Adaptive amino acid substitutions enable transmission of an H9N2 avian influenza virus in guinea pigs

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H9N2 is the most prevalent low pathogenic avian influenza virus (LPAIV) in domestic poultry in the world. Two distinct H9N2 poultry lineages, G1-like (A/quail/Hong Kong/G1/97) and Y280-like (A/Duck/Hong Kong/Y280/1997) viruses, are usually associated with binding affinity for both α 2,3 and α 2,6 sialic acid receptors (avian and human receptors), raising concern whether these viruses possess pandemic potential. To explore the impact of mouse adaptation on the transmissibility of a Y280-like virus A/Chicken/Hubei/214/2017(H9N2) (abbreviated as WT), we performed serial lung-to-lung passages of the WT virus in mice. The mouse-adapted variant (MA) exhibited enhanced pathogenicity and advantaged transmissibility after passing in mice. Sequence analysis of the complete genomes of the MA virus revealed a total of 16 amino acid substitutions. These mutations distributed across 7 segments including PB2, PB1, PA, NP, HA, NA and NS1 genes. Furthermore, we generated a panel of recombinant or mutant H9N2 viruses using reverse genetics technology and confirmed that the PB2 gene governing the increased pathogenicity and transmissibility. The combinations of 340 K and 588 V in PB2 were important in determining the altered features. Our findings elucidate the specific mutations in PB2 contribute to the phenotype differences and emphasize the importance of monitoring the identified amino acid substitutions due to their potential threat to human health.
The capacity of AIVs to transmit among mammals appears to require multiple viral features, such as human receptor binding, increased polymerase activity and high thermostability of HA.2-4,22,23,25,26 Human receptor binding specificity, specifically leucine (L) at position 226 in HA receptor binding site, is critical for direct transmission of avian H9N2 viruses in ferrets.20 Increased viral polymerase activity mediates adaptation of AIVs to a mammalian host.26 The high thermostability of HA facilitates H5 AIV transmission via respiratory droplets in mammals.27

The transmission of AIVs to mammals appears to acquire human receptor binding preference.28-30 Y439 (A/Duck/Hong Kong/Y439/1997), G9 (A/Chicken/Hong Kong/G9/1997), G1 (A/quail/Hong Kong/G1/97) and Y280 (A/Duck/Hong Kong/Y280/1997) are four different H9N2 poultry lineages. The G1 and Y280 poultry lineages are usually associated with both avian and human receptor binding affinity and could potentially transmit between mammals.5 Mice have been widely applied to study mammalian adaptation of AIVs. Serial passage of AIVs in mammals can result in adaptive changes that confer enhanced pathogenicity and transmissibility in mammals.31-33 Although the pathogenicity and transmissibility of H9N2 AIVs have been characterized previously,33-35 the molecular features that account for H9N2 airborne transmissibility in mammals are not clear. In the current study, a Y280-like H9N2 virus transmitted among guinea pigs after mouse adaption. To explore which gene-specific mutations contribute to altered phenotype, we generated recombinant and mutant viruses using reverse genetics technology.

Materials and Methods

Ethics statement. The ethics statement was described in our previous work.36 Briefly, all animal studies were conducted in strict accordance with the Guidelines of Animal Welfare of World Organization for Animal Health and the protocols approved by the Hubei Provincial Animal Care and Use Committee (approval number SYXX 2016-0004).

Viruses. The wild type H9N2 virus used in this study was isolated from chickens in 2017, in China, and named A/chicken/Hubei/214/2017 (abbreviated as WT). A single amino acid substitution in PB2 was generated by using A Quick Change XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The WT and MA were the parental viruses. We used the WT as the backbone to generated the recombinant reassortant viruses (WT-PB2MA, WT-PB1att, WT-PAatt, WT-NPatt, WT-HAatt, WT-NAatt, WT-NSatt) and the mutant viruses (WT-PB2Δ490 and WT-PB2Δsav) as described previously.37 The recombinant reassortant viruses each contained one gene from the MA virus and the mutant viruses each was a single amino acid substitution in the PB2 of WT. The viruses were propagated in 9-day-old specific pathogen free (SPF) embryonated eggs and stored at −80°C.

