Molecular, antigenic, and pathogenic characterization of H5N8 highly pathogenic avian influenza viruses isolated in the Democratic Republic of Congo in 2017

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Abstract

In May 2017, high mortality of chickens and Muscovy ducks due to the H5N8 highly pathogenic avian influenza virus (HPAIV) was reported in the Democratic Republic of Congo (DR Congo). In this study, we assessed the molecular, antigenic, and pathogenic features in poultry of the H5N8 HPAIV from the 2017 Congolese outbreaks. Phylogenetic analysis of the eight viral gene segments revealed that all 12 DR Congo isolates clustered in clade 2.3.4.4B together with other H5N8 HPAIVs isolated in Africa and Eurasia, suggesting a possible common origin of these viruses. Antigenically, a slight difference was observed between the Congolese isolates and a representative virus from group C in the same clade 2.3.4.4. After the intranasal inoculation of a representative DR Congo virus, high pathogenicity was observed in chickens and Muscovy ducks but not in Pekin ducks. Viral replication was higher in chickens than in Muscovy duck and Pekin duck organs; however, neurotropism was pronounced in Muscovy ducks. Our data confirmed the high pathogenicity of the DR Congo virus in chickens and Muscovy ducks, as observed in the field. National awareness and strengthening surveillance in the region are needed to better control HPAIVs.

Introduction

Since the detection of the H5N1 highly pathogenic avian influenza virus (HPAIV) A/goose/Guangdong/1/1996 (Gs/GD) in China in 1996, the progeny of this Gs/GD-like virus, classified in different genetic clades based on the H5 hemagglutinin (HA) gene, has spread worldwide [8, 9]. The co-circulation of this virus with other avian influenza viruses (AIVs) in poultry and wild bird populations has led to reassortment with viruses carrying different neuraminidase (NA) genes to generate H5Nx viruses [6].

In 2010, an HPAIV, reassorted with an N8 gene isolated from a mallard duck classified in clade 2.3.4.4, was reported in wild birds in China [5]. In late 2014, the descendants of this H5N8 HPAIV caused several outbreaks in Pekin ducks, chickens, geese, and wild birds in South Korea; consequent outbreaks were reported in Japan, China, and some European countries, as well as in Canada and the United States by the end of the year [7, 15, 26, 30]. During the spread of this reassortant H5N8 HPAIV of clade 2.3.4.4, two distinct groups were identified: the viruses of group icA were detected in eastern Asia and North America [30] also detected in Europe [21], and the group B viruses were detected in China, South Korea, and the Russian Federation and later found in the Middle East [12], Europe [11], and in West Africa [28]. Lastly, early in 2017, the group B viruses caused outbreaks in eastern and central African countries, including Uganda, Cameroon, and the Democratic Republic of Congo (DR Congo) [23, 32, 34, 36]. The migratory waterfowls have been pointed out to play an important role in the global spread of HPAIVs due to the long-distance seasonal movements along their migration routes; furthermore, the presence of other sedentary waterfowls have been suggested to facilitate the intra-continent dissemination of the virus [3, 10].

In reported global HPAI outbreaks caused by the H5N8 virus of clade 2.3.4.4, the mortality of naturally infected Muscovy ducks (*Cairina moschata*) was only sporadically

described; however, in our previous study of the outbreaks that occurred in the DR Congo [32], high mortality was reported in this species. Although most of the studies regarding the pathogenicity of HPAIVs have been conducted in Pekin ducks, these two duck species differ genetically; even though they have a common ancestor and share the characteristics of anseriform [2, 16]. No study has assessed the antigenic characteristics and pathogenicity in poultry of the H5N8 HPAIVs of clade 2.3.4.4B newly reported in eastern and central African countries. Therefore, this study aims to characterize the molecular, antigenicity, and pathogenicity in poultry of the H5N8 HPAIVs isolated in the DR Congo to understand their features.

Materials and Methods

Virus isolation

In our previous study, four representative H5N8 HPAIV isolates from swab samples collected from 22 birds during the outbreaks that occurred in Ituri Province, DR Congo, were genetically analyzed at the Istituto Zooprofilattico Sperimentale delle Venezie in Italy [32]. For this study, medium aliquots of all swab samples were sent to the Laboratory of Microbiology, Faculty of Veterinary Medicine at Hokkaido University in Japan for further analysis. The samples were inoculated into 10-day-old embryonated chicken eggs obtained from conventional chicken flocks tested free of avian influenza virus antibody. The harvested allantoic fluids were subjected to the hemagglutination (HA) test according to the OIE manual (https://www.oie.int/standard-setting/terrestrial-manual/access-online/). In total, eight H5N8 HPAIVs were isolated and characterized, in addition to the four isolates described in the previous study (Table 1). The viral titers of the isolates were determined as the 50% egg

