The PB2 E627K mutation contributes to the high polymerase activity and enhanced replication of H7N9 influenza virus

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Human infection by H7N9 influenza virus was first identified in China in March 2013. As of 12 August 2013, a total of 135 documented cases with 44 fatalities had been reported. Genetic and laboratory analyses of the novel H7N9 viruses isolated from patients indicate that these viruses possess several polymerase gene mutations previously associated with human adaptation and potential pandemic capabilities. However, the function of these mutations in the emergence and pathogenicity of the viruses is not well known. In this study, we demonstrate that the PB2 E627K mutation, which occurs in over 70% of the H7N9 patient isolates, promotes the replication of H7N9 virus by enhancing PB2 polymerase activity and enhances virulence in mice. Our results show the PB2 E627K mutation has played an important role in this H7N9 influenza outbreak and in the pathogenicity of the H7N9 virus.

INTRODUCTION

On 29 March 2013 the Chinese Center for Disease Control and Prevention confirmed the first case of human infection with H7N9 influenza A virus (Gao et al., 2013b). As of 12 August 2013, 135 cases with 44 fatalities had been confirmed, causing worldwide concern (Zhang et al., 2013). Patients infected with H7N9 viruses have a rapidly progressive pneumonia, leading to respiratory failure and acute respiratory distress syndrome (Gao et al., 2013a). Phylogenetic analysis suggests that the novel H7N9 virus is a triple reassortant and that its viral genes are of avian origin. The surface glycoprotein haemagglutinin (HA) was derived from the H7N3 virus from domestic ducks in Zhejiang, whereas the neuraminidase (NA) was derived from the wild bird H7N9 virus in South Korea. All six internal genes show high similarity to the poultry H9N2 virus (Gao et al., 2013b; Kageyama et al., 2013). Human infections with H7N9 virus have not been reported previously, and animal infections with H7N9 viruses had not been detected in China before this outbreak.

The influenza virus is a continuous threat to human health. In addition to the annual seasonal epidemic, the influenza virus occasionally causes pandemics. During the past century, several pandemics have occurred, including 1918 (H1N1), 1957 (H2N2), 1968 (H3N2) and 2009 (pH1N1); all the pandemic viruses bear HA and NA genes of avian or pig origin (Liu et al., 2013). Owing to the low fidelity of the viral RNA-dependent polymerase, it is easy for viruses to acquire adaptive mutations that allow transmission to hosts of different species. In modern times, direct human infections with avian influenza viruses occur only sporadically, including with the H7N2 (Ostrowsky et al., 2012), H7N3 (Skowronski et al., 2006; Tweed et al., 2004), H7N7 (Fouchier et al., 2004), H9N2 (Blair et al., 2013; Peiris et al., 1999) and H5N1 (de Jong et al., 1997) subtypes. Most human infections result in mild illness and conjunctivitis, except for the H5N1 subtype, which is associated with greater than 50% mortality. Patients infected with the H7N9 subtype have been detected in over 10 provinces of China, and most experienced a severe clinical syndrome.

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The GenBank/EMBL/DDBJ accession numbers for the H7N9 PB2 influenza virus sequences are AGK84850, AGK84856, AGK84859, AG051410, AG051398, AG051442, AGJ73498, AGL44433, AGI60293, AGN94649, AGN9457, AGJ51961, and AGM16245.
At this time, no investigations have revealed evidence of sustained spread of this virus in humans. However, the possibility of limited human-to-human spread cannot be excluded in a few small clusters of human H7N9 virus infections (Qi et al., 2013).

Further analysis of the H7N9 gene segments has shown the presence of signature amino acids associated with adaptation to the human host and with virulence, such as the Q226L mutation in HA, which is associated with increased binding to mammalian-like receptors in the human upper airway, and the E627K and D701N mutations in PB2, which are associated with enhanced replication and virulence (Kageyama et al., 2013; Li et al., 2005; Liu et al., 2013). During the past 2 months, many studies have shown that H7N9 viruses have a mixed lineage. The percentage of viruses containing the PB2 E627K mutation was calculated. The percentage of H7N9 patient isolates containing PB2 E627K is high, at 71.4 % [PB2 627K was found in 10 of 14 human H7N9 isolates and only four isolates downloaded from the National Center for Biotechnology Information (NCBI) were PB2 627E], and the fatality rate was approximately 30 % (http://www.who.int/influenza/human_animal_interface/influenza_h7n9/Data-Reports/en/). All of the 37 H7N9 viruses isolated from avian or environmental sources contain PB2 627E (Zhang et al., 2013). Together, these data suggest that the PB2 E627K mutation plays an important role in the occurrence of H7N9 from avian sources in human hosts.

