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Molecular characterization of a virulent strain of Newcastle disease virus isolated from a diseased chicken in Kyrgyzstan in 2016

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Abstract

Newcastle disease (ND) is a contagious viral disease affecting birds throughout the world and causing economic losses in the poultry industry. At present, little is known about the genetic causes of the ND outbreak that occurred in commercial poultry farms in Kyrgyzstan in 2016. In the current study, we isolated and confirmed a strain of ND virus (NDV) isolated from one out of two samples obtained from an unvaccinated flock. Based on phylogenetic analyses, this NDV strain was clustered in the class II subgenotype VIId and was closely related to the Chinese NDV isolate. Phylogenetic analyses also revealed that the isolated NDV strain has an origin different from the four NDV strains previously identified in Kyrgyzstan. According to a multibasic amino acid (aa) sequence at the F0 proteolytic cleavage site (^{112}R-R-Q-K-R-F^{117}), the NDV isolate was determined as a virulent strain. Several mutations in the neutralizing epitopes (notably, E^{347}→K) and the global head were observed in the hemagglutinin-neuraminidase protein of the new isolate. The present study represents the first molecular characterization of the coding region of NDV genome obtained from Kyrgyzstan in 2016. This essential information may be useful for ND diagnostics and control.

Key Words: Kyrgyzstan, Newcastle disease, Genotype, Genome characterization

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Introduction

Newcastle disease virus (NDV) causes high levels of morbidity and mortality in poultry, which results in enormous economic losses[2]. Since ND was first described in Indonesia in 1926 and in the Newcastle upon Tyne in England in 1927[12], four worldwide ND panzootics have occurred[22].

NDV or avian orthoavulavirus 1 (APMV-1) belongs to the genus *Orthoavulavirus* of the family *Paramyxoviridae* under order *Mononegavirales*[3]. NDV possesses an enveloped, single-stranded, and negative-sense RNA virus genome of approximately 15,200 nucleotides. The viral genome is composed of six open reading frames encoding six major proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large RNA-directed RNA polymerase protein (L)[2]. In addition, two nonstructural proteins including V and W are expressed by RNA editing of the P mRNA and present only in virus-infected cells[21,31].

NDVs are divided into two distinct genetic clades, class I and class II, based on their complete sequences or full F protein sequences[16]. Class I and class II contain at least nine (I–IX) and eighteen (I–XVIII) genotypes, respectively[9]. All the NDV strains in class I and most of the genotype I and II viruses within class II are mainly avirulent strains; while virulent NDV strains belong to class II genotypes III to IX and XI to XVI[7]. Regarding pathogenicity indices for chicken, NDV can be divided into three major pathotypes (i.e., velogenic, mesogenic, and lentogenic)[10]. The velogens are highly pathogenic and are further divided into viscerotropic velogenic NDV which causes intestinal infection with high mortality, and neutropic velogenic NDV which produces neurological and respiratory symptoms with high mortality. Mesogens are less pathogenic, with acute neurological and respiratory signs. Lentogens cause mild respiratory tract infection and are commonly used as live vaccines[24]. The pathotype of NDV is also identified based on the difference in cleavage site sequence of the F protein, which is a major precursor glycoprotein (F0) on the NDV particle. The F0 protein is cleaved by host cell proteases into two disulfide-linked subunits (F1 and F2). All the velogenic and mesogenic NDV strains have an amino acid (aa) sequence of ^{112}R/K-R-Q-R/K-R-F^{117} in the F cleavage site, whereas the lentogenic viruses carry ^{112}G/E-K/R-Q-G/E-R-L^{117} motif[1,27].

In the past few decades, the predominant virus strains responsible for NDV outbreak worldwide came from genotypes V, VI, and VII of class II[1,17]. Notably, genotype VII of class II NDV strains have become particularly important strains associated with the most recent outbreaks in Europe, Africa, the Middle East, South America, and Asia[39]. To date, genotype VII has been further divided into nine (VII a–k) subgenotypes[23].

