Study on the mechanism of increased virulence of Newcastle disease virus in ducks

（ニューカッスル病ウイルスのアヒルにおける病原性増強機序に関する研究）

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General introduction

Newcastle disease (ND) is one of the most prevalent poultry diseases and represents a serious impediment for poultry production throughout the world (Suarez, 2020). The causative agent is virulent strains of Avian orthoavulavirus 1 (AOAV-1), referred to as Newcastle disease virus (NDV), which belongs to the subfamily *Avulavirinae* of the family *Paramyxoviridae* (ICTV, 2020). There are 22 species of the subfamily *Avulavirinae*, commonly referred to as avian paramyxovirus (APMV) 1–22 using former nomenclature; APMV-1 is synonymous with AOAV-1. Among APMVs, only APMV-1 is known to cause serious clinical symptoms in avian species (Suarez, 2020). In this thesis, the term 'NDV' is used to refer to AOAV-1/APMV-1, regardless of the pathogenicity.

NDV is an enveloped virus with a negative-strand, non-segmented RNA genome. There are three sizes of NDV genome; 15,186, 15,192, and 15,198 (Czegledi *et al*., 2006), restricted by the "rule of six" which requires that the genomes of paramyxoviruses be a polyhexameric length for efficient replication (Kolakofsky *et al*., 2005). The NDV genome contains six genes (3′-N-P-M-F-HN-L-5′), which encode nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and RNA-dependent RNA polymerase protein (L), respectively. In addition, the nonstructural V protein is also encoded by the P gene as a result of an RNA editing mechanism (Steward *et al*., 1993). The envelope proteins HN and F are involved in the entry of the virus into host cells. The interaction of F with HN, which binds receptors, triggers conformational changes in F, leading to membrane fusion between the viral envelope and the host cell membrane (Bose *et al*., 2015; Chang *et al*., 2012). The viral genome RNA encapsidated by the NP is released into the cytoplasm by membrane fusion and serves as a template for transcription and replication by the RNA-dependent RNA polymerase complex containing L and P (Jordan *et al*., 2018). During the late stage of infection, M associates with the cell membrane and acts as a scaffold

for viral assembly, facilitating the budding of viral particles (El Najjar *et al*., 2014). V suppresses the host's innate immune system by inhibiting type I IFN signaling, contributing to efficient viral replication (Huang *et al*., 2003; Qiu *et al*., 2016). HN protein also possesses neuraminidase enzyme activity that degrades sialic acid receptors, contributing to the release of progeny viruses and the suppression of self-attachment (Mirza *et al*., 1994).

NDV infects a wide range of avian species; chickens show especially high susceptibility. Affected poultry with virulent NDVs displays a variety of symptoms that vary from NDV strains, including respiratory symptoms, depression, nervous manifestations, or diarrhea, and often fatal outcomes (Suarez, 2020). According to the severity of disease in chickens, NDV strains are categorized into three pathotypes: lentogenic (avirulent), mesogenic (intermediate virulence), and velogenic (highly virulent). The World Organization for Animal Health (OIE) have defined the virulent NDVs that viruses with an intracerebral pathogenicity index (ICPI) exceeding 0.7 or containing phenylalanine at position 117 along with multiple basic amino acids at the fusion protein cleavage site (OIE, 2021). The inactive F0 precursor is cleaved into the functionally active F1 and F2 by host proteases. This step is essential for the viral life cycle. The F proteins of velogenic NDVs have polybasic amino acids (e.g., R-R-Q-K-R) at their cleavage sites that are recognized by ubiquitous furin-like proteases, causing systemic infection and high mortality in chickens. In contrast, lentogenic viruses have a cleavage site consisting of a monobasic amino acid sequence (e.g. G-R-Q-G-R) that is cleaved by trypsin-like proteases. Since these proteases are mainly expressed in the respiratory system and digestive tract, the replication of lentogenic viruses is limited in these organs, leading to low virulence.

Based on the full F gene nucleotide sequence, NDV strains are classified into two distinct clades: class I and class II (Dimitrov *et al*., 2019). Class I NDV strains comprise lentogenic strains, which are mainly maintained in wild or domestic waterfowl, and are occasionally detected in

chickens in live bird markets (LBMs) (Miller *et al*., 2010; Zaib *et al*., 2018; Zhu *et al*., 2014). Class I NDV strains comprise a single genotype, which can be subdivided into three sub-genotypes, 1.1.1, 1.1.2, and 1.2 (Dimitrov *et al*., 2019), whereas class II NDV strains are more diverse and are subdivided into 21 genotypes (I–XXI) (Dimitrov *et al*., 2019). Class II NDV includes both poultry and wild waterfowl isolates including non-virulent and virulent strains (Czegledi *et al.*, 2006; Miller *et al*., 2010).

Since lentogenic NDV is frequently isolated from wild birds belonging to the order *Anseriformes*, these species are considered to be natural reservoirs (Dimitrov *et al*., 2016; Zaib *et al*., 2018). On the other hand, most velogenic NDV strains have been isolated from poultry, or sometimes from domestic waterfowls such as ducks and geese (Zaib *et al*., 2018). Moreover, phylogenetically diverse NDV, including velogenic NDVs, have been isolated from chickens, ducks, pigeons, and various other bird species in LBMs (Dimitrov *et al*., 2016; Kang *et al*., 2016a; Ogali *et al*., 2020). The spillover of NDVs from poultry to wild birds has also been reported in China (Xiang *et al*., 2017), suggesting that velogenic NDV is likely to spread from poultry to wild birds, raising concern about the potential transboundary dissemination of the virus.

Although the origin and natural reservoirs of velogenic NDV are not clear, the possibility that velogenic NDV emerges from lentogenic predecessor has been reported. Velogenic NDV isolates found in Ireland and Australia during an outbreak in the 1990s were genetically related to the previous lentogenic viruses found in wild waterfowls in each region (Alexander *et al*., 1992; Gould *et al*., 2001). Several studies experimentally demonstrated that lentogenic viruses acquired a virulent phenotype after consecutive passages in chickens (Meng *et al*., 2016; Shengqing *et al*., 2002; Tsunekuni *et al*., 2010). These findings support the notion that velogenic NDV has emerged from wild bird-originating lentogenic virus by circulating in poultry populations.

Gallinaceous avian species such as chickens and turkeys show high susceptibility to velogenic

NDVs, whereas *Anseriformes*, known as waterfowls such as ducks and geese, are generally resistant (Kang *et al*., 2014; Miller *et al*., 2013; Zhang *et al*., 2011). The difference between chickens and ducks in NDV susceptibility is considered to be related to the innate immune system. Retinoic acid-inducible gene-like receptors (RLRs) such as retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) detect viral negative-sense RNA, and act as sensors of viral infections (Reikine *et al*., 2014). RLRs stimulate signaling pathways that lead to the transcription of type I interferon (IFN)-α and β (Liu *et al*., 2017). Waterfowl such as ducks and geese express both RLRs, whereas chickens express only MDA5 (Barber *et al*., 2010; Sun *et al*., 2013). The V protein of NDV is known to block the IFN production and the signaling pathway by targeting multiple host factors, including MDA5 (Childs *et al*., 2007; Sun *et al*., 2019). Therefore, RIG-I is likely to contribute to an effective antiviral response in waterfowl (Anis *et al*., 2013; Kang *et al*., 2015; Kang *et al*., 2016b; Sun *et al*., 2013; Zaib *et al*., 2018).

However, ND does occasionally occur in *Anseriformes*. Severe ND outbreaks have sporadically occurred in domestic ducks and geese in China, and velogenic NDVs with high virulence in waterfowls have been isolated (Chen *et al*., 2015; Dai *et al*., 2014; Jinding *et al*., 2005; Liu *et al*., 2015; Wu *et al*., 2015; Xu *et al*., 2017; Zhang *et al*., 2011; Zou *et al*., 2005). The mechanisms for how NDV acquired high pathogenicity in ducks remain unclear.

This thesis provides biological and molecular bases for the virulence of NDV in ducks, as domesticated *Anseriformes*. In Chapter I, the authors report that successive passages of verogenic NDV in ducks resulted in amino acid substitutions in several viral proteins, which enhanced replication and virulence in ducks. In Chapter II, the author reports that the amino acid substitutions in F and M protein of duck-passaged NDV strain contributed to the enhancement of the virulence in ducks.

Chapter I

Analysis of biological and genetic changes of Newcastle disease virus during passages of velogenic strain in domestic ducks

Abstract

Velogenic Newcastle disease virus (NDV) strains, which show high mortality in chickens, generally do not cause severe disease in waterfowl such as ducks. To elucidate the difference in the pathogenic mechanisms of NDV between chickens and ducks, a chicken-derived velogenic strain (9a5b) was passaged in domestic ducks five times in their air sacs, followed by 20 times in their brains. Eventually, d5a20b acquired higher intracerebral and intranasal pathogenicity in ducks. The intracerebral pathogenicity index (ICPI) value increased from 1.10 to 1.88. All one-week-old ducks intranasally inoculated with the passaged virus (d5a20b) died by 5 days post-inoculation, whereas 70% of the ducks inoculated with parental 9a5b survived for 8 days. The d5a20b strain replicated in broader systemic tissues in ducks as compared with the 9a5b strain. The velogenic profile of 9a5b in chickens was maintained after passaging in ducks. The d5a20b suppressed IFN-β gene expression in duck embryo fibroblasts and more rapidly replicated than 9a5b. A total of 11 amino acid substitutions were found in the P, V, M, F, HN, and L proteins of d5a20b. These results suggest that chicken-derived velogenic NDVs have the potential to become virulent in both chickens and ducks during circulation in domesticated waterfowl populations.

Introduction

Susceptibility to NDV varies between avian species, and waterfowl such as ducks and geese are generally resistant to velogenic NDV (Miller *et al*., 2013; Kang *et al*., 2014; Zhang *et al*., 2011). However, fatal ND cases in *Anseriformes* have been sporadically reported. In 2007, a velogenic NDV was isolated from a dead mallard duck found in field in Serbia (Vidanović *et al*., 2011). Since the late 1990s, lethal ND outbreaks in domestic ducks and geese have sporadically occurred in China (Chen *et al*., 2015; Jinding *et al*., 2005; Liu *et al*., 2015; Wu *et al*., 2015; Xu *et al*., 2017; Zhang *et al*., 2011; Zou *et al*., 2005). During the ND outbreaks in duck farms in Jiangsu province, China, in 2008, infected ducks showed a drop in egg production, diarrhea, and neurological symptoms, with 80% morbidity and 30%–50% mortality rates (Liu *et al*., 2015). The molecular basis of how these velogenic NDVs acquire high virulence in waterfowls remains unclear.

In a previous study, the lentogenic NDV Goose/Alaska/415/91 (Alaska/415) which was isolated from wild goose was passaged in chickens (Shengqing *et al*., 2002). After nine passages in the chicken air sac, followed by five passages in the chicken brain, the passaged virus, 9a5b, became highly virulent in chickens. The amino acid sequence at the F cleavage site changed from the lentogenic type E-R-Q-E-R (Alaska/415) to the velogenic type K-R-Q-K-R (9a5b). These results showed that lentogenic NDVs maintained in wild waterfowl have the potential to become velogenic during circulation in poultry populations.

Similarly, it could be postulated that NDVs with virulence for waterfowl had emerged from ordinary velogenic NDVs during circulation among domestic birds. To clarify whether velogenic NDVs are able to enhance the virulence for ducks, in the present study, the 9a5b strain was additionally passaged in the air sac and brain of domestic duck (*Anas platyrhynchos* var. *domesticus*). The replicability and pathogenicity of the passaged viruses in ducks were confirmed by *in vitro* and *in vivo* approaches.

