Whole-genome sequence and genesis of an avian influenza virus H5N1 isolated from a healthy chicken in a live bird market in Indonesia: Accumulation of mammalian adaptation markers in avian hosts

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28

29 **Abstract**

30 **Background.**

31

32 Influenza A viruses are a major pathogen that causes significant clinical and economic harm to
33 many animals. In Indonesia, the highly pathogenic avian influenza (HPAI) H5N1 virus has been
34 endemic in poultry since 2003 and has caused sporadic deadly infections in humans. The genetic
35 bases that determine host range have not yet been fully elucidated. We analyzed whole-genome
36 sequence of a recent H5 isolate to reveal the evolution toward its mammalian adaptation.

37

38 **Methods.**

39

40 We determined the whole-genome sequence of A/chicken/East Java/Av1955/2022 (hereafter,
41 "Av1955") from a healthy chicken in April 2022 and conducted phylogenetic and mutational
42 analysis.

43

44 **Results.**

45

46 Phylogenetic analysis revealed that Av1955 belonged to the H5N1 clade 2.3.2.1c (Eurasian
47 lineage). The six gene segments (PB1, PB2, HA, NP, NA, and NS) out of the eight segments
48 derived from viruses of H5N1 Eurasian lineage, one (PB2) from the H3N6 subtype and the
49 remaining one (M) from H5N1 clade 2.1.3.2b (Indonesian lineage). The donor of the PB2
50 segment was a reassortant among three viruses of H5N1 Eurasian and Indonesian lineages and
51 the H3N6 subtype. The HA amino acid sequence contained multiple basic amino acids at the
52 cleavage site. Mutation analysis revealed that Av1955 possessed the maximal number of
53 mammalian adaptation marker mutations.

54

55 **Conclusions.**

56

57 Av1955 was a virus of H5N1 Eurasian lineage. The HA protein contains an HPAI H5N1-type
58 cleavage site sequence, while the virus was isolated from a healthy chicken suggesting its low
59 pathogenicity nature. The virus has increased mammalian adaptation markers by mutation and
60 intra- and inter-subtype reassortment, gathering gene segments possessing the most abundant
61 maker mutations among previously circulating viruses. The increasing mammalian adaptation
62 mutation in avian hosts suggests that they might be adaptive to infection in mammalian and
63 avian hosts. It highlights the importance of genomic surveillance and adequate control measures
64 for H5N1 infection in live poultry markets.

65

66 **Keywords:**

67 Highly pathogenic avian influenza H5N1, Clade 2.3.2.1c Eurasian lineage, Low pathogenic
68 avian influenza H3N6, Whole-genome sequence, Human health, Inter-subtype reassortment,
69 Intra-subtype reassortment, Mammalian adaptation marker mutation

Introduction

Influenza A viruses are a major pathogen that causes significant clinical and economic harm to various species, including poultry, pigs, horses, marine mammals, and humans (Peiris et al., 2007; Webster et al., 1992). The surface antigenicity of the virus particles divides them into 18 hemagglutinin (HA) (H1 to H18) and 11 neuraminidase (NA) (N1 to N11) subtypes (Tong et al., 2013). Highly pathogenic avian influenza (HPAI) H5N1 viruses are capable of sporadic human infection. They have the potential to cause severe sickness with a high case fatality rate among people who have been hospitalized and proven to have the virus. To date, there have been 861 confirmed disease cases in people, and 455 have led to death (WHO, 2021). Indonesia reported 200 confirmed human cases and 168 fatalities. The 168 fatalities account for over one-third of all deaths among all affected countries. However, Indonesia had only two cases in 2014 and 2015, zero in 2016, and one in 2017, the last (WHO, 2015; WHO, 2019; WHO, 2021).

The HPAI H5N1 subtype was first discovered in Indonesia in 2003 (Li et al., 2004), spreading to numerous regions and killing over 16 million chickens by the end of 2007 (Lam et al., 2008; Putri et al., 2019). The sequence of the hemagglutinin gene classified it into clade 2.1. This clade subsequently branched into clades 2.1.1 to 2.1.3 in Indonesia (WHO/OIE/FAO/H5N1, 2008). Clade 2.1.3 further branched into clades 2.1.3.1 to 2.1.3.3; then clade 2.1.3.2 into clades 2.1.3.2a and b (WHO/OIE/FAO/H5N1, 2014). Clade 2.1.1 viruses were predominantly isolated from HPAI-infected chickens during outbreaks between 2003 and 2005. Clades 2.1.2 and 2.1.3 were isolated from birds between 2003 and 2005 and humans in 2005. Clade 2.1.3.2, a branch of clade 2.1.3, became prevalent in poultry and humans in 2007. Clade 2.1.3.2b, a descendant of clade 2.1.3.2, became prevalent from 2010 to 2012.

An incursion of a new HPAI H5N1 clade, clade 2.3.2.1c, from the mainland of Southeast Asia occurred in Indonesia in 2012 (Dharmayanti et al., 2014). The clade became prevalent as early as 2013. Shimizu et al. (2016) reported the isolations of three avian influenza viruses in East Java, Indonesia: H5N1 clade 2.3.2.1c (Av154) from an HPAI outbreak in a turkey farm in 2013, H5N1 clade 2.1.3.2b (Av240) from an ill chicken at a live poultry market in 2014, and H3N6 (Av39) from a mildly ill duck at a live poultry market in 2013 (Shimizu et al., 2016). Clade 2.3.2.1c was designated as Eurasian lineage, while clade 2.1.3.2b was designated as Indonesian lineage based on the place of emergence. Since 2016, only Eurasian lineage had been isolated in East Java, Indonesia (K Rahardjo, 2019, unpublished data).

