HOKKAIDO UNIVERSITY

Title	Studies on the control of avian influenza and Newcastle disease
Author(s)	Augustin, Tshibwabwa TWABELA
Degree Grantor	北海道大学
Degree Name	博士(獣医学)
Dissertation Number	甲第14623号
Issue Date	2021-06-30
DOI	https://doi.org/10.14943/doctoral.k14623
Doc URL	https://hdl.handle.net/2115/82523
Type	doctoral thesis
File Information	Augustin_TWABELA.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Studies on the control of avian influenza and Newcastle disease

(鳥インフルエンザおよびニューカッスル病の制御に関する研究)

Augustin Tshibwabwa Twabela

Table of Contents

Table of Contents i

Table of figures and tables iii

Abbreviations v

Notes viii

Preface ix

Chapter I 1

Molecular, antigenic, and pathogenic characterization of H5N8 high pathogenicity avian influenza viruses isolated in the Democratic Republic of the Congo in 2017 1

Introduction 2

Materials and Methods 4

Results 12

Discussion 27

Brief summary 31

Chapter II 32

A new variant among Newcastle disease viruses isolated in the Democratic Republic of the Congo in 2018 and 2019 32

Introduction 33

Materials and Methods 35

Results 43

Discussion 52

Brief summary 55

Chapter III 56

Effect of baloxavir marboxil and peramivir for the treatment of high pathogenicity avian influenza in chickens 56

Introduction 57

Materials and Methods 59

Results 64

Discussion 74

Brief summary 78

Conclusion 79

Acknowledgements 81

References 84

Table of figures and tables

Figure 1. Phylogenetic tree based on the HA gene segment containing 83 sequences (1,562 nucleotides) of H5N8 HPAIVs isolated in the DRC. 13

Figure 2. Phylogenetic tree based on the NA gene of H5N8 HPAIVs. 14

Figure 3. Phylogenetic tree based on the PB2 gene of H5N8 HPAIVs. 15

Figure 4. Phylogenetic tree based on the PB1 gene of H5N8 HPAIVs. 16

Figure 5. Phylogenetic tree based on the PA gene of H5N8 HPAIVs. 17

Figure 6. Phylogenetic tree based on the NP gene of H5N8 HPAIVs. 18

Figure 7. Phylogenetic tree based on the M gene of H5N8 HPAIVs. 19

Figure 8. Phylogenetic tree based on the NS gene of H5N8 HPAIVs. 20

Figure 9. Antigenic cartography of H5Nx HPAIVs of clades 2.3.4.4 and 2.3.4. 24

Figure 10. The pathogenicity of a representative H5N8 HPAIV (Mdk/CD/KAF1/17) in poultry intranasally inoculated with 0.1 mL of $10^{6.0}$ EID_{50}. 26

Figure 11. The map of the DRC divided in provinces. The blue shaded region indicates the sampling sites. 36

Figure 12. Phylogenetic tree of class II NDV based on the full-length nucleotide sequence of the F gene containing 133 sequences including the DRC strains. 44

Figure 13. The phylogenetic tree based on the whole-genome sequence of NDV class II. 45

Figure 14. The survival of chickens infected with the DRC field strain NDV/Ck/DRC/PL219/2019. 51

Figure 15. Effects of the simultaneous treatment using multiple doses of BXM or PR in chickens infected with an HPAIV, BS/Akita/1/16. 65

Figure 16. Effects of delayed treatment with multiple-dose administration of BXM or PR in chickens infected with HPAIV, BS/Akita/1/16. 67

Figure 17. Effects of the simultaneous treatment with a single dose of BXM in chickens infected with HPAIV, BS/Akita/1/16. 69

Figure 18. Drug concentration in the blood of chickens and Pekin ducks..... 71

Table 1. H5N8 HPAIV isolated in the DRC in 2017 5

Table 2. Antigenic analysis of clade 2.3.4.4 group icA, B, C, and D H5Nx viruses 9

Table 3. Estimates of evolutionary divergence over sequence pairs between the DRC viruses and related representative viruses from clade 2.3.4.4b..... 22

Table 4. Characteristics of the NDV isolated in the DRC in 2018 and 2019..... 37

Table 5. Estimates of evolutionary divergence over sequence pairs among genotypes of class II NDVs. 47

Table 6. The antigenic relatedness between the DRC isolates and vaccine strains. 49

Table 7. Virus recovery in tracheal and cloacal swabs and antibody response in the serum of the chickens treated with different doses of BXM at single administration..... 73

Abbreviations

AA	amino acid
AAALAC	Association for Assessment Accreditation of Laboratory Animal Care
AI	avian influenza
AIV(s)	avian influenza virus(es)
ABSL-3	animal biosecurity level 3
AMED	Japan Agency for Medical Research and Development
APMV-1	avian paramyxovirus type 1
BLAST	basic local alignment search tool
BXA	baloxavir acid
BXM	baloxavir marboxil
CPE	cytopathogenic effect
dpi	day post inoculation
DRC	Democratic Republic of the Congo
EID_{50}	50% egg infectious dose
F	fusion
FCS	fetal calf serum
g	gram
HA	hemagglutinin
HAt	hemagglutination test
HI	hemagglutination inhibition test
HN	hemagglutinin-neuraminidase
hpa	hour post administration
HPAI	high pathogenicity avian influenza
HPAIV(s)	high pathogenicity avian influenza virus(es)
ICPI	intracerebral pathogenicity index
GTR	general time reversible
J-GRID	Japan Initiative for Global Research Network on Infectious Diseases
JICA	Japan International Cooperation Agency
kg	kilogram

L	large
M	matrix
ML	maximum likelihood
MLD_{50}	50% mean lethal dose
MDT	mean death time
MDCK	Madin-Darby Canine Kidney
MEM	minimum eagle medium
mg	milligram
mL	milliliter
µg	microgram
µM	micromolar
nd	not determined
ng	nanogram
NA	neuraminidase
ND	Newcastle disease
NDV(s)	Newcastle disease virus(es)
NP	nucleoprotein
NS	nonstructural
OIE	World Organization for Animal Health
PBS	phosphate buffered saline
P	phosphoprotein
PA	polymerase acid
PB1	polymerase basic 1
PB2	polymerase basic 2
PR	peramivir
RNA	ribonucleic acid
SATREPS	Science and Technology Research Partnership for Sustainable Development
SPSS	statistic package for social science
$TCID_{50}$	50% tissue culture infectious dose
VTM	viral transport medium

Notes

This thesis contains three articles published as follows

1. **Twabela A.,** Okamatsu M., Tshilenge G., Mpiana S., Masumu J., Nguyen L.T., Matsuno K., Monne I., Zecchin B., Sakoda Y. Molecular, antigenic, and pathogenic characterization of H5N8 highly pathogenic avian influenza viruses isolated in the Democratic Republic of Congo in 2017. **Arch. Virol. 2020, 165, 87–96. doi: 10.1007/s00705-019-04456-x**

   Copyright © 2017 Archives of Virology published by Springer-Verlag GmbH Austria, part of Springer Nature.

2. **Twabela A.,** Nguyen L.T., Masumu J., Mpoyo P., Mpiana S., Sumbu J., Okamatsu M., Matsuno K., Isoda N., Zecchin B., Monne I., Sakoda Y. A new variant among Newcastle disease viruses isolated in the Democratic Republic of the Congo in 2018 and 2019. **Viruses. 2021, 13, 151. doi: 10.3390/v13020151.**

   Copyright © 2021 Viruses published by MDPI.

3. **Twabela A.,** Okamatsu M., Matsuno K., Isoda N., Sakoda Y. Evaluation of baloxavir marboxil and peramivir for the treatment of high pathogenicity avian influenza in chickens. **Viruses. 2020, 12, 1407. doi: 10.3390/v12121407**

   Copyright © 2020 Viruses published by MDPI.

Preface

In the last 10 decades, the global human population is constantly increasing; leading to an extensive livestock production to satisfy the daily consumption and the protein intake. This demand has promoted the extensive livestock farming worldwide for meat production and related goods. Poultry is one of the animal categories most widely exploited due to the short production cycle and the small space required for raising a large number of population. For this reason, the poultry farming systems have been developed in both advanced and developing countries using different level of biosecurity concerning the environmental interaction. Low biosecurity in the poultry farms usually leads to the introduction and spread of the pathogens in high dense populations, and affect negatively the productivity.

Among the infectious diseases that affect poultry, avian influenza (AI) and Newcastle disease (ND) are known to cause severe damage to the poultry industry. Therefore, preventive measures such as improvement of biosecurity and effective prophylaxis can minimize the occurrence of these diseases. However, in most cases, especially in the traditional and small-scale poultry farming systems, these measures are rarely applied. Besides the poultry population, wild birds play an important role as reservoirs and carriers of the AI and ND viruses, being causative agents of the diseases [1,2]. These wild birds are also susceptible to some AI virus (AIV) and Newcastle disease virus (NDV) strains. Considering this fact, it is clear that all avian species are at risk for the infection and need to be protected.

Apart from the damage caused by the AI and ND to the poultry industry and wild bird populations, many AIV strains have shown their zoonotic potential acquired by mutation and interspecies adaptation, threatening on the public health [3-6]. For the preparedness for any eventuality and to prevent the occurrence of such events, it is recommended to implement an effective surveillance for the detection and characterization of circulating viral strains to identify possible mutations that can lead to the virulence change, cross-species transmission, and pandemic in humans.

In the Democratic Republic of the Congo (DRC), the poultry industry either in the industrial or traditional system is important for food safety and economy. The increase of poultry farms with a low biosecurity system is critical in terms of AIV and NDV infection. Additionally, in the rural areas, farmers keep their poultry close to the human habitats resulting in a permanent contact between humans and poultry enhancing the potential risk of zoonotic infection by the AIVs, even though no case of such infection has been reported in the DRC. The first case of high pathogenicity AI (HPAI) in the DRC was detected in backyard farms [7], this confirms the vulnerability of these farming systems that expose birds to the infection. One year after the first HPAI outbreak, an outbreak of ND was reported in backyard farms, with high mortality in chickens. These situations required establishing an effective surveillance for both AI and ND to prevent further incursion and spread of the viruses in the poultry populations, and to monitor the zoonotic potential of AIVs.

The stamping out of infected birds with HPAI viruses (HPAIVs) is highly recommended to reduce the spread of the AIV in case of an outbreak [8]; whereas the active surveillance and the biosecurity enhancement in the farms are the standard

measures. However, the therapeutic options should be envisaged for the treatment of some bird species that might escape from the traditional control measures. The valuable captive wild bird populations might be subjected to the treatment when infected with HPAIVs. Therefore, effective and appropriate antiviral drugs need to be evaluated for this purpose.

Regarding the above situations, the present study aimed to provide detailed traits of AIV and NDV isolated in the DRC, and to evaluate the antiviral drugs that can be used for the treatment of HPAIV infection in captive wild birds. Under this concept, this thesis covers three topics divided into three chapters. Chapter I is related to the characterization of the H5N8 HPAIVs isolated in the DRC in 2017; Chapter II focuses on the NDVs isolated in the DRC in 2018 and 2019. Considering the importance of valuable captive birds that can be killed by HPAIV or subjected to the stamping out due to the infection, Chapter III evaluates the antiviral drugs that can be used for the treatment of captive wild birds for the conservation of the breed.

Chapter I

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

Introduction

In 1996, the H5N1 HPAIV A/goose/Guangdong/1/1996 (Gs/GD) emerged in China. The descendants of this Gs/GD-like virus, classified in different genetic clades based on the H5 HA gene, spread globally [9,10]. The co-circulation of this virus with other AIVs in poultry and wild bird populations has led to reassortment with viruses carrying different neuraminidase (NA) genes to generate H5Nx viruses [11].

In 2010, a reassortant of an HPAIV with an N8 gene isolated from a mallard duck classified in the major clade 2.3.4.4, was reported in wild birds in China [12]. In late 2014, the progeny 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 [13-16]. During the evolution of this reassortant H5N8 HPAIV of major clade 2.3.4.4, two distinct clades were identified: the viruses of clade 2.3.4.4c were detected in eastern Asia and North America [14] also detected in Europe [17], and the clade 2.3.4.4b viruses were detected in China, South Korea, and the Russian Federation and later found in the Middle East [18], Europe [19], and in West Africa [20]. Lastly, early in 2017, the clade 2.3.4.4b viruses caused outbreaks in eastern and central African countries, including Uganda, Cameroon, and the DRC [7,21-23]. 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 [24,25].

In reported global HPAI outbreaks caused by the H5N8 virus of major clade 2.3.4.4, the mortality of naturally infected Muscovy ducks (*Cairina moschata*) was only sporadically described; however, in the previous study of the outbreaks that occurred in the DRC [7], 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 [26,27]. Furthermore, 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 DRC to understand their features.

Materials and Methods

Virus isolation

In the previous study, four representative H5N8 HPAIV isolates from swab samples collected from 22 birds in Ituri Province, DRC, were genetically analyzed at the Istituto Zooprofilattico Sperimentale delle Venezie in Italy [7]. For this study, medium aliquots of all swab samples were sent to the Laboratory of Microbiology, Faculty of Veterinary Medicine, 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 AIV antibody. The harvested allantoic fluids were subjected to the hemagglutination test (HAt) according to the World Organization for Animal Health (OIE) manual [28]. 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 infectious dose (EID_{50}) and calculated using the Reed and Muench method [29]. The isolates were stored at −80°C until further use.

Table 1. H5N8 HPAIV isolated in the DRC 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 previously [7]. 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.

Sequencing and molecular analysis

Viral RNA was extracted from fresh infectious allantoic fluid using the TRIzol LS reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The extracted RNA was subjected to next-generation sequencing. Briefly, MiSeq libraries were prepared using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) and sequenced using the MiSeq Reagent Kit v3 (600 cycles) (Illumina, San Diego, CA, USA). Sequence reads were mapped to reference sequence of AIV A/mallard duck/Netherlands/43/2006 (H5N2) accession number KX979501 for HA gene, and the consensus sequence was rebuilt until all mismatches were solved using the CLC Genomic Workbench, version 12.0 (CLC bio, Aarhus, Denmark). All viral sequences were submitted to GenBank, and accession numbers are provided in Table 1.

For phylogenetic analysis, the nucleotide sequence datasets of eight viral gene segments from representative major clade 2.3.4.4 viruses, including outgroup viruses downloaded from the Global Initiative on Sharing All Influenza Data and GenBank, were created and aligned. The maximum likelihood (ML) was applied to construct the phylogenetic trees using the best-fit general time reversible (GTR) model of the nucleotides substitution with gamma-distributed rate variation among sites (with 4 rate categories, Γ4) included in MEGA 7 software [30].

The evolutionary divergence over sequences pairs between the DRC viruses and other related viruses of the clade 2.3.4.4b was calculated in MEGA 7 using the Kimura's method [31] with 48 sequences grouped in 9 regions representing the origin of sequences

including the DRC, 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 (AA) sequences in the antigenic sites of the HA protein for all the DRC isolates and compared to other representative viruses of major clade 2.3.4.4 according to the H3 numbering as described previously [32].

Antiserum preparation and antigenic analysis

Two isolates A/Muscovy duck/DR Congo/KAF1/2017 (Mdk/CD/KAF1/17) and A/Muscovy duck /DR Congo/NYA14/2017 (Mdk/CD/NYA14/17) were selected based on their differences in deduced AA sequences of the HA protein. The antigen was prepared as described previously [33]. 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 (PBS) 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 viruses from clade 2.3.4.4a, c, and e. 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 [34]; 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 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. In addition, the serum against A/mallard/Hokkaido/24/2009 (H5N1) (Mal/Hok/2009) an unclassified low pathogenicity avian influenza isolated in wild birds in japan [37] was included in the in the panel. 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 titers as described previously [35]. 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 [36].

Table 2. Antigenic analysis of clade 2.3.4.4 a, b, c, and e H5Nx viruses

Clade	Group	Virus	Subtype	Antisera						
				Mdk/CD KAF1/17	Mdk/CD NYA14/17	Bs/Aki/ 1/16	Dk/VN/ 1151/14	Ck/Kmm/ 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	e	BS/Akita/1/16	H5N6	640	640	1,280	640	640	160	40
2.3.4.4	a	Dk/VTN/HU1-1151/14	H5N6	1,280	2,560	2,560	2,560	1,280	1,280	80
2.3.4.4	c	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 DRC. Underlined numbers indicate homologous titers for each virus and the corresponding antiserum.

Animal experiments

To investigate the pathogenicity of the DRC 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 the Laboratory of Microbiology, Faculty of Veterinary Medicine, Hokkaido University, Japan; 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 (CPE) after inoculating viral suspensions on Madin-Darby Canine Kidney (MDCK) cell monolayers; viral titers were calculated as the 50% tissue culture infectious dose ($TCID_{50}$).

Ethical statement

All animal experiments were performed in the animal biosafety level 3 (ABSL3) facility at the Faculty of Veterinary Medicine of Hokkaido University, Sapporo, Japan; approval number 16-0105 and 18-0037 for the antiserum preparation and the pathogenicity assessment, respectively. The experiments were conducted under the guidelines of the Institutional Animal and Committee of Hokkaido University, certified by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) since 2007.

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 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 DRC 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 DRC clustered along with the other African and Eurasian H5N8 HPAIVs in major clade 2.3.4.4b (Figure 1). The same pattern was observed for the NA gene and the other internal gene segments (Figure 2 to 8). All eight gene segments of the DRC 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) when analyzed in the basic local alignment search toll (BLAST). The viruses isolated in Egypt, South Africa and some in Cameroon clustered separately from the DRC viruses, thus showing higher genetic distances; suggesting possible multiple introductions of these viruses into Africa

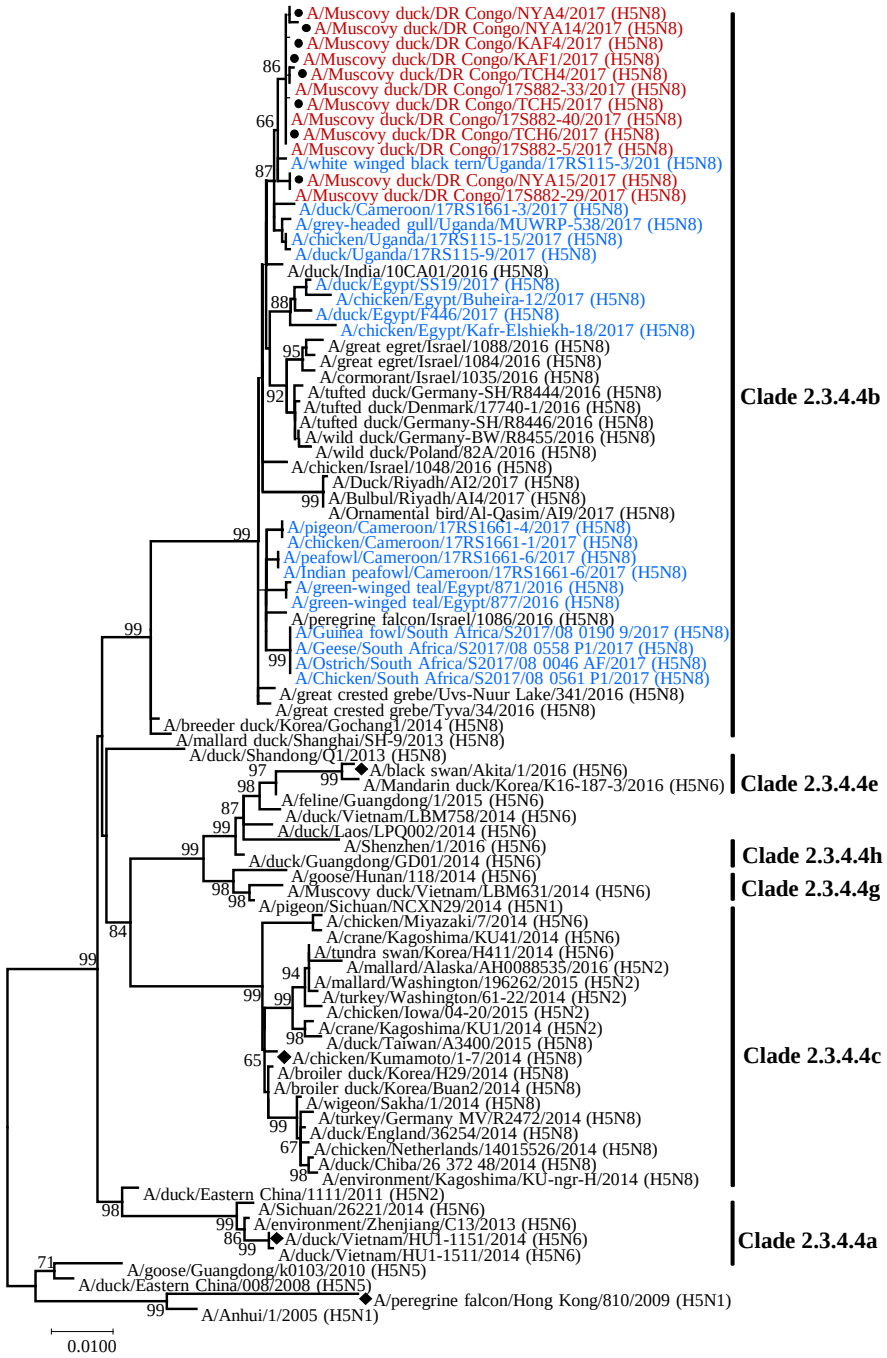

Figure 1. Phylogenetic tree based on the HA gene segment containing 83 sequences (1,562 nucleotides) of H5N8 HPAIVs isolated in the DRC.

