Acta virologica **65**: 149 – 159, 2021 doi: 10.4149/av_2021_210

Complete genome and molecular characterization of genotype VII velogenic Newcastle disease virus isolated in China

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Received August 16, 2020; revised January 6, 2021; accepted March 31, 2021

Summary. – Circulation of dominant genotype VII of Newcastle disease virus (NDV) causes significant economic losses to the poultry industry in China. Although most of genotype VII NDV has frequently been isolated in China to date, the genome sequence difference between duck-origin and chicken-origin NDVs remains largely unknown. In this study, a NDV strain of Chicken/China/HB/2017 (HB), isolated during an outbreak in China, was subjected to genetic, biological, phylogenetic and the pathogenicity characterization. The complete genome of HB strain is 15,192 nucleotides (nt) long and consisting of six genes in the order of 3'-NP-P-M-F-HN-L-5'. Several amino acid mutations were identified in the functional domains of F and HN proteins, including fusion peptide, heptad repeat region, transmembrane domains, and neutralizing epitopes. Phylogenetic analysis based on the F gene revealed that the HB strain and three other duck-origin NDV strains in China were grouped under subgenotype VII.1.1 and shared 99.1~99.2% nucleotide identity. Additionally, the challenge experiment results showed that the strain was highly pathogenic with 100% morbidity and mortality. Virus shedding was detected from 2 days post-infection until the fifth day. In conclusion, this study offers our understanding of circulating strains of NDV and genes involved in virulence and evolution between different hosts.

Keywords: Newcastle disease virus; China; complete genome; genotype VII; mutations

Introduction

Newcastle disease (ND), caused by virulent strains of Newcastle disease virus (NDV), is a highly contagious and widespread avian disease that results in severe economic losses to the poultry industry (Absalon *et al*., 2014). The NDV is a member of the genus *Avula virus* of family *Paramyxoviridae* (Amarasinghe *et al*., 2018). Historically, NDVs has been classified into two classes, class I and class II. Class II viruses with at least 18 genotypes display a higher degree of genetic diversity, virulence and broader host range than class I viruses (Dimitrov *et al*., 2016). The genome size of class I strains is 15,198 bp, while the class II strains 15,186 or 15,192 bp long. The genome of NDV contains six genes in the order of 3'-NP-P-M-F-HN-L-5' that encode six major proteins (Czegledi *et al*., 2006). According to their pathogenicity in chickens, NDVs have been classified into three major pathotypes including lentogenic, mesogenic, and velogenic strains (Kristeen-Teo *et al*., 2017; Liu *et al*., 2011). Previous studies have demonstrated that the sequence of cleavage sites in the F protein is the

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Abbreviations: HR = heptad repeat region; ICPI = intracerebral pathogenicity index; MDT = mean death time; MEGA = Molecular Evolutionary Genetics Analysis; ND = Newcastle disease; NDV = Newcastle disease virus; OIE = Office International Des Epizooties; UTR = untranslated region

primary molecular determinant of NDV pathogenicity (Panda *et al*., 2004). This sequence is 112R/K-R-Q-R/K-R-F117 in velogenic viruses and 112G/E-K/R-Q-G/E-R-L117 in lentogenic viruses. The replication of NDV strictly follows "the rule of six" where efficient replication only takes place if the genome size is a multiple of six nucleotides. In addition, RNA editing occurs in the transcription of P gene to produce the other proteins, namely, V and W (Wang *et al*., 2019). NDV has been endemic in China since it was first described in village chicken flocks in 1946 (Liu *et al*., 2003). In the past 20 years, genotype VII NDV has been continuously detected in China and it is now the predominant pathogen responsible for most outbreaks of ND. NDV has a wide host range, in addition to poultry species, at least 250 species of birds can be infected (Xiang *et al*., 2020; Xu *et al*., 2019). Among poultry, chickens and turkeys are the most susceptible, and ducks and geese are the least susceptible. In general, duck species may show few or no clinical symptoms, even when exposed to NDV strains virulent for chickens. Previous studies have revealed that duck-origin NDV isolate can infect chickens and ducks and can be transmitted to naive contact chickens and ducks (Steward *et al*., 1993). Although many genotype VII NDVs from ducks and chickens have been isolated to date, the genome sequence differences between duck-origin and chicken-origin NDV have not been studied. To provide further molecular basis of the NDV transmission from water fowl to land fowl, we have isolated NDV strain (HB) from a vaccinated commercial chicken farm in Hebei province. Its molecular and biological characteristics were evaluated in *in vitro* and *in vivo* experiments.

Materials and Methods

Virus collection and isolation. The tissue samples (brain, lung, liver and spleen) were collected from one vaccinated chicken in a commercial farm of Baoding city, Hebei Province, Northern China, in 2017. Both, the 9-day-old SPF embryonated chicken eggs (Ringpu Biological Company of Baoding city, Northern China) and DF-1 cells (our laboratory) were used to isolate the virus. The mean death time (MDT) in 9-day-old SPF embryonated chicken eggs and intracerebral pathogenicity index (ICPI) in one-day-old SPF chickens were assessed in accordance with OIE manual of diagnostic tests. The hemagglutination (HA) positive allantoic fluid was collected and identified by standard hemagglutination inhibition (HI) and reverse transcription polymerase chain reaction (RT-PCR) assays.

Immunofluorescence assay (IFA). NDV-infected DF-1 cells were fixed with 4% paraformaldehyde at 4°C for 30 min then washed three times with PBS and permeabilized with 0.2% Triton X-100 for 10 min at room temperature. After blocking with 5% bovine serum albumin (BSA) at 37°C for 1 hr, the cells were incubated with chicken anti-NDV polyclonal antibody for 1 hr at 37°C. After washing for three times with PBS, the cells were incubated with FITC-rabbit anti-chicken secondary antibody for 1 hr. The stained cells were examined using a fluorescence microscope.

