

The substitution of T271A in PB2 protein could enhance the infectivity and pathogenicity of Eurasian avian-like H1N1 swine influenza viruses in mice

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Research

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Abstract

Background

Currently, Eurasian avian-like H1N1 (EA H1N1) swine influenza viruses (SIVs) are widely prevalent in pigs in China, with sporadic human cases reported as well. As one of the key molecular makers detected in avian H5N1 and H7N9 viruses and pandemic H1N1 2009 virus, contributions of T271A in PB2 protein to the EA H1N1 viruses are still unknown. In this study, we investigated the effects of residue 271 in PB2 protein on the viral properties of EA H1N1 viruses.

Methods

Infectivity, replication, virulence and pathogenicity of the recombinant viruses containing A or T in position 271 in PB2 protein were studied in cells and mice.

Results

The results showed that the substitution PB2-T271A increased the viral replication in mammalian and avian cell lines. In addition, the mutation enhanced the viral infectivity, virulence and pathogenicity in mice. The viral titers of lung tissue in the rgHuN_{271A} virus were higher than that of the rgHuN_{271T} at 1, 4, and 7 dpi. The MID₅₀ of the rgHuN_{271A} and rgHuN_{271T} virus were 10^{1.1} TCID₅₀ and 10^{1.9} TCID₅₀, respectively. Besides, the substitution of PB2-T271A enhanced the viral polymerase activity in mammalian cells.

Conclusions

The pathogenicity and replication of EA H1N1 virus containing 271A in PB2 protein were higher than the EA H1N1 virus containing 271T in PB2 protein *in vivo* and *in vitro*. Therefore, the PB2-T271A mutation should be continually monitored in influenza viruses circulating in pigs and humans.

Background

The contributions of molecular markers to the adaptation, virulence and transmission of influenza A virus have been broadly studied. These molecular markers include a series of substitutions in hemagglutinin (HA), neuraminidase (NA), and polymerase basic protein 2 (PB2). Amongst them, mutations in the polymerase complex (PB2, PB1 and PA) play an important role in the avian influenza viruses breaking the species barrier and increase their fitness in the mammalian hosts. The host-adaptive mutations of E627K and D701N in PB2 protein have been well recognized as the molecular biomarkers that enhance the viral replication, pathogenesis, and virulence of H5N1, H7N7, and H7N9 influenza viruses in mammals [1–4].

In 2007, to discover host-associated genomic signatures, a total of 23,581 full genome sequences of avian and human influenza viruses were analyzed [5]. Many conservative residues between human and avian influenza viruses were identified as potential host-associated markers, including PB2-T271A [5–7].

In avian influenza viruses, the 271 site in the PB2 protein was threonine (T); nevertheless, the 271 site resulted in alanine (A) in human influenza viruses. Subsequent studies showed that PB2 residue 271A could enhance viral polymerase activity and the viral growth of 2009 H1N1 pandemic influenza viruses in mammalian cells [8–10]. Besides, the PB2-T271A mutation could contribute to the efficient transmission of 2009 H1N1 pandemic influenza virus in guinea pigs [9]. Similar results were found in H7N9 and H5N1 influenza viruses [11–13]. Besides, the combination of the substitutions PB2-T271A and 590/591 SR increased the viral replication and adaptation of the 2009 pandemic H1N1 and triple reassortant swine influenza viruses (SIVs) in mammals [14].

Eurasian avian-like H1N1 (EA H1N1) SIVs have been predominant in pigs in China since 2005 [15]. After a long term evolution, the EA H1N1 viruses were reported to have the potential to cause an influenza pandemic [16]. As a matter of fact, sporadic human cases of infection with EA H1N1 viruses have been continuously reported in China [17–20]. In a previous study, the substitution of D701N in the PB2 protein was shown to enhance the viral replication and pathogenicity of EA H1N1 viruses [21]. Besides, the substitution Q357K in the NP protein plays an important role in the virulence phenotype of EA H1N1 viruses [22]. Our previous study reported that the pathogenicity of two human-isolated EA H1N1 viruses (A/Hunan/42443/2015 (H1N1, HuN) and A/Jiangsu/1/2011 (H1N1, JS1) viruses) were divergent [19]. Sequence analysis showed that the two viruses had different amino acids at the 271 site, with A and T in HuN and JS1 viruses, respectively. Whether the 271 residue contributed to the viral virulence is still to be shown. Thus, we analyzed the effects of the residue 271 on the PB2 protein of HuN EA H1N1 SIVs *in vivo* and *in vitro*.

Methods

Cell culture

Madin–Darby canine kidney cells (MDCK), human embryonic kidney cells (293T), human type II alveolar epithelial cells (A549), chicken embryo fibroblasts cells (DF-1) and porcine kidney cells (PK15) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), HEPES (10 mM; Invitrogen), penicillin (100 units/ml), and streptomycin (100 µg/ml; Invitrogen), and were incubated in a humidified atmosphere of 5% CO₂ at 37°C.

Site-directed mutagenesis

The eight gene plasmids of HuN virus were constructed and stored by the Chinese National Influenza Center. A plasmid with a signal mutation A271T in the PB2 protein was constructed using the forward primer 5'-GTAAGAAGAGCAACAGTGTAGCAG-3' and the reverse primer 5'-CTGCTGACACTGTTGCTCTTAC-3'. To ensure the presence of the point mutation and the absence of unwanted mutations, the viral cDNA was sequenced.

Virus rescue and virus titration

As previously described [23], the eight plasmids containing the cDNA of the HuN_{271A} (with residue 271A in PB2 protein) or HuN_{271T} (with residue 271T in PB2 protein) virus were co-transfected into co-cultured 293T/MDCK cells on six-well plates using SuperFect transfection reagent (Qiagen, Valencia, CA, USA). After 48 hours, cultured supernatants were inoculated into 9-day-old specific pathogen free (SPF) embryonated eggs. To avoid unwanted mutations, we sequenced both the recombinant viruses. Virus titration was performed in MDCK cells. The 50% tissue culture infective dose (TCID₅₀) was calculated by the Reed-Muench formula [24].

Virus growth kinetics study

To detect the growth kinetics of both recombinant viruses, A549, DF-1 and PK15 cells were inoculated with the viruses at the multiplicity of infection (MOI) of 0.01. After incubating at 37°C for 1 hour, the supernatants were removed and cells were washed twice with PBS. DMEM containing TPCK-trypsin, penicillin and streptomycin was added. Supernatants were collected at specified time points of 0, 12, 24, 36, 48, 60, 72, 84, and 96 hours post inoculation (hpi) and stored at -80 °C until use. Virus titers were determined by a TCID₅₀ assay in MDCK cells.

