

**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***

Running title: PB2 271A and 590/591 SR for replication and virulence

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Supplementary Materials and Methods and Figures

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Generation of recombinant viruses by reverse genetics

The parental virus 1930-TX98-PB2 was generated in the backbone of a classical A/Swine/Iowa/15/30 (1930) H1N1 virus by replacing its swine-origin PB2 gene (containing 271T, 590G, 591Q and 627K) with an avian-origin PB2 (containing 271A, 590S, 591R and 627E) from the triple reassortant A/Swine/Texas/4199-2/98 (TX/98) H3N2 virus as described previously (1). A Gene tailor Site-Directed Mutagenesis Kit was used with the pHW-TX98-PB2 plasmid to generate the respective mutated plasmids with single (pHW-TX98-PB2-A271T), double (pHW-TX98-PB2-S590A-R590A), or triple (pHW-TX98-PB2-A271T-S590A-R590A) amino acid substitutions. All plasmid mutations were confirmed by sequencing (primers are available upon request). Three recombinant viruses with single, double, or triple amino acid substitutions were generated in the backbone of the classical 1930 virus by using reverse genetics: 1930-TX98-PB2-271T, 1930-TX98-PB2-590A591A and 1930-TX98-PB2-271T590A591A. The parental and recombinant viruses were plaque-purified and passaged thrice in MDCK cells and then confirmed by sequencing.

Growth dynamic of parental and mutated viruses

We assessed the growth characteristics of the parental and mutated viruses by performing plaque assays and growth-curve analysis. A monolayer of either MDCK (MOI = 0.001), A549 (MOI = 0.1), swine PK15 cells in 6-well plates was infected with different viruses. Supernatants from infected cells were collected at different time points (i.e., 12h, 24h, 36h and 48h) post-infection (pi) and titrated by infecting MDCK cells in 96-well plates (2). The 50% tissue culture-infective dose per milliliter (TCID₅₀/mL) was calculated by using the method of Reed and Muench. The size of plaques formed by different viruses was compared.

Analysis of viral vRNA, mRNA and cRNA by primer extension assays

Intracellular viral vRNA, cRNA, and mRNA were quantified as described previously (3). Briefly, MDCK cells were infected with parental and mutated viruses at an MOI = 3. Total RNA was isolated from cell lysates at 4h, 8h and 12h pi by using the RNeasy kit; the time points were chosen to ensure that the early and late stage of viral replication cycles was included. Total RNA (5 µg for detection of viral RNA and 2 µg for detection of cellular 5S rRNA) was mixed with an excess of DNA primers, labeled at the 5' end with 30 µCi γ -32P ATP and T4 polynucleotide kinase, and denatured by heating to 95°C for 3 min. For primer extension, the mixture was cooled to 50°C and 100 U of SuperScript III reverse transcriptase was added. The mixture was incubated for 90 min at 50°C. Two PB1 gene-specific primers of the 1930-TX98-PB2 virus were used in the separate reverse transcription reactions: 5'-TGATTTCTGAATCTGGGAGGA-3' (to detect vRNA) and 5'-GGAACAGGATATACCATGGA-3' (to detect mRNA and cRNA). A specific primer to detect cellular 5S rRNA (5'-TCCCAGGCGGTCTCCCATCC-3') was included as an internal control for the amount of total RNA. Transcription products were separated on a 8% polyacrylamide gel containing 7 M urea in TBE buffer and were detected by autoradiography. The amount of RNA was quantified by densitometry and the relative viral RNAs amount was normalized using ratios to 5S rRNA.

Western Blots

MDCK cells were infected the parental and mutated viruses at an MOI=3. Infected cell were harvested at 4h, 8h and 12h pi and lysed in lysis buffer (PBS, 1% Triton X-100, one protease inhibitor cocktail tablets per 10ml PBS). Cell lysates was mixed with the gel-loading buffer (50 mM Tris pH 6.8, 2% SDS, 10% glycerol, 2% 2-mercaptoethanol, 0.1% bromphenol blue) and heated for 10 min at 100°C. Fifteen µl of samples were loaded onto 12% SDS-PAGE

gel and separated by electrophoresis, and then electrophoretically transferred to a polyvinylidene difluoride membrane in a transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The blots were blocked with 5% nonfat milk for overnight at 4°C and incubated with the first antibody (goat polyclonal antibodies against influenza PB2 and PB1, and GAPDH, Santa Cruz Biotechnology Inc.) for 1 hour at room temperature. The blots were then washed three times for 10 min in PBS containing 0.05% Tween-20. The blots were further incubated with the second antibody rabbit anti-goat IgG-HRP (Santa Cruz Biotechnology Inc) for 1 hour at room temperature. After washing three times, blots were incubated with Amersham HyperfilmTM ECL reagent (GE Healthcare, Rockford, UK) for 60s and exposed to high performance chemiluminescence film. Protein bands were identified by prestained protein marker (Fermentas, Inc). The amount of protein expression was quantified by densitometry and the relative PB2 and PB1 amount was normalized using ratios to GAPDH.

