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Molecular epidemiology and pathogenicity of animal influenza viruses isolated in Japan

（日本で分離された動物インフルエンザウイルスの
分子疫学と病原性に関する研究）

Katsushi Kanehira

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Abbreviations

A A(H1N1)pdm09: pandemic A(H1N1) 2009

D dpi: days post-inoculation

E ELISA: enzyme-linked immunosorbent assay

G GISAID: Global Initiative on Sharing Avian Influenza Data

H HA: hemagglutinin

HI: hemagglutination inhibition

HPAIV: highly pathogenic avian influenza virus

hpi: hours post-inoculation

M MDCK: Madin-Darby canine kidney

MDT: mean death time

MP: matrix protein

N NA: neuraminidase

NIAH: National Institute of Animal Health

NIID: National Institute of Infectious Disease

NP: nucleoprotein

NS: nonstructural protein

O OIE: Office international des epizooties

P PA: polymerase acidic protein

PB1: polymerase basic 1 protein

PB2: polymerase basic 2 protein

PBS: phosphate buffered saline

PRRSV: porcine reproductive and respiratory syndrome virus

R RT-PCR: reverse transcription polymerase chain reaction

S SIV: Swine influenza virus

V vRNA: viral RNA

W WHO: World Health Organization

Unit Abbreviations

E EID_{50}: 50% chicken embryo infectious dose

H h: hour

M μg: microgram

min: minute

ml: milliliter

μl: microliter

R rpm: round per minute

Abbreviations of virus strains

Part 1

B	BD14/10: A/swine/Binh Duong/03-14/2010 (H3N2)
	BD16/10: A/swine/Binh Duong/02-16/2010 (H1N2)
	Bri07: A/Brisbane/59/2007 (H1N1)
E	Ehi02: A/swine/Ehime/1/2002 (H3N2)
G	Gun12: A/swine/Gunma/1/2012 (H1N2)
	Gun13: A/swine/Gunma/1/2013 (H1N2)
H	Hir05: A/Hiroshima/52/2005 (H3N2)
I	Iba13: A/swine/Ibaraki/1/2013 (H1N2)
	Iow30: A/swine/Iowa/15/1930 (H1N1)
K	Kyo79: A/swine/Kyoto/3/1979 (H1N1)
M	Miy13: A/swine/Miyazaki/2/2013 (H3N2)
N	Nag00: A/swine/Nagano/2000 (H3N2)
	NC99: A/New Caledonia/20/1999 (H1N1)
	NY04: A/New York/55/2004 (H3N2)
O	Osa08: A/swine/Osaka-C/12-20/2008 (H3N2)
P	Pan99: A/Panama/2007/1999 (H3N2)
S	Sai05: A/swine/Saitama/01/2005 (H1N2)
	SI06: A/Solomon Islands/3/2006 (H1N1)
	Syd97: A/Sydney/5/1997 (H3N2)
T	Toc08: A/swine/Tochigi/1/2008 (H1N2)
	Tsu05: A/duck/Tsukuba/67/2005 (H1N1)
U	Uru07: A/Uruguay/716/2007 (H3N2)
W	Wad69: A/swine/Wadayama/5/1969 (H3N2)
	Wuh95: A/Wuhan/359/1995 (H3N2)
	Wyo03: A/Wyoming/03/2003 (H3N2)

Part 2

A	Aki08: A/whooper swan/Akita/1/2008 (H5N1)
	Anh05: A/Anhui/1/2005 (H5N1)
B	Ban10: A/chicken/Bangladesh/1151-9/2010 (H5N1)
C	C48: A/duck/Chiba/26-372-48/2014 (H5N8)
	C61: A/duck/Chiba/26-372-61/2014 (H5N8)
E	E1010: A/chicken/Egypt/1063/2010 (H5N1)
	E1212: A/chicken/Egypt/121/2012 (H5N1)

	E1510: A/chicken/Egypt/1553-26/2010 (H5N1)
G	Goc14: A/breeder duck/Korea/Gochang1/2014 (H5N8)
	Bua14: A/broiler duck/Korea/Buan2/2014 (H5N8)
H	Hma07: A/chicken/Hmawbi/517/2007 (H5N1)
I	Ind07: A/chicken/Indonesia/demak1631-56/2007 (H5N1)
K	KT2407: A/village chicken/Kyaing Tong/2433/2007 (H5N1)
	Kum14: A/chicken/Kumamoto/1-7/2014 (H5N8)
M	Miy07: A/chicken/Miyazaki/K11/2007 (H5N1)
P	Pyi06: A/chicken/Pygyitagon/204/2006 (H5N1)
S	SA61: A/tern/South Africa/1961 (H5N3)
	Shi02: A/whistling swan/Shimane/580/2002 (H5N3)
T	TG1513: A/duck/Tien Giang/15421/2013 (H5N1)
	TG8913: A/chicken/Tien Giang/8932/2013 (H5N1)
	Tha06: A/chicken/Thailand/PC-170/2006 (H5N1)
V	Vie11: A/duck/Vietnam/NCVD-672/2011 (H5N1)
Y	Yam04: A/chicken/Yamaguchi/7/2004 (H5N1)

Preface

Influenza A virus belongs to the genus *Influenzavirus A* of the family *Orthomyxoviridae*. The influenza A virus genome comprises eight negative-sense RNA segments, which is viral RNA (vRNA), named as PB2, PB1, PA, HA, NP, NA, MP, and NS depending on the assigned representative coding-protein in each vRNA (1). Previous phylogenetic analyses revealed that the genes of influenza A virus envelope proteins, hemagglutinin (HA) and neuraminidase (NA), evolve rapidly and continually produce variants, whereas viral internal protein genes acquire mutations very gradually (2, 3). The HA and NA variations are exploited to subtype the influenza A virus.

Influenza A viruses have been isolated from several species, including humans, pigs, horses, dogs, cats, bats, marine mammals, and various domestic and wild birds (4). This pathogen frequently crosses the species barrier between animals and humans, and it has been well-established that waterfowl act as a direct or indirect source of influenza viruses (4). Accordingly, influenza A viruses of 16 HA (H1–H16) and 9 NA (N1–N9) subtypes have been detected in waterfowls (5, 6). The combination of the HA and NA subtypes includes all known influenza A virus subtypes isolated from humans and animals, except for newly detected viruses from bats subtypes (H17N10 and H18N11) (7). Despite mutations of vRNA in the replication, the genome segmentation is a distinctive feature that permits the reassortment of viral genomic segments in cells infected concurrently with different influenza viruses. Reassortment can theoretically result in 256 different gnomic variations from two parental viruses (1) and has been the genetic basis for the appearance of strains of previous human outbreaks, such as H2N2 “Asian” influenza in 1957 and H3N2 “Hong Kong” influenza in 1968 (8, 9).

Swine influenza virus (SIV), an influenza A virus isolated from pigs, is a major causative agent of respiratory disease in the swine industry (10). Infection by SIV results in weight loss in pigs and imposes an economic distress on pig farmers (11). The direct cost of swine influenza was estimated to be between US$3.23 and US$10.31 per pig in the USA and £7 per pig in the UK (4). Three predominant SIV subtypes possessing different combinations of HA and NA genomic segments, H1N1, H1N2, and H3N2, are currently in circulation worldwide, including in Japan

(10). In most cases, the SIVs are geographically restricted and their diversity is also regionally dependent (12). Pigs and humans have shared influenza A viruses since at least 1918, when the "Spanish" influenza virus caused a devastating pandemic among humans (6). In Japan, classical H1N1 SIVs originating from the "Spanish" influenza virus are considered to have infected pigs in the late 1970s (13). Reassortment between a classical H1N1 SIV and a virus originating from human epidemic H3N2 virus generated an H1N2 SIV possessing 7 genomic segments from the classical SIV and the NA segment from the human epidemic virus (14). This has become the predominant SIV since then (15), and H3N2 strains originating from the human epidemic strain has been isolated sporadically in Japan (16). The pandemic A(H1N1) 2009 (A(H1N1)pdm09) virus, a reassortant between a Eurasian avian-like H1N1 SIV and a North American triple reassortant SIV, introduced into humans and spread to large proportion of world (17). Since then, transmission of A(H1N1)pdm09 viruses from human to pig has been reported worldwide, including in Japan (18-21). Reassortant viruses between A(H1N1)pdm09 viruses and SIVs have also been isolated from pigs (21-35). Three novel reassortant SIVs between A(H1N1)pdm09 viruses and endemic SIVs, isolated from symptomatic pigs in Japan in 2013, were analyzed phylogenetically and serologically, and the results have been presented in part 1.

Avian influenza viruses with a high pathogenicity against chickens are defined as highly pathogenic avian influenza virus (HPAIV) (36). Historically, HPAIVs have only belonged to the H5 and H7 HA subtypes with few exceptions, although many strains of H5 and H7 avian influenza virus have a low pathogenicity (36-39). The Asian subtype H5 HPAIV lineage originates from A/goose/Guangdong/1/1996 (H5N1) which was isolated from a sick goose during an outbreak in Guangdong Province, China, in 1996 (40). Since 2003, it has spread across Eurasia, and in 2005, it entered Africa (41, 42). Since 2004, HPAIVs of the Asian lineage of the H5N1 subtype has caused sporadic but highly pathogenic avian influenza outbreaks in poultry farms in Japan (43-45). After a 3-year interval, in April 2014, an outbreak in a broiler chicken farm was confirmed in the Kumamoto prefecture in Japan. An H5N8 HPAIV isolated from pooled cloaca swabs were analyzed for investigation of its genetic origin, serological property, and infectivity and pathogenicity against chickens and ducks as mentioned in part 2.

Part 1

Phylogenetic and serological analyses of reassortant swine influenza viruses isolated in 2013 in Japan

Introduction

SIV causes a highly contagious respiratory disease that is typically characterized by high fever, anorexia, inactivity, abdominal breathing, and dyspnea (10). SIV, along with porcine reproductive and respiratory syndrome virus (PRRSV) and *Mycoplasma hyopneumoniae*, is a major pathogen detected in 10–22-week-old pigs with clinical signs of porcine respiratory disease complex. Notably, SIV infections can impair reproductive ability; coinfection by SIV and PRRSV or *M. hyopneumoniae* can seriously compromise reproductive ability (11, 46). In addition, because the tracheal epithelium in pigs expresses receptors for avian and human influenza viruses, domestic pigs may be mixing vessels for the emergence of novel reassortant viruses having the potential to cause human pandemics (47).

Despite limited subtypes, the broad susceptibility of pigs to influenza viruses is also reflected in the predominant SIV lineages. The classical H1N1 SIVs originating from the "Spanish" influenza virus that caused the devastating human pandemic persisting from 1918 to 1919. These linages were predominant in European and North American pigs until the 1990s (6). Since 1979, the classical H1N1 swine lineage has largely been replaced in Europe by an H1N1 virus of avian origin that possesses a genome comprising entirely of avian virus genes. This avian H1N1 lineage has since become endemic to Europe (48). Notably, H1N2 SIVs have cocirculated with avian origin H1N1 SIVs among European pigs; the HA genes of these H1N2 lineages are genetically and antigenically distinct from the avian origin H1N1 SIVs; moreover, the N2 variants of the NA genes in the H1N2 SIVs are derived from a human epidemic strain (49). In 1997, in a H3N2 lineage that originated solely from a human epidemic strain in the USA, two types of reassortant H3N2 SIVs appeared. One of these reassortant H3N2 lineage was a double reassortant containing genes from (i) classical swine and (ii) seasonal human influenza viruses (50), whereas the other was a triple reassortant H3N2 virus containing genes from (i) seasonal human influenza,

(ii) classical swine H1N1, and (iii) avian influenza viruses (51). A(H1N1)pdm09, a quadruple reassortant containing six genes from a triple reassortant H1N1 virus and two genes – an NA and an M genomic segments – from an avian origin SIV found in Europe and Asia (52) has since spread among human populations from 2009. Moreover, A(H1N1)pdm09 viruses have been isolated from pigs in several areas worldwide (18, 53). In addition, A(H1N1)pdm09 viruses have reportedly independently reassorted with endemic SIVs in several regions worldwide (21-35).

Outbreaks of classical H1N1 swine influenza were first observed in the late 1970s in Japan (13). A H1N2 SIV lineage possessing the NA genomic segment from the human epidemic H3N2 and all other genomic segments from classical H1N1 SIV were generated by reassortment soon after the invasion of classical H1N1 (14). This lineage became and has remained the predominant SIV in Japan (15); and although human-like H3N2 strains originating from a human epidemic strain have been isolated sporadically (16), A(H1N1)pdm09 viruses have been repeatedly isolated from pigs in since late 2009 (19). Notably, a reassortant SIV that contained A(H1N1)pdm09 genomic segments and an endemic SIV genomic segment was isolated from a healthy pig during active monitoring (54). Since then, three reassortants containing A(H1N1)pdm09 virus and endemic SIV genomic segments were isolated independently from symptomatic pigs. In this part, the three isolates of this reassortant SIV were analyzed phylogenetically and serologically to acquire information for livestock hygiene and public health purposes.

Materials and Methods

Viruses. Madin-Darby canine kidney (MDCK) cells were used in the Gunma Prefectural Livestock Health Laboratory to isolate A/swine/Gunma/1/2013 (Gun13) (H1N2) from the lung of a dead pig, in the Ibaraki Prefectural Livestock Hygiene Center to isolate A/swine/Ibaraki/1/2013 (Iba13) (H1N2) from the lung of an autopsied pig with respiratory symptoms, and in the Miyazaki Prefectural Livestock Hygiene Center to isolate A/swine/Miyazaki/2/2013 (Miy13) (H3N2) from a nasal swab taken from a piglet with respiratory symptoms.

Other SIV strains that were used as the reference strains in hemagglutination inhibition

(HI) assays are listed in Table 1-1. These reference SIVs were propagated in embryonated chicken eggs or in MDCK cells to be used in HI assays.

Nucleotide sequence and genetic analysis. Gun13, Iba13, Miy13, and three reassortant reference viruses, A/swine/Saitama/01/2005 (Sai05) (H1N2), A/swine/Ehime/1/2002 (Ehi02) (H3N2), and A/swine/Nagano/2000 (Nag00) (H3N2), were used in this study and subjected to vRNA extraction using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). Full coding sequences of whole-genome segments from Gun13, Iba13, and Miy13 (except for HA of Sai05 and Nag00, and HA and NA segments of Ehi02) were determined by direct sequencing of reverse transcription polymerase chain reaction (RT-PCR) products using the ABI genetic analyzer (ABI PRIZM 3100; Life Technologies, Grand Island, NY, USA). These sequences and reference virus sequences (including HA sequences of Sai05 and Nag00) from NCBI GenBank, each comprising eight genetic segments, were used in phylogenetic analyses by neighbor joining method with 1,000 bootstrap replicates to determine the genetic relationships among these three reassortant viruses and reference viruses using two software programs, BioEdit software version v. 7.0.5.3 (55) and MEGA 5 (56). In addition, the deduced amino acid sequence of the HA protein antigenic sites of the HA proteins were compared among the viruses.

Antisera. Hyperimmune antisera against a formalin-inactivated virus were prepared in chickens. Briefly, allantoic fluid or supernatant from MDCK cell cultures that contained infectious virus particles was inactivated using 0.05% or 0.1% formalin and concentrated by ultracentrifugation. Samples with inactivated viruses were then subjected to differential centrifugation through a sucrose density gradient. Each concentrated antigen with a protein content of 50 or 100 μg was intramuscularly inoculated into a chicken, and each chicken was inoculated at least four times with either incomplete or complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA). Experimental procedures and animal care were in accordance with the guidelines of Animal Care and Use and Biosafety Committees of National Institute of Animal health (NIAH). Post-infection ferret antisera against human seasonal influenza strains and homologous inactivated antigens were kindly provided by the National Institute of Infectious Disease (NIID), Japan.

Serological analysis. HI assays were used to assess the antigenic reactivities of each H1 and H3 influenza A virus as described previously (26). Briefly, hyperimmune antisera were treated for 20 h with a receptor-destroying enzyme from *Vibrio cholera* (RDE II; Denka Seiken Co., Ltd., Tokyo, Japan) to remove non-specific inhibitors of hemagglutination. Complement system proteins and the receptor-destroying enzyme in these mixtures were then heat inactivated at 56 °C for 30 min. The subsequent antisera were absorbed with packed guinea pig red blood cells for 60 min at room temperature. For each treated antiserum, a dilution series was generated via serial 2-fold dilutions with phosphate buffered saline (PBS) pH 7.4 from a 1:20 dilution. Each dilution series was used for the HI assays. Guinea pig erythrocytes were resuspended in PBS (0.5% v/v) and used in each HI assay. A cutoff value of 1:20 was adopted to prevent false positive results because of non-specific reactions.