Materials and methods

Viruses and viral passaging

The velogenic NDV strain, 9a5b, was previously generated from the lentogenic NDV isolate Goose/Alaska/415/91 via passaging in chickens (Shengqing *et al*., 2002). 9a5b was further passaged 20 times in duck brains after 5 serial passages in duck air sacs as follows. Domestic ducks (Cherry Valley var., Takahashi artificial hatchery, Osaka, Japan) were purchased and reared in the Tottori University Animal Experimentation Building. Allantoic fluid containing a $10^{8.6}$ 50% egg infectious dose (EID_{50})/0.2 mL of 9a5b was inoculated into the air sacs of two 2-day-old ducks (Table 1). The ducks were euthanized with isoflurane (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) at 3 days post-inoculation (dpi), and the lungs were aseptically collected. Air sac passages in three to four 2-day-old ducks were performed in the same manner using 0.2 mL of 10% lung tissue suspensions as the inoculum, and the passaged strain (d5a) was obtained after five passages. Subsequent intracerebral passages were performed using 0.05 mL of 10% brain tissue suspensions. The inoculated birds were observed for a maximum of 6 days. Since it is known that ducks increase the resistance to NDV as they grow (Dai *et al*., 2014), the age of the inoculated ducks was gradually increased from 1- to 34-day-old during passaging to allow for the efficient selection of NDV variants pathogenic for ducks. Throughout the passaging study, the lungs or brains of the ducks showing clinical signs were preferentially used as the inoculum. The recovered viruses were identified by the number of passages in air sacs (a) and brains (b); for example, the designation ‘d5a20b’ indicates that the virus was passaged 5 times in the air sac (a) and 20 times in the brain (b) of ducks (d).

Viruses were propagated in 10-day-old embryonated chicken eggs (Aoki breeder farm, Tochigi, Japan) for 48 h at 37°C. The allantoic fluid was harvested and stored at −80°C. The 50% tissue culture infectious dose ($TCID_{50}$) and EID_{50} of the virus stocks were determined in Madin-Darby bovine kidney (MDBK) cells and 10-day-old embryonated chicken eggs, respectively.

Cell lines

MDBK cells were grown in Eagle's Minimum Essential Medium (E-MEM; Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Beit-Haemek, Israel), and maintained in E-MEM supplemented with 0.3% bovine serum albumin (BSA; Sigma-Aldrich, MO, USA). Duck embryo fibroblasts (DEFs) and chicken embryo fibroblasts (CEFs) were prepared from 12-day-old embryonated duck eggs (Takahashi artificial hatchery) and 10-day-old embryonated chicken eggs (Aoki breeder farm), respectively. The DEFs and CEFs were cultured in Dulbecco's minimal essential medium (DMEM; Thermo Fisher Scientific) supplemented with 10% FBS and then maintained in DMEM supplemented with 0.3% BSA.

Pathogenicity index tests

The intracerebral pathogenicity index (ICPI) in 1-day-old specific-pathogen-free (SPF) chickens (Nisseiken Co., Ltd., Tokyo, Japan) and 2-day-old domestic ducks (Takahashi artificial hatchery) were determined according to the OIE protocol (OIE, 2021) with slight modifications. Groups of 10 birds were intracerebrally inoculated with $10^{6.7}$ EID_{50}/0.05 mL of the virus and scored for clinical symptoms every 24 h for 8 days. The score was 0 if normal, 1 if sick, and 2 if dead. The ICPI value is the mean score per bird per observation. An index of 2.00 means that all birds died within 24 h, and an index of 0.00 means that no bird showed any clinical sign during the 8-day observation period.

Experimental infection of 1-week-old ducks and chickens

Ten 1-week-old domestic ducks (Takahashi artificial hatchery) or SPF chickens (Nisseiken) were intranasally inoculated with 10^7 EID_{50}/0.2 mL of the virus. At 3 and 5 dpi, two birds from each group

were euthanized and multiple organs (brain, trachea, lung, liver, spleen, pancreas, kidney, and colorectum) were sampled for virus titration. Since chickens inoculated with d5a20b died at 4 dpi, so their organs were collected at that time instead of at 5 dpi. Tissue suspensions were prepared from each collected organ, and viral titers were determined by chicken egg inoculation using Reed & Müench's method (1938). The remaining six birds were observed daily for a maximum of 8 days.

Virus replication kinetics

Viruses were inoculated at a multiplicity of infection (MOI) of 0.001 into DEFs or CEFs, which were cultured in 6-well plates. To calculate the MOI, the $TCID_{50}$ titer was converted to the PFU titer by multiplying the $TCID_{50}$ titer by 0.7 (Bryan *et al.*, 1957). After 1 h incubation, the cells were washed with PBS and covered with DMEM containing 0.3% BSA at 37°C. The supernatants in each well were collected and replaced with equal volumes of fresh medium at 8-h intervals until 64 h post-inoculation (hpi). The virus titer of each sample was measured in MDBK cells, and the $TCID_{50}$ was calculated using the method of Reed & Müench (1938).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Viruses were inoculated at an MOI of 1 into DEFs or CEFs cultured in 6-well plates. At 6, 12, 24, and 48 h post-inoculation, total cellular RNA was extracted with RNeasy mini kit (Qiagen, Hilden, Germany). Resuspended RNA was quantified on a Nanodrop spectrophotometer (Thermo Fisher Scientific), and was then reverse-transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen). The cDNA was amplified through qPCR with primer sets specific to the *IFN-β* and *GAPDH* genes (Table 2) on a CFX96 real-time PCR cycler (Bio-Rad, Hercules, CA, USA) with iQ SYBR Green Supermix (Bio-Rad). Relative expression levels of *IFN-β* mRNA were calculated by cycle quantification (Cq) values using the standard curve generated with cDNA. Data were

normalized to the *GAPDH* mRNA levels in each sample and are expressed relative to the level of the mock treatment group. Data were analyzed by Tukey's multiple comparisons test at each time point using BellCurve for Excel (Social Survey Research Information Co., Tokyo, Japan).

Sequencing of the complete viral genome

Viral genomic RNA was extracted from the infected allantoic fluid with a QIAamp Viral RNA Mini Kit (Qiagen), and cDNA synthesis was conducted using PrimeScript reverse transcriptase (Takara Bio, Shiga, Japan). PCR was performed with specific primers and the KOD Dash DNA polymerase (Toyobo, Osaka, Japan). The amplified products were directly sequenced using the Big Dye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3130 Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocol. Obtained complete genome sequences of d5a and d5a20b were submitted to GenBank (accession numbers: LC589003 and LC589004, respectively).

Comparative analysis of putative amino acid sequences

The amino acids that were substituted in the passaged NDVs were compared to NDV isolates, which were reported to be virulent in waterfowls. Nucleotide and deduced amino acid sequences of NDV isolates were obtained from GenBank, and their accession numbers are shown in Table 4. The amino acid sequences were aligned using the MEGA-X software (Kumar *et al*., 2018).

Results

Passage of velogenic NDV in domestic ducks

The velogenic 9a5b virus was passaged in the air sacs and in the brains of domestic ducks. The parental 9a5b and the first air sac-passaged virus, d1a, induced no clinical signs or mortality in the inoculated ducks (Table 1). After the third passage, some of the inoculated ducks died, and all three 2-day-old ducks died at the fifth passage. The obtained d5a was then used as the inoculum for the first intracerebral passage. All three inoculated 3-day-old ducks intracerebrally inoculated with d5a survived for 3 days. The virus was recovered from pooled brain suspensions at a titer of $10^{4.7}$ EID_{50}/g. To efficiently select the variant with high mortality, younger 1-day-old ducks were used for the next 2 passages; the obtained d5a2b killed all 3 ducks via the intracerebral route of infection. After 8 passages in the brains of 7 - to 11-day-old ducks (data not shown), the passaged virus, d5a10b, showed high intracerebral pathogenicity in 10-day-old ducks, showing 100% mortality (Table 1). Finally, the d5a19b strain killed 2 of the 3 inoculated 34-day-old ducks at 4 and 6 dpi, and the d5a20b strain was obtained from pooled brain suspensions of the dead birds.

Intracerebral pathogenicity of the parental and passaged NDVs in domestic ducks and chickens

ICPI values in domestic ducks and chickens were determined to assess the intracerebral pathogenicity of each passaged virus (Table 3). In ducks, the ICPI scores of 9a5b, d5a, and d5a20b were 1.10, 1.63, and 1.88, respectively. The ducks inoculated with 9a5b showed depression, tremors, and torticollis at 3 dpi, and all birds died at 4–7 dpi. The d5a-inoculated ducks exhibited depression and tremors, and died at 2–4 dpi. All ducks with d5a20b were severely depressed and emaciated at 1 dpi, and died at 2 dpi. In chickens, the ICPI scores of these strains were 1.78, 1.85, and 1.89, respectively, indicating that these were velogenic NDVs according to the criteria set by OIE (OIE,

2021). The chickens inoculated with each virus died by 1–3 dpi; they showed severe depression but no distinct neurological symptoms as was also seen in the d5a20b-inoculated ducks.

Intranasal pathogenicity of the parental and passaged NDVs in 1-week-old domestic ducks and chickens

To investigate the intranasal pathogenicity of the passaged viruses, six 1-week-old domestic ducks and chickens were each intranasally inoculated with the virus and observed for 8 days (Fig. 1). The survival rates of the ducks inoculated with 9a5b, d5a, and d5a20b viruses were 67%, 33%, and 0%, respectively (Fig. 1A). The 9a5b-inoculated ducks showed little or no clinical signs, and 2 birds died at 6 dpi. Among the d5a-inoculated ducks, one bird died suddenly at 4 dpi, and three birds died at 6–8 dpi after exhibiting neurological symptoms such as leg paralysis, head tremor, and torticollis. The remaining two birds lived to 8 dpi, but presented with signs of depression and neurological symptoms. The ducks inoculated with d5a20b exhibited severe lethargy and neurological symptoms at 3–4 dpi, and all 6 birds died at 5 dpi.

All chickens inoculated with 9a5b or d5a20b died within 3–4 dpi (Fig. 1B). As observed in ducks, chickens with 9a5b tended to die without showing apparent clinical signs, whereas most chickens with d5a20b exhibited neurological symptoms before death.

Viral replication in organs of the infected 1-week-old domestic ducks and chickens

Viral titers in the organs of domestic ducks and chickens intranasally inoculated with each virus were determined to assess their tissue tropism (Figs. 2 and 3). At 3 dpi, 9a5b was detected in all organs except the brain (tissue samples from one or all two birds were virus-positive) (Fig. 2A). 9a5b was not detected in the trachea, liver, or spleen at 5 dpi (Fig. 2B), and had lower titers in the lungs, pancreas and colon compared to samples obtained at 3 dpi (Fig. 2A). The passaged strains d5a

and d5a20b replicated in almost all of the tested duck organs at 3 and 5 dpi (Fig. 2). d5a20b was detected in all tested brains at 3 and 5 dpi (#5, #6, #11, and #12), whereas 9a5b and d5a were not detected in some ducks (#1–3, #8 and #9).

Since the chickens inoculated with d5a20b (bird ID; #19, 20) died by 4 dpi, their organs were sampled at that time instead of at 5 dpi (Fig. 3B). Both 9a5b and d5a20b were detected in all the tested chicken organs at 3–5 dpi (Fig. 3). At 3 dpi, 9a5b was recovered at similar or higher titers than d5a20b from all organs except the brain (Fig. 3A).

Viral replication kinetics in DEF and CEF cells

The replication kinetics of 9a5b, d5a, and d5a20b were examined in DEF and CEF cells (Fig. 4). In DEF cells, d5a and d5a20b efficiently replicated at earlier time points (8–32 hpi) than 9a5b; notably, d5a20b replicated with a two orders higher titer than that of 9a5b at 16 hpi (Fig. 4A). The d5a strain replicated with titer level intermediate between those of 9a5b and d5a20b at 16–32 hpi. On the other hand, all three viruses replicated at approximately the same titer levels in CEF cells (Fig. 4B).

Quantification of IFN-β gene expression in infected DEFs and CEFs

The expression levels of the *IFN-β* gene in DEF and CEF cells inoculated with each virus were analyzed by RT-qPCR (Fig. 5). At 6 to 12 hpi, no significant *IFN-β* expression upregulation was observed in both DEFs and CEFs compared to that of the mock infection groups. The *IFN-β* expression level in DEFs peaked at 24 hpi for all viruses, and d5a induced the significantly highest level (38.77-fold), followed by 9a5b (17.38-fold) and d5a20b (8.42-fold) (Fig. 5A). At 48 hpi, the *IFN-β* expression level was decreased from the level detected at 24 hpi for each viral strain (by 5.37-fold for 9a5b, 10.43-fold for d5a, and 2.78-fold for d5a20b) in DEFs. No statistically significant

difference in *IFN-β* gene expression level was observed between the d5a20b and mock infection groups of DEFs throughout the experiment.

