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BRIEF REPORT

Identification of four genotypes of H3N2 swine influenza virus in pigs from southern China

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Abstract In 2011, four H3N2 swine influenza viruses (SIVs) were isolated from nasal swabs of four pigs (800 nasal swabs were collected from pigs showing influenza-like symptoms) in Guangdong province, China. Four different genotypes of H3N2 appeared among pigs in southern China, including wholly human-like H3N2 viruses, intermediate (1975) double-reassortant human H3N2 viruses (resulting from reassortment between an early human lineage and a recent human lineage), recent double-reassortant human H3N2 viruses, and avian-like H3N2 viruses. Because pigs can support the reassortment of human and avian influenza viruses, our surveillance should be enhanced as a part of an overall pandemic preparedness plan.

Keywords Influenza virus · H3N2 subtype · Genetic analysis · Reassortment · Swine

Currently, three main subtypes of the influenza A virus have been found to be present in different swine populations worldwide: H1N1, H1N2, and H3N2 [5, 9]. The occurrence of the 2009 H1N1 influenza pandemic provided evidence that pigs can function as intermediate hosts or "mixing vessels," which allows the generation of novel viruses that can subsequently infect humans [3, 10, 13]. Reassortment among swine, avian, and human viruses has been reported to occur in pigs [13, 17]. Therefore, influenza monitoring in Chinese swine populations is of great significance. H3N2 subtype influenza viruses first appeared in humans and caused a major influenza pandemic in 1968 in southern China. Recently, an increasing number of H3N2 influenza viruses have been isolated from pigs in this region.

In order to better understand the prevalence and genetic evolution of the H3N2 influenza virus in pigs in southern China, 800 nasal swabs were collected from pigs showing influenza-like symptoms. These samples were collected between January 2010 and February 2012, from the following seven cities located in the Guangdong province in China: Guangzhou, Huizhou, Zhuhai, Zhanjiang, Shantou, Zhongshan, and Yangjiang. The isolation of viruses from nasal swabs in Madin-Darby canine kidney cells and subtyping experiments were carried out according to previously reported protocols [2, 8]. In this manner, nine SIV strains were isolated, including four H1N1, one H1N2, and four H3N2 strains. H3N2 SIVs are potential threats to human health [6]; therefore, the objective of this study was to genotype and characterize the four H3N2 isolates (A/swine/Guangdong/L21/2011 [H3N2], A/swine/Guangdong/L22/2011 [H3N2], A/swine/Guangdong/L23/2011 [H3N2], and A/swine/Guangdong/L5/2011 [H3N2]).

RT-PCR analysis was performed to amplify the full-length coding regions of all eight viral RNAs for sequence analysis. Viral RNA was isolated using TRIzol reagent (GIBCO-BRL), and reverse transcription was performed using an influenza virus oligonucleotide universal primer, 5′-AGCAAAAGCAGG-3′. A series of primers were designed to amplify the eight genes for sequencing. PCR