The sequences were aligned together with representative H5Nx strains isolated in other African, European, and Asian countries, and in the United States of America. Red color represents viruses isolated in the DRC, black circles indicate viruses included in this study; black rhombus sign indicates the virus used in the antigenic analysis for clade 2.3.4.4a, c, e and a low pathogenicity avian influenza virus in clade 2.3.4; and blue color are the representative H5N8 viruses isolated in other African countries. The numbers below or above the node indicate the bootstraps values ≥ 60%.

Figure 2. Phylogenetic tree based on the NA gene of H5N8 HPAIVs.
The dataset contains 70 nucleotide sequences (1,444 nucleotides in length). The red color indicates viruses isolated in the DRC, the black circles indicate the viruses reported in this study, and blue color are the representative H5N8 viruses isolated in other African countries. The numbers below or above the node indicate the bootstraps values ≥ 60%.

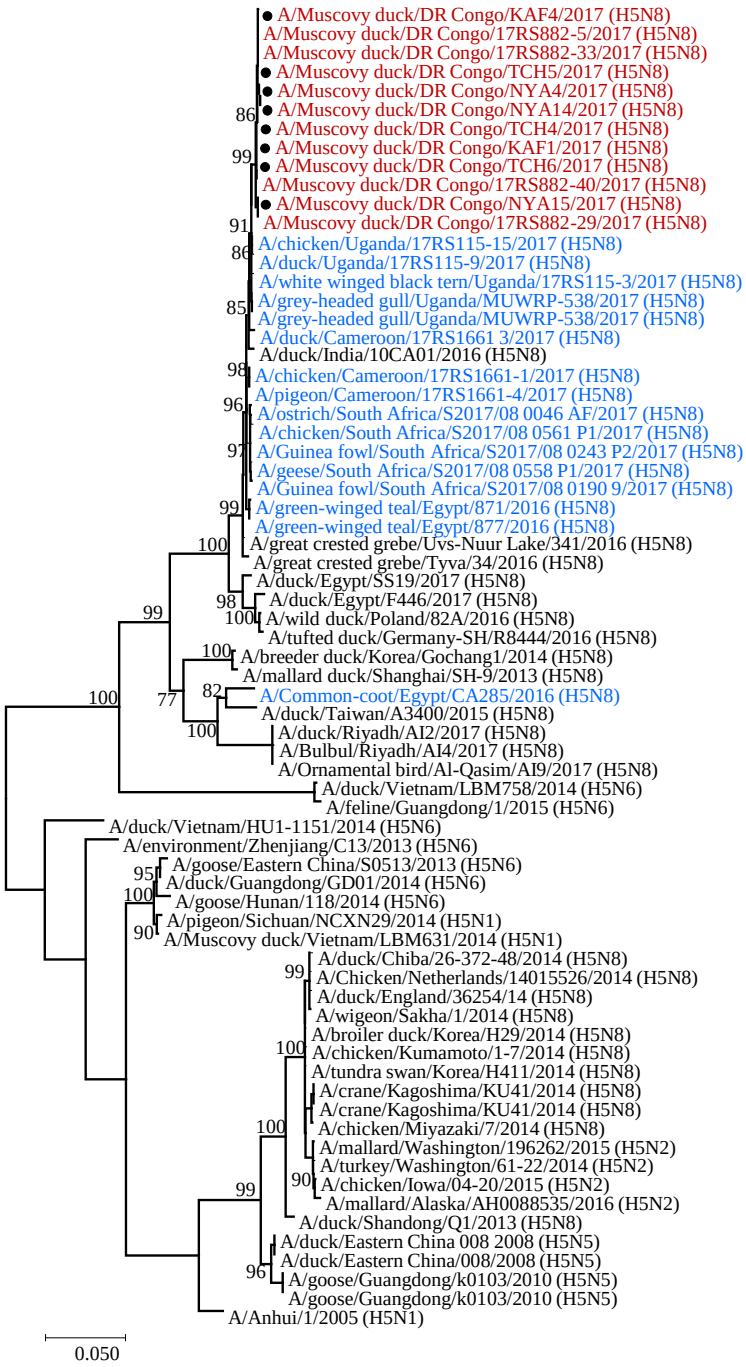

Figure 3. Phylogenetic tree based on the PB2 gene of H5N8 HPAIVs.

The dataset contains 68 nucleotide sequences (2,329 nucleotides in length). The red color indicates viruses isolated in the DRC, the black circles indicate the viruses reported in this study, and blue color are the representative H5N8 viruses isolated in other African countries. The numbers below or above the node indicate the bootstraps values ≥ 60%.

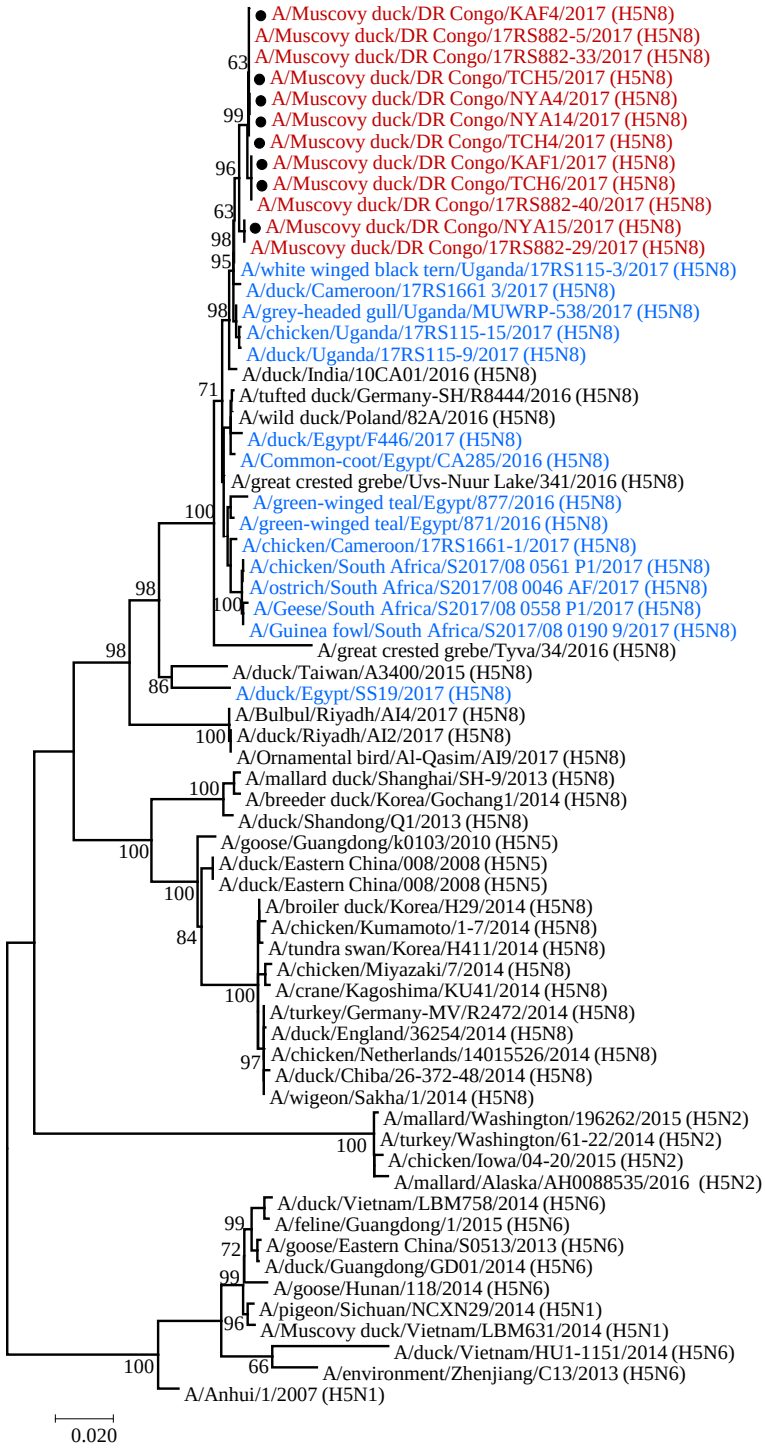

Figure 4. Phylogenetic tree based on the PB1 gene of H5N8 HPAIVs.

The dataset contains 68 nucleotide sequences (2,329 nucleotides in length). The red color indicates viruses isolated in the DRC, the black circles indicate the viruses reported in this study, and blue color are the representative H5N8 viruses isolated in other African countries. The numbers below or above the node indicate the bootstraps values ≥ 60%.

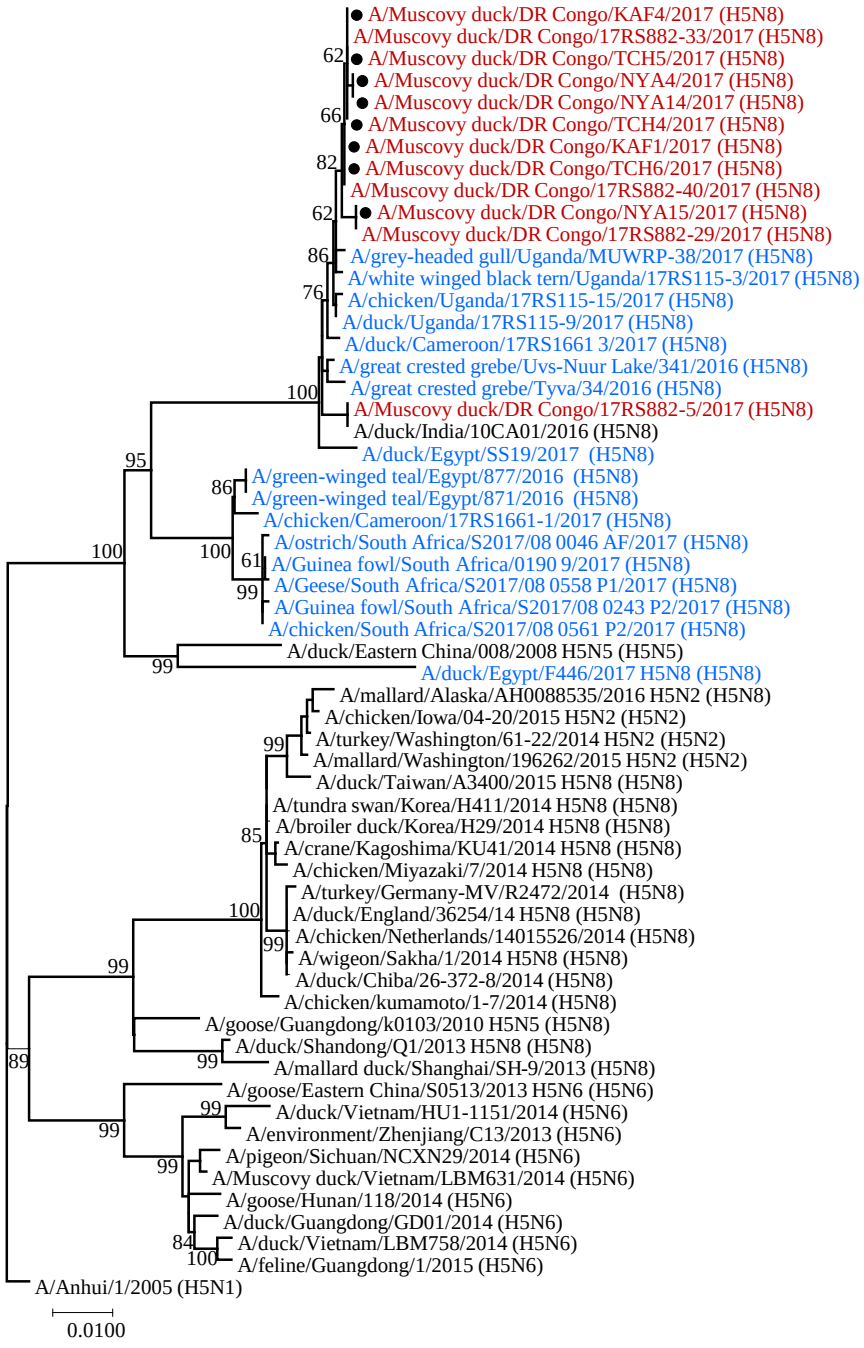

Figure 5. Phylogenetic tree based on the PA gene of H5N8 HPAIVs.

The dataset contains 68 nucleotide sequences (2,218 nucleotides in length). The red color indicates viruses isolated in the DRC, the black circles indicate the viruses reported in this study, and blue color are the representative H5N8 viruses isolated in other African countries. The numbers below or above the node indicate the bootstraps values ≥ 60%.

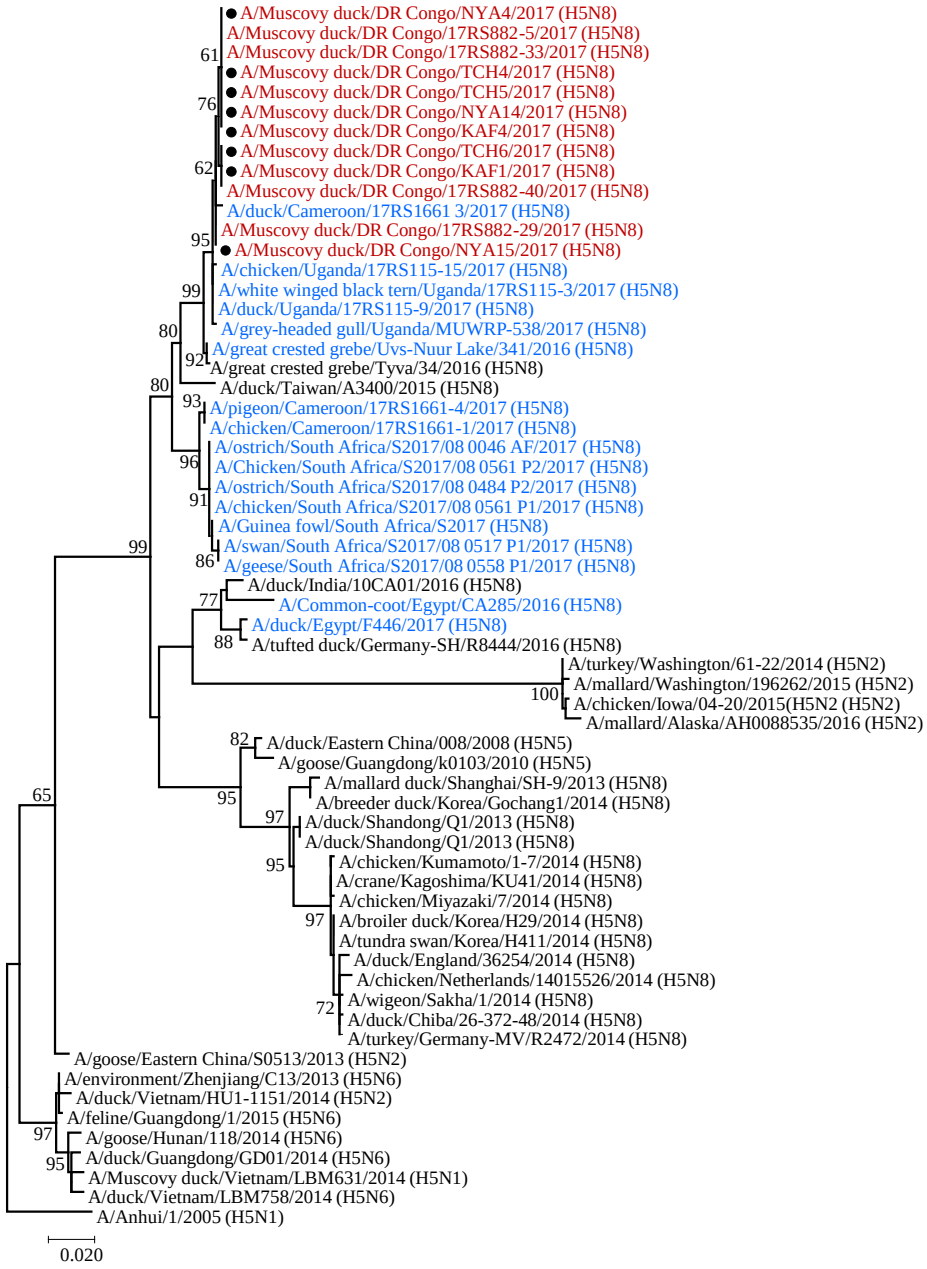

Figure 6. Phylogenetic tree based on the NP gene of H5N8 HPAIVs.

The dataset contains 68 nucleotide sequences (1,1551 nucleotides in length). The red color indicates viruses isolated in the DRC, the black circles indicate the viruses reported in this study, and blue color are the representative H5N8 viruses isolated in other African countries. The numbers below or above the node indicate the bootstraps values ≥ 60%.

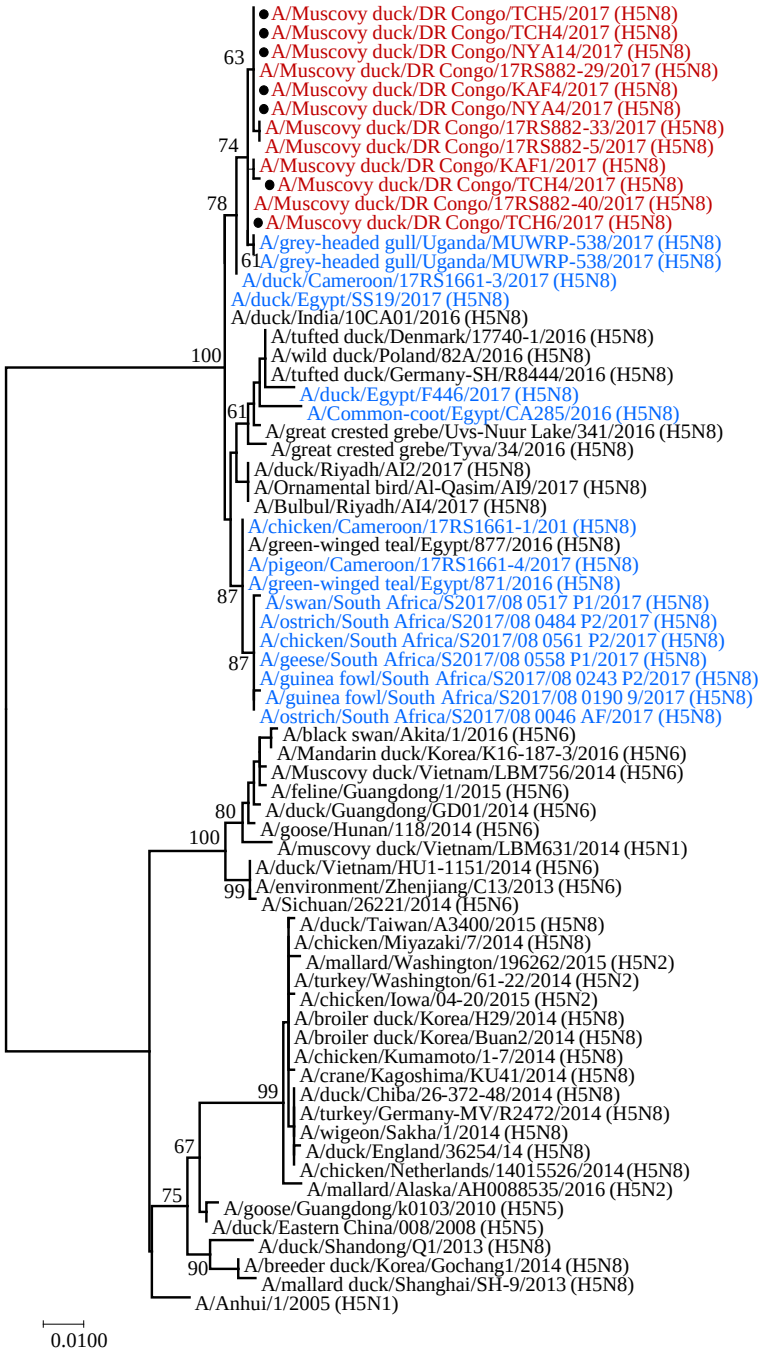

Figure 7. Phylogenetic tree based on the M gene of H5N8 HPAIVs.

The dataset contains 68 nucleotide sequences (1,013 nucleotides in length). The red color indicates viruses isolated in the DRC, the black circles indicate the viruses reported in this study, and blue color are the representative H5N8 viruses isolated in other African countries. The numbers below or above the node indicate the bootstraps values ≥ 60%.

