Enhanced pathogenicity and neurotropism of mouse-adapted H10N7 influenza virus are mediated by novel PB2 and NA mutations

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Abstract

The H10 subtype of avian influenza viruses (AIVs) circulates globally in wild birds and poultry, and this subtype has been shown to be increasingly prevalent in China. Among the various H10 viruses, H10N7 AIVs have caused repeated mammalian and human infections. To investigate their genetic adaptation in mammals, we generated a mouse-adapted avian H10N7 variant (A/mallard/Beijing/27/2011-MA; BJ27-MA) which exhibited increased virulence in mice compared to wild-type virus and acquired neurotropism. Sequencing showed the absence of the widely recognized mammalian adaptation markers of E627K and D701N in PB2 in the mouse-adapted strain; instead, five amino acid mutations were identified: E158G and M631L in PB2; G218E in haemagglutinin (H3 numbering); and K110E and S453I in neuraminidase (NA). The neurovirulence of the BJ27-MA virus necessitated the combined presence of the PB2 and NA mutations. Mutations M631L and E158G of PB2 and K110E of NA were required to mediate increased virus replication and severity of infection in mice and mammalian cells. PB2-M631L was functionally the most dominant mutation in that it strongly upregulated viral polymerase activity and played a critical role in the enhancement of virus replication and disease severity in mice. K110E mutation in NA, on the other hand, significantly promoted NA enzymatic activity. These results indicate that the novel mutations in PB2 and NA genes are critical for the adaptation of H10N7 AIV in mice, and they could serve as molecular signatures of virus transmission to mammalian hosts, including humans.

INTRODUCTION

At present, avian influenza viruses (AIVs) – which contributed to the 1918 H1N1, 1957 H2N2 and 1968 H3N2 virus pandemics [1] through viral reassortment – cause great economic loss to the global poultry industry. H5N1 and H9N2 influenza viruses, as the two major subtypes circulating in poultry, are prime candidates for potentially causing another human influenza outbreak [2, 3]. In addition, recently reported avian H7N9 virus infections in humans raised further concerns about the potential subtypes that could cause possible pandemics [4]. Thus, contingency planning for the prevention and management of AIV infections in humans should be based on a broad range of possible subtypes.

The H10 subtype of AIV was first isolated from chickens in Germany in 1949 [5, 6]. Subsequently, viruses bearing the H10 haemagglutinin (HA) and a different neuraminidase (NA) started to circulate widely in wild birds and domestic poultry around the world [7–9]. In recent years, H10 subtype AIVs have become increasingly prevalent in China [10]. Since 1984, repeated infections or deaths of mammals with this subtype have been reported [11–13]. Human cases of H10 virus infections have also been documented...
Inoculation (p.i.; data not shown), indicating a significant activity and inappetence; all infected mice died by 5 days post-
played severe clinical symptoms of respiratory distress, inac-
decreased activity and a ruffled coat. At P9 and P10, mice dis-
P5, infected mice showed mild clinical signs, including
human infection with a novel reassortant H10N8 virus were
and February 2014, two fatal cases and one severe case of
break in a chicken farm in Australia [9]. In 2014
mammals, including humans. In 2010, H10N7 caused an out-

tic H10N7 influenza virus in mammals, we serially passaged this low-pathogenic avian-derived H10N7 virus (BJ27) in mice. We found that mouse-adapted H10N7 virus acquired increased pathogenicity with high mortality and neurovirulence. The well-known mammalian adaptation markers PB2-E627K and PB2-D701N [28, 29] were not found in any of the 30 clones, indicating that other viable adaptations were present in the BJ27-MA virus. Here, five conserved amino acid mutations that could be linked to increased pathogenicity were identified in three virus segments of the BJ27-MA virus as PB2-E158G, PB2-M631L, HA-G218E (H3 numbering), NA-K110E and NA-S453I.

