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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-

49 sense, non-segmented, and single-stranded RNA viruses (Munir et al., 2012, Alexander,
50 2003, Rehman et al., 2018).

51 The clinical manifestations of NDV strains vary from subclinical infection to 100 percent
52 mortality according to the degree of strain virulence and host susceptibility (Jindal et al.,
53 2009). NDVs are categorized as velogenic (high mortality; MDT (mean death time) <60 h),
54 mesogenic (respiratory signs, occasionally nervous signs; MDT 60–90 h), lentogenic (sub
55 clinical to mild respiratory infects; MDT >90 h) and asymptomatic enteric (inapparent
56 infection) (Cattoli et al., 2011, Miller et al., 2013), based on their pathogenicity in the host.
57 The pathogenicity of new isolates of NDV is determined by calculating the MDT, intra-
58 cerebral pathogenicity index (ICPI), and/or intravenous pathogenicity index (IVPI). All
59 NDVs exhibiting an ICPI of ≥ 0.7, an IVPI of ≥ 1.40, and/or the amino acid sequence of
60 ^{112}R/K-R-Q-R/K-R;F^{117} at the F-protein cleavage site are virulent and must be reported to the
61 World Organization for Animal Health (Kim et al., 2007, OIE., 2012, Samadi et al., 2014).

62 Historically, phylogenetic analyses of the nucleotide sequences of NDV strains have revealed
63 that one serotype of NDV consists of two distinct classes (class I and class II). Class II
64 viruses are primarily responsible for the outbreaks observed in commercial poultry and pet
65 birds (Fan et al., 2015) and are comprised of 18 (I–XVIII) genotypes, containing the majority
66 of the sequenced NDVs isolated from wild birds, pet birds and poultry (Kang et al., 2016,
67 Zhang et al., 2011). A phylogenetic analysis of NDVs recovered from outbreaks in China
68 revealed that genotype VII is the predominant genotype in chickens and waterfowl (Rui et al.,
69 2010, Liu et al., 2003), and these NDVs are considered to be enzootic due to their spread
70 around the globe (Kang et al., 2016).

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

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

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

81 **Materials and Methods**

82 **Virus isolates and experimental birds**

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

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

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

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

100 **Pathogenicity assessments**

101 The pathogenic potential of the chicken and duck-origin isolates was determined individually
102 by assessing the MDT in 10-day-old chick embryos and the ICPI in 1-day-old chicks. Briefly,
103 allantoic fluid containing the NDV isolate was diluted 10 times in phosphate buffered saline
104 (PBS) and inoculated into 10-day-old chick embryos to determine the MDT as described by
105 Alexander (2003). The 50% egg lethal dose (ELD_{50}) was calculated by the Reed and Muench
106 (1938) method. For ICPI, one-day-old chicks were inoculated intracerebrally with 0.1 ml of
107 10-fold diluted virus. The inoculated chicks were observed for 10 days for mortality (scored
108 as 2), sickness/paralysis (scored as 1) or continued health (scored as 0). Total scores were
109 determined, and the mean daily score was calculated to obtain the ICPI (Alexander and
110 Swayne, 1998).

111 **Virus growth kinetics**

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

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

120 Pathogenicity in chickens and ducks

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

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

133 Primer design

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

139 **RNA extraction and RT-PCR**

140 Viral genomic RNA was extracted from the allantoic fluid using TRIzol reagent (Invitrogen,
141 San Diego, USA) following the manufacturer's instructions. Reverse transcription was
142 performed at 42 °C for 1 h using 32.5 µl of viral RNA suspension, 1 µl of random primers, 1
143 µl of RNase inhibitor, 1.5 µl of M-MLV RTase, 10 µl of 5× M-MLV Buffer, and dNTPs at
144 2.5 mM (Promega, USA) for a total volume of 50 µl. Ten pairs of primers, listed in
145 Additional file 1: Table S1, were used to obtain the complete genome sequence of each virus.
146 Amplification was performed in a PCR machine with the previously prepared cDNA as a
147 template in a 50-µl reaction volume containing each primer at 20 pmol and 2 U of Taq PCR
148 mix (Vigorous Biotechnology Corporation, Beijing, China). NDV-positive strain cDNA and
149 sterile water were used as the positive and negative control, respectively. Reactions were
150 performed according to the following protocol: 94 °C for 5 min followed by 32 cycles of 94
151 °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.

