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Isolation and genetic analysis of human origin H1N1 and H3N2 influenza viruses from pigs in China

Hai Yu [a,1], Gui-Hong Zhang [b,1], Rong-Hong Hua [a], Qiang Zhang [a], Tian-Qiang Liu [a], Ming Liao [b,*], Guang-Zhi Tong [a,*]

[a] *National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, No. 427 Maduan Street, Harbin 150001, PR China*
[b] *College of Veterinary Medicine, Southern China Agricultural University, Guangzhou 510642, PR China*

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Abstract

Influenza A viruses of subtypes H1N1 and H3N2 have been reported widely in pigs, associated with clinical disease. These mainly include classical swine H1N1, avian-like H1N1, and human-like or avian-like H3N2 viruses. From 2005 to 2006, we carried out swine influenza virus surveillance in eight provinces of China. Here we report, for the first time, the isolation and genetic analysis of a human-like influenza H1N1 virus from a pig in a farm of Guangdong province of southern China, a host suspected to generate new pandemic strains through genetic reassortment. Each of the eight gene segments is of human origin. Phylogenetic analysis indicates that these genes form a human lineage, suggesting that this virus is the descendant of recent human H1N1 influenza viruses. In addition, four swine H3N2 viruses were also isolated. The three H3N2 viruses from Guangdong province are descendants of recent human viruses, while an H3N2 virus from Heilongjiang province derives from early human viruses. Isolation and genetic analysis of human H1N1 and H3N2 influenza viruses from pigs in China provides further evidence about the interspecies transmission of human influenza viruses to pigs and emphasizes the importance of reinforcing swine influenza virus surveillance.

Keywords: Swine influenza; Human H1N1 influenza virus; Human H3N2 influenza virus; Genetic analysis

Swine influenza is an acute respiratory disease caused by influenza A virus within the Orthomyxoviridae family. The primary clinical manifestations of viral infection are fever and acute respiratory distress. Currently, three main subtypes of influenza viruses are circulating in the swine population throughout the world: H1N1, H3N2, and H1N2 [1,2].

China, especially southern China, is regarded as an epicenter of pandemic influenza viruses throughout history [3]. The tracheal epithelium in pigs expresses the receptors for avian influenza viruses and human influenza viruses, suggesting the pigs as an intermediate host of interspecies transmission or as mixing vessels for the emergence of new isolate with human pandemic potential [4–6]. In the past, a number of influenza viruses have been isolated from pigs in China. These mainly included classical and avian-like swine H1N1 viruses, H3N2 viruses similar to human A/Hong Kong/2/68(A/HK/2/68), A/Victoria/3/75, and A/Sydney/5/97 viruses, avian-like swine H9N2 viruses, and double-reassortant H1N2 virus containing genes similar to those of human and swine viruses [7–10].

From 2005 to 2006, we carried out swine influenza virus surveillance in eight provinces of China, intending to learn the overall information about the prevalence of swine influenza and provide necessary datum for swine influenza control, and possibly also some useful information for the prediction and preparedness of future human influenza

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[*] Corresponding authors. Fax: +86 451 82734181.
E-mail addresses: mliao@scau.edu.cn (M. Liao), gztong@hvri.ac.cn (G.-Z. Tong).
[1] These authors contributed equally to this study.

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pandemics. In this paper, we describe, for the first time, the isolation and genetic analysis of a human-like H1N1 virus from a pig in a farm of Guangdong province of southern China. In addition, we also depict the isolation and genetic analysis of four swine H3N2 influenza viruses.

Materials and methods

Sampling of pigs. A total of 500 samples, including nasal swabs, lungs, and trachea were collected from eight provinces (Heilongjiang, Henan, Shandong, Guangdong, Zhejiang, Anhui, Jiangxi, and Beijing) in China from 2005 to 2006. Samples were collected into transport medium containing antibiotics [8,11] and kept at 4 °C until transported to the laboratory.

Virus isolation and identification. For virus isolation, strict measures were followed to prevent aberrant contamination. Gloves and injection syringes were sterile, work surface was exposed to ultraviolet ray for enough time, and 10-day-old SPF embryonated chicken eggs were handled by using iodine tincture and alcohol. Then samples were inoculated into amnionic and allantoic cavities of eggs, and the eggs were incubated for 48–72 h at 35 °C. Virus isolate was passaged and identified by hemagglutination inhibition (HI) test and neuraminidase inhibition (NI) test using a panel of reference sera (National Reference Laboratory for Avian Influenza, Harbin Veterinary Research Institute, CAAS, China).