The PB2 E627K mutation contributes to high polymerase activity and enhanced replication of H7N9

To test the contribution of PB2 E627K to H7N9 polymerase activity, we performed a luciferase reporter assay. We used WSN (Influenza A/WSN/1933 (H1N1)) virus as a reference strain. The polymerase activity of H7N9 627K was approximately 20-fold higher than that of H7N9 627E in 293T cells (Fig. 1a). However, there was not as big a difference between the polymerase activity of H7N9 627K and 627E in the chicken embryo fibroblast (CEF) cell line DF-1 as that in mammalian cells (Fig. 1b). The polymerase activity pattern of PB2 627K/E in H7N9 was similar to the activity observed in H5N1 cases (Mänz et al., 2012). To further confirm the contribution of PB2 627K to H7N9 replication, we compared the replication kinetics of H7N9 viruses containing PB2 627K and 627E in the A549 human alveolar epithelial cell line and primary CEIs. The virus titre of H7N9 was approximately 100-fold higher than that of H7N9 PB2 K627E from 12 h onwards after infection in A549 cells. The viral titre of H7N9 PB2 K627E did not increase during the infection, suggesting that the virus is not well adapted to the A549 cell line (Fig. 1c). There was no apparent difference in viral titre between H7N9 and H7N9 PB2 627E in CEIs (Fig. 1d). Taken together, these results indicate that PB2 627K enhances polymerase activity and viral replication in mammalian cells.

Polymerase activity of H7N9 with PB2 627K is higher than with PB2 627E and is capable of promoting virus replication at 33 °C

Although the mechanism of how PB2 E627K exerts its effects is not yet clear, one possible function of PB2 627K is to facilitate replication of avian viruses in the human upper respiratory tract, which generally has a temperature of 33 °C. In contrast, the temperature of the avian intestinal tract is closer to 41 °C, a temperature at which PB2 627E facilitates efficient viral replication (Hatta et al., 2007; Steel et al., 2009). To address whether the PB2 E627K mutation influences polymerase activity at a lower temperature, we tested the polymerase activity of WSN and H7N9 viruses with PB2 627K or 627E at 33 °C in 293T cells. The polymerase activity of H7N9 was still relatively high and about 1.5-fold higher than that of WSN at 33 °C. It was obvious that the polymerase activity of H7N9 viruses with
PB2 627K was about 60-fold higher than that of H7N9 viruses with PB2 627E (Fig. 2a). These data suggest that the H7N9 viruses may have the ability to replicate well in the human upper respiratory tract at 33 °C. To test the contribution of PB2 627K to viral replication at 33 °C, the replication kinetics of H7N9 were tested in A549 cells. As shown in Fig. 2b, compared with H7N9 PB2 K627E, H7N9 grew well in A549 cells at 33 °C. These data show that H7N9 is more adapted to mammalian cells than H7N9 PB2 K627E at 33 °C.

**PB2 627K contributes to the replication and transcription processes in mammalian cells**

As we have demonstrated that PB2 627K contributes to the high polymerase activity and enhanced replication of H7N9 viruses in mammalian cells, we further investigated whether the higher activity associated with PB2 627K was related to an increase in transcription (mRNA) and/or replication (cRNA and vRNA synthesis) by using a quantitative PCR-based assay. H7N9 viruses harbouring PB2 627K had a higher transcription activity at 33 °C, as demonstrated by a >10-fold higher level of PB2 627K than PB2 627E mRNA. The cRNA of H7N9 viruses harbouring PB2 627K was about threefold higher than that of H7N9 with PB2 627K, although the vRNA level of H7N9 with PB2 627K was slightly higher than that of H7N9 with PB2 627E (Fig. 3b). Conversely, the H7N9 viruses containing PB2 627K had a higher replication and transcription activity at 37 °C, as demonstrated by an approximately twofold increase in the level of all three kinds of RNA of PB2 627K compared with that of PB2 627E (Fig. 3a). These data indicate that PB2 627K enhances polymerase activity by regulating the viral replication and transcription process in mammalian cells.
PB2 627K enhances the replication of H7N9 in mice

To further determine the pathogenicity and replication of H7N9 harbouring 627K or 627E in mammals, mice were infected with 10^6 median 50 % egg-infectious dose (EID_{50}) of both viruses, and organs were harvested at 3 and 5 days post-infection. The body mass of mice infected with H7N9 decreased by approximately 20 %, while the body mass of mice infected with H7N9 PB2 K627E did not decrease (Fig. 4a). The replication of H7N9 and H7N9 PB2 K627E was detected in nasal turbinates and lungs but not in other organs (brain, spleen and kidneys). The viral titres of H7N9 in nasal turbinates and lungs was significantly higher than the titres of H7N9 PB2 K627E (Fig. 4b, c). The replication of H7N9 was similar in nasal turbinates and the lung, whereas the viral titre of H7N9 PB2 K627E in nasal turbinates was lower than that in the lung. Our data suggest that PB2 627K promotes viral replication in mice, and H7N9 virus containing PB2 627K is better adapted to the upper and lower respiratory tracts than virus containing PB2 627E.