Cloning and sequencing of RT-PCR products. The viral nucleotide sequences of Newcastle disease virus APMV-1/duck/China/WF00D/2003 strain (JN599167.1), NDV/chicken/China/JS-3-05-Ch/2012 strain (JN618349.1) and APMV-1/duck/China/Md/CH/LGD/1/2005 strain (KM885167.1) published in GenBank, were used to design 12 pairs of primers for amplifying the entire genome of the virus in SnapGene software (https://www.snapgene.com/). Total nucleic acids were extracted from the 9-day-old embryonated SPF eggs of a single passage using the TaKaRa MiniBEST universal viral RNA/DNA extraction kit Ver5.0, used for reverse transcription and PCR amplification with PrimeSTAR HS polymerase (Takara, Japan). The PCR was done under the following conditions in a thermal cycler: 1 cycle of 3 min at 94°C; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min 30 s; and 1 cycle of 10 min at 72°C. The amplified products mixed with 6× Super GelRed prestain loading buffer (US Everbright Inc, China) were analyzed by electrophoresis in a 1% agarose gel. Furthermore, PCR products were subcloned into pEASY-blunt cloning vectors (Trangen, China) and subsequently subjected to Sanger Sequencing reactions (Sangon Biotech, China).

Sequence analyses and phylogenetic studies. Sequences were compiled using the SEQMAN program in DNASTAR (Sixth edition, DNASTAR, Inc, USA). Confirmations of identity were performed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The consensus amino acid sequence was derived from NDV strains of different genotypes as described previously (Wang *et al*., 2016). Phylogenetic analysis was done using the complete genome sequences or the sequence of each structural gene (NP, F and HN) from strains of NDV available from GenBank and the phylogenetic trees were constructed using the distance-based neighbor-joining method in the MEGA 7 (Kumar *et al*., 2016).

Pathogenicity in chickens. Twenty 4-week-old SPF chickens were randomly allocated to two experimental groups, consisting of HB-infected group (n = 10) and mock-infected (control) group (n = 10). The infected group was challenged by the intramuscular injection with 0.1 ml virus suspension containing 10^5 EID_{50}/0.1 ml, while the control group was inoculated via the same route with the same amount of sterile phosphate-buffered saline (PBS). The birds of the two groups were reared in different isolators and were monitored daily for characteristic clinical signs of Newcastle disease. The dead birds were necropsied and examined for the lesions immediately. The time of death was recorded and used to plot the survival curves and to calculate the mean death time. For virus titration, tissue samples were homogenized in PBS containing antibiotics and the supernatant was serially diluted 10-fold and inoculated in DF-1 cells, in duplicate wells for each dilution. The virus titers

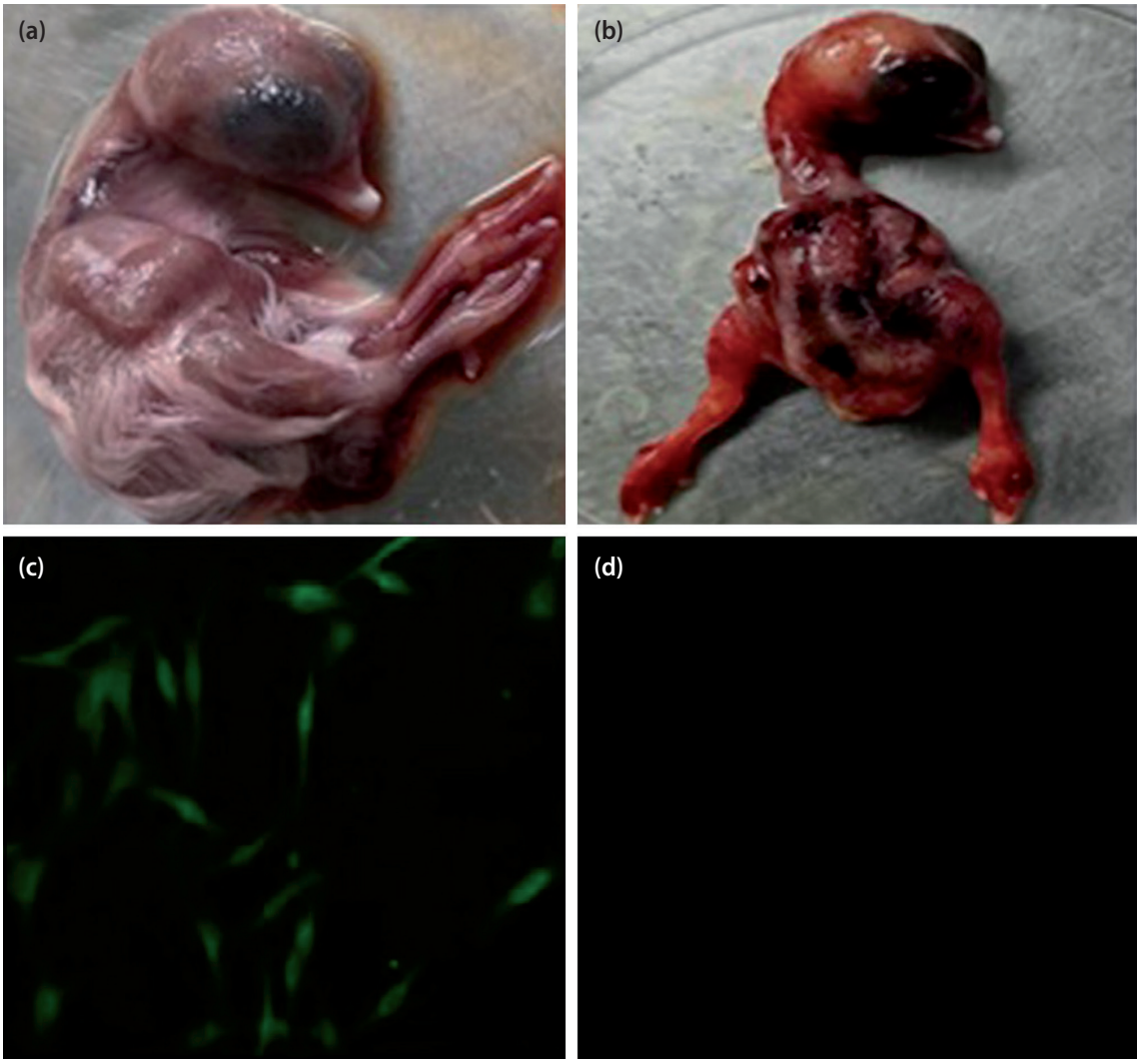

Fig. 1

The isolation and identification results of NDV HB isolate

(a) The SPF embryonated chicken egg inoculated with PBS as negative control. **(b)** The SPF chicken embryo inoculated NDV-HB solution (5^{th} passage), after about 50 h post-inoculation. **(c)** Immunofluorescence showed positive green fluorescence signal in DF-1 cells infected with NDV isolate HB (magnification: 200 ×). **(d)** Non-infected DF-1 cells showed no fluorescent signal (magnification: 200 ×).

were determined by IFA as described above and the $TCID_{50}$ per 100 mg of tissue was calculated.

