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RESEARCH ARTICLE

Epidemiology and Genotypic Diversity of Eurasian Avian-Like H1N1 Swine Influenza Viruses in China

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Abstract

Eurasian avian-like H1N1 (EA H1N1) swine influenza virus (SIV) outside European countries was first detected in Hong Kong Special Administrative Region (Hong Kong, SAR) of China in 2001. Afterwards, EA H1N1 SIVs have become predominant in pig population in this country. However, the epidemiology and genotypic diversity of EA H1N1 SIVs in China are still unknown. Here, we collected the EA H1N1 SIVs sequences from China between 2001 and 2018 and analyzed the epidemic and phylogenic features, and key molecular markers of these EA H1N1 SIVs. Our results showed that EA H1N1 SIVs distributed in nineteen provinces/municipalities of China. After a long-time evolution and transmission, EA H1N1 SIVs were continuously reassorted with other co-circulated influenza viruses, including 2009 pandemic H1N1 (A(H1N1)pdm09), and triple reassortment H1N2 (TR H1N2) influenza viruses, generated 11 genotypes. Genotype 3 and 5, both of which were the reassortments among EA H1N1, A(H1N1)pdm09 and TR H1N2 viruses with different origins of *M* genes, have become predominant in pig population. Furthermore, key molecular signatures were identified in EA H1N1 SIVs. Our study has drawn a genotypic diversity image of EA H1N1 viruses, and could help to evaluate the potential risk of EA H1N1 for pandemic preparedness and response.

Keywords Eurasian avian-like H1N1 (EA H1N1) swine influenza viruses (SIV) · Epidemiology · Genotypes · Molecular markers

Introduction

Pigs are considered as "mixing vessel" of human and avian influenza viruses, since their respiratory tract contained both human-type and avian-type receptors (Ito *et al.* 1997; Suzuki *et al.* 1997). When infecting more than one co-circulating viruses, reassortments occurred and novel reassortant viruses were generated (Scholtissek *et al.* 1985). Currently, several lineage of swine influenza viruses (SIVs) were circulating in pigs globally, including classical swine influenza viruses (CS), Eurasian avian-like influenza viruses (EA), triple reassortment influenza viruses (TR), 2009 pandemic H1N1 viruses (A(H1N1)pdm09) and several reassorted SIVs (Chen *et al.* 2013; Smith *et al.* 2009; Yang *et al.* 2016).

The EA SIVs, with all eight genes from avian influenza virus gene pool, were firstly recognized in 1979 in Europe (Pensaert *et al.* 1981; Scholtissek *et al.* 1983). Since 1979, EA SIVs appeared to have a selective advantage over CS H1N1 and had replaced the CS SIVs in European swine populations (Campitelli *et al.* 1997). In China, CS H1N1

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viruses were the predominant viruses in pigs before 2002. Since 2002, the prevalence of EA H1N1 SIVs were continuously increasing (Vijaykrishna *et al.* 2011). At present, EA H1N1 SIVs were predominant in pigs in China. Reassorments among EA SIVs and other co-circulated SIVs were continuously occurred and various genotypes were generated. Recent studies reported that the EA H1N1 SIVs circulated in pigs in China can bind to human-type receptors and most of them can transmitted via respiratory droplets among ferrets model (Yang *et al.* 2016). In addition, the majority of human population had low immunity to EA H1N1 viruses (Vijaykrishna *et al.* 2011). Actually, human infections with EA H1N1 SIVs have been reported in Europe and Asia (Gregory *et al.* 2003; Yang *et al.* 2012). In Chinese mainland, different genotypes EA H1N1 SIVs have been reported to infect humans (Wang *et al.* 2013; Xie *et al.* 2018; Yang *et al.* 2012; Zhu *et al.* 2016, 2019). Thus, the EA H1N1 SIVs were proposed as one of the most potential zoonotic influenza viruses to cause the next pandemic (Yang *et al.* 2016).

Previous studies on SIVs in China revealed that after a long-time evolution and transmission, the reassortant EA SIVs were dominant and the number of reassortant viruses was increasing during 2007–2009 (Vijaykrishna *et al.* 2011). In 2010, two novel double reassortants EA H1N1 SIVs were isolated (Sun *et al.* 2016). The EA H1N1 SIVs variants were reassorted with A(H1N1)pdm09 or TR H1N2 viruses, which were transmitted efficiently from pig to pig and from pig to ferret (Zhu *et al.* 2011). The surveillance in pigs from 2010 to 2013 in China isolated 139 EA H1N1 SIVs from ten provinces, which were divided into 5 genotypes (Yang *et al.* 2016). Up to now, no reports have updated the epidemiology and genotypic diversity of EA SIVs in pigs in China. Thus, we performed a systematic analysis on the epidemic characteristics, the variety and prevalence of genotypes and the key molecular markers of EA SIVs in China.