Clade 2.3.2.1c caused only one fatal infection in humans in Indonesia, the last case in 2017. The genetic bases that determine host range have not yet been fully elucidated. Influenza virus evolves by the mutation of the genes and by the reassortment of the gene segments. Mertens et al. (2013) identified 149 phenotypic marker mutations related to host tropism or increased pathogenicity in mammals from previously reported literature. Recently, Rehman et al. (2022) reported detections of avian influenza A/H5 viruses in poultry at live bird markets in East Java, Indonesia. In this study, we determined the whole-genome sequence of one of the detected viruses to identify the clade/lineage and some characteristic features of the amino acid sequences of the viral proteins. We compared the phenotypic marker mutations with its ancestral viruses to reveal the evolution toward its mammalian adaptation.

Materials and Methods

Ethical approval

The Animal Care and Use Committee at the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia gave their approval for every step of this study (Approval no. 1.KE.028.03.2021).

Virus

Rehman et al. (Rehman et al., 2022) conducted a cross-sectional study from March 2021 to April 2022, collecting 600 tracheal and cloaca swab samples from live bird markets in East Java, Indonesia. The authors isolated an avian influenza A/H5 virus from a tracheal swab sample collected on 5 March 2022 from a healthy chicken, which showed no symptoms of lethargy, shortened neck, retracted feather, diarrhea, torticollis, and dyspnea. For this research, they provided a one-mL aliquot of an allantoic fluid harvested from the A/H5 virus-infected egg. The harvest was positive for the hemagglutination test with chicken red blood cells. It was also positive for 1-step TaqMan real-time RT-PCR tests targeting the influenza A virus M gene and H5 HA gene using sets of primers and probes as previously described (Shimizu et al., 2016) (data, not shown). All procedures were performed in the BSL3 laboratory of the Institute of Tropical Disease, Airlangga University. We named the virus A/chicken /East Java/Av1955/2022 (hereafter, "Av1955").

Whole-genome sequencing

We performed genome analysis by next-generation sequencing as previously described (Novianti et al., 2019). Total RNA was isolated from the allantoic harvest using a QIAamp viral Minikit (Qiagen, Tokyo, Japan). Linear polyacrylamide was used as a transporter rather than tRNA. A TruSeq RNA sample preparation kit version 2 was utilized to compile an RNA library (Illumina, Japan). The library was loaded in the flow cell of the 300-cycle MiSeq reagent kit version 2 (Illumina, USA). A MiSeq system (Illumina) sequenced the barcode-labeled multiplex library, which had two runs of 150 bp each. The system created the FASTQ files of sequence reads removing the primer and adaptor sequences. For analysis, the files were imported into CLC Genomics Workbench version 8.1 (CLC bio, Japan). The reads were mapped to the genomes of 27 reference viruses of influenza type A virus, including all subtypes of HA (H1 to H18) and NA (N1 to N11). Tentative complete 8-segment genome sequences were constructed from the assembled consensus with the best coverage and common sequences of type A influenza viruses at the 5' end (12 nucleotides [nt]) and 3' end (13 nt) of the genome segments. The reads were mapped again to the tentative complete genome sequence to assemble Av1955 consensus sequences. The assembled sequences covered 97.4 to 99.5% of the complete genome-segments mostly lacking the 5' and 3' consensus sequences. The sequences of the 8 genome-segments were submitted to the GISAID database with isolate ID: EPI_ISL_13690275.

Genetic analysis

158   BLAST analysis in the GISAID EpiFlu database (https://gisaid.org) on 10 August 2022
159   identified viruses with the highest identity to the nucleotide sequence of one or more gene
160   segments of Av1955.
161
162   The genetic information processing software Genetyx v14 (Genetyx Co., Tokyo, Japan)
163   generated the phylogenetic trees using the RAxML with 100 bootstrap replicates. The trees are
164   rooted in the A/South Carolina/1/1918(H1N1) (HA: GenBank AF117241) or A/Brevig
165   Mission/1/1918(H1N1) (NA: AF250356, PB2: DQ208309, PB1: DQ208310, PA: DQ208311,
166   NP: AY744935, M: AY130766, NS: AF333238).
167
168   The amino acid sequences of the viral proteins were decoded from the genome nucleotide
169   sequences and analyzed for nonsynonymous mutations. The putative host adaptation mutations
170   were analyzed according to the evaluation of phenotypic markers described by Mertens et al.
171   (2013).
172

173   **Isolate ID (EPI_ISL) in the GISAID database of viruses used as a reference**

174   A/duck/East Java/Av39/2013 (H3N6) (hereafter, Av39): EPI_ISL_307026; A/chicken/East
175   Java/Av240/2014 (H5N1 Indonesian lineage) (HA clade 2.1.3.2b) (hereafter, Av240):
176   EPI_ISL_307019; A/turkey/East Java/Av154/2013 (H5N1 Eurasian lineage) (HA clade 2.3.2.1c)
177   (hereafter, Av154): EPI_ISL_307002; A/eagle/Jakarta Timur/20616-206-III/2016 (H5N1)
178   (hereafter, Eg20616): EPI_ISL_266799; A/chicken/East Java/Av1210/2017 (H5N1) (hereafter,
179   Av1210): EPI_ISL_401823; A/chicken/East Java/Spg119/2018 (H5N1) (hereafter, Spg119):
180   EPI_ISL_365528; and A/chicken/East Java/Av1534/2019 (H5N1) (hereafter, Av1534):
181   EPI_ISL_401824.
182
183   We noted the accession number of each genome segment of the viruses in Table S1.
184

