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GENETIC DIVERSITY AND EVOLUTION

Genesis and Spread of Newly Emerged Highly Pathogenic H7N9 Avian Viruses in Mainland China

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ABSTRACT The novel low-pathogenic avian influenza A H7N9 viruses (LPAI H7N9 viruses) have been a threat to public health since their emergence in 2013 because of the high rates of mortality and morbidity that they cause. Recently, highly pathogenic variants of these avian influenza A H7N9 viruses (HPAI H7N9 viruses) have emerged and caused human infections and outbreaks among poultry in mainland China. However, it is still unclear how the HPAI H7N9 virus was generated and how it evolved and spread in China. Here, we show that the ancestor virus of the HPAI H7N9 viruses originated in the Yangtze River Delta region and spread southward to the Pearl River Delta region, possibly through live poultry trade. After introduction into the Pearl River Delta region, the origin LPAI H7N9 virus acquired four amino acid insertions in the hemagglutinin (HA) protein cleavage site and mutated into the HPAI H7N9 virus in late May 2016. Afterward, the HPAI H7N9 viruses further reassorted with LPAI H7N9 or H9N2 viruses locally and generated multiple different genotypes. As of 14 July 2017, the HPAI H7N9 viruses had spread from Guangdong Province to at least 12 other provinces. The rapid geographical expansion and genetic evolution of the HPAI H7N9 viruses pose a great challenge not only to public health but also to poultry production. Effective control measures, including enhanced surveillance, are therefore urgently needed.

IMPORTANCE The LPAI H7N9 virus has caused five outbreak waves in humans and was recently reported to have mutated into highly pathogenic variants. It is unknown how the HPAI H7N9 virus originated, evolved, and disseminated in China. In this study, we comprehensively analyzed the sequences of HPAI H7N9 viruses from 28 human and 21 environmental samples covering eight provinces in China that were taken from November 2016 to June 2017. The results show that the ancestor virus of the HPAI H7N9 viruses originated in the Yangtze River Delta region. However, the insertion of four amino acids into the HA protein cleavage site of an LPAI H7N9 virus occurred in late May 2016 in the Pearl River Delta region. The mutated HPAI H7N9 virus further reassorted with LPAI H7N9 or H9N2 viruses that were cocirculating in poultry. Considering the rapid geographical expansion of the HPAI H7N9 viruses, effective control measures are urgently needed.

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Since the emergence of a novel avian influenza A H7N9 virus in the Yangtze River Delta region in spring 2013, human cases of infection with H7N9 viruses have occurred every winter-spring season in mainland China (1). So far, there have been a total of five epidemic seasons. The fifth epidemic season started earlier than the first four, and the epidemic was the largest one to date (2). As of 14 July 2017, there have been over 1,550 laboratory-confirmed human cases of avian influenza A H7N9 virus infection in China, with the fatality rate being approximately 39% (http://www.chinaivdc.cn/cnic/en/).

In the first four epidemic waves, the avian influenza A H7N9 viruses circulating in poultry were classified as low-pathogenic avian influenza A H7N9 viruses (LPAI H7N9 viruses) because the infected poultry were asymptomatic (2, 3). In late February 2017, Guangdong Province in China officially reported that two patients were infected with an H7N9 avian influenza A virus that appeared to be highly pathogenic (highly pathogenic influenza A H7N9 viruses [HPAI H7N9 viruses]) (4–6). The insertion of 4 amino acids (Lys-Arg-Thr-Ala) into the hemagglutinin (HA) cleavage site connecting the HA1 and HA2 peptide regions appears to have induced the H7N9 virus to become highly pathogenic for fowl of the order *Galliformes* (6, 7). A similar virus was detected in one patient from Taiwan Province; he was a businessman returning from Guangdong Province (8). As of 14 July 2017, a total of 28 human cases of HPAI H7N9 virus infection have been reported, with 14 of these cases being fatal (http://www.chinaivdc.cn/cnic/en/).

Several outbreaks among poultry were also reported in mainland China from March 2017 to June 2017 in Hunan, Hebei, Henan, Tianjin, Shaanxi, and Heilongjiang Provinces and Inner Mongolia (http://www.moa.gov.cn/, http://www.oie.int/). Approximately 60,000 poultry have died due to HPAI H7N9 virus infections, and another 300,000 chickens were culled. Thus, the H7N9 viruses have not only caused a threat to public health, but they have also become a great burden in the poultry industry.

Since the emergence of H7N9 avian influenza viruses, the influenza virus HA genes have evolutionarily diversified into two main lineages: the Yangtze River Delta lineage and the Pearl River Delta lineage (9). The HPAI H7N9 viruses were reported to belong to the Yangtze River Delta lineage (6). However, it is still unknown when, where, and how the HPAI H7N9 viruses were generated. Furthermore, an assessment of the geographic spread of HPAI H7N9 viruses is urgently needed for use in risk assessment. In this study, human and environmental isolates of HPAI H7N9 viruses collected from December 2016 to June 2017 were used to trace the evolution and spread of the HPAI H7N9 viruses.

RESULTS

HPAI H7N9 epidemics in China. The 28 human cases of HPAI H7N9 virus infection evaluated in this study were detected in Guangdong, Guangxi, Hunan, Hebei, Henan, Shaanxi, and Taiwan Provinces (Fig. 1A). As shown in Fig. 1B, the majority of cases of HPAI H7N9 virus infection occurred in the winter-spring season of 2016 and 2017. Of these 28 cases, 21 were from Guangdong and Guangxi Provinces. HPAI H7N9 viruses were also detected in live poultry markets in Guangdong, Beijing, and Shaanxi Provinces and on farms in Inner Mongolia and Hebei Provinces. HPAI H7N9 viruses have affected 35.3% (12/34) of the provinces in China to date (Fig. 1A). The sequences of the viruses from 28 human and 21 environmental samples collected during the period from November 2016 to June 2017 in eight provinces in China were included for analysis.

