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Imported pigs may have introduced the first classical swine influenza viruses into Mainland China

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ABSTRACT

Objectives: The first classical swine influenza A H1N1 viruses were isolated in Mainland China in 1991. To aid surveillance of swine influenza viruses as part of pandemic preparedness, we sought to identify their origin.
Methods: We sequenced and phylogenically analyzed 19 swine influenza viruses isolated in 1991 and 1992 in China and compared them with viruses isolated from other regions during the same period.
Results: All 19 swine influenza viruses analyzed in our study shared the highest similarity with the classical swine influenza virus A/Swine/Maryland/23239/1991 (H1N1). Phylogenetic trees of eight segmented genes exhibited similar topology, with all segments in the cluster of classical swine influenza viruses. In addition, antigenic analysis also indicated that the tested isolated were related to classical swine influenza isolates.
Conclusions: Classical swine H1N1 influenza viruses were predominant in Beijing pig herds during this period. Since both antibody and virus detections did not indicate the presence of CS H1N1 before 1991 in Mainland China, we combined with the data on pigs imported to and exported from China and concluded that these viruses might spread to China via pigs imported from North America and that they could affect the genetic evolution and transmission dynamics of swine influenza viruses in Hong Kong.
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1. Introduction

The influenza A virus belongs to the *Orthomyxovirus* family, which has a viral genome of 8 segments of single-stranded negative-sense RNA that encode 12 or 13 functional proteins (Chen et al., 2001; Jagger et al., 2012; Webster et al., 1992; Wise et al., 2009). Influenza A viruses have been isolated from a variety of host species, including wild and domestic birds and marine and terrestrial mammals (Alexander, 1982; Brown, 2000; Webster et al., 1992). Swine-origin influenza A H1N1 virus causes a respiratory disease that is a great economic concern for the swine industry and a threat to public health. Because a pig's respiratory tract has receptors for both avian influenza viruses (*N*-acetylneuraminic acid-α2,3-galactose) and human influenza viruses (*N*-acetylneuraminic acid-α2,6-galactose), pigs are an important intermediate host and a "mixing vessel" for genetic reassortment (Ma et al., 2008). Reassortment can occur when various sources and lineages of influenza A viruses co-infect pigs (Castrucci et al., 1993); thus, pigs may play a significant role in influenza A viral evolution and pandemic virus emergence.

Swine influenza viruses (SIVs) were recognized as early as 1918, at the same time as the 1918 Spanish flu H1N1 subtype caused the pandemic in humans. However, it was not until 1930 that the SIV known as the classical swine H1N1 virus (CS H1N1) was first isolated (Shope, 1931). Subsequently, CS H1N1 was predominant in pigs in North America (Chambers et al., 1991; Hinshaw et al., 1978; Chen et al., 2009; Liu et al., 2009) and was also found in pigs in Europe (Blakemore and Gledhill, 1941; Kaplan and Payne, 1959; Nardelli et al., 1978) and Asia (Guo et al., 1992; Kupradinun et al., 1991; Shortridge and Webster, 1979; Yamane et al., 1978). Surveillance information for SIVs in Asia is very limited, especially before 1998. There were only 1003 full-length SIV sequences deposited in GenBank before 1991, with almost all of the 457 Asian SIVs were isolated around 1980s. SIV surveillances in China have been conducted since 1970s (Guo et al., 1992), however, it is not until 1991 the first classical swine influenza isolate was reported in Mainland China, with other 18 SIVs isolated successively during 1991 and 1992 in the same region, Beijing city (data not shown). After this documented SIV (Guo et al., 1992), other SIVs data were

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Table 1
Subtypes of swine influenza viruses in this study and HI titrations with postinfection ferret antisera against influenza A (H1N1) virus isolates of swine and human origin.

