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Delayed peak of human infections and ongoing reassortment of H7N9 avian influenza virus in the newly affected western Chinese provinces during Wave Five

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ABSTRACT

Objectives: Eight additional provinces in western China reported human infections for the first time during the fifth wave of human H7N9 infections. The aim of this study was to analyze the epidemiological and virological characteristics of this outbreak.
Methods: The epidemiological data of H7N9 cases from the newly affected western Chinese provinces were collected and analyzed. Full-length genome sequences of H7N9 virus were downloaded from the GenBank and GISAID databases, and phylogenetic, genotyping, and genetic analyses were conducted.
Results: The peak of human infections in the newly affected western Chinese provinces was delayed by 4 months compared to the eastern Chinese provinces, and both low pathogenic (LP) and highly pathogenic (HP) H7N9-infected cases were found. The LP- and HP-H7N9 virus belonged to 10 different genotypes (including four new genotypes), of which G11 and G3 were the dominant genotypes, respectively. Almost all of these viruses originated from eastern and southern China and were most probably imported from neighboring provinces. Genetic characteristics of the circulating viruses were similar to those of the viruses from previously affected provinces during Wave Five.
Conclusions: A delayed peak of human infections was observed in the newly affected western Chinese provinces, and reassortment has been ongoing since the introduction of H7N9 viruses. This study highlights the importance of continued surveillance of the circulation and evolution of H7N9 virus in western China.

Introduction

Since the first human infection with avian influenza virus (AIV) subtype H5N1 in 1997 (Subbarao et al., 1998), several other new subtypes of AIV in humans have been reported in recent years (Chen et al., 2014; Gao et al., 2013; Pan et al., 2016; Peiris et al., 1999; Tong et al., 2018). Human infection with H7N9 AIV was initially identified in a Chinese pneumonia patient in 2013 (Gao et al., 2013), and China has experienced five waves of human H7N9 infection thus far (Quan et al., 2018; Su et al., 2017; Wang et al., 2017). During the first four waves between March 2013 and September 2016, a total of 798 human infections with a case fatality rate (CFR) of 40.6% (324 deaths) were reported from 21 provinces/regions/municipalities (Kile et al., 2017). However, a total of 766 human cases with 288 deaths were reported in the fifth wave during October 2016 to September 2017 (Kile et al., 2017; Quan et al., 2018). The number of reported human cases was the largest among the five waves, with almost as many as the combined number from the first four waves. Furthermore, a novel, highly pathogenic (HP) H7N9 AIV variant emerged and co-circulated with low

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pathogenic (LP) H7N9 AIV, which caused 28 human infections (Yang et al., 2019; Zhang et al., 2017).

Since the emergence of H7N9 AIV, two outbreak sources have been established in China, including the Yangtze River Delta and Pearl River Delta regions, of which the Yangtze River Delta region is considered as the original source of H7N9 outbreaks (Wang et al., 2016). Human H7N9 infections were also first reported in the two outbreak sources and then in the surrounding regions during the fifth wave (Quan et al., 2018; Su et al., 2017). Notably, in addition to the substantial increase in the total number of human cases, an obvious geographical dissemination of human cases was also observed. Eight provinces in western China reported human H7N9 cases for the first time, indicating a western spread of H7N9 viruses in humans (Yang et al., 2017; Yang et al., 2019).

Previous studies have analyzed and compared the epidemiological characteristics of the first four waves and the fifth wave