Molecular dating of HPAI virus H7 and N9 genes. To elucidate the origin and timing of the HPAI H7N9 virus HA genes, we integrated the genetic divergence of the HA genes of both the LPAI and HPAI H7N9 viruses onto the same time scale. As expected, the HA genes of H7N9 viruses can be divided into two main lineages: the

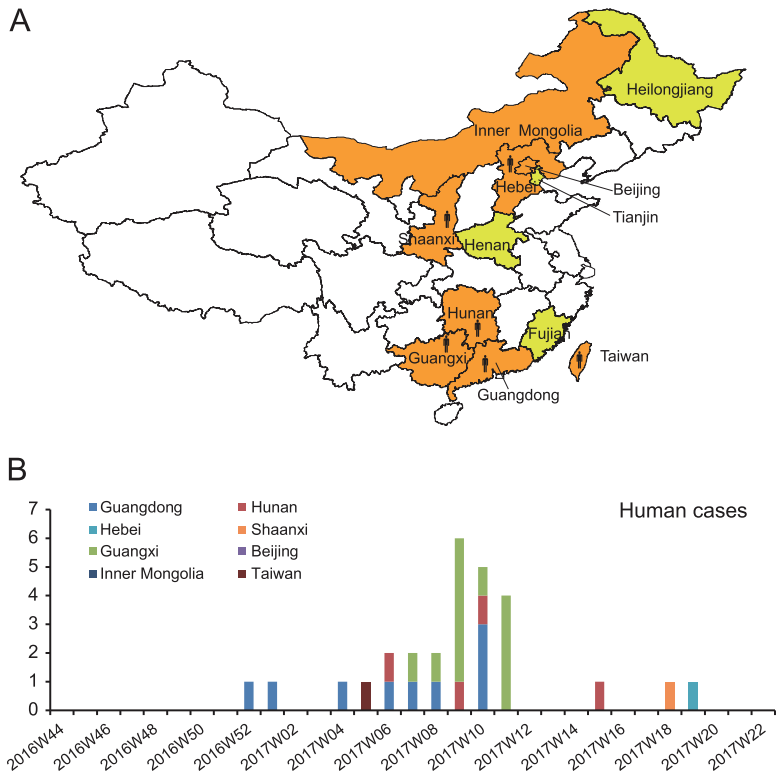

FIG 1 Geographic distribution and prevalence of HPAI H7N9 viruses. (A) Map showing the HPAI H7N9 virus-positive regions in China. Both orange and yellow indicate regions where HPAI H7N9 viruses were detected; provinces with available HPAI H7N9 sequences are highlighted in orange, while those with unavailable sequences are shown in yellow. Provinces marked with a human figure are the regions where human cases were reported. (B) Weekly virus numbers (indicated on the *y* axis) and geographic information for the HPAI H7N9 viruses isolated from humans. The different colors indicate the isolation locations. The designations on the *x* axis indicate the year and the week (W) of the year.

Yangtze River Delta lineage and the Pearl River Delta lineage. All of the HPAI H7N9 viruses, regardless of isolation source, grouped into a single cluster, named the HPAI H7N9 virus cluster. The outgroup viruses of the HPAI H7N9 virus cluster were exclusively categorized into the Yangtze River Delta lineage, indicating that all HPAI H7N9 viruses originated from the Yangtze River Delta lineage (Fig. 2A). The median time to the most recent common ancestor (tMRCA) among these HPAI virus H7 genes was estimated to be late May 2016 (95% highest posterior density [HPD], March 2016, July 2016) (Fig. 2).

As shown in Fig. 2B, all of the avian and human HPAI H7N9 virus isolates were rooted from the LPAI H7N9 viruses isolated in Guangdong Province. This result verifies that the HPAI H7N9 viruses originated from the LPAI H7N9 viruses, such as A/Environment/Guangdong/15120/2016(H7N9)-like or A/Environment/Guangdong/15123/2016(H7N9)-like viruses. Additionally, these results indicate that Guangdong Province may be the location where the LPAI H7N9 virus acquired a 4-amino-acid insertion and mutated into the HPAI H7N9 viruses. Furthermore, several subclusters were observed in the HPAI H7N9 virus cluster. Expect for the HPAI H7N9 viruses isolated from Guangdong Province, the other HPAI H7N9 viruses, including those from Guangxi, Hunan, and Shaanxi Provinces and Inner Mongolia, were exclusively categorized into subclusters with viruses from Guangdong Province. All outgroup viruses of these subclusters were detected in Guangdong Province, indicating that all HPAI H7N9 viruses may have originated in Guangdong Province and subsequently spread to other regions.

In contrast to the groupings based on H7 genes, HPAI H7N9 viruses analyzed on the basis of their N9 genes fell into one major group and two minor groups (see Fig. S1 in the supplemental material). This finding indicates that reassortment events may have occurred among the N9 genes of HPAI H7N9 and LPAI H7N9 viruses. The N9 genes in