Results

Genomic analysis of A/swine/Gunma/1/2013 (H1N2) and A/swine/Ibaraki/1/2013 (H1N2). Three influenza A viruses, Gun13, Iba13, and Miy13, were subjected to genomic sequencing and phylogenetic analyses. The neighbor-joining method was used to classify ancestries of whole-genome segments of these viruses. The HA gene of Gun13 and Iba13 was found to have originated from a classical H1 HA swine lineage that has been circulating only among Japanese SIVs (Figure 1-1A). These HA genes clustered together and formed a sub-lineage within the Japanese strains. Notably, these HA genes were distinguishable from the HA gene sequence of A/swine/Gunma/1/2012 (Gun12) (H1N2), which was isolated in the same prefecture in Japan as Gun13 the year before (2012) (54). The HA gene of Gun12 belonged to an A(H1N1)pdm09 lineage. The NA genes of Gun13 and Iba13 were most closely related to the NA genes of human-like H1N2 swine viruses, and in contrast to the HA genes, were distinguishable from each other within the lineage (Figure 1-1B). Phylogenetic analyses of the six internal genes of Gun13 and Iba13, with the exception of the NP genes, revealed that each internal gene was of A(H1N1)pdm09 origin (Figure 1-1C, Table 1-2; phylogenetic trees of PB2, PB1, PA, and NS are

not shown). The NP gene of Gun13 or Iba13 was most closely related to the corresponding genes from the Japanese H1N2 SIV lineage (Figure 1-1D).

Genomic analysis of A/swine/Miyazaki/2/2013 (H3N2). The HA and NA genes of Miy13 descended from human seasonal influenza viruses and showed the highest homology with corresponding genes from human and human-like swine H3N2 viruses, which had been isolated in 2000 (Figure 1-1B, E, Table 1-2). All internal viral protein genes of Miy13 were the most closely related to corresponding genes from A(H1N1)pdm09 viruses (Figure 1-1C, D, Table 1-2; phylogenetic trees of PB2, PB1, PA, and NS are not shown).
Taken together, Gun13, Iba13, and Miy13 were each reassortants between A(H1N1)pdm09 viruses and viruses that persist in the Japanese pig population.

Antigenic analysis and comparison of deduced amino acid sequences of antigenic sites of A/swine/Gunma/1/2013 (H1N2) and A/swine/Ibaraki/1/2013 (H1N2). The HI cross-reactivities of Gun13 and Iba13 viruses across a panel of 13 distinct antisera generated against A(H1N1)pdm09 viruses, seasonal human lineage H1 viruses, or avian H1 virus were examined (Table 1-3). Gun13 and Iba13 reacted similarly with each antisera. The Gun13 virus reacted with the Sai05 and A/swine/Tochigi/1/2008 (Toc08) (H1N2) antisera at HI titers of 1,280 and 160, respectively. These titers were a 4-fold less dilute than the reactive HI titers of 5,120 and 640 of the native antigens Sai05 HA and Toc08 HA, respectively. Similarly, the Iba13 virus reacted with the Toc08 and A/swine/Binh Duong/02-16/2010 (BD16/10) (H1N2) antisera at HI titers of 320 and 160, respectively, which were 2- and 4-fold less dilute than the reactive titers of the respective native antigen, respectively. Cross-reactivity between the Gun13 and Iba13 viruses and antisera against A/swine/Iowa/15/1930 (Iow30) (H1N1), A/swine/Kyoto/3/1979 (Kyo79) (H1N1), A/duck/Tsukuba/67/2005 (Tsu05) (H1N1), or each of three seasonal human-lineage viruses, A/New Caledonia/20/1999 (NC99) (H1N1), A/Solomon Islands/3/2006 (SI06) (H1N1), and A/Brisbane/59/2007 (Bri07) (H1N1), was minimal or undetectable.

Deduced amino acid residues in the HA protein antigenic sites of Gun13, Iba13, and reference viruses tested in the HI assays were compared for identifying amino acid residues that

confer differential antigenic specificities (Table 1-4). Between Gun13 and Iba13 the HA nucleotide and putative amino acid sequences were 96.9% and 95.9% identical, respectively. Eleven amino acid substitutions were observed between their antigenic sites in the HA1 domain. Amino acid residues substituted from Gun13 to Iba13 were as follows: P158Q in the Sa site; R152K, G155S, L188I, Q189R, and P194T in the Sb site; R141S, F165V, and K234E in the Ca site; and S74R and G115E in the Cb site. Gun13 reacted most strongly with anti-Sai05 among the antisera examined; Gun13 HA1 and Sai05 HA1 differed at 17 amino acid residues within the antigenic domain. Anti-Toc08 antiserum also reacted with the Gun13 virus at a titer 4-fold less dilute than the reactive titer of the Toc08 virus, and 20 substitutions between the antigenic sites of Gun13 HA1 and Toc08 HA1 were identified. Iba13 HA1 and Toc08 HA1 differed at 19 amino acid residues within the antigenic region. Although only a 4-fold reduction from the homologous titer was observed with the antiserum against BD16/10, 28 amino acid substitutions in the antigenic region were identified between Iba13 and BD16/10.

Antigenic analysis and comparison of deduced amino acid sequences of antigenic sites of A/swine/Miyazaki/2/2013 (H3N2). Antigenic analysis of Miy13 was also performed using a panel of 13 antisera generated against four individual human-like H3 swine viruses, eight individual seasonal human-like H3 viruses, or an avian H3 virus (Table 1-5). The Miy13 virus reacted with anti-A/Wyoming/03/2003 (Wyo03) (H3N2) serum at the same titer as the Wyo03 virus. Miy13 reacted with the anti-A/Wuhan/359/1995 (Wuh95) (H3N2) serum at a 2-fold higher titer than the Wuh95 virus. Notably, the Nag00 virus, which was isolated from an asymptomatic pig in active surveillance in slaughtering center of Nagano prefecture, is one of the SIVs with an HA gene that is most homologous with the Miy13 HA gene. When tested with anti-A/swine/Binh Duong/03-14/2010 (BD14/10) (H3N2), Wuh95, Wyo03, or A/New York/55/2004 (NY04) (H3N2) antisera, the reactive Nag00 HI titer was always 2-fold lower than the reactive Miy13 titer. Moreover, the reactive Nag00 titer was 2-fold higher than the reactive Miy13 titer with anti-A/swine/Osaka-C/12-20/2008 (Osa08) (H3N2), anti-A/Hiroshima/52/2005 (Hir05) (H3N2), and anti-A/Uruguay/716/2007 (Uru07) (H3N2); 8-fold higher with anti-A/Panama/2007/1999 (Pan99) (H3N2); and 16-fold higher with anti-A/Sydney/5/1997 (Syd97) (H3N2). Although

similar tendency was observed in serological reactivities of Miy13 and Nag00 against antisera tested, differences of HI titers against almost all antisera were revealed.

Variations in deduced amino acid residues in the HA1 domains of 15 H3-subtype HA proteins are listed in Table 1-6. The antigenic sites in the HA1 domain of the HA proteins from Miy13 and Nag00 differed at 11 amino acid residues: four each in sites A and B, two in C, and one in E. Moreover, Miy13 HA lost a potential glycosylation site (AA133) in site A, whereas the corresponding amino acid of Nag00 HA had retained the potential glycosylation site. The antigenic sites in HA1 of HA proteins from Miy13 and Wyo03 presumably differed at 17 amino acid residues: six in site A, five in B, three in C, one in D, and two in E.

Discussion

Two H1N2 influenza viruses (Gun13 and Iba13) and one H3N2 influenza virus (Miy13) were isolated from different Japanese pigs over a relatively short period in 2013. They were each identified as a reassortant between A(H1N1)pdm09 viruses and viruses that had persisted in the Japanese pig population. The possibility that genetic reassortment occurred in the MDCK cells during virus isolation is very low because, although the primer sets that can amplify a broad spectrum of type A influenza genes including A(H1N1)pdm09 viruses, endemic SIVs and human seasonal viruses were used to amplify genomic segments by RT-PCR for direct sequencing. No mixed DNA sequence, amplified by RT-PCR, was identified indicating that there was a single strain of virus in each supernatant of inoculated cell culture.

Comparison of the genetic constellation of three H1N2–two H1N2 strains described here and Gun12, a virus isolated in 2010 in Japan (54)– indicated that frequent reassortment events involving A(H1N1)pdm09 viruses have occurred in the Japanese pig population. The NA genes of Gun13, Iba13, and Gun12 were found to be related to human-like swine H1 viruses. Moreover, two of these NA genes –Gun13 NA and Gun12 NA– clustered together and these two were distinct from Iba13. Notably, the HA and NP genes of Gun13 and Iba13 were all of classical swine H1 virus origin and each Gun13-Iba13 gene pair formed a cluster in the respective tree. These NA, HA, and NP genes each may have developed from viruses directly or indirectly descending from

the reassortant virus A/swine/Ehime/1/1980 (H1N2), which includes the N2 of human influenza virus and seven other genomic segments from classical H1N1 viruses that have been circulating in Japan (15, 47). Moreover, when BLAST was used to compare the HA and NA genes of Miy13 with the swine H3N2 virus dataset, the highest similarities found were with the corresponding genes of Nag00, an H3N2 human-like swine virus isolated in Japan. Taken together, these H3N2 findings indicate that Miy13 inherited the HA and NA genes from an offspring of Nag00 or an allied virus. Collectively, at least four reassortant viruses have originated independently within individual animals among the Japanese pig population.

Vaccine efficacy, specifically in protecting domestic pigs against SIV, depends mostly on antigenic homology between a vaccine strain and an endemic strain (57). Based on the HI assays, there was little cross-reactivity between the current SIV vaccine strain Kyo79 (H1N1) and A/swine/Wadayama/5/1969 (H3N2; Wad69) and the reassortants described here. The relatively large number of amino acid substitutions separating the reassortants from the vaccine strains is consistent with their weak HI cross-reactivity. Therefore, detailed studies are needed to confirm that current vaccines effectively protect pigs from infection by these reassortants.

The gene constellations of Gun13 and of Iba13 are rare in that they indicate reassortment between A(H1N1)pdm09 virus and an epidemic SIV. Specifically, all Gun13 and Iba13 internal gene segments, except the NP genes, are of A(H1N1)pdm09 origin, though the genes encoding the surface antigens and NP are of epidemic SIV origin. Another case of this constellation has been reported in A/swine/Indiana/240218/2010 (H1N2) (23). The most commonly observed constellations of reassortants between A(H1N1)pdm09 virus and epidemic SIV are those comprising the six internal genes from an A(H1N1)pdm09 virus and both genes encoding the surface antigens from an epidemic SIV (24, 26, 27, 34). Notably, this is also the constellation of Miy13. Another common A(H1N1)pdm09 reassortant constellation comprises the six internal genes and the HA gene from an A(H1N1)pdm09 virus and the NA gene from an SIV (25, 28, 31, 32, 35). This is the constellation of Gun12. There might be a selective advantage in pig hosts for reassortants that contain all internal segments, as a set, from A(H1N1)pdm09 viruses. A reassortant virus, A/swine/Guangdong/1361/2010 (H1N1), that comprises all the internal genes from A(H1N1)pdm09 viruses and the surface genes from Eurasian avian-like SIV is efficiently

transmitted among pigs (34). Similarly, intact A(H1N1)pdm09 viruses are also efficiently transmitted among pigs (58, 59). Therefore, possession of all six internal segments from an A(H1N1)pdm09 virus might not interfere with efficient transmission of an SIV reassortant from pig to pig.

Gun13 and Gun12(54) were independently isolated from two different pigs that did and did not have respiratory symptoms. Nevertheless, the Gun13 and Gun12 genomic segments were found to be more than 97.7% identical, except for the HA and NP sequences. Similarly, Miy13 was isolated from a pig with respiratory symptoms. However, Nag00, which is probably the origin of the Miy13 HA and NA genes, was isolated during active monitoring, and infection with Nag00 seems not to cause symptoms in the field (unlike with Miy13). Taken together, these findings indicate that the presence of some of A(H1N1)pdm09 internal gene segments may facilitate or drive a change in the pathogenicity of SIV for pigs. Although many reassortants between A(H1N1)pdm09 virus and epidemic SIV have been isolated from symptomatic pigs (22, 23, 27, 28, 31-33, 35), few such reassortants have been isolated from asymptomatic pigs (23, 24, 26). Although the primary route of SIV transmission is believed to be pig-to-pig contact via nasopharyngeal exposure, aerogenic transmission caused by respiratory symptoms may contribute to the spread of SIVs in large, densely populated pig herds (10). Moreover, aerogenic transmission probably accounts for the transmission of SIV infections to farms with high biosecurity standards (10). The mechanisms that define the pathogenicity of reassortants between A(H1N1)pdm09 virus and endemic SIV must be elucidated from the viewpoint of SIV epidemiology.

Whereas Miy13 had strong antigenic reactivities with antisera generated for seasonal human-lineage viruses isolated until 2003, Miy13 had little reactivities with antisera with other viruses isolated after 2004. In addition, antisera generated with some seasonal human-lineage H1 virus strains did not react with either Gun13 or Iba13. These results suggest that human individuals, particularly those young, probably do not possess antibodies that can recognize Gun13, Iba13, or Miy13. A reassortant, A/swine/Guangdong/1361/2010, has been reported to infect human tissue *ex vivo* (34). In addition, several human cases of infection with reassortant viruses containing the A(H1N1)pdm09 M genomic segment in the backbone of a triple reassortant H3N2 of swine origin have been identified (60). The M genomic segment from the A(H1N1)pdm09 virus is commonly

present among the vast majority of reassortants between A(H1N1)pdm09 viruses and SIVs (22-25, 27-29, 31-35). Each of the four such reassortants identified in Japan, Gun13, Iba13, Miy13, and Gun12, have an M genomic segment from A(H1N1)pdm09 viruses. The genomic constellation of these four reassortants (Gun12, Gun13, Iba13, and Miy13) and the relative lack of human immunity against these viruses indicates that these reassortants or other novel reassortants between A(H1N1)pdm09 virus and endemic SIV could emerge and spread in the human population. Therefore, they pose a potential risk to public health.

Brief Summary

In 2013, three reassortant SIVs, two H1N2 and one H3N2 subtypes, were isolated from symptomatic pigs in Japan. Each contained genes from the pandemic A(H1N1)2009 viruses and endemic SIVs. Phylogenetic analysis revealed that the two H1N2 viruses, A/swine/Gunma/1/2013 and A/swine/Ibaraki/1/2013, were reassortants that contained genes from three distinct lineages: (i) H1 and NP genes derived from a classical swine H1 HA lineage uniquely circulating among SIVs in Japan, (ii) NA genes from human-like H1N2 swine viruses, and (iii) other genes from pandemic A(H1N1)2009 viruses. The H3N2 virus, A/swine/Miyazaki/2/2013, comprised genes from two sources: (i) HA and NA genes derived from human and human-like H3N2 swine viruses and (ii) other genes from pandemic A(H1N1)2009 viruses. Phylogenetic analysis also indicated that each of the reassortants may have developed independently in Japanese pigs. The antigenic reactivities of A/swine/Miyazaki/2/2013 with antisera generated for some seasonal human-lineage viruses isolated during or before 2003 were high, but A/swine/Miyazaki/2/2013 reactivities with antisera against viruses isolated after 2004 were much lower. In addition, antisera against some strains of seasonal human-lineage H1 viruses did not react with either A/swine/Gunma/1/2013 or A/swine/Ibaraki/1/2013. These findings indicate that the emergence and spread of these reassortant SIVs is a potential public-health risk.

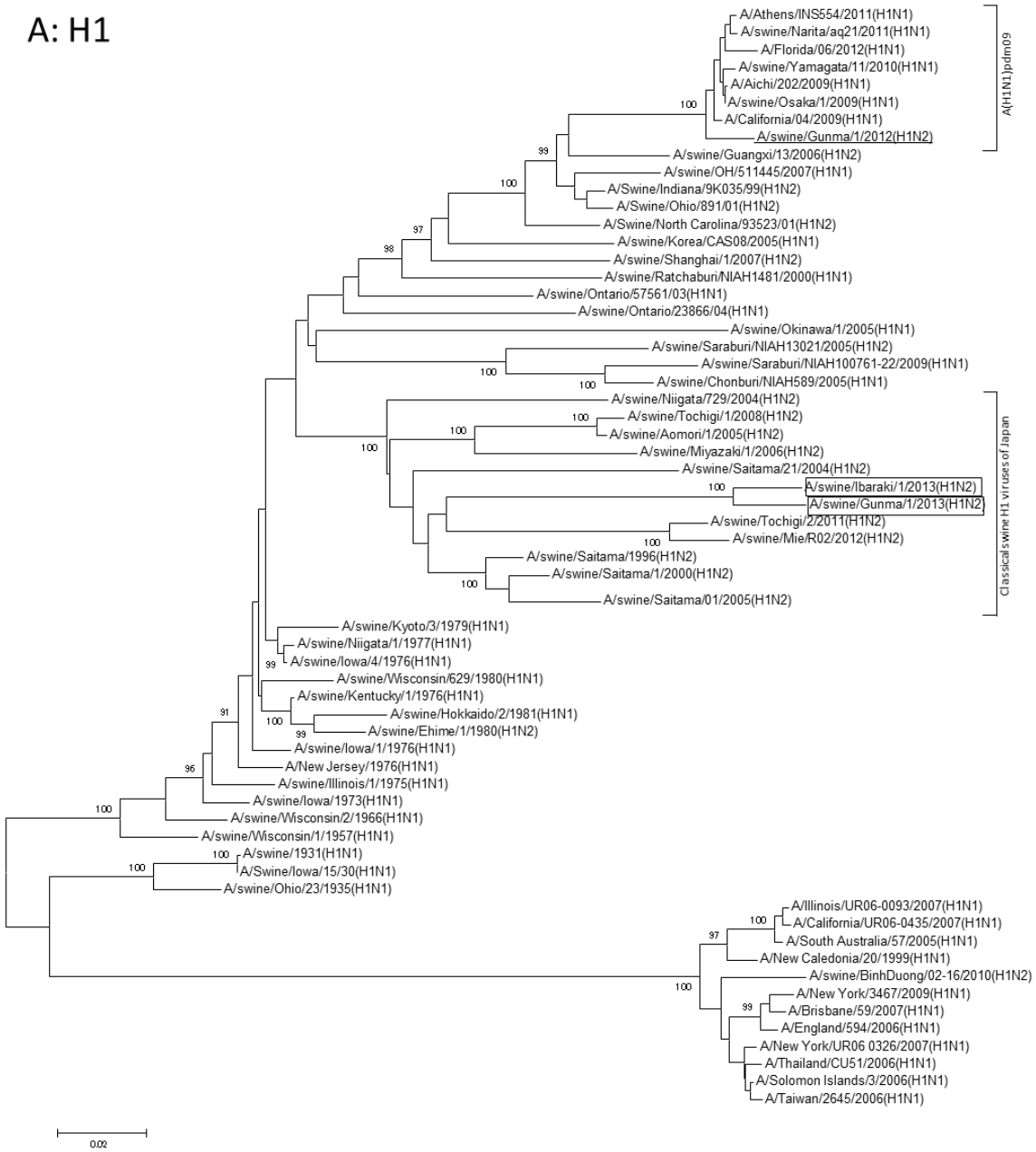

Figure 1-1. Phylogenetic trees of H1 (A) and H3 (E) HA, N2 NA (B), MP (C), and NP (D) genes from swine and human virus lineages. Swine viruses analyzed in part 1 are shown in rectangular boxes. The A/swine/Gunma/1/2012 virus is underlined. Bootstrap values greater than 90 are shown.

