Pathogenicity of modified bat influenza virus with different M genes and its reassortment potential with swine influenza A virus

Jianmei Yang, Jinhua Lee, Jingjiao Ma, Yuekun Lang, Jerome Nietfeld, Yuhao Li, Michael Duff, Yonghai Li, Yuju Yang, Haixia Liu, Bin Zhou, David E. Wentworth, Juergen A. Richt, Zejun Li and Wenjun Ma

Abstract
In our previous studies, the reassortant virus containing only the PR8 H1N1 matrix (M) gene in the background of the modified bat influenza Bat09:mH1mN1 virus could be generated. However, whether M genes from other origins can be rescued in the background of the Bat09:mH1mN1 virus and whether the resulting novel reassortant virus is virulent remain unknown. Herein, two reassortant viruses were generated in the background of the Bat09:mH1mN1 virus containing either a North American or a Eurasian swine influenza virus M gene. These two reassortant viruses and the reassortant virus with PR8 M as well as the control Bat09:mH1mN1 virus replicated efficiently in cultured cells, while the reassortant virus with PR8 M grew to a higher titre than the other three viruses in tested cells. Mouse studies showed that reassortant viruses with either North American or Eurasian swine influenza virus M gene did not enhance virulence, whereas the reassortant virus with PR8 M displayed higher pathogenicity when compared to the Bat09:mH1mN1 virus. This is most likely due to the fact that the PR8 H1N1 virus is a mouse-adapted virus. Furthermore, reassortment potential between the Bat09:mH1mN1 virus and an H3N2 swine influenza virus (A/swine/Texas/4199-2/1998) was investigated using co-infection of Madin–Darby canine kidney cells, but no reassortant viruses were detected. Taken together, our results indicate that the modified bat influenza virus is most likely incapable of reassortment with influenza A viruses with in vitro co-infection experiments, although reassortant viruses with different M genes can be generated by reverse genetics.

INTRODUCTION
Influenza virus is an important respiratory pathogen belonging to the family Orthomyxoviridae. There are four types of influenza viruses (A–D) distinguished by the antigenic differences in their major internal virus proteins, nucleoprotein (NP) and matrix protein (M1). Influenza A viruses (IAVs) are divided into different subtypes based on the genetic and antigenic differences in their surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). There are 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9) of IAV found in waterfowl and shorebirds which are considered to be the natural reservoirs of IAVs [1]. IAVs can infect a broad range of host species including terrestrial and aquatic birds, domestic animals, sea animals and humans, and are responsible for seasonal influenza epidemics and pandemics in humans. Two major mechanisms involved in the rapid evolution of IAVs are antigenic drift and shift (reassortment). At least three pandemic influenza viruses were generated by reassortment, which were responsible for 1957 Asian (H2N2), 1968 Hong Kong (H3N2) and 2009 pandemics in human history.

Two novel genome sequences of influenza A-like virus designated HL17NL10 and HL18NL11 were recently acquired from bat specimens by next-generation sequencing [2, 3]. However, no infectious virus has been isolated from bats. Studies have shown that neither the HA nor the NA of bat influenza A-like viruses have the same functions as canonical HA and NA of IAVs [4–7]. In contrast, the internal
genes of bat influenza A-like viruses have been demonstrated to be functional to support virus replication by generating modified/chimeric bat viruses, which contain six internal genes of bat influenza A-like virus and two surface gene HA and NA ORFs of prototypic IAVs with bat virus packaging signals [8, 9]. All these data indicate that proper receptors or substrates are needed in order to grow an infectious bat influenza A-like virus [10].

Because bats migrate and are distributed worldwide, and interact with other species including humans and domestic animals such as pigs, which are considered mixing vessels for IAV reassortment, there is potential for IAV and bat influenza A-like virus co-infections. However, the potential for reassortment between bat influenza A-like viruses with other IAVs is not understood. Although previous studies including our own have shown that modified/chimeric bat influenza viruses fail to reassort with lab- and/or mouse-adapted A/Puerto Rico/8/1934 (PR8) and A/WSN/1933 (WSN) viruses by co-infection of Madin–Darby canine kidney (MDCK) cells [8, 9], whether modified/chimeric bat viruses can reassort with other subtypes of IAVs remains unclear. Interestingly, when we investigated the rescue efficiency of internal protein coding gene segment reassortment between modified Bat09 : mH1mN1 and PR8 viruses (this modified bat virus contains six internal genes from the HL17NL10 A/little yellow-shouldered bat/Guatemala/164/2009 virus and two surface HA and NA ORFs from the PR8 virus flanked by bat virus packaging signals, Fig. 1), the results showed that five gene segments (NP, NS, PB2, PB1 and PA) could not be replaced by the respective gene from the PR8 virus, whereas the M gene segment of PR8 could replace the Bat09 M segment [8]. However, the pathogenicity of the rescued reassortant virus [PR8(M)] with the PR8 M gene and whether M genes from other lineages can be rescued remain unknown.