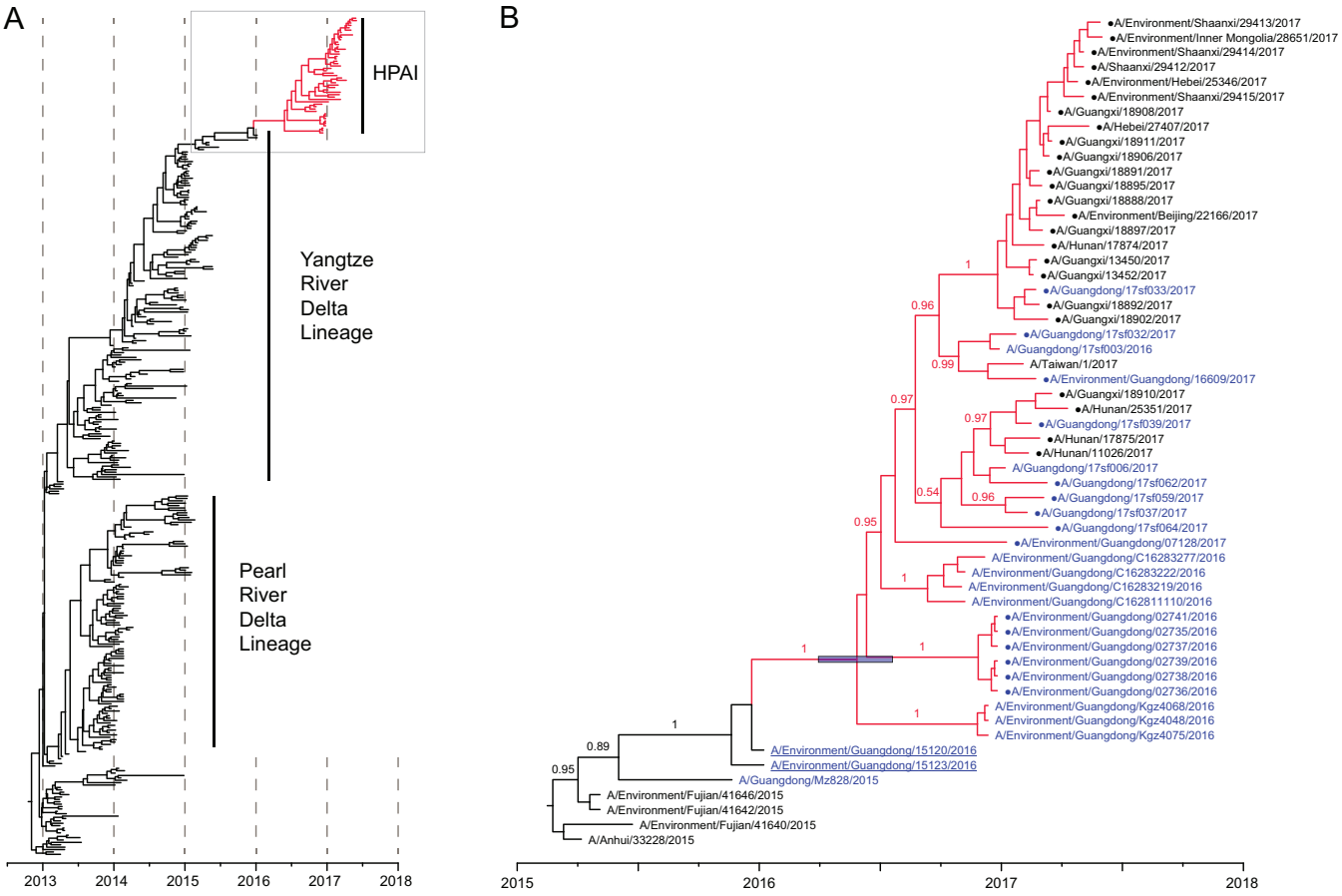

FIG 2 Genomic divergence and evolutionary analysis of H7N9 avian influenza A virus H7 gene sequences. (A) A Bayesian maximum clade credibility (MCC) phylogeny of HA genes of influenza A H7N9 viruses. The phylogenetic clades that include avian influenza A H7N9 viruses were obtained from the dated phylogeny of HA gene segments constructed by a molecular phylogenetic analysis and were further aligned onto the same time scale. The branches in black and red represent LPAI and HPAI H7N9 viruses, respectively. (B) Representative clades in the gray box in panel A. The branches in black and red represent LPAI and HPAI H7N9 viruses, respectively. All viruses, including the LPAI and HPAI H7N9 viruses, which were isolated in Guangdong Province are highlighted in blue. The blue bar shown in the tree indicates the 95% highest posterior density of tMRCA of HPAI H7N9 viruses. LPAI H7N9 virus strains A/Environment/Guangdong/15120/2016(H7N9) and A/Environment/Guangdong/15123/2016(H7N9), which were likely the ancestor of HPAI H7N9 viruses, are underlined. The viruses isolated and sequenced in this study are highlighted with a solid circle at the beginning of the virus name.

all of our sampled viruses belong to the Yangtze River Delta lineage, except for the N9 gene of the A/Guangdong/17sf064/2017(H7N9) virus. As for the major N9 gene group, the outgroup viruses of the HPAI H7N9 viruses were also A/Environment/Guangdong/15120/2016(H7N9)-like or A/Environment/Guangdong/15123/2016(H7N9)-like LPAI H7N9 viruses. Results from a Bayesian maximum clade credibility (MCC) phylogenetic analysis of N9 genes also support the hypothesis that Guangdong Province was the genesis location of HPAI H7N9 viruses.

Genetic characterization of six internal HPAI H7N9 virus genes. Comparing the full genome sequences, we found that the HA and NA genes of these 49 HPAI H7N9 viruses shared over 99% identity at the nucleotide level (Fig. 3). In contrast, the six internal genes of the HPAI H7N9 viruses showed distinct diversity (Fig. 3), with the basic polymerase 2 (PB2), PB1, acidic polymerase (PA), nucleoprotein (NP), matrix (M), and nonstructural protein (NS) genes of the 41 viruses sharing 94.0 to 100%, 94.5 to 100%, 95.4 to 100%, 92.5 to 100%, 95.8 to 100%, and 94.2 to 100% identity, respectively, at the nucleotide level.

The six internal genes of the HPAI H7N9 viruses were grouped with those of the LPAI H7N9 or H9N2 viruses (Fig. S2). The PB2, M, and NS genes each formed two groups in the phylogenetic trees (Fig. S2A, E, and F). The PA and NP genes each formed three groups in the phylogenetic trees (Fig. S2C and D), and the PB1 gene formed four groups

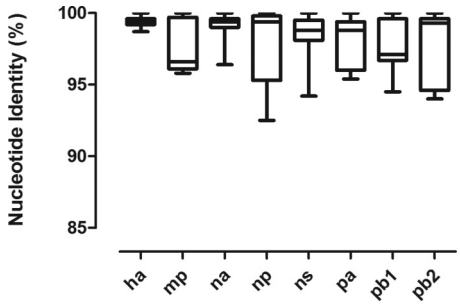

FIG 3 Nucleotide sequence similarity for each gene segment among all HPAI H7N9 virus sequences. The bottoms and tops of the boxes are the first and third quartiles, respectively, and the band inside the box is the median. The ends of the whiskers represent the minimum and maximum of all of the data.

(Fig. S2B). Based on the genomic diversity of these six internal genes, we classified the HPAI H7N9 viruses into 14 genotypes (Fig. 4). All 14 of these virus genotypes were detected in Guangdong Province and its neighboring provinces, Hunan and Guangxi Provinces. Viruses belonging to genotype II were detected in six out of eight provinces. The viruses isolated in the provinces of Shaanxi, Hebei, and Beijing and in Inner Mongolia, which are located in northern China, were exclusively genotype II viruses (Fig. 4). This result indicates that the internal genes from HPAI H7N9 viruses that were generated in Guangdong Province have continued reassorting with the internal genes from LPAI H7N9 or H9N2 viruses.