83 infectious dose (EID_{50}) and calculated using the Reed and Muench method [27]. The isolates
84 were stored at −80°C until further use.
85
86 **Sequencing and molecular analysis**
87 Viral RNA was extracted from fresh allantoic fluid using the TRIzol LS reagent (Life
88 Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The extracted
89 RNA was subjected to next-generation sequencing; briefly, MiSeq libraries were prepared using
90 the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA,
91 USA) and sequenced using the MiSeq Reagent Kit v3 (600 cycles) (Illumina, San Diego, CA,
92 USA). Sequence reads were mapped to reference sequence of avian influenza virus A/mallard
93 duck/Netherlands/43/2006 (H5N2) accession number KX979501 for HA gene, and the
94 consensus sequence was rebuilt until all mismatches were solved using the CLC Genomic
95 Workbench, version 12.0 (CLC bio, Aarhus, Denmark). All viral sequences were submitted to
96 GenBank, and accession numbers are shown in Table 1.
97 For phylogenetic analysis, the nucleotide sequence datasets of eight viral gene segments
98 from representative clade 2.3.4.4 viruses, including outgroup viruses downloaded from the
99 Global Initiative on Sharing All Influenza Data and GenBank, were created and aligned. The
100 maximum likelihood was applied to construct the phylogenetic trees using the best-fit general
101 time reversible model of the nucleotides substitution with gamma-distributed rate variation
102 among sites (with 4 rate categories, Γ4) included in MEGA 7 software [20].
103 The evolutionary divergence over sequences pairs between the DR Congo viruses and
104 other related viruses of the clade 2.3.4.4B was calculated in MEGA 7 using the Kimura's method
105 [19] with 48 sequences grouped in 9 regions representing the origin of sequences including DR
106 Congo, Uganda, Cameroon, South Africa, Egypt, India, Middle East, Europe, and East Asia.

The GENETYX network version 12.0 (Genetyx Co., Tokyo, Japan) was used to assess the position of the deduced amino acid sequences in the antigenic sites of the HA protein for all the DR Congo isolates and compared to other representative viruses of clade 2.3.4.4 according to the H3 numbering as described previously [35].

Antiserum preparation and antigenic analysis

Two isolates (Mdk/CD/KAF1/17 and Mdk/CD/NYA14/17) were selected based on their differences in deduced amino acid sequences of the HA protein. The antigen was prepared as described previously [18]. For antiserum production, 500 μg of the antigen mixed with Freund's complete and Freund's incomplete adjuvant for the first and the second inoculation, respectively was injected intramuscularly into the thigh muscle of a naïve chicken twice at 14-day intervals; 14 days later, a booster with 1 ml of the antigen mixed with phosphate buffered saline was injected intravenously. Seven days after the booster, whole blood was collected from the chicken for serum preparation. The antisera, and their corresponding antigens, were included in a panel of representative clade 2.3.4.4 viruses from groups icA, C, and D. Then, cross-reactivity was assessed using the hemagglutination inhibition (HI) test (Table 2). The A/chicken/Kumamoto/1-7/2014 (H5N8) virus (Ck/Kmm/1-7/14; accession number: AB932556) was isolated from a chicken in Japan; the A/black swan/Akita/1/2016 (H5N6) virus (BS/Akita/1/16; accession number: LC198528) was isolated from a black swan in Japan. The A/duck/Vietnam/HU1-1151/2014 (H5N6) (Dk/VTN/HU1-1151/16; accession number: LC041313) virus was isolated from a Pekin duck during surveillance activity in Vietnam, and the A/peregrine falcon/Hong Kong/810/2009 (H5N1) (PF/HK/810/09; accession number: AB521159) virus was isolated from a peregrine falcon in Hong Kong. We included also in the panel the antiserum against A/mallard/Hokkaido/24/2009 (H5N1) (Mal/Hok/24/09); an unclassified low pathogenic avian

influenza virus isolated in wild birds in Japan [37]. To visualize the antigenic constellation of these viruses, the antigenic map was built using the ACMACS website (https://acmacs-web.antigeniccartography.org/). The antigenic cartography methods were applied using the cross-HI test results as described previously [29]. The distance between two antigens was calculated using ACMACS, and the difference was considered significant if the distance between two antigens was more than two antigenic units for a given combination [4].

Animal experiments

To investigate the pathogenicity of DR Congo H5N8 viruses in poultry, 0.1 ml of one representative isolate (Mdk/CD/KAF1/17) at $10^{6.0}$ EID_{50} was inoculated intranasally to eight, six-week-old chickens (*Gallus gallus domesticus*, Julia) witch were obtained from Hokkai Starchick, Hokkaido, Japan; eight, four-week-old Muscovy ducks (*Cairina moschata*) hatched in our laboratory; and eight, four-week-old Pekin ducks (*Anas platyrhynchos domesticus,* Cherry Valley) obtained from Takikawa Shinseien, Hokkaido, Japan. All bird species used for animal experiments were conventional and obtained from flocks free of antibody against AIVs by HI test before the experiment. At three days post inoculation (dpi), four birds from each experimentally infected group were sacrificed, and organ samples, including the trachea, lung, kidney, colon, pectoral muscle, and brain, were collected for virus titration. The remaining birds in each group were monitored for clinical observation until 14 dpi.

For virus titration, the collected organs were homogenized using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) to make 10% (w/v) suspensions in viral transport medium. Viral infectivity was observed as the cytopathogenic effect after inoculating viral suspensions on Madin-Darby Canine Kidney cell monolayers; viral titers were calculated as the 50% tissue culture infectious dose ($TCID_{50}$).

All animal experiments were conducted in the animal biosafety level 3 (ABSL3) laboratory at the Faculty of Veterinary Medicine, Hokkaido University, Japan.

Statistical analysis

To compare viral replication titers in chicken, Muscovy duck, and Pekin duck organs, the one-way analysis of variance Tukey test was performed using the Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM Corp, Armonk, NY, USA). The difference was considered significant when the *p* value was less than 0.05.

