Title; Potential of Genotype VII Newcastle Disease Viruses to Cause Differential Infections in Chickens and Ducks

Comparative pathobiology of genotype VII NDV in ducks and chickens

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Summary

Newcastle disease (ND), caused by Newcastle disease virus (NDV), is one of the most infectious and economically important diseases in the poultry industry worldwide. While infections are reported in a wide range of avian species, the pathogenicity of chicken-origin virulent NDV isolates in ducks remains elusive. In this study, two NDV strains were isolated

and biologically characterized from an outbreak in chickens and apparently healthy ducks. Pathogenicity assessment indices, including the mean death time (MDT), intracerebral pathogenicity index (ICPI), and cleavage motifs in the fusion (F) protein, indicated that both isolates were velogenic in nature. However, both isolates showed differential pathogenicity in ducks. The chicken-origin isolate caused high (70%) mortality, whereas the duck-origin virus resulted in low (20%) mortality in 4-week-old ducks. Intriguingly, both isolates showed comparable disease pathologies in chickens. Full genome sequence analysis showed that the virus genome contains 15192 nucleotides and features that are characteristic of velogenic strains of NDV. A phylogenetic analysis revealed that both isolates clustered in class II and genotype VII. There were several mutations in the functionally important regions of the fusion (F) and haemagglutinin-neuraminidase (HN) proteins, which may be responsible for the differential pathogenicity of these viruses in ducks. In summary, these results suggest that NDV strains with the same genotype show differential pathogenicity in chickens and ducks. Furthermore, chicken-origin virulent NDVs are more pathogenic to ducks than duck-origin viruses. These findings propose a role for chickens in the evolution of viral pathogenicity and the potential genetic resistance of ducks to poultry viruses.

Key words: Newcastle disease virus; sequencing; phylogenetic analysis; pathogenicity; ducks

Introduction

Newcastle disease (ND) is highly contagious and one of the most devastating diseases for many avian species, particularly commercial poultry (Alexander, 2003, Aldous et al., 2014). It is caused by the avian avulavirus 1 (AAvV-1, formerly avian paramyxovirus 1), also known as Newcastle disease virus (NDV), which belongs to the genus *Avulavirus* in the family *Paramyxoviridae*, order *Mononegavirales*, encompassing a diverse group of negative-

sense, non-segmented, and single-stranded RNA viruses (Munir et al., 2012, Alexander, 2003, Rehman et al., 2018).

The clinical manifestations of NDV strains vary from subclinical infection to 100 percent mortality according to the degree of strain virulence and host susceptibility (Jindal et al., 2009). NDVs are categorized as velogenic (high mortality; MDT (mean death time) <60 h), mesogenic (respiratory signs, occasionally nervous signs; MDT 60–90 h), lentogenic (sub clinical to mild respiratory infects; MDT >90 h) and asymptomatic enteric (inapparent infection) (Cattoli et al., 2011, Miller et al., 2013), based on their pathogenicity in the host. The pathogenicity of new isolates of NDV is determined by calculating the MDT, intra-cerebral pathogenicity index (ICPI), and/or intravenous pathogenicity index (IVPI). All NDVs exhibiting an ICPI of ≥0.7, an IVPI of ≥1.40, and/or the amino acid sequence of ^{112}R/K-R-Q-R/K-R;F^{117} at the F-protein cleavage site are virulent and must be reported to the World Organization for Animal Health (Kim et al., 2007, OIE., 2012, Samadi et al., 2014).

Historically, phylogenetic analyses of the nucleotide sequences of NDV strains have revealed that one serotype of NDV consists of two distinct classes (class I and class II). Class II viruses are primarily responsible for the outbreaks observed in commercial poultry and pet birds (Fan et al., 2015) and are comprised of 18 (I–XVIII) genotypes, containing the majority of the sequenced NDVs isolated from wild birds, pet birds and poultry (Kang et al., 2016, Zhang et al., 2011). A phylogenetic analysis of NDVs recovered from outbreaks in China revealed that genotype VII is the predominant genotype in chickens and waterfowl (Rui et al., 2010, Liu et al., 2003), and these NDVs are considered to be enzootic due to their spread around the globe (Kang et al., 2016).

