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Letter

Genetic characterization and pathogenicity of a reassortant Eurasian avian-like H1N1 swine influenza virus containing an internal gene cassette from 2009 pandemic H1N1 virus

Shuaiyong Wang [a,1], Manzhu Wang [a,1], Lingxue Yu [a,1], Juan Wang [a], Jiecong Yan [a], Xinli Rong [a], Yanjun Zhou [a], Tongling Shan [a], Wu Tong [a], Guoxin Li [a], Hao Zheng [a], Guangzhi Tong [a,b], Hai Yu [a,b,*]

[a] Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, No.518, Ziyue Road, Minhang District, Shanghai, 200241, China
[b] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, 225009, China

Dear editor,

Swine influenza virus (SIV) is a member of the *Orthomyxoviridae* family, *influenza A virus* genus, which can cause the swine influenza—an acute and highly contagious respiratory disease in pigs (Brown, 2000; Kothalawala et al., 2006). The SIV was first observed in 1918 in the United States and the progenitor of the SIV was the H1N1 influenza virus which caused the Spanish influenza pandemic of 1918 (Shope, 1931). Due to the high transmission rate and 100% morbidity, swine influenza has become widespread worldwide and caused huge economic losses to the pig industry (Kyriakis et al., 2017).

Since the porcine airway epithelium contains both α2,6-linked sialic acids (mammalian influenza virus receptor) and α2,3-linked sialic acids (avian influenza virus receptor), pigs have been considered as a mixing vessel for occurring potential influenza virus reassortment derived from different host species (Sun et al., 2016). The 2009 pandemic H1N1 (pdm/09) virus was a reassortant SIV which caused a human influenza pandemic (Smith et al., 2009). Since the emergence of the pdm/09 virus, reassortants between pdm/09 virus and enzootic SIVs have been detected in swine around the world (Vijaykrishna et al., 2010). China has the most abundant SIVs ecosystem, the genetic lineages mainly include classical swine H1N1, Eurasian avian-like H1N1 (EA H1N1), triple reassortant H1N2, pdm/09 and H3N2 lineages, and the subtypes are predominantly H1N1, H1N2 and H3N2 (Vijaykrishna et al., 2011). The introduction of pdm/09 virus has increased its reassortment with Eurasian avian-like H1N1 (EA H1N1) which were the predominant SIV circulating in swine in China (Liang et al., 2014).

EA H1N1 SIVs were first detected in Belgium in 1979 and the first identification of EA H1N1 SIVs in China was reported in 2001 (Pensaert et al., 1981; Vincent et al., 2014). Various types of reassortants between EA H1N1 SIVs and pdm/09 viruses have been detected among swines in China and some of which have acquired mutations responsible for enhanced virus infectivity to humans (Wang et al., 2013; Zhu et al., 2016; Li et al., 2019). Zoonotic infections with EA H1N1 SIV also have been reported in Europe (Parys et al., 2021). Thus, the research on EA H1N1 SIV should be paid enough attention. In this study, we reported the genetic characterization of a reassortant EA H1N1 SIV which isolated from a pig farm in Shandong province of north China, and further studied the pathogenicity of the reassortant virus in mice.

The reassortant EA H1N1 SIV, designated A/swine/Shandong/L1/2018(H1N1) (SD/18, Genbank accession no. ON030710–ON030717), was detected in the lungs of pigs with typical symptoms of swine influenza, such as fever and cough, from a pig farm in Shandong province of north China. Cytopathic effects of MDCK cells appeared on 24 h after inoculation with the virus isolate and the viral supernatant was harvested on 48 h after inoculation. The hemagglutination (HA) test was performed to test the antibody titers as described previously (Killian, 2014), and the HA titers of the viral supernatant reached 32 HAU/50 μL as measured with 0.5% chicken red blood cells.

Viral RNA was extracted from the supernatant using a RNeasy Mini kit (Qiagen, Germany) and cDNA synthesis was performed by using SuperScript®III First-Strand Synthesis System (Invitrogen, USA) with Uni-12 primer according to the manufacturer's protocol. All eight segments were amplified by PCR with specific primers (Supplementary Table S1) and cloned into the pMD-18T vector, and then sequenced by Sangon Biotech Company (Shanghai, China). To elucidate the genetic characteristics of SD/18, we performed full-length genome sequencing of all eight segments and compared them with SIV sequences from GenBank. BLAST results indicated that hemagglutinin (HA) and neuraminidase (NA) gene segments of SD/18 shared the highest nucleotide identity with A/swine/Jiangsu/s16/2011(H1N1) and A/swine/Shaanxi/s1/2011(H1N1), with

* Corresponding author.
E-mail address: haiyu_008@126.com (H. Yu).
[1] Shuaiyong Wang, Manzhu Wang, and Lingxue Yu contributed equally to this study.