Animal experiments

To evaluate the contribution of the substitution PB2-T271A to pathogenicity of the EA H1N1 SIVs, we compared the infectivity, replication and virulence of recombinant viruses in mice. Eight-to ten-week-old SPF C57BL/6J female mice were used in the experiment. Mice were randomly assigned. Each group had five mice. The mice were anesthetized with pentobarbital sodium and inoculated intranasally with 50 µl of 10-fold serial dilutions of each recombinant virus in phosphate-buffered saline (PBS), with doses ranging from 10¹-10⁶ TCID₅₀. Mock mice were inoculated with 50 µl PBS. Body weight were recorded daily for 14 days post inoculation (dpi). When the mice lost 30% of their body weight, they were killed humanely. The 50% mouse lethal dose (MLD₅₀) was calculated based on the survival rate of the mice. Serum from the surviving mice was collected on the 14th day after post-infection, and we detected hemagglutinin inhibiting (HI) antibody titers to calculate the 50% mouse infective dose (MID₅₀).

To evaluate the tropism and replication capacity of both recombinant viruses, we inoculated mice with 10⁵ TCID₅₀ viruses. Three mice were euthanized at 1, 4, and 7 dpi, respectively. Respiratory tract samples containing lung, trachea, and nasal turbinate were collected. The right lobes of the lungs were pumped with 10% formaldehyde solution, which were used for histological analysis. The viral titers were determined by TCID₅₀ assay in MDCK cells.

Histopathological analysis

The right lungs were fixed and dehydrated. The tissue blocks were placed in a mold containing melted paraffin wax and solidified into wax blocks. Lung tissues were sectioned at 4–5 µm thick. Then hematoxylin and eosin staining (H&E) was performed to observe viral infection.

Quantification of cytokines in mice lungs

The severity of influenza virus in human and mice was closely related to the expression of cytokines and chemokines, including interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), chemokine (C-C motif) ligand 5 (CCL5), and interferon α 1 (IFN- α 1) [25-29]. RNA was extracted from the mice lungs (QIAamp Viral RNA Mini Kit). Levels of cytokine and chemokine proteins, including IL-6, IL-8, IL-10, CCL5, and IFN- α 1 were detected by quantitative PCR. Quantitative PCR was conducted by the reagent of TB Green Premix Ex Taq (TaKaRa Biotech, Dalian, China). The LightCycler 96 was used to detect the amplification (Roche, USA).

Polymerase activity assay

Monolayers of 293T cells were transfected with a firefly luciferase reporter plasmid, a *Renilla* luciferase expressing plasmid and four plasmids expressing the polymerase genes PB2, PB1, PA and NP. The *Renilla* luciferase was used as an internal transfection control for the dual luciferase assay. After 24 hours of transfection, luciferase activity was measured using EnVision 2104 Multilabel Reader (PerkinElmer, USA) and the assay was performed in triplicate.

Western blot assay

293T cells transfected with plasmids in the polymerase activity assay were lysed with passive lysis buffer (Promega, Madison, USA). Cells lysates were mixed with SDS-PAGE sample loading buffer (Beyotime Biotech, China) and heated at 95 °C for 10 min. The samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, Billerica, USA). The membrane was blocked with 5% nonfat milk for 2 hours and then incubated with anti-PB2 (GTX125926, GeneTex, Irvine, USA). The primary antibody was incubated overnight at 4 °C. The membrane was incubated with goat anti-rabbit secondary antibody for 1 hour. The images were obtained by using the MiniChemi Imaging and Analysis System (Sage Creation, China).

Statistical analysis

The student's t test and two-way ANOVA were calculated using GraphPad Prism software 6.0. A *P* value < 0.05 was considered to be statistically significant.

Results

The substitution of PB2-T271A enhanced the viral replication in cells

To determine the effect of PB2-T271A substitution on virus replications *in vitro*, we performed growth kinetics analysis of both recombinant viruses in A549, PK15, and DF-1 cells (Fig. 1). It was obvious that the rgHuN_{271T} virus rarely replicated in DF-1 cells. Nevertheless, the rgHuN_{271A} virus had significantly better growth advantages than the rgHuN_{271T} virus in DF-1 cells during 36 to 96 hpi (*P* < 0.01) (Fig. 1a). Furthermore, the replication titers of rgHuN_{271A} virus were significantly higher than those of rgHuN_{271T}.

virus from 36 to 96 hpi in A549 cells ($P < 0.01$) (Fig. 1b). In PK15 cells, both recombinant viruses could replicate efficiently (Fig. 1c). Generally, the replication titers of rgHuN_{271A} virus were higher than the rgHuN_{271T} virus (Fig. 1c). There were statistical differences between the two recombinant viruses at 36, 48, 72, 84, and 96 hpi ($P < 0.01$ or $P < 0.05$). These results indicated that the mutation PB2-T271A could enhance the viral replication in mammalian and avian cell lines.

The substitution of PB2-T271A enhanced viral morbidity and mortality in mice

To evaluate the effects of the PB2-T271A mutation on pathogenicity in mice, the recombinant viruses at the indicated dose (10^1 - 10^6 TCID₅₀) were inoculated intranasally with five mice in each group. At the inoculation dose of 10^1 - 10^3 TCID₅₀ with the recombinant rgHuN_{271A} or rgHuN_{271T} viruses, we observed that no mice died and the body weight loss was <10% (Fig. 2a-b). When inoculated with 10^4 TCID₅₀ of the recombinant viruses, the body weight loss of the rgHuN_{271A} group mice was approximately 20%. However, the body weight loss of the rgHuN_{271T} group mice was <10% (Fig. 2a-b). At the inoculation dose of 10^5 TCID₅₀, four-fifths of the mice of the rgHuN_{271A} virus died (Fig. 2c). Two mice died at 7 dpi; another two mice died at 8 dpi. Meanwhile, no mice died at the inoculation dose of 10^5 TCID₅₀ with rgHuN_{271T} virus (Fig. 2d). When infection with the highest dose of 10^6 TCID₅₀ of the recombinant viruses, all mice of rgHuN_{271A} virus died at 6 dpi and four mice of rgHuN_{271T} virus died at 8 dpi; one mouse survived until the end of the experiment (Fig. 2c-d). To evaluate the virulence of the recombinant viruses, we calculated the MLD₅₀. The MLD₅₀ of the rgHuN_{271A} virus was $10^{4.5}$ TCID₅₀ (Fig. 2c). Nevertheless, the MLD₅₀ of the rgHuN_{271T} virus was $10^{5.7}$ TCID₅₀ (Fig. 2d). The MID₅₀ values of the rgHuN_{271A} and rgHuN_{271T} virus were $10^{1.1}$ TCID₅₀ and $10^{1.9}$ TCID₅₀, respectively (Table 1). These results indicated that the substitution T271A in the PB2 protein could increase the virulence of the EA H1N1 SIVs in mice.