Upregulation in *IFN-β* expression was observed as of 24 hpi in d5a- and d5a20b-infected CEFs (by 4.49- and 3.76-fold, respectively) (Fig. 5B). These viruses induced the maximum increase in the *IFN-β* expression level in CEFs at 48 hpi with around similar levels detected in the d5a-infected (11.96-fold) and d5a20b-infected (9.86-fold) cells. However, 9a5b did not cause significant *IFN-β* expression upregulation in CEFs at any time point.

Genetic comparisons among the passaged viruses and field isolates

The complete genome sequences of d5a and d5a20b were determined, and the deduced amino acid sequences were compared with those of the parental 9a5b (Table 4). The following six amino acid substitutions were found in the six viral proteins in d5a: Q229H in P, G230C in V, N123D in M, I142M in F, G538R in HN, and F1844I in L protein, and these substitutions were maintained in d5a20b. After consecutive passages in the brain, an additional 5 amino acids in the proteins of d5a20b were found to have substitutions: Q44R and D342N in M, E304K in F, and F1676Y and R2024H in L protein. Of these mutations, the substitution of Q44R in M was consistent with seen in the field isolate HN1007, which causes ND in domestic ducks in China (Wen *et al*., 2016). The other 4 mutations were not observed in field NDVs virulent for waterfowls.

Disccusion

In this study, to reveal the pathogenic mechanisms of NDV in waterfowls, the velogenic NDV 9a5b strain was passaged in domestic ducks. As a result, the passaged d5a20b strain showed increased virulence, causing 100% mortality in 1-week-old ducks (Table 3; Fig. 1). These results demonstrate that chicken-derived velogenic NDV increases its virulence in ducks during repeated transmission among ducks.

The parental 9a5b strain was previously generated by passaging the lentogenic waterfowl isolate Goose/Alaska/415/91 in chicken air sacs and brains (Shengqing *et al*., 2002; Takakuwa *et al*., 1998). Unexpectedly, the parental strain 9a5b showed moderate pathogenicity to ducks with an ICPI of 1.10 and a mortality rate of 33% in 1-week-old ducks. The 9a5b strain acquired molecular features that are considered to be critical factors for viral pathogenicity, including the multi-basic F cleavage site motif (ERQER/L to KRQKR/F) and a shorter HN protein (caused by a newly generated stop codon), during in chicken passages (Shengqing *et al*., 2002; Tsunekuni *et al*., 2010). These features likely played a role in the increased pathogenicity of 9a5b in ducks.

NDVs with virulence for ducks tended to replicate in the brain, whereas most of the velogenic NDVs without virulence in ducks were not（Dai *et al*., 2013; Kang *et al*., 2014）. Similarly, 9a5b acquired high pathogenicity in chickens accompanied by an increased replication efficiency in chicken brains by passaging (Shengqing *et al*., 2002). Furthermore, nonpurulent encephalitis was observed in 9a5b-inoculated chickens, but not in ducks in the pathological analysis (Anis *et al*., 2013). Related to these results, in the present study, most of the 1-week-old ducks inoculated with d5a and d5a20b exhibited neurological symptoms, and these viruses were detected in the brains as of an early time point (3 dpi) (Fig. 2). Therefore, the brain is presumed to be one of the critical organs involved in the pathogenicity of NDV in ducks, as well as in chickens.

Anis *et al*. (2013) reported an earlier and higher IFN-β expression in the lungs of 9a5b-infected ducks than in chickens. Consistent with this report, we found that the *IFN-β* gene expression level in 9a5b-infected DEFs peaked earlier than that in CEFs (Fig. 5). Indeed, 9a5b-infected ducks showed more rapid viral clearance and a higher survival rate than those of chickens. By contrast, Kang *et al*. (2016) reported that velogenic NDVs induced higher expression levels of innate immune-related genes in CEFs than in DEFs. This discrepancy is likely related to the different degree of viral host adaptation in duck cells; indeed, 9a5b replicated well in DEFs, whereas the velogenic NDVs studied by Kang *et al*. (2016) replicated with much lower titers in DEFs than in CEFs. Therefore, the innate immune response to velogenic NDVs in waterfowl is likely to vary according to the degree of host adaptation, and cannot be generalized.

The course of infection of 9a5b and its passaged strains varied from multiple aspects. Both d5a and d5a20b were detected in systemic duck organs at 5 dpi, and these ducks had higher mortality than those infected with 9a5b. Moreover, d5a20b exhibited more rapid replication in DEFs. Interestingly, the *IFN-β* expression level in DEFs infected with each virus was not correlated with the number of passages; *IFN-β* expression was not significantly upregulated in d5a20b-infected cells, whereas d5a induced higher levels of *IFN-β* than the parental strain 9a5b (Fig. 5A). We postulate that d5a20b suppressed some IFN gene-stimulating pathway in duck cells, contributing to its higher virulence *in vivo*.

The V protein of d5a and d5a20b contains a G230C substitution located adjacent to the highly conserved cysteine-rich C-terminal domain (CTD) (Steward *et al*., 1995) (Table 4). The V protein of NDV has been shown to inhibit not only MDA5 but also phosphorylated STAT1 (Qiu *et al*., 2016). STAT1 is phosphorylated upon type I IFN stimulation to induce the expression of IFN-activated genes. The CTD is critical for the degradation of phosphorylated STAT1 by V protein, which consequently blocks type I IFN signaling (Qiu *et al*., 2016). Therefore, the G230C substitution,

which adds a cysteine residue adjacent to the CTD, may affect the anti-MDA5 and/or STAT1 activity of the V protein. Further studies are therefore needed to verify whether the amino acid at position 230 in V protein is responsible for stimulating or interfering host innate immunity in ducks.

To date, almost all of the NDV strains that are pathogenic in waterfowl belong to class II genotype VII or IX (Chen *et al*., 2015; Dai *et al*., 2014; Jinding *et al*., 2005; Kang *et al*., 2014; Meng *et al*., 2018; Wu *et al*., 2015; Zhang *et al*., 2011; Zou *et al*., 2005). These viruses do not always form species-specific phylogenetic lineages, and often show close genetic relationships to chicken isolates (Kang *et al*., 2014; Meng *et al*., 2018; Wu *et al*., 2015; Xu *et al*., 2017). Taken together with the fact that class II viruses (especially those of genotype VII) are more predominant than class I viruses in chickens (Dimitrov *et al*., 2016; Miller *et al*., 2015), virulent NDV to waterfowl is likely to have emerged by transmission from chickens. However, the Goose/Alaska/415/91 strain, the progenitor of 9a5b, belongs to the class I subgenotype 1.2 of NDV (Shengqing *et al*., 2002; Takakuwa *et al*., 1998), suggesting that class I viruses also have the capability to acquire pathogenicity for waterfowl. In this study, passaging was conducted with artificial infection routes, and viruses were directly inoculated into the air sacs or brains of ducks. Therefore, further validation is needed to clarify whether class I NDV also acquires increased pathogenicity through natural infection routes such as oral and nasal routes. Recently, a number of class I NDV have been isolated from chickens and domestic waterfowls, especially in LBMs in Korea and China (Choi *et al*., 2012; Li *et al*., 2019; Wu *et al*., 2015; Zhu *et al*., 2014), which raises concern about the potential emergence of further virulent strains to waterfowl.

In conclusion, this study demonstrated that chicken-adapted velogenic NDVs are able to enhance the virulence in ducks. The results suggest that NDVs with virulence in ducks can emerge during viral circulation between chickens and ducks. Certain circumstances, such as LBMs, where different bird species can easily have contact with each other, may have driven the emergence of

duck-adapted viruses. Recently, a number of spillovers of NDVs from domestic birds to wild birds have been reported around the world (Duan *et al*., 2014; Ferreira *et al*., 2019; Kim *et al*., 2012; Xiang *et al*., 2017). To prevent virulent NDV transmission to migratory waterfowls and transboundary viral spread, biosecurity in poultry markets and farms should be improved.

Legends for figures

Figure. 1. Survival rate of 1-week-old domestic ducks (A) and chickens (B) inoculated with the passaged viruses.

Six birds were each intranasally inoculated with 10^7 EID_{50} of 9a5b, d5a, and d5a20b strains and then monitored for survival daily for 8 days.

Figure 2. Viral recovery from the organs of one-week-old domestic ducks.

One-week-old ducks were intranasally inoculated with 10^7 EID_{50} of the virus, and organs were collected at 3 and 5 dpi from two birds in each group. Virus titres in organs at 3dpi (A) and 5 dpi (B) were determined. The limit of detection (1.5 log EID_{50}/g) is represented by the broken line.

Figure 3. Viral recovery from the organs of one-week-old chickens.

One-week-old chickens were intranasally inoculated with 10^7 EID_{50} of the virus, and their organs were collected at 3 and 5 dpi from two birds in each group. Since chickens inoculated with d5a20b died by 4 dpi (#19 and #20), the organs were collected at that time point instead of at 5 dpi. Virus titres in organs at 3 dpi (A) and 4–5 dpi (B) were determined. The limit of detection (1.5 log EID_{50}/g) is represented by the broken line.

Figure 4. Replication kinetics of passaged viruses in fibroblasts.

Duck embryo fibroblasts (DEFs; A) and chicken embryo fibroblasts (CEFs; B) cells were inoculated with each virus at a multiplicity of infection of 0.001. The supernatant was harvested every 8 h until 64 h post-inoculation. Viral titres were determined by measuring the $TCID_{50}$ in MDBK cells. The limit of detection (2.1 log $TCID_{50}$/ mL) is represented by the broken line. Each virus titre is

expressed as the mean of three separate experiments, and error bars represent their corresponding standard deviations (mean ± SD). Asterisks indicate significant differences in the virus titres compared to 9a5b at each time point; *P* values were calculated using two-tailed, unpaired *t*-tests (*$P < 0.05$, **$P < 0.01$).

Figure 5. Relative *IFN-β* expression levels in infected fibroblasts.

Relative *IFN-β* mRNA expression levels in duck embryo fibroblasts (DEFs) (A) and chicken embryo fibroblasts (CEFs) (B) following infection with each virus at a multiplicity of infection of 1 at 6, 12, 24, and 48 hpi. Data are normalized to the *GAPDH* mRNA levels in each sample and are expressed relative to the level in the mock treatment group. Each value represents the mean ± SD (n = 3). Data were analysed using Tukey's multiple comparisons test at each time point (*$P < 0.05$, **$P < 0.01$).

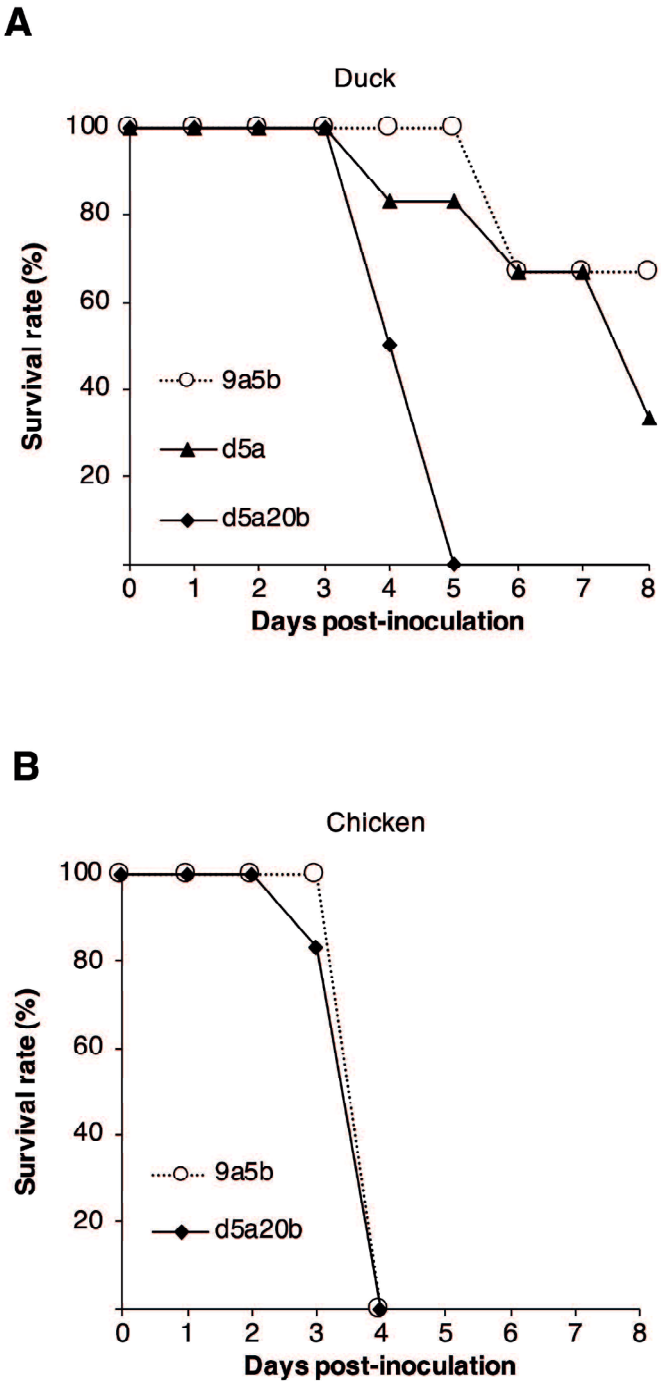
A
Duck
100
80
60
40
20
Survival rate (%)
9a5b
d5a
d5a20b
0
1
2
3
4
5
6
7
8
Days post-inoculation
B
Chicken
100
80
60
40
20
Survival rate (%)
9a5b
d5a20b
0
1
2
3
4
5
6
7
8
Days post-inoculation