Figure 1-1. (continued)

C: MP

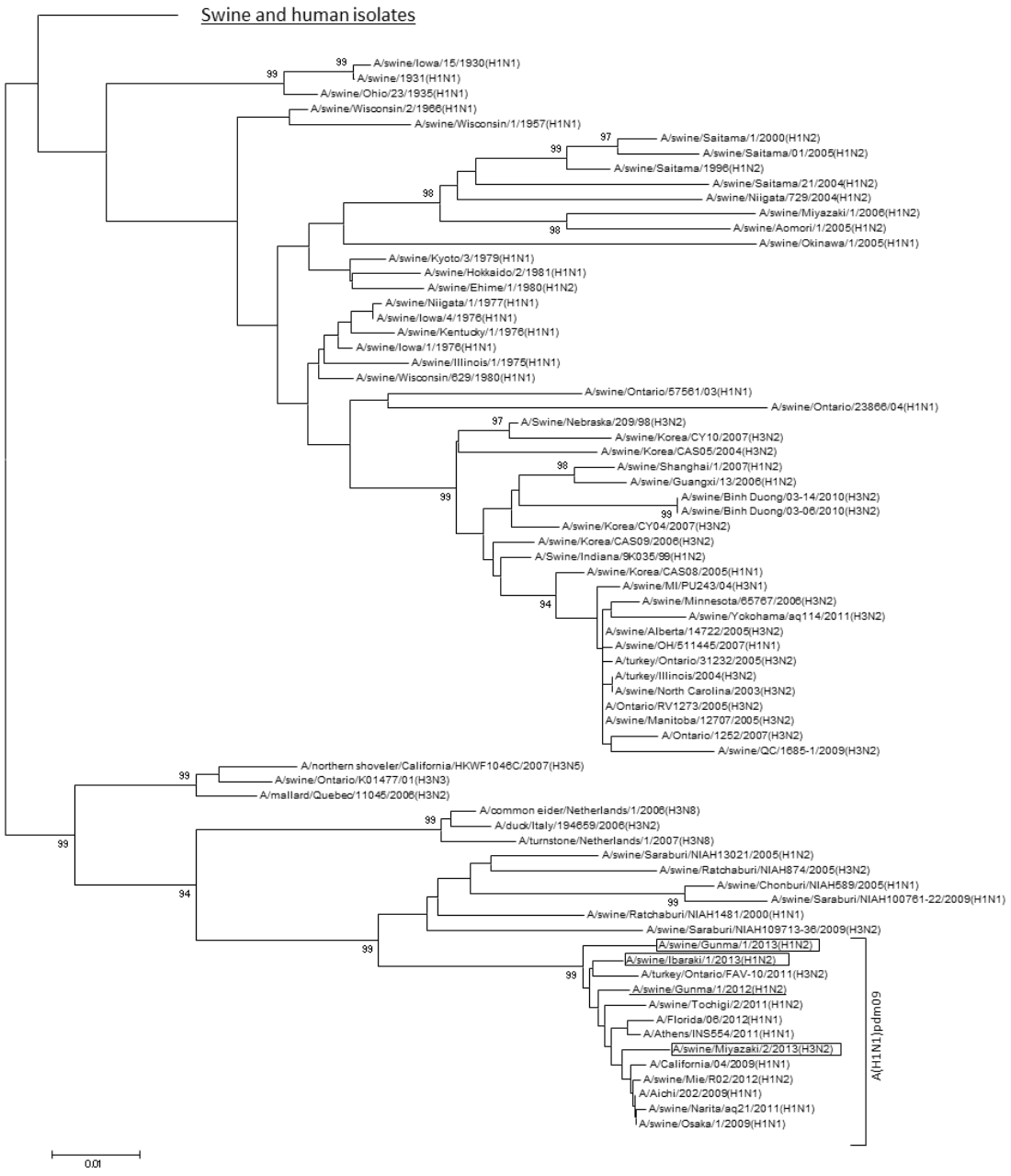

Figure 1-1. (continued)

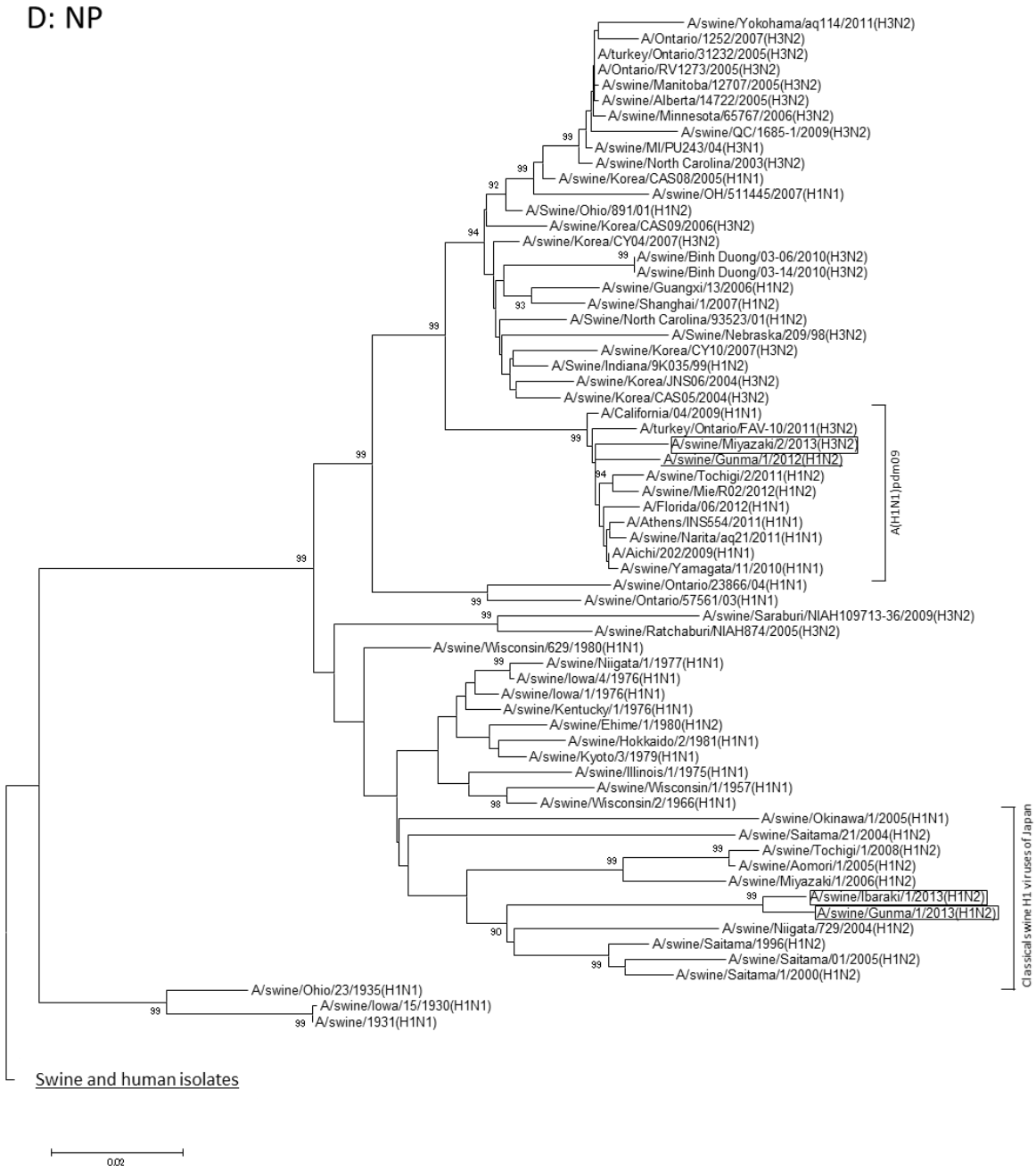

Figure 1-1. (continued)

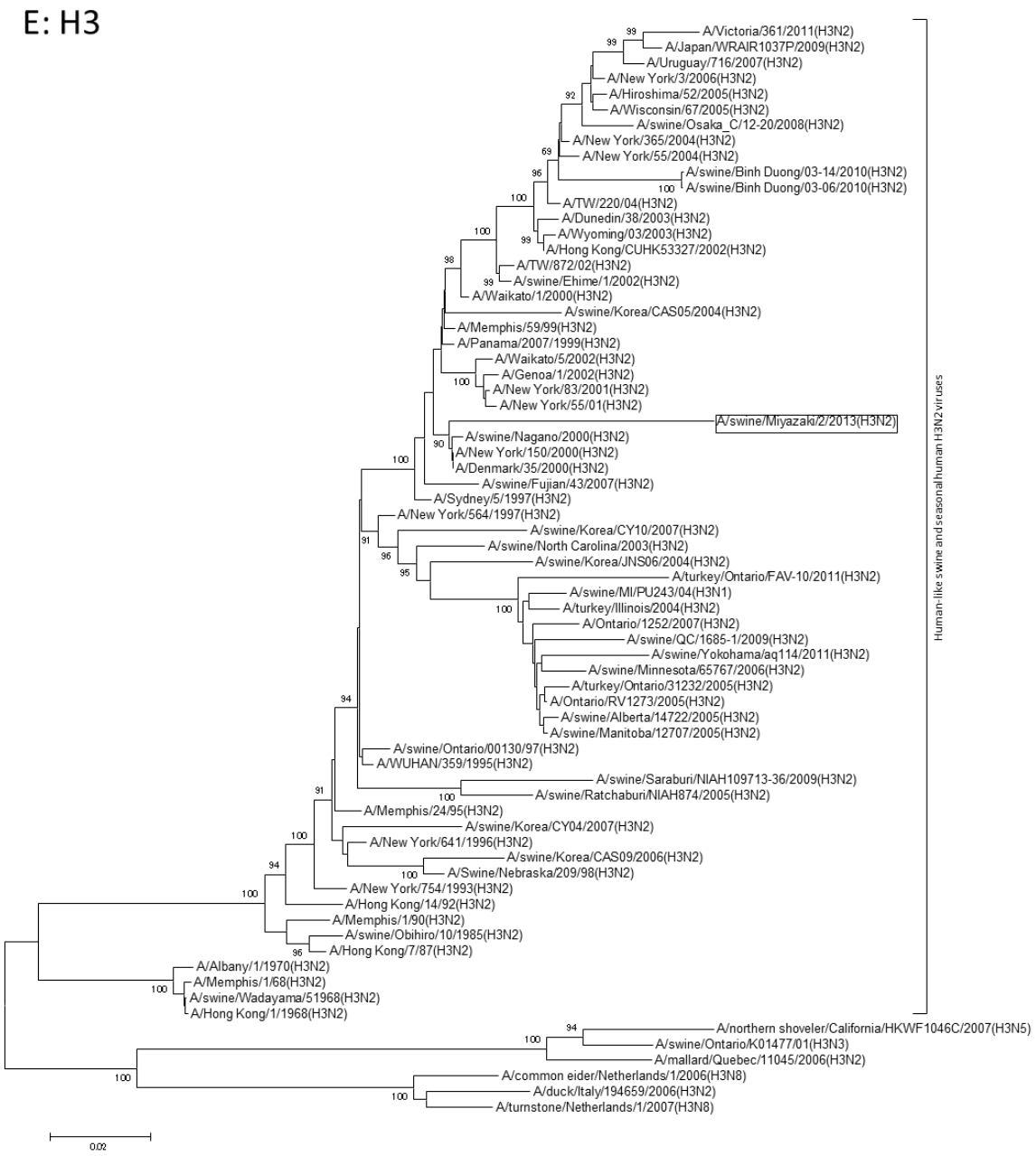

Figure 1-1. (continued)

Table 1-1. Viruses used in Part 1

Virus	Abbreviation	Subtype	Genetic lineage of HA gene	Country of origin	HA gene
H1 viruses					
A/swine/Gunma/1/2013	Gun13	H1N2	Classical swine	Japan	AB921005
A/swine/Ibaraki/1/2013	Iba13	H1N2	Classical swine	Japan	AB921004
A/swine/Iowa/15/1930	Iow30	H1N1	Classical swine	USA	AF091308
A/swine/Kyoto/3/1979	Kyo79	H1N1	Classical swine	Japan	AB434384
A/swine/Niigata/729/2004	Nii04	H1N2	Classical swine	Japan	AB600850
A/swine/Saitama/01/2005	Sai05	H1N2	Classical swine	Japan	AB762401
A/swine/Tochigi/1/2008	Toc08	H1N2	Classical swine	Japan	AB514929
A/swine/Tochigi/2/2011	Toc11	H1N2	Classical swine	Japan	AB741007
A/swine/Binh Duong/02-16/2010	BD16/10	H1N2	Human-like swine	Vietnam	AB762408
A/California/04/2009	Cal09	H1N1	A(H1N1)pdm09	USA	FJ966082
A/swine/Narita/aq21/2011	Nar11	H1N1	A(H1N1)pdm09	Japan (Denmark)*	AB741039
A/New Caledonia/20/1999	NC99	H1N1	Seasonal human	New Caledonia	CY033622
A/Solomon Islands/3/2006	SI06	H1N1	Seasonal human	Solomon Islands	EU124177
A/Brisbane/59/2007	Bri07	H1N1	Seasonal human	Australia	CY058487
A/duck/Tsukuba/67/2005	Tsu05	H1N1	Avian	Japan	n.a.
H3 viruses					
A/swine/Miyazaki/2/2013	Miy13	H3N2	Human-like swine	Japan	AB921006
A/swine/Nagano/2000	Nag00	H3N2	Human-like swine	Japan	AB762409
A/swine/Wadayama/5/1969	Wad69	H3N2	Human-like swine	Japan	D21183
A/swine/Ehime/1/2002	Ehi02	H3N2	Human-like swine	Japan	AB762411
A/swine/Osaka-C/12-20/2008	Osa08	H3N2	Human-like swine	Japan	AB762413
A/swine/Binh Duong/03-14/2010	BD14/10	H3N2	Human-like swine	Vietnam	AB598503
A/swine/Yokohama/aq114/2011	Yok11	H3N2	Human-like swine (Triple reassortant SIV: cluster IV)	Japan	AB741023
A/Wuhan/359/1995	Wuh95	H3N2	Seasonal human	China	CY112821
A/Sydney/5/1997	Syd97	H3N2	Seasonal human	Australia	CY039079
A/Panama/2007/1999	Pan99	H3N2	Seasonal human	Panama	DQ508865
A/Wyoming/03/2003	Wyo03	H3N2	Seasonal human	USA	EU268227
A/New York/55/2004	NY04	H3N2	Seasonal human	USA	CY033638
A/Hiroshima/52/2005	Hir05	H3N2	Seasonal human	Japan	EU501660
A/Uruguay/716/2007	Uru07	H3N2	Seasonal human	Uruguay	EU716426
A/Victoria/361/2011	Vic11	H3N2	Seasonal human	Australia	KC306165
A/budgerigar/Aichi/1/1977	Aic77	H3N8	Avian	Japan	n.a.

n.a.: not available.

*Isolated from swine imported from Denmark.