![Figure 2](image2.png)

**Fig. 2.** Polymerase activity and replication kinetics of H7N9 harbouring PB2 627K or 627E at 33 °C. (a) Polymerase activity of H7N9 and H7N9 627E in 293T cells at 33 °C. Fifty nanograms each of PB1, PB2, PA and NP in pCAGGS; 100 ng of human pPolI-NP-luc; and 10 ng of pRLSV40 (Promega) were co-transfected into 293T cells, which were harvested 24 h after transfection. All data were normalized to the activity of the WSN sample. (b) Replication kinetics of H7N9 harbouring PB2 627K or 627E at 33 °C. A549 cells were infected with viruses at an m.o.i. of 0.01. At 12, 24, 48 and 72 h post-inoculation, the supernatants were harvested and virus titres were determined in eggs.

![Figure 3](image3.png)

**Fig. 3.** Quantification of viral RNA levels of H7N9RNP complexes containing PB2 627K or 627E at 37 °C and 33 °C. 293T cells were co-transfected with expression plasmids encoding NP, PA, PB1 and PB2 together with pPolI-NA plasmid and incubated at (a) 37 °C or (b) 33 °C. Total cellular RNA was isolated 24 h post-transfection and was subjected to quantitative RT-PCR for segment 6 (NA gene) transcripts. All data were normalized to viral RNA, cRNA and mRNA of the H7N9 sample. Results are means ± sd from three independent assays.
PB2 E627K contributes to high polymerase activity in mammalian cells, but not in avian cells. Using a luciferase reporter assay, we demonstrated that PB2 E627K promotes the polymerase activity of H7N9 in mammalian cells, but not in avian cells. In addition, viral titre studies in mice demonstrate that PB2 E627K contributes to the enhanced replication of H7N9 in vivo. Many potential mechanisms of the high polymerase activity associated with PB2 E627K have been proposed. Amino acid E627 lies on the surface of a polymerase PB2 subunit and is involved in the interaction with the host factor importin-α, with the differential use of importin-α isoforms governs cell tropism and host adaptation of influenza virus based on studies investigating the transmission of avian influenza H5N1 (Gabriel et al., 2008, 2011). Some studies have shown that the enhanced interaction between NP and PB2 mediated by PB2 E627K contributes to the high polymerase activity in mammalian cells but not in avian cells (Labadie et al., 2007; Rameix-Welti et al., 2009). Some studies suggested that the adaptive mutation PB2 E627K was mediated by an inhibitory or stimulatory factor in host cells (Mehle & Doudna, 2008; Moncorge et al., 2010). The crystallography indicated that the amino acid PB2 627 was in the C-terminal RNA binding domain and the PB2 627K had higher RNA binding activity than the PB2 627E (Kuzuhara et al., 2009). More experiments should be performed to test this mechanism in the H7N9 virus. The stability of influenza virus is temperature dependent. To cause a pandemic, a virus needs to maintain a stable state and replicate well at a lower temperature (33 °C), which is the approximate temperature of the human upper respiratory tract. PB2 627K improves polymerase activity at lower temperatures and is thought to confer stability on viruses, which allows the virus to replicate better at the lower temperature (Steel et al., 2009). We tested the polymerase activity of PB2 E627K at 33 °C. H7N9 harbouring PB2 E627K had relatively high polymerase activity at 33 °C. The viral titre...
in nasal turbinates also indicated that H7N9 is well adapted to the upper respiratory tract. In addition, PB2 627K may improve polymerase activity by regulating the transcription and replication processes at different temperatures.

Our study characterized the H7N9 virus (harbouring PB2 627K) and found that it possessed higher polymerase activity and improved replication in mammalian cells when compared to H7N9 harbouring PB2 K627E. More importantly, PB2 627K promoted the replication of H7N9 in nasal turbinates and lungs in mice and can cause illness. Our data indicate that the PB2 627K plays an important role in the outbreak and pathogenicity of H7N9.

METHODS

Facility. All experiments with live H7N9 viruses were conducted within the enhanced animal biosafety level 3 (ABSL3 +) facility in the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences, approved for such use by the Ministry of Agriculture of China and the China National Accreditation Service for Conformity Assessment.