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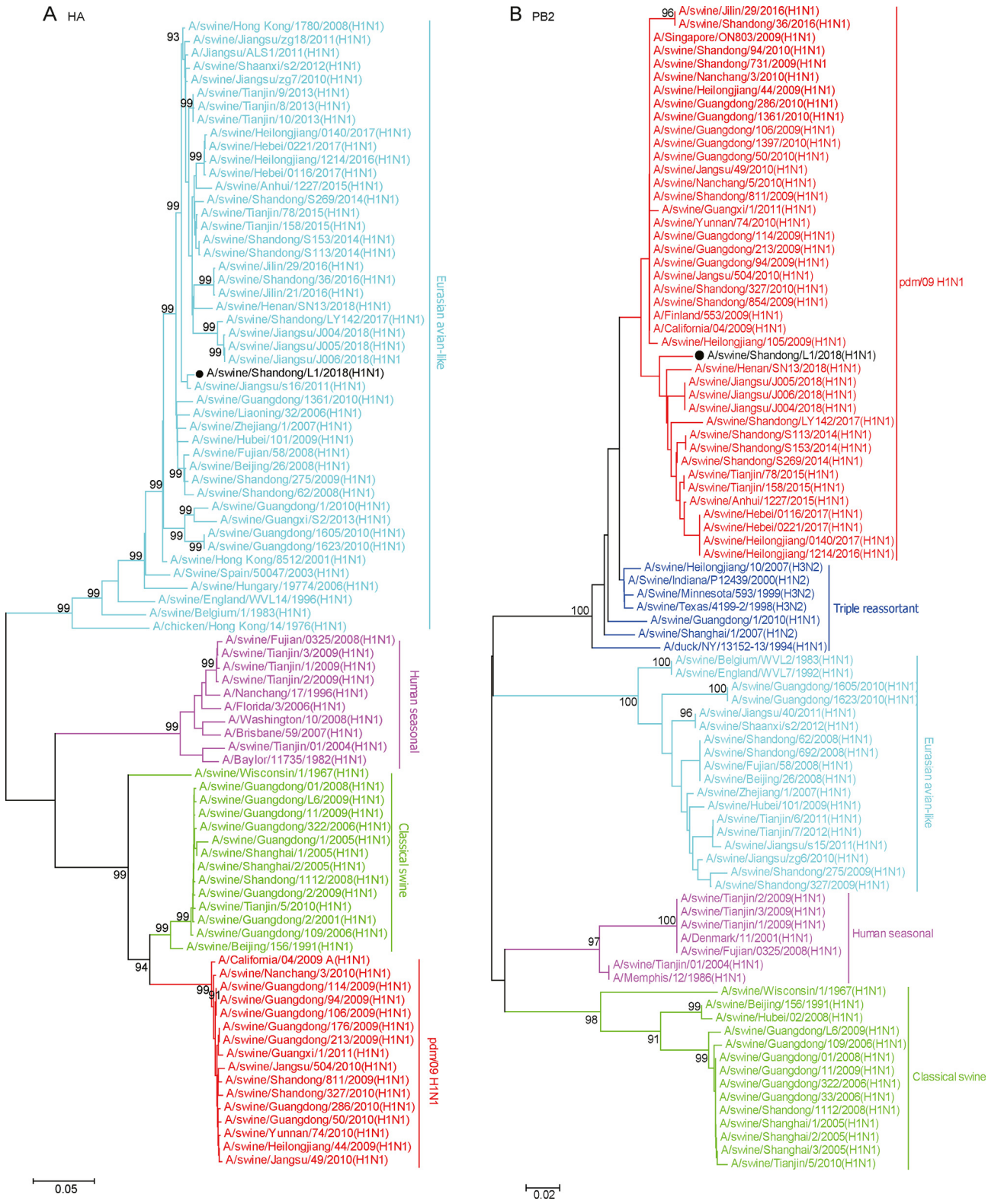

