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by Resti Yudhawati

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Original Article

Comparison of Virulence and Lethality in Mice for Avian Influenza Viruses of Two A/H5N1 and One A/H3N6 Isolated from Poultry during Year 2013-2014 in Indonesia

Resti Yudhawati[1,2], Rima R Prasetya[2], Jezzy R Dewantari[2], Aldise M Nastri[2], Krisnoadi Rahardjo[2], Arindita N Novianti[2], Muhammad Amin[1], Fedik A Rantam[3], Emmanuel D Poetranto[2], Laksmi Wulandari[1,2], Maria I Lusida[2], Soetjipto[2], Gatot Soegiarto[2], Yohko K Shimizu[4], Yasuko Mori[4], and Kazufumi Shimizu[2,4]*

[1]*Department of Pulmonology and Respiratory Medicine, Faculty of Medicine, [2]Indonesia-Japan Collaborative Research Center for Emerging and re-emerging Infectious Diseases, Institute of Tropical Disease, and [3]Department of Virology and Immunology, Faculty of Veterinary Medicine / Stem Cell Research and Development Center, Airlangga University, Surabaya, Indonesia; and [4]Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Japan*

SUMMARY: In Indonesia, the highly pathogenic avian influenza A/H5N1 virus has become endemic and has been linked with direct transmission to humans. From 2013 to 2014, we isolated avian influenza A/H5N1 and A/H3N6 viruses from poultry in Indonesia. This study aimed to reveal their pathogenicity in mammals using a mouse model. Three of the isolates, Av154 of A/H5N1 clade 2.3.2.1c, Av240 of A/H5N1 clade 2.1.3.2b, and Av39 of A/H3N6, were inoculated into BALB/c mice. To assess morbidity and mortality, we measured body weight daily and monitored survival for 20 d. Av154- and Av240-infected mice lost 25% of their starting body weight by day 7, while Av39-infected mice did not. Most of the Av154-infected mice died on day 8, while the majority of the Av240-infected mice survived until day 20. A 50% mouse lethal dose was calculated to be 2.0×10^1 50% egg infectious doses for Av154, 1.1×10^5 for Av240 and $> 3.2 \times 10^6$ for Av39. The Av154 virus was highly virulent and lethal in mice without prior adaptation, suggesting its high pathogenic potential in mammals. The Av240 virus was highly virulent but modestly lethal, whereas the Av39 virus was neither virulent nor lethal. Several mammalian adaptive markers of amino acid residues were associated with the highly virulent and lethal phenotypes of the Av154 virus.

INTRODUCTION

In Indonesia, the highly pathogenic avian influenza A/H5N1 virus has been endemic in poultry since 2003 and causes sporadic infection in humans (1). Indonesia is a country with high cumulative number of human infections with the virus, recording 200 cases with 168 mortalities from 2003 to 2019, which is the highest mortality rate in the world (2). Viruses of H5 HA clade 2.1 had been exclusively circulating in poultry until 2012 in Indonesia. Incursion of viruses of clade 2.3.2.1c was reported for the first time in September 2012 (3). In September 2013, we isolated a virus of clade 2.3.2.1c, Av154, from an outbreak at a turkey farm in East Java, Indonesia (4). In February 2014, we isolated a virus of clade 2.1.3.2b, Av240, from an ill chicken at a live poultry market. To indicate the place of emergence, the virus of clade 2.1.3.2b was identified as being of Indonesian lineage since it evolved from clade 2.1 in Indonesia whereas the virus of clade 2.3.2.1c was identified as being of Eurasian lineage since it evolved in Eurasia. In addition, we isolated an avian influenza A/H3N6 virus, Av39, from a mildly ill duck at a live poultry market in June 2013 (4). This was the first isolate of avian influenza A/H3N6 virus in Indonesia. A previous serological study suggested a high prevalence of subclinical infection with avian influenza A/H5N1 viruses among workers in live poultry markets in Indonesia; 84% and 34% were positive for antibody activity against Av154 of Eurasian lineage and Av240 of Indonesian lineage, respectively; none of the workers had had severe acute respiratory illness during the previous year (4).

