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**Contribution of mutation I142M in fusion protein and Q44R in matrix protein of Newcastle
disease virus to virulence in ducks**

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Running head

FACTOR OF NDV VIRULENCE IN DUCK

ABSTRACT

Although verogenic Newcastle disease viruses (NDVs) generally cause subclinical infection in waterfowls such as ducks, NDVs with high virulence in waterfowl have been sporadically reported. We previously reported that the NDV d5a20b strain, which is obtained by serial passaging of the velogenic 9a5b strain in domestic ducks, showed increased virulence in ducks (Hidaka *et al.*, 2021). The d5a20b strain had 11 amino acid substitutions in its P/V, M, F, HN, and L proteins as compared to 9a5b. In the present study, we generated a series of recombinant (r) NDVs with these amino acid substitutions to identify the molecular basis of virulence of NDV in ducks, and evaluated their influences on virulence and *in vitro* viral properties. 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*.

KEYWORD

duck, Newcastle disease virus (NDV), virulence, waterfowl

INTRODUCTION

Newcastle disease virus (NDV) is a pathogen of Newcastle disease (ND), which causes severe economic losses in the poultry industry worldwide. NDV belongs to the subfamily *Avulavirinae* of the family *Paramyxoviridae*, and was recently named as Avian orthoavulavirus 1 [20]. NDV has a negative single-stranded RNA genome containing six genes in the order 3'-NP-P-M-F-HN-L-5', which encodes nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large protein (L). Additionally, the non-structural proteins V and W are expressed by RNA editing during P gene transcription [43]. 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 [6, 9]. The viral genome RNA encoded by the NP serves as a template for transcription and replication by the polymerase complex containing L and P [24]. V suppresses the host's innate immune system by inhibiting type I IFN signaling, contributing to efficient viral replication [18, 36]. 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 [15].

NDVs are classified into three pathotypes based on their virulence in chickens: lentogenic (avirulent), mesogenic (moderately virulent), and velogenic (highly virulent) [31]. The amino acid sequence of the F cleavage site is a major determinant of the virulence of NDV [34]. 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. Although the cleavability of F is the most important virulence determinant of NDV, various other viral proteins are intricately involved in

virulence in chickens. The increased functional activity of several viral proteins correlates with virulence, such as polymerase activity [12], receptor binding and neuraminidase activity [19], and anti-immune activity by the V protein [1, 48].

The susceptibility to NDV varies among bird species. Waterfowls, such as ducks and geese, are generally resistant to velogenic NDV. Waterfowls infected with velogenic NDV do not show serious symptoms, whereas chickens develop severe disease with high mortality [2, 31]. This difference in susceptibility to NDV infection could be explained by differences in the innate immune system between waterfowls and chickens [38]. Ducks and geese have functional retinoic acid-inducible gene-I (RIG-I), which chickens do not [4]. RIG-I is a cytoplasmic RNA sensor that recognizes virus-derived RNAs and induces IFN production. Another RIG-I-like receptor, the melanoma differentiation-associated gene 5 (MDA5), is expressed in both chickens and ducks. The V protein of NDV inhibits IFN production by targeting multiple host factors, including MDA5 [10, 45]. Therefore, the expression of RIG-I in waterfowls may contribute to resistance against NDV infection [38, 44].

However, fatal ND cases have been sporadically reported. In 2007, a velogenic NDV was isolated from a dead mallard duck found in field in Serbia [47]. Since the late 1990s, lethal ND outbreaks in domestic ducks and geese have sporadically occurred in China [23, 26, 51, 53, 54]. 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 [26]. Morbidity and mortality rates in affected flocks were approximately 80% and 30%–50%, respectively. The molecular basis of how these velogenic NDVs acquire high virulence in waterfowls remains unclear.

In our previous study, to investigate the virulence of NDV, the lentogenic NDV Goose/Alaska/415/91 (Alaska/415) isolated from wild goose was passaged in chickens [42]. 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. More recently, we passaged 9a5b five times in duck air sacs, followed by 20 times in the duck brain [17]. 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) [21] were grown in Eagle's minimum essential medium (MEM; Thermo Fisher Scientific, Waltham, MA, U.S.A.) 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.

112

113 *Plasmid construction*

114 The full-length antigenome cDNA of the NDV strain 9a5b [42] was constructed from six cDNA
115 fragments. cDNA was synthesized using PrimeScript reverse transcriptase (Takara Bio, Shiga,
116 Japan) and amplified using the KOD-Plus-DNA polymerase (TOYOBO, Osaka, Japan). These
117 cDNA fragments were assembled using the unique restriction enzyme sites (Fig. 1) and cloned into
118 the plasmid vector pATX-dribo (kindly provided by Dr. Hideki Ebihara, Mayo Clinic). The 5'-end
119 of the leader region and 3'-end of the trailer region of antigenomic cDNA was connected to the T7
120 RNA polymerase promoter adjacent to the ribozyme sequence derived from the hepatitis delta virus.
121 The resulting clone was named pATX-dribo 9a5b. The full-length antigenomic cDNA of d5a20b,
122 designated pATX-dribo d5a20b, was prepared based on pATX-dribo 9a5b by exchanging the cDNA
123 with the corresponding regions of the d5a20b cDNA using the In-Fusion HD Cloning Kit (Takara
124 Bio). Using this procedure and overlapping PCR, full-length antigenome cDNAs containing various
125 amino acid substitution(s) found between 9a5b and d5a20b were also generated (Fig. 1). The cDNA
126 fragments containing substitution(s) were prepared using overlapping PCR.