The substitution of PB2-T271A enhanced the viral replication in mice

To evaluate the replication of the recombinant viruses in the respiratory tracts of mice, we collected the tracheal, nasal turbinate, and lung tissue samples from mice at 1, 4, and 7 dpi with the inoculated dose of 10^5 TCID₅₀. Both recombinant viruses could replicate well in the respiratory tract tissues. The rgHuN_{271A} viral titers of nasal turbinate were higher than that of rgHuN_{271T}, with no statistical difference (Fig. 3). There was no significant change in the viral titer of trachea tissues between the two recombinant viruses. However, the viral titers of lung tissues in the rgHuN_{271A} virus were significantly higher than those of the rgHuN_{271T} at 1, 4, and 7 dpi ($P < 0.01$) (Fig. 3). These data showed that the substitution PB2-T271A could increase the replication of EA H1N1 viruses in mice.

The substitution of PB2-T271A enhanced viral pathogenicity in mice

To compare the pulmonary histopathological damage induced by the recombinant viruses, the H&E staining was performed with lung tissue sections (Fig. 4). At 1 dpi, the lung damage of both recombinant viruses was rarely observed (Fig. 4a-b). The cell exfoliation and local bronchitis occurred in the mice lung

of the rgHuN_{271A} virus group compared to rgHuN_{271T} viruses at 4 dpi (Fig. 4c-d). Interstitial bronchial pneumonia and focal alveolar damage of the lung were manifested at 7 dpi in the group of rgHuN_{271A} virus; however, scattered pathological lesions of the lungs were observed in rgHuN_{271T} virus (Fig. 4e-f). These results indicated that the substitution PB2-T271A could increase the viral pathogenicity in mice.

The substitution of PB2-T271A enhanced the proinflammatory response in mice

To compare the proinflammatory response of the recombinant rgHuN_{271A} and rgHuN_{271T} viruses, we detected the cytokines and chemokine expression levels at 1, 4, and 7 dpi including IL-6, IL-8, IL-10, CCL5, and IFN- α 1 (Fig. 5). The recombinant rgHuN_{271A} virus induced higher levels of IL-6, IL-8, IL-10, and IFN- α 1 than those of rgHuN_{271T} virus at 1, 7 dpi (Fig. 5), even if there was no statistic difference. These results indicated that the mutation of T271A in PB2 protein could increase proinflammation response in mice.

The substitution of PB2-T271A enhanced the viral polymerase activity in mammalian cells

To measure the impact of mutation PB2-T271A in the recombinant viruses on the transcription and replication of viral RNA in 293T cells, the luciferase reporter assay was conducted. From the results, we can see that the PB2-T271A substitution significantly increased the polymerase activity ($P < 0.01$). This was increased approximately four-fold compared to the polymerase activity of rgHuN_{271T} virus (Fig. 6). The PB2 proteins of the rgHuN_{271A} and rgHuN_{271T} viruses were expressed at similar levels in 293T cells by Western blot analysis (Fig. 6).

Discussion

As a molecular maker, PB2-T271A was able to enhance the polymerase activity and viral replication in mammal cells for the 2009 pandemic H1N1 and H7N9 influenza virus [9, 11]. On the other hand, as a zoonotic influenza virus, EA H1N1 viruses circulating in pigs in China were considered most likely to cause the next pandemic [16]. However, studies of the molecular markers in the EA H1N1 viruses were limited in number. In this study, we systematically analyzed the contributions of PB2-271 substitution of EA H1N1 SIVs both *in vivo* and *in vitro*. We found that PB2-T271A substitution enhanced the viral replication ability and polymerase activity of EA H1N1 viruses in cell lines, and more significantly, it increased the infectivity, replication, and virulence of EA H1N1 SIVs in mice.

Since 2005, EA H1N1 viruses have been predominant in China [15]. Several molecular makers played an important role in EA H1N1 viruses. The PB2-R251K, PB2-D701N, and NP-Q357K of EA H1N1 viruses contributed to the viral replication and pathogenicity in mice [21, 22, 30]. Furthermore, these mutations enhanced the polymerase activity. The PB2-T271A substitution showed similar results. It enhanced the viral replication *in vivo* and *in vitro* (Fig. 1–2). In addition, the residue 271 in PB2 was located near the cap-binding domain, thus affecting the interaction between PB2 and the cap structure [8, 31]. This might increase the viral polymerase activity and vRNA synthesis [32].

PB2-271A and PB2-271T are conserved in human influenza viruses and avian influenza viruses, respectively (Table 2) [10]. The sequence analysis showed that 99.6% of human H1N1 influenza viruses possessed A at position 271 in PB2 protein. However, 98.2% of avian H1N1 influenza viruses contained T at position 271 in PB2. In swine populations, the prevalence of A (71.0%) was higher than that of T (22.4%) for H1N1 SIVs (Table 2). Considering the higher virulence exhibited by 271A viruses than 271T viruses in mice, surveillance of the EA H1N1 viruses should be strengthened, especially for those with 271A residues.

Conclusions

In this study, we evaluated the effect of the PB2-T271A mutation on EA H1N1 SIVs *in vivo* and *in vitro*. The substitution PB2-T271A increased the viral polymerase activity and growth properties in mammalian cells, and the infectivity and virulence in mice. Thus, substantial attention should be paid on the PB2-T271A mutation.

