博士論文

Epidemiological surveillance and virological analyses of highly pathogenic H5N1 avian influenza viruses in Indonesia.

（インドネシアにおける高病原性 H5N1 鳥インフルエンザウイルスの疫学的調査及びウイルス学的解析）

今村剛朗

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病因病理学専攻　ウイルス感染分野

指導教員：河岡義裕

申請者：今村剛朗

共同研究者：山田晋弥、岩附(堀本)研子、Chairul A. Nidom

Abstract

Highly pathogenic avian influenza (HPAI) H5N1 viruses have been circulating in Indonesia since 2003, and sporadic human H5N1 cases were reported until 2017. However, there are no reports on the epidemiology or virological characteristics of the currently circulating HPAI H5N1 viruses, and their potential to cause a human endemic or pandemic is unknown. With the aim of elucidating the characteristics of the currently circulating HPAI H5N1 viruses in Indonesia, I conducted epidemiological surveillance of poultry and swine in Indonesia between 2010 and 2016. Two distinct sub-clades of H5N1 viruses, clade 2.1.3 and clade 2.3.2.1d, were identified, and the clade 2.3.2.1d strains appear to have recently become dominant among poultry. Clade 2.3.2.1d strains showed mammalian-adaptive traits including high viral polymerase activity and moderate replication capability in human lung epithelial cells, and high virulence in BALB/c mice. My findings indicate that further, continuous surveillance of HPAI H5N1 viruses in Indonesia is warranted in order to detect the emergence of viruses with endemic or pandemic capability.

Among the Indonesian HPAI H5N1 viruses analyzed, I identified two genetically closely related viruses with different replication efficiency and pathogenicity in mammalian hosts. Further analysis of these two viruses revealed two amino acid mutations in the viral

PB2 protein (PB2-109A and PB2-758T) that enhanced viral replication efficiency in human lung epithelial cells and pathogenicity in BALB/c mice. The mechanism by which these two PB2 mutations enhance virulence in mammalian hosts has not yet been elucidated, but my findings are valuable for assessing the risk of avian-to-human transmission of highly pathogenic H5N1 avian influenza field isolates.

Preface

Influenza viruses are members of the family *Orthomyxoviridae*, which have a negative-sense, single-stranded, segmented RNA genome [1, 2]. There are four genera of influenza viruses: A, B, C, and D. Human influenza A and B viruses cause annual seasonal epidemics. Wild aquatic birds are natural reservoirs of influenza A viruses, which are zoonotic pathogens infecting humans as well as various other animal species. The natural reservoirs of influenza B viruses are unknown. Influenza B viruses circulate mainly among humans, but there have been several reports of infection in seals [3, 4]. Influenza C viruses cause mild respiratory illness among humans, especially children [5-7]. Influenza D viruses primarily affect cattle and their zoonotic potential has not yet been clarified [8, 9].

Influenza A virus is composed of eight segments of single-stranded, negative-sense RNA that each encodes one or more viral proteins (Fig. 1) [1, 10, 11]. Replication and transcription of viral RNA require three polymerase proteins [polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1) and polymerase acidic protein (PA)] , which are encoded by the three largest segments. The seventh and eighth segments each encode two viral proteins that are essential to the nuclear export of newly synthesized viral ribonucleoprotein (vRNP) complexes, virion assembly, or interactions with the host immune

response. The surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) are encoded by the fourth and the sixth segment, respectively. HA is critical to viral binding and internalization to the host cell, whereas NA promotes the release of viruses from the cell surface (Fig. 2).

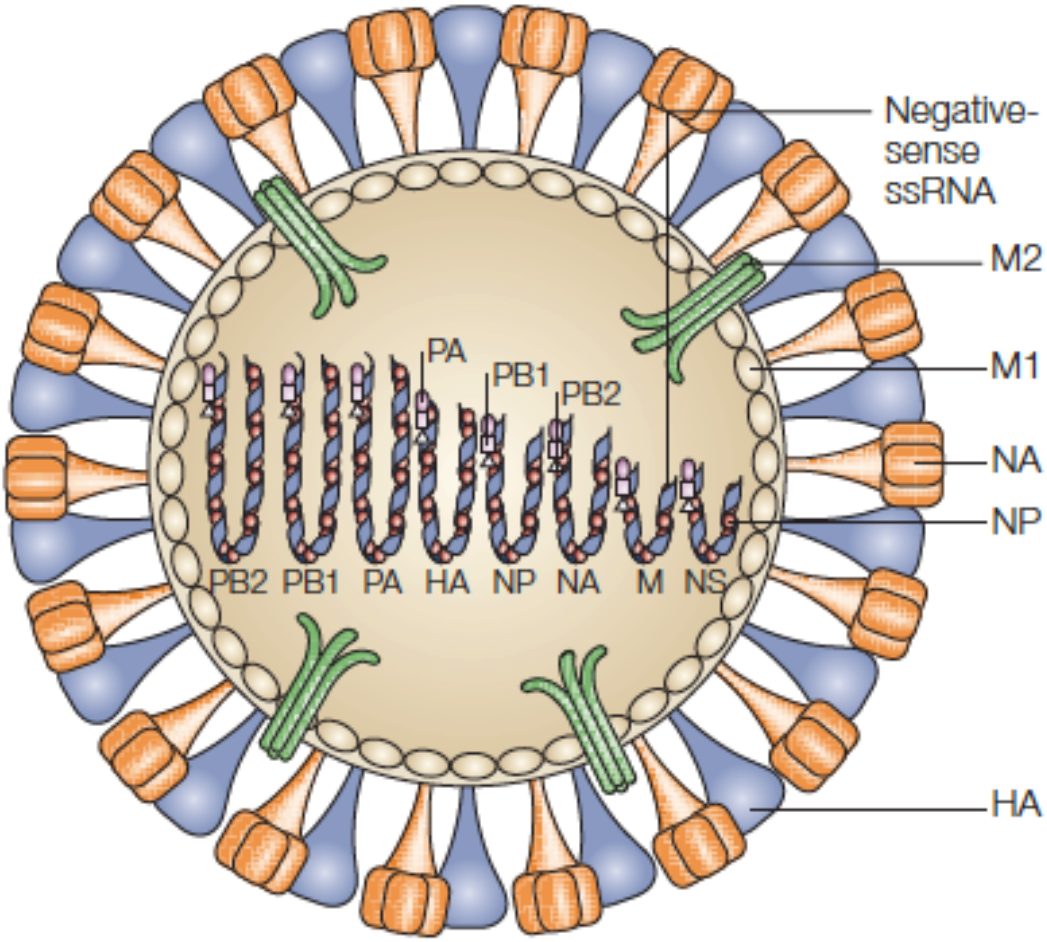

Figure 1. The structure of influenza virus

Influenza A virus is composed of eight segments of single-stranded, negative-sense RNA within a viral envelope. The viral envelope is coated with matrix protein (M1), and embedded with surface glycoproteins HA and NA, and M2 ion-channel protein. The viral RNA segments are associated with nucleoprotein (NP) and three polymerase proteins (PB2, PB1, and PA). The figure was cited from [11].

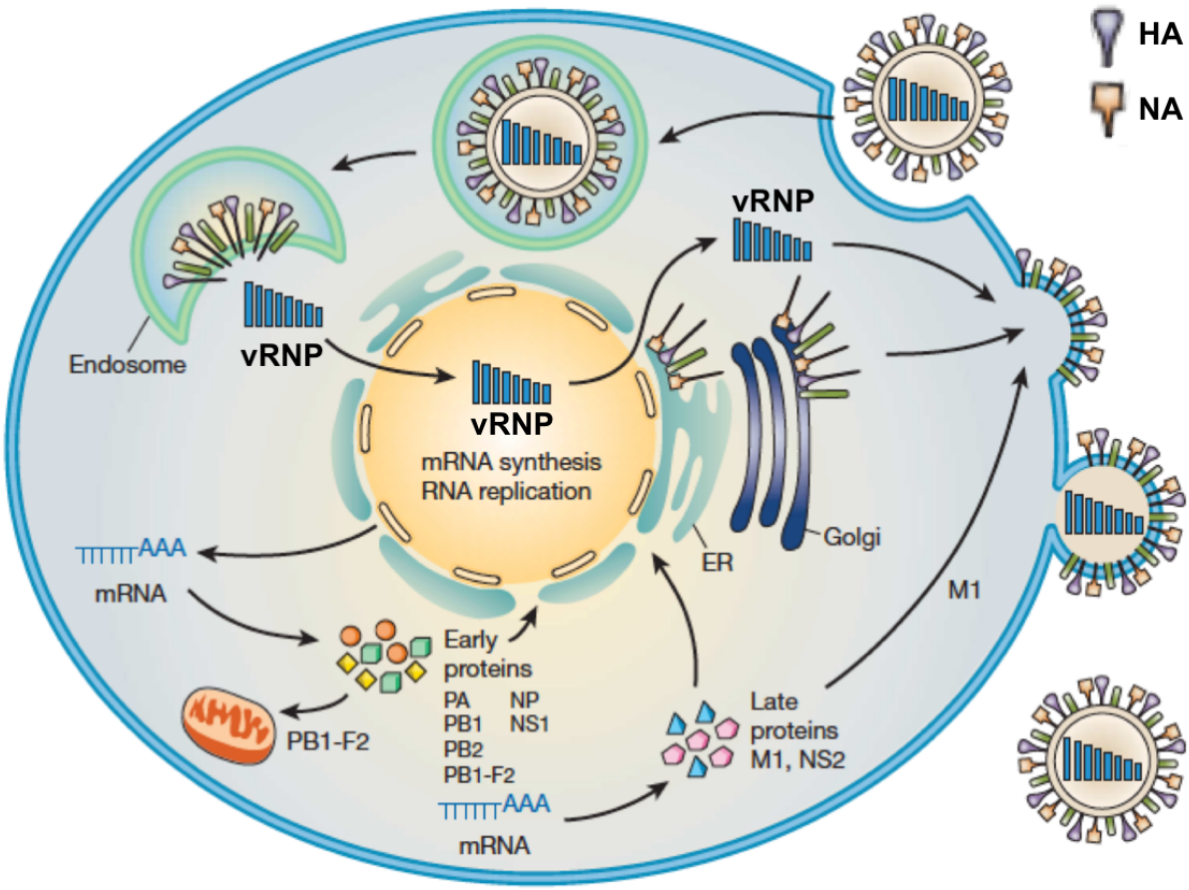

Figure 2. Influenza virus life cycle

Influenza A virus binds to the cell surface and enters the cell via HA-mediated endocytosis. Then, the viral nucleoprotein (vRNP) is uncoated in the cytoplasm, and subsequently imported into the nucleus, where replication and transcription occur. After messenger RNA (mRNA) exportation and translation in the cytoplasm, newly synthesized viral proteins are transported back to the nucleus and formed into vRNP. The assembly and budding of progeny virions occur at the plasms membrane. The figure was adapted from [12].

On the basis of the antigenicity of the two surface glycoproteins, HA and NA, influenza A viruses are classified into 18 HA subtypes (H1–H18) and 11 NA subtypes (N1–N11). Wild aquatic birds are natural reservoir for most subtypes of influenza A viruses, except for subtype H17N10 and H18N11 which are only found in bats [1, 2, 13, 14]. Among humans, influenza A viruses of the H1N1, H1N2, H2N2, H3N2, and H3N8 subtypes have established circulation at least once [2].

Influenza A viruses evolve via accumulation of mutations and rearrangements of their viral RNA segments, which is termed reassortment [2]. These processes lead to the introduction of new influenza A viruses into human populations, causing global epidemics, called pandemics. Among the four recorded influenza pandemics, H1N1 Spanish influenza in 1918–1919, H2N2 Asian influenza in 1957, and H3N2 Hong Kong influenza in 1968 involve avian influenza viruses [2, 12]. The H1N1 pandemic in 2009 originated from reassortment of swine, avian, and human influenza A viruses [12].

Most subtypes of influenza A viruses are maintained asymptomatically in the Orders *Anserifomes* (i.e., ducks, geese, and swans) and *Charadriiformes* (i.e., gulls, terns, surfbirds, and sandpipers) [15]. However, there exist highly pathogenic avian influenza (HPAI) viruses that cause significant mortality in chickens [16]. All HPAI viruses known to

111 date belong to either the H5 or H7 subtype. One characteristic feature of the HA of HPAI
112 viruses is the presence of polybasic amino acids at the HA cleavage site. Low pathogenic
113 avian influenza (LPAI) viruses possess a single arginine residue at the cleavage site, which
114 can be cleaved in limited organs, resulting in localized infection in the respiratory or intestinal
115 tract in poultry. In contrast, the polybasic cleavage site can be cleaved by ubiquitous
116 proteases, broadening viral tissue tropism and resulting in systemic infections and enhanced
117 virulence in poultry [2, 11, 17, 18].
118 In 1996, an HPAI virus of the H5N1 subtype was isolated from a farmed goose in
119 Guangdong province, China [19]. In 1997, an outbreak of H5N1 viruses among poultry and
120 18 cases of human infection were reported in Hong Kong [2, 20, 21]. Since then, HPAI H5N1
121 viruses have caused numerous outbreaks in poultry worldwide, especially in China, Southeast
122 Asia, and Africa [22]. Transmission of HPAI H5N1 viruses to humans has been monitored by
123 the WHO, and a total of 860 human infection cases, including 454 lethal cases, have been
124 reported as of September, 2018 [23, 24]. Thus, aside from their damage to the poultry
125 industry, HPAI H5N1 viruses pose an imminent threat to humans due to their potential to
126 cause epidemics and pandemics.
127

Materials and Methods

Ethics and biosafety

All experiments with H5N1 viruses were performed in biosafety level 3 (BSL3) laboratories at the University of Tokyo, which were approved for such use by the Ministry of Agriculture, Forestry, and Fisheries, Japan. All experiments with mice were performed in accordance with the University of Tokyo's Regulations for Animal Care and Use and were approved by the Animal Experiment Committee of the Institute of Medical Science, the University of Tokyo (P15-10).

Cells

Madin-Darby canine kidney (MDCK) cells were cultured in minimal essential medium (MEM) containing 5% newborn calf serum (NCS), vitamins, essential amino acids, and antibiotics. MDCK cells expressing the PB2 protein of A/Puerto Rico/8/34 (H1N1, PR8) were cultured in the same medium as MDCK cells with blasticidin supplementation. A549 human lung adenocarcinoma epithelial cells were cultured in Ham's F-12K (Kaighn's) medium containing 10% fetal bovine serum (FBS) and antibiotics. Human embryonic kidney 293T cells and DF-1 chicken-embryo fibroblast cells were grown in Dulbecco’s modified

145 Eagle medium (DMEM) containing 10% FBS and antibiotics. MDCK, A549, and 293T cells
146 were incubated at 37℃ with 5% CO_2. DF-1 cells were incubated at 39℃, with 5% CO_2.
147 Normal human bronchial epithelial (NHBE) cells (Lonza, Walkersville, MD) were cultured
148 and differentiated in an air-liquid interface system [25, 26]. Briefly, the apical surface of the
149 cells was exposed to a humidified 95% air/5% CO_2 environment, and the basolateral medium
150 was changed every two days.
151
152 **Sample collection and virus isolation**
153 Tracheal, cloacal, nasal, or fecal swab samples were collected from poultry and
154 swine in various locations in Indonesia from 2010 through 2016 (Table 2). The collected
155 samples were inoculated into 10-day-old embryonated eggs, and the allantoic fluid was tested
156 for hemagglutination using chicken red blood cells. Hemagglutination-positive allantoic fluid
157 underwent reverse transcription PCR (RT-PCR) and direct sequencing. Sequence-confirmed
158 H5N1 samples were stored at -80℃.
159
160
161

Isolation of viral RNA, RT-PCR, and generation of viruses by reverse genetics

Viral RNA was extracted from allantoic fluid of embryonated chicken eggs by using the QIAamp viral RNA minikit (Qiagen, Hilden, Germany). Extracted RNA was reverse-transcribed with Superscript Ⅲ (Invitrogen, Carlsbad, CA) and universal primers specific for influenza A virus genes to generate cDNA. The resulting products were PCR-amplified by using Phusion High-Fidelity DNA polymerase (New England BioLabs, Ipswich, MA) with specific primers for each virus gene and cloned into the viral expression plasmid pHH21 [27]. Mutations in the PB2 and NA genes were generated by PCR amplification of the respective construct with primers possessing the desired mutations. Primer sequences are listed in Table 1. All constructs were sequenced to ensure the absence of unwanted mutations. All avian, reassortant, and mutant viruses were generated in 293T cells by use of plasmid-based reverse genetics, as described previously[27]. The supernatant of transfected 293T cells was collected after an incubation time of less than 24 hours in order to avoid the emergence of mammalian-adaptive mutations, and the supernatant was inoculated into nine- or ten-day-old embryonated chicken eggs. After an incubation of up to 48 hours, the allantoic fluid of the inoculated eggs was harvested and stored at -80℃. The virus titers were determined by using plaque assays in MDCK, A549, and DF-1 cells, respectively.

179 **Table 1. List of primers**

Primer	Sequence	Description
A-u12g	5'-AGCGAAAGCAGG-3'	RT-PCR
uniPB2-1	5'-AGCGAAAGCAGGTC-3'	RT-PCR
uniPB2-2341R	5'-AGTAGAAACAAGGTCGTTT-3'	RT-PCR
uniPB1-1	5'-AGCGAAAGCAGGCA-3'	RT-PCR
uniPB1-2341R	5'-AGTAGAAACAAGGCATTT-3'	RT-PCR
uniPA-1	5'-AGCGAAAGCAGGTAC-3'	RT-PCR
uniPA-2233R	5'-AGTAGAAACAAGGTACTT-3'	RT-PCR
uniHA-1	5'-AGCAAAAGCAGGGG-3'	RT-PCR
H5-u12+20F	5'-AGCAAAAGCAGGGGTTCAATCTGTCAAAATGG-3'	RT-PCR
H5-u13+20R	5'-AGTAGAAACAAGGGTGTTTTTAACTACAATCTG-3'	RT-PCR
uniNP-1	5'-AGCGAAAGCAGGGTA-3'	RT-PCR
uniNP-1565R	5'-AGTAGAAACAAGGGTATTTTT-3'	RT-PCR
uniNA-1	5'-AGCGAAAGCAGGAGT-3'	RT-PCR
uniNA-1413R	5'-AGTAGAAACAAGGAGTTTTTT-3'	RT-PCR
uniM-1	5'-AGCGAAAGCAGGTAG-3'	RT-PCR
uniM-1027R	5'-AGTAGAAACAAGGTAGTTTTT-3'	RT-PCR
WSNM-1F	5'-AGCAAAAGCAGGTAGATATTGAAAGATGAG-3'	RT-PCR
WSNM-1027R	5'-AGTAGAAACAAGGTAGTTTTTTACTCCAGC-3'	RT-PCR
uniNS-1	5'-AGCGAAAGCAGGGTG-3'	RT-PCR
uniNS-890R	5'-AGTAGAAACAAGGGTGTTTT-3'	RT-PCR
Bm-HA-1	5'-TATTCGTCTCAGGGAGCAAAAGCAGGGG-3'	pHH21 cloning
Bm-HA-u12+9F	5'-TATTCGTCTCAGGGAGCAAAAGCAGGGGTTCAATCTG-3'	pHH21 cloning
Bm-HA-u13+5R	5'-ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTTTAACT-3'	pHH21 cloning
Bm-NA-1	5'-TATTCGTCTCAGGGAGCAAAAGCAGGAGT-3'	pHH21 cloning
Bm-NA-u12+2F	5'-TATTCGTCTCAGGGAGCAAAAGCAGGAGTTC-3'	pHH21 cloning
Bm-NA-1413R	5'-ATATCGTCTCGTATTAGTAGAAACAAGGAGTTTTTT-3'	pHH21 cloning
Bm-NA-u13+5R	5'-ATATCGTCTCGTATTAGTAGAAACAAGGAGTTTTTTGAACA-3'	pHH21 cloning
Bm-PB2-1	5'-TATTCGTCTCAGGGAGCGAAAGCAGGTC-3'	pHH21 cloning
Bm-PB2-u12+9F	5'-TATTCGTCTCAGGGAGCGAAAGCAGGTCAAATATATT-3'	pHH21 cloning
Bm-PB2-2341R	5'-ATATCGTCTCGTATTAGTAGAAACAAGGTCGTTT-3'	pHH21 cloning
Bm-PB2-u13+6R	5'-ATATCGTCTCGTATTAGTAGAAACAAGGTCGTTTTTAAAC-3'	pHH21 cloning
Bm-PB1-1	5'-TATTCGTCTCAGGGAGCGAAAGCAGGCA-3'	pHH21 cloning
Bm-PB1-2341R	5'-ATATCGTCTCGTATTAGTAGAAACAAGGCATTT-3'	pHH21 cloning
Bm-PA-1	5'-TATTCGTCTCAGGGAGCGAAAGCAGGTAC-3'	pHH21 cloning
Bm-PA-2233R	5'-ATATCGTCTCGTATTAGTAGAAACAAGGTACTT-3'	pHH21 cloning
Ba-NP-1	5'-TATTGGTCTCAGGGAGCAAAAGCAGGGTA-3'	pHH21 cloning
Ba-NP-u12+15F	5'-TATTGGTCTCAGGGAGCAAAAGCAGGGTAGATAATCACTCACCG-3'	pHH21 cloning
Ba-NP-1565R	5'-ATATGGTCTCGTATTAGTAGAAACAAGGGTATTTTT-3'	pHH21 cloning
Ba-NP-u13+1R	5'-ATATGGTCTCGTATTAGTAGAAACAAGGGTATTTTTC-3'	pHH21 cloning
Bm-M-1	5'-TATTCGTCTCAGGGAGCAAAAGCAGGTAG-3'	pHH21 cloning
Bm-M-u12+2F	5'-TATTCGTCTCAGGGAGCAAAAGCAGGTAGAT-3'	pHH21 cloning
Bm-M-1027R	5'-ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTT-3'	pHH21 cloning
Bm-M-u13+2R	5'-ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTTAC-3'	pHH21 cloning
Bm-NS-1	5'-TATTCGTCTCAGGGAGCAAAAGCAGGGTG-3'	pHH21 cloning
Bm-NS-890R	5'-ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT-3'	pHH21 cloning
PB2-ClaI-F-1	5'-ACAATCGATGCCGCCACCATGGAGAGAATAAAAGAA-3'	pCAGGS cloning
PB2-ClaI-F-2	5'-ACAATCGATGCCGCCACCATGGAACGAATAAAAGAA-3'	pCAGGS cloning
PB2-ClaI-F-4	5'-ACAATCGATGCCGCCACCATGGAGAGAATAAAAGAATTAAG-3'	pCAGGS cloning
PB2-Sph-F-1	5'-ACAGCATGCGCCGCCACCATGGAGAGAATAAAAGAAC-3'	pCAGGS cloning
PB2-NheI-R-1	5'-ACAGCTAGCCTAATTGATGGCCATCCGAATT-3'	pCAGGS cloning
PB2-NheI-R-3	5'-ACAGCTAGCCTAATTGGTGGCCATCCGAATTCTTTTGGTCGC-3'	pCAGGS cloning
PB2-NheI-R-4	5'-ACAGCTAGCCTAATTGGTGGCCATCCGAATT-3'	pCAGGS cloning
PB1-ClaI-F-1	5'-ACAATCGATGCCGCCACCATGGATGTCAATCCGACT-3'	pCAGGS cloning
PB1-ClaI-F-8	5'-ACAATCGATGCCGCCACCATGGATGTCAATCCGACC-3'	pCAGGS cloning
PB1-ClaI-F-9	5'-ACAATCGATGCCGCCACCATGGATGTCAACCCGACT-3'	pCAGGS cloning
PB1-NheI-R-1	5'-ACAGCTAGCCTATTTTTGCCGTCTGAGCTCTT-3'	pCAGGS cloning
PB1-NheI-R-5	5'-ACAGCTAGCCTATTTTTGCCGT-3'	pCAGGS cloning
PB1-NheI-R-7	5'-ACAGCTAGCCTATTTTTGCCG-3'	pCAGGS cloning
PA-ClaI-F-1	5'-ACAATCGATGCCGCCACCATGGAAGATTTTGTGCGACA-3'	pCAGGS cloning
PA-ClaI-F-2	5'-ACAATCGATGCCGCCACCATGGAAGACTTTGTGCGACAAT-3'	pCAGGS cloning
PA-ClaI-F-4	5'-ACAATCGATGCCGCCACCATGGAAGATTTTGTGCG-3'	pCAGGS cloning
PA-ClaI-F-5	5'-ACAATCGATGCCGCCACCATGGAAGACTTTGTGCG-3'	pCAGGS cloning
PA-SphI-F-1	5'-ACAGCATGCGCCGCCACCATGGAAGATTTTGTGCG-3'	pCAGGS cloning
PA-NheI-R-1	5'-ACAGCTAGCCTATTTCAGTGCATGTGCGAGGAA-3'	pCAGGS cloning
PA-NheI-R-2	5'-ACAGCTAGCCTATTTCAGTGCATGTGTGAGGAA-3'	pCAGGS cloning
PA-NheI-R-5	5'-ACAGCTAGCCTATTTCAGTGCATGTGCGAGGA-3'	pCAGGS cloning
PA-NheI-R-6	5'-ACAGCTAGCCCTATTTCAGTGCA-3'	pCAGGS cloning

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181

Table 1. List of primers *(continued)*

Primer	Sequence	Description
PA-SphI-R-1	5'-ACAGCATGCCTATTTCAGTGCA-3'	pCAGGS cloning
PA-XhoI-R-1	5'-ACACTCGAGCTATTTCAGTGCA-3'	pCAGGS cloning
NP-ClaI-F-1	5'-ACAATCGATGCCGCCACCATGGCGTCTCAAGGCACCAAA-3'	pCAGGS cloning
NP-ClaI-F-2	5'-ACAATCGATGCCGCCACCATGGCGTCTCAAGGCACC-3'	pCAGGS cloning
NP-NheI-R-1	5'-ACAGCTAGCTTAATTGTCATACTCCTCTGCATT-3'	pCAGGS cloning
NP-NheI-R-2	5'-ACAGCTAGCTTAATTGTCATATTCCTCTGCATTGTCTC-3'	pCAGGS cloning
NP-NheI-R-3	5'-ACAGCTAGCTTAATTGTCATACTCCTCTGCATTGTCTC-3'	pCAGGS cloning
UT521-UT541-HA-LPAI-F	5'-CTCAGAAATAGCCCTCAAAGAGAAACGAGAGGACTATTTGGAGC-3'	HA mutagenesis pHH21
UT521-UT541-HA-LPAI-R	5'-CCAAATAGTCCTCTCGTTTCTCTTTGAGGGCTATTTCTGAGCCC-3'	HA mutagenesis pHH21
UT14-HA-LPAI-F	5'-CACACACGTCTCTGGTCTATTTGGAGCTATAGCAGG-3'	HA mutagenesis pHH21
UT14-HA-LPAI-R	5'-CACACACGTCTCTGGACTGTTTGGAGCTATAGCAGG-3'	HA mutagenesis pHH21
UT17-HA-LPAI-F	5'-CACACACGTCTCTGTCCTCTAGTCTCTCTTTGAGGGGCTATTTCTGAGCCCTGTCGC-3'	HA mutagenesis pHH21
UT17-HA-LPAI-R	5'-CACACACGTCTCTGTCCTCTAGTCTCTCTTTGAGGACTATTTCTGAGCCCAGTTGC-3'	HA mutagenesis pHH21
UT64-HA-LPAI-F	5'-CTCAGAAATAGTCCTCAAAGAGAGACAAGAGGACTGTTTGGAGC-3'	HA mutagenesis pHH21
UT64-HA-LPAI-R	5'-CAAACAGTCCTCTTGTCTCTCTTTGAGGACTATTTCTGAGCCCA-3'	HA mutagenesis pHH21
UT167-UT173-HA-LPAI-F	5'-AATAGTCCTCAAAGAGAGACAAGAGGACTGTTTGGGGCTA-3'	HA mutagenesis pHH21
UT167-UT173-HA-LPAI-R	5'-CAAACAGTCCTCTTGTCTCTCTTTGAGGACTATTTCTGAGCCCA-3'	HA mutagenesis pHH21
NA-521-I32L-F	5'-GTTACAAATTGGGAACATAATCTCCTTATGGGTCAGTCAT-3'	NA mutagenesis pHH21
NA-521-I32L-R	5'-CCCTTTCTGAATTGAATGACTGACCCATAAGGAGATTATGT-3'	NA mutagenesis pHH21
NA-521-N50S-F	5'-TCAACACCAAGCTGAATCAATCAGCAATACTAAACCTCTTAC-3'	NA mutagenesis pHH21
NA-521-N50S-R	5'-CTCAGTAAGAGGTTTAGTATTGCTGATTGATTCAGCTTGGTGTT-3'	NA mutagenesis pHH21
NA-521-K53N-F	5'-TCAACACCAAGCTGAATCAATCAACAATACTAACCCTCTTAC-3'	NA mutagenesis pHH21
NA-521-K53N-R	5'-CTCAGTAAGAGGGTTAGTATTGTTGATTGATTCAGCTTGGTGTT-3'	NA mutagenesis pHH21
NA-521-T111I-F	5'-TCATGCTCCCACTTGGAATGCAGAATTTTCTTCTTAACTCAGGG-3'	NA mutagenesis pHH21
NA-521-T111I-R	5'-AAGGCTCCCTGAGTTAAGAAGAAAATTCTGCATTCCAAGTGGG-3'	NA mutagenesis pHH21
NA-521-L120M-F	5'-TCAGGGAGCCTTGATGAATGACAAGCACTCCAACGGAACTGTC-3'	NA mutagenesis pHH21
NA-521-L120M-R	5'-TTTGACAGTTCCGTTGGAGTGCTTGTCATTCATCAAGGC-3'	NA mutagenesis pHH21
NA-521-M143V-F	5'-GAACATTAATGAGTTGTCCTGTGGGTGA-3'	NA mutagenesis pHH21
NA-521-M143V-R	5'-AGCCTCACCCACAGGACAACTCATTAAT-3'	NA mutagenesis pHH21
NA-521-major01-F	5'-TCAATCAGCAATACTAACCCTCTTACTGAGAAAGCTGTGGCTTC-3'	NA mutagenesis pHH21
NA-521-major01-R	5'-CTCAGTAAGAGGGTTAGTATTGCTGATTGATTCAGCTTGGTGTT-3'	NA mutagenesis pHH21
NA-541-L32I-F	5'-GTTACAAATTGGGAACATAATCTCAATATGGGTCAGTCAT-3'	NA mutagenesis pHH21
NA-541-L32I-R	5'-CCCTTTCTGAATTGAATGACTGACCCATATTGAGATTATGT-3'	NA mutagenesis pHH21
NA-541-S50N-F	5'-TCAACACCAAGCTGAATCAATCAACAATACTAACCCTCTTAC-3'	NA mutagenesis pHH21
NA-541-S50N-R	5'-CTCAGTAAGAGGGTTAGTATTGTTGATTGATTCAGCTTGGTGTT-3'	NA mutagenesis pHH21
NA-541-N53K-F	5'-TCAACACCAAGCTGAATCAATCAGCAATACTAAACCTCTTAC-3'	NA mutagenesis pHH21
NA-541-N53K-R	5'-CTCAGTAAGAGGTTTAGTATTGCTGATTGATTCAGCTTGGTGTT-3'	NA mutagenesis pHH21
NA-541-I111T-F	5'-TCATGCTCCCACTTGGAATGCAGAACTTTCTTCTTAACTCAGGG-3'	NA mutagenesis pHH21
NA-541-I111T-R	5'-AAGGCTCCCTGAGTTAAGAAGAAAGTTCTGCATTCCAAGTGGG-3'	NA mutagenesis pHH21
NA-541-M120L-F	5'-TCAGGGAGCCTTGCTGAATGACAAGCACTCCAACGGAACTGTC-3'	NA mutagenesis pHH21
NA-541-M120L-R	5'-TTTGACAGTTCCGTTGGAGTGCTTGTCATTCAGCAAGGC-3'	NA mutagenesis pHH21
NA-541-V143M-F	5'-GAACATTAATGAGTTGTCCTATGGGTGA-3'	NA mutagenesis pHH21
NA-541-V143M-R	5'-AGCCTCACCCATAGGACAACTCATTAAT-3'	NA mutagenesis pHH21
NA-chimera1-R	5'-GGCTCTCTAATAACAAACACATCCCCCTTGGAACCG-3'	NA mutagenesis pHH21
NA-chimera2-F	5'-AGGACAACAATATAAGGATCGGTTCC-3'	NA mutagenesis pHH21
PB2-521-A109V-02-F	5'-TAGGAATGGGCCAACGACAAGTGCAGTCCATTATCCAAAGGTTT-3'	PB2 mutagenesis pHH21
PB2-521-A109V-02-R	5'-GTATGTTTTGTAAACCTTTGGATAATGGACTGCAC-3'	PB2 mutagenesis pHH21
PB2-521-A292T-F	5'-AGCACACAAATTGGTGGGACAAGAATGGTGGACATCCTTA-3'	PB2 mutagenesis pHH21
PB2-521-A292T-R	5'-GCCTAAGGATGTCCACCATTCTTGTCCCACCAATTTGTGTG-3'	PB2 mutagenesis pHH21
PB2-521-K369N-F	5'-GAGGAATTCACAATGGTTGGGCGGAATGCAACAGCT-3'	PB2 mutagenesis pHH21
PB2-521-K369N-R	5'-TTGCTTTCCTCAGGATAGCTGTTGCATTCCGCCCAA-3'	PB2 mutagenesis pHH21
PB2-521-L447Q-F	5'-CAAAAGTGCTATTTCAGAACTGGGGAATTGAACCCA-3'	PB2 mutagenesis pHH21
PB2-521-L447Q-R	5'-CAATTCCCCAGTTCTGAAATAGCACTTTTGCGTCCT-3'	PB2 mutagenesis pHH21
PB2-521-T758I-F	5'-ACAGCGACCAAAAGAATTCGGATGGCCATCAATTAGTGTCG-3'	PB2 mutagenesis pHH21
PB2-521-T758I-R	5'-AATTCGACACTAATTGATGGCCATCCGAATTCTTTTGGTCGC-3'	PB2 mutagenesis pHH21
PB2-541-V109A-F	5'-AATGGGCCAACGACAAGTGCAGCCCATTATCCAAAGGTTTAC-3'	PB2 mutagenesis pHH21
PB2-541-V109A-02-R	5'-GTATGTTTTGTAAACCTTTGGATAATGGGCTGCAC-3'	PB2 mutagenesis pHH21
PB2-541-T292A-F	5'-AGCACACAAATTGGTGGGGCAAGAATGGTGGACATCCTTA-3'	PB2 mutagenesis pHH21
PB2-541-T292A-R	5'-GCCTAAGGATGTCCACCATTCTTGCCCCACCAATTTGTGTG-3'	PB2 mutagenesis pHH21
PB2-541-N369K-F	5'-GAGGAATTCACAATGGTTGGGCGGAAGGCAACAGCT-3'	PB2 mutagenesis pHH21
PB2-541-N369K-R	5'-TTGCTTTCCTCAGGATAGCTGTTGCCTTCCGCCCAA-3'	PB2 mutagenesis pHH21
PB2-541-Q447L-F	5'-CAAAAGTGCTATTTCTGAACTGGGGAATTGAACCCA-3'	PB2 mutagenesis pHH21
PB2-541-Q447L-R	5'-CAATTCCCCAGTTCAGAAATAGCACTTTTGCGTCCT-3'	PB2 mutagenesis pHH21
PB2-541-I758T-F	5'-ACAGCGACCAAAAGAATTCGGATGGCCACCAATTAGTGTCG-3'	PB2 mutagenesis pHH21
PB2-541-I758T-R	5'-AATTCGACACTAATTGGTGGCCATCCGAATTCTTTTGGTCGC-3'	PB2 mutagenesis pHH21
PB2-chimera1-R	5'-CTTCCTCTTCCTTCGTGACGG-3'	PB2 mutagenesis pHH21
PB2-chimera2-F	5'-CAAAAGAACAAGTGGATCATCCGTC-3'	PB2 mutagenesis pHH21

Primers used for RT-PCR, pHH21 cloning, pCAGGS cloning, and introduction of amino acid mutations are listed above.

Viral replication assay

Triplicate wells of confluent A549 cells or DF-1 cells were infected with viruses at a multiplicity of infection (MOI) of 0.0002, and incubated for 1 hour. The infected virus loads were calculated by using virus titers measured in the respective A549 cells and DF-1 cells. The incubation temperature was set to 33℃ or 37℃ for A549 cells (temperatures of the human lower and upper respiratory tract), and to 39℃ for DF-1 cells (body temperature of birds). After the 1-hour-incubation, A549 cells were further incubated in F-12K containing 0.3% bovine serum albumin (BSA) at 33℃ or 37℃. DF-1 cells were further incubated in DMEM containing 0.3% BSA at 39℃. Supernatant was harvested at 24-hour-intervals, and frozen at -80℃. Virus titers in the collected supernatant at each time point were determined by use of plaque assays in MDCK cells; 6- (1-tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin was added for replication efficiency analysis and plaque assays of reassortant virus strains possessing the HA segment of PR8 or a monobasic cleavage site.

Mouse experiments

Six-week-old, female, specific-pathogen-free (SPF) BALB/c mice (SLC, Japan) were used for mouse experiments. To analyze the pathogenicity of H5N1 viruses isolated

during surveillance activities, four to five mice/group were anesthetized with isoflurane and intranasally inoculated with $1x10^3$ plaque-forming units (PFU) of virus in a 50-μl volume, using virus titers measured in MDCK cells. The mice were monitored daily for clinical signs of infection and changes in body weight and mortality for 12 days. To determine the dose lethal to 50% of mice (MLD_{50}), four or five mice/group were intranasally inoculated with 10^1 to 10^6 PFU of virus in a 50-μl volume. The mice were monitored and checked for changes in body weight and mortality for 14 days post-infection. MLD_{50} values were calculated by using the method of Reed & Muench [28]. For virus replication in mouse organs, groups of mice (six mice/group) were intranasally infected with $1x10^3$ PFU of virus in a 50-μl volume. Three mice in each group were euthanized on days three and six post-infection, respectively. Organs (brain, liver, kidney, spleen, colon, heart, trachea, lungs, and nasal turbinate) were collected, and their virus titers were measured by use of plaque assays in MDCK cells.

Mini-replicon assay

A549 cells and DF-1 cells were transfected with 0.1 μg each of the pCAGGS constructs encoding the polymerase proteins PB1, PA, nucleoprotein (NP) and wild-type or mutant PB2, together with 0.1 μg each of the pHH21 constructs encoding firefly luciferase

220 and Renilla luciferase as an internal control [29, 30]. The transfected A549 cells were
221 incubated either at 33℃ for 48 hours, or at 37℃ for 24 hours, respectively. The transfected
222 DF-1 cells were incubated at 39℃ for 24 hours. After incubation, the cells were lysed and the
223 luciferase activity was determined by using the dual-luciferase system detector kit (Promega,
224 Madison, WI). The luciferase activity values were normalized to the Renilla activity.
225
226 **HA receptor binding assay**
227 Virus binding to sialic acid receptors was analyzed using a solid-phase binding
228 assay with the Octet RED instrument from ForteBio (Menlo Park, CA, USA), as previously
229 described [31, 32]. The receptor analogs used were *N*-acetylneuraminic acid linked to
230 galactose by either an α2,3-linkage (Neu5Ac(alpha2-3)LacNAc-PGA-Biotin) or an α2,6-
231 linkage (Neu5Ac(alpha2-6)LacNAc-PGA-Biotin) (Tokyo Chemical Industry, Tokyo, Japan),
232 and they were loaded onto streptavidin biosensors. Replication-incompetent PB2-knockout
233 virus strains possessing the PR8 strain backbone with the HA and NA segments of the target
234 virus strains were generated by using reverse genetics. The HA cleavage site of the target
235 HPAI H5N1 virus strains was transformed from polybasic into monobasic so that the receptor
236 binding assay could be conducted in the BSL2 facility. The generated virus strains were

propagated in MDCK cells expressing the PB2 protein of PR8. Generated viruses were concentrated by ultracentrifugation at 28000 rotations per minute (rpm) for 2 hours using a 25% sucrose cushion, and 5 μ g of concentrated virus was used for each receptor binding assay. A/Vietnam/1203/2004 (H5N1) bound to Sia α 2,3Gal (pink), and A/Kawasaki/173/2001 (H1N1) bound to Sia α 2,6Gal served as controls. An analyzed virus strain that was not loaded with glycoprotein served as a baseline.