To address these questions, we first tried to rescue reassortant viruses in the background of the modified Bat09 : mH1mN1 virus by replacing the bat M gene with either North American classical M or Eurasian M genes that exist in North American swine influenza viruses (SIVs) and then further characterized these novel reassortant viruses using the Bat09 : mH1mN1 virus as a control. In addition, we also determined reassortment potential between the modified Bat09 : mH1mN1 and an H3N2 A/swine/Texas/4199-2/1998 (TX98) by co-infection of MDCK cells.

**RESULTS**

**Generation and characterization of reassortant viruses with different origin M genes**

A reassortant virus PR8(M) was rescued in the genetic background of modified bat influenza Bat09 : mH1mN1 virus in 2009 virus and two surface HA and NA ORFs from the PR8 virus flanked by bat virus packaging signals, Fig. 1), the results showed that five gene segments (NP, NS, PB2, PB1 and PA) could not be replaced by the respective gene from the PR8 virus, whereas the M gene segment of PR8 could replace the Bat09 M segment [8]. However, the pathogenicity of the rescued reassortant virus [PR8(M)] with the PR8 M gene and whether M genes from other lineages can be rescued remain unknown.

![Fig. 1. Schematic diagram of rescued reassortant viruses with different M genes. Three reassortant viruses (PR8(M), TX98(M) and KS-107824(M)) were generated in the background of the modified Bat09 : mH1mN1 by replacing the bat M gene with the M gene from different origins. Black bars represent genes from the HL17NL10 A/little yellow-shouldered bat/Guatemala/164/2009 (Bat09 HL17NL10); white bars represent HA or NA coding regions from A/Puerto Rico/8/1934 (PR8); grey bars represent M genes from the PR8, A/swine/Texas/4199-2/98 (TX98) and A/swine/Kansas/11-107824/2011 (KS-107824) viruses, respectively.](https://example.com/fig1)

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our previous study [8], in which the bat influenza M gene was replaced with the M gene from the PR8 H1N1 virus (Fig. 1). Two reassortant viruses [TX98(M) and KS-107824 (M)] depicted in Fig. 1 were generated by reverse genetics in the genetic background of the Bat09 : mH1mN1 virus with either a North American swine influenza classical M gene from the H3N2 A/swine/Texas/4199-2/1998 (TX98) or a Eurasian swine M gene from the H3N2 A/swine/Kansas/11-107824/2011 (KS-107824) virus in this study. Both novel reassortant viruses as well as the PR8(M) virus using the modified bat influenza virus Bat09 : mH1mN1 as a control were further characterized. All four viruses caused clear cytopathic effects in infected MDCK cells and formed obvious plaques. The TX98(M) virus formed significantly larger size of plaques, while the KS-107824(M) virus formed significantly smaller size of plaques at 48 h post-infection (p.i.) when compared to the control Bat09 : mH1mN1 virus (Table 1).

Furthermore, we determined growth dynamics of four reassortant viruses in MDCK, swine testis (ST) and A549 cells. All four viruses were able to replicate in each cell line. In general, the PR8(M) virus replicated more efficiently in each cell line than the other three viruses. For example, the PR8(M) virus grew to a significantly higher titre in MDCK cells at 48 h p.i. than both TX98(M) and KS-107824(M) viruses; the PR8(M) virus grew to significantly higher titres in A549 cells at 24 and 36 h p.i. than the other three tested viruses; and the PR8(M) virus grew to a significantly higher titre in ST cells at 12 and 24 h p.i. than the KS-107824(M) virus (Fig. 2). In addition, we also investigated the growth properties of the modified bat Bat09 : mH1mN1 virus in primary bat cells from the Sturnira lilium species including lung, liver and kidney cells (kindly provided by Dr Adolfo García-Sastre at the Icahn School of Medicine at Mount Sinai, NY, USA) using the MDCK cells as a control. Results show that these bat cells do not support replication of the chimeric bat virus, as virus titres of the Bat09 : mH1mN1 virus in each bat cell line did not increase over time (12, 24 and 36 h p.i.) (data not shown). This fact suggests that internal genes of the bat virus most likely do not affect viral fitness in cells from their natural host.