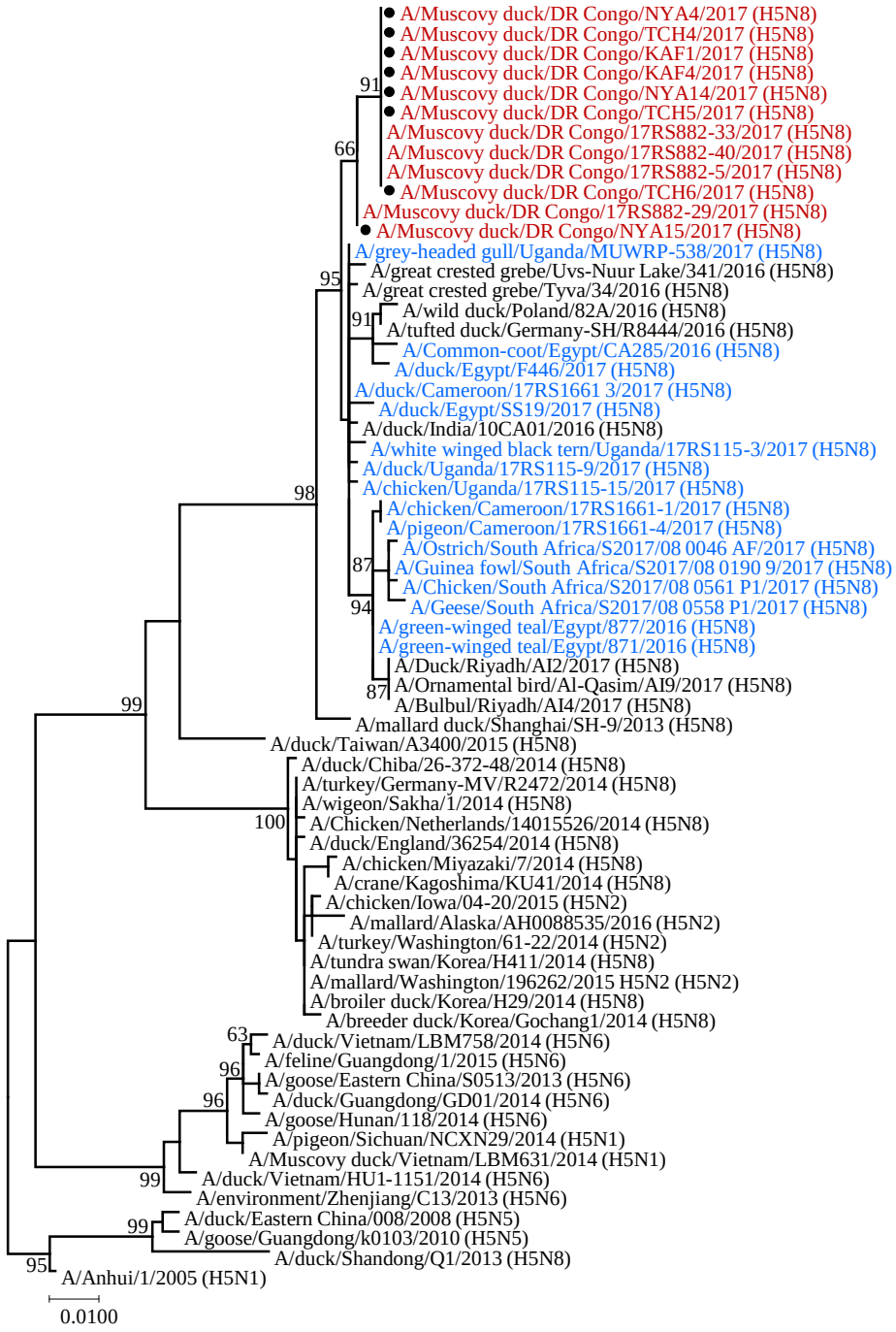

Figure 8. Phylogenetic tree based on the NS gene of H5N8 HPAIVs.

The dataset contains 68 nucleotide sequences (876 nucleotides in length). The red color indicates viruses isolated in the DRC, the black circles indicate the viruses reported in this study, and blue color are the representative H5N8 viruses isolated in other African countries. The numbers below or above the node indicate the bootstraps values ≥ 60%.

The genetic distance between the DRC viruses and related viruses from Africa and Eurasia was estimated by evolutionary divergence over their sequences. The result revealed a genetic distance between the DRC viruses and those from Cameroon, South Africa and Egypt with the distance of 0.81%, 1.42%, and 1.64%, respectively (Table 3).

The DRC isolates were aligned with other representative H5N8 HPAIV isolates of clade 2.3.4.4b from Africa, Asia, and Europe; the position of the deduced AA sequences in the HA protein was identified. All representative clade 2.3.4.4b viruses included in the analysis had similar sequences in the critical position of the antigenic sites. The multibasic AA in the cleavage site of the HA protein of the DRC viruses displayed the common motif (LREKRRKR/GLF) of the HPAIV strains as observed for other clade 2.3.4.4b H5N8 HPAIVs [20]. No deletions or insertions were observed in the nucleotide sequence of the HA protein of the DRC isolates when compared to the representative viruses of the same clade 2.3.4.4b.

Table 3. Estimates of evolutionary divergence over sequence pairs between the DRC viruses and related representative viruses from clade 2.3.4.4b

Origin of sequences	No. of sequences included	Number of Base Substitutions Per Site								
		DRC	Ugd	Cmr	SA	Egy	Ind	ME	EU	EA
DR Congo (DRC)	12									
Uganda (Ugd)	4	0.45								
Cameroon (Cmr)	4	0.81	0.79							
South Africa (SA)	5	1.42	1.01	0.71						
Egypt (Egy)	7	1.64	1.59	1.51	1.71					
Indian (Ind)	1	0.64	0.61	0.74	0.78	1.48				
Middle East (ME)	7	1.22	1.17	1.22	1.19	1.97	1.09			
Europe (EU)	4	1.01	0.99	1.02	1.26	1.67	0.88	1.18		
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. Analyses were conducted using the Kimura 2-parameter model [30]. 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 1,562 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Antigenic features of the DRC viruses

Four representative isolates from the DRC and a panel of H5Nx HPAIVs in the clade 2.3.4.4a, c, and e, isolated in Asia were analyzed, and their corresponding antisera were used for the cross-HI test (Table 2). The antigenic cartography (Figure 9) derived from the cross-HI test results revealed that despite antigenic clustering of the major clade 2.3.4.4 viruses, a moderate difference was noticed between the two DRC viruses (Mkd/CD/KAF1/17 and Mkd/CD/TCH6/17) and the BS/Akita/1/16 virus with three antigenic units distant. However, no difference was observed between the DRC 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 DRC viruses in clade 2.3.4.4b and e; but not with the viruses in clade 2.3.4.4c and a in the same genetic major clade 2.3.4.4. Furthermore, a significant antigenic divergence was noticed between all viruses of major 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.

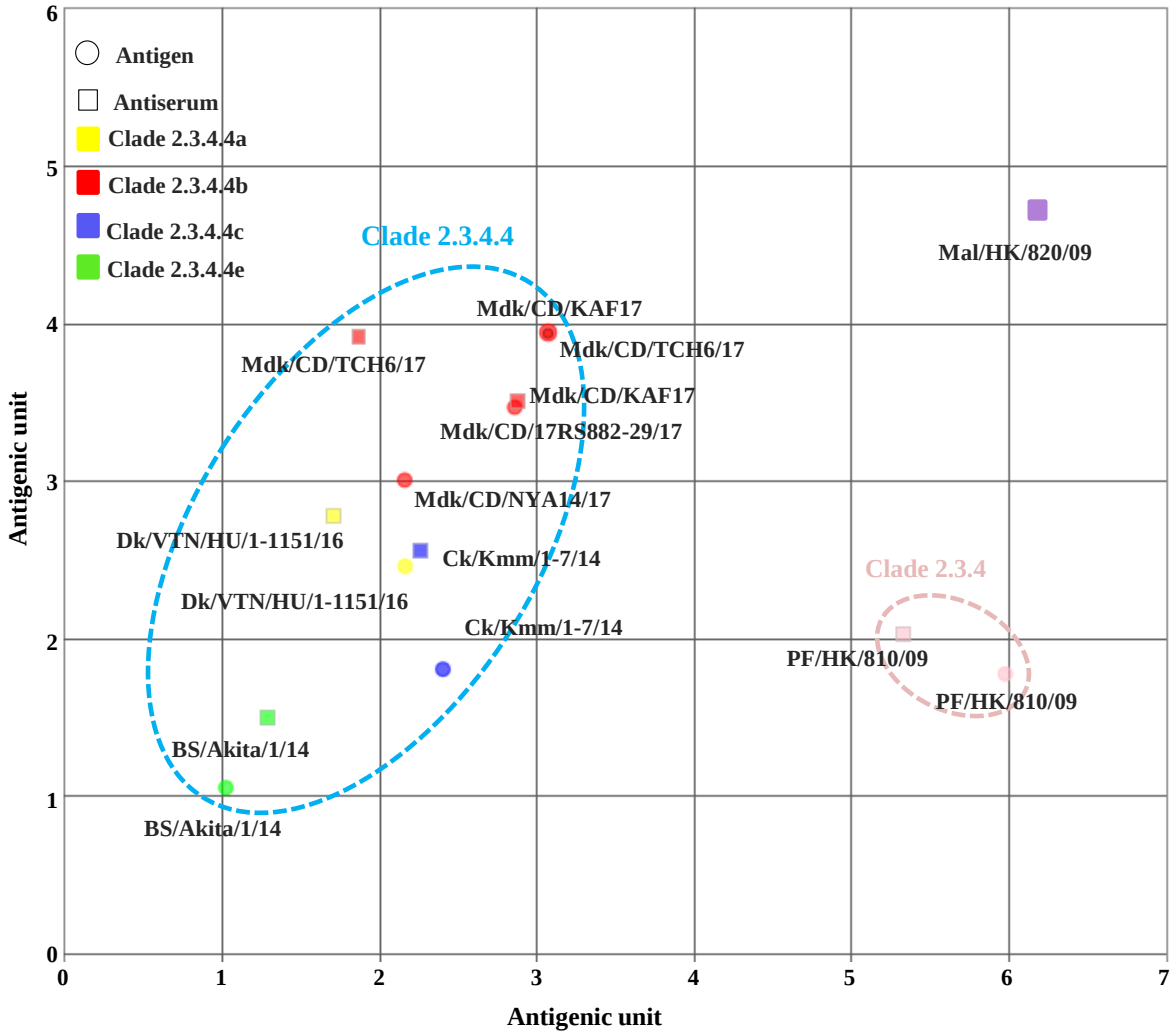

Figure 9. Antigenic cartography of H5Nx HPAIVs of major clades 2.3.4.4 and 2.3.4. The square and the circle indicate the antiserum and the antigen, respectively. The yellow, red, blue and green colors indicate clade 2.3.4.4a, b, c, and e viruses, respectively. The pink and purple colors indicate the clade 2.3.4 and a H5N1 LPAIV, 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 major clades 2.3.4.4 and 2.3.4, respectively.

Pathogenicity of the DRC 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 10a). 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 10b). 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.

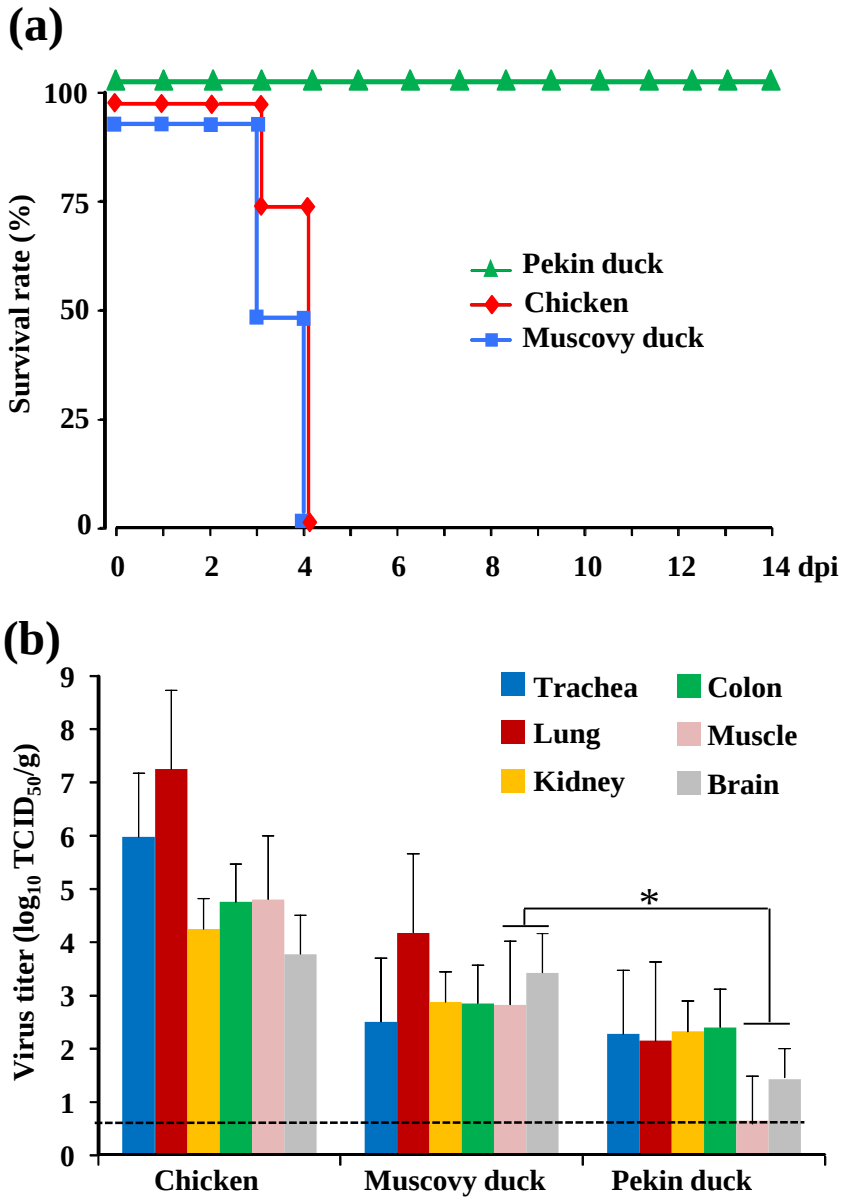

Figure 10. The pathogenicity of a representative H5N8 HPAIV (Mdk/CD/KAF1/17) in poultry intranasally inoculated with 0.1 mL of $10^{6.0}$ EID_{50}.

(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.

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 DRC, Uganda, and Cameroon. In the previous report [7], the outbreaks that occurred in the DRC was linked to those reported in Uganda in January 2017 given that no sequence data of the virus was available to ascertain their relationship at that time; the availability of sequences of viruses from Uganda and Cameroon [22,23] 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 DRC 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 [22] 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 DRC 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 DRC 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 [37,38]. The diverse introduction of these viruses could be explained by the presence of different flyways of migratory birds reaching the African continent [39]. Nevertheless, the possibility of a single introduction of the virus in Africa cannot be excluded 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 the ability to exclude the role of poultry trade in the introduction of the viruses in certain African regions.

The low reactivity of two representative DRC viruses with the antisera raised against the clade 2.3.4.4e virus indicated a slight antigenic difference between the DRC viruses belonging to clade 2.3.4.4b and the virus of clade 2.3.4.4e. Such a small antigenic variation among viruses of different major clade 2.3.4.4 was observed in the study by Hiono et al [40]. However, the isolates from the DRC 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 [41] ; since the DRC does not apply vaccination against AIV, it can be assumed that this slight antigenic variation occurred naturally during the evolution of the virus. However, the distinguishable antigenic distance between the major 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.

The pathogenicity assessment clearly reflected what was observed in the field during the outbreaks in the DRC [7] and Uganda [22], where the high mortality of Muscovy ducks (locally called ducks) and in chickens was reported. In the case of the DRC outbreaks, the high mortality of Muscovy ducks was linked to the high density of these ducks preferentially raised in areas near the lake [21]. In the present study, the viral replication was observed 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 [42]. 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. [43], where the Muscovy ducks were challenged with H5N6 HPAIVs belonging to clade 2.3.4.4e, 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. This result contrasted with that observed with viruses isolated in Germany [44] and China [45], 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. The results of the present study 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. [45].

The results from the present experimental study confirmed field observations regarding the pathogenicity of the DRC 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 the major 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 of AIVs in both wild birds and poultry that can help to understand the virus spread and evolution. Sharing the information from such surveillance at the regional and global levels will allow for a better understanding of AIVs in the region. Enhancing biosecurity in the poultry sector, achieving earlier diagnoses, and culling infected birds to control the spread of infection are essential to manage the landscape of HPAIVs.

Brief summary

AI is still threatening the bird populations, and the aquatic wild birds as the main source of AIVs are responsible for spreading the AIVs globally. During the circulation in the bird population, AIVs acquire mutations that may lead to virulence changing and host species adaptation. The detection and characterization of circulating AIVs are key elements for good surveillance. This study characterized the HPAIVs that caused the outbreak in poultry in the DRC for the first time. The genetic analysis revealed that these viruses clustered with those isolated in African countries and the Eurasia suggesting the same origin and no reassortment occurred during their evolution. Antigenically, there was no difference among the DRC isolates and representative viruses of the major clade 2.3.4.4. The DRC isolates increased the virulence in Muscovy ducks, this phenomenon was hardly observed in previous outbreaks caused by AIVs of the same clade 2.3.4.4. Continuous surveillance in wild birds and poultry is required to monitor the evolution of the IAVs in the DRC and the region; such data sharing will contribute to the global surveillance of AIVs.

Chapter II

A new variant among Newcastle disease viruses isolated in the Democratic Republic of Congo in 2018 and 2019

Introduction

ND, which is caused by the NDV, is a highly contagious and devastating infectious disease that affects poultry and wild birds worldwide. The NDV is also known as the avian paramyxovirus type 1 (APMV-1) and has been recently classified in the family *Paramyxoviridae,* subfamily *Avulavirinae,* and genus *Metaavulavirus* [46]. The NDV has a negative-sense RNA genome comprising 15,186 –15,198 nucleotides for the whole genome and encodes for six proteins in the following order: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large polymerase (L) [47,48]. In addition, two nonstructural proteins, V and W, are generated during P gene translation through RNA editing [49]. The HN and F proteins are surface glycoproteins that play an important role in the infection, pathogenicity, and antigenicity of NDV [50,51]. Based on the genome size and nucleotide sequences of the F and L genes, NDV strains have been divided into class I and class II [52]. Class II, NDVs have higher genetic diversity, including 20 recently identified genotypes named I to XXI (excluding XV, as it contains recombinant viruses), which comprise virulent and avirulent strains [53].

More than 250 avian species have been identified to be susceptible to NDV with varying pathogenicity [54]. However, poultry populations possess high susceptibility to NDV, and the pathogenicity varies by strain. Strains are classified based on their pathogenicity as highly virulent (velogenic strain), moderately virulent (mesogenic strain), and avirulent (lentogenic strain) [55]. The virulence of NDV is primarily attributed to the function of the F gene [56]; however, other genes modulate the capacity of virus replication which can lead to virulence [57]. The characteristics and

epidemiological data on NDVs circulating in poultry and wild bird populations within a specific region provide useful information for ND control and prevention.

In the DRC, the poultry industry impacts the economy and public health through food safety. The production is mostly based on commercial and modern chicken farming systems with broilers and layers; however, small-scale and backyard farming systems are employed in rural areas. Owing to its high commodity value, poultry production has a vital role in the economy.

During the last two decades, outbreaks and resulting mortality attributed to NDV infection in poultry have been frequently reported across various farming systems in the DRC. Most case reports were based on clinical manifestations, whereas few laboratory-confirmed cases were notified [58]. No investigations were performed to confirm or characterize the reported cases; therefore, there is lack of epidemiological and genetic profiling of NDV strains circulating in poultry populations of the DRC. This study aimed to characterize NDVs isolated in the DRC during the period 2018–2019. The phylogenetic, pathogenic, and antigenic traits of NDVs were evaluated to elucidate the features of newly isolated strains and to provide a basis for the development of mitigation measures for the control and prevention of ND throughout the DRC.

Materials and Methods

Sample collection

Tracheal and cloacal swab samples were collected from diseased chickens during two different outbreaks that occurred in 2018 and 2019 in the Tshuapa and Kinshasa provinces of the DRC, respectively (Figure 11; Table 4). All chickens were raised in traditional farming systems without any vaccination history. The collected swabs were put into a viral transport medium (VTM) (Teknova, 2290 Bert Dr, Hollister, CA 95023, USA), and aliquots were shipped on dry ice to Hokkaido University, Sapporo, Japan, for the diagnosis of AI and ND.

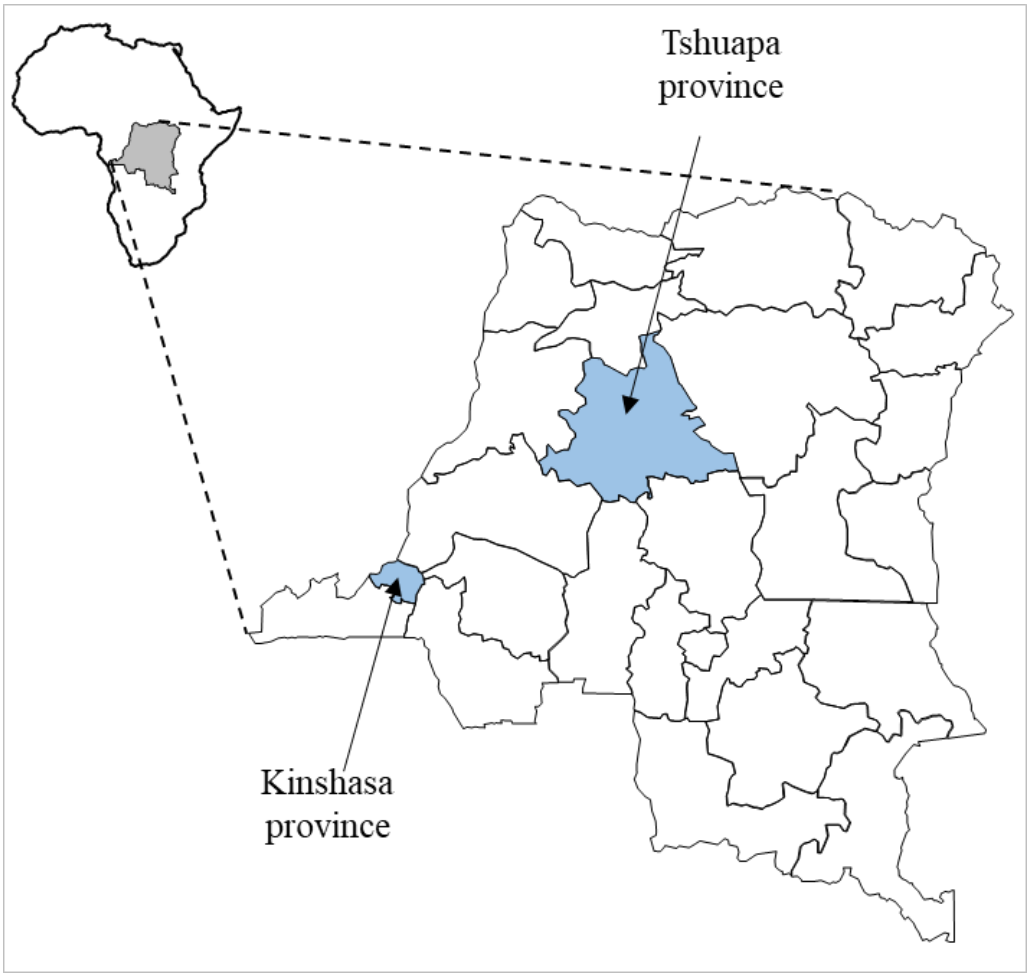

Figure 11. The map of the DRC divided into provinces. The blue shaded regions indicate the sampling sites.