Immunofluorescence assay

After viral infection or plasmid transfection, A549 cells were fixed with 4% paraformaldehyde and then permeabilized with 0.2% Triton X-100 [33]. After blocking, antigens were probed with mouse anti-WSN NP 3/1 monoclonal antibody, mouse anti-Aichi NP 2S 347/4 monoclonal antibody, or mouse anti-PB2-18/1 monoclonal antibody, followed by Alexa Fluor 488 anti-mouse IgG (Life Technologies, Carlsbad, CA, United States). Nuclei were stained with Hoechst 33342 (Life Technologies, Carlsbad, CA, United States). The cells were then imaged by using a laser-scanning microscope (LSM780 system, Carl Zeiss) and analyzed with Zen software (Carl Zeiss).

Chapter 1: Epidemiological surveillance and characterization of highly pathogenic H5N1 avian influenza viruses isolated in Indonesia between 2010 and 2016

Introduction

HPAI H5N1 viruses have been circulating in poultry in Indonesia since 2003 [34-36]. Poultry production in Indonesia is diverse, ranging from backyard poultry farms to large industrial integrated poultry farms. Uncontrolled movement of live poultry and poor biosecurity practices, including trades in live bird markets, continue to be a problem, and the surveillance system for HPAI is still not well organized [37]. Despite the Indonesian government's attempt to eradicate H5N1 viruses through culling of infected chickens and poultry vaccination, HPAI H5N1 viruses are still widely circulating in Indonesia [34, 35, 38, 39]. Along with the enzootic state among poultry, 200 human H5N1 cases in Indonesia, including 168 lethal cases, have been reported to the WHO as of September, 2018 (Fig. 3) [23]. The latest human H5N1 case in Indonesia was reported in September, 2017, which resulted in a fatal outcome [40]. HPAI H5N1 viruses in Indonesia thus pose a clear and present threat to humans.

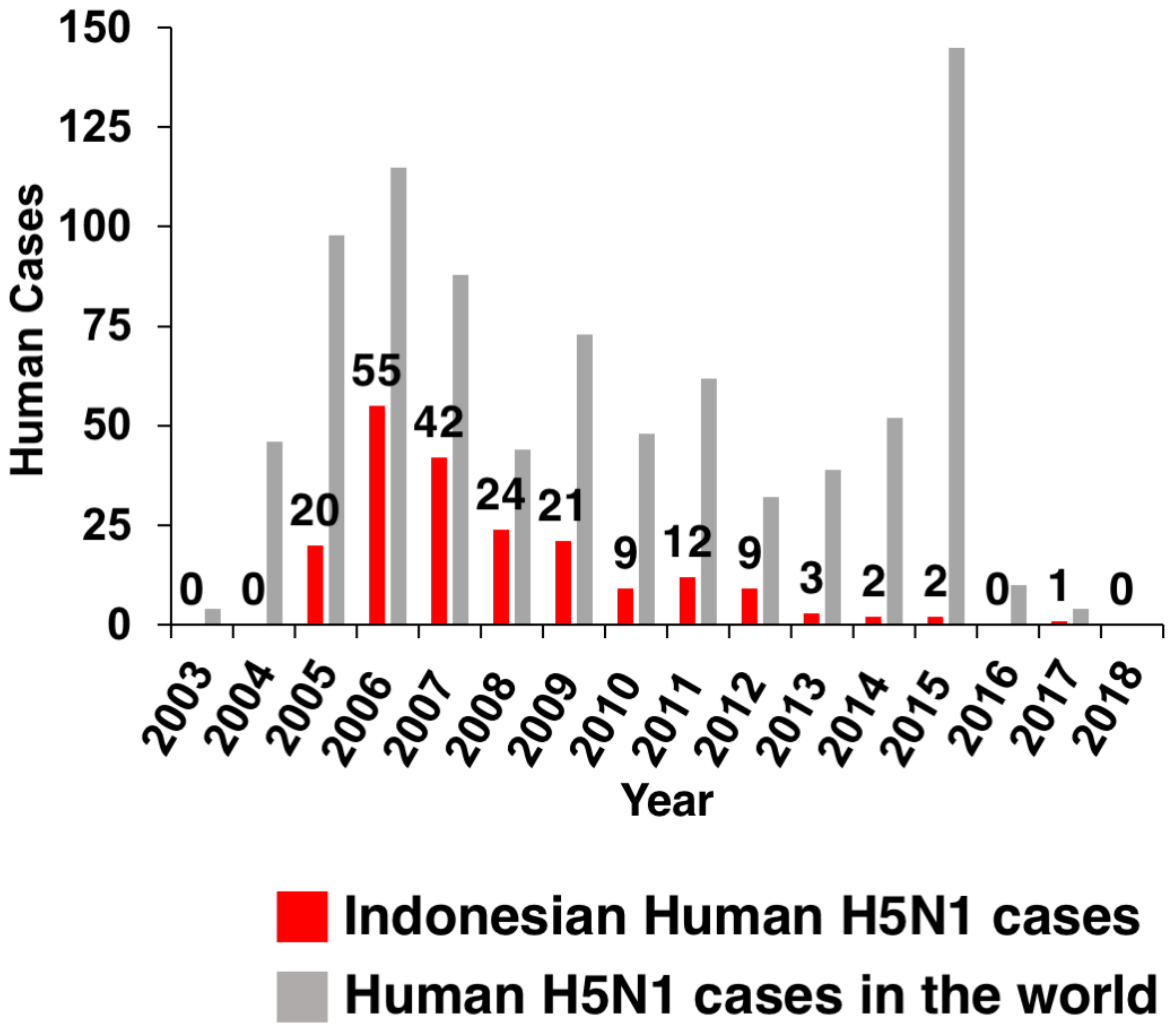

Figure 3. Human H5N1 cases in Indonesia

Human H5N1 cases reported to the WHO are shown. The red bar indicates human H5N1 cases detected in Indonesia, and the grey bar shows the number of human H5N1 cases worldwide. The numbers in the figure are the numbers of Indonesian human cases reported each year. The data are from a WHO report released on September 21, 2018 [23].

286 Various molecular changes have been identified that facilitate avian influenza virus
287 infection of humans. Three influenza virus proteins—PB2, HA, and non-structural protein
288 (NS1)—are recognized as major determinants of virulence, pathogenicity, and host range
289 restriction [10, 41, 42].
290 The HA protein mediates virus attachment and the fusion of the viral and
291 endosomal membranes [1]. The receptor-binding specificity of the HA protein plays an
292 important role in host range restriction. In general, avian influenza viruses bind preferentially
293 to sialic acid linked to galactose by an α 2,3-linkage (Sia α 2,3Gal), whereas human
294 influenza viruses have higher affinity for Sia α 2,6Gal [43-45]. Acquisition of Siaα2,6Gal
295 recognition capability is thought to be essential for avian influenza viruses to possess
296 pandemic potential and transmit readily among humans [46, 47].
297 The PB2 protein is a component of the viral polymerase complex along with PB1,
298 PA, and NP. Among many amino acid mutations, PB2-627, PB2-701, and PB2-591 are
299 recognized as major determinants of pathogenicity and host range restriction [48-50].
300 Despite the current circulation of HPAI H5N1 viruses among poultry in Indonesia
301 and human H5N1 cases being continuously reported until 2017, there are few epidemiological
302 reports on Indonesian HPAI H5N1 viruses. As of October, 2018, the latest efforts to

303 molecularly characterize circulating viruses were conducted on HPAI H5N1 viruses isolated
304 in 2012 and 2013 [51, 52]. Little is known about the recent Indonesian HPAI H5N1 viruses,
305 including their potential to become endemic or cause global pandemics.
306 With the aim to elucidate the current situation with respect to HPAI H5N1 virus
307 circulation in Indonesia, our lab conducted epidemiological surveillance of poultry and swine
308 in Indonesia from 2010 through 2016. We isolated HPAI H5N1 viruses from collected
309 samples and characterized them, including assessing their human epidemic and pandemic
310 potential.
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Results

Isolation of HPAI H5N1 viruses

From 2010 through 2016, we conducted epizoologic surveillance of HPAI viruses of the H5N1 subtype among poultry and swine in Indonesia. From the 2,534 samples collected, 39 HPAI H5N1 virus strains were isolated utilizing embryonated chicken eggs (Table 2, Fig. 4). More than 50% of the samples were collected in East Java. To elucidate the current situation regarding HPAI H5N1 virus circulation in Indonesia, we characterized 39 of these HPAI H5N1 virus strains.

Table 2. Collected field samples and HPAI H5N1 virus strains isolated from them

		Year											
		2010		2011		2012		2013		2014		2015		2016	
		No. of swabs	(H5N1 positive)	No. of swabs	(H5N1 positive)	No. of swabs	(H5N1 positive)	No. of swabs	(H5N1 positive)	No. of swabs	(H5N1 positive)	No. of swabs	(H5N1 positive)	No. of swabs	(H5N1 positive)
		503	***(11)***	***147***	***(0)***	***411***	***(5)***	***56***	***(4)***	***854***	***(6)***	***124***	***(1)***	***439***	***(12)***
Chicken	***Total***	***233***	***(11)***	***95***	***(0)***	***4***	***(0)***	***40***	***(4)***	***615***	***(4)***	***82***	***(1)***	***372***	***(8)***
	East Java	54	(1)	0	(0)	4	(0)	40	(4)	341	(4)	82	1	332	(8)
	Central Java	6	(1)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	40	(0)
	West Java	38	(2)	85	(0)	0	(0)	0	(0)	83	(0)	0	(0)	0	(0)
	Kalimantan	34	(5)	0	(0)	0	(0)	0	(0)	191	(0)	0	(0)	0	(0)
	Sulawessi	40	(1)	10	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
	Sumatra	61	(1)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
Duck	***Total***	***9***	***(0)***	***50***	***(0)***	***2***	***(2)***	***12***	***(0)***	***214***	***(2)***	***27***	***(0)***	***55***	***(4)***
	East Java	0	(0)	4	(0)	2	(2)	12	(0)	168	(1)	27	(0)	53	(4)
	Central Java	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	2	(0)
	West Java	0	(0)	40	(0)	0	(0)	0	(0)	25	(0)	0	(0)	0	(0)
	Kalimantan	0	(0)	0	(0)	0	(0)	0	(0)	21	(1)	0	(0)	0	(0)
	Sulawessi	9	(0)	6	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
	Sumatra	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
Quail	***Total***	***0***	***(0)***	***1***	***(0)***	***3***	***(3)***	***4***	***(0)***	***0***	***(0)***	***0***	***(0)***	***0***	***(0)***
	East Java	0	(0)	0	(0)	5	(5)	4	(0)	0	(0)	0	(0)	0	(0)
	Central Java	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
	West Java	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
	Kalimantan	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
	Sulawessi	0	(0)	1	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
	Sumatra	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
Other Avians	***Total***	***0***	***(0)***	***1***	***(0)***	***0***	***(0)***	***0***	***(0)***	***25***	***(0)***	***15***	***(0)***	***12***	***(0)***
	East Java	0	(0)	0	(0)	0	(0)	0	(0)	24	(0)	1	(0)	6	(0)
	Central Java	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	6	(0)
	West Java	0	(0)	1	(0)	0	(0)	0	(0)	1	(0)	14	(0)	0	(0)
	Kalimantan	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
	Sulawessi	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
	Sumatra	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
Swine	***Total***	***261***	***(0)***	***0***	***(0)***	***400***	***(0)***	***0***	***(0)***	***0***	***(0)***	***0***	***(0)***	***0***	***(0)***
	East Java	201	(0)	0	(0)	190	(0)	0	(0)	0	(0)	0	(0)	0	(0)
	Central Java	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
	West Java	36	(0)	31	(0)	210	(0)	0	(0)	0	(0)	0	(0)	0	(0)
	Kalimantan	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
	Sulawessi	0	(0)	91	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
	Sumatra	24	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)

Listed in the table are the animal species and locations from which swab samples were collected, as well as the HPAI H5N1 virus strains isolated from those samples.

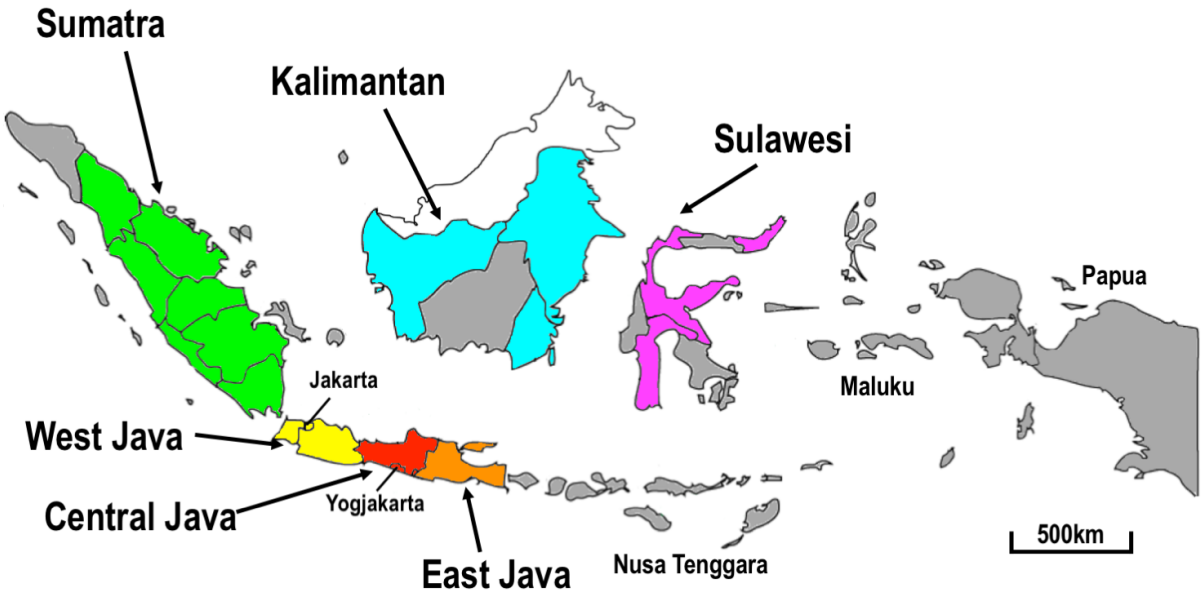


Figure 4. Map of Indonesia

Colored area shows the provinces where sampling for H5N1 virus was conducted between 2010 and 2016; West Java (yellow), Central Java (red), East Java (orange), Sumatra (green), Kalimantan (blue) and Sulawesi (pink).

362 **Phylogenetic analysis of the HA and PB2 genes**

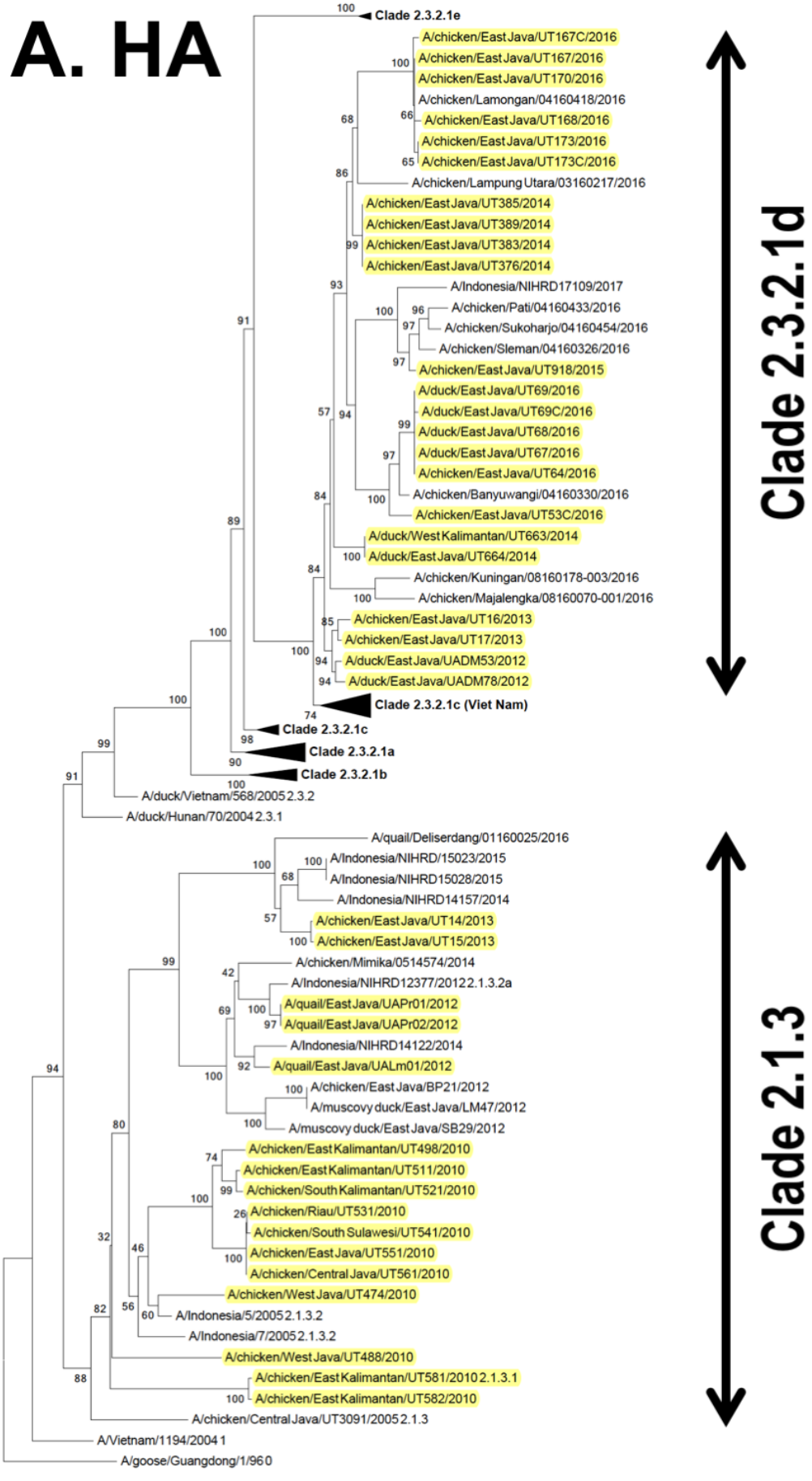
363 The nucleotide sequences of the eight segments of the isolated HPAI H5N1 virus
364 strains were analyzed by using the Sanger sequencing. To investigate the evolution of HPAI
365 H5N1 viruses in Indonesia between 2010 and 2016, phylogenetic trees of the eight viral
366 segments'genes of the 39 strains from our surveillance were constructed by using the
367 Neighbor-Joining method and MEGA 7 (Fig. 5A-H) [53-57]. Phylogenetic analysis of the
368 HA genes revealed that the 39 H5N1 virus strains were classified into two subclades, clade
369 2.1.3 and clade 2.3.2.1d (Fig. 5A) [58, 59]. Clade 2.1.3 virus strains had been circulating in
370 Indonesia since 2003 [34, 35], and 16 H5N1 virus strains from our surveillance, which were
371 isolated between 2010 and 2013, belonged to clade 2.1.3. The remaining 23 H5N1 virus
372 strains were classified into clade 2.3.2.1d [59], which has been circulating in Indonesia since
373 2012 [51]. In our surveillance, after 2014, all H5N1 virus strains belonged to clade 2.3.2.1d,
374 and no H5N1 virus strain belonging to clade 2.1.3 was identified.

375 Phylogenetic analysis of the NA, PB1, PA, NP, M, and NS genes revealed the same
376 pattern as that observed with HA; the isolated H5N1 virus strains being classified into two
377 subgroups corresponding to either clade 2.1.3 or clade 2.3.2.1d (Fig5. B, D-H).

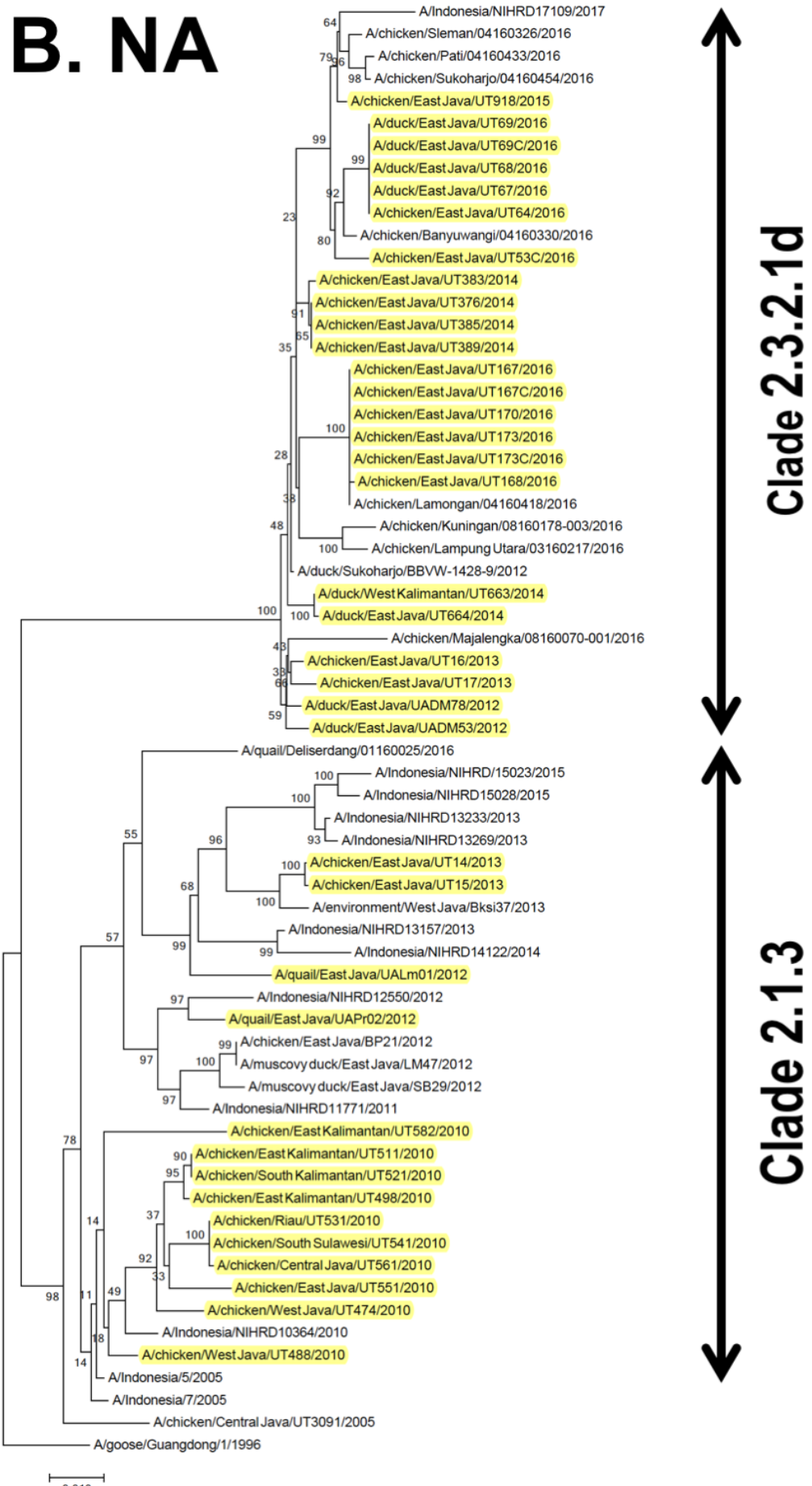
Phylogenetic analysis of the PB2 genes of our 35 H5N1 virus strains revealed the formation of three distinct sub-clusters (Fig. 5C). One sub-cluster comprised clade 2.1.3 strains, and the other two sub-clusters contained clade 2.3.2.1d strains. One sub-cluster of the clade 2.3.2.1d strains included strains isolated between 2013 and 2016, whereas the other clade 2.3.2.1d sub-cluster consisted of six strains of clade 2.3.2.1d virus collected at the same live bird market on the same day in March, 2016. NCBI nucleotide BLAST analysis revealed that these six strains' PB2 genes were 99% identical to another three Indonesian H5N1 viruses isolated in 2016: A/chicken/Lamongan/04160418/2016, A/chicken/Sukoharjo/04160454/2016, and A/chicken/Pati/04160433/2016. The BLAST analysis also revealed that these six strains' PB2 genes were 93%–94% identical to the PB2 genes of the following non-H5N1 viruses: A/duck/Mongolia/47/2001 (H7N1), A/duck/Shimane/188/1999 (H1N1), A/mallard/Jiangxi/7376/2003 (H6N2), A/duck/Malaysia/F118-08-04/2004 (H5N2), and A/environment/Korea/W152/2006 (H7N7). In the phylogenetic tree of PB2 genes, three clade 2.3.2.1d Indonesian HPAI H5N1 strains with PB2 reassortment already listed above (A/chicken/Pati/04160433/2016, A/chicken/Sukoharjo/04160454/2016, and A/chicken/Lamongan/04160418/2016) were located in the vicinity of these six strains.

In addition to the 39 isolated H5N1 strains, there were several reassortant Indonesian H5N1 viruses reported in the databases. Three clade 2.1.3 strains (A/muscovy duck/East Java/SB29/2012, A/muscovy duck East Java/LM47/2012, and A/chicken/East Java/BP21/2012) possessed reassortant PB2, PB1, and NS segments, as previously reported [52]. Phylogenetic analysis also revealed that reassortment between clade 2.1.3 and clade 2.3.2.1d had already occurred in Indonesia in 2016, which, to our knowledge, has not been reported. Two clade 2.3.2.1d strains isolated in 2016, A/chicken/Kuningan/08160178-003/2016 and A/chicken/Majalengka/08160070-001/2016, possessed PB1, PA, NP, HA, and NA segments belonging to clade 2.3.2.1d, but their PB2, M, and NS segments belonged to clade 2.1.3.

These findings suggest that reassortment of viral segments has sporadically occurred among both clade 2.1.3 and clade 2.3.2.1d H5N1 virus strains in Indonesia since at least 2012 [52].


A. HA
Clade 2.3.2.1e
A/chicken/East Java/UT167C/2016
A/chicken/East Java/UT167/2016
A/chicken/East Java/UT170/2016
A/chicken/Lamongan/04160418/2016
A/chicken/East Java/UT168/2016
A/chicken/East Java/UT173/2016
A/chicken/East Java/UT173C/2016
A/chicken/Lampung Utara/03160217/2016
A/chicken/East Java/UT385/2014
A/chicken/East Java/UT389/2014
A/chicken/East Java/UT383/2014
A/chicken/East Java/UT376/2014
A/Indonesia/NIHRD17109/2017
A/chicken/Pati/04160433/2016
A/chicken/Sukoharjo/04160454/2016
A/chicken/Sleman/04160326/2016
A/chicken/East Java/UT918/2015
A/duck/East Java/UT69/2016
A/duck/East Java/UT69C/2016
A/duck/East Java/UT68/2016
A/duck/East Java/UT67/2016
A/chicken/East Java/UT64/2016
A/chicken/Banyuwangi/04160330/2016
A/chicken/East Java/UT53C/2016
A/duck/West Kalimantan/UT663/2014
A/duck/East Java/UT664/2014
A/chicken/Kuningan/08160178-003/2016
A/chicken/Majalengka/08160070-001/2016
A/chicken/East Java/UT16/2013
A/chicken/East Java/UT17/2013
A/duck/East Java/UADM53/2012
A/duck/East Java/UADM78/2012
Clade 2.3.2.1c (Viet Nam)
Clade 2.3.2.1c
Clade 2.3.2.1a
Clade 2.3.2.1b
A/duck/Vietnam/568/2005 2.3.2
A/duck/Hunan/70/2004 2.3.1
A/quail/Deliserdang/01160025/2016
A/Indonesia/NIHRD/15023/2015
A/Indonesia/NIHRD15028/2015
A/Indonesia/NIHRD14157/2014
A/chicken/East Java/UT14/2013
A/chicken/East Java/UT15/2013
A/chicken/Mimika/0514574/2014
A/Indonesia/NIHRD12377/2012 2.1.3.2a
A/quail/East Java/UAPr01/2012
A/quail/East Java/UAPr02/2012
A/Indonesia/NIHRD14122/2014
A/quail/East Java/UALm01/2012
A/chicken/East Java/BP21/2012
A/muscovy duck/East Java/LM47/2012
A/muscovy duck/East Java/SB29/2012
A/chicken/East Kalimantan/UT498/2010
A/chicken/East Kalimantan/UT511/2010
A/chicken/South Kalimantan/UT521/2010
A/chicken/Riau/UT531/2010
A/chicken/South Sulawesi/UT541/2010
A/chicken/East Java/UT551/2010
A/chicken/Central Java/UT561/2010
A/chicken/West Java/UT474/2010
A/Indonesia/5/2005 2.1.3.2
A/Indonesia/7/2005 2.1.3.2
A/chicken/West Java/UT488/2010
A/chicken/East Kalimantan/UT581/2010 2.1.3.1
A/chicken/East Kalimantan/UT582/2010
A/chicken/Central Java/UT3091/2005 2.1.3
A/Vietnam/1194/2004 1
A/goose/Guangdong/1/96 0
0.010
Clade 2.3.2.1d
Clade 2.1.3

412


B. NA
A/Indonesia/NIHRD17109/2017
A/chicken/Sleman/04160326/2016
A/chicken/Pati/04160433/2016
A/chicken/Sukoharjo/04160454/2016
A/chicken/East Java/UT918/2015
A/duck/East Java/UT69/2016
A/duck/East Java/UT69C/2016
A/duck/East Java/UT68/2016
A/duck/East Java/UT67/2016
A/chicken/East Java/UT64/2016
A/chicken/Banyuwangi/04160330/2016
A/chicken/East Java/UT53C/2016
A/chicken/East Java/UT383/2014
A/chicken/East Java/UT376/2014
A/chicken/East Java/UT385/2014
A/chicken/East Java/UT389/2014
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A/chicken/East Java/UT167C/2016
A/chicken/East Java/UT170/2016
A/chicken/East Java/UT173/2016
A/chicken/East Java/UT173C/2016
A/chicken/East Java/UT168/2016
A/chicken/Lamongan/04160418/2016
A/chicken/Kuningan/08160178-003/2016
A/chicken/Lampung Utara/03160217/2016
A/duck/Sukoharjo/BBVW-1428-9/2012
A/duck/West Kalimantan/UT663/2014
A/duck/East Java/UT664/2014
A/chicken/Majalengka/08160070-001/2016
A/chicken/East Java/UT16/2013
A/chicken/East Java/UT17/2013
A/duck/East Java/UADM78/2012
A/duck/East Java/UADM53/2012
A/quail/Deliserdang/01160025/2016
A/Indonesia/NIHRD/15023/2015
A/Indonesia/NIHRD15028/2015
A/Indonesia/NIHRD13233/2013
A/Indonesia/NIHRD13269/2013
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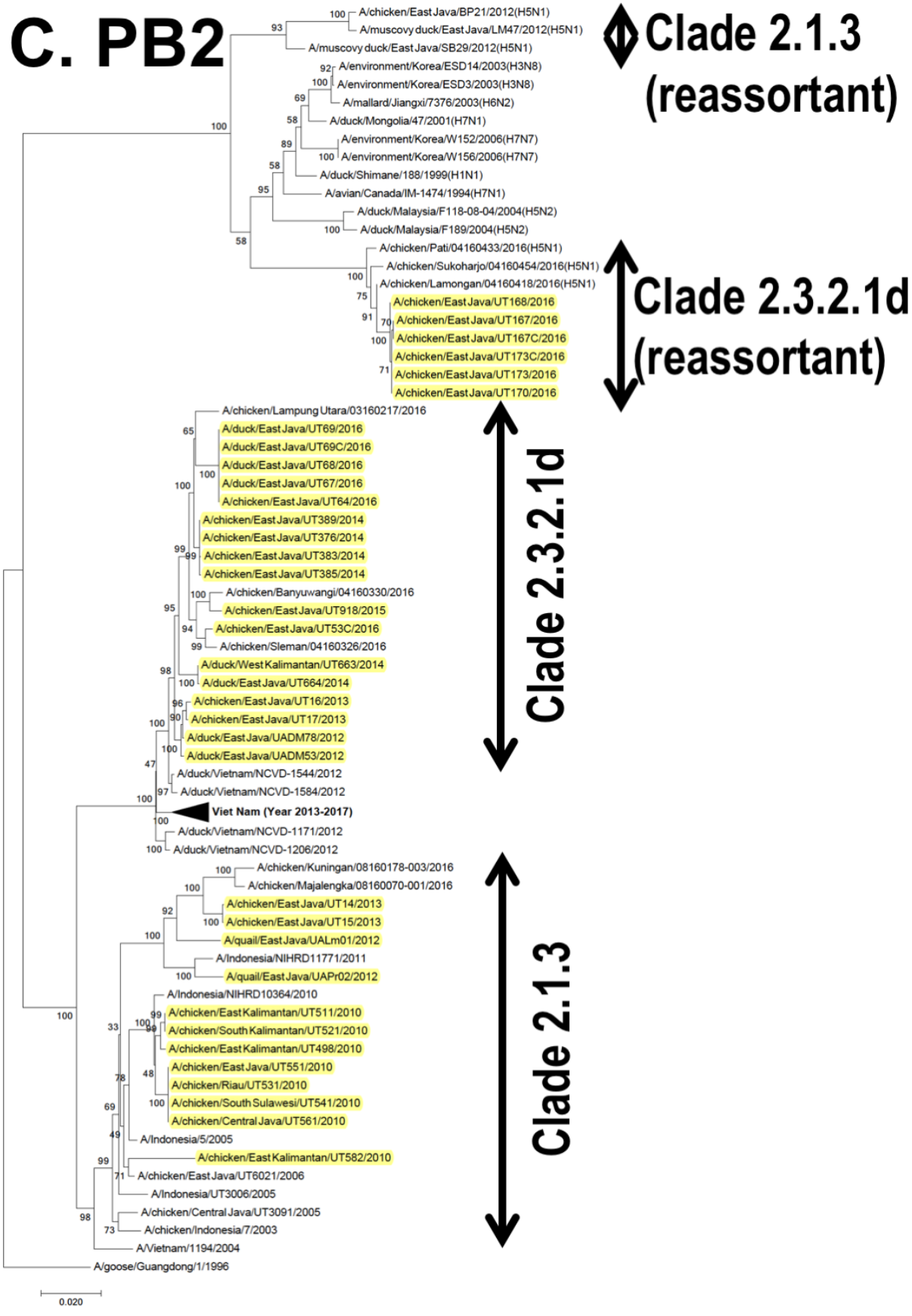
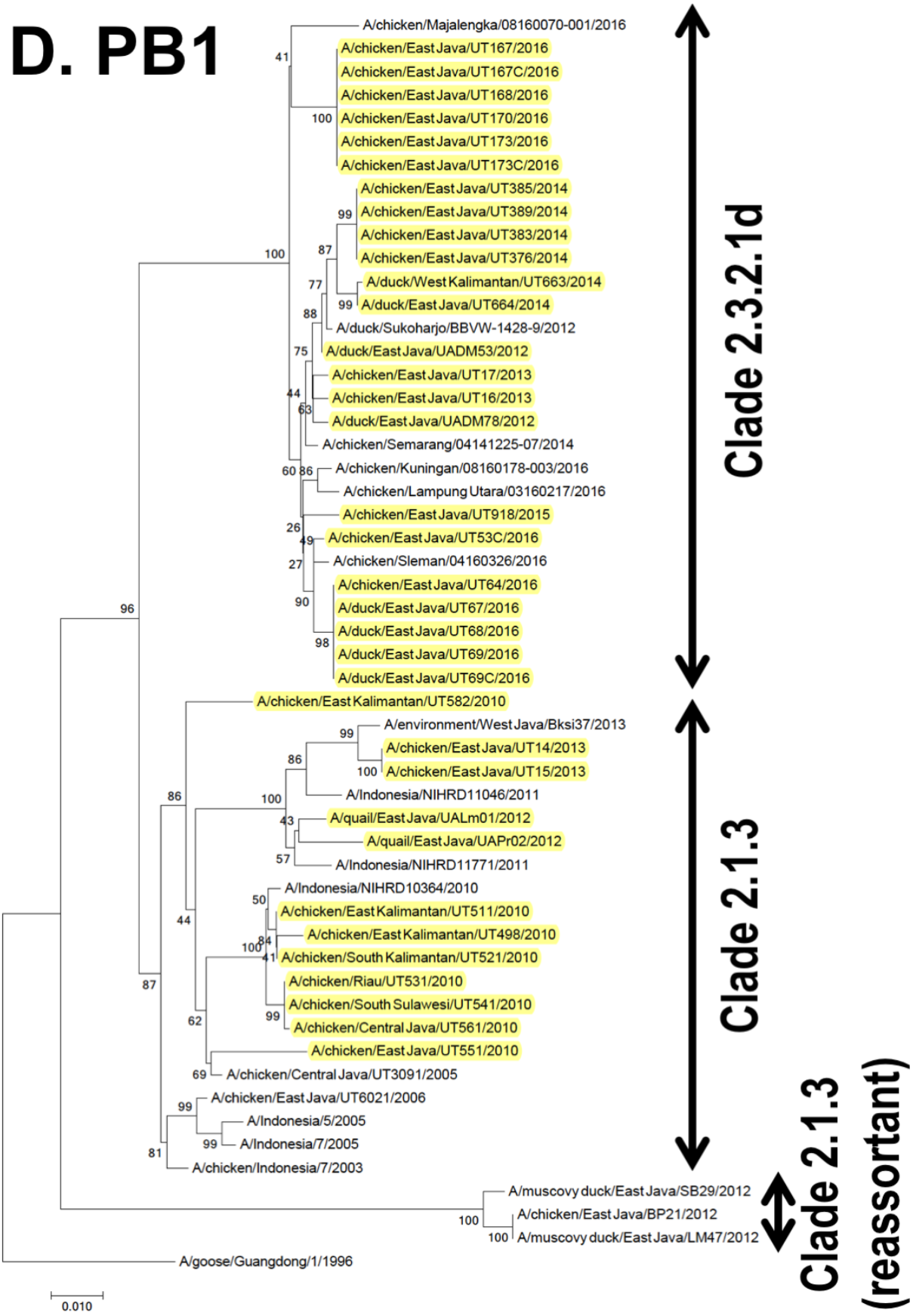
A/Indonesia/NIHRD14122/2014
A/quail/East Java/UALm01/2012
A/Indonesia/NIHRD12550/2012
A/quail/East Java/UAPr02/2012
A/chicken/East Java/BP21/2012
A/muscovy duck/East Java/LM47/2012
A/muscovy duck/East Java/SB29/2012
A/Indonesia/NIHRD11771/2011
A/chicken/East Kalimantan/UT582/2010
A/chicken/East Kalimantan/UT511/2010
A/chicken/South Kalimantan/UT521/2010
A/chicken/East Kalimantan/UT498/2010
A/chicken/Riau/UT531/2010
A/chicken/South Sulawesi/UT541/2010
A/chicken/Central Java/UT561/2010
A/chicken/East Java/UT551/2010
A/chicken/West Java/UT474/2010
A/Indonesia/NIHRD10364/2010
A/chicken/West Java/UT488/2010
A/Indonesia/5/2005
A/Indonesia/7/2005
A/chicken/Central Java/UT3091/2005
A/goose/Guangdong/1/1996
0.010
Clade 2.3.2.1d
Clade 2.1.3