### Table 1. Sizes of plaques formed by each indicated virus in MDCK cells at 48 h p.i.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Plaque size*</th>
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<tr>
<td>Bat09 : mH1mN1</td>
<td>1.09±0.06</td>
</tr>
<tr>
<td>PR8(M)</td>
<td>1.38±0.05</td>
</tr>
<tr>
<td>TX98(M)</td>
<td>2.62±0.11**</td>
</tr>
<tr>
<td>KS-107824(M)</td>
<td>0.64±0.19**</td>
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*Thirty plaques formed by each virus were randomly selected, and the diameter of each plaque was measured. The plaque size is represented as the mean of diameter ± SEM (mm).

**P<0.01.

### Eurasian SIV M gene does not enhance the pathogenicity of the modified bat influenza Bat09 : mH1mN1 virus

The Eurasian M gene has been shown to play a critical role in the virulence and transmissibility of the 2009 pandemic H1N1 virus [11–13] and is currently dominating and replacing the classical M gene in North American SIVs [14, 15], suggesting that it has potential to change virulence and transmissibility of a novel virus by reassortment. Therefore, we firstly evaluated the pathogenicity of the KS-107824(M) virus in mice using the modified bat influenza Bat09 : mH1mN1 virus as a control. In contrast to control mice, animals intranasally infected with 2.5×10⁵ TCID₅₀/mouse of each virus showed obvious clinical signs such as depression, decreased activities, ruffled fur and weight loss. Both viruses induced severe weight loss (Fig. 3a) and caused a high mortality in infected mice (Fig. 3b). The KS-107824(M) caused 87.5 % mortality (7/8) in infected mice, while the Bat09 : mH1mN1 virus resulted in 100 % mortality (8/8). Both viruses replicated efficiently in mouse lungs and grew to a high titre (greater than 8 log₁₀ TCID₅₀/ml) at both 3 and 5 days p.i. No significant difference was observed in virus titres detected between mice infected with Bat09 : mH1mN1 and KS-107824(M) (Fig. 3c). The results indicate that the Eurasian SIV M does not enhance the pathogenicity of the modified bat influenza Bat09 : mH1mN1 virus in mice.

### PR8 M gene enhances virulence of the modified bat influenza Bat09 : mH1mN1 virus in mice

Since the Eurasian SIV M did not enhance the pathogenicity of the modified bat influenza Bat09 : mH1mN1 virus in mice that were infected with a high dose (2.5×10⁵ TCID₅₀/mouse) of each virus, we investigated whether the M gene from other origins affects the pathogenicity of the modified bat influenza Bat09 : mH1mN1 virus. Groups of mice were infected with a low dose (5×10⁴ TCID₅₀/mouse) of each virus including PR8(M), TX98(M) and KS-107824(M) as well as the Bat09 : mH1mN1 as a control. The TX98(M), KS-107824(M) and Bat09 : mH1mN1 viruses induced slight weight loss in infected mice that then recovered (Fig. 3d), whereas the reassortant PR8(M) virus caused severe weight loss and 62.5 % mortality (five of eight) in infected mice (Fig. 3e). All four viruses could replicate in mouse lungs with a virus titre ranging from 5.3 to 9.0 log₁₀ TCID₅₀/ml⁻¹. A higher virus titre was detected in the modified bat influenza Bat09 : mH1mN1 virus-infected mice and a lower virus titre was found in the TX98(M) virus-infected animals at both 3 and 5 days p.i., and a significant difference was observed between Bat09 : mH1mN1 and TX98(M) groups at both days (Fig. 3f).

Histopathological analysis showed that all four viruses induced typical influenza-induced pneumonia in infected mice, such as bronchoalveolar epithelial cell degeneration and necrosis, and interstitial pneumonia when compared to the control mice (Fig. 4b). More severe lung damage was observed in each infection group at 5 days p.i. than at 3 days p.i. (Fig. 4a). In contrast, the PR8(M) virus caused more severe histopathological lung damage than the other three
viruses at both days, and a significant difference in the histopathological scores was found between PR8(M)- and Bat09 : mH1mN1-infected groups at 3 days p.i. (Fig. 4a). NP antigens of IAV were detected in lung tissues of each mouse from each infection group at 3 days p.i. by immunohistochemistry (IHC) staining (Fig. 4b).