Results

Virus isolation and identification

The supernatants of clinical samples (200 µl) were inoculated in to 9-day-old embryonated SPF chicken eggs and DF-1 cells. The SPF chicken embryos inoculated with NDV HB solution died at about 50 h post-inoculation. After dissection, it was found that there was an extensive intratissue hemorrhage, tissue adhesion, and even dissolution (Fig. 1a, b). The cells inoculated with supernatant of clinical samples showed a visible CPE that consisted of enlarged, densely granular cells that occurred either singly or in clusters, with evidence of cell shrinkage and detachment. To date, the isolate has been serially propagated in DF-1 cells for 43 passages. The propagation of NDV HB in DF-1 cells was also confirmed by IFA staining using chicken anti-NDV antibodies (Fig. 1c, d).

Table 1. Detailed information on an NDV HB strain and biological characteristics

Virus	Pathogenicity		Virus titer		
	ICPI	MDT	EID_{50}/ml	$TCID_{50}$/ml	$TCID_{50}$/ml
HB	1.86	53.7	$10^{8.15}$	$10^{7.05}$	$10^{6.95}$

Table 2. Genome length characteristics of isolated HB strain

Region	Gene start	3'-UTR	ORF length (nt)	5'-UTR	Gene end	Nucleotide length (nt)	Amino acid length (aa)
Leader						55	
NP	56-65	66	122-1591	217	1798-1808	1753	489
P	1810-1819	83	1893-3080	180	3250-3260	1451	395
M	3262-3271	34	3296-4390	112	4493-4502	1241	364
F	4504-4513	46	4550-6211	84	6285-6295	1792	553
HN	6327-6336	91	6418-8133	195	8318-8328	2002	571
L	8376-8385	11	8387-15001	77	15069-15078	6703	2204
Trailer						113	
Whole genome						15192	

Table 3. Nucleotide (nt) and amino acid (aa) sequence identities (%) between HB strain, vaccine, and closely related strains

Gene	Strain (genotype)	V4 (I)[b]		La Sota (II)[b]		Mukteswar (III)[b]		Herts (IV)[a]		Md/CH/LGD/1/200514/02 (VII)[a]	
		nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Complete genome	HB	83.8		82.9		85.6		87.5		99.2	
NP	HB	85.4	91.2	85.6	92.0	87.8	93.9	88.8	92.1	99.9	97.8
P	HB	82.7	82.6	82.7	82.3	84.5	93.3	84.8	67.5	99.6	99.0
M	HB	87.7	91.0	84.6	88.2	87.4	89.9	88.2	71.5	99.7	99.2
F	HB	82.5	82.3	84.8	84.3	84.2	63.1	86.6	69.4	97.1	95.8
HN	HB	81.6	87.2	85.2	88.1	82.3	62.3	84.4	65.1	99.3	98.4
L	HB	87.1	93.6	nd[c]	nd	nd	nd	89.1	73.8	99.3	98.9

[a]Nucleotide sequences for comparison with Chinese isolate were from GenBank, complete genome sequences of Md/CH/LGD/1/200514/02 (KM885167) and Herts (AY741404). [b]Vaccine strains widely-used in China. [c]No data.

Biological characterization

The pathogenicity assessment was conducted according to the guidelines provided by the OIE, and the results showed that MDT of HB strain was 53.7 h (Table 1). Intracerebral inoculation of HB in one-day-old chicks resulted in an ICPI of 1.86, and the strain had a cleavage site motif of ^{112}RRQKRF^{117}, which is typical for velogenic NDV strains. The results of HA and HI assays with the isolated virus showed that the isolate was HA positive with the titer of $9\log^2$. Virus titration was done on different types of cells, to understand the virus growth characteristics. We have used embryonated eggs, BHK-21 and DF-1 cell lines. The HB strain presented almost same $TCID_{50}$ on DF-1 and BHK-21 cells. The EID_{50} on embryonated eggs and $TCID_{50}$ on cells of HB viruses presented only a little difference. The HB strain showed preferential replication on eggs as indicated by ND properties.

Complete genome sequence analysis of the isolates

The isolated genome of HB strain is 15,192 bp long and follows the rule of six for a competent viral replication. The obtained nucleotide data were submitted to

Table 4. Amino acid mutations in the functional domains of the F protein

Strains	Signal peptide (1–31aa)				HRa (143–185aa)	HRb (268–299aa)	HRc (471–500aa)					Trans-membrane domain (501–521aa)	
	11	24	27	28	(156–172)	272	480	488	489	494	499	508	520
Consensus[a]	V	S	C	L	ESIAATNEAVHEVTDGL	N	K	L	D	K	S	T	V
HB	P	G	R	P	**RF**I**G**A**VIGS**V**ALGVATA**[b]	Y	R	I	E	R	Y	I	G
Duck/Fujian/FP/02	P	G	R	P	**RF**I**G**A**VIGS**V**ALGVATA**	K	R	L	E	R	S	T	G
Md/CH/LGD/1/2005	P	G	R	P	-	K	R	L	E	R	S	T	G
BP01	P	G	R	P	-	K	R	L	E	R	S	T	G
La Sota	-[b]	-	-	-	-	-	-	-	-	-	-	-	
V4	-	-	-	-	ESIAATNEAVHEVTD**N**L	-	-	-	-	-	-	-	

[a]The consensus amino acid sequences were derived from 100 velogenic, mesogenic and lentogenic NDV strains from GenBank. [b]The bold letters indicate amino acid mutations.

the GenBank (Acc. No. MK342603). A comparison of the nucleotide and amino acid sequences among the HB and selected class II reference strains representing genotypes I-IV and VII is presented in Table 3. Analyses of the complete genome sequences revealed that HB shared 99.2% nucleotide identity with NDV strain Md/CH/LGD/1/2005 (genotype VIId). However, the lowest nucleotide identity was observed with widely used vaccine strain La Sota (82.9%, genotype II). In addition, the analyses of the coding sequences of each gene showed that among all the genes, the NP gene was the most conserved with sequence identities that ranged from 85.4% to 99.0% (nt) and 91.2% to 97.8% (aa) in all strains (Table 3). In contrast, the HN gene was the most variable with sequence identities that ranged from 81.6% to 99.3% (nt) and 62.3%–98.4% (aa). In contrast to early genotypes (genotypes I-IV) of NDV strains, six nucleotide (1648TCCCAC1653 for HB) insertion at the 5'-UTR of the NP gene was observed in the HB strain. The leader sequence was 55 nt, and the trailer sequence was 113 nt long. Sequences of intergenic regions are between 1–47 nt. In addition, the 3' terminus of the leader (TGTTTGGT) and the 5' terminus of the trailer (ACCAAACA) sequences are identical in HB strains.

