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BRIEF REPORT

Amino acid substitutions occurring during adaptation of an emergent H5N6 avian influenza virus to mammals

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Abstract Avian influenza viruses (AIVs) are known to cross species barriers, and emergent highly pathogenic H5N6 AIVs pose a serious threat to human health and the poultry industry. Here, we serially passaged an H5N6 virus 10 times in BALB/c mice. The pathogenicity of the wild-type 6D2 (WT-6D2) and mammal-adapted 6D2 strain (MA-6D2) were compared. The viral titer in multiple organs and the death rate for MA-6D2 were significantly higher than for WT-6D2. We provide evidence that the mutations HA A150V, NA R143K and G147E, PB2 E627K, and PA A343T may be important for adaptation of H5N6 AIVs to mammals.

Keywords Avian influenza virus · H5N6 · Serial passage · Mammal adaption · Pathogenicity

Introduction

Since 1997, the H5 subtype of highly pathogenic avian influenza viruses (HPAIV) has caused substantial economic losses in the poultry industry worldwide. Recently, emergent H5 strains such as H5N1, H5N2, H5N5, H5N6, and H5N8 have been identified [1–3]. In 2014, an outbreak of H5N6 avian influenza A occurred in China, Laos, and Vietnam, with a high mortality rate in birds [4, 5]. Human infections with H5N6 have also been reported sporadically [6–8], and there have been six laboratory-confirmed cases of human infection with avian-origin H5N6 virus as of January 6, 2016 [9]. All of the reported cases occurred in China, four of which were fatal due to severe pneumonia, raising concerns about a potential human pandemic.

The natural hosts of AIV are waterfowl, but on rare occasions, AIVs can be transmitted to non-natural host species including domestic poultry, lower mammals and humans. The ability of AIV to cross species barriers underscores the importance of elucidating the mechanism of avian-human transmission and the factors that influence AIV pathogenicity and replication capacity. AIV strains that cross species barriers undergo rapid molecular changes on multiple levels to facilitate zoonotic spread [10]. Serial passage in murine hosts has become a common method for determining the amino acid substitutions that occur during adaption to mammalian hosts [11, 12]. By studying these changes, we may be able to elucidate the specific molecular

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adaptations that account for changes in the replication capacity and virulence of the virus.

In 2014, we isolated an H5N6 virus, A/duck/Zhejiang/6D2/2013 (6D2; GenBank: KJ807773-88), from poultry. This virus was highly pathogenic to chickens but mildly pathogenic to mice [13]. Here, we have serially passaged the 6D2 virus 10 times in a murine host and sequenced the viral genome, revealing amino acid substitutions associated with adaptation to a mammalian host.

Materials and methods

Serial passage in BALB/c mice

Six-week-old BALB/c mice (three per group) were inoculated intranasally with 50 μL of phosphate-buffered saline (PBS) containing 10^6 EID_{50} of A/duck/Zhejiang/6D2/2013 (6D2). To collect the passaged virus, the mice were sacrificed 3 days postinfection (dpi), and the lungs were collected, washed three times with 1 mL of PBS, manually homogenized by grinding, and then centrifuged. The supernatant from the lung homogenate was collected and used to inoculate the next group of mice. The virus was passaged 10 times in naive BALB/c mice. At each passage, three mice were anesthetized with pentobarbital natricum and inoculated intranasally with 50 μL of the virus supernatant from the previous passage.

RNA extraction, reverse transcription, polymerase chain reaction, and sequencing

To investigate the molecular changes that occurred during each passage, an aliquot of virus was retained for sequencing. A Viral RNA Mini Kit (QIAGEN) was used to extract viral RNA according to the manufacturer's protocol. All of the gene segments were amplified as described previously [14]. Fragment sequencing was performed using the Big Dye Terminator v3.0 Cycle Sequencing Ready Reaction Kit, and the BioEdit software version 7.0.9.0 was used to analyze the resulting sequences.

Evaluating viral replication and pathogenicity in mice

We first compared the viral titer of the wild-type 6D2 P0 virus (WT-6D2) and the mammalian-adapted 6D2 (MA-6D2) virus in different organs in a murine host. Six-week-old BALB/c mice (three per group) were inoculated intranasally with 10^6 EID_{50} of WT-6D2 or MA-6D2 virus in 50 μL of PBS. The mice were sacrificed at 3 dpi and their brain, spleen, liver, kidney, heart and lungs were harvested, homogenized, diluted, and inoculated into 9-day-old embryonated eggs to determine the viral titer (EID_{50}). At 3 dpi, mouse lungs were collected, fixed in formalin, embedded in paraffin, and cut into 4 um-thick sections, half of which were used for immunohistochemistry staining and the other half for haematoxylin and eosin (H&E) staining. To determine the MLD_{50} (50 % mouse lethal dose), we inoculated six groups of six-week-old BALB/c mice (five mice per group) with 50 μL of tenfold serial dilutions of WT-6D2 or MA-6D2 and observed the mice for 14 days. MLD_{50} values were calculated by the Reed and Muench method [15].

