Journal of Animal and Veterinary Advances 10 (18): 2331-2342, 2011
ISSN: 1680-5593

Isolation and Phylogenetic Analysis of H9N2 Swine Influenza Virus from Sick Pigs in Southern China in 2010

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Abstract: In January, 2010 before swine were infected with foot and mouth disease in Guangdong, some pigs have shown flu-like symptoms; cough, sneeze, runny nose and fever. Researchers collected the nasopharyngeal swab of all sick pigs as much as possible. One H9N2 influenza viruses were isolated from the pig population. The complete genome of one isolate, designated A/Swine/Guangdong/L1/2010(H9N2) was sequenced and compared with sequences available in GenBank. The results of analyses indicated that the sequence of A/Swine/Guangdong/L1/2010(H9N2) was similar to those of several avian influenza viruses. According to phylogenetic analysis of the complete gene sequences, A/Swine/Guangdong/L1/2010(H9N2) possibly originated from the reassortment of avian influenza viruses.

Key words: Influenza A virus, phylogenetic analysis, H9N2, swine, fever, China

INTRODUCTION

Influenza A viruses are a group of viruses causing mainly respiratory tract infections which belongs to the family Orthomyxoviridae. It is one of the major pathogens that threaten human health. The influenza A virus contains eight single-stranded RNA segments of negative sense and encodes at least 11 proteins. The virus is further classified into 16 Hemagglutinin (HA) and 9 Neuraminidase (NA) sub-types based on antigenic differences (Fouchier *et al*., 2005). Avian influenza H9N2 virus continues to threaten the poultry population worldwide which results in egg production losses and low immunity (Alexander, 2000; Guo *et al*., 2000; Naeem *et al*., 1999). H9N2 virus was remarkable because it not only infected avian and pigs but also infected human (Ito *et al*., 1998; Butt *et al*., 2005). Recently, the study confirmed that H9N2 virus can cause a significant morbidity and mortality to pigs (Cong *et al*., 2007). Some mice got acute respiratory distress syndrome induced by H9N2 virus (Deng *et al*., 2010).With luck, the H9N2 virus did not result in a serious illness in human. However, we are wise it will continue to be cautious about the virus.

In February of 2010, the Foot and Mouth Disease (FMD) caused rampant epidemic diseases in pig population of Guangdong which caused a great damage to farmers (Paton *et al*., 2010). Before the outbreak of FMD, many pigs have a severe outbreak of influenza-like disease occurred in different intensive pig farms of Guangdong province. Many pigs have similar clinical symptoms; cough, sneeze, runny nose and fever. These clinical symptoms last for 3-8 days then some pigs have sick of FMD. May be the influenza virus lowers pig immunity to common illnesses so, some pigs will get FMD. Researchers collected the nasopharyngeal swab of all of flu-like pigs as much as possible to perform virus isolation.

MATERIALS AND METHODS

A total of 246 samples were submitted to the Virology Laboratory, College of Veterinary Medicine, South China Agricultural University. All individual samples positive (87/246) by rRT-PCR were subjected to virus isolation by inoculating them into 10 days old specific-pathogen-free embryonated chicken eggs via the allantoic route. The inoculated eggs were incubated at 37°C for 72 h. Embryonic death was monitored every 12 h and then allantoic fluid were harvested under aseptic conditions and stored at -70°C for reserved. Sub-type identification were conducted through RT-PCR and through standard hemagglutination inhibition and neuraminidase inhibition assays. One influenza viruses were isolated and named; A/swine/Guangdong/L1/ 2010 (H9N2) (SW/GD/L1/2010) (Fig. 1).

Viral RNA from the isolates propagated in 10 days old embryonated eggs was extracted by lysing the viruses with TRIzol reagent (Invitrogen). The RNA was reverse-transcribed into single-stranded DNA with M-MLV reverse transcriptase (Promega). Full-length PCR amplification of eight RNA segments was performed with a set of primer.

The *HA*, *NA*, *NP*, *M* and *NS* gene segments of the viruses were amplified using segment-specific primers designed by Hoffmann *et al*. (2001), the other genes were amplified by primer of the laboratory.