Materials and Methods

Surveillance Data and Genomic Sequences

The genomic sequences of Eurasian avian-like swine influenza A virus isolated in China were searched from the public database GenBank and Global Initiative on Sharing All Influenza Data (GISAID) databases from 2001 to 2018, including 293 *HA*, 274 *NA*, 271 *PB2*, 273 *PB1*, 269 *PA*, 272 *NP*, 276 *M*, 272 *NS*. A total of 266 full genomic sequences of EA SIVs were downloaded (Supplementary Table S1). Spatial and temporal data of these 266 EA SIVs were collected and analyzed.

Phylogenetic Analysis

All available EA H1N1 SIVs genomic sequences were aligned with MAFFT v7.222 (Kazutaka and Standley 2013). Phylogenetic trees were generated by applying maximum likelihood (ML) method with general time-reversible (GTR) model. The robustness of ML topology was determined by 1000 bootstrap replicates.

Genotypic Diversity

To identify the genotypic diversity of EA H1N1 SIVs, genotypes were defined based on the clade distributions of their internal gene segments. From the ML phylogenies, each gene segment was assigned to the specific lineages to generate a genotype.

Genotypes Definition

According to the ML phylogenies, each gene segment was categorized into lineages circulating in swine in China, as follows: EA H1N1, A(H1N1)pdm09, CS H1N1, and TR H1N2. Then the eight gene segments of each EA H1N1 virus could be derived from different lineages. The gene combination of distinct lineages was classified as a specific genotype.

Results

The Description Analysis of EA SIVs Sequences in China during 2001–2018

In all 293 *HA* sequences, 93.9% (275) of EA SIVs were EA H1N1 SIVs and 6.1% (18) were EA H1N2 SIVs. From 2001 to 2018, 275 EA H1N1 SIVs sequences were collected from nineteen provinces/municipalities of China (Fig. 1A). Hong Kong contributed the most EA H1N1 SIVs sequences, followed by Guangdong and Shandong provinces. The first EA H1N1 sequence was reported in Hong Kong in early 2001. Subsequently, more EA H1N1 SIVs sequences were reported and peaked during 2009–2011 (Fig. 1B). Human cases infected with EA H1N1 SIVs were reported from Jiangsu, Hebei, Hunan, Yunnan, and Fujian provinces (Table 1). These results indicated that the EA H1N1 SIVs were currently prevalent in pigs and sporadically cause human infections in China.

Phylogenetic Analysis of EA H1N1 SIVs

To determine the evolution of EA H1N1 SIVs in China during 2001–2018, phylogenetic analyses of all eight gene