185   **Results**

186   **HA and NA subtypes of Av1955**

187   The BLAST analysis revealed that the HA sequence of Av1955 was most close to Spg119 of
188   H5N1 (the sharing identity, 97.7%) and the NA to Av1210 of H5N1 (97.3%) among the viruses
189   in the GISAD database (Table 1). The HA and NA segments were most close to Av154 of H5N1
190   Eurasian lineage (HA clade 2.3.2.1c) (95.1% and 96.2%, respectively) among the three isolates
191   (Av39, Av240, and Av154) during 2013-2014 in East Java (Table 1). In the phylogenies, the HA
192   and NA segments are on the same branch with Av154 (Figure 1). These results clearly indicate
193   that Av1955 is a virus of the H5N1 Eurasian lineage (HA clade 2.3.2.1c).
194
195   **Genesis of Av1955**

196   The BLAST analysis revealed that the genome sequence of Av1955 was most close to Spg119
197   for the PB2 segment (the sharing identity, 98.0%), HA (97.7%), NP (98.3%), and NS (98.5%); to

198   Av1534 for the PB1 (97.9%) and PA (98.1%); to Av1210 for the NA (97.3%); and to Eg20616
199   for the M (98.9%) among the viruses in the GISAD database (Table 1). These results indicated
200   that Av1955 acquired the PB2, HA, NP, and NS segments from an Spg119-like virus; the PB1
201   and PA from an Av1534-like virus; the NA from an Av1210-like virus; and the M from an
202   Eg2061-like virus. These four putative donors of the segments are H5N1 Eurasian lineage since
203   all HA and NA closely cluster with Av154 of H5N1 Eurasian lineage in the phylogenies of HA
204   and NA (Figure 1).
205
206   Av1955 was most close to Av39 for the PB2 (93.2%); to Av154 for the PB1 (95.9%), PA
207   (96.0%), HA (95.2%), NP (97.0%), NA (96.2%), and NS (97.2%); and Av240 for the M (98.1%)
208   among the three isolates in East Java during 2013-2014. In the phylogenies, the PB2 of Av1955
209   closely clusters with Spg119 and Av39; all of the PB1, PA, HA, NP, and NA cluster with
210   Eg20616, Av1210, Spg119, Av1534, and Av154; the M with Eg20616, Spg119, and Av154; and
211   the NS with Av1210, Spg119, Av1534, and Av154.
212
213   The PB2, M, and NS of Eg20616 closely cluster with Av240, while the other six segments with
214   Av154, indicating that Eg206166 is a reassortant between Av240 and Av154; Eg20616 acquired
215   the PB2, M and NS segments from an Av240-like virus, and the other six segments from an
216   Av154-like virus.
217
218   The PB2 and M of Spg119 closely cluster with Av39 and Av240, respectively, while the other
219   six segments with Av154, indicating that Spg119 is a triple reassortant between Av39, Av240,
220   and Av154; Spg119 acquired the PB2 segment from an Av39-like virus, the M segment from an
221   Av240-like virus, and the other six segments from an Av154-like virus.
222
223   From the described results, we proposed a model of the genesis of Av1955 by multi-steps of
224   inter- and intra-subtype reassortment, as illustrated in Figure 2.
225
226   **Characteristic amino acid sequences of Av1955**

227   Table 2 summarizes the findings of an analysis of Av1955 amino acid sequences of the receptor
228   binding, cleavage, and glycosylation sites in HA, deletions in NA and NS1, truncation of PB1-
229   F2, and amantadine and rimantadine resistant mutations in M2. At the receptor binding region of
230   the HA protein, the amino acid sequences were E-186, G-221, Q-222, and G-224 (H5
231   numbering), which are consistent with the avian type specifically binding 2,3-linked sialic acid
232   receptor. There were no mutations in this region among the seven reference viruses. The amino
233   acid sequence at the cleavage site of the HA protein was PQRE-RRRKR for Av1955, Av154,
234   and the four donor strains and was PQRESRRKKR for Av240; all of these possessed five
235   consecutive basic amino acid residues as typical highly pathogenic avian influenza A viruses.
236   Av39 had PEKQT----R at the site, only one basic amino acid residue R, as specific low
237   pathogenic viruses. A glycosylation site, NST, was present at 154-156 in Av240, while the
238   particular sequence was absent in seven other viruses, including Av1955 as a result of
239   substitution(s) to GST for Av39; DNA for Av154; NNA for Eg20616, Av1210, Spg119, and
240   Av1534; and CNA for Av1955.
241

242 There were deletions in NA at 49-68 and NS at 80-84 with Av1955 and all reference viruses
243 except Av39. The Av1955 PB1-F2 was truncated at 25, while that of the reference viruses,
244 except for Av39 and Av240, was truncated at 57. Av39 and Av240 possessed 87 open reading
245 frames for PB1-F2, almost full of 90. The M2 of Av1955 was a resistant type to amantadine and
246 rimantadine.

247

248 **Mammalian adaptation markers in the amino acid sequences of Av1955**

249 Martens et al. (2013) identified 149 phenotypic marker mutations related to the host tropism or
250 increased pathogenicity in mammals from previously reported literature. They distribute to PB2
251 (34), PB1 (16), PB1-F2 (1), PA (18), HA (38), NP (10), NA (2), M1 (6), M2 (4), NS1 (16), and
252 NS2 (4). We analyzed the presence or absence of these mutations in Av1955 and seven reference
253 viruses. Table 3 summarizes the results showing the substitutions positive for at least one of the
254 eight viruses. There were no marker mutations in NP and NA proteins. Av1955 had 11 markers
255 in PB2, while the donor of Spg119 had 10. The increase indicates that the virus acquired one
256 marker mutation in the PB2 (R318K) by a substitution mutation. A marker increase also
257 occurred with NS1 (7 to 8 by P215T).

