Acta virologica **55**: 219 – 226, 2011 doi:10.4149/av_2011_03_219

Molecular characterization and pathogenicity of swine influenza H9N2 subtype virus A/swine/HeBei/012/2008/(H9N2)

ZHANG RUI-HUA[1], CUI HONG-YU[1], XU MING-JU[1], LI KAI[1], CHEN HUA-LAN[2], WANG CUN-LIAN[1], WEI DONG[1], LI CUN-XIN, XU TONG[1*]

[1]Department of Veterinary Medicine, College of Animal Science, HeBei North University, Zhangjiakou,075131, Hebei, P.R. China; [2]China Animal Influenza Laboratory of Ministry of Agriculture, National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute of CAAS, Harbin 150001, P.R. China

Received November 15, 2010; accepted August 4, 2011

Summary. – The H9N2 subtype influenza virus (IV) is a remarkable member of the influenza A viruses because it can infect not only chickens, ducks and pigs, but also humans. Pigs are susceptible to both human and avian influenza viruses and have been proposed to be intermediate hosts for the generation of pandemic influenza viruses through reassortment or adaptation to the mammalian host. To further understand the genetic characteristics and evolution, we investigated the source and molecular characteristics of the H9N2 subtype swine influenza virus (SIV), and observed its pathogenicity in BALB/c mice. The BALB/c mice were inoculated intranasally with 100 median mouse infectious dose of A/swine/HeBei/012/2008/(H9N2) viruses to observe the pathogenicity. The HA, NP, NA and M gene were cloned, sequenced and phylogenetically analyzed with related sequences available in GenBank. The infected mice presented with inactivity, weight loss and laboured respiration, while the pathological changes were characterized by diffuse alveolar damage in the lung. The nucleotide and deduced amino acid sequence of HA, NP, NA and M gene was similar with that of A/chicken/Hebei/4/2008(H9N2). The HA protein contained 6 glycosylation sites and the motif of HA cleavage site was PARSSR GLF, which is characteristic of low pathogenic IV. In the HA, NP, M and NA gene phylogenetic trees, the isolate clustered with A/chicken/Hebei/4/2008(H9N2). The isolate possibly came from A/chicken/Hebei/4/2008(H9N2) and was partially varied during its cross-species spread.

Keywords: swine influenza virus; H9N2 subtype; sequence analysis; pathogenicity

Introduction

The RNA genome of SIV is divided into eight segments numbered one to eight. Segments number 4, 5, 6, and 7 encode HA, NP, NA, and M, respectively. The other segments and genes are important for other parts of the virion structure or function (Yin and Liu, 1997; Brown, 2000). Since each segment functions as individual gene coding for one of the viral proteins, it enables a mutation to easily jump to another subtype. Among all eight genes, the HA and NA genes of influenza A virus mutate at highest frequencies (Fitch *et al*., 1997, 2000). It has been generally believed that influenza adaptation to a new host requires a long evolutionary process (Webster *et al*., 1992; Katz, 2003; Peiris *et al.,* 2007), but the IV spread from poultry and human to swine has occurred under the natural conditions (Peiris *et al*., 2001; Xu *et al*., 2004). Therefore, IV might spread among different species, even resulting in death of the infected host (Kendal *et al*., 1977; Wentworth *et al*., 1994, 1997). It was reported that the SIV virus spread to humans and led to illness and death in 1976 (Top and Russell, 1977), this strain of the IV has been spreading among humans, swine and poultry. The swine is considered as the

*Corresponding author. E-mail: xutong1969@sohu.com; fax: +86-313-4029336.

Abbreviations: EID_{50} = median egg infectious dose; IV = influenza virus; SIV = swine influenza virus; SIV H9N2 = swine influenza H9N2 subtype virus; p.i. = post inoculation

mixer for producing new virus by gene rearrangement of human, poultry and swine influenza (Scholtissek, 1990). In recent years, the increasing number of infection and death cases caused by H5N1, H9N2, and H7N7 virus in humans has brought public interest to the IV genetics and variation in different host species.

The frequent occurrence of influenza outbreaks among humans, swine, and birds has not only caused a huge economic loss but also posed a severe threat to human health. Due to low pathogenicity of H9N2, the studies of influenza currently focus mostly on the highly pathogenic influenza viruses, such as H5N1. However, the novel H1N1 IV, which HA cleavage site appears as low virulence, emerged in humans in Mexico in early 2009 (Brown *et al*., 1998). As of June 18, 2010, the World Health Organization (WHO) had announced that the H1N1 influenza cases have been found in 214 countries and regions worldwide, and more than 18,336 patients died from influenza A/H1N1 (WHO, 2009). The epidemic of H1N1 influenza has alarmed the world: SIV with low virulence may infect humans and cause serious public health problems. Therefore, it is urgent to further supervise the epidemic and molecular evolution of SIV. H9N2 SIV was isolated from the Hebei province of China in 2008. The genes were cloned, sequenced, phylogenetically analyzed and compared with other sequences available in GenBank. In addition, the pathogenicity in mice was investigated, with the objectives to understand the molecular evolution characteristics and pathogenic condition of mammals, and to facilitate studies of the pathogenesis of H9N2 swine influenza disease in animals and humans.