Results

Phylogenetic and genetic analyses

Full-length sequences of the eight gene segments of 12 DR Congo H5N8 HPAIV isolates were analyzed with other H5Nx viruses of clade 2.3.4.4. The phylogenetic tree based on the HA gene segment showed that all 12 viruses isolated in the DR Congo clustered along with the other African and Eurasian H5N8 HPAIVs in group B of clade 2.3.4.4 (Figure 1). The same pattern was observed for the NA gene and the other internal gene segments (Supplementary Figure 1, from A to G). All eight gene segments of the DR Congo isolates exhibited a close relationship with viruses from Uganda and one virus from Cameroon, toward which they showed the highest similarity (99.53% and 99.35%, respectively). The viruses isolated in Egypt, South Africa and some in Cameroon clustered separately from the DR Congo viruses, thus showing higher genetic distances; suggesting possible multiple introductions of these viruses into Africa.

The genetic distance between the DR Congo viruses and related viruses from Africa and Eurasia was estimated by evolutionary divergence over their sequences, the result revealed a

genetic distance between the Congolese viruses and those from Cameroon, South Africa and Egypt with the distance of 0.81%, 1.42% and 1.64%, respectively (Table 2).

The DR Congo isolates were aligned with other representative H5N8 HPAIV isolates of clade 2.3.4.4 group B from Africa, Asia, and Europe; the position of the deduced amino acid sequences in the HA protein was identified. All representative clade 2.3.4.4, group B viruses included in the analysis had similar sequences in the critical position of the antigenic sites (data not shown). The multibasic amino acids in the cleavage site of the HA protein of the DR Congo viruses displayed the common motif (LREKRRKR/GLF) of the HPAIV strains as observed for other clade 2.3.4.4, group B H5N8 HPAIVs [28]. No deletions or insertions were observed in the nucleotide sequence of the HA protein of the DR Congo isolates when compared to the representative viruses of the same clade 2.3.4.4 group B.

Antigenic features of the DR Congo viruses

Four representative isolates from the DR Congo and a panel of clade 2.3.4.4 H5Nx HPAIVs, groups icA, C, and D, isolated in Asia were analyzed, and their corresponding antisera were used for the cross-HI test (Table 3). The antigenic cartography (Supplementary Figure 2) derived from the cross-HI test results revealed that despite antigenic clustering of the clade 2.3.4.4 viruses, a moderate difference was noticed between the two DR Congo viruses (Mkd/CD/KAF1/17 and Mkd/CD/TCH6) and the BS/Akita/1/16 virus with three antigenic units distant. However, no difference was observed between the DR Congo viruses and the Ck/Kmm/1-7/14 or Dk/VTN/HU1-1151/16. On the other hand, the antisera raised against the Mdk/CD/KAF1/17 and Mdk/CD/NYA14/17 viruses were antigenically different from the BS/Akita/1/16 virus. This result suggested that there is a slight antigenic difference between the DR Congo viruses in group B and viruses in group C; but not with the viruses in groups icA and

D in the same genetic clade 2.3.4.4. Furthermore, a significant antigenic divergence was noticed between all viruses of clade 2.3.4.4 with the virus PF/HK/810/09 of clade 2.4.3 and the antiserum raised against the unclassified virus Mal/Hok/24/09.

Pathogenicity of DR Congo H5N8 HPAIVs in chickens, Muscovy ducks, and Pekin ducks

After intranasal inoculation with $10^{6.0}$ EID_{50} of Mdk/CD/KAF1/17, all chickens showed depression from 2 dpi and developed lethargy progressively; one chicken died at 3 dpi and three died at 4 dpi (Figure 2A). Two Muscovy ducks died suddenly at 3 dpi; two others at 3 dpi showed a lack of appetite, torticollis, dorsal decubitus, and leg pedaling before they died at 4 dpi. However, no Pekin ducks died during the observation period; only one duck showed neurological signs at 2 dpi (head shaking) as well as a lack of appetite.

Virus recovery from bird organs

High virus titers were recovered from all harvested chicken organs (trachea, lung, kidney, colon, muscle, and brain), with more efficient replication in respiratory organs including the lung and trachea ($10^{7.2}$ and $10^{5.8}$ $TCID_{50}$/g, respectively) (Figure 2B). In Muscovy ducks, the virus replicated in all tested organs, although the virus titers were lower than in chicken organs; the highest virus titers were found in the lung and brain ($10^{4.2}$ and $10^{3.4}$ $TCID_{50}$/g, respectively). In contrast, viral replication in Pekin ducks also varied among each individual, with the highest virus loads observed in the trachea, lung, kidney, and colon ($10^{2.2}$, $10^{2.1}$, $10^{2.2}$, and $10^{2.3}$ $TCID_{50}$/g, respectively); these titers were similar to those observed in Muscovy ducks. However, in the brain and muscle of Pekin ducks, the virus titers were significantly lower ($p = 0.014$) than the titers in Muscovy ducks.

