Austin Journal of Infectious Diseases

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Austin Publishing Group

Research Article

The Single E627K Amino Acid Substitution in PB2 Enhances the Pathogenicity of Wild-Bird-Origin H6N6 Subtype Avian Influenza Virus in Mice

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Received: July 17, 2020; **Accepted:** August 26, 2020; **Published:** September 02, 2020

Abstract

Avian Influenza Viruses (AIVs) are harbored by wild waterfowl as a natural host, and there is a species barrier restricting virus transmission from birds to mammals, including humans. However, it has been reported that, through genetic mutations, AIVs occasionally infect mammals and acquire high pathogenicity. The Amino Acid (aa) substitution of glutamic acid to lysine at position 627 (E627K) in polymerase basic protein 2 (PB2) is one of the well-known factors underlying mammalian adaptation. Although this substitution was previously observed in mammalian-adapted H5, H7, and H9 AIV subtypes, the impact of this mutation on the mammalian adaptation of other AIV subtypes is not fully verified. Here, we isolated the low pathogenic AIV subtype H6N6 from a wild bird fecal sample in Tokachi Subprefecture, Hokkaido, Japan. We passaged this H6N6 subtype in BALB/c mice four times and acquired the mouse-adapted virus. Whole-genome sequence analysis showed that the adapted virus had only one aa substitution (E627K) in PB2. The adapted virus-inoculated mice tended to show increased weight loss and mortality compared with the original virus-inoculated mice. The viral titer in the lungs of the adapted virus-inoculated mice was significantly higher than that of the original virus-inoculated mice. Additionally, the virus isolated from the lung of the original virus-inoculated mice with serious symptoms harbored the E627K substitution. Our findings indicate the possibility that the PB2 E627K substitution in H6N6 subtype AIV rapidly appears in mammalian hosts and contributes to the enhanced pathogenicity of this virus.

Keywords: Avian Influenza; E627K; H6 Subtype; Mammalian Adaptation; Pathogenicity; PB2

Abbreviations

aa: Amino Acid; **AIV:** Avian Influenza Virus; **PB2**: Polymerase Basic Protein 2; **dpi:** Days Postinoculation; **EID_{50}:** 50% Egg Infectious Dose; **IAV:** Influenza A Virus; **PCR:** Polymerase Chain Reaction

Introduction

Influenza A Virus (IAV) is a negative-sense single-stranded RNA virus belonging to the family Orthomyxoviridae with an eight-segmented genome. Wild aquatic birds are natural hosts of most IAV subtypes, and such avian IAVs (AIVs) occasionally cross the species barrier and infect mammals, including humans [1]. Several Amino Acid (aa) substitutions in the Polymerase Basic Protein 2 (PB2) subunit of the AIV RNA polymerase have been identified as important factors contributing to increased virulence and adaptation in mammalian hosts [1]. E627K, which has been detected in human-adapted highly pathogenic H5 and H7 AIV subtypes, is one of the most widely known substitutions associated with enhanced pathogenicity in mammalian hosts [2-4]. In addition, the contribution of the E627K substitution in H9N2 subtype AIV to the enhanced virulence in mice has been shown [5]. This substitution contributes to the improvement in PB2 polymerase activity at a lower temperature, which works to the advantage of efficient virus proliferation in the respiratory tracts of mammals [4,6]. As mentioned above, although the impact of the E627K substitution in H5, H7, and H9 AIV subtypes on mammalian adaptation has been reported, the effect of a single aa substitution at this position in other AIV subtypes remains poorly understood.

In recent years, H5, H7, H9, and H10 AIV infections have reportedly caused diseases in humans [7-11]. In addition to these subtypes, the first human case of H6N1 subtype AIV infection was reported in Taiwan in 2013 [12,13], suggesting the possibility of an epidemic among humans when it becomes adapted to them. In the present study, to elucidate the mammalian adaptation mechanism of H6 subtype AIV, the wild-bird-origin AIV subtype H6N6 was passaged in mice, and the pathogenicity and aa sequences of the passaged viruses were evaluated.

Materials and Methods

Viruses

The H6N6 subtype AIV strain A/avian/Japan/14UO0177/2014 was isolated from a wild bird fecal sample in Tokachi Subprefecture, Hokkaido, Japan, in 2014. The virus was propagated in the allantoic cavity of 10-day-old embryonated chicken eggs, and the collected allantoic fluid was used as the original virus solution. The original virus was intranasally inoculated into BALB/c mice generated in

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Citation: Miura H, Ozeki Y, Omatsu T, Katayama Y, Imai K, Mizutani T, et al. The Single E627K Amino Acid Substitution in PB2 Enhances the Pathogenicity of Wild-Bird-Origin H6N6 Subtype Avian Influenza Virus in Mice. Austin J Infect Dis. 2020; 7(1): 1041.