**No reassortment between the Bat09 : mH1mN1 and H3N2 TX98 SIV by co-infection of MDCK cells**

Our previous study showed that no reassortment was detected when the MDCK cells were co-infected with both the modified bat influenza Bat09 : mH1mN1 and a lab-adapted PR8 H1N1 virus [8]. Herein, we determined reassortment potential between the Bat09 : mH1mN1 and an H3N2 TX98 SIV by co-infecting the MDCK cells (m.o.i. ratio of Bat09 : mH1mN1 and TX98 was 4:2). Single plaques were picked and further analysed to determine the origin of each gene segment by the specific reverse transcription (RT)-PCR assay. A total of 101 single viruses were analysed. The results showed that 78 isolates were the parental modified bat Bat09 : mH1mN1 virus and 23 isolates were the parental TX98 H3N2 virus; no reassortant virus was detected. Our data indicate that reassortment potential between the modified Bat09 : mH1mN1 and TX98 H3N2 viruses is extremely low or not possible under the co-infection condition.

**DISCUSSION**

One of the most important characteristics of IAVs is the ability to exchange genomic RNA segments when two or more IAVs infect the same cell or host, resulting in novel genotypes and phenotypes of virus that may have zoonotic potential [16, 17]. Previous studies have shown that the packaging signals of most gene segments of the bat virus are not compatible with those of canonical IAVs, and it is thought to be one of major reasons to block reassortment between both viruses [8, 9, 18]. Interestingly, we are able to rescue reassortant viruses with different origins of unmodified M gene (from PR8, North American classical and Eurasian swine M gene) in the background of the modified Bat09 : mH1mN1 virus. These data indicate that the M gene packing signals of bat virus and conventional IAVs are compatible, and both bat and canonical IAV M genes encode functional M1 and M2 proteins, although they show a low identity at amino acid level. However, we could not rescue the recombinant virus with bat M gene in the background of the PR8 virus [8]. Furthermore, another study showed that the NS1 gene, not the NEP gene, from bat HL17NL10 influenza A-like virus can be rescued in the PR8 backbone [19]. These data suggest that multiple factors such as interactions of protein–protein and protein–RNA are also critical for reassortment of IAVs [8]. Since the M gene packing signals of both bat virus and conventional IAVs are compatible, it would be interesting to identify the minimal sequence or nucleic acids for efficient packaging in future research.

Swine are considered to be the mixing vessel for avian, human and swine influenza viruses. If the bat influenza A-like virus or a modified bat virus was able to infect pigs, it would be possible for reassortant viruses to be produced through reassortment with currently circulating SIVs. This is why both North American classical and Eurasian M genes from SIVs circulating in North American swine herds [14, 20] were selected to generate reassortant viruses in the background of the Bat09 : mH1mN1 virus. Both rescued reassortant viruses containing either the North American classical or the Eurasian M gene displayed similar or even lower replication and virulence in various cells and in mice when compared to the control Bat09 : mH1mN1 virus. These results indicate that replacement of the M gene of SIVs does not enhance pathogenicity of the modified Bat09 : mH1mN1 virus. In contrast, the reassortant virus with the PR8 M gene showed more efficient replication in cells and enhanced virulence in mice than the other two reassortant viruses with either North American classical or Eurasian M genes as well as the control modified Bat09 : mH1mN1 virus.

**Fig. 2.** Growth dynamics of each rescued reassortant virus. Monolayers of MDCK (m.o.i. 0.001), A549 (m.o.i. 0.01) and ST cells (m.o.i. 0.01) were infected with each reassortant virus at the indicated m.o.i., and samples were collected at indicated time points. Each data point on the curve indicates the mean±SEM of three independent experiments. *P<0.05; **P<0.01; ***P<0.001.
mH1mN1 virus. This is most likely because PR8 is a mouse-adapted virus. It is important to note that the reassortant virus also has both HA and NA ORFs from the PR8 virus, as previous studies have shown that interaction of M1 with both HA and NA is critical in triggering the budding process, resulting in the formation and release of virus particles [21, 22]. This fact most likely explains the enhanced replication of the PR8 (M) virus compared with the other three viruses in cells. Furthermore, our previous study demonstrated that the right combination of NA and M genes enhances virus replication and transmissibility as was seen with 2009 pandemic H1N1 Eurasian NA and M genes, which were critical for replication and transmissibility of a North American triple reassortant H1N1 virus in pigs [12]. Both HA and NA associate with M1 via their transmembrane domain [21, 22]. When the M sequence of the bat, PR8, TX98 and KS-107824 viruses was compared at the amino acid level, identities among the M1 proteins and the M2 proteins were determined to be 79.8–97.2% and 44.1–86.6%, respectively. There were 7 amino acid differences (I15V, V41A, I115V, S116A, A137T, N231D and A239T, 97.2% identity) in the M1 protein and 16 amino acid differences (E14G, G16E, R18K, G21D, T27I, I28A, N31S, T39I, C50Y, G61R, K70E, K78Q, E79K, A86V, S93N and E95V, 86.6% identity) in the M2 protein between the TX98 and PR8 viruses. However, the specific amino acids that are critical in affecting the interaction between NA/HA and M1 and how this affects virus replication and virulence need to be elucidated in future studies.