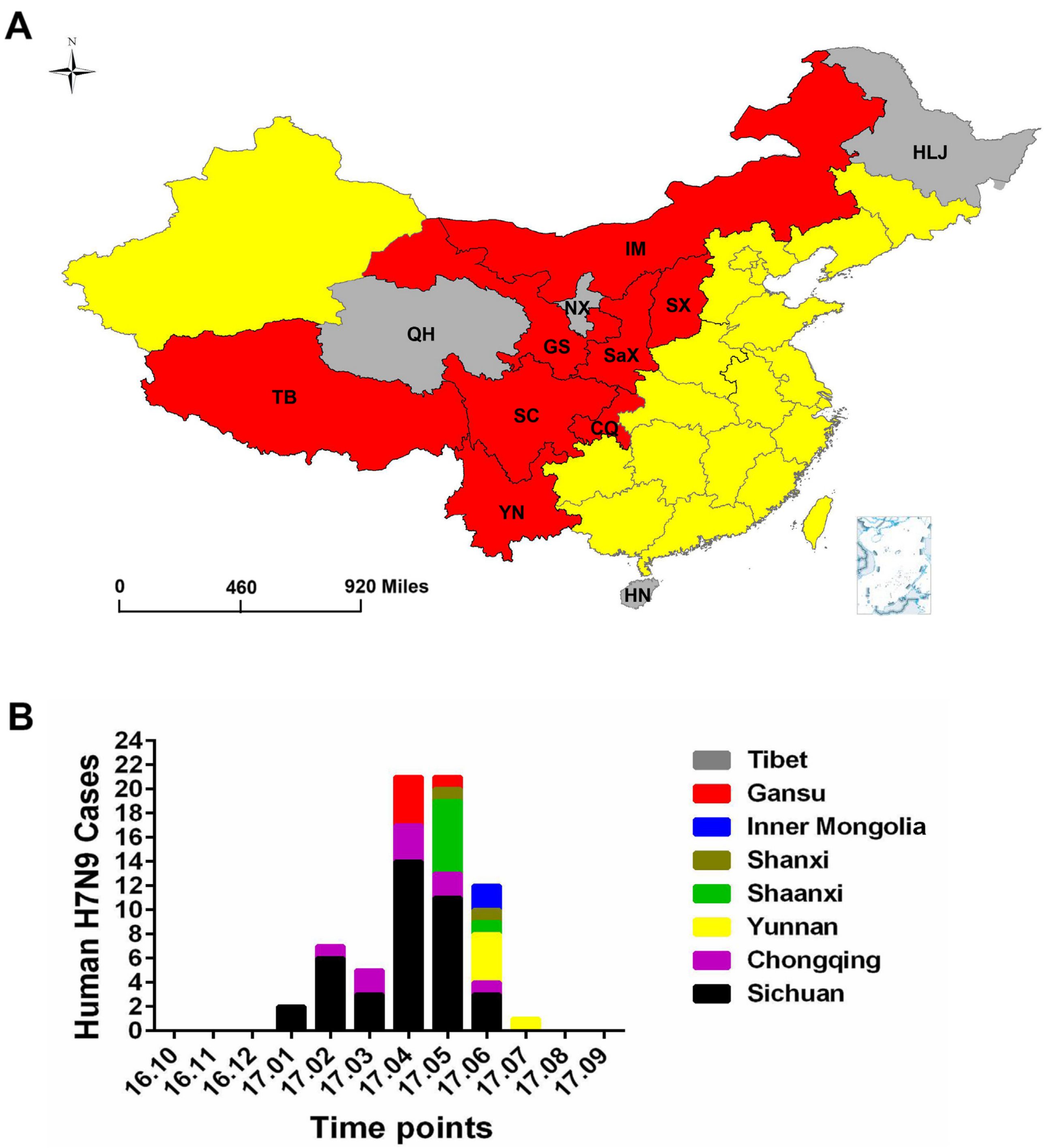

Figure 1. Spatial and temporal distributions of human H7N9 infections in the newly affected western Chinese provinces during Wave Five. (A) The geographical spread of human H7N9 infections during Wave Five. The provinces reporting human infections during the first four waves, the eight provinces reporting H7N9 cases for the first time during Wave Five, and the four provinces without human H7N9 cases are colored in yellow, red, and gray, respectively. (B) The numbers of human H7N9 cases in the newly affected western Chinese provinces during Wave Five are listed by month. Abbreviations in the map are as follows: CQ, Chongqing; GS, Gansu; HLJ, Heilongjiang; HN, Hainan; IM, Inner Mongolia; NX, Ningxia; QH, Qinghai; SaX, Shaanxi; SC, Sichuan; SX, Shanxi; TB, Tibet; YN, Yunnan.

(Su et al., 2017; Wang et al., 2017). Furthermore, a recent study showed the westward spread of HP-H7N9 virus among humans, and described the epidemiological features and a human case of HP-H7N9 infection in Shaanxi Province (Yang et al., 2018). However, the epidemiological and virological characteristics of the H7N9 viruses in the newly affected western Chinese provinces have not been characterized. This study was performed to characterize and analyze the epidemiology of human infections and virology data from these provinces during Wave Five.