Materials and Methods

Bacterial strains and experimental animals Escherichia coli DH5a were conserved by Microbiology Lab of Hebei North University; SPF female BALB/c mice (6–8-week-old) and SPF chicken embryos come from Beijing Laboratory Animal Research Center, Beijing, P.R. China.

Virus isolation and identification Total 20 nasal swabs and 12 materials of lung and trachea were obtained from pigs suspected to die of swine influenza in 2008. The samples processed by conventional method were inoculated into 10-day-old chicken embryos (0.1 ml/embryo). Inoculated embryos were incubated at 37°C for 96 hrs. The allantoic fluid was then harvested and tested for hemagglutination activity. If the allantoic fluid showed hemagglutination activity, the infuenza virus subtype was identified by China Animal Influenza Laboratory of Ministry of Agriculture (Harbin, P.R. China) by means of hemagglutination inhibition and neuraminidase inhibition tests.

The pathogenicity of H9N2 isolate in BALB/c mice Six- to eight-week-old female specific pathogen-free BALB/c mice were divided into 3 groups of 16 mice each. During the experiment, animals had access to food and water ad libitum. Sixteen mice in the first group were all inoculated with A/swine/HeBei/012/2008/(H9N2) virus, which had been diluted 1:10 by sterile saline, by the natural routes of infection (50 μl, intraocular and intranasal). Eight mice in the second group were inoculated in the same way as the first group. The remaining 8 mice were not infected, but lived together with the H9N2-infected mice, in order to investigate whether the virus was transmitted from the inoculated mice to the neighbors by direct contact. The mice of the third group received diluted noninfectious allantoic fluid in place of the virus and served as mock-infected controls. All manipulations were performed under bio-safety level 3+ laboratory conditions. The study was approved by the Animal Care Committee of Hebei North University (Zhangjiakou, Hebei Province, P.R. China).

The clinical signs in infected mice were observed daily. Eight mice in the first and third group were monitored daily for morbidity, as measured by weight loss, and mortality for 14 days post inoculation (p.i.). The infected mice were necropsied timely and gross pathologic changes were observed when they died. Two of the remaining mice in the first group and living together with infected mice in second group were sacrificed on days 2, 6, and 14 p.i. The main organs were collected for virus isolation, histopathological observation, and demonstration of SIV by immunohistochemical methods.

Virus titration Virus titration was performed as previously described (Lu *et al.,* 1999). The lungs, brains, spleens, kidneys, livers, and hearts were collected, weighed, and homogenized by using a mortar and pestle in cold PBS plus antibiotics on days 2, 6, and 14 p.i. Clarified homogenates were titrated for viral infectivity in embryonated chicken eggs from initial dilutions of 1:10 (lung) or 1:2 (other organs). Viral titers were expressed as mean $\log_{10}$ EID_{50}/gram.

Histopathologic and immunohistochemical analysis Tissues were fixed in 10% neutral buffered formalin solution, sectioned, and stained with hematoxylin-and-eosin. Duplicate sections were processed for immunohistochemistry by using biotin-streptavidin method essentially as described previously (Zaki *et al.*, 1995). A monoclonal antibody to influenza A nucleoprotein was used as the primary antibody.

RT-PCR Viral RNA was extracted from the infected allantoic fluid of SPF embryos using the RNA-SOLV® reagent RNA isolation solvent (Omega Bio-teck, Lilburn, GA) according to the manufacturer´s instructions. The HA, NA, NP, and M genes were RT-PCR amplified with specific primers for IV of H9 subtype. The primers were as follows:

HA F: 5′-AGT AGT ATC ACT AAT AAC TAT AAT AC-3′ and HA R:5′-AGG CGA CAG TCG AAT AAA TGG TGA GG-3′;

NA F: 5′-GCA GGA GTG AAC ATG AAT CCA AAT C-3′ and NA R: 5′-ATT GCG AGA GCT TAT ATA GGC ATG AAG-3′;

NP F: 5′-CCG AGT GAC ATC AAC ATC ATG ACG TCT C-3′ and NP R: 5′-TCT CTA ATT GTC ATA CTC CTC TGC ATT G-3′;

M F: 5′-TTA TTA CTC CAG CTC TAT CTT GAC-3′ and M R: 5′-GAA AGA TGA GCG TTC TAA CCG AGG-3′

The RT-PCR amplification was performed with the Onestep RNA PCR Kit (AMV) (TaKaRa, Dalian, P.R. China).