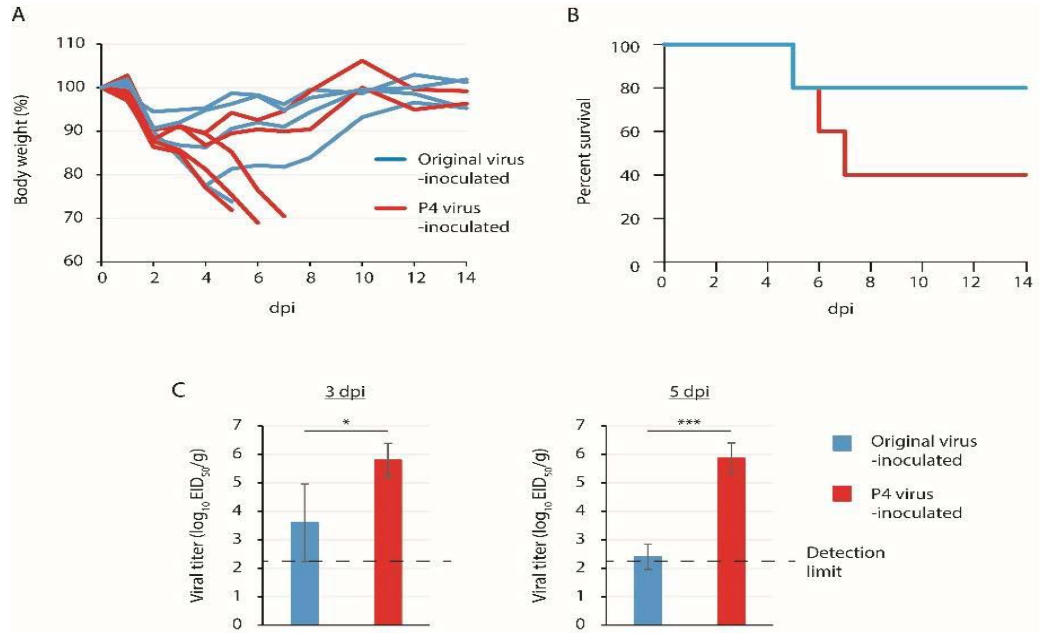

Figure 1: Evaluation of the pathogenicity of the P4 virus in mice.
(A-C) The original and P4 viruses were intranasally inoculated into mice. (A) Body weight changes and (B) survival rates were monitored daily for 14 days after virus inoculation (n=5). (C) The viral titers in the lungs were evaluated at 3 (n=5) and 5 dpi (n=4). Student's t-test (A, C) and log-rank test (B) were performed to analyze statistical significance; *P < 0.05, ***P < 0.001.

our laboratory under light anesthesia with isoflurane (Intervet K.K., Tokyo, Japan), and the lung was harvested at 3 Days Postinoculation (dpi). The lung homogenate was prepared as previously described [14] and inoculated into mice. This passaging procedure was repeated three more times (for a total of four passages after the original virus inoculation), and the acquired lung homogenate was inoculated into the allantoic cavity of eggs. The collected allantoic fluid was named P4 virus solution. All animal experiments were approved by the Institutional Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine and performed in compliance with the institutional guidelines.

Mouse infection study

BALB/c mice were intranasally inoculated with the original or P4 virus solution. The amount of inoculated virus was 10^4 50% tissue culture infective dose in 50 µl/mouse. Body weights and survival rates were monitored daily for 14 days after the virus inoculation (n=5). The mice were euthanized when a 25% reduction in body weight was observed. To evaluate the viral titer in the lung, mice in each group were euthanized on 3 and 5 dpi (n=4-5). The viral titer in the lung (50% Egg Infectious Dose (EID_{50})/g) was calculated using the Behrens-Kärber method [15].

Virus genome sequence analysis

RNAs were extracted from the original and P4 viruses, and next-generation sequencing was performed as previously described [16]. RNAs extracted from the lung of the original virus-inoculated mice with severe symptoms were transcribed into cDNA using FastGene Scriptase II (Nippon Genetics Co., Ltd., Tokyo, Japan), and the partial PB2 genome (the 1078th-1986th nucleotides containing the 627th aa-coding codon) was amplified using the following primers: 5'-TAYGARGARTTCACAATGGT-3' and 5'-ATATGGTCTCGTATTAGTAGAAACAAGGTCGTTT-3'. Polymerase Chain Reaction (PCR) was conducted under the following conditions: an initial denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, annealing at 52°C for 30 s, and 70°C for 3 min, and a final extension at 72°C for 10 min. The sequence analysis of amplified PCR products was performed as previously described [17]. The obtained nucleotide sequence data were analyzed using BioEdit software, and the genetic mutation and translated aa substitution site were identified.

Statistical analysis

P values were calculated using the following statistical analyses with GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA). Student's t-test and log-rank test were performed to analyze the body weight changes and viral titers in the lung, and the survival rate, respectively. P-values less than 0.05 were considered statistically significant.

Results

First, to identify aa substitutions retained in the P4 virus, next-generation sequencing was conducted. The whole-genome sequence analysis of the original and P4 viruses showed that the P4 virus had only one aa substitution (E627K) in PB2. Next, these original and P4 viruses were inoculated into mice, and body weight changes and survival rates were monitored. After 5 dpi, 25% weight loss/death was observed in 1/5 mice in the original virus-inoculated group, whereas these events were observed in 3/5 in the P4 virus-inoculated group (Figure 1A, B). However, no statistical difference was found between the two groups. The viral titer in the lungs of the P4 virus-inoculated group was $\geq 10^2$ and $\geq 10^3$ EID_{50}/g higher than that of the original virus-inoculated group at 3 and 5 dpi, respectively (Figure 1C).

