel evolution.

The HP H7N9 emerged in southern China (Guangdong) during epidemic wave 4. It was subsequently rapidly spread throughout northern China from its original place during wave 5 (BF = 6.07 and PP = 0.73), most likely via live poultry trafficking [28], and then seldom detected in the South. During the periods (same as waves, https://www.fao.org/ag/againfo/programmes/en/empres/H7N9/situation_update.html) 6 to 9, it was mainly maintained as a local transmission within northern China. Meanwhile, H7N9 was also disseminated from the North to Northwest subregions of China with a high PP (0.60) and vice versa (0.72). Notably, the migration route from the North to the Southwest has a BF = 3.54 and a PP = 0.61, implying that the HP H7N9 viruses isolated in Yunnan in 2021 emigrated from northern China in an unknown way. The HP H7N9 in Southwest China was first introduced from adjacent Southern in Period 5 [3]. Nonetheless, the virus has been reintroduced in Period 9 from Northern (Fig. 2A). The HP H7N9 viruses have had an elevated level of intra-subregion transmission in the Northern since 2017 and in the Southwest since 2020 (Fig. 2B). In summary, despite the reintroduction of H7N9 into Southwest China, it is still endemic and spreading within the northern subregion of China. The northern subregion has become a novel epicenter of HP H7N9 dissemination. Its spatial distribution must be closely monitored for timely and effective prevention and control response.

Computational analyses of pathogen genomes are increasingly used to unravel epidemics' transmission dynamics and dispersal history [29]. Previous research indicates that the spread of H5N1, H5N6, and H7N9 viruses among domestic chickens is geographically continuous at a national level and is most likely related to the intensity of China's live poultry trade [28]. Unlike the H5N1 virus, wild bird migration has not been associated with the spatial spread of H7N9 [28], and HP H7N9 rarely causes infections in wild birds [30]. Additionally, unlike the early waves of H7N9, which emerged and spread from live bird markets (LBM), the HP H7N9 has been mostly isolated from poultry farms in recent years [6–8, 16]. Therefore, the transportation of live poultry, poultry products, or even other occasional factors may contribute to the cross-subregional spread of the HP H7N9 virus. There is currently no evidence to prove whether it is direct or indirect of HP H7N9 spreading from northern China to southwestern China, given the inherent sample bias. Despite the lockdowns and movement control measures taken under the shadow of COVID-19 in China, the cross-subregional dissemination of HP H7N9 was not yet halted during 2020. Further surveillance studies in poultry and wild birds are required to monitor the geographical distribution and expansion of the HP H7N9 virus.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11262-023-01974-4.

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Author contributions DH performed research, analyzed data, and wrote the paper; XL and MG designed the study; XW analyzed data; YY and YL reviewed the manuscript; XW and SH Contributed new methods.

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Data availability We collected all the H7N9 sequences and metadata from the GISAID database, as the methods described.

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.

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