Discussion

The phylogenetic analysis based on the eight viral gene segments revealed that all 12 viruses collected in the four different territories of the Ituri province were genetically similar to other isolates from Uganda and one from Cameroon, suggesting a common origin of the virus that caused outbreaks in the DR Congo, Uganda, and Cameroon. In our previous report [32], we linked the outbreaks that occurred in the DR Congo to those reported in Uganda in January 2017 given that no sequence data of the virus was available to reveal their relationship at that time; the availability of sequences of viruses from Uganda and Cameroon [23, 34] showed clearly the close relationship between these H5N8 HPAIVs, even though some Cameroon isolates showed different topology; suggesting the origin of all the viruses circulating in these regions from a common progenitor. This indicated that the DR Congo virus could have been introduced by wild waterfowl, given the location of the outbreaks in the wetlands and at the edge of the Albert Lake, areas that are suitable for hosting the wild waterfowl. The detection of the virus in wild birds in Ugandan outbreaks during the same period [23] suggests the role of wild waterfowl in the introduction and spread of the H5N8 virus in this region due to close contact between poultry raised in backyards and wild birds, especially given the low or complete absence of biosecurity in the traditional raising systems applied in these areas. The phylogenetic analysis based on the HA, NA, and internal genes of the viruses from the DR Congo showed a close relationship to those isolated from Uganda and one from Cameroon. However, the different clustering of Egyptian, South African and some Cameroon strains together with higher genetic distances from the DR Congo viruses observed in the evolutionary analysis indicate a different evolutionary pattern suggesting possible diverse introduction route of clade 2.3.4.4B H5N8 HPAIVs into the African continent. More specifically, the viruses detected in the eastern and central African regions may have reached Africa following a path different from that reached by viruses detected

in Egypt and South Africa [17, 22]. The diverse introduction of these viruses could be explained by the presence of different flyways of migratory birds reaching the African continent [25]. Nevertheless, we can't exclude the possibility of a single introduction of the virus in Africa with further spread in different countries due to intra-continental wild birds and poultry movements. However, the unavailability of data about the poultry products movement from outside the Africa limits our ability to exclude the role of poultry trade in the introduction of the viruses in certain African regions.

The low reactivity of two representative DR Congo viruses with the antisera raised against the clade 2.3.4.4, group C virus indicated a slight antigenic difference between the DR Congo viruses belonging to group B and the virus of the group C of clade 2.3.4.4. Such a small antigenic variation among viruses of different groups in the same clade 2.3.4.4 was observed in the study by Hiono et al., [14]. However, the isolates from the DR Congo used in the antigenic analysis did not show major mutations in the most relevant antigenic sites. Vaccination against AIVs has been reported to be one of the major factors that develop antigenic variants of AIVs [24] ; since the DR Congo does not apply vaccination against AIV, we can assume that this slight antigenic variation occurred naturally during the evolution of the virus. However, the distinguishable antigenic distance between the clade 2.3.4.4 viruses, the clade 2.4.3, and the unclassified virus revealed clearly the divergence genetic and antigenic evolution of these viruses leading.

Our pathogenicity assessment clearly reflected what was observed in the field during the outbreaks in the DR Congo [32] and Uganda [23], where the high mortality of Muscovy ducks (locally called ducks) and in chickens was reported. In the case of the DR Congo outbreaks, the high mortality of Muscovy ducks was linked to the high density of these ducks preferentially raised in areas by the lake [36]. In the present study, we observed viral replication in all

harvested chicken and Muscovy duck organs, reflecting the systemic infection characteristic of the HPAIV. However, in the Muscovy duck, the effective virus replication was observed in the lung and brain. In addition, neurological signs, such as head shaking, torticollis, dorsal decubitus, and leg pedaling before death, were also observed, as in the study by Anis and coauthors in hybrid duck species [1]. Taken together, the clinical signs, tissue tropism, and virus replication in Muscovy ducks infected with the H5N8 HPAIV 2.3.4.4B could explain the high mortality observed in both field and laboratory conditions, which were the same as that observed in chickens. Compared to the study conducted by Uchida et al. [33], where the Muscovy ducks were challenged with H5N6 HPAIVs belonging to clade 2.3.4.4C, only one strain caused 50% mortality at 5 dpi, while two strains did not kill the inoculated birds. In addition, the neurological signs as well as the virus titer in the brain were observed in only two of the eight birds infected. For the Pekin duck, no death was noticed during the observation period following H5N8 HPAIV infection. Our result contrasted with that observed with viruses isolated in Germany [13] and China [31], where two of 10 and one of eight Pekin ducks, respectively, died during the observation period after being inoculated with clade 2.3.4.4B H5N8 HPAIVs. Our results showed lower viral replication in Pekin duck organs than in Muscovy duck organs, and the preferential organs were the colon, kidney, and lung, not the brain as observed in the study by Sun et al., [31].

The results from our experimental study confirmed field observations regarding the pathogenicity of the DR Congo clade 2.3.4.4B H5N8 HPAIV in Muscovy ducks. However, further studies are needed to understand this phenomenon, especially whether it is related to the host factors of the Muscovy duck and/or the adaptation of the virus, given that other viruses in clade 2.3.4.4 yielded different pathogenic effects in Muscovy ducks. The need to raise awareness in Africa is critical, where most countries lack permanent, effective, and continuous monitoring

298 of AIVs in both wild birds and poultry that can help to understand the virus spread and evolution.
299 Sharing the information from such surveillance at the regional and global levels will allow for a
300 better understanding of AIVs in the region. Enhancing biosecurity in the poultry sector,
301 achieving earlier diagnoses, and culling infected birds to control the spread of infection are
302 essential to manage the landscape of HPAIVs.