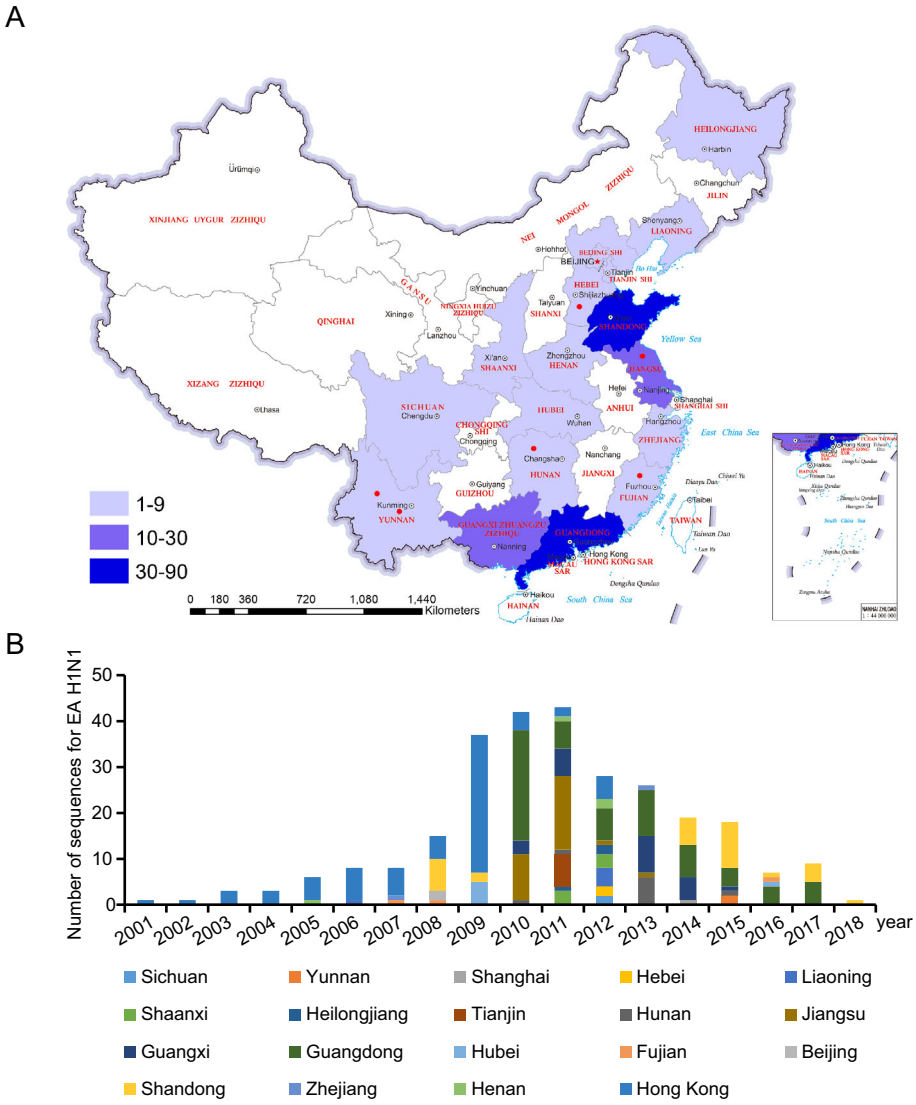
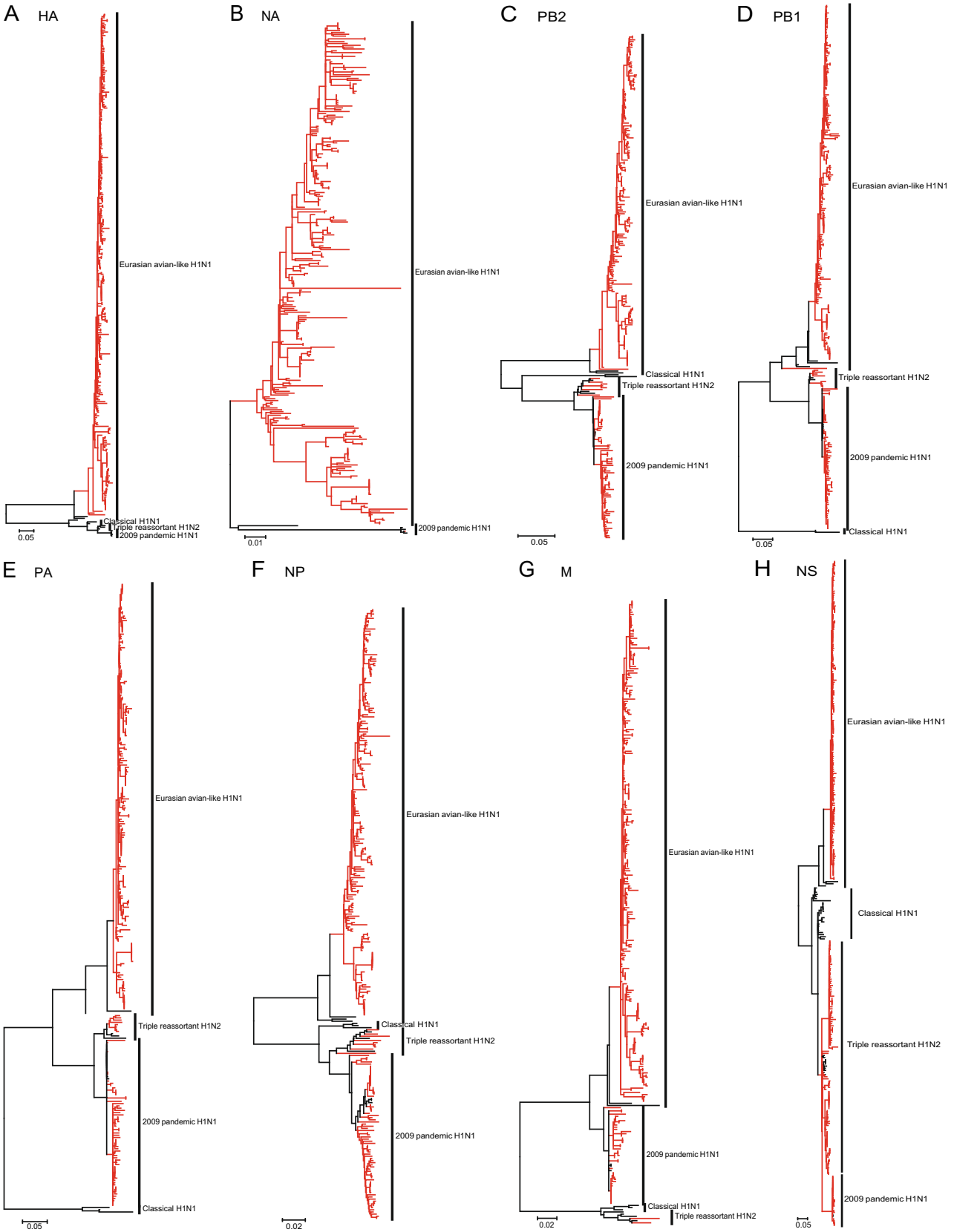