Abbreviations

EA H1N1: Eurasian avian-like H1N1; SIVs: swine influenza viruses; MDCK: Madin–Darby canine kidney cells; 293T: human embryonic kidney cells; A549: human type II alveolar epithelial cells; DF-1: chicken embryo fibroblasts cells; PK15: porcine kidney cells; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; PBS: phosphate-buffered saline; SPF: specific pathogen free; DPI: days post inoculation; HPI: hours post inoculation; TCID₅₀: 50% tissue culture infections dose; MOI: multiplicity of infection; MLD₅₀: 50% mouse lethal dose; MID₅₀: 50% mouse infective dose; HI: hemagglutinin inhibiting; H&E: hematoxylin and eosin; IL-6: interleukin-6; IL-8: interleukin-8; IL-10: interleukin-10; CCL5: chemokine (C-C motif) ligand 5; IFN- α 1: interferon α 1

Declarations

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Availability of data and materials

The datasets used in this study are available from the corresponding authors on reasonable request.

Authors' contributions

YLS, DYW, and WFZ designed the study; ZMF, WFZ, LJZ, YKC, RBG, XYL and JL performed the experiments; YLS, WFZ and ZMF analyzed the data and discussed the results; ZMF wrote the manuscript; YLS and WFZ revised the manuscript.

Ethics approval and consent to participate

The experimental protocols in mice were approved by the Ethics Committee of the National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention (20170811024).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Zhu W, Li L, Yan Z, Gan T, Li L, Chen R, et al. Dual E627K and D701N mutations in the PB2 protein of A(H7N9) influenza virus increased its virulence in mammalian models. *Sci Rep.* 2015;5:14170.
2. Chen H, Bright RA, Subbarao K, Smith C, Cox NJ, Katz JM, et al. Polygenic virulence factors involved in pathogenesis of 1997 Hong Kong H5N1 influenza viruses in mice. *Virus Res.* 2007;128(1–2):0–163.
3. Steel J, Lowen AC, Mubareka S, Palese P, Baric RS. Transmission of Influenza Virus in a Mammalian Host Is Increased by PB2 Amino Acids 627K or 627E/701N. *PLoS Pathog.* 2009;5(1):e1000252.
4. Le QM, Sakai-Tagawa Y, Ozawa M, Ito M, Kawaoka Y. Selection of H5N1 Influenza Virus PB2 during Replication in Humans. *J Virol.* 2009;83(10):5278–81.
5. Finkelstein DB, Mukatira S, Mehta PK, Obenauer JC, Su X, Webster RG, et al. Persistent host markers in pandemic and H5N1 influenza viruses. *J Virol.* 2007;81(19):10292–9.
6. Chen GW, Chang SC, Mok CK, Lo YL, Kung YN, Huang JH, et al. Genomic signatures of human versus avian influenza A viruses. *Emerg Infect Dis.* 2006;12(9):1353–60.
7. Miotto O, Heiny A, Tan TW, August JT, Brusic V. Identification of human-to-human transmissibility factors in PB2 proteins of influenza A by large-scale mutual information analysis. *BMC*

- Bioinformatics. 2008;9(Suppl 1):18.
8. Bussey KA, Bousse TL, Desmet EA, Kim B, Takimoto T. PB2 Residue 271 Plays a Key Role in Enhanced Polymerase Activity of Influenza A Viruses in Mammalian Host Cells. *J Virol*. 2010;84(9):4395–406.
 9. Zhang Y, Zhang Q, Gao Y, He X, Kong H, Jiang Y, et al. Key molecular factors in hemagglutinin and PB2 contribute to efficient transmission of the 2009 H1N1 pandemic influenza virus. *J Virol*. 2012;86(18):9666–74.
 10. Hayashi T, Wills S, Bussey KA, Takimoto T. Identification of Influenza A Virus PB2 Residues Involved in Enhanced Polymerase Activity and Virus Growth in Mammalian Cells at Low Temperatures. *J Virol*. 2015;89(15):8042–9.
 11. Liu Q, Zhou B, Ma W, Bawa B, Ma J, Wang W, et al. Analysis of Recombinant H7N9 Wild-Type and Mutant Viruses in Pigs Shows that the Q226L Mutation in HA Is Important for Transmission. *J Virol*. 2014;88(14):8153–65.
 12. Ngai KL, Chan MC, Chan PK. Replication and transcription activities of ribonucleoprotein complexes reconstituted from avian H5N1, H1N1pdm09 and H3N2 influenza A viruses. *PLoS One*. 2013;8(6):e65038.
 13. Wen L, Chu H, Wong BH, Wang D, Li C, Zhao X, et al. Large-scale sequence analysis reveals novel human-adaptive markers in PB2 segment of seasonal influenza A viruses. *Emerg Microbes Infect*. 2018;7(1):47.
 14. Liu Q, Qiao C, Marjuki H, Bawa B, Ma J, Guillossou S, et al. Combination of PB2 271A and SR polymorphism at positions 590/591 is critical for viral replication and virulence of swine influenza virus in cultured cells and in vivo. *J Virol*. 2012;86(2):1233–7.
 15. Vijaykrishna D, Smith GJD, Pybus OG, Zhu H, Bhatt S, Poon LLM, et al. Long-term evolution and transmission dynamics of swine influenza A virus. *Nature*. 2011;473(7348):519–22.
 16. Yang H, Chen Y, Qiao C, He X, Zhou H, Sun Y, et al. Prevalence, genetics, and transmissibility in ferrets of Eurasian avian-like H1N1 swine influenza viruses. *Proc Natl Acad Sci U S A*. 2016;113:392–7.
 17. Yang H, Qiao C, Tang X, Chen Y, Xin X, Chen H. Human infection from avian-like influenza A (H1N1) viruses in pigs, China. *Emerg Infect Dis*. 2012;18(7):1144–6.
 18. Qi X, Cui L, Jiao Y, Pan Y, Li X, Zu R, et al. Antigenic and genetic characterization of a European avian-like H1N1 swine influenza virus from a boy in China in 2011. *Arch Virol*. 2012;158(1):39–53.
 19. Zhu W, Zhang H, Xiang X, Zhong L, Yang L, Guo J, et al. Reassortant Eurasian Avian-Like Influenza A(H1N1) Virus from a Severely Ill Child, Hunan Province, China, 2015. *Emerg Infect Dis*. 2016;22(11):1930–6.
 20. Xie JF, Zhang YH, Zhao L, Xiu WQ, Chen HB, Lin Q, et al. Emergence of Eurasian Avian-Like Swine Influenza A (H1N1) Virus from an Adult Case in Fujian Province, China. *Virol Sin*. 2018;33(3):282–6.
 21. Liu S, Zhu W, Feng Z, Gao R, Guo J, Li X, et al. Substitution of D701N in the PB2 protein could enhance the viral replication and pathogenicity of Eurasian avian-like H1N1 swine influenza viruses. *Emerg Microbes Infect*. 2018;7(1):75.