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Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

Research involving Animals: The animal experiments were approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (Approval numbers 16-0105 and 18-0037 for the antiserum preparation and the pathogenicity assessment, respectively). The experiments were performed according to the guidelines of the committee. The Faculty of Veterinary Medicine, Hokkaido University, is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, which it has maintained since 2007.

Informed consent: Not Applicable

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439

Figure legends

Fig. 1 Phylogenetic tree based on the HA gene segment containing 83 sequences (1,562 nucleotides) of H5N8 HPAIVs isolated in the DR Congo were aligned together with representative H5Nx strains isolated in other African, European, and Asian countries, and in the United States of America. Bold letters represent viruses isolated in the DR Congo, black circles indicate viruses included in this study; black rhombus sign indicates the virus used in the antigenic analysis for each group in the clade 2.3.4.4 and a low pathogenic avian influenza virus in clade 2.3.4; and in gray highlight are the representative H5N8 viruses isolated in other African countries. The numbers below or above the node indicate the bootstraps values ≥ 60%.

Fig. 2 The pathogenicity of a representative DR Congo H5N8 HPAIV (Mdk/CD/KAF1/17) in poultry intranasally inoculated with 0.1 ml of $10^{6.0}$ EID_{50}. (A) The survival rates of chickens, Muscovy ducks, and Pekin ducks (four birds in each group species). (B) The virus titers in the organs of chickens, Muscovy ducks, and Pekin ducks collected at 3 dpi. Bars indicate the standard error of means. Statistical significance was calculated using the Tukey test. * *($p<0.05$)* indicates statistically significant differences of viral titers between groups. Dotted line indicates the detection limit of the test.

Supplementary data

Sup. Fig. 1 Phylogenetic trees of the NA and internal gene segments of H5Nx HPAIVs: (A) 1,444 nucleotides of the NA gene; (B) 2,329 nucleotides of the PB2 gene; (C) 2,328 nucleotides of the PB1 gene; (D) 2,218 nucleotides of the PA gene; (E) 1,551 nucleotides of the NP gene; (F) 1,013 nucleotides of the M gene; and (G) 876 nucleotides of the NS gene of H5N8 HPAIVs isolated in the DR Congo were aligned together with representative H5Nx strains isolated in other African, European, and Asian countries, and in the United States of America. The dataset contain 70 and 65 nucleotide sequences for NA and internal genes respectively. The bold letters represent viruses isolated in the DR Congo, the black circles indicate the viruses reported in this study, and in gray highlight are the representative H5N8 viruses isolated in other African countries. The numbers below or above the node indicate the bootstraps values ≥ 60%.

Sup. Fig. 2 Antigenic cartography of H5Nx HPAIVs of clades 2.3.4.4 and 2.3.4. The square and the circle indicate the antiserum and the antigen, respectively. The blue, red, green and yellow colors indicate group icA, B, C, and D viruses, respectively. The spacing between grid lines is one antigenic unit of distance, which equals a two-fold difference in the cross-HI test. The dashed circle in blue and pink represents the genetic clades 2.3.4.4 and 2.3.4, respectively.

Table 1. H5N8 highly pathogenic avian influenza viruses isolated in the Democratic Republic of Congo in 2017

Isolate	Isolate abbreviation	Sampling date	Sampling site		Accession number
			Latitude	Longitude	
A/Muscovy duck/DR Congo/KAF1/2017	Mdk/CD/KAF1/17	13 May	1.559	30.559	MK631786 to MK631793
A/Muscovy duck/DR Congo/KAF4/2017	Mdk/CD/KAF4/17	13 May	1.559	30.559	MK636723 to MK636730
A/Muscovy duck/DR Congo/17RS882-40/2017*	Mdk/CD/17S882-40/17	13 May	1.559	30.559	MG607403, MG607407, MG607411, MG607415, MG607419, MG607423, MG607427, MG607431
A/Muscovy duck/DR Congo/NYA4/2017	Mdk/CD/NYA4/17	14 May	1.532	30.527	MK636731 to MK636738
A/Muscovy duck/DR Congo/NYA14/2017	Mdk/CD/NYA14/17	14 May	1.532	30.527	MK636739 to MK636746
A/Muscovy duck/DR Congo/NYA15/2017	Mdk/CD/NYA15/17	14 May	1.532	30.527	MK636747 to MK636754
A/Muscovy duck/DR Congo/17RS882-33/2017*	Mdk/CD/17S882-33	14 May	1.532	30.527	MG607402, MG607406, MG607410, MG607414, MG607418, MG607422, MG607426, MG607430
A/Muscovy duck/DR Congo/TCH4/2017	Mdk/CD/TCH4/17	14 May	1.455	30.485	MK636755 to MK636762
A/Muscovy duck/DR Congo/TCH5/2017	Mdk/CD/TCH5/17	14 May	1.455	30.485	MK636763 to MK636770
A/Muscovy duck/DR Congo/TCH6/2017	Mdk/CD/TCH6/17	14 May	1.455	30.485	MK636771 to MK636778
A/Muscovy duck/DR Congo/17RS882-5/2017*	Mdk/CD/17S882-5/17	14 May	1.455	30.485	MG607404, MG607408, MG607412, MG607416, MG607420, MG607424, MG607428, MG607432
A/Muscovy duck/DR Congo/17RS882-29/2017*	Mdk/CD/17S882-29/17	15 May	1.599	30.606	MG607401, MG607404, MG607409, MG607413, MG607417, MG607421, MG607425, MG607429

*indicates a virus published in our previous paper [26]. For the eight newly studied viruses, the accession numbers are given in the following gene order: PB2, PB1, PA, HA, NP, NA, M, and NS.