Methods

Geographical and temporal distribution analyses

The geographical distribution analysis was performed as reported previously (Quan et al., 2018). In summary, provinces

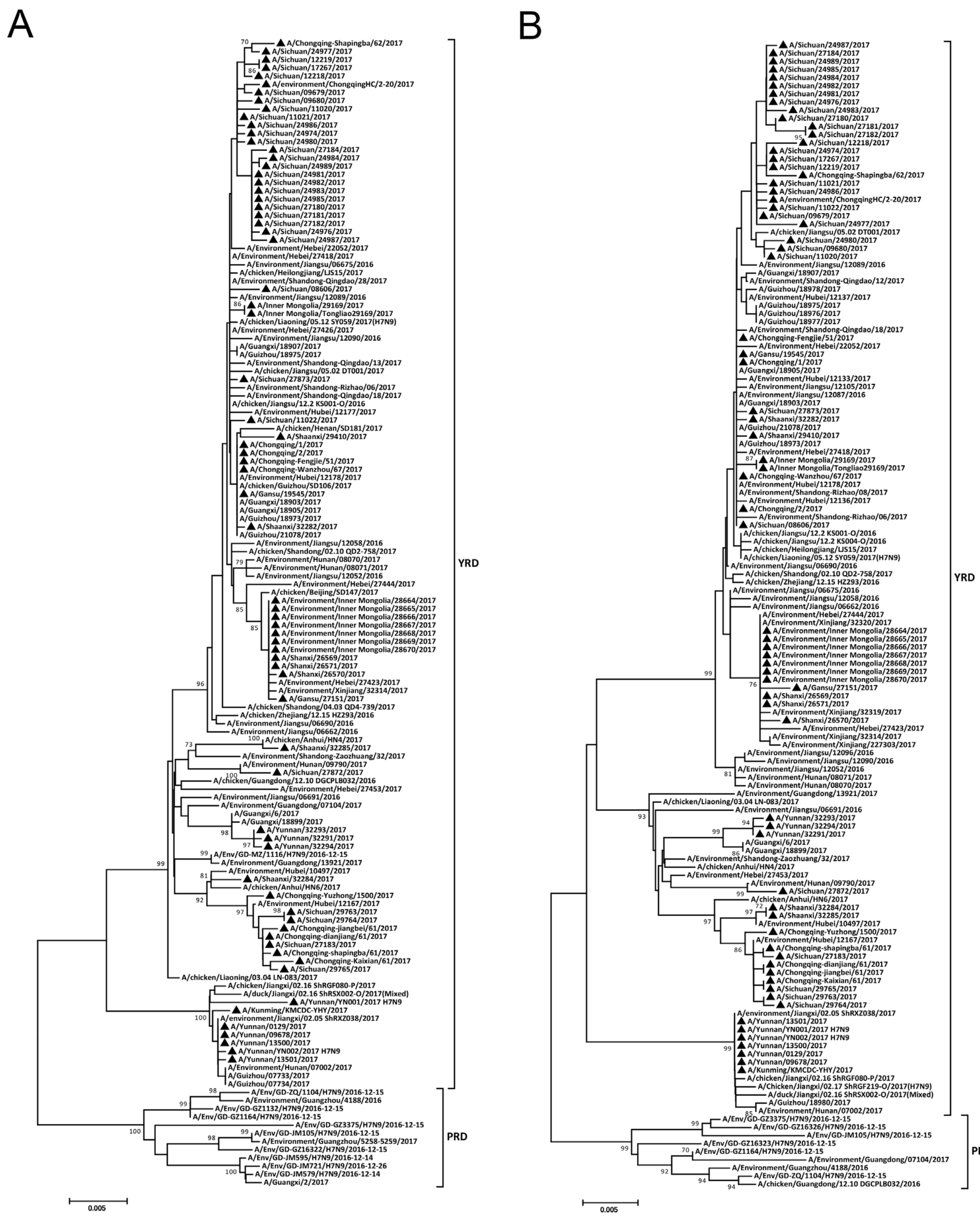

Figure 2. Phylogenetic analysis of the HA and NA genes of LP-H7N9 viruses in the newly affected western Chinese provinces during Wave Five. Panels A and B show the phylogenetic trees of HA and NA genes of human- and environment-derived LP-H7N9 viruses from the newly affected western Chinese provinces (labeled with black triangles) and representative poultry- or environment-derived LP-H7N9 viruses from other provinces during Wave Five, respectively. YRD: Yangtze River Delta; PRD: Pearl River Delta.