Figure 1

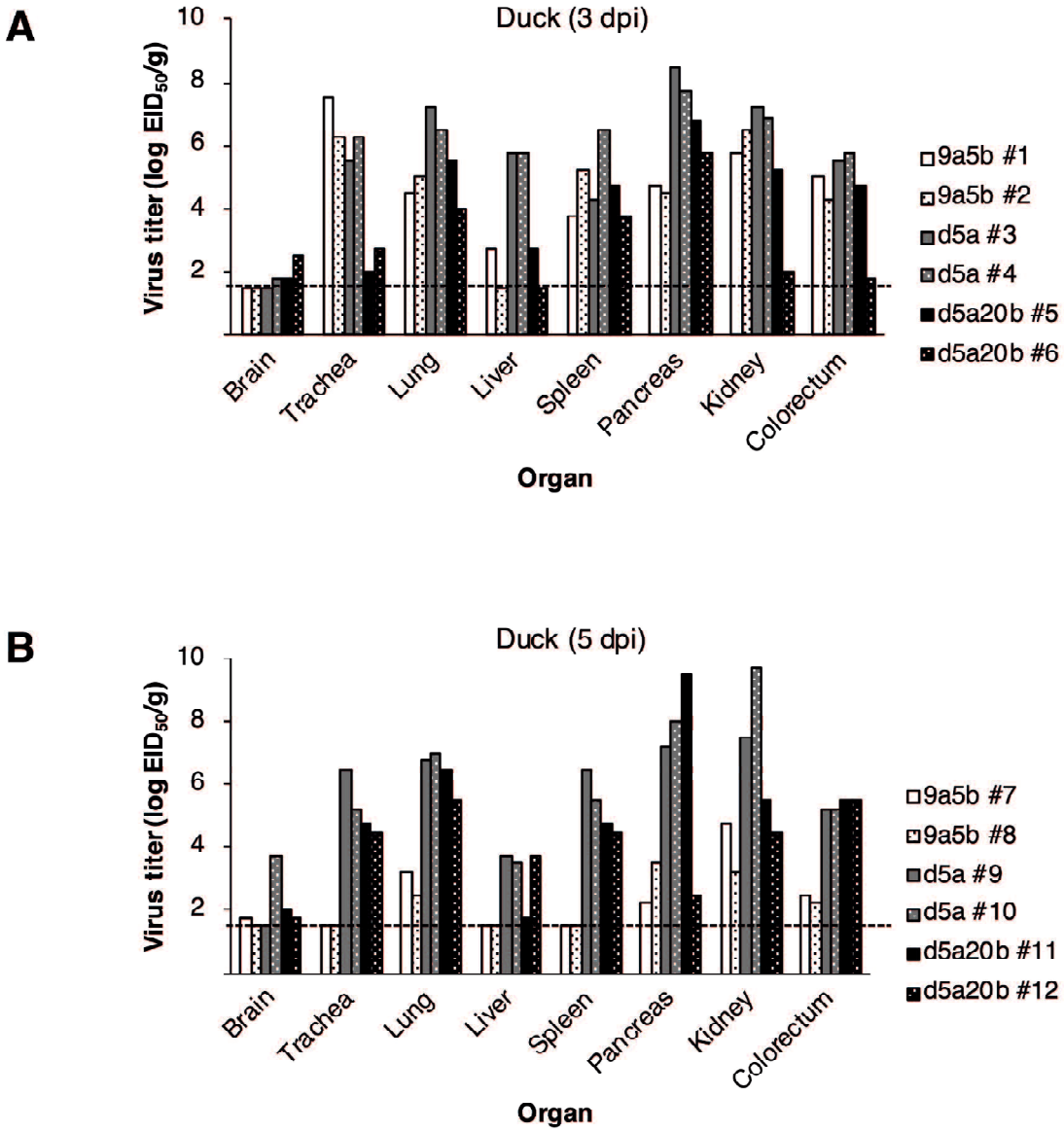
A
Duck (3 dpi)
Virus titer (log EID50/g)
10
8
6
4
2
Brain
Trachea
Lung
Liver
Spleen
Pancreas
Kidney
Colorectum
Organ
9a5b #1
9a5b #2
d5a #3
d5a #4
d5a20b #5
d5a20b #6
B
Duck (5 dpi)
Virus titer (log EID50/g)
10
8
6
4
2
Brain
Trachea
Lung
Liver
Spleen
Pancreas
Kidney
Colorectum
Organ
9a5b #7
9a5b #8
d5a #9
d5a #10
d5a20b #11
d5a20b #12

Figure 2

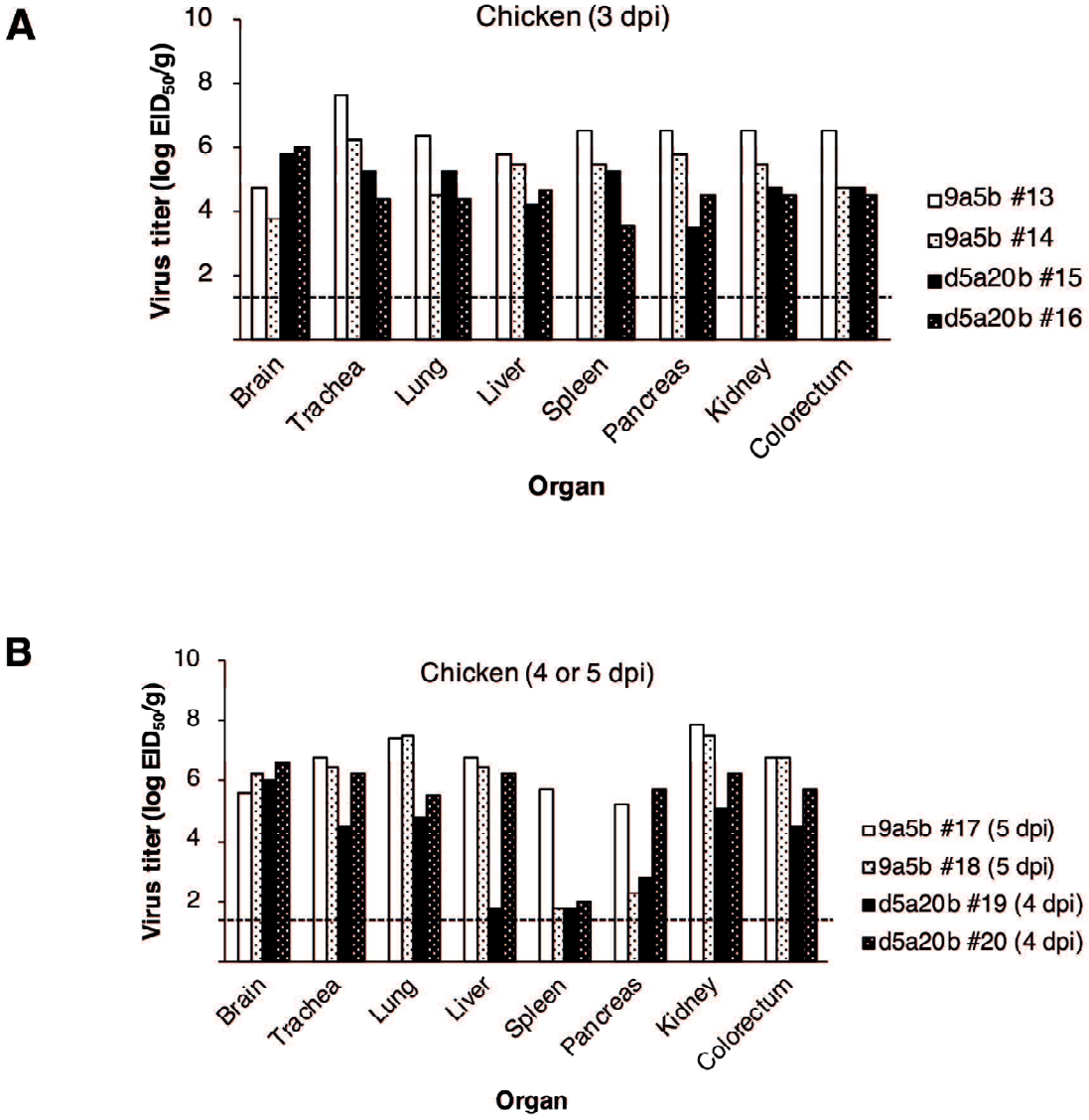
A
Chicken (3 dpi)
Virus titer (log EID50/g)
10
8
6
4
2
Brain
Trachea
Lung
Liver
Spleen
Pancreas
Kidney
Colorectum
Organ
9a5b #13
9a5b #14
d5a20b #15
d5a20b #16
B
Chicken (4 or 5 dpi)
Virus titer (log EID50/g)
10
8
6
4
2
Brain
Trachea
Lung
Liver
Spleen
Pancreas
Kidney
Colorectum
Organ
9a5b #17 (5 dpi)
9a5b #18 (5 dpi)
d5a20b #19 (4 dpi)
d5a20b #20 (4 dpi)

Figure 3

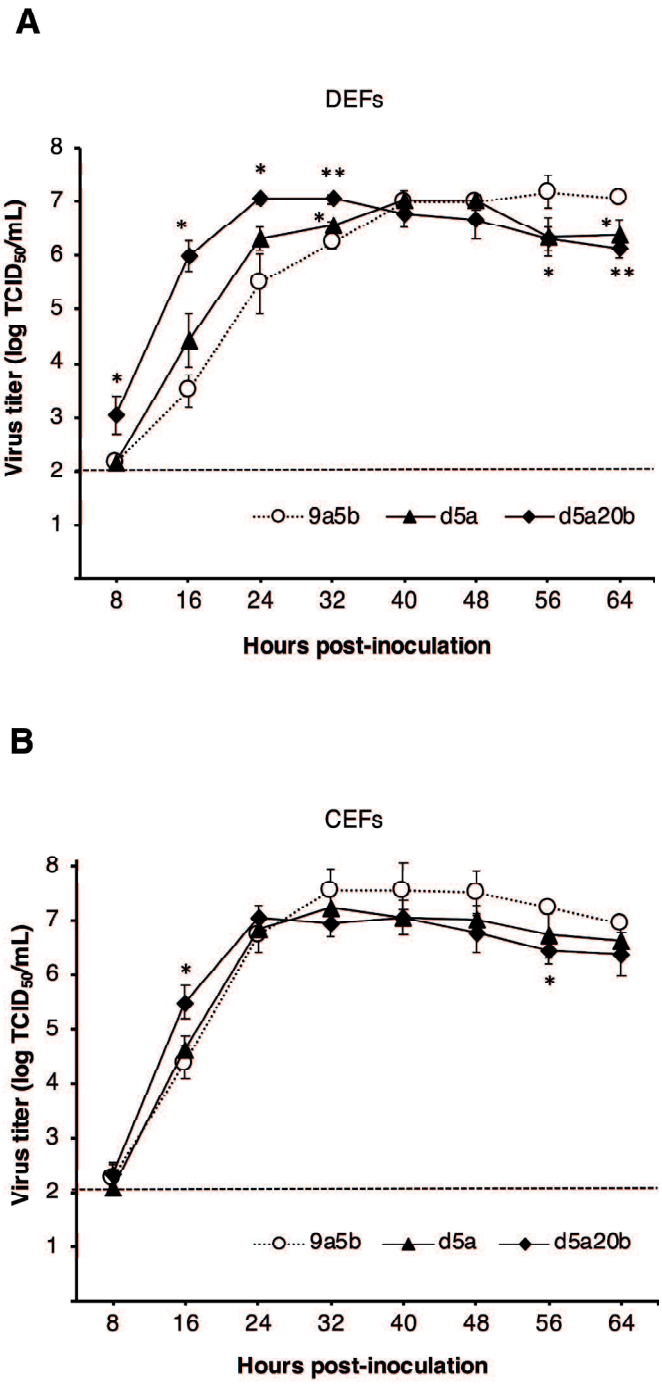
A
DEFs
Virus titer (log TCID50/mL)
Hours post-inoculation
9a5b
d5a
d5a20b
B
CEFs
Virus titer (log TCID50/mL)
Hours post-inoculation
9a5b
d5a
d5a20b