Table 1-2. Origin of viral gene segments

	Sub type	segment							
		1	2	3	4	5	6	7	8
		PB2	PB1	PA	HA	NP	NA	MP	NS
A/swine/Ibaraki/1/2013	H1N2	pdm09	pdm09	pdm09	cSIV	cSIV	hlS H1	pdm09	pdm09
A/swine/Gunma/1/2013	H1N2	pdm09	pdm09	pdm09	cSIV	cSIV	hlS H1	pdm09	pdm09
A/swine/Gunma/1/2012[†]	H1N2	pdm09	pdm09	pdm09	pdm09	pdm09	hlS H1	pdm09	pdm09
A/swine/Miyazaki/2/2013	H3N2	pdm09	pdm09	pdm09	hlS H3N2	pdm09	hlS H3N2	pdm09	pdm09
A/swine/Nagano/2000	H3N2	hlS H3N2	hlS H3N2	hlS H3N2	hlS H3N2	hlS H3N2	hlS H3N2	hlS H3N2	hlS H3N2

[†]This virus was isolated at Gunma Prefectural Institute of Public Health and Environmental Sciences.
pdm09: pandemic A(H1N1) 2009 virus, cSIV: classical swine influenza virus, hlS: human-like swine influenza virus

Table 1-3. Hemagglutination inhibition (HI) titers with swine and human H1 viruses

virus	HI titers of sera from chicken† and ferret‡ infected with												
	Classical SIV						A(H1N1)pdm09 virus		Seasonal human-lineage virus				Avian virus
	Iow30†	Kyo79†	Nii04†	Sai05†	Toc08†	Toc11†	Cal09†	Nar11†	BD16/10†	NC99‡	SI06‡	Bri07‡	Tsu05†
Gun13	20	40	40	1280	160	320	80	320	80	<20	<20	<20	40
Iba13	20	40	160	320	320	160	40	160	160	<20	<20	<20	80
Classical SIV													
Iow30	**5120**	640	80	40	320	<20	20	80	80	<20	<20	<20	640
Kyo79	5120	**5120**	80	640	640	<20	160	<20	80	<20	<20	<20	320
Nii04	160	320	**1280**	320	320	320	640	40	160	<20	<20	<20	320
Sai05	320	640	320	**5120**	160	160	80	160	80	<20	<20	<20	640
Toc08	2560	1280	320	1280	**640**	320	2560	80	80	<20	<20	<20	640
Toc11	1280	640	160	640	160	**2560**	2560	640	80	<20	<20	<20	640
A(H1N1)pdm09 virus													
Cal09	2560	1280	80	320	160	80	**1280**	1280	160	<20	<20	<20	160
Nar11	5120	1280	320	640	160	160	5120	**5120**	80	20	20	20	1280
Seasonal human-lineage virus													
BD16/10	20	20	20	<20	80	<20	<20	80	**640**	<20	<20	<20	20
NC99	40	80	40	<20	160	<20	<20	80	160	**640**	80	80	20
SI06	40	40	20	<20	320	<20	<20	<20	640	160	**640**	640	40
Bri07	80	40	20	<20	160	<20	<20	160	160	80	320	**1280**	80
Avian virus													
Tsu05	2560	80	80	<20	320	<20	<20	40	160	<20	<20	<20	**1280**

HI titers between native antigens and the respective antisera are indicated in bold and underlined.

Table 1-4. Variation in deduced amino acid residues of antigenic sites in HA proteins from H1 viruses

	Amino acid position in HA1[†]																				
	Sa								Sb												
	124[‡]	125	154	156	158	159	160	161	152	155	183	184	185	186	188	189	192	193	194
	(124)	(125)	(155)	(157)	(159)	(160)	(161)	(162)	(153)	(156)	(184)	(185)	(186)	(187)	(189)	(190)	(193)	(194)	(195)
Gun13	S	G	G	S	P	K	I	S	R	G	T	S	N	D	L	Q	Q	N	P
Iba13	-[§]	-	-	-	Q	-	-	-	K	S	-	-	-	-	I	R	-	-	T
BD16/10	P	N	N	L	-	N[¶]	L	-	V	-	N	I	G	-	R	A	H	T	E
Toc08	P	D	-	T	-	-	L	-	K	N	-	-	A	-	Q	S	-	-	A
Iow30	P	N	E	-	-	-	L	-	K	N	-	-	T	-	Q	S	-	-	A
Kyo79	P	N	-	-	-	-	L	-	K	N	-	-	A	-	Q	S	-	-	A
Nii04	P	N	-	-	-	-	L	-	K	N	-	N	D	-	R	S	-	-	A
Sai05	P	N	-	-	-	-	-	-	K	N	-	N	A	-	Q	T	-	-	A
Toc11	P	L	-	-	-	-	L	N	K	N	-	-	T	-	Q	S	-	-	N
Cal09	P	N	-	-	-	-	L	-	K	N	-	-	A	-	Q	S	-	-	A
Nar11	P	N	-	-	-	-	L	-	K	N	-	T	A	-	Q	S	-	-	A
NC99	P	N[¶]	N	L	-	N[¶]	L	-	G	-	N	I	G	N	R	A	H	T	E
SI06	P	N	N	L	-	N[¶]	L	-	G	-	N	I	G	-	R	A	H	K	E
Bri07	P	N[¶]	N	L	-	N[¶]	L	-	G	-	N	I	G	N	K	A	H	T	E

	Ca													Cb				
	136	137	138	140	141	165	167	169	202	203	204	221	234	71	72	73	74	115
	(137)	(138)	(139)	(141)	(142)	(166)	(168)	(170)	(203)	(204)	(205)	(222)	(235)	(71)	(72)	(73)	(74)	(115)
Gun13	S	Q	D	A	R	F	N	G	T	S	T	D	K	S	K	V	S	G
Iba13	-	-	-	-	S	V	-	-	-	-	-	-	E	-	-	-	R	E
BD16/10	-	H	N	K	S	A	-	E	S	-	H	-	E	I	S	R	E	E
Toc08	-	Y	A	V	N	I	-	-	S	-	-	- /G	E	-	T	-	-	E
Iow30	P	Y	A	-	S	V	-	-	S	-	K	G	E	L	T	-	-	E
Kyo79	P	Y	A	-	N	V	-	-	S	-	-	G	E	F	I	-	-	E
Nii04	P	Y	A	K	N	V	-	-	-	P	-	N	E	-	T	-	-	E
Sai05	P	-	A	-	N	A	-	-	S	-	-	G	-	-	T	-	-	E
Toc11	P	H	A	T	K	V	-	-	S	-	-	-	E	-	T	-	-	E
Cal09	P	H	A	-	K	I	D	-	S	-	R	-	E	-	T	A	-	E
Nar11	P	H	A	-	K	I	D	-	-	-	R	-	E	-	T	A	-	E
NC99	-	H	N	K	S	V	-	E	S	-	H	-	E	I	S	K	E	E
SI06	-	H	N	E	S	A	-	E	S	-	H	-	E	I	S	R	E	E
Bri07	-	H	N	E	S	A	-	E	S	-	H	-	E	I	S	K	E	E

Sa, Sb, Ca and Cb are the antigenic sites of H1 HA protein.

[†]Amino acid positions where the substitutions were observed are indicated.

[‡]H1 HA numbering of A/swine/Gunma/1/2013 (of A/California/04/2009).

[§]Amino acid residues differing from HA protein of A/swine/Gunma/1/2013 (H1N2) are shown.

[¶]N-glycosylation site predicted by NetNGlyc 1.0 (www.cbs.dtu.dk/services/NetNGlyc/).

Table 1-5. Hemagglutination inhibition (HI) titers with swine and human H3 viruses

virus	HI titers of sera from chicken† and ferret‡ infected with												
	Classical SIV				Seasonal human-lineage virus								Avian virus
	Wad69†	Osa08†	BD14/10†	Yok11†	Wuh95‡	Syd97‡	Pan99‡	Wyo03‡	NY04‡	Hir05‡	Uru07‡	Vic11‡	Aic77†
Miy13	80	1280	160	160	1280	40	80	2560	160	40	40	20	<20
Nag00	20	2560	80	160	640	640	640	1280	80	80	80	20	<20
Human-like SIV													
Wad69	**10240**	20	160	20	20	<20	<20	80	<20	<20	<20	<20	80
Ehi02	80	10240	320	640	640	320	1280	5120	640	320	320	80	40
Osa08	40	**20480**	160	160	20	<20	320	320	320	80	80	160	<20
BD14/10	20	1280	**1280**	80	160	40	160	640	40	40	40	20	20
Yok11	160	2560	320	**10240**	160	20	40	160	160	80	40	40	<20
Seasonal human-lineage virus													
Wuh95	160	160	320	2560	**640**	160	160	160	20	20	20	<20	20
Syd97	640	320	320	2560	2560	**5120**	2560	2560	80	160	320	<20	<20
Pan99	80	5120	80	40	160	80	**640**	640	20	20	20	<20	<20
Wyo03	320	10240	160	80	160	160	320	**2560**	1280	160	640	<20	<20
NY04	320	2560	160	20	20	<20	20	1280	**10240**	320	640	20	<20
Hir05	160	10240	320	<20	<20	40	40	160	640	**2560**	1280	40	20
Uru07	320	2560	160	40	<20	20	<20	160	1280	640	**2560**	80	<20
Vic11	80	5120	640	640	40	20	20	160	640	640	640	**1280**	20
Avian virus													
Aic77§	320	640	640	40	<20	<20	<20	<20	20	20	20	<20	**1280**

HI titers between native antigens and the respective are indicated in bold and underlined.

§Chicken RBCs were used for these HI tests.

Table 1-6. Variation in deduced amino acid residues of antigenic sites in HA proteins from H3 viruses

	Amino acid position in HA1[†]																								
	A															B								
	122[†]	126	128	131	133	135	137	138	140	142	143	144	145	146		155	156	157	158	186	188	189	192	193
Miy13	N	N	T	A	N	A	T	A	K	R	S	S	N	S		Y	Q	L	R	S	D	N	I	S
Nag00	-[§]	-	-	-	-[¶]	T	S	-	-	-	-	I	K	-		H	-	-	K	-	-	S	-	-
Pan99	-	-	-	-	-[¶]	T	S	-	-	-	-	N	K	-		H	-	-	K	-	-	S	-	-
Wyo03	-	-	A	T	-	T	S	-	-	-	-	N	K	-		T	H	-	K	G	-	S	-	-
Wad69	T	T	-	T	-	G	N	-	-	G	P	D	S	G		T	K	S	G	-	N	Q	T	-
Ehi02	-	-	-	-	-[¶]	T	S	-	-	-	-	N	K	-		H	-	-	K	G	-	S	-	-
Osa08	-	-	-	T	-	T	S	S	-	-	-	N	K	-		T	H	-	N	G	-	-	-	F
BD14/10	S	-	-	T	-	K	S	S	-	-	-	D	S	-		T	H	-	N	G	Y	-	-	-
Yok11	Q	-	-	-	D	S	Y	-	-	E	-	V	K	-		H	N	-	D	G	-	R	T	N
Wuh95	-	-	-	-	D	T	Y	-	-	G	-	V	K	-		H	K	-	E	-	-	S	T	-
Syd97	-	-	-	-	-[¶]	T	Y	-	-	S	-	I	K	-		H	-	-	K	-	-	S	T	-
NY04	-	-	-	T	-	T	S	S	-	-	-	N	-	-		T	H	-	K	V	-	-	-	R
Hir05	-	-	-	T	-	T	S	-	-	-	-	N	-	-		T	R	-	K	G	-	-	-	F
Uru07	-	-	-	T	-	T	S	S	I	-	-	N	-	-		T	H	-	K	G	-	-	-	F
Vic11	-	-	-	T	-[¶]	T	S	-	I	-	-	N[¶]	-	-		T	-	-	N	V	-	K	-	F

	B (continued)			C						D								E		
	196	198	199	50	53	54	275	276	278	201	202	209	213	219	220	226	242	63	78	83
Miy13	A	A	S	R	N	S	G	R	N	R	V	S	V	S	R	V	I	N[¶]	A	E
Nag00	-	S	-	-	D	-	-	K	-	-	-	-	-	-	-	-	-	-[¶]	G	-
Pan99	-	-	-	-	D	-	-	K	-	-	-	-	-	-	S	-	-	-[¶]	G	-
Wyo03	-	-	-	G	D	-	-	K	-	-	I	-	-	-	-	-	-	-[¶]	G	K
Wad69	V	-	-	K	-	N	D	T	I	-	-	-	I	-	-	L	V	D	V	T
Ehi02	-	-	P	G	D	-	-	K	-	-	I	-	-	-	-	- /I	-	-[¶]	G	K
Osa08	-	-	-	E	D	-	-	K	S	-	I	-	-	-	-	I	-	-[¶]	G	K
BD14/10	-	-	-	G	-	-	-	K	-	I	I	N	-	-	-	I	-	-[¶]	G	K
Yok11	V	E	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-[¶]	D	G
Wuh95	V	-	-	-	D	-	-	N	-	-	-	-	-	-	-	I	-	-[¶]	G	-
Syd97	-	-	-	-	D	-	-	K	-	-	-	-	-	-	-	I	-	-[¶]	G	-
NY04	-	-	-	G	D	-	-	K	-	-	I	-	-	-	-	I	-	-[¶]	G	K
Hir05	-	-	-	G	D	-	-	K	-	-	I	-	-	-	-	I	-	-[¶]	G	K
Uru07	-	-	-	E	D	-	-	K	-	-	I	-	-	-	-	I	-	-[¶]	G	K
Vic11	-	S	-	E	D	-	-	K	-	-	I	-	-	Y	-	I	-	-[¶]	G	K

A, B, C, D and E are the antigenic sites of H3 HA protein.

[†]Amino acid positions where the substitutions were observed are indicated.

[‡]H3 HA numbering.

[§]Amino acid residues differing from HA protein of A/swine/Miyazaki/2/2013 are shown.

[¶]N-glycosylation site predicted by NetNGlyc 1.0 (www.cbs.dtu.dk/services/NetNGlyc/).

Part 2

Characterization of H5N8 influenza A virus isolated from chickens during an outbreak of severe avian influenza in Japan in April 2014

Introduction

The H5 HPAIVs have caused sporadic HPAI outbreaks on poultry farms in Japan (43-45). The causative viruses of those outbreaks belong to Asian subtype H5N1 HPAIVs which are genetically related to the virus isolated from a 1996 outbreak in geese in Guangdong province, China (40). The poultry trade and migration of wild birds are believed to have caused the spread of the virus, and it has affected poultry production and public health mainly in Asian countries (61).

In April 2014, an HPAI outbreak on a broiler chicken farm was confirmed in Kumamoto prefecture in Japan. As a biosecurity control measure, approximately 114,000 chickens were culled on that farm and an epidemiologically related farm. After several control measures were implemented, Kumamoto prefecture declared the end of the outbreak on May 8, 2014 (62). Since January 2014, H5N8 HPAIVs have caused a series of outbreaks in broiler and breeder ducks and broiler and layer chickens in Korea (62). Genetically similar viruses have also been isolated from dead wild birds, including the Baikal teal *Anas formosa* in Korea (63). Although approximately 14 million domestic birds were culled, Korean outbreaks caused by H5N8 HPAIV have not yet ended (62, 64). H5N8 HPAIVs were also isolated from ducks in China during a 2013–2014 monitoring (65). Since the fall of 2014, several HPAI outbreaks, caused by H5N8 HPAIVs, have occurred in poultry in Europe (62, 64, 66, 67). These viruses were also isolated from wild bird specimens in several prefectures (62, 64, 68) and this was followed by disease outbreaks in poultry in Miyazaki, Yamaguchi, Okayama and Saga prefectures in Japan from December 2014 to January 2015(62, 64). Concurrent with the outbreaks in poultry in Japan, HPAIVs possessing the related Asian subtype of the H5 gene spread more broadly and were isolated from wild and domestic birds in various parts of the world including Chinese Taipei (H5N2, H5N3, and H5N8), Canada (H5N2), and the USA(H5N1, H3N2, and H5N8) (64, 69-71).

In this part, A/chicken/Kumamoto/1-7/2014 (Kum14) (H5N8), a representative strain from the outbreak in Kumamoto prefecture, was analyzed to determine its genetic origin,

infectivity in poultry, and serological properties related to risk assessment in the poultry industry. In addition, deduced amino acid sequences were analyzed to assess the risk that this strain poses to public health.

Materials and Methods

Viruses. Kum14 was isolated in an embryonated egg inoculated with a pooled cloacal swab from five dead chickens obtained from the Kumamoto HPAI outbreak. A/duck/Chiba/26-372-48/2014 (C48) (H5N8) and A/duck/Chiba/26-382-61/2014 (C61) (H5N8) were isolated from wild duck fecal specimens from Chiba prefecture in November 2014 by the Municipal Livestock Hygiene Center of Chiba prefecture and subjected to analysis. A/breeder duck/Korea/Gochang1/2014 (Goc14) (H5N8) and A/broiler duck/Korea/Buan2/2014 (Bua14) (H5N8) were kindly provided by Dr. Youn-Jeong Lee, Animal and Plant Quarantine Agency, Korea.

Genetic analysis of A/chicken/Kumamoto/1-7/2014. RNA was extracted from infectious allantoic fluid from an embryonated egg using an RNeasy Mini Kit. A cDNA library was prepared using an NEBNext Ultra^{TM} RNA Library Prep kit (New England Biolabs, Beverly, MA, USA) and was analyzed using a Miseq second-generation sequencer (Illumina, Inc., San Diego, CA, USA) with Reagent Kit v2 (Illumina, Inc.). The whole-genome sequence of Kum14 was determined by a two-step sequence-mapping method using a CLC genome workbench (CLC bio, Inc., Aarhus, Denmark) as follows. First, partial and temporary template sequences for mapping data were selected depending on the Kum14 subtype determined before the whole-genome mapping. The sequence data for the cDNA library were mapped onto the temporarily templates, and partial sequences of all eight genomic segments of Kum14 were obtained. The top hits in a NCBI BLAST analysis for full-length sequences matching the longest partial sequences of the eight genomic segments of Kum14 were selected as the second templates. The sequence data for the cDNA library were assembled by mapping on the second templates to establish whole-genome sequences.