Key amino acid mutations occurring in HPAI H7N9 viruses. To determine if the HPAI H7N9 viruses had attained any key molecular substitutions associated with increased virulence and transmissibility in mammals or with antiviral drug resistance, we analyzed the sequences of all the sampled HPAI H7N9 viruses. As shown in Table S3, all of the HPAI H7N9 viruses had obtained a 4-amino-acid insertion in the HA cleavage site (positions 320 to 321). However, a substitution from Gly (G) to Arg (R) at position 320 occurred in only a subset of the HPAI H7N9 viruses. All of the LPAI H7N9 and HPAI H7N9 viruses sampled before 2017 possessed 320G, except for one virus isolated from a human (A/Guangdong/17sf003/2016). In contrast, the majority (31/33) of HPAI H7N9 virus isolates in this study that were sampled in 2017 had 320R mutations. This result further indicates that the HPAI H7N9 viruses originated from LPAI H7N9 viruses.

Additionally, several changes associated with receptor specificity were observed in the HPAI H7N9 viruses (Table 1). The G186V and Q226L (H3 numbering) substitutions in the HA protein have previously been reported to be associated with a switch in receptor specificity from avian-type receptor specificity (α2-3Gal) to human-type receptor specificity (α2-6Gal) (10–12), and G186V alone in the HA protein can also increase the affinity of the virus for the human-type receptor (7, 10). All 21 environmental isolates and 25/28 human isolates in this study had both the G186V and L226Q substitutions (Table 1).

Several mutations conferring adaptations for mammalian infection have occurred in the PB2 protein of HPAI H7N9 viruses. For example, the T271A, Q591K, E627K, and D701N substitutions in the PB2 protein, which are associated with increased polymerase activity or enhanced virulence in mice, occurred in 1, 1, 14 (3 mixed), and 4 of the 28 viruses from human samples, respectively. In contrast, no such mutations were detected in the HPAI H7N9 viruses from environmental samples. These phenomena are frequently observed when an avian virus is transmitted to a mammalian host (13–16), and they further confirm that the source of human infection with the HPAI H7N9 viruses is poultry populations. Additionally, residues in the PA and NP proteins of several H7N9 viruses showed changes in their amino acid preference from avian to human (Table 1). In PA, 1/28 and 2/28 HPAI H7N9 viruses isolated from humans had A404S and S409N, respectively, whereas no viruses sampled from the environment had these mutations. Similar results were observed for positions 33 and 109 in the NP protein. All sampled

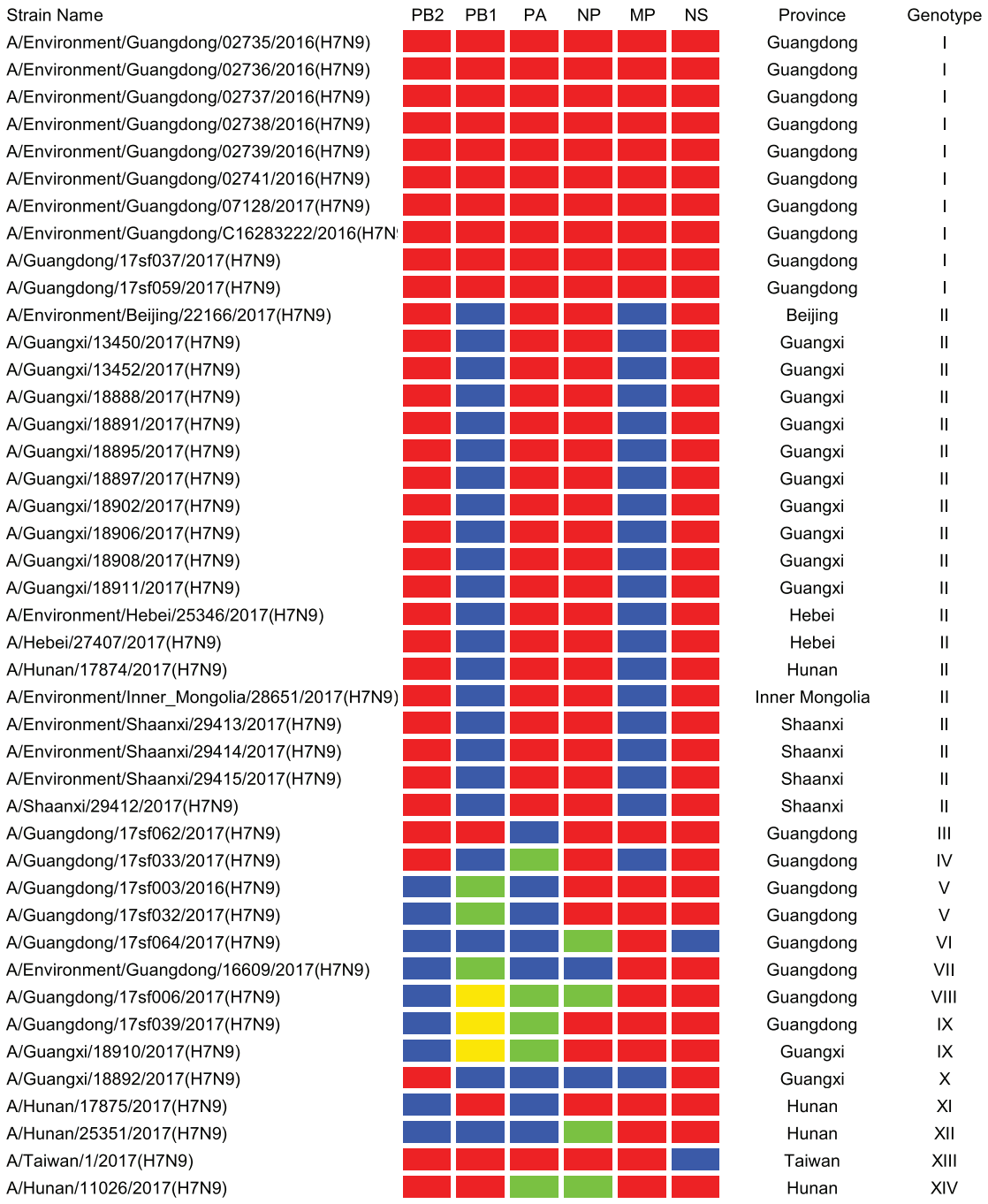

FIG 4 Genotypes of HPAI H7N9 avian influenza viruses. The six internal gene segments are indicated at the top. The colors of the bars represent the groups in the trees in Fig. S2 in the supplemental material.