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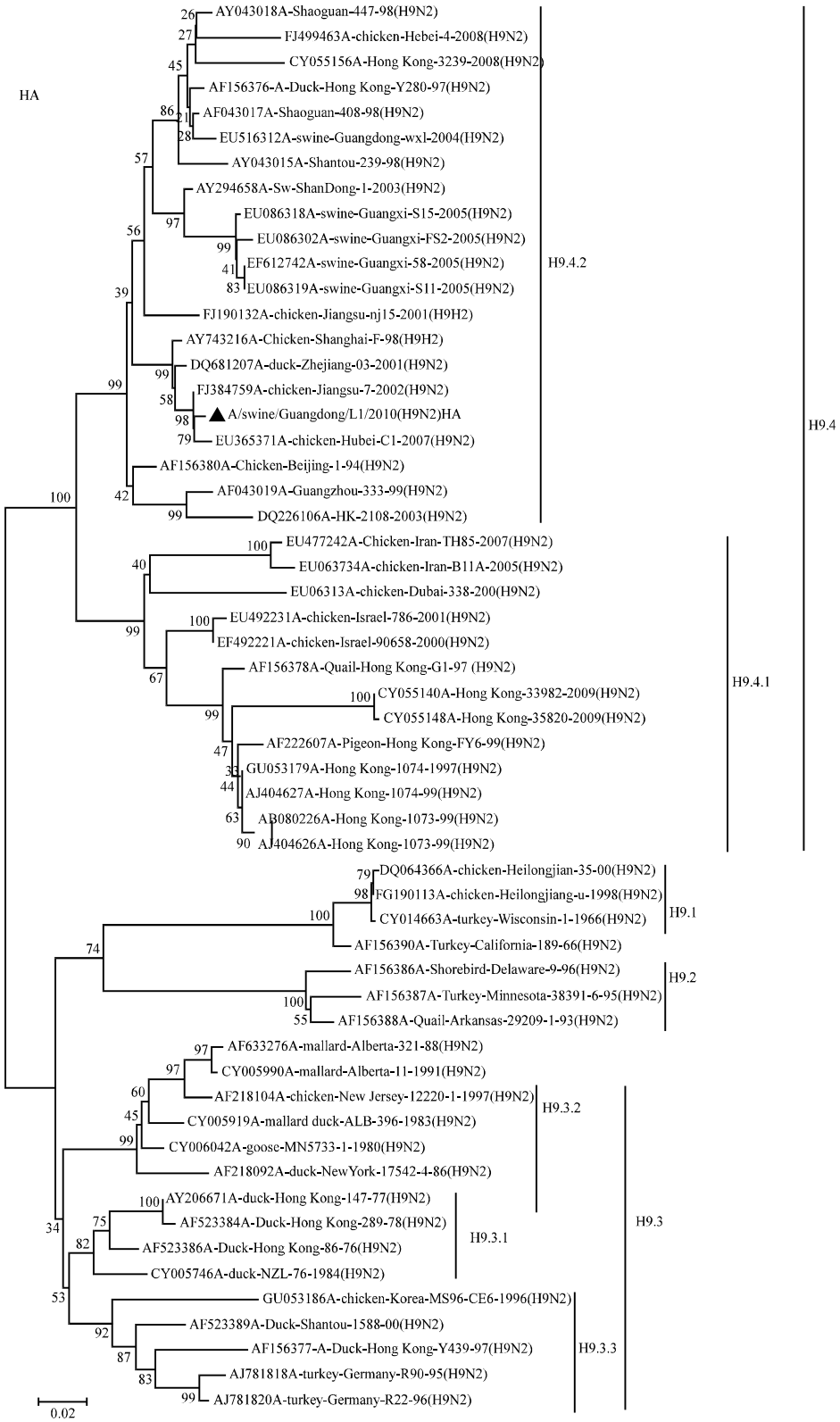

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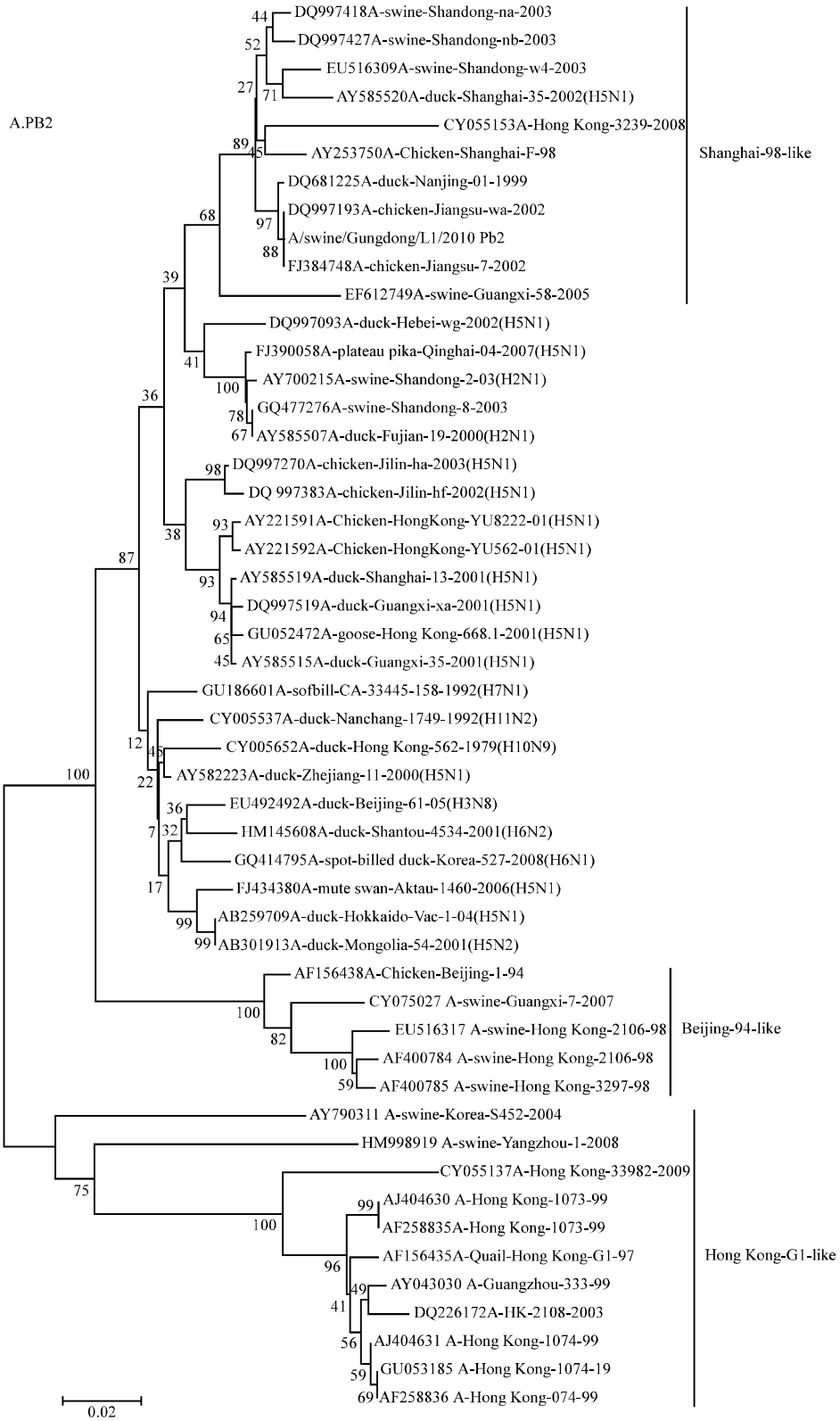

