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Different pathogenicity of two strains of clade 2.3.4.4c H5N6 highly pathogenic avian influenza viruses bearing different PA and NS gene in domestic ducks

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ABSTRACT

H5Nx clade 2.3.4.4 highly pathogenic avian influenza viruses (HPAIVs) have been disseminated to wide geographic regions since 2014. In 2016, five distinct genotypes (C-1 to C-5) of clade 2.3.4.4c H5N6 HPAIVs were detected in South Korea. In this study, we evaluated the pathogenicity, susceptibility to infection, and transmissibility of the two strains representing the C-1 and C-4 genotypes of the H5N6 viruses, which have different PA and NS gene, in domestic ducks. Although the susceptibility to infection of domestic ducks to the two strains was similar, the C-4 genotype virus induced higher mortality in ducks than C-1 genotype virus. A higher titer of viral shedding were detected in ducks challenged with the C-4 genotype virus compared with the C-1 genotype virus. These results indicated that the reassortment of HPAIVs with prevailing low pathogenic avian influenza viruses could effect on the pathogenicity in ducks.

1. Introduction

The A/Goose/Guangdong/1/96 (Gs/Gd) lineage of H5 highly pathogenic avian influenza viruses (HPAIVs) have caused continuous outbreaks in Asian countries since their first detection in 1996 (Lee et al., 2017a; Sonnberg et al., 2013). These Gs/Gd H5 HPAIVs have evolved into a number of genetically distinct multiple clades and subclades (Sonnberg et al., 2013). Since 2008, various novel subtypes of Gs/Gd lineage H5 HPAIVs belonging to clade 2.3.4, including H5N2, H5N3, H5N5, H5N6, and H5N8, have been detected in wild and poultry in China. Subsequently, the HPAIV clade 2.3.4.4 has evolved into four distinct subgroups (A–D) (Lee et al., 2016a). Among them, subgroup A and B H5N8 viruses spread to various regions of Asia, Europe, and North America (Lee et al., 2017a). Migratory wild waterfowl were most probably responsible for the long-distance transmission of HPAIVs (The Global Consortium for H5N8 and Related Influenza Viruses, 2016).

In China, subgroup C and D clade 2.3.4.4 H5N6 HPAIVs have become endemic in duck species and replaced H5N1 between 2014 and 2016 (Bi et al., 2016). At least 34 distinct genotypes of H5N6 viruses, derived by reassortment with various low pathogenic avian influenza viruses (LPAIVs), have been reported in China. These H5N6 HPAIVs were also detected from mammalian species, such as pig, cat and humans, and therefore present a potential threat to public health (Bi et al., 2016). In October 2016, novel H5N6 viruses that originated from the G1.1.9 genotype virus in China, belonging to subgroup C, caused outbreaks in South Korea and subsequently evolved into five distinct genotypes (C-1 to C-5) (Kwon et al., 2017a; Lee et al., 2017b). Although the C-1 genotype was only detected in wild birds, the C-4 genotype was the dominant genotype in poultry and wild birds during the outbreak in South Korea (Ministry of Agriculture, Food and Rural Affairs, Republic of Korea).

Although most HPAIVs strains induce 100% mortality in gallinaceous species, the mortality is varied and usually low in duck species, including domestic ducks, mallard (*Anas platyrhynchos*) and mandarin duck (*Aix galericulata*) (Kang et al., 2017; Kim et al., 2009; Kwon et al., 2017b). Previous studies indicated that the distribution of domestic

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duck density and wild waterfowl migration were important factors in HPAIV outbreaks (Gilbert et al., 2010; Hill et al., 2015). Further, many studies indicated that duck species could be infected by HPAIVs, especially clade 2.3.4.4 viruses, and shed a high titer of viruses without clinical signs (Hiono et al., 2017; Lee et al., 2016b; Pantin-Jackwood et al., 2017; Sun et al., 2016). Therefore, duck species may be important as a healthy reservoir in HPAIV epidemiology.

The information about the pathogenicity and susceptibility to infection of novel strains in duck species was important for the HPAIV control strategy. Although the C-1 genotype virus was first identified in South Korea and was genetically similar to the Chinese origin viruses, the C-4 genotype viruses which had different PA and NS genes became the dominant genotype in poultry and wild birds (Ministry of Agriculture, Food and Rural Affairs, Republic of Korea). To determine if the biological characteristic in ducks have changed for these two genotypes showing different epidemiologic characteristics, we compared the pathogenicity, susceptibility to infection, and transmissibility between two viruses representing two genotypes, C-1 and C-4, of the H5N6 clade 2.3.4.4 virus in two-week-old domestic Pekin ducks (Anas platyrhynchos domesticus). Further, we compared the complete genome sequences of these two strains to identify the possible molecular determinants responsible for the increased pathogenicity in ducks.

2. Materials and methods

2.1. Animals

We used 45 two-week-old domestic ducks that were acquired from a breeding duck farm. All the ducks were tested for influenza virus infection by viral RNA detection and serological testing prior to their use in the studies. Oral and cloacal swabs from each bird were analyzed by matrix gene-based real-time RT-PCR, as described previously (Spackman et al., 2003). The serum samples collected from birds were serologically analyzed for influenza type A nucleoprotein (NP)-specific antibody by using a commercial competitive NP-ELISA kit for multi-species (Bionote, Korea), in accordance with the manufacturer's instructions, and for H5 subtype hemagglutinin (HA) protein-specific antibody by using a hemagglutination inhibition (HI) assay. All birds were negative for viral RNA detection and serological testing. All birds used in this study were housed in self-contained isolation units (2–3 ducks per isolation unit) ventilated under negative pressure with HEPA-filtered air. Commercial poultry feed and fresh water were provided daily. All experiments were performed in a biosafety level 3 (BSL3) facility at Konkuk University. All animal procedures performed in this study were reviewed, approved, and supervised by the Institutional Animal Care and Use Committee (IACUC) (No. KU16225) and the Institutional Biosafety Committee (No. 2016-019) of Konkuk University.