Table 4. Characteristics of the NDVs isolated in the DRC in 2018 and 2019

Isolate	Date of isolation	Host	Location	ICPI	MDT	Cleavage site amino acid motif	Accession number
NDV/Ck/DRC/18VIR3696/2018	May 2018	Chicken	0.2843 S 0.8851 E	nd	nd	^{112}RRQKR|F^{117}	MW363929
NDV/Ck/DRC/PL33/2018	May 2018	Chicken	0.2843 S 20.8851 E	1.84	54.4	^{112}RRQKR|F^{117}	MW363930
NDV/Ck/DRC/PL219/2019	July 2019	Chicken	-4.2436 S 15.5362 E	1.92	57.2	^{112}RRQKR|F^{117}	MW363931

ICPI: intracerebral pathogenicity index; MDT: mean death time; nd: not determined.

Virus isolation and identification

The swab supernatant was inoculated in 10-day-old embryonated chicken eggs obtained from a conventional chicken flock that tested negative for the NDV antibody via the HI test [59] using the antigen NDV/duck/Hokkaido/1/1977 strain available in library of Microbiology laboratory. After 72 h of incubation at 37°C, the presence of NDV was confirmed in the allantoic fluid by a HAt . All HAt-positive samples were then subjected to the HI test against a hyperimmune antiserum anti-NDV from the library of Microbiology laboratory, according to the OIE guidelines [59].

Sequencing, phylogenetic, and genetic analyses

For sequencing, viral RNA was extracted from the infectious allantoic fluid using TRIzol LS reagent, and the whole-genome sequence was obtained using next-generation sequencing as described in chapter I. The reads were mapped to the reference sequence of NDV, and the consensus sequence was reconstructed until the mismatches were solved using the CLC Genomic Workbench, version 12.0 (CLC bio, Aarhus, Denmark)

For phylogenetic analysis, two different datasets were prepared. One contained the pilot dataset of the complete F gene sequence proposed by Dimitry et al. [53], in which three sequences of the DRC isolates were inserted. The other dataset contained the whole-genome sequence of the representative NDV downloaded from GenBank (n = 68), which belonged to each of the 20 genotypes, and three full genome sequences of the DRC isolates were inserted. The two datasets were aligned in BioEdit version 7.0.5.3, and the ML method using the GTR model with gamma distribution was run in MEGA 7 [30] to construct the phylogenetic tree.

The identities of the DRC and other NDV isolates were assessed using BLAST; and changes in the AA position of the F protein were assessed using GENETYX v.15.0.1 (GENETYX Corp. Tokyo, Japan). The evolutionary divergence among sequence pairs of Congo NDVs and other viruses of different genotypes in class II was calculated in MEGA 7 [30].

Pathogenicity assessment of NDV isolates

The pathogenicity of the two DRC isolates (NDV/Ck/DRC/PL33/2018 and NDV/Ck/DRC/PL219/2019) was determined based on the Intracerebral Pathogenicity Index (ICPI) and mean death time (MDT) assays, as recommended by the OIE [59]. Briefly, for the ICPI assessment, 0.05 mL fresh infectious allantoic fluid was diluted 10-fold with sterile PBS and then intracerebrally inoculated in 10 24-hours-old chicks hatched in the Laboratory of Microbiology, Faculty of Veterinary Medicine, Hokkaido University, Japan; the eggs were obtained from an NDV-unvaccinated flock. The inoculated chicks were monitored for clinical signs and mortality every 24 h for 8 days and were scored as 0 for normal, 1 for sick, and 2 for dead. For the MDT, 0.1 mL of 10-fold diluted allantoic fluid (10^{-5} to 10^{-10}) was inoculated in 10-day-old embryonated chicken eggs from the same NDV-unvaccinated flock. The eggs were incubated for 120 h at 37°C and were candled every 8 h for embryo viability. The number of dead embryos was recorded to calculate the mean lethal dose (MLD) that can kill all inoculated embryos. The MDT was obtained as the mean time of survival after inoculation of the virus with the MLD titer.

Antigenic characterization

Hyperimmune antisera against two DRC field isolates (NDV/Ck/DRC/PL33/2018 and NDV/Ck/DRC/PL219/2019) and two vaccine strains (I-2 and Hitchner B1) were prepared in 4-week-old chickens immunized thrice with 0.1% formalin-inactivated allantoic fluid, as previously described [41]. A cross-HI test was performed against the homologous and heterologous antigen in a 96-well microtitration plate using 1% chicken red blood cells [59]. Additionally, the serum neutralizing test (SNT) was performed as previously described [60]. Briefly, the prepared antisera were inactivated at 56°C for 30 min and diluted 10-fold in serum-free minimum Eagle's medium (MEM, Nissui Pharmaceutical, Tokyo, Japan) containing 0.3 mg/mL L-glutamine (Wako Chemicals, Tokyo, Japan), 100 U/mL penicillin G (Meiji Seika Pharma, Tokyo, Japan), 0.1 mg/mL streptomycin (Meiji Seika Pharma, Tokyo, Japan), and 8 µg/mL gentamicin (TAKATA Pharmaceutical Co., Ltd. Saitama, Japan). The antigen was also diluted in serum-free MEM to obtain the 10^2 EID_{50}. The diluted antisera were mixed at equal volumes with a homologous or heterologous antigen, and the mixture was incubated for 1 h at 37°C. Then, 100 µL of the mixture was added in the confluent monolayer of the chicken embryo fibroblast cells that had been prepared 3 days before. After 1 h of incubation, cells were washed three times with PBS, and then MEM with serum was added. The plate was incubated for 6 days at 37°C in 5% CO_2. Then the CPE was observed in each well, and the neutralizing titer was calculated using the Reed and Muench method [29]. The antigenic relatedness was determined by the coefficient of antigenic similarity (*R*) between each pair of strain according to the method of Archetti and Horsfall [61], where an *R*-value of $0.67 < R < 1.5$ indicated no significant antigenic difference, $0.5 < R < 0.67$

indicated a minor difference, and $R < 0.5$ indicated a major difference between the two virus strains.

Vaccine potency test

To evaluate the potency of an ND vaccine used locally in the DRC to protect poultry from newly isolated viruses, 4-week-old chickens (hybrid-breed Julia and Boris brown, which were hatched and reared in the Laboratory of Microbiology, Faculty of Veterinary Medicine, Hokkaido University, Japan, and tested free of the NDV antibody) were categorized into three groups of 10 birds each. Two groups were assigned for vaccination, and the chickens were inoculated with either the I-2 or Hitchner B1 (B1) vaccine by the eye drop method. The I-2 vaccine strain, which is used in the DRC for small-scale chicken farms [62], was prepared according to the user manual [63]. Briefly, the I-2 vaccine strain master seed that was shipped with the swab samples from the DRC was inoculated into 10-day-old embryonated chicken eggs from an unvaccinated flock and tested negative for the NDV antibody. After 3 days' incubation at 37°C, the allantoic fluid was collected and clarified by centrifugation for 5 min at 2500 rpm. The allantoic fluid containing 10^9 EID_{50}/mL titer was mixed at equal volumes with a sterile stabilizer of 2% gelatin (Wako Chemicals, Tokyo, Japan) in distillated water. For the vaccination, 30 µL of the prepared vaccine was inoculated by the eye drop method. The B1 was purchased in Japan (Nisseiken Co., Ltd. Tokyo, Japan) and was used following the manufacturer's instructions. The remaining group of chickens was assigned to the control group that received no vaccination. At 7 days after the first vaccination, a boost vaccination was performed by eye drop; 15 days after the first shot and when the antibody titer reached 128 HI or more against each vaccine strain according to HI assay [59], 100

µL 10^2 EID_{50} of infectious allantoic fluid of the NDV/Ck/DRC/PL219/2019 isolate was intranasally inoculated in the chickens of all three groups. The chickens were monitored daily for 14 days to record the survival and clinical signs. Tracheal and cloacal swab samples were collected at 0, 3, 5, 7, and 14 days post-infection (dpi) for virus shedding assessment. All swabs were placed separately into 2 mL of the VTM. After vortex and clarification by centrifugation for 5 min at 2000 rpm, the supernatant of each swab was inoculated into four 10-day-old chicken embryonated eggs that were obtained from an unvaccinated flock. After 3 days of incubation at 37°C, the infectivity of each swab was confirmed by an HAt of the inoculated allantoic fluid.

Ethical Statement

For samples shipment, the approval was obtained from Central Veterinary Laboratory of Kinshasa, DRC, number: 534/ADM/LVC/SA/YB/148/2018 of June 13, 2018.

The animal experiments were conducted in the ABSL3 facility of the Faculty of Veterinary Medcine, Hokkaido University, Sapporo, Japan; which is certified by AAALAC International since 2007. The approval mumber 18-0035 and 18-0037 were obtained for antiserum preparation and challenge experiments, respectivelly.

Results

Virus isolation and identification

All three HAt-positive samples were identified as NDV by HI test using antiserum prepared against NDV/duck/Hokkaido/1/1977, which was available in the serum library of Microbiology Laboratory. Among these viruses, two were isolated in the Tshuapa province and one was isolated in the Kinshasa province (Figure 11, Table 4). All samples were collected from sick chickens in the traditional farming system. The nucleotide sequence for the whole-genome of the Congo isolates was submitted to the GenBank database under accession numbers provided in Table 4.

Phylogenetic and genetic analyses

Phylogenic analysis based on the complete F gene sequence revealed that the virus NDV/Ck/DRC/PL219/2019 was clustered in genotype VII with other Asian, some African, and European strains; however, a specific clustering of only Asian/Middle East strains in subgenotype VII.2 was observed. The two viruses NDV/Ck/DRC/18VIR3696/2018 and NDV/Ck/DRC/PL33/2018 that were isolated in the Tshuapa province in 2018 were clustered independently from the other NDV strains in the public database, thus forming a divergent branch in the phylogenetic tree (Figure 12). The same clustering for the F gene was observed in the phylogenetic tree that had been constructed using the whole-genome sequence (Figure 13).

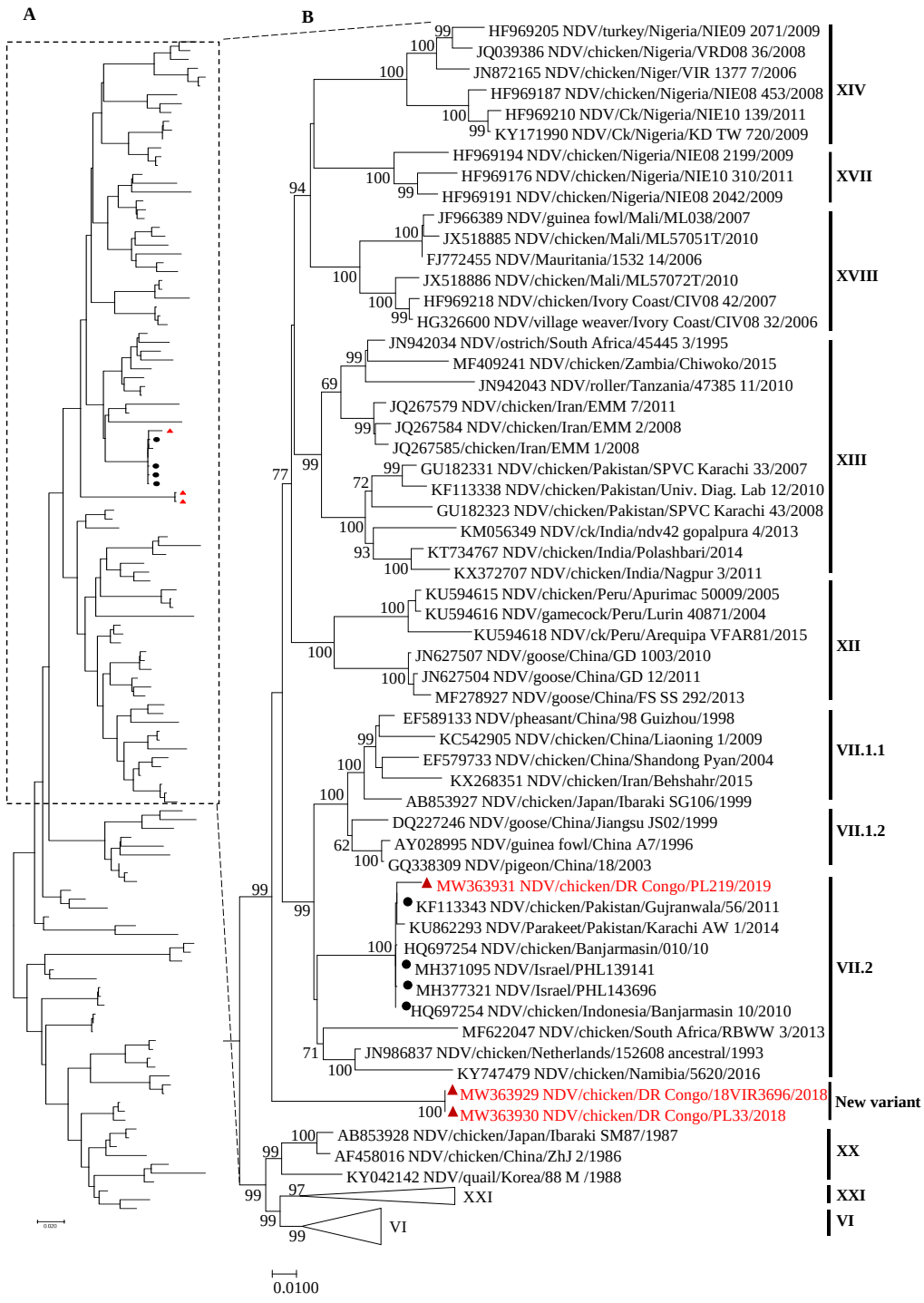

Figure 12. Phylogenetic tree of class II NDV based on the full-length nucleotide sequence of the F gene containing 133 sequences including the DRC strains.

The analysis was performed in MEGA7 v 7.0.23 using the ML method based on the GTR model with 1,000 bootstrap replicates. (A) The entire phylogenetic tree in which the taxa tips are not shown. (B) Part of the tree is shown with all taxa tips indicated; the vertical bar indicates the genotype or subgenotype of each strain group or individual. The triangle and red color indicate the DRC isolates. The black circle indicates the strain in the top five hits of the BLAST search result of the DRC strain NDV/Ck/DR Congo/PL219/2019.

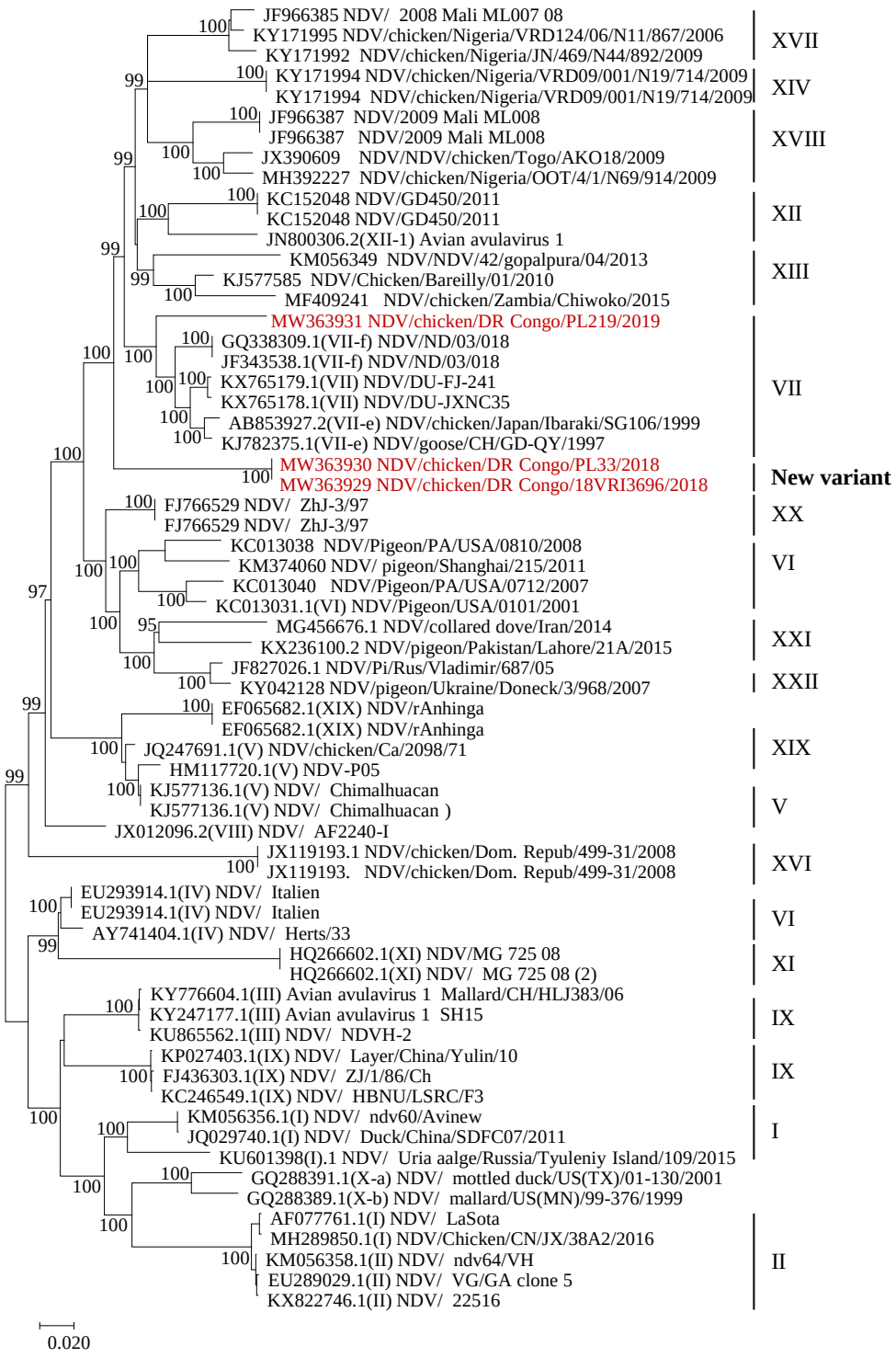

Figure 13. The phylogenetic tree based on the whole-genome sequence of the NDV class II.

The tree was constructed in MEGA7 v 7.0.23 using ML method based on GTR model with 1,000 bootstrap replicates. The dataset contains 64 tax

The similarities of the complete F gene sequence between the DRC isolates and other strains were assessed using BLAST search. For the NDV/Ck/DCR/18VIR3696/2018 and NDV/Ck/DRC/PL33/2018 strains, the identity with the 10 top-hit strains was 90.53%–88.85%, and contained mostly strains from India and Indonesia. For the isolate NDV/Ck/DRC/PL219/2019, the identity was 98.00%–96.00%, with the top-hit strains coming from Pakistan and Israel (data not shown). To confirm the divergence that was observed in the phylogenetic tree, an evolutionary distance analysis of the three DRC isolates was performed with other NDVs of different genotypes. The result revealed that the genetic distance between the two strains NDV/Ck/DRC/18VIR3696/2018 and NDV/Ck/DRC/PL33/2018 and that of other viruses from all 20 genotypes was >10% (Table 5), which indicated a genetic divergence that has been assigned as a new variant of class II NDVs.

Table 5. Estimates of evolutionary divergence over sequence pairs among genotypes of class II NDVs.

Genotype	Number of Base Substitutions Per Site																		
	DRC	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**XI**	**XII**	**XIII**	**XIV**	**XVI**	**XVII**	**XVIII**	**XIX**	**XX**
DRC (*n* = 2)																				
I (*n* = 12)	20.09																			
II (*n* = 3)	21.67	14.20																		
III (*n* = 3)	19.30	12.79	14.66																	
IV (*n* = 2)	15.84	13.02	14.81	10.09																
V (*n* = 6)	15.72	18.56	19.98	16.33	14.24															
VI (*n* = 21)	15.10	19.85	21.10	18.72	16.03	15.25														
VII (*n* = 14)	13.74	19.11	21.75	17.61	14.95	15.54	13.89													
VIII (*n* = 3)	14.46	15.19	1.709	13.63	10.89	11.39	12.09	12.72												
IX (*n* = 3)	19.22	12.16	13.12	9.36	9.53	15.81	18.04	17.22	12.72											
X (*n* = 4)	22.56	12.55	12.07	14.11	14.07	19.60	20.95	20.27	16.54	12.81										
XI (*n* = 3)	25.94	2153	23.24	19.43	14.76	21.38	24.24	24.69	19.74	17.62	22.44									
XII (*n* = 6)	14.89	20.33	23.40	19.15	16.58	15.56	13.77	12.45	13.36	18.71	21.37	25.65								
XIII (*n* = 12)	13.20	19.61	22.14	18.96	15.49	15.37	14.57	11.76	13.02	17.41	20.78	24.42	11.89							
XIV (*n* = 6)	16.49	23.57	26.30	22.92	19.51	18.32	17.13	15.01	15.93	22.49	23.98	29.69	14.50	14.05						
XVI (*n* = 3)	17.64	17.53	19.81	16.35	13.38	14.83	16.13	16.57	11.74	15.62	18.32	22.85	16.97	16.47	19.85					
XVII (*n* = 3)	14.29	19.52	22.99	19.20	16.54	15.98	15.84	13.58	14.30	17.78	21.64	23.91	13.08	12.46	13.59	18.08				
XVIII (*n* = 6)	13.82	19.84	21.98	18.57	15.74	15.50	14.05	12.65	13.25	17.60	20.86	23.87	12.00	11.57	13.74	16.97	10.92			
XIX (*n* = 3)	18.26	21.96	22.35	20.02	17.40	09.59	17.79	17.92	14.47	18.97	22.40	24.93	18.81	18.03	21.03	18.40	18.84	18.27		
XX (*n* = 6)	13.30	16.82	19.14	16.17	13.28	12.97	09.14	11.89	09.69	14.91	18.85	21.43	12.54	12.53	16.15	13.52	13.17	12.13	16.18	
XXI (*n* = 10)	16.25	20.55	22.55	19.57	17.03	16.76	11.73	15.30	13.62	18.66	22.12	25.12	16.09	16.19	19.24	17.68	17.88	16.00	19.20	10.47

The number of base substitutions per averaged across all sequence pairs between groups is shown. Analyses were conducted using the maximum composite likelihood model.