Table 2. Estimates of evolutionary divergence over sequence pairs between the DR Congo viruses and related representative viruses from clade 2.3.4.4B

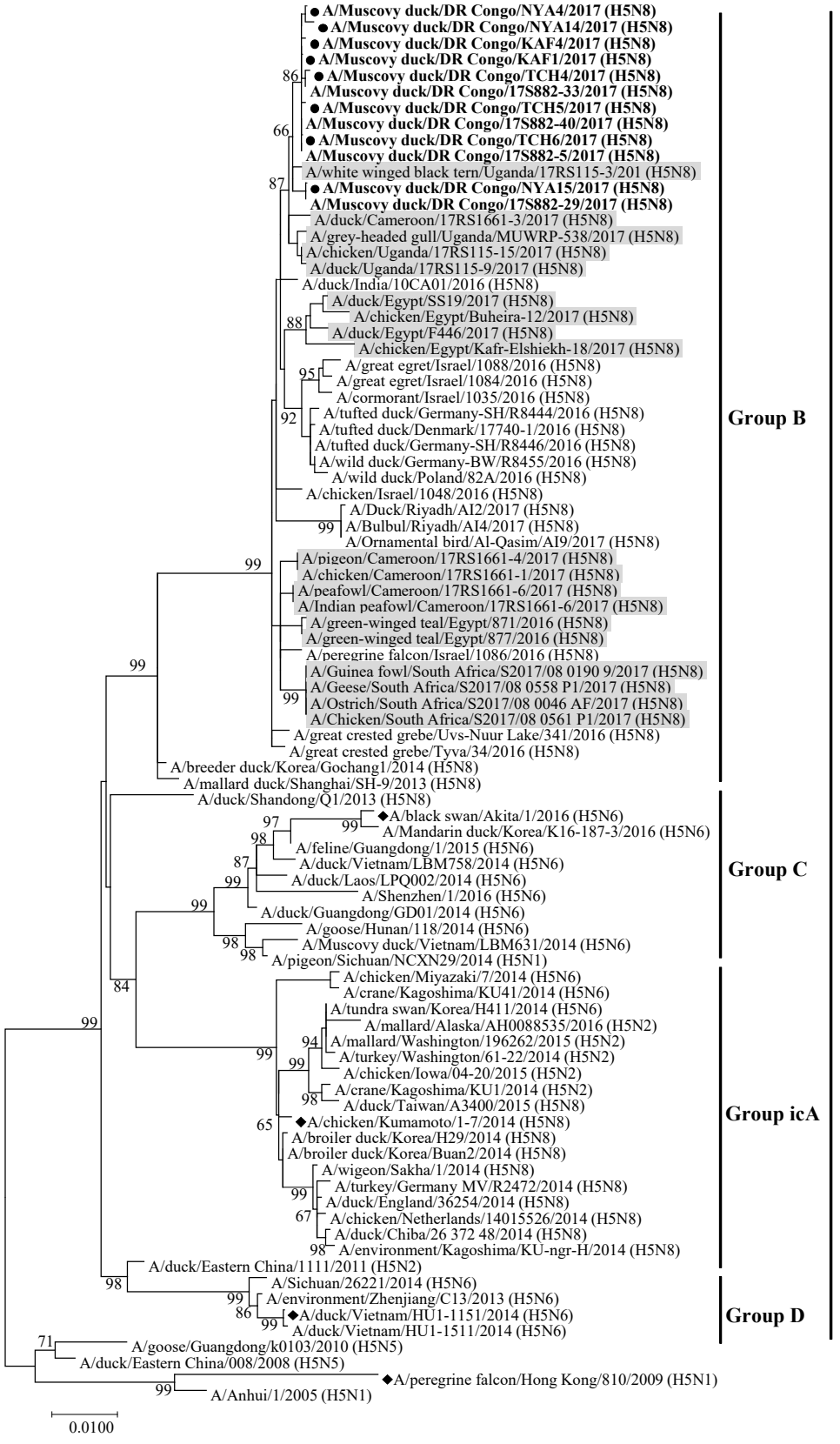
Origin of sequences	No. of sequences included	DRC	Ugd	Cmr	SA	Egy	Ind	ME	EU	EA
DR Congo (DRC)	12		*0.12*	*0.19*	*0.23*	*0.20*	*0.19*	*0.20*	*0.24*	*0.20*
Uganda (Ugd)	4	0.45		*0.17*	*0.23*	*0.20*	*0.17*	*0.18*	*0.23*	*0.19*
Cameroon (Cmr)	4	0.81	0.79		*0.18*	*0.17*	*0.19*	*0.18*	*0.24*	*0.17*
South Africa (SA)	5	1.42	1.01	0.71		*0.22*	*0.22*	*0.22*	*0.28*	*0.21*
Egypt (Egy)	7	1.64	1.59	1.51	1.71		*0.17*	*0.19*	*0.22*	*0.19*
Indian (Ind)	1	0.64	0.61	0.74	0.78	1.48		*0.19*	*0.23*	*0.18*
Middle East (ME)	7	1.22	1.17	1.22	1.19	1.97	1.09		*0.20*	*0.19*
Europe (EU)	4	1.01	0.99	1.02	1.26	1.67	0.88	1.18		*0.24*
East Asia (EA)	4	1.36	1.33	1.23	1.32	1.81	1.17	1.58	1.44	

The numbers of base substitutions per site from averaging over all sequence pairs between groups are shown. Standard error estimate(s) are shown in italic above the diagonal and were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Kimura 2-parameter model [20]. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 48 nucleotide sequences. Codon positions included were 1st+2nd+3rd. All positions containing gaps and missing data were eliminated. There were a total of 1562 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [21].