Mouse-adapted H10N7 virus exhibited enhanced pathogenicity and neurovirulence

Two groups of 11 BALB/c mice were infected with 10^{5.5} TCID_{50} of BJ27 or BJ27-MA virus to compare viral pathoge-
nicity. BJ27-MA virus caused dramatic weight loss in infected mice and all died by 6 days p.i., while mice infected with BJ27 showed modest weight loss of 8.7 % and began to recover from 7 days p.i. (Fig. 1a, b). To determine whether the difference in pathogenicity between BJ27 and BJ27-MA virus was due to altered viral replication, groups of three BALB/c mice were euthanized at 3 and 5 days p.i., and virus titres in lung and brain were determined. As shown in Fig. 1 (c) and (d), BJ27-MA virus replicated to a higher titre in the lungs than wild-type BJ27 virus at 3 and 5 days p.i. Furthermore, BJ27-MA virus was isolated from the brains of infected mice with mean titres of 2.1 log_{10} TCID_{50} ml^{-1} at 3 days p.i. and 2.8 log_{10} TCID_{50} ml^{-1} at 5 days p.i. No virus was isolated from the brains of BJ27-virus-infected mice. Therefore, the mouse-adapted BJ27 virus has acquired neurotropism, which could contribute to the severity of infection in mice.

Genetic changes in adapted BJ27-MA virus

To identify the potential segments and amino acid substitutions that are responsible for the increased pathogenicity and replication of BJ27-MA virus in mice, the consensus sequences of 30 virus clones were determined. Interestingly, the most common mammalian adaptation determinants of PB2-E627K and PB2-D701N [28, 29] were not found in any of the 30 clones, indicating that other viable adaptations were present in the BJ27-MA virus. Here, five conserved amino acid mutations that could be linked to increased pathogenicity were identified in three virus segments of the BJ27-MA virus as PB2-E158G, PB2-M631L, HA-G218E (H3 numbering), NA-K110E and NA-S453I.

PB2-E158G mutation resides in the amino-terminal nucleo-
protein (NP) binding region (1–269 aa) [24] and PB2-
M631L lies in the PB2–PB1 and PB2–NP interaction regions [26]. HA-G218E is located close to the 220-loop of the globular head HA1 domain [30]. NA-K110E and NA-S453I reside in the amino-terminal and carboxyl-terminal regions of NA protein, respectively; both are located in the interface of the tetrameric structure of NA protein [31].

PB2 and NA segments in BJ27-MA virus conferred increased pathogenicity and replication capacity in mice

To identify virus segments from the BJ27-MA virus that confer increased pathogenicity in mice, a series of recombi-
nant viruses were generated by reverse genetics based on wild-type BJ27 (rBJ27) and BJ27-MA (rBJ27-MA) viruses. Recombinant viruses rBJ27-PB2, rBJ27-HA and rBJ27-NA were constructed in an rBJ27 virus background with the substituted segments of PB2, HA and NA from the rBJ27-
MA virus. Mice infected with recombinant viruses were monitored over 14 days for weight loss and survival. As shown in Fig. 2(a, b), similar to wild-type BJ27 virus infection, all mice infected with rBJ27 and rBJ27-HA viruses survived with a maximum of 6.7 and 8.8% weight loss, respectively. By contrast, mice infected with rBJ27-MA and rBJ27-PB2 viruses showed 25 to 31% weight loss and 100% mortality by 6 days p.i. rBJ27-NA virus infection resulted in a moderate increase in pathogenicity with 40% mortality.

The 50% mouse lethal dose (MLD$_{50}$) values also showed the same descending order of viral virulence: rBJ27-MA, rBJ27-PB2 (both MLD$_{50}$ 4.75 log$_{10}$ TCID$_{50}$) > rBJ27-NA (5.75 log$_{10}$ TCID$_{50}$) > rBJ27 and rBJ27-HA (>6.5 log$_{10}$ TCID$_{50}$; Table 1). None of these segment recombinant viruses were neurotropic, although they were recovered from lungs and extrapulmonary organs (kidney and/or spleen; Table 1). Significantly higher viral titres were found in the lungs of mice infected with rBJ27-PB2 and rBJ27-NA compared to those infected with rBJ27 (P<0.05). Thus, the adaptive PB2 and NA segments of BJ27-MA conferred increased virulence of wild-type BJ27 virus in mice.