In the past, waterfowl such as geese and ducks were considered the natural reservoirs of avirulent NDVs and thought to be resistant to virulent strains of NDV (Alexander, 2003,

Rosenberger et al., 1975). However, continuous outbreaks of ND by genotype VII viruses have been noted in waterfowl since 1997 (Phan et al., 2013).

This study attempted to understand the pathogenicity of duck- and chicken-origin viruses in chicken and ducks. To this end, we chose two genotype VII isolates of NDV and compared their pathogenicity in chicken and ducks. The results demonstrated that both viruses differed in their pathogenicity in ducks. To elucidate the differences in their pathogenicity, we characterized these viruses biologically and genetically and compared them with other genotype VII strains of NDV.

Materials and Methods

Virus isolates and experimental birds

The two NDV strains used in this study were isolated from an outbreak on a commercial chicken farm and from apparently healthy ducks in Shandong, China. These strains recovered from chickens and ducks were designated Ch/CH/SD/2008/128 and Du/CH/SD/2009, respectively. To ensure homogeneity, both isolates were plaque-purified three times to prepare working stocks. These purified viruses were grown in the allantoic cavities of 10-day-old specific pathogen-free (SPF) embryonated chicken eggs purchased from Merial (Beijing, China) and were incubated at the laboratory facilities of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences. Virus stocks were quantified using haemagglutination (HA) assays and were stored at -80 °C until use.

To investigate the pathogenicity of these two isolates, day-old SPF chicks were obtained from Merial (Beijing, China) and were maintained at the Shanghai Veterinary Research Institute, China. All ducklings were purchased from Jiangyin (a farm) and had no prior history of disease or vaccination against NDV. To ensure cleanliness, all ducklings were screened by a

HA inhibition (HI) assay, and only serologically negative ducklings were selected for the challenge experiment.

All animal experiments were approved by the Institutional Animal Care and Use Committee, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

Pathogenicity assessments

The pathogenic potential of the chicken and duck-origin isolates was determined individually by assessing the MDT in 10-day-old chick embryos and the ICPI in 1-day-old chicks. Briefly, allantoic fluid containing the NDV isolate was diluted 10 times in phosphate buffered saline (PBS) and inoculated into 10-day-old chick embryos to determine the MDT as described by Alexander (2003). The 50% egg lethal dose (ELD_{50}) was calculated by the Reed and Muench (1938) method. For ICPI, one-day-old chicks were inoculated intracerebrally with 0.1 ml of 10-fold diluted virus. The inoculated chicks were observed for 10 days for mortality (scored as 2), sickness/paralysis (scored as 1) or continued health (scored as 0). Total scores were determined, and the mean daily score was calculated to obtain the ICPI (Alexander and Swayne, 1998).

Virus growth kinetics

The growth kinetics of both viruses were determined under multiple cycle growth conditions in chicken embryo fibroblast (CEF) and duck embryo fibroblast (DEF) cells. The CEF and DEF cells were grown in Dulbecco's minimal essential medium with 10% foetal bovine serum at 37 °C and were inoculated with the NDV isolates at a multiplicity of infection (MOI) of 0.01 plaque-forming units. The supernatant was collected at 12-h intervals and replaced by equal volumes of fresh medium until 120 h post infection (hpi). Virus titres were

measured in CEF and DEF cells by following the Reed and Muench (1938) and are expressed as the 50% tissue culture infective dose ($TCID_{50}$).

Pathogenicity in chickens and ducks

To examine the pathogenicity of the Ch.CH/SD/2008/128 and Du/CH/SD/2009/134 viruses in ducks and chicken, four-week-old chickens (n=52) and Peking ducks (n=52) were divided into 4 groups (consisting of 13 chicks or ducks). All groups were inoculated with the same dose (10^6 ELD_{50}) of Ch/CH/SD/2008/128, Du/CH/SD/2009/134 or PBS in a volume of 200 µl via the intramuscular route. As a control, we inoculated a group of birds with ZJ1, which is a previously well-characterized strain of NDV. All birds in the infected groups were observed for clinical signs and mortality.

To assess histopathological changes in the intestine, trachea, lungs, and spleen, we euthanized three chickens and Peking ducks from each group (showing clinical signs or death from infected groups) at 3 days post infection (dpi). These tissues were fixed in 10% neutral buffered formalin, and 4-µm sections were prepared and examined for histopathological changes. This experiment was performed three times to validate the results.