Because the potential risk of infection with avian influenza viruses and resulting disease in humans is not fully understood, it is important to study the pathogenicity of the viruses in a mammal model. The

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*Corresponding author: Mailing address: Center for Infectious Diseases, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe City, Hyogo 650-0017, Japan. Tel: +81-78-382-6272, E-mail: shimizu.kazufumi@gmail.com

Table 1. Fifty percent mouse lethal dose of Av154, Av240, and Av39 avian influenza virus isolates[1)]

Virus Isolate	Hemagluti-nation Titer	50% Egg Infectious Dose (EID_{50})	50% Mouse Lethal Dose (MLD_{50})	EID_{50} per MLD_{50}
Av154	480	2.0×10^8/mL	1.0×10^7/mL	2.0×10^1
Av240	1,280	3.2×10^8/mL	3.0×10^3/mL	1.1×10^5
Av39	160	3.2×10^7/mL	< 10/mL	$> 3.2 \times 10^6$

[1)]: Fifty percent mouse lethal dose was calculated from the data shown in Fig. 2 by using the method of Reed and Muench (8). Av154: A/turkey/East Java/Av154/2013(H5N1) clade 2.3.2.1.c Eurasian lineage, Av240: A/chicken/East Java/Av240/2014(H5N1) clade 2.1.3.2b Indonesian lineage, Av39: A/duck/East Java/Av39/2013(H3N6).

mouse model has been used for many years to assess pathogenicity, because mice are susceptible to avian A/H5N1 viruses without prior virus adaptation. BALB/c mice have α2,3-linked sialic acid residues which act as receptors for avian influenza virus, in the ciliated airway epithelial cells and type II alveolar epithelial cells (5), in addition to α2,6-linked sialic acid residues for human influenza virus (6) and, therefore, avian influenza virus can infect them concomitant with pathological changes. In this study, we aimed to reveal the pathogenicity of three isolates of avian influenza type A viruses in mammals using a mouse model.

MATERIALS AND METHODS

Mouse model: BALB/c female mice were provided by the Stem Cell Research and Development Center, Airlangga University, Surabaya, Indonesia. They were raised with standard feed and water in sterilized conditions. The cage was placed in a ventilated micro-isolator enclosure under negative pressure with HEPA-filtered air in a biosafety cabinet. After completion of the experiments, all surviving mice were sacrificed by injection of a high dose ketamine (100 mg/kg body weight) and xylasin (10 mg/kg body weight) intraperitoneally.

Virus: As virus inoculum, we used viruses from three isolates, Av154 of A/H5N1 Eurasian lineage (A/turkey/East Java/Av154/2013, H5 HA clade 2.3.2.1c), Av240 of A/H5N1 Indonesian lineage (A/chicken/East Java/Av240/2014, H5 HA clade 2.1.3.2b), and Av39 of A/H3N6 (A/duck/East Java/Av39/2013), to assess their pathogenicity in mice. Each virus was propagated in 10-day-old embryonated chicken eggs for 2 days at 37°C. The allantoic fluids were harvested and tested for hemagglutination activity and infectivity (hemagglutination titers and 50% egg infectious doses (EID_{50}) are shown in Table 1). Infectious allantoic harvests were pooled and the aliquots were stored in a freezer at −80°C until use. The whole-genome sequences of the viruses were determined (manuscript in preparation) and submitted to the GISAID database with Isolate IDs as follows: A/turkey/East Java/Av154/2013, EPI_ISL_307002; A/chicken/East Java/Av240/2014, EPI_ISL_307019; A/duck/East Java/Av39/2013, EPI_ISL_307026.