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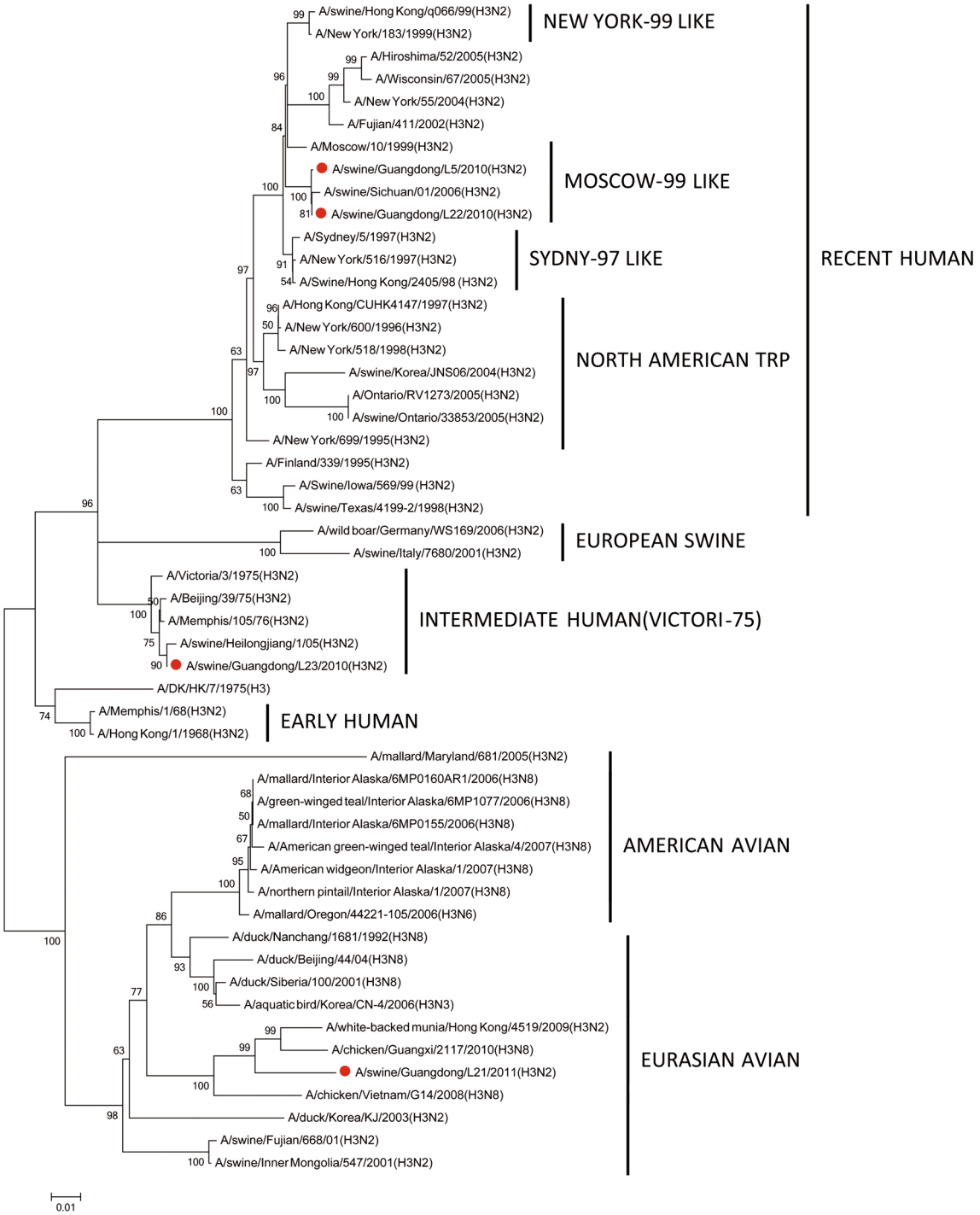

Fig. 1 Phylogenetic trees for the H3N2 HA gene of influenza A viruses. The analysis was based on nucleotide sequences of the open reading frame of the HA gene. The phylogenetic trees were generated by the neighbor-joining method using the MEGA program (version 5.0)

products were purified using an Agarose Gel DNA Purification Kit (TaKaRa, Dalian), cloned into the pMD18-T vector, and sequenced (TaKaRa, Dalian). The GenBank accession numbers of the isolates used in this study are JX494706–JX494713, JX494714–JX494721, JX414231–JX414238, and JX096501–JX096508. Comparisons of nucleotide and deduced amino acid sequences were made using DNAStar 7.0 software, and phylogenetic trees were generated by neighbor-joining (NJ) methods using the MEGA 5.0 program. Bootstrap values were calculated based on 1,000 replicates of the alignment. Glycosylation sites on the hemagglutinin of these viruses were predicted by "NetNGlyc 1.0 Server'' (http://www.cbs.dtu.dk/services/NetNGlyc).

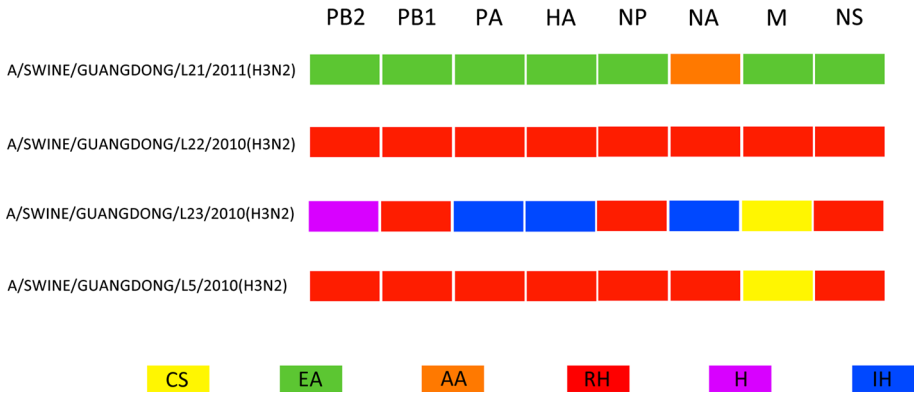

Fig. 2 Identification of the genotypes of four isolated H3N2 swine influenza viruses. CS, EA, AA, RH, H and IH stand for classical SIV, Eurasian avian-like SIV, American AIV, recent human influenza virus, human influenza virus, and intermediate human influenza virus, respectively