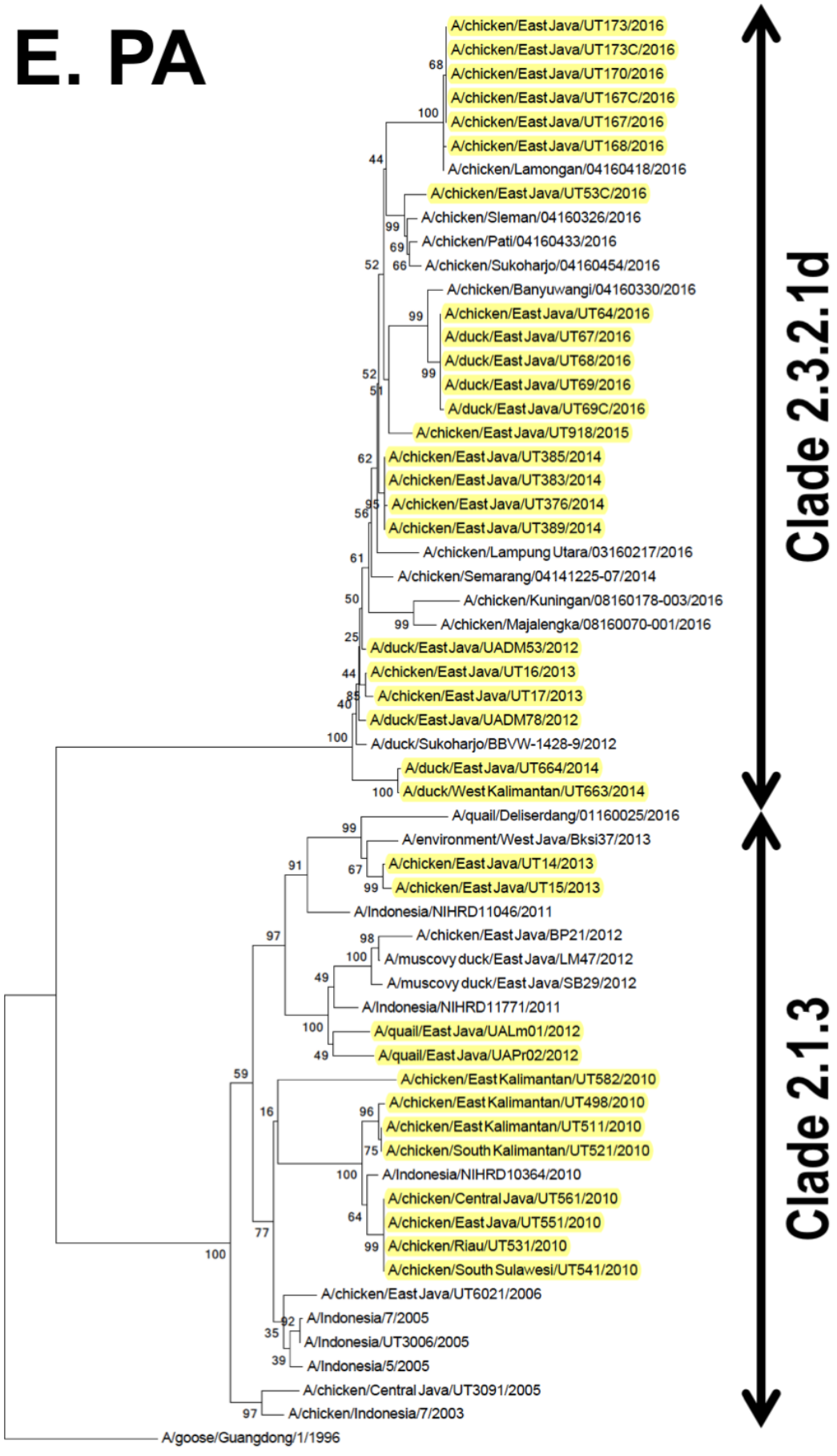

C. PB2
A/chicken/East Java/BP21/2012(H5N1)
A/muscovy duck/East Java/LM47/2012(H5N1)
A/muscovy duck/East Java/SB29/2012(H5N1)
A/environment/Korea/ESD14/2003(H3N8)
A/environment/Korea/ESD3/2003(H3N8)
A/mallard/Jiangxi/7376/2003(H6N2)
A/duck/Mongolia/47/2001(H7N1)
A/environment/Korea/W152/2006(H7N7)
A/environment/Korea/W156/2006(H7N7)
A/duck/Shimane/188/1999(H1N1)
A/avian/Canada/IM-1474/1994(H7N1)
A/duck/Malaysia/F118-08-04/2004(H5N2)
A/duck/Malaysia/F189/2004(H5N2)
A/chicken/Pati/04160433/2016(H5N1)
A/chicken/Sukoharjo/04160454/2016(H5N1)
A/chicken/Lamongan/04160418/2016(H5N1)
A/chicken/East Java/UT168/2016
A/chicken/East Java/UT167/2016
A/chicken/East Java/UT167C/2016
A/chicken/East Java/UT173C/2016
A/chicken/East Java/UT173/2016
A/chicken/East Java/UT170/2016
A/chicken/Lampung Utara/03160217/2016
A/duck/East Java/UT69/2016
A/duck/East Java/UT69C/2016
A/duck/East Java/UT68/2016
A/duck/East Java/UT67/2016
A/chicken/East Java/UT64/2016
A/chicken/East Java/UT389/2014
A/chicken/East Java/UT376/2014
A/chicken/East Java/UT383/2014
A/chicken/East Java/UT385/2014
A/chicken/Banyuwangi/04160330/2016
A/chicken/East Java/UT918/2015
A/chicken/East Java/UT53C/2016
A/chicken/Sleman/04160326/2016
A/duck/West Kalimantan/UT663/2014
A/duck/East Java/UT664/2014
A/chicken/East Java/UT16/2013
A/chicken/East Java/UT17/2013
A/duck/East Java/UADM78/2012
A/duck/East Java/UADM53/2012
A/duck/Vietnam/NCVD-1544/2012
A/duck/Vietnam/NCVD-1584/2012
Viet Nam (Year 2013-2017)
A/duck/Vietnam/NCVD-1171/2012
A/duck/Vietnam/NCVD-1206/2012
A/chicken/Kuningan/08160178-003/2016
A/chicken/Majalengka/08160070-001/2016
A/chicken/East Java/UT14/2013
A/chicken/East Java/UT15/2013
A/quail/East Java/UALm01/2012
A/Indonesia/NIHRD11771/2011
A/quail/East Java/UAPr02/2012
A/Indonesia/NIHRD10364/2010
A/chicken/East Kalimantan/UT511/2010
A/chicken/South Kalimantan/UT521/2010
A/chicken/East Kalimantan/UT498/2010
A/chicken/East Java/UT551/2010
A/chicken/Riau/UT531/2010
A/chicken/South Sulawesi/UT541/2010
A/chicken/Central Java/UT561/2010
A/Indonesia/5/2005
A/chicken/East Kalimantan/UT582/2010
A/chicken/East Java/UT6021/2006
A/Indonesia/UT3006/2005
A/chicken/Central Java/UT3091/2005
A/chicken/Indonesia/7/2003
A/Vietnam/1194/2004
A/goose/Guangdong/1/1996
0.020
Clade 2.1.3 (reassortant)
Clade 2.3.2.1d (reassortant)
Clade 2.3.2.1d
Clade 2.1.3

414

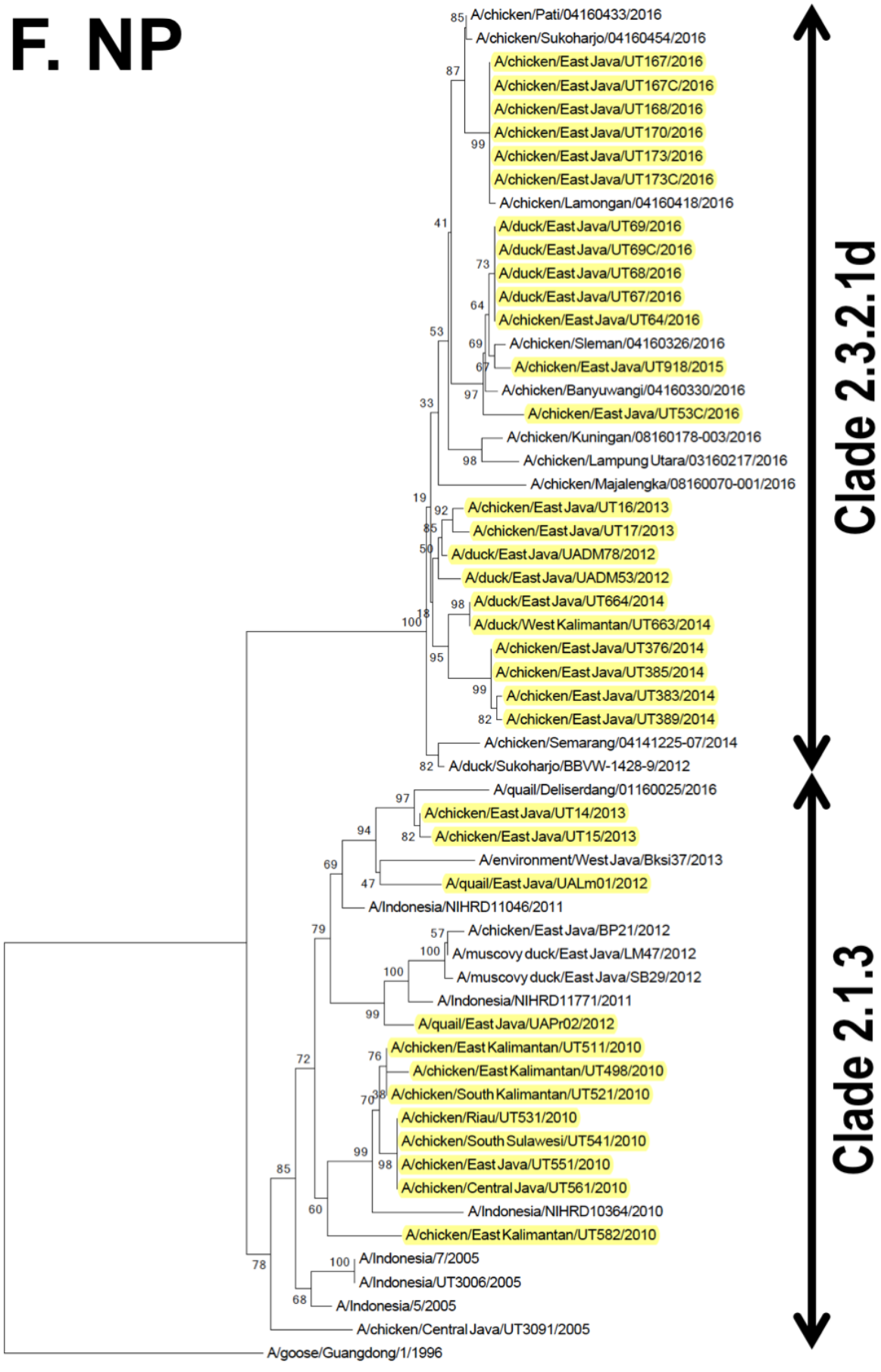

D. PB1
A/chicken/Majalengka/08160070-001/2016
A/chicken/East Java/UT167/2016
A/chicken/East Java/UT167C/2016
A/chicken/East Java/UT168/2016
A/chicken/East Java/UT170/2016
A/chicken/East Java/UT173/2016
A/chicken/East Java/UT173C/2016
A/chicken/East Java/UT385/2014
A/chicken/East Java/UT389/2014
A/chicken/East Java/UT383/2014
A/chicken/East Java/UT376/2014
A/duck/West Kalimantan/UT663/2014
A/duck/East Java/UT664/2014
A/duck/Sukoharjo/BBVW-1428-9/2012
A/duck/East Java/UADM53/2012
A/chicken/East Java/UT17/2013
A/chicken/East Java/UT16/2013
A/duck/East Java/UADM78/2012
A/chicken/Semarang/04141225-07/2014
A/chicken/Kuningan/08160178-003/2016
A/chicken/Lampung Utara/03160217/2016
A/chicken/East Java/UT918/2015
A/chicken/East Java/UT53C/2016
A/chicken/Sleman/04160326/2016
A/chicken/East Java/UT64/2016
A/duck/East Java/UT67/2016
A/duck/East Java/UT68/2016
A/duck/East Java/UT69/2016
A/duck/East Java/UT69C/2016
A/chicken/East Kalimantan/UT582/2010
A/environment/West Java/Bksi37/2013
A/chicken/East Java/UT14/2013
A/chicken/East Java/UT15/2013
A/Indonesia/NIHRD11046/2011
A/quail/East Java/UALm01/2012
A/quail/East Java/UAPr02/2012
A/Indonesia/NIHRD11771/2011
A/Indonesia/NIHRD10364/2010
A/chicken/East Kalimantan/UT511/2010
A/chicken/East Kalimantan/UT498/2010
A/chicken/South Kalimantan/UT521/2010
A/chicken/Riau/UT531/2010
A/chicken/South Sulawesi/UT541/2010
A/chicken/Central Java/UT561/2010
A/chicken/East Java/UT551/2010
A/chicken/Central Java/UT3091/2005
A/chicken/East Java/UT6021/2006
A/Indonesia/5/2005
A/Indonesia/7/2005
A/chicken/Indonesia/7/2003
A/muscovy duck/East Java/SB29/2012
A/chicken/East Java/BP21/2012
A/muscovy duck/East Java/LM47/2012
A/goose/Guangdong/1/1996
0.010
Clade 2.3.2.1d
Clade 2.1.3
Clade 2.1.3
(reassortant)

415

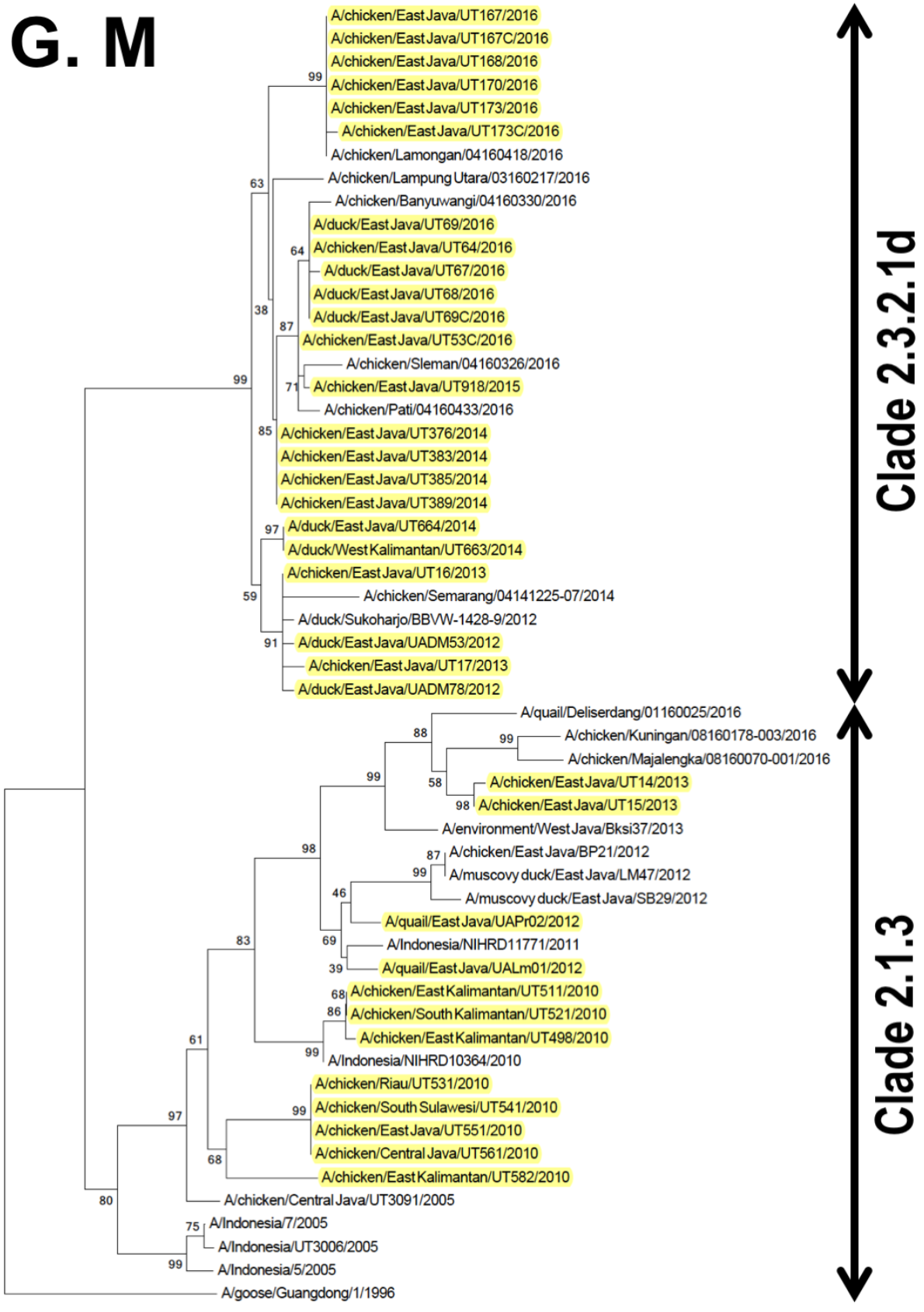
416


E. PA
A/chicken/East Java/UT173/2016
A/chicken/East Java/UT173C/2016
A/chicken/East Java/UT170/2016
A/chicken/East Java/UT167C/2016
A/chicken/East Java/UT167/2016
A/chicken/East Java/UT168/2016
A/chicken/Lamongan/04160418/2016
A/chicken/East Java/UT53C/2016
A/chicken/Sleman/04160326/2016
A/chicken/Pati/04160433/2016
A/chicken/Sukoharjo/04160454/2016
A/chicken/Banyuwangi/04160330/2016
A/chicken/East Java/UT64/2016
A/duck/East Java/UT67/2016
A/duck/East Java/UT68/2016
A/duck/East Java/UT69/2016
A/duck/East Java/UT69C/2016
A/chicken/East Java/UT918/2015
A/chicken/East Java/UT385/2014
A/chicken/East Java/UT383/2014
A/chicken/East Java/UT376/2014
A/chicken/East Java/UT389/2014
A/chicken/Lampung Utara/03160217/2016
A/chicken/Semarang/04141225-07/2014
A/chicken/Kuningan/08160178-003/2016
A/chicken/Majalengka/08160070-001/2016
A/duck/East Java/UADM53/2012
A/chicken/East Java/UT16/2013
A/chicken/East Java/UT17/2013
A/duck/East Java/UADM78/2012
A/duck/Sukoharjo/BBVW-1428-9/2012
A/duck/East Java/UT664/2014
A/duck/West Kalimantan/UT663/2014
A/quail/Deliserdang/01160025/2016
A/environment/West Java/Bksi37/2013
A/chicken/East Java/UT14/2013
A/chicken/East Java/UT15/2013
A/Indonesia/NIHRD11046/2011
A/chicken/East Java/BP21/2012
A/muscovy duck/East Java/LM47/2012
A/muscovy duck/East Java/SB29/2012
A/Indonesia/NIHRD11771/2011
A/quail/East Java/UALm01/2012
A/quail/East Java/UAPr02/2012
A/chicken/East Kalimantan/UT582/2010
A/chicken/East Kalimantan/UT498/2010
A/chicken/East Kalimantan/UT511/2010
A/chicken/South Kalimantan/UT521/2010
A/Indonesia/NIHRD10364/2010
A/chicken/Central Java/UT561/2010
A/chicken/East Java/UT551/2010
A/chicken/Riau/UT531/2010
A/chicken/South Sulawesi/UT541/2010
A/chicken/East Java/UT6021/2006
A/Indonesia/7/2005
A/Indonesia/UT3006/2005
A/Indonesia/5/2005
A/chicken/Central Java/UT3091/2005
A/chicken/Indonesia/7/2003
A/goose/Guangdong/1/1996
0.010
Clade 2.3.2.1d
Clade 2.1.3

417

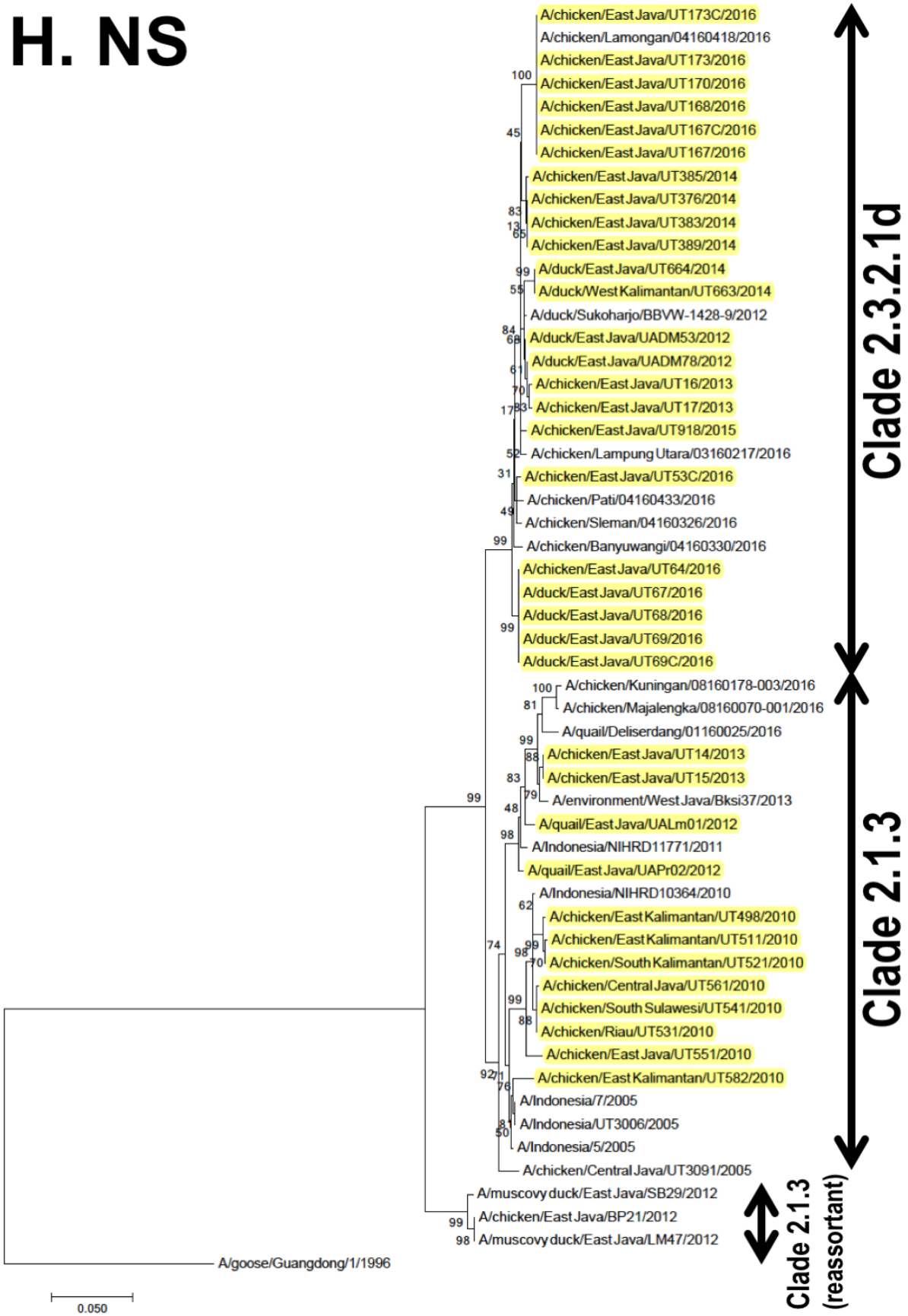

F. NP
A/chicken/Pati/04160433/2016
A/chicken/Sukoharjo/04160454/2016
A/chicken/East Java/UT167/2016
A/chicken/East Java/UT167C/2016
A/chicken/East Java/UT168/2016
A/chicken/East Java/UT170/2016
A/chicken/East Java/UT173/2016
A/chicken/East Java/UT173C/2016
A/chicken/Lamongan/04160418/2016
A/duck/East Java/UT69/2016
A/duck/East Java/UT69C/2016
A/duck/East Java/UT68/2016
A/duck/East Java/UT67/2016
A/chicken/East Java/UT64/2016
A/chicken/Sleman/04160326/2016
A/chicken/East Java/UT918/2015
A/chicken/Banyuwangi/04160330/2016
A/chicken/East Java/UT53C/2016
A/chicken/Kuningan/08160178-003/2016
A/chicken/Lampung Utara/03160217/2016
A/chicken/Majalengka/08160070-001/2016
A/chicken/East Java/UT16/2013
A/chicken/East Java/UT17/2013
A/duck/East Java/UADM78/2012
A/duck/East Java/UADM53/2012
A/duck/East Java/UT664/2014
A/duck/West Kalimantan/UT663/2014
A/chicken/East Java/UT376/2014
A/chicken/East Java/UT385/2014
A/chicken/East Java/UT383/2014
A/chicken/East Java/UT389/2014
A/chicken/Semarang/04141225-07/2014
A/duck/Sukoharjo/BBVW-1428-9/2012
A/quail/Deliserdang/01160025/2016
A/chicken/East Java/UT14/2013
A/chicken/East Java/UT15/2013
A/environment/West Java/Bksi37/2013
A/quail/East Java/UALm01/2012
A/Indonesia/NIHRD11046/2011
A/chicken/East Java/BP21/2012
A/muscovy duck/East Java/LM47/2012
A/muscovy duck/East Java/SB29/2012
A/Indonesia/NIHRD11771/2011
A/quail/East Java/UAPr02/2012
A/chicken/East Kalimantan/UT511/2010
A/chicken/East Kalimantan/UT498/2010
A/chicken/South Kalimantan/UT521/2010
A/chicken/Riau/UT531/2010
A/chicken/South Sulawesi/UT541/2010
A/chicken/East Java/UT551/2010
A/chicken/Central Java/UT561/2010
A/Indonesia/NIHRD10364/2010
A/chicken/East Kalimantan/UT582/2010
A/Indonesia/7/2005
A/Indonesia/UT3006/2005
A/Indonesia/5/2005
A/chicken/Central Java/UT3091/2005
A/goose/Guangdong/1/1996
0.010
Clade 2.3.2.1d
Clade 2.1.3

418


G. M
A/chicken/East Java/UT167/2016
A/chicken/East Java/UT167C/2016
A/chicken/East Java/UT168/2016
A/chicken/East Java/UT170/2016
A/chicken/East Java/UT173/2016
A/chicken/East Java/UT173C/2016
A/chicken/Lamongan/04160418/2016
A/chicken/Lampung Utara/03160217/2016
A/chicken/Banyuwangi/04160330/2016
A/duck/East Java/UT69/2016
A/chicken/East Java/UT64/2016
A/duck/East Java/UT67/2016
A/duck/East Java/UT68/2016
A/duck/East Java/UT69C/2016
A/chicken/East Java/UT53C/2016
A/chicken/Sleman/04160326/2016
A/chicken/East Java/UT918/2015
A/chicken/Pati/04160433/2016
A/chicken/East Java/UT376/2014
A/chicken/East Java/UT383/2014
A/chicken/East Java/UT385/2014
A/chicken/East Java/UT389/2014
A/duck/East Java/UT664/2014
A/duck/West Kalimantan/UT663/2014
A/chicken/East Java/UT16/2013
A/chicken/Semarang/04141225-07/2014
A/duck/Sukoharjo/BBVW-1428-9/2012
A/duck/East Java/UADM53/2012
A/chicken/East Java/UT17/2013
A/duck/East Java/UADM78/2012
A/quail/Deliserdang/01160025/2016
A/chicken/Kuningan/08160178-003/2016
A/chicken/Majalengka/08160070-001/2016
A/chicken/East Java/UT14/2013
A/chicken/East Java/UT15/2013
A/environment/West Java/Bksi37/2013
A/chicken/East Java/BP21/2012
A/muscovy duck/East Java/LM47/2012
A/muscovy duck/East Java/SB29/2012
A/quail/East Java/UAPr02/2012
A/Indonesia/NIHRD11771/2011
A/quail/East Java/UALm01/2012
A/chicken/East Kalimantan/UT511/2010
A/chicken/South Kalimantan/UT521/2010
A/chicken/East Kalimantan/UT498/2010
A/Indonesia/NIHRD10364/2010
A/chicken/Riau/UT531/2010
A/chicken/South Sulawesi/UT541/2010
A/chicken/East Java/UT551/2010
A/chicken/Central Java/UT561/2010
A/chicken/East Kalimantan/UT582/2010
A/chicken/Central Java/UT3091/2005
A/Indonesia/7/2005
A/Indonesia/UT3006/2005
A/Indonesia/5/2005
A/goose/Guangdong/1/1996
0.0050
Clade 2.3.2.1d
Clade 2.1.3

419

420


H. NS
A/chicken/East Java/UT173C/2016
A/chicken/Lamongan/04160418/2016
A/chicken/East Java/UT173/2016
A/chicken/East Java/UT170/2016
A/chicken/East Java/UT168/2016
A/chicken/East Java/UT167C/2016
A/chicken/East Java/UT167/2016
A/chicken/East Java/UT385/2014
A/chicken/East Java/UT376/2014
A/chicken/East Java/UT383/2014
A/chicken/East Java/UT389/2014
A/duck/East Java/UT664/2014
A/duck/West Kalimantan/UT663/2014
A/duck/Sukoharjo/BBVW-1428-9/2012
A/duck/East Java/UADM53/2012
A/duck/East Java/UADM78/2012
A/chicken/East Java/UT16/2013
A/chicken/East Java/UT17/2013
A/chicken/East Java/UT918/2015
A/chicken/Lampung Utara/03160217/2016
A/chicken/East Java/UT53C/2016
A/chicken/Pati/04160433/2016
A/chicken/Sleman/04160326/2016
A/chicken/Banyuwangi/04160330/2016
A/chicken/East Java/UT64/2016
A/duck/East Java/UT67/2016
A/duck/East Java/UT68/2016
A/duck/East Java/UT69/2016
A/duck/East Java/UT69C/2016
A/chicken/Kuningan/08160178-003/2016
A/chicken/Majalengka/08160070-001/2016
A/quail/Deliserdang/01160025/2016
A/chicken/East Java/UT14/2013
A/chicken/East Java/UT15/2013
A/environment/West Java/Bksi37/2013
A/quail/East Java/UALm01/2012
A/Indonesia/NIHRD11771/2011
A/quail/East Java/UAPr02/2012
A/Indonesia/NIHRD10364/2010
A/chicken/East Kalimantan/UT498/2010
A/chicken/East Kalimantan/UT511/2010
A/chicken/South Kalimantan/UT521/2010
A/chicken/Central Java/UT561/2010
A/chicken/South Sulawesi/UT541/2010
A/chicken/Riau/UT531/2010
A/chicken/East Java/UT551/2010
A/chicken/East Kalimantan/UT582/2010
A/Indonesia/7/2005
A/Indonesia/UT3006/2005
A/Indonesia/5/2005
A/chicken/Central Java/UT3091/2005
A/muscovy duck/East Java/SB29/2012
A/chicken/East Java/BP21/2012
A/muscovy duck/East Java/LM47/2012
A/goose/Guangdong/1/1996
0.050
Clade 2.3.2.1d
Clade 2.1.3
Clade 2.1.3
(reassortant)

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422

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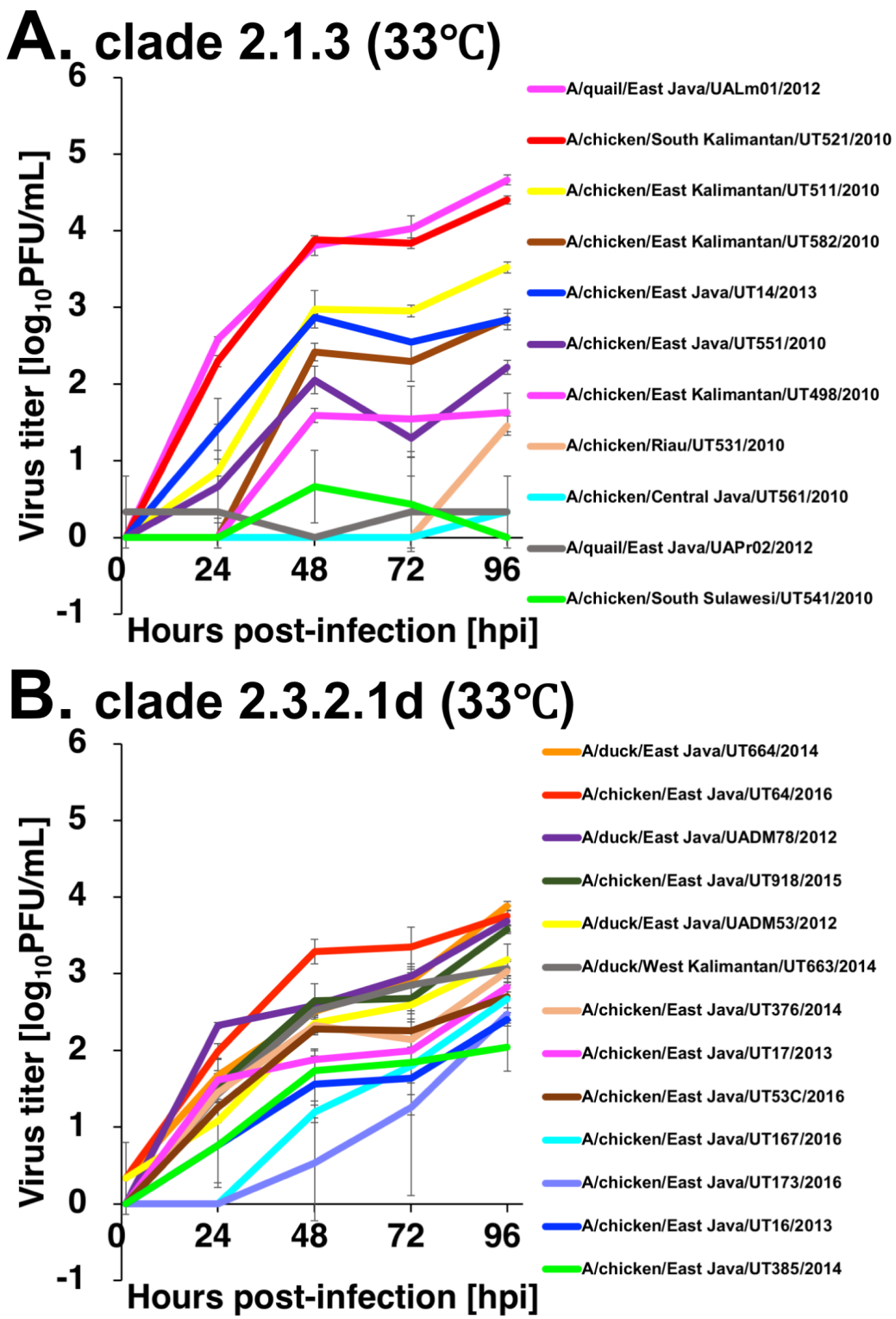
Figure 5. Phylogenetic trees of the eight gene segments of Indonesian H5N1 virus strains

Phylogenetic trees of the HA (A), NA (B), PB2 (C), PB1 (D), PA (E), NP (F), M (G), and NS (H) genes of Indonesian H5N1 virus strains were constructed by using the Neighbor-Joining method and MEGA 7. The trees were rooted to A/goose/Guangdong/1/1996. Virus strains colored in yellow indicate strains isolated during our surveillance activities between 2010 and 2016.

Replication efficiency in cultured cells

To assess the replicative ability of the isolated viruses in human respiratory cells, we compared the growth properties of 24 HPAI H5N1 viruses at an MOI of 0.0002 in A549 cells incubated at either 33℃ or 37℃. The replication efficiency of the clade 2.1.3 viruses differed among the strains (Fig. 6A, C). In contrast, clade 2.3.2.1d strains grew to a moderate degree without significant differences in replication efficiency among the strains (Fig. 6B, D). All 23 viruses replicated well in DF-1 cells incubated at 39℃ (Figure 7A–C).


