Adaptive Amino Acid Substitutions Enhance the Virulence of a Novel Swine-Origin Canine H1N1 Influenza Virus in Mice

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Abstract

Swine-origin H1N1 influenza virus caused an outbreak in human in 2009 (H1N1/pdm09). We isolated several novel reassortant influenza A viruses bearing the H1N1/pdm09 segments (PB2, PB1, PA and NP) from dogs, which showed high similarity with H1N1 swine influenza virus in the previous study. To understand the molecular basis of swine-origin H1N1 reassortant canine influenza virus adaptation to new host species, the mouse-adapted variants of H1N1 canine virus were obtained by serial lung-to-lung passages in BALB/c mice. After thirteen passages, the virus significantly enhanced replication in MDCK cells and became lethal to mice. Six adaptive mutations mapped to two in the ribonucleoprotein (RNP) complex (PB2-E578D, PA-T97I), two in hemagglutinin (HA-N198D and A227E), two in the non-structural protein 1 and nuclear export protein (NS1-A53D and NEP-R42K), indicating that they may contribute to the increased virulence and replication. The results provided valuable insights into how the swine-origin influenza virus adapt to a new host that deserves more attentions.

Introduction

Influenza A virus (IAV) is an important pathogen with wide range of mammalian and avian species. In particular, the acquisition of adaptive mutations by repeated infection and replication could accelerate the evolution of the viral genome and adapt to the new hosts. During the last two decades, IAVs finished the 'host jump' from horse to dog (H3N8) and avian to dog (H3N2)[42], and viral adaptation will be responsible for the host jump. In addition, there were more reports of self-limited infections in dogs, including H9N2, H6N1, H5N2, H5N1, even the human influenza viruses [4, 6, 16, 21, 24, 26, 31, 34, 36, 37, 44, 46]. In our previous study, two reassortant Eurasian avian-like H1N1 swine influenza virus genotypes possessing four internal segments from H1N1 pdm/09 lineage (PB2, PB1, PA and NP) were individually introduced from swine into canines, which had potential infection and transmission for humans[2, 39]. However, not much is known about the mutations required for swine-origin H1N1 canine viruses to overcome species barriers and adapt to new hosts.

Adaptation is considered a primary impetus in evolution, and the process of natural selection in IAVs appears to be mimicked by experimental adaptation in mice[1]. Numerous studies have identified the amino acid substitutions associated with HA, NP, NA, M and NS genes and the polymerase genes during mouse adaptation could be critical determinants of virulence, tissue tropism and pathogenicity [8, 10, 22, 23, 25, 27, 29, 30, 40, 41, 43]. The HA protein determined the binding specificity by mediating the attachment of viruses to host-cell sialyloligosaccharide receptors via binding sites, which was a primary determinant for mouse lung virulence[41]. The viral ribonucleoprotein (vRNP) complex contributed to the host species adaptation by increasing polymerase activity and virus replication efficiency in mammals [3, 22, 29, 30, 45]. For example, the Glu-to-Lys substitution at position 627 (E627K) or the Asp-to-Asn substitution at position 701 (PB2-D701N) were identified as key determinants for adaptation of avian influenza virus to a new host species [1, 7, 15]. To gain a better understanding of viral determinants critical to the pathogenesis of the novel swine-origin H1N1 CIV bearing H1N1 pdm/09 segments in a mammalian model, we generated a mouse-adapted variant A/canine/Guangxi/LZ57 /2015 (LZ57) by serial lung-to-lung passages in mice and identified six adaptive mutations, located in the PB2 (E578D) and PA (T97I) of the polymerase complex, the HA (N198D and A227E), NS1 (A53D) and NEP (R42K) which will provide new insight into factors that affect the virulence and replication in mammals.

Materials And Methods

Virus and cells

Madin-Darby canine kidney (MDCK) cells and human embryonic kidney cells (293T) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Corning) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1× penicillin-streptomycin liquid (Solarbio, Beijing, China) at 37°C, 5% CO₂. The swine-origin H1N1 A/Canine/Guangxi/LZ57/2015 (LZ57) influenza virus was originated from the purification of A/Canine/Guangxi/LZ56/2015 (LZ56) [2] by plaque assay for three times. The LZ57 virus naturally harbored a 135-amino acid truncation in the effector domain of NS1 protein, which exhibited low pathogenicity in mice based on previous studies (data not shown).