**Combined PB2 and NA segments of BJ27-MA virus contributed to neurovirulence**

Influenza virus replication in the central nervous system (CNS) often leads to a fatal outcome [32–34]. Although mouse-adapted BJ27-MA virus was able to efficiently replicate in the murine brain, none of the single-segment recombinant viruses described above were found in the brain of infected mice (Table 1). Next, we generated three double-segment recombinant viruses in an rBJ27 backbone: rBJ27-PB2/HA virus; rBJ27-PB2/NA virus; and rBJ27-HA/NA virus. As shown in Table 2, only rBJ27-PB2/NA virus was found in infected murine brains at 3 and 5 days p.i. with MLD$_{50}$ and viral load similar to those infected with rBJ27-MA at each time point. Viral NP could be detected in...
neurons of mice infected with rBJ27-MA and rBJ27-PB2/NA viruses (Fig. 3). These data demonstrated that the combined PB2 and NA segments of rBJ27-MA contributed to its neurovirulence in mice.

PB2-M631L, PB2-E158G and NA-K110E contributed to severe BJ27-MA virus infection

To pinpoint the contribution of individual single mutations in PB2 and NA to the increased pathogenicity of BJ27-MA, four point-mutant viruses were generated with the rBJ27 backbone.
as rBJ27-PB2/E158G, rBJ27-PB2/M631L, rBJ27-NA/K110E and rBJ27-NA/S453I. Virus rBJ27-PB2/M631L showed the highest virulence, with all of the infected mice dying before 8 days p.i. (Fig. 2c, d). rBJ27-PB2/E158G and rBJ27-NA/K110E caused moderate weight loss of around 13.3% without fatality. Virus NA-S453I and wild-type rBJ27 were the least pathogenic and caused 7.2–8.1% weight loss. PB2-M631L virus had the lowest MLD50 (4.75 log10 TCID50) relative to all other viruses (>6.5 log10 TCID50) (Table 1). Compared with rBJ27, the viral titres of rBJ27-PB2/M631L, rBJ27-PB2/E158G and rBJ27-NA/K110E in murine lungs were significantly higher at 3 and/or 5 days p.i. (Table 1). Virus rBJ27-PB2/M631L produced the highest viral titre. However, none of the four point-mutation viruses showed extrapulmonary infection in liver, spleen, kidney or brain.

Histopathological staining of infected lungs at 5 days p.i. showed that the severity of tissue damage was correlated with pathogenicity (Fig. 2e). rBJ27-PB2/M631L and rBJ27-MA elicited the most severe lung lesions of oedema, inflammatory infiltrates, interstitial pneumonia and bronchopneumonia. Lungs from rBJ27-PB2/E158G and rBJ27-NA/K110E virus infection showed less severe bronchopneumonia. Almost no lung lesion was detected from groups infected with rBJ27 and rBJ27-NA/S453I viruses except for some thickening of the alveolar wall and mild infiltration of inflammatory cells.

We next compared the replication of the four mutant viruses in MDCK and human pulmonary adenocarcinoma (A549) cells, infected at an m.o.i. of 0.01 or 0.1, respectively, for 72 h. In MDCK cells, rBJ27-MA virus showed higher replication than rBJ27, rBJ27-PB2/E158G and rBJ27-PB2/M631L viruses. However, rBJ27-MA virus showed higher replication than rBJ27, rBJ27-PB2/E158G and rBJ27-PB2/M631L viruses in human pulmonary adenocarcinoma (A549) cells. Therefore, rBJ27-MA virus was the most pathogenic virus in both murine and human cells.

**Table 1. Pathogenicity and replication of BJ27 (H10N7) recombinant and mutant viruses in mice**

<table>
<thead>
<tr>
<th>Virus</th>
<th>MLD50 (log10 TCID50)</th>
<th>Lung 3 days p.i.</th>
<th>Lung 5 days p.i.</th>
<th>Brain 3 days p.i.</th>
<th>Brain 5 days p.i.</th>
<th>Spleen 3 days p.i.</th>
<th>Spleen 5 days p.i.</th>
<th>Kidney 3 days p.i.</th>
<th>Kidney 5 days p.i.</th>
<th>Liver 3 days p.i.</th>
<th>Liver 5 days p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>rBJ27</td>
<td>&gt;6.5</td>
<td>4.5±0.3</td>
<td>4.7±0.1</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>rBJ27-MA</td>
<td>4.75</td>
<td>6.5±0.3†</td>
<td>6.9±0.3†</td>
<td>2.1±0.4†</td>
<td>2.8±0.6†</td>
<td>2.3, 1.8§</td>
<td>2.3, 1.8§</td>
<td>0/3</td>
<td>2.9±0.3†</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>rBJ27-PB2</td>
<td>4.75</td>
<td>6.2±0.4†</td>
<td>6.6±0.3†</td>
<td>0/3</td>
<td>0/3</td>
<td>2.3†</td>
<td>2.3†</td>
<td>0/3</td>
<td>2.2±0.6†</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>rBJ27-NA</td>
<td>&gt;6.5</td>
<td>5.0±0.4</td>
<td>5.4±0.3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>rBJ27-NA/S453I</td>
<td>5.75</td>
<td>5.5±0.1†</td>
<td>6.1±0.3†</td>
<td>0/3</td>
<td>0/3</td>
<td>1.8†</td>
<td>1.8†</td>
<td>0/3</td>
<td>2.9±0.3†</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>rBJ27-PB2/NA/K110E</td>
<td>&gt;6.5</td>
<td>5.2±0.4†</td>
<td>5.5±0.3†</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>rBJ27-NA/S453I</td>
<td>&gt;6.5</td>
<td>4.9±0.4†</td>
<td>5.3±0.1†</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

