

# Comparison of the protective antigen variabilities of prevalent Newcastle disease viruses in response to homologous/heterologous genotype vaccines

Yonghua Li,<sup>\*,1</sup> Zaib Ur Rehman,<sup>\*,†,1</sup> Mengjiao Li,<sup>\*</sup> Zahid Manzoor,<sup>†</sup> Wei Liu,<sup>\*</sup> Xusheng Qiu,<sup>\*</sup> Yingjie Sun,<sup>\*</sup> Ying Liao,<sup>\*</sup> Lei Tan,<sup>\*</sup> Cuiping Song,<sup>\*</sup> Weiwei Liu,<sup>\*</sup> Shengqing Yu,<sup>\*</sup> Chan Ding,<sup>\*,‡</sup> and Chunchun Meng<sup>\*,2</sup>

<sup>\*</sup>Shanghai Veterinary Research Institute (SHVRI), Chinese Academy of Agricultural Sciences (CAAS), Shanghai 200241, China; <sup>†</sup>Faculty of Veterinary and Animal Sciences, PMAS Arid Agriculture University, Rawalpindi 46300 Pakistan; and <sup>‡</sup>Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou University, Yangzhou, 225009, PR China

**ABSTRACT** The genotype VII Newcastle disease virus (NDV) vaccine has begun to replace the traditional genotype II NDV vaccine and is widely used in the commercial poultry of China. However, the effect of homologous and heterogeneous anti-NDV serum on the evolution of prevalent NDV is unknown. To understand the effect of genotype II and VII anti-NDV serum on the evolution of genotype VII NDV strains, ZJ1 (waterfowl origin) and CH/SD/2008/128 (ND128; chicken origin) were used for serial passage of 30 generations in DF-1 cells without anti-NDV serum or with genotype II and VII anti-NDV serum independently. The F and HN genes of the 2 viruses were amplified for the 10th, 20th, and 30th generations of each serial passage group and compared with their respective original viruses. We found that there was only one mutation at position 248 in the F gene of ZJ1 due to the serum pressure of

genotype VII anti-NDV. Similarly, mutations at residue 527 of the F gene, and position 9 and 319 of the HN gene of ND128 were noted in both anti-NDV serum groups. The results show that the nonsynonymous (NS)-to-synonymous (S) ratio of the F gene of ZJ1 virus was 1.6, and for the HN gene, it was 2.5 in the anti-II serum group. In the anti-VII serum group, the NS/S ratio for the F gene was 2.1, and for the HN gene, it was 2.5. The NS/S ratio of the F gene of the ND128 virus was 0.8, and for the HN gene, it was 3 in the anti-II serum group. Furthermore, the NS/S ratio of the F gene was 0.8, and the HN gene was 2.3 in the anti-VII group. Taken together, our findings highlight that there was no significant difference in the variation of protective antigens in genotype VII NDV under the selection pressure of homologous and heterogeneous genotype NDV inactivated vaccines.

**Key words:** Newcastle disease virus, HN gene, F gene, immune selection, genetic variation

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## INTRODUCTION

Newcastle disease (ND) is a widespread and well-recognized disease, which involves remarkable economic losses to the poultry industry by inducing digestive, nervous, and respiratory signs. Newcastle disease (ND) is caused by *Avian orthoavulavirus 1* (AOAV-1), also known as Newcastle disease virus (NDV) (Alexander, 2003; Meng et al., 2018), a member of the order *Mononegavirales*, family *Paramyxoviridae*, and genus *Orthoavulavirus*

(Dimitrov et al., 2019). The genome of NDV is nonsegmented and single-stranded with negative-sense RNA of 15.2 kb, which encodes 6 proteins, including the nucleocapsid, fusion (F) protein, hemagglutinin-neuraminidase (HN) protein, matrix protein, phosphoprotein, and large polymerase (Alexander, 2003; Rehman et al., 2018).

Based on phylogenetic analyses of the nucleotide sequences, NDV strains are divided into 2 distinct classes (class I and class II) of one serotype. Outbreaks of the ND are primarily caused by class II viruses (Fan et al., 2015). Among the class II NDVs, genotype VII is the predominant genotype in chicken and waterfowl and is responsible for the 4th and 5th panzootics of ND worldwide (Miller et al., 2015; Dimitrov et al., 2016). Intensive vaccination, biosecurity, and surveillance programs are practiced to protect birds from ND. Most of the commercially available vaccines are based

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<sup>1</sup>These authors contributed equally to this work.