Top 50 hits of BLAST against the EpiFlu database of Global Initiative on Sharing Avian Influenza data (GISAID) against nucleotide sequences of each genomic segment of Kum14 (performed on 13 August 2014) and five H5N8 HPAIVs belonging to clade 2.3.4.4 isolated in Japan and Europe in the fall of 2014, C48, C61, A/turkey/Germany-MV/R472/2014 (H5N8), A/duck/England/36254/2014 (H5N8), and A/chicken/Netherland/14015526/2014 (H5N8) were used for phylogenetic analysis to determine the genetic origins of Kum14 and the genetic relationship among the viruses. The sequences of each of the eight genomic segments were aligned by MAFFT v. 7.215 (72). Phylogenetic trees were constructed by the maximum-likelihood method using MEGA 5. The phylogeny test options used to construct the trees were 1,000 bootstrap replicates, complete deletion of gaps/missing data, and nearest neighbor interchange for the heuristic method. The Hasegawa-Kishino-Yano nucleotide substitution model was used for HA, NA, PB, and NP; the Tamura-Nei model was used for PB1; the Kimura 2-parameter model was used for M; and the Tamura 3-parameter model was used for NS because they were selected by MEGA 5 as the best models. Gamma distribution was selected as the best setting for each genomic segment for the rate among sites.

Experimental infection of chickens and ducks. Following the guidelines established by the Office international des epizooties (OIE) (36), 0.2 ml of 10-fold-diluted Kum14 infectious allantoic fluid was inoculated intravenously into eight 5-week-old chickens. They were observed at 16, 17, 24, 32, 40, 48, 56 and 64 h post-inoculation (hpi). Five- and 10-week-old specific pathogen-free white leghorn chickens (L-M-6 strain), were obtained from Nissei-Bio Co., Ltd. (Yamanashi, Japan). Four-week-old Cherry Valley strain domestic ducks were obtained from Hamada Co. Ltd. (Saitama, Japan). All animal experiments were performed in biosafety level 3 facilities and in accordance with the guidelines of the Animal Care and Use and Biosafety Committees of NIAH.

Challenge doses of 10^6, 10^4, and 10^2 50% chicken embryo infectious doses (EID_{50}) per 100 µl of diluted in PBS were prepared to investigate infectivity. Groups of six 10-week-old and three 5-week-old chickens were inoculated intranasally with 10^6 EID_{50} per 100 µl of Kum14. Groups of three 10-week-old and three 5-week-old chickens were inoculated intranasally with 10^4 and 10^2 EID_{50} per

100 μl of Kum14, respectively. The chickens were observed every 24 h for 16 days to determine the mean death time (MDT). Tracheal and cloacal swabs were taken at 1, 3, 4, 7, 10, and 14 days post-inoculation (dpi) and at death. They were then dipped into collection medium (MEM containing 0.5% BSA, 25 μg of Fungizon per ml, 1,000 units of penicillin and 1,000 μg of streptomycin per ml, 0.01 M HEPES, and 8.8 mg of $NaHCO_3$ per ml). Groups of four 4-week-old ducks were inoculated intranasally with 10^6, 10^4, and 10^2 EID_{50} per 100 μl of Kum14. The ducks were observed every 24 h for 14 days to determine the MDT, and swabs were taken at 1, 3, 4, 7, 10, and 14 dpi and were dipped into the collection medium. Swabs were removed from the collection medium and the medium was stored at −80°C until titration.

To investigate tissue dissemination of the virus in chickens, six 10-week-old chickens were inoculated intranasally with 10^6 EID_{50} per 100 μl of Kum14. Three chickens were euthanized at 1 and 3 dpi. The blood, brain, trachea, lung, heart, liver, kidneys, pancreas, spleen, duodenum, rectum, pectoral major muscle, biceps femoris muscle and bursa of Fabricius were collected. Tissues were minced using a Precellys homogenizer (Bertin Technologies, Orleans, France) to prepare a 10% (w/v) emulsion, and the emulsion supernatant was stored at −80°C until titration. Frozen samples were thawed and centrifuged at 3,000 rpm for 5 min at 4°C. Supernatants were subjected to viral titration in embryonated eggs, and EID_{50} was calculated using the Reed and Munch method (73). Mean virus titers were assessed for significance by using a one-way analysis of variance with Bonferroni's *post hoc* test using GraphPad Prism (GrapPad Software Inc., San Diego, CA, USA). A *P*-value of <0.05 was considered statistically significant. Sera were collected from surviving chickens and ducks and were analyzed by HI assays using the Kum14 antigen and enzyme-linked immunosorbent assay (ELISA) by commercial kit targeting anti-NP antibodies (Influenza A Ab Test, IDEXX, Laboratories, Westbrook, ME, USA).

Preparation of antigens and antisera. Viruses which possessed the H5 HA antigen of A/chicken/Indonesia/demak1631-56/2007 (Ind07) (H5N1), A/chicken/Egypt/1553-26/2010 (E1510) (H5N1), A/chicken/Bangladesh/1151-9/2010 (Ban10) (H5N1), A/duck/Vietnam/NCVD-672/2011 (Vie11) (H5N1), A/chicken/Thailand/PC-170/2006 (Tha06) (H5N1), and A/chicken/Pygyitagon/204/2006 (Pyi06) (H5N1) were reconstructed by reverse genetics using the HA gene in which the cleavage sites had been replaced by low pathogenic avian influenza virus type HA

cleavage sites and A/Puerto Rico/8/1934 (H1N1) derived NA and internal genes, as described previously (74). Viruses prepared by reverse genetics and wild-type Kum14, Goc14, Bua14, A/chicken/Egypt/121/2012 (E1212) (H5N1), A/chicken/Egypt/1063/2010 (E1010) (H5N1), A/chicken/Miyazaki/K11/2007 (Miy07) (H5N1), A/whooper swan/Akita/1/2008 (Aki08) (H5N1), A/duck/Tien Giang/15421/2013 (TG1513) (H5N1), A/chicken/Tien Giang/8932/2013 (TG8913) (H5N1), A/chicken/Hmawbi/517/2007 (Hma07) (H5N1), A/village chicken/Kyaing Tong/2433/2007 (KT2407) (H5N1), A/chicken/Yamaguchi/7/2004 (Yam04) (H5N1), A/tern/South Africa/61 (SA61) (H5N3), and A/whistling swan/Shimane/580/2002 (Shi02) (H5N3) were inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs and incubated at 37°C. Hyperimmune antisera against formalin inactivated Kum14, KT2407, Ind07, E1212, E1510, Miy07, Ban10, Aki08, Shi10, Vie11 Yam04, Tha06, TG8913, Pyi06, SA61, and Shi02 were prepared in chickens as described in Part 1. A/Anhui/1/2005 (Anh05) (H5N1) antigen and ferret antiserum against Anh05 and Kum14 were kindly provided by NIID.

Antigen characterization. HI assays (74) were performed for antigenic characterization of Kum14 and other H5 viruses. Briefly, treatment with RDE II and inactivation were performed as described in Part 1. Subsequently, chicken and ferret antisera were absorbed with packed chicken red blood cells for 60 min at room temperature. For each treated antiserum, a dilution series was generated via serial 2-fold dilutions with PBS from 1:20 dilution. Each dilution series was used for HI assays with 0.5% chicken red blood cells. A cutoff value of 1:20 was adopted to prevent false positive results because of non-specific reactions in the HI assays.

Results

Genetic analysis of A/chicken/Kumamoto/1-7/2014. Whole-genome sequences of Kum14 were successfully determined with the exception of 5 bases at the 3'-non-coding end of the PA genomic segment which could not be covered with the complementary DNA library of vRNA of Kum14. Mixed infection was not observed by analysis with the Miseq sequencer and single-nucleotide polymorphisms were detected at three sites, G/A at nucleotide position (nt) 1,837

of PA, T/G at nt 2,127 of PB1, and G/A at nt 897 of NA, by setting the nucleotide content threshold at 10%. The mixture at nt 1,837 of the PA segment resulted in a polymorphism of glutamate/lysine at amino acid position 613 of the PA protein, and the other two substitutions were synonymous.

It has been proposed that Kum14 is an HPAIV because it contains a protease cleavage site comprising multiple basic amino acids, PLRERRRKR/GLF. Phylogenetic analysis of the HA gene (Figure 2-1) indicated that Kum14 possesses clade 2.3.4.4 H5 of Asian lineage, which was previously classified as clade 2.3.4.6 (75) and then re-classified in 2015 as 2.3.4.4 by the World Health Organization (WHO)/OIE/Food and Agriculture Organization H5N1 Evolution Working Group (76) and is similar to the Korean strains (63). Genomic analysis of Kum14 sequences showed that all genomic segments shared a high degree of sequence identity (>99.59% identity) with Bua14, A/baikal teal/Korea/Donglim3/2014 (H5N8), and A/mallard/Korea/W452/2014 (H5N8). These are H5N8 HPAIV strains isolated during the Korea HPAI outbreaks that had started a few months before the Japanese outbreak in April 2014 (Figure 2-1 and 2-2). In addition, Kum14 shared a common ancestor with H5N8 viruses isolated in November 2014 from wild-duck feces in Chiba prefecture in Japan (C48 and C61) and from domestic and wild birds in Germany, England, and the Netherlands in the fall of 2014 (Figure 2-1 and 2-2, indicated by asterisks). However, bootstrap analysis of each segment demonstrated that the cluster comprising the H5N8 isolates from the fall of 2014 was clearly distinguishable from the one that included Kum14, with bootstrap values of 99% for HA, PB1, and PA; 98% for NA and PB2; and 94% for NP. The phylogenetic analysis of all eight segments was performed to investigate the ancestor of each genomic segment of Kum14. The PB2, HA, NP, and NA segments of Kum14 were believed to have originated from a H5N8 HPAI virus, such as A/duck/Jiangsu/k1203/2010 (H5N8), which was isolated in 2010 in Jiangsu, China. Conversely, the PB1, PA, M, and NS segments were believed to have originated from a H5N2 virus, such as A/duck/Eastern China/1111/2011 (H5N2) or A/goose/Eastern China/1112/2011 (H5N2).

Receptor preference of HA protein of HPAIVs is closely related to public-health risk. The single amino acid substitutions A138S (77), G186V (78), Q226L (79, 80), Q196H (81), Q196R, S227R (82), and N224K (79), and the combination of amino acid substitutions Q226L and

G228R (83) in the HA protein of influenza A virus have been reported to increase the affinity of HA for the human receptor, sialic acid linked to galactose by alpha 2,6 linkages. These substitutions were not observed in the HA of Kum14. Loss of the glycosylation site at position 158 (H3 numbering), which links to the ability to transmit ferret to ferret by aerosol (79, 84-86), was found in the HA of Kum14 as the result of a T/S160A substitution.

Some amino acid residues involved in promoting viral replication and pathogenicity in mice were found in viral proteins of Kum14. Serine 66 of the PB1-F2 protein of Kum14 has been identified as being associated with increased viral replication in mouse cells (87). Presence of asparagine 30 and glutamate 215 in the M1 protein and serine 42 in the NS1 protein, related to increasing pathogenicity in mice (88, 89), were found in Kum14. Amino acid substitutions that are latently related to pathogenicity in mammals, including lysine 627 and asparagine 701, independently influence the virulence and transmission ability in mammals (90, 91). The combination of alanine 271, serine 590, and arginine 591 in PB2, which increases replication and virulence in cultured cells and mice (92), were not conserved in Kum14.

Regarding anti-influenza drug resistance, the S31N substitution in M2 protein, which has been shown to confer viral resistance to amantadine and remantadine (93), was found in Kum14. NA inhibitors, which control influenza A virus infection by inhibiting the release step in the virus life cycle, are considered to be effective against Kum14 because known mutations (E119G, Q136K, D151A, D198G, I222R, H274Y, R292K, N294S and R371K; N2 numbering) were not found in the NA protein of the virus (94).

Experimental infection studies with chickens and ducks. Following the guidelines established by the OIE, the survival of eight 5-week-old chickens inoculated intravenously with 0.2 ml of the 10-fold-diluted Kum14-infected allantoic fluid was observed. Six chickens died within 24 hpi, and the remaining two died at 40 and 64 hpi, respectively (Figure 2-3). Depression was observed in three chickens before they died at 24, 40, and 64 hpi. A mild or moderate level of cyanosis of the combs and legs was observed in six of the eight chickens.

To assess susceptibility, virus shedding, symptoms, and lethality when poultry were infected with Kum14 via a natural route, 5- and 10-week-old chickens and 4-week-old domestic

ducks were inoculated intranasally with 100-fold serially diluted Kum14. All chickens inoculated with 10^6 EID_{50} of Kum14 died following infection, whereas chickens inoculated with 10^4 and 10^2 EID_{50} of Kum14 survived the observation period for 16 days, regardless of their age (Figure 2-4A and 2-4B). All inoculated ducks survived for the observation period (Figure 2-4C). The MDT of the 10-week-old chickens inoculated with 10^6 EID_{50} of Kum14 was 175.3 h, whereas the MDT of the three 5-week-old chickens inoculated with 10^6 EID_{50} of Kum14 was 112 h. Depression, respiratory distress, and mild, moderate, or severe levels of cyanosis were observed in all moribund chickens within 24 h of their death. Unilateral leg paresis was observed in two ducks inoculated with 10^4 and 10^6 EID_{50} of Kum14.

Ducks inoculated with either 10^6 or 10^4 EID50 Kum14 all showed anti-NP serum antibodies collected at 14 dpi, and identified by ELISA. Serum from three of the four ducks inoculated with 10^6 EID_{50} of virus and two of four ducks inoculated with 10^4 EID_{50} of Kum14 had an HI titer > 10 against the Kum14 antigen at 14 dpi. Anti-NP antibodies were not detected in any of the ducks inoculated with 10^2 EID_{50} of Kum14 or in chickens surviving up to 16 dpi.
Virus shedding was detected from 3 dpi onward in tracheal and cloacal swabs of 10- and 5-week-old chickens inoculated intranasally with 10^6 EID_{50} of Kum14 (Table 2-1A and B). Peak tracheal virus shedding was observed at 4 dpi in both age groups, with no significant difference in the viral titers. One of the 10-week-old chickens died at 308 hpi, and this was the longest survival time for any chicken inoculated with 10^6 EID_{50} of Kum14. Severe cyanosis and depression had been observed beginning at 6 dpi in this chicken.

Chickens inoculated intranasally with 10^6 EID_{50} of Kum14 were sacrificed to examine tissue dissemination of Kum14. A titer of at least $10^{1.07}$ EID_{50} of Kum14 per ml was detected in all tissues tested at 3dpi, although the virus was not detected in any tissue tested at 1 dpi (Table 2-2). In domestic ducks inoculated intranasally with 10^6 and 10^4 EID_{50} of Kum14, the virus shedding was detected earlier in the trachea than in the cloaca, although the shedding was maintained for longer time in the cloaca than in the trachea (Table 2-1C). The titer of the peak of viral shedding from the cloaca of a duck ($10^{7.2}$ EID_{50}/ml) was higher than the titers of any samples obtained from the chickens, although there was no significant difference between the mean peak titers of ducks

and chickens. Low-level virus shedding was detected sporadically in cloacal swabs of ducks inoculated with 10^2 EID_{50} of Kum14.

Serological cross-reactivity of A/chicken/Kumamoto/1-7/2014. The HI cross-reactivities of Kum14 and 21 other H5 avian influenza viruses with a panel of 16 distinct chicken antisera generated against H5 viruses were examined (Table 2-3A). Kum14 and viruses belonging to clade 2.3.4.4, Bua14 and Goc14, reacted similarly with the panel of antisera examined. Kum14 and Bua14 reacted with Kum14 antisera at an HA titer of 320, and weakly reacted with Ban10, Tha06, SA61, and Shi02 antisera at HA titers of 20, 20, 40, and 80, respectively. Although Goc14 reacted with the same antisera as Kum14 and Bua14, the HA titers for the Goc14 antigen with Kum14, Ban10, and Tha06 antiserum were 2-fold lower than the HA titers for the Kum14 antigen and the same series of antisera. Kum14, Bua14, and Goc14 antigens did not react with any other antiserum tested. Chicken antisera against Kum14 reacted with antigens belonging to clade 2.3.4.4. In addition, the antisera reacted with Miy07, Ban10, Vie11, Yam04, Tha06, TG8913, and Shi02 antigens with lower HI titers compared with the titers against clade 2.3.4.4 antigens. HI assay was also performed using ferret antisera against Kum14 and Anh05, which belongs to clade 2.3.4 and is a pre-pandemic H5N1 vaccine candidate strain used by the WHO Global Influenza Program (Table 2-3B). No serological cross-reactivity between Kum14 and Anh05 was detected. Ferret antisera generated against Kum14 reacted differently with H5 antigens than with chicken antisera. Namely, Goc14, Miy07, Vie11, and Tha06 antigens, which reacted with the chicken antiserum against Kum14, did not react with the ferret antiserum. E1510, which did not react with the chicken antiserum against Kum14, reacted with the ferret antiserum. The ferret antiserum against Kum14 reacted with the Yam04 antigen at an 8-fold higher HI titer than the homologous antigen, although the chicken antisera reacted with Yasm04 at a 4-fold lower titer.