The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 128 nucleotide sequences of the pilot dataset, including three DRC strains. The included condon positions were 1st, 2nd, 3rd, and noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 v 7.0.23. The number in brackets indicates the number of taxa in each group. DRC indicates new variant NDV isolated from the DRC.

Pathogenicity of the DRC isolates

Molecular assessment of the cleavage site of the F protein revealed that all three DRC isolates possessed the AA combination motif ^{112}RRQKR|F^{117} of the velogenic strains of NDV. In addition, the ICPI of NDV/Ck/DRC/PL33/2018 and NDV/Ck/DRC/PL219/2019 was 1.84 and 1.95, respectively. The MDT of NDV/Ck/DRC/PL33/2018 and NDV/Ck/DRC/PL219/2019 was 54.4 and 57.2 h, respectively (Table 4). Considered together, these results indicate the velogenic pathotype of the DRC strains.

Antigenic characterization

To evaluate the vaccine's efficacy, it is necessary to determine the antigenic relatedness between the field isolates and vaccine strain. For these newly isolated strains, the antigenicity between field isolates and vaccine strains frequently used in the DRC was compared. The cross-HI test revealed no antigenic difference between the field and vaccine strains, given that the HI titers differed only 2- to 4-fold. *R*-values of > 0.7 obtained from the SNT also showed that there was no antigenic difference between the viruses (Table 6a and b). These results indicated that the newly isolated NDVs in the DRC are antigenically closely related to the vaccine strains used in the DRC that were evaluated in this study.

Table 6. The antigenic relatedness between the DRC isolates and vaccine strains.

(a)

Virus	Antiserum to virus			
	PL33/18	PL219/19	I-2	B1
NDV/Ck/DRC/PL33/2018	1,024	512	256	512
NDV/Ck/DRC/PL219/2019	512	512	512	512
I-2 vaccine	512	512	512	512
B1 vaccine	256	1,024	1,024	512

(b)

Virus	Antiserum to virus			
	PL33/18	PL219/19	I-2	B1
NDV/Ck/DRC/PL33/2018	**1.00**	1.00	0.98	0.99
NDV/Ck/DRC/PL219/2019	0.99	**1.00**	0.89	0.95
I-2 vaccine	0.98	0.98	**1.00**	0.97
B1 vaccine	0.90	1.00	0.99	**1.00**

(a) The HI titer between homologous and heterologous viruses; the underline indicates the homologous titer. (b) The *R*-value was obtained from cross-SNT titers between the field and vaccine strains; bold indicates the homologous *R*-value.

Vaccine potency against the field isolate

To ensure the antigenic relatedness between DRC field isolates and the vaccine strains, the animal experiment was conducted by vaccinating and subsequently infecting naïve chickens with the NDV/Ck/DRC/PL219/2019 strain. All the vaccinated chickens in the I-2 and B1 groups survived the infection, and no clinical signs were observed during the 14 day period. However, in the control group, all chickens died at 3 dpi (Figure 14). To assess the virus shedding from surviving chickens, swab samples collected at 3, 5, 7, and 14 dpi were inoculated in eggs. No virus was detected in any of swabs from the chickens in the vaccinated groups (data not shown). This result indicates that the vaccines efficiently protected the chickens from infection and suppressed virus shedding from infected birds.

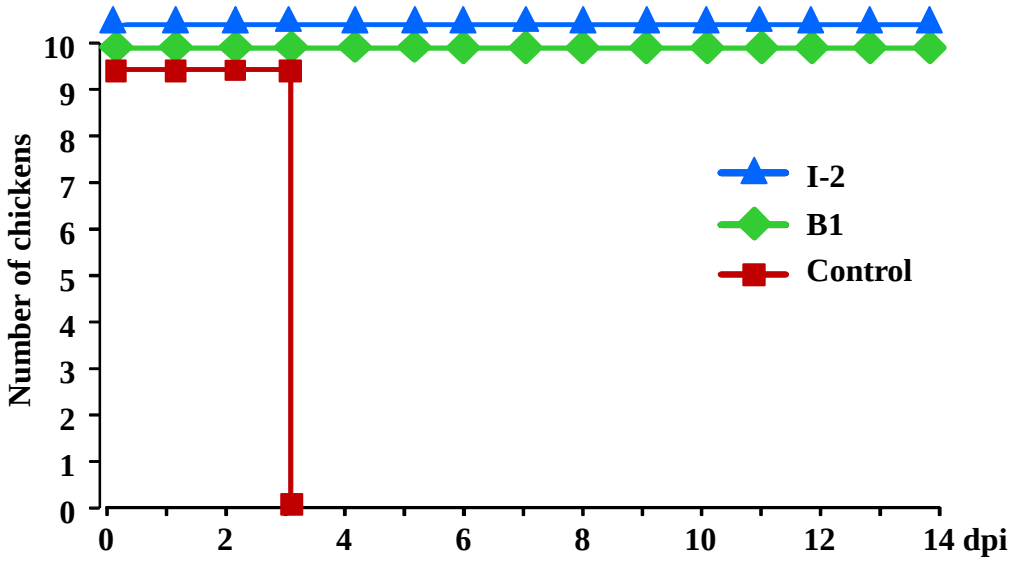

Figure 14. The survival of chickens infected with the DRC field strain NDV/Ck/DRC/PL219/2019. In two groups of 10 birds each, chickens were vaccinated twice by eye drop at 7 days interval with either I-2 or B1 vaccine. In another group, 10 unvaccinated chickens were assigned as the control. Fifteen days post-vaccination, all chickens were infected intranasally with 10^6 EID_{50}/100 mL of infectious allantoic fluid and were monitored for 14 days.

Discussion

In the DRC, ND has been reported for a long time in commercial and traditional poultry farms. The reports were mainly clinically based observations that were made during the outbreaks, and few laboratory-based cases were notified [58]. Thus, there is no information on the genetic profile or virological characteristics of circulating NDV strains. In this study, detailed features of three NDV isolates from two different outbreaks were obtained. Phylogenetic analysis of the complete F gene sequence based on a recent classification by Dimitrov et al. [53] revealed that the viruses NDV/Ck/DRC/18RVI3696/2018 and NDV/Ck/DRC/PL33/2018 isolated in the Tshuapa province were highly divergent from the NDV/Ck/DRC/PL219/2019 virus isolated in Kinshasa belonging to genotype VII. The isolates from the Tshuapa province genetically diverged from all other NDV sequences that are currently available in the referenced public databases.

The topology and the length of the branches of the phylogenetic tree and the genetic distance between these two isolates from Tshuapa province as well as the complete dataset of sequences from all existing genotypes indicates that these isolates may represent a new variant of the NDV in class II. However, these isolates could not have been assigned to a new genotype, given their isolation from a single outbreak, because isolation should occur from multiple independent outbreaks, as recommended in the new classification criteria proposed by Dimitrov et al. [53]. The high divergence of these viruses could be owing to virus evolution in naïve poultry populations, which has led to several mutations during the virus replication rather than owing to vaccine immune pressure, which has been reported to increase the occurrence of mutations in the NDV

genome [64]. In the countryside of the Tshuapa province, vaccination is rarely administered in indigenous poultry, excluding the possibility of vaccine pressure. It is also hypothesize that this NDV variant is a native strain that has never been identified before to be compared with other NDVs circulating in the region. Moreover, the isolate NDV/Ck/DRC/PL219/2019 clustered with other African NDVs in the large genotype VII, but not with NDVs from neighboring countries. However, this virus was closely related to Indonesian and Pakistani strains that clustered together in subgenotype VII.2. It is assumed that this virus has been introduced from Asia and/or the Middle East considering their close relatedness. This situation can be explained by the fact that in the DRC, especially in the Kinshasa province where this virus was isolated, the poultry industry and related businesses are conducted mainly by Indian, Pakistani, and Lebanese communities, which may have introduced the virus via live chickens or contaminated materials. A BLAST search of the NDV/Ck/DRC/PL219/2019 isolate produced a top-10 hit among viruses from Pakistan and Israel, which supports the assumption that this virus was introduced from Asia, based on its sequence identity. However, the lack of genetic data for the NDVs circulating in neighboring countries cannot be denied [65] that makes difficult to identify more closely related viruses in the region. However, considering the transboundary character of NDV, these velogenic isolates from the DRC can spread across the region given the porosity and the poor control measures between countries borders.

This study demonstrated that two variants of NDV isolated in the DRC are antigenically closely related to the I-2 vaccine strain that is mostly used in the traditional farming system of the DRC [62] and to the B1 strain that is widely used against ND.

Furthermore, the I-2 and B1 vaccine strains were evaluated for their potential ability to protect chickens from newly isolated NDVs in the DRC. The two vaccine strains efficiently protected chickens against lethal infection and suppressed virus shedding from vaccinated birds. Cumulatively, these findings indicate that despite the new variants of NDV detected in the DRC, ND can still be well controlled by an effective vaccination program that utilizes the currently available vaccines. However, although an effective vaccine is available, the ND still endemic mainly in the traditional poultry farming systems probably due to the lack of adequate veterinary services in rural zones making it difficult to access the vaccine by farmers [62]. This study demonstrates that it is necessary to conduct epidemiological surveys to elucidate the endemicity of ND in the DRC so that the appropriate strategies can be applied for the control and prevention of the disease throughout the DRC.

Brief summary

NDV causes severe disease in birds; poultry populations are the most sensitive to the infection. Although there are effective prevention measures such as biosecurity in farms and vaccination for susceptible species, the disease still endemic, especially in developing countries. The epidemiological data and characteristics of NDV strains circulating in a particular area are critical to control the ND. In the DRC, NDV is endemic; however, there is no information about the NDVs circulating in the country. This study provided the genetic, antigenic, and pathogenic characteristics of three NDVs isolated from chickens during two different outbreaks that occurred in backyards in 2018 and 2019. Genetically, the two NDVs isolated in the same outbreak in 2018 were classified as a new variant given their divergence with other viruses in the current public database. The other virus isolated in 2019 was classified in genotype VII, clustering with Asian and some African NDVs; suggesting its introduction into the DRC from Asia. No antigenic difference was noticed between the DRC isolates and the vaccine strains, and the challenge experiment to chickens revealed the vaccine potency against the Congo NDVs, indicating that ND can be well controlled in the DRC using the current vaccine with an effective vaccination program.

Chapter III

Evaluation of baloxavir marboxil and peramivir for the treatment of high pathogenicity avian influenza in chickens

Introduction

HPAI is one of the most devastating avian viral diseases; HPAIV infection induces an acute disease with high mortality reaching 100% in chickens [66,67]. Aside from chickens, HPAI also affects other poultry populations such as ducks, turkeys, and quails [68]. While aquatic wild birds form the natural reservoir of HPAIV [1,2], other wild bird species have been reported to be susceptible to HPAIV infection, which sometimes can become fatal [69,70]. Therefore, it is necessary to not only protect poultry but also wild bird species from HPAIV infection.

HPAI vaccination among chickens and domestic ducks has been implemented as a preventive measure and infection control in some countries [71,72]; however, stamping out of affected population has remained to be the global standard recommendation [8]. Since vaccines must be updated frequently following antigenic changes of epidemic viruses [73,74] and may lead to the silent spread of HPAIV with asymptomatic infections [75]; therefore, in many countries, vaccination against HPAI is prohibited for use in poultry [71,76]. Active surveillance of wild birds and improvement of biosecurity in farms are also performed to monitor and prevent HPAI in addition to stamping out [8]. Wild birds in captivity, such as birds in zoos, may need to be covered by these strategies as well because of their potential susceptibility to HPAIV infection [70].

To protect captive wild birds at risk from HPAIV infection in the zoo or sanctuary, both stamping out and emergency vaccination are conducted [69,77]. However, in the case of valuable captive birds, such as rare species and breeds, culling may not be an option for conservation and economical purposes. Thus, additional approaches are needed in order to treat birds infected with HPAIV using antiviral drugs. Anti-influenza drugs have been intensively developed for human treatment [78,79]; however, only few

studies have assessed their effectivity on avian species; for instance, oseltamivir, a pro-drug for NA inhibition, has been evaluated for preventative and therapeutic effects against AIV infection in poultry. However, several limitations were noticed in this study: a lack of clinical signs in chickens when challenged with a low pathogenic avian influenza virus [80]; presence of virus growth but lack of clinical signs in eggs used for HPAIV infection [81]; and partial protection of chickens infected with HPAIV [82]. Furthermore, the metabolism of oseltamivir in bird species was not evaluated for the production of an active drug form for influenza treatment as seen in humans or mice.

In this study, two anti-influenza drugs used in humans were evaluated for the potential treatment of HPAI in rare birds. This included baloxavir marboxil (BXM), a novel cap-dependent endonuclease inhibitor that acts as a pro-drug, and peramivir (PR), a NA inhibitor used as a control as its active form has a direct anti-influenza effect for the treatment of AIV infection. The antiviral efficacy of these drugs was evaluated using the chicken model, which demonstrates severe HPAI infection, and the metabolism of BXM in both chickens and Pekin ducks was assessed to determine the effective dosage that can be applied for other bird species.

Materials and Methods

Antiviral drugs

A 20 mg tablet of BXM, was used for oral administration; BXM is metabolized in the liver to baloxavir acid (BXA), which is the active form of BXM. 10 mg/mL solution of PR, was administered via intramuscular injection. Both drugs were purchased from Shionogi & Co., Ltd, Osaka, Japan.

Virus

An HPAIV BS/Akita/1/16 was used as a challenge strain in chickens. The virus was propagated in 10-day-old chicken embryonated eggs, and the infectious allantoic fluid was used for infection. The inoculum contained $10^{5.3}$ EID_{50}, which is equivalent to 10 chicken lethal doses per 100 µL (10 CLD_{50}/100 µL).

Animal experiments

For all challenge experiments, 6-week-old chickens (*Gallus gallus domesticus*, Julia strain; 0.5 kg body weight, obtained from Hokkai Starchick, Hokkaido, Japan) were used in this study. The chickens were housed in conventional conditions without vaccination and were tested as free of AIV antibody by HI test [83] against the challenge BS/Akita/1/16 virus. Three experiments were carried out to mimic the different field conditions of infection during an HPAI outbreak and subsequent treatment. For drug administration, a BXM tablet was crushed into powder and was further dissolved in 2 mL of sterile PBS for oral administration, whereas PR liquid was injected in the thigh muscle.

Experiment 1. Simultaneous treatment with BXM or PR in the chicken model

Chickens were divided into three groups of eight birds each (BXM, PR, and no treatment as control). The chickens were infected intranasally with 100 µL of the lethal A/BS/Akita/1/16 virus. A dose of 20 mg/kg was immediately administered orally for BXM and intramuscularly for PR the treatment was repeated every 12 h for 5 days. The 20 mg/kg dose was determined based on the dose of 1 mg/kg used in humans for clinical treatment [84]; however, because of the high dose of the inoculum (10 CLD_{50}) of the challenge virus, the doses were adjusted accordingly.

Experiment 2. Delayed treatment with BXM or PR in the chicken model

Chickens were divided the same as in Experiment 1. However, the treatment for all chickens started 24 h after the infection when the first clinical signs such as depression and lack of appetite appeared. Drugs were then administered twice a day for 5 days.

Experiment 3. Assessment of the minimum dose of BXM in the chicken model

A range of five doses (0.1, 0.5, 2.5, 12.5, and 62.5 mg/kg) was prepared to determine the minimum dose of BXM at a single administration; this as the dose of 20 mg/kg used in Experiments 1 and 2 was higher compared with that used for human and mouse administration. Therefore, chickens were divided into 6 groups (8 birds each) depending on the dosage, with one group serving as the control; four chickens were assigned for organ sampling at 3 dpi, and the remaining chickens were assigned for clinical observation for 14 days. Chickens were infected with 100 µL of the inoculum, and a single dose of BXM was orally administered immediately after the infection.

In all three experiments, organ samples including the trachea, lung, kidney, colon, and brain were collected at 3 dpi from four of eight chickens in each group for virus recovery. The remaining chickens were assigned for 14 days of clinical observation.

In addition to sample collection from organs at 3 dpi, swabs were collected from the surviving chickens at 0, 3, 5, 7, and 14 dpi in the clinical observation groups. Briefly, tracheal and cloacal swabs were collected and put separately in 2 mL of VTM containing MEM and 0.5% BSA fraction V (Roche, Basel, Switzerland). After vortexing and centrifugation, the supernatant was kept in aliquots at –80°C until use. At 14 dpi before euthanasia, blood was collected from the surviving chickens for antibody response assessment in serum against the challenge virus by HI test [83].

Assessment of BXA and PR kinetics in chickens and Pekin ducks

To assess the metabolism of BXM and the kinetics of BXA and PR for the determination of the appropriated dosage in bird species, three chickens were assigned for each drug, and a single dose of 20 mg/kg was administered orally and intramuscularly for BXM and PR, respectively. For BXM, the blood was collected at 0, 8, 24, and 48 h post-administration (hpa), whereas for PR, it was collected at 0, 0.5, 2, and 8 hpa. The duration of blood sampling was different for BXM and PR treatment given the long plasma half-life previously reported for BXM in humans [85] and the shorter plasma half-life reported for PR in mice [86] and humans [87].

To assess the BXM metabolism and BXA blood concentration in different bird species, Pekin ducks were used in the study as well. Three 6-week-old chickens and three 4-week-old Pekin ducks (*Anas platyrhynchos domesticus*, Cherry Valley; obtained from

Takikawa Shinseien, Hokkaido, Japan) received a single dose of 2.5 mg/kg of BXM; blood samples were then collected at 0, 8, 24, 48, 72, 96, and 120 hpa.

Blood was collected in heparinized tubes, and plasma was obtained after centrifugation at 3,000 rpm for 5 minutes. For the BXA stability, 3 μM of dichlorovinyl dimethyl phosphate (Wako Chemicals, Tokyo, Japan) was added to the plasma before storage at –80°C for further analysis. The BXA and PR plasma concentration was determined using a validated liquid chromatography-tandem mass spectrometry method at Sumika Chemical Analysis Service, Ltd., (Osaka, Japan) as previously described [88]. The drug plasma concentration was calculated as a mean standard deviation (SD) of three individual values plotted in the graph.

Virus recovery in organs and shedding in swabs of chickens

To evaluate the virus replication in infected chickens after the treatment, organs collected at 3 dpi in all three challenge experiments were homogenized using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) to make a 10% (w/v) of suspension in VTM. Viral infectivity was assessed based on the CPE observed in the monolayer of MDCK cells maintained in MEM supplemented with 10% nonimmobilized fetal calf serum (FCS; SAFC Biosciences, Lenexa, KS, USA); cells were then incubated at 37°C in 5% CO_2. Briefly, tenfold dilutions of the organ supernatant with VTM were performed in a FCS-free MEM; each dilution was inoculated in 4 wells and incubated for 1 h at 37°C. Cells were later washed twice with PBS, and 100 μL of FCS-free MEM was added and incubated for 72 h at 37°C in 5% CO_2. The $TCID_{50}$ was calculated according to Reed and Muench [29], which was set as the viral titer of the sample.

To measure the virus shedding of chickens after infection and treatment with BXM, tenfold dilutions of swab suspension in VTM were each inoculated into four 10-day-old chicken embryonated eggs obtained from a conventional flock tested free of AIV. After a 48 hs incubation at 35°C, the HAt using 1% chicken red blood cells [83] was performed to assess the infectivity titer calculated as EID_{50} [29].

Statistical analysis

To compare the virus replication between the treatment groups, one-way analysis of variance with Tukey test was performed using SPSS version 20.0 (IBM Corp, Armonk, NY, USA). The difference was considered significant at a p-value < 0.05.

Ethical statement

All animal experiments were performed in the ABSL3 facility at the Faculty of Veterinary Medicine of Hokkaido University, Sapporo, Japan; approval number 18-0090, under the guidelines of the Institutional Animal and Committee of Hokkaido University, certified by the AAALAC International since 2007.