Adaption of H1N1 canine influenza virus in mice

The LZ57 virus was adapted to mice by serial lung-to-lung passages (13 passages) of the wild-type LZ57 virus (LZ57-WT) for generating the mouse-adapted virus (LZ57-MA). Briefly, groups of six-week-old female BALB/c mice (n=6) were inoculated intranasally with 50 µl of LZ57-WT virus (10^6 PFU). Mice were euthanized at 3 days post-inoculation (d.p.i.). The harvested lung tissue (labeled as passage 1, MAP1) was homogenized and centrifuged. Fifty microliters (50 µl) was used to intranasally inoculate the next naive mouse in the series until the 13th passage (MAP13). After 13 passages, the virus in the lung homogenate was purified for three times by plaque assay on MDCK cells. The bigger plaque was identified and inoculated on MDCK cells for preparing a virus stock. The final virus was designated as LZ57-MA.

Mouse experiments

To evaluate the pathogenicity of LZ57-MA viruses, groups of 6-week-old female BALB/c mice (Charles River, Beijing) were lightly anesthetized and inoculated intranasally with 10^6 PFU of H1N1 influenza viruses in a volume of 50 µl. The control group was inoculated intranasally with 50 µl phosphate-buffered saline (PBS). Three mice in each group were euthanized and lung tissues were collected to assess viral replication in MDCK cells on 3 d.p.i. Clinical symptoms, body weight and survival were monitored and recorded until 14 d.p.i.. Mice were euthanized when the body weight loss was above 25% of the pre-inoculation weight. The 50% mouse-lethal dose (MLD_{50}) was determined by inoculating groups of five BALB/c mice intranasally with 10-fold serial dilutions of each virus (from 10^2 to 10^5 PFU per mouse). As described above, the healthy status and body weight were monitored daily for 14 d.p.i.. The MLD_{50} were calculated by the method of Reed-Muench. All of the animal experiments described in this study were approved by the Guangxi University Animal Care & Welfare Committee (GXU2020-08).

Genome sequencing and alignment

Viral RNA of the LZ57-WT and LZ57-MA variant (the 13th passage purified by plaque assay) was extracted using QIAamp Viral RNA Mini Kit (Qiagen Germantown, MD, USA) following manufacturer's instructions. Reverse transcription and amplification of the full-length genome were performed using segment-specific primers [2]. The PCR products were purified and sequenced by the method of Sanger (Shanghai, China) and aligned using the Lasergene sequence analysis software package (DNASTar, Madison, WI).

Viral growth kinetics

To explore the differences of replication between wild-type and mouse-adapted viruses in MDCK cells, the monolayer MDCK cells were infected with recombinant viruses at a multiplicity of infection (M.O.I.) of 0.01 and incubated at 37°C for 1 h. Cells were cultured with serum-free DMEM including 1 µg/ml of TPCK trypsin. Cell supernatants were harvested at 12, 24, 36, 48, and 60 hours post-infection (h.p.i.) and titrated in MDCK cells by plaque assays. Each sample consisted to three replicates and analyzed using the Reed and Muench method.

Prediction of HA protein structure

To understand the effect of mutations of HA protein to the structure and function, the partial structure models of HA complex of LZ57-WT and LZ57-MA were generated by the online prediction platform I-TASSER (<https://zhanglab.dcm.b.med.umich.edu/I-TASSER/>). PyMOL software (<http://www.Py mol.org /pymol/>) was used to analysis the predicted result and form the visual images.

Results

Adaption of the H1N1 CIV to mice

To produce the mouse-adapted swine-origin H1N1 CIV, the LZ57-WT virus was serially passaged in BALB/c mouse lungs. The body weight and virus titer in lungs were evaluated from 1 to 13 passages (Table 1). After ten passages, the mice displayed a continuous trend of body weight loss and high replication in mouse lung (6.23 Log_{10} PFU/ml). In order to obtain the stable mouse-adapted virus, the H1N1 CIV was passaged until the 13th passage. As a result, the weight loss, survival rate and viral titer in mouse lungs exhibited markedly virulence, compared to the wild-type virus, indicating that virus populations of mouse-adapted variants

have acquired mutations that affected the increased virulence. The mouse-adapted variants of the 13th passage were selected and purified by plaque assay. Three bigger plaque-picked clones with the single-phenotype were sequenced and determined the consistency of genome sequences. One of them was selected to represent the mouse-adapted LZ57 virus (LZ57-MA) for subsequent mouse study, the results showed there were obvious clinical symptoms, body weight loss in the LZ57-MA-infected mice, which led to die at 4 d.p.i. and higher virus titer of 6.87 Log₁₀ PFU/ml in the lungs ($p<0.01$) (Fig. 1).