22. Zhu W, Feng Z, Chen Y, Yang L, Liu J, Li X, et al. Mammalian-adaptive mutation NP-Q357K in Eurasian H1N1 Swine Influenza viruses determines the virulence phenotype in mice. *Emerg Microbes Infect.* 2019;8(1):989–99.
23. Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG. A DNA transfection system for generation of influenza A virus from eight plasmids. *Proceedings of the National Academy of Sciences.* 2000;97(11):6108–6113.
24. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. *Am J Hyg.* 1938;27:493–7.
25. Zhou J, Wang D, Gao R, Zhao B, Song J, Qi X, et al. Biological features of novel avian influenza A (H7N9) virus. *Nature.* 2013;499(7459):500–3.
26. Perrone LA, Plowden JK, Garcia-Sastre A, Katz JM, Tumpey TM. H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice. *PLoS Pathog.* 2008;4(8):e1000115.
27. Zeng H, Belser JA, Goldsmith CS, Gustin KM, Veguilla V, Katz JM, et al. A(H7N9) virus results in early induction of proinflammatory cytokine responses in both human lung epithelial and endothelial cells and shows increased human adaptation compared with avian H5N1 virus. *J Virol.* 2015;89(8):4655–67.
28. Gao H, Sun Y, Hu J, Qi L, Wang J, Xiong X, et al. The contribution of PA-X to the virulence of pandemic 2009 H1N1 and highly pathogenic H5N1 avian influenza viruses. *Sci Rep.* 2015;5:8262.
29. Turianova L, Lachova V, Svetlikova D, Kostrabova A, Betakova T. Comparison of cytokine profiles induced by nonlethal and lethal doses of influenza A virus in mice. *Exp Ther Med.* 2019;18(6):4397–405.
30. Cai M, Zhong R, Qin C, Yu Z, Wen X, Xian J, et al. The R251K Substitution in Viral Protein PB2 Increases Viral Replication and Pathogenicity of Eurasian Avian-like H1N1 Swine Influenza Viruses. *Viruses.* 2020;12(1).
31. Gabriel G, Czudai-Matwich V, Klenk HD. Adaptive mutations in the H5N1 polymerase complex. *Virus Res.* 2013;178(1):53–62.
32. Sekaran SD, Ngai KLK, Chan MCW, Chan PKS. Replication and Transcription Activities of Ribonucleoprotein Complexes Reconstituted from Avian H5N1, H1N1pdm09 and H3N2 Influenza A Viruses. *PLoS One.* 2013;8(6):e65038.
33. Karber G. 50% end point calculation. *Arch Exp Pathol Pharmak.* 1931;162:480–3.

Tables

Table 1 Seroconversions of the C57BL/6J mice inoculated with the recombinant rgHuN_{271A} or rgHuN_{271T} viruses.

viruses	Inoculation doses (logTCID ₅₀)	HI titers					MID ₅₀ (logTCID ₅₀)
		Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	
271A	6	ND	ND	ND	ND	ND	1.1
	5	ND	ND	ND	ND	1280	
	4	ND	1280	1280	1280	1280	
	3	1280	1280	1280	1280	1280	
	2	640	640	640	160	160	
	1	320	160	-	-	-	
271T	6	ND	ND	ND	ND	1280	1.9
	5	640	1280	1280	1280	640	
	4	640	320	1280	640	640	
	3	160	320	320	320	160	
	2	10	80	80	-	-	
	1	-	-	-	-	-	
3	-	-	-	-	-	-	

Notes: The serum of mice was collected at 14 dpi. The HI assay was performed by calculating the 50% mouse lethal dose (MID₅₀) according to the Karber method [33]. ND: Not determined because of mice death, the results were treated as positive. The HI titer “-” was considered as negative.

Table 2 The percentage of 271T and 271A in PB2 protein of H1N1 influenza viruses.

Hosts	Total no. sequences*	Percentage of amino acid at position 271 in PB2 (%)	
		T	A
Human	24556	0.11 (2701)	99.6 (21855)
Avian	596	98.15 (585)	1.68 (10)
Swine	2450	22.39 (549)	70.98 (1739)

*All the sequences of PB2 were downloaded from the public database of Global Initiative on Sharing All Influenza Data (<http://platform.gisaid.org>).

Figures

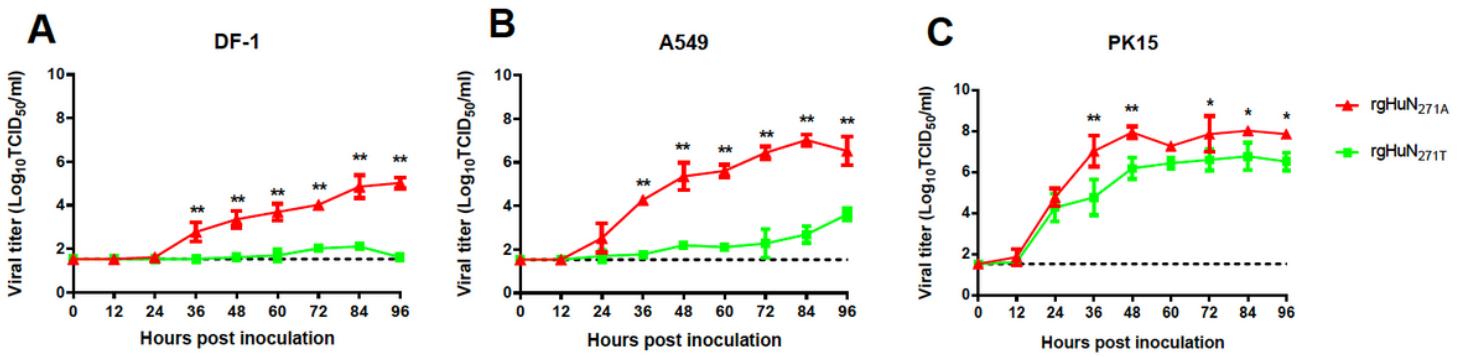


Figure 1

Replication kinetics of the two recombinant viruses in DF-1, A549 and PK15 cells. Cells were inoculated at a multiplicity of infection 0.01 with the rgHuN271A and rgHuN271T viruses in triplicate. The supernatants were collected at the indicated time points for titration in MDCK cells. a DF-1 cells. b A549 cells. c PK15 cells. * indicated $P < 0.05$ when comparing the growth kinetics of the rgHuN271A and rgHuN271T viruses. ** $P < 0.01$.