Discussion

The infectivity of Kum14 in chickens appeared to be relatively low when compared with previous studies of H5 HPAIVs from chickens in Japan (95, 96). Five- and 10-week-old chickens inoculated

intranasally with 10^4 and 10^2 EID_{50} of Kum14 showed no evidence of infection. The survival period of chickens infected with Kum14 also appeared to be longer than that of chickens inoculated with H5 HPAIVs previously isolated in Japan (95, 96). In addition, virus shedding from chickens infected with Kum14 was relatively low compared with previous H5 HPAIVs from chickens in Japan (95, 96). A previous study suggested that adaptation of the H5 virus in waterfowl may reduce the amount of virus excreted from chickens and decrease transmission efficiency from infected to naïve chicken (96). Based on this previous observation and the fact that many genetically related viruses to Kum14 (Figure 2-1, 2-2) have been repeatedly isolated from waterfowl (62, 64, 65), Kum14 and related viruses could be more adapted to waterfowl than to chickens and it is not inconsistent with relatively low pathogenicity of Kum14 against chickens. The lack of symptoms caused by Kum14 infection, despite the high infectivity and the virus shedding pattern in ducks suggested that waterfowl could be efficiently reserved or carry Kum14 and related viruses. Although a challenge dose of 10^4 EID_{50} of Kum14 was enough to infect ducks, a challenge with a lower-dose of the virus may also infect ducks. Three of the four ducks inoculated with 10^2 EID_{50} of Kum14 sporadically shed a small amount of virus in the cloaca, although none of them were seroconverted. This is consistent with a previous study in which AIV-infected ducks shed virus in their feces but produced very low levels of serum antibodies (97), and this may be an advantage of waterfowl as a reservoir or carrier of Kum14 and related viruses. In the experimental infection of domestic ducks with 10^6 EID_{50} of Kum14, virus shedding with a very high titer ($10^{7.2}$ EID_{50}/ml) in the cloaca of one duck at 4 dpi was detected. This suggested that some waterfowl may act as super-spreaders of the virus. It is critical and with some urgency that an investigation of the infection dynamics and mechanisms is conducted. This should include persistent infection and virus shedding in waterfowl infected with Kum14-related viruses to understand the rapid and global spread of H5 HPAIV belonging to clade 2.3.4.4 (64, 69-71).

To estimate the area from which area the Kum14 was introduced, phylogenetic data of Kum14 and related viruses have to be discussed. All eight genomic segments of Kum14 have highest sequence homologies (>99%) with each segment of Korean H5N8 viruses, isolated from wild and domestic birds shortly before isolation of Kum14. This result suggests that Kum14 and these Korean H5N8 viruses share an ancestor pool of virus and carrier route. Analysis of all eight

genomic segments of Kum14 suggested that the ancestor of Kum14 was generated by a reassortant event between two Asian sub-linages of HPAIVs, which occurred in East Asia or a related area. As shown in Figures 2-1 and 2-2, several viruses appear to share an ancestor virus with Kum14 because each genomic segment of the viruses is closely related to each other (indicated with asterisk). In those H5N8 viruses, the presence of A/duck/Beijing/FS01/2013 (H5N8), isolated from a dead domestic duck in China in November 2013, indicates that H5N8 HPAIV possessing a common genetic ancestor of all eight genomic segments was present in East Asia at least in late 2013. Although the PB2 genomic segment of A/duck/Beijing/FS01/2013 (H5N8) has not been included in Figure 2-2B because the length of submitted sequence of the PB2 genomic segment of the virus in the database was too short for analyzing in the phylogenetic tree, 1,776 of 1,784-nt submitted PB2 sequence were 99.55% identical to PB2 of Kum14. For a detailed analysis of these H5N8 influenza A viruses indicated with an asterisk in Figure 2-1 and 2-2, comparison of HA genes is suitable because of its rapid genetic evolution (3). As shown in Figure 2-1, H5N8 HPAIVs possessing a common genetic ancestor with Kum14 (indicated with the asterisk) are distinguished by several clusters. Because the cluster comprising HPAIVs isolated in Beijing is clearly distinguishable from the remaining viruses, including Kum14, the possibility that Kum14 was introduced directly from Beijing virus pool appears to be relatively low. On the other hand, viruses genetically related to Kum14 (Figure 2-1 and 2-2) has spread to broad areas of the Eurasian and North American continents in 2014 (62, 64, 66, 67, 69-71). In November 2014, two viruses, C48 and C61, sharing common ancestors with Kum14, were isolated in Chiba prefecture, Japan, and HPAIVs more closely related to C48 and C61 than to Kum14 were isolated in Europe. The 2010–2011 H5N1 HPAIV of outbreaks in Japan were considered to have been carried by wild migratory birds. They carried the viruses from their nesting lakes in Siberia in the summer and transmitted them through at least three routes via China, Korea, and Russia to Japan (98). This suggests the presence of a migration route for birds carrying H5N8 from East Asia to Russia. In addition, an estimated 4,800,000 wild ducks, geese and swans migrate between Russia and European countries (99). These previous studies suggesting the presence of a migration route of waterfowl carrying H5N8 HPIVs explains why closely related viruses were isolated concurrently in European countries and Japan. In addition, an investigation of when H5N8 HPAIVs related to

Kum14 were introduced into the nesting lakes of migratory birds is an important and intriguing issue to understanding from which area the Kum14 was carried.

HPAIVs cause public-health concerns worldwide. Since the first 1997 outbreak of H5N1 HPAIV in humans (100), H5 HPAIV infections have been confirmed in over 660 individuals and more than 330 of them died (101). Although the loss of the glycosylation at position 158 of HA increases the transmission efficiency among mammals (79, 84-86), Kum14 and many strains of H5 HPAIV, including Japanese strains of previous outbreaks (95, 96) also lost the glycosylation of the site. However, in recent reports, HPAIVs with HA belonged to clade 2.3.4.4 and isolated from wild and domestic waterfowl could bind to both the avian receptor, sialic acid linked to galactose by alpha 2,3 linkages, and the human receptor (102, 103). Whether these observations are applicable to clade 2.3.4.4 isolates from chickens, including Kum14, needs to be investigated to assess the potential risk of human infection by a virus belonged to that clade. Mutations in the M1 and NS1 proteins, related to increased pathogenicity in mice (88, 89), were found in Kum14, whereas most of the previous H5 isolates, including H5N1 viruses isolated in Japan, also possess these amino acid residues. In addition, amino acid substitutions related to resistance of the NA inhibitor were not conserved in Kum14. Although the observations made in this study do not suggest that a Kum14 virus should be considered a public-health risk in comparison to previous H5 HPAIVs, further investigation is required for an appropriate assessment.

Brief Summary

An H5N8 subtype of HPAIV, A/chicken/Kumamoto/1-7/2014 (Kum14) (H5N8), was isolated from a chicken farm in Japan during an outbreak in April 2014. Phylogenetic analysis of the virus revealed that it belonged to HA clade 2.3.4.4. All eight genomic segments shared high sequence homologies with H5N8 subtype HPAIVs, A/broiler duck/Korea/Buan2/2014 (H5N8) and A/baikal teal/Korea/Donglim3/2014 (H5N8), which were isolated in Korea in January 2014. Intranasal experimental infection of chickens and ducks with Kum14 was performed to assess the pathogenicity of the virus in chickens and the potential for waterfowl to act as a virus reservoir and carrier. A high-titer virus challenge (10^6 EID_{50}/animal) was lethal in chickens, but they were unaffected by lower virus doses (10^2 EID_{50} or 10^4 EID_{50}/animal). A virus challenge of all doses examined was found to asymptomatically infect ducks. These results suggest that migratory waterfowl act as carriers of Kum14 into Japan. HI assays revealed that Kum14 possessed relatively low cross-reactivity with H5 viruses belonging to clades other than clade 2.3.4.4. Specific and effective serological diagnostics for the HPAIVs belonging to clades 2.3.4.4 need to be prepared to control prospective outbreaks.

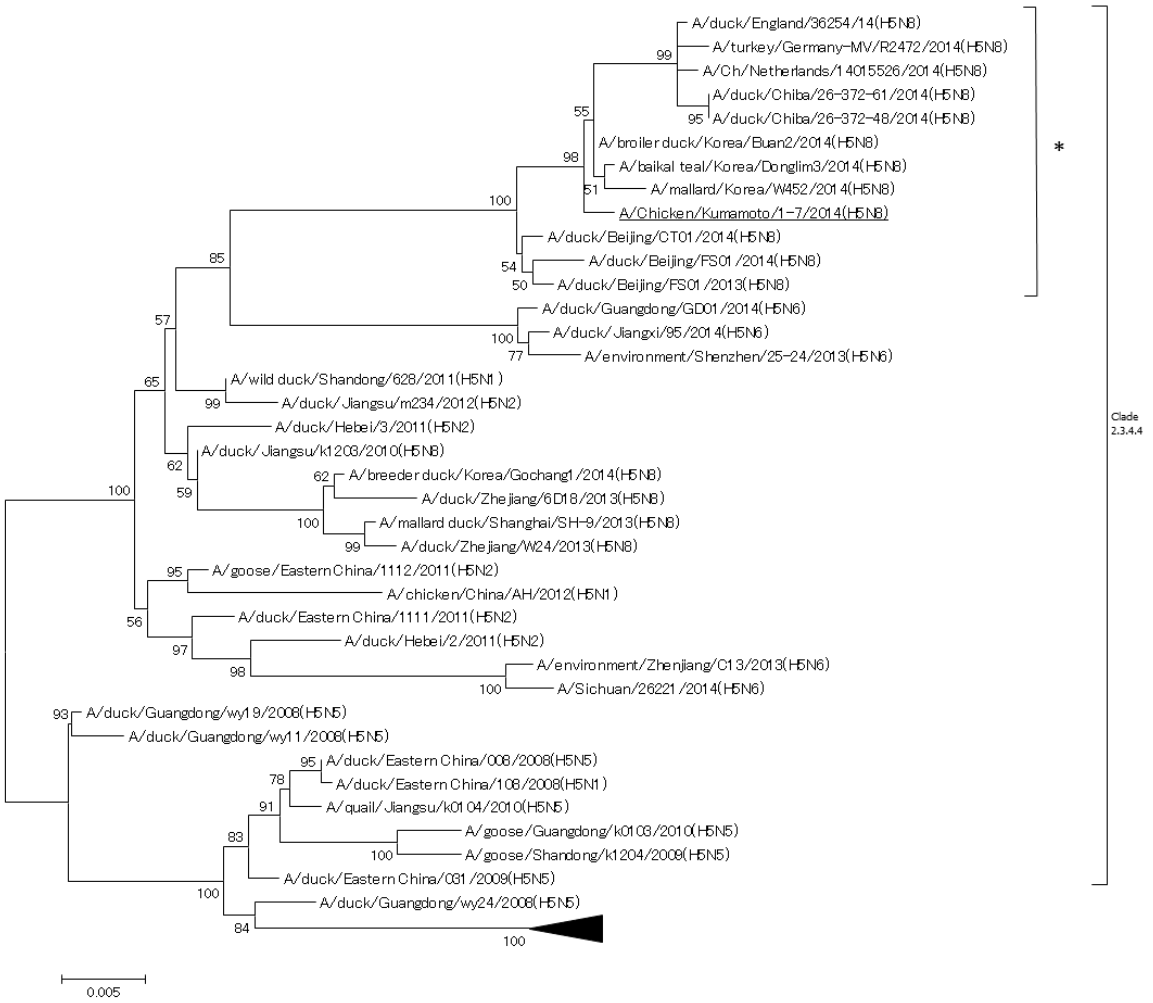

Figure 2-1. Phylogenetic tree of hemagglutinin (HA) gene of the isolates belonging to clade 2.3.4.4. A/chicken/Kumamoto/1-7/2014 (H5N8) is underlined. MEGA 5 was used to create the tree using the maximum likelihood method and bootstrapped with 1000 replicates. Scale bar indicates distance units between sequence pairs. Viruses indicated with asterisk share common genetic ancestors of all genomic segments, with an exception of A/duck/Beijing/FS01/2013 (H5N8), which PB2 sequence in GISAID was too short to hit in BLAST for selection of reference sequences.

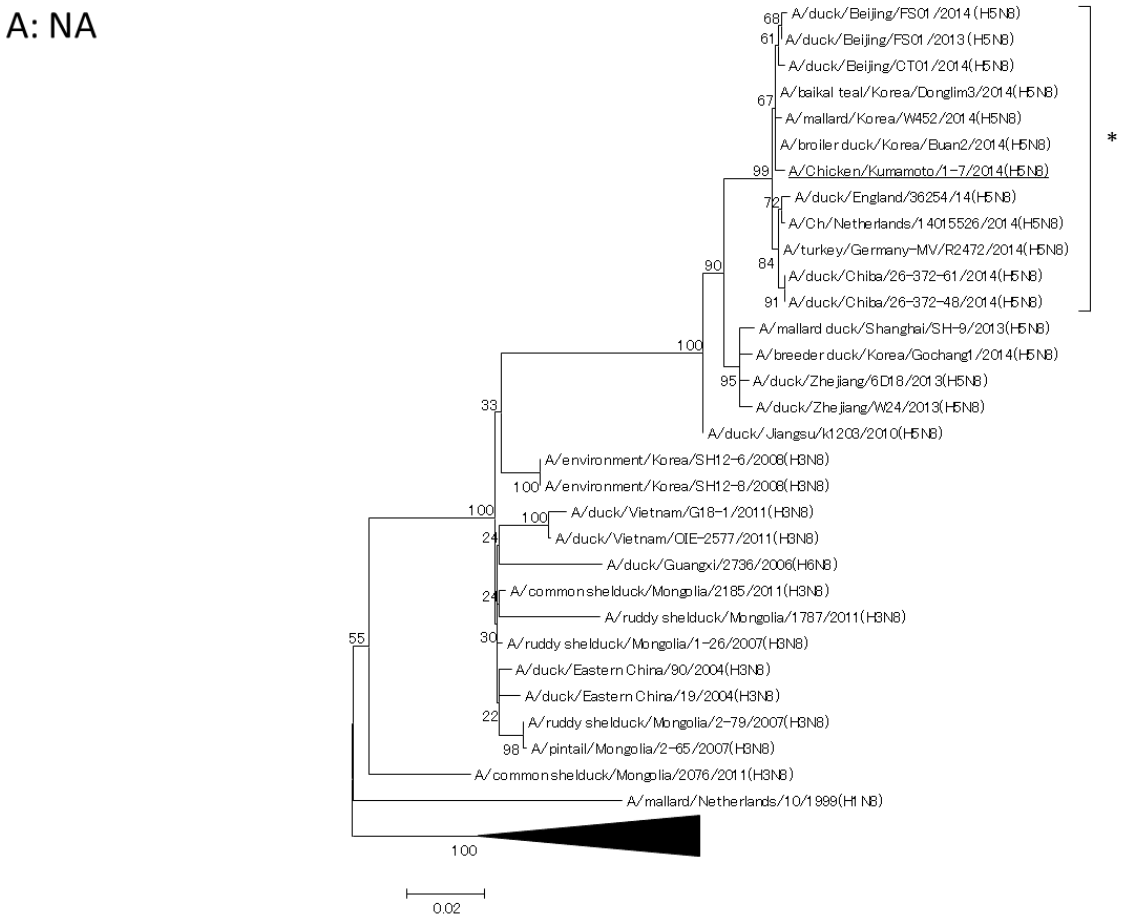

Figure 2-2. Phylogenetic analysis of NA (A), PB2 (B), PB1 (C), PA (D), NP (E), MP (F) and NS (G) genes of A/chicken/Kumamoto/1-7/2014 (H5N8) and other viruses.
A/chicken/Kumamoto/1-7/2014 (H5N8) is underlined. The trees were created as described in Figure 2-1 legend.