with human H7N9 cases across the five waves were analyzed based on the data from the National Health and Family Planning Commission of the People's Republic of China (http://www.nhfpc.gov.cn/jkj/new_index.shtml), FluTrackers (https://flutrackers.com/forum/forum/china-h7n9-outbreak-tracking), and the World Health Organization (http://www.who.int/influenza/human_animal_interface/avian_influenza/archive/en/). In addition, human H7N9 cases reported from the newly affected western Chinese provinces during Wave Five were analyzed by month.

Genetic analysis

The gene sequences were edited using DNASTAR (version 7.0) and BioEdit (version 7.0.5.2). Nucleotide and amino acid sequences of the H7N9 viruses during Wave Five were downloaded from the GenBank and GISAID (Global Initiative on Sharing All Influenza Data) databases, and aligned using MEGA (version 7.0). Biologically important amino acid residue mutations associated with pathogenicity, replication, transmissibility, host adaptation, and drug

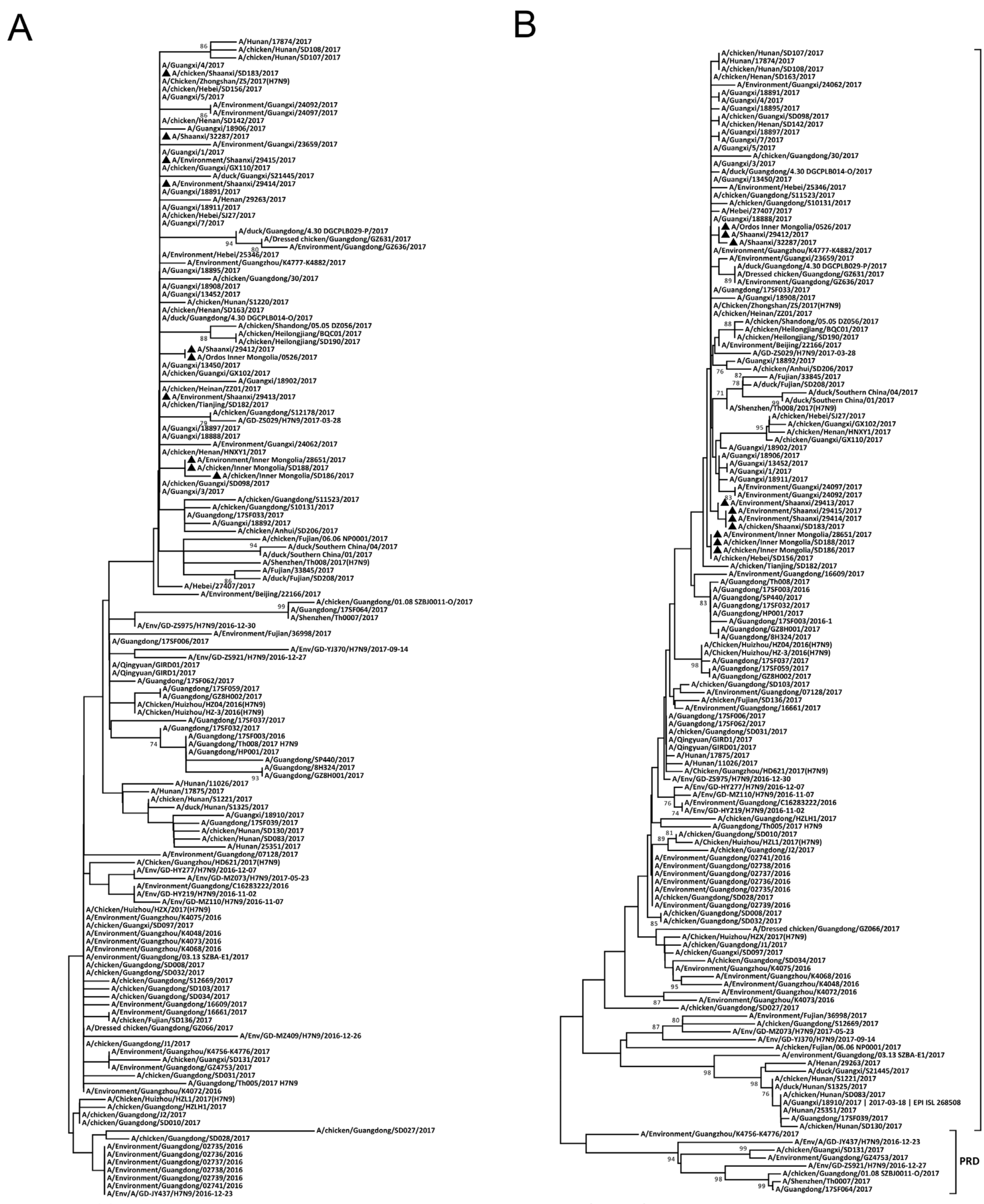

Figure 3. Phylogenetic analysis of the HA and NA genes of HP-H7N9 viruses in the newly affected western Chinese provinces during Wave Five. Panels A and B show the phylogenetic trees of HA and NA genes of the available HP-H7N9 viruses from the newly affected western Chinese provinces (labeled with black triangles) and other provinces during Wave Five, respectively. YRD: Yangtze River Delta; PRD: Pearl River Delta.

resistance were further inspected (Bi et al., 2015; Mok et al., 2014; Shi et al., 2014; Shi et al., 2013; Song et al., 2014; Yamayoshi et al., 2015; Yamayoshi et al., 2018; Yang et al., 2017).

Phylogenetic and genotyping analyses

Full-length genome sequences of H7N9 virus were downloaded from the GenBank and GISAID databases, and phylogenetic and genotyping analyses were done as reported previously (Cui et al., 2014; Quan et al., 2018). In general, for each gene, multiple sequence alignment was performed using Muscle (Edgar, 2004). The phylogenetic analysis was performed using RAxML (Stamatakis, 2014), and 1000 bootstrap replicates were run. For phylogenetic trees of the internal genes, different lineages of H7N9 AIVs were classified if they had a common ancestor no later than 2013. Generally, based on the classification of different lineages of each internal gene and bootstrap values of >70%, the genotype of each H7N9 AIV was designated and genotypes from the western Chinese provinces during Wave Five were analyzed in detail.