We then assessed the clinical outcome of infecting mice with the wild-type 6D2 P0 virus (WT-6D2) and the mammalian-adapted 6D2 (MA-6D2) virus. Six-week-old BALB/c mice (six per group) were infected intranasally with either WT-6D2 or MA-6D2 (10^6 EID_{50} in 50 μL of PBS). Control mice were inoculated with the same volume of PBS. The body weight and survival rate of the mice were recorded every day until 14 dpi. The relative pathogenicity of WT-6D2 and MA-6D2 was evaluated by comparing the effects of the virus on clinical symptoms, viral titers, tissue pathology, body weight, and survival rate.

Results

The viral titer of MA-6D2 was higher than WT-6D2 in the brain, liver, kidney, heart, and lungs (Table S1, Fig. S1), indicating that viral adaptation expanded the organ tropism of 6D2 and that MA-6D2 had acquired the ability to replicate more efficiently in mice. The amount of virus required to reach the MLD_{50} was 6 logs greater than the EID_{50} for WT-6D2 and only 2.68 logs greater than the EID_{50} for MA-6D2, indicating that MA-6D2 was more pathogenic than WT-6D2.

In terms of clinical observations, the mice inoculated with MA-6D2 had more severe signs of illness, including ruffled fur, hunched posture, and reduced activity levels compared to mice infected with WT-6D2. Upon histological examination, there were no clinically significant pathology findings in the lungs of the WT-6D2-infected mice (Fig. 1A), and only a limited number of infected cells were identified by immunohistochemistry (Fig. 1C). However, there were obvious signs of bronchointerstitial pneumonia (fluid leakage and lymphocyte infiltration) in the lungs of mice inoculated with MA-6D2 (Fig. 1B), and infected epithelial cells (stained brown) were identified using immunohistochemistry (Fig. 1D). Substantial weight loss was observed in the MA-6D2-infected mice, compared to modest weight loss in the group infected with WT-6D2 (Fig. 2A). Consistent with these findings, 100 % mortality was observed in the mice infected with MA-6D2 by 8 dpi,

