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**Supplementary Data**

**Mutual antagonism of mouse-adaptation mutations in HA and PA proteins on H9N2 virus replication**

Liping Ma a,b,d, Huabin Zheng a,b,d, Xianliang Ke a,b, Rui Gui a,b,d, Zhongzi Yao a,b,d, Jiasong Xiong a,b,d, Quanjiao Chen a,b,c,\*

a CAS Key Laboratory of Special Pathogens and Biosafety, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, 430207, China

b Center for Biosafety Mega-Science, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, 430207, China

c Hubei Jiangxia Laboratory, Wuhan, 430207, China

d University of Chinese Academy of Sciences, Beijing, 100049, China

\*Corresponding author

E-mail: chenqj@wh.iov.cn (Q. Chen).

ORCID: 0000-0001-8716-6510

**Fig. S1** Distribution of the six mutations of HA and PA proteins in clinically isolated H9N2 strains. (**A, B**) The proportion of mutation sites of HA (**A**) and PA (**B**) proteins in mammalian- and avian-derived strains. A total of 26 HA and 26 PA amino acid sequences of mammalian H9N2 strains as well as 849 HA and 849 PA amino acid sequences of avian H9N2 isolates, were downloaded from the GISAID database.

**Fig. S2** Lung histopathology of mice infected with rP0-P3HA, rP0-P5PA, rP0-HA-L226Q, rP0-HA-T511I, and rP0-HA-A528V viruses at 3 d.p.i. The images were gained at the ×10 magnification and the bars of the enlarged images represented 100 μm.

**Fig. S3** The receptor-binding properties of 3W3 HA recombinant viruses. Five rescued viruses containing single-point or triple-point HA mutation were tested using the solid-phase direct binding assay with trisaccharide receptors to determine their receptor binding preferences. Red and blue represent avian- and human-origin receptors, respectively. OD450, optical density at 450 nm.

**Fig. S4** Back titration for tested recombinant viruses. **A** virus titer of 11 key viruses’ stocks. The virus titer was obtained by plaque assay in MDCK cells, and indicated with grey. The copy number of the viruses’ vRNA was obtained by absolute quantitative qPCR detecting the vRNA of *NP* gene (presented as the means ± standard deviations, n = 3), pHW2000-NP as the standard, and marked with blue. **B** The fold of plaque and the vRNA copy number of rP0 virus relative to each tested virus was showed with grey and blue, respectively (presented as the means ± standard deviations, n = 3). The mean values of the fold were indicated above the columns.

**Fig. S5.** Heat map of recombinant viruses with synergistic effects on viral replication. A549 and DF-1 cells were infected with the indicated recombinant viruses at MOI 0.01. The virus titers at various time points were normalized with those of the rP0 virus and visualized based on the Log2 fold-change of rP0. Each cell represents the fold change in virus titer (PFU/mL) at that time point compared with that of the rP0 virus after Log2 normalization. Each experiment was performed three times. The viral titers of the first two viruses (except for rP0) of each pattern are compared with those of the third virus of each pattern, while the viral titers of the third virus of each pattern are compared with those of rP0. Two-tailed unpaired *t*-test, \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001. P97, PA-T97I; P545, PA-I545V; P594, PA-S594G; H511, HA-T511I; H528, HA-A528V.

**Table S1.** The primer sequences used in this study.

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| --- | --- | --- |
| Purpose | Primer sequence (5′**–**3′) a，b | |
| Forword | Reverse |
| pHW2000-PB2 construction | ACCTCCGAAGTTGGGGGGGAGCGAAAGCAGGTCAAATATATTCAATATGG | TTTTGGGCCGCCGGGTTATTAGTAGAAACAAGGTCGTTTTTAAACAATTCG |
| pHW2000-PB1 construction | ACCTCCGAAGTTGGGGGGGAGCGAAAGCAGGCAAACTATTTGAATGGA | TTTTGGGCCGCCGGGTTATTAGTAGAAACAAGGCATTTTTTCATGAAGG |
| pHW2000-PA construction | ACCTCCGAAGTTGGGGGGGAGCGAAAGCAGGTACTGATCCAAAATGG | TTTTGGGCCGCCGGGTTATTAGTAGAAACAAGGTACTTTTTTGGACAG |
| pHW2000-HA construction | ACCTCCGAAGTTGGGGGGGAGCAAAAGCAGGGGAATTTCACAACCACTCAAGATG | TTTTGGGCCGCCGGGTTATTAGTAGAAACAAGGGTGTTTTTGCTAATTATATAC |
| pHW2000-NP construction | ACCTCCGAAGTTGGGGGGGAGCAAAAGCAGGGTAGATAATCACTCACTGAGTGA | TTTTGGGCCGCCGGGTTATTAGTAGAAACAAGGGTATTTTTCTTCAATTGTC |
| pHW2000-NA construction | ACCTCCGAAGTTGGGGGGGAGCAAAAGCAGGAGTGAAAATGAATCC | TTTTGGGCCGCCGGGTTATTAGTAGAAACAAGGAGTTTTTTCTAAAATTGCG |
| pHW2000-M construction | ACCTCCGAAGTTGGGGGGGAGCAAAAGCAGGTAGATGTTTAAAGATG | TTTTGGGCCGCCGGGTTATTAGTAGAAACAAGGTAGTTTTTTCAGTCAGC |
| pHW2000-NS construction | ACCTCCGAAGTTGGGGGGGAGCAAAAGCAGGGTGACAAAAACATAATGG | TTTTGGGCCGCCGGGTTATTAGTAGAAACAAGGGTGTTTTTTATCATTA |
| HA-L226Q mutation | CAACGGTC**A**GATGGGAAGAATCAATTATTACTGGT | TCTTCCCATC**T**GACCGTTGACAAGAGGCCTTGGTC |
| HA-T511I mutation | GAAGGAA**T**TTACAAAATCCTAACCATTTATTCGA | GATTTTGTAA**A**TTCCTTCAGATTCCAGCTTGACC |
| HA-A528V mutation | GTGATTG**T**AATGGGGTTTGCTGCCTTCTTGTT | ACCCCATT**A**CAATCACAAGGGATGAGGCGACA |
| PA-T97I mutation | TGCAACA**T**CACGGGTGTCGAAAAACCTAAAT | CACCCGTG**A**TGTTGCAGATACTATTCACCAC |
| PA-I545V mutation | TCCTTGAA**G**TAGGGGATATGCTCCTGCGAAC | TCCCCTA**C**TTCAAGGACACAATACTTTTCCCA |
| PA-S594G mutation | GATTGAG**G**GTATGATTGAAGCTGAATCCTCCG | CAATCATAC**C**CTCAATCTGTTGGAGAGACTGG |
| qPCR detection of NP vRNA | AACTGCTGGCCTTACCCATCTGAT | AGAGAGCACATCCTGGGGTCCATC |

1. The underlined sequences indicate homologous sequence to the corresponding gene;
2. The changed nucleotides are in boldface.

**S1 Dataset.** Sheet 1: HA protein molecular characteristics of mammalian H9N2 viruses. Sheet 2: PA protein molecular characteristics of mammalian H9N2 viruses. Sheet 3: HA protein molecular characteristics of avian H9N2 viruses. Sheet 4: PA protein molecular characteristics of avian H9N2 viruses.

**S2 Dataset.** Quantification of the viral titers for three repeats of all the 64 recombinant viruses normalized to rP0 virus.