Table 1
Pathogenicity of the mouse-adapted H1N1 CIV by serial lung-to-lung passages in mice

Virus	Changes of body weight (%) ^a	Viral titer in lung [(Log ₁₀ PFU/ml)±SD] ^b
LZ57-WT	11.86	5.69±0.88
The 3rd passage	2.07	5.67±0.42
The 6th passage	16.42	6.01±0.24
The 10th passage	N/A	6.32±0.28
The 13th passage	N/A	6.87±0.15 ^{**}
^a N/A represents not available, indicating that mice were euthanized when the body weight loss was above 25%.		
^b ^{**} The lung titer in the LZ57-MP13 group were significantly higher than the LZ57-WT group($P<0.01$).		

Table 1
Comparative analysis of amino acids between LZ57-WT and LZ57-MA

Protein	Position	Changes of amino acids in influenza viruses ^b						
		LZ57-WT	LZ57-MA	Human(H1N1) (2009-2020)	Swine(H1N1) (2009-2020)	Canine	Avian (H3N2)	Equine (H3N8)
PB2	578	E	D	E(3983/3988) G(4/3988) Q(1/3988)	E(1575/1576) D(1/1576)	E(152/152)	E(263/263)	E(120/120)
PA	97	T	I	T(4266/4273) I(7/4273)	T(1598/1615) A(12/1615) S(3/1615) I(1/1615) K(1/1615)	T(162/164) A(1/164) N(1/164)	T(291/291)	T(108/109) I(1/109)
HA ^a	198	N	D	A(11357/11957) E(534/11957) T(25/11957) V(25/11957) N(7/11957) G(4/11957) S(4/11957) P(1/11957)	A(3076/3891) E(177/3891) N(572/3891) T(27/3891) S(17/3891) V(12/3891) D(4/3891) K(4/3891) I(1/3891) P(1/3891)	H3N2: A(153/155) V(2/155) H3N8: A(1/196) E(195/196) H5:P(3/3) H9N2:T(1/1) H6N1:G(1/1) H1N1:A(2/2)	A(381/389) T (5/389) N(1/389) S (1/389) V (1/389)	E(818/844) A(13/844) G(13/844)
	227	A	E	E(11815/11863) A(20/11863) K(10/11863) G(9/11863) D(3/11863) Q(3/11863) P(2/11863) H(1/11863)	A(2698/3889) E(1022/3889) T(94/3889) S(57/3889) G(12/3889) V(3/3889) K(2/3889) Q(1/3889)	H3N2:S(155/155) H3N8:S(196/196) H5:S(5/5) H9N2:Q(1/1) H6N1:R(1/1) H1N1:E(2/2) ^c	S(386/389) P(3/389)	S(832/843) P(9/843) A(1/843) T(1/843)

^aH3 numbering.

^bThe isolates observed in nature containing specific amino acid, which come from Influenza Research Database (<https://www.fludb.org/>).

^cA/canine/Beijing/cau2/2009(H1N1)(JN540086); A/canine/Beijing/cau9/2009(H1N1)(JN540094)

Protein	Position	Changes of amino acids in influenza viruses ^b							
		LZ57-WT	LZ57-MA	Human(H1N1) (2009-2020)	Swine(H1N1) (2009-2020)	Canine	Avian (H3N2)	Equine (H3N8)	
NS1	53	A	D	D(2490/2607)	D(870/1243)	D(163/163)	D(228/230)	D(86/96)	
				N(101/2607)	E(313/1243)		N(2/230)	N(7/96)	
				E(11/2607)	N(40/1243)			G(3/96)	
				Y(2/2607)	K(8/1243)				
				G(2/2607)	G(7/1243)				
				A(1/2607)	A(2/1243)				
					S(2/1243)				
	Y(1/1243)								
NEP	42	R	K	R(792/801)	R(693/697)	R(95/97)	R(119/120)	R(43/43)	
				G(3/801)	G(2/697)		K(1/97)		K(1/120)
				K(3/801)	K(2/697)		G(1/97)		
				T(2/801)					