* Mean virus titre in sample (log10 TCID50 ml-1) ± s0. The lower limit of detection was 100.75 TCID50 ml-1 for each sample.
† Virus titre of corresponding strains was significantly higher than that of rBJ27 (P<0.05, ANOVA).
‡ The number of samples with recovered viruses versus the number of total collected samples.
§ The number(s) shows virus titre in an individual infected mouse.

**Table 2. Pathogenicity and neurovirulence of double-segment recombinant H10N7 viruses in mice**

<table>
<thead>
<tr>
<th>Virus</th>
<th>MLD50 (log10 TCID50)</th>
<th>Average virus titre in brain*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lung 3 days p.i.</td>
</tr>
<tr>
<td>rBJ27</td>
<td>&gt;6.5</td>
<td>0/3†</td>
</tr>
<tr>
<td>rBJ27-MA</td>
<td>4.75</td>
<td>2.1±0.4</td>
</tr>
<tr>
<td>rBJ27-PB2/HA</td>
<td>5.25</td>
<td>0/3</td>
</tr>
<tr>
<td>rBJ27-PB2/NA</td>
<td>4.75</td>
<td>1.8, 2.3§</td>
</tr>
<tr>
<td>rBJ27-HA/NA</td>
<td>5.5</td>
<td>0/3</td>
</tr>
</tbody>
</table>

* Mean virus titre in sample (log10 TCID50 ml-1) ± s0. Lower limit of detection was 100.75 TCID50 ml-1 in the brain.
† The number of samples with recovered viruses versus the number of total collected samples.
‡ The number(s) shows virus titre in an individual mouse.

**Fig. 3. Neurotropism of recombinant BJ27 (H10N7) viruses in murine brain.** Sections of brains taken from mice 5 days p.i. with 105.5 TCID50 of indicated viruses were immunostained for viral NP (open arrow). Scale bar, 400 µm.
yield (up to 56-fold higher) than the parental rBJ27 from 24 to 72 h p.i., and PB2-M631L mutation increased the replication of rBJ27 virus at 36 h p.i. (both \( P<0.05 \)) (Fig. 4a). The replication abilities of the rBJ27-PB2/E158G, rBJ27-NA/K110E and rBJ27-NA/S453I viruses were similar to those of the rBJ27 virus. In A549 cells, the rBJ27-MA virus also showed higher yield from 24 to 60 h p.i., and the rBJ27-PB2/M631L, rBJ27-PB2/E158G and rBJ27-NA/K110E viruses produced more progeny virus at 24 or 36 h p.i. than the rBJ27 virus (all \( P<0.05 \)) (Fig. 4b). The growth characteristics of wild-type and mutant H10N7 viruses were also determined in embryonated chicken eggs. The virus titres were between 7.75 and 8.25 \( \log_{10} \) 50% egg infectious dose (EID\(_{50}\)) ml\(^{-1}\). There was no significant difference of the virus titres in eggs infected with these viruses (data not shown). In summary, PB2-M631L and PB2-E158G and NA-K110E mutations in the rBJ27 virus backbone conferred enhanced replication abilities as compared to wild-type rBJ27 virus in mice and mammalian cells, with the PB2-M631L mutation being the most potent determinant.