RT-PCR product cloning, identification and sequencing The RT-PCR products were separated in a 0.8% agarose gel by electrophoresis, and amplicons of the appropriate sizes were purified using an Agarose Gel DNA extraction kit (Shanghai Sheng Gong Bioengineering Co., Ltd., Shanghai, P.R. China). The RT-PCR products were cloned into the pMD-18T carrier (Takara, Dalian, P.R. China) and transformed into *E. coli* DH5α. Positive plasmid were sequenced on a ABI Prism® 377XL DNA sequencer.

Sequence analysis and phylogenetic analysis Besides the isolated viruses described above, we also utilized the sequences of other H9N2 viruses that were deposited in GenBank. The homology analyses of nucleotide and deduced amino acid sequence for HA, NA, NP, M gene were performed using DNAMAN Sequence Analysis Software package (Lynnon Biosoft, Quebec, Canada). The phylogenetic trees based on the nucleotide sequences were generated using the Software package described above.

Results

Virus isolation and identification

The total of 32 samples included nasal swabs and tissue materials. These were inoculated into SPF chicken embryos as well as consecutively blind passaged for 3 generations, and the allantoic fluid was collected for hemagglutination test. The allantoic fluids with the hemagglutination titers between 2^6 and 2^9 were selected, and the subtype was identified by China Animal Influenza Laboratory of Ministry of Agriculture. The isolate was then identified as swine influenza A H9N2 virus by means of hemagglutination inhibition and neuraminidase inhibition tests, and was designated as A/swine/HeBei/012/2008/ (H9N2).

The pathogenicity of the H9N2 isolate in BALB/c mice

All 8 observed mice in the first group presented inactivity, lethargy, ruffled fur, inappetence and weight loss. The weight of the infected mice decreased obviously, and was only 2/3 of the weight compared to that of the mock-infected mice (15.8 ± 1.5 vs. 21.3 ± 0.4 g) on day 6 p.i. with the A/swine/HeBei/012/2008/(H9N2) virus ($p < 0.01$). Three mice died between days 3 and 6 p.i. The H9N2-infected mice in the second group showed similar clinical signs as those in the first group from days 2 p.i, but the remaining 8 non-infected mice, living together with infected-mice, did not present abnormal signs; no mice in the control group showed abnormal symptoms.

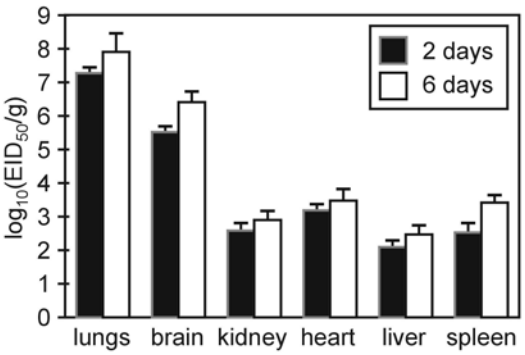

Fig. 1

Replication of influenza A (H9N2) virus in the tissues of mice infected with H9N2 virus

Mice were infected with A/swine/HeBei/012/2008/(H9N2) virus, tissues were collected on days 2 and 4 (b) p.i. and the virus was titrated in embryonated eggs. Viral titers are expressed as log10 EID_{50}/g. The limit of virus detection was ≤101.1 EID_{50}/g for all other tissues.

Replication of the H9N2 Virus in mouse tissues

We examined the kinetics of virus replication on days 2, 6, and 14 p.i in brain, lung, kidney, spleen, heart, and liver tissue following infection with the H9N2 isolate. As shown in Fig. 1, the H9N2 viral infection resulted in detectable virus in all the organs above on day 2 p.i. However, none of the H9N2 viruses could be detected in these tissues on day 14. Moreover, viral infection resulted in higher titers of the virus in the lungs than other organs on days 2 and 6 p.i. The peak viral titer appeared on day 6 p.i, reaching 7.2 $\log_{10}$ EID_{50}/g in the lungs. However, the virus had not been isolated from any organs of the mice in co-habitation infection group and the control group.

Gross and histopathological lesions in H9N2-infected mice

Gross observation of the infected mice demonstrated lungs to be highly edematous, with profuse areas of hemorrhage and congestion, and a lot of bloody foam-like liquid flowing out of the bronchus. The lung wet-weight of infected mice increased significantly ($p < 0.01$) and was 3–5 times that of normal lung wet-weight (0.1–0.2g/0.3–0.67g, normal/infected). Liver can be seen to have a slight congestion; heart, spleen, brain and kidney did not show apparent changes. The organs of the non-inoculated mice in co-habitation infection group were normal as well as those of the control group

Histopathological lesions mainly occurred in the lungs. It was characterized by diffuse alveolar damage in lung. In Fig. 2a: (1) there were a large number of red blood cells, inflammatory cells and edema fluid in the alveoli; Moreover, alveolar wall thickening and alveolar interstitial edema were