HPAI H7N9 viruses, regardless of human or avian origin, had 31N in the M2 protein, indicating their reduced susceptibility to rimantadine and amantadine (17).

HPAI H7N9 viruses with an R292K mutation in the NA protein have been reported to be resistant to multiple neuraminidase inhibitor-based drugs (7), and all three of the viruses from the initial cases of HPAI H7N9 virus infection were reported to have acquired an R292K mutation in the NA protein (6, 8). In addition to these three viruses, another two human HPAI H7N9 virus isolates also had this mutation. Other NA protein mutations that are associated with drug resistance occurred in only two human isolates

TABLE 1 Key molecular markers of HPAI H7N9 viruses from humans or the environment

Gene	Function	Mutation	Amino acid(s)	No. (%) of viruses from: Humans	Environment
HA[a]	Alters receptor specificity	G186V/N/K	I	3 (11)	0 (0)
		G186V/N/K	V	25 (89)	21 (100)
		K193T	K	28 (100)	21 (100)
		N224K	N	28 (100)	21 (100)
		Q226L	H	1 (4)	0 (0)
		Q226L	L	1 (4)	0 (0)
		Q226L	Q	25 (89)	21 (100)
		Q226L	Q/L	1 (4)	0 (0)
		G228S	G	28 (100)	21 (100)
	Cleavage peptides	PEVP**KG**K**R**TA**R** ↓ G[b]		0 (0)	15 (71)
		PEVP**KRKR**TA**R** ↓ G		26 (93)	6 (29)
		PEGP**KRKR**TA**R** ↓ G		1 (4)	0 (0)
		PEVP**KRKR**AA**R** ↓ G		1 (4)	0 (0)
NA[c]	Reduces drug sensitivity	E119V	E	27 (96)	21 (100)
		E119V	V	1 (4)	0 (0)
		A246T	A	27 (96)	21 (100)
		A246T	A/V	1 (4)	0 (0)
		H274Y	H	27 (96)	21 (100)
		H274Y	Y	1 (4)	0 (0)
		R292K	R	23 (82)	21 (100)
		R292K	K	4 (14)	0 (0)
		R292K	R/K	1 (4)	0 (0)
PB2	Restores polymerase activity at 37°C	T271A	T	27 (96)	15 (100)
		T271A	A	1 (4)	0 (0)
	Enhances 627K and 701N function	K526R	K	8 (29)	1 (7)
		K526R	R	20 (71)	14 (93)
	Restores polymerase activity	M535L	M	8 (29)	1 (7)
		M535L	L	20 (71)	14 (93)
	Efficient replication in mammalian and avian cells and higher virulence in mice	A588V	A	17 (61)	13 (87)
		A588V	T	2 (7)	1 (7)
		A588V	V	9 (32)	1 (7)
	Enhances virulence in mice	Q591K	Q	27 (96)	15 (100)
		Q591K	Q/K	1 (4)	0 (0)
	Increases virulence in mammalian models	E627K	E	13 (46)	15 (100)
		E627K	K	11 (39)	0 (0)
		E627K	E/K	3 (11)	0 (0)
		E627K	E/V	1 (4)	0 (0)
		D701N	D	24 (86)	15 (100)
		D701N	N	4 (14)	0 (0)
	Host signature amino acids (avian to human)	A199S	A	27 (96)	15 (100)
		A199S	T	1 (4)	0 (0)
		T271A	T	27 (96)	15 (100)
		T271A	A	1 (4)	0 (0)
		K702R	K	25 (89)	14 (93)
		K702R	R	3 (11)	1 (7)
PB1	Increases transmission in ferrets	I368V	L	1 (4)	0 (0)
		I368V	V	27 (96)	15 (100)
PB2-F2		Length change	25 aa[d]	1 (4)	0 (0)
		Length change	34 aa	3 (11)	0 (0)
		Length change	76 aa	14 (50)	6 (40)
		Length change	87 aa	2 (7)	0 (0)
		Length change	90 aa	8 (29)	9 (60)
PA	Host signature amino acids (avian to human)	V100A	A	15 (54)	14 (93)
		V100A	V	13 (46)	1 (7)
		A404S	A	27 (96)	15 (100)
		A404S	S	1 (4)	0 (0)
		S409N	N	26 (93)	15 (100)
		S409N	S	2 (7)	0 (0)

(Continued on next page)

TABLE 1 (Continued)

Gene	Function	Mutation	Amino acid(s)	No. (%) of viruses from: Humans	Environment
NP	Host signature amino acids (avian to human)	V33I	V	27 (96)	15 (100)
		V33I	I	1 (4)	0 (0)
		I109V	I	27 (96)	15 (100)
		I109V	V	1 (4)	0 (0)
M1	Impacts growth and transmission in guinea pig model	P41A	A	27 (96)	15 (100)
		P41A	S	1 (4)	0 (0)
	Alters virulence in mice	T215A	A	28 (100)	15 (100)
	Host signature amino acids (avian to human)	V115I	V	27 (96)	15 (100)
		V115I	I	1 (4)	0 (0)
M2	Reduces susceptibility to licensed anti-influenza virus medications	S31N	N	28 (100)	15 (100)
NS1	Alters virulence in mice	P42S	S	28 (100)	15 (100)
	Alters the antiviral response in the host	N205S	S	28 (100)	15 (100)
	PDZ motif	Positions 227–230	Deletion	27 (96)	15 (100)
		Positions 227–230	KPEV	1 (4)	0 (0)
NS2	Alters the antiviral response in the host	T48A	A	28 (100)	15 (100)

[a]The H7 numbering system was used.
[b]Boldface letters indicate basic amino acids, and the downward-pointing arrows indicate the cleavage site.
[c]The N2 numbering system was used.
[d]aa, amino acids.

(Table 1), one each with E119V and H274Y. No antiviral drug resistance-related substitutions were detected in the HPAI H7N9 viruses isolated from the environment.