A. clade 2.1.3 (33℃)
Virus titer [log10PFU/mL]
6
5
4
3
2
1
0
-1
0
24
48
72
96
Hours post-infection [hpi]
A/quail/East Java/UALm01/2012
A/chicken/South Kalimantan/UT521/2010
A/chicken/East Kalimantan/UT511/2010
A/chicken/East Kalimantan/UT582/2010
A/chicken/East Java/UT14/2013
A/chicken/East Java/UT551/2010
A/chicken/East Kalimantan/UT498/2010
A/chicken/Riau/UT531/2010
A/chicken/Central Java/UT561/2010
A/quail/East Java/UAPr02/2012
A/chicken/South Sulawesi/UT541/2010
B. clade 2.3.2.1d (33℃)
Virus titer [log10PFU/mL]
6
5
4
3
2
1
0
-1
0
24
48
72
96
Hours post-infection [hpi]
A/duck/East Java/UT664/2014
A/chicken/East Java/UT64/2016
A/duck/East Java/UADM78/2012
A/chicken/East Java/UT918/2015
A/duck/East Java/UADM53/2012
A/duck/West Kalimantan/UT663/2014
A/chicken/East Java/UT376/2014
A/chicken/East Java/UT17/2013
A/chicken/East Java/UT53C/2016
A/chicken/East Java/UT167/2016
A/chicken/East Java/UT173/2016
A/chicken/East Java/UT16/2013
A/chicken/East Java/UT385/2014

461

462

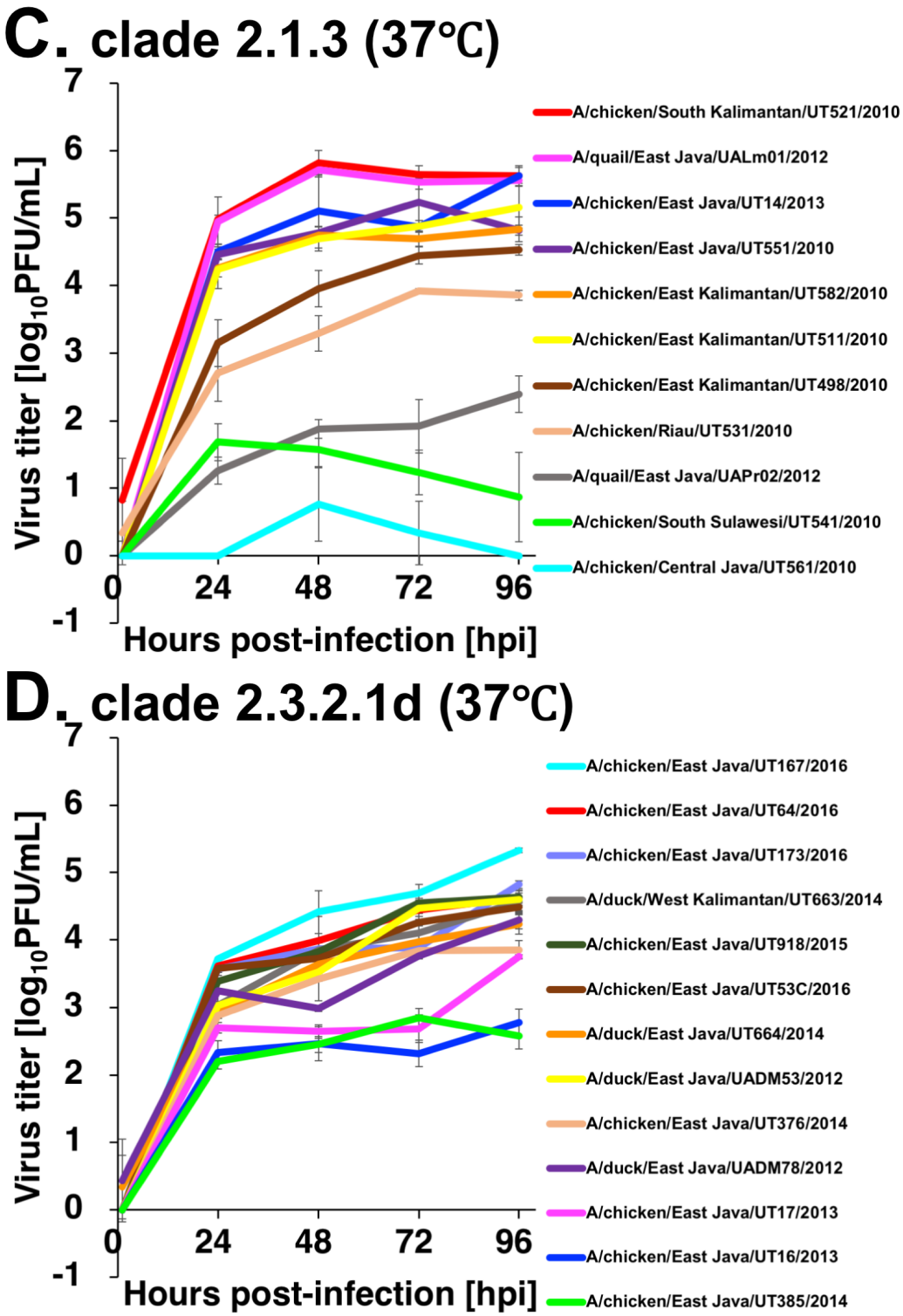
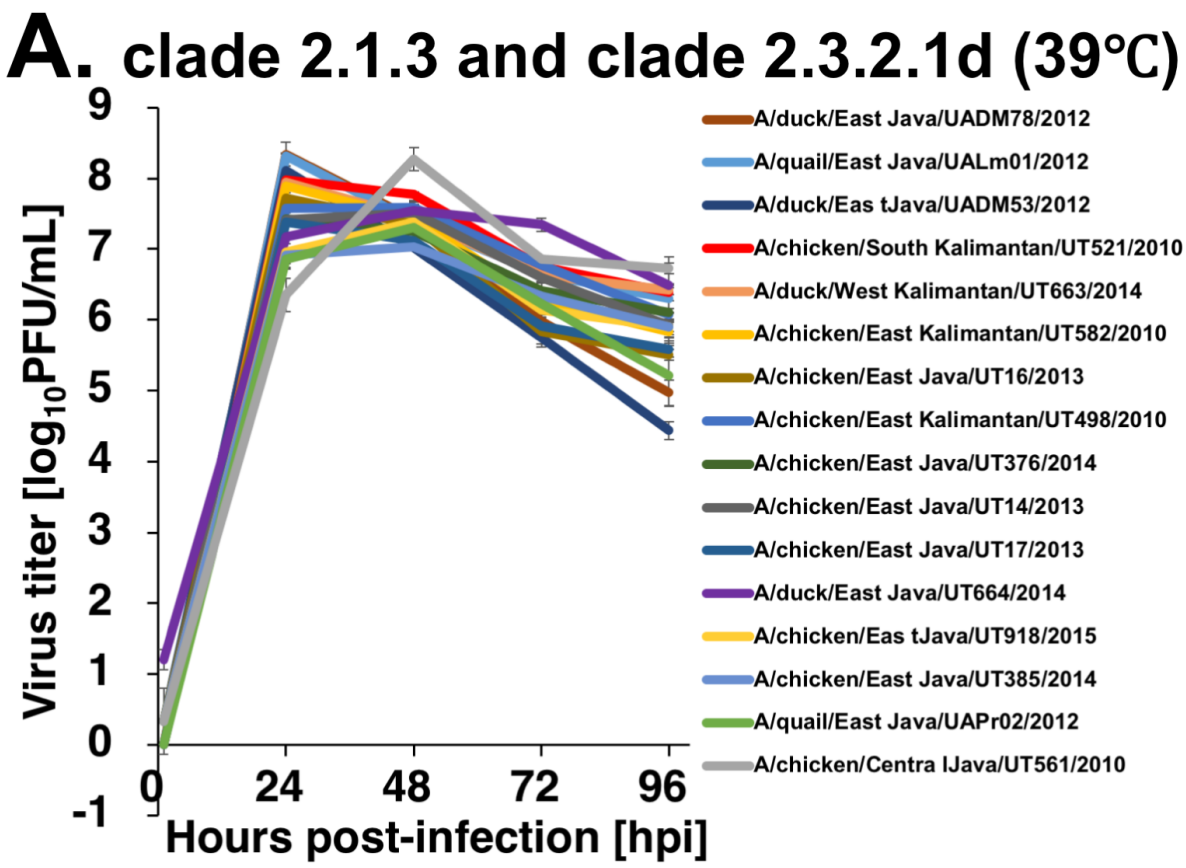
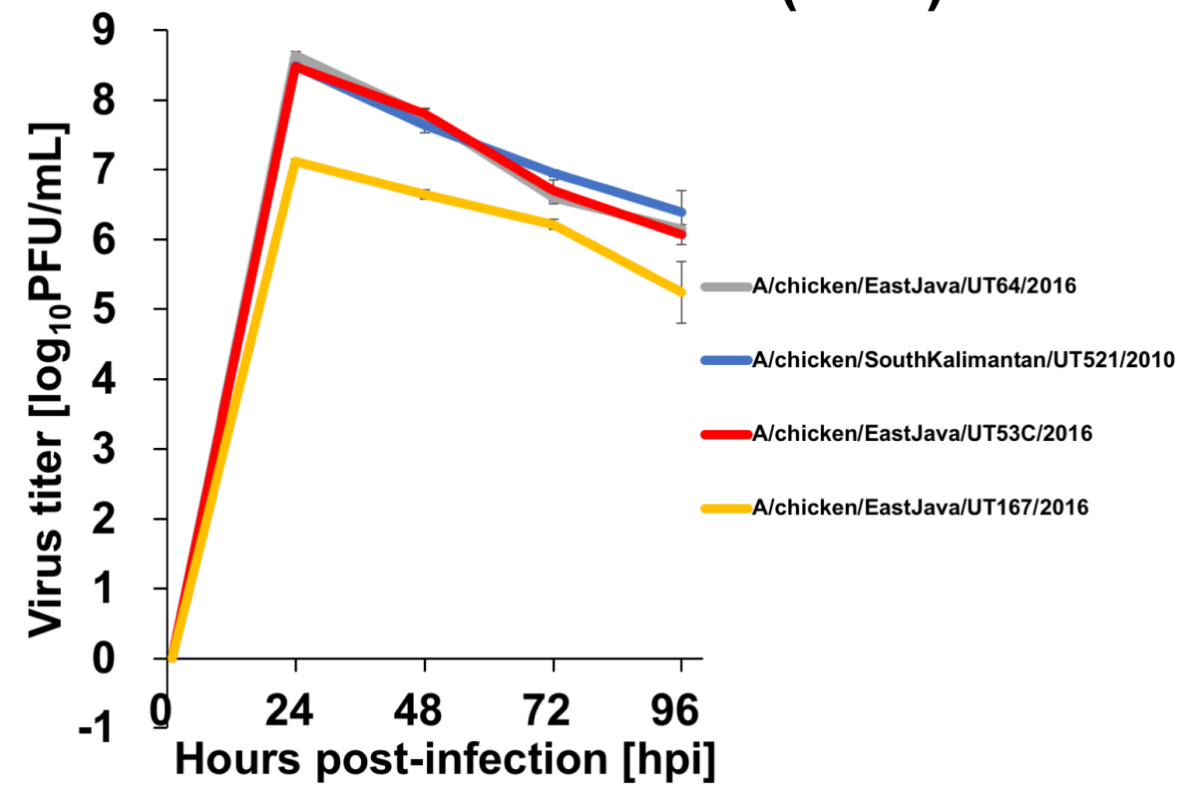


Figure 6. Virus growth in A549 cells incubated at 33℃ and 37℃

A549 cells were infected with clade 2.1.3 and clade 2.3.2.1d strains at an MOI of 0.0002 and then incubated at either 33℃ or 37℃ for 96 hours. The growth of clade 2.1.3 strains at 33℃ (A) and 37℃ (C), and the growth of clade 2.3.2.1d strains at 33℃ (B) and 37℃ (D) are shown. The data presented are the mean ± s.d. of triplicate experiments. The detection limit of this experiment is 1 $\log_{10}$PFU/ml.


A. clade 2.1.3 and clade 2.3.2.1d (39℃)
Virus titer [log10PFU/mL]
9
8
7
6
5
4
3
2
1
0
-1
0
24
48
72
96
Hours post-infection [hpi]
A/duck/East Java/UADM78/2012
A/quail/East Java/UALm01/2012
A/duck/Eas tJava/UADM53/2012
A/chicken/South Kalimantan/UT521/2010
A/duck/West Kalimantan/UT663/2014
A/chicken/East Kalimantan/UT582/2010
A/chicken/East Java/UT16/2013
A/chicken/East Kalimantan/UT498/2010
A/chicken/East Java/UT376/2014
A/chicken/East Java/UT14/2013
A/chicken/East Java/UT17/2013
A/duck/East Java/UT664/2014
A/chicken/Eas tJava/UT918/2015
A/chicken/East Java/UT385/2014
A/quail/East Java/UAPr02/2012
A/chicken/Centra lJava/UT561/2010


B. clade 2.3.2.1d in 2016 (39℃)
Virus titer [log10PFU/mL]
9
8
7
6
5
4
3
2
1
0
-1
0
24
48
72
96
Hours post-infection [hpi]
A/chicken/EastJava/UT64/2016
A/chicken/SouthKalimantan/UT521/2010
A/chicken/EastJava/UT53C/2016
A/chicken/EastJava/UT167/2016

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471

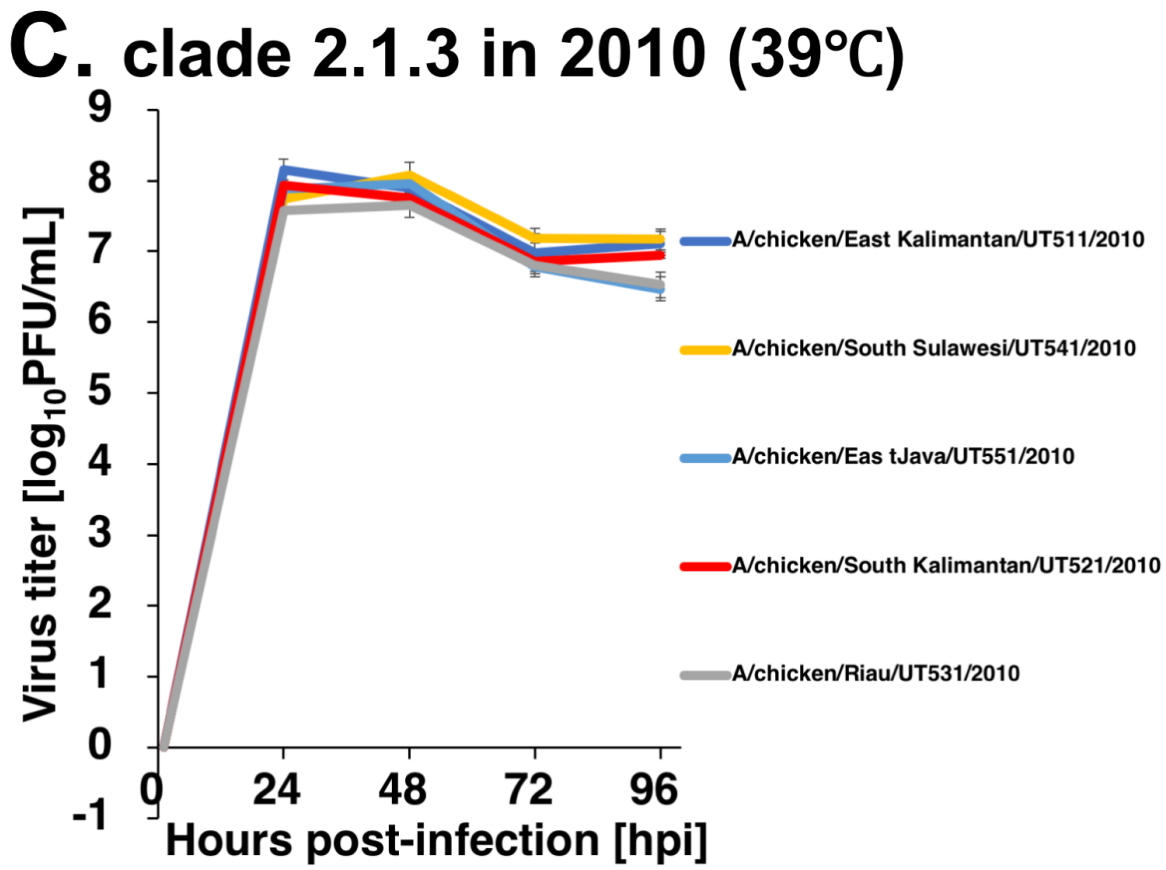


Figure 7. Virus growth in DF-1 cells at 39℃

Viral growth in DF-1 cells was analyzed by infecting the cells with virus at an MOI of 0.0002 and then incubated them at 39℃ for 96 hours. Three independent experiments were conducted; shown is the growth property analysis of clade 2.1.3 and clade 2.3.2.1d strains isolated between 2010 and 2015 (A), of clade 2.3.2.1d strains isolated in 2016 (B), and of additional clade 2.1.3 strains isolated in 2010 (C). A/chicken/East Java/UT521/2010 was included as a reference strain, which showed similar growth properties in all three experiments. The data presented are the mean ± s.d. of triplicate experiments. The detection limit of this experiment is 1 $\log_{10}$PFU/ml.

We also analyzed the replicative ability of H5N1 viruses in normal human bronchial epithelial (NHBE) cells (Figure 8A, B). Monolayers of NHBE cells were cultured and differentiated at the air-liquid interface, and then infected with three H5N1 virus strains, A/chicken/South Kalimantan/UT521/2010 (clade 2.1.3), A/chicken/East Kalimantan/UT498/2010 (clade 2.1.3), and A/chicken/East Java/UT376/2014 (clade 2.3.2.1d) at a viral concentration of 1000 PFU/well. A/California/04/2009 (H1N1 pdm09) virus was also used as a representative human influenza virus. A/California/04/2009 replicated efficiently in NHBE cells incubated at both 33℃ and 37℃. In contrast, the three H5N1 viruses showed inferior replicative efficiency in NHBE cells compared to A/California/04/2009, especially when incubated at 33℃. There was no significant difference in viral replication efficiency between A/chicken/South Kalimantan/UT521/2010 and A/chicken/East Java/UT376/2014. A/chicken/East Kalimantan/UT498/2010 replicated similarly to A/chicken/South Kalimantan/UT521/2010 and A/chicken/East Java/UT376/2014 at 37℃, but little viral replication was observed at 33℃.

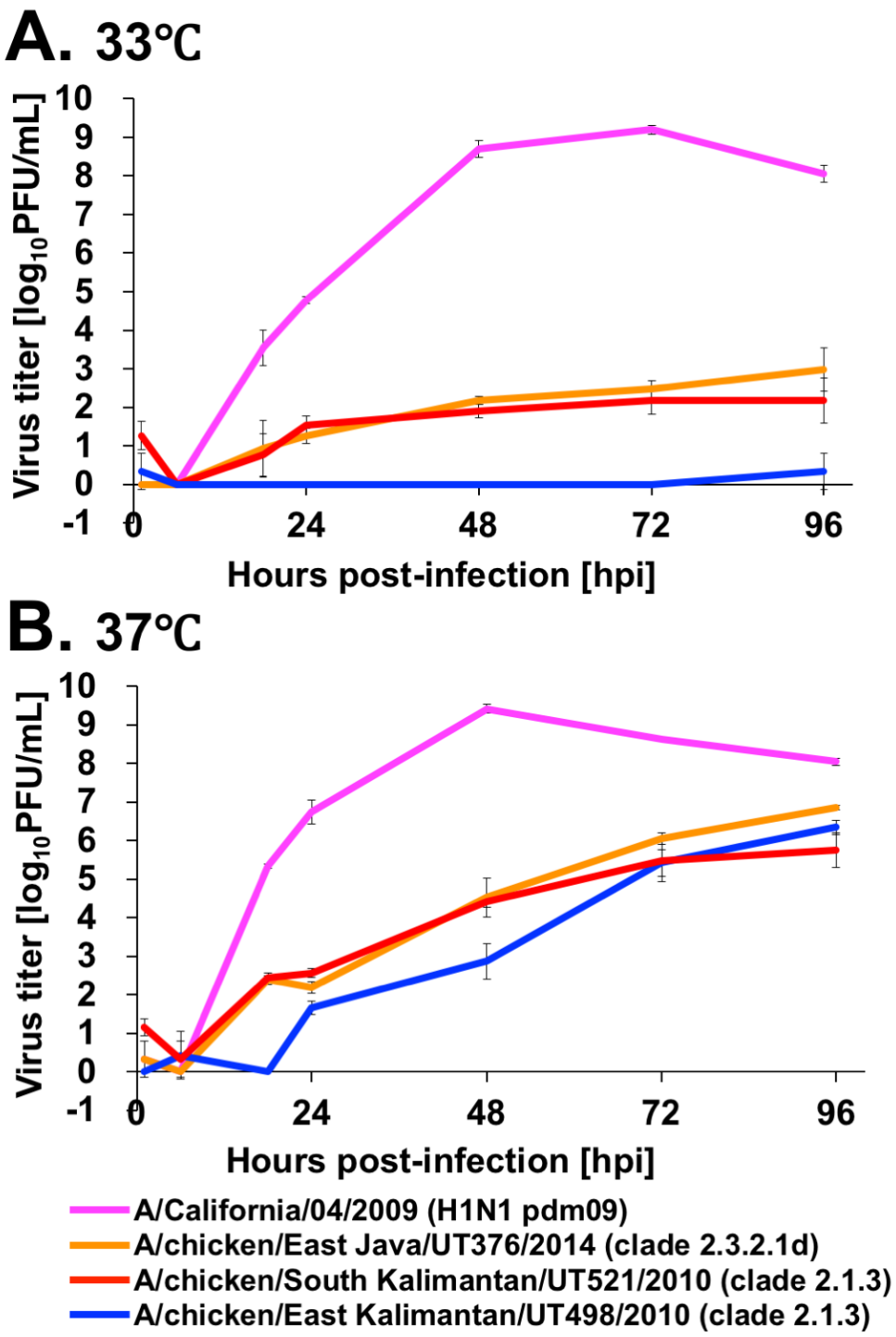
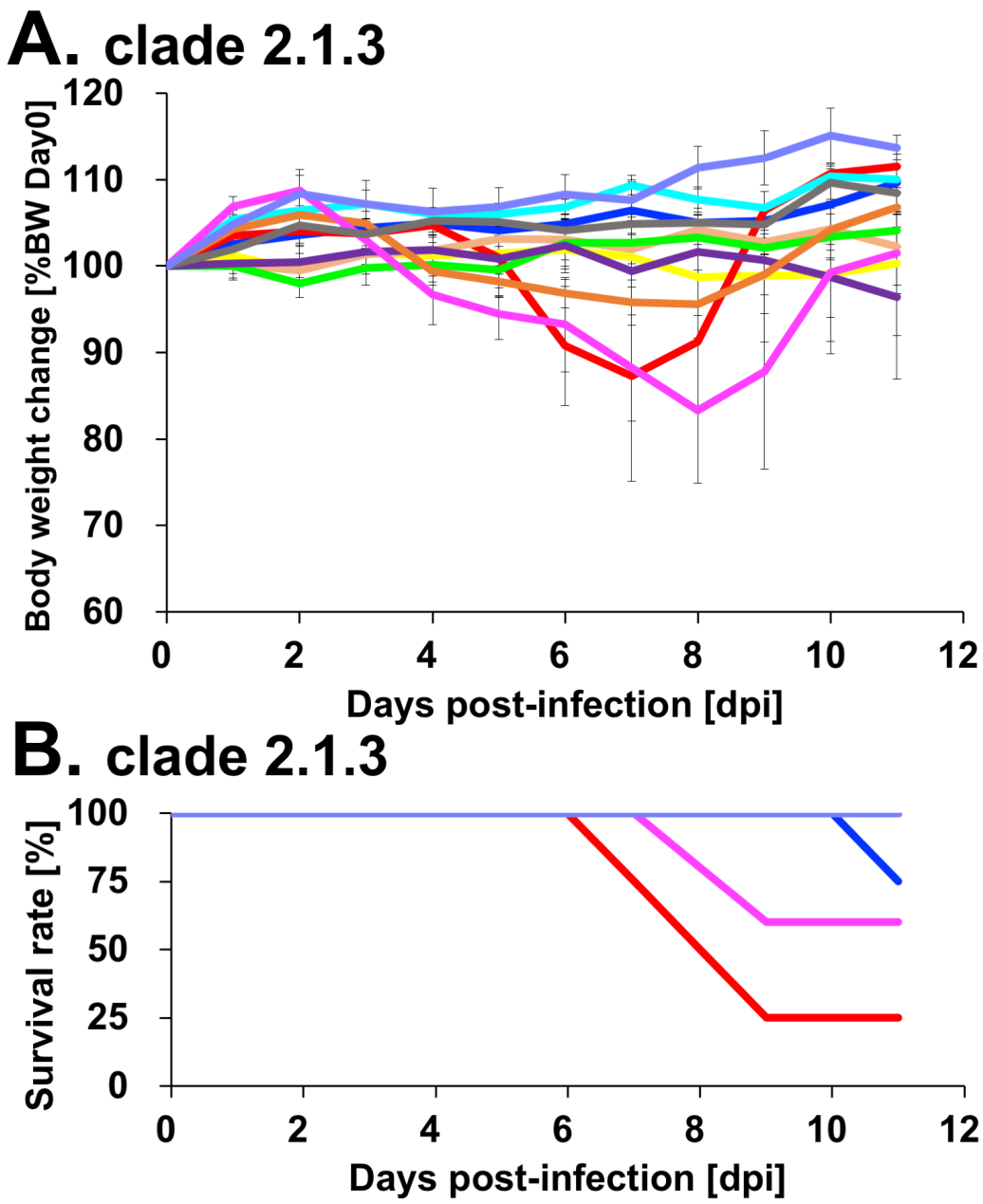


Figure 8. Virus growth in NHBE cells incubated at 33℃ and 37℃

Differentiated NHBE cells were infected with A/chicken/East Kalimantan/UT498/2010 (clade 2.1.3), A/chicken/South Kalimantan/UT521/2010 (clade 2.1.3), A/chicken/East Java/UT376/2014 (clade 2.3.2.1d), and A/California/04/2009 (H1N1 pdm09) at a viral concentration of 1000 PFU/well, and then incubated at either 33℃ (A) or 37℃ (B) for 96 hours. The data presented are the mean ± s.d. of triplicate experiments. The detection limit of this experiment is 1 $\log_{10}$PFU/ml.

516 **Pathogenicity in BALB/c mice**

517 To examine viral virulence in mammalian hosts, 6-week-old female BALB/c mice
518 were intranasally inoculated with H5N1 viruses at a concentration of $1x10^3$ PFU, and body
519 weight changes and the mortality of the inoculated mice were recorded for 12 days (Fig. 9A-
520 D). The pathogenicity of the clade 2.1.3 viruses differed among the strains, and the mortality
521 rate of the BALB/c mice ranged from 0% to 75% (Fig. 9A, B). The clade 2.1.3 strains
522 possessing high replication efficiency in A549 cells (i.e., A/chicken/South
523 Kalimantan/UT521/2010 and A/quail/East Java/UALm01/2012) (Fig. 6A, B) also showed
524 high pathogenicity in BALB/c mice. In contrast, all clade 2.3.2.1d virus strains caused a
525 uniform decrease in body weight and a mortality rate of 100%, except for A/chicken/East
526 Java/UT167/2016 (Fig. 9C, D). A/chicken/East Java/UT167/2016 belonged to clade 2.3.2.1d,
527 but possessed a PB2 segment that is different from other clade 2.3.2.1d viruses (reassortant
528 PB2 segment from hereafter) and lacked the three PB2 mammalian-adaptive amino acids (i.e.,
529 PB2-147T-339T-588T) [60]. BALB/c mice infected with A/chicken/East Java/UT167/2016
530 showed little body weight change and their mortality rate was 25%.
531
532


A. clade 2.1.3
Body weight change [%BW Day0]
120
110
100
90
80
70
60
0
2
4
6
8
10
12
Days post-infection [dpi]
B. clade 2.1.3
Survival rate [%]
100
75
50
25
0
0
2
4
6
8
10
12
Days post-infection [dpi]
A/chicken/East Kalimantan/UT498/2010
A/chicken/East Kalimantan/UT511/2010
A/chicken/South Kalimantan/UT521/2010
A/chicken/Riau/UT531/2010
A/chicken/South Sulawesi/UT541/2010
A/chicken/East Java/UT551/2010
A/chicken/Central Java/UT561/2010
A/chicken/East Kalimantan/UT582/2010
A/quail/East Java/UALm01/2012
A/quail/East Java/UAPr02/2012
A/chicken/East Java/UT14/2013

533

534

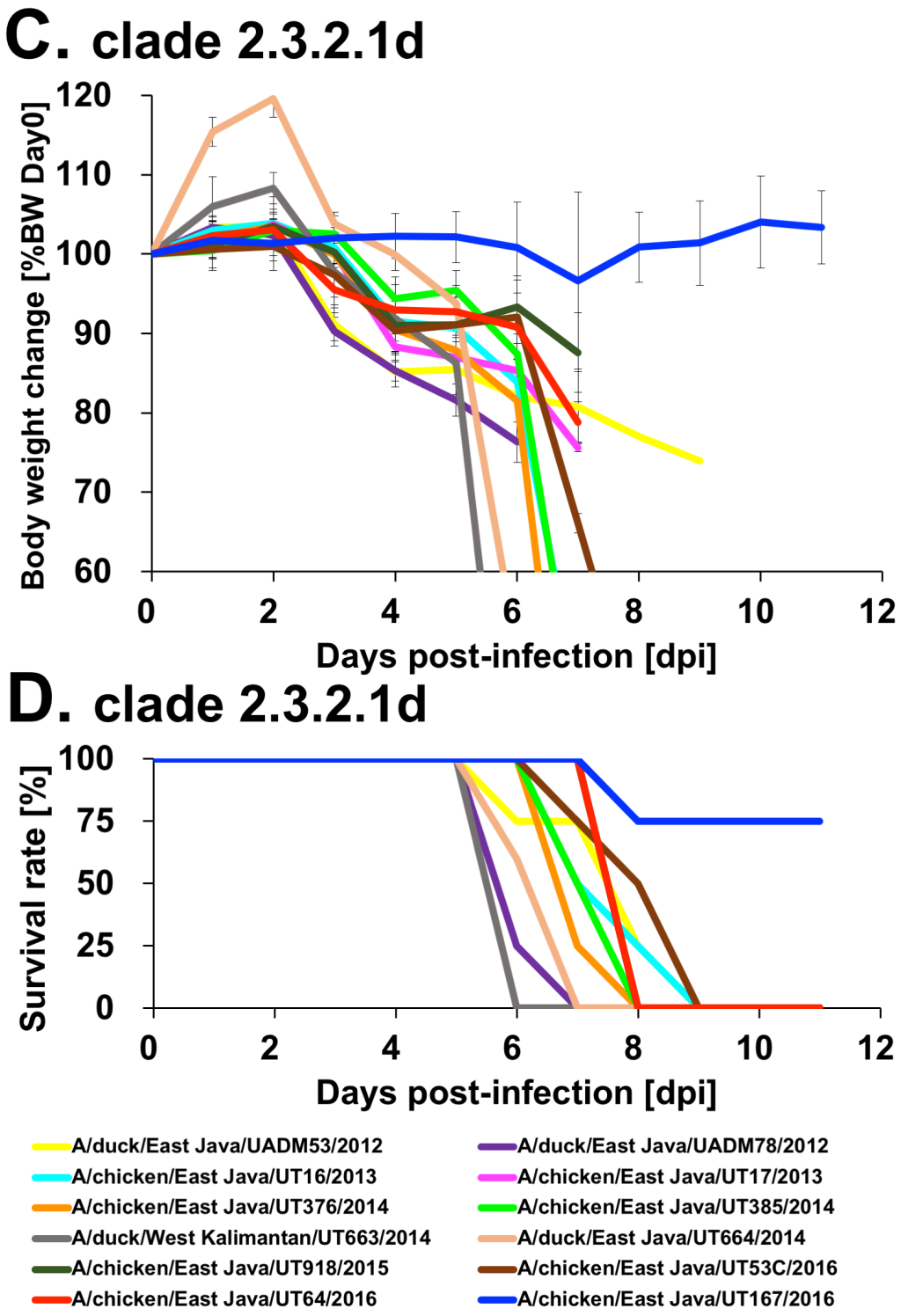


Figure 9. Pathogenicity in BALB/c mice

6-week-old female BALB/c mice were intranasally inoculated with $1x10^3$ PFU/animal of H5N1 virus strains, and their body weight change and survival rate were observed for 12 days. Body weight change (A) and survival rate (B) of mice infected with clade 2.1.3 viruses, and body weight change (C) and survival rate (D) of mice infected with clade 2.3.2.1d viruses are shown. The values for body weight change are the mean ± s.d. of live BALB/c mice.

Mammalian-adaptive amino acid mutations in the PB2 protein of clade 2.3.2.1d strains

Sequence analysis of PB2 genes revealed that clade 2.3.2.1d strains possessed a set of three amino acids known to increase virulence in mammalian hosts: PB2-147T, -339T, and -588T [60]. These three PB2 amino acids, originally found in H5N1 strains from Viet Nam, increase viral polymerase activity and replication efficiency in mammalian cells, and also enhance pathogenicity in mice [60]. However, the corresponding amino acids of the six strains (A/chicken/East Java/UT167/2016, A/chicken/East Java/UT167C/2016, A/chicken/East Java/UT168/2016, A/chicken/East Java/UT170/2016, A/chicken/East Java/UT173/2016, A/chicken/East Java/UT173C/2016) with a reassortant PB2 segment were PB2-147I, 339K, and 588A. None of the isolated virus strains possessed the well known mammalian-adaptive amino acid mutations PB2-591K, -627K, or -701N [48-50].

Viral polymerase activity

Viral polymerase activity is an important determinant of viral pathogenicity and host-range specificity. To assess the viral polymerase activity of the isolated H5N1 virus strains, a mini-replicon assay in A549 cells and DF-1 cells was performed on seven H5N1 virus strains (three strains belonging to clade 2.1.3, and four strains to clade 2.3.2.1d).

559 A/Puerto Rico/8/34 (H1N1) (PR8) was also included in the analysis as a mammalian strain

560 (Fig. 10A-C). Among the analyzed virus strains including PR8, the difference in viral

561 polymerase activity in DF-1 cells incubated at 39℃ was less than two fold. In A549 cells,

562 Indonesian H5N1 viruses possessed lower viral polymerase activity compared with that of

563 PR8, and this difference was more pronounced when the A549 cells were incubated at 33℃.

564 Clade 2.3.2.1d strains showed increased viral polymerase activity in A549 cells compared

565 with that of clade 2.1.3 strains, especially at 33℃. Among the clade 2.1.3 strains,

566 A/chicken/East Java/UT14/2013, isolated in 2013, possessed higher viral polymerase activity

567 in A549 cells than A/chicken/South Kalimantan/UT521/2010 or A/chicken/South

568 Sulawesi/UT541/2010. As to clade 2.3.2.1d strains, A/chicken/East Java/UT17/2013 and

569 A/chicken/East Java/UT64/2016 showed similar viral polymerase activity in A549 cells.

570 However, the clade 2.3.2.1d strains A/chicken/East Java/UT167/2016 and A/chicken/East

571 Java/UT173/2016, which have a reassortant PB2 segment, showed low viral polymerase

572 activity in A549 cells, similar to that of clade 2.1.3 strains.

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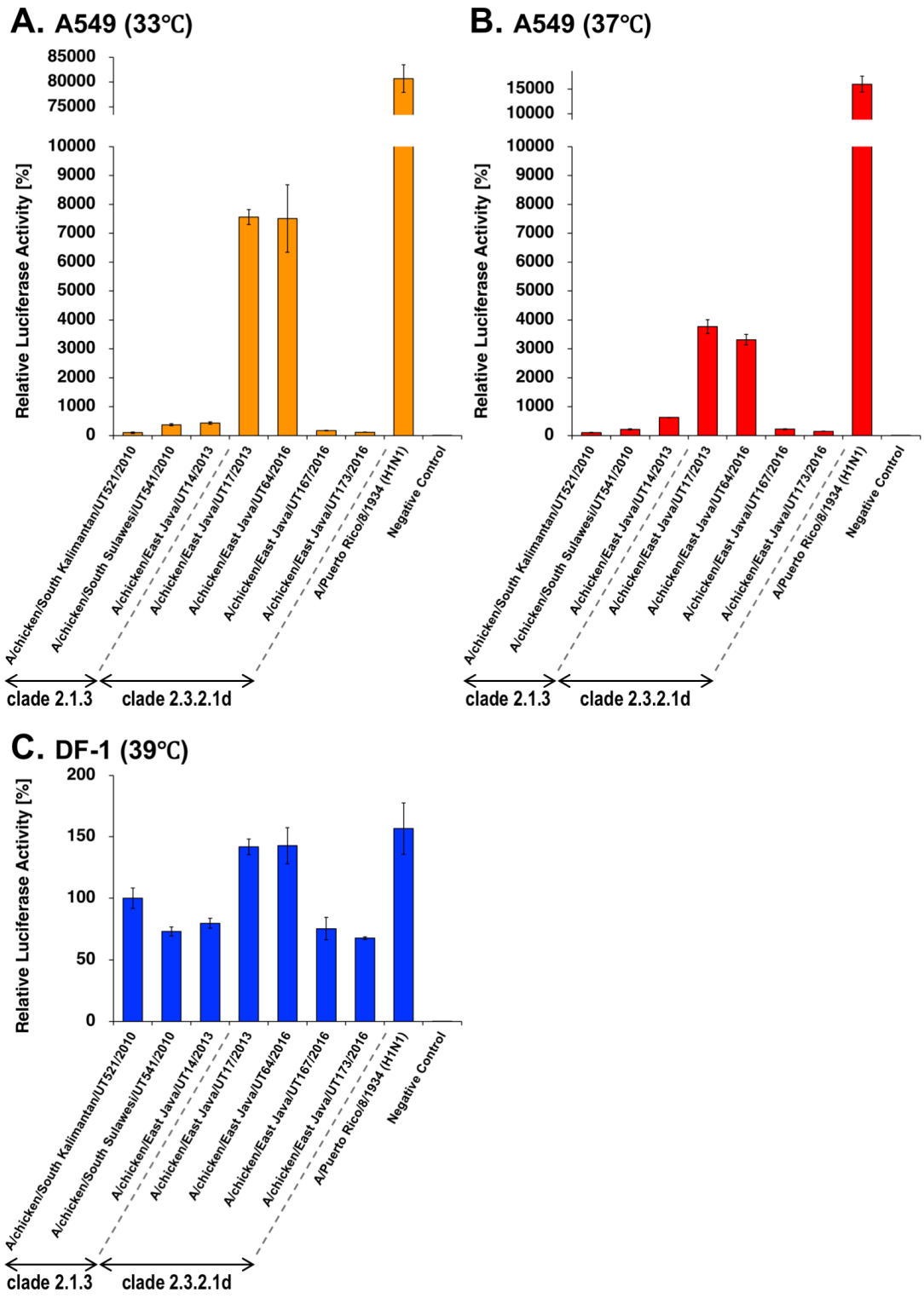

Figure 10. Viral polymerase activity

A549 cells and DF-1 cells were transfected with 0.1 μg each of the pCAGGS constructs encoding PB2, PB1, PA, NP, firefly luciferase, and Renilla luciferase. A549 cells were incubated at 33°C for 48 hours (A), or at 37°C for 24 hours (B), and DF-1 cells were incubated at 39°C for 24 hours (C); then, the mini-replicon assay was performed. The data presented are the mean ± s.d. of triplicate experiments.

583 **Amino acid mutations in the HA protein of clade 2.1.3 and clade 2.3.2.1d strains**

584 Sequencing of the HA genes of the isolated viruses revealed multiple amino acid
585 mutations located around the receptor binding site. Among the clade 2.1.3 strains,
586 A/chicken/South Kalimantan/UT521/2010 and A/chicken/South Sulawesi/UT541/2010
587 possessed HA-137A and HA-193R, which are known to increase receptor binding affinity for
588 human-type sialic acid-linked galactose (Sia α 2,6Gal) [46, 61, 62]. A/chicken/South
589 Kalimantan/UT521/2010 and A/chicken/South Sulawesi/UT541/2010 also harbored unique
590 amino acid mutations (appearing in less than 40% of virus strains belonging to clade 2.1.3)
591 inside and around the receptor binding site (HA-144N, -187D, and -188A) (Fig. 11A). Clade
592 2.3.2.1d strains possessed the following previously reported mammalian-adaptive amino acid
593 mutations: HA-101N, -137A, -159N, -160A, and -193R [46, 61-64]. Clade 2.3.2.1d strains
594 also harbored unique amino acid mutations (appearing in less than 40% of virus strains
595 belonging to clade 2.3.2.1d) inside and around the receptor binding site (HA-120R, 145P, -
596 193K, and -222R) (Fig. 11B). Clade 2.3.2.1d strains have lost the glycosylation site at HA-
597 158, which is also known to increase human-type receptor binding affinity [46, 62, 65].
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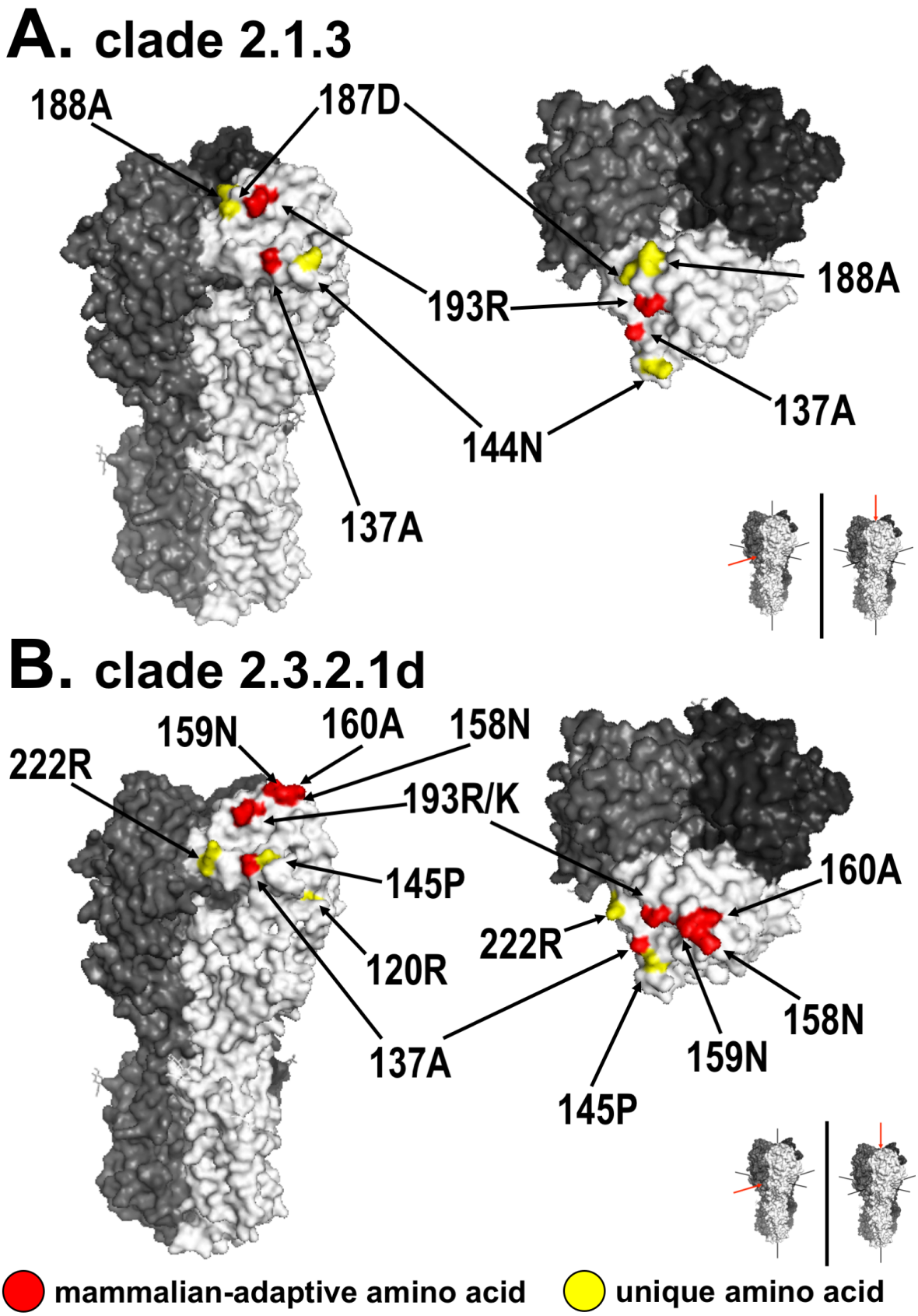
599
600


A. clade 2.1.3
188A
187D
193R
188A
137A
144N
137A
B. clade 2.3.2.1d
159N
160A
158N
222R
193R/K
145P
160A
120R
222R
158N
137A
159N
145P
mammalian-adaptive amino acid
unique amino acid

601

602

Figure 11. Characteristic amino acids around the HA receptor binding site on the structural model of HA trimer

A structural model of the HA trimer was created by PyMOL using protein data obtained from the Protein Data Bank (ID: 4KTH) [66]. Among amino acids of isolated Indonesian H5N1 viruses belonging to clade 2.1.3 (A) or clade 2.3.2.1d (B), the previously reported mammalian-adaptive amino acids are colored in red, while unique amino acids are colored in yellow. The amino acid HA-101N was located on the surface of internal side of HA trimer on the model, thus was not shown in the figure. All the amino acids' positions are described in H3 numbering.