Virus inoculation: Av154 and Av240 viruses were serially diluted from 10^{-1} to 10^{-6} and Av39 was diluted from 10^0 to 10^{-3} with 0.2% bovine serum albumin (BSA) in Tris-buffered saline containing glucose (TGS; 25 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4-$12H_2O$, 5.6 mM glucose, pH7.4). After being lightly anesthetized with ketamine (20 mg/kg body weight) and xylasin (2 mg/kg body weight) via intraperitoneal injections, BALB/c mice (10-week-old females, n = 5) were inoculated intranasally with 50 µL of each dilution of the viruses. The control mice (n = 10) were mock inoculated with 50 µL of 0.2% BSA in TGS. All groups of mice were observed for survival and their body weights, an alternative indicator of infection, were measured daily for a period of 20 days after inoculation. The 50% mouse lethal dose (MLD_{50}) was determined by assessing the number of dead and surviving mice on day 20, according to the method of Reed and Muench (7). All procedures were performed in the BSL3 laboratory of the Institute of Tropical Disease, Airlangga University.

This study was approved by the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Universitas Airlangga; the document identifier is 515-KE. All treatments for mice were administered under anesthesia and all necessary measures were taken to minimize animal suffering.

Analysis of amino acid sequences: The amino acid sequences of the viral proteins decoded from the genome nucleotide sequences were analyzed for mutations that could putatively confer the observed viral phenotypes, according to the evaluation of phenotypic markers described by Mertens et al. (8); similar or identical sequences containing mutations that were previously reported were also analyzed.

RESULTS

Loss of body weight by infection: After virus inoculation, we measured body weight loss, a sensitive indicator of pathogenic viral infection in mice. Fig. 1A illustrates the changes in the body weight of mice after intranasal inoculation with serial 10-fold dilutions of Av154, ranging from 10^{-1}-10^{-6}. At the dilution of 10^{-1} (russet line), weight loss began on the first day after inoculation, decreasing day by day to 75% of the initial body weight until the mice died. At the dilutions from 10^{-2}-10^{-5}, the body weight decreased to less than 80% of the initial measurement and most mice died. At the dilution of 10^{-6} (purple line), no significant differences compared to control mice were seen. Fig. 1B illustrates the changes after inoculation with serial 10-fold dilutions of Av240 ranging from 10^{-1}-10^{-6}. At the dilutions of 10^{-1} (russet line) and 10^{-2} (red line), weight loss began on the first day and decreased day by day to 75% of the initial body weight at around day 7 to 14

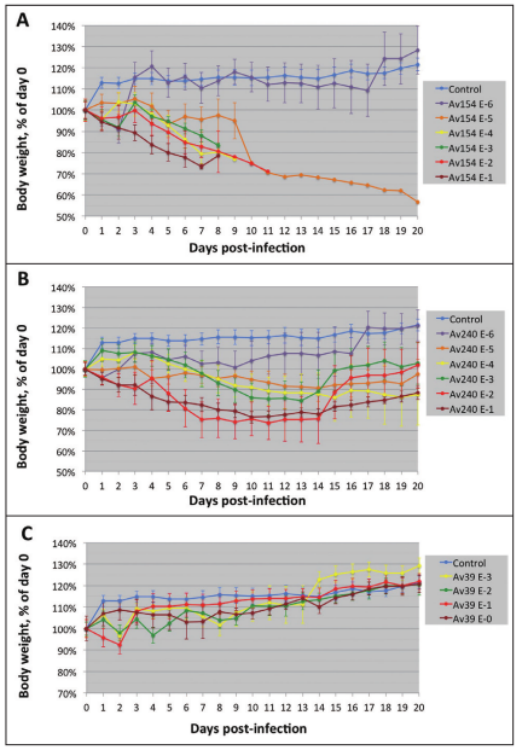

Fig. 1. (Color online) Weight loss of mice infected with Av154, Av240, and Av39 of avian influenza virus isolates. BALB/c female mice (n = 5) were inoculated intra nasally with a 50 µL of serial 10-fold dilutions of each virus and the body weights were measured daily for 20 days. The average body weights (% of day 0) were plotted with standard errors: Av154 E-1 to Av154 E-6 represent for the mice inoculated with 10^{-1} to 10^{-6} dilution, respectively, of A/turkey/East Java/Av154/2013(H5N1) Eurasian lineage virus pool (A), Av240 E-1 to Av240 E-6 with 10^{-1} to 10^{-6} dilution, respectively, of A/chicken/East Java/Av240/2014(H5N1) Indonesian lineage virus pool (B), and Av39 E-0 to Av39 E-3 with 10^{0} to 10^{-3} dilution, respectively, of A/duck/East Java/Av39/2013(H3N6) (C). Control represents for mock-control mice (n = 10) inoculated with the diluent (0.2% bovine serum albumin in Tris-buffered saline containing glucose) (A, B, and C).