Analysis of viral RNA. Viral gene sequencing and analysis were carried out as follows. In brief, viral RNA was directly extracted from infected allantoic fluids using RNeasy Mini Kit (Qiagen, Chatsworth, CA) and reverse transcription (RT) were carried out under standard conditions using Uni12 (AGCAAAAGCAGG) primer. PCR was performed using specific primers for eight genes (primer sequences are available on request). PCR products were purified with the QIA quick PCR purification Kit (Qiagen, Inc.) and cloned into pMD18-T vector (TaKaPa, Dalian), then sequenced using synthetic oligonucleotides by Invitrogen Company. Sequence data were edited and analyzed using Bioedit software. The phylogenetic trees were generated with the MEGALIGN program (DNASTAR, Madison, WI) by using the Clustal alignment algorithm.

Results

Virus isolation and identification

Isolating swine influenza viruses is relatively difficult, which depend on the time of sampling, the route of inoculation, the adaptation of swine influenza virus to SPF eggs, and so on. Among 500 samples, one virus was identified as H1N1 subtype and four viruses were identified as H3N2 subtype. The five swine influenza viruses were designated as A/Swine/Guangdong/96/06(H1N1), A/Swine/Heilongjiang/1/05(H3N2), A/Swine/Guangdong/164/06(H3N2), A/Swine/Guangdong/165/06(H3N2), and A/Swine/Guangdong/166/06(H3N2).

Homology analysis of nucleotide sequences

The eight gene segments of the swine influenza virus isolates were sequenced, and their homologies were determined by comparison with sequences available in GenBank (Table 1). The H1N1 virus (A/Swine/Guangdong/96/06) was similar to recent human H1N1 influenza viruses in all eight gene segments, with homologies ranging from 98.8% to 99.6%. The three H3N2 isolates from Guangdong province were also closely related to recent human H3N2 influenza viruses in all eight gene segments, with homologies ranging from 99.3% to 99.6%. But interestingly, the H3N2 isolate from Heilongjiang province

Table 1
Genetic homology of the swine influenza viruses isolated in China with related sequences available in GenBank

Viruses	Gene	Virus with the highest identity	Identity (%)	GenBank Accession No.
A/Swine/Guangdong/96/06	HA	A/Dunedin/2/00(H1N1)	99.6	CY011584
	NA	A/Canterbury/43/00(H1N1)	99.4	CY010094
	PB1	A/New York/233/00(H1N1)	99.2	CY002646
	PB2	A/New York/443/01(H1N1)	99.4	CY003479
	PA	A/New York/443/01(H1N1)	99.1	CY003477
	NP	A/New York/234/00(H1N1)	99.3	CY002651
	M	A/New York/443/01(H1N1)	98.8	CY003473
	NS	A/New York/443/01(H1N1)	99.0	CY003476
A/Swine/Guangdong/164/06 A/Swine/Guangdong/165/06 A/Swine/Guangdong/166/06	HA	A/Moscow/10/99(H3N2)	99.3	DQ487341
	NA	A/Hong Kong/1144/99(H3N2)	99.5	AF382332
	PB1	A/Moscow/10/99(H3N2)	99.4	DQ487328
	PB2	A/New York/431/00(H3N2)	99.6	CY003247
	PA	A/Moscow/10/99(H3N2)	99.4	DQ487327
	NP	A/Moscow/10/99(H3N2)	99.5	DQ487330
	M	A/Hong Kong/1143/99(H3N2)	99.5	AF386766
	NS	A/Moscow/10/99(H3N2)	99.6	DQ487332
A/Swine/Heilongjiang/1/05	HA	A/Swine/Ange-Gardien/150/90(H3N2)	99.5	U07146
	NA	A/Swine/Colorado/1/77(H3N2)	99.9	CY009302
	PB1	A/Memphis/105/76(H3N2)	99.2	CY009066
	PB2	A/Beijing/39/75(H3N2)	99.1	CY006051
	PA	A/Memphis/106/76(H3N2)	99.1	CY008697
	NP	A/Beijing/39/75(H3N2)	99.4	CY006047
	M	A/Beijing/39/75(H3N2)	99.3	CY006045
	NS	A/Swine/Colorado/1/77(H3N2)	99.6	M80960