DISCUSSION

Following the first reported human case of HPAI H7N9 virus infection in Guangdong Province in late February 2017, HPAI H7N9 viruses were also detected in poultry in this region (6). Since then, HPAI H7N9 viruses have rapidly spread to other regions. In late March 2017, a poultry farm in Hunan Province became the first to report an HPAI H7N9 virus outbreak. Subsequently, HPAI H7N9 virus outbreaks have occurred in increasingly more regions, including Hebei, Henan, Tianjin, and Shaanxi Provinces (Fig. 1). At the beginning of June, Inner Mongolia, which is located near the north end of China, reported an HPAI H7N9 virus outbreak in a poultry farm (http://www.moa.gov.cn/). Currently, human infections with HPAI H7N9 viruses have been reported mostly in southern China (Guangdong and Guangxi Provinces). However, other provinces have also sporadically reported human cases due to the spread of HPAI H7N9 viruses.

The Yangtze River Delta region is well recognized as the original source of the H7N9 outbreaks (9, 18). Multiple introductions of LPAI H7N9 viruses from the Yangtze River Delta region to the Pearl River Delta region have been previously observed, and these have caused the establishment of an additional outbreak source (9). The molecular evolutionary analysis conducted in our study shows that the ancestor of the HPAI H7N9 virus originated in the Yangtze River Delta region and later crossed into the Pearl River Delta region. The LPAI H7N9 virus imported from the Yangtze River Delta region acquired a 4-amino-acid insertion in approximately late May 2016 in Guangdong Province. The generated HPAI H7N9 viruses have continued to reassort with LPAI H7N9 viruses (both the NA and the six internal genes) or H9N2 viruses (the six internal genes), generating multiple virus genotypes (Fig. 5). One viral genotype (genotype II) has spread to the neighboring provinces and continued northward. These genotypes are almost certainly further disseminating to wider geographic regions due to distant poultry movement along trade routes.

The amino acid insertion in the HA cleavage site, together with the G320R mutation, resulted in the presence of multiple basic amino acids in the cleavage site (PKRKRTA R↓G; Table 1; see also Table S3 in the supplemental material), which could facilitate systemic virus replication in poultry and mammals, including humans (19–21). A recent

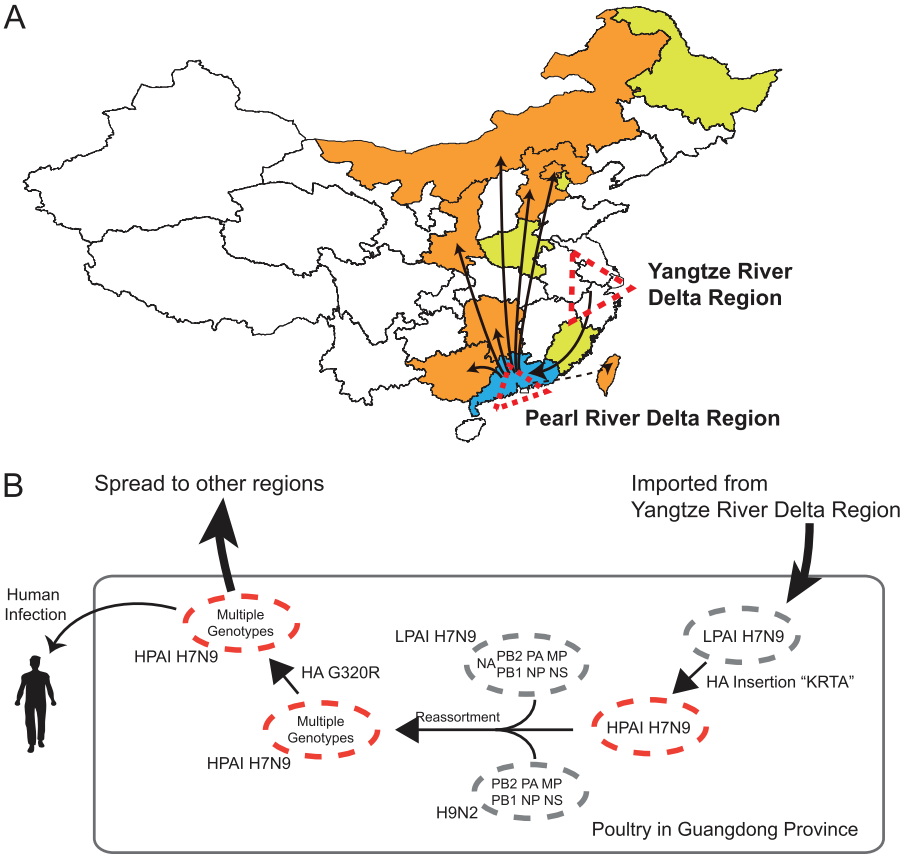

FIG 5 Geographic dissemination (A) and evolutionary pathways (B) of the HPAI H7N9 viruses. (A) The two outbreak sources of the Yangtze River Delta region and Pearl River Delta region are shown in dashed red triangles. The geographic spread of the viruses is indicated by arrows. The dashed arrow indicates the migrated H7N9 infection case. (B) The formation and dynamic reassortments of the HPAI H7N9 influenza viruses are illustrated with thin arrows in the gray rectangle. Dashed gray or red circles represent the LPAI or HPAI H7N9 viruses, respectively. The locations of human infections with HPAI H7N9 viruses are indicated with cartoon figures. Thick arrows point to the geographic spread of H7N9 viruses.

study found no significant difference between the rates of death caused by HPAI H7N9 and LPAI H7N9 viruses (22). In our study, although the difference was not significant ($P = 0.2449$), the rate of fatalities caused by HPAI H7N9 viruses (14/28, 50.0%) was higher than that caused by LPAI H7N9 viruses (592/933, 38.8%) in human infections. Furthermore, HPAI H7N9 viruses have been reported to retain dual receptor binding properties, in contrast to the HPAI H5N1 viruses, which cannot bind sialic acid α2,6 human-type receptors (7, 23). As such, the HPAI H7N9 viruses are able to infect humans more easily than the HPAI H5N1 viruses can.

All three of the first reported HPAI H7N9 viruses (100%) had an R292K mutation in the NA protein, which is known to confer resistance to multiple drugs (5, 6, 8). This has raised great concern about the clinical treatment of individuals infected with these viruses. In our study, 17.9% of HPAI H7N9 human isolates (5/28 isolates, including the isolates causing the first three cases) possessed the antiviral resistance-conferring R292K mutation, and no antiviral drug resistance mutations were detected in the environmental isolates (Table 1). These results indicate that neuraminidase inhibitor antiviral drugs could still be effective for the treatment of HPAI H7N9 virus-infected patients. Early antiviral therapy, ideally, within 48 h of symptom onset, is still highly recommended for patients with suspected or confirmed HPAI H7N9 virus infection (24).