2.2. Viruses

The A/Mandarin_duck/Korea/K16-187-3/2016 (K16-187-3) [EpiFlu isolate ID: EPI_ISL_238148], belonging to the C-1 genotype, and the A/duck/Korea/ES2/2016 (ES2) [EpiFlu isolate ID: EPI_ISL_239262], belonging to C-4 genotype, of the clade 2.3.4.4 subgroup C H5N6 viruses were used in this study. The K16-187-3 virus was isolated from fecal samples from mandarin duck at 28th October 2016 and it was first report of H5N6 HPAIV detection from wild birds in South Korea in 2016 (Kwon et al., 2017a). The ES2 virus was isolated from a commercial duck farm with high mortality at 16th November 2016 and it is first outbreak of H5N6 HPAIV in poultry farm (Lee et al., 2017b). The ES2 virus was provided by the Animal and Plant Quarantine Agency, South Korea. These viruses were propagated by using 9- to 11-day-old embryonated chicken eggs and the second egg passage viruses were used for experimental inoculation of ducks. The 50% egg infectious dose (EID_{50}) of virus stocks were calculated by the method of Reed and Muench using serial 10-fold dilutions in phosphate-buffered saline (PBS) and 9- to 11-day-old embryonated chicken eggs. The viral titers of virus stocks were determined as $10^{8.5}EID_{50}$/mL for K16-187-3 virus (C-1 genotype) and $10^{9.0}$ EID_{50}/mL for ES2 virus (C-4 genotype).

2.3. Experimental design

Twenty ducks were used for the challenge experiment for each virus and the control group contained five ducks. To evaluate the mean bird infectious dose (BID_{50}) and the mean bird lethal dose (BLD_{50}), the ducks were divided into three groups for each virus (5–7 ducks). Each duck was inoculated via the intranasal (0.1 mL) and choanal cleft (0.1 mL) with 10^2, 10^4, or 10^6 EID_{50} in 0.2 mL of each virus. To evaluate transmissibility, three uninoculated ducks were co-housed with the birds inoculated with 10^6 EID_{50} at 1 day post inoculation (dpi). Each un-inoculated duck was co-housed with one inoculated duck. Five sham ducks were housed in isolated cages and inoculated with 0.2 mL of PBS via the intranasal and choanal cleft.

The ducks were monitored daily for illness or death until 14 dpi by veterinarians. Oropharyngeal and cloacal swabs were collected in 1 mL PBS from the virus-inoculated birds at 1–7 and 10 dpi to detect viral shedding. For histological examination, two ducks challenged with 10^6 EID_{50} of each virus and two sham inoculated ducks were euthanized and eight organs (brain, trachea, heart, lung, liver, spleen, pancreas, and kidney) were collected at 3 dpi. Necropsies were performed on ducks that died during the experiment and the same organs were collected. Blood samples were collected at 14 dpi to determine seroconversion.

2.4. Virus detection and quantification

To assess viral shedding, the oropharyngeal and cloacal swab samples collected at 1–7 and 10 dpi were suspended in 1 mL PBS. From this suspension, 200 μL was used for the extraction of RNA by using MagNA Pure 96 DNA and Viral NA Small Volume Kit on the MagNA Pure 96 instrument (Roche Applied Sciences, Germany) in accordance with the manufacturer's instructions. The amount of influenza virus RNA was quantified by the cycle threshold (**Ct**) value using rRT-PCR (Spackman et al., 2003).

To extrapolate the Ct values to infectious units, known titers of the HPAI virus from egg allantoic fluid were subjected to 10-fold serial dilutions and viral RNA was extracted from these dilutions and quantified by rRT-PCR, as described above. The resulting standard curve showed a high correlation ($r^2 > 0.99$) and was used to convert the Ct values to EID_{50} equivalents/mL. The detection limit was $10^{1.4}$ EID_{50} equivalents/mL (Ct value 35).

2.5. Serology

To confirm the seroconversion of birds, serum samples were collected 1 day prior to infection and 2 weeks after infection. To detect homologous anti-H5 antibodies, serum samples collected from wild birds were treated with receptor-destroying enzymes and tested for HI antibodies to the virus by using homologous antigens inactivated by formalin with 1% turkey red blood cells (RBC). We used the commercially available multispecies competitive NP-ELISA Kit (Bionote, Korea) to detect anti-influenza A NP-specific antibodies as a means to identify influenza A virus infection, including non-H5 influenza. The ELISA was performed in accordance with the manufacturer's instructions.

2.6. Histopathology and immunohistochemistry

Paraffin sections were cut (5 μm), dewaxed, and stained with hematoxylin and eosin. Duplicate sections were subjected to immunohistochemical analysis to examine the distribution of influenza virus antigens in individual tissues. Briefly, sections were stained with a mouse monoclonal antibody against influenza A virus nucleoprotein

(MCA-400, AbD Serotec, Dusseldorf, Germany), followed by a biotinylated goat anti-mouse IgG secondary antibody. The bound antibodies were detected by using an avidin-biotin detection system (Ventana Medical Systems, Tucson, AZ, USA). The RedMap kit (Ventana Medical Systems, Tucson, AZ, USA) served as the substrate chromogen.