<sup>2</sup>Corresponding author: [mengcc@shvri.ac.cn](mailto:mengcc@shvri.ac.cn)

on genotype II LaSota and B1; genotype I I2, V4, and PHY-LMV42; and Ulster lentogenic strains (Miller et al., 2010; Dimitrov et al., 2017). However, outbreaks of the ND are still reported in commercial poultry (Dimitrov et al., 2017). This situation suggests that the existing genotype II vaccines are not providing 100% protection from NDV infection and cannot prevent the shedding of the pathogenic virus (Miller et al., 2013; Liu et al., 2017), which may play a role in the spread and evolution of virulent NDV. This may be due to significant differences between the prevailing NDV strains and the current vaccine strains in their biology, serology, and genetics (Bello et al., 2020). Keeping in mind the genetic difference, a genotype (VII)-matched vaccine was developed (Hu et al., 2009), which has been extensively used in China since 2015 and has significantly decreased the isolation of genotype VII NDVs from poultry.

In viruses, the mutation rate is the rate at which nucleotide changes are made during genomic replication, whereas the substitution rate is the fixed mutation in a population (Peck and Lauring, 2018). The mutation rate of RNA viruses is high and can be explained by the genomic replication of these viruses by the RNA-dependent RNA polymerase (Choi, 2012). A higher mutation rate in RNA viruses may enable them to escape immune responses (Sanjuan and Domingo-Calap, 2016) and may lead to constant changes in the antigenicity, pathogenicity, or other characteristics of the virus. F and HN are the main proteins that are involved in the pathogenicity of NDV, and mutations in the functionally important part of these proteins may lead to increased pathogenicity in poultry (Meng et al., 2018).

Due to extensive vaccination of genotype VII in commercial poultry, the field strains of the genotype VII NDV are facing huge antibody selection pressure. To date, the effect of genotype II and VII vaccination on the mutation rate of NDV strains circulating in the commercial poultry premises is not well known. Here, the present study was planned to address this question. Two strains of genotype VII NDV (ZJ1, ND128) were selected and passaged for 30 consecutive generations under genotype II and VII hyperimmune serum, and the F and HN genes were sequenced after every tenth generation. Then, the sequences of the passaged viruses were compared with the wild-type (primary) viruses, and the mutation rate was determined.

## MATERIALS AND METHODS

### *Virus Propagation*

A wild-type velogenic NDV isolate, ZJ1, was originally isolated from geese in 2000 (Goose/China/ZJ1/2000; GB AF431744.3) and was obtained from Professor Xiufan Liu from Yangzhou University (Yangzhou, China) and stored at  $-80^{\circ}\text{C}$ . The ND128 (CH/SD/2008/128; KJ600785.1) was isolated in our laboratory from an outbreak of ND in chickens, and the biological characteristics of the virus have already been published (Meng et al., 2018). The

original viruses were inoculated into 10-day-old specific pathogen-free (SPF) chicken embryos, which were purchased from Merial (Beijing, China). The chicken embryos that died within 24 h after inoculation were discarded, and the remaining were observed every 8 h. The dead chicken embryos were placed in a refrigerator at  $4^{\circ}\text{C}$  for the constriction of their blood vessels to avoid hemolysis, and the virus was harvested. Allantoic fluid containing the virus was harvested and filtered through  $0.22\ \mu\text{m}$  filter and then inactivated at  $56^{\circ}\text{C}$  for 30 min and stored at  $-20^{\circ}\text{C}$  until use. The mean death time (MDT), hemagglutination assay (HA), intracerebral pathogenicity index (ICPI), and 50% tissue culture infective dose ( $\text{TCID}_{50}$ ) of the viruses was determined as described previously (Meng et al., 2018).

### *Preparation of Anti-NDV Chicken Serum*

The commercial genotype II (Veken Biotechnology Development Company, Harbin, Heilongjiang, China) and genotype VII (Yibang Bioengineering Co., Ltd, Qingdao, Shandong, China) killed NDV vaccines were injected in the subcutaneous tissue of the neck of 3-week-old SPF chickens according to the manufacturer's instructions. Three weeks after the first immunization, booster immunization was performed, and 2 wk later, blood was drawn from the wing vein of chickens and placed at  $37^{\circ}\text{C}$  to separate the serum and test the NDV-specific hemagglutination inhibition (HI) value.