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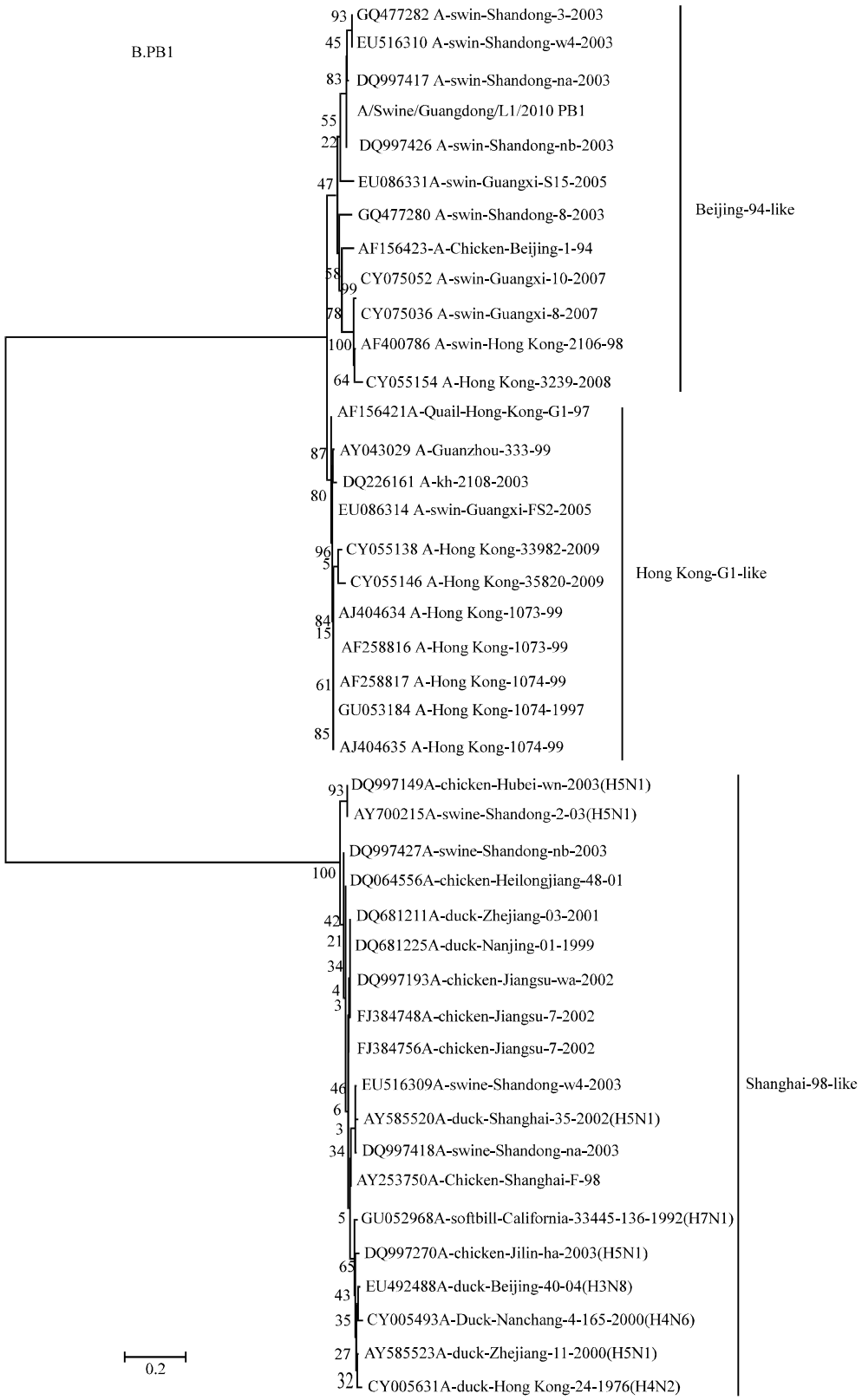