258

259 The markers of the donor segments were the same number as the recipient, Av1955, with PB1
260 (8), PA (3), HA (5), M1 (3), M2 (0), and NS2 (1). The markers in the transferred proteins were
261 the maximum among the four donor viruses except for the HA. The HA donor, Spg119, had 5
262 marker mutations in HA, while Av1210 and Av1534 had 6 and 7, respectively. Av1955 acquired
263 39 mammalian adaptation markers in its proteins, an 8% increase from 36 of Spg119 (the donor
264 of 3 genome segments).

265

266 **Discussion**

267 Sequencing and phylogenetic analysis (Figure 1) identified the HA and NA of Av1955
268 (A/chicken/East Java/Av1955/2022) as an H5N1 subtype of a Eurasian lineage (HA clade
269 2.3.2.1c). However, Av1955 possesses the PB2 and M genome segments derived from viruses of
270 the H3N6 subtype and H5N1 Indonesian lineage, respectively. The phylogenies indicated that
271 the donor of the PB2 segment, Spg119, was a triple-reassortant among H3N6 and H5N1
272 Indonesian and Eurasian lineages. The donor of the M segment, Eg20616, was a double-
273 reassortant between H5N1 Indonesian and Eurasian lineages. The genesis of Av1955 might
274 involve two steps of genome-segment reassortment (Figure 2). It is worth noting that the M
275 genome segment of the Indonesian lineage has remained in the virus of Eurasian lineage after the
276 7-year disappearance of the H5N1 Indonesian lineage; the last isolate was in 2015 in East Java
277 (A Nastri, 2019, unpublished data).

278

279 There were no mammalian adaptation mutations in the receptor binding region of the HA
280 protein among the seven reference viruses and Av1955. Their amino acid sequences were E-186,
281 G-221, Q-222, and G-224 (H5 numbering), consistent with the avian type specifically binding to
282 a 2,3-linked sialic acid receptor on the cell surface of avian hosts. If the mutations occur, viruses
283 are unable to infect avian hosts. The HA protein of Av1955 contains an HPAI H5N1-type
284 cleavage site sequence with multiple basic amino acid residues (PQRERRRKR). In contrast, the
285 virus was isolated from a healthy chicken suggesting its low pathogenicity nature. All of the

286 H5N1 isolates in East Java, Indonesia, since 2013 were from sick poultry except a few
287 reassortants possessing a PB2 genome segment derived from avirulent subtypes like H3N6;
288 Spg119, A/chicken/East Java/Spg119/2018 (H5N1), was one of the exceptions isolated from a
289 healthy chicken (K Rahardjo, 2019, unpublished data). The virus obtained its PB2 genome from
290 an H3N6 virus, as shown in Figure 2. Av1955 lost a glycosylation site at 154-156 on the HA
291 protein; the loss has been reported to increase pathogenicity in mice (Suguitan et al., 2012).
292 Av1955 has deletions at 49-68 on the NA and 80-84 on the NS1, both of which have been shown
293 to increase virulence in mice (Matsuoka et al., 2009; Zhou et al., 2009; Long et al., 2008). The
294 Av1955 PB1-F2 truncated at 25 of the total length, 90. The deletion of the main body has been
295 related to increased virulence in mammals (Mettier et al., 2021). Av1955 has amino acid residues
296 of A-27 and N-31 of the M2 protein, which are known to render the phenotype of resistance to
297 amantadine and rimantadine (Belshe et al., 1988; Lan et al., 2010; Pinto et al., 1992). The M
298 genome segment originated from the Av240-like virus of the H5N1 Indonesian lineage (HA
299 clade 2.1.3.2b). Av240 had already acquired the resistant mutations, V27A and N31N, while one
300 of the ancestors of Indonesian lineage, A/Indonesia/5/2005 (H5N1) (HA clade 2.1.3.2), had not
301 yet. It was reported that these mutations occurred during 2003-2008 in human and avian
302 infections in Indonesia (Dharmayanti et al., 2010). Since amantadine had been subscribed to
303 influenza patients but never used for poultry in Indonesia, the avian viruses possessing the M
304 segment with resistant mutations might come from human-to-avian transmission.

305

306   Av1955 possesses 39 mammalian adaptation marker mutations out of 149 identified by
307 Martens et al. (2013), while the four viruses of the genome-segment donor have 34-36 and the
308 three ancestral viruses isolated during 2013-2014 have 27-34. The virus has increased the
309 number of the markers by substitution mutations and intra- and inter-subtype reassortment
310 gathering gene segments possessing the most abundant maker mutations among circulating
311 viruses. The increasing mammalian adaptation mutation in avian hosts suggests that they might
312 be adaptive to infection in mammalian and avian hosts. It highlights the importance of genomic
313 surveillance and adequate control measures for H5N1 infection in live poultry markets. In
314 contrast, we have not identified the remaining 110 mammalian adaptation marker mutations in
315 the eight avian viruses analyzed in this study. One of the most studied mutations, PB2 E627K,
316 has been known to release the restriction of PB2 activities in replication in mammalian cells
317 (Subbarao et al., 1993), including human volunteers (Clements et al., 1992). It is a plausible
318 explanation that this kind of mammalian adaptation mutation arises only in the mammalian host
319 after the transmission.

320

321 **Conclusions**

322 Av1955 was a virus of the H5N1 Eurasian lineage (HA clade 2.3.2.1c). The virus has increased
323 mammalian adaptation markers by substitution mutation and intra- and inter-subtype
324 reassortment, gathering gene segments possessing the most abundant maker mutations among
325 circulating viruses. The increasing mammalian adaptation mutation in avian hosts suggests that
326 they might be adaptive to infection in mammalian and avian hosts. It highlights the importance
327 of genomic surveillance and adequate control measures for H5N1 infection in live poultry
328 markets.