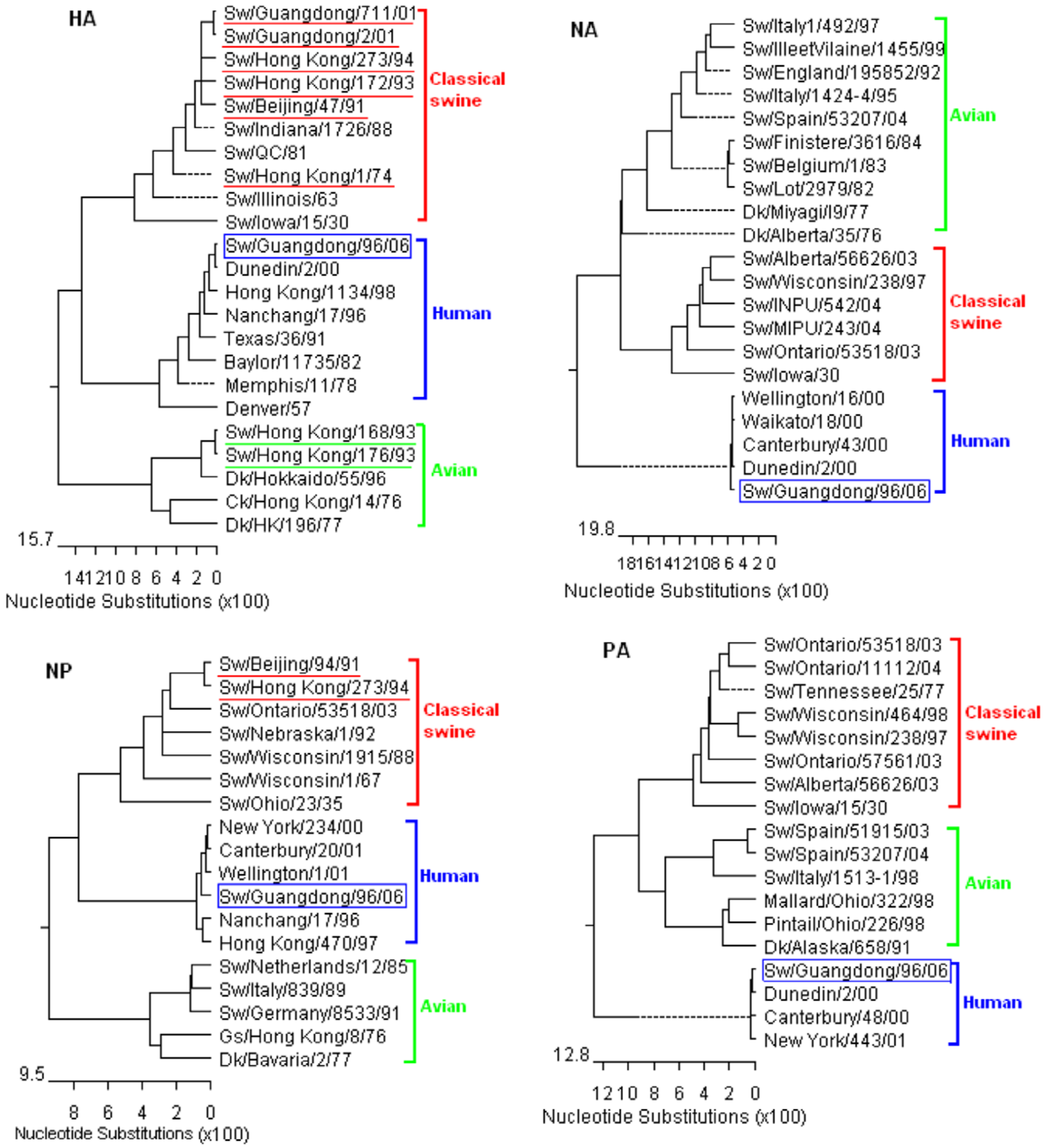

Fig. 1. Phylogenetic trees of the HA gene (981 bp from positions 84 to 1064), NA gene (1323 bp from positions 93 to 1415), NP gene (1342 bp from positions 46 to 1387) and PA gene (2136 bp from positions 40 to 2175) of the H1N1 influenza A viruses. The tree was generated by using MEGALIGN software (DNASTAR). Horizontal distances are proportional to genetic distance. Vertical lines are for spacing branches and labels. Abbreviations used in virus designation are as follows: Sw, swine; Dk, duck; Ck, chicken, Gs, goose; Tk, turkey. The underlined isolates are swine H1N1 influenza viruses that have been isolated in China before. The virus in open box is the swine H1N1 virus isolated and sequenced in this study.

was closely related to early human H3N2 influenza viruses in each of the eight gene segments.

Phylogenetic relationship of swine H1N1 influenza viruses from China

To characterize the gene segments of the H1N1 swine influenza virus more precisely, the phylogenetic trees were generated using the nucleotide sequences of the HA, NA, NP, and PA genes available in GenBank and the information from the trees was analyzed.

Phylogenetic analysis of the HA gene of the H1N1 influenza viruses shows that all of the H1N1 swine viruses isolated in China can be segregated into three lineages, including classical swine strains, human strains, and avian strains (Fig. 1). In the past, most of the H1N1 swine influenza viruses, isolated in China, were closely related to classical swine or avian influenza viruses. Classical swine lineage mainly includes A/Swine/Guangdong/711/01, A/