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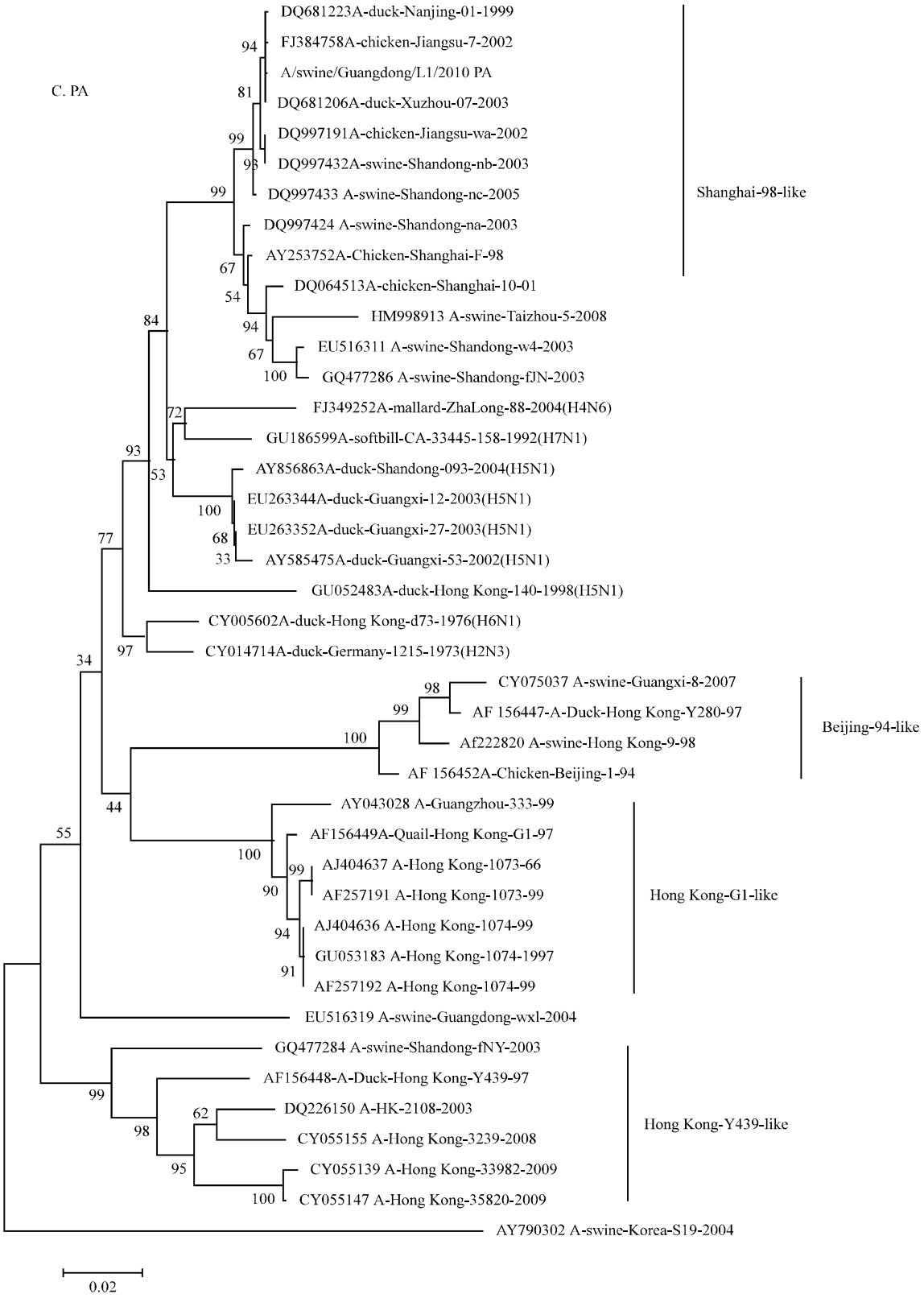

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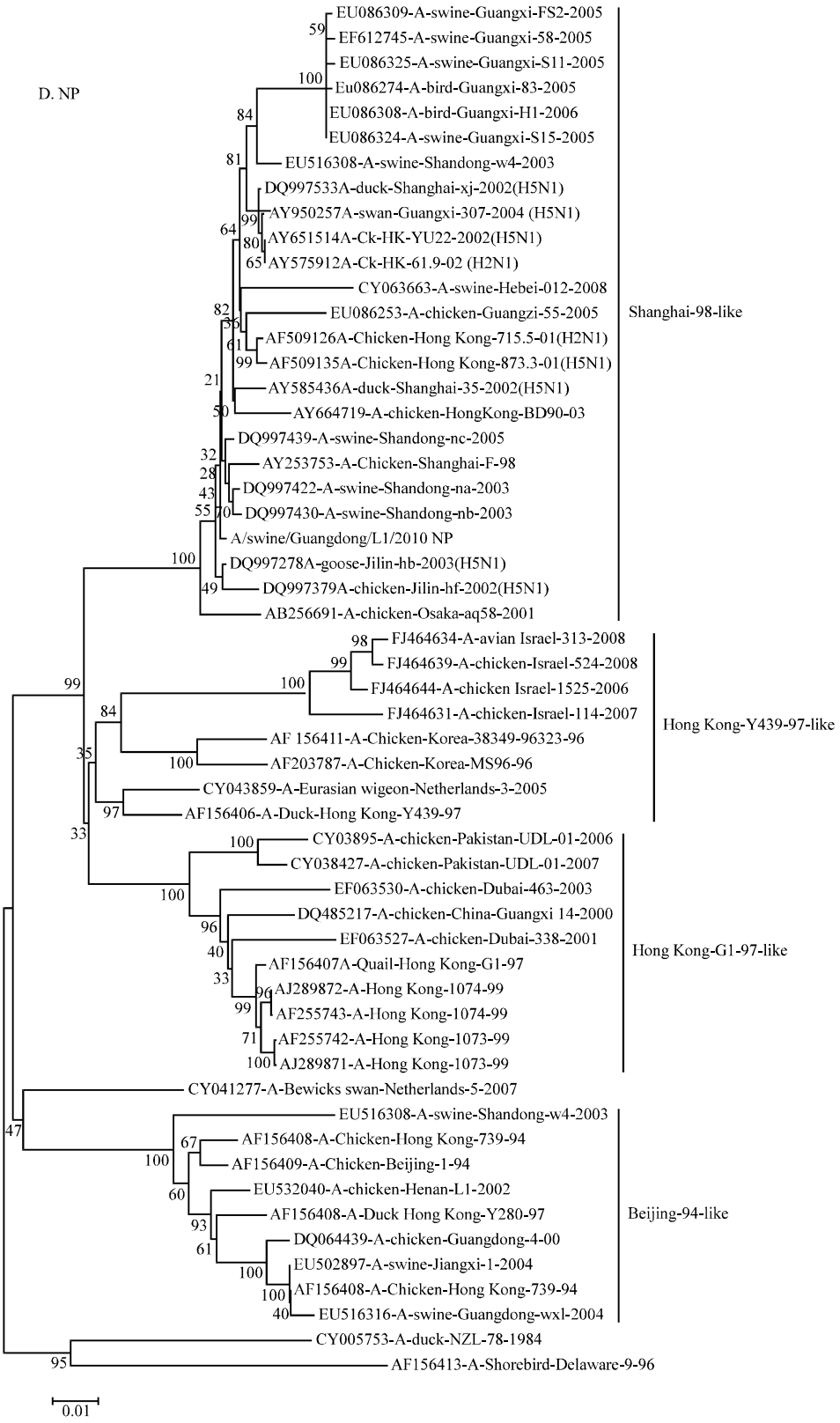