2.7. Sequence analysis

For the molecular analysis of the challenged viruses, RNA was extracted from virus-propagated allantoic fluids by using the RNeasy kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. Eight genes from each virus were amplified by using two-step reverse-transcription PCR, as previously described (Hoffmann et al., 2001). Nucleotide sequencing was performed by using the BigDye Terminator v3.1 Cycle Sequencing Kit and the products were analyzed using the ABI PRISM 3730xl genetic analyzer (Applied Biosystems, Foster City, CA). The nucleotide sequences for the complete coding regions of H5N2 HPAIV were aligned by using MUSCLE (Edgar, 2004). The complete coding regions of each segment were aligned and used for subsequent single-nucleotide polymorphism (SNP) analysis using the Geneious v8.1.2 program (Katoh and Toh, 2008).

For the phylogenetic analysis, a maximum likelihood tree was computed by using MEGA 6 with the Hasegawa-Kishino-Yano model of nucleotide substitutions and γ-distributed rate variation among sites with four rate categories (Tamura et al., 2013). The statistical analysis of the phylogenetic tree was assessed using a bootstrap analysis with 1000 replicates.

3. Results

3.1. Infectivity, mortality, and clinical signs

Sham ducks and ducks inoculated with $10^{2.0}$ EID_{50} of both C-1 and C-4 genotype viruses did not show viral shedding, seroconversion, and clinical signs. All ducks inoculated with $10^{4.0}$ EID_{50} and $10^{6.0}$ EID_{50} of both viruses excreted virus and all the surviving birds showed seroconversion in the HI test. Ducks were considered to be infected if they showed viral shedding, seroconversion, or mortality. The BID_{50} of both viruses was $10^{3.0}$ EID_{50} in ducks (Table 1).

Although the two strains showed the same BID_{50} in ducks, the BLD_{50} of the C-4 genotype virus ($10^{4.0}$ EID_{50}) was much lower than that of the C-1 genotype virus (> $10^{6.0}$ EID_{50}). The C-1 genotype virus caused mortality in two out of five ducks infected with $10^{6.0}$ EID_{50} dose, with a mean death time (MDT) of 3.5 days. However, the C-4 genotype virus caused mortality in three out of five ducks infected with $10^{4.0}$ EID_{50} dose, all five ducks infected with $10^{6.0}$ EID_{50} dose, and all three ducks co-housed with $10^{6.0}$ EID_{50} dose-infected ducks, with a MDT of 6.7–7.3 days. All dead ducks showed severe lethargy and low feed and water consumption at 1–2 days before death, except for one bird inoculated with $10^{6.0}$ EID_{50} dose of the C-4 genotype virus, which showed severe neurological signs, torticollis, from 7 dpi and died at 14 dpi (Table 1, Fig. 1).

Although two out of the three ducks co-housed with $10^{6.0}$ EID_{50} dose C-1 genotype virus-inoculated ducks became infected, all three ducks co-housed with $10^{6.0}$ EID_{50} dose C-4 genotype virus-inoculated ducks became infected. As one $10^{6.0}$ EID_{50} dose C1 genotype virus-infected duck died at 2 dpi, it was suspected that the matching co-housed birds were not exposed to a sufficiently high viral dose and were exposed for a shorter period than the other co-housed birds. A significant difference in transmissibility was not detected in this study (Table 1).

These results indicated that although ducks have similar susceptibility to infection to these two strains, the C-4 genotype virus induced higher mortality in ducks than C-1 genotype virus.

3.2. Viral shedding

Viral shedding was detected in all ducks inoculated with $10^{4.0}$ EID_{50} and $10^{6.0}$ EID_{50} and ducks co-housed with ducks inoculated with $10^{6.0}$ EID_{50} of both viruses. The viral RNA was detected from 1 dpi to 10 dpi, and more often and in higher titers, from the swab samples from the oropharynx than the cloaca. The peak viral shedding from the oropharynx occurred 3–5 days after infection with both viruses. A higher titer of viral shedding was detected in ducks exposed to the C-4 genotype virus than those exposed to the C-1 genotype virus. The peak amount of viral shedding from the oropharynx was between $10^{4.58}$ EID_{50} equivalent/mL (Ct = 24.07) to $10^{3.21}$ EID_{50} equivalent/mL (Ct = 28.79) in the ducks exposed to the C-1 genotype of virus and from $10^{5.31}$ EID_{50} equivalent/mL (Ct = 21.54) to $10^{3.89}$ EID_{50} equivalent/mL (Ct = 26.44) in the ducks exposed to the C-4 genotype of virus (Fig. 2).

3.3. Gross lesions, histopathology, and immunohistochemistry

All dead birds were necropsied to identify gross lesions. Multi-focal necrotic foci or hemorrhage in the pancreas and necrotic foci in the cardiac muscle were observed in all dead ducks infected with both viruses. One of two dead bird infected by C-1 genotype and four of nine dead birds infected by C-4 genotype virus presented with more severe gross lesions, such as swollen liver, severe ascites, and severe necrosis in the cardiac muscle (Fig. 3).

Eight organs (brain, trachea, heart, lung, liver, spleen, pancreas, and kidney) were collected from all necropsied ducks and histopathologic examination and immunohistochemistry (IHC) were performed. Although the severity of lesions and degree of virus antigen expression appears to be slightly more severe in C-1 genotype virus inoculated ducks sacrificed at 3 dpi than in the C-4 genotype virus inoculated ducks, mortality was higher in C-4 genotype virus infected ducks than C-1 genotype virus infected ducks after 3 dpi and the severe histologic lesion and antigen expression were detected in dead ducks. These results indicated that although both of viruses caused systemic infection and induced lesions, C-4 genotype virus induced severe lesions that can cause death in more ducks than C-1 genotype virus after 3 dpi.