Primer design

Ten pairs of primers were designed based on the full-length nucleotide sequences of the NDV isolates ZJ1, NA-1, SD09, and SDWF02 (GenBank: AF431744.3, DQ659677.1, and HM188399.1, respectively) to amplify the complete genome sequences of these recently isolated NDV strains. All primers used in this study are provided in Additional file 1: Table S1.

RNA extraction and RT-PCR

Viral genomic RNA was extracted from the allantoic fluid using TRIzol reagent (Invitrogen, San Diego, USA) following the manufacturer's instructions. Reverse transcription was performed at 42 °C for 1 h using 32.5 μl of viral RNA suspension, 1 μl of random primers, 1 μl of RNase inhibitor, 1.5 μl of M-MLV RTase, 10 μl of 5× M-MLV Buffer, and dNTPs at 2.5 mM (Promega, USA) for a total volume of 50 μl. Ten pairs of primers, listed in Additional file 1: Table S1, were used to obtain the complete genome sequence of each virus. Amplification was performed in a PCR machine with the previously prepared cDNA as a template in a 50-μl reaction volume containing each primer at 20 pmol and 2 U of Taq PCR mix (Vigorous Biotechnology Corporation, Beijing, China). NDV-positive strain cDNA and sterile water were used as the positive and negative control, respectively. Reactions were performed according to the following protocol: 94 °C for 5 min followed by 32 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s; and a final elongation step of 10 min at 72 °C.

The 3'- and 5'-termini of the viral genome sequences were amplified by a modified 3' and 5' rapid amplification of cDNA ends (RACE) procedure as described previously (Meng et al., 2012). For 3'-RACE, viral genomic RNA was ligated to anchor primer F (Table S1) using T4 RNA ligase according to the manufacturer's instructions (New England Biolabs, Beverly, MA, USA). The ligated RNA was purified and reverse-transcribed using the complementary anchor primer R (Table S1). PCR amplification was carried out using anchor primer R and the antisense NP-gene specific reverse primers 3-LR and 3-SR. To determine the sequence of the genomic 5'-terminal end, primer 5-LR was used to generate single-stranded cDNA as described in the manufacturer's instructions (Thermoscript RT-PCR System Kit, Invitrogen). Residual RNA was removed after cDNA synthesis. The cDNA was purified using a PCR purification kit (TaKaRa, Dalian, China) and subsequently ligated to anchor primer F using

T4 RNA ligase as described above. The resulting anchor-primer-ligated cDNA was amplified using 5-LF/SF and anchor primer R.

Gel extraction of PCR products and nucleotide sequencing

Approximately 50 µl of each PCR reaction mixture was loaded onto a 1% agarose gel and electrophoresed for 40 minutes. PCR products of the expected length were purified using a gel extraction kit (TianGen Biotech Beijing, China) and ligated to the T-easy vector and transformed into DH5α *Escherichia coli* competent cells. Recombinant plasmids containing the amplified product from each PCR fragment were purified using the Tiangen Spin Plasmid Purification Kit (TianGen, Beijing, China) and sequenced in both directions using universal T7 and SP6 primers. Three different recombinant plasmids from each PCR fragment were selected for sequencing. Sequencing was conducted by Sanggong Biotechnology (Sanggong, Shanghai, China).

Phylogenetic analysis

To assess the phylogenetic relationships between the isolates in this study with previously characterized NDV strains from different parts of world, full genome sequences were acquired from GenBank (http://www.ncbi.nlm.nih.gov/) for each known genotype of NDV (Qiu et al., 2017). All sequences were aligned and analysed using the ClustalW multiple alignment algorithm in the MegAlign program of the DNASTAR software suite (version 3.1; DNAstar, Madison, WI, USA). A phylogenetic tree was constructed using MEGA4.0 software (Molecular Evolutionary Genetics Analysis, version 4.0) by the neighbour-joining method (1000 replicates for bootstrap). The evolutionary distances between the different sequences were computed by the pairwise distance method using the maximum composite likelihood model (Zhang et al., 2011).

Structural presentation of HN proteins

The crystal structures of the HN proteins of NDV were downloaded from the protein data bank (PDB) under ID number 3TIE, as described previously (Yuan et al., 2011), to model the structures of the mutations. The HN proteins from both viruses were merged to show the exact substitutions in their structures. All structural annotations were generated using PyMOL (version 1.7.4, Schrödinger).