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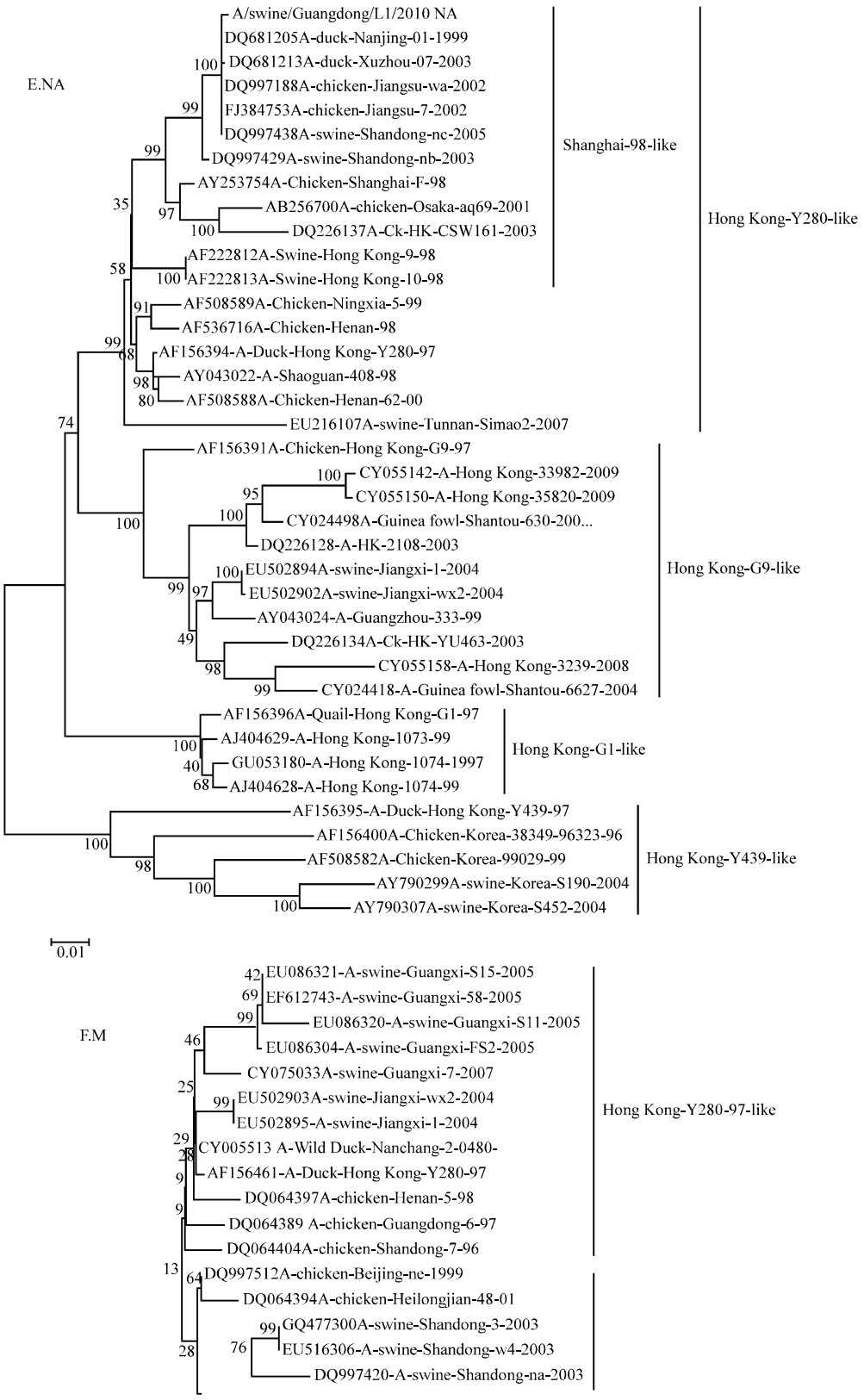