Histological lesions were prominently observed in the lung, liver,

Table 1
Mortality, mean death time, viral shedding, serology, mean bird infectious dose, and mean bird lethal dose of the C-1 and C-4 genotypes of H5N6 viruses.

Challenge virus	Challenge dose log (EID_{50})	Number of infected birds/total	Mortality (MDT)	Viral shedding (positive/total)	HI assay (positive/total) (mean titer)	BID_{50} log (EID_{50})	BLD_{50} log (EID_{50})
K16-187-3 H5N6 (C-1 genotype)	2.0	0/5	0/5	0/5	0/5	3.0	> 6.0
	4.0	5/5	0/5	5/5	5/5 (6.6)		
	6.0	7/7	2/5 (3.5 days)	7/7	3/3 (6.7)		
	Contact exposed	2/3	0/3	2/3	2/3 (5.0)		
ES2 H5N6 (C-4 genotype)	2.0	0/5	0/5	0/5	0/5	3.0	4.0
	4.0	5/5	3/5 (7.3 days)	5/5	2/2 (6.5)		
	6.0	7/7	4/5 (7.5 days)	7/7	1/1 (9.0)		
	Contact exposed	3/3	3/3 (6.7 days)	3/3	N/A		

EID_{50}, mean egg infectious dose; MDT, mean death time; BID_{50}, mean bird infectious dose; BLD_{50}, mean bird lethal dose.

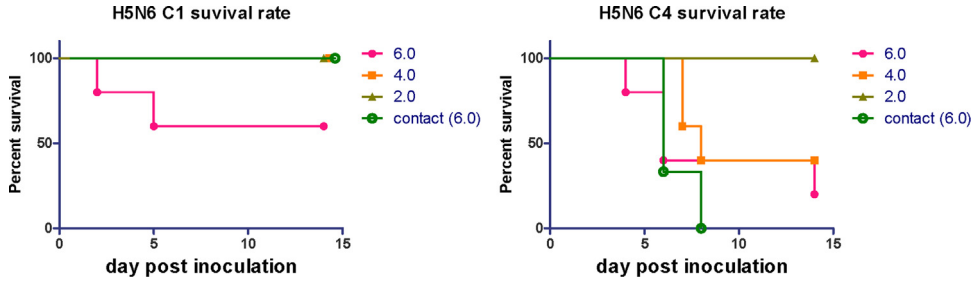

Fig. 1. The survival rate of ducks inoculated with the C-1 (A) and C-4 (B) genotypes of the H5N6 virus. Each duck was inoculated via the intranasal and choanal cleft with 10^2, 10^4, or 10^6 egg infectious dose (EID_{50}) in 0.2 mL of each virus. The ducks were monitored daily for illness or death.

spleen, pancreas, kidney, heart, and brain. Edema and thrombosis were seen in the lung. Edema and necrosis were found in the liver, spleen, and kidney. Focal to multifocal necrosis, with or without non-suppurative inflammation were observed in the heart and brain. Severe vacuolation and necrosis of exocrine glands were observed in the pancreas (Fig. 4). Immunohistochemistry revealed that avian influenza virus antigens were seen in the white blood cells of blood vessels and the parenchymal cells of numerous organs. The antigens were detected in the alveolar macrophages in the lung, macrophages in the spleen, Kupffer cells in liver, exocrine gland cells in the pancreas, tubular epithelium in the kidney, myocytes in the heart, and neuron and glial cells in the brain (Fig. 5). The viral antigen was detected in tissues that showed histological lesions (Table 2). No histological lesion and viral antigen staining was detected in tissues collected from sham-inoculated ducks. These results indicated that H5N6 HPAIV circulated by blood to organs and induced gross and histological lesions.

3.4. Sequence analysis

The genome sequence of eight genes and 11 proteins of two strains of H5N6 HPAIVs were compared to identify the genetic changes associated with altered pathogenicity. All genes showed high nucleotide identity (99.55–100%) and were clustered together in phylogenetic analysis, except the PA (92.49%) and NS (97.09%) genes. The PA gene of the C-1 genotype virus was clustered with China H5N6 viruses belonging to G1.1.9 genotype, but the PA gene of the C-4 genotype virus was clustered with LPAIVs isolated in Mongolia. The NS gene of the C1 genotype virus was clustered with H5N6 viruses belonging to the G1.1 genotype isolated from China and Vietnam, but the NS gene of the C4 genotype virus was clustered with Chinese H5N6 viruses belonging to the G1.1.9 genotype (Supplemental Figure).

A total of 14 amino acid changes were detected in the PB2 (5), PB1 (5), PB1-F (1), HA (2), NP (1), NA (0), M1 (0) and M2 (0) proteins.