Virus	Subtype	Titer of ferret antiserum to antigen*				
		A/California/07/2009	A/Brisbane/59/2007	A/Jiangsu/1/2011	A/swine/Beijing/156/1991	A/New Jersey/8/1976
A/California/07/2009	H1N1	**640**	–	–	40	–
A/Brisbane/59/2007	H1N1	–	**320**	–	–	–
A/Jiangsu/1/2011	H1N1	⩾2560	–	**1280**	⩾2560	640
A/New Jersey/8/1976	H1N1	1280	–	80	1280	**640**
A/swine/Beijing/156/1991	H1N1	1280	–	320	⩾**2560**	640
A/swine/Beijing/139/1991	H1N1	1280	–	160	⩾2560	1280
A/swine/Beijing/205/1992	H1N1	⩾2560	–	160	⩾2560	1280
A/swine/Beijing/217/1992	H1N1	640	–	160	⩾2560	1280
A/swine/Beijing/48/1991	H1N1	⩾2560	–	160	⩾2560	1280
A/swine/Beijing/24/1991	H1N1	1280	–	80	⩾2560	1280
A/swine/Beijing/257/1992	H1N1	⩾2560	–	160	⩾2560	1280
A/swine/Beijing/110/1991	H1N1	1280	–	160	1280	1280
A/swine/Beijing/216/1992	H1N1	⩾2560	–	160	⩾2560	1280
A/swine/Beijing/121/1991	H1N1	⩾2560	–	160	⩾2560	1280
A/swine/Beijing/149/1991	H1N1	⩾2560	–	160	⩾2560	640
A/swine/Beijing/130/1991	H1N1	⩾2560	–	320	⩾2560	⩾2560
A/swine/Beijing/126/1991	H1N1	⩾2560	–	320	⩾2560	2560
A/swine/Beijing/133/1991	H1N1	⩾2560	–	160	⩾2560	1280
A/swine/Beijing/155/1991	H1N1	640	–	80	⩾2560	1280
A/swine/Beijing/215/1992	H1N1	⩾2560	–	320	⩾2560	1280
A/swine/Beijing/47/1991	H1N1	⩾2560	–	320	⩾2560	1280
A/swine/Beijing/234/1992	H1N1	⩾2560	–	320	⩾2560	1280
A/swine/Beijing/134/1991	H1N1	⩾2560	–	320	⩾2560	⩾2560

Swine influenza viruses analyzed in this study were listed in *italic*; *–, HI titer of less than 20; Homologous titers are indicated in bold.

derived from incident samples in disparate geographical regions. Although systematic SIVs surveillance began in southern China in 1998 (Vijaykrishna et al., 2011), the origin of SIVs isolated in Mainland China in 1991 is still unknown. In order to address this question, a total of 19 SIV strains isolated in Beijing from 1991 to 1992 were completely sequenced and analyzed (Table 1).

2. Materials and methods

2.1. Virus propagation and sequencing

To reproduce the viruses, 0.2 mL viral stock were inoculated in 9-day-old embryonated chicken-specific pathogen-free eggs and incubated at 35 °C for 48 h. The allantoic fluids were harvested, and viral RNA was extracted using QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to manufacture's instructions. Gene segments were amplified using the Qiagen® OneStep RT-PCR Kit. Polymerase chain reaction products were purified from agarose gel using the QIAquick Gel Extraction Kit (Qiagen). Complete genome sequencing was performed with an ABI 3730XL automatic DNA analyzer (Applied Biosystems, Foster City, CA, USA) using the ABI BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's recommendations. All sequences generated and used in this article were submitted to the Global Initiative on Sharing Avian Influenza Data (GISAID).

2.2. Phylogenetic analysis

In order to estimate the genetic diversity of the viruses, phylogenetic trees were constructed for each gene segment independently. Multiple sequence alignments were performed with the ClustalW program using MEGA software version 5.05. The phylogenetic relationships were estimated from the nucleotide sequences of each H1N1 swine influenza viral gene relative to selected H1N1 subtype influenza reference strains obtained from GenBank. Finally, a phylogenetic tree was drawn using the neighbor-joining method with MEGA software version 5.05.

2.3. serological assays

The hemagglutination inhibition (HI) tests were carried out according to standard protocols by using 0.5% turkey red blood cells (Kendal AP and Pereira MS, 1982; WHO, 2009). Five ferret antisera against the human and swine H1N1 isolates A/California/07/2009 (A(H1N1)pdm09), A/Brisbane/59/2007 (seasonal H1N1), A/Jiangsu/1/2011 (human Euroasian avian-like H1N1) (Zu et al., submitted for publication), A/swine/Beijing/156/1991 (CS H1N1), and A/New Jersey/8/1976 (CS H1N1) were raised. Prior to the HI assay, serum samples were treated with a 1:5 (vol/vol) of receptor destroying enzyme (RDE, Sigma, USA) at 37 °C for 18 h, and followed by heat inactivated at 56 °C for 30 min to remove nonspecific serum inhibitors. Serum samples were titrated in 2-fold dilutions in phosphate-buffered saline and tested at an initial dilution of 1:10. HI antibody titers ⩽20 were defined as serological negative.

3. Results

3.1. Homology analysis of nucleotide sequences

Eight gene segments of the 19 viruses were fully sequenced and phylogenetically characterized. H1N1 isolates in this study shared 94.6–100% of their genes. These results suggest that these viruses possess a similar genetic constellation sharing common evolutionary lineages. Basic Local Alignment Search Tool analysis showed that each gene segment of the viruses shared the most similarities with A/Swine/Maryland/23239/1991 (H1N1) (Table 1). Thus, A/Swine/Maryland/23239/1991 was selected as a reference strain for analysis owing to its classical swine influenza lineage and its circulation in North America in the same period as the investigated viruses.

3.2. Phylogenetic analysis of the isolated viruses

To determine the evolutionary relationships of the swine H1N1 viruses isolated in this study, gene sequences were compared with those of other influenza viruses isolated from humans and pigs in

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