F protein analysis

F protein is type I membrane glycoprotein that is considered as a vital factor of pathogenicity. The 100 NDV isolates (obtained in the GenBank), closely related strains and vaccine strains (La Sota, V4), were used in the molecular studies as reference sequences. The F protein cleavage site of HB was 112RRQKRF117, which was typical for velogenic NDV. Unlike to most avian paramyxovirus type 1, NDV-HB F protein has four potential N-glycosylation sites, G1-85(NRT), G2-191(NNT), G3-366(NTS), G5-471(NNS). Analysis of amino acids in the functional domain of the F protein showed that HB had 2 amino acid mutations in signal peptide, 20 mutations in the heptad repeat region (HR), and 2 in the transmembrane domain, when compared with consensus amino acid sequences derived from NDV strains of different genotypes and vaccine strains La Sota and V4 (Table 4). In the signal peptide and HRa, no amino acid difference was identified between HB and subgenotype VIId Duck/Fujian/FP/02strain. There are 14 amino acid mutations between HB and other subgenotype VIId strains (Md/CH/LGD/1/2005, BP01) in HRa. Furthermore, analysis of the remaining regions showed one mutation in HRb (position 272), 2 mutations in HRc (position 488 and 499) and one mutation in transmembrane domain (position 508) between HB and the three isolates, respectively. The mutated amino acids tend to be Y and I.

HN protein analysis

The HN protein of HB consisted of 571 aa, which is similar to Md/CH/LGD/1/2005 and BP01, but different from duck/China/Fujian/FP/02 (571 aa) strain. Amino acid sequence analysis of HN of the four isolates showed 98.4–98.6% similarity to each other. However, only 62.3–88.1% identity was observed when compared to other different NDV genotypes (I-IV). Seven amino acid mutations of HB were located in the neutralizing epitopes compared with consensus strains and vaccine strains (La Sota and V4). However, there are only 4 amino acid mutations with the closely related NDV strains (Duck/Fujian/FP/02, Md/CH/LGD/1/2005, BP01). All mutations are summarized in Table 5. The sialic acid binding sites and cysteine residues in HB were completely conserved as occurs with most NDVs.

Table 5. Amino acid mutations in the functional domains and neutralizing epitopes of HN protein

Virus (genotype)	HN protein						
	33	332–333	340	346–353	494	514	569
Consensus[a]	I	GK	Y	DEQDYQIR	G	I	D
HB	M	WI	H	R353G	D	V	-
Duck/Fujian/FP/02	M	-	-	-	D	V	-
Md/CH/LGD/1/2005	M	-	-	-	D	V	-
BP01	M	-	-	-	D	V	-
La Sota	-	-	-	-	-	-	-
V4	-	-	-	-	-	-	-

[a]The consensus amino acid sequences were derived from NDV vaccine strains (Mukteswar, La Sota, HB isolate V4).

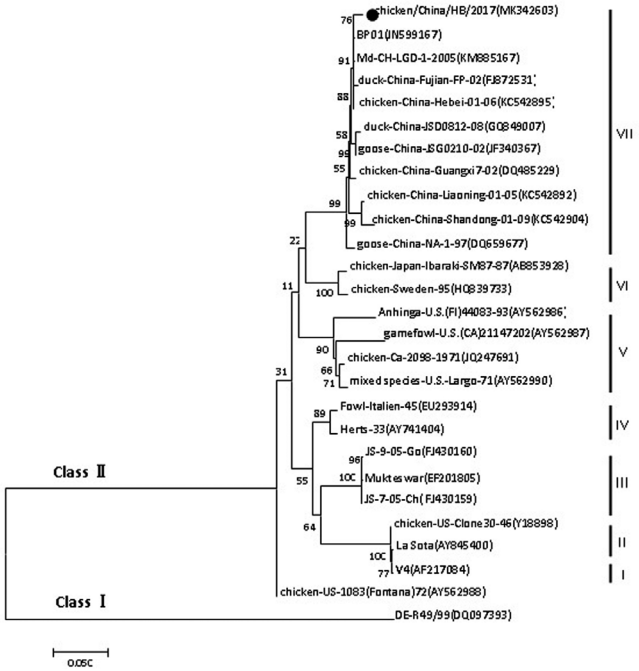

Fig. 2

Phylogenetic tree of the nucleotide sequences of NDV isolates based on the complete genome

All the reference sequences used in this study were obtained from the GenBank database. The tree was constructed by the neighbor-joining method with 1,000 bootstrap replications using MEGA 7 software. The isolate identified in this study is indicated by black dot.

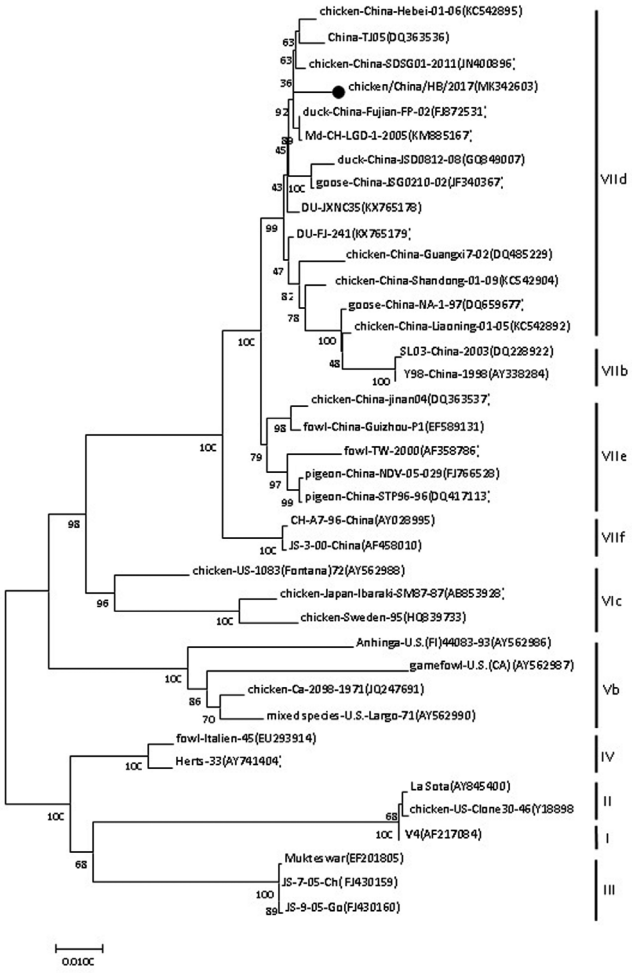

Fig. 3

Phylogenetic tree of the nucleotide sequences of NDV isolates based on the F gene

All the reference sequences used in this study were obtained from the GenBank database. The tree was constructed by the neighbor-joining method with 1,000 bootstrap replications using MEGA 7 software. The isolate identified in this study is indicated by black dot.