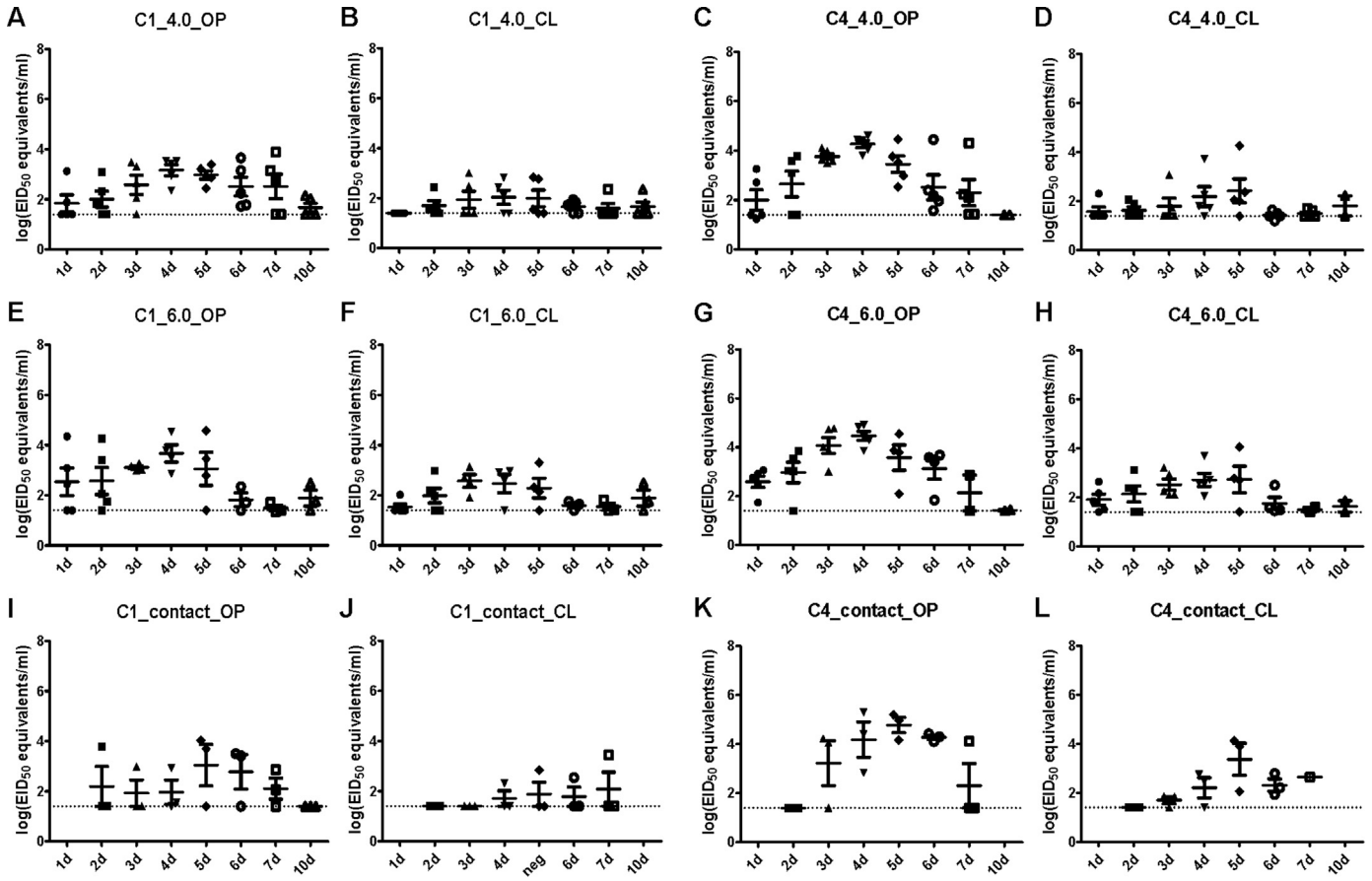

Fig. 2. Viral titers from the oropharyngeal (OP) and cloacal (CL) swabs collected from ducks inoculated with 10^4 (A, B) or 10^6 (E, F) EID_{50}/mL of C-1 genotype of H5N6 virus, inoculated with 10^4 (C, D) or 10^6 (G, H) EID_{50}/mL of C-4 genotype of H5N6 virus and co-housed with inoculated birds (I–L). The amount of influenza virus RNA was quantified by the cycle threshold (Ct) value using rRT-PCR and the Ct values were converted to EID_{50} equivalents/mL using standard curve.