From the early 1940s, the implementation of NDV vaccination has been employed to control NDV outbreaks in the poultry industry worldwide[9]. While NDV vaccines have also been applied in Kyrgyzstan, sporadic outbreaks of the disease have been reported. In a previous study, NDV isolates obtained in 2005 in Kyrgyzstan were found to belong to subgenotype VIId based on phylogenetic analysis of the partial F gene[4]. However, information on the molecular characteristics of Kyrgyz NDV isolates is limited. The aim of the current study was to characterize an NDV isolate obtained in Kyrgyzstan in 2016. This characterization will not only be helpful for analyzing the genetic nature of NDV in Kyrgyzstan, but also that of NDV in other countries. In addition, the genetic profile of the isolate used in this study will advance the currently available genomic data on APMV-1.

Materials and methods

Ethics statement: This article does not contain any studies with human participants performed by any of the authors. Collection of chicken tissue samples was conducted by the Kyrgyz

Table 1. Primers and probe used in this study

Name	Primer sequence (5′–3′)	Fragment size (bp)	Position	Reference
APMV F-1F	TATACACCTCRTCBCARACRGG	403	4698–4720[a]	this study
APMV F-1R	ACRAAYTGCTGCATCTTCCC		5080–5100[a]	
APMV M-1F	AGTGATGTGCTCGGACCTTC	296	3935–3957[b]	this study
APMV M-1R	CCTGAGGAGAGGCATTTGCTA		4210–4230[b]	
M+4100[c]	AGTGATGTGCTCGGACCTTC	101	4100–4120	[39]
M-4200[c]	CCTGAGGAGAGGCATTTGCTA		4180–4200	
APMV-Probe[c]	FAM-TTCTCTAGCAGTGGGACAGCCTGC-TAMRA		4169–4192	

[a] Numbers correspond to positions within the LaSota strain genome (JF950510).
[b] Numbers correspond to positions within the Chiken/China/Liaoning/02/2005 genome (KC542893).
[c] Primers and probe sequences were used as previous described and validated[39].

Research Institute of Veterinary Medicine under institutional approval, and permission from the chicken owners.

Sample collection and virus isolation: Lung tissue samples were obtained post-death from two diseased chickens of 4 months of age in an unvaccinated broiler flock in the Kyrgyz Republic on September 2016. The samples were collected by the Kyrgyz Research Institute of Veterinary Medicine and transferred to Obihiro University of Agriculture and Veterinary Medicine. The lungs were homogenized in Dulbecco's Modified Eagle's medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with kanamycin (1 mg/ml), gentamycin (100 µg/ml), and amphotericin B (5 µg/ml) to prepare 20% homogenates. A total of 200 µl supernatant of lung homogenate was inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs (3 eggs/sample). After incubating at 37°C for up to 5 days with daily candling and inspections, allantoic fluids were examined for hemagglutination (HA) activity using 0.5% chicken red blood cells according to the Manual for the laboratory diagnosis and virological surveillance of influenza[37]. Allantoic fluids positive with HA activity were harvested and stocked for further studies.

Total RNA extraction, reverse transcription polymerase chain reaction (RT-PCR), and quantitative real-time RT-PCR: RNA was extracted from the HA positive allantoic fluids using ISOGEN II (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. Preparation of cDNA was performed using random primers (Invitrogen, Carlsbad, CA, USA) and M-MLV Reverse Transcriptase (Invitrogen) under the following condition: 25°C for 10 min, 37°C for 60 min, and 65°C for 10 min.