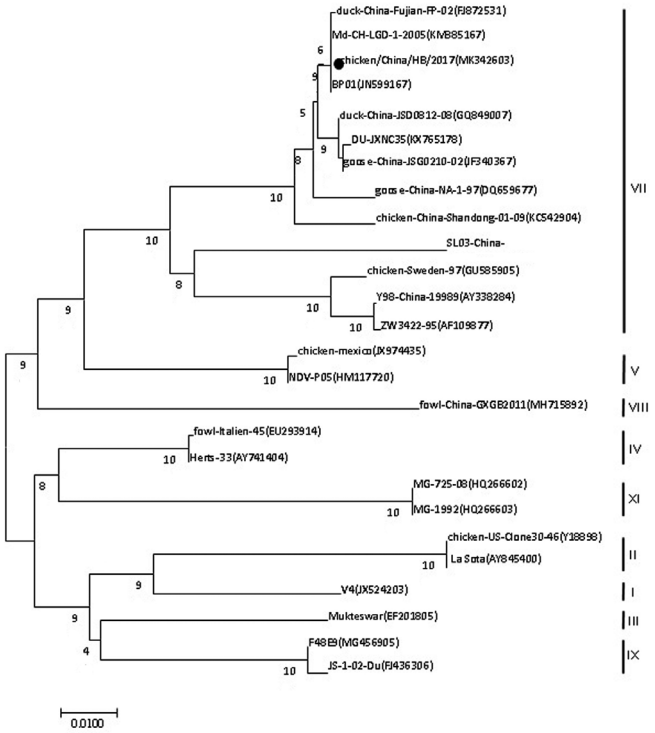

Fig. 4

Phylogenetic tree of the nucleotide sequences of NDV isolates based on the NP gene

All the reference sequences used in this study were obtained from the GenBank database. The tree was constructed by the neighbor-joining method with 1,000 bootstrap replications using MEGA 7 software. The isolate identified in this study is indicated by black dot.

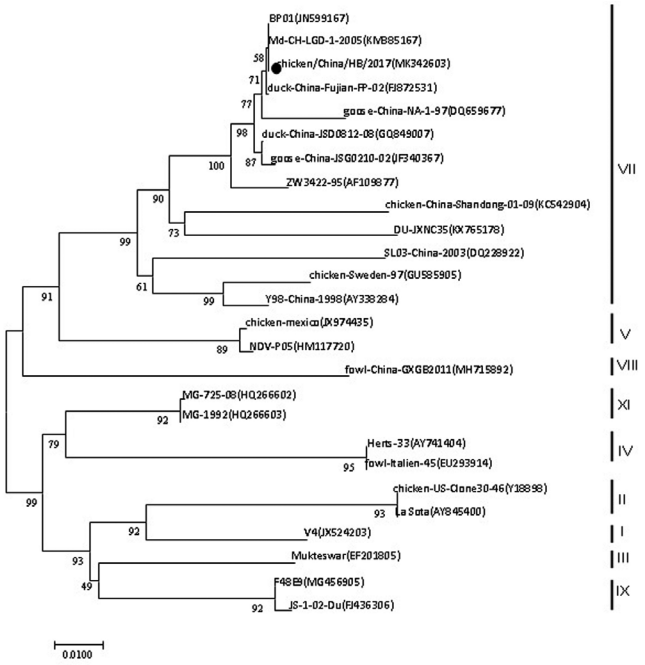

Fig. 5

Phylogenetic tree of the nucleotide sequences of NDV isolates based on the HN gene

All the reference sequences used in this study were obtained from the GenBank database. The tree was constructed by the neighbor-joining method with 1,000 bootstrap replications using MEGA 7 software. The isolate identified in this study is indicated by black dot.

Four potential glycosylation sites at positions 119 (NNS), 341 (NNT), 433 (NKT), 481 (NIS) were identified in the HN protein of HB.

Phylogenetic analysis

ClustalW of DNAStar software used to perform sequence alignment on multiple genotype VIIstrains showed that the F gene sequence of NDV HB shared 91.1–97.2% nucleotide identity with the NDVs belonging to subgenotypes VIIb, VIId, VIIe and VIIf, available in GenBank. However, only 82.2–86.6% nt identity was observed when compared the other different genotypes (I-IV strain, Table 3). Phylogenetic analysis based on the F gene revealed that HB strain phylogenetically clustered together with genotype VIId sublineage of genotype VII strains but were clearly distinct from viruses representing other known NDV genotypes in class II. Additionally, according to the updated phylogenetic classification system, the HB strain is within subgenotype VII.1.1 (Fig. 3). The HB strain shared the highest homology with Md/CH/LGD/1/2005, Duck/Fujian/FP/02, and BP01 strain and was in the same branch of the genetic evolution tree. To confirm the robustness of the genetic groupings and topology of the phylogenetic tree obtained by the F gene sequences, we performed a phylogenetic analysis based on the complete genome sequence and the complete ORFs of NP and HN (Fig. 2, 4, and 5). Similar to the results obtained in the analysis of the F gene, all these phylogenetic reconstructions resulted in HB strain forming an isolated branch with genotype VII, separate from the viruses of other known class II genotypes. HB strain remained in the same branch with Md/CH/LGD/1/2005, duck/China/Fujian/FP/02 and BP01 strains.