Phylogenetic analysis of the sequences of all eight viral RNA segments demonstrated that the A/swine/Guangdong/L21/2011 (H3N2) virus was entirely of avian influenza virus (AIV) lineage. The HA and NA gene segments of this H3N2 SIV belonged to the H3 AIV lineage, which was present in southern China, and the M gene was phylogenetically close to those of the H5N1 AIVs. A/Swine/Guangdong/L22/2011 and A/Swine/Guangdong/L5/2011 showed a low level of sequence identity to A/Swine/Guangdong/L23/2011; the two isolates formed a cluster along with human isolates collected in the 2000s, whereas A/Swine/Guangdong/L23/2011 represented an intermediate (1975) swine virus that arose be reassortment between an early human lineage and a recent human lineage. Interestingly, the M gene from A/Swine/Guangdong/L23/2011 and A/Swine/Guangdong/L5/2011 was clustered with classical SIV, indicating that reassortment was common between the H3N2 human and H1N1 SIVs in the pig population of southern China (Fig. 1, Supplementary Figures 1–7). The reassortment of these two isolates is shown in Figure 2. Therefore, three of the four swine influenza isolates obtained in this study were identified as reassortants.

The results of the molecular analysis of the HA1 genes of these H3N2 influenza viruses are shown in Figure 3. Comparing the sequences of the avian-like, 1975 human-origin, and 2000 human-origin H3N2 SIVs, we found that there were seven glycosylation sites (8, 22, 38, 63, 126, 165, and 285) in the HA1 genes of the 1975 human-origin H3N2 SIVs. In addition to these seven glycosylation sites, three other glycosylation sites at positions 122, 133, and 246 were found in the 2000 human-origin isolates. There were five glycosylation sites (8, 22, 38, 165, and 285) in the HA1 genes of H3N2 SIVs of avian origin. In comparison with the A/swine/Guangdong/L21/2011 (H3N2) strain, both H3N2 viruses of human origin had the same substitutions, i.e., Q226L and G228S, whereas those with Q-226 and G-228 (avian and equine viruses, respectively) recognized sialic acid α2, 3-galactose (SAα2, 3 Gal).

The 2009 H1N1 pandemic virus was a re-assortant of swine origin, containing HA, NP, and NS segments from a classical swine (North American) lineage, PB2 and PA segments from an avian (North American) lineage, the PB1 segment from a human seasonal H3N2 virus, and NA and MP segments from a Eurasian swine lineage [4]. Recently, sporadic cases of human infection with novel reassortant viruses of swine origin have been reported in the United States [12]. The number of isolates used in the present study was not sufficient, but we were able to demonstrate that the re-assortment of influenza virus was common in pigs in southern China. The special geographical location and lifestyle in southern China, where pigs, humans, wild aquatic birds, and domestic poultry are frequently in contact, facilitates interspecies transmission and generation of new reassortant genotypes of influenza viruses, which may have the potential to threaten human health [11, 16].

In this study, amino acid sequence analysis of the HA1 gene revealed multiple changes in the surface proteins of swine influenza viruses. Most of these changes between swine viruses were detected in regions other than the proposed antigenic sites. In this study, the three human-like swine H3N2 isolates had 7–10 glycosylation sites in the HA1 gene, although not all of these sites may be used. Of these sites, positions 122, 133, and 246 were unique to the circulating human lineage. In addition, the swine viruses of human (earliest human, early human, and recent human) lineages had more glycosylation sites than those of the avian lineage. For instance, human-lineage virus variants with a larger number of glycosylation sites co-circulated in the epidemic area and appear to have prevailed at the end of the outbreak [7, 15]. For H3N2 viruses, residues 226 and 228 on the receptor-binding domain of the HA1 molecule were shown to play a critical role in determining receptor specificity [1, 14]. The three human-like swine H3N2 isolates possessed L/I226 and S228, which are usually detected in human viruses, whereas Q226 and G228 are generally found in avian-like SIVs. These results enhance our understanding of the genetic characteristics of different genotypes of swine influenza A (H3N2) viruses in southern China.