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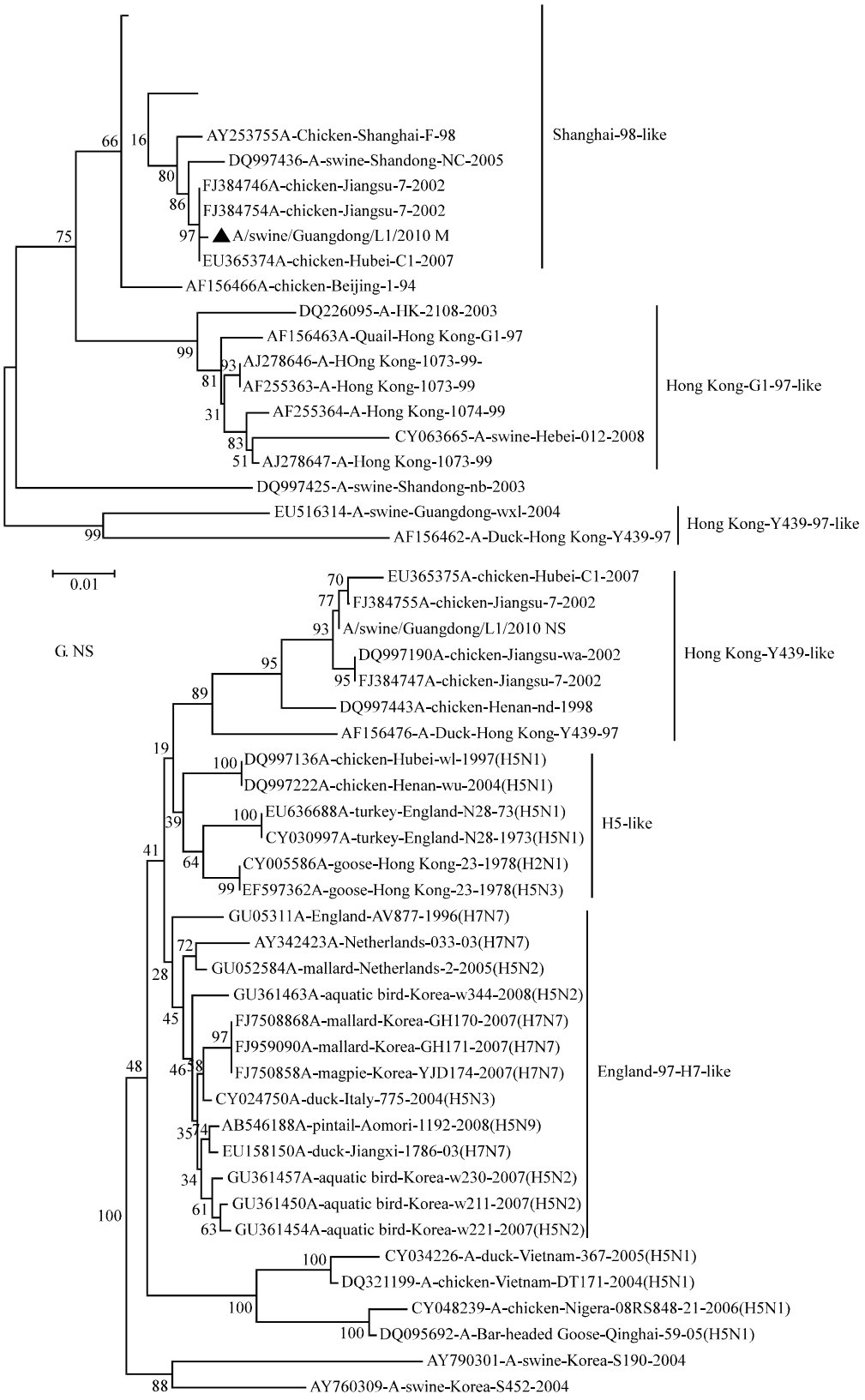

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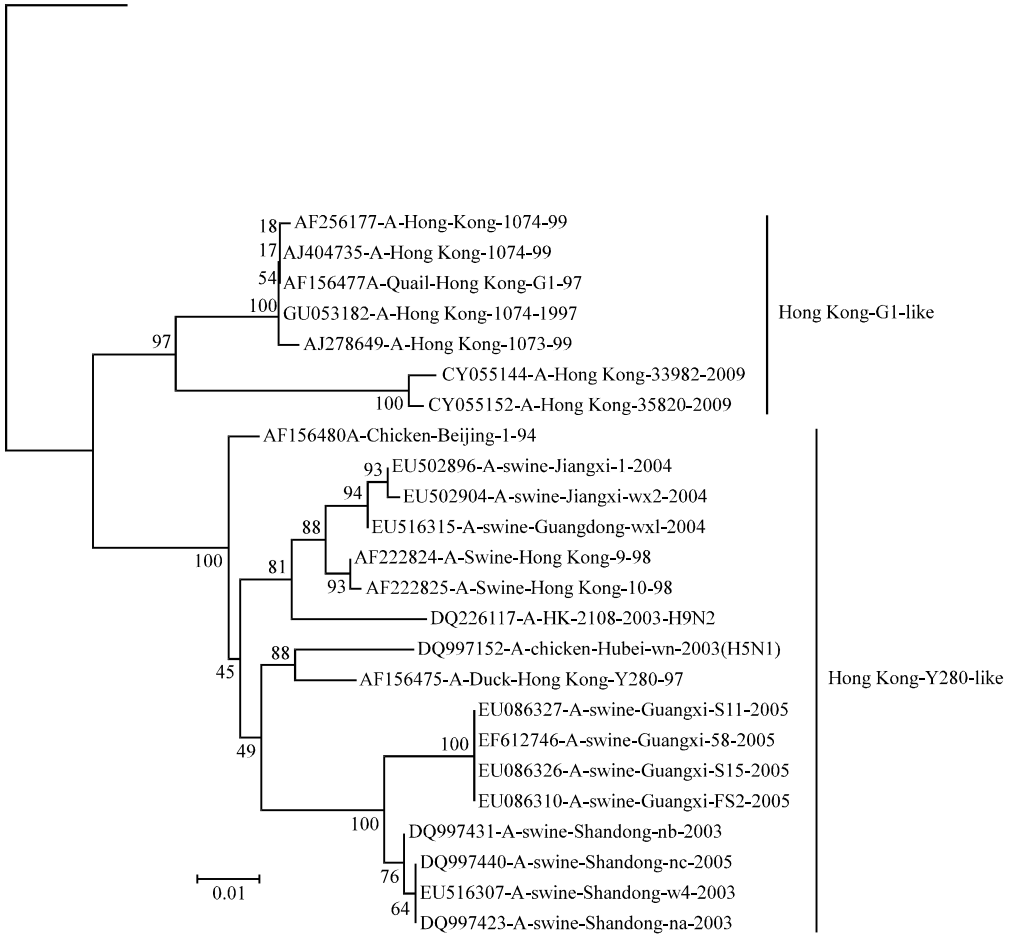