### *Determination of Anti-NDV Chicken Serum Concentration for Use in Cell Culture*

DF-1 cells in good condition were inoculated into a twelve-well plate with a cell density of up to 70%. The test was performed to measure the optimum dilution of the anti-NDV serum for the subsequent serial passages. Two control wells were used: one well contained the virus, and the other only contained antiserum. The remaining 10 wells were infected with 100  $\text{TCID}_{50}$  virus. After 1 h of inoculation, cells were washed 3 times with phosphate buffer solution and Dulbecco's modified Eagle's medium (DMEM) containing anti-NDV serum diluted at ratios of 1:10, 1:20, 1:30, 1:40, and 1:50. Then, the plates were observed under a microscope 72 h after the virus infection, and the minimum dilution factor of the serum with cytopathic changes was recorded. The minimum concentration of the serum that prevents the NDV-specific cytopathic (CPE) was used in the following passages. Similar procedures were performed for both the anti-NDV serum (genotype II and VII) and those with both of the viruses (ZJ1 and ND128).

### *Serial Passages of Genotype VII NDVs under Anti-NDV Serum Conditions*

DF-1 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) at approximately 70 to 80% confluence in 6 well plates.

The 9 wells were infected with 100 TCID<sub>50</sub> ZJ1 and ND128, respectively. After 1 h of inoculation, cells were washed 3 times with phosphate buffer solution, and DMEM containing genotype II anti-NDV (anti-II) serum (3 wells in group 2) and genotype II anti-NDV (anti-VII) serum (3 wells in group 3) was added. The cells in group 1 (3 wells) were inoculated without anti-NDV serum. The cell supernatant was collected 72 h (70–80% cells showed cytopathic effects) after inoculation and centrifuged at 1300 g for 5 min, and the upper layer was collected for use in the next passage. For the next passage, the cellular supernatant containing the virus was diluted 10<sup>3</sup> times and used for the following inoculation. Similarly, viruses were serially passaged to 30 generations, and the viral RNA was extracted and sequenced for the 10th, 20th, and 30th generations.

### **Amplification and Sequencing of the Full-Length F and HN Genes of NDV**

For the amplifications of the F and HN genes of the ZJ1 and ND128 viruses, RNA was isolated from the cellular lysate and supernatant using Trizol (Invitrogen, Waltham, Massachusetts, A.) according to the manufacturer's instructions. Then, 2 µg of the total RNA was used for the reverse transcription using M-MLV reverse transcriptase (200 U), random hexamers, 1X RT buffer, RNase inhibitor (20 U), DTT (0.01 M), and 1 µL of a 10 mM dNTP mixture with a final reaction volume of 25 µL, as described previously (Meng et al., 2018). The temperature conditions of the reverse transcription were 37°C for 2 h and 70°C for 5 min. Primers were designed for the ZJ1 and ND128 on the basis of their sequences and are presented in Table 1. The F and HN genes of the ZJ1 and ND128 viruses were amplified by PCR using cDNA as a template. The amplification conditions were 94°C for 3 min followed by 30 cycles at 94°C for 30 s, 62°C for 30 s, 72°C for 60 s, and a final extension step at 72°C for 5 min. After PCR, the product was electrophoresed on a 1% agarose gel and observed under UV light. After electrophoresis, the target band was cut and melted, and DNA was purified (Magen's Gel DNA Mini Recovery Kit), ligated to pMD19-T vector, and transformed to DH5α competent cells. The recombinant plasmids containing the amplified products of the F and HN genes of the viruses from each PCR fragment were purified using the Tiangen Spin Plasmid Purification Kit (Tiangen, Beijing, China) and sequenced by Sanggong Biotechnology (Sanggong, Shanghai, China).

**Table 1.** Primer sequences used to amplify the F and HN genes of the ZJ1 and ND128 viruses.

Gene type	Forward primer(5'-3')	Reverse primer(5'-3')	Gen bank accession number
ND128 F gene	ACGGGTAGAAGAGTCTGGATCCC	CTTGACAGATTATCGGAATTGACGCAC	KJ600785.1
ND128 HN gene	ACGGGTAGAACGGTCAGAGGAG	ATAAAGTGATAGTGGGCGGGACTC	KJ600785.1
ZJ1 F gene	ACGGGTAGAAGAGTCTGGAT	AGTCTTCTACTTGACAGGTT	AF431744.3
ZJ1 HN gene	ACGGGTAGAACGGTCAGAGGAG	ATAAAGTGATAGTGGGCGGGACTCA	AF431744.3

### **Sequence Analysis of the Parent and Passaged NDVs**

Sequences analyses of the F and HN gene open reading frames (ORF) of the ZJ1 and ND128 viruses were conducted and compared with their original sequences downloaded (No. mentioned in Table S1) from the GenBank using the Lasergene 7 (DNASTAR) software package (DNASTAR, Madison, WI), and the results were recorded.