Fig. 1 The description analysis of EA H1N1 SIVs sequences in China during 2001–2018. **A** Geographic distributions of EA H1N1 SIVs sequences in China. EA H1N1 SIVs affected regions are highlighted in blue. Blue from light to dark indicated the virus EA H1N1 SIVs number increased from 1 to 90. The red circles indicated human cases of infection with EA H1N1 SIVs. **B** Temporal distribution of EA H1N1 SIVs sequences in swine in China during 2001–2018.

Table 1 Human cases infected with EA H1N1 SIVs in China.

Year	Province	Strain name	Genotype	References
2011	Jiangsu	A/Jiangsu/1/2011	1	Yang *et al.* (2012)
2012	Hebei	A/Hebei-Yuhua/SWL1250/2012	1	Wang *et al.* (2013)
2015	Hunan	A/Hunan/42443/2015	3	Zhu *et al.* (2016)
2015	Yunnan	A/Yunnan-Longyang/SWL1982/2015	3	Zhu *et al.* (2019)
2015	Yunnan	A/Yunnan-Wuhua/SWL1869/2015	3	Zhu *et al.* (2019)
2016	Fujian	A/Fujian-Cangshan/SWL624/2016	5	Xie *et al.* (2018)

segments were performed. Phylogenetic tree of hemagglutinin (*HA*) showed that the EA H1N1 SIVs isolated from pigs in China formed a monophyletic group (Fig. 2A, red branches). We used the swine H1 clade classification tool to classify the clade of *HA* (Anderson *et al.* 2016) and found that all the *HA* sequences of EA H1N1 SIVs were classified as 1C.2.3. In contrast, the other genes of EA H1N1 SIVs, including the neuraminidase (*NA*), basic polymerase 2 (*PB2*), basic polymerase 1 (*PB1*), polymerase (*PA*), nucleoprotein (*NP*), matrix (*M*), and nonstructural protein (*NS*), were demonstrated distinct diversity (Fig. 2B–2H). The *NA* genes were derived from EA H1N1 and A(H1N1)pdm09. Origins of the *PB2*, *PB1*, *PA*, *NP* and *M* genes included EA H1N1, A(H1N1)pdm09 and TR H1N2. The *NS* genes were derived from EA H1N1, A(H1N1)pdm09, and TR H1N2 respectively. These results


A HA
Eurasian avian-like H1N1
Classical H1N1
Triple reassortant H1N2
2009 pandemic H1N1
0.05
B NA
Eurasian avian-like H1N1
2009 pandemic H1N1
0.01
C PB2
Eurasian avian-like H1N1
Classical H1N1
Triple reassortant H1N2
2009 pandemic H1N1
0.05
D PB1
Eurasian avian-like H1N1
Triple reassortant H1N2
2009 pandemic H1N1
Classical H1N1
0.05
E PA
Eurasian avian-like H1N1
Triple reassortant H1N2
2009 pandemic H1N1
Classical H1N1
0.05
F NP
Eurasian avian-like H1N1
Classical H1N1
Triple reassortant H1N2
2009 pandemic H1N1
0.02
G M
Eurasian avian-like H1N1
2009 pandemic H1N1
Classical H1N1
Triple reassortant H1N2
0.02
H NS
Eurasian avian-like H1N1
Classical H1N1
Triple reassortant H1N2
2009 pandemic H1N1
0.05

◀**Fig. 2** Phylogenies of *HA* (**A**), *NA* (**B**), *PB2* (**C**), *PB1* (**D**), *PA* (**E**), *NP* (**F**), *M* (**G**), and *NS* (**H**) of EA H1N1 SIVs from 2001 to 2018. The phylogenetic analysis was performed by MEGA 7.0 with maximum likelihood (ML) method. The bootstrap was 1000. The red indicated the sequences of EA H1N1 SIVs. The black indicated the reference sequences.

indicated that dynamic reassortments occurred between the EA H1N1 and co-circulated SIVs in pigs.