Results

Biological characteristics

The biological properties of recently isolated NDV strains were assessed by HA and *in vivo* assays. The HA assay was performed in V-bottomed titration plates with chicken red blood cells, and the results indicated that the isolates had HA titres of 2^8 and 2^9 (Table 1). The chicken isolate Ch/CH/SD/2008/128 showed an ICPI of 1.9, an MDT of 55 h, an ELD_{50} of $10^{8.31}$, and a $TCID_{50}$ of $10^{7.8}$ per ml. The ICPI, MDT, ELD_{50} and $TCID_{50}$ values for the duck isolate Du/CH/SD/2009/134 were 1.81, 59 h, 7.16 and 7.35, respectively (Table 1). All of these biological characteristics indicated that both isolates obtained in this study were velogenic.

Growth kinetics

The multi-cycle growth kinetics of the Ch/CH/SD/2008/128 and Du/CH/SD/2009/134 NDV isolates were examined *in vitro* in CEF and DEF cells infected at an MOI of 0.01 (Fig. 1). The growth of Du/CH/SD/2009/134 in CEF cells was lower than the growth of Ch/CH/SD/2008/128. Replication of the duck-origin virus was higher in DEF cells than the chicken-origin virus. Despite the differences between primary cells and established cell lines, the titres of both viruses reached a maximum at 36 hpi.

Pathogenicity of the isolates in chicken and ducks

Four groups of chickens and four groups of ducks, consisting of 10 birds each, were infected with either Ch/CH/SD/2008/128, Du/CH/SD/2009/134, ZJ1, or PBS in a volume of 200 µl via the intramuscular route. The survival rates in all groups are shown in Fig. 2. These outcomes indicate that Ch/CH/SD/2008/128, Du/CH/SD/2009/134, and ZJ1 caused 70%, 20% and 10% mortality, respectively (Fig. 2A) in ducks. Intriguingly, all isolates, regardless of the species of origin, caused 100% mortality in chickens (Fig. 2B).

To assess the tissue damage and histopathological changes induced by the different isolates in chickens and ducks, tissue samples from the intestine, trachea, lungs and spleen were examined at three dpi. Representative histopathological illustrations of the different tissues are shown in Fig. 3. Infected ducks showed the infiltration of heterophils, macrophages and lymphocytes in the mucosa and lamina propria of the intestine. Mild enteritis, along with necrosis, was also observed in the intestine (Fig. 3 E, I, M). Furthermore, ducks infected with Ch/CH/SD/2008/128 showed dropout of the epithelium and broken villi (Fig. 3 M), which were less frequently observed in the other groups. There were obvious histopathological changes in the respiratory system, including tracheitis, the proliferation of goblet cells, heterophilic infiltration in the tracheal mucosa (Fig. 3 F, J, N), and interstitial pneumonia in the lungs (Fig. 3 G, K, O). Pathological lesions were more severe in the Ch/CH/SD/2008/128-infected ducks than in Du/CH/SD/2009/134- and ZJ1-infected ducks. Histopathology of the spleen indicated that Ch/CH/SD/2008/128 caused marked lymphocyte depletion, necrosis and fibrin deposits resulting from necrosis (Fig. 3 D, H, L, P). Similar to the observed mortality levels in chickens, histopathological analyses showed comparable lesions in all infected chickens, indicating the ability of both NDV strains to cause pathology in chickens (data not shown).

Genetic analysis of NDV isolates

The genomic features of the Du/CH/SD/2009/134 and Ch/CH/SD/2008/128 strains are given in Table 2. Both isolates had a genome length of 15192 nucleotides. The structural genes of both the NDV strains had the same start, end, and intergenic positions (Table 2). All observed characteristics of these isolates are representative of virulent NDVs. The complete genomes and amino acid sequences of Du/CH/SD/2009/134 and Ch/CH/SD/2008/128 were compared with those of other genotype VII viruses (Table 3). The genomic sequence of Du/CH/SD/2009/134 showed 96.8%, 97.3%, 96.6%, 97.0%, 98.1%, 97.4%, 97.2%, 95.6%, and 96.5% similarity with Ch/CH/SD/2008/128, BPO1, chicken/TC/9/2011, ZJ1, SD09, SDWF02, GM, NA-1, and China/Guangxi9/2003, respectively. Ch/CH/SD/2008/128 showed 98.2%, 97.8%, 97.8%, 96.2%, 96.1%, 96.6%, 96.0% and 96.7% similarity with BPO1, chicken/TC/9/2011, ZJ1, SD09, SDWF02, GM, NA-1, and China/Guangxi9/2003, respectively (Table 3).