**PB2-M631L and PB2-E158G mutations enhanced polymerase activity of BJ27-MA virus in human cells**

Polymerase activity, performed by ribonucleoprotein (RNP), has been shown to catalyze viral transcription and genomic replication, which correlate with viral replication and pathogenicity in hosts [35]. PB2 is one of the components of RNP. To evaluate whether the mutations of PB2-E158G and PB2-M631L affect viral polymerase activity, we generated three mutant RNP complexes in the background of the RNP of rBJ27, and measured their polymerase activities in human embryonic kidney (293T) cells by a luciferase minigenome assay (Fig. 5). RNP polymerase activity with single E158G or M631L mutation was 28 or 62 times higher, respectively, than with wild-type rBJ27 RNP complex; combined E158G and M631L mutations induced 75 times higher activity than with the wild-type PB2 (all \( P<0.05 \)). Western blots using protein lysates derived from 293 T cells transfected with the different PB2 mutant plasmids in RNP polymerase assays showed comparable PB2 protein expression, indicating that the differences in polymerase activity were not due to protein expression levels (Fig. 5). Collectively, our data suggest that enhanced polymerase activities resulting from PB2-M631L and PB2-E158G mutations contribute to increased viral replication in mice and mammalian cells, while the single M631L mutation in PB2 appears to be a major contributor.

**NA-K110E increased NA enzymatic activity**

As there are two amino acid mutations in the NA protein, including K110E and S453I, we evaluated their effects on NA enzymatic activity, as described previously [36]. Based on \( K_m \) values, we found that the NA-K110E mutation significantly increased substrate affinity, as did the mutant segment (rBJ27-NA with double mutations) (Table 3). Similarly, \( V_{\text{max}} \), which was determined by the specific activity and the amount of enzyme in the reaction mixture, was significantly higher with the K110E mutation than with wild-type rBJ27 virus (\( P<0.05 \)). The \( V_{\text{max}} \) of rBJ27-NA/S453I virus was higher (although not significantly) than that of rBJ27 virus. Thus, NA-K110E mutation significantly improved NA enzymatic activity.

**DISCUSSION**

In this study, the H10N7 AIV had no known virulence-related mutations. However, serial passages of avian H10N7 virus in mice resulted in a dramatic acquisition of pathogenicity, with increased viral replication, viral dissemination that extended to the brain and high mortality rates. Five conserved mutations were identified in the PB2, HA and NA genes of the passaged BJ27-MA virus (PB2-E158G, PB2-M631L, HA-G218E, NA-K110E and NA-S453I). The mutations in PB2 and NA genes significantly upregulated viral polymerase activity and NA enzymatic activity, respectively; their combined presence in BJ27-MA virus was necessary for neurovirulence. In particular, M631L mutation in PB2 was a

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**Fig. 4.** Growth kinetics of mutant BJ27 (H10N7) viruses in MDCK and A549 cells. Confluent MDCK (a) or A549 (b) cells were infected with viruses as indicated at an m.o.i. of 0.01 or 0.1, respectively. Data are presented as means±SD of three independent experiments. *, the value is significantly different from that of rBJ27 (\( P<0.05 \)).
major molecular determinant for the overall increase in virulence of the mouse-adapted H10N7 virus.

The PB2 gene plays important roles in the adaptation of influenza viruses from avian to mammalian through increasing polymerase activity and viral replication [37]. Polymerases of avian origin generally have impaired function in human and other mammalian cells [35]. To overcome this natural restriction, avian polymerases need to acquire mutations that can improve activity in mammalian hosts. E627K or D701N in PB2 is a common adaptive change in AIVs that causes mammalian adaptation [28, 29, 38]. In our mice adaptation experiments, we found that nine H5N1 and two pandemic H1N1/2009 viruses possessed PB2-M631L but not E627K or D701N mutation, implying that PB2-M631L could be functionally important and independent of E627K or D701N. During the pH1N1/2009 virus outbreak in humans, instead of the PB2-E627K mutation, the PB2-G590S/Q591R mutation was found to be responsible for the increased polymerase activity in human cells [39]. Therefore, PB2-M631L could be a novel functional mutation in H10N7 virus adaptation in mammalian hosts.