Pathogenicity in chickens

The clinical pathological characteristics and the virulence of NDV HB were assessed in chickens. Some NDV HB-inoculated birds exhibited observable depression, ruffled feathers and green diarrhea from the 2nd day post-infection. At 3 dpi, all birds in the treated group presented

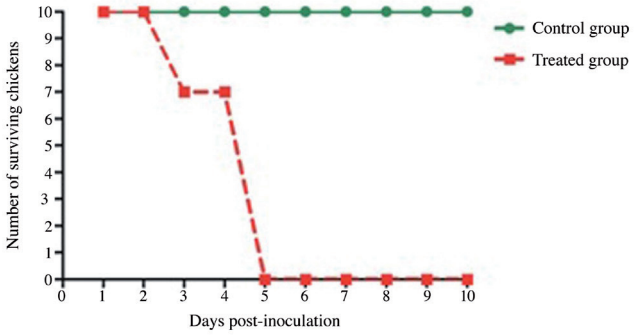

Fig. 6

Survival curve of NDV HB infected chickens
Three birds of the treated group died 3 days post-inoculation, and the remaining birds died 5 days post-inoculation.

clinical symptoms, such as lethargy, head twitch, severe diarrhea, even paralysis. The birds all died by day 5 post-inoculation (Fig. 6). Gross lesions associated with velogenic viscerotropic NDV infection were observed, including spleen necrosis, glandular gastric hemorrhage, multiple lymph follicular swelling and bleeding, variegated kidney. The viral titer was highest in the spleen, ranging from $10^{3.77}$ to $10^{5.25}TCID_{50}$/0.1mg tissue (Table 6). Additionally, all birds inoculated with NDV HB shed the virus and was found in oral and cloacal swabs at 2, 3, and 5 dpi, with virus titers ranging from $10^{2.3}$ to $10^{4.4}TCID_{50}$/0.1 ml and from $10^{2.6}$ to $10^{4.1}TCID_{50}$/0.1 ml, respectively.

Discussion

NDV was first isolated in 1926, and remains a worldwide epidemic and an important limiting factor in the development of commercial poultry production and the establishment of trade links to date (Berezin *et al.*, 2019; Welch *et al.*, 2019). Thus, it should be routinely monitored all over the world. In this study, the HB strain had high similarity to duck-origin NDV strains (Md/CH/LGD/1/2005, BP01, and Duck/Fujian/FP/02). The phylogenetic analysis revealed that the four isolates clustered into class II genotype VII and keeps in a closely connected position in the phylogenetic tree. Previous studies have demonstrated that the genotype IX of NDV strain YF18 could be transmitted from infected ducks to chickens (Kang *et al.*, 2016). The ducks are more resistant to the disease, and they may be infected without overt symptoms, but they may spread the virus in farms or live-bird markets by the shedding of the virus via oropharyngeal and cloacal route (Zhang *et al.*, 2020). Considering the factor of high homology with duck-origin NDV and high pathogenicity, we assumed that the HB may be transmitted from infected ducks to vaccinated domestic chickens. Therefore, it is necessary to do more investigation to identify the source of the virus, and enhance active surveillance for NDV in waterfowl to assess the potential for virus shedding and transmission.

Infection of chickens with NDV HB results in characteristic velogenic viscerotropic Newcastle disease. In the challenge study, when in the 4-week-old SPF chickens infected with NDV strain HB at dose of $10^5 EID_{50}$, all of the virus-infected chickens died between 3 and 5 dpi and both the morbidity and mortality were 100%, indicated that HB had high pathogenicity in chickens. In addition, on the 3rd dpi, the virus replicated in all tissue samples, confirming the systemic nature of the caused infection. Viral tissue tropism is an important factor determining the virulence and broad tropism results in more severe pathogenicity or higher virulence for many viruses. Additionally, viral titer of the spleen and caecum samples in the treated group was higher than other tissue samples, which may be related to the typical clinical signs of velogenic viscerotropic NDV strains. The results of virus distribution and virus replication sites are similar to previously reported in studies with SG10 and other vNDV (Jin *et al.*, 2017; Moura *et al.*,

Table 6. Virus titer from different tissue samples after infection with NDV HB strain

Tissue sample	Control group		Treated group	
	Virus titer range ($log10TCID_{50}$/0.1 mg tissue) ±SD	Negative rate (100%)	Virus titer range ($log10TCID_{50}$/0.1 mg tissue) ± SD	Positive rate (100%)
Spleen	ND[a]	100	3.77 ±0.12[b]-5.25 ±0.48	100
Lungs	ND	100	2.97 ± 0.36-4.04 ±0.34	100
Caecum	ND	100	3.40 ± 0.21-4.65 ±0.29	100
Kidney	ND	100	1.86 ± 0.16-3.48 ±0.28	100
Trachea	ND	100	2.47 ± 0.17-3.84 ±0.17	100
Brain	ND	100	2.23 ± 0.28-3.18 ±0.25	100

[a]Not detected

2016), which revealed that typical Newcastle disease is successfully replicated by the pathogenicity experiments in SPF chickens.

Glycoprotein F mediates the fusion of the viral envelope to the plasma membrane in the entry process of the virus into the host cell. It is considered to be closely related to the virulence of NDV strains (Liu *et al*., 2019b). The F gene contains four main domains including signal peptide, fusion peptide, heptad repeats region (HR), and transmembrane domain (Ren *et al*., 2019). In this study, compared with 100 consensus NDVs including vaccine strains La Sota and V4, the F protein of HB strain had 4, 20, and 2 amino acid mutations in the signal peptide, heptad repeat region and transmembrane domain, respectively. These mutations are mainly concentrated in the signal peptide and HR region. Notably, according to previous reports, the amino acid at position 27 of the signal peptide could affect viral virulence in chickens, and amino acid mutations of the HR region could affect the fusion activity of NDV (Wang *et al*., 2016), which may partially explain the difference between the current strains and the current vaccine strains. Generally, there were six potential glycosylation sites at positions 85, 191, 366, 447, 471 and 541, which were highly conserved in F protein of most NDVs. Notably, HB only had four potential glycosylation sites, unlike commonly used vaccine strains. In another study, it has been shown that some mutations at glycosylation sites of F protein could increase the virulence and pathogenicity of NDV (Ganar *et al*., 2014). It is also evident that mutation in the cytoplasmic domain of F protein can lead to the production of a hyperfusogenic virus via increasing viral replication and pathogenicity in chickens (Samal *et al*., 2020). Whether the losses at the position 447 and 541 of potential glycosylation sites F protein could change the antigenicity of HB needs to be further studied.