Fig. 1: Phylogenetic relationships of the *HA* gene of A/swine/Guangdong/L1/2010(H9N2) compared to genetically related influenza viruses. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Vertical distances are for spacing branches and labels. The phylogenetic trees were generated by using the neighbor-joining alogrithm. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Phylogenetic trees for H9N2 swine influenza virus isolated from southern China; a) PB2; b) PB1; c) PA; d) NP; e) NA; f) M and g) NS

Phylogenic analysis was based on nucleotides 100-1,435 (1,336 bp) of the *HA* gene, 40-1,352 (1,313 bp) of NA, 129-2,122 (1,994 bp) of PB2, 234-2,034 (1,801 bp) of PB1, 154-2,034 (1,989 bp) of PA, 79-1,410 (1,332 bp) of NP, 34-1007 (974 bp) of M and 50-872 (823 bp) of NS. Multiple alignments were constructed by using the ClustalW multiple alignment program of the software BioEdit (Version 7.0.5.3).

Phylogenic trees were generated by neighbor-joining bootstrap analysis (1,000 replicates) using the Tamura-Nei algorithm in MEGA Version 4.0. The nucleotide sequences for each gene segment of the eight H9N2 viruses were aligned with ClustalW which showed that the corresponding gene segments of the eight viruses had high nucleotide similarity (Table 1).

Table 1: Sequence homology of each gene from A/Swine/Guangdong/L1/2010(H9N2) and reference virus sequences available in GenBank

Genes	Region	Virus with the highest degree of homology	Nucleotide sequence identity (%)
HA	100-1435	A/chicken/Jiangsu/7/2002 (H9N2) [FJ384759]	99.7
NA	40-1352	A/chicken/Jiangsu/7/2002 (H9N2)[FJ384753]	99.9
PB2	129-2122	A/chicken/Jiangsu/7/2002 (H9N2) [FJ384748]	99.1
PB1	234-2034	A/swine/Shandong/nb/2003(H9N2) [DQ997426]	99.8
PA	154-2034	A/duck/Xuzhou/07/2003(H9N2) [DQ681206]	99.9
NP	79-1410	A/goose/Jilin/hb/2003(H5N1) [DQ997278]	99.6
M	34-1007	A/chicken/Hubei/C1/2007(H9N2) [EU365374]	99.8
NS	50-872	A/chicken/Jiangsu/7/2002(H9N2) [FJ384755]	99.9

RESULTS AND DISCUSSION

To investigate all of the detailed genetic characteristics, there was compared the deduced amino acid sequences of the Hemagglutinin (*HA1*) gene from the swine H9N2 isolates against the representatives of three lineages (avian, swine and human) available in GenBank. The cleavage site motif of the virus was PARSSR/GL which was the same with A/chicken/Beijing/1/1994 and A/HongKong/1074/99b (Table 2) and had an amino acid difference from cleavage site sequence of Guangxi-like swine influenza and Shandong-like swine influenza virus (Shi *et al*., 2008). The receptor-binding site was also analyzed in cluding the amino acids at position 183, 190, 226, 227 and 228. The 183 of receptor-binding site of A/swine/Guangdong/L1/2010 was aspartic acid which have high conservative with other verses with the exception of Quail/HK/G1/97, DK/HK/Y439/97(Guan *et al*., 1999) and Hong Kong human influenza virus. There have different amino acids at position 190. Judging from the 226 and 228 of the virus, the A/swine/Guangdong/L1/2010 confers receptor specificity similar to avian influenza virus.

Because the glycosylation sites can impact the function of HA, reasearchers analyzed the potential glycosylation sites of the H9N2 virus. There are seven sites (N-X-T/S in which X may be any amino acid except proline) in HA, six were located in HA1 and two sites were in HA2. The 11, 123, 280, 287 and 474 positions of potential glycosylation sites were highly conserved compared with other H9 virus.

Table 2: Amino acids at the cleavage site and the receptor-binding sites of HA of H9N2 viruse

Viruses*	Amino acid sequence at cleavage site	Amino acid residue** 183	190	226	227	228
SW/GD/L1/2010	PARSSR/GL	N	V	Q	Q	G
SW/GX/58/2005	PARASR/GL	N	T	Q	Q	G
SW/GX/FS2/2005	PARASR/GL	N	A	M	Q	G
SW/GX/S15/2005	PARASR/GL	N	V	Q	Q	G
SW/GX/S11/2005	PARASR/GL	N	T	Q	Q	G
SW/SD/1/2003	PARLSR/GL	N	A	L	Q	G
SW/GD/WXL/2004	PARSSR/GL	N	A	Q	Q	G
CK/HB/C1/2007	PARSSR/GL	N	V	Q	Q	G
CK/JS/7/2002	PARSSR/GL	N	V	Q	Q	G
CK/HB/4/2008	PARSSR/GL	N	V	Q	Q	G
CK/BJ/1/94	PARSSR/GL	N	V	Q	Q	G
DK/NJ/01/1999	PARSSR/GL	N	T	Q	Q	G
DK/ZJ/03/2001	PARSSR/GL	N	T	Q	Q	G
Quail/HK/G1/97	PARSSR/GL	H	E	L	Q	G
DK/XZ/07/2003	PARSSR/GL	N	V	Q	Q	G
DK/HK/Y439/97	PAASNR/GL	H	E	Q	Q	G
DK/HK/Y280/97	PARSSR/GL	N	T	L	Q	G
CK/SH/F/98	PARSSR/GL	N	A	Q	Q	G
HK/1073/97	PARSSR/GL	H	E	L	Q	G
HK/1074/99	PARSSR/GL	H	E	L	Q	G
HK/1073/99	PARSSR/GL	H	E	L	Q	G
HK/3239/2008	PSRSSR/GL	N	A	L	Q	G