H9N2 adaptation in mice. The mouse-adapted H9N2 virus was derived from series of sequential lung-to-lung passages of the WT virus in mice as described previously.35 Briefly, groups of three five-week-old female BALB/c mice were anesthetized with ether and intranasally inoculated with 50 μL of a 10^6 EID₀₅ solution of the WT virus. Lungs were harvested and homogenized in 0.7 mL of PBS at 3 dpi. The supernatants were subsequently used to inoculate into naive mice. The infected mice died at 3 dpi at the fourth passage. The mouse-adapted virus (MA) was isolated from the homogenized lung tissue supernatants using 9-day-old SPF embryonated eggs for subsequent use in pathogenicity and transmissibility studies.

Sequence analysis. The viral gene sequences were acquired as described in our previous work.36 In brief, viral RNA was extracted from allantoic fluid using TRIzol reagent (Invitrogen) and reverse transcribed into cDNAs using the primer Uni12 (5′-AGC RAA AGC AGG-3′) primers, an RT reagent kit and viral genes were amplified using a PCR kit (Takara, Japan) according to the manufacturer’s protocol. The PCR products of the eight segments of the viruses were amplified by PCR using specific virus primers as described by Hoffmann et al.38 The PCR products were purified and sequenced by Sangon Biotech Company. Amino acid substitutions between the WT and MA viruses were identified. All the sequence data were analyzed with the SeqMan program (DNASTAR, Madison, WI). All reference sequences used in this study were obtained from the National Center for Biotechnology Information (NCBI) GenBank database.

Receptor binding specificity assay. The receptor-binding specificities of the WT and MA viruses were determined by HA assays with 1% chicken red blood cells (cRBCs) as described in our previous work.36. The HA assays, sialic acid residues were enzymatically removed from cRBCs by incubating the cells with 50 μL of a 10^6 EID₀₅ solution of the WT virus. Lungs were harvested and homogenized in 0.7 mL of PBS at 3 dpi. The supernatants were subsequently used to inoculate into naive mice. The infected mice died at 3 dpi at the fourth passage. The mouse-adapted virus (MA) was isolated from the homogenized lung tissue supernatants using 9-day-old SPF embryonated eggs for subsequent use in pathogenicity and transmissibility studies.

Cell culture and growth curves. The virus growth curve experiment was performed as described in our previous work.39,40 Madin-Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Auckland, New Zealand). The growth kinetics of the WT and MA viruses were determined by inoculating MDCK cells at a multiplicity of infection (MOI) of 0.001 50% tissue culture infectious dose (TCID₅₀) per cell. One hour after inoculation (hpi), the cells were washed twice with PBS, and fresh medium supplemented with 1 μg/mL tetracycline (TPCK) and trypsin (Sigma, St. Louis, MO, USA) was added. The supernatants were sampled at 24, 36, and 48 hpi. The HA titers were determined by calculating the lg TCID₅₀/mL in MDCK cells. The TCID₅₀ values were calculated according to the method of Reed and Muench.
eggs (EID50/gram). Briefly, the lung tissues were weighed, and 0.1 grams of each tissue was placed into 1 ml of P
and MA peaked at 106.7 TCID50/mL and 105 TCID50/mL at 36 hpi, respectively (Fig. 2). The virus titer of MA was
ity of the WT and MA viruses, we tested the growth curve of WT and MA in MDCK cells. The virus titers of WT
receptor binding specificity of the two viruses as previously described36. Briefly, the receptor binding affinity
are representative of at least 3 separate experiments. The error bars indicate the standard deviation.
(ANOVA) with GraphPad Prism software (San Diego, CA, USA). All assays were run in triplicate, and the data
receptor-binding specificity is an important factor for cross-species transmission of AIVs41,42. We thus measured
(Fig. 3).