In addition, we demonstrated that no reassortment occurred between modified Bat09: mH1mN1 and the H3N2 TX98 swine virus by co-infection of MDCK cells. This result is consistent with findings of previous co-infection studies using modified/chimeric bat virus and lab/mouse-adapted H1N1

![Fig. 3. Weight loss, survival rate and virus replication in mice infected with each indicated virus at a high dose of 2.5×10^4 TCID_{50}/mouse (a–c) and at a low dose of 5×10^2 TCID_{50}/mouse (d–f). (a, d) Mean body weight of surviving mice in each group is represented as percentage of the original weight on day 0. (b, e) Survival rate of mice infected with indicated reassortant virus. (c, f) Virus titres in mouse lungs on 3 and 5 days p.i. The data are represented as mean±SEM.](image-url)
viruses [8, 9]. Reassortant viruses with the M gene from either the H3N2 TX98 or the mouse-adapted H1N1 PR8 virus in the Bat09 : mH1mN1 backbone can be generated by reverse genetics, but these reassortant viruses could not be detected by mimicking the process that occurred in nature through coinfection with both viruses. This is probably due to the fact that when multiple compatible genes are present, an ideal eight-gene segment constellation will be selected that is critical for virus ribonucleoprotein complexes packaging into the virions [23, 24]. For example, there are two M genes with compatible packaging signals possible in our study, but only one was selected. These data indicate that bat influenza A-like viruses most likely cannot reassort with conventional IAVs in nature, although bat M gene of the modified Bat09 : mH1mN1 virus can be replaced by other M genes from different origins by reverse genetics.

**METHODS**

**Cells**

Human embryonic kidney 293 T cells were maintained in Opti-modified minimal Eagle’s medium supplemented with 10 % FBS (HyClone) and 1 % antibiotics (Invitrogen). MDCK cells were maintained in minimum essential medium (MEM) supplemented with 5 % FBS, 1× L-glutamine (Invitrogen), MEM vitamins (Invitrogen) and 1 % antibiotics. Human lung carcinoma epithelial cells (A549) and swine testis (ST) cells were maintained in Dulbecco’s modified Eagle’s medium containing 7 % FBS, 1× L-glutamine and 1 % antibiotics. Cells were inoculated with the respective virus in MEM-infecting medium containing 0.3 % BSA (Sigma), 1 μg ml⁻¹ tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) and 1 % antibiotics.
Virus rescue

The full-length M genes of A/swine/Texas/4199-2/1998 (H3N2) and A/swine/Kansas/11-107824/2011 (H3N2) were amplified using influenza virus universal primers and cloned into pHH2000 vector as described previously [25–27]. The reassortant viruses with North American swine influenza M or Eurasian M gene were generated in the background of the Bat09 : mH1mN1 virus by replacing bat M gene using eight-plasmid reverse genetics as described previously [8, 12, 28]. Briefly, 1 µg plasmid for each gene was mixed with 4 µl Lipofectin 2000 (Life Technologies) and incubated at room temperature for 5 min, and then 250 µl Opti-MEM was added and incubated at room temperature for 15 min. The transfection mixture was transferred to 90% confluent human embryonic kidney 293 T cell monolayers in a six-well plate and incubated at 37°C with 5% CO₂ for 2–3 days. The supernatant was collected and passaged on MDCK cells three times until they showed cytopathic effect. Titres of rescued viruses were determined by TCID₅₀ on MDCK cells.