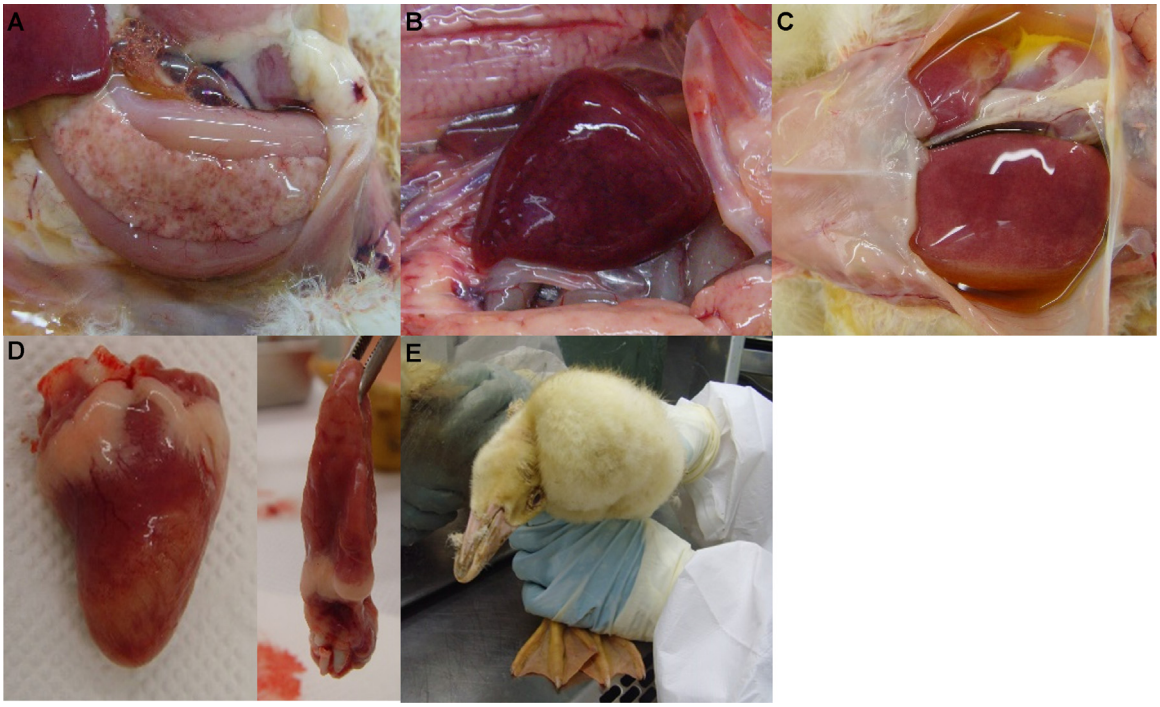

Fig. 3. Clinical signs and gross lesions of dead ducks inoculated by the C-4 genotype of the H5N6 virus. (A) Multi-focal pancreatic hemorrhage (B) Necrotic foci in the spleen. (C) Swollen liver and severe ascites. (D) White necrotic foci in cardiac muscle. (E) Torticollis.

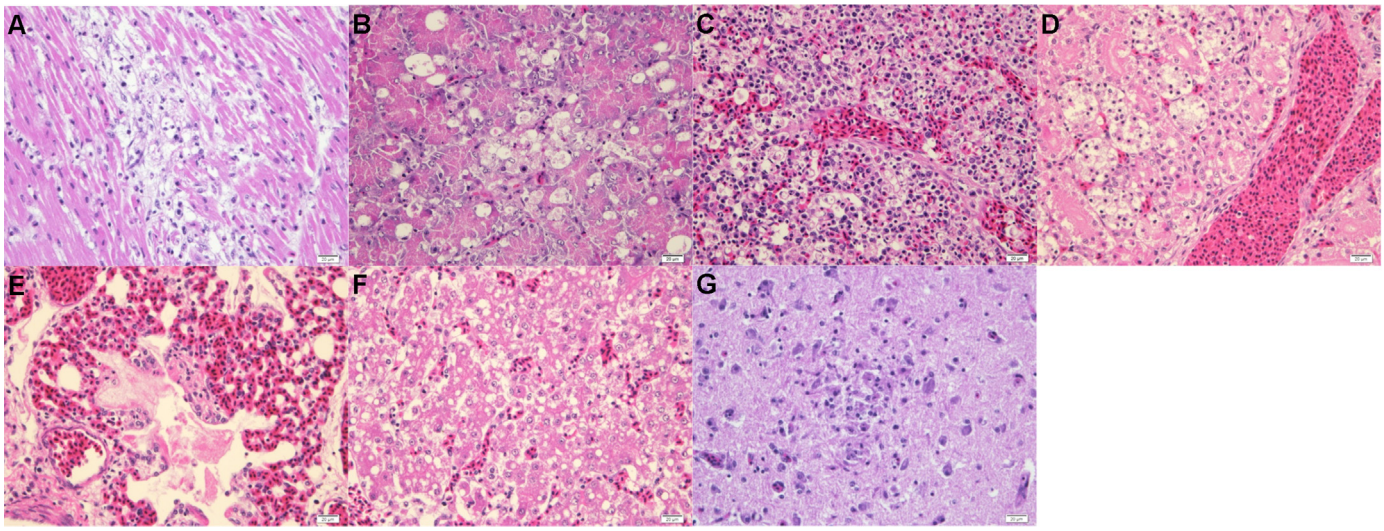

Fig. 4. Histopathology of ducks inoculated with the C-4 genotype of the H5N6 virus. (A) The moderate myocardial necrosis and non-suppurative myocarditis in the heart. (B) The severe vacuolation and necrosis of exocrine glands in the pancreas. (C) The necrosis in the spleen. (D) The necrosis of tubules in the kidney. (E) The edema in an atrium of the lung. (F) The hepatocytes necrosis in the liver. (G) Moderate neuronal necrosis and gliosis in the brain.

However, 23 amino acid changes in the PA protein (96.7% identity), 11 amino acid changes in the NS1 protein (95.1%) and 2 amino acid changes in NEP (98.3%) were detected (Table 3).

4. Discussion

Novel subtypes of clade 2.3.4.4 H5Nx HPAIVs were generated by reassortment between H5N1 HPAIV and LPAIV in duck species. These viruses usually showed relatively low pathogenicity in duck species, including wild waterfowl, compared with viruses of another clades (Bi et al., 2016; Lee et al., 2017a; Qi et al., 2014). Clade 2.3.4.4 subgroup B H5N8 viruses evolved into at least nine genotypes including H5N5 subtype (Świętoń and Śmietanka, 2018; Woo et al., 2017). Thirty-four distinct genotypes of H5N6 viruses were reported in China and five more distinct genotypes were detected in South Korea, including the viruses used in this study (Bi et al., 2016; Lee et al., 2017b). However, the effect of reassortment of HPAIVs on epidemiology and pathogenicity in duck species has not been fully defined.

In this study, we identified the different pathogenicity of two viruses representing two genotypes of H5N6 HPAIVs, C-1 and C-4, which contain different PA and NS genes in domestic ducks. Domestic ducks showed similar sensitivity to these viruses, but the C-4 genotype resulted in higher mortality than the C-1 genotype. While lower MDT and more severe histologic lesion at 3dpi were observed in ducks challenged with C-1 genotype virus than C-4 genotype virus, the higher mortality, more severe lesions and a higher titer of viral shedding were observed