HA receptor binding assay

To evaluate the receptor binding properties of the HA of recent Indonesian H5N1 virus strains, I performed a receptor binding assay on seven H5N1 virus strains - three clade 2.1.3 strains (A/chicken/South Kalimantan/UT521/2010, A/chicken/South Sulawesi/UT541/2010, and A/chicken/East Java/UT14/2013) and four clade 2.3.2.1d strains (A/chicken/East Java/UT17/2013, A/chicken/East Java/UT64/2016, A/chicken/East Java/UT167/2016, and A/chicken/East Java/UT173/2016). Despite amino acid mutations around the HA receptor binding site, all seven Indonesian H5N1 virus strains recognized Sia α 2,3Gal, but did not bind to Sia α 2,6Gal (Figure 12A-G).

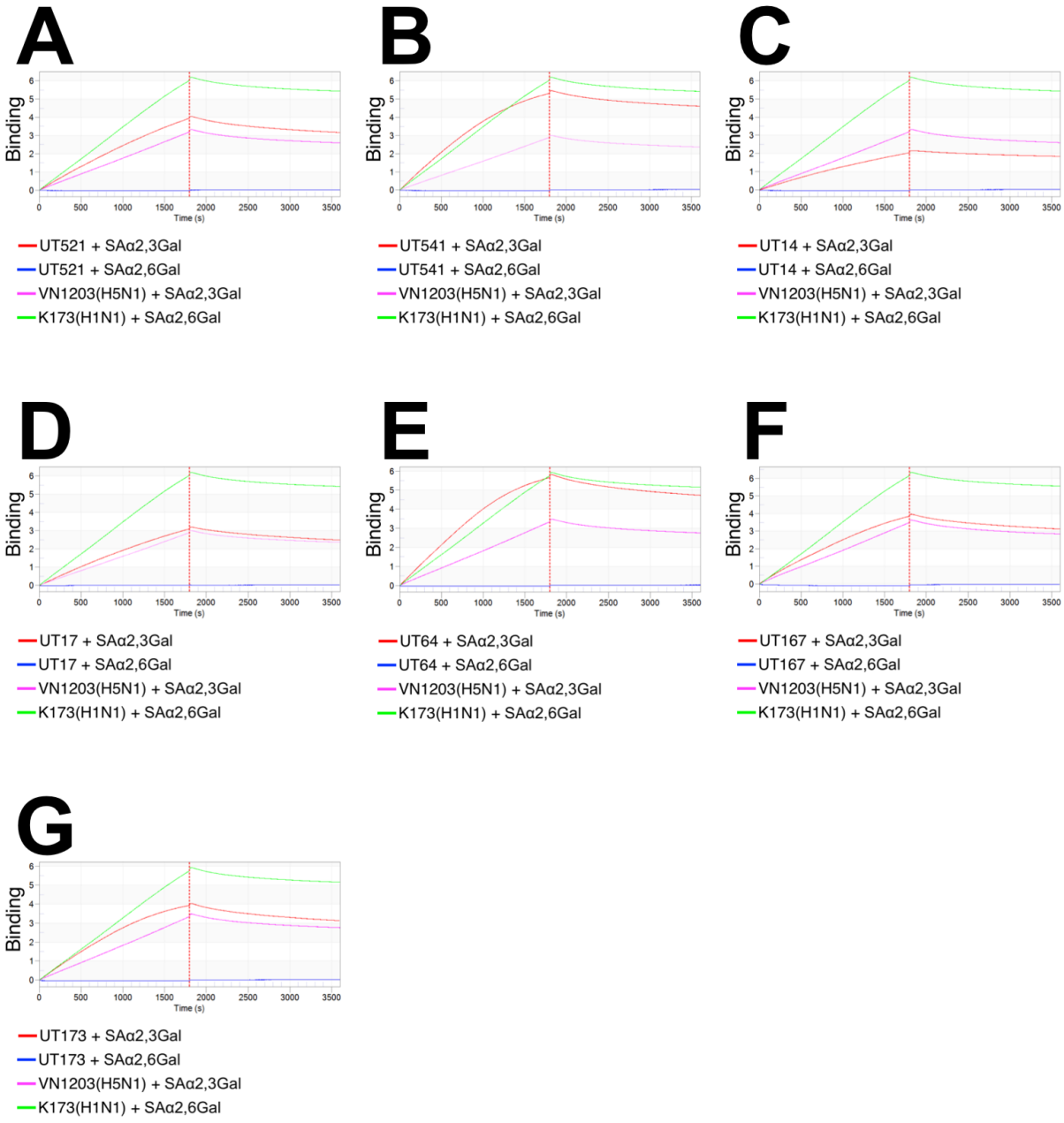


Figure 12. HA receptor binding assay

The binding of each virus strain to Sia α 2,3Gal (red) or Sia α 2,6Gal(blue) was analyzed. Analyzed virus strains are as follows: A/chicken/South Kalimantan/UT521/2010 (UT521) (A), A/chicken/South Sulawesi/UT541/2010 (UT541) (B), A/chicken/East Java/UT14/2013 (UT14) (C), A/chicken/East Java/UT17/2013 (UT17) (D), A/chicken/East Java/UT64/2016 (UT64) (E), A/chicken/East Java/UT167/2016 (UT167) (F), and A/chicken/East Java/UT173/2016 (UT173) (G). A/Vietnam/1203/2004 (VN1203) (H5N1) bound to Sia α 2,3Gal (pink), and A/Kawasaki/173/2001 (K173) (H1N1) bound to Sia α 2,6Gal (green) served as controls. All virus strains bound to Sia α 2,3Gal, but did not bind to Sia α 2,6Gal.

Discussion

During our surveillance activities in Indonesia between 2010 and 2016, HPAI H5N1 virus strains were constantly isolated from poultry and waterfowl. During this timeframe, HPAI H5N1 virus strains were also listed on databases such as the influenza virus research (IVR) and global initiative on sharing all influenza data (GISAID) databases. Therefore, it can be stated that HPAI H5N1 viruses are constantly circulating in poultry and waterfowl in Indonesia.

Clade 2.1.3 strains had been circulating in Indonesia since 2003 [34, 36, 38]. After the emergence of clade 2.3.2.1d strains in 2012, both clade 2.1.3 and clade 2.3.2.1d strains were isolated between 2012 and 2013, suggesting co-circulation of these two different H5N1 subclades in Indonesia during that period [51].

During our surveillance, only clade 2.3.2.1d virus strains were isolated after 2014. As of October 29, 2018, the HA genes of 36 HPAI H5N1 virus strains, which were isolated between 2014 and 2018, were listed on the IVR and GISAID databases. Of these 36 virus strains, 31 were isolated from avian hosts, and five were isolated from humans. Among the 31 isolated from avian hosts, only two strains belong to clade 2.1.3; the latest clade 2.1.3 strain was isolated in 2016, in Deliserdang, Northern Sumatra.

664 Clade 2.3.2.1d strains are suspected to have been introduced into Indonesia in 2012
665 via an unknown route [67]. In the phylogenetic tree of HA genes, Indonesian clade 2.3.2.1d
666 strains are located in close proximity to clade 2.3.2.1c strains isolated in Viet Nam between
667 2012 and 2017 (Fig. 5A). NCBI nucleotide BLAST analysis of clade 2.3.2.1d strains,
668 A/duck/East Java/UADM53/2012, and A/chicken/East Java/UT17/2013 revealed multiple
669 H5N1 strains isolated in Viet Nam in 2012 and 2013 with 99% identicality.
670 Our surveillance data and the databases possess sampling bias and, therefore,
671 cannot stand alone to make an assertive argument. That being said, the possibility exists that
672 clade 2.3.2.1d virus strains have become dominant among poultry in Indonesia since their
673 introduction to Indonesia in 2012. Our characterization analysis revealed no significant
674 difference in virus replication efficiency or in viral polymerase activity in DF-1 cells between
675 clade 2.1.3 strains and clade 2.3.2.1d strains. Our data thus cannot provide an explanation for
676 the suspected dominance of clade 2.3.2.1d strains over clade 2.1.3 strains in poultry.
677 The HA receptor binding assay showed that neither clade 2.1.3 nor clade 2.3.2.1d
678 Indonesian H5N1 viruses have acquired the ability to bind to α2,6-linked
679 sialylglycopolymers, which is essential for efficient infection and transmission among
680 humans [44-46]. Human influenza virus strains are reported to preferentially bind to longer

681 α 2,6-linked sialylglycopolymers; however, only short unbranched sialylglycopolymers were
682 used in our HA receptor binding assay [68, 69]. Hence, we cannot exclude the possibility that
683 the HA of Indonesian H5N1 viruses has acquired some level of human-type receptor binding
684 capability. Our characterization analysis revealed that clade 2.3.2.1d strains possess high
685 pathogenicity in BALB/c mice, resulting in 100% mortality when inoculated at a viral
686 concentration of $1x10^3$ PFU per animal. This high virulence likely resulted from the presence
687 of three PB2 amino acids (PB2-47T, -339T, and -588T), as previously reported [60]. The viral
688 polymerase activity of the clade 2.3.2.1d strains in A549 cells was also increased compared
689 with that of clade 2.1.3 strains. While the replication efficiency of clade 2.1.3 strains varied
690 among strains infecting A549 cells, clade 2.3.2.1d strains showed moderate replication
691 efficiency in these cells at both 33℃ and 37℃, without significant differences among the
692 virus strains.
693 When discussing characterization differences between clade 2.1.3 and clade
694 2.3.2.1d strains, we are limited by sampling biases, especially with respect to the sampling
695 locations. The analyzed clade 2.1.3 strains were collected in various areas of Indonesia
696 between 2010 and 2013, mostly from chickens. In contrast, the analyzed clade 2.3.2.1d strains
697 were collected mostly in East Java between 2013 and 2016, and their hosts were either

698 chickens or ducks. Therefore, it is possible that the uniform viral characteristics, high
699 virulence in BALB/c mice, and moderate replication efficiency in A549 cells of the observed
700 clade 2.3.2.1d strains are limited to such strains circulating in East Java, and do not represent
701 clade 2.3.2.1d strains from other areas of Indonesia.

702 It is difficult to assess the infectivity and virulence of Indonesian H5N1 virus strains
703 in humans. Human H5N1 cases in Indonesia have been regularly reported to the WHO, but
704 the number of cases has drastically decreased since 2010; only five human cases have been
705 reported since 2014 (Fig. 3). Interestingly, among the five H5N1 virus strains isolated from
706 these human hosts, four strains belonged to clade 2.1.3, and only one strain, isolated in 2017,
707 belonged to clade 2.3.2.1d. In Viet Nam, where clade 2.3.2.1c strains are circulating, the last
708 human cases of H5N1 infection were reported in 2014.

709 Our analysis of the Indonesian clade 2.3.2.1d strains revealed that they possess
710 strong virulence in BALB/c mice and that their viral polymerase activity is higher in A549
711 cells than that of clade 2.1.3 strains. Clade 2.3.2.1d strains can replicate to a moderate degree
712 in A549 cells, but their replication efficiency was not superior to that of some clade 2.1.3
713 strains despite having higher polymerase activity in A549 cells. The dissociation between the
714 observed viral characteristics and the decrease in reported human H5N1 cases might be due to

715 the inability of clade 2.3.2.1d strains to efficiently infect human hosts. Another possibility is
716 that the clade 2.3.2.1d viruses establish subclinical or mild infections that are overlooked, and
717 the number of infected patients is actually underestimated [70-72].

718 During our surveillance, we detected HPAI H5N1 virus clade 2.3.2.1d strains with
719 reassortant PB2 segments of unknown origin. The identified reassortant virus strains did not
720 possess higher replication efficiency or pathogenicity in mammalian hosts compared to other
721 clade 2.3.2.1d strains currently circulating in Indonesia. However, the possibility of human-
722 adapted HPAI H5N1 virus strains emerging through reassortment remains. This risk warrants
723 the establishment of further surveillance of HPAI viruses in Indonesia.

724 According to reported epidemiological data, the number of human H5N1 infection
725 cases in Indonesia has remained low for the past five years, after the emergence of the clade
726 2.3.2.1d strains. The clade 2.3.2.1d strains, however, were revealed to possess moderate
727 replication capability in A549 cells, and their polymerase activity in A549 cells is high
728 compared to the previously dominant clade 2.1.3 strains. Moreover, clade 2.3.2.1d strains are
729 highly virulent in BALB/c mice. The possibility cannot be dismissed that Indonesian H5N1
730 virus strains might acquire efficient infectivity and transmissibility to humans through viral
731 reassortment and amino acid mutations. Continuation and reinforcement of HPAI surveillance

732 in Indonesia is crucial for prevention and early detection of such human-adapted HPAI H5N1

733 viruses.

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Chapter 2: Amino acid substitutions in the PB2 protein of an H5N1 avian influenza virus enhance its replication efficiency and pathogenicity in mammalian hosts.

Introduction

HPAI H5N1 viruses have been enzootic among poultry worldwide since 1996, and 860 human cases of HPAI H5N1 infection have been reported to the WHO as of October, 2018, with a high mortality rate [19, 22, 23]. However, so far, human HPAI H5N1 cases have been limited to those with close contact with infected poultry, and it is suspected that efficient human-to-human transmission of HPAI H5N1 has not yet taken place in nature. In order for HPAI H5N1 viruses to overcome host range restriction and induce epidemics or global pandemics, they need to acquire mammalian-adaptive qualities such as efficient infection and replication in humans, human-to-human transmissibility, and evasion of human immune responses to viral infection [2, 10].

Among the influenza A viral proteins, PB2 is considered a major determinant of virulence, pathogenicity, and host range restriction [10]. The PB2 protein is an essential component of the viral polymerase complex, together with the PB1, PA, and NP proteins. Various mammalian-adaptive mutations of PB2 have been identified, including PB2-627K,

766 PB2-701N, and PB2-591K [48-50]. HPAI H5N1 virus strains possessing such mammalian-
767 adaptive PB2 segments are already circulating worldwide. For example, HPAI H5N1 clade
768 2.2.1 viruses currently circulating in Egypt typically possess PB2-627K [73-75]. HPAI H5N1
769 clade 2.3.2.1c viruses circulating in Viet Nam and clade 2.3.2.1d viruses currently circulating
770 in Indonesia possess three amino acids in PB2, namely PB2-47T, -339T, and -588T, which
771 together confer high viral polymerase activity and virulence in mammalian hosts [60].
772 Our characterization analysis of Indonesian HPAI H5N1 virus strains isolated
773 between 2010 and 2016 revealed two genetically closely related strains belonging to clade
774 2.1.3 with different replication efficiency and pathogenicity in mammalian hosts:
775 A/chicken/South Kalimantan/UT521/2010 (UT521) and A/chicken/South
776 Sulawesi/UT541/2010 (UT541) (Fig. 13). UT521 possessed high replication efficiency in
777 A549 cells and high pathogenicity in BALB/c mice (Fig. 6A, 6C, 9A, 9B). In contrast, UT541
778 possessed low replication efficiency and low pathogenicity. Sequencing analysis identified 36
779 different amino acids between UT521 and UT541 (Table 3). The amino acid sequences of the
780 Indonesian clade 2.1.3 strains listed in the IVR and GISAID databases were also analyzed.
781 Amino acids prevalent among more than 40% of Indonesian clade 2.1.3 strains were defined
782 as major amino acids, whereas amino acids, of which less than 10% of Indonesian clade 2.1.3

783 strains are possessed, were defined as unique amino acids. In Table 3, the unique amino acids
784 are colored in yellow. Our goal was to identify the viral factors responsible for the observed
785 differences in viral character between the two strains by using reassortant and mutant HPAI
786 H5N1 viruses.
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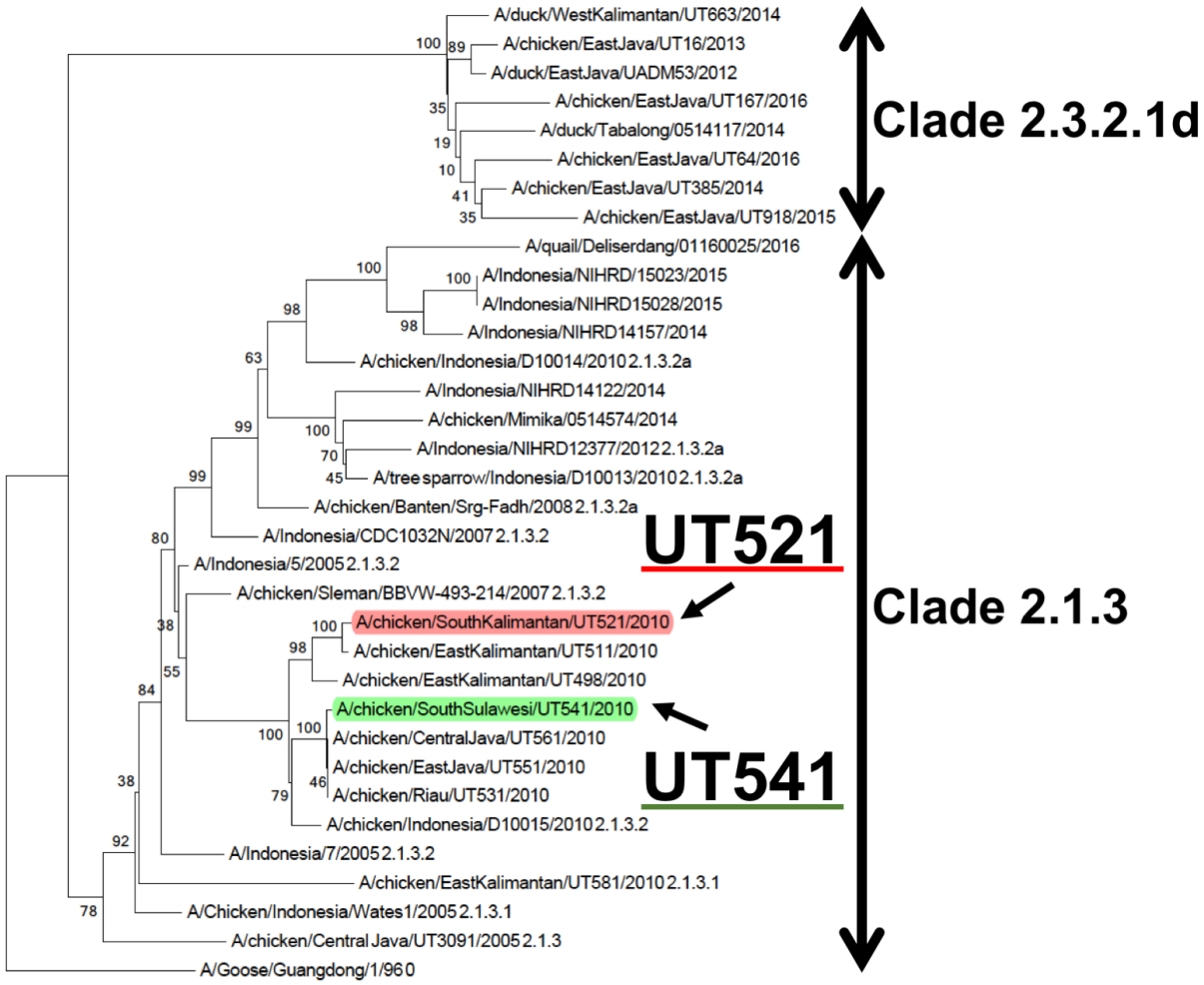


Figure 13. Phylogenetic tree of the HA genes of Indonesian H5N1 strains

The phylogenetic tree of the HA genes was constructed using the neighboring-joining method and MEGA 7. The tree was rooted to A/goose/Guangdong/1/96. UT521 (colored in red) and UT541 (colored in green), which both belong to clade 2.1.3, are located close together in the phylogenetic tree of the HA genes.

Table 3. Amino acids differences between UT521 and UT541

Virus	HA							NA						NS1					NS2
	86	129	140	214	373	511	528	32	50	53	111	120	143	53	126	188	207	210	36
A/chicken/SouthKalimantan/UT521/2010	T	L	N	T	R	I	A	I	N	K	T	L	M	D	R	Q	F	S	K
A/chicken/SouthSulawesi/UT541/2010	N	S	S	A	K	V	S	L	S	N	I	M	V	N	K	R	L	P	E

Virus	PB2					PB1			PA				M2				
	109	292	369	447	758	117	331	339	294	396	535	602	20	31	58	90	95
A/chicken/SouthKalimantan/UT521/2010	A	A	K	L	T	T	K	V	A	D	Q	A	I	S	E	Y	E
A/chicken/SouthSulawesi/UT541/2010	V	T	N	Q	I	A	E	I	D	N	H	V	T	G	G	H	A

□ major amino acid

■ unique amino acid

Nucleotide sequences were analyzed by direct sequencing. Thirty-six amino acids were different between UT521 and UT541. Unique amino acids are colored in yellow in the table. Both UT521 and UT541 possessed unique amino acid of PB2 at position 369, and the major amino acid was arginine. All the amino acids' positions are described in H5 numbering.

Results

Generation by reverse genetics and characterization of A/chicken/South Kalimantan/UT521/2010 and A/chicken/South Sulawesi/UT541/2010

UT521 and UT541 were generated by using reverse genetics [27]. RG1 indicates the reverse genetics strain of UT521, and RG17 indicates the reverse genetics strain of UT541. All virus strains with RG in the name were generated by means of reverse genetics. To test whether there were any differences between the wild-type virus strains and the reverse genetics-generated virus strains, their viral growth capability in A549 and DF-1 cells was analyzed. There was no significant difference in replication efficiency between the wild-type and reverse genetics-generated virus strains (Figure 14A-C). MLD_{50} values in BALB/c mice were also determined; while both the wild-type and reverse genetics-generated strains of UT541 had MLD_{50} values higher than $1x10^6$ PFU/animal, RG1, the reverse genetics-generated strain of UT521, had an MLD_{50} value of $1x10^{3.8}$ PFU/animal, in contrast to the wild-type UT521 strain's MLD_{50} value of $1x10^{2.8}$ PFU/animal. Together, these results demonstrate that the reverse genetics-generated strains possessed similar viral properties to those of the wild-type strains and were therefore suitable for further analysis.

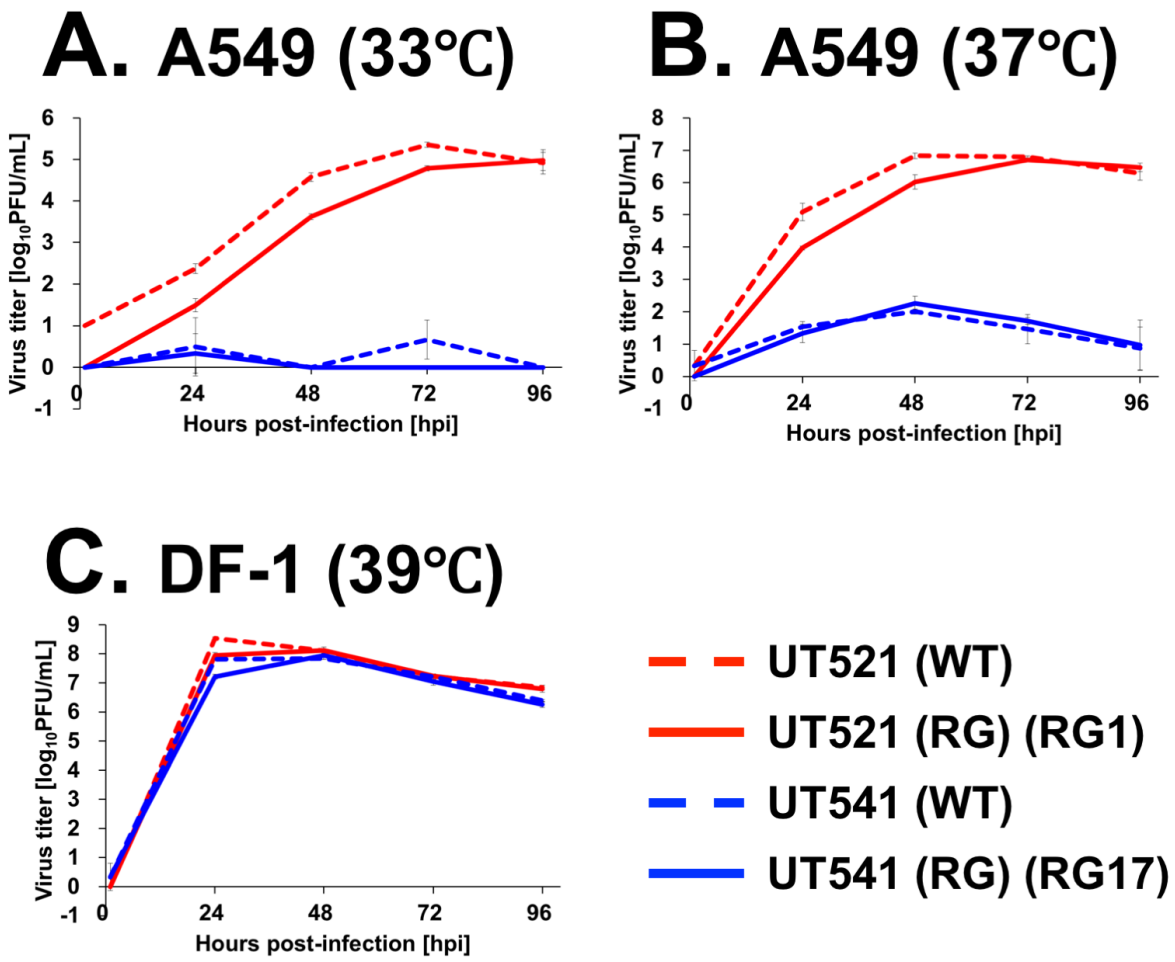


Figure 14. Viral replication efficiency of wild-type and reverse genetics-generated viruses in A549 and DF-1 cells

The viral replication efficiency of wildtype and reverse genetics-generated strains of UT521 and UT541 was analyzed in A549 cells incubated at 33℃ (A) and at 37℃ (B), and in DF-1 cells incubated at 39℃ (C). Wild-type virus strains are indicated as UT521 (WT) and UT541 (WT). RG1 represents the reverse genetics-generated strain of UT521, and RG17 represents the reverse genetics-generated strain of UT541. The data presented are the mean ± s.d. of triplicate experiments.

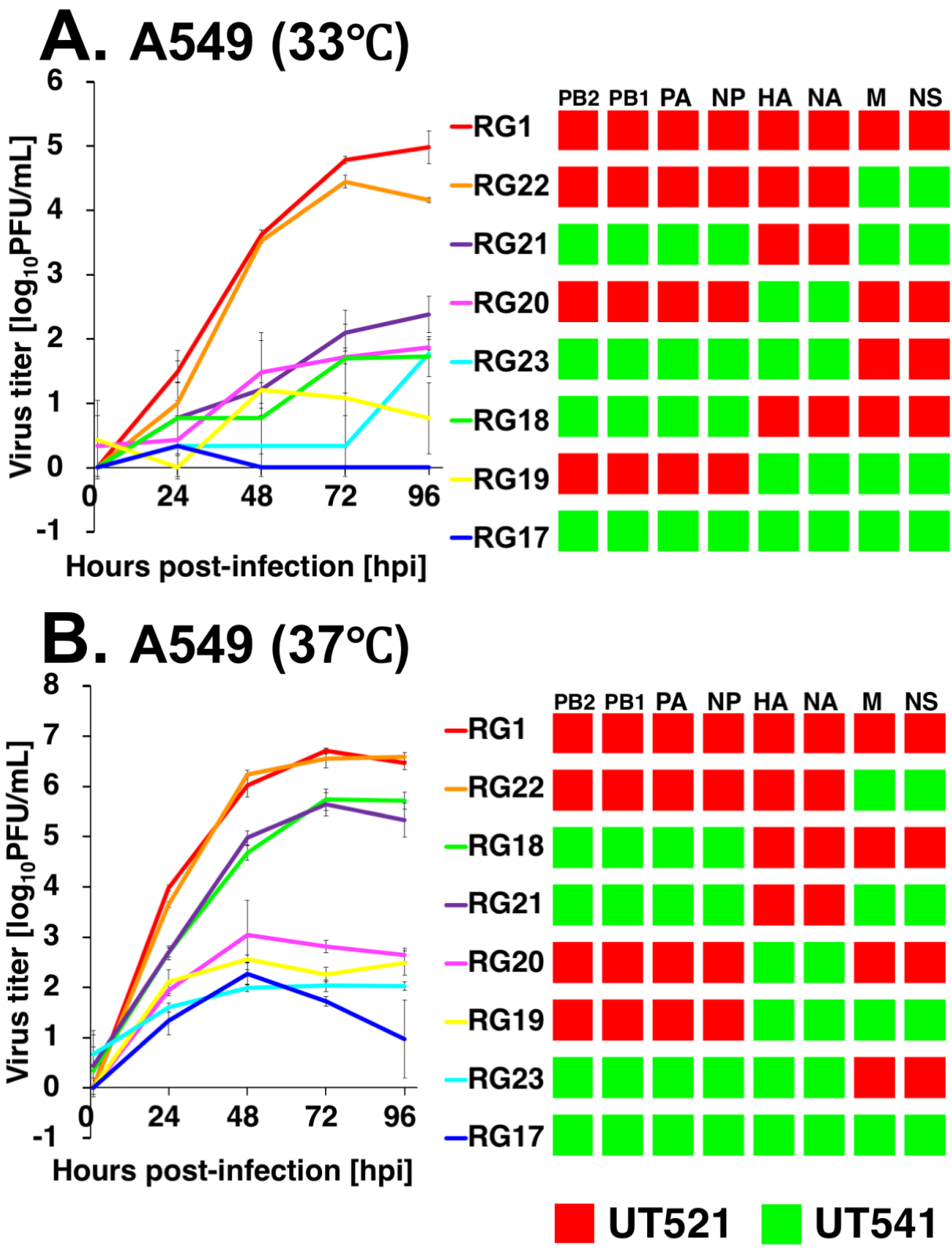
The effect of viral surface glycoproteins and the polymerase complex on viral replication efficiency in A549 cells

To elucidate which of the eight viral RNA segments is responsible for the difference in replication efficiency between UT521 and UT541, reassortant viruses were produced by reverse genetics, and their growth properties in A549 cells incubated at 33℃ and 37℃ were analyzed.

First, the eight viral RNA segments were categorized into three groups: the polymerase complex consisting of the PB2, PB1, PA, and NP segments; the surface glycoprotein group consisting of the HA and NA segments, and the remaining group consisting of the M and NS segments. Viruses with different combinations of the three groups were generated by reverse genetics and their growth properties were analyzed in A549 and DF-1 cells.

In A549 cells incubated at 37℃, viruses possessing the surface glycoproteins of UT521 (RG 1, RG18, RG21, and RG22) showed high replication efficiency (Fig. 15B). Among the virus strains possessing the surface glycoproteins of UT521, virus strains with the polymerase complex of UT521 (RG1 and RG22) showed higher replication efficiency than those with the polymerase complex of UT541 (RG18 and RG21). The enhancing effect of

possessing both the polymerase complex and the surface glycoproteins of UT521 was more pronounced in A549 cells incubated at 33℃ than at 37℃ (Fig. 15A). All of the generated reassortant viruses grew well in DF-1 cells incubated at 39℃ (Fig. 15C).


A. A549 (33℃)
Virus titer [log10PFU/mL]
Hours post-infection [hpi]
PB2 PB1 PA NP HA NA M NS
RG1
RG22
RG21
RG20
RG23
RG18
RG19
RG17
B. A549 (37℃)
Virus titer [log10PFU/mL]
Hours post-infection [hpi]
PB2 PB1 PA NP HA NA M NS
RG1
RG22
RG18
RG21
RG20
RG19
RG23
RG17
UT521
UT541

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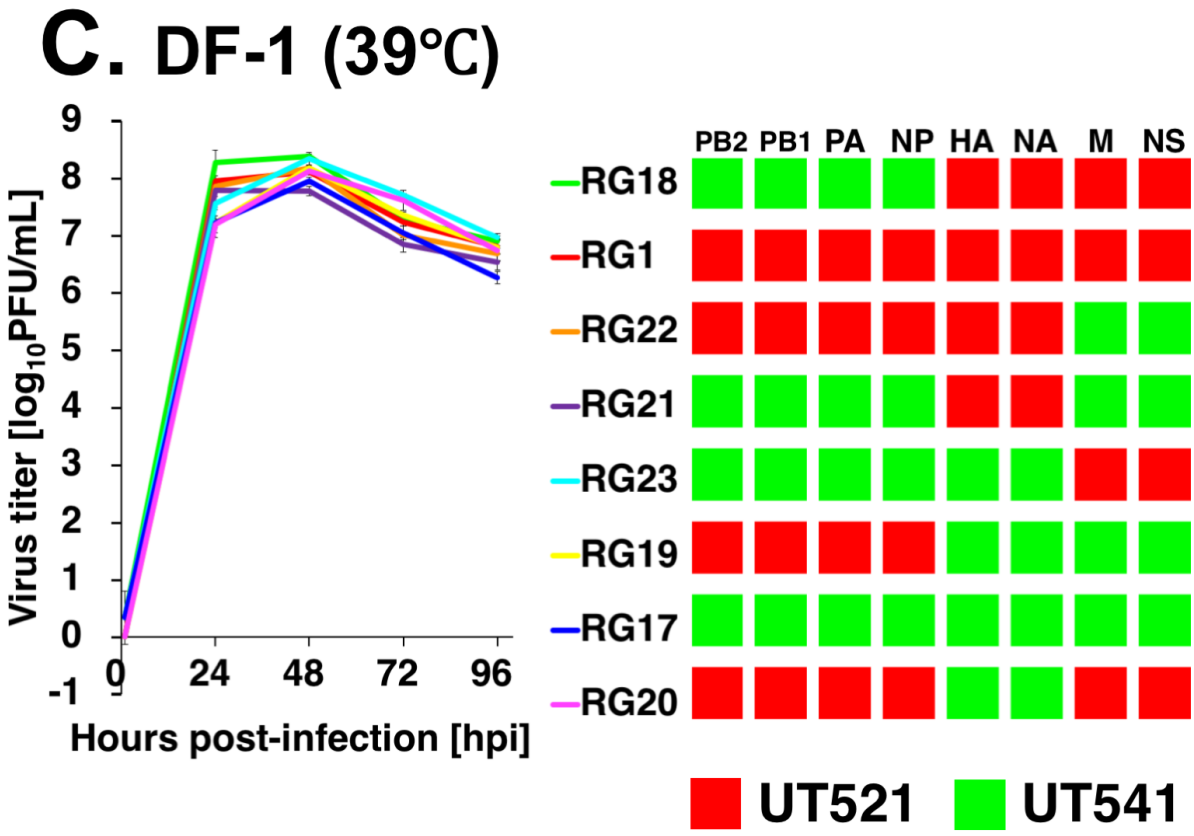


Figure 15. Effect of the viral polymerase complex and surface glycoproteins on viral replication in A549 cells

Viral replication efficiency of reassortant virus strains was analyzed in A549 cells incubated at 33℃ (A) and 37℃ (B), and in DF-1 cells incubated at 39℃ (C). The data presented are the mean ± s.d. of triplicate experiments.

The effect of the NA segment on viral replication efficiency in A549 cells

When the effect of the surface glycoproteins HA and NA on viral growth was analyzed, it was revealed that virus strains with the NA segment of UT541 possessed lower replication efficiency in A549 cells incubated at 37℃ (RG17, RG20, RG33 and RG34), compared to virus strains with the NA segment of UT521 (Fig. 16A). All of the generated reassortant viruses grew well in DF-1 cells incubated at 39℃ (Fig. 16B).

The NA segments of UT521 and UT541 differ by six amino acids located at positions 32, 50, 53, 111, 120, and 143 (Table 3). Viruses with single amino acid mutations introduced into the NA segment were generated by reverse genetics. Viral growth property analysis revealed that virus strains possessing unique amino acids of UT541 strain (RG17, RG33, RG48, RG52, RG53, RG54, RG77, RG78 and RG79), namely NA-32-L, NA-111I, and NA-120M, showed lower replication efficiency in A549 cells compared to virus strains possessing either major amino acids or unique amino acids of UT521 strain (Fig. 17A). All of the generated reassortant viruses grew well in DF-1 cells incubated at 39℃ (Fig. 17B).