E158G and M631L were identified to mediate the promotion of polymerase activity, viral pathogenicity and replication in mice. PB2-E158G was reported to be a pathogenic determinant of pandemic H1N1 and avian H5 influenza viruses in mice [25]. PB2-M631L is a novel and dominant pathogenic mutation that has not been described previously. The structure of PB2 shows that position 631 is close to position 627 and located at the PB2-PB1 and PB2-NP interaction regions [26]. Sequence analysis of PB2 from AIVs found that there were five and 41 AIVs with PB2-E158G and PB2-M631L, respectively. Based on available sequences of human isolates, we found that nine H5N1 and two pandemic H1N1/2009 viruses possessed PB2-M631L but not E627K or D701N mutation, implying that PB2-M631L could be functionally important and independent of E627K or D701N. During the pH1N1/2009 virus outbreak in humans, instead of the PB2-E627K mutation, the PB2-G590S/Q591R mutation was found to be responsible for the increased polymerase activity in human cells [39]. Therefore, PB2-M631L could be a novel functional mutation in H10N7 virus adaptation in mammalian hosts.

**Table 3.** NA enzyme kinetics of mutant H10N7 viruses*

<table>
<thead>
<tr>
<th>Virus</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$</th>
<th>$V_{max}$ ratio†</th>
</tr>
</thead>
<tbody>
<tr>
<td>rBJ27</td>
<td>28.7±4.1</td>
<td>0.53±0.10</td>
<td>1.00</td>
</tr>
<tr>
<td>rBJ27-MA</td>
<td>14.8±1.8†</td>
<td>0.90±0.09†</td>
<td>1.70†</td>
</tr>
<tr>
<td>rBJ27-NA</td>
<td>15.6±2.3†</td>
<td>0.86±0.06†</td>
<td>1.62†</td>
</tr>
<tr>
<td>rBJ27-NA/K110E</td>
<td>17.8±1.5†</td>
<td>0.78±0.02†</td>
<td>1.47†</td>
</tr>
<tr>
<td>rBJ27-NA/S453I</td>
<td>20.7±2.2</td>
<td>0.66±0.14</td>
<td>1.25</td>
</tr>
</tbody>
</table>

*A standardized virus dose of $10^6$ TCID$_{50}$ ml$^{-1}$ was used for the NA kinetics assay.
†The value of corresponding strains was significantly different from that of rBJ27 ($P<0.05$, ANOVA).
‡The ratio of the recombinant viruses versus rH10N7 virus $V_{max}$ values.

NA cleaves sialic acid from glycans on host cells and emerging virions, thus allowing unhindered release of progeny virus from infected cells [40]. Several studies found that amino acid mutations or deletions in NA can affect NA enzymatic activity, which correlates with viral replication and pathogenicity in vitro or in vivo [41–43]. Here, NA-K110E in BJ27-MA virus, acting as a novel mammalian mutation, significantly increased NA activity, which may subsequently contribute to the increased replication and pathogenicity of rBJ27-MA virus in mice. We found that the two NA mutations (K110E and S453I) are located at the interface of the tetrameric structure of NA [31], which may affect the formation of tetrmer.

BJ27-MA virus had G218E mutation on the HA segment. This mutation was reported to affect both the receptor specificity and the pH of fusion, which played an important role in the enhanced virulence of H3N2 human influenza virus in mice [44]. In this study, the mutation of HA-G218E had an undetectable effect on the virulence of H10N7 virus in mice, indicating that the function of this mutation is subtype specific. A similar phenomenon is found in the study of the residue PA-224S, which has totally different roles in H5N1 and H1N1 influenza viruses [45, 46].

Neurovirulence is not commonly observed in the adaptation of low pathogenic AIVs in mice [22–24, 27]. In addition to the passaged H10N7 virus, another related virus (H10N8) was reported to acquire neurotropism after two passages in mice [47], which suggests that the H10 subtype is more likely to gain the ability to replicate in the mammalian brain. Clinically, CNS disease is a common extra-respiratory complication in humans induced by influenza virus. Patients with CNS manifestations are more likely to experience severe illness [32–34]. In Australia and Texas, USA, 9.7 and 8.8% of hospitalized children infected with pH1N1/2009 virus developed neurological complications, respectively [48, 49]. Zhang et al. also...
found that viral replication in murine brain positively correlates with pathogenicity in mice [50]. The collective evidence indicates that high pathogenicity for both the lung and brain could cooperate to promote mortality in humans post-avian-virus infection. The molecular mechanism of influenza virus causing infection in the CNS is unclear. HA, NA and PB2 genes have been separately found to be critical to the neurovirulence of H1N1 or H5N1 viruses in mammalian hosts [51–54]. The absence of an oligosaccharide chain at position 146 of NA in H1N1 led to higher local concentrations of plasm migraine and increased HA cleavage, leading to the gaining of neurovirulence [54]. However, in this study, we have demonstrated that the virus with a single adapted PB2, HA or NA segment is not able to infect the CNS. Only when it is combined with PB2/NA, but not PB2/HA or NA/HA, can the virus replicate in the brain to a similar level to the rBJ27-MA virus, indicating that the synergistic effect of PB2 and NA is important for H10N7 neurovirulence. The PB2-mediated increased polymerase activity and the enhanced NA activity may contribute to efficient infection in the mouse CNS.