Table 3. Antigenic analysis of clade 2.3.4.4, group icA, B, C, and D H5Nx viruses.

Clade	Group	Virus	Subtype	Antisera						
				Mdk/CD KAF1/17	Mdk/CD NYA14/17	Bs/Aki/ 1/16	Dk/VN/ 1151/14	Ck/Kum/ 1-7/14	PF/HK/ 810/09	Mal/Hok/ 24/09
2.3.4.4	B	**Mdk/CD/KAF1/17**	H5N8	2,560	2,560	80	640	640	640	40
2.3.4.4	B	**Mdk/CD/NYA14/17**	H5N8	2,560	5,120	640	2,560	1,280	640	80
2.3.4.4	B	**Mdk/CD/TCH6/17**	H5N8	2,560	2,560	80	640	640	640	40
2.3.4.4	B	**Mdk/CD/17S882-29/17**	H5N8	2,560	2,560	320	640	640	640	40
2.3.4.4	C	BS/Akita/1/16	H5N6	640	640	1,280	640	640	160	40
2.3.4.4	D	Dk/VTN/HU1-1151/14	H5N6	1,280	2,560	2,560	2,560	1,280	1,280	80
2.3.4.4	icA	Ck/Kmm/1-7/14	H5N8	640	1,280	640	640	1.280	640	40
2.3.4	-	PF/HK/810/09	H5N1	320	80	40	160	<20	5,120	20

Bold font indicates representative viruses isolated in the DR Congo. Underlined numbers indicate homologous titers for each virus and the corresponding antiserum.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● A/Muscovy duck/DR Congo/KAF4/2017 (H5N8)
● A/Muscovy duck/DR Congo/KAF1/2017 (H5N8)
● A/Muscovy duck/DR Congo/TCH4/2017 (H5N8)
A/Muscovy duck/DR Congo/17S882-33/2017 (H5N8)
● A/Muscovy duck/DR Congo/TCH5/2017 (H5N8)
A/Muscovy duck/DR Congo/17S882-40/2017 (H5N8)
● A/Muscovy duck/DR Congo/TCH6/2017 (H5N8)
A/Muscovy duck/DR Congo/17S882-5/2017 (H5N8)
A/white winged black tern/Uganda/17RS115-3/201 (H5N8)
● A/Muscovy duck/DR Congo/NYA15/2017 (H5N8)
A/Muscovy duck/DR Congo/17S882-29/2017 (H5N8)
A/duck/Cameroon/17RS1661-3/2017 (H5N8)
A/grey-headed gull/Uganda/MUWRP-538/2017 (H5N8)
A/chicken/Uganda/17RS115-15/2017 (H5N8)
A/duck/Uganda/17RS115-9/2017 (H5N8)
A/duck/India/10CA01/2016 (H5N8)
A/duck/Egypt/SS19/2017 (H5N8)
A/chicken/Egypt/Buheira-12/2017 (H5N8)
A/duck/Egypt/F446/2017 (H5N8)
A/chicken/Egypt/Kafr-Elshiekh-18/2017 (H5N8)
A/great egret/Israel/1088/2016 (H5N8)
A/great egret/Israel/1084/2016 (H5N8)
A/cormorant/Israel/1035/2016 (H5N8)
A/tufted duck/Germany-SH/R8444/2016 (H5N8)
A/tufted duck/Denmark/17740-1/2016 (H5N8)
A/tufted duck/Germany-SH/R8446/2016 (H5N8)
A/wild duck/Germany-BW/R8455/2016 (H5N8)
A/wild duck/Poland/82A/2016 (H5N8)
A/chicken/Israel/1048/2016 (H5N8)
A/Duck/Riyadh/AI2/2017 (H5N8)
A/Bulbul/Riyadh/AI4/2017 (H5N8)
A/Ornamental bird/Al-Qasim/AI9/2017 (H5N8)
A/pigeon/Cameroon/17RS1661-4/2017 (H5N8)
A/chicken/Cameroon/17RS1661-1/2017 (H5N8)
A/peafowl/Cameroon/17RS1661-6/2017 (H5N8)
A/Indian peafowl/Cameroon/17RS1661-6/2017 (H5N8)
A/green-winged teal/Egypt/871/2016 (H5N8)
A/green-winged teal/Egypt/877/2016 (H5N8)
A/peregrine falcon/Israel/1086/2016 (H5N8)
A/Guinea fowl/South Africa/S2017/08 0190 9/2017 (H5N8)
A/Geese/South Africa/S2017/08 0558 P1/2017 (H5N8)
A/Ostrich/South Africa/S2017/08 0046 AF/2017 (H5N8)
A/Chicken/South Africa/S2017/08 0561 P1/2017 (H5N8)
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A/duck/Shandong/Q1/2013 (H5N8)
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A/Mandarin duck/Korea/K16-187-3/2016 (H5N6)
A/feline/Guangdong/1/2015 (H5N6)
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A/Shenzhen/1/2016 (H5N6)
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A/goose/Hunan/118/2014 (H5N6)
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A/pigeon/Sichuan/NCXN29/2014 (H5N1)
A/chicken/Miyazaki/7/2014 (H5N6)
A/crane/Kagoshima/KU41/2014 (H5N6)
A/tundra swan/Korea/H411/2014 (H5N6)
A/mallard/Alaska/AH0088535/2016 (H5N2)
A/mallard/Washington/196262/2015 (H5N2)
A/turkey/Washington/61-22/2014 (H5N2)
A/chicken/Iowa/04-20/2015 (H5N2)
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A/duck/Taiwan/A3400/2015 (H5N8)
◆A/chicken/Kumamoto/1-7/2014 (H5N8)
A/broiler duck/Korea/H29/2014 (H5N8)
A/broiler duck/Korea/Buan2/2014 (H5N8)
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A/turkey/Germany MV/R2472/2014 (H5N8)
A/duck/England/36254/2014 (H5N8)
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A/Sichuan/26221/2014 (H5N6)
A/environment/Zhenjiang/C13/2013 (H5N6)
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A/duck/Vietnam/HU1-1511/2014 (H5N6)
A/goose/Guangdong/k0103/2010 (H5N5)
A/duck/Eastern China/008/2008 (H5N5)
◆A/peregrine falcon/Hong Kong/810/2009 (H5N1)
A/Anhui/1/2005 (H5N1)
0.0100
Group B
Group C
Group icA
Group D
Clade 2.3.4.4