Results

Geographical spread of human H7N9 infections towards western China during Wave Five

During the first four waves, human cases were mainly reported in the provinces/municipalities of eastern China around the two established outbreak sources (Su et al., 2017; Wang et al., 2017). However, aside from the previously affected 21 provinces/municipalities, there were eight provinces in western China that reported human H7N9 cases for the first time during Wave Five, including Sichuan (thirty-nine cases), Chongqing (nine cases), Tibet (three cases), Shaanxi (seven cases), Inner Mongolia (two cases), Shanxi (two cases), Yunnan (five cases) and Gansu (five cases) (Figure 1A). Human cases in these provinces were reported during January to July 2017, and peaked in April and May (Figure 1B). During this time period, human cases were reported nearly every month in Sichuan and Chongqing provinces, while for the other six provinces, H7N9 cases were reported starting in April, and mainly in May and June.

Phylogenetic and genotyping analyses of the LP-H7N9 isolates in the newly affected western Chinese provinces

Phylogenetic analysis of the LP-H7N9 isolates in the newly affected western Chinese provinces showed that the HA and NA genes both belonged to the Yangtze River Delta lineage (Figure 2). HA genes of the isolates from each of the newly affected western Chinese provinces clustered with different isolates from various districts, including eastern, central, and southern China. For example, isolates from Sichuan, Chongqing, and Shaanxi provinces showed high homology with each other, and mainly clustered with isolates from Hubei and Hebei provinces; isolates from Yunnan Province mainly clustered with viruses from Guangxi and Guizhou

Table 1
Key molecular markers of H7N9 viruses from humans and the environment in the newly affected western Chinese provinces.