In summary, our mouse adaptation study clearly shows that avian H10N7 virus can readily become highly virulent and neurotropic after limited passages in mice. We demonstrate that this enhanced pathogenicity is mediated by specific mutations in PB2 and NA genes; in particular, PB2-M631L is a novel and critical determinant of virulence.

**METHODS**

**Viruses and cells**

The H10N7 virus A/mallard/Beijing/27/2011 (BJ27) was isolated in Beijing, China, and propagated in the allantoic cavities of 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs (Merial, Beijing, China) at 37°C for 72 h. Allantoic fluid containing virus was harvested, aliquoted and frozen at −80°C for later use. MDCK, A549 and 293 T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% FBS (Gibco), 100 units ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin at 37°C in 5% CO₂ atmosphere.

**Adaptation of the BJ27 virus in mice**

Six 6-week-old female BALB/c mice (Beijing Experimental Animal Center) were anaeasthetized with Zoletil 50 (tiletamine-zolazepam; Virbac, Garros, France) and inoculated intranasally with 50 µl 10⁻⁵ TCID₅₀ of viruses in 50 µl PBS. Mice in each group were euthanized at 3 days p.i.; lungs, brains, spleens, kidneys and livers were collected, weighed and 10% (v/v) homogenates were prepared with cold PBS. Virus titres in cleared homogenates were determined by TCID₅₀ assay on MDCK cells.

**Mouse experiments**

Groups of eleven 6-week-old female BALB/c mice (Beijing Experimental Animal Center) were anaesthetized with Zoletil 50 (tiletamine-zolazepam; Virbac, Garros, France) and inoculated intranasally with 10⁻⁵ TCID₅₀ of viruses in 50 µl PBS. Three mice in each group were euthanized at 5 days p.i.; lungs, brains, spleens, kidneys and livers were collected, weighed and 10% (v/v) homogenates were prepared with cold PBS. Virus titres in cleared homogenates were determined by TCID₅₀ assays in MDCK cells. The remaining five mice in each group were monitored for weight loss and mortality for 14 days. Mice that lost more than 30% of their body weight were humanely euthanized and count as mortality. To determine the MLD₅₀, groups of three 6-week-old female mice were anaesthetized with Zoletil 50 and inoculated intranasally with 50 µl of 10-fold serial dilutions of viruses in PBS. The mice were monitored for 14 days. MLD₅₀ was calculated and expressed in TCID₅₀. For histopathology and immunohistological analysis, mouse lungs and brains collected at 5 days p.i. were fixed in 10% phosphate-buffered formalin, embedded in paraffin and, then cut into 5 mm thick sections and stained with

**Sequence analysis**

Viral RNA was extracted from allantoic fluid containing plaque-purified BJ27-MA. The eight virus genes were amplified by reverse-transcription PCR (RT-PCR) and sequenced by Sanger sequencing. Adaptive mutations arising from serial passages were identified by comparing consensus BJ27-MA and wild-type BJ27 sequences.

**Plasmid construction and virus rescue**

The eight gene segments of BJ27 and BJ27-MA were amplified by RT-PCR and cloned into the expression plasmid, PHW2000, a bi-directional polI/polII plasmid used for reverse genetics. Single mutations of interest in the PB2 and NA genes were introduced by PCR-based site-directed mutagenesis with primer pairs containing point mutations. All constructs were sequenced to confirm mutational changes.