Figure 1. Pathogenicity in mice. Five mice per group were intranasally inoculated with 10^6.0 EID50 of PBS,
weights were monitored daily for 14 days. The values are the average scores of the overall body weight loss with respect to the initial body weights, ± standard deviations (SDs). (B) The survival percentages were calculated by observing the infected mice. (C) Lungs were collected from mice inoculated with 10^6.0 EID50 WT or MA at 1, 3, 5 and 7 dpi (n = 3), virus titers were determined in 9-day-old SPF embryonated eggs (EID50/gram). Briefly, the lung tissues were weighed, and 0.1 grams of each tissue was placed into 1 ml of PBS containing 100 U/ml penicillin, to make 10% weight/volume lung homogenates (*P < 0.05; **P < 0.01).

Mouse experiments. Mouse experiments were performed as described in our previous work39. Groups of five six-week-old female BALB/c mice (Merial Vital Laboratory Animal Technology Company, Beijing, China) were anesthetized with ether and intranasally inoculated with 50 μL of 10^6 EID50 solution of the test virus or PBS. The weight loss and mortality of mice in these groups were monitored daily for 14 days. Mice that lost >30% of their original body weight were humanely euthanized.

Guinea pig experiments. Guinea pig experiments were performed as described in our previous work36. Hartley strain female guinea pigs weighing 300 to 350 g (Merial Vital Laboratory Animal Technology Company, Beijing, China), confirmed to be seronegative for influenza viruses prior to the experiment, were used in these studies. In the transmission studies, groups of three guinea pigs were anesthetized with ether and intranasally inoculated with 300 μL of 10^6.0 EID50 solution of the test virus and housed in a cage placed in an isolator. The next day, three naive guinea pigs were individually paired and cohoused with an infected guinea pig for the direct contact transmission studies, and another naive guinea pig was housed in a wire frame cage adjacent to the infected guinea pig for the aerosol transmission studies. The distance between the cages of the infected and aerosol-contact guinea pigs was 5 cm. To monitor virus shedding, nasal washes were collected from all animals at 2, 4, 6, and 8 dpi and titrated.

Statistics analysis. Statistically significant differences were determined using one-way analysis of variance (ANOVA) with GraphPad Prism software (San Diego, CA, USA). All assays were run in triplicate, and the data are representative of at least 3 separate experiments. The error bars indicate the standard deviation.

Results
The adapted H9N2 virus exhibits enhanced pathogenicity. We studied the pathogenicity of the MA virus in mice. Mice inoculated with the MA virus rapidly lost more than 30% of their original weight and succumbed to death at 5 dpi (Fig. 1), its MDL50 was 10^4.5 EID50/mL. In contrast, the WT-inoculated mice experienced no substantial body weight loss and had nonlethal infections (Fig. 1A,B). These results show that a series of lung-to-lung passages of the H9N2 virus resulted in substantially increased virulence in mice.

Additionally, we also tested the viral titers of WT and MA in the lungs of the mice. In the MA-infected mice, the titers were 10^3.5 EID50/gram, 10^3.4 EID50/gram and 10^2.3 EID50/gram at 1, 3 and 5 dpi respectively which were 10 fold higher than those of WT (*P = 0.01; **P < 0.05; ***P < 0.01; n = 3). No virus shedding was detected in the lungs of the WT-infected mice at 5 and 7 dpi (Fig. 1C). These results suggest that MA showed advantageous growth properties in the lungs of infected mice compared to WT.

In summary, based on the results of mice studies, the MA virus exhibited increased virulence and advantageous growth ability compared to the WT virus.

The adapted H9N2 virus replicates to higher titers in MDCK cells. To evaluate the replicative capacity of the WT and MA viruses, we tested the growth curve of WT and MA in MDCK cells. The virus titers of WT and MA peaked at 10^6.7 TCID50/mL and 10^7 TCID50/mL at 36 hpi, respectively (Fig. 2). The virus titer of MA was 10 fold higher than that of WT (**P < 0.01, n = 3), suggesting MA replicate more efficiently than WT.