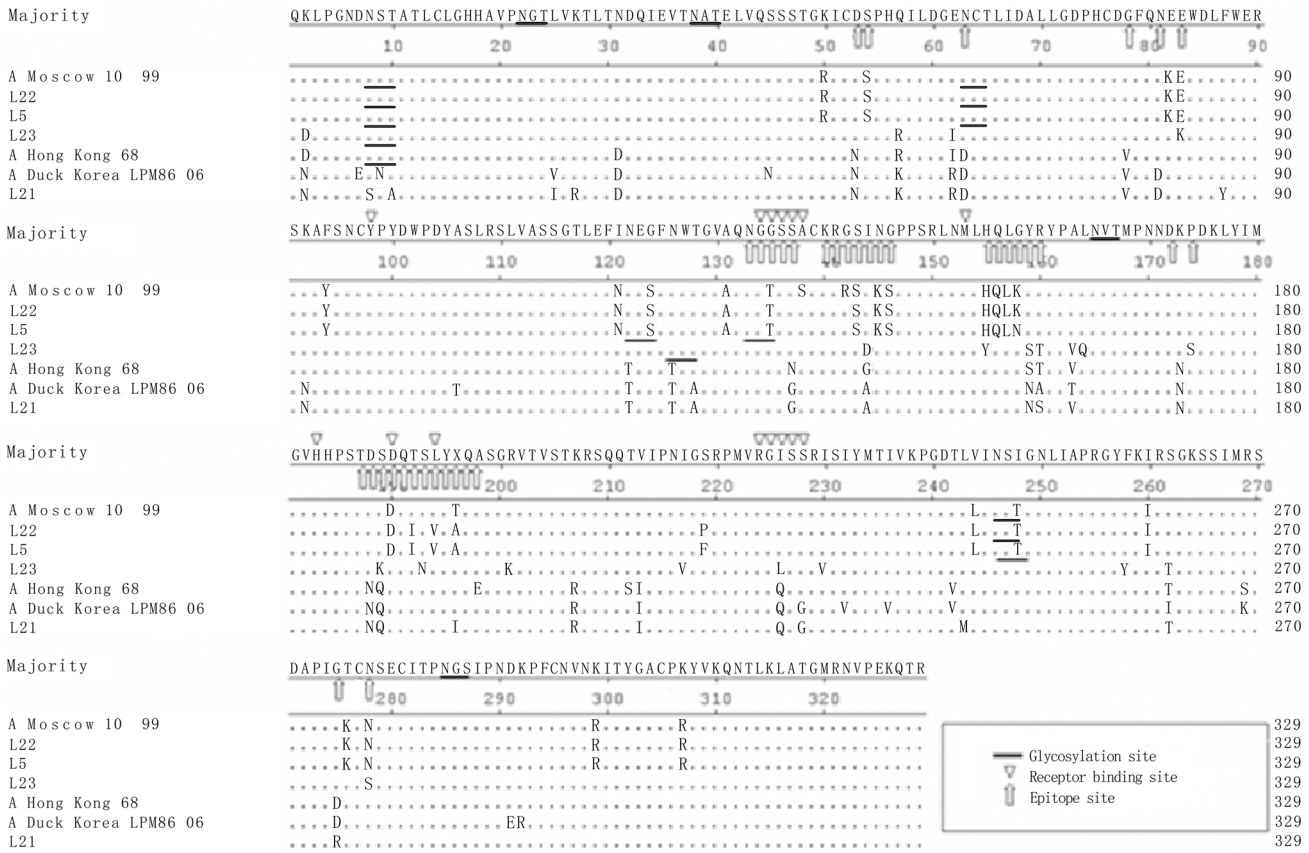

Fig. 3 Molecular analysis of the HA1 proteins of three isolated H3N2 viruses and the respective reference H3N2 viruses. Dots represent amino acids that are identical to those in the consensus sequence. Only amino acids that are different from those in the consensus sequence are indicated. Numbering starts at the N-terminus of HA1. Overlined residues are potential glycosylation sites. Receptor-binding sites are shown as open inverted triangles (∇). Amino acid residues mapped previously to defined epitope sites are shown as open arrows (⇑). Some of the receptor-binding residues are also in known antigenic sites. For these sites, symbols for both are shown

In this study, we have summarized and reported, for the first time, the coexistence of entirely human-like H3N2 viruses (A/swine/Guangdong/L22/2011 [H3N2]), intermediate (1975) human double-reassortant H3N2 viruses (A/swine/Guangdong/L23/2011 [H3N2]), recent human double-reassortant H3N2 viruses (A/swine/Guangdong/L5/2011 [H3N2]), and avian-like H3N2 viruses (A/swine/Guangdong/L21/2011 [H3N2]) in pigs in southern China. Given the evidence that pigs can support the reassortment of human and avian influenza viruses, it is prudent to enhance surveillance for different genotypes of H3N2 SIVs in pigs as a part of an overall pandemic preparedness plan. In addition, the potential of avian-like H3N2 SIV or novel H3 reassortant viruses infecting the human population should also be taken into consideration. Southern China is known to have been a critical region for human influenza pandemics throughout history [11]. Both the 1957 and 1968 pandemic influenza viruses emerged in this area [11]. For the above-described reasons, the appearance of AIVs among pigs contributes to concerns about both veterinary and human health.

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Conflict of interest None.

Ethical approval All animal research was conducted under the guidance of the Centers for Disease Control and Prevention's Institutional Animal Care and Use Committee and performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The animal research in our study was approved by the Guangdong Province Animal Disease Control Center.

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