Unlike the LPAI H7N9 viruses, the HPAI H7N9 viruses are not silently circulating in chickens. Given that the LPAI and HPAI H7N9 viruses currently cocirculate in poultry,

TABLE 2 Difference between the HA1 amino acid sequence of the virus isolates tested and that of candidate vaccine strain A/Guangdong/17sf003/2016

Virus	Amino acid at the following HA1 position[a]:																														
	11	22	37	64	82	86	112	115	120	150	151	156	163	164	165	169	177	203	205	211	214	217	218	227	261	266	267	282	292	317
A/Guangdong/17sf003/2016	S	R	N	P	E	V	P	F	I	A	A	T	K	E	S	I	V	S	V	R	V	Q	S	I	R	A	N	N	A	V
A/Taiwan/1/2017	.	.	.	.	.	.	.	.	T	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	T	.
A/Shaanxi/29412/2017	.	K	.	.	G	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Hunan/25351/2017	P	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	I	.	.	.	.	H	.	.	G	.	.	S	.	.
A/Hunan/17875/2017	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G
A/Hunan/17874/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	Y	.	G	.	.	.	.	.
A/Hunan/11026/2017	.	.	.	.	.	.	.	.	.	.	.	A	R	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Hebei/27407/2017	.	K	.	S	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Guangxi/18911/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Guangxi/18910/2017	P	.	.	.	.	I	.	.	.	.	.	.	R	.	.	.	I	.	.	.	.	.	.	.	G	.	.	S	.	.
A/Guangxi/18908/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Guangxi/18906/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	Y	.	.	.	.	.	.	G	.	.	.	.	.
A/Guangxi/18902/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	Q/L	.	.	G	.	.	.	.	.
A/Guangxi/18897/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Guangxi/18895/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Guangxi/18892/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Guangxi/18891/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	L	.	.	G	.	.	.	.	.
A/Guangxi/18888/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Guangxi/13452/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Guangxi/13450/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Guangdong/17sf064/2017	.	.	.	.	.	.	.	.	.	.	V	.	R	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	A/T	.
A/Guangdong/17sf062/2017	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	I	I/R	.	.	.	.	G	.	.	.	.	.
A/Guangdong/17sf059/2017	.	.	.	.	.	.	.	.	.	.	.	.	R	K	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Guangdong/17sf039/2017	P	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	I	.	.	.	.	.	.	.	G	.	.	S	.	.
A/Guangdong/17sf037/2017	.	.	.	.	.	.	.	.	.	T	.	.	R	K	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Guangdong/17sf033/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	.	.	G	.	S/N	.	.	.
A/Guangdong/17sf032/2017	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A/Guangdong/17sf006/2017	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Environment/Shaanxi/29415/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Environment/Shaanxi/29414/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Environment/Shaanxi/29413/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Environment/Inner Mongolia/28651/2017	.	K	.	.	.	A	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Environment/Hebei/25346/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Environment/Guangdong/Kgz4075/2016	.	.	.	.	.	.	S	L	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Environment/Guangdong/Kgz4068/2016	.	.	.	.	.	.	S	L	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Environment/Guangdong/Kgz4048/2016	.	.	.	.	.	.	S	L	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Environment/Guangdong/C16283277/2016	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Environment/Guangdong/C16283222/2016	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Environment/Guangdong/C16283219/2016	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
A/Environment/Guangdong/C162811110/2016	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	L	G	.	.	.	.	.
A/Environment/Guangdong/16609/2017	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	T	.
A/Environment/Guangdong/07128/2017	.	.	.	.	.	.	.	.	.	.	.	.	R	.	N	.	.	.	.	.	I	.	.	.	G	.	.	.	.	.
A/Environment/Guangdong/02741/2016	.	.	S	.	.	.	S	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.
A/Environment/Guangdong/02739/2016	.	.	S	.	.	.	S	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.
A/Environment/Guangdong/02738/2016	.	.	S	.	.	.	S	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.
A/Environment/Guangdong/02737/2016	.	.	S	.	.	.	S	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.
A/Environment/Guangdong/02736/2016	.	.	S	.	.	.	S	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.
A/Environment/Guangdong/02735/2016	.	.	S	.	.	.	S	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.
A/Environment/Beijing/22166/2017	.	K	.	.	.	.	.	.	.	.	.	.	R	.	.	V	.	.	.	.	.	.	.	.	G	.	.	.	.	.

[a]The H7 numbering system was used. Periods indicate the same amino acid as in A/Guangdong/17sf003/2016, and sites with mixtures are separated by a slash.

the situation will likely be more serious and complicated now than it was before. Previously, the asymptomatic circulation of LPAI H7N9 viruses in poultry increased the difficulty of monitoring virus spread, which led to the risk that endemic LPAI H7N9 viruses in poultry could facilitate the spillover of the virus into humans and cause human infections and deaths. In contrast, the HPAI H7N9 virus is easier to monitor, but it has already had a heavy impact on Chinese poultry production and has also caused human infections and deaths. Both the LPAI and HPAI H7N9 viruses could spread to the majority of regions in China via poultry trade and movements. Notably, some avian influenza viruses, such as the HPAI H5N1 and H5N8 viruses, are able to transmit from domestic poultry to wild waterfowl; as a consequence, bird migration led to the subsequent intercontinental spread of those viruses. Thus, the possibility that the HPAI H7N9 viruses may also transmit to other regions by bird migration pathways cannot be excluded.

Vaccination is the most effective measure for infectious disease control and prevention. Coordination of the development of candidate influenza vaccine viruses (CVVs) by the World Health Organization (WHO) is an essential component in the overall global strategy for influenza pandemic preparedness. WHO proposed an H7N9 vaccine candidate [an A/Anhui/1/2013(H7N9)-like virus] soon after the human infections with H7N9 virus were first reported; however, the H7N9 viruses continue to evolve both genetically and antigenically, necessitating the development of new CVVs. Two additional vaccine candidates were later proposed by WHO for H7N9 vaccine development [A/Hunan/2560/2016(H7N9)-like and A/Guangdong/17SF003/2016(H7N9)-like viruses] in March 2017 (http://apps.who.int/iris/bitstream/10665/258733/1/WER9233-460-475.pdf?ua = 1). All of the HPAI H7N9 viruses analyzed in this study are genetically similar to the vaccine virus A/Guangdong/17SF003/2016(H7N9). However, several amino acid differences were observed in the HA1 proteins, including R22K, K163R, I169V, and R261G (Table 2). Whether or not these differences affect virus antigenicity urgently needs to be studied to improve preparedness for HPAI H7N9 virus infections.