To assess the selection pressure on the viral quasispecies, nonsynonymous (NS, amino acid changing) and synonymous (S, amino acid preserving) mutation ratios (NS/S) were calculated as described previously (Shpaer and Mullins, 1993; Venugopal et al., 1998).

## **RESULTS**

### **Determination of the Highest Anti-NDV Serum Concentration**

The HI titer of the anti-II serum was 10, whereas for the genotype VII, it was 9. The results of the virus neutralization test indicated that the virus replications were completely inhibited at a 1:20 dilution of anti-II serum, but virus replication was noted at dilution of 1:30. Similarly, for anti-VII serum, virus growth was completely inhibited at 1:10 dilution. Thus, dilutions of 1:30 and 1:20 were used for the anti-II and anti-VII serum respectively, for virus consecutive passage.

### **Biological Characteristics of Parent and Mutated Strains**

The biological properties of parent and mutated strains were assessed using hemagglutination assay HA and in vivo assays. The HA was performed in the V-bottomed titration plates with chicken red blood cells, and the results indicate that the parent and mutant strains has the same HA titers (Table 2). Both the viruses show the almost same MDTmean death time and intracerebral pathogenicity index (ICPI), indicating the well stability of the virus under anti-II or anti-VII NDV serum (Table 2). Compare to parent viruses, the TCID<sub>50</sub> of the passaged viruses were increased (Table 2).

### **Analysis of Sequence Alignment Results**

Both ZJ1 and ND128 were continuously passaged to the 30th generations under the anti-II serum and anti-VII serum as well as without anti-NDV serum. The sequencing results of the F gene and HN gene after every

**Table 2.** Comparison the biological characteristics of the parent and mutant strains.

Strain	HA	MDT(h)	ICPI	TCID <sub>50</sub>
ZJ1	2 <sup>6</sup>	54	1.89	7.67
ZJ1-30th <sup>a</sup>	2 <sup>6</sup>	52	1.88	7.87
ZJ1-mutant 1 <sup>b</sup>	2 <sup>6</sup>	57	1.87	7.87
ZJ1-mutant 2 <sup>c</sup>	2 <sup>6</sup>	55	1.92	8.00
ND128	2 <sup>8</sup>	55	1.90	7.80
ND128-30th <sup>a</sup>	2 <sup>8</sup>	57	1.87	8.25
ND128-mutant 1 <sup>b</sup>	2 <sup>8</sup>	53	1.91	8.00
ND128-mutant 2 <sup>c</sup>	2 <sup>8</sup>	56	1.88	8.16

ZJ1-30th<sup>a</sup> = ZJ1 without anti-serum passage for 30 generation.

ZJ1-mutant 1<sup>b</sup> = ZJ1 under genotype II anti-serum passage for 30 generation.

ZJ1-mutant 2<sup>c</sup> = ZJ1 under genotype VII anti-serum passage for 30 generation.

ND128-30th<sup>a</sup> = ND128 without anti-serum passage for 30 generation.

ND128-mutant 1<sup>b</sup> = ND128 under genotype II anti-serum passage for 30 generation.

ND128-mutant 2<sup>c</sup> = ND128 under genotype VII anti-serum passage for 30 generation.

Abbreviations: HA, hemagglutination assay; **ICPI**, intracerebral pathogenicity index; **MDT**, mean death time; **TCID<sub>50</sub>**, 50% tissue culture infective dose.

**Table 3.** Amino acid mutations in the F and HN proteins of ZJ1 with (anti-II and anti-VII) or without antiserum.

	Well	GEN	F gene			HN gene			
			248	345	116	185	189	419	420
Anti-II serum	1	10			G → R				
		20							
		30							
	2	10			G → R				
		20		I → T		Y → F	S → T		
		30							
	3	10			G → R				
		20							
		30							
Anti-VII serum	1	10			G → R				
		20		I → T	G → R				
		30	D → R			Y → F	S → T		
	2	10		I → T	G → R				
		20		I → T	G → R				
		30	D → R			Y → F	S → T	S → F	Y → N
	3	10		I → T	G → R				
		20		I → T	G → R				
		30	D → R			Y → F	S → T	S → F	Y → N
Without anti-serum	1	10							
		20		I → T	G → R				
		30				Y → F			
	2	10		I → T	G → R				
		20		I → T	G → R				
		30				Y → F		S → F	Y → N
	3	10		I → T	G → R				
		20		I → T	G → R				
		30				Y → F	S → T	S → F	Y → N