*CK: Chicken; DK: Duck; SW: Swine; BJ: Beijing; XZ: Xuzhou; JS: Jiangsu; NJ: Nanjing; ZJ: Zhejiang; GX: Guangxi; HK: Hong Kong; HB: Hubei; SD: Shandong; SH: Shanghai; GD: Guangdong. **Numbered according to H3 HA numbering. Amino acids at positions H183, E190, Q226, Q227 and G228 of the receptor-binding sites were consider conserved at these positions in the avian virus consensus sequence

Analysis of the deduce NA amino acid, a deletion (62-64aa) was found in the virus which was the same with Shandong-like swine influenza virus and A/chicken /shanghai/F /98 virus. Although, we still do not know what the deletion will have an affect on the virus, a shorter NA stalk is in contact with increased virulence in poultry (Deshpande *et al*., 1985). The three amino acid deletion lead to the loss of one potential glycosylation site (Asn61), other potential glycosylation sites were retained. On the basis of the inferred phylogenies for this virus, using previously described H9 sequences and according to the recent classification for H9 subtype proposed by (Liu *et al*., 2009).

The virus identified in this study was grouped in H9.4.2 lineage which resides in the same clade with A/chicken/Beijing/94 and A/Guangzhou/333/99 along with A/chicken/Shanghai/F/98. Sequence homology with A/chicken/Jiangsu/7/2002 was found to be 99.7%. It still has a high homology with A/chicken/Beijing/94 (96.6%) which can cause a high pathogenicity to poultry. A/chicken/Hebei/4/2008 can induce acute respiratory distress syndrome in mice (Deng *et al*., 2010) which has a high homology (92.7%) with the virus.

The phylogeny of the *NA* genes showed that the SW/GD/L1/2010 was close to Shanghai/98-like lineage. Compared with other sequence of GenBank, the NA has a most high homology with A/chicken/Jiangsu/7/2002, with 99.9%. It has low homology with HK/Y439-like lineage. Phylogenetic trees of the nucleotide sequences of PB2, PA, NP and M were clustered to Shanghai/98 lineage; PB1 was grouped in Beijing/94 lineage; NS was likely originated from Hong Kong/Y439 lineage (supplemental material).

In summary, the H9N2 virus was a multiple reassortant virus. All the eight genes were originated from avian origin. Hong Kong/G1-like and Beijing/94-like viruses have been prevalent mainly in quails and chickens in southern China in 1990s (Guo *et al*., 2000; Xu *et al*., 2007). Shanghai/98-like viruses are a natural reassortant virus which was different from Hong Kong/G1-like and Beijing/94-like viruses, establishing an independent Shanghai/98-like clade in the phylogenetic tree (Lu *et al*., 2005). Hong Kong/Y439-like viruses were show up in china. The H9N2 swine influenza virus of the study was generated by gene reassortment of Beijing/94 lineage, Hong Kong/Y439 lineage and Shanghai/98 lineage. The other H9N2 swine influenza viruses as previously reported did not include the Hong Kong/Y439 lineage.

This reassortant H9N2 swine influenza virus 1st appears in china. Although, most H9N2 virus show a low pathogenicity in poultry, pigs and human, it can cause the production loses to agriculture. So, far pigs are reared in abundance and being the major source of protein for an increasingly affluent population are raised in increasing numbers. Swine influenza already circulated in pig population and induced severe damage. Just as FMD infected pigs in southern China, we must be on the alert. Swine flu vaccine did not yet apply to pigs of China but avian flu vaccine was used in Chinese poultry in long run. Maye this makes the influenza virus genic mutation or reassortant to resist immune selective pressure. It is probable that avian influenza virus crossed the interspecies barrier to pigs even human. In addition, Pigs can therefore function as intermediate hosts or mixing vessels in establishing new influenza virus lineages by supporting coinfection, replication and reassortment among human, avian and swine influenza viruses (Peiris *et al*., 2001; Brown, 2000). The finding provides further evidence about the interspecies transmission of avian influenza viruses to pigs and emphasizes the importance of reinforcing swine influenza virus surveillance, especially before the emergence of highly pathogenic FMD in pigs in southern china. In view of the complicated environment of China, serological testing and epidemiological surveillance will be needed.

Nucleotide sequence accession numbers: Nucleotide sequences from the A/Swine/Guangdong/L1/2010(H9N2) isolate have been submitted to GenBank with accession numbers HQ893759-HQ893766.

CONCLUSION

In this study, the finding provides further evidence about the interspecies transmission of avian influenza viruses to pigs and emphasizes the importance of reinforcing Swine Influenza Virus (SIV) surveillance, especially before the emergence of highly pathogenic FMDs in pigs in Guangdong.

ACKNOWLEDGEMENT

This study was supported by the National Basic Research Programe (973 project) from China Ministry of Science and Technology (Grant No. 2011CB504703-1) and International Cooperative Projects (Grant No. S2011ZR0429) and China Guangdong hundred thousand engineering.

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