Phylogenetic analysis

To investigate the genetic nature of the Du/CH/SD/2009/134 and Ch/CH/SD/2008/128 NDV isolates and obtain epidemiological insights, a phylogenetic analysis was performed using the complete genomes of known isolates belonging to class I and all genotypes of class II. The clustering patterns of both isolates revealed their grouping in class II and genotype VII (Fig. 4).

Sequence analysis of the F and HN proteins

The amino acid sequence at the F protein cleavage site is considered the main determinant of NDV pathogenicity. Therefore, we analysed the proteolytic cleavage site motifs for F_0 in the isolated NDV strains. The results demonstrated that both NDV strains shared the motif

characteristic of typical virulent strains and carried K^{101} and V^{121} substitutions, which are typical of genotype VII. The F_0 cleavage site motif of both NDV isolates was ^{112}RRQKRF^{117}. Substitutions were observed in the deduced amino acid sequence of Du/CH/SD/2009/134 at positions 129, 179, 181 and 396 from valine to glycine, valine to glycine, lysine to glutamate and isoleucine to methionine, respectively (Table 4). The deduced amino acid sequences of Ch/CH/SD/2008/128 and Du/CH/SD/2009/134 were different at positions 16, 97, 129, 179, 181, 396, 480, 527, 543, and 551.

Previous genomic comparisons of different NDV strains revealed that the length of the HN protein of NDV is variable and can be one of at least twelve lengths: 570, 571, 572, 577, 578, 580, 581, 582, 585, 586, 615 and 616 amino acids (Romer-Oberdorfer et al., 2003, Zhang et al., 2014, Jin et al., 2016). In this study, both isolated strains carried an HN of 571 amino acids, which is a feature of velogenic NDV (Maminiaina et al., 2010). Several specific substitutions were observed in the HN proteins of Du/CH/SD/2009/134 and Ch/CH/SD/2008/128 at amino acid positions 9, 102, 138, 141, 216, 309, 323, 331, 355, 477, 479, and 514 (Table 5). The mutations in the HN proteins of these viruses are depicted in the crystal structure in green (Fig. 5).

Nucleotide sequence accession numbers

The generated sequence data are available from GenBank under the accession numbers KJ600785 and KJ600786.

Discussion

ND is one of the most devastating avian diseases, infecting many different species of birds and causing high mortality and morbidity (Alexander, 2003). ND is endemic in many parts of the world, including Asia, and is considered a major economic issue due to investments in vaccination and biosecurity in countries where it is theoretically controlled. The first outbreak

of ND in China was reported in 1946, and since then, a strict ND vaccination programme has been adopted in commercial as well as rural poultry, which has significantly reduced outbreaks of ND. However, ND remains enzootic in different parts of the country and is considered a major disease in poultry (Liu et al., 2007).

Historically, four panzootics of ND have been observed since 1926. The first epizootic was caused by genotypes II and IV, and the second and third were caused by genotypes V and VI. Genotype VII was responsible for the most recent fourth pandemic of ND and is the most predominant genotype in China (Rui et al., 2010). Traditionally, waterfowl such as ducks and geese are considered the natural carriers or reservoirs for NDV (Dimitrov et al., 2016, Alexander, 2003). Some reports suggest the potential role of waterfowl in the evolution of NDV, but epidemiological and virological studies of circulating strains of NDV in ducks are limited.