Figure 4

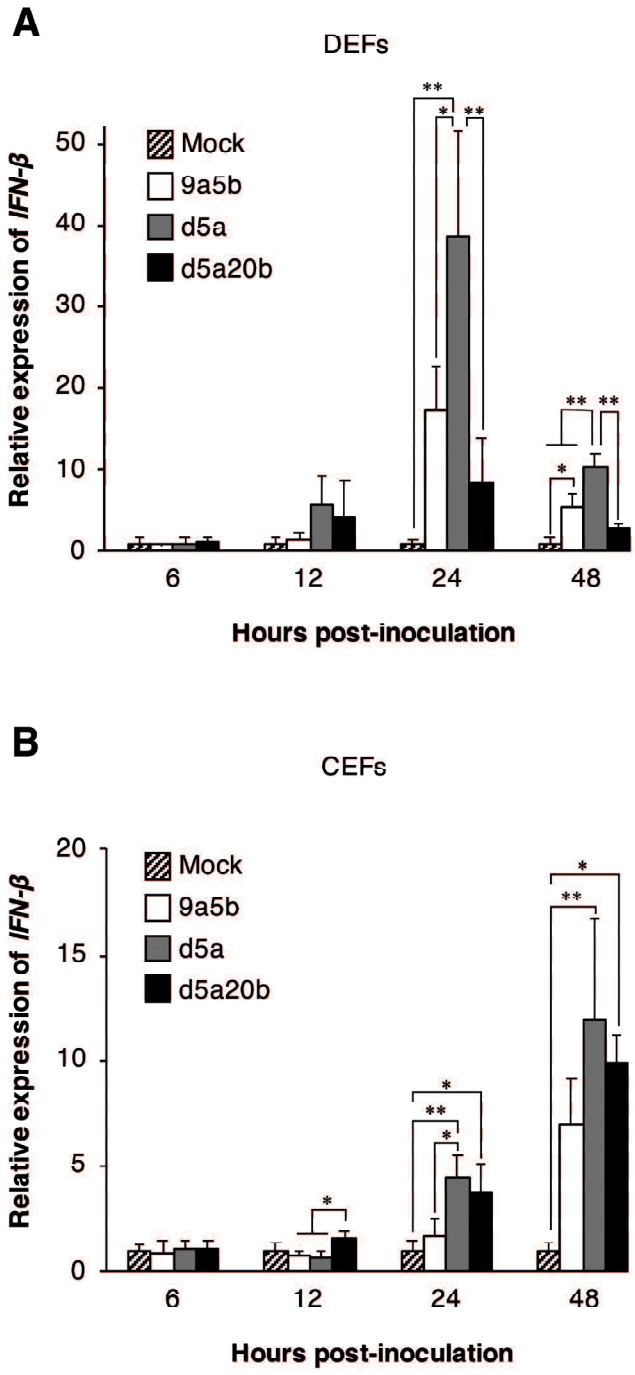
A
DEFs
Relative expression of IFN-β
Mock
9a5b
d5a
d5a20b
Hours post-inoculation
B
CEFs
Relative expression of IFN-β
Mock
9a5b
d5a
d5a20b
Hours post-inoculation

Figure 5

Table 1. Passage history for the 9a5b strain in domestic ducks

Inoculation route	Inoculated virus	Age of inoculated ducks (days)	Observation period (days)	Mortality (dead/total)	Virus titer[a] (log EID_{50}/g)	
					Lung	Brain
Air sacs	9a5b (parent)	2	3	0/2	6.3	NT[b]
	d1a	2	3	0/4	6.6	NT
	d2a	2	3	2/4	7.6	≤1.5
	d3a	2	3	1/3	8.3	≤1.8
	d4a	2	3	3/3	8.3	2.6
Intracerebral	d5a	3	3	0/3	NT	4.7
	d5a1b	1	3	1/3	NT	5.5
	d5a2b	1	3	3/3	NT	6.5
	d5a10b	10	3	3/3	NT	5.5
	d5a19b	34	6	2/3	NT	5.5

[a] Virus titer of pooled tissue suspension.

[b] Not tested.

Table 2. Primer sequences for quantitative real-time polymerase chain reaction

Gene	Primer sequence	Product size (bp)
Duck		
IFN-β	5′-ATCAACGCGCACTTTTTCCC-3′	120
	5′-TGCTTGGACTGCTGAGGATG-3′	
GAPDH	5′-GTCGGAGTCAACGGATTTGG-3′	249
	5′-CTGCCCACTTGATGTTGCTG-3′	
Chicken		
IFN-β	5′-CTCTTGCTTCTGCCAGCTCT-3′	86
	5′-TGGAGGCTTTTCCAAGAGAA-3′	
GAPDH	5′-CCTCTCTGGCAAAGTCCAAG-3′	176
	5′-GGTCACGCTCCTGGAAGATA-3′	

Table 3. Intracerebral pathogenicity of the parental and passaged NDVs

Bird species (Age)	Inoculated virus	Clinical signs	The number of birds								ICPI
			1	2	3	4	5	6	7	8 (dpi)	
Duck (2-day-old)	9a5b	Normal	10	10	8	0	0	0	0	0	1.10
		Sick	0	0	2	8	4	2	0	0	
		Dead	0	0	0	2	6	8	10	10	
	d5a	Normal	10	2	0	0	0	0	0	0	1.63
		Sick	0	5	1	0	0	0	0	0	
		Dead	0	3	9	10	10	10	10	10	
	d5a20b	Normal	0	0	0	0	0	0	0	0	1.88
		Sick	10	0	0	0	0	0	0	0	
		Dead	0	10	10	10	10	10	10	10	
Chicken (1-day-old)	9a5b	Normal	6	0	0	0	0	0	0	0	1.78
		Sick	4	2	0	0	0	0	0	0	
		Dead	0	8	10	10	10	10	10	10	
	d5a	Normal	4	0	0	0	0	0	0	0	1.85
		Sick	4	0	0	0	0	0	0	0	
		Dead	2	10	10	10	10	10	10	10	
	d5a20b	Normal	0	0	0	0	0	0	0	0	1.89
		Sick	9	0	0	0	0	0	0	0	
		Dead	1	10	10	10	10	10	10	10	

Table 4. Comparison of deduced amino acid residues between the subjected viruses and field isolates virulent for waterfowls

	Amino acid residue at homologous positions of the substitution in the passaged NDVs											GenBank accession no.	Reference
	P	V	M			F		HN	L				
	229	230	44	123	342	142	304	538	1676	1844	2024		
9a5b[a]	Q	G	Q	N	D	I	E	G	F	F	R	AB524406	Shengqing *et al*. (2002)
d5a[a]	H[b]	C	Q	D	D	M	E	R	F	I	R	LC589003	Hidaka *et al*. (2021)
d5a20b[a]	H	C	R	D	N	M	K	R	Y	I	H	LC589004	Hidaka *et al*. (2021)
HN1007	Q	G	R	N	D	I	E	N	F	F	E	KX761866	Wen *et al*. (2016)
F48E8	Q	G	Q	N	D	I	E	N	F	F	E	FJ436302	Dai *et al*. (2013)
XBT14	Q	G	Q	N	D	I	E	N	F	F	E	JX677561	Fu *et al*. (2017)
NDV/Duck/China/GD09-2/2009	Q	G	Q	N	D	I	E	N	F	F	E	HQ317394	Zhang *et al*. (2011)
Duck/CH/GD/NH/10	Q	G	Q	N	D	I	E	N	F	F	E	KF219497	Kang *et al*. (2014)
ZJ/2/86/Ch, ZhJ-2/86	ND[c]	ND	ND	ND	ND	I	E	ND	ND	ND	ND	AF458016	Wan *et al*. (2004)
FP1/02	Q	G	Q	N	D	I	E	N	F	F	E	FJ872531	Shi *et al*. (2011)
Ch/CH/SD/2008/128	Q	G	Q	N	D	I	E	N	F	F	E	KJ600785	Meng *et al*. (2018)
Du/CH/SD/2009/134	Q	G	Q	N	D	I	E	N	F	F	E	KJ600786	Meng *et al*. (2018)
Goose/China/Jilin/MHK-1/2010	Q	G	Q	N	D	I	E	N	F	F	E	KM408752	Chen *et al*. (2015)
SF02	Q	G	Q	N	D	I	E	N	F	F	E	AF473851	Zou *et al*. (2005)
Goose/Guangdong/2010	Q	G	Q	N	D	I	E	N	F	F	E	KC551967	Sun *et al*. (2013)
XJ/2/97/Ch	Q	G	Q	N	D	I	E	N	F	F	E	JN618348	Wan *et al*. (2004)
JS/1/97/Go	ND	ND	ND	ND	ND	I	E	ND	ND	ND	ND	AF456435	Wan *et al*. (2004)
Md/CH/LGD/1/2005	Q	G	Q	N	D	I	E	N	F	F	E	KM885167	Wu *et al*. (2015)
Ck/CH/LHLJ/1/06	Q	G	Q	N	D	I	E	N	F	F	E	KU140419	Xu *et al*. (2017)
Go/CH/LHLJ/1/06	Q	G	Q	N	D	I	E	N	F	F	E	KJ607169	Xu *et al*. (2017)
CK/CH/HN/1/07	ND	ND	ND	ND	ND	I	E	ND	ND	ND	ND	FJ480799	Wu *et al*. (2015)
Duck/Jiangsu/JSD0812/2008	Q	G	Q	N	D	I	E	N	F	F	E	GQ849007	Dai *et al*. (2014)
Go/CH/HLJ/LL01/08	Q	G	Q	N	D	I	E	N	F	F	E	GU143550	Wu *et al*. (2015)
Duck/CH/GD/ SS/10	Q	G	Q	N	D	I	E	N	F	F	E	KF219498	Kang *et al*. (2014)
Duck/China/Guizhou/SS1/2014	Q	G	Q	N	D	I	E	N	F	F	E	KP742770	Duan *et al*. (2015)
QH-1/79	ND	ND	ND	ND	ND	I	ND	ND	ND	ND	ND	AF378250	Wan *et al*. (2004)

[a] The d5a20b and its progenitor strains.

[b] Amino acid residues consistent with d5a20b are highlighted.

[c] Not determined.

Chapter II

Identification of viral proteins and the amino acid residues involved in virulence of Newcastle disease virus for domestic ducks

Abstract

The Newcastle disease virus (NDV) d5a20b strain, which is obtained by serial passaging of the velogenic 9a5b strain in domestic ducks, showed increased virulence in ducks. The d5a20b strain had 11 amino acid substitutions in its P/V, M, F, HN, and L proteins as compared to 9a5b. In order to identify the molecular basis of virulence of NDV in ducks, a series of recombinant (r) NDVs were generated with these amino acid substitutions, and evaluated their influences on virulence and *in vitro* viral properties. As a result, each of the single amino acid substitutions in either the F protein I142M or the M protein Q44R contributed to the enhancement of intracerebral and intranasal pathogenicity in domestic ducks. The cell-cell fusion activity of the virus with F I142M was five times higher than that of the parental r9a5b. The virus with M Q44R rapidly replicated in duck embryo fibroblasts. Additionally, the rM+F+HN strain, which has the same amino acid sequences as d5a20b in M, F, and HN proteins, showed the highest level of virulence and replication efficiency among the generated recombinant viruses, nearly comparable to rd5a20b. These results suggest that multiple factors are involved in the high growth ability of NDV in duck cells, leading to increased virulence *in vivo*.