An in-house quantitative real-time RT-PCR was performed to examine the presence of NDV M gene in a Roche LightCycler Nano Instrument (Roche Life Sciences, Mannheim, Germany). A control plasmid for the NDV M gene quantification was prepared. The partial M gene (296 bp) was amplified using a pair of primers, APMV M-1F and APMV M-1R (Table 1), then inserted into a pGEM-T Easy vector (Promega Corporation, Madison, USA). The constructed plasmid recovered from the transformed *E. coli* was serially diluted and applied to real-time PCR to obtain a standard curve. A mixture of 10 µl of EagleTaq Master Mix with ROX (Roche Life Sciences), 1 µl of a probe (5 µM), 1 µl each of forward and reverse primers (10 µM) previously described (Table 1)[38], 6 µl of distilled water and 2 µl of cDNA template was placed and amplified with the following thermal condition: 45 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 60 s. The obtained standard curve demonstrated a high accuracy ($R^2 > 0.99$) and suggested that the assay can detect 500 copies (Cq value: 35.8). Tested samples showing Cq

Table 2. Nucleotide sequence identities (%) of individual genes of Chicken/Kyrgyzstan/2016/1-16 isolate with representative strains of each genotype

Gene	Strain	Nucleotide identity (%) with Chicken/Kyrgyzstan/2016/1-16						
		Ulster (AY562991)[a]	LaSota (AY845400)	Mukteswar (EF201805)	Hert/33 (AY741404)	Largo/71 (AY562990)	IT-227/82 (AJ880277)	NA-1 (DQ659677)
	Genotype	I	II	III	IV	V	VI	VII
NP		87.21	84.62	86.19	89.52	89.93	90.54	95.78
P		83.41	81.64	84.00	86.53	87.87	86.44	94.44
M		85.84	84.29	86.84	88.21	89.13	91.05	95.89
F		85.31	83.15	85.67	87.90	87.72	89.65	95.84
HN		81.51	76.49	82.19	84.80	87.40	88.20	94.55
L		87.10	85.30	87.61	88.70	90.20	90.99	95.72

The highest identity for each gene is underlined.
[a] GenBank accession number.

values less than or equal to 35.8 were regarded as positive.

A conventional RT-PCR was performed to confirm the presence of the NDV F gene using a thermocycler (MyCycler, Bio-Rad, Foster City, CA, USA) with a 20 µl reaction mixture: 2 µl cDNA, 0.1 µl TAKARA Ex-Taq polymerase (Takara Bio Inc., Kyoto, Japan), 1 µl each of forward and reverse primers (Table 1), 1.6 µl 2.5 mM mixture of dNTPs, 2 µl 10X buffer for PCR, and 12.3 µl water. Reactions were performed according to the following thermal condition: 94°C for 5 min, 40 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 40 s, and then 1 cycle of 72°C for 10 min.

Nucleotide sequencing and phylogenetic analyses: The nucleotide sequence of the full-length viral genome was determined by Next Generation Sequencing (NGS) method using a MiSeq bench-top sequencer (Illumina, San Diego, CA, USA) as we previously reported[33]. Next, a creation of FASTQ formatted sequence data was performed using MiSeq Reporter program (Illumina), and the contiguous sequences or contig assemblies from the short sequences were read by CLC Genomic Workbench version 6.5.1 (CLC bio, Aarhus, Denmark) for *de novo* assembly. To obtain the full-length NDV sequence, the consensus sequence of assembled contigs was determined using BLAST.

The nucleotide and deduced aa sequences were aligned and analyzed using CLUSTAL W, Ver. 1.4 in the BioEdit Package, Ver. 7.2 software[14]. The homology of nucleotide and aa sequences was determined using GENETYX Ver. 10 software (GENETYX Corp., Tokyo, Japan) and compared with other sequences available in GenBank identified by BLAST homology searches (http://www.ncbi.nlm.nih.gov/). A maximum likelihood method with 1,000 bootstrap replicates using the Kimura 2-parameter model and General Time Reversible model was used to establish phylogenetic trees in MEGA6 software[32].