127 To generate helper plasmids expressing the RNP components, the open reading frames (ORFs)
128 of NP, P, and L genes of the lentogenic NDV strain Alaska/415, the parent virus of 9a5b [42], were
129 amplified using RT-PCR and cloned into the plasmid vector pcDNA3.1 (Thermo Fisher Scientific).
130 The resulting plasmids were named pcDNA-415NP, pcDNA-415P, and pcDNA-415L, respectively.
131 Primer sequences used for plasmid construction are available upon request.

132

133 *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, U.S.A.) 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 (TCID₅₀) and 50% egg infectious dose (EID₅₀) 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 [33] with slight modifications. Ten birds were intracerebrally inoculated with 10^{6.7} EID₅₀/0.05 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 10⁷ EID₅₀/0.2 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 TCID₅₀ titer was converted to the PFU titer by multiplying the TCID₅₀ titer by 0.7 [8]. Culture supernatants were collected at 8, 16, 24, 32, 48, and 64 hr post-inoculation (hpi), and virus titers (TCID₅₀) were determined in MDBK cells using the method of Reed and Muench [37].

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. 1). 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 ($>10^8$ EID₅₀/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. 1). The ICPIs of r9a5b and rd5a20b were 1.33 and 1.94, comparable to those of wild-type 9a5b and d5a20b, respectively [17]. 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. 2). 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. 3A). Among the recombinant NDVs with a single protein of d5a20b, rM showed higher viral titers than r9a5b at all time points (Fig. 3B). rM123 also showed high growth, comparable to that of rM (Figs. 3B and 3C). rM44 replicated slightly higher titers than r9a5b at earlier time points (16–24 hpi) and lower titers after 32 hpi (Fig. 3C). 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. 3).

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. 4). 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.

DISCUSSION

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. 1 and 2).

All recombinant NDVs carrying F I142M showed increased syncytium formation in DEFs (Fig. 4). 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) [46]. 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 [6, 9]. As the HRA region undergoes a large conformational change during the fusion process, the component amino acid residues are important for fusion activity [41]. 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 [28, 50]. 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. 3C). 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 [39], whereas T458D and

G459D of the G7 strain had a negative impact [22]. 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) [11]. 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. 1 and 2), but did not reflect changes in the virus titers of supernatants in tissue cultures *in vitro* (Fig. 3C). Cell-cell fusion *in vivo* is presumed to be associated with the virulence of other paramyxoviruses, such as measles virus and Sendai virus [27, 29]. Syncytia has also been reported in the bronchial lumen of chickens infected with velogenic NDV [32]. 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 [13]. 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 [30, 40]. Duan *et al.* (2014) 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 [14]. In this study, recombinant NDV rM44 showed higher growth at early time points *in*

vitro (Fig. 3C) and virulence (Figs. 1 and 2) compared to r9a5b. The amino acid at position 44 of M was included in the putative NoLS in a previous study [13]. 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. 1 and 2). In addition, the virulence of rM+F+HN was nearly comparable to that of rd5a20b (Figs. 1 and 2). Thus, multiple amino acid substitutions in M and envelope proteins may synergistically enhance virulence in ducks. Notably, M N123D promoted replication *in vitro* (Fig. 3C), 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 [5]. 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 [7, 16]. 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 [3, 38]. In our previous study [17], 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 [1, 48]. However, the virulence of rP+L, including G230C in V, was similar to that of parental r9a5b (Figs. 1 and 2). 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 (Supplementary Table 1). 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 [49]. 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 [47, 52]. Thus, to prevent the outbreak of lethal ND in waterfowl, improvement of biosecurity in farms and surveillance of wild birds for ND are important.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

316

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FIGURE LEGENDS

Figure 1. Genome constitution and intracerebral pathogenicity index (ICPI) in ducks of recombinant Newcastle disease viruses (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 2. Survival rates of one-week-old ducks intranasally inoculated with recombinant Newcastle disease virus

Five (rM44) or six (other viruses) birds were intranasally inoculated with 10^7 50% egg infectious dose of the virus and then monitored for survival daily for eight days.

Figure 3. Growth kinetics of recombinant Newcastle disease viruses (NDVs) in duck embryo fibroblasts (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 50% tissue culture infectious dose (TCID₅₀) in Madin-Darby bovine kidney cells. The limit of detection was 2.1 log TCID₅₀/ml. Each virus titer was expressed as

the mean of three or more separate experiments, and error bars represent the corresponding standard error of the mean (mean $\pm$ 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 4. 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).

Figure 1

Figure 2

519 **Figure 3**

523 **Figure 4**

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Supplementary Table 1 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	[25]
AY935490	02-1334	2002	Australia	Chicken	II	I	1.61	ND	[25]
FJ430159	JS/7/05/Ch	2005	China	Chicken	II	III	ND	2.88	[35]
FJ430160	JS/9/05/Go	2005	China	Goose	II	III	1.61	1.33	[35]
KX761866	HN1007	2010	China	Duck	II	III	1.94	ND	[49]

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

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

^c Not determined.