Pathogenicity And Growth Characteristics Of Mouse-adapted Virus

To evaluate the virulence of the mouse-adapted H1N1 viruses in vivo, the MLD₅₀ of mouse-adapted and wild type viruses were detected using a BALB/c mouse model. After infecting mice with serial 10-fold dilutions of mouse-adapted and wild type viruses shown in Fig. 2a, the MLD₅₀ of parental and mouse-adapted virus were >6 (LZ57-WT) and 2.75 Log₁₀ PFU (LZ57-MA) respectively, suggesting that there was clear disparity in virulence. In contrast, the mouse-adapted LZ57-MA virus was able to replicate to significantly higher level than LZ57-WT virus in MDCK cells (Fig. 2b). The trend was kept throughout the time course ($P<0.01$). The highest titer of the mouse-adapted virus reached to 8.47 Log₁₀ PFU/ml. In addition, the LZ57-MA virus formed relatively larger plaques when compared with those of LZ57-WT ($P<0.05$), suggesting that the adaptive mutations might be the key factor to affect the replicative capability in new hosts (Fig. 2c).

Amino acid substitutions involved in the adaption of H1N1 CIV in mice

To understand the molecular basis responsible for the increased virulence during the adaptation of an H1N1 canine influenza virus to mice, the full-length genome sequences of both the LZ57-WT and LZ57-MA were sequenced. Sequence analysis identified the six acquired mutations of the mouse-adapted virus in the polymerase proteins (PB2-E578D and PA-T97I), hemagglutinin (HA-N198D and HA-A227E), non-structural protein 1 (NS1-A53D), and nuclear export protein (NEP-R42K). The statistical results of different-origin influenza viruses at specific position showed that the residues of PB2-578E, PA-97T and NEP-42R were commonly present in most influenza viruses which still kept advantageous selection. All of six adaptive mutations could be found in swine H1N1 virus, but some of them were rare, including PB2-578D, PA-97I, HA-198D and NEP-42K (Table 2). In particular, the residue of PA-97I had been detected in some human, swine and equine isolate. Interestingly, the amino acid at HA-227 varied depending on the origin of virus. The residue of glutamic acid at 227 in HA was detected in majority of human (99.6%) and swine viruses (26.3%), but most of canine, avian and equine viruses possessed mainly serine at this position, suggesting the amino acid might be involved in the host specificity.

Discussion

The experimental infection of mouse lung with influenza viruses have been shown to be a valuable mammalian model, which allows the competition of different mutants in mice and resulted in the selection of predominant genotypes to adapt to a new host. Herein, we serially passaged a swine-origin H1N1 canine virus by lung-to-lung in BALB/c mice for studying the molecular basis of adaptation to the new host species. After 13 passages in mice, the mouse-adapted virus caused severe disease resulting in 100% mortality at 4 d.p.i. and showed growth advantage in MDCK cells. Six amino acid substitutions in five viral proteins (PB2-E578D, PA-T97I, HA-N198D, HA-A227E, NS1-A53D, NEP-R42K) in mouse-adapted virus were identified, which contributed to the increased virulence and pathogenicity in mice. Studies of mouse adaptation of novel swine-origin H1N1 canine influenza virus shed light on possible virulence factor to mammalian hosts.