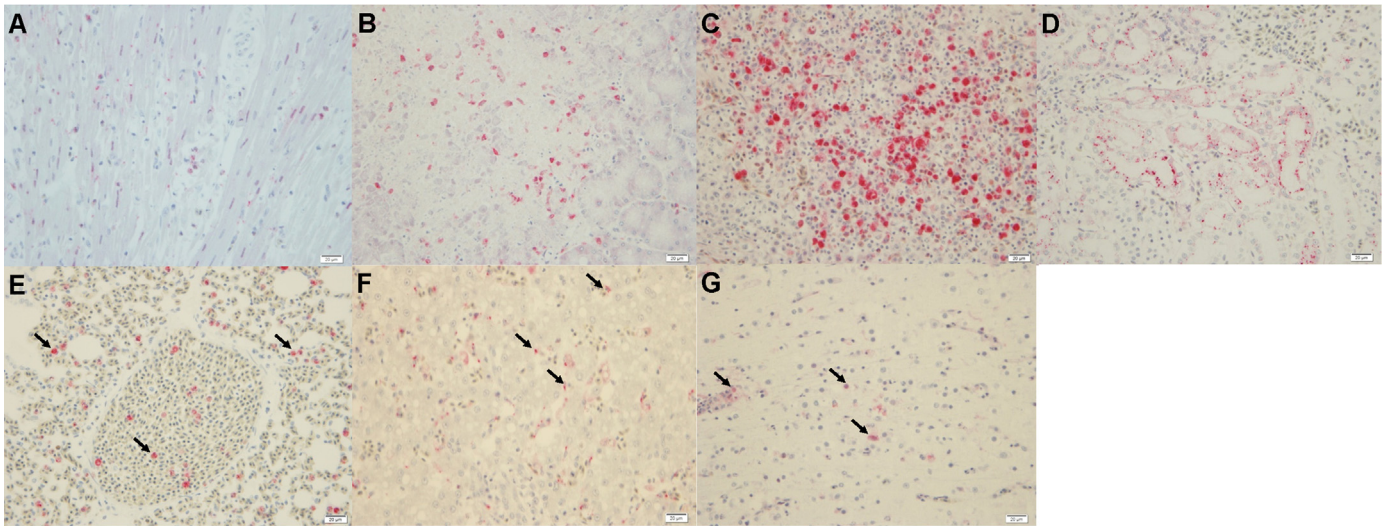

Fig. 5. Immunohistochemical detection of viral antigen in ducks inoculated with the C-4 genotype of the H5N6 virus. (A) Viral antigen in myocytes in the heart. (B) Viral antigen in necrotic exocrine pancreatic cells in the pancreas. (C) Viral antigen in cells in the spleen. (D) Viral antigen in tubular epithelium in the kidney. (E) Viral antigen in alveolar macrophages and white blood cells (arrows) in the lung. (F) Viral antigen in Kupffer cells (arrows) in the liver. (G) Viral antigen in neuron and glial cells (arrows) in the brain.

Table 2
The viral antigen distribution determined by immunohistochemistry and histopathology of dead or sacrificed ducks inoculated with C-1 and C-4 genotype H5N6 viruses.

Exposed virus	Individual number	Inoculation dose (log (EID_{50}))	History	Histology	Tissue							
					Trachea	Liver	Spleen	Pancreas	Lung	Kidney	Heart	Brain
C1 genotype virus	#1	6.0	Died at 2 dpi	Histopathology[a]	–	NT	+ +	+	+ +	–	+ +	–
				IHC[b]	–	NT	+ +	+	+ +	+	+ +	+
	#2	6.0	Sacrificed at 3 dpi	Histopathology	+	+	+	+	+	+	+	+
				IHC	–	+ +	+ +	+	–	+ +	+	+
	#3	6.0	Sacrificed at 3 dpi	Histopathology	+	+ +	+	+ +	+	+	+	+ +
				IHC	+	+ +	+ +	+	+ +	+	+	+
	#4	6.0	Died at 5 dpi	Histopathology	+	+ +	+ +	+ +	+ +	+	+ +	+ +
				IHC	+	+ +	+ +	+ +	+ +	+ +	+ +	+
C4 genotype virus	#1	6.0	Sacrificed at 3 dpi	Histopathology	–	+	+	–	+	+	+	–
				IHC	+	+	+	–	–	+ +	–	–
	#2	6.0	Sacrificed at 3 dpi	Histopathology	+	+	+	+ +	+	+	+	+
				IHC	+	+	+	+	+	+	+	+
	#3	6.0	Died at 4 dpi	Histopathology	+	NT	+ +	+ + +	+ +	+	+ +	+
				IHC	–	NT	+ +	+ +	+ +	+	+ +	–
	#4	Contact exposed	Died at 6 dpi	Histopathology	+	+ +	+ +	+ + +	+ +	+	+ +	+
				IHC	+	+ +	+ +	+ + +	+ +	+ +	+ +	+
	#5	Contact exposed	Died at 6 dpi	Histopathology	+ +	+	+ +	+ + +	+ + +	+	+ +	+ +
				IHC	+	+ +	+ + +	+ +	+ +	+ +	+	+ +
	#6	6.0	Died at 6 dpi	Histopathology	+	+ +	+ +	+ +	+ +	+	+ +	+
				IHC	–	+ +	+ +	+	+	+ +	+	+ +
	#7	6.0	Died at 6 dpi	Histopathology	+	+ +	+	+ +	+ +	+	+ +	+
				IHC	–	+ +	+ +	+	+	+ +	+	+
	#8	4.0	Died at 7 dpi	Histopathology	–	+ +	+	+	–	+	+ +	+
				IHC	–	+ +	+ +	–	–	+	–	–
	#9	4.0	Died at 7 dpi	Histopathology	–	+ +	+ +	+	+	+ +	+ +	+ +
				IHC	+	+ +	+ +	+	+	+ +	+	+
	#10	4.0	Died at 8 dpi	Histopathology	+	+ +	+ +	+ +	+ +	+	+ +	+
				IHC	+	+ +	+ +	+ +	+	+ +	+ +	+
	#11	Contact exposed	Died at 8 dpi	Histopathology	+	+	+ +	+ +	+	+	+ +	+
				IHC	+	+ +	+ +	+ +	+	+ +	+ +	+

EID_{50}, mean egg infectious dose; IHC, Immunohistochemistry; NT, not tested.
[a] – = no lesion, + = mild lesion, + + = moderate lesion, + + + = severe lesion.
[b] = no positive signal, + = weak signal, + + = moderate signal, + + + = strong signal.