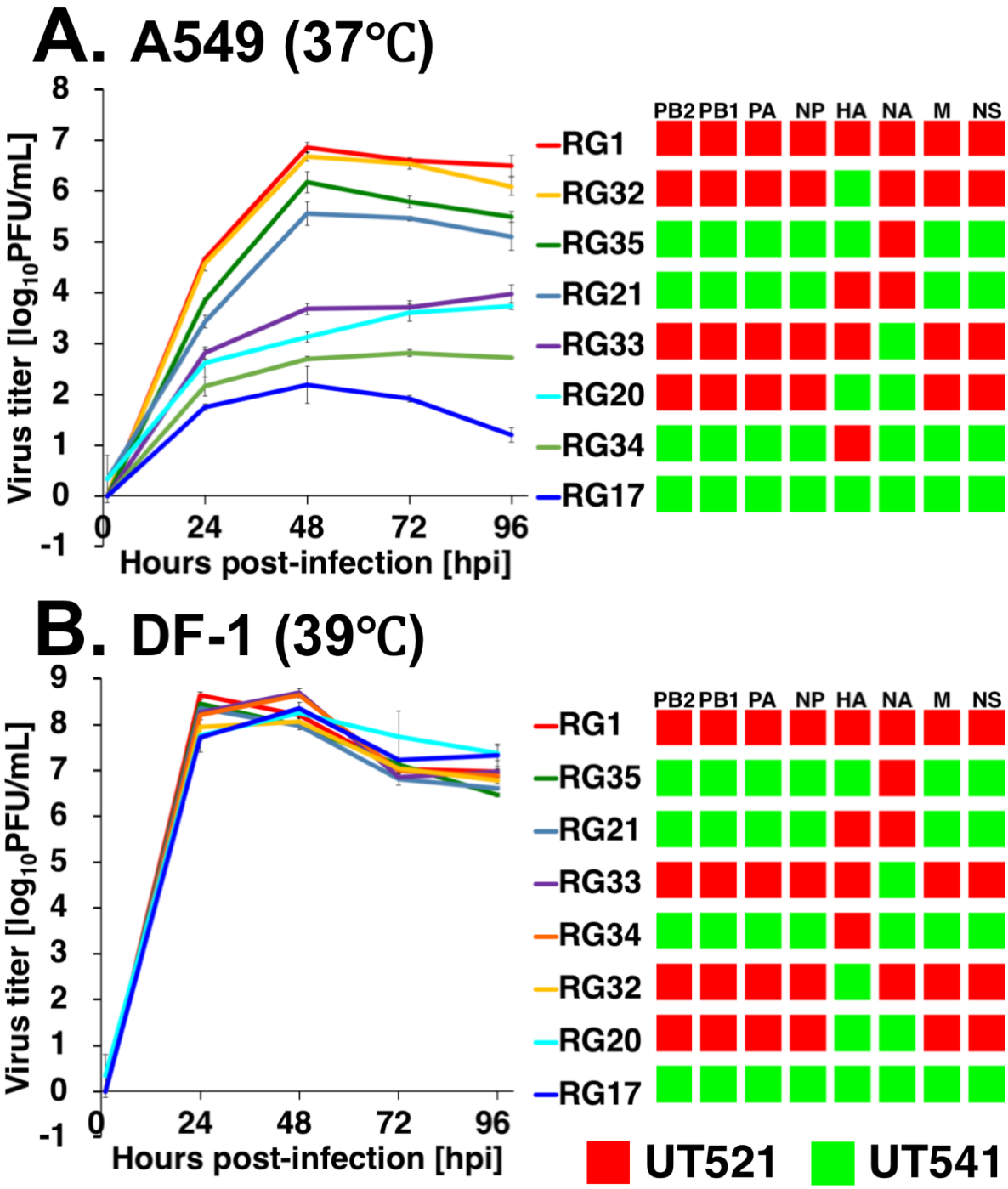
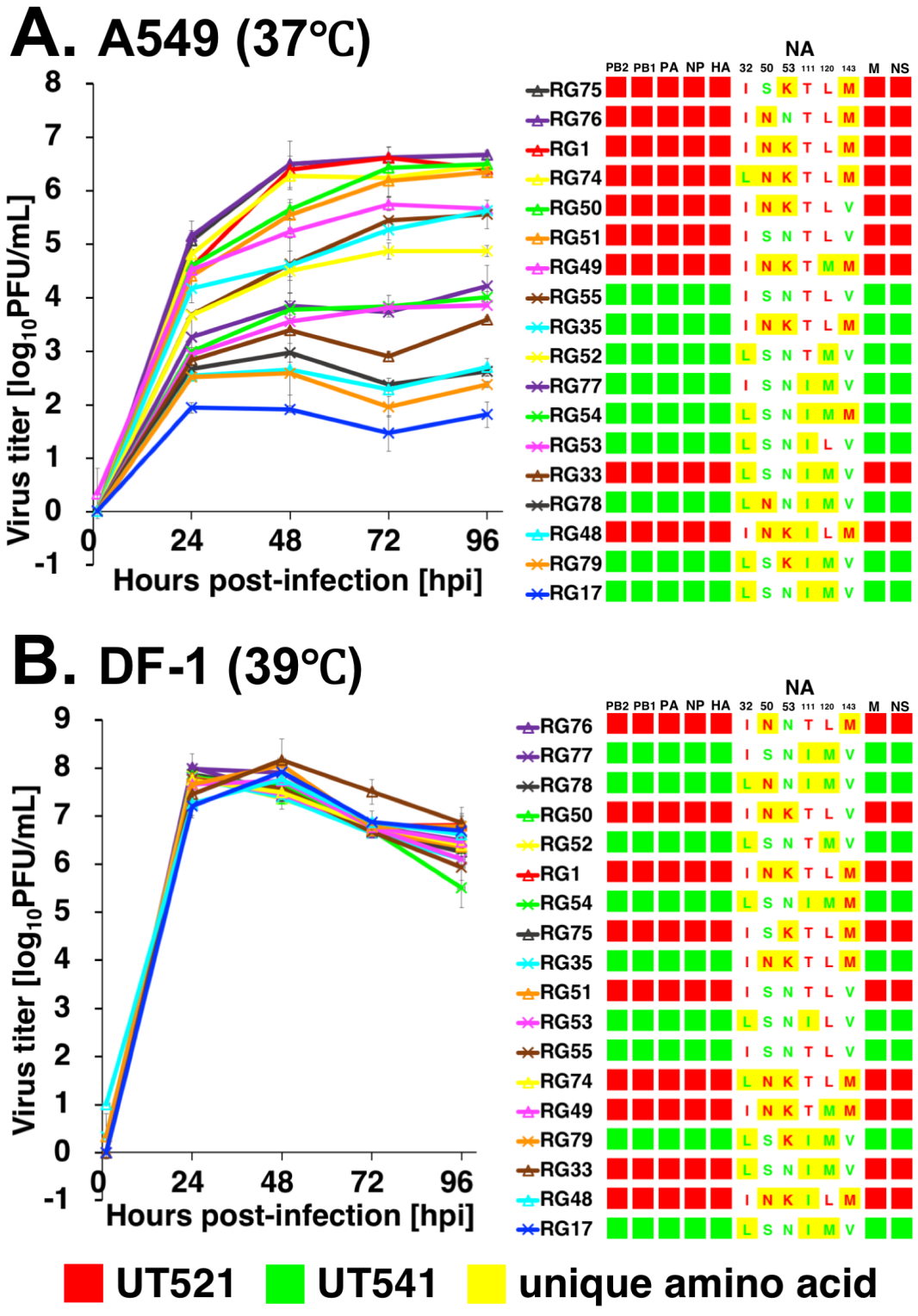


Figure 16. Effect of the NA segment on viral replication in A549 cells

Viral replication efficiency of reassortant virus strains was analyzed in A549 cells incubated at 37℃ (A) and in DF-1 cells incubated at 39℃ (B). The data presented are the mean ± s.d. of triplicate experiments.



925

926 **Figure 17. Viral replication efficiency of mutant NA strains in A549 cells**

927 Viral replication efficiency of virus strains with mutant NA segments was analyzed in A549

928 cells incubated at 37℃ (A) and in DF-1 cells incubated at 39℃ (B). Unique amino acids are

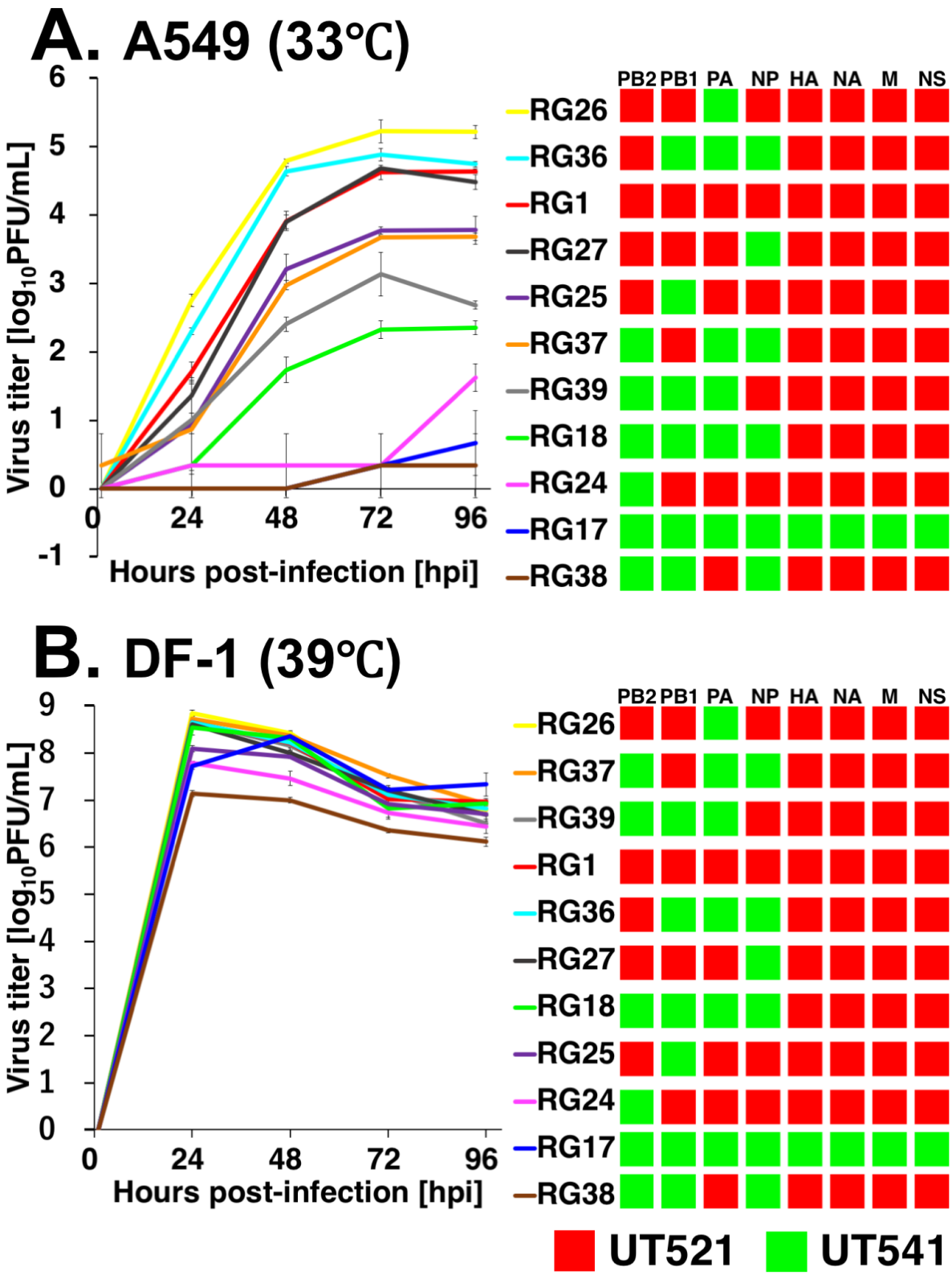
929 colored in yellow. The data presented are the mean ± s.d. of triplicate experiments.

930

The effect of the PB2 segment on viral replication efficiency in A549 cells

The growth property analysis of the reassortant viruses in A549 cells incubated at 33℃ revealed that both the viral polymerase complex and the surface glycoproteins of UT521 are required for high viral replication efficiency (Fig. 15A, B). In addition, the NA segment of UT521 was found to be essential for high viral replication efficiency in A549 cells (Fig. 16A).

When the effect of the polymerase complex (i.e., the PB2, PB1, PA, and NP segments) on viral growth was analyzed, it was revealed that virus strains with the PB2 segment of UT521 possessed higher replication efficiency in A549 cells incubated at 33℃ (RG1, RG26, RG27 and RG36), compared to virus strains with the PB2 segment of UT541 (Fig. 18A). Viral growth property analysis of virus strains with reassortant NA and PB2 segments revealed that both the NA and PB2 segments of UT521 are essential for efficient viral replication in A549 cells incubated at 33℃ (RG1 and RG70) (Fig. 19A). All of the generated reassortant viruses grew well in DF-1 cells incubated at 39℃ (Fig. 18B, 19B). We conducted further analysis focusing on the PB2 segment.



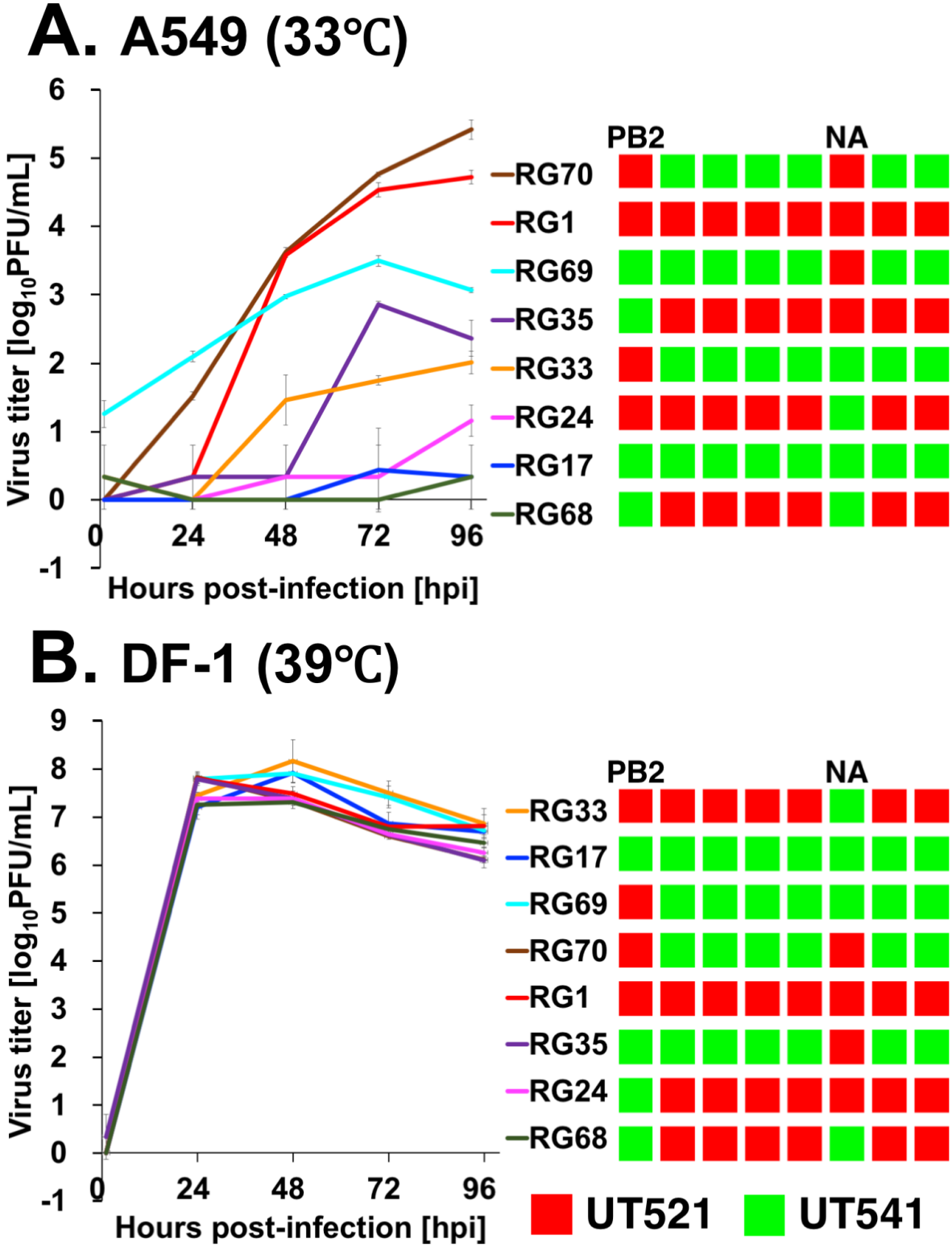
948

949 **Figure 18. Effect of the PB2 segment on viral replication in A549 cells at 33℃**

950 Viral replication efficiency of reassortant virus strains was analyzed in A549 cells incubated

951 at 33℃ (A) and in DF-1 cells incubated at 39℃ (B). The data presented are the mean ± s.d.

952 of triplicate experiments.



953

954 **Figure 19. Effect of the NA and PB2 segments on viral replication in A549 cells at 33℃**

955 Viral replication efficiency of reassortant virus strains was analyzed in A549 cells incubated

956 at 33℃ (A) and in DF-1 cells incubated at 39℃ (B). The data presented are the mean ± s.d.

957 of triplicate experiments.

958

959 The PB2 segments of UT521 and UT541 differ by five amino acids located at
960 positions 109, 292, 369, 447, and 758 (Table 3). Viruses with single amino acid mutations
961 introduced into the PB2 segment were generated by reverse genetics. Analysis of the growth
962 property of the generated mutant viruses in A549 cells revealed the following findings. When
963 introduced into the polymerase complex of UT521, PB2-A109V (exchange from unique to
964 major amino acid) resulted in the greatest decrease in viral replication efficiency (RG1 and
965 RG59), followed by PB2-T758I (exchange from unique to major amino acid) (RG1 and
966 RG58) (Fig. 20A). When introduced into the polymerase complex of UT541, PB2-V109A
967 (exchange from major to unique amino acid) resulted in the greatest increase in viral
968 replication efficiency (RG18 and RG64), followed by PB2-I758T (exchange from major to
969 unique amino acid) (RG18 and RG63) (Fig. 20A). From the findings above, two unique
970 amino acids of UT521, PB2-109A and PB2-758T, were identified to confer higher replication
971 efficiency in A549 cells incubated at 33℃ (Fig. 20A). Simultaneous introduction of amino
972 acid mutations into both PB2 loci revealed a synergistic effect on viral replication efficiency
973 (RG80 and RG81) (Fig. 21A). All of the reassortant and mutant virus strains produced by
974 reverse genetics replicated well in DF-1 cells incubated at 39℃ (Fig. 20B, 21B).

When plotted in a structural model of the polymerase complex (Protein Data Bank ID: 4WSB) using PyMOL (DeLano Scientific, San Carlos, CA, USA), PB2-109A was located on the surface of the polymerase complex (Fig. 22). The function of PB2-109A/V has not been reported and remains unknown. PB2-758T is reported to be part of the nuclear localization signal (NLS), and could not be plotted on the structural model because the published crystal structure of the polymerase complex did not include the NLS region [76].

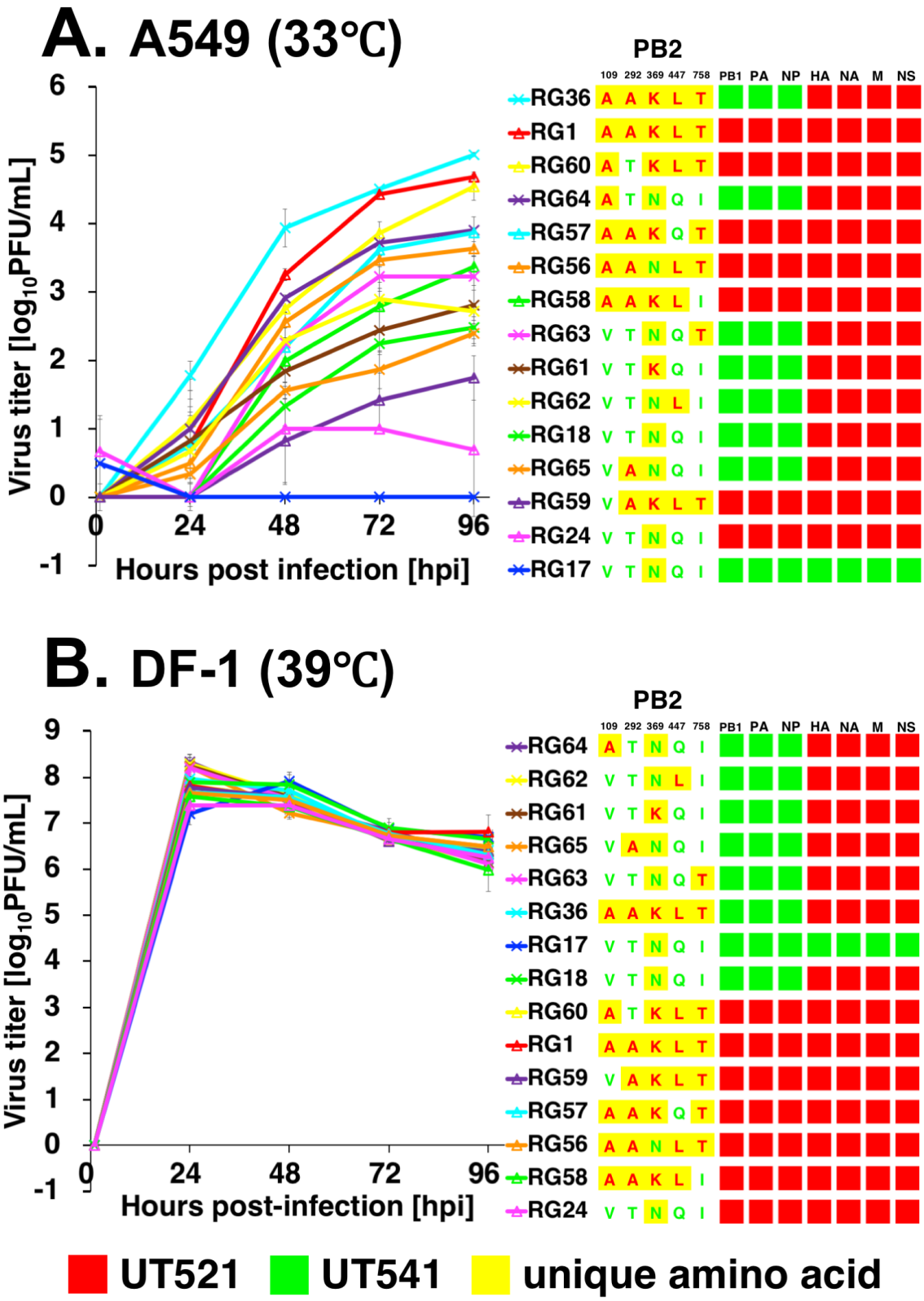
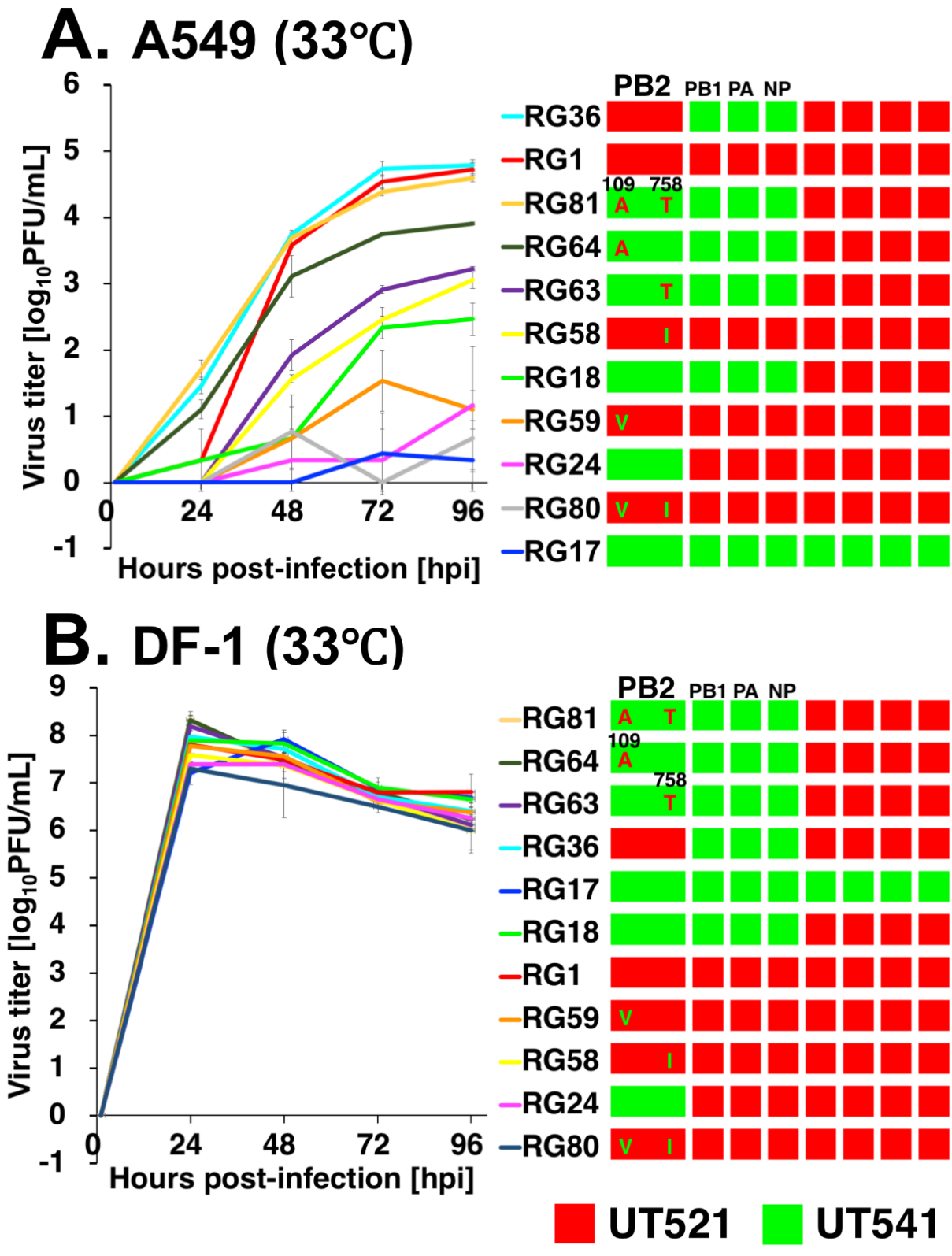


Figure 20. Viral replication efficiency of mutant PB2 strains in A549 cells

Viral replication efficiency of virus strains with mutant PB2 segments was analyzed in A549 cells incubated at 33℃ (A) and in DF-1 cells incubated at 39℃ (B). The data presented are the mean ± s.d. of triplicate experiments.



997

998 **Figure 21. Effect of PB2-109A and PB2-758T on viral replication in A549 cells at 33℃**

999 Viral replication efficiency of PB2 mutant virus strains was analyzed in A549 cells incubated

1000 at 33℃ (A) and in DF-1 cells incubated at 39℃ (B). The data presented are the mean ± s.d.

1001 of triplicate experiments.

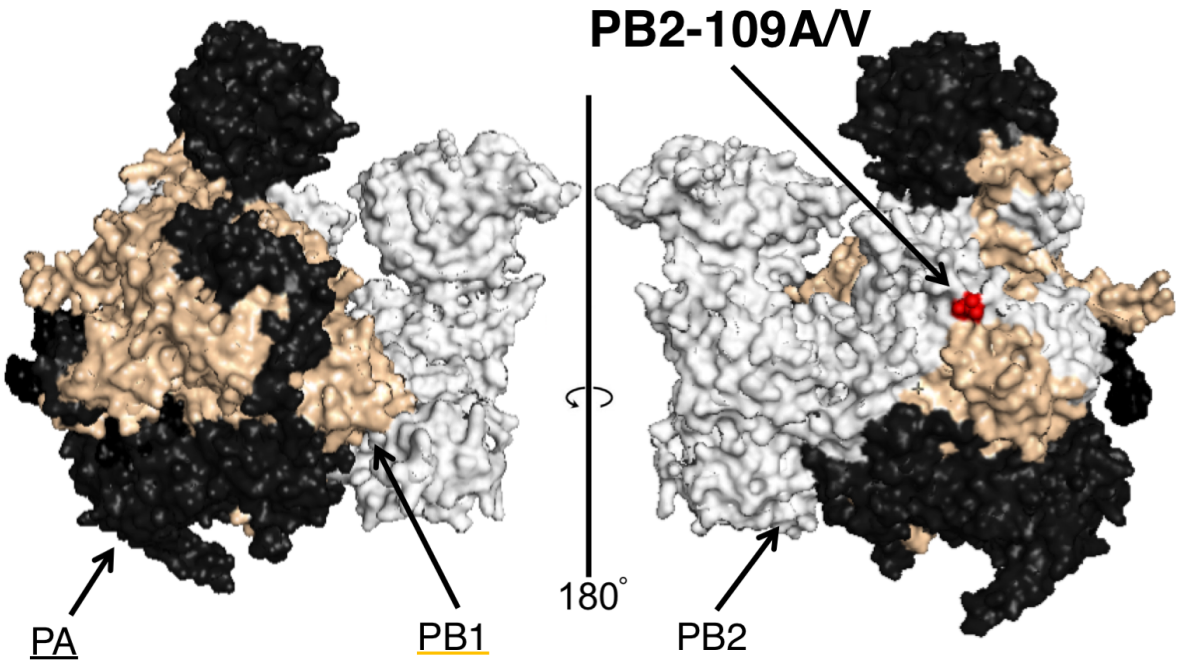


Figure 22. PB2-109A/V location on the structural model of the polymerase complex

A structural model of the polymerase complex was created by PyMOL using protein data obtained from the Protein Data Bank (ID: 4WSB) [76]. The PB2 protein is colored in white, the PB1 protein in wheat, and the PA protein in black. PB2-109A/V is shown on the surface of the polymerase complex by the red spheres. PB2-758T/I could not be plotted on the model because of the model is lacking in data around the region.

The effect of PB2 amino acid mutations on virus pathogenicity in BALB/c mice

Viral MLD_{50} values were obtained in order to evaluate the effect of two PB2 amino acid mutations on virus pathogenicity in BALB/c mice (Table 4). The introduction of the unique amino acid PB2-109A into the polymerase complex of UT541 resulted in greater enhancement of pathogenicity in BALB/c mice compared to the introduction of PB2-758T. In contrast, the introduction of either of the major amino acids PB2-109V or PB2-758I into the polymerase complex of UT521 conferred similar diminishing effects on pathogenicity in BALB/c mice. The simultaneous introduction of PB2-109A and PB2-758T into the polymerase complex of UT541, or the introduction of PB2-109V and PB2-758I into the polymerase complex of UT521 had a synergistic effect on pathogenicity in BALB/c mice.

To investigate viral replication in BALB/c mice, mice were inoculated with virus at a concentration of $1x10^3$ PFU/animal, and organs were harvested on days 3 and 6 post-inoculation to determine virus titers in the organs (Table 4, Fig. 23A-I). Wild-type UT521 was notable for its replication capability in both respiratory and non-respiratory organs, indicating that wild-type UT521 possesses an invasive trait. In the lungs, wild-type UT521, RG1 (UT521 reverse genetics-generated strain), RG36, and RG81 showed higher replication efficiency than the rest of the analyzed viruses, indicating that the combination of PB2-109A

and PB2-758T is important for high BALB/c pathogenicity. Among the non-respiratory organs analyzed, RG1 replication was identified in the spleen and heart, RG36 in the spleen, and RG81 in the brain (Fig. 23A-I).

Table 4. BALB/c MLD_{50} values for PB2 reassortant and mutant viruses

Virus	PB2	PB1	PA	NP	HA	NA	M	NS	BALB/c MLD_{50} [PFU/animal]	Virus Titer in Lungs [log_{10}PFU/g] Day3 #1	Day3 #2	Day3 #3	Day6 #1	Day6 #2	Day6 #3
UT521(WT)									$1x10^{2.8}$	6.67	6.38	6.51	7.97	6.54	6.48
RG1									$1x10^{3.8}$	3.80	2.90	3.34	5.83	5.30	5.56
RG59	V								$1x10^{4.5}$	2.85	2.90	-	2.78	-	-
RG58	I								$1x10^{4.7}$	3.93	3.15	3.40	3.00	3.15	3.18
RG80	V I								$> 1x10^{6}$	2.30	2.48	2.60	2.30	3.32	2.48
RG24									$1x10^{5.5}$	3.00	-	-	-	-	3.85
RG18									$> 1x10^{6}$	2.48	2.78	2.78	2.60	4.92	2.70
RG64	A								$1x10^{4.5}$	3.34	2.78	3.04	3.58	4.61	5.78
RG63	T								$1x10^{6.0}$	2.30	3.54	3.23	3.83	4.59	4.62
RG81	A T								$1x10^{4.5}$	4.46	4.41	3.23	5.48	4.49	5.93
RG36									$1x10^{4.0}$	5.53	5.89	5.84	6.45	6.07	6.10
RG17									$> 1x10^{6}$	2.30	2.30	2.30	2.60	2.00	2.00
UT541(WT)									$> 1x10^{6}$	3.04	3.11	2.90	2.95	3.43	3.43

-: detection limit <2 [log_{10}PFU/g]

UT521 UT541

MLD_{50} values in BALB/c mice and viral growth in mouse organs were analyzed. PB2-109A and PB2-758T conferred high virulence in BALB/c mice. Wild-type UT521 replicated well in the lungs and in non-respiratory organs. RG1, RG36, and RG81 replicated well in the lungs. These three strains also grew in some non-respiratory organs. The detection limit was 2 log_{10}PFU/g.

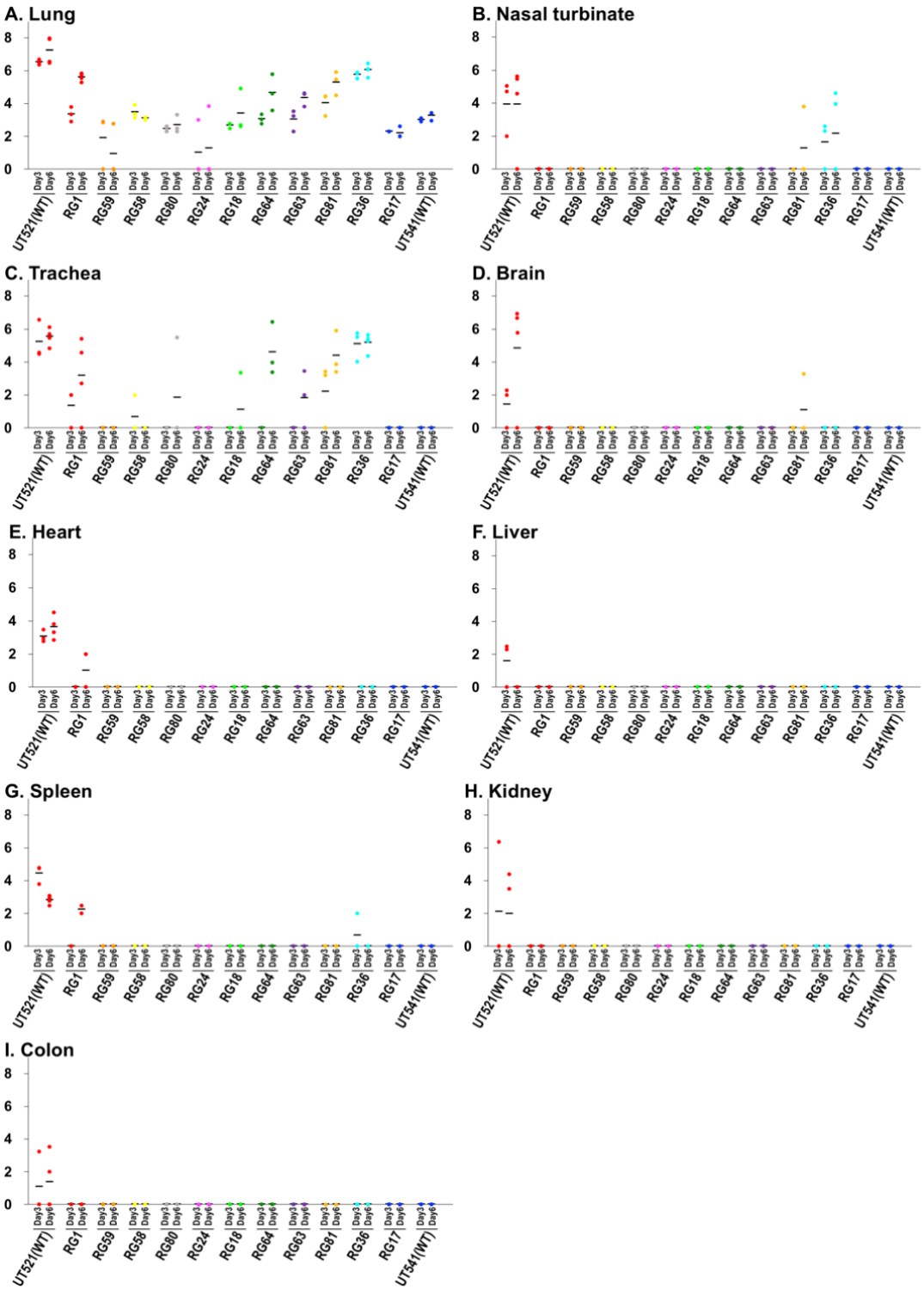


Figure 23. Viral replication in BALB/c mouse organs

BALB/c mice were intranasally inoculated with H5N1 virus strains at a concentration of $1x10^3$ PFU/animal; organs were harvested on days 3 and day 6 post-inoculation, and a plaque assay was conducted in MDCK cells. The figures show virus titers in the lungs (A), nasal turbinate (B), trachea (C), brain (D), heart (E), liver (F), spleen (G), kidney (G) and colon (I) on day 3 and day 6. The detection limit was 2 $\log_{10}$PFU/g.

Mini-replicon assay

To evaluate the effect of the identified PB2 amino acid mutations on viral polymerase activity, a mini-replicon assay was performed in A549 and DF-1 cells. Although the viral replication efficiency in A549 cells of the virus strain possessing the polymerase complex of UT521 (RG1) was higher than that of the virus strain with the polymerase complex of UT541 (RG17), the viral polymerase activity in A549 cells was higher with the UT541 polymerase complex than with the UT521 polymerase complex (Fig. 24). The respective introduction of the unique PB2 amino acids PB2-109A and PB2-758T into the UT541 polymerase complex increased the viral polymerase activity in A549 cells. The respective introduction of the major PB2 amino acids PB2-109V and PB2-758I into the UT521 polymerase complex had little effect on the polymerase activity in A549 cells. There was no significant difference in viral polymerase activity in DF-1 cells among the evaluated viruses.