329

330 **Acknowledgments**

331 We gratefully acknowledge the authors and the originating and submitting laboratories of the
332 sequences from GISAID's EpiFlu database (Table S1), on which this research is based. We are
333 thankful for the assistance of Virology and Biomolecular Laboratory staff, Faculty of Veterinary
334 Medicine Universitas Airlangga were acknowledged for their cooperation in the study. We
335 sincerely thank the Ministry of Research, Technology and Higher Education of Indonesia for
336 their advice to carry out this study in Indonesia.

337

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Figure 1

Phylogenetic analysis of the eight gene segments of Av1955

The genetic information processing software Genetyx v14 (Genetyx Co., Tokyo, Japan) generated the phylogenetic trees using the RAxML with 100 bootstrap replicates. The tree is rooted in the A/Brevig Mission /1/1918 or A/South Carolina/1/1918 (H1N1). Four viruses with the highest identity to the nucleotide sequence of one or more gene segments of A/ck/EJ/Av1955/2022 (bold and pink color) were included in the phylogenies; these were A/eg/JT/20616-206-III/2016 (H5N1), A/ck/EJ/Av1210c/2017 (H5N1), A/ck/EJ/Spg119/2018 (H5N1) and A/ck/EJ/Av1534/2019 (H5N1). Three viruses isolated from poultry in East Java in 2013-2014 were also included; A/dk/EJ/Av39ac/2013 (H3N6) (bold and green), A/ck/EJ/Av240/2014 (H5N1-Ind) (bold and brown), and A/tk/EJ/Av154/2013 (H5N1-Eur). (bold and blue). Bold and black highlighted the virus at the nearest position to A/ck/EJ/Av1955c/2022 in each of the eight genome segments. "ck": chicken, "eg": eagle, "dk": duck, "tk": turkey, "EJ": East Java, "JT": Jakarta Timur, "–Ind": Indonesian lineage (HA clade 2.1.3.2b), and "-Eur": Eurasian lineage (HA clade 2.3.2.1c).