In the present study, we isolated two velogenic NDVs from chicken and ducks and performed pathotypic and genetic characterization. Pathogenicity assessments, including MDT (<59), ELD_{50} (7.19 and 8.31) and ICPI (>1.81), showed that both isolates were virulent. In an animal infection experiment, Ch/CH/SD/2008/128 and Du/CH/SD/2009/134 caused 70% and 20% mortality in ducks, respectively. The mortality patterns in chickens were the same for both viruses. These unusual mortality patterns caused by different host-origin isolates of the same genotype (VII) compelled us to find genomic differences by studying the whole genome of each virus. The whole genome sequences of the Du/CH/SD/2009/134 and Ch/CH/SD/2008/128 NDV isolates were determined and analysed to examine their pathological and phylogenetic relationships with other strains. Both isolates had a genome consisting of 15192 base pairs. Genetic analysis of these isolates showed that both shared a similarity of 96%. Both isolates had an F protein cleavage site motif, which determines the

NDV pathotype, of ^{112}RRQKRF^{117}, a characteristic of typical velogenic strains of genotype VII.

To further explore the molecular basis of the mortality caused by both isolates in ducks, we compared the F and HN genes, which are major contributors to pathogenicity (Kim et al., 2011). The hydrophobic transmembrane region of the F protein is located at amino acid residues 500–553 of F_0. This hydrophobic base and partially hydrophilic rim are important for transport and processing of the F protein (Plemper et al., 2003, Gravel et al., 2011). Several studies have shown potential roles for the cytoplasmic and transmembrane domains of the NDV fusion protein based on its structure and function (Sergel and Morrison, 1995, Kim et al., 2011, Gravel et al., 2011). Tyrosine-containing signals in the cytoplasmic domains of viral membranes are important for targeted protein delivery (Brewer and Roth, 1991, Weise et al., 2010) and may be important for virus assembly because they guide these proteins to cholesterol-rich membrane domains (Dolganiuc et al., 2003). The NDV fusion protein cytoplasmic domain is located at amino acids 523–553, which is a highly conserved region.

In the present study, comparison of the F protein of Ch/CH/SD/2008/128 with that of Du/CH/SD/2009/134 and 5 other viruses of genotype VII revealed point substitutions at amino acid positions of 16, 97, 129, 179, 181, 396, 480, 527, 543, and 551. The Y527A tyrosine mutation in the F protein results in hyperfusogenic phenotypes characterized by increased replication, pathogenicity (Samal et al., 2013), and immunogenicity (Manoharan et al., 2016). Cleavage of the tyrosine mutant F protein is higher than that of the wild type, suggesting this mutation affects the transport and expression of the F protein (Samal et al., 2013). The increased pathogenicity of Ch/CH/SD/2008/128 in our study may also be attributable to the T551A mutation, which increases the fusion index (Samal et al., 2013). Based on these observations, we hypothesized that these three mutations in the cytoplasmic

329 domain region of Ch/CH/SD/2008/128 contributed to the increased pathogenicity of this
330 virus in ducks.

331 The HN, another multifunctional glycoprotein, is comprised of the cytoplasmic domain, a
332 transmembrane region, a stalk region and a globular head (Peeters et al., 2001). The stalk
333 region supports the globular head (Kolakofsky and Lamb, 2007) and mediates the fusion
334 process (Deng et al., 1995, Tanabayashi and Compans, 1996). All antigenic sites, receptor
335 binding and neuraminidase activity are controlled by the globular region. Two receptor
336 binding sites are present on the globular head. Site I is involved in receptor binding and
337 neuraminidase activity, and site II is associated with receptor binding and fusion (Crennell et
338 al., 2000, Iorio et al., 2001, Zaitsev et al., 2004, Jin et al., 2017). A mutation in the stalk
339 region of the HN protein modulates the fusion process and expression of the HN-F complex
340 on the cellular surface (Iorio et al., 2009, Mirza and Iorio, 2013). Previous studies have
341 revealed that the amino acid residues at 169, 174, 175, 192, 198, 236, 258, 299, 317, 401,
342 416, 498, 516, 517, 519, 526, 552, 553, and 557 are important for HN protein function
343 (presented in red in Fig. 5B) (Takimoto et al., 2002, Connaris et al., 2002, Estevez et al.,
344 2011, McGinnes and Morrison, 2006, Zaitsev et al., 2004, Rangaswamy et al., 2017). In the
345 present study, we identified amino acid substitutions at positions 9, 323, 331 and 514 when
346 we compared the Ch/CH/SD/2008/128 and Du/CH/SD/2009/134 isolates. The crystal
347 structure of the HN protein showed that the amino acid substitution at 514 may change
348 fusion, receptor binding and tissue tropism in ducks, leading to high mortality. These point
349 mutations in the structure of the HN protein may be involved in promoting fusion, tissue
350 tropism or virulence, as a point mutation in the HN protein can alter viral pathogenicity (Liu
351 et al., 2015).