Results

Isolation of Kyrgyz NDV

Allantoic fluid inoculated with one of the two samples from diseased chickens showed HA activity at a titer of 1:128. Results from real-time RT-PCR and conventional RT-PCR confirmed the presence of the NDV M and F genes in this sample. The aa sequence of F-protein cleavage site was determined as ^{112}R-R-Q-K-R-F^{117}. The NDV isolate obtained was designated as Chicken/Kyrgyzstan/2016/1-16 (Kyr/1-16).

Full-length sequence of Kyr/1-16 was obtained from NGS data except for a 94 bp nucleotide sequence between positions 1647 and 1740. The nucleotide sequence data of Kyr/1-16 was deposited into GenBank under accession number MK423875.

The NDV isolate Kyr/1-16 was genetically

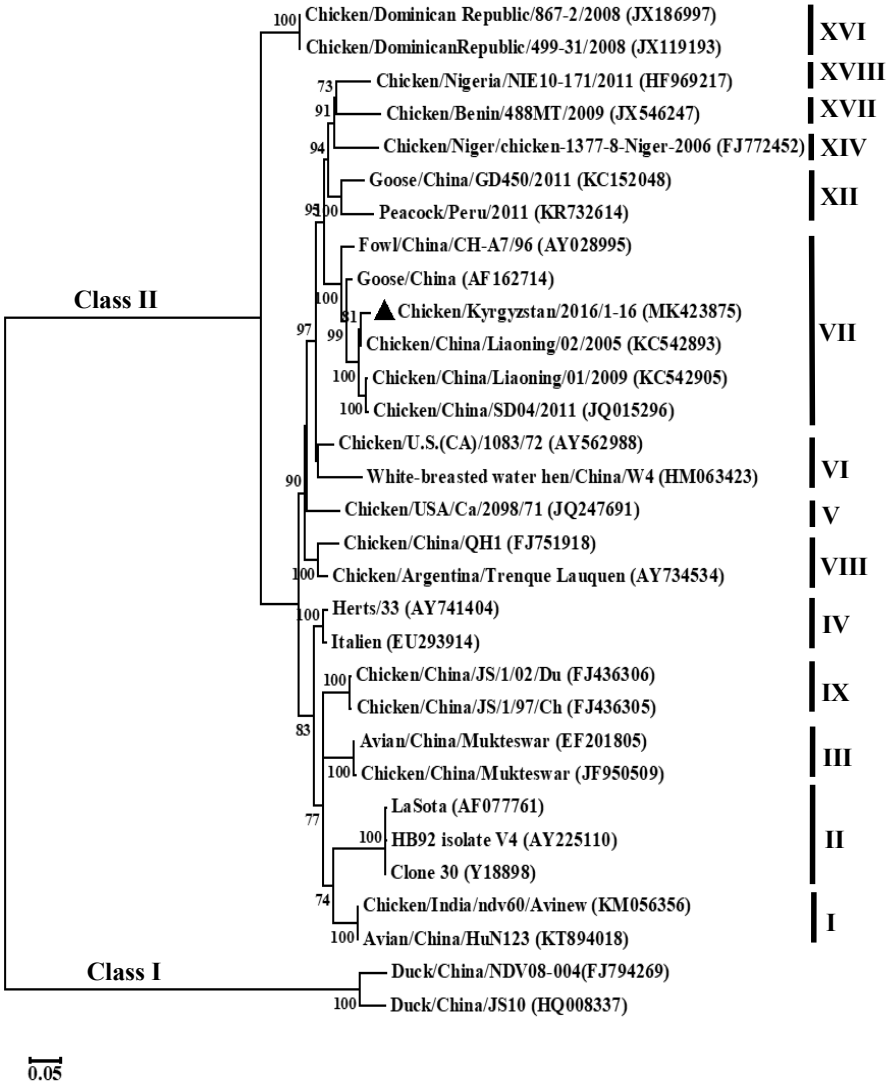

Fig. 1. Phylogenetic tree of the full-length sequence of F gene from Kyr/1-16 compared with the sequences from other NDV strains downloaded from GenBank. The tree was constructed using the maximum likelihood method (1,000 bootstrap replicates) in MEGA6 software. Bootstrap values are shown at the nodes. The sequence determined in this study is marked with a triangle.

compared with selected class II reference strains representing genotypes I–VII. We found that Kyr/1-16 shares the highest nucleotide identity with the NA-1 strain of genotype VII for the NP (95.78%), P (94.44%), M (95.89%), F (95.84%), HN (94.55%), and L (95.72%) genes (Table 2), suggesting that the Kyr/1-16 strain is closely related to genotype VII.