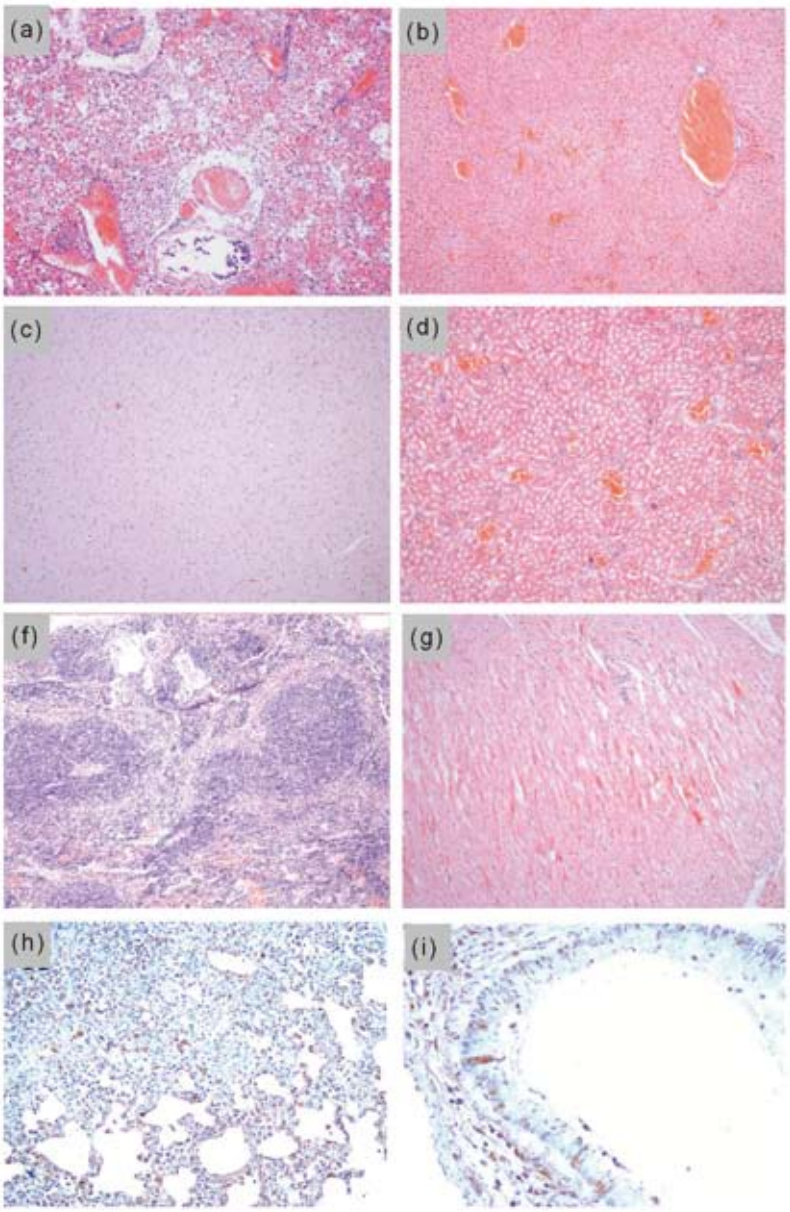

Fig. 2

Histolopathologic changes and immunostaining in tissues of mice infected with H9N2 virus

Tissues were processed for H&E (a, b, c, d, e, and g) or lung sections were stained by IHC methods to demonstrate SIV (h and i). (a) H&E staining of the lungs of H9N2-infected mice showing red blood cells, inflammatory cells and edema fluid in the alveoli; Edema and thickening of alveolar walls; Edema of alveolar wall interstitium; Blood vessels and trachea surrounded by inflammatory cells; (b) H&E staining of the liver tissues of H9N2-infected mice showing congestion, red blood cell deposited in the lobular central vein (*solid arrow*) and hepatic cord; (c) H&E staining of the brain tissues of H9N2-infected mice showing congestion (*solid arrow*); (d) H&E staining of the kidney tissues of H9N2-infected mice showing congestion (*solid arrow*), tubular epithelial cell swelling, lumen narrowing, tubular epithelium stretched in the lumen to form the apophysis; (f) H&E staining of the splenic tissues of H9N2-infected mice showing normal histology; (g) H&E staining of the heart of H9N2-infected mice showing Congestion and hemorrhage in myocardial fibers (*solid arrow*). (h) SIV antigen in the nuclei and cytoplasm of type II pneumocytes (*arrow*) and (i) bronchial epithelium (*solid arrow*). Magnifications: x100 (a), x100 (b), x100 (c), x100 (d), x100 (f), x100 (g), x100 (h), x200 (i).

present; (2) there was interstitial edema around small blood vessels, surrounded with a large number of inflammatory cells (open arrow); (3) small bronchial mucous epithelium dropout (solid arrow), and a large number of inflammatory cells were observed in the bronchial lumen. Immunohistochemical analysis demonstrated the presence of viral antigen

in nuclei and cytoplasm of type II pneumocytes and bronchial epithelium (Fig. 2h and i). The pathological changes in the liver showed different degrees of congestion. A number of red blood cells were found in the lobular central vein (solid arrow) and hepatic cord (Fig. 2b); The brain tissue presented mild congestion (Fig. 2c); Kidney showed congestion (solid arrow), tubular epithelial cell swelling, lumen narrowing (Fig. 2d); Splenic white pulp and red pulp were normal (Fig. 2f); Mild congestion and hemorrhage could be seen in myocardial fibers (Fig. 2g).