Introduction

The F proteins of velogenic Newcastle disease viruses (NDVs) have polybasic amino acids at their cleavage sites that are recognized by ubiquitous furin-like proteases, causing systemic infection and high mortality in chickens. Although the cleavability of F is the most important virulence determinant of NDV, various other viral proteins are intricately involved in virulence. The increased functional activity of several viral proteins correlates with virulence, such as polymerase activity (Dortmans *et al*., 2010), receptor binding and neuraminidase activity (Huang *et al*., 2004), and anti-immune activity by the V protein (Alamares *et al*., 2010; Wang *et al*., 2019). These functional changes contributed to the virulence in chickens by increased viral replication, expansion of tissue tropism, and inhibition of immune response.

Waterfowls infected with velogenic NDV generally do not show serious symptoms, whereas chickens develop severe disease with high mortality (Aldous *et al*., 2010; Miller *et al*., 2013). However, fatal ND cases in waterfowls have been sporadically reported. Since the late 1990s, lethal ND outbreaks in domestic ducks and geese have sporadically occurred in China (Jinding *et al*., 2005; Liu *et al*., 2015; Wu *et al*., 2015; Xu *et al*., 2017; Zou *et al*., 2005). The molecular basis of how these velogenic NDVs acquire high virulence in waterfowls remains unclear.

As showed in Chapter I, we passaged chicken-derived velogenic NDV 9a5b strain five times in duck air sacs, followed by 20 times in the duck brain. The resulting virus, designated d5a20b, showed higher virulence in ducks, and had a total of 11 amino acid substitutions in P/V, M, F, HN, and L as compared to 9a5b. These results indicate that some or all of these amino acid substitutions are linked to increased virulence in ducks. To identify the amino acids involved in the virulence in ducks, in this study, we generated recombinant NDVs with the amino acid substitutions found in d5a20b using reverse genetics and evaluated the effects of these substitutions on virulence. We found that the substitutions I142M in F and Q44R in M enhanced the virulence of NDV in ducks.

Materials and methods

Cell lines

Madin-Darby bovine kidney (MDBK) cells and baby hamster kidney cells expressing T7 RNA polymerase (BHK/T7-9; kindly provided by Prof. Nobuyuki Minamoto of Gifu University) (Ito *et al*., 2003) were grown in Eagle's minimum essential medium (MEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Beit-Haemek, Israel). Duck embryo fibroblasts (DEFs) were prepared from 12-day-old embryonated cherry valley duck eggs (Takahashi Artificial Hatchery, Osaka, Japan). The DEFs were cultured in Dulbecco's minimal essential medium (DMEM; Thermo Fisher Scientific) supplemented with 10% FBS.

Plasmid construction

The full-length antigenome cDNA of the NDV strain 9a5b (Shengqing *et al*., 2002) was constructed from six cDNA fragments. cDNA was synthesized using PrimeScript reverse transcriptase (Takara Bio, Shiga, Japan) and amplified using the KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan). These cDNA fragments were assembled using the unique restriction enzyme sites (Fig. 6) and cloned into the plasmid vector pATX-dribo (kindly provided by Dr. Hideki Ebihara, Mayo Clinic). The 5'-end of the leader region and 3'-end of the trailer region of antigenomic cDNA was connected to the T7 RNA polymerase promoter adjacent to the ribozyme sequence derived from the hepatitis delta virus. The resulting clone was named pATX-dribo 9a5b. The full-length antigenomic cDNA of d5a20b, designated pATX-dribo d5a20b, was prepared based on pATX-dribo 9a5b by exchanging the cDNA with the corresponding regions of the d5a20b cDNA using the In-Fusion HD Cloning Kit (Takara Bio). Using this procedure and overlapping polymerase chain reaction (PCR), full-length antigenome cDNAs containing various amino acid substitution(s) found between 9a5b and d5a20b were also generated (Fig. 6). The cDNA fragments containing substitution(s) were prepared using

overlapping PCR.

To generate helper plasmids expressing the RNP components, the open reading frames (ORFs) of NP, P, and L genes of the lentogenic NDV strain Alaska/415, the parent virus of 9a5b (Shengqing *et al.*, 2002), were amplified using reverse transcription PCR (RT-PCR) and cloned into the plasmid vector pcDNA3.1 (Thermo Fisher Scientific). The resulting plasmids were named pcDNA-415NP, pcDNA-415P, and pcDNA-415L, respectively. Primer sequences used for plasmid construction are available upon request.

Virus rescue

BHK/T7-9 cells were co-transfected with 10 μg full-length antigenomic cDNA and helper plasmids expressing RNA components (1 μg pcDNA-415NP, 0.5 μg pcDNA-415P, and 0.5 μg pcDNA-415L) using TransIT LT-1 reagent (Mirus Bio LLC, Madison, WI, USA) and incubated at 37 °C. The culture supernatants were collected at 72 hr post-transfection and inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs to produce virus stocks. The 50% tissue culture infectious dose ($TCID_{50}$) and 50% egg infectious dose (EID_{50}) of the virus stocks were determined in MDBK cells and 10-day-old embryonated chicken eggs, respectively.

Pathogenicity index tests

The intracerebral pathogenicity index (ICPI) in 2-day-old cherry valley ducks (Takahashi artificial hatchery) was determined according to the OIE protocol (OIE *et al.*, 2021) with slight modifications. Ten birds were intracerebrally inoculated with $10^{6.7}$ EID_{50}/0.05 ml of the virus and scored for clinical symptoms every 24 hr for 8 days (0 if normal, 1 if sick, and 2 if dead). The ICPI value is the mean score per bird per observation. It ranges from 0.0 to 2.0, and a large score indicates high pathogenicity.

Experimental infection of 1-week-old ducks

Five or six 1-week-old cherry valley ducks (Takahashi artificial hatchery) were intranasally inoculated with 10^7 EID_{50}/0.2 ml of the virus. Clinical manifestations and survival rates were monitored daily until 8 days post-inoculation (dpi).

Virus replication kinetics

Viruses were inoculated at a multiplicity of infection (MOI) of 0.001 into DEFs in 6-well plates. To calculate the MOI, the $TCID_{50}$ titer was converted to the PFU titer by multiplying the $TCID_{50}$ titer by 0.7 (Bryan *et al.*, 1957). Culture supernatants were collected at 8, 16, 24, 32, 48, and 64 hr post-inoculation (hpi), and virus titers ($TCID_{50}$) were determined in MDBK cells using the method of Reed and Müench (Reed and Müench, 1938).

Cell-cell fusion assay

DEFs in 12-well plates were inoculated with virus at an MOI of 5 and incubated at 37 °C. At 8 hpi, the cells were fixed with methanol and stained with the Giemsa stain solution (FUJIFILM Wako Pure Chemical, Osaka, Japan). The fusion index was determined as the total number of nuclei in the syncytia with three or more nuclei in 10 random fields.

Ethics statements

All animal experiments were approved by the Institutional Animal Care and Use Committee of Tottori University (Approval Number: 29-036) and conducted at a biosafety level 2 Animal Experimental Facility, Tottori University, Japan. All experiments were conducted in accordance with the committee guidelines. Throughout the present study, any birds unable to feed or drink were euthanized and recorded as dead on the following day's observation.

Results

Rescue of recombinant NDVs

To identify the amino acids involved in the virulence of the NDV d5a20b strain in ducks, a series of amino acid substitutions were introduced into the parental 9a5b genome (Fig. 6). The r9a5b strain is an artificial parental virus containing the 9a5b genome, and rd5a20b possessed a total of 11 amino acid substitutions in the 9a5b backbone. The recombinant chimeric viruses rM+F+HN, rF+HN, and rP+L had combinations of the d5a20b-origin amino acid substitutions in the envelope-associated proteins (M, F, and HN) or polymerase-associated proteins (P and L). The chimeric viruses rP, rM, rF, rHN, and rL contained amino acid substitution(s) in the individual proteins. Note that the mutation in the P gene (for Q229H) of rP+L and rP simultaneously induced the substitution G230C in the V protein. The recombinant viruses rM44, rM123, rM342, rF142, and rF304 possessed a single amino acid change in the M or F proteins. All recombinant NDVs were successfully rescued from the transfected BHK/T7-9 cells, showing efficient replication in embryonated chicken eggs ($>10^8$ EID_{50}/ml). Sequencing results confirmed that the stocked viruses had no undesired mutations.

Intracerebral pathogenicity of recombinant NDVs in domestic ducks

To evaluate the pathogenicity of the recombinant NDVs, we conducted ICPI tests in two-day-old domestic ducks (Fig. 6). The ICPIs of r9a5b and rd5a20b were 1.33 and 1.94. rM+F+HN and rF+HN showed higher ICPI values (1.88 and 1.56, respectively) than the parental r9a5b, whereas enhanced virulence was not observed in rP+L. The ICPIs of rM, rF, and rHN were 1.50, 1.65, and 1.06, respectively, suggesting that the amino acid substitutions in M and F contributed to intracerebral pathogenicity in ducks. Among the mutant viruses with single amino acid substitutions, rM44 and rF142 showed ICPI values of 1.64 that were higher than r9a5b. The results indicated that the amino

acid substitutions I142M in F and Q44R in M were independently responsible for the enhanced virulence of d5a20b in ducks.

Pathogenicity in one-week-old ducks via the intranasal route

To evaluate the pathogenicity of the recombinant NDVs via a natural infection route, one-week-old ducks were intranasally inoculated with the selected recombinant viruses. The ducks inoculated with r9a5b survived the observation period without showing any clinical signs (Fig. 7). Two of the six rP+L-inoculated ducks showed leg paralysis at 5 dpi, although all birds survived. All six rd5a20b-inoculated birds were depressed at 3 dpi and dead at 4–5 dpi. Similarly, rM+F+HN-inoculated ducks showed depression and paralysis at 3–4 dpi, and all six ducks died by 6 dpi. rF142 and rM44 induced slower disease progression and lower mortality rates in ducks compared to rd5a20b. Five ducks inoculated with rF142 exhibited depression from 5 dpi, and five of the six birds died at 6–8 dpi. rM44-inoculated birds showed leg paralysis after 3 dpi, and three of the five birds died at 5–8 dpi. These results showed that a single amino acid change in F (I142M) or in M (Q44R) also increased the intranasal virulence of 9a5b in ducks. However, these single amino acid changes were insufficient to induce virulence comparable to that of rd5a20b.

Viral replication kinetics in DEFs

The effects of amino acid changes on NDV replication were assessed using DEFs. rd5a20b and rM+F+HN rapidly replicated at 8–32 hpi compared to r9a5b (Fig. 8A). Among the recombinant NDVs with a single protein of d5a20b, rM showed higher viral titers than r9a5b at all time points (Fig. 8B). rM123 also showed high growth, comparable to that of rM (Figs. 8B and 8C). rM44 replicated slightly higher titers than r9a5b at earlier time points (16–24 hpi) and lower titers after 32

hpi (Fig. 8C). The other recombinant NDVs used in this examination showed similar (rF+HN, rP+L, rF, rHN, rL, rF142, and rF304) or lower (rP and rM342) replication trends than r9a5b (Fig. 8).

Effect of F and HN amino acid substitutions on fusion activity in DEFs

The fusion index of rd5a20b in infected DEFs was 663%, which was significantly higher than that of r9a5b (set as 100%) (Fig. 9). Enhanced fusion activity was also observed in the following viruses with amino acid substitution I142M in F: rM+F+HN, 761%; rF+HN, 436%; rF, 737%; and rF142, 507%. The other tested viruses did not show any significant increase in fusion activity.

Disccusion

In this study, recombinant NDVs with the I142M substitution in F or the Q44R substitution in M contributed to the increased virulence of NDV in two-day- and one-week-old domestic ducks (Fig. 6 and 7).

All recombinant NDVs carrying F I142M showed increased syncytium formation in DEFs (Fig. 9). Position 142 is located in the N-terminus of the N-terminal heptad repeat (HRA) in F and is adjacent to the fusion peptide (FP) (Swanson *et al.*, 2010). The FP of NDV is a highly hydrophobic region that consists of amino acid positions 117–141. Once fusion is triggered, the HRA region, which consists of β-strands and helices, refolds into a coiled-coil, and the FP springs up and inserts into the target lipid membrane (Bose *et al.*, 2015; Chang *et al.*, 2012). As the HRA region undergoes a large conformational change during the fusion process, the component amino acid residues are important for fusion activity (Sergel *et al.*, 2001). Among the *Paramyxoviridae*, amino acid substitutions in the HRA region have been reported to modulate fusion activity by altering the stability of the pre-fusion structure of the F protein (Luque *et al.*, 2007; West *et al.*, 2005). Therefore, the F I142M substitution in NDV also potentially facilitates conformational changes by reducing the stability of the prefusion structure. In addition, I142M did not increase virus replication *in vitro* (Fig. 8C). The previously reported amino acids in F proteins responsible for facilitating cell-cell fusion activity caused various effects on viral replication *in vitro*: Y524A and Y527A of the BC strain increased the expression of F on the cell surface and viral replication (Samal *et al.*, 2013), whereas T458D and G459D of the G7 strain had a negative impact (Ji *et al.*, 2017). These results indicate that enhanced fusion activity of NDV does not necessarily lead to higher viral replicability in poultry tissue cultures.