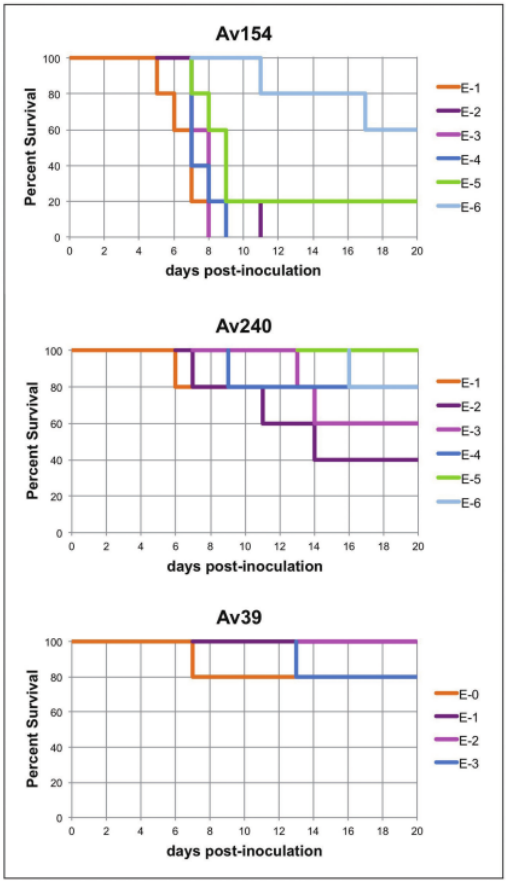

Fig. 2. (Color online) Mortality of mice infected with avian influenza virus isolate Av154, Av240, or Av39. BALB/c female mice (n = 5) were inoculated intra nasally with a 50 µL of serial 10-fold dilutions of the virus pools (the hemagglutination titers and 50% egg infectious doses (EID_{50}) were shown in Table 1) and observed for survival daily for a period of 20 days post-inoculation. Percent survival was plotted for each day. Av154: A/turkey/East Java/Av154/2013(H5N1) clade 2.3.2.1c Eurasian lineage, Av240: A/chicken/East Java/Av240/2014(H5N1) clade 2.1.3.2b Indonesian lineage, Av39: A/duck/East Java/Av39/2013(H3N6). E-0 to E-6: 10^{0} to 10^{-6} dilutions.

post-inoculation. In contrast to the Av154 infection, the majority of the mice survived and regained 90–100% of their initial body weight by day 20. At the dilutions from 10^{-3}-10^{-5}, the body weight decreased to less than 90% on around days 10 to 14 and the majority of mice survived, regaining 90–100% of their body weight by day 20. At the dilution of 10^{-6} (purple line), no significant decrease from the initial body weight was observed. Fig. 1C illustrates the body weight changes after the inoculation of Av39. No significant changes compared to the control group were observed at any of the dilutions tested.

Mortality by virus infection: We monitored the survival rate of inoculated mice for a period of 20 d after inoculation to determine the lethality of the infection. Fig. 2 shows survival curves of mice infected with Av154, Av240, and Av39. Following inoculation with Av154 at dilutions of 10^{-1}-10^{-4}, no mice survived till day 20 and most of them died by day 7 or 8. One mouse survived at the dilution of 10^{-5} and three mice at the dilution of 10^{-6}. In contrast to Av154, the majority of the mice inoculated with any dilution of Av240 survived to day 20, with the exception of the dilution at 10^{-2}, where two mice survived and three died. As for the mice inoculated with Av39, most of them survived, except one died at the 10^{0} and one at the 10^{-3} dilution. From the data shown in Fig. 2, the titers of MLD_{50} were calculated to be 1.0×10^{7}/mL for Av154, 3.0×10^{3}/mL for Av240, and < 10/mL for Av39. The MLD_{50} was calculated to be 20 EID_{50} for Av154, 1.1×10^{5} for Av240, and > 3.2×10^{6} for Av39, as shown in Table 1.