Swine/Hong Kong/273/94, A/Swine/Beiing/47/91, A/Swine/Hong Kong/172/93, and so on. A/Swine/Hong Kong/168/93 and A/Swine/Hong Kong/176/93, had emerged in China, belong to avian lineage. But A/Swine/Guangdong/96/06 was closely related with A/Dunedin/2/00 and lies within the human lineage.

Phylogenetic analyses of NA, NP, and PA genes (Fig. 1) also show clear divisions of each of these genes into different lineages including classical swine lineage, human lineage, and avian lineage, similar to the HA gene. Phylogenetic trees and homology (Table 1) of nucleotide sequence of gene segments of A/Swine/Guangdong/96/06 showed that the HA gene is possibly from A/Dunedin/2/00; the NA gene is possibly from A/Canterbury/43/00; NP, PB1 genes may derive from A/New York/234/00 and A/New York/233/00, respectively; PA, PB2, M, and NS genes may derive from A/New York/443/01. So A/Swine/Guangdong/96/06 is the descendant of recent human H1N1 influenza viruses.

Phylogenetic relationship of swine H3N2 influenza viruses from China

To learn whether the four swine H3N2 isolates are related to the H3 swine viruses previously, we used nucleotide sequences of representative swine viruses isolated in China before to construct the HA tree. Phylogenetic analysis of the HA gene of the H3N2 influenza viruses shows that all of the H3N2 swine viruses isolated in China recently can be segregated into three lineages, including early human strains, recent human strains, and avian strains (Fig. 2). This grouping differs somewhat from that reported by Nerome et al. [12] and Zhou et al. [13], because of the emergence of new H3N2 swine influenza viruses. The early human-derived