Gene	Functions	Mutations	Amino acids	Number (%) of viruses	
				Humans	Environment
HA[a]	Alters receptor specificity	G186V	V	64 (98)	20 (100)
		K193T	T	0 (0)	0 (0)
		N224K	K	0 (0)	0 (0)
		Q226L	L	63 (97)	13 (65)
		G228S	S	0 (0)	0 (0)
	Cleavage peptides	PEIPKGR/GL	NA	62 (95)	13 (65)
		PEVPKRKRTAR/GL	NA	2 (3)	7 (35)
NA[b]	Reduces drug sensitivity	E119V	V	0 (0)	0 (0)
		A246T	T	0 (0)	0 (0)
		H274Y	Y	0 (0)	0 (0)
		R292K	K	4 (6)	0 (0)
		R292K	R/K	1 (1.5)	0 (0)
PB2	Restores polymerase activity at 37 °C	T271A	A	0 (0)	0 (0)
	Enhances 627 K and 701 N function	K526R	R	14 (22)	7 (50)
	Restores polymerase activity	M535L	L	14 (22)	7 (50)
	Efficient replication in mammalian and avian cells and higher virulence in mice	K482R	R	0 (0)	0 (0)
		A588V	V	47 (76)	7 (50)
	Enhances virulence in mice	Q591K	K	1 (1.6)	0 (0)
	Increases virulence in mammalian models	E627K	K	37 (59)	0 (0)
		E627K	E/K	6 (10)	0 (0)
		D701N	N	3 (5)	0 (0)
	Host signature amino acids (avian to human)	A199S	S	0 (0)	0 (0)
		T271A	A	0 (0)	0 (0)
		K702R	R	0 (0)	0 (0)
PB1	Increases transmission in ferrets	I368V	V	60 (95)	12 (100)
PA	Host signature amino acids (avian to human)	V100A	A	11 (17)	7 (50)
		A404S	S	0 (0)	0 (0)
		S409N	N	63 (100)	14 (100)
NP	Host signature amino acids (avian to human)	V33I	I	0 (0)	0 (0)
		I309V	V	0 (0)	0 (0)
M1	Impacts growth and transmission in guinea pig model	P41A	A	63 (100)	13 (93)
	Alters virulence in mice	T215A	A	63 (100)	14 (100)
	Host signature amino acids (avian to human)	V115I	I	0 (0)	0 (0)
M2	Reduces susceptibility to licensed anti-influenza virus medications	S31N	N	63 (100)	14 (100)
NS1	Alters virulence in mice	P42S	S	63 (100)	14 (100)
	Alters the antiviral response in the host	N205S	S	63 (100)	14 (100)

NA, not available.
[a] The H3 numbering system was used.
[b] The N2 numbering system was used.

provinces; isolates from Inner Mongolia possessed high homology with Shanxi Province isolates and mainly clustered with isolates from Hebei and Xinjiang provinces. NA genes of the isolates showed a similar pattern to the HA genes. Isolates in Sichuan and Chongqing provinces mainly clustered with isolates from Hubei and Shandong provinces; isolates from Yunnan Province mainly clustered with isolates from Guangxi and Jiangxi provinces; isolates from Shaanxi, Inner Mongolia, and Shanxi provinces showed high homology and mainly clustered with isolates from Hubei and Xinjiang provinces. The two isolates in Gansu Province clustered with isolates in Shandong and Xinjiang provinces, respectively (Figure 2).

A further analysis of the genotypes of the LP-H7N9 isolates in the newly affected western Chinese provinces was then performed (**Supplementary Material**, Table S1). The results showed that a total of 10 genotypes (G1, G3, G11, G24, G34, G35, G37, G38, G39, and G40) with four new genotypes (G37, G38, G39, and G40) were found in Sichuan, Chongqing, and Shaanxi provinces, among which G11 was the dominant genotype (42/62, 67.7%).