The adapted H9N2 virus display human and avian receptor binding affinity. Human receptor-binding specificity is an important factor for cross-species transmission of AIVs41,42. We thus measured the receptor binding specificity of the two viruses as previously described36. Briefly, the receptor binding affinity was determined by evaluating the ability of WT and MA to agglutinate four types of cRBCs. cRBCs contain avian and human receptors, while cRBCs treated with VCNA contains no receptors (desialylation-cRBCs), and resi-
allyated cRBCs contained either human (α2,6-cRBCs) or avian (α2,3-cRBCs) receptors. The HA titers represent 3 separate experiments. The results showed that the WT and MA viruses bind to both avian and human receptors (Fig. 3).
The adapted H9N2 virus transmits in guinea pigs. To explore the impact of mouse adaptation on the transmissibility of the MA virus, we next measured the transmissibility of WT and MA in guinea pigs following the same procedures as we previously reported. The WT viruses were only detected in the infected group, and no viruses were detected in the contact group or in the aerosol contact group, indicating that no virus transmission occurred (Fig. 4A). The MA viruses transmitted to 2 direct contact guinea pigs and 1 aerosol contact guinea pig (Fig. 4B). These findings demonstrate that the MA virus has acquired transmissibility in the guinea pig model after mouse adaptation.

Sequence analysis in the adapted H9N2 virus. The molecular basis for the increased virulence and transmissibility was investigated by sequencing the complete genomes of WT and MA viruses. Sixteen amino acid substitutions were identified as shown in Table 1, and these mutations were distributed across 7 segments of the influenza genome. These included 3 changes in PB1 proteins, 4 changes in PA proteins, 2 changes in each the PB2, NP, HA and NS1 proteins and a single change in NA protein.

Mutations in PB2 enable H9N2 to transmit among guinea pigs. To confirm which gene-specific mutations contributed to the increased pathogenicity and transmissibility of the MA virus, we used the WT virus as the backbone to generate a panel of recombinant viruses, each containing one gene from the MA virus. The pathogenicity of the recombinant viruses was studied in mice. The recombinant virus containing the PB2 gene from MA (WT-PB2MA) exhibited increased virulence, and the body weight loss and mortality of the infected mice were comparable to those of the MA-infected mice. Whereas the other recombinant viruses WT-PB1MA, WT-PAMA, WT-NPMA, WT-HAMA, WT-NSMA, caused no substantial body weight loss and nonlethal infections (Fig. 5). In addition, we also evaluated the transmissibility of the recombinant viruses. The WT-PB2MA transmitted to 2 direct contact guinea pigs and 1 aerosol contact guinea pig (Fig. 6A), but the other recombinant viruses transmitted to neither the direct contact groups nor the aerosol contact groups (data not shown).

Two amino acid substitutions, 340 K and 588 V, were identified in the PB2 gene of the MA virus. Therefore, we generated variant viruses contained a single amino acid substitution in PB2 in WT backbone (WT-PB2340K and WT-PB2588V). The two variants also displayed increased pathogenicity than the recombinant viruses, with the exception of the WT-PB2MA virus (Fig. 5). In guinea pig study, both WT-PB2340K and WT-PB2588V transmitted...
Figure 4. Horizontal transmissions of the viruses in guinea pigs. Groups of three guinea pigs seronegative for influenza viruses were inoculated with $10^{6.0}$ EID$_{50}$ of the test viruses. The next day, the three inoculated guinea pigs were individually cohoused with a direct-contact guinea pig; in addition, an aerosol contact guinea pig was housed in a wire frame cage adjacent to that of the infected guinea pig. The distance between the cages of the infected and aerosol-contact guinea pigs was 5 cm. Nasal washes were collected from all animals for virus shedding detection every other day beginning on day 2 after the initial infection. Each color bar represents the virus titer in an individual animal. The dashed lines indicate the lower limit of virus detection.

| Segment | Position | WT | MA |
|---------|----------|----|----|
|        |          |    |    |
| PB2     | 340      | R  | K  |
|         | 568      | A  | V  |
| PB1     | 48       | K  | Q  |
|         | 368      | V  | I  |
|         | 628      | M  | L  |
| PA      | 343      | S  | A  |
|         | 356      | K  | R  |
|         | 423      | V  | I  |
|         | 554      | V  | L  |
| NP      | 217      | V  | I  |
|         | 239      | V  | M  |
| HA      | 235      | M  | Q  |
|         | 254      | K  | R  |
| NA      | 72       | R  | K  |
| NS1     | 127      | N  | T  |
|         | 216      | T  | P  |

Table 1. Amino acid substitutions in the MA virus.