Cloned and sequenced HA, NA, NP, and M gene

HA, NA, NP, and M genes were amplified from A/swine/HeBei/012/2008/(H9N2) SIV, cloned into pMD-18 T vector, and sequenced. The nucleotide sequence data from this study were deposited in the GenBank (the accession numbers are CY063662, CY063664, CY063663, and CY063665).

The sequence analysis of HA, NA, NP, and M gene HA

The proteolytic cleavage site was RSSR↓GLF, representing low pathogenicity, and differed from the sequence RERRRKK↓GLF characteristic for the high pathogenicity. Analysis of the potential glycosylation sites in the HA of the H9N2 virus isolates revealed six sites. Glycosylation sites 1 (aa 27 to 29), 2 (aa 80 to 82), 3 (aa 139 to 141), 4 (aa 296 to 298), 5 (aa 303 to 305), 6 (aa 490 to 492) were: NST, NPS, NVS, NTT, NVS, and NGT, respectively. The nucleotide sequence and deduced amino acid sequence of the HA gene was compared for sequence identity with the HA gene sequences of representative H9N2 strains from GenBank. The nucleotide sequence of HA gene from this strain shared 99% sequence identity with the HA gene from A/chicken/Hebei/4/2008(H9N2), and 83% sequence identity with the HA gene from A/mallard/Switzerland/WV3080036/2008(H9N2). The deduced amino acid sequence of the HA gene from this strain shared 99% sequence identity with the HA gene from A/chicken/Hebei/4/2008(H9N2), and 90% sequence identity with the HA gene from A/mallard/Switzerland/WV3080036/2008(H9N2). The HA phylogenetic tree is shown in the figure (Fig. 3 HA) and shows that the HA gene of this H9N2 virus shared the same clade with A/chicken/Hebei/4/2008(H9N2).

NA

ORF including the start codon and stop codon of NA was determined to encode 467 amino acids. Analysis of the potential glycosylation sites in the NA of the H9N2 virus isolates revealed five sites. Glycosylation sites 1 (aa 44 to 46), 2 (aa 66 to 68), 3 (aa 83to 85), 4 (aa 143 to 145), 5 (aa 231 to 235) were: NPS , NST, NWS, NGT, and NGT, respectively.

The homologies of the nucleotide sequence and deduced amino acid sequence of the NA gene from this H9N2 virus and the NA gene from A/chicken/Hebei/4/2008(H9N2) and A/chicken/korea/38349-p96323/96(H9N2) were 86%–99% and 98%–100%, respectively. The NA phylogenetic tree is shown in the figure (Fig. 3 NA). The NA gene of this H9N2 virus was closely related to the A/chicken/Hebei/4/2008(H9N2), and unrelated to the A/chicken/korea/38349-p96323/96.

NP

ORF of the NP was determined to encode 498 amino acids. The nucleotide sequence and deduced amino acid sequence of NP gene from this strain both shared 99% sequence identity with the NP gene from A/chicken/Hebei/4/2008(H9N2), and 95% and 98%, sequence identity with the NP gene from A/swine/Guangxi/S11/2005(H9N2), respectively. The NP phylogenetic tree is shown in the figure (Fig. 3 NP). The NP gene of this H9N2 virus shared the same clade with the A/chicken/Hebei/4/2008(H9N2).

M

The matrix protein (M) gene encodes two viral proteins, M1 and M2. M1 coding region was 6–764 bp and was determined to encode 252 amino acids. M2 coding region containing 2 parts: region 6–31 bp and region 730–987 bp was determined to encode 97 amino acids following RNA splicing. M2 protein has amino acids (Thr11, Gly14, Glu16 Ser20, Tyr57) (Zhou *et al.,* 1999), which have poultry IV character. The nucleotide sequence and deduced amino acid sequence of M gene from this strain both shared 99% sequence identity with the M gene from A/chicken/Hebei/4/2008(H9N2), and 93% and 94% sequence identity with the M gene from A/swine/Guangxi/S11/2005(H9N2), respectively. The phylogenetic tree of the M protein is shown in the figure 3 (Fig. 3 M). The M gene of this H9N2 virus was closely related to that of A/chicken/Hebei/4/2008(H9N2).