Phylogenetic analysis and genotyping of Kyrgyz *NDV*

Phylogenetic analyses of the full-length F and HN genes was performed for Kyr/1-16 and the NDV strains representing all the genotypes within NDV class I and class II. The results on the F gene clearly indicated that Kyr/1-16 is clustered into genotype VII in class II. Furthermore, Kyr/1-16 was found to be closely related to the Chinese NDV isolate, Chicken/China/Liaoning/02/2005 (KC542893), which belongs to genotype VII (Fig. 1). Analyses of the HN gene showed similar results (data not shown). It was also found that Kyr/1-16 retains K^{101} and V^{121} in the F protein (See accession number MK423875 in GenBank), which is a characteristic of genotype VII[19]. Based on the

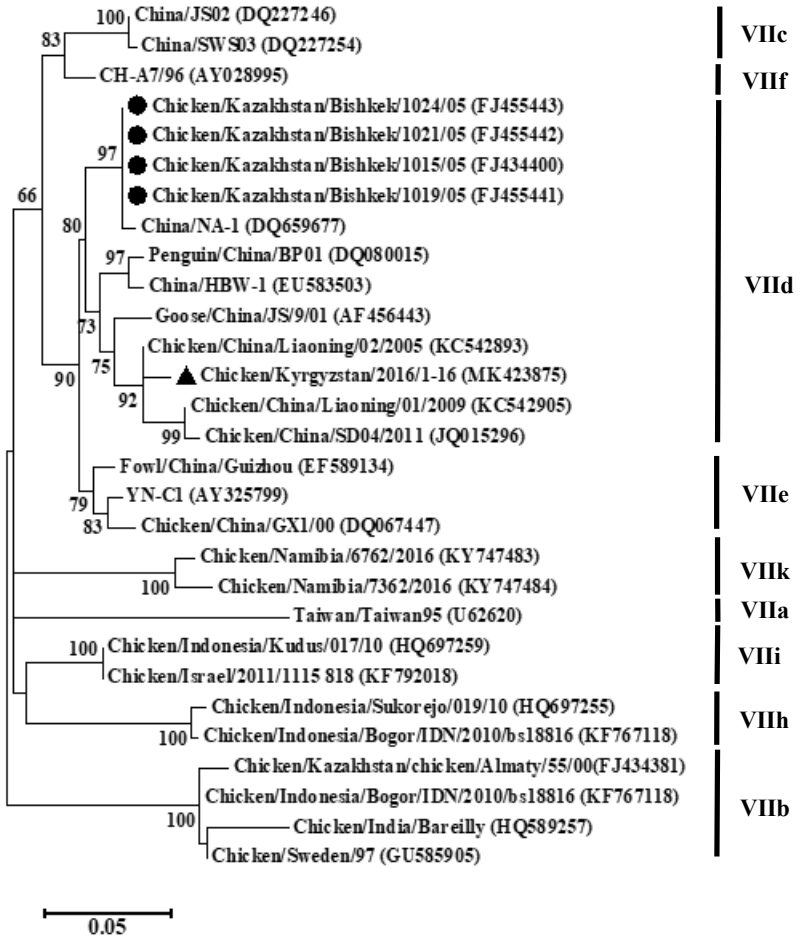

Fig. 2. Phylogenetic tree of the F variable region (47–421 nucleotide position) sequence from Kyr/1-16 compared with the sequences from other NDV strains downloaded from GenBank. The tree was constructed using the maximum likelihood method (1,000 bootstrap replicates) in MEGA6 software. Bootstrap values are shown at the nodes. The sequence determined in this study is marked with a triangle. The four NDV strains previously identified in Kyrgyzstan are marked with circle.