Amino acid substitutions in viral polymerase complex played a crucial role in overcoming the host barrier [3, 8, 13, 14, 17, 28, 32, 35, 45]. The substitution PA-T97I has been identified as a key factor for the increased polymerase activity and virulence of the mouse-adapted virus whereas the mutation of PB2-E578D did not affect the virulence in mice [30]. Additionally, an isoleucine residue at PA position 97 was implicated in the adaptation of H6N1 and H7N9 avian influenza viruses in mice [3, 47]. In addition, the mouse-adapted rLZ57-MA acquired N198D and A227E mutations within the receptor binding site (RBS) of HA protein. The amino acid residues in the RBS region were critical for the recognition of different-origin influenza virus and alteration of cell receptor [20, 33]. The mutation of Asn-to-Asp at position 198 (H3 numbering) in the H1 HA1 domain of the mouse-adapted virus resulted in loss of a glycosylation sites, which was known to be a common mechanism for acquisition of virulence [25]. The mutation of HA-A227E occurred frequently in human H1N1 influenza viruses (Table 2), which located between amino acids 226 and 228 that played a key role in receptor specificity and host range restriction [38]. In contrast, previous study demonstrated that the combining the T200A and E227A mutations in the swine H1 protein had a synergistic effect that mainly contributed to the receptor binding affinity [5]. Nevertheless, the combination of 219I and 227E in the new pandemic H1 protein resulted in the disruption of interaction with the α 2-6 sialylated glycans [18]. In our study, the view of the receptor-binding site (RBS) in the crystal structure showed that the substitution of the residues at position 198 (N198D) and 227 (A227E) resulted in the interaction between A227E and 220R (220-loop), or 198D and 199H (190-helix) by the potential hydrogen bond (Fig. 3), suggesting that double mutations in HA might play crucial role in receptor binding specificity; how these mutations affected the receptor-binding preference still remained to be further investigated. The NS1-A53D substitution commonly existed in all species and highly conserved. The NEP-R42K mutation could be observed in other species, including avian-origin CIV and avian H3N2 virus (Table 2). None of them was implicated in increased virulence. More studies clarified that NS1 was a multifunctional protein that played a key role in inhibition of host innate immune response and viral replication [9, 11, 12, 27]. The adaptive mutation in NS1 protein required during mouse adaptation was associated to host switching [27]. The acquisition of adaptive mutations in NEP facilitated the avian influenza viruses escaped the restriction of viral genome by enhancing the polymerase activity to cross the species barrier [19].

In conclusion, four single-gene reassortants possessing the adaptive mutations required during the wild-type swine-origin H1N1 virus adaptation could exhibit various virulence and replicative capability in mice and MDCK cells respectively. Our data suggested

that the involved multiple amino acid substitutions in viral proteins contributed to the adaptation. How these adaptive amino acids played a synergistic role in different segments lead to increase virulence and replication still remained unknown, but the clarification of the key determinants of host adaptation and underlying mechanism will be essential for understanding the interspecies transmission of influenza A viruses.

Declarations

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Conflict of Interest: All authors declare that they have no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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Figures

Figure 1

The increased virulence of the mouse-adapted H1N1 canine virus in mice. Six-week-old female BALB/c mice were inoculated intranasally with 10^6 PFU/50 μ l dose of each virus or the mocked PBS. The changes of body weights were shown (a) and mortality of LZ57-MA was evaluated based on the survival rates (b). The replication in mouse lungs was tittered by plaque assay (c). The asterisk indicates a *P*-value (**P*<0.05; ***P*<0.01; ****P*<0.001) compared to the wild-type virus.

Figure 2

Pathogenicity of the wild-type and mouse-adapted virus in mice and viral replication in MDCK cells. Groups of six-week-old female BALB/c mice were inoculated intranasally with 50 μ l medium containing 10^2 , 10^3 , 10^4 , 10^5 PFU/ml of LZ57-WT and LZ57-MA, shown in a. Viral replication and plaque phenotype of the wild-type and mouse-adapted virus in MDCK cells were shown in b and c. Cells were inoculated with LZ57-WT and LZ57-MA viruses at a multiplicity of infection (MOI) of 0.01. The viruses were inoculated with 10-fold serial dilutions of virus at 37°C for 1h. Infected cells were stained with 0.8% (w/v) crystal violet solution at 60 h.p.i..

Figure 3

Comparison of amino acid mutations between the wild-type (a) and mouse-adapted canine H1N1 virus (b). Three components of the HA receptor-binding pocket: the 130-loop (131-135aa), 190-helix (184-191aa), and 220-loop (218-225aa) are shown in cyan, purple and blue, respectively. The amino acids at position 198 and 227 identified in the LZ57-MA virus were mapped on the HA crystal structure. The red arrow indicates the mutation of amino acids.