Amino acid sequence comparison: Table 2 summarizes the results of the analysis of amino acid sequences of each virus for receptor-binding,

Table 2. Amino acid sequence comparison of the receptor binding site, glycosylation site, and cleavage site in HA and deletions in NA and NS1

Amino acid mutation (H5 numbering)			Amino acid residue[1] Av154[2]	Av240[3]	Av39[4]	Phenotype
HA	Substitution at receptor binding site (12)	E186G/D	E	E	E	Increased virus binding to α2,6 (13, 14,15)
		G221D	G	G	G	Change in receptor binding affinity from avian to human receptors (16,17)
		Q222L	Q	Q	Q	Change in receptor binding recognition from α2,3 to α2,6 (18,19,20), Increased virus binding to α2,6 (21), Airborne transmissible in mammals (22,23)
		G224S	G	G	G	Increased virus binding to α2,6 (21, 24) , Airborne transmissible in mammals (22)
	Loss of glycosylation site	154-156	**Lost** (DNA)	Not lost (NST)	**Lost** (GST)	Increase virus binding to α2,6 and pathogenicity in mice (24,25,26)
	Multiple basic amino acids in cleavage site	321-330	PQRE-**RRRKR↓**G	PQRES **RRKKR↓**G	PEKQT ----R↓G	Increased virulence in mice (27,28,29, 30,31,32)
NA	Deletion	49-68	**Deleted**	**Deleted**	Not deleted	Enhance virulence in mice (33,34)
NS1	Deletion	80-84	**Deleted**	**Deleted**	Not deleted (TIASV)	Enhance virulence in mice associated with D92E shift (35)

[1]: Mammalian adaptive amino acid residue is highlighted by bold and light grey of background color.
[2]: Av154: A/turkey/East Java/Av154/2013(H5N1) clade 2.3.2.1.c Eurasian lineage.
[3]: Av240: A/chicken/East Java/Av240/2014(H5N1) clade 2.1.3.2b Indonesian lineage.
[4]: Av39: A/duck/East Java/Av39/2013(H3N6).

Table 3. Amino acid substitutions related to mammalian adaptation (8)

Amino acid substitution (H5 numbering)		Amino acid residue[1] Av154[2]	Av240[3]	Av39[4]	Phenotype
PB2	T339K	T	T	**K**	Enhanced polymerase activity, Increased virulence in mice
	R368Q	**Q**	R	R	Better polymerase activity and Enhance virulence in mice
	K526R	K	**R**	K	Increased polymerase activity, Increased virulence in mammals and birds
	V667I	V	V	**I**	Enhanced transmission
	K702R	K	K	**R**	Human host marker
PB1	R207K	**K**	R	**K**	Increased polymerase activity in mammalian cells
PA	A404S	**S**	A	A	Human host marker
HA	N154D	**D**	N	G	Airborne transmissibility in mammals
	S155N	**N**	S	S	Increased virus binding to α2,6, Increased replication in mammals
	T156A	**A**	T	T	Increased virus binding to α2,6, Airborne transmissible in mammals
	K189R	**R**	M	N	Increased virus binding to α2,6, Increased replication in mammals
	V210I	V	V	**I**	Increased virus binding to α2,6
	A263T	**T**	A	S	Increased virulence in mammals
M1	V15I/T	**I**	**I**	V	Increased virulence in mammals
M2	V27A	I	**A**	V	Reduced susceptibility to amantadine and rimantadine
	S31N/G	S	**N**	S	Reduced susceptibility to amantadine and rimantadine
	L55F	L	L	**F**	Enhanced transmission
NS1	D87E	**E**	D	D	Increased virulence in mammals
	T/D92E	**E**	D	D	Increased virulence in mammals, Escape of antiviral host response
	T/D/V/R/A127N	T	A	**N**	Increased virulence in mammals
NS2	A47T	**T**	A	A	Increased IFN antagonism