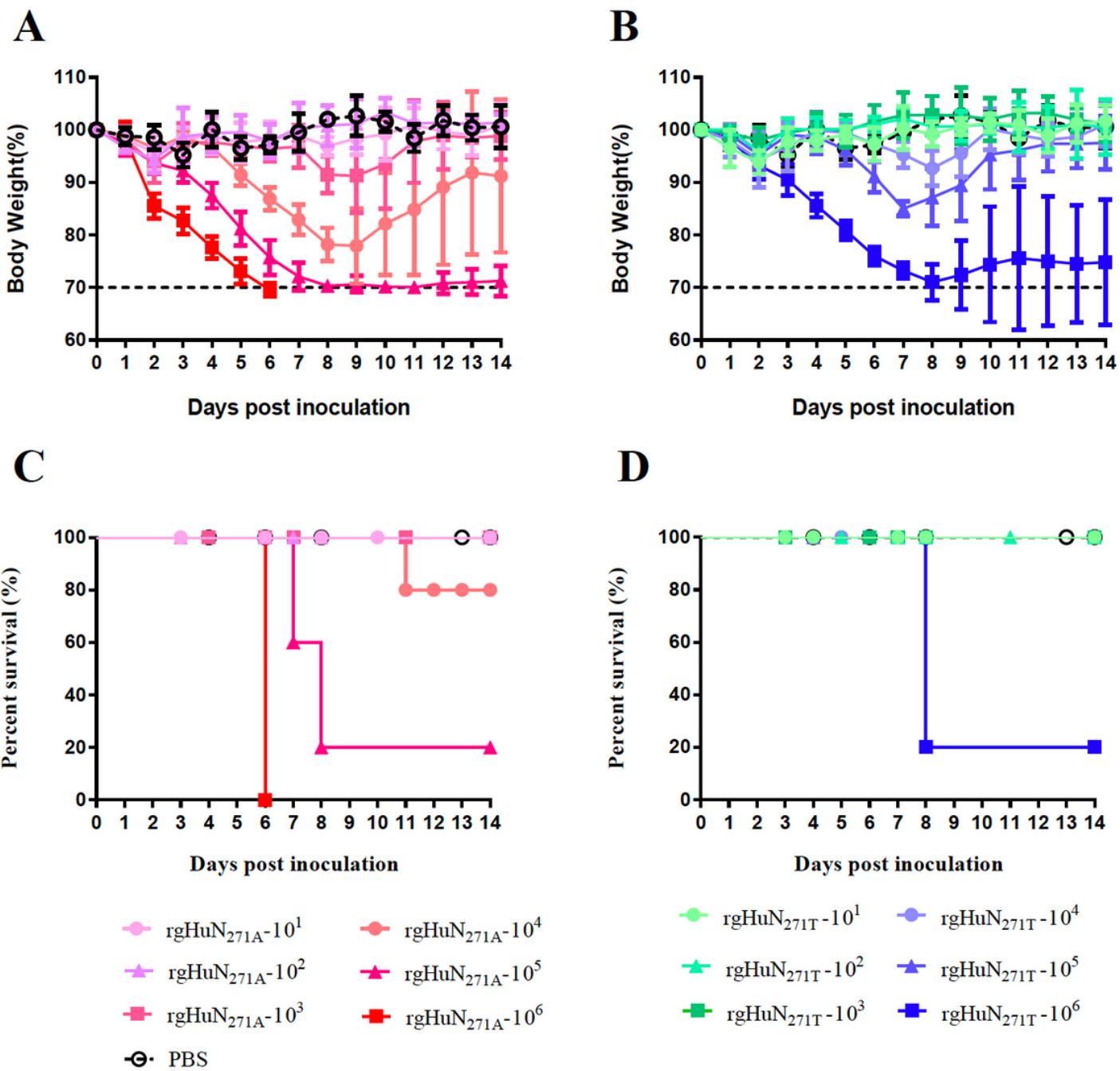


Figure 2

Body weight changes and survival rates of the rgHuN271A and rgHuN271T viruses in mice. Mice were inoculated intranasally with 50 μ l at the dose of 10¹-10⁶ TCID₅₀ of the recombinant viruses rgHuN271A or rgHuN271T. The mice of the control group were inoculated intranasally with 50 μ l PBS. a and b Body weight changes and survival rate of rgHuN271A virus in mice. c and d Body weight changes and survival rate of rgHuN271T virus in mice. Body weight and survival were recorded daily for 14 dpi.