Genotypic Diversity of EA H1N1 SIVs

Reassortant genotypes of the EA H1N1 SIVs could then be defined based on the clade distributions of their internal gene segments. The EA H1N1 SIVs isolated from both pigs and humans were classified into 11 distinct genotypes, from genotype 1 to genotype 11. Genotype 1 viruses accounted for 55.3% of the EA H1N1 SIVs. And the eight gene segments of this genotype viruses were exclusively originated from EA H1N1 (Fig. 3). EA H1N1 and TR H1N2 viruses produced double reassortment viruses and generated genotypes 2, 6, and 10 (Fig. 3). The introductions of the A(H1N1)pdm09 into pigs continuously provided their internal genes to EA H1N1 SIVs and generated novel genotypes 4, 7, and 8 (Fig. 3). Reassortments of EA H1N1, A(H1N1)pdm09 and TR H1N2 generated triple reassortment viruses and formed genotypes 3, 5, 9, and 11 (Fig. 3). These results indicated that the EA H1N1 SIVs circulated in China exhibited a high genotypic diversity.

Genotype 1 was widely prevalent from 2001 to 2013 (Fig. 4A). From 2009 to 2013, the genotypes 2 and 4 were co-circulated with genotype 1 (Fig. 4A). Since 2013, genotypes 3 and 5 have gradually replaced genotypes 1, 2, and 4 in swine populations (Fig. 4A). Guangdong Province had the largest number of genotypes, with a total of 7 genotypes (Fig. 4B). In addition, Hong Kong and Guangxi ranked second and third, respectively (Fig. 4B). This result indicated that genotype 3 and 5 have become predominant in pig population.

Molecular Characteristics of EA H1N1 SIVs

We next analyzed the molecular characteristics which were associated with mammalian adaptations, receptor binding ability, virulence or transmission and antiviral resistance of all EA H1N1 SIVs (Table 2).

The influenza virus *HA* gene was a major factor that determines the receptor binding property and the host range. It was well known that E190D and G225E could increase the receptor-binding affinity to human type α-2,6-linked sialic acid receptors (Tumpey *et al.* 2007). And majority EA H1N1 SIVs contained HA-190D (85.9%) and 225E (84.1%) mutations. Studies have shown that adaptive mutations of influenza A viruses were identified mostly in viral polymerase complexes (Chen *et al.* 2007). Mutations PB2-E627K and D701N not only enhanced the viral polymerase activity but also increased the virulence of H7N9 and H5N1 avian influenza viruses in mammals (Chen *et al.* 2007; Steel *et al.* 2009; Zhu *et al.* 2015). The substitution PB2-D701N could also enhance viral replication and pathogenicity of EA H1N1 viruses in mice (Liu *et al.* 2018). 70.0% of the EA H1N1 SIVs posed N at position 701 in the PB2 protein. Whereas, 98.5% of all EA H1N1 SIVs at 627 site were E. Another PB2-Q591K mutation was shown to enhance polymerase activity, replication and virulence in mice in H5N1 influenza virus (Yamada *et al.* 2010). The percentage of 591K residue in