152 The 3'- and 5'-termini of the viral genome sequences were amplified by a modified 3' and 5'
153 rapid amplification of cDNA ends (RACE) procedure as described previously (Meng et al.,
154 2012). For 3'-RACE, viral genomic RNA was ligated to anchor primer F (Table S1) using T4
155 RNA ligase according to the manufacturer's instructions (New England Biolabs, Beverly,
156 MA, USA). The ligated RNA was purified and reverse-transcribed using the complementary
157 anchor primer R (Table S1). PCR amplification was carried out using anchor primer R and
158 the antisense NP-gene specific reverse primers 3-LR and 3-SR. To determine the sequence of
159 the genomic 5'-terminal end, primer 5-LR was used to generate single-stranded cDNA as
160 described in the manufacturer's instructions (Thermoscript RT-PCR System Kit, Invitrogen).
161 Residual RNA was removed after cDNA synthesis. The cDNA was purified using a PCR
162 purification kit (TaKaRa, Dalian, China) and subsequently ligated to anchor primer F using

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

165 **Gel extraction of PCR products and nucleotide sequencing**

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

175 **Phylogenetic analysis**

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

186 **Structural presentation of HN proteins**

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

192 **Results**

193 **Biological characteristics**

194 The biological properties of recently isolated NDV strains were assessed by HA and *in vivo*
195 assays. The HA assay was performed in V-bottomed titration plates with chicken red blood
196 cells, and the results indicated that the isolates had HA titres of 2^8 and 2^9 (Table 1). The
197 chicken isolate Ch/CH/SD/2008/128 showed an ICPI of 1.9, an MDT of 55 h, an ELD_{50} of
198 $10^{8.31}$, and a $TCID_{50}$ of $10^{7.8}$ per ml. The ICPI, MDT, ELD_{50} and $TCID_{50}$ values for the duck
199 isolate Du/CH/SD/2009/134 were 1.81, 59 h, 7.16 and 7.35, respectively (Table 1). All of
200 these biological characteristics indicated that both isolates obtained in this study were
201 velogenic.

202 **Growth kinetics**

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

209 **Pathogenicity of the isolates in chicken and ducks**

210 Four groups of chickens and four groups of ducks, consisting of 10 birds each, were infected
211 with either Ch/CH/SD/2008/128, Du/CH/SD/2009/134, ZJ1, or PBS in a volume of 200 µl
212 via the intramuscular route. The survival rates in all groups are shown in Fig. 2. These
213 outcomes indicate that Ch/CH/SD/2008/128, Du/CH/SD/2009/134, and ZJ1 caused 70%,
214 20% and 10% mortality, respectively (Fig. 2A) in ducks. Intriguingly, all isolates, regardless
215 of the species of origin, caused 100% mortality in chickens (Fig. 2B).

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

233 **Genetic analysis of NDV isolates**

234 The genomic features of the Du/CH/SD/2009/134 and Ch/CH/SD/2008/128 strains are given
235 in Table 2. Both isolates had a genome length of 15192 nucleotides. The structural genes of
236 both the NDV strains had the same start, end, and intergenic positions (Table 2). All observed
237 characteristics of these isolates are representative of virulent NDVs. The complete genomes
238 and amino acid sequences of Du/CH/SD/2009/134 and Ch/CH/SD/2008/128 were compared
239 with those of other genotype VII viruses (Table 3). The genomic sequence of
240 Du/CH/SD/2009/134 showed 96.8%, 97.3%, 96.6%, 97.0%, 98.1%, 97.4%, 97.2%, 95.6%,
241 and 96.5% similarity with Ch/CH/SD/2008/128, BPO1, chicken/TC/9/2011, ZJ1, SD09,
242 SDWF02, GM, NA-1, and China/Guangxi9/2003, respectively. Ch/CH/SD/2008/128 showed
243 98.2%, 97.8%, 97.8%, 96.2%, 96.1%, 96.6%, 96.0% and 96.7% similarity with BPO1,
244 chicken/TC/9/2011, ZJ1, SD09, SDWF02, GM, NA-1, and China/Guangxi9/2003,
245 respectively (Table 3).

246 **Phylogenetic analysis**

247 To investigate the genetic nature of the Du/CH/SD/2009/134 and Ch/CH/SD/2008/128 NDV
248 isolates and obtain epidemiological insights, a phylogenetic analysis was performed using the
249 complete genomes of known isolates belonging to class I and all genotypes of class II. The
250 clustering patterns of both isolates revealed their grouping in class II and genotype VII (Fig.
251 4).

252 **Sequence analysis of the F and HN proteins**

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

256 characteristic of typical virulent strains and carried K^{101} and V^{121} substitutions, which are
257 typical of genotype VII. The F_0 cleavage site motif of both NDV isolates was $^{112}RRQKRF^{117}$.
258 Substitutions were observed in the deduced amino acid sequence of Du/CH/SD/2009/134 at
259 positions 129, 179, 181 and 396 from valine to glycine, valine to glycine, lysine to glutamate
260 and isoleucine to methionine, respectively (Table 4). The deduced amino acid sequences of
261 Ch/CH/SD/2008/128 and Du/CH/SD/2009/134 were different at positions 16, 97, 129, 179,
262 181, 396, 480, 527, 543, and 551.