Phylogenetic and genotyping analyses of the HP-H7N9 isolates in the newly affected western Chinese provinces

HP-H7N9 human infections were found in Inner Mongolia and Shaanxi provinces (Quan et al., 2018; Yang et al., 2017; Yang et al., 2018), and so the phylogenetic and genotyping analyses were also done with the available HP-H7N9 viruses in the GenBank and GISAID databases, both from human cases and live poultry markets (LPMs). Consistent with previous studies (Quan et al., 2018; Yang et al., 2017), all HA genes of HP-H7N9 virus belonged to the Yangtze River Delta lineage (Figure 3A). HA genes of the HP-H7N9 isolates from Inner Mongolia and Shaanxi provinces possessed >99.8% identity to each other (**Supplementary Material**, Table S2), and showed the highest homology with isolates from Guangxi Province (Figure 3A). In addition, they showed >98.5% identity with the rest of the HP-H7N9 viruses (**Supplementary Material**, Table S2), and had a common ancestor with the HP-H7N9 viruses in Guangdong Province. All NA genes of HP-H7N9 isolates from Inner Mongolia and Shaanxi provinces belonged to the Yangtze River Delta lineage, as did most of the HP-H7N9 isolates (Figure 3B). The NA genes also showed the highest homology with isolates from Guangxi Province, and shared >99.6% identity to each other. Furthermore, the six HP-H7N9 isolates from Inner Mongolia and Shaanxi provinces all belonged to genotype G3 (**Supplementary Material**, Table S1).

Molecular characterization of H7N9 isolates in the newly affected western Chinese provinces during Wave Five

A further analysis of the key molecular substitutions of H7N9 isolates in the newly affected western Chinese provinces during Wave Five was then performed (Table 1). The G186V and Q226L (H3 numbering) substitutions associated with receptor specificity were observed in most of the isolates from both humans and the environment. Several substitutions associated with adaptations for mammalian infection were observed in the PB2 protein. The K526R, M535L, and A588V substitutions in the PB2 protein were frequently present in isolates from humans and the environment. Q591K, E627K, and D701N substitutions in the PB2 protein occurred in the isolates from humans, while not in the isolates from the environment samples. The R292K substitution in the NA protein conferring resistance to neuraminidase inhibitors occurred in some human-derived H7N9 viruses, but not in the environment-derived isolates. Host signature-associated mutations in the PB2 (A199S, T271A, K702R), PA (A404S), NP (V33I, I309V), and M1 (V151I) proteins were not observed in the original H7N9 viruses, while V100A and S409N substitutions in the PA proteins were present in the viruses from humans and from the environment. In addition, all six HP-H7N9 isolates possessed a 4-amino-acid insertion in the cleavage site of the HA protein, forming the new cleavage site of PEVPKRKRTAR/GL, one of the four motif patterns in HP-H7N9 viruses (Quan et al., 2018).

Discussion

During the first four waves, human H7N9 infections were mainly concentrated in the areas around the outbreak sources of the Yangtze River Delta and Pearl River Delta regions (Su et al., 2017; Wang et al., 2017). During the fifth wave, the reported human cases in eight additional provinces in western China indicated a geographical spread of H7N9 viruses in humans (Quan et al., 2018; Yang et al., 2017), which further posed an increasing threat. The peak of human cases in the newly affected western Chinese provinces was in May and June of 2017, which was a delay of 4 months compared to the overall temporal distribution of human cases during Wave Five (Quan et al., 2018; Yang et al., 2017). The differences in temperature and humidity among these provinces may serve as one of the main reasons, as they have been identified to be critical for influenza survival, transmission, and seasonality (Lowen et al., 2007; Lowen and Steel, 2014; Shaman and Kohn, 2009; Shaman et al., 2010). Although HP-H7N9 virus was first identified in Guangdong Province (Zhang et al., 2017; Zhou et al., 2017), it has now been identified in poultry from the south (Guangdong) to the north (Heilongjiang) and also the west of China (Inner Mongolia and Shaanxi), with the common ancestor from Guangdong HP-H7N9 viruses (Quan et al., 2018; Yang et al., 2017; Yang et al., 2018). Unlike LP-H7N9 viruses, which cause asymptomatic infections and circulate silently in chickens, HP-H7N9 viruses are highly pathogenic and cause severe diseases in chickens (Liu et al., 2014; Quan et al., 2018). These are quite important for the future surveillance of H7N9 virus.