B: PB2

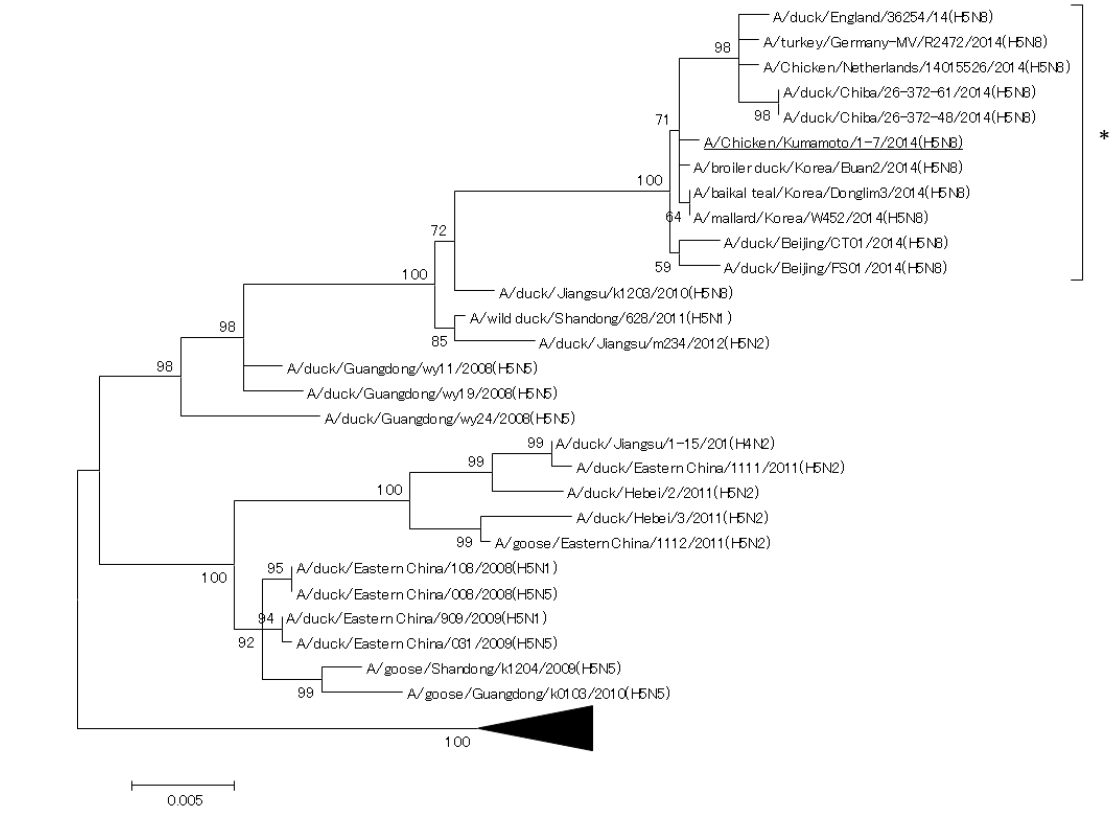

Figure 2-2. (continued)

C: PB1

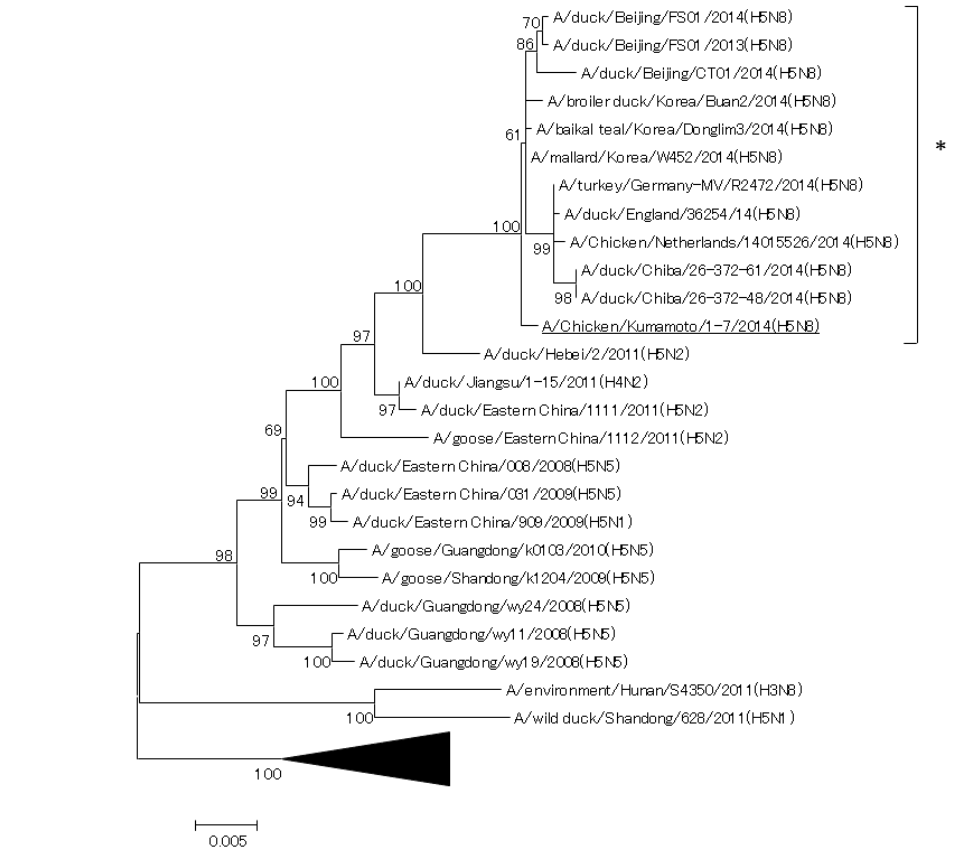

Figure 2-2. (continued)

D: PA

Figure 2-2. (continued)

E: NP

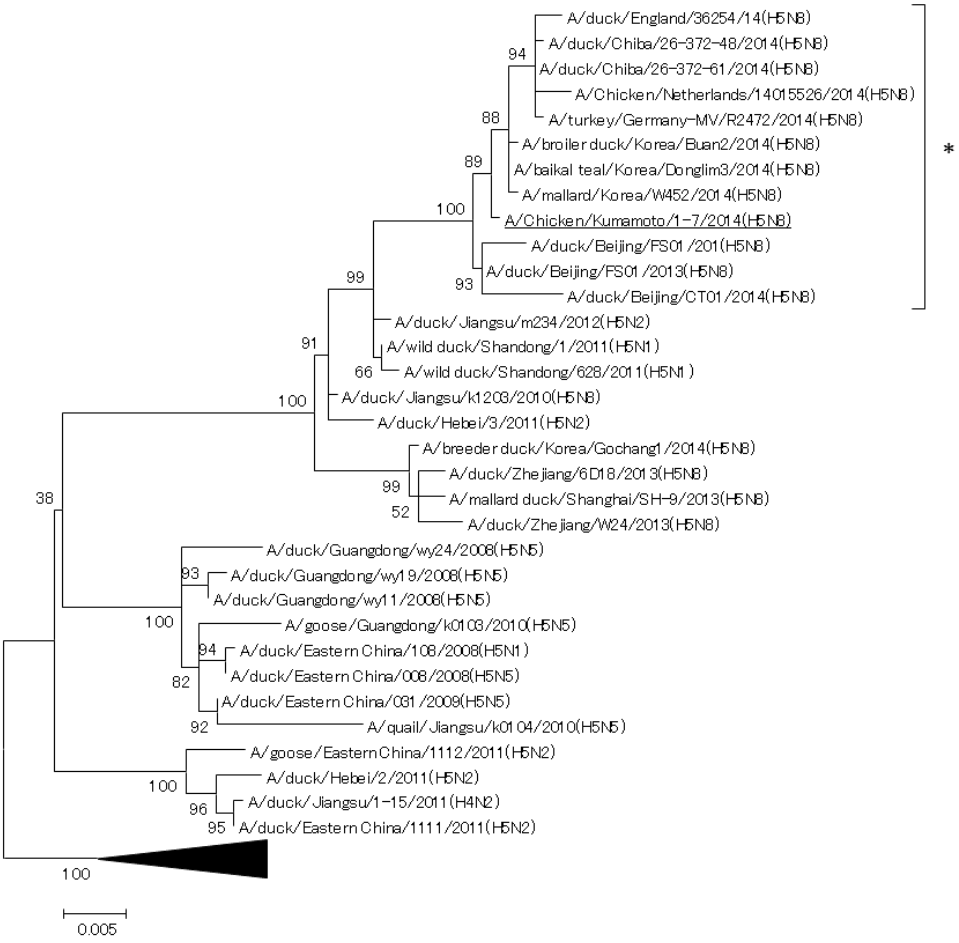

Figure 2-2. (continued)

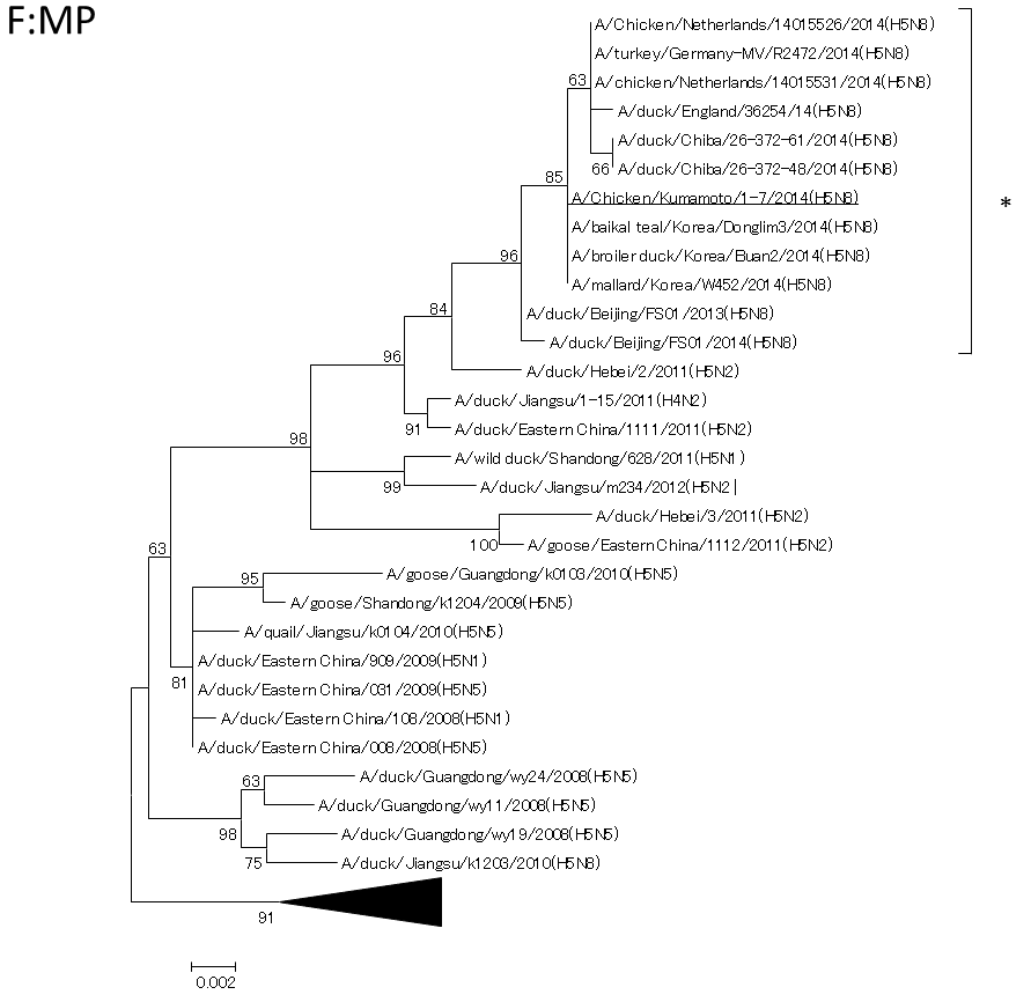

Figure 2-2. (continued)

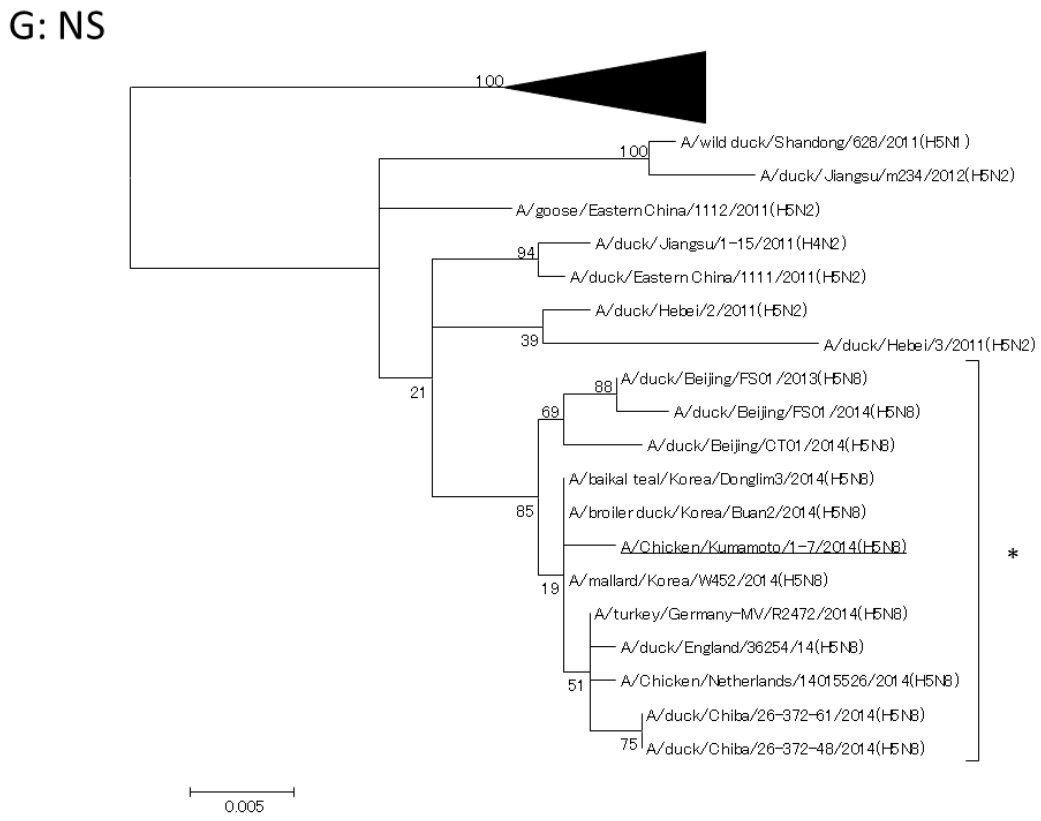

Figure 2-2. (continued)

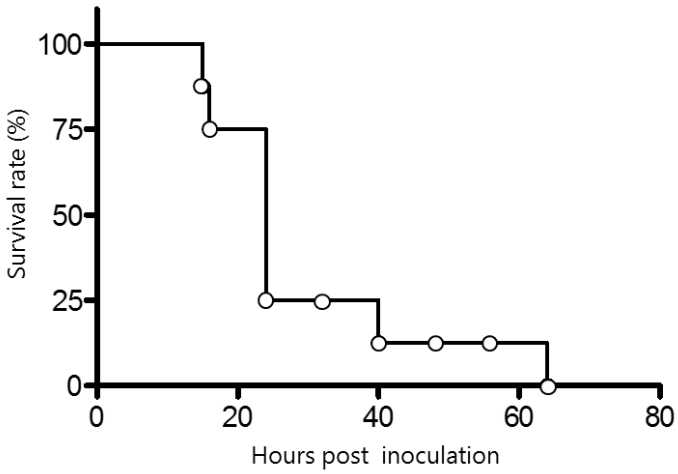

Figure 2-3. Survival rate of chickens intravenously inoculated with A/chicken/Kumamoto/1-7/2014 (H5N8). Eight 5-week old chickens were intravenously inoculated with 0.2 ml of 10-fold diluted infective allantoic fluid. Observation were performed at 16, 17, 24, 32, 40, 48, 56 and 64 hpi (circle symbols).

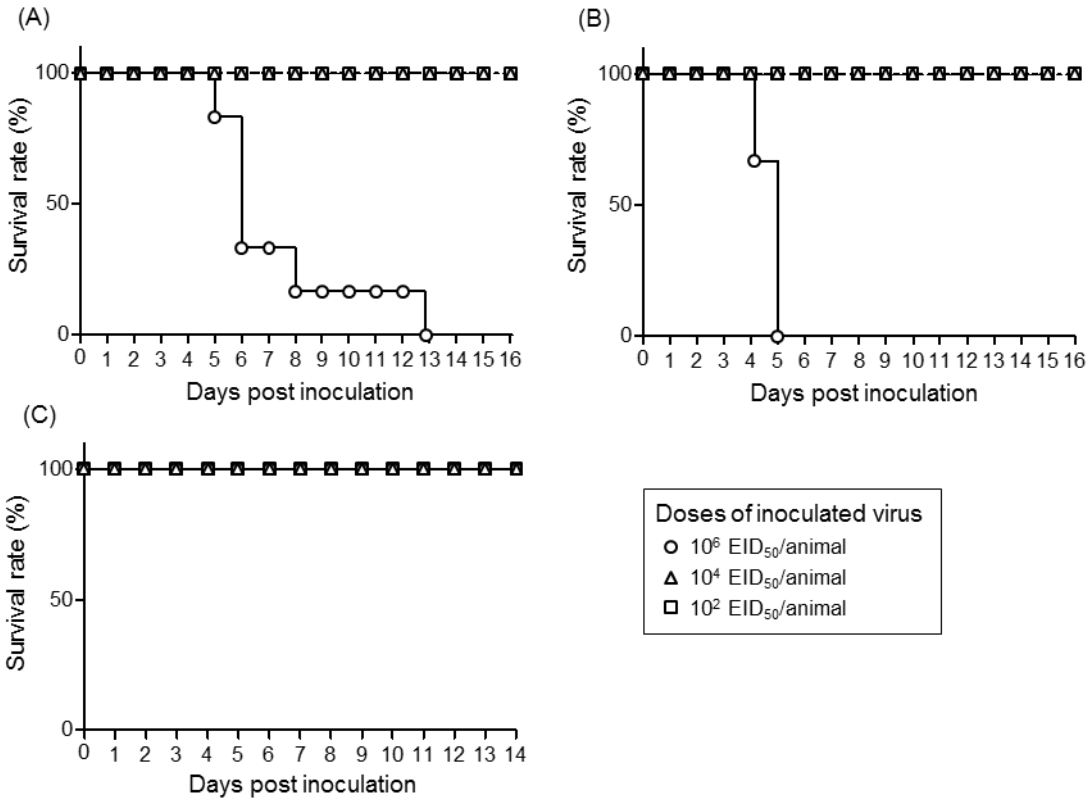

Figure 2-4. Survival rate of (A) 10-week old chickens, (B) 5-week old chickens and (C) 4-week old domestic ducks intranasally inoculated with A/chicken/Kumamoto/1-7/2014 (H5N8). The circle, triangle and square symbols represent survival rates of animals inoculated with 10^6 EID_{50}/animal, 10^4 EID_{50}/animal and 10^2 EID_{50}/animal of virus, respectively.