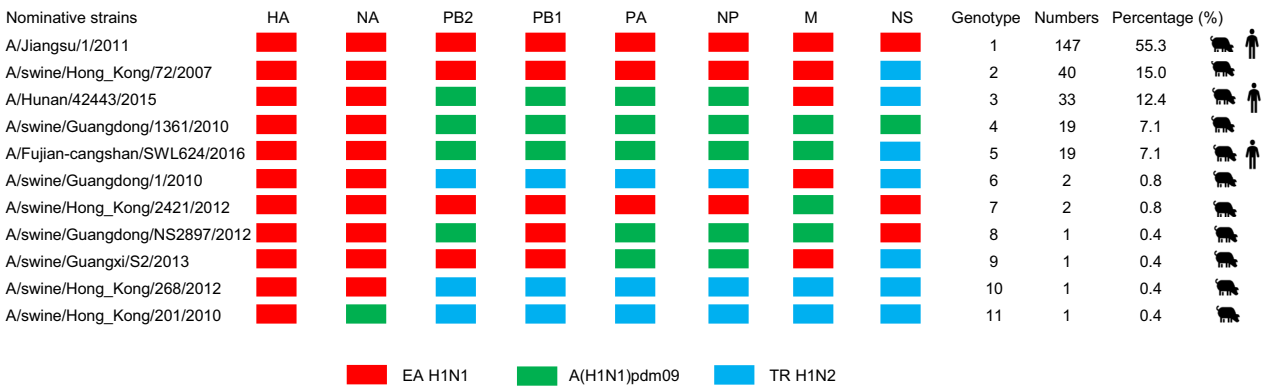


Fig. 3 Genotypes of EA H1N1 SIVs identified in China. The name of representative viruses are showed (left). All the eight gene segments of EA H1N1 SIVs are at the top of the graph. Origin of each gene segment is indicated by a colored block for representing the different swine influenza virus lineages. The 11 distinct genetic constellations are labeled as genotype 1 to 11. EA H1N1, Eurasian avian-like H1N1; A(H1N1)pdm09, 2009 pandemic H1N1; TR H1N2, triple reassortment H1N2.

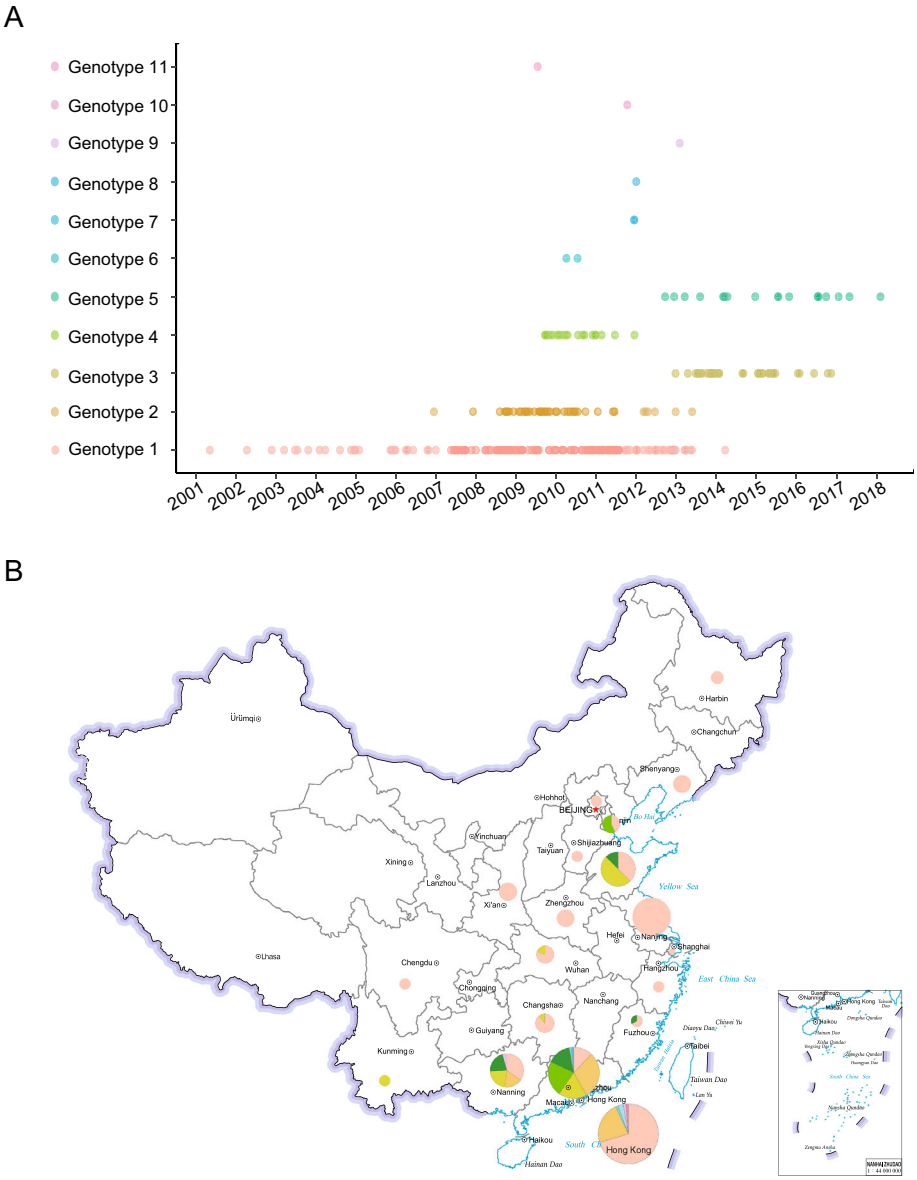


Fig. 4 Development and prevalence of EA H1N1 genotypes during 2001–2018. **A** 11 distinct genotypes are listed on the left. The colored circles represent the corresponding genotypes and the isolated time. **B** The distribution of EA H1N1 genotypes in each province. The size of the circle indicated the number of EA H1N1 SIVs. The different colors represent the different genotypes which refer to **A**.