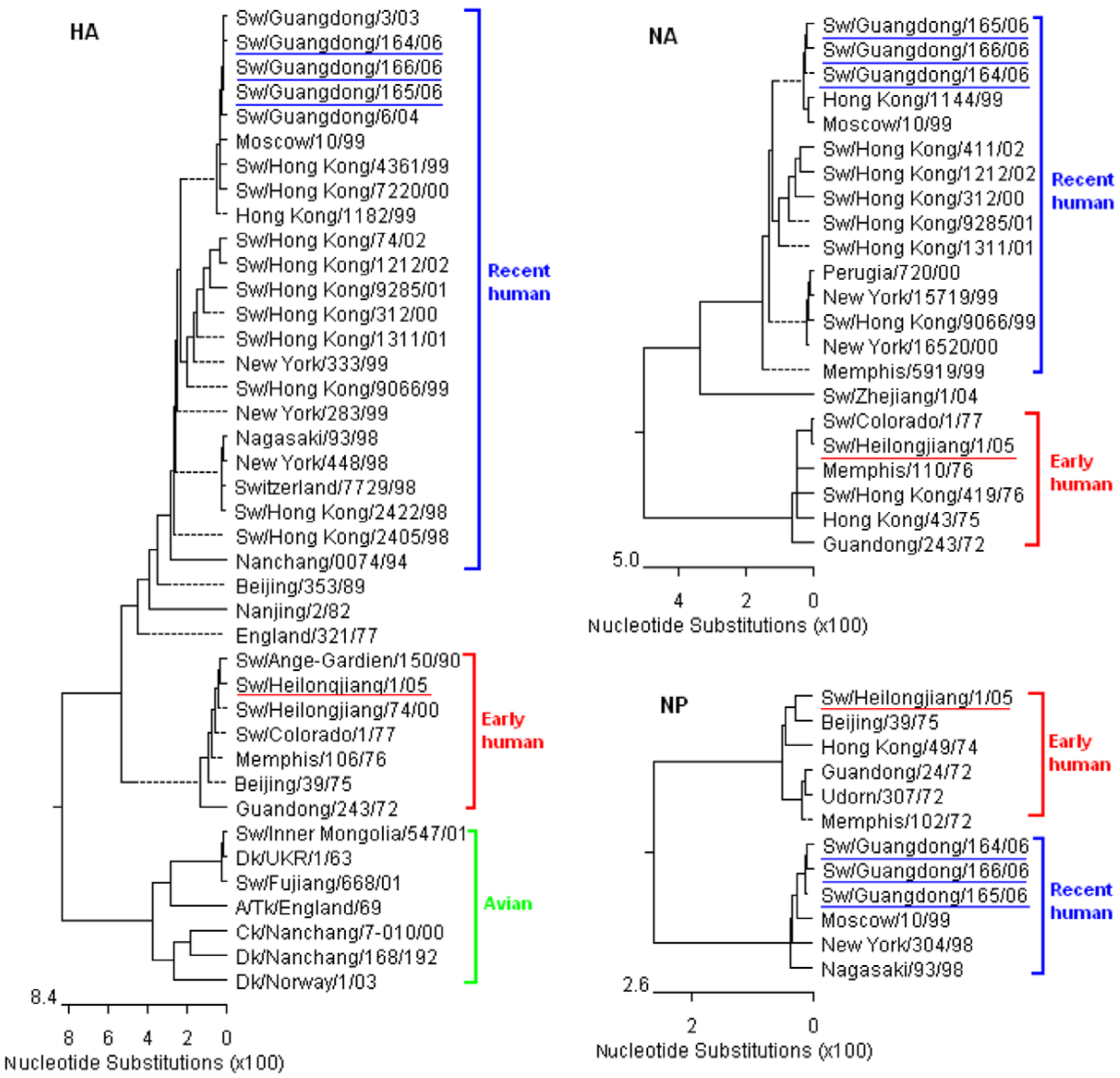

Fig. 2. Phylogenetic trees of HA gene (1621 bp from positions 77 to 1697), NA gene (1310 bp from positions 88 to 1397) and NP gene (1520 bp from positions 24 to 1543) of the H3N2 influenza A viruses. The method used and abbreviations are as given in the legend to Fig. 1. The underlined viruses are the swine H3N2 viruses isolated and sequenced in this study.

swine influenza viruses, A/Swine/Heilongjiang/74/00 and A/Swine/Heilongjiang/1/05, were closely related to Memphis76-like viruses and appeared to have established a separate lineage. The recent human lineage includes three sublineages represented by Moscow99-like, New York99-like, and Nagasaki98-like viruses. The three H3N2 isolates from Guangdong province belong to Moscow99 sublineage. In addition, two isolates, A/Swine/Inner Mongolia/547/01 and A/Swine/Fujian/668/01, form an avian lineage.

Phylogenetic analysis of the NA and NP genes shows that the NA and NP genes of swine H3N2 influenza viruses isolated in China recently can only be separated into two lineages (Fig. 2), which is different from HA gene because of the lack of sequence data in GenBank. The three isolates from Guangdong province belong to recent human lineage. And A/Swine/Heilongjiang/1/05 seems to be located in early human lineage. So it is apparent from phylogenetic trees and homology (Table 1) of the nucleotide sequence that the three viruses from Guangdong province are descendants of recent human viruses, while interestingly, the virus from Heilongjiang province derives from early human viruses.