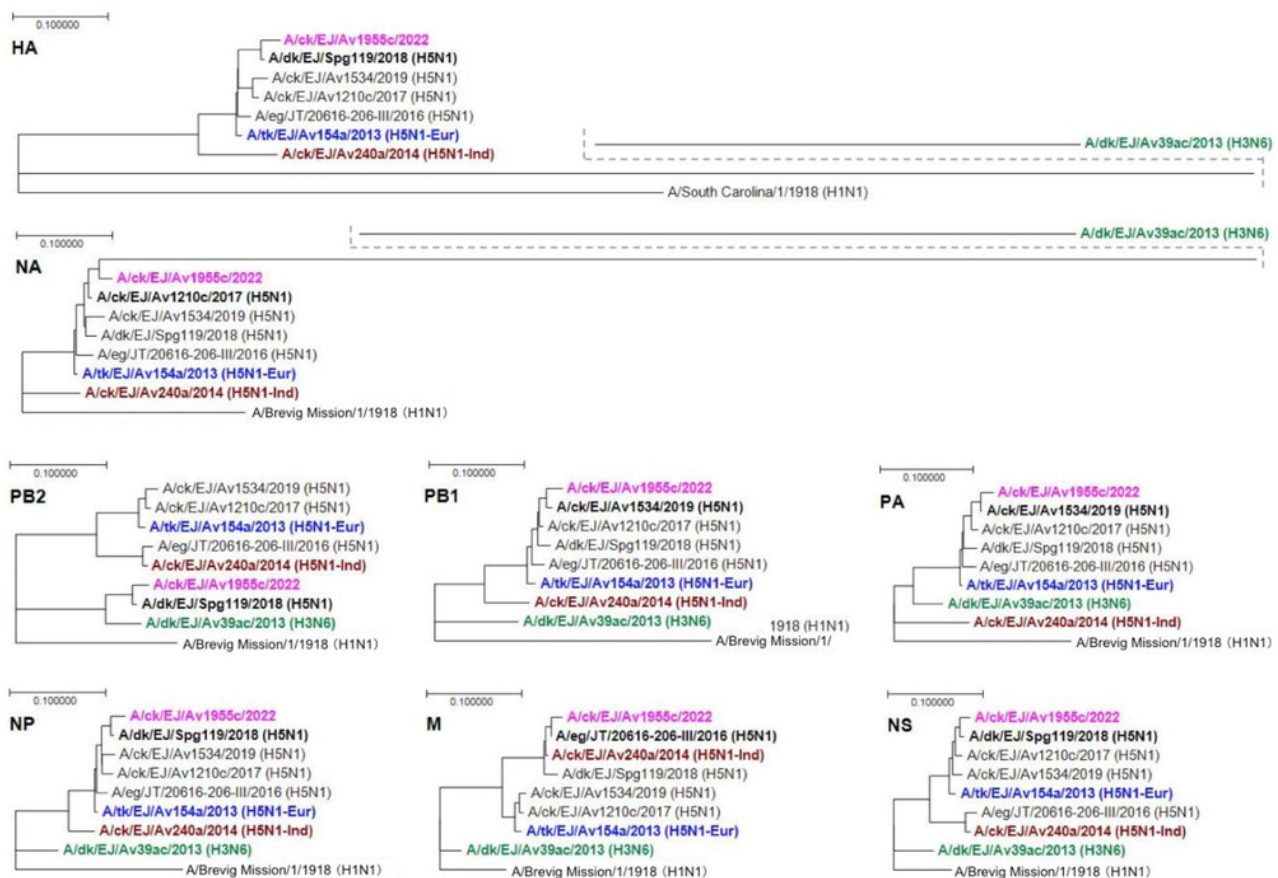
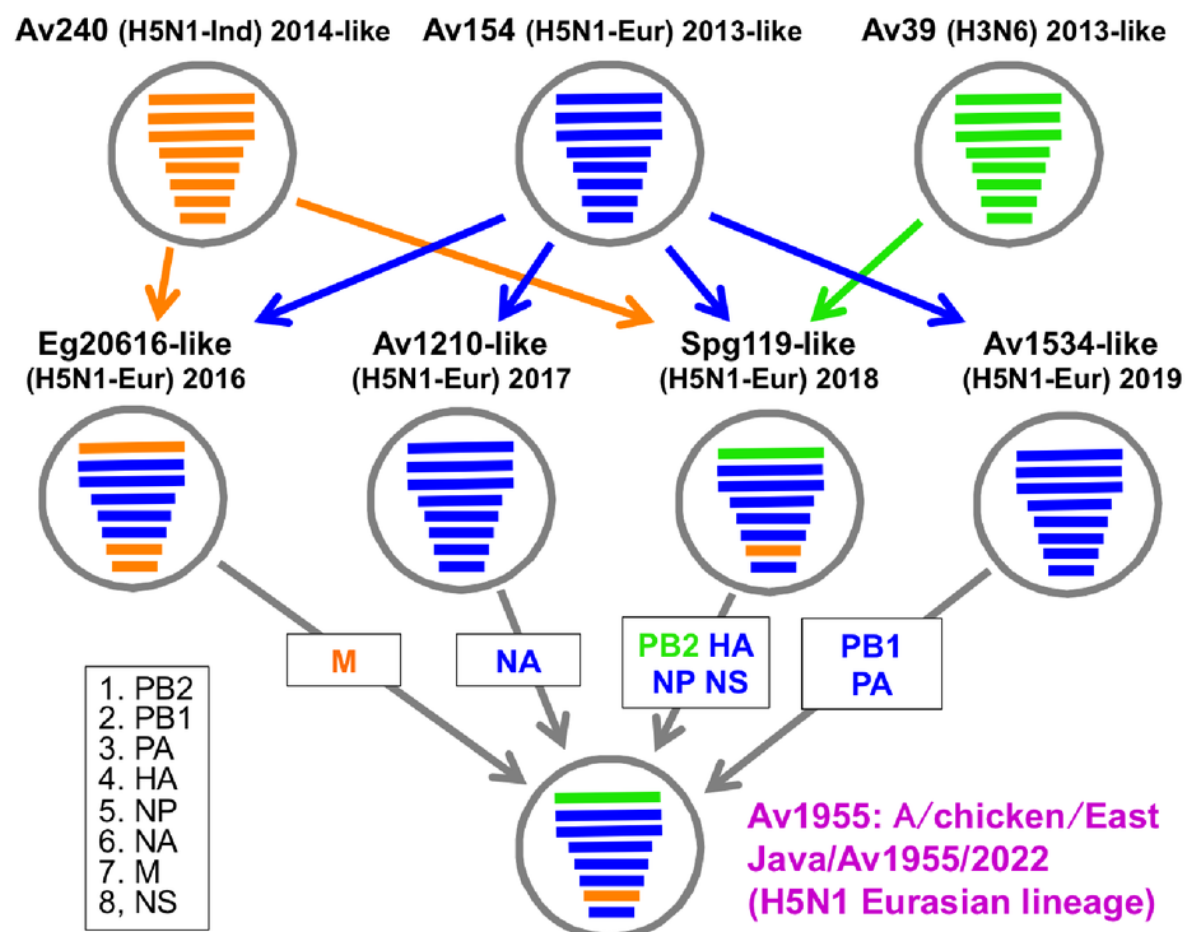



Figure 2

The genesis of Av1955 by multiple reassortments

Av39: A/duck/East Java/Av39/2013 (H3N6), **Av240**: A/chicken/East Java/Av240/2014 H5N1 Indonesian lineage), **Av154**: A/turkey/East Java/Av154/2013 (H5N1 Eurasian lineage), **Eg20616:** A/eagle/Jakarta Timur/20616-206-III/2016 (H5N1 Eurasian lineage), **Av1210**: A/chicken/East Java/Av1210/2017 (H5N1 Eurasian lineage), **Spg119**: A/chicken/East Java/Spg119/2018 (H5N1 Eurasian lineage), and **Av1534**: A/chicken/East Java/Av1534/2019 (H5N1 Eurasian lineage). Isolate ID (EPI_ISL) in the GISAID database of the viruses was noted in Materials and Methods.

Viruses with the highest identity to the nucleotide sequence of one or more gene segments of Av1955

2

Genome segment	Identity to the eight genome segments of Av1955, %[a]							
	Isolates in 2013-2014[b]			Viruses with the highest identity[b]				Av1955
	Av39 H3N6	Av240 H5N1-Ind	Av154 H5N1-Eur	Eg20616 H5N1	Av1210 H5N1	Spg119 H5N1	Av1534 H5N1	
PB2	**93.2**	85.2	85.3	85.2	85.2	**_98.0_**	85.0	100.0
PB1	87.6	91.3	**95.9**	96.0	97.4	96.7	**_97.9_**	100.0
PA	90.9	87.6	**96.0**	95.3	96.8	95.4	**_98.2_**	100.0
HA	53.8	87.0	**95.2**	94.6	94.4	**_97.7_**	93.8	100.0
NP	90.3	93.5	**97.0**	96.7	96.6	**_98.3_**	96.6	100.0
NA	54.0	87.7	**96.2**	95.0	**_97.3_**	96.0	95.5	100.0
M	90.4	**98.1**	93.8	**_98.9_**	93.3	97.0	93.4	100.0
NS	88.8	93.0	**97.2**	92.4	97.9	**_98.5_**	97.2	100.0

4 [a] "Bold" indicates the highest identity to Av1955 among Av39, Av154, and Av240. " Bold and
5 underlined" indicate the highest identity to Av1955 among viruses in the EpiFlu database of
6 GISAID with whole-genome sequence
7 [b] Virus short name: Av39, A/duck/East Java/Av39/2013; Av240, A/chicken/East
8 Java/Av240/2014; Av154, A/turkey/East Java/Av154/2013; Eg20616, A/eagle/Jakarta
9 Timur/20616-206-III/2016; Av1210, A/chicken/East Java/Av1210/2017; Spg119,
10 A/chicken/East Java/Spg119/2018; and Av1534, A/chicken/East Java/Av1534/2019.