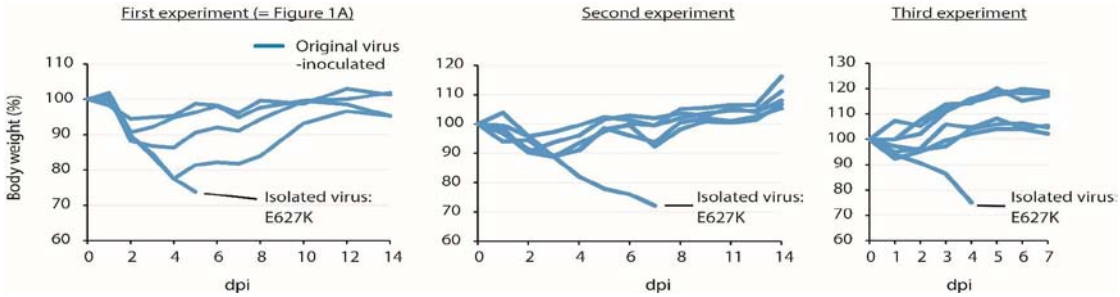

Figure 2: Evaluation of the pathogenicity and aa substitution in the original virus.
The original virus was intranasally inoculated into mice, and body weight changes were monitored. The result of the first experiment is the same as that observed in the original virus-inoculated group shown in Figure 1A. Substitutions at the 627th aa in the viruses isolated from the three mice with severe body weight reduction and clinical symptoms were shown. n=5, 6, and 7 in the first, second, and third experiments, respectively.

As shown in Figure 1A and B, a 20% death rate was observed even in the original virus-inoculated group. Therefore, we inoculated the original virus into mice two more times and monitored body weight changes to confirm the reproducibility. As a result, ~15% of mice showed severe body weight reductions and clinical symptoms in the second and third experiments. The lungs were harvested from these mice, and the aa sequence at position 627 was analyzed. As expected, the E627K substitution was observed in all of the viruses isolated from the three tested mice (Figure 2).

Discussion

In the current experiment, the E627K substitution, which is one of the well-known mammalian adaptation markers, was observed in the wild-bird-origin H6N6 AIV subtype after its inoculation into mice. Similar to other mammalian-adapted strains, including H5, H7, and H9 subtypes, this single aa substitution contributed to the enhanced pathogenicity of H6N6 subtype AIV in mice. The D701N mutation in PB2 is also a well-known mammalian adaptation marker. The D701N substitution enhances the binding of PB2 to importin, which promotes PB2 transport into the nucleus and facilitates virus proliferation in mammalian cells [18]. Although the effect of the single D701K substitution on virus proliferation and pathogenicity in mammalian hosts may be smaller than that of the single E627K substitution [19,20], dual E627K and D701N substitutions enhance viral polymerase activity and virulence in mammalian hosts compared with the single E627K substitution [19]. In the current study, the E627K substitution rapidly appeared in the original virus-inoculated mice, whereas the D701N substitution was not observed even in the P4 virus. Similar to this report, a previous virus transmission study using ferrets showed that the E627K substitution was more likely to occur than the D701N mutation [19]. Therefore, it is possible that, as the passaging of the current H6N6 subtype AIV is repeated in mammalian hosts, mammalian adaptation-related substitutions other than E672K occur, including D701N. In our study, the mouse was used as a mammalian model. However, the mouse is considered as a poor model for virus transmission because it does not show sneezing and running nose. This point is one of the limitations of our research. On the other hand, the ferret shows a sneeze reflex and can be available for IAV transmission study [21]. Hence this more suitable animal model should be used in the future study for further understanding of the mammalian adaptation mechanisms of the current H6N6 subtype AIV.

Although a human infection case of H6N1 subtype AIV was reported in 2013, this virus did not retain either E627K or D701N in PB2 [13]. However, our finding indicates the possible occurrence of human-adapted H6 AIV subtypes harboring the E627K substitution with the potential to threaten human health. Hence, conducting AIV surveillance and monitoring aa substitutions related to mammalian adaptation in not only H7, H5, and H9 subtypes but also other AIV subtypes to control human AIV infection cases are important.

Conclusion

We showed that the E627K substitution appeared when the wild-bird-origin H6N6 AIV subtype was infected into mice, and this single aa substitution resulted in enhanced pathogenicity in mice. Our finding indicates the potential emergence of mammalian-adapted viruses of not only H5, H7, and H9 strains but also the H6 AIV subtype. The present study contributes to a better understanding of the mammalian adaptation mechanisms in H6 subtype AIV.

Acknowledgments

We thank Sachiko Matsuda for her technical assistance. We would like to thank Enago (https://www.enago.jp) for the English language editing. This research was partially funded by JSPS Kakenhi, grant number 17K19878, from the Japan Society for the Promotion of Science, Japan.

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