Growth kinetics of reassortant viruses

To evaluate growth kinetics of reassortant viruses, monolayers of MDCK (m.o.i. 0.001), A549 (m.o.i. 0.01) or ST (m.o.i. 0.01) cells were infected with each virus at the indicated m.o.i. The supernatants from infected cells were collected at different time points (12, 24, 36 and 48 h p.i.) and titrated on MDCK cells in 96-well plates. The virus titres were determined as TCID₅₀ per millilitre by the Reed and Muench method [29]. Plaque assays were also performed on MDCK cells to compare the size of plaques formed by different reassortant viruses.

Mouse experiments

To evaluate the virulence of the modified Bat09 : mH1mN1 virus and the reassortant KS-107824(M) virus with Eurasian SIV M gene in mice, a total of 42 female mice aged 6–7 weeks were randomly allocated to three groups (14 mice per group). Each mouse in the infection groups was intranasally inoculated with 2.5×10⁴ TCID₅₀ of each virus in 50 µl fresh MEM medium while under light anaesthesia by inhalation of 4% isoflurane, whereas each mouse in the mock group was inoculated with fresh MEM. Three mice from each group were euthanized and necropsied on both 3 and 5 days p.i. to determine virus replication in mouse lungs. The remaining eight mice in each group were kept to monitor body weights daily, and clinical signs were observed twice daily after onset of disease for 14 days. If the mice lost 25% or more of their initial body weight, they were humanely euthanized and necropsied. During necropsy, lung tissues were collected for virus titration and histopathological examination. The right part of the lung was frozen at −80°C for virus titration, and the left part of the lung was fixe in 10% phosphate-buffered formalin for histopathologic examination. For virus titration, a 10% lung homogenate was prepared in cold fresh MEM medium by using a Mini Bead Beater-8 (Biospec Products). The homogenate was centrifuged at 3500 g for 10 min, and the supernatant was titrated by infecting MDCK cells in 96-well plates. For the histopathologic examination, fixed lung tissues were processed routinely and stained with haematoxylin and eosin (H&E). For detection of viral NP in lung sections, a rabbit anti-H1N1 (2009 flu pandemic) NP polyclonal antibody was used in the IHC staining. Lung lesions were examined by a veterinary pathologist in a blinded fashion and given a score of 0–3 to reflect the severity of bronchial epithelial injury as described previously [30].

The pathogenicity of four reassortant viruses was also investigated in mice that were infected with a low dose of each virus (5×10⁴ TCID₅₀/mouse). The same procedure was applied as described above.

Co-infection study to assess reassortment potential between modified Bat09 : mH1mN1 and TX98 viruses

To investigate the reassortment potential between modified bat influenza virus and IAV, monolayer MDCK cells were co-infected with the modified Bat09 : mH1mN1 and TX98 H3N2 viruses. The modified Bat09 : mH1mN1 virus contains the HA and NA coding regions from the H1N1 A/ Puerto Rico/8/1934 virus with bat virus packaging signals and six internal genes from the HL17NL10 A/little yellow-shouldered bat/Guatemala/164/2009 influenza A-like virus. For co-infection study, the MDCK cells were infected with the modified Bat09 : mH1mN1 virus at an m.o.i. of 4 and with the TX98 virus at an m.o.i. of 2. After 1 h incubation, the supernatant was removed and the infected cells were washed with fresh MEM, and then the infection medium supplemented with 1 µg ml⁻¹ TPCK-trypsin (Worthington) was added. The supernatant was collected after 24 h incubation and was used to perform plaque assays on MDCK cells to pick up single plaques. The single plaque was purified and amplified for further analysis to determine each gene origin by using specific RT-PCR (primers are available upon request). For the RT-PCR assay, RNAs were extracted from each amplified single virus using the QIAamp Viral RNA Mini kit (Qiagen), and then each segment was amplified by specific primers using SuperScript III One-Step RT-PCR System (Invitrogen).

Statistical analysis

Virus titres, plaque sizes, mouse weight and microscopic pneumonia scores were analysed by ANOVA in GraphPad Prism version 5.0 (GraphPad Software). A value of P≤0.05 was considered to be significant. Those response variables shown to have a significant effect by treatment group were subjected to comparison for all pairs using a Tukey–Kramer test. Pairwise mean comparisons between infected and control groups were made using Student’s t-test.

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Conflicts of interest
W. M., J. M., B. Z. and D. W. are inventors of the patent entitled ‘modified influenza viruses and their uses’ owned by Kansas State University and the J. Craig Venter Institute.

Ethical statement
Mouse experiments were conducted in Biosafety Level 2+ facilities and approved by the Institutional Animal Care and Use Committee (IAUC no. 3339) at Kansas State University.

References