Table 2-1. Virus shedding and antibody detection in chickens and ducks intranasally inoculated with A/chicken/Kumamoto/1-7/2014(H5N8)

(A) 10 week-old chickens

		Infectivity titers in chicken embryos				Antibody	
		Trachea swab		Cloaca swab			
Infection Dose	Day	Shedding	Titer (log_{10} EID_{50}/ml)	Shedding	Titer (log_{10} EID_{50}/ml)	HI[§]	ELISA[¶]
10^6 EID_{50}/chicken(N=6)[†]	1	0/6	ND	0/6	ND	-	-
	3	4/6	1.72±0.638 (<0.324-3.32)	6/6	0.66±0.129 (<0.324-1.07)	-	-
	4	5/6	2.45±0.808 (<0.532-5.02)	5/6	1.12±0.055 (1.017-1.299)	-	-
	7	2/2	3.70±0.375 (3.32-4.70)	0/2	ND	-	-
	10	1/1	3.20 (3.20)	0/1	ND	-	-
10^4(N=3)	1-16[‡]	ND	ND	ND	ND	0/3	0/3
10^2(N=3)	1-16[‡]	ND	ND	ND	ND	0/3	0/3

Titers are indicated mean±standerd error (range). ND; Not detected. -; Not tested.

†; All animals inoculated with 10^6 EID_{50} Kum14 were dead by13 dpi.

‡; Days 1, 3, 4, 7, 10, 14 and 16.

§; Number of serum samples (at 16 dpi) where HI titers against A/chicken/Kumamoto/1-7/2014 antigen were greater than 10.

¶; Number of serum samples (at 16 dpi) which were determined as positive by Influenza A virus antibody test kit (IDEXX Laboratories, Inc.)

(B) 5 week-old chickens

		Infectivity titers in chicken embryos				Antibody	
		Trachea swab		Cloaca swab			
Infection Dose	Day	Shedding	Titer (log_{10} EID_{50}/ml)	Shedding	Titer (log_{10} EID_{50}/ml)	HI[§]	ELISA[¶]
10^6 EID_{50}/chicken(N=3)[†]	1	0/3	ND	0/3	ND	-	-
	3	2/3	1.80±0.271 (1.53-2.07)	1/3	1.53 (1.53)	-	-
	4	3/3	4.42±0.949 (2.53-5.53)	3/3	1.13±0.446 (<0.324-1.87)	-	-
10^4(N=3)	1-16[‡]	ND	ND	ND	ND	0/3	0/3
10^2(N=3)	1-16[‡]	ND	ND	ND	ND	0/3	0/3

Titers are indicated mean±standerd error (range). ND; Not detected. -; Not tested.

†; All animals inoculated 10^6 EID_{50} Kum14 were dead until 5 dpi.

‡; Days 1, 3, 4, 7, 10, 14 and 16.

§; Number of serum samples (at 16 dpi) where HI titers against A/chicken/Kumamoto/1-7/2014 antigen were greater than 10.

¶; Number of serum samples (at 16 dpi) which were determined as positive by Influenza A virus antibody test kit (IDEXX Laboratories, Inc.)

(C) 4 week-old ducks

		Infectivity titers in chicken embryos				Antibody	
		Trachea swab		Cloaca swab			
Infection Dose	Day	Shedding	Titer (log_{10} EID_{50}/ml)	Shedding	Titer (log_{10} EID_{50}/ml)	HI[†]	ELISA[‡]
10^6 EID_{50}/duck(N=4)	1	1/4	1.32	0/4	ND	-	-
	3	4/4	3.65±0.468 (2.32-4.53)	4/4	2.73±0.312 (2.07-3.32)	-	-
	4	4/4	3.92±0.609 (2.20-5.07)	4/4	4.46±0.961 (2.87-7.20)	-	-
	7	1/4	<0.299 (<0.299)	2/4	1.32±0.00 (1.32)	-	-
	10	0/4	ND	1/4	<0.324 (<0.324)	-	-
	14	0/4	ND	1/4	1.017 (1.017)	3/4	4/4
10^4(N=4)	1	0/4	ND	0/4	ND	-	-
	3	2/4	2.82±1.50 (1.32-4.32)	1/4	3.20 (3.20)	-	-
	4	4/4	4.15±0.874 (2.32-6.53)	3/4	1.24±0.583 (<0.324-2.32)	-	-
	7	2/4	4.20±0.333 (3.87-4.53)	2/4	2.60±0.271 (2.32-2.87)	-	-
	10	0/4	ND	4/4	<0.480±0.0786 (<0.324-<0.699)	-	-
	14	0/4	ND	1/4	<0.699 (<0.699)	2/4	4/4
10^2(N=4)	1	0/4	ND	1/4	<0.324 (<0.324)	-	-
	3	0/4	ND	1/4	<0.324 (<0.324)	-	-
	4	0/4	ND	0/4	ND	-	-
	7	0/4	ND	2/4	<0.387±0.0625 (<0.324-<0.449)	-	-
	10	0/4	ND	0/4	ND	-	-
	14	0/4	ND	0/4	ND	0/4	0/4

ND; Not detected. -; Not tested.

†; Number of serum samples (at 14 dpi) where HI titers against A/chicken/Kumamoto/1-7/2014 antigen were greater than 10.

‡; Number of serum samples (at 14 dpi) which were determined as positive by Influenza A virus antibody test kit (IDEXX Laboratories, Inc.)

Table 2-2. Tissue dissemination in chickens intranasally inoculated with A/chicken/Kumamoto/1-7/2014(H5N8)

	Virus titers in organs of infected chickens (log_{10} EID_{50}/ml)					
	Day 1 after inoculation			Day 3 after inoculation		
Organ/tissue	1	2	3	4	5	6
Blood	-	-	-	1.07	2.20	2.02
Brain	-	-	-	2.32	3.45	3.53
Trachea	-	-	-	2.87	3.20	3.32
Lung	-	-	-	4.20	5.32	4.87
Heart	-	-	-	4.32	4.32	3.53
Liver	-	-	-	3.32	3.32	4.07
Kidney	-	-	-	3.70	5.07	4.32
Pancreas	-	-	-	2.02	4.95	3.32
Spleen	-	-	-	4.07	5.07	5.32
Duodenum	-	-	-	2.53	4.53	3.20
Rectum	-	-	-	3.07	4.87	3.53
Pectoral major muscle	-	-	-	2.20	2.87	2.53
Biceps femoris muscle	-	-	-	3.02	3.53	3.20
Bursa of Fabricius	-	-	-	3.38	4.53	3.32

Chickens inoculated with 10^6 EID_{50}/chicken of virus.

Numbers 1 to 6 indicate animal identification number. Values indicate virus titer (log_{10} EID_{50}/ml).

A dash indicates that virus was not detected**.**

Table 2-3. Serological cross-reactivities among the H5 viruses tested by hemagglutination inhibition (HI) assay.

		Hyperimmune serum raised in chickens																Ferret infection serum	
Antigen	HA clade	Kum14	KT2407	Ind07	E1212	E1510	Miy07	Ban10	Aki08	Shi10	Vie11	Yam04	Tha06	TG8913	Pyi06	SA61	Shi02	Kum14	Anh05
Kum14	2.3.4.4	**320**	<10	<10	<10	<10	<10	20	<10	<10	<10	<10	20	<10	<10	40	80	**80**	<10
Bua14	2.3.4.4	320	<10	<10	<10	<10	<10	20	<10	<10	<10	<10	20	<10	<10	40	80	80	<10
Goc14	2.3.4.4	160	<10	<10	<10	<10	<10	10	<10	<10	<10	<10	10	<10	<10	40	40	<10	<10
Anh05	2.3.4	<10	1280	640	40	40	320	20	80	80	20	640	1280	2560	2560	80	2560	<10	**320**
Hma07	2.3.4	<10	1280	80	10	40	40	<10	80	20	40	320	640	1280	1280	20	1280	<10	20
KT2407	2.3.4.2	<10	**1280**	80	10	80	20	<10	640	20	20	160	320	1280	1280	10	640	<10	10
Ind07	2.1.3	<10	1280	**1280**	10	80	40	<10	1280	80	320	1280	640	20	320	80	640	<10	20
E1212	2.2.1.1(classical‡)	<10	<10	160	**160**	<10	160	640	320	40	20	320	160	<10	40	20	320	<10	<10
E1010	2.2.1.1 (variant‡)	<10	320	80	<10	640	10	<10	160	10	<10	320	80	<10	80	<10	10	<10	<10
E1510	2.2.1.1 (variant‡)	<10	320	320	<10	**1280**	<10	<10	640	<10	<10	320	80	<10	160	<10	10	10	<10
Miy07	2.2	20	640	80	10	<10	**320**	640	80	40	<10	320	640	640	640	40	1280	<10	<10
Ban10	2.2.2	80	20	160	20	<10	640	**1280**	40	10	10	640	80	10	320	40	640	40	10
Aki08	2.3.2	<10	320	80	10	80	<10	10	**1280**	640	320	160	80	<10	320	20	160	<10	<10
Shi10	2.3.2.1	<10	40	80	20	160	<10	10	1280	**1280**	640	320	80	<10	320	20	160	<10	<10
TG1513	2.3.2.1	<10	<10	40	<10	160	<10	20	640	640	160	320	40	<10	160	20	160	<10	<10
Vie11	2.3.2.1B	40	10	40	10	20	<10	<10	640	10	**320**	160	80	20	80	10	20	<10	10
Yam04	2.5	80	320	1280	20	20	1280	2560	2560	160	320	**2560**	1280	80	640	160	1280	640	20
Tha06	1	20	640	80	<10	20	10	640	20	<10	20	160	**640**	640	640	80	640	<10	20
TG8913	1.1	40	640	40	10	20	40	<10	20	10	20	80	640	**1280**	640	40	1280	10	<10
Pyi06	7	<10	1280	160	10	40	40	<10	160	<10	80	320	640	1280	**1280**	20	1280	<10	10
SA61		<10	40	20	<10	<10	80	640	10	<10	80	160	320	320	160	**320**	1280	20	<10
Shi02	EA-nonGsGD	20	640	160	<10	<10	160	640	160	<10	80	640	1280	1280	1280	320	**5120**	80	10

HA clade nomenclature depends on the report (Influenza Other Respir Viruses 8(3):384-388) and a new clade designated 2.3.4.4 in January 2015 by WHO/OIE/FAO H5 Working Group.

†: Homologous HI titers are indicated in bold and underlined.

‡: Two antigenically divergent H5N1 Egyptian sublineages.

General Conclusion

Animal influenza viruses affect domestic animals and cause economic losses to the livestock industry (4). In addition, the pathogens bidirectionally cross the species barrier between animals and humans (4). Investigation of biological properties of isolated strains of animal influenza viruses is indispensable to understand animal disease and risks to public health, based on the concept of One Health (104, 105). In this study, three strains of SIVs and one of the HPAIVs isolated from symptomatic animals in Japan were analyzed.

All three SIVs analyzed in Part 1 were reassortant viruses between A(H1N1)pdm09 viruses and epidemic SIVs, which circulated in the Japanese pig population. Phylogenetic analysis of eight genomic segments of these reassortant viruses suggested that reassortant events that participated with A(H1N1)pdm09 viruses occurred frequently. Because (i) the reassortant SIVs were isolated from symptomatic pigs and (ii) antigenic cross-reactivities against vaccine strains of the isolated SIVs in this study were relatively low, those SIVs pose a potential risk to the livestock industry. The frequent reassortant events in pigs and emergence of reassortant SIVs possessing genomic segments of a human-origin virus in Japan are shown in this study. Because pigs and humans have historically shared influenza viruses (4), SIV monitoring is required to prevent an emerging pandemic strain from those reassortant SIVs.

A/chicken/Kumamoto/1-7/2014 (H5N8) (Kum14), which was isolated in an influenza outbreak in a poultry farm in Kumamoto prefecture, Japan, was analyzed in part 2. This virus was classified as clade 2.3.4.4 H5 HPAIVs by sequence analysis of the HA gene. The virus ancestor was thought to have been generated by a reassortant event between two HPAIVs that occurred in East Asia or related areas. All eight genomic segments of Kum14 were found to be most closely related with H5N8 HPAIVs, which were isolated from domestic and wild birds in Korea shortly before Kum14 isolation. Experimental infection indicated that ducks can be infected with a relatively low dose of Kum14 compared with chickens. In addition, Kum14 pathogenicity against chickens was relatively low compared with previous Japanese H5N1 HPAIVs, suggesting that finding of Kum14 infected chickens in the field by observation would be difficult. Additionally, serological cross-reactivities between H5N8 HPAIVs of clade 2.3.4.4 and H5 viruses of other

clades were low or not detected in the HI assay. These results suggest that migratory waterfowl could act as the carrier of Kum14 into Japan and that specific and effective virological and serological diagnostics for the HPAIVs belonging to clades 2.3.4.4 need to be prepared to control prospective outbreaks.

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Summary in Japanese (和文要旨)

動物インフルエンザウイルスは家畜・家禽に感染し、生産性の低下、対策コストの発生等を通じて家畜・家禽産業に悪影響を与える病原体である。また、潜在的に人間に感染する可能性をもち、歴史上記録のある人間のインフルエンザパンデミックの病原体は動物インフルエンザウイルスに遺伝的関連があることが明らかになっており、警戒すべき人獣共通感染症病原体でもある。本研究では日本で分離された 3 株の豚インフルエンザウイルスと 1 株の高病原性鳥インフルエンザウイルスについて遺伝系統学的及びウイルス学的解析を実施した。

2013 年、いずれも症状を示したブタから 2 株の H1N2 亜型豚インフルエンザウイルス(SIV)と 1 株の H3N2 亜型 SIV が分離された。これらの SIV は遺伝子である RNA セグメントが 2009 年に人間でパンデミックを起こした H1N1 亜型ウイルス(A(H1N1)2009 ウイルス)と日本のブタで循環している豚インフルエンザウイルス(SIV)とに由来するリアソータントウイルスであることが遺伝系統学的解析により明らかになった。すなわち、2 株の H1N2 亜型 SIV(A/swine/Gunma/1/2013 及び A/swine/Ibaraki/1/2013)は(1)HA 及び NP セグメントを日本のブタで循環する古典的 H1 亜型 SIV に、(2)NA セグメントを人 H1N2 亜型近縁 SIV に、(3)その他の RNA セグメントを A(H1N1)2009 ウイルスに由来するリアソータントウイルスであることが示された。一方、H3N2 亜型 SIV(A/swine/Miyazaki/2/2013)は(1)HA 及び NA セグメントを人季節性ウイルスに近縁な H3N2 亜型 SIV に、(2)その他の RNA セグメントを A(H1N1)2009 ウイルスに由来するリアソータントウイルスであることが示された。これら 3 株の SIV は、日本のブタにおいて、それぞれ独立して遺伝子再集合により発生したものであると考えられた。A/swine/Miyazaki/2/2013(H3N2)の人季節性ウイルスに対する血清学的反応性は、2003 年以前の株に対しては高いものの、2004 年以降の株に対してはより低かった。また H1 亜型人季節性ウイルス株に対する抗血清の幾つかは A/swine/Gunma/1/2013(H1N2)及び A/swine/Ibaraki/1/2013(H1N2)に対して反応性を示さなかった。これらの結果は本研究で解析した 3 株のウイルスのようなリアソータント SIV の出現やまん延が公衆衛生上のリスクとなることを示している。

2014 年 4 月、熊本県の養鶏場における高病原性鳥インフルエンザ発生事例において、死亡鶏のクロアカスワブより H5N8 亜型高病原性鳥インフルエンザウイルス A/chicken/Kumamoto/1-7/2014 が分離され、遺伝系統学的解析の結果、本ウイルスの HA は

クレード 2.3.4.4 に分類されることが明らかとなった。本ウイルスは、全 RNA セグメントが 2014 年 1 月に韓国において分離された A/broiler duck/Korea/Buan2/2014、A/baikal teal/Korea/Donglim3/2014 に高い配列相同性を示し、これらのウイルスと共通の祖先をもつことが強く示唆された。A/chicken/Kumamoto/1-7/2014 を経鼻接種したニワトリは 10^6EID_{50} では全羽死亡したが 10^4EID_{50} 以下では感染が認められなかった。一方でアヒルは 10^4EID_{50} 以上のウイルス投与で感染したものの、明確な症状を示さなかった。これらのことは渡りをおこなう水禽類が A/chicken/Kumamoto/1-7/2014 を日本に持ち込んだ可能性を示唆している。また、A/chicken/Kumamoto/1-7/2014 は clade 2.3.4.4 以外の H5 亜型ウイルスに対して血清学的交差性が低いことが明らかになった。現在、clade 2.3.4.4 H5 亜型高病原性鳥インフルエンザウイルスは世界の広い範囲でまん延しており、今後も日本に侵入する可能性が高い。Clade2.3.4.4 H5 亜型高病原性鳥インフルエンザウイルスを効率的に検出する診断法に利用可能な抗血清とウイルス学的検査法の整備が必要と考えられた。