Discussion

The investigation of IV demonstrates that the HA homology in the same subtype is about 80%–100%, and the inter-subtype homology is below 68.5% (Nobusawa *et al.,* 1991). The HA gene from A/swine/HeBei/012/2008/(H9N2) in this study shared 99% sequence identity with the HA gene from A/chicken/Hebei/4/2008(H9N2), which suggested that they were closely related to each other and may be derived from the same strain progenitors. But the HA gene from the strain in this study also shared 83% sequence identity with the HA gene from A/mallard/Switzerland/

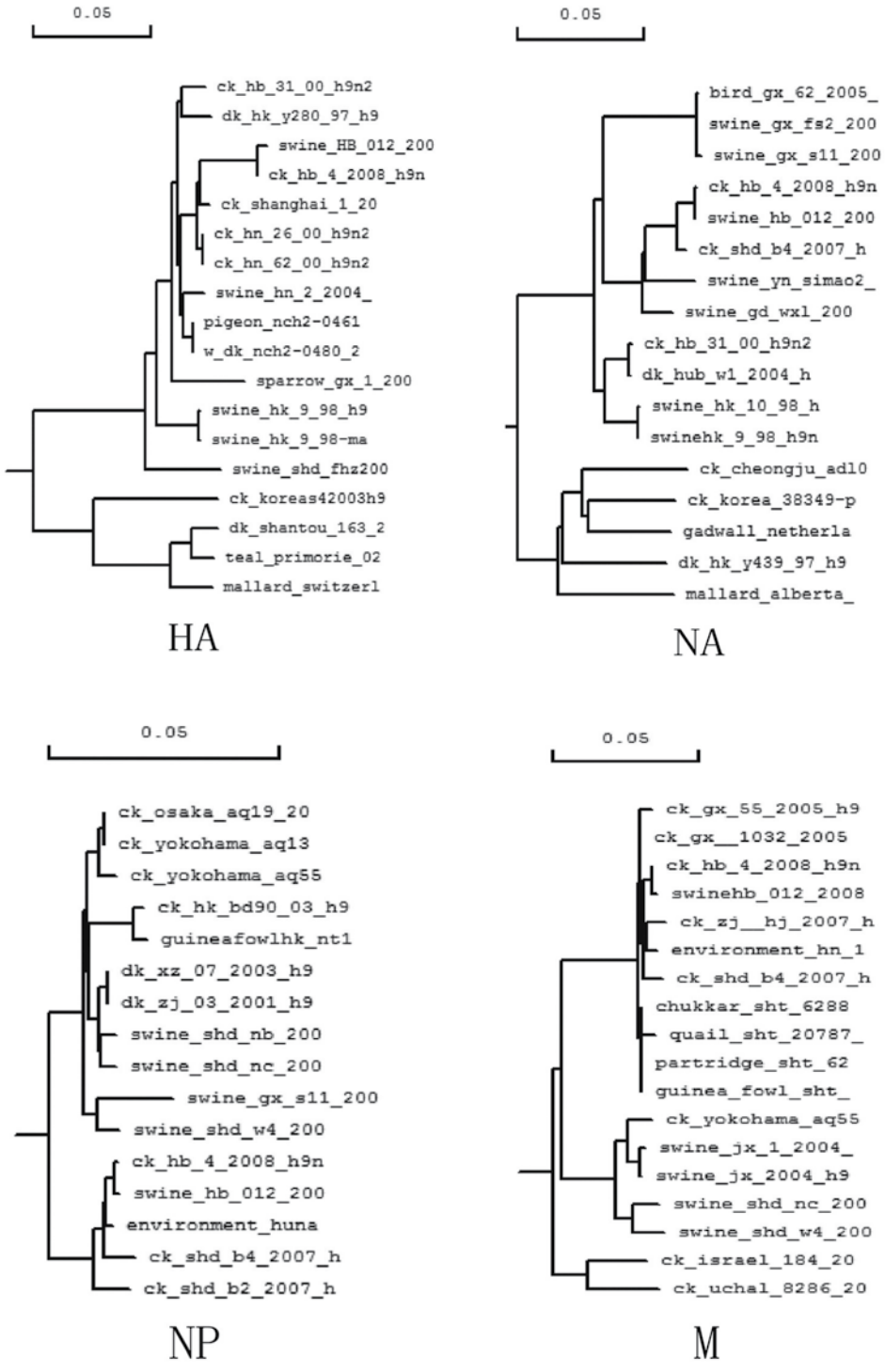

Fig. 3

Phylogenetic tree of the HA, NA, NP, and M gene of A/HeBei/012/2008/(H9N2)

Phylogenetic analysis of HA, NA, NP, and M gene of A/HeBei/012/2008/(H9N2) virus circulating in China. Reference strains were obtained from the GenBank. CK = chicken; DK = duck.