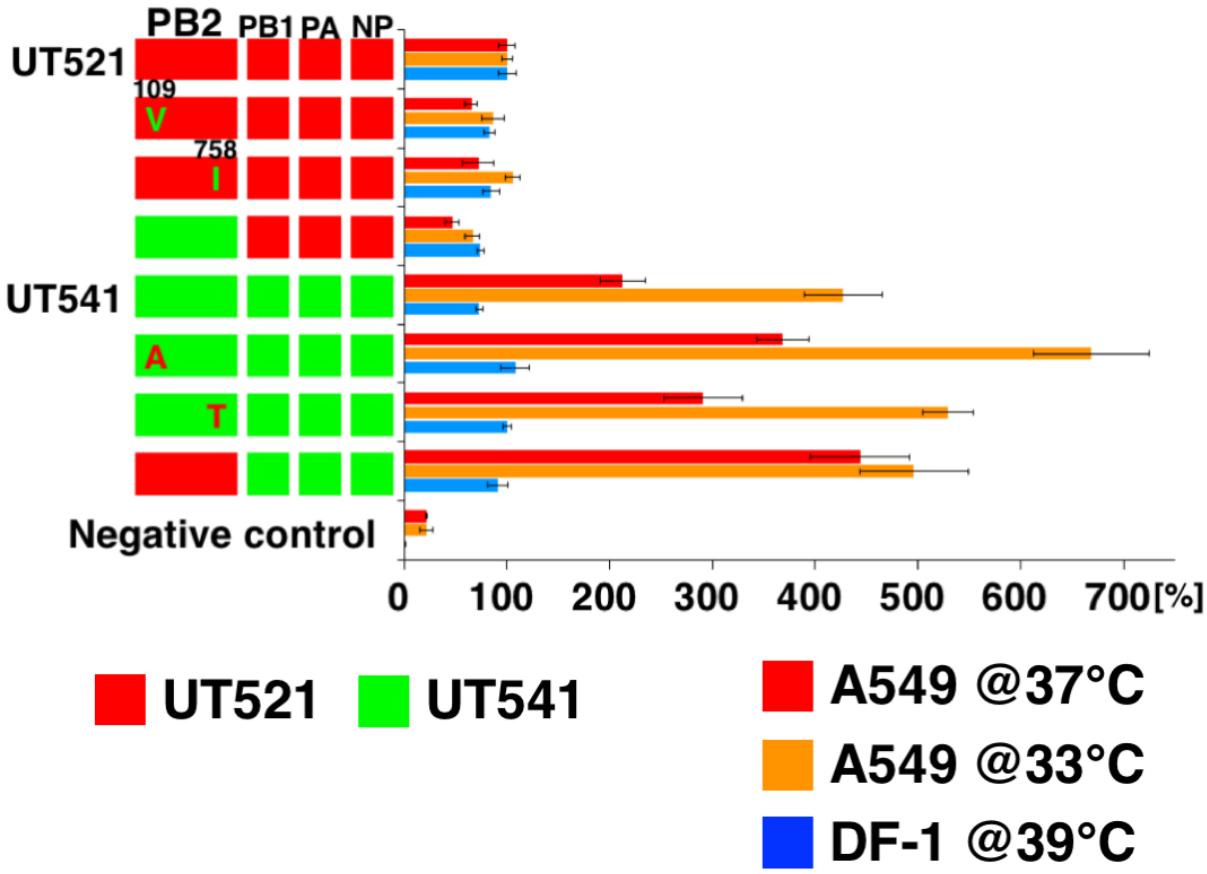
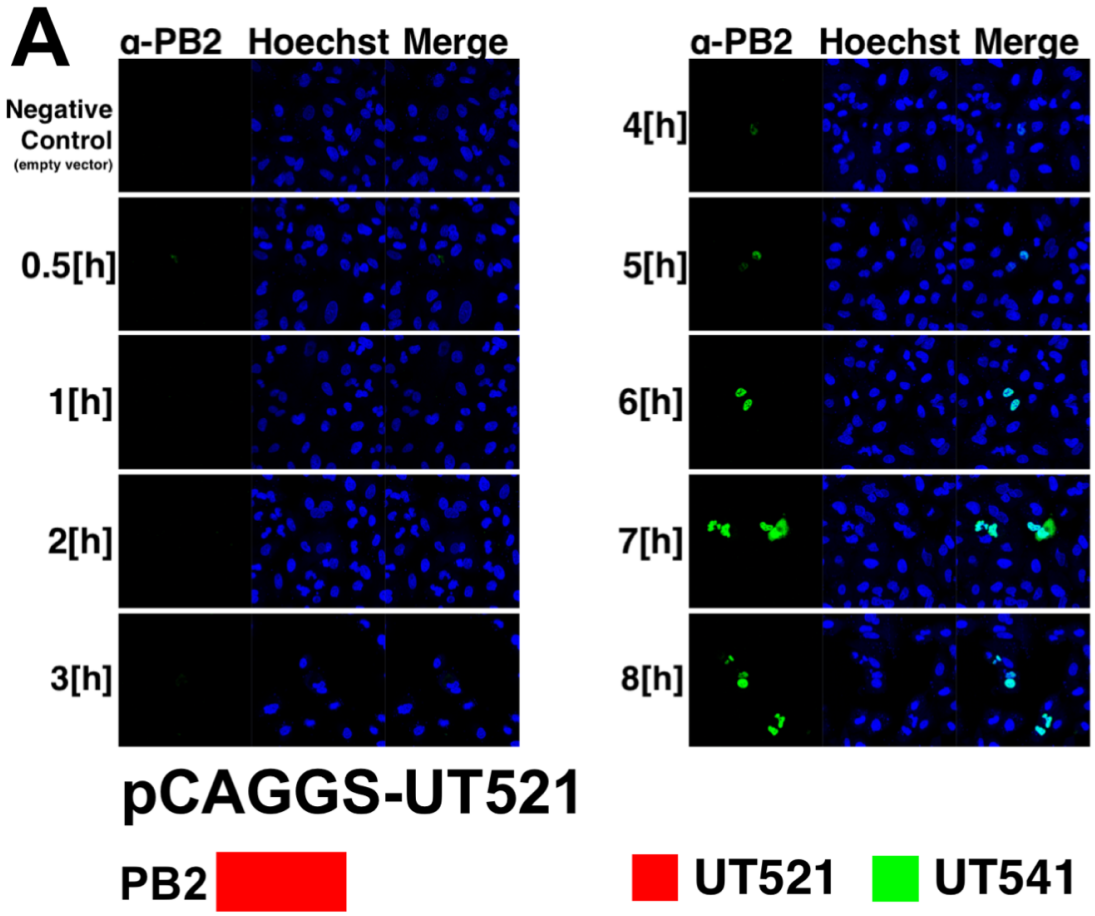


Figure 24. The effect of PB2-109A and PB2-758T on viral polymerase activity

Viral polymerase activity was measured by use of a mini-replicon assay. The polymerase activity of UT521 was set to 100%. UT541 showed higher polymerase activity compared to UT521 in A549 cells incubated at 33℃ or 37℃. The respective introduction of the unique PB2 amino acids PB2-109A and PB2-758T into UT541 increased polymerase activity in A549 cells. The respective introduction of the major amino acids PB2-109V and PB2-758I into UT521 had little effect on polymerase activity in A549 cells. No significant difference in polymerase activity was identified among the analyzed strains in DF-1 cells. The data presented are the mean ± s.d. of triplicate experiments.

PB2 nuclear localization

PB2-758I is known to be a part of the NLS [77]. To evaluate the effect of PB2 amino acid mutations on nuclear import, pCAGGS plasmid DNA encoding wild-type and mutant PB2 protein was transfected into A549 cells incubated at 33℃. The transfected A549 cells were fixed and stained with a mouse monoclonal antibody against PB2 protein (PB2-18/1) and observed using a laser-scanning microscope. Nuclear accumulation of the PB2 protein was observed from 4 hours post-transfection. There was no appreciable difference in the timing or pattern of the nuclear accumulation of PB2 protein (Fig. 25 A-F).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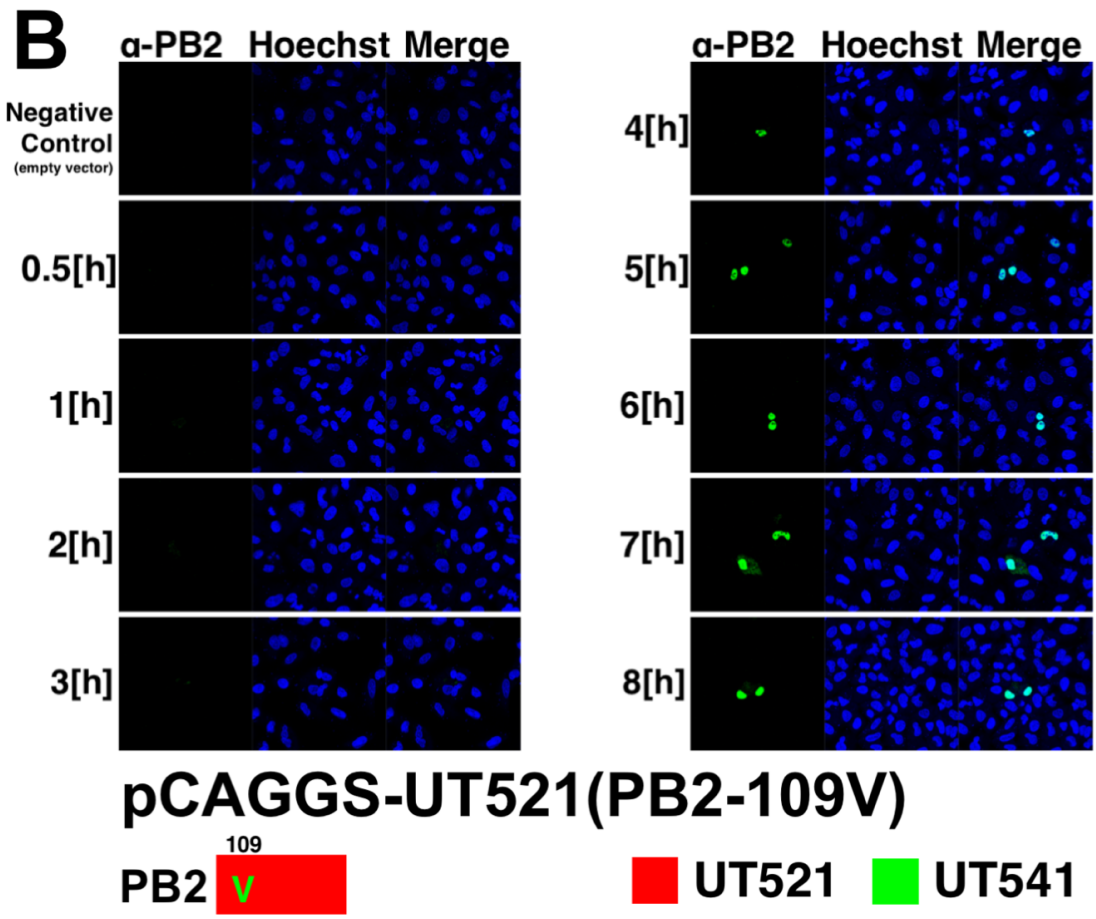
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UT521
UT541

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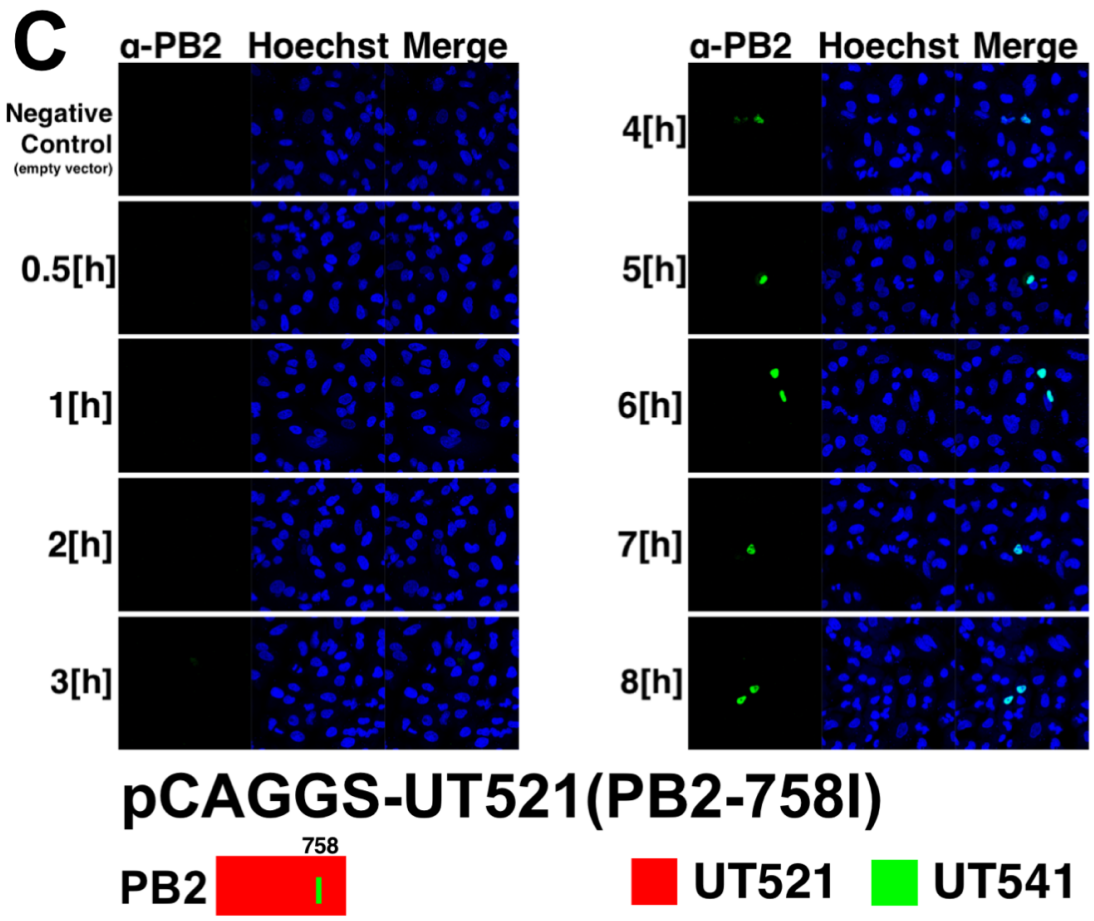
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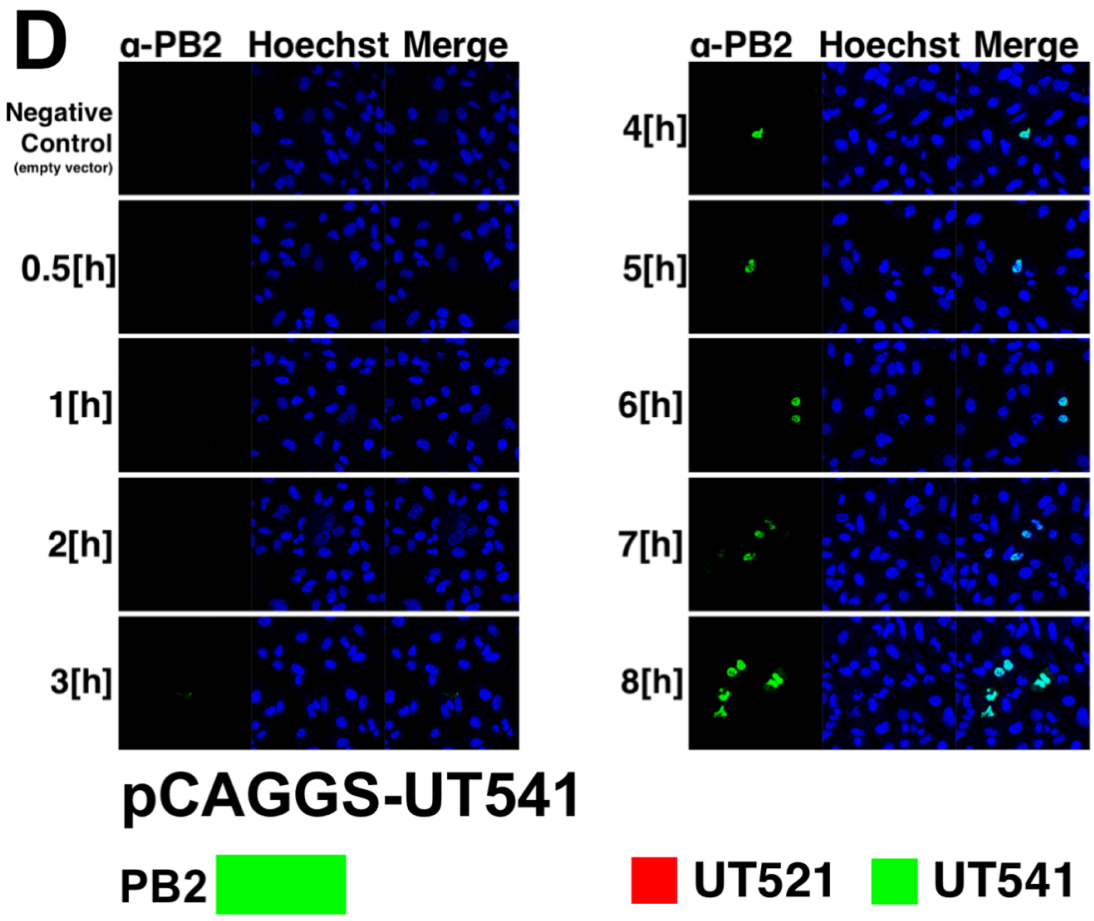
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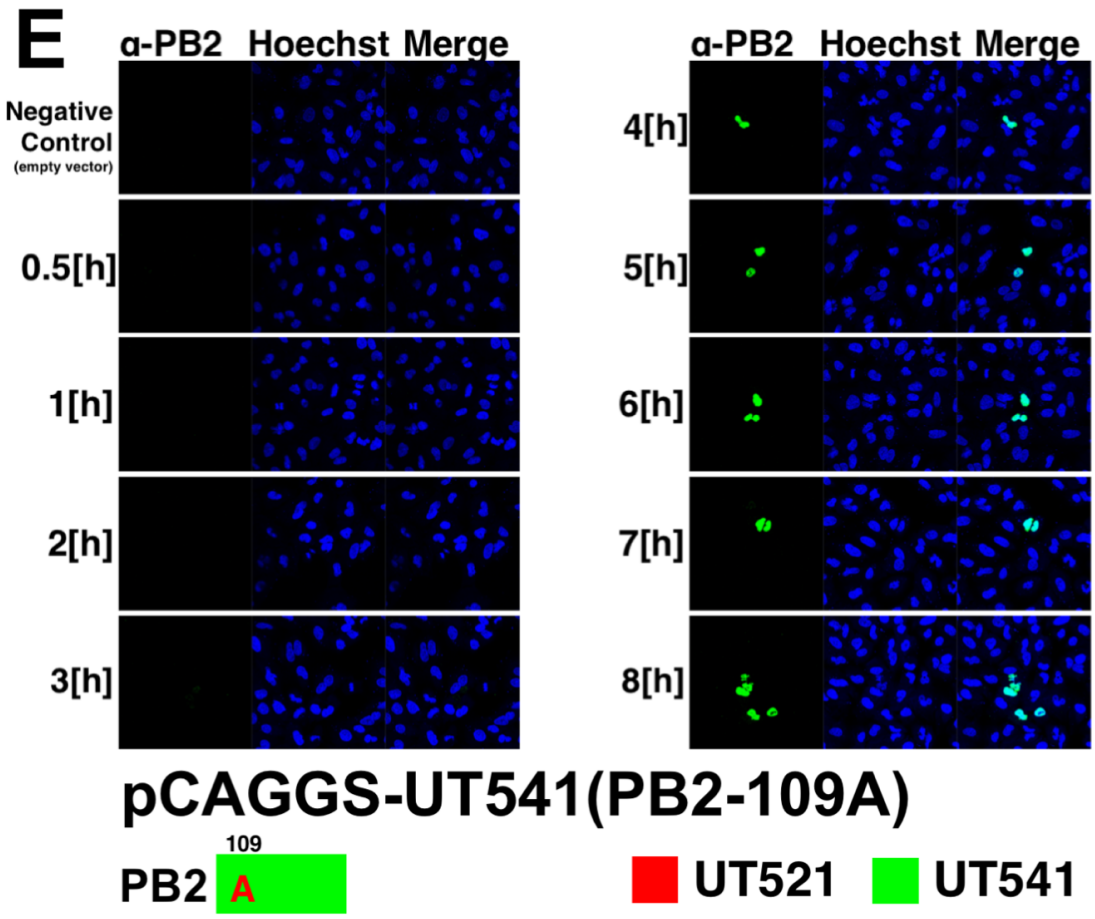
pCAGGS-UT541
PB2
UT521
UT541

1117


E
α-PB2 Hoechst Merge
Negative Control (empty vector)
0.5[h]
1[h]
2[h]
3[h]
α-PB2 Hoechst Merge
4[h]
5[h]
6[h]
7[h]
8[h]
pCAGGS-UT541(PB2-109A)
109
PB2 A
UT521
UT541

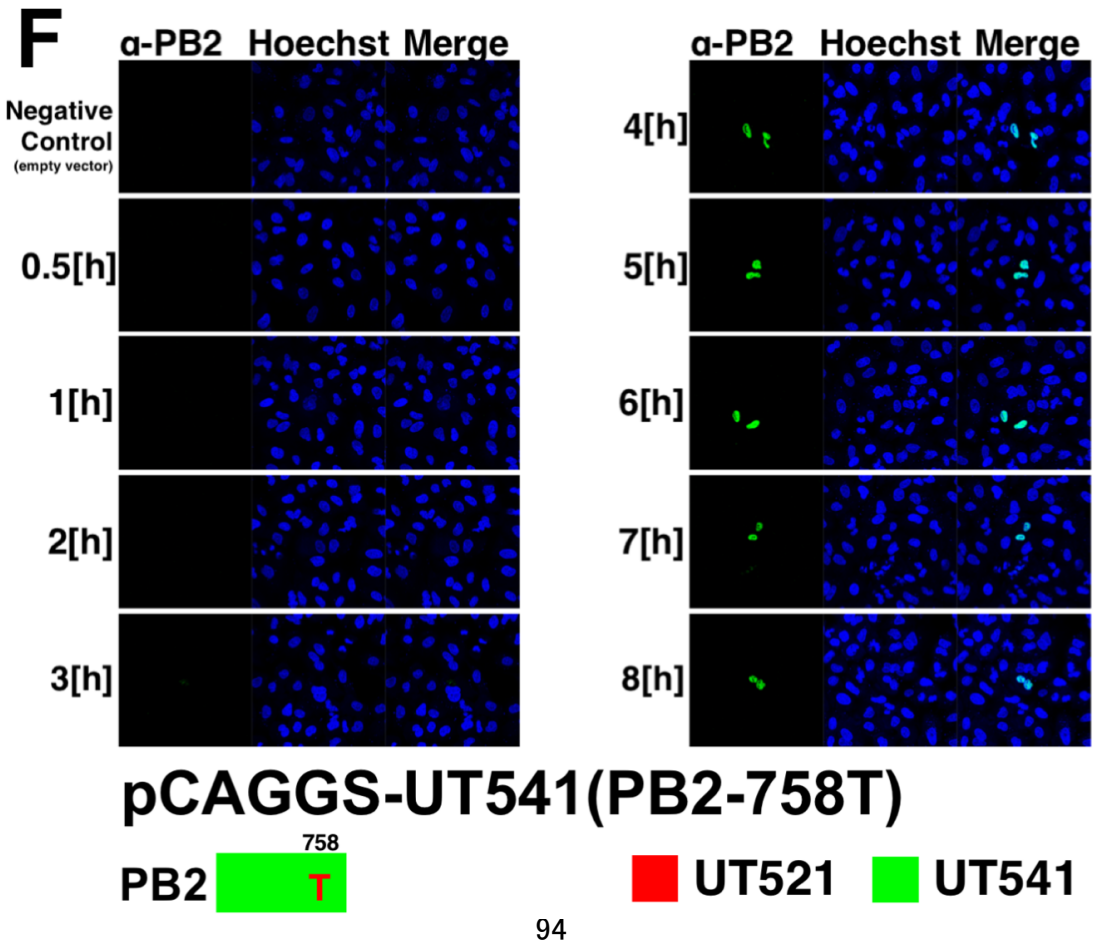

F
α-PB2 Hoechst Merge
Negative Control (empty vector)
0.5[h]
1[h]
2[h]
3[h]
α-PB2 Hoechst Merge
4[h]
5[h]
6[h]
7[h]
8[h]
pCAGGS-UT541(PB2-758T)
758
PB2 T
UT521
UT541

Figure 25. Viral PB2 protein nuclear localization

pCAGGS plasmid DNA encoding wild-type and mutant PB2 protein (0.25 μg) was transfected into A549 cells incubated at 33℃ (A-F). The transfected A549 cells were fixed and stained with a mouse monoclonal antibody against PB2 and observed by using a laser-scanning microscope.

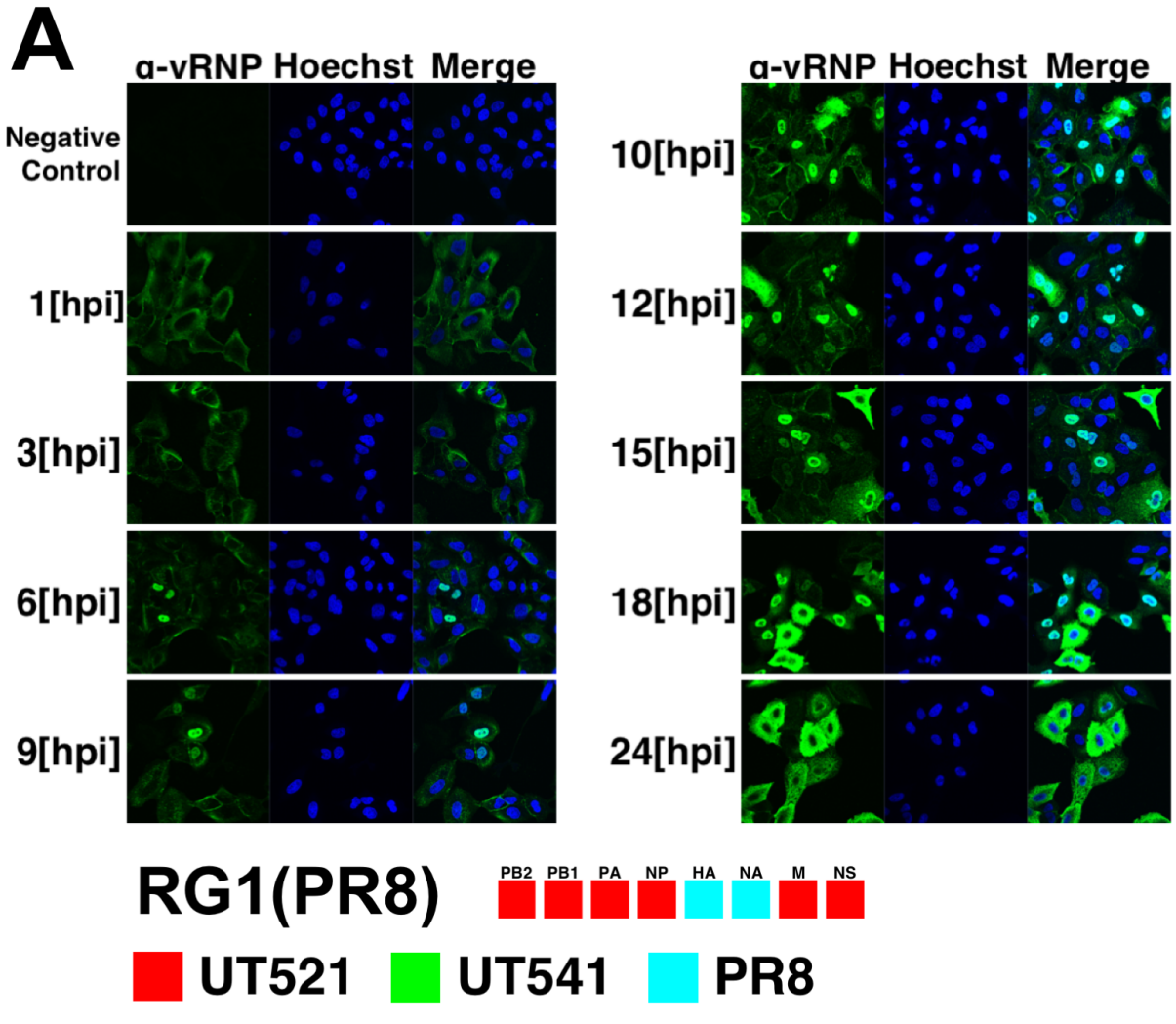
vRNP intracellular localization

To investigate the effect of the PB2 amino acid mutations PB2-109A and PB2-758T on viral intracellular localization, A549 cells were infected with reassortant virus strains at an MOI of 100 and incubated at 33℃ for 24 hours. The A549 cells were then fixed and stained with the mouse anti-WSN NP 3/1 monoclonal antibody, which was previously reported to recognize vRNP complexes [78]. The vRNP intracellular localization in infected A549 cells was observed by using a laser-scanning microscope. In order to conduct the experiment in the BSL2 facility, reassortant viruses possessing he HA and NA segments of PR8 and the rest of the genes from the H5N1 backbone were generated by using reverse genetics.

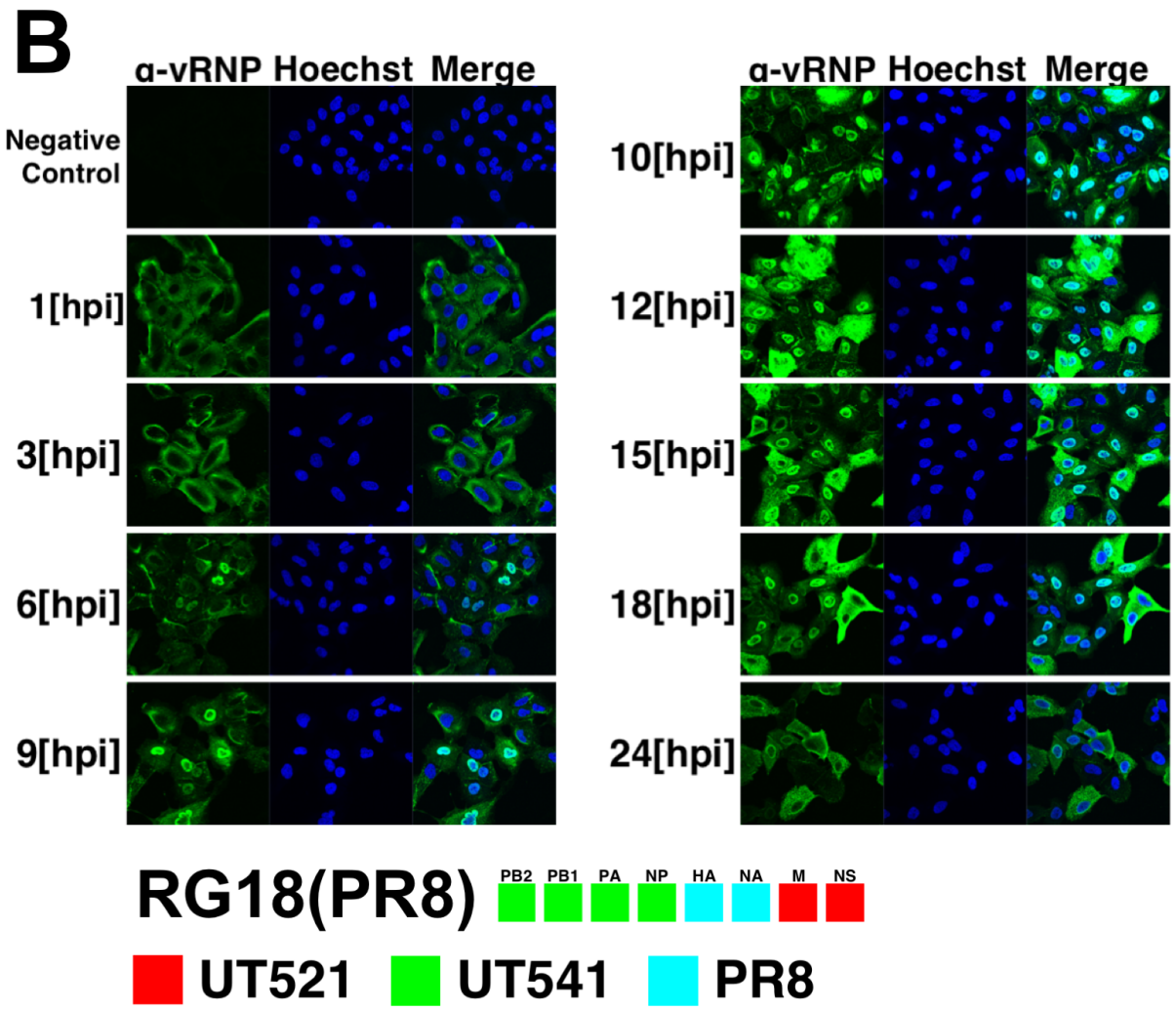
Immunofluorescence analysis of the reassortant viruses revealed that the vRNP first appeared in the cytoplasm, then, 6 hours post-infection, nuclear localization of the vRNP was observed. Fluorescence associated with the vRNP in the cytoplasm gradually intensified, and at 24 hours post-infection, the vRNP was mostly located in the cytoplasm. No significant difference was observed between the analyzed virus strains (Fig. 26).

The replication efficiency of the recombinant mutant virus strains in A549 cells incubated at 33℃ was analyzed, at an MOI of 10 (Fig. 27). Although the enhancing effect of PB2-109A and PB2-758T on viral growth could still be observed, the difference in virus titers

among the analyzed virus strains was small compared with the viral growth analysis of the H5N1 strains that did not bear HA and NA segments from PR8, infected at the low MOI of 0.0002 (Fig. 21A, Fig. 27).


A
α-vRNP Hoechst Merge
α-vRNP Hoechst Merge
Negative Control
1[hpi]
3[hpi]
6[hpi]
9[hpi]
10[hpi]
12[hpi]
15[hpi]
18[hpi]
24[hpi]
RG1(PR8)
PB2 PB1 PA NP HA NA M NS
UT521
UT541
PR8

1174


B
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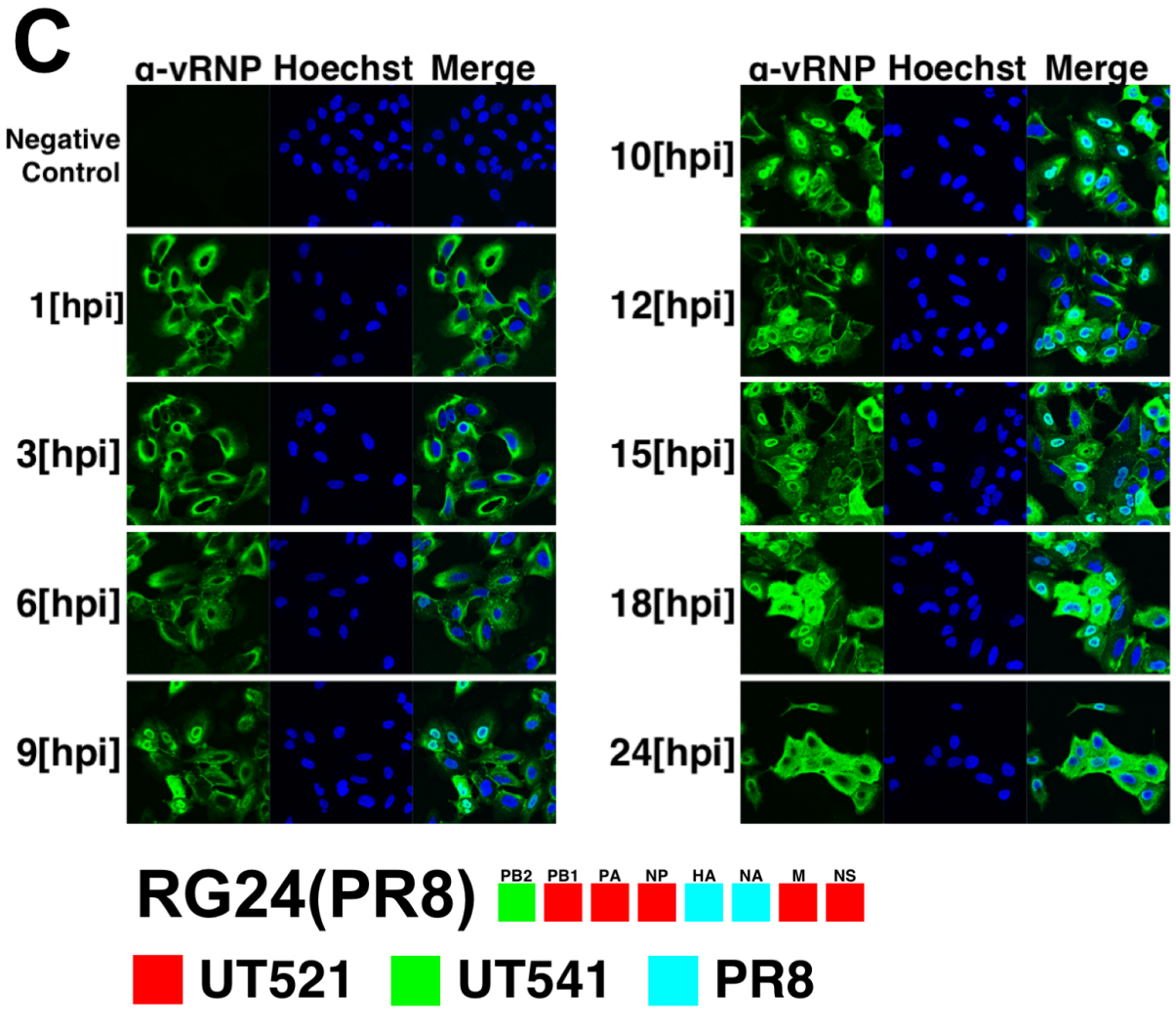
Negative Control
1[hpi]
3[hpi]
6[hpi]
9[hpi]
α-vRNP Hoechst Merge
10[hpi]
12[hpi]
15[hpi]
18[hpi]
24[hpi]
RG18(PR8)
PB2 PB1 PA NP HA NA M NS
UT521
UT541
PR8

1175


C
α-vRNP Hoechst Merge
Negative Control
1[hpi]
3[hpi]
6[hpi]
9[hpi]
α-vRNP Hoechst Merge
10[hpi]
12[hpi]
15[hpi]
18[hpi]
24[hpi]
RG24(PR8)
PB2 PB1 PA NP HA NA M NS
UT521
UT541
PR8

1176

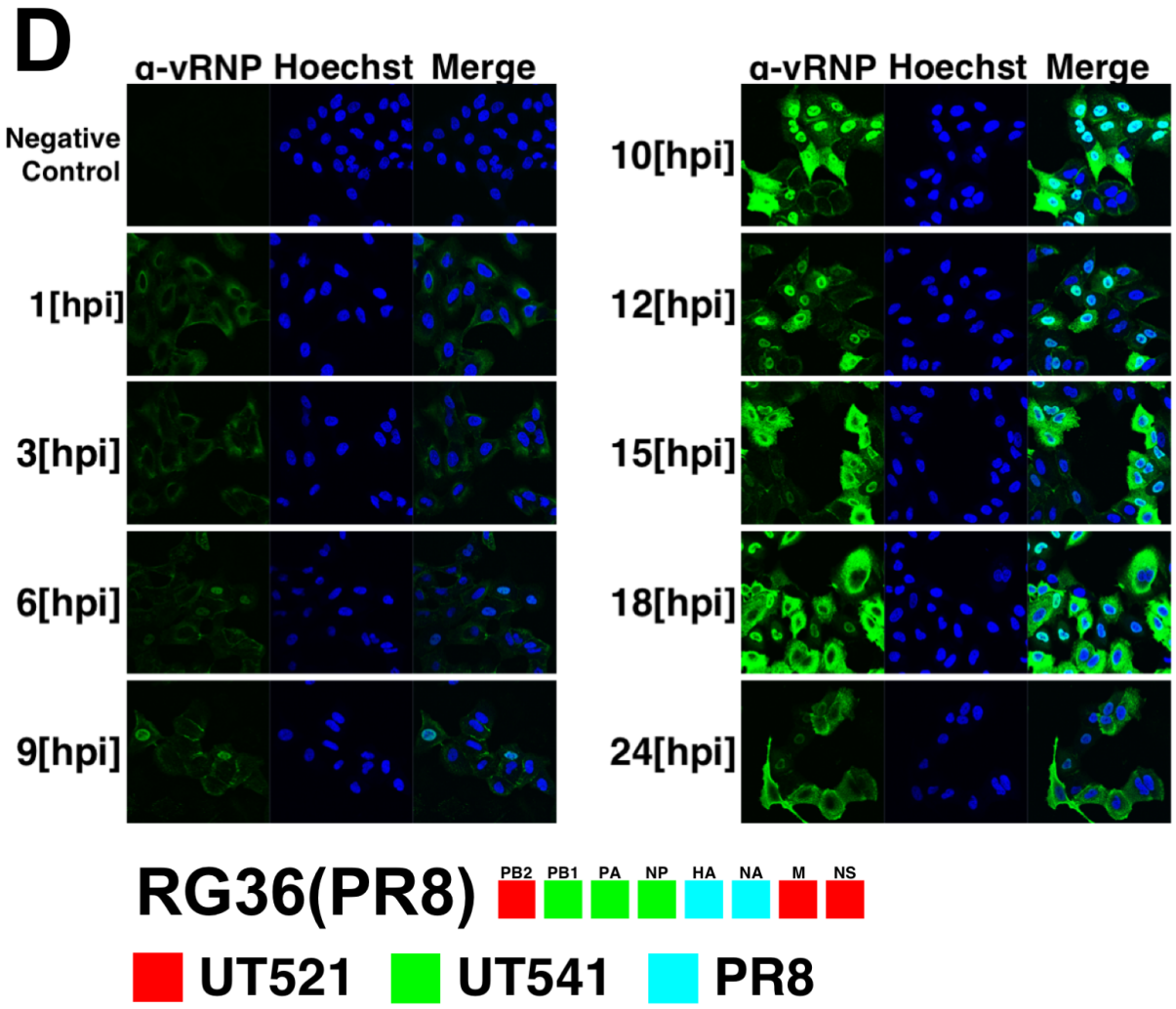


Figure 26. Immunofluorescence analysis of the intracellular localization of vRNP

A549 cells were infected with reassortant virus strains bearing the HA and NA segments of PR8 at an MOI of 100 and incubated at 33℃ for 24 hours. The A549 cells were then fixed at different time points and stained with a vRNP-recognizing monoclonal antibody (anti-WSN NP 3/1) and observed by using a laser-scanning microscope.