Discussion

Influenza A viruses of subtypes H1N1 and H3N2 have been reported widely in pigs, associated with clinical disease. In this study, recent molecular evolution of H1N1 and H3N2 influenza viruses in pigs in China was depicted. The HA, NA, and NP genes of swine H1N1 influenza viruses can be segregated into three lineages, including classical swine strains, avian strains and human strains (Fig. 1). Our H1N1 virus belongs to human strains and is firstly reported in mainland China. While the HA gene of swine H3N2 influenza viruses can be segregated into three lineages, including early human strains, recent human strains, and avian strains (Fig. 2). The three H3N2 isolates from Guangdong province belong to recent human strains, while interestingly, the H3N2 isolate from Heilongjiang province belongs to early human strains.

Swine influenza was first observed in 1918 at the time of the human pandemic and the virus was isolated and identified in 1930 by Shope [14]. This virus was the prototype strain of a group of viruses now known as classical swine influenza viruses. Serological and virologic surveillance has shown that classical swine H1N1 is prevalent throughout the major pig population of the world [1,8,15,16]. Since 1979, classical swine influenza viruses have been replaced by avian-like H1N1 viruses that are antigenically distinguishable from classical swine H1N1 viruses in Europe. All of gene segments of the prototype viruses were of avian origin indicating that transmission of a whole avian virus into pigs had occurred [17]. Human H1N1 viruses can infect pigs and pig to pig transmission has been demonstrated under experimental conditions. Serological surveillance studies worldwide suggest that the prevailing human H1N1 strains are readily transmitted to pigs [1], but there are a few reports about isolation of the human-like swine H1N1 viruses [18,19].

In China, classical swine H1N1 virus were the predominant influenza viruses circulating in northern, central (Henan and Jiangxi), and southern (Guizhou and Guangdong) provinces [20]. Since 1993, avian-like swine influenza viruses had been isolated from pigs and circulated with classical H1N1 viruses [8]. But there were no reports about the isolation and genetic characterization of human H1N1 influenza virus in pigs in China. In the spring of 2006, an outbreak of acute respiratory disease occurred in a commercial pig farm in Guangdong province of China. Most of the weanling and grower pigs in the herd showed influenza-like symptom. A total of 60 samples including nasal swabs, lungs, and trachea were collected from this farm and were inoculated into SPF embryonated chicken eggs, and a swine influenza virus was obtained through 3–4 passages in SPF embryonated chicken eggs. The isolate was designated as A/Swine/Guangdong/96/06(H1N1) and was human-like swine H1N1 influenza virus.

Infections of pigs with human H3N2 influenza viruses can occur under natural conditions. Shope (1938) presented serological evidence that human to pig transmission could occur. Around 1970, following the human ‘Hong Kong’ flu pandemic, the human H3N2 virus was transmitted to pigs [1]. This human-like swine H3N2 virus continued to circulate, particularly in Europe and Asia, but only sporadically caused clinical sign. It has only started causing clinical disease since 1984, probably as a result of reassortment with the avian-like swine H1N1 virus [21]. Recently, two antigenically distinct H3N2 reassortants were isolated from infected animals: a double-reassortant virus containing genes similar to those of human and swine influenza viruses, and a triple-reassortant virus containing genes similar to those of human, swine, and avian influenza viruses, which caused serious illness and reproductive losses in sows [13]. In this study, our four swine H3N2 viruses were related closely to recent or early human H3N2 viruses in all gene segments and had not undergone genetic reassortment.

Pigs may act as an intermediate host of interspecies transmission or as mixing vessels for the emergence of new isolate with human pandemic potential. Human influenza viruses that infect pigs may become a new swine influenza virus [4–6]. China, especially southern China is regarded as an epicenter for the emergence of pandemic influenza viruses [3]. The 1957 and 1968 pandemics originated in this region [22]. Now we report the isolation of human-like H1N1 and H3N2 viruses from pigs in China. So the isolation and genetic analysis of human H1N1 and H3N2 influenza virus from pigs emphasizes the importance of reinforcing swine influenza virus surveillance in China.

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