Results

Effects of multiple doses of BXM or PR in the simultaneous treatment of HPAIV infection

To evaluate the protective effect of BXM and PR in chickens following HPAIV infection, bird survival was monitored, and viral replication in the organs was measured. No deaths were recorded in BXM; one chicken died at 2 dpi in PR group with treatment at 20 mg/kg, whereas all chickens died by 3 dpi in the control group (Figure 15a). Viral replication in the organs of the BXM-administrated chickens has been noticed to decrease compared with that in the control group ($p < 0.001$); however, viral replication in the PR and control groups was determined to be the same (Figure 15b). As per these findings, BXM treatment was able to completely suppress viral replication, enabling the chickens to survive infection, whereas only partially protective efficacy was observed with PR treatment because of insufficient inhibition of viral replication.

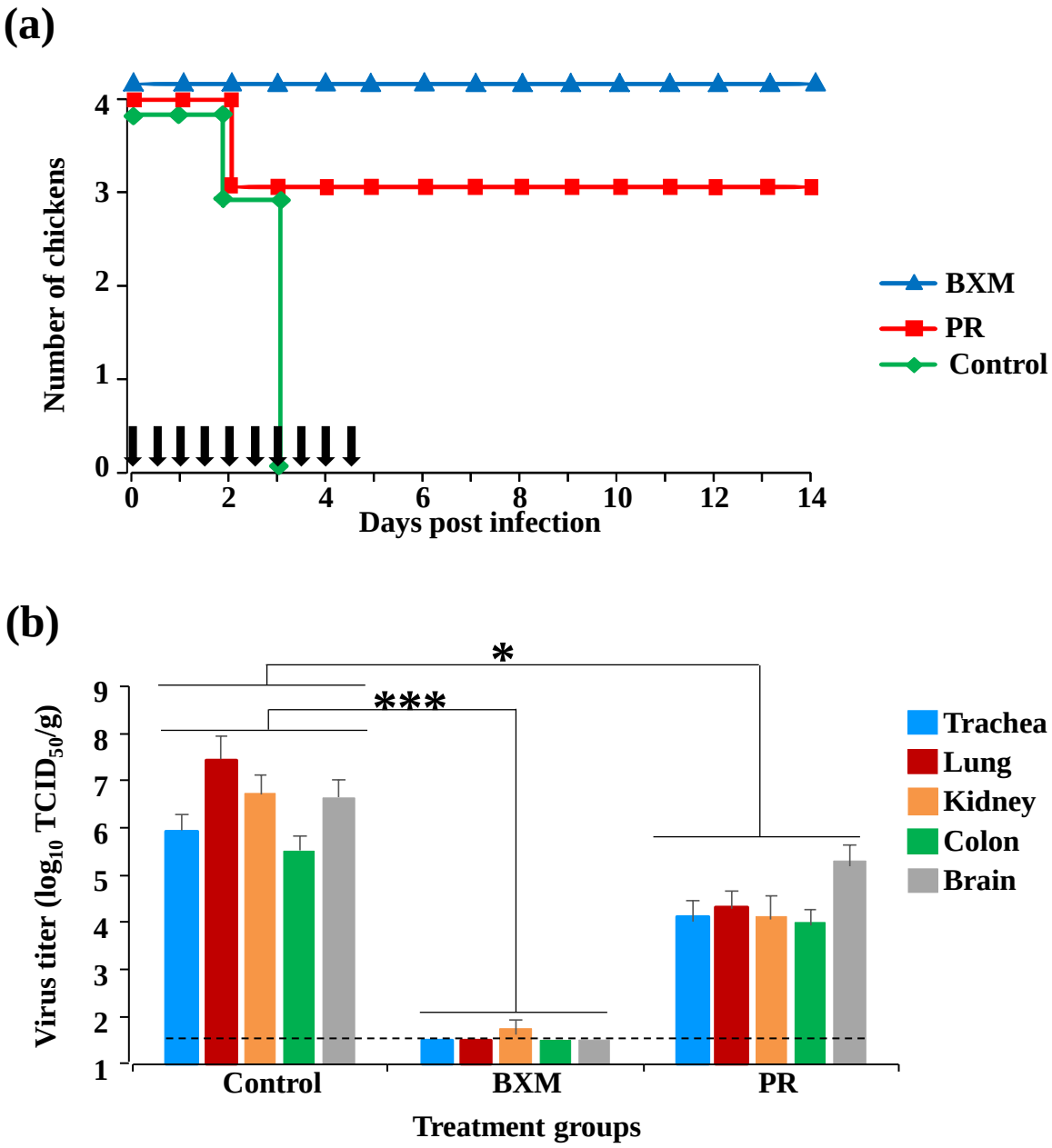

Figure 15. Effects of the simultaneous treatment using multiple doses of BXM or PR in chickens infected with an HPAIV, BS/Akita/1/16.

Eight chickens per group were infected with 10 CLD_{50}/100 µL ($10^{5.3}$ EID_{50}) of the HPAIV and treated immediately with 20 mg/kg of either BXM or PR; the untreated group was assigned as the control. (**a**) Survival of half the infected birds was followed until 14 dpi; vertical black arrows indicate the treatment schedule. (**b**) Virus recovery from organs collected at 3 dpi from four chickens in the control and each treatment group was assessed in monolayer MDCK cells. The black dotted line indicates the detection limit ($10^{1.5}$ $TCID_{50}$/g), whereas bar indicates the standard deviation (n = 4). *(∗, $p < 0.05$; ∗∗∗, $p < 0.001$).*

Effects of multiple doses of BXM or PR in the delayed treatment of HPAIV infection

The simultaneous treatment of chickens with either BXM or PR demonstrated the protective effects of both two drugs; a further experiment was carried out to assess the protective potential of both of these drugs starting at notification of clinical signs at 24 hpi, which mimics the field situation of an HPAI outbreak. All the chickens in the three groups (BXM, PR, and control) succumbed to HPAI infection. However, in the BXM group, one chicken survived up to 6 dpi, whereas in the PR group, three chickens survived until 4 dpi, compared with those in the control group who had all died by 3 dpi (Figure 16a). Virus replication in chicken organs at 3 dpi was significantly lower in the BXM-treated group than that in the control group ($p < 0.05$), although there was no significant difference between the PR and control group (Figure 16b). Despite the mortality of chickens observed in the three groups, BXM treatment had prolonged the survival of one chicken and suppressed viral replication to a greater extent than that compared to using PR.

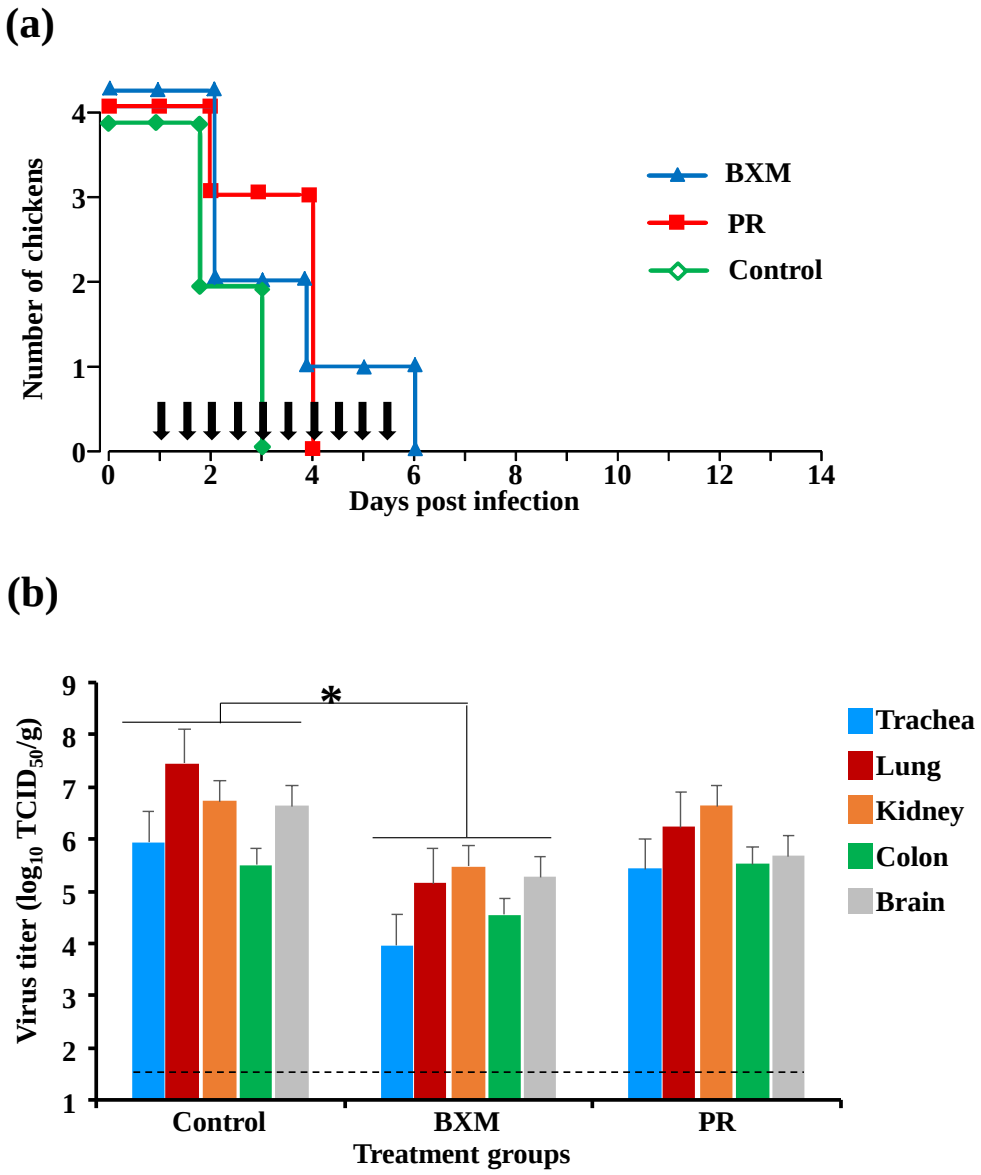

Figure 16. Effects of delayed treatment with multiple-dose administration of BXM or PR in chickens infected with HPAIV, BS/Akita/1/16.

Eight chickens per group were infected with 10 CLD_{50}/100 µL ($10^{5.3}$ EID_{50}) of the HPAIV and treated with 20 mg/kg of either BXM or PR for 24 hs to 5 days post-infection; the untreated group was kept as the control. (**a**) Survival and clinical signs of half the infected birds was followed until 14 dpi; vertical black arrows indicate the treatment schedule. (**b**) Virus recovery from organs collected at 3 dpi from four chickens of each group was assessed in monolayer MDCK cells. The viral titer calculated as the average (n = 4) of each organ in the treatment group was compared to that of the control group; bar indicates the standard deviation. (*, $p < 0.05$).

Protective effect of a single dose of BXM in the simultaneous treatment of HPAI in the chicken model

Multiple administration of BXM (20 mg/kg) has provided complete protection in chickens against the lethal infection of HPAIV, whereas the same dose of PR has only partially achieved slight inhibition of virus growth (Figures 15a–b). Therefore, it was important to determine the minimum dose that could protect chickens from the lethal infection. When chickens were infected with 10 CLD_{50}/100 µL of BS/Akita/1/16 followed immediately by a single administration of different doses of BXM (0.1, 0.5, 2.5, 12.5, and 62.5 mg/kg), all the chickens in the groups administered with 2.5, 12.5, and 62.5 mg/kg survived for 14 days. However, the dosage of 0.1 and 0.5 mg/kg BXM only protected one of the four chickens treated (Figure 17a). Viral replication in chicken organs was completely inhibited in chickens treated with 2.5, 12.5, and 62.5 mg/kg BXM (Figures 17e–g), although low-titer virus was detected in the lung and brain of two chickens treated with 2.5 mg/kg BXM (Figure 17e). In the 0.1 and 0.5 mg/kg BXM-treated groups, the viral replication was completely inhibited in the organs of two of the four chickens (Figures 17c–d) compared with those in the control group where virus replication was relatively high (Figure 17b).

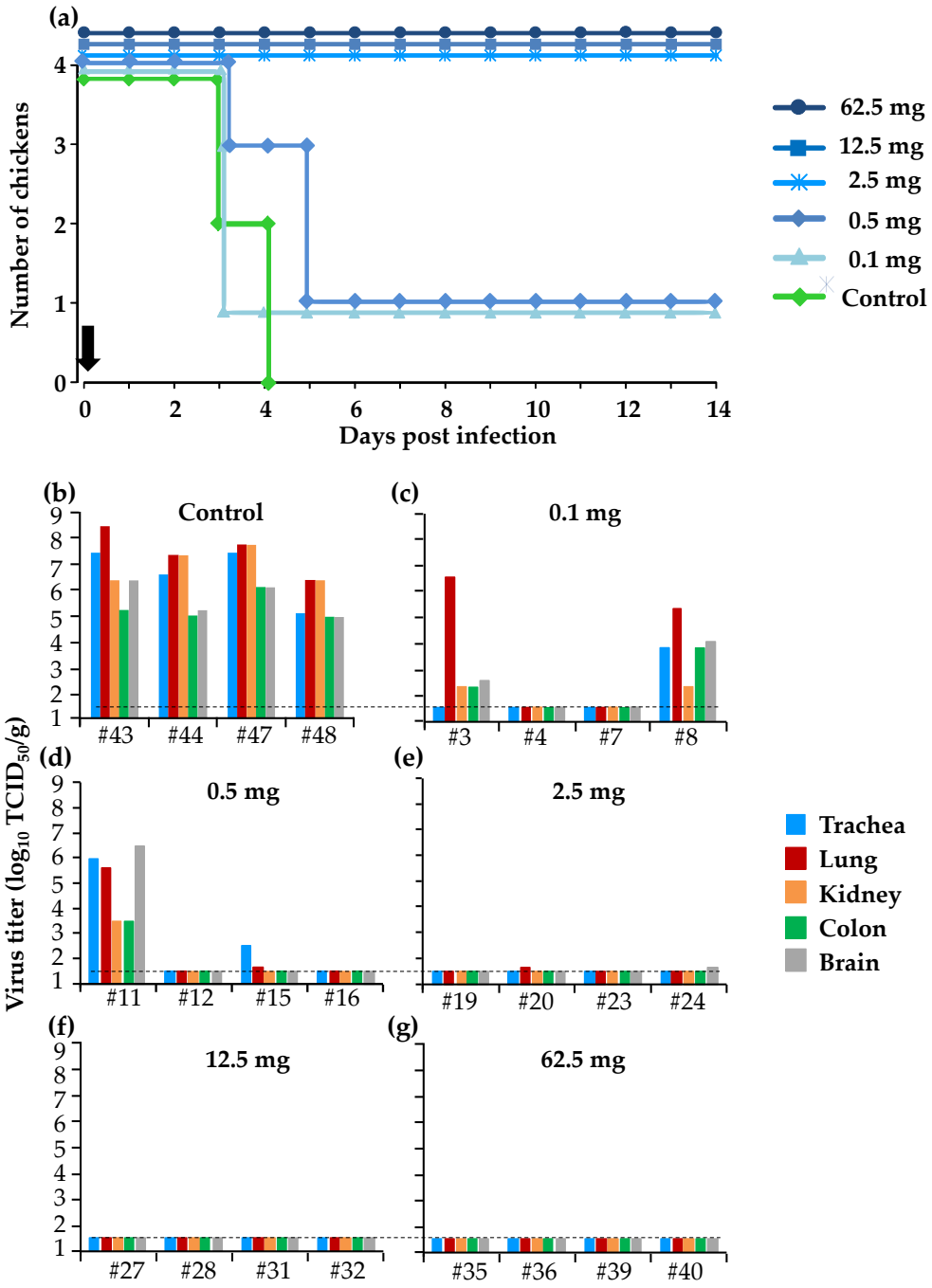

Figure 17. Effects of the simultaneous treatment with a single dose of BXM in chickens.

Eight chickens per treatment group were infected with 10 CLD_{50}/100 µL (5.3 $\log_{10}$ EID_{50}) of the HPAIV, BS/Akita/1/16, and they were immediately treated with different doses of BXM or untreated to serve as the control. (**a**) Survival of half the birds in each group was monitored until 14 dpi; the vertical black arrow indicates the treatment schedule. (**b**–**g**) Viral recovery from organs collected at 3 dpi from four chickens was assessed in MDCK cells; the black dotted line indicates the detection limit (1.5 $\log_{10}$ $TCID_{50}$/g).

Kinetics of BXA and PR in chickens and Pekin ducks

BXM and PR are drugs developed for the treatment of influenza in humans, and there is no available data yet concerning their dosage and kinetics in avian species. Given that the dosage of 20 mg/kg used in Experiments 1 and 2 was higher compared with that used clinically in humans [89,90], the concentration of the two drugs in blood was measured to adjust for appropriate dosage for birds. The mean plasma concentration for 20 mg/kg of BXA was 8808.9, 715.3, and 80.4 ng/mL at 8, 24, and 48 hpa, respectively. For PR, the mean plasma concentration was 27809.7, 7422.9, and 390.8 ng/mL at 0.5, 2, and 8 hpa, respectively (Figure 18a). This relatively high concentration was a consequence to the higher dose of drugs administered to chickens compared with their body weight (20 mg/kg).

After adjusting the BXM dosage to 2.5 mg/kg in chickens, the kinetics of BXA was assessed in chickens and ducks to compare the BXM metabolism in different bird species. In chickens, the maximum plasma concentration (C_{max}) was 171.3 ng/mL at 8 hpa, which decreased gradually to 0.14 ng/mL at 120 hpa. In ducks, the C_{max} was 122.8 ng/mL at 8 hpa, which decreased to 0.4 ng/mL at 120 hpa (Figure 18b). No significant difference was observed in the plasma concentration between the two species, indicating that the metabolism of BXM is similar between chickens and Pekin ducks.

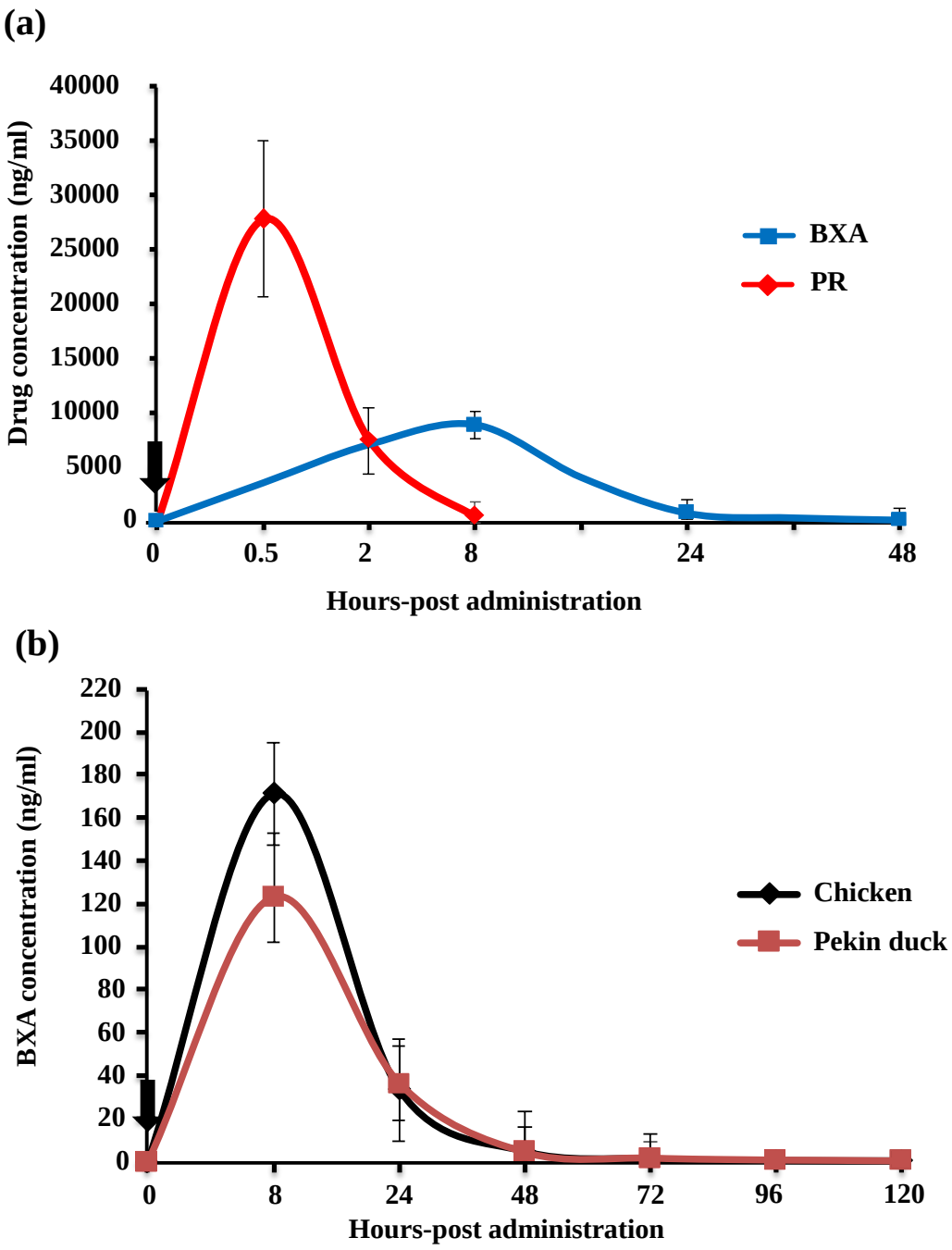

Figure 18. Drug concentration in the blood of chickens and Pekin ducks.

(**a**) Three chickens per group received 20 mg/kg of BXM or PR. The blood was collected at 0, 8, 24, and 48 hpa for BXM and at 0, 0.5, 2, and 8 hpa for PR. (**b**) BXA plasma concentration in chickens and Pekin ducks administered with 2.5 mg/kg of BXM. Blood was collected at 0, 8, 24, 48, 72, 96, and 120 hpa. The curve shows the mean plasma concentration of BXA and PR at different time points. Bar indicates the standard deviation (n = 3). The black vertical arrow indicates the drug administration schedule.