EA H1N1 SIVs was 0.4%. In addition to key adaptive signatures in PB2 protein, several other mutations in PB1, PA and NP were implicated in enhanced viral polymerase activity including PB1-L473V (Xu *et al.* 2011), PA-K356R (Xu *et al.* 2016), NP-Q357K (Zhu *et al.* 2019) and so on. Theses mutations in EA H1N1 SIVs were PB1-L473V (99.6%), PA-K356R (25.7%), and NP-Q357K (31.5%), respectively. Furthermore, it was also reported that M1-P41A mutation in EA H1N1 SIVs increased the transmission in guinea pigs (Campbell *et al.* 2014). We found that 98.6% was A at position 41 in M1 protein in EA H1N1 SIVs. In our study, we analyzed the percentage of these mutations in all eight gene segments of EA H1N1 SIVs. We found that some molecular markers were already widespread in EA H1N1 SIVs, such as PB1-L473V (99.6%), and M1-P41A (98.6%). There were still some molecular markers including PB2-E627K (0.4%) and PB2-K526R (5.6%) that account for a very small proportion in EA H1N1 SIVs. Therefore, continuous surveillance needs to be implemented.

Antiviral drugs M2 and neur-aminidase inhibitors played important role in influenza treatment (Babu *et al.* 2001; Hay *et al.* 1985). It was known that M2-S31 N mutation was resistant to amantadine and rimantadine by changing in the transmembrane channel domain (Shiraishi *et al.* 2003). We found that 98.9% of EA H1N1 SIVs posed 31N in the M2 protein. It suggested that the EA H1N1 SIVs were resistant to amantadine and rimantadine widely. H274Y and N294S mutations caused resistance to neur-aminidase inhibitors (Ives *et al.* 2002). However, these substitutions were not observed in all EA H1N1 SIVs,

Table 2 Prevalence of key molecular markers in EA H1N1 SIVs in China.

Gene	Phenotypic characteristic(s)	Mutation	Percentage	
HA[a]	Altered the receptor specificity	E190D	E (0%)	D (85.9%)
		G225E	G (12.7%)	E (84.1%)
NA[b]	Resistance to NA inhibitors	H274Y	H (100%)	Y (0%)
		N294S	N (100%)	S (0%)
PB2	Altered the virulence in mice	D9N	D (98.1%)	N (1.1%)
		L89V	L (0.4%)	V (98.9%)
		E158G	E (100%)	G (0%)
	Mammalian host adaption	D256G	D (100%)	G (0%)
	Enhance viral polymerase activity	T271A	T (68%)	A (30.8%)
	Enhance the 627 k and 701 N function	K526R	K (94.4%)	R (5.6%)
	Restored the polymerase activity	M535L	M (100%)	L (0%)
	Enhance the viral polymerase activity,	Q591K	Q (66.2%)	K (0.4%)
	increase the virulence in mice	E627K	E (98.5%)	K (0.4%)
		D701N	D (30%)	N (70%)
	Altered the virulence in mice	A676T	A (2.2%)	T (96%)
PB1	Enhance the viral polymerase activity	L473V	L (0.4%)	V (99.6%)
	Altered the virulence in mice	R198K	R (0.8%)	K (99.2%)
PA	Enhance the viral polymerase activity	L336M	L (72.9%)	M (26.8%)
	Enhance the viral polymerase activity, increase the virulence in mice	K356R	K (74.4%)	R (25.7%)
	Species-associated signature positions	S409N	S (17.5%)	N (81.0%)
NP	Enhance the viral polymerase activity	A150R	A (0%)	R (98.9%)
	Enhance the viral polymerase activity,	N319K	N (85.6%)	K (14.4%)
	altered the virulence in mice	Q357K	Q (66.7%)	K (31.5%)
M1	Increase the transmission in guinea pigs	P41A	P (0%)	A (98.6%)
	Altered the virulence in mice	T215A	T (0%)	A (100%)
M2	Resistance to adamantine derivatives	S31N	S (0.7%)	N (98.9%)
NS1	Altered the virulence in mice	D92E	D (97.1%)	E (1.5%)
	Altered the antiviral response in host	N205S	N (10.2%)	S (54.2%)
		G210R	G (12.7%)	R (56.7%)

[a]The H3 numbering system was used.