HN protein is a glycoprotein which plays an important role in the recognition of sialic acid-containing receptors on cell surfaces as well as in the promotion of F protein fusion activity (Ji *et al*., 2018). In this study, three unique features were found in the HN gene of HB. First, comparing the HN protein between HB strain and vaccine strains La Sota and V4, we found that there are several amino acid mutations in the functional domains and neutralizing epitopes of the HN protein of the Chinese isolate. The mutations (N263K, I514V) in these neutralizing epitopes were related to neutralizing escape variants (Choi *et al*., 2013; Snoeck *et al*., 2013). One amino acid mutation (I33M) in transmembrane domain was related to affecting virus fusion promotion activities; one amino acid mutation (R353G) in neutralizing epitopes was not found in another genotypes VII of NDVs. Among them, the mutation R353G lies in the globular head. As it was reported, both receptor recognition and neuraminidase properties of HN lie in the globular head, which is also suggested to be the antibody binding site (Liu *et al*., 2019a). Therefore, this mutation may affect its antigenicity and increase the possibility that NDV infects chickens. Second, most of NDVs contain six potential glycosylation sites at positions 119, 341, 433, 481, 508 and 501, but HB only had four potential glycosylation sites at positions 119, 341, 433 and 508, which are similar to the F protein. We eventually found that HB strain contains about 10 specific amino acid residues that are different from most of other genotype VII NDV strains, especially those encoded in the first 75 nucleotides at the N-terminal end. The differences in HN that make the HB strain more hydrophobic may make entry into the host cell easier.

In conclusion, we confirmed that the strain HB was a velogenic NDV of genotype VII and had the high pathogenicity in chickens. In addition, the present study systematically analyzed the whole genome sequence of the NDV HB isolate from China. Several unique mutations were identified in the neutralizing epitopes and functional domains of the isolated strain. These findings will further expand the epidemiological data of NDV in China and may be important to improve the current understanding of NDV epidemiology and evolution.

Acknowledgments. This work was partly supported by the Earmarked Fund for Hebei Lays Innovation Team of Modern Agro-industry Technology Research System (HBCT2018150210) and by grants for one-hundred outstanding innovative talents from Hebei institution of higher learning (SLRC2017039). The authors would like to thank College of Animal Medicine, Agricultural University of Hebei, Baoding, China, for providing facilities.

References

Absalon AE, Mariano-Matias A, Garcia LJ, Morales-Garzon A, Toscano-Contreras A, Lucio-Decanini E, Cortes-Espinosa DV (2014): Complete genome analysis of velogenic Newcastle disease virus reference strain „Chimalhuacan": evolution of viral lineages in Mexico. Virus Genes 49, 233–236. https://doi.org/10.1007/s11262-014-1082-8

Amarasinghe GK, Arechiga Ceballos NG, Banyard AC, Basler CF, Bavari S, Bennett AJ, Blasdell KR, Briese T, Bukreyev A, Cai Y, Calisher CH, Campos Lawson C, Chandran K, Chapman CA, Chiu CY, Choi KS, Collins PL, Dietzgen RG, Dolja VV, Dolnik O, Domier LL, Durrwald R, Dye JM, Easton AJ, Ebihara H, Echevarria JE, Fooks AR, Formenty PBH, Fouchier RAM, Freuling CM, Ghedin E, Goldberg TL, Hewson R, Horie M, Hyndman TH, Jiang D, Kityo R, Kobinger GP, Kondo H, Koonin EV, Krupovic M, Kurath G, Lamb RA, Lee B, Leroy EM, Maes P, Maisner A, Marston DA, Mor SK, Muller T, Muhlberger E, Ramirez VMN, Netesov SV, Ng TFF, Nowotny N, Palacios G, Patterson JL, Paweska JT, Payne SL, Prieto K, Rima BK, Rota P, Rubbenstroth D, Schwemmle M, Siddell S, Smither SJ, Song Q, Song T, Stenglein MD, Stone

DM, Takada A, Tesh RB, Thomazelli LM, Tomonaga K, Tordo N, Towner JS, Vasilakis N, Vazquez-Moron S, Verdugo C, Volchkov VE, Wahl V, Walker PJ, Wang D, Wang LF, Wellehan JFX, Wiley MR, Whitfield AE, Wolf YI, Ye G, Zhang YZ, Kuhn JH (2018): Taxonomy of the order Mononegavirales: update 2018. Arch. Virol. 163, 2283–2294. https://doi.org/10.1007/s00705-018-3814-x

Berezin V, Abdukhakimova D, Trenozhnikova L, Bogoyavlenskiy A, Turmagambetova A, Issanov A, Azizan A (2019): Antiviral activities of extremophilic actinomycetes extracts from Kazakhstan's unique ecosystems against influenza viruses and paramyxoviruses. Virol. J. 16, 150. https://doi.org/10.1186/s12985-019-1254-1

Choi KS, Kye SJ, Kim JY, Lee HS (2013): Genetic and antigenic variation of shedding viruses from vaccinated chickens after challenge with virulent Newcastle disease virus. Avian Dis. 57, 303–306. https://doi.org/10.1637/10379-092112-ResNote.1

Czegledi A, Ujvari D, Somogyi E, Wehmann E, Werner O, Lomniczi B (2006): Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications, Virus Res. 120, 36–48. https://doi.org/10.1016/j.virusres.2005.11.009

Dimitrov KM, Ramey AM, Qiu X, Bahl J, Afonso CL (2016): Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. Infect. Genet. Evol. 39, 22–34. https://doi.org/10.1016/j.meegid.2016.01.008

Ganar K, Das M, Sinha S, Kumar S (2014): Newcastle disease virus: Current status and our understanding. Virus Res. 184, 71–81. https://doi.org/10.1016/j.virusres.2014.02.016

Ji Y, Liu T, Du Y, Cui X, Yu Q, Wang Z, Zhang J, Li Y, Zhu Q (2018): A novel genotype VII Newcastle disease virus vaccine candidate generated by mutation in the L and F genes confers improved protection in chickens. Vet. Microbiol. 216, 99–106. https://doi.org/10.1016/j.vetmic.2018.01.021

Jin JH, Cheng JL, He ZR, Ren YC, Yu XH, Song Y, Yang HM, Yang YL, Liu T, Zhang GZ (2017): Development and application of a triplex real-time PCR assay for simultaneous detection of avian influenza virus, Newcastle disease virus, and duck Tembusu virus. Front. Microbiol. 8, 1607.