[1]: Mammalian adaptive amino acid residue is highlighted by bold and light grey of background color.
[2]: Av154: A/turkey/East Java/Av154/2013(H5N1) clade 2.3.2.1.c Eurasian lineage.
[3]: Av240: A/chicken/East Java/Av240/2014(H5N1) clade 2.1.3.2b Indonesian lineage.
[4]: Av39: A/duck/East Java/Av39/2013(H3N6).

glycosylation, and cleavage sites in the HA protein. At the receptor binding site, all viruses had E-186, G-221, Q-222, and G-224 (H5 numbering), which were compatible with the avian receptor α2,3-linked sialic acid. Av240 had a glycosylation site NST at position 154–156, while N at 154 was substituted with D for Av154 and G for Av39, losing the glycosylation site. For Av154 and Av240, the amino acid sequences at the cleavage sites were PQRE-RRRKR and PQRESRRKKR, respectively, possessing five consecutive basic amino acid residues, a typical characteristic of highly pathogenic avian influenza A viruses. For Av39, the amino acid sequence was PEKQT----R with only one basic amino acid residue R, a typical feature of viruses possessing low pathogenicity. In addition, Table 2 compares deletions in the NA and NS1 proteins; Av154 and Av240 had deletions at 49–68 of NA and 80–84 of NS1, while Av39 did not have those deletions.

Mertens et al. (8) listed 152 phenotypic markers or amino acid substitutions for avian influenza viruses related to pathogenicity in mammals and transmission from birds to mammals. We compared the reported phenotypic markers or amino acid substitutions with those of our three isolates; Table 3 summarizes the results. In total, there were 12 amino acid residues related to adaptation to a mammalian host in Av154, 4 in Av240, and 7 in Av39. Only Av154 had the adaptation markers of Q-368 in PB2; S-404 in PA; D-154, N-155, A-156, R-189, and T-263 in HA; E-87 and E-92 in NS1; and T-47 in NS2.

DISCUSSION

In this study, we compared the morbidity and mortality in mice due to infection with the three isolates of avian influenza type A viruses that we isolated from 2013 to 2014 in East Java, Indonesia. The isolates showed distinctive differences in pathogenicity in mice; Av154 was highly virulent and lethal with an MLD_{50} of 20 EID_{50}, Av240 was highly virulent and modestly lethal with 1.1×10^5, and Av39 was neither virulent nor lethal with $> 3.2 \times 10^6$ (Figs. 1 and 2). Several studies have reported the variations in pathogenicity of different strains of avian influenza viruses in mouse models (9–11).In terms of infection with influenza virus, oligosaccharides terminated by α2,3-linked sialic acid (SA) in the epithelial cell receptor are the preferential target for avian strains and those terminated by α2,6-linked SA are the preferential target for human strains. It has been reported that in the mouse often used as a model for studying influenza viruses, the α2,3-linked SA receptor is expressed in the ciliated airway and type II alveolar epithelial cells, which is targeted for infection by avian influenza viruses (5,6). We confirmed infection of the three viruses in murine lungs through the detection of viral messenger RNA using RT-PCR with oligo(dT)18VN as the primer for reverse transcription (unpublished data). The deduced amino acid sequences of HA indicated that all of the three viruses had the amino acid residues E-186, G-221, Q-222, and G-224 at the receptor binding site of HA (12) (Table 2), suggesting their α2,3-SA binding specificity (13–21). Av154 has the amino acid residues N-155, A-156, and R-189 in HA (Table 3); these residues are mammalian adaptive markers for increased virus binding to α2,6-SA. According to Ha Y et al. (22), avian H5 hemagglutinins were capable of binding to avian and human receptors, and Maines et al. (23) showed that a single amino acid mutation of K189R resulted in increased binding to α2,6-linked sialic acid without a loss of binding to α2,3-linked sialic acid. This explains in part the high lethality shown by Av154, because the α2,6-linked SA receptor is also expressed in mouse epithelial cells (6). Unlike Av154, Av240 has an N-linked glycosylation site at position 154–156; viruses with a glycosylation site at this position were relatively less virulent and loss of the carbohydrate at position 154 increased the binding affinity to the receptors, particularly SA-α-2,6-Gal (24,25). In addition, viruses with loss of the 154N glycosylation site showed increased pathogenicity, systemic spread, and pulmonary inflammation in mice (26), which corresponded to the genetic traits of the highly virulent Av154.