Plasmids. PB1, PB2, PA and NP from A/Anhui/1/2013 (H7N9) were commercially synthesized and cloned into the vector pCAGGS with restriction enzyme EcoRI (TaKaRa). Viral cDNAs from the A/WSN/33 (H1N1) virus were kindly provided by Professor Hans Klenk (Marburg University, Germany) and cloned into the vector pCAGGS (kindly provided by Dr Jun-ichi Miyazaki, Osaka University, Japan). The pPolI-NP-luc and pPolI-NA were also provided by Professor Hans Klenk. The avian pPolI-NP-luc was constructed by replacing the human pPolI-NP-luc promoter by the avian promoter.

Cells. Human embryonic kidney 293T cells and immortalized CEFs (DF-1) were purchased from the ATCC and maintained in Dulbecco’s modified Eagle’s medium (HyClone) supplemented with 10 % FBS (Gibco) plus penicillin and streptomycin. Alveolar basal epithelial cells (A549) were maintained in F-12K Nutrient Mixture (Gibco) plus penicillin and streptomycin. Madin–Darby canine kidney cells were grown in minimum essential medium with Eagle’s salts containing 4 % FBS, 4 mM l-glutamine, and antibiotics.

Reverse genetics. An eight-plasmid reverse genetics system was used to generate H7N9 and H7N9-PB2 K627E viruses. As described previously, cDNA from the human-infecting H7N9 influenza virus A/Anhui/1/2013 (AH1) was inserted into the bidirectional transcription vector pB8. We introduced the mutation PB2 K627E into the AH1-PB2 plasmid by site-directed mutagenesis with the QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s protocol. The plasmids used for virus rescue and the genes from the rescued viruses were fully sequenced to confirm the absence of unwanted mutations. Virus rescue was performed as previously described (Li et al., 2005). Briefly, 293T cells were co-transfected with 0.5 μg of each of the eight plasmids mixed with 10 μl Lipofectamine LTX (Invitrogen) according to the manufacturer’s instructions. Eight hours later, the DNA-transfection mixture was replaced by Opti-MEM (Gibco). The supernatant was harvested and injected into 10-day-old specific-pathogen-free embryonated eggs for virus propagation after 48 h. The rescued virus was detected by haemagglutination assay.

Viral growth kinetics. Viruses were inoculated into A549 or CEF monolayers at an m.o.i. of 0.01. One hour after infection, the cells were replaced with fresh Opti-MEM and incubated at 33 °C and 37 °C, respectively. Culture supernatant was collected at the indicated time points post-infection and titrated in eggs. The growth data shown are the average results of three independent experiments.

Luciferase reporter assay. Fifty nanograms each of PB1, PB2, PA and NP; 100 ng of pPolI-NP-luc; and 10 ng of pRLSV40 (Promega) were co-transfected into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were harvested 24 h after transfection at 37 °C and 33 °C. Luciferase assays were performed using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer’s protocol. The firefly and Renilla luciferase activities were measured using a microplate luminometer (Veritas). The ratio of firefly luciferase activity to Renilla luciferase activity was calculated to represent the efficiency of the transcription/replication of the viral-like reporter RNA. All experiments were performed in triplicate. Results are presented as the mean ± SD.

Strand-specific real-time RT-PCR assay. Fifty nanograms each of PB1, PB2, PA and NP in pCAGGS and 100 ng of pPolI-NA (provided by Professor Hans Klenk) were co-transfected into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were harvested 24 h after transfection at 37 °C and 33 °C, and the total cellular RNA was extracted with TRIzol RNA isolation reagents (Invitrogen). RNA (0.5 μg of each sample) was reverse-transcribed by using the strand-specific tagged primers (Kawakami et al., 2011) for the NA gene using a ReverTra Ace qPCR RT kit (Toyobo) according to the manufacturer’s instructions. Real-time quantitative PCR (qPCR) was performed with SYBR Green Real-time PCR Master Mix (Toyobo) according to the manufacturer’s instructions on an ABI PRISM 7900HT. The qPCR cycle conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The vRNA, cRNA and mRNA levels were expressed relative to GAPDH mRNA as ratios.

Mouse infection. To evaluate the virulence of H7N9 influenza virus in a mammalian host, two groups (n=11) of 6–7-week-old female BALB/c mice (Vital River) were inoculated with 10^6 EID<sub>50</sub> of the H7N9 virus or the H7N9-PB2 K627E virus in a volume of 50 μl. Three mice were euthanized at 3 and 5 days post-infection, respectively, and their nasal turbinates, lungs, spleen, kidneys and brain were suspended in 1 ml of cold sterile PBS and subsequently homogenized for viral titration. The other mice were weighed and observed for signs of disease for 2 weeks.

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PB2 E627K promotes high polymerase activity of H7N9