Virus shedding and antibody response in chickens

To determine how long the surviving chickens could shed virus following infection and single treatment with different doses of BXM, swabs were collected at 0, 3, 5, 7, and 14 dpi, and these were inoculated in eggs. No virus was recovered from swabs of surviving chickens treated with 0.1, 0.5, 2.5, 12.5, and 62.5 mg/kg; however, high virus titers from 2.7 to 4.2 $\log_{10}$ EID_{50}/mL were recovered from the swabs collected on three chickens that had died by 5 dpi in the 0.1 and 0.5 mg/kg treatment groups (Table 7). Furthermore, to ascertain whether the surviving chickens were infected, the antibody response against the challenge virus was examined. The HI titers for all surviving chickens ranged from 32 to 64 HI (Table 7). These results indicate that although infection was established in chickens by inoculation with HPAI virus, viral excretion and clinical symptoms were predominantly suppressed by BXM activity at a dose of 2.5 mg/kg or higher. Together, chicken survival and virus recovery can indicate that the protective effects of BXM were dose-dependent, and the minimum dose that conferred full protection to chickens from a lethal dose challenge of HPAIV infection is 2.5 mg/kg.

Table 7. Virus recovery in tracheal and cloacal swabs and antibody response in the serum of the chickens treated with different doses of BXM at single administration.

Treatment (mg/kg)	Chicken ID	Virus recovery from swabs on days post-challenge (log_{10} EID_{50}/mL) [a]					HI titer in serum [b] on days post challenge	
		0	3	5	7	14	0	14
		T/C	T/C	T/C	T/C	T/C		
Control	# 41	– /–	5.2 / 4.8	†	†	†	<2	†
	# 42	– /–	4.9 / 4.0	†	†	†	<2	†
	# 45	– /–	5.5 / 4.3	†	†	†	<2	†
	# 46	– /–	5.3 / 4.5	†	†	†	<2	†
0.1	# 1	– /–	4.2 / 3.5	†	†	†	<2	†
	# 2	– /–	– /–	– /–	– /–	– /–	<2	64
	# 5	– /–	3.8 / 2.7	†	†	†	<2	†
	# 6	– /–	4.2 / 3.5	†	†	†	<2	†
0.5	# 9	– /–	3.4 / 2.7	†	†	†	<2	†
	# 10	– /–	3.5 / 3.5	3.7 / 3.2	†	†	<2	†
	# 13	– /–	3.2 / 3.0	2.6 / 3.0	†	†	<2	†
	# 14	– /–	– /–	– /–	– /–	– /–	<2	64
2.5	# 17	– /–	– /–	– /–	– /–	– /–	<2	64
	# 18	– /–	– /–	– /–	– /–	– /–	<2	64
	# 21	– /–	– /–	– /–	– /–	– /–	<2	64
	# 22	– /–	– /–	– /–	– /–	– /–	<2	64
12.5	# 25	– /–	– /–	– /–	– /–	– /–	<2	32
	# 26	– /–	– /–	– /–	– /–	– /–	<2	64
	# 29	– /–	– /–	– /–	– /–	– /–	<2	64
	# 30	– /–	– /–	– /–	– /–	– /–	<2	64
62.5	# 33	– /–	– /–	– /–	– /–	– /–	<2	64
	# 34	– /–	– /–	– /–	– /–	– /–	<2	64
	# 37	– /–	– /–	– /–	– /–	– /–	<2	64
	# 38	– /–	– /–	– /–	– /–	– /–	<2	64

[a] The virus titer in tracheal and cloacal swab; (#) indicates the identification for each chicken; (–/–) indicates under detection limit (0.5 log_{10} EID_{50}/mL) for both T and C swab supernatant. [b] The antibody titer in the serum before and after infection.; (†) indicates that chicken died during the observation period; (T) and (C) indicate tracheal and cloacal swabs, respectively.

Discussion

HPAI remains to be a major concern for poultry and wild bird populations. Currently, there is no approved drug for the treatment of HPAIV infection in birds although other preventive measures can be applied, mostly for the poultry populations. To protect captive wild birds from HPAIV infection, studies have been carried out to establish its treatment and prevention using different models. Lee et al. [81], Kaleta et al. [80], and Meijer et al. [82] have evaluated the use of oseltamivir, although several limitations were noticed to apply it efficiently for AI treatment. Other studies have also evaluated the use of poultry vaccine for the prevention of HPAI caused by H5 and H7 virus strains in zoo birds [77,82,91]; however, an effective immune response was not induced in all of the vaccinated birds, which only implies that a proportion of birds remain at risk for HPAIV infection. Additionally, vaccine to AI is usually subtype- or strain-specific, whereas the AI outbreak is not predictable for such a vaccine specificity; in the case of emergency vaccination for a specific AI strain, the birds will still be exposed to infection during the seroconversion period. Moreover, in some countries, vaccine use for AI is prohibited for poultry and in wild bird populations. In this present study, the chickens were used, the most susceptible avian species to AIV, as an animal model to test for HPAIV infection, which has been known for its high virus replication in the organs and high mortality, mimicking a natural HPAI outbreak in the field. This model demonstrated that both BXM and PR were efficacious for the protection of chickens in simultaneous treatment, although in the group administered with PR, one out of four chickens died and virus replication was not completely inhibited. Taken together, these results provide an additional approach to overcome the limitations of vaccination

and other traditional measures used to protect captive wild birds in case of an HPAI outbreak.

Initially, a dose of 20 mg/kg of BXM or PR was administered to chickens; in the simultaneous treatment, the survival of birds and virus titer in organs differed between the BXM and PR groups, with clear beneficial effects for BXM (Figures 15a and b). Moreover, with delayed treatment, even though a net difference was not observed in both survival and the virus recovery, one chicken in the BXM group survived up to 6 dpi and the virus titer was lower compared with those in the control group (Figure 15a). These results indicate that BXM possessed greater protective effectiveness compared with that of PR; therefore, BXM was used for further experiments. The dosage of 20 mg/kg to chickens has been determined to be higher, compared to the dosage used in humans [84] and mice; therefore, this dose was adjusted this dose to 2.5 mg/kg, which was determined to be the minimum dose that protected 100% of infected chickens with complete inhibition of virus replication in the organs as well as virus shedding, although the virus was recovered at low titer in organs from two chickens in this treatment group (Figure 16e). This remnant viral population could be due to slower elimination of the virus from these chickens given that no virus was recovered in the swabs collected at 3 to 14 dpi from four chickens in the same group (Table 7). In summary, although dosage guidelines were provided in this study, it may not be possible to determine the appropriate dosage for each bird species without examining the concentration of BXA in blood. Nevertheless, administering 62.5 mg/kg to chickens, which is approximately 60-fold higher than that used in humans and mice, did not exhibit any side effects; this finding

suggests that for treatment purposes, the proposed dosage of 2.5 mg/kg or more can be administered to other bird species in case of HPAIV infection.

No virus was recovered in swabs collected from chickens treated with 2.5 mg/kg of BXM, although the antibody response revealed that these chickens were infected (Table 7). On the other hand, plasma concentration of BXA for the 2.5 mg/kg dose at 48 hs was 4.48 and 4.28 ng/mL in chickens and Pekin ducks, respectively, which is similar to the 6.92 and 6.85 ng/mL plasma concentrations reported to be effective for inhibition of virus replication in humans [85] and mice [92], respectively. This suggests that a single dose of 2.5 mg/kg can prevent virus replication and shedding from infected birds. However, HAIV is reported to persist up to 15 days in the environment after an outbreak, with the possibility of infecting other healthy birds in the flock [93-95]. Therefore, it would be necessary to administer four consecutive doses of 2.5 mg/kg at 48 h interval to an entire flock to maintain the BXA blood concentration above 4.28 ng/mL, which was assumed as the antiviral effect in chickens in this study; this would ensure full protection for all birds.

In summary, in this study, BXM and PR were demonstrated to be effective for the treatment HPAI virus infection in the simultaneous treatment. However, for the delayed treatment reflecting real-life field situation, the antiviral efficacy was limited, indicating that early administration would be essential in case of HPAI outbreak. The BXM treatment could be the first choice for protecting valuable wild birds from culling or death during an HPAI outbreak, whereas PR as a second choice might be used even though the antiviral effects were limited in this study. Using the chicken and duck models, the administration of 2.5 mg/kg of BXM induced sufficient plasma concentration of BXA to

protect birds from HPAI infection for 48 h. Further studies may be essential to optimize the dosage of BXM for treating HPAI in the wild bird species by evaluating the metabolism of the BXM for a variety of species despite the similarities observed in chickens and Pekin ducks in the current experiments.

Brief summary

In this study, two human antiviral drugs, BXM and PR, were evaluated in chickens as a model for the treatment of HPAIV infection in other bird species. At the dose of 20 mg/kg, BXM protected all chickens infected with an HPAIV; the virus replication in the chicken's organs, as well as the virus shedding in the swabs after the treatment, was completely suppressed. In contrast, the protective effect of PR was partially observed; the virus was detected in the chicken's organs and swabs after the treatment. To determine the appropriate dose to use for the treatment of HPAIV infection in other avian species, 2.5 mg/kg was determined as the minimum effective dose at a single administration in the simultaneous treatment. The blood kinetics of BXM at 2.5 mg/kg was similar in chickens and Pekin ducks. This suggests that BXM can be used as a prophylactic and therapeutic agent for HPAIV infection in other valuable bird species.

Conclusion

This study aims to provide relevant information that can be used to implement the effective control of AI and ND in bird populations. The intensive exploitation of poultry worldwide increases the number of susceptible avian species leading to the active circulation of AIVs and NDVs. The close contact between humans and poultry exposes the people to the AIVs of zoonotic potential; the virulent strains of AIV and NDV cause severe damage to the poultry industry. On the other hand, some valuable wild captive birds susceptible to AIV need to be protected from the natural death or stamping out during an HPAIV outbreak, and this might require antiviral drugs that can be used for their treatment.

Regarding the essential information for preventing AIV and NDV infection, this study focused on the DRC where outbreaks of HPAIV and NDV were recently reported. The AIV and NDV isolated during the outbreaks were characterized and detailed information was provided for establishing the adequate control measures not only in the DRC but also in the African continent given the transboundary character of the AI and ND.

This study demonstrated that migratory wild birds from Eurasia introduced the HPAIV that caused the outbreak, and the virus spread within the continent probably by cross-country migratory birds or cross-border poultry movements. The laboratory experimental study revealed that the HPAIV that caused the outbreak in the DRC increased the virulence in Muscovy ducks compared to the other viruses in the same clade 2.3.4.4b as observed in the field situation; suggesting that during the evolution, this virus changed its pathogenic character for different birds species.

Regarding the NDV detected in the DRC in 2018 and 2019, the study revealed two different genotypes of velogenic strains are circulating in the country. Among them, a new variant of the NDV was identified and the other isolated was closely related to the Asian strains, suggesting its origin. The antigenicity and vaccine potency of the NDVs isolated in the DRC demonstrated that the ND can well be controlled using the current vaccine; however, this required the implementation of a good vaccination program. Therefore, there is a need to assess further factors that allow the ND to be endemic in the DRC.

Although standard key elements for the control measures for the HPAIV infection, such as the surveillance, early diagnosis, and stamping out of infected birds, the vaccination can be applied additionally in some countries. Except for these control measures, this study evaluated the possibility of the treatment for HPAIV infection in valuable captive wild birds with two human antiviral drugs namely BXM and PR. In chicken as a model, BXM was effective to protect chickens infected with an HPAIV when the treatment started earlier than the onset of clinical signs; whereas PR showed limited therapeutic effects. The BXM treatment is expected to be applied in captive wild birds during an outbreak of HPAI to prevent the extinguishing of the rare breed.

In this thesis, the important information about the AIV and NDV isolated in the DRC was provided; this information is the baseline to implement effective control measures for both AIV and NDV infections in the DRC. Moreover, an effective treatment was suggested by using the chicken model, and this treatment may be used for some valuable wild captive birds infected with HPAIV. The findings in the three chapters will contribute to the control of AIV and NDV infection in the field.

Acknowledgements

This PhD work would not be achieved without the supervision, contribution, team working and collaboration of many people.

First, I would like to express my great appreciation to my principal supervisor Professor Yoshihiro Sakoda, Laboratory of Microbiology, Department of Infectious Disease, Faculty of Veterinary Medicine, Hokkaido University, Japan. Your continuous scientific and social support was immeasurable to achieve this work.

I express my gratitude to my vice-superior, Associate Professor Masatoshi Okamatsu and Associate Professor Norizaku Isoda, both in the Laboratory of Microbiology, Department of Infectious Disease, Faculty of Veterinary Medicine, Hokkaido University, Japan. Without your guidance, experience sharing, and encouragement throughout this study, it would not have been achieved easily.

My sincere acknowledgments to my academic advisors, Professor Ayato Takada, Division of Global Epidemiology, and Doctor Keita Matsuno, Unit of risk Analysis and Management; both in the Research Center for Zoonosis Control, Hokkaido University, Japan. Your guidance was greatly important to improve the scientific quality of this work.

I express my great acknowledgment to Professor Jean Jacque Muyembe, Director of the Institute of Biomedical Research, Ministry of Public Health; Professor Justin Masumu, Central Veterinary Laboratory of Kinshasa; and Professor Steve Mundeke Ahuka, Institute of Biomedical Research, Ministry of Public Health; all in the DRC. Your administrative and scientific contributions were greatly important to achieve this study.

I wish to express profound appreciation to Dr. Constant Sibitali Nsiyu Kysta, Dr. Julienne Sumbu Walandila, Dr. Serge Mpina Tshipambe, and Mr. Alex Madidi; from the

Central Veterinary Laboratory of Kinshasa; and Dr. Ezekiel Bushu Mulinde from the Veterinary Laboratory of Goma; all in the DRC. Your unwavering support for field activities was greatly important to achieve this study.

Many thanks to Dr Isabella Monne, Bianca Zecchin, and all staff from the Istituto Zooprofilattico Sperimentale Delle Venezie, Padova, Italy, for the great scientific collaboration.

To all the members and colleagues of the Laboratory of Microbiology, Department of Infectious Disease, Faculty of Veterinary Medicine; your collaboration and unwavering support were of inestimable value to produce this work.

This study would not be achieved without the financial contribution of the Environment Research and Technology Development, the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID), the Japan Agency for Medical Research and Development (AMED), the Agency for Medical Research and Development (AMED), and the Science and Technology Research Partnership for Sustainable Development (SATREPS).

I deeply appreciate the great contribution of the Advanced Training Program For Fostering Global Leaders on Infection Diseases Control funded by the Japan International Cooperation Agency (JICA), to provide financial and administrative support throughout the preparation of this study.

I wish to express my sincere mourning for the animals, chickens, ducks, egg embryos, and cells that were sacrificed to achieve this study.

To you my lovely wife Nancy Marthe Kapenda, my children Gracy Bwakudiyi, Allegracia Kansele, and Mickellange Twabela. You allowed me to leave you physically

during all the study period to achieve this goal. Please receive my heartfelt gratitude and acknowledgment for your daily support and encouragement.

References

1. Webster, R.G.; Bean, W.J.; Gorman, O.T.; Chambers, T.M.; Kawaoka, Y. Evolution and ecology of influenza A viruses. Microbiol. Rev. 1992, 56, 152–179.
2. Ottis, K.; Bachmann, P.A. Isolation and characterization of ortho- and paramyxoviruses from feral birds in Europe. Zentralbl Veterinarmed B 1983, 30, 22–35.
3. Pohlmann, A.; Hoffmann, D.; Grund, C.; Koethe, S.; Hüssy, D.; Meier, S.M.; King, J.; Schinköthe, J.; Ulrich, R.; Harder, T.; Beer, M. Genetic characterization and zoonotic potential of highly pathogenic Avian influenza virus A(H5N6/H5N5), Germany, 2017-2018. Emerg. Infect. Dis. 2019, 25, 1973–1976.
4. Widdowson, M.A.; Bresee, J.S.; Jernigan, D.B. The global threat of animal influenza viruses of zoonotic concern: then and now. J. Infect. Dis. 2017, 216, S493–S498.
5. Arbi, M.; Souiai, O.; Rego, N.; Larbi, I.; Naya, H.; Ghram, A.; Houimel, M. Historical origins and zoonotic potential of avian influenza virus H9N2 in Tunisia revealed by Bayesian analysis and molecular characterization. Arch. Virol. 2020, 165, 1527–1540.
6. Simulundu, E.; Nao, N.; Yabe, J.; Muto, N.A.; Sithebe, T.; Sawa, H.; Manzoor, R.; Kajihara, M.; Muramatsu, M.; Ishii, A., et al. The zoonotic potential of avian influenza viruses isolated from wild waterfowl in Zambia. Arch. Virol. 2014, 159, 2633–2640.
7. Twabela, A.T.; Tshilenge, G.M.; Sakoda, Y.; Okamatsu, M.; Bushu, E.; Kone, P.; Wiersma, L.; Zamperin, G.; Drago, A.; Zecchin, B., Monne, I. Highly pathogenic avian influenza A(H5N8) virus,Democratic Republic of the Congo, 2017. Emerg. Infect. Dis. 2018, 24, 1371–1374.
8. Swayne, D.E.; Spackman, E. Current status and future needs in diagnostics and vaccines for high pathogenicity avian influenza. Dev. Biol. 2013, 135, 79-94.
9. Donis, R.O.; Smith, G.; Brown, I.H.; Capua, I.; Cattoli, G.; Chen, H.; Cox, N.; Davis, C.T.; Donis, R.O.; Fouchier, R.A.M., et al. Continuing progress towards a unified nomenclature for the highly pathogenic H5N1 avian influenza viruses: Divergence of clade 2·2 viruses. Influenza Other Resp. 2009, 3, 59–62.
10. Donis, R.O.; Smith, G.J.D.; Perdue, M.L.; Brown, I.H.; Chen, H.; Fouchier, R.A.M.; Kawaoka, Y.; Mackenzie, J.; Shu, Y.; Capua, I., et al. Toward a unified nomenclature

system for highly pathogenic avian influenza virus (H5N1). Emerg. Infect. Dis. 2008, 14, e1.

11. Claes, F.; Morzaria, S.P.; Donis, R.O. Emergence and dissemination of clade 2.3.4.4 H5Nx influenza viruses - How is the Asian HPAI H5 lineage maintained. Curr. Opin. Virol. 2016, 16, 158–163.
12. Chen, Z.; Liu, X.; Zhao, M.; Liu, X.; Zhu, Y.; Zhang, Y.; Liu, W.; Zhao, K.; Duan, Z.; Gu, M., et al. Characterization of three H5N5 and one H5N8 highly pathogenic avian influenza viruses in China. Vet. Microbiol. 2013, 163, 351–357.
13. Lee, D. H.; Bahl, J., Torchetti, M. K.; Killian, M. L.; Ip, H. S.; DeLiberto, T. J.; Swayne, D. E. Highly Pathogenic Avian Influenza Viruses and Generation of Novel Reassortants, United States, 2014–2015. Emerg. Infect. Dis. 2016, 22, 1283–1285.
14. Lee, D. H.; Torchetti, M. K.; Winker, K.; Ip, H. S.; Song, C. S.; Swayne, D. E. Intercontinental spread of Asian-origin H5N8 to North America through Beringia by migratory birds. J. Virol. 2015, 89, 6521–6524.
15. Kang, H.M.; Lee, E.K.; Song, B.M.; Jeong, J.; Choi, J.G.; Jeong, J.; Moon, O.K.; Yoon, H.; Cho, Y.; Kang, Y.M., et al. Novel reassortant influenza A(H5N8) viruses among inoculated domestic and wild ducks, South Korea, 2014. Emerg. Infect. Dis. 2015, 21, 298–304.
16. Pohlmann, A.; Starick, E.; Harder, T.; Grund, C.; Hoper, D.; Globig, A.; Staubach, C.; Dietze, K.; Strebelow, G.; Ulrich, R.G., Schinköthe, J., Teifke, J. P., Conraths, F. J., Mettenleiter, T. C., Beer, M. Outbreaks among wild birds and domestic poultry caused by reassorted influenza A(H5N8) clade 2.3.4.4 viruses, Germany, 2016. Emerg. Infect. Dis. 2017, 23, 633–636.
17. Lee, D.H.; Bertran, K.; Kwon, J.H.; Swayne, D.E. Evolution, global spread, and pathogenicity of highly pathogenic avian influenza H5Nx clade 2.3.4.4. J. Vet Sci 2017, 18, 269–280.
18. Ghafouri, S.A.; GhalyanchiLangeroudi, A.; Maghsoudloo, H.; KH Farahani, R.; Abdollahi, H.; Tehrani, F.; Fallah, M.H. Clade 2.3.4.4 avian influenza A (H5N8) outbreak in commercial poultry, Iran, 2016: the first report and update data. Trop. Anim. Health Prod. 2017, 49, 1089–1093.