352 Our *in vivo* studies also support our hypothesis regarding the tissue tropism and pathogenicity
353 of natural point mutations in F and HN because both viruses had the same F_0 cleavage site,

but survival rates and other histopathological changes in different tissues, including the intestine, trachea, lungs and spleen, of ducks challenged with Ch/CH/SD/2008/128 and Du/CH/SD/2009/134 were quite different. The differential pathogenicities of these isolates suggested that NDV evolves in its natural host (duck) to increase its survival rate. Similar results describing NDV persistent infection due to a modification in the genome have been shown in an ovarian cell line (Rangaswamy et al., 2017), but persistent infection in ducks, geese, or chickens has yet to be studied. Our chicken-origin NDV isolate did not evolve in ducks, and thus, it caused high mortality.

In conclusion, our results demonstrate that apparently healthy ducks may be carriers of velogenic NDV strains, which cause high mortality in chickens, and similarly, chicken-origin NDV isolates can cause high mortality in ducks. Strikingly, we observed a sudden increase in the virulence of the chicken-origin NDV isolate in ducks due to point mutations in the HN and F proteins but not at the cleavage site. Duck-origin NDVs cause lower mortality in ducks, whereas chicken-origin NDVs, which belong to the same genotype, cause higher tissue damage and mortality in ducks. This may be due to a point mutation in the NDV F and HN proteins. Our experiment also laid the foundation to study the role of the HN and F proteins in the tissue tropism and pathogenicity of genotype VII NDV isolates in ducks.

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Conflict of Interest

None of the authors of this study has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the article.

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558 **Table 1**

559 Pathogenicity indexes for the NDV isolates in this study

Isolate name	Origin	Host	[a]HA	[b]$TCID_{50}$	[c]ELD_{50}	[d]MDT (h)	[e]ICPI
Ch/CH/SD/2008/128	Shandong, China	Chicken	2^8	7.80	8.31	55h	1.90
Du/CH/SD/2009/134	Shandong, China	Duck	2^9	7.35	7.16	59h	1.81

560 [a]HA=Haemagglutination

561 [b]$TCID_{50}$=50% tissue culture infective dose

562 [c]ELD_{50}=50% embryo lethal dose

563 [d]MDT (h)=Mean death time in embryonated eggs (hours)

[e]ICPI=Intracerebral pathogenicity index in day-old chicks

Table 2

Genomic features of the NDV isolates Du/CH/SD/2009/134 and Ch/CH/SD/2008/128

Gene	Gene start		Gene end		Intergenic sequences
	Sequences	Start	Sequences	End	
NP	ACGGGTAGAA	56	CCCAAGGTAT	1798	TAGAAAAAAAT
P	ACGGGTAGAA	1810	CATT(C)aAGAAAT	3250	TAAGAAAAAAT
M	ACGGGTAGAA	3262	TCTAGCAAAT	4493	TAGAAAAAAC
F	ACGGGTAGAA	4504	GTAGAAGACT	6285	TAAGAAAAAACTACTGGGAACAAGCAACCAAAGAGCAATAC
HN	ACGGGTAGAA	6327	ATCACTTTAT	8318	TAAGAAAAAATACAGAAAGCATTGAGATGTAAGGGAAAACAACCAACAAGAGGGAAC
L	ACGGGTAGGA	8376	TAGAAAAAAG	15079	

Note: [a]The Ch/CH/SD/2008/128 gene end sequences is CATCAGAAAT, the Du/CH/SD/2009/134 is CATTAGAAAT.