In summary, H7N9 viruses present characteristics different from those of other zoonotic influenza viruses, such as H5N1, and they pose greater risks not only for humans but also for poultry. Recently, three amino acid mutations in HA (V186N/K, K193T, G228S) have been reported to confer a switch in specificity for human-type receptors and may aid in the efficient transmission of influenza A virus in mammals (25). Although none of these three mutations (V186N/K, K193T, G228S) was detected in the HPAI H7N9 viruses examined in this study (Table 1), viruses should be closely monitored for these mutations. Besides, effective control measures, such as the permanent closure of live poultry markets and central slaughtering, the prevention of interregional poultry movements, and the implementation of powerful active systematic surveillance in poultry and humans, must be enacted immediately and strictly.

MATERIALS AND METHODS

Sample collection and transportation. Respiratory specimens from patients with suspected H7N9 infection and environmental samples from live poultry markets or farms (Table 3) were collected by local centers for disease control and prevention according to national influenza surveillance guidelines. All samples were collected in individual vials, placed in transport medium with antibiotics, and packed on ice before being sent to the laboratory for further processing. Reverse transcription-PCRs (RT-PCRs) for detecting subtypes H5, H7, and H9 were conducted using the original samples (26). H7N9-positive samples were inoculated into 9- to 11-day-old, specific-pathogen-free, embryonated chicken eggs for 48 to 72 h at 37°C for virus isolation by the Chinese National Influenza Center (CNIC) and the Guangdong Province Center for Disease Control and Prevention. Whole-genome sequencing of H7N9-positive samples or isolated viruses was performed by CNIC. HPAI H7N9 viruses were identified on the basis of these sequencing results and then further analyzed in this study.

Virus sequencing. RNA was extracted using an RNeasy minikit (Qiagen, Hilden, Germany) and used to resubtype the viruses with the following sequencing procedures. The extracted RNA was subjected to reverse transcription and amplification using a SuperScript III One-Step RT-PCR system (Thermo Fisher, Waltham, MA, USA) as previously described (27). Whole-genome sequencing of influenza A virus (28) was implemented on a MiSeq high-throughput sequencing platform (Illumina, Inc., San Diego, CA, USA) with

TABLE 3 Information on environmental samples used in this study

Site	Location	No. of samples	Sample collection date (yr-mo-day)	Virus(es)
Live poultry market 1	Guangdong	40	2016-12-26	A/Environment/Guangdong/02739/2016, A/Environment/Guangdong/02738/2016, A/Environment/Guangdong/02737/2016, A/Environment/Guangdong/02736/2016, A/Environment/Guangdong/02741/2016, A/Environment/Guangdong/02735/2016
Live poultry market 2	Guangdong	18	2017-01-09	A/Environment/Guangdong/07128/2017
Live poultry market 3	Guangdong	40	2017-02-22	A/Environment/Guangdong/16609/2017
Live poultry market 4	Beijing	27	2017-04-06	A/Environment/Beijing/22166/2017
Live poultry market 5	Shaanxi	50	2017-05-05	A/Environment/Shaanxi/29415/2017 A/Environment/Shaanxi/29414/2017
Live poultry market 6	Shaanxi	6	2017-05-30	A/Environment/Shaanxi/29413/2017
Farm 1	Hebei	15	2017-04-26	A/Environment/Hebei/25346/2017
Farm 2	Inner Mongolia	20	2017-06-02	A/Environment/Inner Mongolia/28651/2017

a paired read length of 150 bp. Data analysis was predominantly conducted using CLC Genomics Workbench (v7.5.1) software. Low-quality reads were trimmed using the CLC trimmer, with the quality limit being set at 0.05. The filtered reads were *de novo* assembled using the CLC program under default parameters. Contigs with coverage of over 10 were extracted and analyzed by BLAST analysis against the sequences in a database containing all influenza A virus nucleotide sequences collected from the National Center for Biotechnology Information (NCBI) and the Global Initiative on Sharing All Influenza Data (GISAID) databases. Sequences with the highest similarity were selected as references for read mapping under the parameters of a length fraction of 0.8 and a similarity fraction of 0.8. The influenza A virus genome sequences were obtained by extracting from the mapping results the consensus sequences, which were those with a coverage depth of at least 100 times at each site on the eight segments.

Evolutionary analysis. The full-genome sequences of HPAI H7N9 viruses from 25 human and 14 environmental samples were obtained in this study. Phylogenetic analyses were conducted using sequences available from NCBI and GISAID (see Table S2 in the supplemental material).

To estimate the time to the most recent common ancestor (tMRCA) of the HA and NA genes of the HPAI H7N9 viruses, nonredundant sub-data sets were selected to run a time-measured Bayesian Markov chain Monte Carlo (MCMC) analysis by the use of BEAST (v1.84) software (29). The SRD06 substitution model (30) and the uncorrelated relaxed molecular clock model were used, and the Bayesian SkyGrid coalescent was set as the tree prior. The Bayesian MCMC was run twice for up to 5×10^7 steps with samples for each 5,000 steps to achieve convergence. The Tracer (v1.6) program was used to examine effective sample size (ESS) values greater than 200. The maximum clade credibility tree with median node heights was constructed after a burn-in of the beginning states. For the six internal genes, neighbor-joining (NJ) trees were constructed by use of the MEGA (v7.0) program with the Kimura 2-parameter model and 1,000 bootstraps.

Accession number(s). Newly determined sequences were deposited in GISAID under accession numbers EPI1013162 to EPI1013273, EPI1018081 to EPI1018176, EPI1022602 to EPI1022697, and EPI1022701 to EPI1022708. See also Table S1 in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JVI.01277-17.

SUPPLEMENTAL FILE 1, PDF file, 1.0 MB.

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