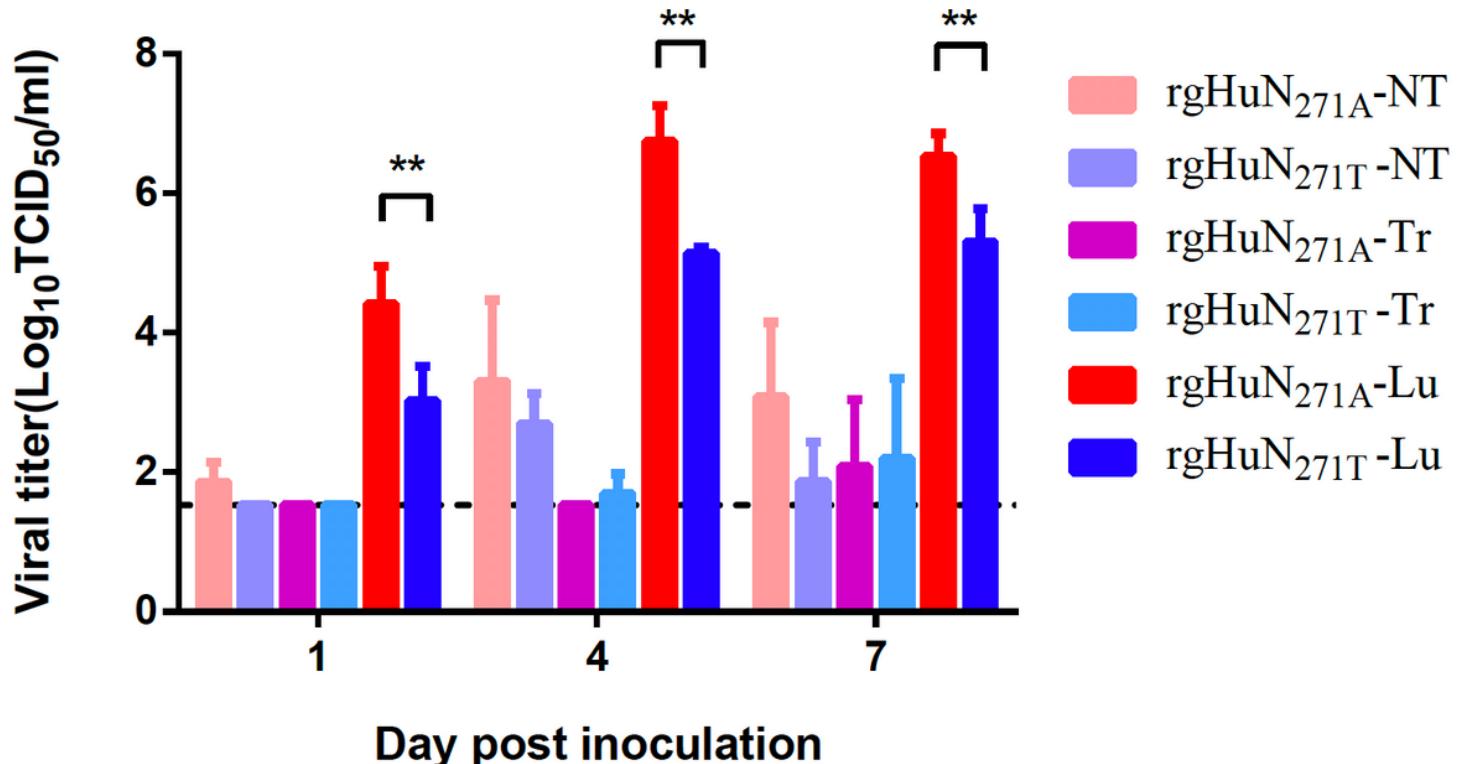


Figure 3

Viral replication titers of respiratory tract samples in mice. Anatomical group mice were inoculated intranasally with 50 μ l of 10⁵ TCID₅₀ of the recombinant viruses. At 1, 4, and 7 dpi, we collected the lung (Lu), trachea (Tr), and nasal turbinate (NT) of mice. **P < 0.01.

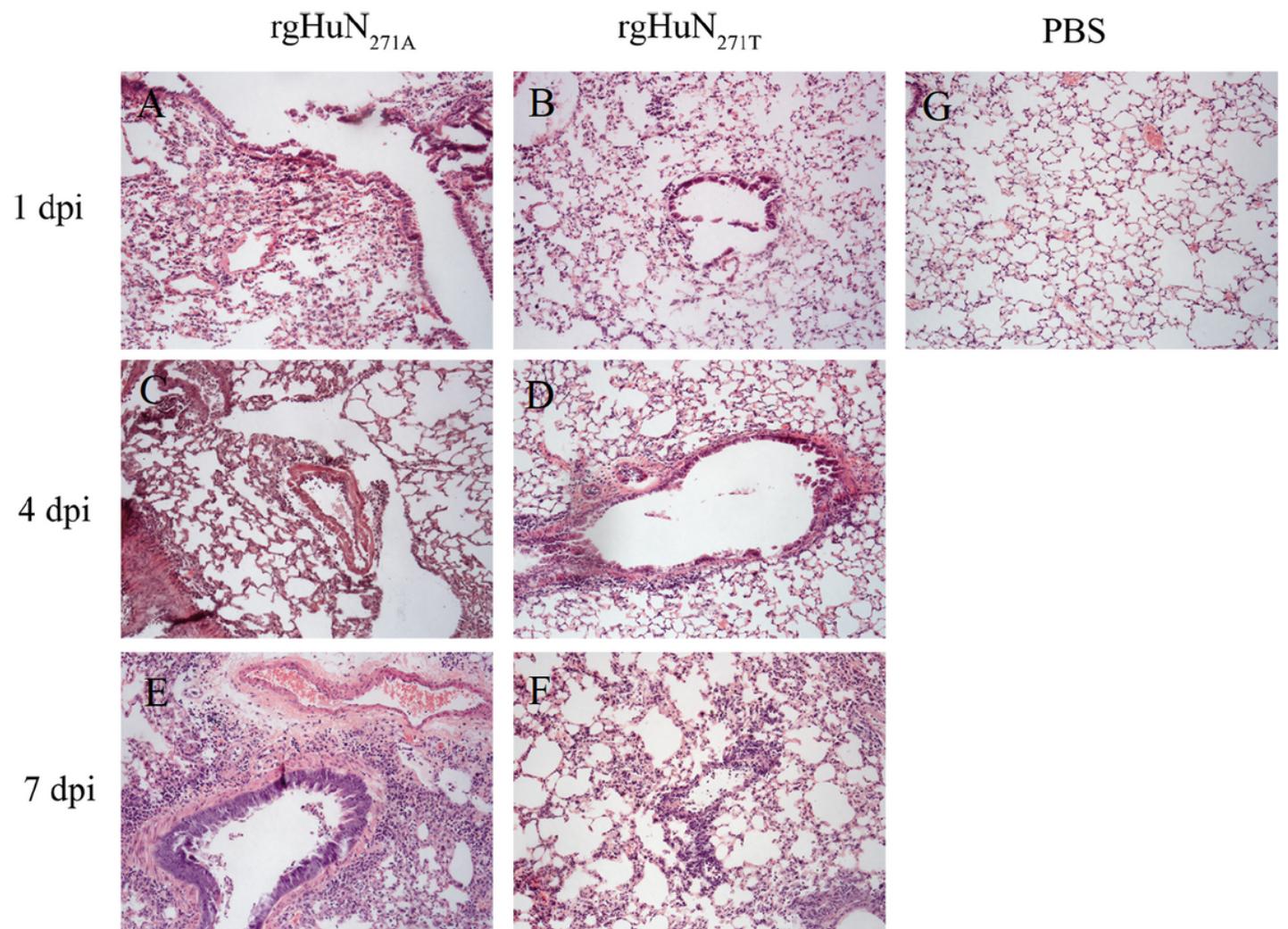


Figure 4

The lungs histopathology changes with the recombinant viruses by the H&E staining. The mice lungs were collected with 10% neutral buffered formalin at 1, 4, and 7 dpi. a-g show the results of H&E staining.