Fig. 1. Phylogenetic and pathogenic analysis of the reassortant EA H1N1 SIV (A/swine/Shandong/L1/2018, SD/18). **A** Phylogenetic tree of *HA* gene for SD/18 suggested it belongs to EA H1N1 lineage. **B** Phylogenetic tree of the *PB2* gene for SD/18 suggested it belongs to 09/pdm H1N1. The black dots indicate the viruses characterized in this study. Phylogenetic trees were performed using the maximum likelihood method with 1000 bootstrap replicates in MEGA7. **C** Body weight change and (**D**) survival curves of the mice after viral infection. Experimental groups of mice were infected with 10^5 $TCID_{50}$ of SD/18 in a volume of 50 μL, and mice in the control group were inoculated with 50 μL of PBS. **E** Virus titers were titrated with MDCK cells. At 3, 5, and 7 dpi, the lungs of two groups of mice were collected and titrated with MDCK cells. **F** Histopathology of the lungs from mice inoculated with SD/18 viruses, or PBS at 3, 5, and 7 dpi by H&E staining. Scale bar = 100 μm. More details about these experiments are provided in Supplementary Material.

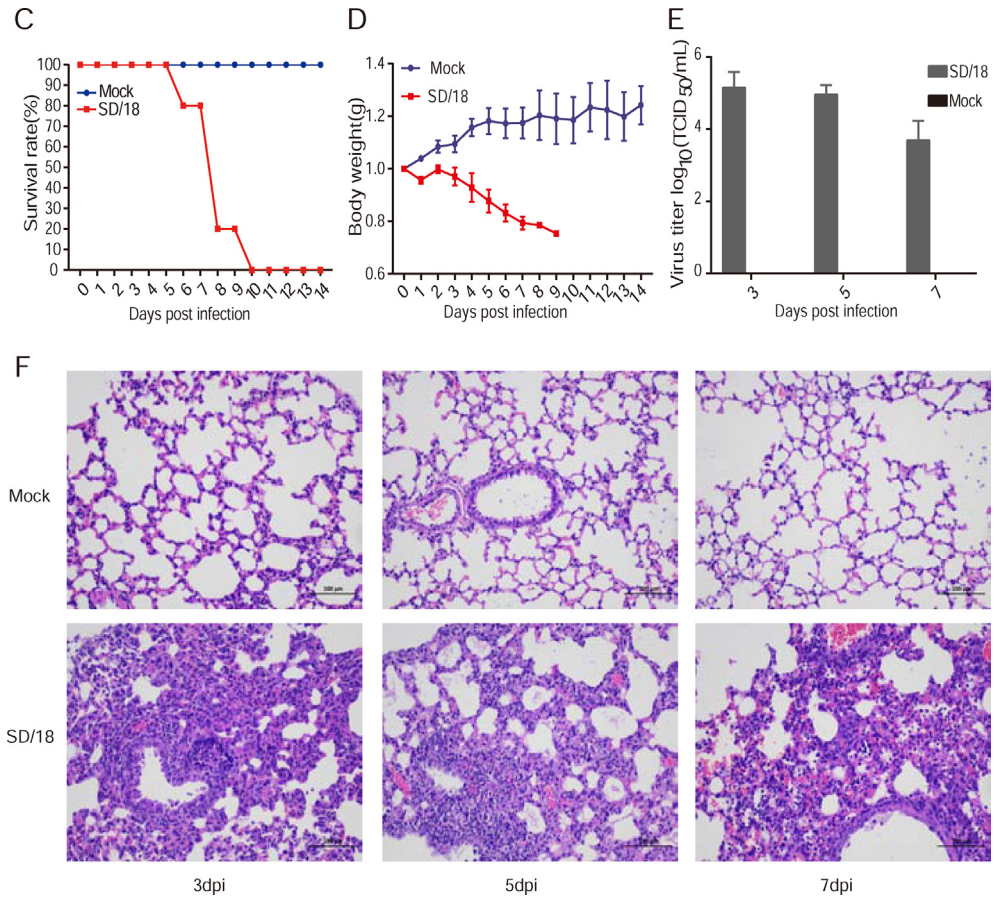

Fig. 1. (*continued*)

an identity of 99.16% and 99.08%, respectively. The results suggested that SD/18 belongs to the lineage of EA H1N1 SIV.