[b]The N2 numbering system was used.

which suggested the neur-aminidase inhibitors could be used for human infections with EA H1N1 viruses.

Discussion

The EA H1N1 SIV outside European countries was detected in Hong Kong in 2001. Currently, EA H1N1, CS H1N1, TR H1N2 and A(H1N1)pdm09 influenza viruses co-circulated in pigs in China (Chen *et al.* 2013). The co-circulation of these lineage viruses in pigs resulted in an increased number of novel reassortment viruses. A total of 11 different genotypes were identified. Genotype 1 viruses have all of their eight gene of avian-origin, which were widely prevalent from 2001 to 2013. Higher genetic diversity was detected between 2009 and 2014, with all genotypes were identified. This might have been caused by strengthened surveillance of SIVs since A(H1N1)pdm09 virus pandemic. During this period, genotype 1, 2, and 4 were prevalent in China, nevertheless, genotype 7–11 were detected only one time. Since 2013, genotype 3 and 5 have become increasingly prevalent and have a selective advantage over original EA H1N1 viruses. Although Hong Kong contributed the largest number of EA H1N1 viruses and had the second largest number of genotypes, most of the swine slaughtered in Hong Kong come from provinces in Chinese mainland (Vijaykrishna *et al.* 2011). This indicates most of swine influenza viruses detected in Hong Kong were imported from Chinese mainland.

The prevalence of EA SIVs in pigs could cause the human infections. In China, human infections with EA H1N1 SIVs were reported occasionally. The first human

infection with EA H1N1 SIVs was identified in Jiangsu Province in 2011 (Yang *et al.* 2012). Thereafter, another 3-year-old boy was identified as EA H1N1 SIV case in Hebei Province in 2012 (Wang *et al.* 2013), which belonged to genotype 1. In 2015, one and two human cases with EA H1N1 SIVs infections were reported in Hunan and Yunnan provinces, respectively (Zhu *et al.* 2016, 2019), which were classified as genotype 3. Fujian Province reported its first EA H1N1 human case in 2016 (Xie *et al.* 2018), which was listed as genotype 5. These were consistent with the prevalence of EA H1N1 SIVs circulating in pig populations during the same period.

The available full genome sequences may have some bias. Hong Kong contributed the largest number of EA H1N1 sequences in the public database. It might be due to systematic surveillance since 1998 in Hong Kong (Vijaykrishna *et al.* 2011). Since 2007, swine influenza virus surveillance began to been implemented in Guangdong, Guangxi, Shandong and other provinces in Northern China (He *et al.* 2018; Liu *et al.* 2009; Sun *et al.* 2016; Yang *et al.* 2016; Zhu *et al.* 2011). Therefore, although all EA H1N1 sequences from the public database have exclusively been included for analysis, they might not cover all of the evolutionary image of the viruses.

In our study, some important molecular signatures were analyzed. The specific amino acid mutations of HA could switch the receptor preference from avian-type receptors to human-type receptors. It was noted that majority of all EA H1N1 SIVs posed 190D (85.9%) and 225E (84.1%), which were preferentially bind to the human-type receptors and caused human infections. Some adaptive mutations including PB2-T271A, PB2-Q591K, PB2-E627K, and PB2-D701 N were able to enhance viral polymerase activity and further facilitated pathogenicity in mice (Bussey *et al.* 2010; Yamada *et al.* 2010; Zhu *et al.* 2015). The EA H1N1 viruses beard 271A (30.8%), 591K (0.4%), and 701N (70.0%) in the PB2 protein, respectively. Through the analysis of antiviral resistance molecular characteristics, we found that EA H1N1 SIVs were resistant to amantadine and rimantadine. However, they were sensitive to neur-aminidase inhibitors. Hence, we should pay more attention to these molecular signatures and identify their effects on pathogenicity, transmission and antiviral resistance of EA H1N1 SIVs.

Taken together, our finding suggested that dynamic reassortments among EA H1N1 SIVs and other swine influenza viruses were continuously occurring in pigs. Occasionally, it has caused human infections, therefore, we should strengthen the monitoring of EA H1N1 SIVs.

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Author Contributions YS, DW, and WZ designed the study; YS, WZ, ZF, LY, JL, and LZ analyzed the data and discussed the results; ZF wrote the manuscript; YS and WZ finalized the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declared that they have no conflicts of interest.

Animal and Human Rights Statement This article does not contain any studies with human or animal subjects performed by any of the authors.

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