10th generations of virus were compared with the wild-type virus and recorded. The nonsynonymous mutations are displayed in [Tables 1](#) and [2](#). It can be seen from [Table 2](#) that a mutation was found in the F protein of the ZJ1 virus at position 248 in the anti-VII serum group. Similarly, a mutation at position 345 was found which was not stable up to 30th generation, and it also occurred in the group without antiserum. The HN protein of ZJ1 mutations were noted at positions 116, 185, 189, 419, and 420. The mutation at position 116 was found in the HN protein in all of the groups after the 10th or 20th generation but could not be stably inherited to the 30th generation. The mutation at position 185 was noted after the 20th generation of the ZJ1 virus in the anti-II serum group and after the 30th generation in the anti-VII group as well as in the group without anti-

NDV serum ([Table 3](#)). The S189T mutations in HN noted in all of the groups were observed after the 20th or 30th generation. Similarly, S419F and Y420N mutations in the HN gene occurred after the 30th generations in the anti-VII serum groups and the group without anti-NDV serum.

As indicated in [Table 4](#), comparison of the wild-type and passaged viruses revealed 2 substitutions in the F protein of the ND128 virus at positions 526 and 527. The amino acid substitution at 526 was stable up to the 30th generation and was noted in all the groups, whereas substitution at H527Y was noted only in the anti-NDV serum groups. In addition to this, there were 4 mutations at positions 9, 265, 319, and 477 in the HN gene of the ND128 virus, G265E, and F477L, which are stably inherited to the 30th generation. G9R and R319V

**Table 4.** Amino acid mutations in the F and HN proteins of ND128 with (anti-II and anti-VII) or without anti-serum.

	Well	GEN	F gene		HN gene			
			526	527	9	265	319	477
Anti-II serum	1	10	M→I	H→Y	G→R	G→E	R→V	
		20	M→I	H→Y		G→E		F→L
		30	M→I	H→Y		G→E		F→L
	2	10	M→I	H→Y	G→R	G→E		
		20	M→I	H→Y		G→E	R→V	F→L
		30	M→I	H→Y		G→E	R→V	F→L
	3	10	M→I	H→Y	G→R	G→E	R→V	F→L
		20	M→I	H→Y		G→E	R→V	F→L
		30	M→I	H→Y		G→E		F→L
Anti-VII serum	1	10	M→I	H→Y	G→R	G→E	R→V	F→L
		20	M→I	H→Y	G→R	G→E	R→V	F→L
		30	M→I	H→Y		G→E	R→V	F→L
	2	10	M→I	H→Y	G→R	G→E		
		20	M→I	H→Y	G→R	G→E	R→V	F→L
		30	M→I	H→Y		G→E		F→L
	3	10	M→I	H→Y	G→R	G→E		
		20	M→I	H→Y	G→R	G→E		F→L
		30	M→I	H→Y		G→E		F→L
Without anti-serum	1	10	M→I					
		20	M→I		G→R			
		30	M→I					F→L
	2	10			G→R	G→E		
		20	M→I		G→R	G→E	R→V	F→L
		30	M→I			G→E		F→L
	3	10			G→R	G→E		
		20	M→I		G→R	G→E		F→L
		30	M→I			G→E		F→L

**Table 5.** Comparison of NS/S ratios of the F gene during continuous passage with or without anti-NDV serum.

	ZJ1 F gene												ND128 F gene											
	Without anti-serum				Anti-II serum				Anti-VII serum				Without anti-serum				Anti-II serum				Anti-VII serum			
	1	2	3	All	1	2	3	All	1	2	3	All	1	2	3	All	1	2	3	All	1	2	3	All
NS <sup>1</sup>	2	2	2	6	3	4	3	10	5	5	5	15	1	1	1	3	2	2	2	6	3	2	2	7
S <sup>2</sup>	1	2	2	5	2	2	2	6	3	2	2	7	2	2	2	6	2	2	3	7	3	3	3	8
NS/S	2	1	1	1.2	1.5	1	1.5	1.6	1.6	2.5	2.5	2.1	0.5	0.5	0.5	0.5	1	1	0.6	0.8	1	0.6	1	0.8

<sup>1</sup>NS represents nonsynonymous mutation.<sup>2</sup>S represents synonymous mutation.**Table 6.** Comparison of NS/S ratios of the HN gene during continuous passage with or without anti-NDV serum.