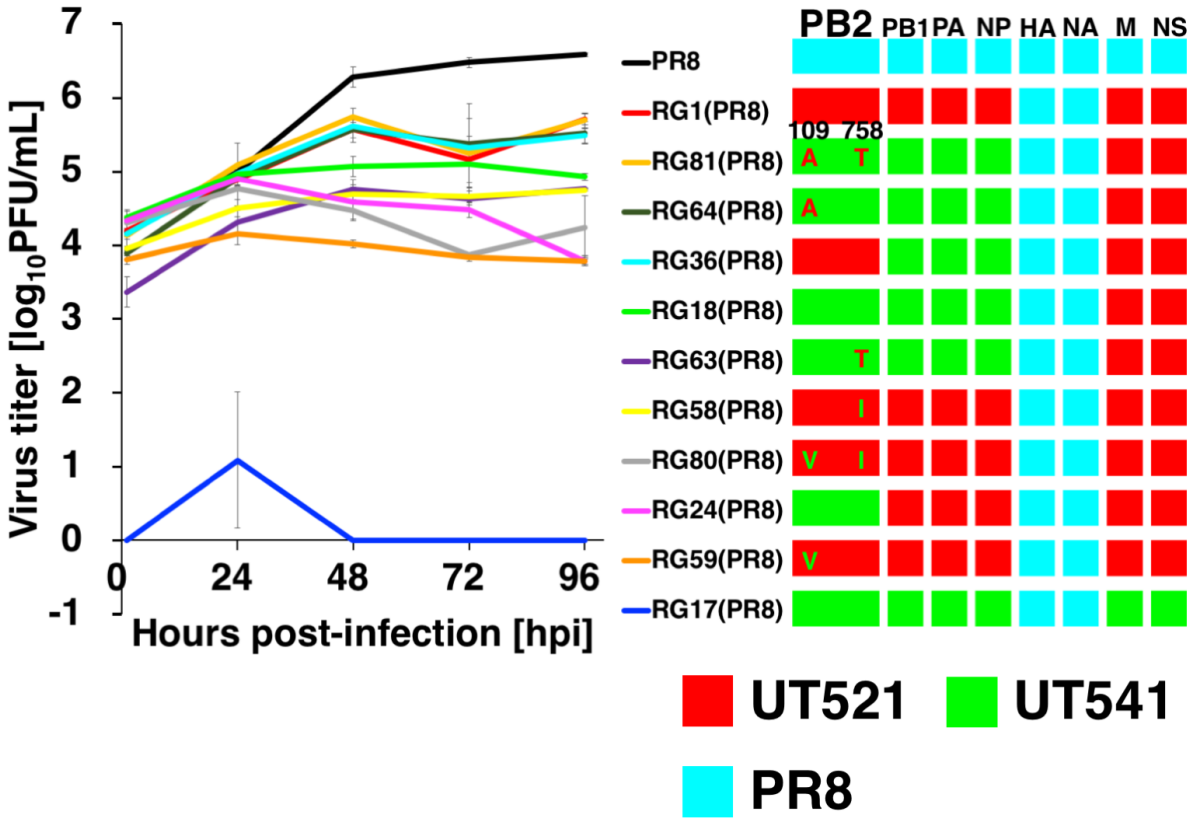


Figure 27. Replication efficiency of recombinant mutant virus strains in A549 cells incubated at 33℃

Recombinant viruses possessing an H5N1 backbone with mutant PB2 segments and HA and NA segments from PR8 were generated by using reverse genetics. For example, the virus strain possessing the UT521 backbone with the HA and NA segments of PR8 is indicated as RG1(PR8) in the figure above. The viral replication efficiency of the generated recombinant mutant virus strains was analyzed in A549 cells incubated at 33℃, at an MOI of 10, utilizing virus titers measured in MDCK cells and supplementation with 1 μg/mL of 6- (1-tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin. The data presented are the mean ± s.d. of triplicate experiments.

Discussion

In this study, we found that two unique amino acids of the PB2 segment, PB2-109A and PB2-758T, confer high replication efficiency in A549 cells, especially at 33℃, and high pathogenicity in BALB/c mice. Although we attempted to elucidate the mechanism by which these amino acids enhance virulence, we were unable to do so.

The two unique PB2 amino acids were identified and analyzed using reassortant and mutant viruses generated by reverse genetics. Although the replication efficiency in A549 and DF-1 cells did not differ between the wild-type H5N1 viruses and their counterpart reverse genetics-generated viruses, a difference in pathogenicity in mice was observed between the wild-type UT521 virus and the reverse genetics-generated UT521 virus. Wild-type UT521 virus possessed higher mouse pathogenicity compared to the reverse genetics-generated strain of UT521 (RG1), as assessed by measuring MLD_{50} values. Moreover, wild-type UT521 showed replicative ability in non-respiratory organs, whereas the replication of RG1 was limited to the respiratory organs, heart, and spleen. Sequencing of all of the viral genomes of RG1 revealed no nucleotide mutation from wild-type UT521. The reason for this observed phenomenon remains unclear, but it has been speculated that a quasispecies state of

1218 wild-type UT521 might confer higher virulence and an invasive trait in infected BALB/c mice
1219 [79, 80].

1220 Unique amino acids in the NA segment of UT541 were responsible for its low
1221 replication efficiency in A549 cells. NA-32L is located in the stalk region, and NA-111I and
1222 NA-120L are thought to be located in the head domain, based on analyses of the NA 3D
1223 model. The mechanism by which these amino acids lower the viral replication efficiency in
1224 A549 cells is unknown.

1225 Two unique PB2 amino acids of UT521, PB2-109A and PB2-758T, were found to
1226 enhance virulence in mammalian hosts. The degree of enhancement, however, differed
1227 between PB2-109A and PB2-758T. Briefly, the introduction of the unique amino acid PB2-
1228 109A conferred greater enhancement of viral replication efficiency in A549 cells incubated at
1229 33℃ and of BALB/c mouse pathogenicity compared to the introduction of PB2-758T.
1230 Similarly, the introduction of the major amino acid PB2-109V resulted in greater
1231 diminishment of viral replication and pathogenicity compared to the introduction of PB2-
1232 758I, but its effect on BALB/c mouse pathogenicity was less pronounced. Together, the two
1233 amino acids had a synergistic effect.

The replication efficiency in A549 cells incubated at 33℃ was greater for viruses possessing the polymerase complex of UT521 compared to those with the polymerase complex of UT541. However, the mini-replicon assay revealed that the viral polymerase activity of UT521 was considerably lower than that of UT541. This inconsistency between viral replication efficiency and polymerase activity might indicate that UT521 uses a replication efficiency-enhancing mechanism that does not involve viral polymerase activity, or that UT541 is more sensitive to inhibition of viral replication. PB2-109A and PB2-758T each increased the polymerase activity when introduced into the polymerase complex of UT541, which is consistent with their enhancement of viral replication efficiency and pathogenicity. However, although the introduction of the major amino acids PB2-109V and PB2-758I into the polymerase complex of UT521 reduced the replication efficiency in A549 cells and virus pathogenicity in BALB/c mice, the introduction of those major amino acids had little effect on viral polymerase activity. These findings indicate that the enhancing effects of PB2-109A and PB2-758T on viral replication efficiency and pathogenicity result from a mechanism other than viral polymerase activity modification.

Since PB2-758T/I is reported to be part of the NLS, pCAGGS plasmid DNA encoding the viral PB2 protein was transfected into A549 cells for immunofluorescence

analysis. The analysis of mutant PB2 protein, however, revealed no difference in nuclear localization efficiency among the analyzed PB2 protein [76]. The effect of PB2-758T/I on the observed differences in viral replication efficiency and pathogenicity could, therefore, not be attributed to the efficiency of viral PB2 protein nuclear localization.

The function of PB2-109A/V has not yet been reported in the literature. PB2 is a major component of the viral polymerase complex along with PB1, PA, and NP, functioning in viral replication, cap-snatching and viral transcription, and the formation of newly synthesized vRNP complexes. Newly synthesized vRNP complexes are assembled and packaged into progeny virions [1, 76]. To evaluate whether PB2-109A/V and PB2-758T/I have any involvement in the viral replication cycle, immunofluorescence analysis of the intracellular localization of the vRNP complexes was conducted. This analysis, however, did not reveal any significant differences in the intracellular localization patterns of the analyzed PB2 reassortant viruses. I suspect that vRNP observed in the nucleus represents accumulated newly synthesized vRNP complexes. Analysis of the intracellular localization of vRNP complexes originating from infected viruses has been attempted by inhibiting new viral protein production using cycloheximide or 5,6-dichloro-1-b-D-ribofuranosyl-benzimidazole (DRB) [81-84]. In this study, the addition of cycloheximide or DRB, however, prevented the

nuclear localization of the vRNP complexes and the intracellular localization of incoming vRNP complexes could not be evaluated (data not shown). In the immunofluorescence analysis of the intracellular localization of vRNP complexes, redistribution of the vRNP complexes into the cytoplasm was observed, which likely represented the nuclear export of newly synthesized vRNP complexes. No significant difference was observed at this stage of the viral cell cycle among the analyzed PB2 reassortant viruses.

On a structural model of the polymerase complex, PB2-109A/V was plotted on the surface. The location of PB2-109A/V on the model was adjacent to where PB2 protein interacts with PB1 protein, so it is possible that PB2-109A/V plays a role in polymerase complex stability. PB2-109A/V might also interact with viral or host factors. The plotted location of PB2-109A/V on the polymerase complex was not in the vicinity of areas reported to be involved in cap-snatching or vRNA replication activity [76].

The viral PB2 segment is also reported to interact with the host immune system [85-87]. Therefore, it is possible that PB2-109A and PB2-758T provide H5N1 viruses with the ability to escape from host immune responses, which in turn provides higher replication efficiency and pathogenicity in mammalian hosts. Measurement of interferon-β or other host

1284 immune system mediators would help further elucidate the functions of the amino acid

1285 mutations PB2-109A and PB2-758T.

1286

Conclusion

Our epidemiological surveillance of poultry and swine in Indonesia between 2010 and 2016 revealed that two sub-clades of viruses, clade 2.1.3 and clade 2.3.2.1d, were circulating in Indonesia. Our data, together with information from databases, suggest the possibility that clade 2.3.2.1d strains have become dominant in Indonesian poultry. Characterization analysis revealed that clade 2.3.2.1d strains possess mammalian-adaptive traits, including high viral polymerase activity and moderate replication capability in human lung epithelial cells, as well as high virulence in BALB/c mice. Our findings support the continued surveillance of HPAI H5N1 viruses in Indonesia, in order to detect the emergence of viruses with endemic or pandemic capability.

Through a virological analysis of two genetically closely related viruses with different replication efficiency and pathogenicity in mammalian hosts, we identified two amino acid mutations in the viral PB2 protein, PB2-109A and PB2-758T, that enhance viral replication efficiency in human lung epithelial cells and pathogenicity in BALB/c mice. Although the mechanism by which these two PB2 mutations enhance virulence in mammalian hosts has not yet been elucidated, our findings are of value for assessing the risk of avian-to-human transmission of highly pathogenic H5N1 avian influenza field isolates.

Acknowledgements

I would like to express my greatest appreciation for the keen insights and passionate instruction of Prof. Yoshihiro Kawaoka, Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo and Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison.

I am deeply grateful for the great mentorship, profound patience, and ceaseless encouragement from Dr. Shinya Yamada, Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo.

I would like to express my gratitude for the critical devotion to our collaborative research and warm hospitality of Prof. Chairul A. Nidom, Avian Influenza Research Center Laboratory, Professor Nidom Foundation, and Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.

I owe my gratitude to Dr. Kiyoko Iwatsuki-Horimoto, Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, for her instruction and support both in the laboratory and in the field.

I would like to thank Drs. Yuko Sakai-Tagawa, Masaki Imai, Maki Kiso, and Seiya Yamayoshi, Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo for their technical assistance and advice.

I would also like to express my appreciation to Dr. Reina Yamaji, World Health Organization, Geneva, and to Reviany V. Nidom, Setyarina Indrasari, Kuncoro P. Santoso, and the other laboratory staff and students of the Avian Influenza Research Center Laboratory, Professor Nidom Foundation, and Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia for their hard work and sincere dedication to epidemiological surveillance in Indonesia.

This work was supported by the NIAID-funded Center for Research on Influenza Pathogenesis (CRIP) (HHSN272201400008C).

References

1. Shaw, M. and P. Palese, *Orthomyxoviridae, p 1151–1185. Fields virology*. 2013, Lippincott Williams & Wilkins, Philadelphia, PA.
2. Wright, P., G. Neumann, and Y. Kawaoka, *Orthomyxoviruses. Fields Virology, eds Knipe DM, et al*. 2013, Lippincott Williams & Wilkins, Philadelphia, PA).
3. Osterhaus, A.D., G.F. Rimmelzwaan, B.E. Martina, T.M. Bestebroer, and R.A. Fouchier, *Influenza B virus in seals*. Science, 2000. **288**(5468): p. 1051-3.
4. Bodewes, R., D. Morick, G. de Mutsert, N. Osinga, T. Bestebroer, S. van der Vliet, S.L. Smits, T. Kuiken, G.F. Rimmelzwaan, R.A. Fouchier, and A.D. Osterhaus, *Recurring influenza B virus infections in seals*. Emerg Infect Dis, 2013. **19**(3): p. 511-2.
5. Jelley, L., A. Levy, Y.M. Deng, N. Spirason, J. Lang, I. Buettner, J. Druce, C. Blyth, P. Effler, D. Smith, and I.G. Barr, *Influenza C infections in Western Australia and Victoria from 2008 to 2014*. Influenza Other Respir Viruses, 2016. **10**(6): p. 455-461.
6. Matsuzaki, Y., K. Sugawara, C. Abiko, T. Ikeda, Y. Aoki, K. Mizuta, N. Katsushima, F. Katsushima, Y. Katsushima, T. Itagaki, Y. Shimotai, S. Hongo, Y. Muraki, and H. Nishimura, *Epidemiological information regarding the periodic epidemics of influenza C virus in Japan (1996-2013) and the seroprevalence of antibodies to different antigenic groups*. J Clin Virol, 2014. **61**(1): p. 87-93.
7. Atkinson, K.V., L.A. Bishop, G. Rhodes, N. Salez, N.R. McEwan, M.J. Hegarty, J. Robey, N. Harding, S. Wetherell, R.M. Lauder, R.W. Pickup, M. Wilkinson, and D. Gatherer, *Influenza C in Lancaster, UK, in the winter of 2014-2015*. Sci Rep, 2017. **7**: p. 46578.
8. Su, S., X. Fu, G. Li, F. Kerlin, and M. Veit, *Novel Influenza D virus: Epidemiology, pathology, evolution and biological characteristics*. Virulence, 2017. **8**(8): p. 1580-1591.
9. Hause, B.M., M. Ducatez, E.A. Collin, Z. Ran, R. Liu, Z. Sheng, A. Armien, B. Kaplan, S. Chakravarty, A.D. Hoppe, R.J. Webby, R.R. Simonson, and F. Li, *Isolation of a novel swine influenza virus from Oklahoma in 2011 which is distantly related to human influenza C viruses*. PLoS Pathog, 2013. **9**(2): p. e1003176.
10. Neumann, G., H. Chen, G.F. Gao, Y. Shu, and Y. Kawaoka, *H5N1 influenza viruses: outbreaks and biological properties*. Cell Res, 2010. **20**(1): p. 51-61.
11. Horimoto, T. and Y. Kawaoka, *Influenza: lessons from past pandemics, warnings from current incidents*. Nat Rev Microbiol, 2005. **3**(8): p. 591-600.

12. Neumann, G., T. Noda, and Y. Kawaoka, *Emergence and pandemic potential of swine-origin H1N1 influenza virus*. Nature, 2009. **459**(7249): p. 931-9.
13. Tong, S., Y. Li, P. Rivailler, C. Conrardy, D.A. Castillo, L.M. Chen, S. Recuenco, J.A. Ellison, C.T. Davis, I.A. York, A.S. Turmelle, D. Moran, S. Rogers, M. Shi, Y. Tao, M.R. Weil, K. Tang, L.A. Rowe, S. Sammons, X. Xu, M. Frace, K.A. Lindblade, N.J. Cox, L.J. Anderson, C.E. Rupprecht, and R.O. Donis, *A distinct lineage of influenza A virus from bats*. Proc Natl Acad Sci U S A, 2012. **109**(11): p. 4269-74.
14. Tong, S., X. Zhu, Y. Li, M. Shi, J. Zhang, M. Bourgeois, H. Yang, X. Chen, S. Recuenco, J. Gomez, L.M. Chen, A. Johnson, Y. Tao, C. Dreyfus, W. Yu, R. McBride, P.J. Carney, A.T. Gilbert, J. Chang, Z. Guo, C.T. Davis, J.C. Paulson, J. Stevens, C.E. Rupprecht, E.C. Holmes, I.A. Wilson, and R.O. Donis, *New world bats harbor diverse influenza A viruses*. PLoS Pathog, 2013. **9**(10): p. e1003657.
15. Webster, R.G., W.J. Bean, O.T. Gorman, T.M. Chambers, and Y. Kawaoka, *Evolution and ecology of influenza A viruses*. Microbiol Rev, 1992. **56**(1): p. 152-79.
16. Swayne, D.E. and D.L. Suarez, *Highly pathogenic avian influenza*. Rev Sci Tech, 2000. **19**(2): p. 463-82.
17. Horimoto, T. and Y. Kawaoka, *Pandemic threat posed by avian influenza A viruses*. Clin Microbiol Rev, 2001. **14**(1): p. 129-49.
18. Horimoto, T. and Y. Kawaoka, *Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus*. J Virol, 1994. **68**(5): p. 3120-8.
19. Xu, X., Subbarao, N.J. Cox, and Y. Guo, *Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong*. Virology, 1999. **261**(1): p. 15-9.
20. Claas, E.C., A.D. Osterhaus, R. van Beek, J.C. De Jong, G.F. Rimmelzwaan, D.A. Senne, S. Krauss, K.F. Shortridge, and R.G. Webster, *Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus*. Lancet, 1998. **351**(9101): p. 472-7.
21. Subbarao, K., A. Klimov, J. Katz, H. Regnery, W. Lim, H. Hall, M. Perdue, D. Swayne, C. Bender, J. Huang, M. Hemphill, T. Rowe, M. Shaw, X. Xu, K. Fukuda, and N. Cox, *Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness*. Science, 1998. **279**(5349): p. 393-6.
22. Health), O.T.W.O.f.A., *OIE Situation Report for Highly Pathogenic Avian Influenza*. 2018.

23. Organization), W.W.H. *Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO*. 2018 [cited 2018 25, October, 2018]; Available from: https://www.who.int/influenza/human_animal_interface/2018_09_21_tableH5N1.pdf?ua=1.
24. Organization), W.W.H. *H5N1 highly pathogenic avian influenza: Timeline of major events*. 2014; Available from: https://www.who.int/influenza/human_animal_interface/H5N1_avian_influenza_update20141204.pdf.
25. Davis, A.S., D.S. Chertow, J.E. Moyer, J. Suzich, A. Sandouk, D.W. Dorward, C. Logun, J.H. Shelhamer, and J.K. Taubenberger, *Validation of normal human bronchial epithelial cells as a model for influenza A infections in human distal trachea*. J Histochem Cytochem, 2015. **63**(5): p. 312-28.
26. Jakiela, B., R. Brockman-Schneider, S. Amineva, W.M. Lee, and J.E. Gern, *Basal cells of differentiated bronchial epithelium are more susceptible to rhinovirus infection*. Am J Respir Cell Mol Biol, 2008. **38**(5): p. 517-23.
27. Neumann, G., T. Watanabe, H. Ito, S. Watanabe, H. Goto, P. Gao, M. Hughes, D.R. Perez, R. Donis, E. Hoffmann, G. Hobom, and Y. Kawaoka, *Generation of influenza A viruses entirely from cloned cDNAs*. Proc Natl Acad Sci U S A, 1999. **96**(16): p. 9345-50.
28. Reed, L.J., *A simple method of estimating fifty per cent endpoints*. Am J Hyg., 1938. **27**: p. 493-497.
29. Yamayoshi, S., S. Yamada, S. Fukuyama, S. Murakami, D. Zhao, R. Uraki, T. Watanabe, Y. Tomita, C. Macken, G. Neumann, and Y. Kawaoka, *Virulence-affecting amino acid changes in the PA protein of H7N9 influenza A viruses*. J Virol, 2014. **88**(6): p. 3127-34.
30. Niwa, H., K. Yamamura, and J. Miyazaki, *Efficient selection for high-expression transfectants with a novel eukaryotic vector*. Gene, 1991. **108**(2): p. 193-9.
31. Lin, Y.P., X. Xiong, S.A. Wharton, S.R. Martin, P.J. Coombs, S.G. Vachieri, E. Christodoulou, P.A. Walker, J. Liu, J.J. Skehel, S.J. Gamblin, A.J. Hay, R.S. Daniels, and J.W. McCauley, *Evolution of the receptor binding properties of the influenza A(H3N2) hemagglutinin*. Proc Natl Acad Sci U S A, 2012. **109**(52): p. 21474-9.
32. Kiso, M., K. Iwatsuki-Horimoto, S. Yamayoshi, R. Uraki, M. Ito, N. Nakajima, S. Yamada, M. Imai, E. Kawakami, Y. Tomita, S. Fukuyama, Y. Itoh, K. Ogasawara, T.J.S. Lopes, T. Watanabe, L.H. Moncla, H. Hasegawa, T.C. Friedrich, G. Neumann,

and Y. Kawaoka, *Emergence of Oseltamivir-Resistant H7N9 Influenza Viruses in Immunosuppressed Cynomolgus Macaques*. J Infect Dis, 2017. **216**(5): p. 582-593.

33. Yamayoshi, S., M. Watanabe, H. Goto, and Y. Kawaoka, *Identification of a Novel Viral Protein Expressed from the PB2 Segment of Influenza A Virus*. J Virol, 2016. **90**(1): p. 444-56.

34. Network, T.W.H.O.G.I.P.S., *Evolution of H5N1 avian influenza viruses in Asia*. Emerg Infect Dis, 2005. **11**(10): p. 1515-21.

35. Lam, T.T., C.C. Hon, O.G. Pybus, S.L. Kosakovsky Pond, R.T. Wong, C.W. Yip, F. Zeng, and F.C. Leung, *Evolutionary and transmission dynamics of reassortant H5N1 influenza virus in Indonesia*. PLoS Pathog, 2008. **4**(8): p. e1000130.

36. Li, K.S., Y. Guan, J. Wang, G.J. Smith, K.M. Xu, L. Duan, A.P. Rahardjo, P. Puthavathana, C. Buranathai, T.D. Nguyen, A.T. Estoepangestie, A. Chaisingh, P. Auewarakul, H.T. Long, N.T. Hanh, R.J. Webby, L.L. Poon, H. Chen, K.F. Shortridge, K.Y. Yuen, R.G. Webster, and J.S. Peiris, *Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia*. Nature, 2004. **430**(6996): p. 209-13.

37. Wibawa, H., D. Karo-Karo, E.S. Pribadi, A. Bouma, R. Bodewes, H. Vernooij, Diyantoro, A. Sugama, D.H. Muljono, G. Koch, F.S. Tjatur Rasa, and A. Stegeman, *Exploring contacts facilitating transmission of influenza A(H5N1) virus between poultry farms in West Java, Indonesia: A major role for backyard farms?* Prev Vet Med, 2018. **156**: p. 8-15.

38. Takano, R., C.A. Nidom, M. Kiso, Y. Muramoto, S. Yamada, Y. Sakai-Tagawa, C. Macken, and Y. Kawaoka, *Phylogenetic characterization of H5N1 avian influenza viruses isolated in Indonesia from 2003-2007*. Virology, 2009. **390**(1): p. 13-21.

39. Nidom, C.A., R. Takano, S. Yamada, Y. Sakai-Tagawa, S. Daulay, D. Aswadi, T. Suzuki, Y. Suzuki, K. Shinya, K. Iwatsuki-Horimoto, Y. Muramoto, and Y. Kawaoka, *Influenza A (H5N1) viruses from pigs, Indonesia*. Emerg Infect Dis, 2010. **16**(10): p. 1515-23.

40. Organization), W.W.H. *Influenza at the human-animal interface Summary and assessment, 25 July 2017 to 27 September 2017*. 2017 25, October, 2018]; Available from: http://www.who.int/influenza/human_animal_interface/Influenza_Summary_IRA_HA_interface_09_27_2017.pdf?ua=1.

41. Neumann, G. and Y. Kawaoka, *Host range restriction and pathogenicity in the context of influenza pandemic*. Emerg Infect Dis, 2006. **12**(6): p. 881-6.

42. Neumann, G., K. Shinya, and Y. Kawaoka, *Molecular pathogenesis of H5N1 influenza virus infections*. Antivir Ther, 2007. **12**(4 Pt B): p. 617-26.
43. Rogers, G.N. and J.C. Paulson, *Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin*. Virology, 1983. **127**(2): p. 361-73.
44. Auewarakul, P., O. Suptawiwat, A. Kongchanagul, C. Sangma, Y. Suzuki, K. Ungchusak, S. Louisirirotchanakul, H. Lerdsamran, P. Pooruk, A. Thitithanyanont, C. Pittayawonganon, C.T. Guo, H. Hiramatsu, W. Jampangern, S. Chunsutthiwat, and P. Puthavathana, *An avian influenza H5N1 virus that binds to a human-type receptor*. J Virol, 2007. **81**(18): p. 9950-5.
45. Suzuki, Y., *Sialobiology of influenza: molecular mechanism of host range variation of influenza viruses*. Biol Pharm Bull, 2005. **28**(3): p. 399-408.
46. Mair, C.M., K. Ludwig, A. Herrmann, and C. Sieben, *Receptor binding and pH stability - how influenza A virus hemagglutinin affects host-specific virus infection*. Biochim Biophys Acta, 2014. **1838**(4): p. 1153-68.
47. Yamada, S., Y. Suzuki, T. Suzuki, M.Q. Le, C.A. Nidom, Y. Sakai-Tagawa, Y. Muramoto, M. Ito, M. Kiso, T. Horimoto, K. Shinya, T. Sawada, M. Kiso, T. Usui, T. Murata, Y. Lin, A. Hay, L.F. Haire, D.J. Stevens, R.J. Russell, S.J. Gamblin, J.J. Skehel, and Y. Kawaoka, *Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors*. Nature, 2006. **444**(7117): p. 378-82.
48. Hatta, M., P. Gao, P. Halfmann, and Y. Kawaoka, *Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses*. Science, 2001. **293**(5536): p. 1840-2.
49. Li, Z., H. Chen, P. Jiao, G. Deng, G. Tian, Y. Li, E. Hoffmann, R.G. Webster, Y. Matsuoka, and K. Yu, *Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model*. J Virol, 2005. **79**(18): p. 12058-64.
50. Yamada, S., M. Hatta, B.L. Staker, S. Watanabe, M. Imai, K. Shinya, Y. Sakai-Tagawa, M. Ito, M. Ozawa, T. Watanabe, S. Sakabe, C. Li, J.H. Kim, P.J. Myler, I. Phan, A. Raymond, E. Smith, R. Stacy, C.A. Nidom, S.M. Lank, R.W. Wiseman, B.N. Bimber, D.H. O'Connor, G. Neumann, L.J. Stewart, and Y. Kawaoka, *Biological and structural characterization of a host-adapting amino acid in influenza virus*. PLoS Pathog, 2010. **6**(8): p. e1001034.
51. Dharmayanti, N.L., R. Hartawan, H. Wibawa, A. Balish, R. Donis, C.T. Davis, and G. Samaan, *Genetic characterization of clade 2.3.2.1 avian influenza A(H5N1) viruses, Indonesia, 2012*. Emerg Infect Dis, 2014. **20**(4): p. 671-4.

52. Dharmayanti, N., S.W. Thor, N. Zanders, R. Hartawan, A. Ratnawati, Y. Jang, M. Rodriguez, D.L. Suarez, G. Samaan, Pudjiatmoko, and C.T. Davis, *Attenuation of highly pathogenic avian influenza A(H5N1) viruses in Indonesia following the reassortment and acquisition of genes from low pathogenicity avian influenza A virus progenitors*. Emerg Microbes Infect, 2018. **7**(1): p. 147.
53. Kumar, S., G. Stecher, and K. Tamura, *MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets*. Mol Biol Evol, 2016. **33**(7): p. 1870-4.
54. Kumar, S., M. Nei, J. Dudley, and K. Tamura, *MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences*. Brief Bioinform, 2008. **9**(4): p. 299-306.
55. Saitou, N. and M. Nei, *The neighbor-joining method: a new method for reconstructing phylogenetic trees*. Mol Biol Evol, 1987. **4**(4): p. 406-25.
56. Felsenstein, J., *Confidence limits on phylogenies: an approach using the bootstrap*. Evolution, 1985. **39**(4): p. 783-791.
57. Tamura, K., M. Nei, and S. Kumar, *Prospects for inferring very large phylogenies by using the neighbor-joining method*. Proc Natl Acad Sci U S A, 2004. **101**(30): p. 11030-5.
58. World Health Organization/World Organisation for Animal, H.F. and H.N.E.W.G. Agriculture Organization, *Revised and updated nomenclature for highly pathogenic avian influenza A (H5N1) viruses*. Influenza Other Respir Viruses, 2014. **8**(3): p. 384-8.
59. Jiang, W., G. Hou, J. Li, C. Peng, S. Wang, and J. Chen, *Novel variants of clade 2.3.2.1 H5N1 highly pathogenic avian influenza virus in migratory waterfowl of Hongze Lake*. Vet Microbiol, 2017. **198**: p. 99-103.
60. Fan, S., M. Hatta, J.H. Kim, P. Halfmann, M. Imai, C.A. Macken, M.Q. Le, T. Nguyen, G. Neumann, and Y. Kawaoka, *Novel residues in avian influenza virus PB2 protein affect virulence in mammalian hosts*. Nat Commun, 2014. **5**: p. 5021.
61. Yang, Z.Y., C.J. Wei, W.P. Kong, L. Wu, L. Xu, D.F. Smith, and G.J. Nabel, *Immunization by avian H5 influenza hemagglutinin mutants with altered receptor binding specificity*. Science, 2007. **317**(5839): p. 825-8.
62. Wang, W., B. Lu, H. Zhou, A.L. Suguitan, Jr., X. Cheng, K. Subbarao, G. Kemble, and H. Jin, *Glycosylation at 158N of the hemagglutinin protein and receptor binding specificity synergistically affect the antigenicity and immunogenicity of a live attenuated H5N1 A/Vietnam/1203/2004 vaccine virus in ferrets*. J Virol, 2010. **84**(13): p. 6570-7.

63. Su, Y., H.Y. Yang, B.J. Zhang, H.L. Jia, and P. Tien, *Analysis of a point mutation in H5N1 avian influenza virus hemagglutinin in relation to virus entry into live mammalian cells*. Arch Virol, 2008. **153**(12): p. 2253-61.
64. Gao, Y., Y. Zhang, K. Shinya, G. Deng, Y. Jiang, Z. Li, Y. Guan, G. Tian, Y. Li, J. Shi, L. Liu, X. Zeng, Z. Bu, X. Xia, Y. Kawaoka, and H. Chen, *Identification of amino acids in HA and PB2 critical for the transmission of H5N1 avian influenza viruses in a mammalian host*. PLoS Pathog, 2009. **5**(12): p. e1000709.
65. Imai, M., T. Watanabe, M. Hatta, S.C. Das, M. Ozawa, K. Shinya, G. Zhong, A. Hanson, H. Katsura, S. Watanabe, C. Li, E. Kawakami, S. Yamada, M. Kiso, Y. Suzuki, E.A. Maher, G. Neumann, and Y. Kawaoka, *Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets*. Nature, 2012. **486**(7403): p. 420-8.
66. Shore, D.A., H. Yang, A.L. Balish, S.S. Shepard, P.J. Carney, J.C. Chang, C.T. Davis, R.O. Donis, J.M. Villanueva, A.I. Klimov, and J. Stevens, *Structural and antigenic variation among diverse clade 2 H5N1 viruses*. PLoS One, 2013. **8**(9): p. e75209.
67. Sonnberg, S., R.J. Webby, and R.G. Webster, *Natural history of highly pathogenic avian influenza H5N1*. Virus Res, 2013. **178**(1): p. 63-77.
68. Hidari, K.I., T. Murata, K. Yoshida, Y. Takahashi, Y.H. Minamijima, Y. Miwa, S. Adachi, M. Ogata, T. Usui, Y. Suzuki, and T. Suzuki, *Chemoenzymatic synthesis, characterization, and application of glycopolymers carrying lactosamine repeats as entry inhibitors against influenza virus infection*. Glycobiology, 2008. **18**(10): p. 779-88.
69. Ogata, M., K.I. Hidari, W. Kozaki, T. Murata, J. Hiratake, E.Y. Park, T. Suzuki, and T. Usui, *Molecular design of spacer-N-linked sialoglycopolypeptide as polymeric inhibitors against influenza virus infection*. Biomacromolecules, 2009. **10**(7): p. 1894-903.
70. Shimizu, K., L. Wulandari, E.D. Poetranto, R.A. Setyoningrum, R. Yudhawati, A. Sholikhah, A.M. Nastri, A.L. Poetranto, A.Y. Candra, E.F. Puruhito, Y. Takahara, Y. Yamagishi, M. Yamaoka, H. Hotta, T. Ustumi, M.I. Lusida, Soetjipto, Y.K. Shimizu, G. Soegiarto, and Y. Mori, *Seroevidence for a High Prevalence of Subclinical Infection With Avian Influenza A(H5N1) Virus Among Workers in a Live-Poultry Market in Indonesia*. J Infect Dis, 2016. **214**(12): p. 1929-1936.
71. Le, M.Q., P. Horby, A. Fox, H.T. Nguyen, H.K. Le Nguyen, P.M. Hoang, K.C. Nguyen, M.D. de Jong, R.E. Jeeninga, H. Rogier van Doorn, J. Farrar, and H.F.

Wertheim, *Subclinical avian influenza A(H5N1) virus infection in human, Vietnam*. Emerg Infect Dis, 2013. **19**(10): p. 1674-7.

72. Robert, M., R. Holle du, V. Setiawaty, K.N. Pangesti, and E.R. Sedyaningsih, *Seroprevalence of avian influenza A/H5N1 among poultry farmers in rural Indonesia, 2007*. Southeast Asian J Trop Med Public Health, 2010. **41**(5): p. 1095-103.

73. Neumann, G., C.A. Macken, A.I. Karasin, R.A. Fouchier, and Y. Kawaoka, *Egyptian H5N1 influenza viruses-cause for concern?* PLoS Pathog, 2012. **8**(11): p. e1002932.

74. Watanabe, Y., M.S. Ibrahim, and K. Ikuta, *Evolution and control of H5N1. A better understanding of the evolution and diversity of H5N1 flu virus and its host species in endemic areas could inform more efficient vaccination and control strategies*. EMBO Rep, 2013. **14**(2): p. 117-22.

75. Arafa, A.S., S. Yamada, M. Imai, T. Watanabe, S. Yamayoshi, K. Iwatsuki-Horimoto, M. Kiso, Y. Sakai-Tagawa, M. Ito, T. Imamura, N. Nakajima, K. Takahashi, D. Zhao, K. Oishi, A. Yasuhara, C.A. Macken, G. Zhong, A.P. Hanson, S. Fan, J. Ping, M. Hatta, T.J. Lopes, Y. Suzuki, M. El-Husseiny, A. Selim, N. Hagag, M. Soliman, G. Neumann, H. Hasegawa, and Y. Kawaoka, *Risk assessment of recent Egyptian H5N1 influenza viruses*. Sci Rep, 2016. **6**: p. 38388.

76. Pflug, A., M. Lukarska, P. Resa-Infante, S. Reich, and S. Cusack, *Structural insights into RNA synthesis by the influenza virus transcription-replication machine*. Virus Res, 2017. **234**: p. 103-117.

77. Boivin, S., S. Cusack, R.W. Ruigrok, and D.J. Hart, *Influenza A virus polymerase: structural insights into replication and host adaptation mechanisms*. J Biol Chem, 2010. **285**(37): p. 28411-7.

78. Eisfeld, A.J., E. Kawakami, T. Watanabe, G. Neumann, and Y. Kawaoka, *RAB11A is essential for transport of the influenza virus genome to the plasma membrane*. J Virol, 2011. **85**(13): p. 6117-26.

79. Vignuzzi, M., J.K. Stone, J.J. Arnold, C.E. Cameron, and R. Andino, *Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population*. Nature, 2006. **439**(7074): p. 344-8.

80. Lauring, A.S. and R. Andino, *Quasispecies theory and the behavior of RNA viruses*. PLoS Pathog, 2010. **6**(7): p. e1001005.

81. Banerjee, I., Y. Yamauchi, A. Helenius, and P. Horvath, *High-content analysis of sequential events during the early phase of influenza A virus infection*. PLoS One, 2013. **8**(7): p. e68450.

82. Killip, M.J., M. Smith, D. Jackson, and R.E. Randall, *Activation of the interferon induction cascade by influenza a viruses requires viral RNA synthesis and nuclear export*. J Virol, 2014. **88**(8): p. 3942-52.
83. Amorim, M.-J., E.K. Read, R.M. Dalton, L. Medcalf, and P. Digard, *Nuclear Export of Influenza A Virus mRNAs Requires Ongoing RNA Polymerase II Activity*. Traffic, 2007. **8**(1): p. 1-11.
84. Read, E.K.C. and P. Digard, *Individual influenza A virus mRNAs show differential dependence on cellular NXF1/TAP for their nuclear export*. Journal of General Virology, 2010. **91**(5): p. 1290-1301.
85. Iwai, A., T. Shiozaki, T. Kawai, S. Akira, Y. Kawaoka, A. Takada, H. Kida, and T. Miyazaki, *Influenza A virus polymerase inhibits type I interferon induction by binding to interferon beta promoter stimulator 1*. J Biol Chem, 2010. **285**(42): p. 32064-74.
86. Graef, K.M., F.T. Vreede, Y.F. Lau, A.W. McCall, S.M. Carr, K. Subbarao, and E. Fodor, *The PB2 subunit of the influenza virus RNA polymerase affects virulence by interacting with the mitochondrial antiviral signaling protein and inhibiting expression of beta interferon*. J Virol, 2010. **84**(17): p. 8433-45.
87. Carr, S.M., E. Carnero, A. Garcia-Sastre, G.G. Brownlee, and E. Fodor, *Characterization of a mitochondrial-targeting signal in the PB2 protein of influenza viruses*. Virology, 2006. **344**(2): p. 492-508.