Figure 1.

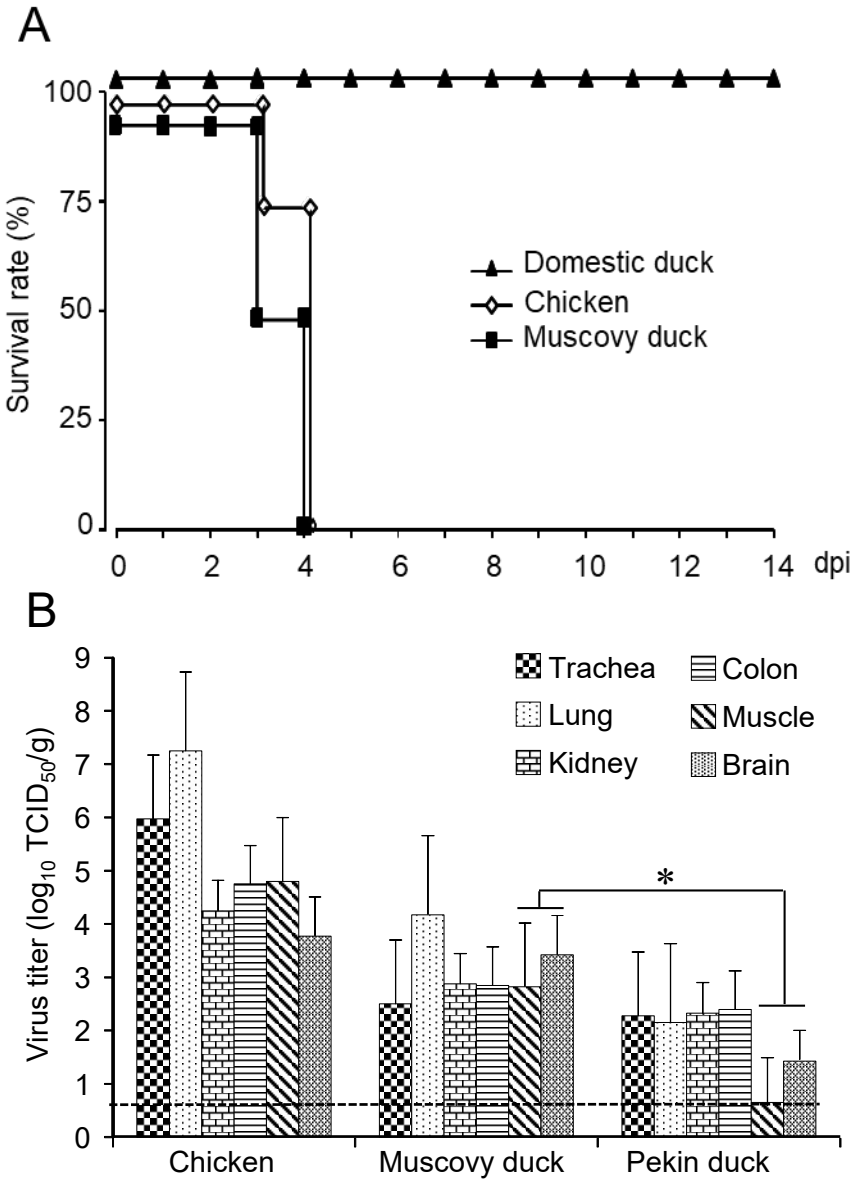
A
100
75
50
25
0
Survival rate (%)
0
2
4
6
8
10
12
14
dpi
Domestic duck
Chicken
Muscovy duck
B
9
8
7
6
5
4
3
2
1
0
Virus titer (log10 TCID50/g)
Trachea
Colon
Lung
Muscle
Kidney
Brain
*
Chicken
Muscovy duck
Pekin duck

Figure 2