WV3080036/2008(H9N2). The representative sequences of H9N2 virus isolated from different hosts were downloaded from GenBank in order to investigate the origin and distribution of A/swine/HeBei/012/2008/(H9N2). The results of sequence analysis and phylogenetic trees show that the investigated H9N2 SIV was separated in different time and place then A/chicken/Hebei/4/2008(H9N2), A/duck/hongkong/y280/97(H9N2), A/pigeon/nangchang/2-04612000(H9N2) and A/sparrow/guangxi/1/2005 (H9N2). As the A/swine/HeBei/012/2008/(H9N2) virus is closely related to these isolates, it can be concluded that the H9N2 influenza viruses from different hosts may have the same origin and have no obvious regional characters. This also proves the notion that IV can spread across species. It also suggests that the virus isolated far away from the prevailing area may have the same origin. However, the variation may occur during its spread, because the virus always tends to avoid the hosts' immune system (Brown, 2000). Therefore, the cross-species spread increases the possibility of variation and improves the variation capability of the virus antigen, which makes it more difficult to prevent and control the influenza.

The motif of the HA cleavage site is PARSSR GLF, which is characteristic of low pathogenic IV. Our data indicates that A/swine/HeBei/012/2008/(H9N2) virus HA has a substitution G226Q. This altered HA doesn´t exhibit the characteristics of avian IV HAs (Vines *et al*., 1998). This mutation may occur during the virus transmission from birds to swine and the adaptation to the new host. However, the amino acid residues (Thr11, Gly14, Glu16, Ser20, Tyr57) related to host specificity of matrix protein 2 were all avian-specific, suggesting the mutation did not occur in related regions. It also indicates that the mutation rate of the external protein HA is higher than that of the internal protein M. It is consistent with the results of the nucleotide homology analysis. HA protein is an important external glycoprotein of influenza virus, which plays an important role in its virulence, host specificity and immune response. The increasing or decreasing number of glycosylation sites can affect the characteristics of virus antigen, receptor binding site and biology (Meng *et al*., 2009). The analysis of the deduced glycosylation sequence indicated that some glycosylation sites were lost in HA of A/swine/HeBei/012/2008/(H9N2). The lost glycosylation sites might have influence on the biological character of the virus, which needs to be further investigated in the future.

BALB/c mice have been successfully used for evaluation of pathogenesis, immunization and antiviral drugs to the H5N1 IV, indicating that BALB/c mice are a suitable animal model to study the highly pathogenic H5 virus (Xu *et al*., 2006, 2009). Because of its low pathogenicity, there are few studies on pathogenicity of H9N2 IV in the BALB/c mouse model. Therefore, the pathogenicity of H9N2 SIV in mice was also investigated in this study. The results showed that A/swine/HeBei/012/2008/(H9N2) virus not only could infect BALB/c mice but also was highly pathogenic to them. However, this result is in contrast with the theory of low pathogenicity due to a specific HA protein cleavage site. The difference may be due to the molecular pathogenesis of IV in poultry and mammals. Moreover, the novel H1N1 flu, that first emerged in Mexico early in 2009, and quickly spread throughout nearly the whole the world, was also confirmed to be a low pathogenic virus by the analysis of the HA cleavage site motif. However, the strain caused fatal infections in some individuals, and resulted in death of thousands of patients. In this study we found that the pathogenicity of A/swine/HeBei/012/2008/(H9N2) virus in mice is in a way similar to the novel H1N1 virus. Because of the fatal H9N2-SIV infections in mice, it may potentially become a virus causing human infection. Therefore, further investigation of the isolate's pathogenesis in mammals has to be conducted in the future. Combined with the sequencing results of A/swine/HeBei/012/2008/(H9N2) HA, NA, NP and M and the phylogenetic tree, it can be found that the virus strain used in this study is closely related to the A/chicken/Hebei/4/2008(H9N2) virus and may be derived from the same strain progenitor. Therefore, it can be concluded that A/swine/HeBei/012/2008/(H9N2) may come from H9N2 avian IV and was partially varied during cross-species spread.

Acknowledgements. This study was supported by the Natural Science Foundation of HeBei province of China (Grant No. C2009001028).

References

Brown IH (2000): The epidemiology and evolution of influenza viruses in pigs. Vet. Microbiol. 4, 29–46. doi.org/10.1016/S0378–1135(00)00164–4

Brown IH, Harris PA, McCauley JW, Alexander DJ (1998): Multiple genetic reassortment of avian and human influenza A viruses European pigs, resulting in the emergence of an H1N2 virus of novel genotype. J. Gen. Virol. 79, 2947–2955

Fitch WM,Bush RM,Bender CA,Cox NJ (1997): Long term trends in the evolution of H (3) HA1 human influenza type A. Proc. Natl. Acad. Sci. USA 94,7712–7718. doi.org/10.1073/pnas.94.15.7712

Fitch WM,Bush RM,Bender CA, Subbarao K, Cox NJ (2000): Predicting the evolution of human influenza A. J. Hered. 91, 183–185. doi.org/10.1093/jhered/91.3.183

Katz JM (2003): The impact of avian influenza viruses on public health. Avian Dis. 47 (Suppl.) 914–920. doi.org/10.1637/0005–2086–47.s3.914