There are two distinct routes for the spread of enveloped viruses between cells. One is the cell-free transmission, where extracellularly released progeny viruses infect target cells. The other is cell-

cell transmission, where viruses efficiently spread from infected cells to surrounding uninfected cells through cell-cell fusion (syncytium formation) (Cifuentes-Muñoz *et al*., 2018). As cell-cell viral spread can skip several infectious events and evade host immunity in the extracellular environment, it is thought to play an important role in viral infection. Thus, we speculate that the enhanced fusion activity of rF142 promoted the spread of viral components via hyperfusogenic activity, resulting in high pathogenicity with cellular dysfunction and organ failure *in vivo* (Figs. 6 and 7), but did not reflect changes in the virus titers of supernatants in tissue cultures *in vitro* (Fig. 8C). Cell-cell fusion *in vivo* is presumed to be associated with the virulence of other paramyxoviruses, such as measles virus and Sendai virus (Ludlow *et al*., 2015; Luque *et al*., 2010). Syncytia has also been reported in the bronchial lumen of chickens infected with velogenic NDV (Mousa *et al*., 2020). It will be interesting to verify whether the increased fusion activity by F I142M is linked to syncytium formation *in vivo*.

The M protein of NDV localizes to the nucleolus by interacting with B23 (NPM1, nucleophosmin) and promotes viral replication (Duan *et al*., 2014a). A putative nucleolar localization signal (NoLS) that interacts with host protein B23 has been identified at amino acid positions 30–60 of M. NoLS is rich in basic amino acids, and arginine in particular promotes localization to nucleoli containing abundant RNA by providing electrostatic interactions with the phosphate groups of RNA (Martin *et al*., 2015; Scott *et al*., 2010). Duan *et al*. (2014b) reported that a single arginine to alanine substitution (R42A) in the NoLS of NDV M reduced the nuclear localization of M and led to decreased viral replication and virulence in chickens. In this study, recombinant NDV rM44 showed higher growth at early time points *in vitro* (Fig. 8C) and virulence (Figs. 6 and 7) compared to r9a5b. The amino acid at position 44 of M was included in the putative NoLS in a previous study (Duan *et al*., 2014a). Thus, the M Q44R, an amino acid substitution with a basic amino acid, might enhance

virulence in ducks by increasing the viral replication efficacy, and its impact on the nuclear localization of M is of further interest.

rF142 and rM44 were highly virulent to ducks compared to r9a5b, but did not reach the full virulence of rd5a20b (Figs. 6 and 7). In addition, the virulence of rM+F+HN was nearly comparable to that of rd5a20b (Figs. 6 and 7). Thus, multiple amino acid substitutions in M and envelope proteins may synergistically enhance virulence in ducks. Notably, M N123D promoted replication *in vitro* (Fig. 8C), likely assisting the high virulence of rd5a20b and rM+F+HN synergistically with F I142M and M Q44R. The amino acid position 123 of M is located at the M dimer contact surface and near the surface of the interaction side of the M dimer with the lipid membrane (Battisti *et al*., 2012). The formation of M dimers and their higher-order oligomers under the lipid membrane is important for the budding of paramyxovirus and its relatives. Mutations in the M dimer interface of canine distemper virus and respiratory syncytial virus have been reported to reduce M dimer formation, leading to a reduction in budding of virus-like particles (Bringolf *et al*., 2017; Förster *et al*., 2015). Therefore, it would be interesting to verify whether M N123D affects M dimer formation and budding efficiency.

In contrast to chickens, ducks are generally resistant to NDV infection, implying the existence of distinctive innate immunity (Anis *et al*., 2013; Rehman *et al*., 2018). In our previous study (Hidaka *et al*., 2021), we found that d5a20b marginally suppressed expression of the *IFN-β* gene in DEFs compared to 9a5b, and we speculated that the amino acid substitution in V is associated with virulence by altering IFN-β signaling suppression activity, as also observed elsewhere (Alamares *et al*., 2010; Wang *et al*., 2019). However, the virulence of rP+L, including G230C in V, was similar to that of parental r9a5b (Figs. 6 and 7). Identifying the substituted amino acid responsible for the low level of *IFN-β* induction of d5a20b is essential to explain the pathogenesis of NDV in ducks.

In this study, F I142M and M Q44R substitutions were identified as determinants of virulence in ducks, and functional changes such as fusogenicity and replication efficiency caused by these substitutions presumably led to high lethality in ducks. Future analysis of the detailed effects of F I142M and M Q44R substitutions will provide further insights into the virulence of NDV in ducks. To determine whether F I142M and M Q44R substitutions were found in other NDVs, we analyzed sequences deposited in GenBank. There were no viruses with F I142M substitution, whereas eight field isolates possessing M Q44R substitution were found. The virulence of five of these eight isolates was reported to be high in chickens (Kattenbelt *et al*., 2006; Qiu *et al*., 2009; Wen *et al*., 2016) (Table 5). Interestingly, one of these isolates, HN1007, was isolated from diseased duck flocks and caused lethal infection with a mortality rate of 10% in domestic ducks (Wen *et al*., 2016). However, as NDVs with F I142M or M Q44R substitution are minor variants in fields, it will be necessary to verify whether the increased virulence in ducks by F I142M and M Q44R substitution is a strain-specific effect. This study revealed that single amino acid changes alone enhance virulence in ducks, suggesting that velogenic NDVs with virulence for waterfowl have the potential to accidentally emerge during infection. The spillovers of NDV from poultry to wild waterbirds have also been reported (Vidanović *et al*., 2007; Xiang *et al*., 2017). Thus, to prevent the outbreak of lethal ND in waterfowl, improvement of biosecurity in farms and surveillance of wild birds for ND are important.

Legends for figures

Figure 6. Genome constitution and intracerebral pathogenicity index (ICPI) in ducks of recombinant NDVs.

The amino acid substitutions introduced into each recombinant NDV are indicated by stars. rd5a20b was generated by introducing all 11 amino acid substitutions into the r9a5b genome backbone. Similarly, 15 recombinant NDVs were generated. The restriction enzyme sites used to construct the full-length antigenome cDNA plasmids are indicated by arrowheads. The ICPI scores in two-day-old ducks of recombinant NDVs are shown on the right side of the genome map for each virus. ND, not determined.

Figure 7. Survival rates of one-week-old ducks intranasally inoculated with recombinant NDVs.

Five (rM44) or six (other viruses) birds were each intranasally inoculated with 10^7 EID_{50} of virus and then monitored for survival daily for eight days.

Figure 8. Growth kinetics of recombinant NDVs in DEFs.

Viral replication curves of recombinant NDVs containing the d5a20b's multiple proteins (A), single protein (B), and single amino acid (C) in DEFs. Cells were inoculated with each virus at a multiplicity of infection of 0.001. Supernatants were harvested at 8, 16, 24, 32, 48, and 64 hpi. Viral titers were determined by measuring the $TCID_{50}$ in MDBK cells. The limit of detection was 2.1 log $TCID_{50}$/ml. Each virus titer is expressed as the mean of three or more separate experiments, and error bars represent the corresponding standard error of the mean (mean ± SEM). Asterisks indicate

significant differences in the virus titers compared to r9a5b at each time point; *P* values were calculated using two-tailed, unpaired *t*-tests (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure 9. Evaluation of the effect of amino acid substitutions on cell fusion activity.

Viruses were inoculated into duck embryo fibroblasts at a multiplicity of infection of 5. At 8 hpi, the cells were fixed and stained. The fusion index is the total number of nuclei in the syncytia (cells containing three or more nuclei) in 10 random fields per well in triplicate. The values are expressed relative to r9a5b, which was taken as 100%. Asterisks indicate significant differences in the fusion index compared to r9a5b. *P* values were calculated using two-tailed, unpaired *t*-tests (* $P < 0.01$, ** $P < 0.001$).

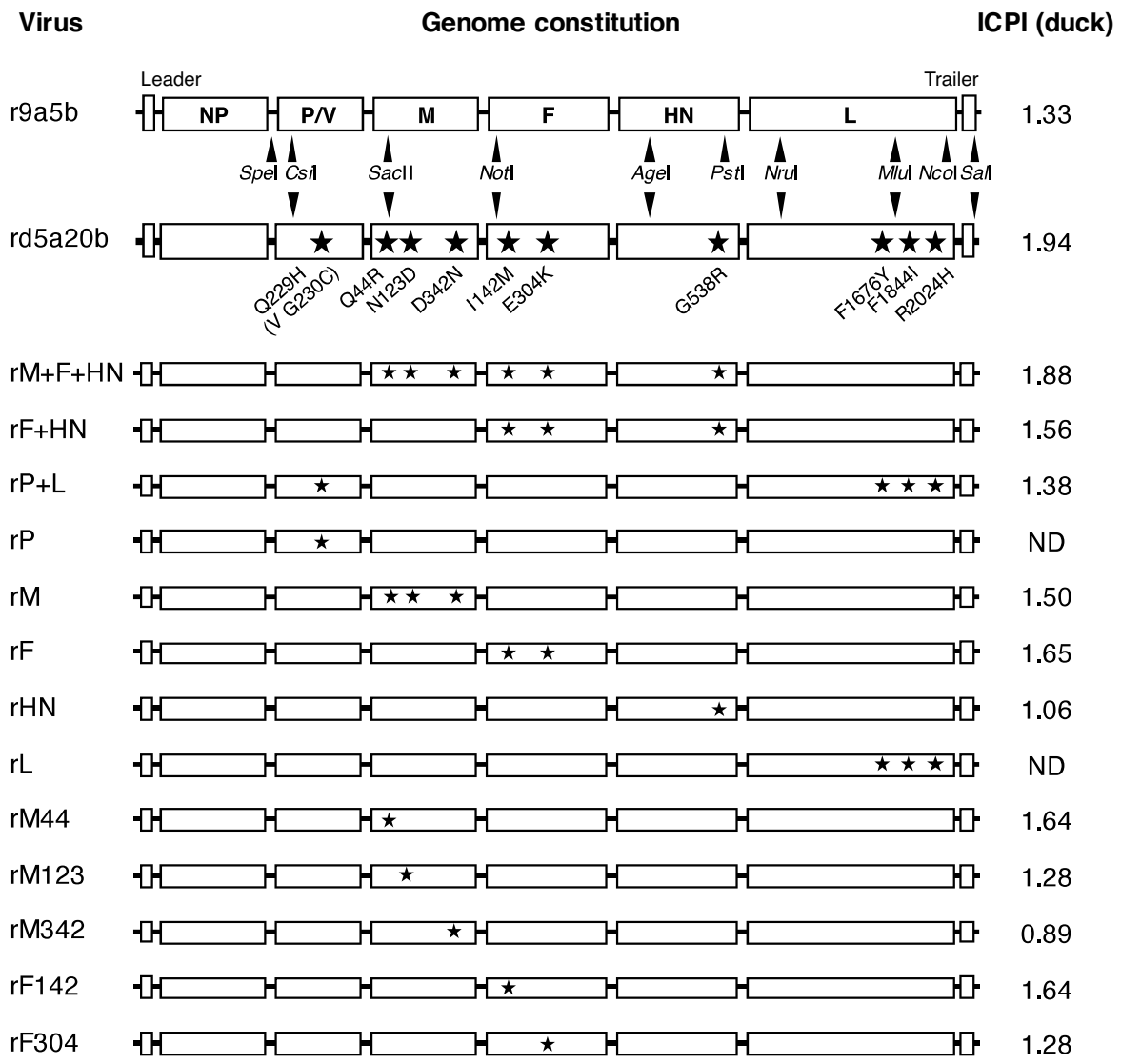
Virus
Genome constitution
ICPI (duck)
Leader
Trailer
NP
P/V
M
F
HN
L
SpeI
CsiI
SacII
NotI
AgeI
PstI
NruI
MluI
NcoI
SalI
r9a5b
1.33
rd5a20b
1.94
Q229H
(V G230C)
Q44R
N123D
D342N
I142M
E304K
G538R
F1676Y
F1844I
R2024H
rM+F+HN
1.88
rF+HN
1.56
rP+L
1.38
rP
ND
rM
1.50
rF
1.65
rHN
1.06
rL
ND
rM44
1.64
rM123
1.28
rM342
0.89
rF142
1.64
rF304
1.28