Table 3
The gene identity and amino acid changes between C-1 and C-4 genotype H5N6 viruses.

Gene segment	Gene identity	Protein	Amino acid identity	Amino acid change		
				Position[a]	C1	C4
PB2	2198/2206 (99.63%)	PB2	754/759 (99.34%)	274	V	A
				282	D	E
				284	S	C
				292	I	T
				722	D	A
PB1	2232/2242 (99.55%)	PB1	752/757 (99.34%)	305	Y	D
				306	H	N
				308	P	K
				387	K	R
				585	F	S
		PB1-F2	88/90 (97.78%)	17	N	S
				44	K	R
PA	1959/2118 (92.49%)	PA	693/716 (96.79%)	N/A	N/A	N/A
HA	1685/1689 (99.76%)	HA	564/566 (99.65%)	387	K	R
				542	A	T
NP	1425/1431 (99.58%)	NP	497/498 (99.80%)	482	N	S
NA	1321/1321 (100%)	NA	100%	–	–	–
M	912/914 (99.78%)	M1	100%	–	–	–
		M2	100%	–	–	–
NS	802/826 (97.09%)	NS1	214/225 (95.11%)	N/A	N/A	N/A
		NEP	118/120 (98.33%)	N/A	N/A	N/A

PB2, polymerase basic 2; PB1, polymerase basic 1; PA, polymerase acidic; HA, hemagglutinin; NP, nucleoprotein; M, matrix; NS, non-structural; PB1-F2, polymerase basic 1-frame 2; NEP, nuclear export protein.
[a] Amino acid number from start codon.

in ducks challenged with the C-4 genotype virus than the C-1 genotype virus after 3 dpi. These results indicated that C-4 genotype virus more efficiently replicated in ducks on the late stage of infection than C-1 genotype virus.

Although H5 HPAIVs showed various pathogenicity in ducks, the molecular determinants for pathogenicity in ducks remained unclear. Previous studies indicated that PA and NS genes of HPAIVs were associated with lethality in ducks (Hulse-Post et al., 2007; Kajihara et al., 2013; Petersen et al., 2013). Another study indicated that the NS gene exerted minimal effects on viral pathogenicity in ducks (Sarmento et al., 2010). The study of Kajihara et al. indicated that multigenic factors were responsible for efficient replication and pathogenicity in ducks because pathogenic changes were only detected in multi-gene reassortment and not in single-gene reassortment (Kajihara et al., 2013). Except for the PA and NS genes, only 14 non-synonymous mutations were detected, and the effects of these mutations have not yet been reported. It was suspected that the PA or NS gene could be contributing factors to viral pathogenicity in ducks, although the mutations in genes other than PA and NS gene may also contribute to the pathogenicity changes. The effect of reassortment and multigenic factors in other genes should be discussed in further studies.

Mutations of the influenza virus, such as in the receptor binding site of HA, the pH-dependent cleavage site of HA, the antiviral protein inhibition site, and the polymerase gene, could change the host preferences, adaptation, and pathogenicity (Li and Cardona, 2010; Manz et al., 2013; Shinya, 2008). The multiple reassortment of HPAIVs with LPAIVs could induce rapid genetic changes that could change host adaptation. Previous studies reported that the circulation of HPAIVs in poultry could change the susceptibility to infection in specific bird species. The H5N2 viruses detected in North America in 2014–15 adapted to chickens during the outbreak (DeJesus et al., 2016; Pantin-Jackwood et al., 2017). In this study, it was suspected that the reassortment of H5N6 viruses changes the replication efficacy and mortality in ducks. The appearance of numerous genotype of HPAIVs could induce various pathogenicity in the specific host, such as that observed in this study and it might facilitate adaptation to the specific host. Further studies should be conducted on the effect of this rapid evolution of HPAIVs by reassortment on epidemiology and disease outbreak.

The low pathogenicity of HPAIVs in ducks could be an obstacle for an eradication strategy because it is hard to be detected without active surveillance. In 2014–2016, the H5N8 HPAIV with low pathogenicity in ducks caused the longest outbreak in South Korea and spread throughout a wide geographic region (Hill et al., 2015; Lee et al., 2015; Song et al., 2017). It is suspected that the HPAIVs of wild migratory birds introduced to duck farms located near the habitat of wild birds with a low biosecurity level in South Korea and had been circulated in duck farms for about 1 year (Hill et al., 2015). The co-circulation of numerous viral phenotypes may be more complicated to control. To detect viruses with low pathogenicity in ducks, active surveillance on duck farms will be needed, although high mortality has been reported on other farms. However, the enhancement of overall biosecurity level of poultry farms should be accompanied with active surveillance to control diseases.

In conclusion, the different pathogenicity of the two viruses bearing different PA and NS gene in ducks was identified. These results indicated that the reassortment of clade 2.3.4.4 HPAIVs with LPAIVs could alter the pathogenicity of the virus in ducks. The evaluation of the pathogenicity of multiple genotypes of viruses in domestic ducks and wild waterfowl are needed to fully understand the pathobiology of HPAIVs.

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Conflict of interest

None.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2019.01.016.

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