Figure 5. The pathogenicity of the rescued viruses. Five mice per group were intranasally inoculated with $10^{6.0}$ EID$_{50}$ of the rescued viruses. (A) Mouse body weights were monitored daily for 14 days. The values are the average scores of the overall body weight loss with respect to the initial body weights, ± standard deviations (SDs). (B) The survival percentages were calculated by observing the infected mice.
to direct contact guinea pigs, but no virus detected in the aerosol contact guinea pigs (Fig. 6B,C). These results suggest the combination of 340K and 588V in PB2 contributed to the aerosol transmissibility of the MA virus.

Discussion

H9N2 AIVs pose a potential threat to public health. In this study, a Y280-like H9N2 virus displayed increased pathogenicity and transmissibility after serial passage in mice. We found that PB2-340K in combination with PB2-588V contributed to the altered features.

Influenza A virus can infect a variety of animal species. The receptor binding specificity of AIV is recognized as an important factor in interspecies transmission. The HA gene of influenza A virus contains receptor binding sites and determines the receptor-binding specificity. AIVs isolates with 226-Leu(L) and 228-Gly(G) (H3 numbering) in HA have been reported to prefer both avian and human receptors. The loss of glycosylation at residue 158 in the HA was also shown to be responsible for H5N1 AIV binding to human receptors. In this study, there was no difference in receptor binding specificity between the WT and MA viruses with the in vivo results for the WT-HAΔMA virus that didn't show difference from the WT, suggesting the HA was not involved in the altered phenotype.

H5N1, H7N9, H9N2 and H5N6 AIVs have been reported to occasionally break the species barrier to infect humans, but they have not been able to disseminate among humans. The major reason is their limited airborne transmissibility among humans. Previous studies found that ferrets and guinea pigs adaptation enabled AIVs to transmit in mammals. In our previous study, mouse adaption could not enable the H5N6 to transmit in the guinea pig model, but it enabled airborne transmission of the H9N2 in this study. We suppose the reason for its airborne transmissibility after mouse adaption might be correlate with its avian and human receptors binding affinity.

Several previous works have studied H9N2 adaptation to chickens or mammals. The HA-363K and PA-672L enabled H9N2 airborne transmission among chickens. The PB2-340K and PB2-588V enabled H9N2 transmission in swine. The PA-343A and PA-346K enabled H9N2 contact transmission in guinea pigs. The loss of glycosylation at 158 in the HA was also shown to be responsible for H5N1 AIV binding to human receptors. In this study, there was no difference in receptor binding specificity between the WT and MA viruses with the in vivo results for the WT-HAΔMA virus that didn't show difference from the WT, suggesting the HA was not involved in the altered phenotype.

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Figure 6. Assessment of amino acid substitutions in PB2 on transmission in guinea pigs. (A–C) Transmissibility of WT-PB2ΔMA, WT-PB2Δ340K and WT-PB2Δ588V. Nasal washes were collected from all animals for virus shedding detection every other day beginning on day 2 after the initial infection. Each color bar represents the virus titer in an individual animal. The dashed lines indicate the lower limit of virus detection.
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**Author contributions**

L.L., C.S. and W.C. were the principal investigator, who designed and supervised the study, and wrote the grant application. L.Y., D.S., C.L., G.K., G.Z., L.J., Z.J., L.Q., Z.W., S.Y., W.H., Z.T., W.G., Z.J. and Z.C. performed the receptor binding specificity assay, cell culture experiments, virus rescue and animal studies. J.M., G.Y. and S.H. designed and supervised the study, and wrote the grant application. L.Y., D.S., C.L., G.K., G.Z., L.J., Z.J., L.Q., Z.W., S.Y., W.H., Z.T., W.G., Z.J. and Z.C. performed the receptor binding specificity assay, cell culture experiments, virus rescue and animal studies. J.M., G.Y., S.H. and Z.Z. designed the work and revised it critically. All authors had read the manuscript and approved its final version.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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