Avian influenza type A viruses are classified into two groups: highly pathogenic and low pathogenic. They differ in the sensitivity of the viral HA protein to host proteases to be cleaved, thereby activating the fusion function. Most of the low pathogenic viruses have a single arginine residue in the HA cleavage site, which can be cleaved by trypsin-like-extracellular proteases and is limited to the airway or intestine (27,28). The highly pathogenic viruses, on the other hand, possess multiple basic amino acid residues in the HA cleavage site, so that HA can be cleaved by various intracellular protease enzymes, such as furin-like-proteases. This cleavage leads to systemic infection, resulting in damage to multiple organs (29–32). Av154 and Av240, which were highly pathogenic, had five consecutive basic amino acid residues in the cleavage site, while the low pathogenic virus Av39 had a single arginine (R) residue (Table 2). This may be the main reason that Av39 was not lethal in mice. Av154 had a deletion of a single residue S at the site proximal to the multiple basic residues common to Av154 and Av240. The deletion was typical for A/H5N1 clade 2.3.2.1 viruses (3) but the biological meaning is not yet understood.

It is known that the level of virulence or severity of illness is in line with the decrease of body weight as well as MLD_{50}. Av154- and Av240-infected mice lost 25% body weight by day 7 with almost the same kinetics as typically seen at the dilution of 10^{-1} (Figs. 1A and 1B), while the body weight of mice infected with any dilution of Av39 increased at a similar rate to mock-infected control mice (Fig. 1C); Av154 and Av240 were highly virulent and Av39 was not virulent. Interestingly, most of the mice inoculated with Av154 died on day 7 or 8 (Fig. 2), while the majority of the mice inoculated with Av240 survived till day 20 and regained body weight (Fig. 2 and Fig. 1B). Thus Av240 showed 5,500-fold less lethality than Av154, as the EID_{50} per MLD_{50} was 110,000 for Av240 and 20 for Av 154 (Table 1). Both Av154 and Av240 had a deletion of 20 amino acids at position 49–68 in the stalk region of NA, which was implicated in enhanced virulence in mice (33,34) (Table 2). This mutation has been observed in highly pathogenic avian influenza A/H5N1 viruses and their human isolates for all we know. Av154 also had deletion

at residues 80 to 84 associated with D92E shift in the NS1 protein (Tables 2 and 3), which has been shown to confer enhanced virulence in chickens and mice (35). Av154 had marker amino acid residues listed by Mertens et al. (8) for increased virulence in mammals of Q-368 in PB2, T-263 in HA, and E-87 and E-92 in NS1. For increased IFN antagonism, it had T-47 in NS2. For increased virus binding to α2,6-SA, it also had N-155, A-156, and R-189 in HA. In contrast, Av240 had none of these mammalian adaptive phenotype markers (Table 3). Lack of these mammalian adaptive markers in Av240 might be responsible for the survival of Av240-infected mice. Further research is needed to reveal its mechanism.

It was shown in this study that the Av154 virus of A/H5N1 clade 2.3.2.1c Eurasian lineage was highly virulent and lethal in mice without prior adaptation, suggesting its highly pathogenic potential in mammals. The Av240 virus of A/H5N1 clade 2.1.3.2b Indonesian lineage was highly virulent and modestly lethal; the majority of the infected mice survived and regained their body weight. Av39 of A/H3N6 was neither virulent nor lethal. Several mammalian adaptive markers of amino acid residues were associated with the highly virulent and lethal phenotypes of the Av154 virus.

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Conflict of interest None to declare.

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