Figure 6

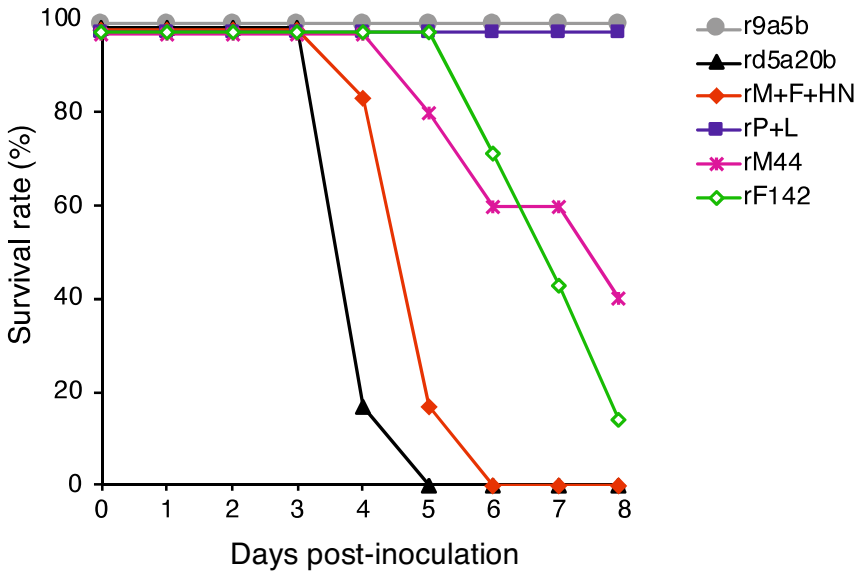
100
80
60
40
20
0
Survival rate (%)
0
1
2
3
4
5
6
7
8
Days post-inoculation
r9a5b
rd5a20b
rM+F+HN
rP+L
rM44
rF142

Figure 7

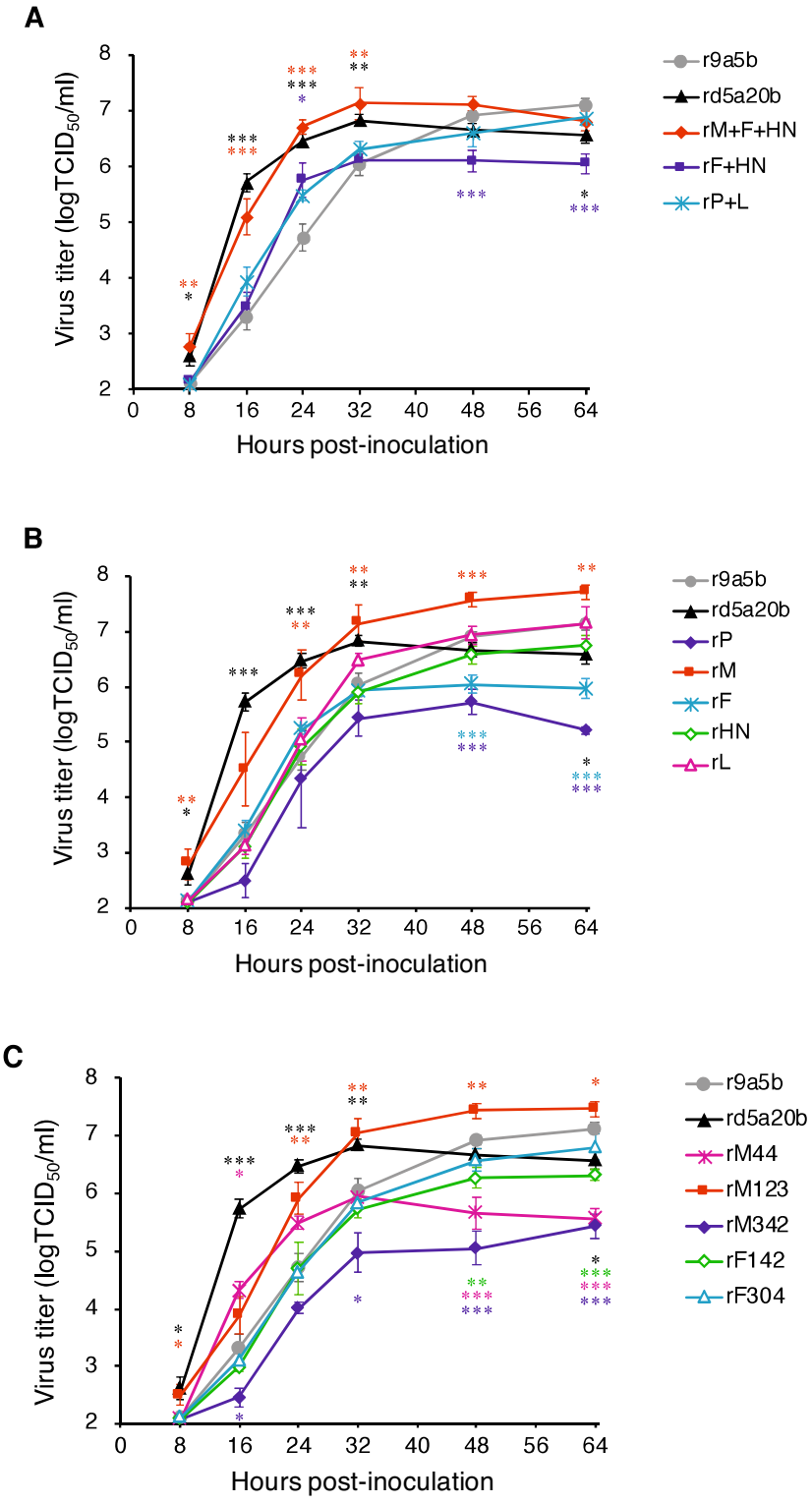
A
Virus titer (logTCID50/ml)
Hours post-inoculation
r9a5b
rd5a20b
rM+F+HN
rF+HN
rP+L
B
Virus titer (logTCID50/ml)
Hours post-inoculation
r9a5b
rd5a20b
rP
rM
rF
rHN
rL
C
Virus titer (logTCID50/ml)
Hours post-inoculation
r9a5b
rd5a20b
rM44
rM123
rM342
rF142
rF304

Figure 8

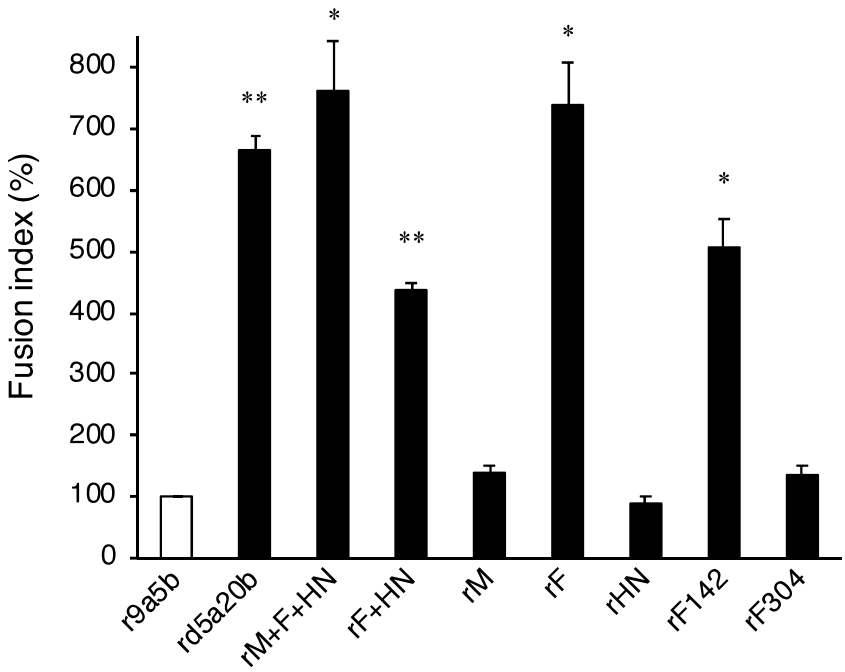
Fusion index (%)
800
700
600
500
400
300
200
100
0
**
*
**
*
*
r9a5b
rd5a20b
rM+F+HN
rF+HN
rM
rF
rHN
rF142
rF304

Figure 9

Table 5 NDV isolates with Q44R substitution in M protein

GenBank accession no.	Isolate	Date isolated	Region isolated	Host isolated	Class	Genotype	ICPI[a]	IVPI[b]	Reference
AY935498	99-1435	1999	Australia	Chicken	II	I	1.69	ND[c]	Kattenbelt *et al.*, 2006
AY935490	02-1334	2002	Australia	Chicken	II	I	1.61	ND	Kattenbelt *et al.*, 2006
FJ430159	JS/7/05/Ch	2005	China	Chicken	II	III	ND	2.88	Qiu *et al.*, 2009
FJ430160	JS/9/05/Go	2005	China	Goose	II	III	1.61	1.33	Qiu *et al.*, 2009
KX761866	HN1007	2010	China	Duck	II	III	1.94	ND	Wen *et al.*, 2016

[a] Intracerebral pathogenicity index (ICPI) score in chickens reported in reference.

[b] Intravenous pathogenicity index (IVPI) in chickens reported in reference.

[c] Not determined.

General conclusion

Velogenic Newcastle disease virus (NDV) is still one of the pathogens that causes serious damage to the poultry industry due to its high contagiousness and virulence to chickens. Although verogenic NDVs generally cause subclinical infection in waterfowls such as ducks, NDVs with high virulence in waterfowl have been sporadically reported. The mechanism of how these velogenic NDVs acquire high virulence in waterfowls remains unclear.

In this study, virulence factors of NDV for ducks were identified in the mutant NDV strain d5a20b, which was obtained by passaging of velogenic NDV in ducks. In Chapter I, a chicken-derived velogenic NDV strain 9a5b was passaged in domestic ducks five times in their air sacs, followed by 20 times in their brains. The d5a20b showed higher pathogenicity accompanying efficient replication in broader systemic tissues in ducks as compared with the 9a5b strain. These results suggest that chicken-derived velogenic NDVs have the potential to become virulent in ducks during circulation in domesticated waterfowl populations. The d5a20b suppressed IFN-β gene expression in duck embryo fibroblasts and more rapidly replicated than 9a5b, indicating that some inhibitory or evasion ability to the immune response during infection perhaps involved in the high virulence of d5a20b in ducks.

The d5a20b strain had 11 amino acid substitutions in its P/V, M, F, HN, and L proteins as compared to 9a5b. In Chapter II, a series of recombinant NDVs with these amino acid substitutions were generated to identify the molecular basis of virulence of NDV in ducks, and evaluated their influences on virulence and *in vitro* viral properties. As a result, each of the single amino acid substitutions in either the F protein I142M or the M protein Q44R contributed to the enhancement of intracerebral and intranasal pathogenicity in domestic ducks. The cell-cell fusion activity of the virus with F I142M was five times higher than that of the parental r9a5b. The virus with M Q44R rapidly replicated in duck embryo fibroblasts. Additionally, the rM+F+HN strain, which has the same amino acid sequences as d5a20b in M, F, and HN proteins, showed the highest level of virulence and

replication efficiency among the generated recombinant viruses, nearly comparable to rd5a20b. These results suggest that multiple factors are involved in the high growth ability of NDV in duck cells, leading to increased virulence *in vivo*.

This study revealed that single amino acid changes alone enhance virulence in ducks, suggesting that velogenic NDVs with virulence for waterfowl have the potential to accidentally emerge during infection. As susceptible hosts of NDV, not only chickens but also waterfowls may need to be monitored for the control of ND. To prevent the outbreak of lethal ND in waterfowls and the viral transmission to migratory waterfowls, which can spread viruses transboundary, biosecurity in poultry markets and farms should be improved. In this study, it was found that amino acid substitutions in the F and M proteins involved in increased virulence of NDV in ducks. Further investigation is required to understand the overall mechanism of how these substitutions increase the virulence of NDV in ducks.

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