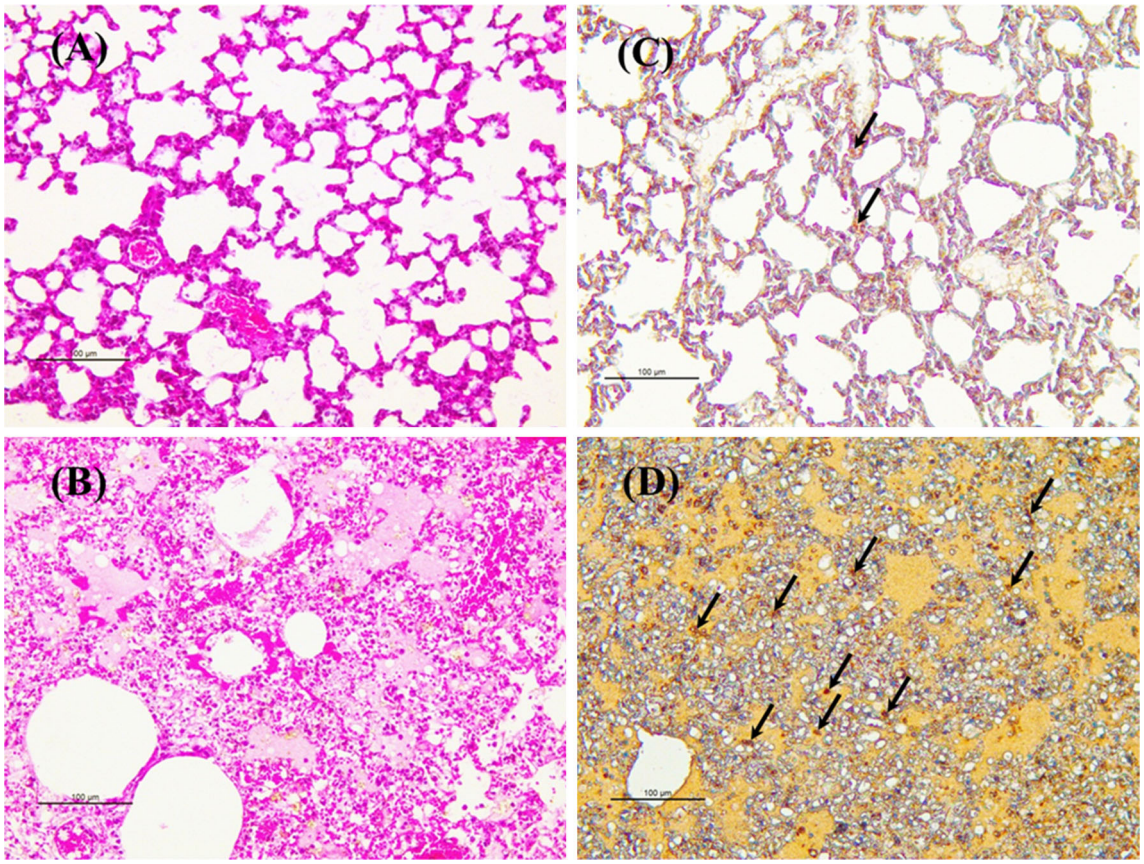

Fig. 1 Tissue pathology in mice infected with WT-6D2 or MA-6D2 at 3 dpi. Representative images are shown of the lungs of mice infected with 10^6 EID_{50} of WT-6D2 or MA-6D2 at 3 dpi. **(A)** No clinically significant pathology findings in the lungs of WT-6D2-infected mice **(B)** Lungs from a mouse infected with MA-6D2. There were obvious features of bronchointerstitial pneumonia, including fluid leakage into the lung tissue and a large number of infiltrating lymphocytes. **(C)** An antibody recognizing the influenza A virus nucleoprotein was used to detect H5N6-infected epithelial cells in the lungs of mice infected with WT-6D2. A limited number of infected cells were found in this group. **(D)** Immunohistochemical staining in the lungs of mice infected with MA-6D2 detected a larger number of infected cells (black arrows)

while 80 % of the mice infected with WT-6D2 survived (Fig. 2B). Together, these results indicated that the MA-6D2 virus caused more-severe disease than the WT-6D2 virus in a mammalian host.

To identify the molecular changes that were associated with increased viral replication and pathogenicity, the genomes of MA-6D2 and WT-6D2 were sequenced and five amino acid substitutions in the HA (A150V), NA (R143K; G147E), PA (A343T), and PB2 (E627K) proteins were identified (Table 1). HA A150V (138 in H3 numbering) was found in P6, near the receptor binding site. NA R143K and G147E, and PA A343T were observed in P2, indicating that these mutations were important for adaptation of 6D2 to a murine host. PB2 E627K is one of the most common amino acid substitutions in mouse-adapted viruses and is often found in H5N1 HPAIV, H7N7 HPAIV, H7N9 LPAIV, and 1918 H1N1 as a marker of virulence in mammalian species, not just in mice [16].

Discussion

For the first time, we report here the laboratory adaptation of H5N6 avian influenza A virus to a murine host. The amino acid substitutions we observed in the MA-6D2 strain are likely to play a key role in its increased virulence and replicative ability in mice.

Amino acid 150 (138 in H3 numbering) in the HA protein is located at the AIV receptor-binding site (Fig. S2), near the 130-loop, which may have influenced the receptor-binding capability of MA-6D2 and facilitated adaptation to a mammalian host. Mutations at this position can also be found in other avian viruses [17–19]. In our study, the HA A150V mutation was detected in P6, appearing later than the amino substitutions in PA and NA.

NA helps progeny viruses leave the host cell by removing terminally linked sialic acid (SA) [20], thus facilitating the spread of the virus. The 150-loop (residues 147–152) (Fig. S3) is located adjacent to the NA active