Characteristic amino acid sequences of Av1955: the receptor binding, cleavage, and glycosylation sites in HA, deletion in NA and NS1, truncation of PB1-F2, and amantadine resistant mutation in M2.

Amino acid mutation			Amino acid sequence[a]								Pheno-type[b]
Pro-tein	Mutation	Site, H5 numbering	Isolates in 2013-2014[c]			Viruses with the highest identity[c]					
			Av39	Av240	Av154	Eg20616	Av1210	Spg119	Av1534	Av1955	
HA	Receptor binding	E186G /D	E	E	E	E	E	E	E	E	[1]
		G221D	G	G	G	G	G	G	G	G	[2]
		Q222L	Q	Q	Q	Q	Q	Q	Q	Q	[3]
		G224S	G	G	G	G	G	G	G	G	[4]
	Multiple basic amino acid	321-330 Cleavage site	PEKQT - - - - R↓G	PQRES**RRKKR**↓G	PQRE -**RRRKR**↓G	PQRE -**RRRKR**↓G	PQRE -**RRRKR**↓G	PQRE -**RRRKR**↓G	PQRE -**RRRKR**↓G	PQRE -**RRRKR**↓G	[5]
	Loss of glycosylation	Loss of 154-156 (NXT/S)	**Lost** (GST)	NST	**Lost** (DNA)	**Lost** (NNA)	**Lost** (NNA)	**Lost** (NNA)	**Lost** (NNA)	**Lost** (CNA)	[6]
NA	Deletion	49-68	Not deleted	**Deleted**	**Deleted**	**Deleted**	**Deleted**	**Deleted**	**Deleted**	**Deleted**	[7]
NS1	Deletion	80-84	TIASV	**Deleted**	**Deleted**	**Deleted**	**Deleted**	**Deleted**	**Deleted**	**Deleted**	[8]
PB1-F2	Truncation	at 12	at 87	at 87	at 57	at 57	at 57	at 57	at 57	**at 25**	[9]
M2	Resistance to amantadine	V27A	V	**A**	I	**A**	I	**A**	V	**A**	[10]
		S31/N/G	S	**N**	S	**N**	S	**N**	S	**N**	[11]

3 [a] "Bold and underlined" indicate the mammalian adaptation markers.

4 [b] **[1]**: Increased virus binding to α2,6 (Glaser et al., 2005); **[2]**: Change in receptor binding affinity

5 from avian to human receptors (Rogers et al., 1983); **[3]**: Change in receptor binding recognition

6 from α2,3 to α2,6 (Connor et al., 1994) , Airborne transmissible in mammals (Maines et al., 2011);

7 **[4]**: Increased virus binding to α2,6, (Imai et al., 2012), Airborne transmissible in mammals

8 (Maines et al., 2011); **[5]**: Increased virulence in mice (Klenk & Garten, 1994; Suguitan et al.,

9 2012; Yudhawati et al., 2020); **[6]**: Increase virus binding to α2,6 and pathogenicity in mice (Wang

10 et al., 2010; Zhang et al., 2015); **[7]**: Enhanced virulence in mice (Matsuoka et al., 2009; Zhou et

11 al., 2009); **[8]**: Enhance virulence in mice associated with D92E shift (Long et al., 2008); **[9]**:

12 Attenuate virulence in mammals (Dharmayantiet al., 2010); **[10]**: Reduce susceptibility to

13 amantadine (Hay et al., 1985); **[11]**: Reduce susceptibility to amantadine and rimantadine (Belshe

14 et al.,1988).

15 [c] Virus short name: See Table 1 note.

Mammalian adaptation markers (Mertens et al., 2013) in the amino acid sequences of Av1955