	ZJ1 HN gene												ND128 HN gene											
	Without anti-serum				Anti-II serum				Anti-VII serum				Without anti-serum				Anti-II serum				Anti-VII serum			
	1	2	3	All	1	2	3	All	1	2	3	All	1	2	3	All	1	2	3	All	1	2	3	All
NS <sup>1</sup>	3	7	8	18	2	6	2	10	4	7	7	18	2	2	1	5	5	7	6	18	5	6	3	14
S <sup>2</sup>	1	3	2	6	2	1	1	4	3	1	3	7	2	2	1	5	2	2	3	6	2	2	2	6
NS/S	3	2.3	4	3	1	6	2	2.5	1.3	7	2.3	2.5	1	1	1	1	2.5	3.5	3	3	2.5	3	1.5	2.3

<sup>1</sup>NS represents nonsynonymous mutation.<sup>2</sup>S represents synonymous mutation.

mutations were noted after the 10th generation; however, in some wells, they could not be stably inherited up to the 30th generation.

### Selection Pressure Result

It is generally considered that the immune selective pressure is present, if the NS/S ratio are higher than 2.5 (Wang and Cui, 2006; Gong and Cui, 2011). As shown

in Table 5, mutations of the F gene of the ZJ1 virus under anti-II serum have an NS/S ratio of 1.6, whereas in the anti-VII serum group, an NS/S ratio of 2.1 was calculated. The NS/S ratio of the anti-II serum and anti-VII serum groups for the ND128 virus were 0.8. It can be concluded that the NS/S ratio of the F gene of the ND128 virus is less than 2.5 with and without anti-NDV serum, and there is no antibody selective pressure.

The NS/S ratio of the HN genes of the ZJ1 and ND128 viruses is given in Table 6. The NS/S ratio was 2.5

for the HN gene of ZJ1 in both the anti-II and anti-VII groups. In addition, the NS/S ratios of the HN gene of ND128 were 3.5 and 2.3 in the anti-II and anti-VII treated groups, respectively. The variability of the HN gene is more dramatic than that of the F gene, and it almost reaches the threshold. However, in the antiserum free group, the ratio of ZJ1 was 3, indicating that the HN gene varies easily, regardless of whether or not there is antibody pressure.

## DISCUSSION

Mutations in the viruses occur during interaction with the host via different evolutionary processes. The mutation rate in the RNA viruses is high due to lack of corrective function in the RNA polymerases during replication of the viruses (Miller et al., 2009; Lauring and Andino, 2010). Multiple factors such as within-host viral and immune kinetics affect the evolution of the viruses (Volkov et al., 2010). The pathogenicity of the virus can change when amino acid mutations occur in the epitope region or due to accumulation of mutations (Gong and Cui, 2011). The HN gene of the ZJ1 in both the antiserum groups had the NS/S ratio at 2.5 which is the critical value of antibody selective pressure. The HN gene of ND128 of the anti-II serum group had a total of 18 mutations, and their NS/S ratio was 3, indicating immune pressure due to antibodies.

The immune pressure of the host induces virus evolution. Point mutation in the viral genome can lead to large changes in pathogenesis (Conenello et al., 2007) and can increase the pathogenicity of NDV (Meng et al., 2018). Our previous study showed that serial passaging of the NDV in the air sacs of chickens led to increased pathogenicity (Meng et al., 2016). The passaging of H9N2 in chicken embryos with maternal antibodies showed the clear selective pressure of antibodies, and the effect of antibodies on the NA gene was less than that of the HA gene (Lou et al., 2009). Similar results of antigenic variation and gene mutations of the H9N2 virus were found by the Su et al. (2020) by serial passaging of the virus in the vaccinated chickens. Studies have shown that the gp85 protein of ALV-J varies significantly and displays clear regularity under the action of immune selective pressure. The 3 core regions located on the hypervariable regions hr1 and hr2 are extremely susceptible to mutation (Venugopal et al., 1998).