Kang Y, Xiang B, Yuan R, Zhao X, Feng M, Gao P, Li Y, Li Y, Ning Z, Ren T (2016): Phylogenetic and pathotypic characterization of Newcastle disease viruses circulating in South China and transmission in different birds. Front. Microbiol. 7, 119. https://doi.org/10.3389/fmicb.2016.00119

Kristeen-Teo YW, Yeap SK, Tan SW, Omar AR, Ideris A, Tan SG, Alitheen NB (2017): The effects of different velogenic NDV infections on the chicken bursa of Fabricius. BMC Vet. Res. 13, 151. https://doi.org/10.1186/s12917-017-1071-y

Kumar S, Stecher G, Tamura K (2016): MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874. https://doi.org/10.1093/molbev/msw054

Liu H, Zhao Y, Zheng D, Lv Y, Zhang W, Xu T, Li J, Wang Z (2011): Multiplex RT-PCR for rapid detection and differentiation of class I and class II Newcastle disease viruses. J. Virol. Methods 171, 149–55. https://doi.org/10.1016/j.jviromet.2010.10.017

Liu XF, Wan HQ, Ni XX, Wu YT, Liu WB (2003): Pathotypical and genotypical characterization of strains of Newcastle disease virus isolated from outbreaks in chicken and goose flocks in some regions of China during 1985–2001. Arch. Virol. 148, 1387–403. https://doi.org/10.1007/s00705-003-0014-z

Liu Y, Chi M, Liu Y, Wen H, Zhao L, Song Y, Liu N, Wang Z (2019a): Roles of the highly conserved amino acids in the second receptor binding site of the Newcastle disease virus HN protein. Virol. J. 16, 164. https://doi.org/10.1186/s12985-019-1273-y

Liu Y, Sun C, Chi M, Wen H, Zhao L, Song Y, Liu N, Wang Z (2019b): Genetic characterization and phylogenetic analysis of Newcastle disease virus from China, Infection, Genetics and Evolution. Infect. Genet. Evol. 75, 103958. https://doi.org/10.1016/j.meegid.2019.103958

Moura VM, Susta L, Cardenas-Garcia S, Stanton JB, Miller PJ, Afonso CL, Brown CC (2016): Neuropathogenic capacity of lentogenic, mesogenic, and velogenic Newcastle disease virus strains in day-old chickens. Vet. Pathol. 53, 53–64. https://doi.org/10.1177/0300985815600504

Panda A, Huang Z, Elankumaran S, Rockemann DD, Samal SK (2004): Role of fusion protein cleavage site in the virulence of Newcastle disease virus. Microb. Pathog. 36, 1–10. https://doi.org/10.1016/j.micpath.2003.07.003

Ren S, Rehman ZU, Shi M, Yang B, Liu P, Yin Y, Qu Y, Meng C, Yang Z, Gao X, Sun Y, Ding C (2019): Hemagglutinin-neuraminidase and fusion proteins of virulent Newcastle disease virus cooperatively disturb fusion-fission homeostasis to enhance mitochondrial function by activating the unfolded protein response of endoplasmic reticulum and mitochondrial stress. Vet. Res. 50, 37. https://doi.org/10.1186/s13567-019-0654-y

Samal S, Khattar SK, Paldurai A, Palaniyandi S, Zhu X, Collins PL, Samal SK (2020): Mutations in the cytoplasmic domain of the Newcastle disease virus fusion protein confer hyperfusogenic phenotypes modulating viral replication and pathogenicity. J. Virol. 94. https://doi.org/10.1128/JVI.01857-19

Snoeck CJ, Owoade AA, Couacy-Hymann E, Alkali BR, Okwen MP, Adeyanju AT, Komoyo GF, Nakoune E, Le Faou A, Muller CP (2013): High genetic diversity of Newcastle disease virus in poultry in West and Central Africa: Co-circulation of genotype XIV and newly defined genotypes XVII and XVIII. J. Clin. Microbiol. 51, 2250–2260. https://doi.org/10.1128/JCM.00684-13

Steward M, Vipond IB, Millar NS, Emmerson PT (1993): RNA editing in Newcastle disease virus. J. Gen. Virol. 74 (Pt 12), 2539–2547. https://doi.org/10.1099/0022-1317-74-12-2539

Wang J, Lv Y, Zhang Y, Zheng D, Zhao Y, Castellan D, Liu H, Wang Z (2016): Genomic characterizations of a newcastle disease virus isolated from ducks in live bird markets

in China. PLoS One 11, e0158771. https://doi.org/10.1371/journal.pone.0158771

Wang X, Jia Y, Ren J, Huo N, Liu H, Xiao S, Wang X, Yang Z (2019): Newcastle disease virus nonstructural v protein upregulates SOCS3 expression to facilitate viral replication depending on the MEK/ERK pathway. Front. Cell Infect. Microbiol. 9, 317. https://doi.org/10.3389/fcimb.2019.00317

Welch CN, Shittu I, Abolnik C, Solomon P, Dimitrov KM, Taylor TL, Williams-Coplin D, Goraichuk IV, Meseko CA, Ibu JO, Gado DA, Joannis TM, Afonso CL (2019): Genomic comparison of Newcastle disease viruses isolated in Nigeria between 2002 and 2015 reveals circulation of highly diverse genotypes and spillover into wild birds. Arch. Virol. 164, 2031–2047. https://doi.org/10.1007/s00705-019-04288-9

Xiang B, Chen R, Liang J, Chen L, Lin Q, Sun M, Kang Y, Ding C, Liao C, Xu C, Ren T (2020): Phylogeny, pathogenicity and transmissibility of a genotype XII Newcastle disease virus in chicken and goose. Transbound Emerg. Dis. 67, 159–170. https://doi.org/10.1111/tbed.13335

Xu X, Ding Z, Li J, Liang J, Bu Z, Ding J, Yang Y, Lang X, Wang X, Yin R, Qian J (2019): Newcastle disease virus-like particles containing the Brucella BCSP31 protein induce dendritic cell activation and protect mice against virulent Brucella challenge. Vet. Microbiol. 229, 39–47. https://doi.org/10.1016/j.vetmic.2018.12.007

Zhang X, Yao M, Tang Z, Xu D, Luo Y, Gao Y, Yan L (2020): Development and application of a triplex real-time PCR assay for simultaneous detection of avian influenza virus, Newcastle disease virus, and duck Tembusu virus. BMC Vet. Res. 16, 203. https://doi.org/10.1186/s12917-020-02399-z