Mammalian adapt. marker mutation		Amino acid at the marker site[b]								Pheno-type[c]
		Isolates in 2013-2014[d]			Viruses with the highest identity[d]					
Protein	Mutation[a]	Av39	Av240	Av154	Eg20616	Av1210	Spg119	Av1534	Av1955	
PB2	T63I	**I**	**I**	**I**	**I**	**I**	*I*	**I**	**I**	**[1]**
	L89V	**V**	**V**	**V**	**V**	**V**	*V*	**V**	**V**	**[1], [2]**
	G309D	**D**	**D**	**D**	**D**	**D**	*D*	**D**	**D**	**[1], [2]**
	R318K	R	R	R	R	R	R	R	**K**	**[2]**
	T339K	**K**	T	T	A	T	*K*	T	**K**	**[1], [2]**
	Q368R	**R**	**R**	Q	**R**	Q	*R*	Q	**R**	**[1], [2]**
	H447Q	**Q**	**Q**	**Q**	**Q**	**Q**	*Q*	**Q**	**Q**	**[1], [2]**
	R477G	**G**	**G**	**G**	**G**	**G**	*G*	**G**	**G**	**[1], [2]**
	I495V	**V**	**V**	**V**	**V**	**V**	*V*	**V**	**V**	**[1], [2]**
	K526R	K	**R**	K	**R**	K	K	K	K	**[2], [3]**
	V667I	**I**	V	V	V	V	V	V	V	**[4]**
	A676T	**T**	**T**	**T**	**T**	**T**	*T*	**T**	**T**	**[2], [3]**
	K702R	**R**	K	K	K	K	*R*	K	**R**	**[4]**
	Subtotal	11	9	6	10	7	*10*	6	11	
PB1	A3V	**V**	**V**	**V**	**V**	**V**	**V**	*V*	**V**	**[1], [2]**
	L13P	**P**	**P**	**P**	**P**	**P**	**P**	*P*	**P**	**[1], [2], [5]**
	R207K	**K**	R	**K**	**K**	**K**	**K**	*K*	**K**	**[2]**
	K328N	**N**	**N**	**N**	**N**	**N**	**N**	*N*	**N**	**[2]**
	S375N	**N**	**N**	**N**	**N**	**N**	S	*N*	**N**	**[1], [2], [5]**
	H436Y	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	*Y*	**Y**	**[1], [2]**
	L473V	**V**	**V**	**V**	**V**	**V**	**V**	*V*	**V**	**[2]**
	M677T	**T**	**T**	**T**	**T**	**T**	**T**	*T*	**T**	**[1]**
	Subtotal	8	7	8	8	8	7	*8*	8	
PA	H266R	**R**	**R**	**R**	**R**	**R**	**R**	*R*	**R**	**[2], [3]**
	A404S	A	A	**S**	**S**	**S**	**S**	*S*	**S**	**[5]**
	S/A515T	**T**	**T**	**T**	**T**	**T**	**T**	*T*	**T**	**[2], [3]**
	Subtotal	2	2	3	3	3	3	*3*	3	

3 **Table 3 (Continued)**

Mammalian adapt. marker mutation	Amino acid at the marker site[b]		marker mutation	Pheno- Isolates in 2013-2014[d]
	Isolates in 2013-2014[d]	Viruses with the highest identity[d]		

Protein	Mutation[a]	Av39	Av240	Av154	Eg20616	Av1210	Spg119	Av1534	Av1955	
HA	D94N	D	S	**N**	**N**	**N**	*N*	**N**	**N**	[6], [7]
	S133A	G	S	**A**	**A**	**A**	*A*	**A**	**A**	[6]
	Q/H/I138 L/N	G	**L**	Q	Q	Q	Q	Q	Q	[1]
	I151T	**T**	I	I	I	I	I	I	I	[6]
	N154D	G	N	**D**	N	N	S	N	G	[4]
	S155N	S	S	**N**	**N**	**N**	*N*	**N**	**N**	[2], [6]
	T156A	T	T	**A**	**A**	**A**	*A*	**A**	**A**	[4], [6]
	T188I	T	T	T	T	**I**	T	**I**	T	[6]
	K189S/R	N	M	**R**	K	**R**	K	**R**	K	[2], [6]
	V210I	**I**	V	V	V	V	V	V	V	[6]
	A263T	S	A	**T**	**T**	**T**	*T*	**T**	**T**	[1]
	Subtotal	2	1	7	5	6	*5*	7	5	
M1	V15I/T	V	**I**	**I**	*I*	**I**	**I**	**I**	**I**	[1]
	N30D	**D**	**D**	**D**	*D*	**D**	**D**	**D**	**D**	[1]
	T215A	**A**	**A**	**A**	*A*	**A**	**A**	**A**	**A**	[1]
	Subtotal	2	2	3	*3*	3	3	3	3	
M2	L55F	**F**	L	L	L	L	L	L	L	[4]
	Subtotal	1	0	0	0	0	0	0	0	
NS1	A/P42S	**S**	**S**	**S**	**S**	**S**	*S*	**S**	**S**	[1], [8]
	D87E	D	D	**E**	D	**E**	*E*	**E**	**E**	[1]
	T/D92E	**E**	**E**	**E**	**E**	**E**	*E*	**E**	**E**	[1]
	L98F	**F**	**F**	**F**	**F**	**F**	*F*	**F**	**F**	[1]
	I101M	**M**	**M**	**M**	**M**	**M**	*M*	**M**	**M**	[1]
	T127N	**N**	A	T	A	T	T	T	T	[1]
	V149A	**A**	**A**	**A**	**A**	**A**	*A*	**A**	**A**	[1], [8]
	N200S	**S**	G	D	G	**S**	*S*	G	**S**	[9]
	P215T	P	P	P	P	P	P	P	**T**	[5]
	Subtotal	7	5	6	5	7	*7*	6	8	
NS2	T48A	**A**	**A**	T	**A**	**A**	*A*	**A**	**A**	[8]
	Subtotal	1	1	0	1	1	*1*	1	1	
Total		34	27	33	35	35	36	34	39	

4   [a] H5 Numbering

5   [b] "Bold and underlined" indicate the mammalian adaptation markers. "Bold, underlined, and italic"

6   indicate the mammalian adaptation markers on the genome segment donor.

7   [c] **[1]**: Pathogenic in mice, Increased virulence in mammals; **[2]**: Increased polymerase activity,

8   Increased replication in mammals; **[3]**: Increased virulence in mammals and birds; **[4]**: Enhanced
9   transmission, Airborne transmissibility in mammals; **[5]**: Human host marker; Mammalian host
10   marker; **[6]**: Increased virus binding to α2,6; **[7]**: Enhanced virus fusion; **[8]**: Antagonism of IFN
11   induction, Escape of antiviral host response; **[9]**: Decreased IFN antagonism.
12   ^{d} Virus short name: See Table 1 note.