Reassortant viruses between BJ27 and BJ27-MA were generated by reverse genetics, as described previously [56]. Briefly, 0.5 µg of each gene segment plasmid was mixed together and incubated with 8 µl TransIT-LT1 reagent (Mirus Bio, USA) at 20°C for 30 min. The TransIT-LT1–DNA mixture was transferred to 70% confluent 293T/MDCK co-cultured monolayers and incubated at 37°C with 5% CO₂. At 6 h post-transfection, the supernatants were replaced with 2 ml Opti-MEM containing 2 µg ml⁻¹ TPCK-treated trypsin (Sigma-Aldrich). At 48 h post-transfection, the cell supernatants were harvested and inoculated into 10-day-old SPF embryonated eggs and incubated for 72 h at 37°C for virus stock preparation. Viral RNA was extracted and analyzed by RT-PCR, and each viral segment was sequenced to confirm identity. Virus titres were determined by TCID₅₀ assay on MDCK cells.
haematoxylin and eosin (H and E) or immunostained with a mouse monoclonal antibody specific for influenza A virus NP (Biorbyt, UK).

**Virus titration and growth kinetics**

The TCID$_{50}$ was determined in MDCK cells with 10-fold serially diluted virus inoculated at 37°C for 72 h. The EID$_{50}$ was determined in 10-day-old embryonated chicken eggs with 10-fold serially diluted virus inoculated at 37°C for 48 h. The TCID$_{50}$ and EID$_{50}$ values were calculated by using Reed and Muench’s method [57].

Selected recombinant viruses were inoculated in MDCK cell monolayers (at an m.o.i. of 0.01) or A549 cell monolayers (at an m.o.i. of 0.1) in serum-free DMEM containing 1 µg ml$^{-1}$ TPCK-treated trypsin and incubated at 37°C with 5% CO$_2$ atmosphere. Cell supernatants were harvested at 12, 24, 36, 48, 60 and 72 h p.i. and titrated using MDCK cells in 96-well plates. Three independent experiments were performed for each virus.

**Polymerase activity assay**

The PB2, PB1, PA and NP gene segments of BJ27, BJ27-MA and BJ27-PB2 mutants were individually inserted into pcDNA3.1 plasmid. PB2, PB1, PA and NP plasmids (125 ng each) were transfected to 60% confluent 293 T cells, together with firefly luciferase reporter plasmid pYH-Luci (10 ng) and internal control plasmid expressing renilla luciferase (2.5 ng). At 24 h post transfection, lysates were prepared with the Dual Luciferase Reporter assay system (Promega) and luciferase activity was measured using the GloMax 96 microplate luminometer (Promega).

**Western blotting**

PB2 expression levels in different transfection groups were determined by Western blotting. Total cell protein lysates were extracted from transfected 293 T cells with CA630 lysis buffer (150 mM NaCl, 1% CA630 detergent, 50 mM Tris base, pH 8.0). Cellular proteins were separated by 12% SDS-PAGE and transferred to a PVDF membrane (Amersham Biosciences, Germany). Each PVDF membrane was blocked with 0.1% Tween 20 and 5% nonfat dry milk in Tris-buffered saline and subsequently incubated with a primary antibody. Primary antibodies used were specific for influenza A virus PB2 (ThermoFisher, USA) and β-actin (Beyotime, China). The secondary antibody was horseradish peroxidase (HRP)–conjugated anti-rabbit or -mouse antibody (Beyotime, China). HRP presence was detected using a mouse monoclonal antibody. Primary antibodies used were specific for influenza A virus NP (Biorbyt, UK). MUNANA produces a fluorescent product. Fluorescence was quantified using a Biotek Synergy H1 plate reader every 3 min over the course of 45 min. Fluorescence curves were then fitted to the Michaelis–Menten equation to determine the values of the Michaelis constant ($K_M$) and the maximum velocity ($V_{max}$). Each experiment comprised triplicate samples of each virus.

**Statistical analyses**

All statistical analyses were performed using GraphPad Prism Software version 5.00 (GraphPad Software, San Diego, CA, USA). The two treatment methods were compared by two-tailed Student’s t-test, and multiple comparisons were carried out by two-way analysis of variance (ANOVA) considering time and virus as factors. Differences were considered statistically significant at $P<0.05$.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

All animal work was approved by the Beijing Association for Science and Technology [approval ID SYXK (Beijing) 2007-0023] and conducted in accordance with the Beijing Laboratory Animal Welfare and Ethics guidelines, as issued by the Beijing Administration Committee of Laboratory Animals, and in accordance with the China Agricultural University Institutional Animal Care and Use Committee guidelines (ID: SKLAB-B-2010-003).

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