Table 3

Comparison of nucleotides and amino acids among NDV isolates Du/CH/SD/2009/134 and Ch/CH/SD/2008/128 and other genotype VII NDV strains

Strains	BPO1	chicken/TC/9/2011	ZJ1	SD09	SDWF02	GM	NA-1	China/Guangxi9/2003	Du/CH/SD/2009/134	Ch/CH/SD/2008/128
BPO1		98.6	99.0	97.3	97.3	97.9	97.2	98.0	97.3	98.2
chicken/TC/9/2011	98.6		98.2	96.8	96.8	97.4	96.8	97.5	96.6	97.8
ZJ1	99.0	98.2		97.0	97.0	97.6	96.9	97.7	97.0	97.8
SD09	97.3	96.8	97.0		98.1	97.8	96.0	96.9	98.1	96.2
SDWF02	97.3	96.8	97.0	98.1		97.7	96.3	96.9	97.4	96.1
GM	97.9	97.4	97.6	97.8	97.7		96.5	97.6	97.2	96.6
NA-1	97.2	96.8	96.9	96.0	96.3	96.5		96.6	95.6	96.0
China/Guangxi9/2003	98.0	97.5	97.7	96.9	96.9	97.6	96.6		96.5	96.7
Du/CH/SD/2009/134	97.3	96.6	97.0	98.1	97.4	97.2	95.6	96.5		96.8
Ch/CH/SD/2008/128	98.2	97.8	97.8	96.2	96.1	96.6	96.0	96.7	96.8	

Table 4

Amino acid changes in the F proteins of genotype VII NDV strains

Virus strain	Amino acid residues									
	16	97	129	179	181	396	480	527	543	551
BPO1	I	D	V	V	K	I	R	Y	T	T
ZJ1	-	-	-	-	-	-	-	-	-	-
SDWF02	-	-	-	-	-	-	-	-	-	-
NA-1	-	-	-	-	-	-	-	-	-	-
China/Guangxi9/2003	-	-	-	-	-	-	-	-	-	-
Du/CH/SD/2009/134	-	-	G	G	E	M	-	-	-	-
Ch/CH/SD/2008/128	V	N	-	-	-	-	K	H	A	A

Table 5

Amino acid changes in the HN proteins of genotype VII NDV strains

Virus strain	Amino acid residues											
	9	102	138	141	216	309	323	331	355	477	479	514
BPO1	V	I	K	I	T	D	N	E	A	I	H	V
ZJ1	-	-	-	-	-	-	-	-	-	-	-	-
SDWF02	-	-	-	-	-	-	-	-	-	-	-	-
NA-1	-	-	-	-	-	-	-	-	-	-	-	-
China/Guangxi9/2003	-	-	-	-	-	-	-	-	-	-	-	-
Du/CH/SD/2009/134	-	-	E	L	I	-	-	-	V	V	-	-
Ch/CH/SD/2008/128	M	T	-	-	-	N	D	K	-	-	Y	I

Figure 1

Growth kinetics of the Ch/CH/SD/2008/128 and Du/CH/SD/2009/134 viruses in CEF and DEF cells. The growth characteristics of these viruses were examined under multiple-cycle growth conditions in these cells.

Figure 2

Survival of 4-week-old ducks and chickens (A) to assess mortality following infection with Ch/CH/SD/2008/128, Du/CH/SD/2009/134 or ZJ1. Each colour indicates a different group on a different day post infection.

Figure 3

Histopathology of the intestine, trachea, lungs, and brain tissues of 4-week-old ducks infected intramuscularly with Ch/CH/SD/2008/128, Du/CH/SD/2009/134 or Herts/33. The ducks were sacrificed at 3 dpi, and the tissues were fixed with formalin, sectioned, and stained with haematoxylin and eosin. A, E, I, and M: infiltration of lymphocytes, dropout of the epithelium, and broken villi (arrow heads); F, J and N: severe inflammation of the tracheal mucosa and submucosa as well as necrosis of the epithelial cells (arrow heads); G, K and O: interstitial pneumonia, congestion and haemorrhages in the lungs; H, L and P; lymphocyte depletion, necrosis and fibrin deposits are shown, respectively.

Figure 4

A phylogenetic tree was constructed with NDV strains based on complete genomic sequences. The viruses highlighted in red were characterized in this study. The phylogenetic tree was constructed using the maximum likelihood method with the Generalized Time Reversible GTR+G+I4 model from the MEGA software package (version 4.0), with 1000 bootstrap trials to assign confidence to the groupings.

Figure 5

660 The crystal structure of the NDV HN protein and the locations of naturally mutated residues.
661 These structures were generated using PyMOL (version 1.7.4). A presents the front view of
662 HN. B shows the important residues involved in receptor binding, tissue tropism and catalytic
663 activity (red colour) and mutations found in the present study (green colour). Amino acid
664 changes of particular interest are encircled in blue.

665