263 Previous genomic comparisons of different NDV strains revealed that the length of the HN
264 protein of NDV is variable and can be one of at least twelve lengths: 570, 571, 572, 577, 578,
265 580, 581, 582, 585, 586, 615 and 616 amino acids (Romer-Oberdorfer et al., 2003, Zhang et
266 al., 2014, Jin et al., 2016). In this study, both isolated strains carried an HN of 571 amino
267 acids, which is a feature of velogenic NDV (Maminiaina et al., 2010). Several specific
268 substitutions were observed in the HN proteins of Du/CH/SD/2009/134 and
269 Ch/CH/SD/2008/128 at amino acid positions 9, 102, 138, 141, 216, 309, 323, 331, 355, 477,
270 479, and 514 (Table 5). The mutations in the HN proteins of these viruses are depicted in the
271 crystal structure in green (Fig. 5).

272 Nucleotide sequence accession numbers

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

275 Discussion

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

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

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

292 In the present study, we isolated two velogenic NDVs from chicken and ducks and performed
293 pathotypic and genetic characterization. Pathogenicity assessments, including MDT (<59),
294 ELD$_{50}$ (7.19 and 8.31) and ICPI (>1.81), showed that both isolates were virulent. In an
295 animal infection experiment, Ch/CH/SD/2008/128 and Du/CH/SD/2009/134 caused 70% and
296 20% mortality in ducks, respectively. The mortality patterns in chickens were the same for
297 both viruses. These unusual mortality patterns caused by different host-origin isolates of the
298 same genotype (VII) compelled us to find genomic differences by studying the whole
299 genome of each virus. The whole genome sequences of the Du/CH/SD/2009/134 and
300 Ch/CH/SD/2008/128 NDV isolates were determined and analysed to examine their
301 pathological and phylogenetic relationships with other strains. Both isolates had a genome
302 consisting of 15192 base pairs. Genetic analysis of these isolates showed that both shared a
303 similarity of 96%. Both isolates had an F protein cleavage site motif, which determines the

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

306 To further explore the molecular basis of the mortality caused by both isolates in ducks, we
307 compared the F and HN genes, which are major contributors to pathogenicity (Kim et al.,
308 2011). The hydrophobic transmembrane region of the F protein is located at amino acid
309 residues 500−553 of F_0. This hydrophobic base and partially hydrophilic rim are important
310 for transport and processing of the F protein (Plemper et al., 2003, Gravel et al., 2011).
311 Several studies have shown potential roles for the cytoplasmic and transmembrane domains
312 of the NDV fusion protein based on its structure and function (Sergel and Morrison, 1995,
313 Kim et al., 2011, Gravel et al., 2011). Tyrosine-containing signals in the cytoplasmic domains
314 of viral membranes are important for targeted protein delivery (Brewer and Roth, 1991,
315 Weise et al., 2010) and may be important for virus assembly because they guide these
316 proteins to cholesterol-rich membrane domains (Dolganiuc et al., 2003). The NDV fusion
317 protein cytoplasmic domain is located at amino acids 523−553, which is a highly conserved
318 region.

319 In the present study, comparison of the F protein of Ch/CH/SD/2008/128 with that of
320 Du/CH/SD/2009/134 and 5 other viruses of genotype VII revealed point substitutions at
321 amino acid positions of 16, 97, 129, 179, 181, 396, 480, 527, 543, and 551. The Y527A
322 tyrosine mutation in the F protein results in hyperfusogenic phenotypes characterized by
323 increased replication, pathogenicity (Samal et al., 2013), and immunogenicity (Manoharan et
324 al., 2016). Cleavage of the tyrosine mutant F protein is higher than that of the wild type,
325 suggesting this mutation affects the transport and expression of the F protein (Samal et al.,
326 2013). The increased pathogenicity of Ch/CH/SD/2008/128 in our study may also be
327 attributable to the T551A mutation, which increases the fusion index (Samal et al., 2013).
328 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,

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

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

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374 **Conflict of Interest**

375 None of the authors of this study has a financial or personal relationship with other people or
376 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)

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

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576 **Table 2**

577 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	TAAGAAAAAACTACTGGG AACAAGCAACCAAAGAGC AATAC
HN	ACGGGTAGAA	6327	ATCACTTTAT	8318	TAAGAAAAAATACAGAAA GCATTGAGATGTAAGGGAA AACAACCAACAAGAGGGA AC
L	ACGGGTAGGA	8376	TAGAAAAAAG	15079	

578 Note: [a]The Ch/CH/SD/2008/128 gene end sequences is CATCAGAAAT, the

579 Du/CH/SD/2009/134 is CATTAGAAAT.

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596 **Table 3**

597 Comparison of nucleotides and amino acids among NDV isolates Du/CH/SD/2009/134 and
598 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	

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611 **Table 4**

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

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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

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632 **Table 5**

633 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

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636 **Figure 1**

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

640 **Figure 2**

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

644 **Figure 3**

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

653 **Figure 4**

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

659 **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.

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