Phylogenetic analysis of the eight gene segments of SD/18 were constructed by the software MEGA7 (www.megasoftware.net) and the reliability of the phylogenetic trees were assessed by bootstrap analysis with 1000 replicates. Reference strains used in this study were listed in Supplementary Table S2. Phylogenetic analysis of the *HA* (Fig. 1A) and *NA* (Supplementary Fig. S1A) gene segments indicated SD/18 was classified into EA H1N1 SIV lineage, which was the predominant subtype virus circulating among swine population in China before 2014. And phylogenetic analysis of polymerase basic 2 (PB2), polymerase basic 1 (PB1), polymerase acidic, nucleoprotein, matrix and nonstructural genes showed they all clustered into the pdm/09 lineage (Fig. 1B, Supplementary Figs. S1B–1F), and all the six internal genes showed high similarity to the reference strains previously isolated in the mainland of China. Taken together, the results of phylogenetic analysis indicated that SD/18 was a reassortant SIV between EA H1N1 SIV and pdm/09 virus, and containing the whole internal gene cassette of pdm/09 virus.

A few key amino acids related to the influenza A virus mammalian adaptation had been detected in previous studies. Based on the results of genetic alignments, multiple substitutions might contribute to mammalian adaptation of SD/18 virus found in this study (Supplementary Fig. S2). We found that *HA* gene of SD/18 virus contained substitution K142 N, which was reported to cause antigenic change (Parys et al., 2021). T271A, G590S, and Q591R in *PB2* gene were also found, which might increase the replication capacity in mammalian cells (Peacock et al., 2020). In addition, we found amino acid substitution T652A in *PB1* gene, which may also have an impact on mammalian adaptation of SD/18 virus (Parys et al., 2021).

To further evaluate the virulence of the reassortant EA H1N1 SIV, we used BALB/c mice as the model. Experimental groups of mice were inoculated with SD/18 at a dose of 10^5 $TCID_{50}$ and the mice in the control group were inoculated with PBS for mock infection. Body weight and clinical signs were recorded daily during the 14-day course of the experiment. It showed that mice in the control group had no obvious clinical symptoms. In contrast, experimental groups of mice inoculated with SD/18 exhibited severe clinical symptoms, including dyspnea, huddling, ruffled fur, hunched posture and drastic weight loss, and all mice died at 9 days post-infection (dpi) (Fig. 1C and D). Viral titers in the lungs of mice at 3, 5, and 7 dpi were titrated with MDCK cells. The results showed that SD/18 could be detected and reach the high level of 10^5 $TCID_{50}$ at 3 dpi and 5 dpi (Fig. 1E). These results suggested that SD/18 could replicate efficiently in mice without adaptation and cause serious clinical symptoms and high mortality.

In addition, we further analyzed the histopathology in the lungs of the mice at 3, 5, and 7 dpi. The H&E staining results showed that no obvious pathological changes were observed in the control group. However, SD/18 induced severe pathological changes of the lungs, characterized by extensive vascular congestion, thickened alveolar walls, significant intra-alveolar edema, with a small amount of inflammatory cell infiltration (Fig. 1F).

Recently, a case of human infection with EA H1N1 SIV was reported in the Netherlands, and the virus could be isolated from human and pigs, which suggested the possibility of EA H1N1 SIV transmission from swine to humans (Parys et al., 2021). In addition, Sun et al., recently reported the genotype 4 reassortant EA H1N1 viruses have acquired increased human infectivity in China (Sun et al., 2020). Taken together, the potential risk of EA lineage SIVs to humans is very high and we need to pay enough attention to the different reassortant EA H1N1 viruses.

In summary, this study reported the genetic characterization of a reassortant EA H1N1 SIV which isolated from swine in north China. Our findings indicated the whole internal gene cassette of pdm/09 virus could be naturally incorporated with *HA* and *NA* genes of EA H1N1 SIV lineage. Moreover, we also evaluated the pathogenicity and replication of the reassortant SIV in mice. The results showed that the virus could replicate efficiently in the lungs of mice and the mortality rate in mice could reach to 100%. On account of the potential risk of a human pandemic, the surveillance of the reassortant EA H1N1 SIV in swine should be strengthened.

Footnotes

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