19. Fusaro, A.; Monne, I.; Mulatti, P.; Zecchin, B.; Bonfanti, L.; Ormelli, S.; Milani, A.; Cecchettin, K.; Lemey, P.; Moreno, A.; Massi, P.; Dorotea, T.; Marangon, S.; Terregino, C. Genetic diversity of highly pathogenic avian influenza A(H5N8/H5N5) viruses in Italy, 2016–17. Emerg. Infect. Dis. 2017, 23, 1543–1547.
20. Selim, A.A.; Erfan, A.M.; Hagag, N.; Zanaty, A.; Samir, A.-H.; Samy, M.; Abdelhalim, A.; Arafa, A.-S.A.; Soliman, M.A.; Shaheen, M.; Ibraheem, E. M.; Mahrous, I.; Hassan, M. K.; Naguib, M. M. Highly pathogenic avian influenza virus (H5N8) clade 2.3.4.4 infection in migratory birds, Egypt. Emerg. Infect. Dis. 2017, 23, 1048–1051.
21. World Organization for Animal Health. Update on Highly Pathogenic Avian Influenza in animals. 2015; 1-1. http://www.oie.int/animal-health-in-the-world-avian-influenza/2015/. Accessed 20 October 2018.
22. Ndumu, D.; Zecchin, B.; Fusaro, A.; Arinaitwe, E.; Erechu, R.; Kidega, E.; Kayiwa, J.; Muwanga, E.; Kirumira, M.; Kirembe, G., Lutwama, J., Monne, I. Highly pathogenic avian influenza H5N8 clade 2.3.4.4B virus in Uganda, 2017. Infect. Genet. Evol. 2018, 66, 269–271.
23. Wade, A.; Jumbo, S.D.; Zecchin, B.; Fusaro, A.; Taiga, T.; Bianco, A.; Rodrigue, P.N.; Salomoni, A.; Kameni, J.M.F.; Zamperin, G.; Nenkam, R.; Foupouapouognigni, Y.; Abdoulkadiri, S.; Aboubakar, Y.; Wiersma, L.; Cattoli, G.; Monne, I. Highly pathogenic avian influenza A(H5N8) virus, Cameroon, 2017. Emerg. Infect. Dis. 2018, 24, 1367–1370.
24. Bodewes, R.; Kuiken, T. Chapter twelve-changing role of wild birds in the epidemiology of avian influenza A viruses. Adv. Virus Res. 2018, 100, 279-307.
25. Fourment, M.; Darling, A.E.; Holmes, E.C. The impact of migratory flyways on the spread of avian influenza virus in North America. BMC Evol. Biol. 2017, 17, 118.
26. Basha, H.A.; Naby, W.S.; Heikal, H. Genetic diversity and phylogenetic relationship among three duck breeds and geese using RAPD markers. Adv. Anim. Vet. Sci. 2016, 4, 462–467.
27. Khan Ahmadi, A.; Rahimi, G.; Vafaei, A.; Sayyazadeh, H. Microsatellite analysis of genetic diversity in Pekin (*Anas platyrhynchos*) and Muscovy (*Cairina moschata)* duck populations. Int. J. Poult. Sci. 2007, 6, 378–382.

28. World Organization for Animal Health. Avian influenza. In manual of diagnostic tests and vaccines for terrestrial animals 2018, Chapter 3.3.4. https://www.oie.int/standardsetting/terrestrial-manual/access-online/2019/. Accessd 15 June 2019.
29. Reed L.J. and Muench, H. A simple method of estimating fifty per cent endpoints. Am. J. Epidemiol. 1938, 27, 493–497.
30. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 2016, 33, 1870–1874.
31. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 1980, 16, 111–120.
32. Wilson, I.A.; Skehel, J.J.; Wiley, D.C. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. Nature 1981, 289, 366–373.
33. Kida, H.; Yanagawa, R. Isolation and characterization of influenza a viruses from wild free-flying ducks in Hokkaido, Japan. Zbt. Bakt. Hyg. Orig. A. 1979, 244, 135–143.
34. Okamatsu, M.; Ozawa, M.; Soda, K.; Takakuwa, H.; Haga, A.; Hiono, T.; Matsuu, A.; Uchida, Y.; Iwata, R.; Matsuno, K., et al. Characterization of highly pathogenic avian influenza virus A(H5N6), Japan, November 2016. Emerg. Infect. Dis. 2017, 23, 691–695.
35. Smith, D.J.; Lapedes, A.S.; de Jong, J.C.; Bestebroer, T.M.; Rimmelzwaan, G.F.; Osterhaus, A.D.M.E.; Fouchier, R.A.M. Mapping the antigenic and genetic evolution of influenza virus. Science 2004, 305, 371–376.
36. Cai, Z.; Zhang, T.; Wan, X.-F. Antigenic distance measurements for seasonal influenza vaccine selection. Vaccine 2012, 30, 448–453.
37. Khomenko, S.; Abolnik, C.; Roberts, L.; Waller, L.; Shaw, K.; Monne. I.; Taylor, J.; Dhingra, M.; Pittiglio, C.; Mugyeom, M.; Roche, X.; Fredrick, K.; Kamata, A.; Okuthe, S.; Kone, P.; Wiersma, L.; Dobschuetz, S.V,; Soumare, B.; Makonnen, Y.; Morzaria, S.; Lubroth, J. 2016–2018 spread of H5N8 highly pathogenic avian influenza (HPAI) in sub-Saharan Africa: epidemiological and ecological. Food and Agriculture Organization of the United Nations Emerging Prevention System, Rome. FOCUS ON No. 12, Aug 2018
38. Moatasim, Y.; Kandeil, A.; Kayali, G.; Ali, M.A.; McKenzie, P.P.; Webby, R.J.; Kayed, A. Genetic characterization of highly pathogenic avian influenza A H5N8 viruses isolated from wild birds in Egypt. J. Gen. Virol. 2017, 98, 1573–1586.

39. Piersma, T.; Lindstrom, A. Migrating shorebirds as integrative sentinels of global. Ibis 2004, 146, 61–69.
40. Hiono, T.; Okamatsu, M.; Matsuno, K.; Haga, A.; Iwata, R.; Nguyen, L.T.; Suzuki, M.; Kikutani, Y.; Kida, H.; Onuma, M.; Sakoda, Y. Characterization of H5N6 highly pathogenic avian influenza viruses isolated from wild and captive birds in the winter season of 2016–2017 in Northern Japan. Microbiol. Immunol. 2017, 61, 387–397.
41. Nguyen, L.T.; Nishi, T.; Shichinohe, S.; Chu, D.H.; Hiono, T.; Matsuno, K.; Okamatsu, M.; Kida, H.; Sakoda, Y. Selection of antigenic variants of an H5N1 highly pathogenic avian influenza virus in vaccinated chickens. Virology 2017, 510, 252–261.
42. Anis, A.; AboElkhair, M.; Ibrahim, M. Characterization of highly pathogenic avian influenza H5N8 virus from Egyptian domestic waterfowl in 2017. Avian Pathol. 2018, 47, 400–409.
43. Uchida, Y.; Mine, J.; Takemae, N.; Tanikawa, T.; Tsunekuni, R.; Saito, T. Comparative pathogenicity of H5N6 subtype highly pathogenic avian influenza viruses in chicken, Pekin duck and Muscovy duck. Transbound. Emerg. Dis. 2019, 66, 1227–1251.
44. Grund, C.; Hoffmann, D.; Ulrich, R.; Naguib, M.; Schinköthe, J.; Hoffmann, B.; Harder, T.; Saenger, S.; Zscheppang, K.; Tönnies, M., et al. A novel European H5N8 influenza A virus has increased virulence in ducks but low zoonotic potential. Emerg. Microbes Infect. 2018, 7, 1–14.
45. Sun, H.; Pu, J.; Hu, J.; Liu, L.; Xu, G.; Gao, G.F.; Liu, X.; Liu, J. Characterization of clade 2.3.4.4 highly pathogenic H5 avian influenza viruses in ducks and chickens. Vet. Microbiol. 2016, 182, 116–122.
46. International committee on taxonomy of viruses. ICTV Taxonomy: 2018b. Release, Available at. https://talk.ictvonline.org/taxonomy/2019. Accessed on 11 November 2020.
47. Lamb, R.A.; Parks, P.G.; Paamyxoviridae. In: Knipe, D.M.; Howley, P.M. (eds) Fields Virology, 6th ed. Lippincott Williams & Wilkins, Philadelphia, USA. 2013,1, 957–995.
48. Samson, A.C.R.; Levesley, I.; Russell, P.H. The 36K polypeptide synthesized in Newcastle disease virus-infected cells possesses properties predicted for the hypothesized ‘V’ protein. J. Gen. Virol. 1991, 72, 1709–1713.
49. Steward, M.; Vipond, I.B.; Millar, N.S.; Emmerson, P.T. RNA editing in Newcastle disease virus. J. Gen. Virol. 1993, 74, 2539–2547.

50. de Leeuw, O.; Peeters, B. Complete nucleotide sequence of Newcastle disease virus: evidence for the existence of a new genus within the subfamily Paramyxovirinae. J. Gen. Virol. 1999, 80, 131–136.
51. Kim, D.; Martinez-Sobrido, L.; Choi, C.; Petroff, N.; García-Sastre, A.; Niewiesk, S.; Carsillo, T. Induction of type I interferon secretion through recombinant Newcastle disease virus expressing measles virus hemagglutinin stimulates antibody secretion in the presence of maternal antibodies. J. Virol. 2011, 85, 200–207.
52. Miller, P.J.; Estevez, C.; Yu, Q.; Suarez, D.L.; King, D.J. Comparison of viral shedding following vaccination with inactivated and live Newcastle disease vaccines formulated with wild-type and recombinant viruses. Avian Dis. 2009, 53, 39–49, 11.
53. Dimitrov, K.M.; Abolnik, C.; Afonso, C.L.; Albina, E.; Bahl, J.; Berg, M.; Briand, FX.; Brown, IH.; Choi, K.S.; Chvala, I.; Diel, D.G.; Durr, P.A.; Ferreira, H.L.; Fusaro, A.; Gil, P.; Goujgoulova, G.V.; Grund, C.; Hicks, J.T.; Joannis, T.M.; Torchetti, M.K.; Kolosov, S.; Lambrecht, B.; Lewis, N.S.; Liu, H.; Liu, H.; McCullough, S.; Miller, P.J.; Monne, I.; Muller, C..;, Munir, M.; Reischak, D.; Sabra, M.; Samal, S.K.; Servan de Almeida, R.; Shittu, I.; Snoeck, C.J.; Suarez, D.L.; Van Borm, S.; Wang, Z.; Wong, F.Y.K. Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. Infect. Gen. Evol. 2019, 74, 103917.
54. Alexander, D.J. Newcastle disease and other avian paramyxoviruses. Revue scientifique et technique (International Office of Epizootics) 2000, 19, 443–462.
55. Dimitrov, K.M.; Ramey, A.M.; Qiu, X.; Bahl, J.; Afonso, C.L. Temporal, geographic, and host distribution of avian paramyxovirus 1 (Newcastle disease virus). Infect. Genet. Evol. 2016, 39,22–34.
56. Nagai, Y.; Klenk, H.D.; Rott, R. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. Virology 1976, 72, 494–508.
57. Dortmans, J.C.F.M.; Koch, G.; Rottier, P.J.M.; Peeters, B.P.H. Virulence of Newcastle disease virus: what is known so far? Vet. Res. 2011, 42, 122.
58. African–Union, Interafrican Bureau for Animal Resources (A.U.-I.B.F.A). Nwecastel Disease 2011. Available online: www.au-ibar.org/2012-10-01-13./348-newcastle-disease. Accessed on 15 October 2020.

59. World Organization for Animal Health (OIE). Newcastle disease virus (Infection with Newcastle disease virus). In the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019. OIE: Paris, France, 2019; pp 964–983. Avialable online: https://www.oie.int/standard-setting/terrestrial-manual/access-online/. Accessed on 10 October 2020.
60. Gravendyck, M.; Tritt, S.; Spenkoch‐Piper, H.; Kaleta, E.F. Antigenic diversity of psittacine herpesviruses: Cluster analysis of antigenic differences obtained from cross‐neutralization tests. Avian Pathol. 1996, 25, 345–357.
61. Archetti , I.; Horsfall , F.L., Jr. Persistent antigenic variation of influenza a viruses after incomplete neutralization in ovo with heterologous immune serum. J. Exp. Med. 1950, 92, 441–462.
62. Lwapa, F.E.I.; Masumu, J.M.; Matala, F.M.; Mukoko, F.N.; Mbao, V.; Moula, N.; Antoine-Moussiaux, N. Participatory assessment of paid vaccination campaigns for village chickens against Newcastle disease in Kongo Central province. Pre. Vet. Med. 2019, 172, 104783.
63. Young, M.B.; Alders, R.; Grimes, S.; Spradbrow, P.B.; Dias, P.; Silva, A.D.; Lobo, Q. Controlling Newcastle disease in village chickens–A laboratory manual; monographs, australian centre for international agricultural research: Canberra, Australia, 2002, 117714, 46–51.
64. Miller, P.J.; King, D.J.; Afonso, C.L.; Suarez, D.L. Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge. Vaccine 2007, 25, 7238–46.
65. Cattoli, G.; Fusaro, A.; Monne, I.; Molia, S.; Le Menach, A.; Maregeya, B.; Nchare, A.; Bangana, I.; Maina, A.G.; N'Goran Koffi, J.N., et al. Emergence of a new genetic lineage of Newcastle disease virus in West and Central Africa–Implications for diagnosis and control. Vet. Microbiol. 2010, 142, 168–176.
66. Capua, I.; Alexander, D.J. Avian influenza: recent developments. Avian Pathol. 2004, 33, 393–404.
67. de Wit, E.; Kawaoka, Y.; de Jong, M.D.; Fouchier, R.A.M. Pathogenicity of highly pathogenic avian influenza virus in mammals. Vaccine 2008, 26 Suppl 4, D54–D58.

68. Alexander, D.J.; Parsons, G.; Manvell, R.J. Experimental assessment of the pathogenicity of eight avian influenza a viruses of H5 subtype for chickens, turkeys, ducks and quail. Avian Pathol. 1986, 15, 647–662.
69. Oh, S.; Martelli, P.; Hock, O.S.; Luz, S.; Furley, C.; Chiek, E.J.; Wee, L.C.; Keun, N.M. Field study on the use of inactivated H5N2 vaccine in avian species. Vet. Rec. 2005, 157, 299–300.
70. Krone, O.; Globig, A.; Ulrich, R.; Harder, T.; Schinköthe, J.; Herrmann, C.; Gerst, S.; Conraths, F.J.; Beer, M. White-tailed sea eagle (*Haliaeetus albicilla*) die-off due to infection with highly pathogenic avian influenza virus, subtype H5N8, in Germany. Viruses 2018, 10, 478.
71. Peyre, M.; Fusheng, G.; Desvaux, S.; Roger, F. Avian influenza vaccines: a practical review in relation to their application in the field with a focus on the Asian experience. Epidemiol. Infect. 2009, 137, 1–21.
72. Swayne, D.E. Impact of vaccines and vaccination on global control of avian influenza. Avian Dis. 2012, 56, 818–828.
73. Ohkawara, A.; Okamatsu, M.; Ozawa, M.; Chu, D.H.; Nguyen, L.T.; Hiono, T.; Matsuno, K.; Kida, H.; Sakoda, Y. Antigenic diversity of H5 highly pathogenic avian influenza viruses of clade 2.3.4.4 isolated in Asia. Microbiol. Immunol. 2017, 61, 149–158.
74. World Health Organization. Antigenic and genetic characteristics of zoonotic influenza viruses and development of candidate vaccine viruses for pandemic preparedness. Weekly Epidemiological Record, 2017, 90, 561–571.
75. Gulbudak, H.; Martcheva, M. A structured avian influenza model with imperfect vaccination and vaccine-induced asymptomatic infection. Bull. Math. Biol. 2014, 76, 2389–2425.
76. Kakogawa, M.; Manubu, O.; Kirisawa, R.; Asakawa, M. Countermeasures for avian influenza outbreaks among captive avian collections at zoological gardens and aquariums in Japan. Microbiol. Exp. 2019, 7, 167–171.
77. Philippa, J.D.W.; Munster, V.J.; Bolhuis, H.v.; Bestebroer, T.M.; Schaftenaar, W.; Beyer, W.E.P.; Fouchier, R.A.M.; Kuiken, T.; Osterhaus, A.D.M.E. Highly pathogenic avian influenza (H7N7): vaccination of zoo birds and transmission to non-poultry species. Vaccine 2005, 23, 5743–5750.

78. Shie, J.J.; Fang, J.M. Development of effective anti-influenza drugs: congeners and conjugates – a review. J. Biomed. Sci. 2019, 26, 84.
79. Principi, N.; Camilloni, B.; Alunno, A.; Polinori, I.; Argentiero, A.; Esposito, S. Drugs for influenza treatment: is there significant news? Front. Med. (Lausanne) 2019, 6, 109–109.
80. Kaleta, E.F.; Blanco Peña, K.M.; Yilmaz, A.; Redmann, T.; Hofheinz, S. Avian influenza A viruses in birds of the order Psittaciformes: reports on virus isolations, transmission experiments and vaccinations and initial studies on innocuity and efficacy of oseltamivir in ovo. Dtsch Tierarztl Wochenschr 2007, 114, 260–267.
81. Lee, D.H.; Lee, Y.N.; Park, J.K.; Yuk, S.S.; Lee, J.W.; Kim, J.I.; Han, J.S.; Lee, J.B.; Park, S.Y.; Choi, I.S.; Song, C.S. Antiviral Efficacy of Oseltamivir against avian influenza virus in avian species. Avian Dis. 2011, 55, 677–679.
82. Meijer, A.; van der Goot, J.A.; Koch, G.; van Boven, M.; Kimman, T.G. Oseltamivir reduces transmission, morbidity, and mortality of highly pathogenic avian influenza in chickens. Int. Congr. 2004, 1263, 495–498.
83. Afonso, C.L., Miller, P. J., Grund, C., Koch, G., Peeters, B., Selleck, P. W.,; & Srinivas, G.B. Manual of diagnosis tests and vaccines for terrestrial animals. 7th ed.;World Organization for Animal Health: Paris, France, 2012, 2, 865–878.
84. Hayden, F.G.; Sugaya, N.; Hirotsu, N.; Lee, N.; de Jong, M.D.; Hurt, A.C.; Ishida, T.; Sekino, H.; Yamada, K.; Portsmouth, S., et al. Baloxavir marboxil for uncomplicated influenza in adults and adolescents. N. Engl. J. Med. 2018, 379, 913–923.
85. Koshimichi, H.; Ishibashi, T.; Kawaguchi, N.; Sato, C.; Kawasaki, A.; Wajima, T. Safety, tolerability, and pharmacokinetics of the novel anti-influenza agent baloxavir marboxil in healthy adults: phase I study findings. Clin. Drug Investig. 2018, 38, 1189–1196.
86. Yun, N.E.; Linde, N.S.; Zacks, M.A.; Barr, I.G.; Hurt, A.C.; Smith, J.N.; Dziuba, N.; Holbrook, M.R.; Zhang, L.; Kilpatrick, J.M.; Arnold, C. S.; & Paessler, S. Injectable peramivir mitigates disease and promotes survival in ferrets and mice infected with the highly virulent influenza virus, A/Vietnam/1203/04 (H5N1). Virology 2008, 374, 198–209.

87. Dillon, R.C.; Witcher, R.; Cies, J.J.; Moore, W.S., 2nd; Chopra, A. Pharmacokinetics of Peramivir in an Adolescent Patient Receiving Continuous Venovenous Hemodiafiltration. J. Pediatr. Pharmacol. Ther. 2017, 22, 60–64.
88. Koshimichi, H.; Tsuda, Y.; Ishibashi, T.; Wajima, T. Population pharmacokinetic and exposure-response analyses of baloxavir marboxil in adults and adolescents including patients with influenza. J. Pharm. Sci. 2019, 108, 1896–1904.
89. McKimm-Breschkin, J.L.; Jiang, S.; Hui, D.S.; Beigel, J.H.; Govorkova, E.A.; Lee, N. Prevention and treatment of respiratory viral infections: Presentations on antivirals, traditional therapies and host-directed interventions at the 5th ISIRV Antiviral Group conference. Antivir. Res. 2018, 149, 118–142.
90. Omoto, S.; Speranzini, V.; Hashimoto, T.; Noshi, T.; Yamaguchi, H.; Kawai, M.; Kawaguchi, K.; Uehara, T.; Shishido, T.; Naito, A., et al. Characterization of influenza virus variants induced by treatment with the endonuclease inhibitor baloxavir marboxil. Sci. Rep. 2018, 8, 9633.
91. Philippa, J.; Baas, C.; Beyer, W.; Bestebroer, T.; Fouchier, R.; Smith, D.; Schaftenaar, W.; Osterhaus, A. Vaccination against highly pathogenic avian influenza H5N1 virus in zoos using an adjuvanted inactivated H5N2 vaccine. Vaccine 2007, 25, 3800–3808.
92. Takeshi Noshi, K.S., Toru Ishibashi, Yoshinori Ando, Hiroshi Ueda, Ryoko Oka, Makoto Kawai, Ryu Yoshida, Akihiko Sato, Takao Shishido, and Akira Naito. Pharmacokinetic and pharmacodynamic analysis of S-033188/S-033447. A novel inhibitor of influenza virus Cap-dependent endonuclease, in mice infected with influenza A virus [P1973]. In: Final Programme of the 27th European congress of clinical Microbiology and Infectious Diseases (Vienna, Australia). 2017, 22–25.
93. Bean, B.; Moore, B.M.; Sterner, B.; Peterson, L.R.; Gerding, D.N.; Balfour, H.H. Survival of influenza viruses on environmental surfaces. J. Infect. Dis. 1982, 146, 47-51.
94. Wood, J.P.; Choi, Y.W.; Chappie, D.J.; Rogers, J.V.; Kaye, J.Z. Environmental persistence of a highly pathogenic avian influenza (H5N1) Virus. Envir. Sci. Technol. 2010, 44, 7515–7520.
95. Yamamoto, Y.; Nakamura, K.; Yamada, M.; Mase, M. Persistence of avian influenza virus (H5N1) in feathers detached from bodies of infected domestic ducks. Appl. Environ. Microbiol. 2010, 76, 5496–5499.