In developing countries, vaccine immunization is an important method to prevent ND. For the survival of the virus, it must escape the antibodies because, otherwise, it will be eliminated by them. Under the long-term selection pressure of vaccine immunity, the F and HN genes, notably located on the surface of virus particles, are facing continuous immune selection pressure. Gong and Cui (2011) studied the effect of anti-VII serum on the mutation of NDV HN and F genes in chicken embryo fibroblast cell cultures. The results showed that under the condition of genotype VII antibodies, the HN gene has 5 amino acid positions with stable NS

mutations, and 3 of them are related to the epitope. The F gene has 2 stable mutations in the anti-NDV serum group. Studies have confirmed that antibody immune selective pressure significantly affects HN gene mutation (Gong and Cui, 2011; Gu, et al., 2011). The 3 positive selection sites (266, 347, and 540) on the HN protein are significantly different between vaccine strains and epidemic strains, indicating that vaccine-induced immune selective pressure can cause HN antigenic variation (Gu et al., 2011). However, as they did not set the heterologous genotype anti-NDV serum as the control, specific antibody pressures were not confirmed.

The results of the present study indicate that the effect of homologous and heterogeneous differences in the NDV vaccine strain and field strains did not significantly affect the mutation rate of the wild strain. Our findings also suggest that the extensive use of genotype VII vaccines will not increase the mutation rate of NDVs circulating in commercial poultry.

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## DISCLOSURES

The authors declare no conflict of interest.

## REFERENCES

- Alexander, D. J. 2003. Newcastle disease. In: Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald, D. E. Swayne, (Eds.), *Disease of Poultry*. Iowa State Press, Ames, IA, pp. 64–87.
- Bello, M. B., S. N. A. Mahamud, K. Yusoff, A. Ideris, M. Hair-Bejo, B. P. H. Peeters, and A. R. Omar. 2020. Development of an effective and stable genotype-matched live attenuated newcastle disease virus vaccine based on a novel naturally recombinant malaysian isolate using reverse genetics. *Vaccines*. 8:270.
- Choi, K. H. 2012. Viral polymerases. *Adv Exp Med Biol*. 726:267–304.
- Conenello, G. M., D. Zamarin, L. A. Perrone, T. Tumpey, and P. Palese. 2007. A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. *PLoS Pathog*. 3:1414–1421.
- Dimitrov, K. M., C. Abolnik, C. L. Afonso, E. Albina, J. Bahl, M. Berg, F. X. Briand, I. H. Brown, K. S. Choi, I. Chvala, D. G. Diel, P. A. Durr, H. L. Ferreira, A. Fusaro, P. Gil, G. V. Goujgoulova, C. Grund, J. T. Hicks, T. M. Joannis, M. K. Torchetti, S. Kolosov, B. Lambrecht, N. S. Lewis, H. Liu, H. Liu, S. McCullough, P. J. Miller, I. Monne, C. P. Muller, M. Mumir, D. Reischak, M. Sabra, S. K. Samal, R. Servan de Almeida, I. Shittu, C. J. Snoeck, D. L. Suarez, S. Van Borm, Z. Wang, and F. Y. K. Wong. 2019. Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. *Infect Genet Evol*. 74:103917.