Kendal AP, Goldfield M, Noble GR, Dowdle WR (1977): Identification and preliminary antigenic analysis of swine influenza–like viruses isolated during an influenza outbreak at Fort Dix, New Jersey. J. Infect. Dis. 136 (Suppl.),

S381–385. doi.org/10.1093/infdis/136.Supplement_3.S381

Lu X, Tumpey TM, Morken T, Zaki SR, Cox NJ, Katz JM (1999): A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. J. Virol. 73, 5903–5911

Meng XQ, Chen YX, Liu Q, Zheng M, Shi KC, Hu J (2009): Cloning and Sequence Analysis of the HA, NP, NA, M and NS Genes of H1N2 Swine Influenza Viruses. Acta Veterinaria et Zootechnica Sinica 40, 221–227 (in Chinese).

Nobusawa E, Aoyama T, Kato H, Suzuki Y, Tateno Y, Nakajima K (1991): Comparison of complete amino acid sequence and receptor-binding properties among 13 serotypes of hemagglutinin of influenza A virus. Virology 182, 475–485. doi.org/10.1016/0042–6822(91)90588–3

Peiris JS, Guan Y, Markwell D, Ghose P, Webster RG, Shortridge KF (2001): Cocirculation of avian H9N2 and contemporary "human" H3N2 influenza A viruses in pigs in southeastern China: potential for genetic reassortment? J. Virol. 75, 9679–9686. doi.org/10.1128/JVI.75.20.9679–9686.2001

Peiris JS, de Jong MD, Guan Y (2007): Avian influenza virus (H5N1): a threat to human health. Clin. Microbiol. Rev. 20, 243–267. doi.org/10.1128/CMR.00037–06

Scholtissek C (1990): Pig as the "mixing vessel" for the creation of new pandemic influenza A viruses. Med. Princ. Pract. 2, 65–271.

Top FH Jr, Russell PK (1977): Swine influenza A at Fort Dix, New Jersey (January–February 1976). IV summary and speculation. J. Infect. Dis. 136 (Suppl.), S376–380. doi.org/10.1093/infdis/136.Supplement_3.S376

Vines A, Wells K, Matrosovich M, Castrucci MR, Ito T, Kawaoka Y(1998): The role of influenza A virus hemagglutinin residues 226 and 228 in receptor specificity and host range restriction. J. Virol. 72, 7626–7631.

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y (1992): Evolution and Ecology of Influenza A Viruses. Microbiol. Rev. 56, 152–179.

Wentworth DE, Thompson BL, Xu X, Regnery HL, Cooley AJ, McGregor MW, Cox NJ, Hinshaw VS (1994):An influenza A(H1N1) virus, closely related to swine influenza virus, responsible for a fatal case of human influenza. J. Virol. 68, 2051–2058.

Wentworth DE, McGregor MW, Macklin MD, Neumann V, Hinshaw VS (1997):Transmission of swine influenza virus to humans after exposure to experimentally infected pigs. J. Infect. Dis. 175, 7–15. doi.org/10.1093/infdis/175.1.7

World Health Organization. Pandemic (H1N1) 2009 – update 110 Available from: http://www.who.int/csr/don/2010_07_23a/en/index.html (accessed July 2010).

Xu T, Qiao J, Zhao L, He G, Li K, Wang J, Tian Y, Wang H (2009): Effect of dexamethasone on acute respiratory distress syndrome induced by the H5N1 virus in mice. Eu. Respir. J. 33, 852–860. doi.org/10.1183/09031936.00130507

Xu, T, Qiao J, Zhao LH, Wang GR, He GM, Li K, Tian Y, Gao M, Wang JL, Wang HY, Dong CG (2006): Acute respiratory distress syndrome induced by avian influenza A (H5N1) virus in mice. Am. J. Respir. Crit. Care Med. 174, 1011–1017. doi.org/10.1164/rccm.200511–1751OC

Xu C, Fan W, Wei R, Zhao H (2004): Isolation and identification of swine influenza recombinant A/Swine/Shangdong/1/2003 (H9N2) virus. Microbes Infect. 6, 919–925. doi.org/10.1016/j.micinf.2004.04.015

Yin Z, Liu JH (1997): Animal Virology. 2nd ed. Beijing : Science Press, pp. 708–709 (in Chinese).

Zaki, SR, Greer PW, Coffield LM, Goldsmith CS, Nolte KB, Foucar K, Feddersen RM, Zumwalt RE, Miller GL, Khan AS, Rollin PE, Ksziaek TG, Nichols ST, Mahy BW, Peters CJ (1995): Hantavirus pulmonary syndrome: pathogenesis of an emerging infectious disease. Am. J. Pathol. 146, 552–579.

Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, Rossow K, Liu L, Yoon K, Krauss S, Webster RG (1999): Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J. Virol. 73, 8851–8856.