Phylogenetic analysis showed that the HA and NA genes of the H7N9 isolates from the newly affected western Chinese provinces all belonged to the Yangtze River Delta region lineage, and the HA genes from the same provinces showed high genetic identities of >97.3% (**Supplementary Material**, Table S3). Isolates from the western Chinese provinces clustered with isolates from various districts, while most of the isolates showed the highest homology with isolates from the neighboring provinces. This indicates that the majority of H7N9 viruses in the newly affected western Chinese provinces might have been imported from the neighboring provinces, most possibly by live poultry or poultry product transport, which has been shown to be a driver of China's H7N9 viral evolution and geographical network propagation (Gao, 2014; Li et al., 2018).

Previous studies have shown that genetic reassortment of AIVs could influence the viral pathogenicity and transmissibility to mammals (Lu et al., 2014; Sun et al., 2011; Zhang et al., 2013), which has been considered as an underlying reason for the substantial increase in human infections during Wave Five (Quan et al., 2018). Based on the previously reported classification method (Cui et al., 2014; Quan et al., 2018), we identified a total of 10 genotypes circulating in the newly affected western Chinese provinces. The predominant circulating genotypes of both LP-H7N9 and HP-H7N9 viruses were the same as those in the previously affected provinces (**Supplementary Material**, Table S1), while four new genotypes (G37–G40) emerged in Sichuan, Chongqing, and Shaanxi provinces. Meanwhile, compared with the most probable source for the viruses in the three provinces, increased numbers of circulating genotypes were observed (**Supplementary Material**, Table S1). This suggests that H7N9 viruses in these newly affected western Chinese provinces have undergone genetic reassortment after the introduction of the H7N9 viruses. Consistent with previous studies (Quan et al., 2018), the dominant genotypes of LP-H7N9 and HP-H7N9 viruses were G11 and G3, respectively, both in the human-derived and environment-derived viruses. Although there is no information on environment-derived isolates before Wave Five in the newly affected western Chinese provinces, these results may also indicate that H7N9 viruses were introduced into these provinces during Wave Five, and further transmitted to humans.

Studies have shown that some molecular substitutions in the proteins of the H7N9 virus are associated with increased virulence and transmissibility in mammals or antiviral drug resistance (Bi et al., 2015; Mok et al., 2014; Shi et al., 2014; Shi et al., 2013; Song et al., 2014; Yamayoshi et al., 2015; Yamayoshi et al., 2018; Yang et al., 2017). The G186V and Q226L substitutions, which have been reported to be associated with a switch in receptor specificity from avian (α2-3Gal) to human type (α2-6Gal) (Shi et al., 2014; Shi et al., 2013; Xu et al., 2013), were found in most of the isolates from humans and the environment, further confirming the ability of these H7N9 viruses to infect humans. Among the substitutions in the PB2 protein associated with increased polymerase activity or enhanced virulence in mice, the K526R, M535L, and A588V substitutions were observed in isolates from both humans and the environment. However, the Q591K, E627K, and D701N substitutions occurred only in the viruses isolated from humans, which are frequently observed in the viruses transmitted from avian to mammalian hosts (Su et al., 2017). The results showed that, with regard to the genetic characteristics analyzed, the viruses in the newly affected western Chinese provinces were similar to the other viruses from Wave Five.

In this study, the epidemiology and virological data from the newly affected western Chinese provinces during Wave Five were analyzed and characterized for the first time. Of concern, the results show a delayed peak of human infections in the provinces of western China, and reassortment has been ongoing since the introduction of H7N9 viruses. However, no evidence of increased transmissibility of H7N9 virus to humans from poultry or environmental exposures was observed. This study highlights the importance of continued surveillance of the circulation and evolution of H7N9 virus in western China.

Author contributions

Yang Yang and Yingxia Liu designed the study. Jin Li and Chuming Chen analyzed the data. Jinli Wei, Huaxin Huang, and Yun Peng helped to collect the data. Yuhai Bi provided scientific input. Yang Yang and Jin Li wrote the paper. All authors read and approved the final manuscript.

Conflict of interest

The authors have declared that no conflicts of interest exist.

Ethical approval

Not applicable.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2019.09.002.

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