Pathogenicity in mice

A total of 70 female BALB/c mice aged 6 to 7 weeks were randomly allocated to 5 groups (14 mice/group). Mice were intranasally inoculated with either 10⁶ TCID₅₀ of virus in 50µL fresh MEM medium or 50 µL fresh MEM (control group) while under light anesthesia by inhalation of 4% isoflurane. Three mice from each group were killed on both 3 and 5 day post inoculation (dpi). The remaining mice (8 per group) were kept so we could monitor their body weights and clinical signs. Weights were recorded daily and general health status was observed twice daily. After the onset of disease, the general health status was observed thrice daily. Severely affected mice (i.e., those with more than 25% body weight loss) were euthanized immediately, and the remaining mice were euthanized on 8 dpi.

During necropsy, the right part of the lung was frozen at -80°C for virus titration, and the left part of the lung was fixed in 10% formalin for histopathologic examination. For virus titration, the 10% lung homogenate was prepared in cold fresh MEM medium by using a Mini Bead Beater-8 (Biospec Products; 16 Bartlesville, OK). The homogenate was centrifuged at 6000 rpm for 5 minutes, and the supernatant was titrated by infecting MDCK cells in 96-well plates. For the histopathologic examination, lung tissues fixed in 10% phosphate-buffered formalin were processed routinely and stained with hematoxylin and eosin. The lungs were examined microscopically; the scoring criteria was based on both the percentage of the lung involved and the histopathologic changes seen, including bronchiolar and alveolar epithelial necrosis, intraalveolar neutrophilic inflammation, peribronchiolar inflammation and bronchiolar epithelial hyperplasia and atypia. The histopathologic changes in lung sections were graded on a scale of 0 to 4 as follows: 0 = normal lung; 1 = minimal change; 2 = mild changes; 3 = moderate changes; and 4 = severe changes. A pathologist scored each slide in a blinded fashion.

Statistical Analysis

Virus titers, weight loss, microscopic lung lesion scores, and mortality were analyzed by using analysis of variance (ANOVA) in GraphPad Prism version 5.0 (GraphPad software Inc. La Jolla, CA); a *P*-value of 0.05 or less was considered significant. Those response variables shown to have a significant effect by treatment group were subjected to comparisons for all pairs by using the Tukey–Kramer test. Pair-wise mean comparisons between inoculated and control groups were made by using the Student's *t*-test.

References

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2. **Richt, J. A., K. M. Lager, B. H. Janke, R. D. Woods, R. G. Webster, and R. J. Webby.** 2003. Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine influenza viruses cocirculating in the United States. *J Clin Microbiol* **41**:3198-205.
3. **Robb, N. C., M. Smith, F. T. Vreede, and E. Fodor.** 2009. NS2/NEP protein regulates transcription and replication of the influenza virus RNA genome. *J Gen Virol* **90**:1398-407.

Supplementary Figure legends

Figure 1: Virus replication in lungs and microscopic lung lesions in mice infected with the parental or mutated viruses on 3 and 5 dpi

a) Virus titers in lungs and b) number of lung lesions in infected mice (3 mice per group). The vertical bars shown indicate the mean of each group, error bars indicate SEM (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Figure 2: Microscopic lung sections from mice infected with the parental and mutated viruses on 5 dpi.

A) Control mice: the bronchioles are lined by normal cuboidal epithelium (arrow) and the alveoli are clear (asterisk). B) The parental 1930-TX98-PB2, C) single 1930-TX98-PB2-271T and D) double mutated 1930-TX98-PB2-590A591A virus infected mice: the alveolar lumen is infiltrated by moderate to large number of neutrophils (asterisk) admixed with cellular debris and edema fluid. There is moderate to marked bronchiolar epithelial necrosis, attenuation and loss (arrow). E) The triple mutated 1930-TX98-PB2-271T590A591A infected mice: the alveoli are mostly clear (asterisk) and very small numbers of neutrophils. There is mild damage to the bronchiolar epithelium (arrow) and the lumen contains sloughed necrotic epithelial cells. Scale bar is 50 μm in all photographs.