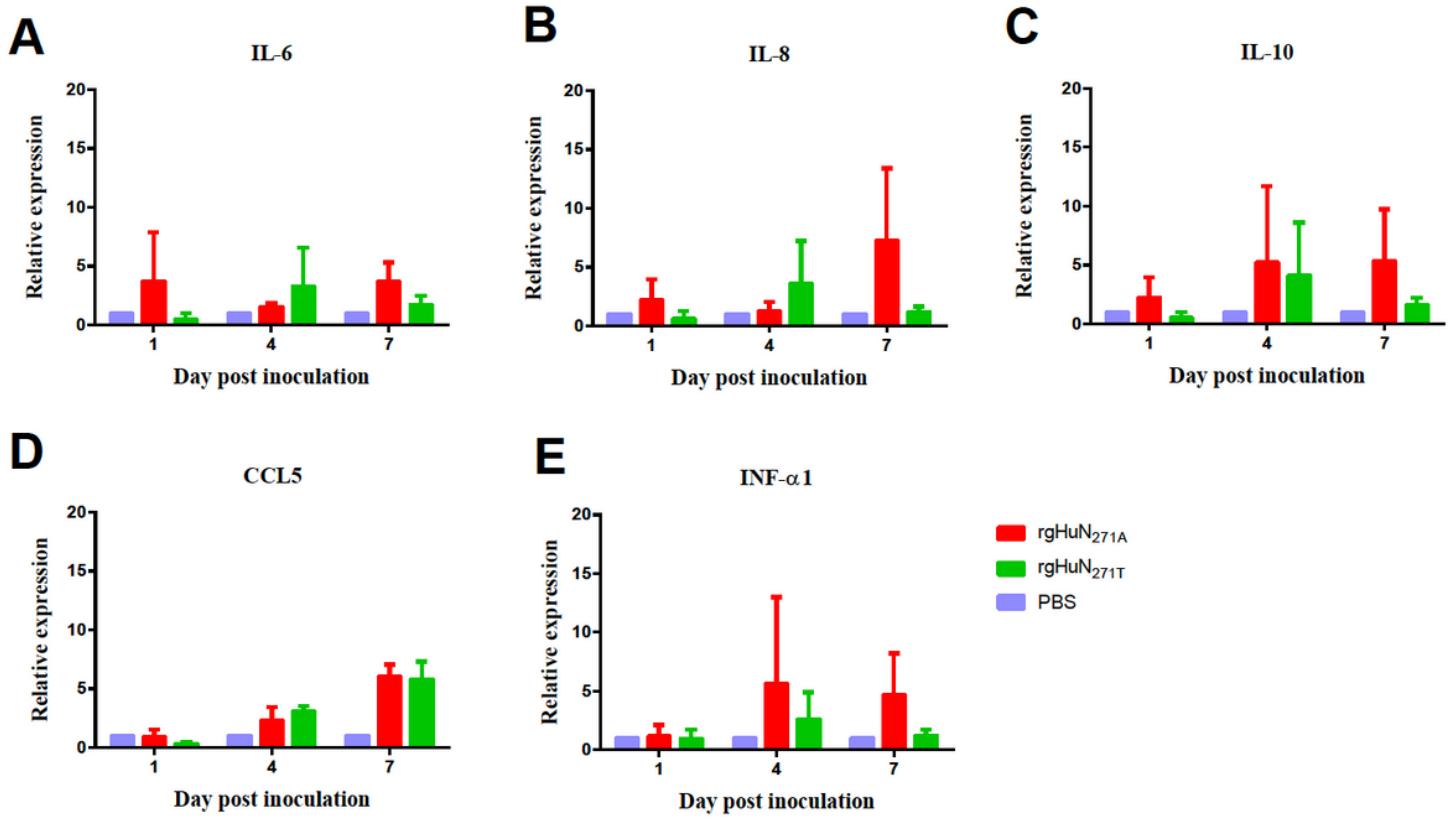


Figure 5

Detection of cytokine proteins in mice lungs infected with the recombinant viruses. Lungs were collected with inoculated 105 TCID50 of the two recombinant viruses at 1, 4, and 7 dpi. IL-6, IL-8, IL-10, CCL5 and IFN- α 1 of lungs in mice were detected.

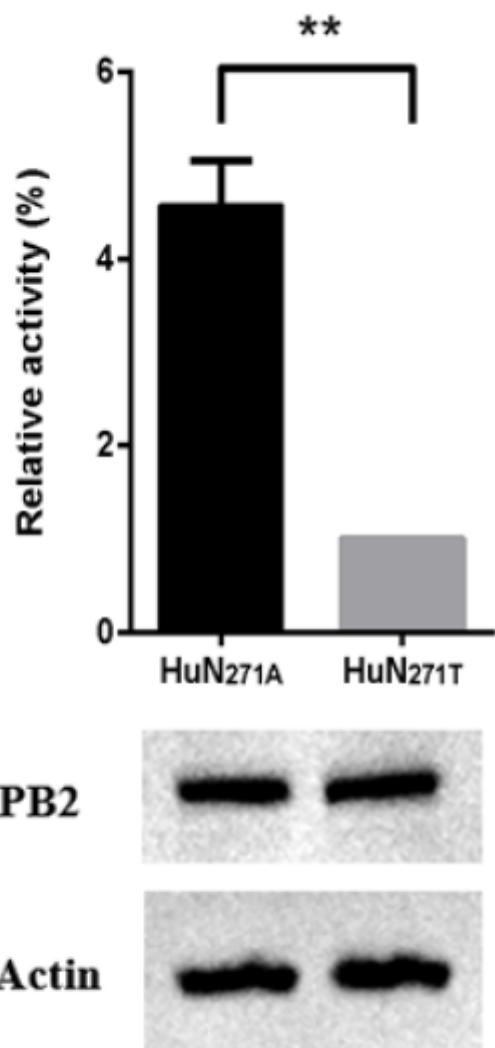


Figure 6

Viral polymerase activity with the two recombinant viruses in 293T cells. A firefly luciferase reporter plasmid, a Renilla luciferase expressing plasmid and the polymerase complex (PB1, PA, NP, and PB2271A or PB2271T) were transfected in 293T cells. After 24 hpi, the polymerase activity was detected. The relative activity (%) means the value of firefly luciferase reporter plasmid was normalized against by the value of Renilla luciferase expressing plasmid. Expression of the rgHuN271A and rgHuN271T PB2 proteins in transfected 293T cells was determined by Western blot assay. **P < 0.01.