- Dimitrov, K. M., C. L. Afonso, Q. Yu, and P. J. Miller. 2017. Newcastle disease vaccines—a solved problem or a continuous challenge? *Vet Microbiol.* 206:126–136.
- Dimitrov, K. M., A. M. Ramey, X. Qiu, J. Bahl, and C. L. Afonso. 2016. Temporal, geographic, and host distribution of avian paramyxovirus 1 (Newcastle disease virus). *Infect Genet Evol.* 39:22–34.
- Fan, S., T. Wang, X. Gao, Y. Ying, X. Li, Y. Li, Y. Li, J. Ma, H. Sun, D. Chu, Y. Xu, S. Yang, Q. Li, Y. Gao, and X. Xia. 2015. Phylogenetic analysis of Newcastle disease viruses isolated from wild birds in the Poyang Lake region of China. *J Vet Med Sci.* 77:1143–1149.
- Gong, Y., and Z. Cui. 2011. Epitope variation in the Newcastle disease virus HN gene under antibody immune selective pressure in cell culture. *Sci China Life Sci.* 54:474–479.
- Gu, M., W. Liu, L. Xu, Y. Cao, C. Yao, S. Hu, and X. Liu. 2011. Positive selection in the hemagglutinin-neuraminidase gene of Newcastle disease virus and its effect on vaccine efficacy. *Virology.* 8:150.
- Hu, S., H. Ma, Y. Wu, W. Liu, X. Wang, Y. Liu, and X. Liu. 2009. A vaccine candidate of attenuated genotype VII Newcastle disease virus generated by reverse genetics. *Vaccine.* 27:904–910.
- Lauring, A. S., and R. Andino. 2010. Quasispecies theory and the behavior of RNA viruses. *PLOS Pathogens.* 6:e1001005.
- Liu, J., J. Zhu, H. Xu, J. Li, Z. Hu, S. Hu, X. W. And, and X. Liu. 2017. Effects of the HN antigenic difference between the vaccine strain and the challenge strain of Newcastle disease virus on virus shedding and transmission. *Viruses.* 9:225.
- Lou, B., X. Zhu, B. Sun, and Z. Cui. 2009. [Mutations of the hemagglutinin gene of H9N2 subtype avian influenza viruses under selective pressure of antibody]. *Wei Sheng Wu Xue Bao.* 49:955–959.
- Meng, C., X. Qiu, S. Yu, C. Li, Y. Sun, Z. Chen, K. Liu, X. Zhang, L. Tan, C. Song, G. Liu, and C. Ding. 2016. Evolution of Newcastle disease virus quasispecies diversity and enhanced virulence after passage through chicken air sacs. *J Virol.* 90:2052–2063.
- Meng, C., Z. U. Rehman, K. Liu, X. Qiu, L. Tan, Y. Sun, Y. Liao, C. Song, S. Yu, Z. Ding, V. Nair, M. Munir, and C. Ding. 2018. Potential of genotype VII Newcastle disease viruses to cause differential infections in chickens and ducks. *Transbound Emerg Dis.* 65:1851–1862.
- Miller, P. J., C. L. Afonso, J. El Attrache, K. M. Dorsey, S. C. Courtney, Z. Guo, and D. R. Kapczynski. 2013. Effects of Newcastle disease virus vaccine antibodies on the shedding and transmission of challenge viruses. *Dev Comp Immunol.* 41:505–513.
- Miller, P. J., E. L. Decanini, and C. L. Afonso. 2010. Newcastle disease: evolution of genotypes and the related diagnostic challenges. *Infect Genet Evol.* 10:26–35.
- Miller, P. J., R. Haddas, L. Simanov, A. Lublin, S. F. Rehmani, A. Wajid, T. Bibi, T. A. Khan, T. Yaqub, S. Setiyaningsih, and C. L. Afonso. 2015. Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features. *Infect Genet Evol.* 29:216–229.
- Miller, P. J., L. M. Kim, H. S. Ip, and C. L. Afonso. 2009. Evolutionary dynamics of Newcastle disease virus. *Virology.* 391:64–72.
- Peck, K. M., and A. S. Lauring. 2018. Complexities of viral mutation rates. *J Virol.* 92:e01031-01017.
- Rehman, Z. U., L. Che, S. Ren, Y. Liao, X. Qiu, S. Yu, Y. Sun, L. Tan, C. Song, W. Liu, Z. Ding, M. Munir, V. Nair, C. Meng, and C. Ding. 2018. Supplementation of vitamin E protects chickens from Newcastle disease virus-mediated exacerbation of intestinal oxidative stress and tissue damage. *Cell Physiol Biochem.* 47:1655–1666.
- Sanjuan, R., and P. Domingo-Calap. 2016. Mechanisms of viral mutation. *Cell Mol Life Sci.* 73:4433–4448.
- Shpaer, E. G., and J. I. Mullins. 1993. Rates of amino acid change in the envelope protein correlate with pathogenicity of primate lentiviruses. *J Mol Evol.* 37:57–65.
- Su, H., Y. Zhao, L. Zheng, S. Wang, H. Shi, and X. Liu. 2020. Effect of the selection pressure of vaccine antibodies on evolution of H9N2 avian influenza virus in chickens. *AMB Exp.* 10:98.
- Venugopal, K., L. M. Smith, K. Howes, and L. N. Payne. 1998. Antigenic variants of J subgroup avian leukosis virus: sequence analysis reveals multiple changes in the env gene. *J Gen Virol.* 79(Pt 4):757–766.
- Volkov, I., K. M. Pepin, J. O. Lloyd-Smith, J. R. Banavar, and B. T. Grenfell. 2010. Synthesizing within-host and population-level selective pressures on viral populations: the impact of adaptive immunity on viral immune escape. *J R So Interface.* 7:1311–1318.
- Wang, Z., and Z. Cui. 2006. Evolution of gp85 gene of subgroup J avian leukosis virus under the selective pressure of antibodies. *Sci China C Life Sci.* 49:227–234.