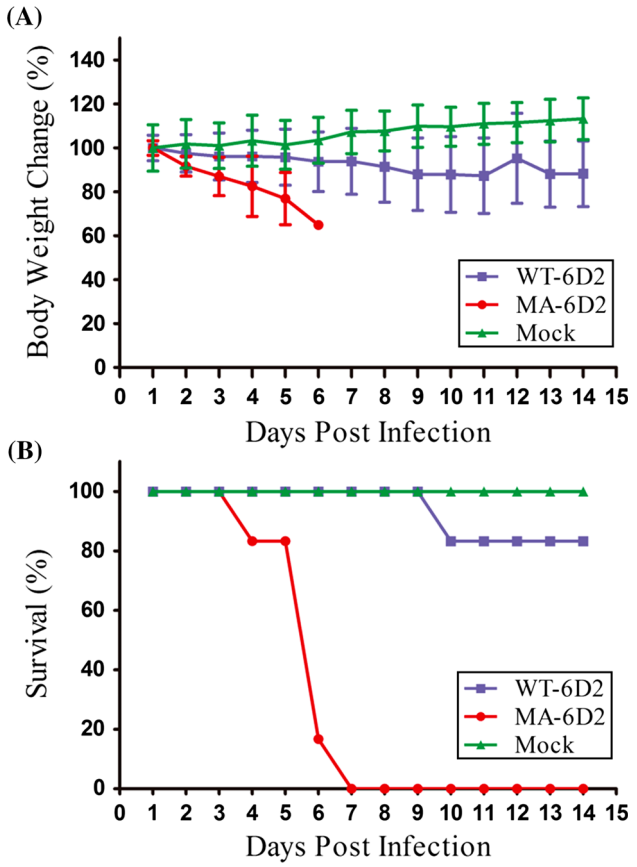

Fig. 2 Weight change and survival rate of mice infected with WT-6D2 or MA-6D2. Six-week-old BALB/c mice (six per group) were inoculated intranasally with 10^6 EID_{50} of WT-6D2 (blue squares), MA-6D2 (red circles), or PBS (mock; green triangles). Changes in body weight and survival rates of the mice were recorded daily until 14 dpi. **(A)** Percent change in body weight from day 0 (pre-infection). **(B)** Survival rate

Table 1 Nucleotide and amino acid sequence differences between wild-type and mammalian-adapted viruses

Segment	Position		P0	P2	P4	P6	P8	P10
HA	449[a]	nt	C	C	C	**T**	**T**	**T**
	150[b]	aa	A	A	A	**V**	**V**	**V**
NA	354[c]	nt	A	**G**	**G**	**G**	**G**	**G**
	360[c]	nt	C	**T**	**T**	**T**	**T**	**T**
	381[c]	nt	A	**G**	**G**	**G**	**G**	**G**
	405[c]	nt	T	**C**	**C**	**C**	**C**	**C**
	411[c]	nt	C	**A**	**A**	**A**	**A**	**A**
	427[a]	nt	C	**A**	**A**	**A**	**A**	**A**
	143[b]	aa	R	**K**	**K**	**K**	**K**	**K**
	428[a]	nt	G	**A**	**A**	**A**	**A**	**A**
	143[b]	aa	R	**K**	**K**	**K**	**K**	**K**
	440[a]	nt	G	**A**	**A**	**A**	**A**	**A**
	147[b]	aa	G	**E**	**E**	**E**	**E**	**E**
PA	1027[a]	nt	G	**A**	**A**	**A**	**A**	**A**
	343[b]	aa	A	**T**	**T**	**T**	**T**	**T**
PB1	882[c]	nt	A	**G**	**G**	**G**	**G**	**G**
PB2	1879[a]	nt	G	G	**A**	**A**	**A**	**A**
	627[b]	aa	E	E	**K**	**K**	**K**	**K**

Viruses collected from passages (P) P0, P2, P4, P6, P8, and P10 were sequenced, and the sequences were aligned to identify nucleotide and amino acid changes. All of the observed mutations are presented. The nucleotide or amino acids identified after substitution are indicated in bold

[a] non-synonymous mutations

[b] synonymous mutations

[c] amino acid substitutions

nt, nucleotide; aa, amino acid

site [21]. Therefore, the mutation NA G147E may have altered the structure of the NA active site and enhanced the spread of the virus to new cells. Maurer-Stroh et al. found that all influenza virus NAs have an insertion at residues 138–147 [22], and these residues, including NA R143K (Fig. S4), facilitate NA assembly and stabilize the tetramers [23].

PA is a crucial component of ribonucleoprotein complexes (RNPs), which are indispensable for transcription and replication of the viral genome [24]. PA can be divided into two domains, the C-terminal domain and the N-terminal domain, and residues 239–716 contain a binding site for PB1 [25]. Alterations to the binding site might alter the ability of PA to bind to PB1, thereby affecting the stability of RNP. This would account for the rapid emergence of the PA A343T mutation in the second passage. This mutation has previously been shown to increase the growth capacity of AIV in human cells and its pathogenicity in the lungs of mice [26, 27].

The PB2 E627K mutation has been shown to play a key role in adaptation to mammals [28] and the enhancement of virulence, both alone [29] and in combination [30] with other amino acid substitutions. In this study, we found that this amino acid substitution also played an important role in adaptation of an H5N6 virus to mice.

Conclusions

Passaging of WT-6D2 in mice increased the virulence and replication capacity of the virus and was associated with well-characterized amino acid substitutions. The association of these specific mutations with mammalian adaptation of H5N6 AIVs requires more in-depth study using reverse genetics to examine the functional changes associated with each mutation. Adaptation of H5N6 strains to mammalian hosts increases the chance of a human pandemic associated with this subtype. To prevent such an outbreak, the

surveillance systems for monitoring AIV transmission to mammalian hosts should be strengthened through long-term monitoring in live-poultry markets and expanded to include early warning systems for HPAIV transmission events.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All of the animal experiments performed were approved by the First Affiliated Hospital, School of Medicine, Zhejiang University (No. 2015-15).

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