nucleotide sequence of the variable region of the F gene (47–421 nucleotide position)[29], Kyr/1-16 was assigned to subgenotype VIId. The four NDV strains isolated in Kyrgyzstan in 2005 (FJ455441–FJ455443, and FJ434400) were also classified in subgenotype VIId, however, they did not form a single cluster with Kyr/1-16 (Fig. 2).

Amino acid substitutions in Kyrgyz NDV

When comparing with previous studies[36,40], there were no sequence changes in Kyr/1-16 at the six potential *N*-glycosylation sites and twelve cysteine residues of the F protein. In addition, the seven neutralizing epitopes, that reportedly play critical roles and are located at positions 72, 74, 75, 78, 79, 157 to 171, and 343 in the F protein[36], were conserved in Kyr/1-16; in contrast, the previous isolates from Kyrgyzstan showed a K to R aa substitution at position 78.

Length of the HN protein in Kyr/1-16 was 571 aa, which is a feature of virulent NDV strains[20]. There are six N-glycosylation sites, including four functional sites (^{119}N-N-S^{121}, ^{341}N-D-T^{343}, ^{433}N-K-T^{435}, and ^{481}N-H-T^{483}), and two non-functional sites (^{508}N-I-S^{510}, and ^{538}N-K-T^{540}) known in the HN protein. Three aa substitutions, D^{342}→N, K^{434}→E, and T^{540}→A, were found in the N-glycosylation site of the HN protein of the Kyr/1-16 isolate. However, there were no sequence changes in Kyr/1-16 at the region

Table 3. Amino acids involved in neutralizing epitopes of representative HN proteins from different genotypes

Virus	Genotype	Neutralizing epitopes of the HN proteins													
		193–201									263	287	321	332	333
Consensus[a]		L	S	G	C	R	D	H	S	H	N	D	K	G	R
Ulster (AY562991)	I	.[b]	.	.	.	.	.	.	.	.	.	.	.	.	K
LaSota (AY845400)	II	.	.	.	.	.	.	.	.	.	.	.	.	.	K
Mukteswar (EF201805)	III	.	.	.	.	.	.	.	.	.	K	.	.	.	.
Hert/33 (AY741404)	IV	.	.	.	.	.	.	.	.	.	S	.	.	.	.
Largo/71 (AY562990)	V	.	.	.	.	.	.	.	.	.	K	.	R	.	.
IT-227/82 (AJ880277)	VI	.	.	.	.	.	.	.	.	.	K	.	.	.	K
NA-1 (DQ659677)	VII	.	.	.	.	.	.	.	.	.	K	.	.	.	K
Kyr/1-16 (MK423875)	VII	.	.	.	.	.	.	.	.	.	K	.	.	.	K

[a] The consensus amino acid sequence was derived from 180 velogenic, mesogenic and lentogenic NDV strains from GenBank.
[b] Same as above sequence.

Table 3 (continued). Amino acids involved in neutralizing epitopes of representative HN protein of different genotypes

Virus	Genotype	Neutralizing epitopes of the HN proteins																				
		346–353								356	494	513–521										569
Consensus[a]		D	E	Q	D	Y	Q	I	R	K	D	R	I	T	R	V	S	S	S	S	D/G
Ulster (AY562991)	I	.[b]	.	.								.	.	.	.	.	.	.	.	.	.	
LaSota (AY845400)	II	.	.	.	.	.	.	.	.	R	G	.	.	.	.	.	.	.	.	.	.	
Mukteswar (EF201805)	III	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Hert/33 (AY741404)	IV	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	
Largo/71 (AY562990)	V	.	.	.	.	.	.	V	.	.	N	.	.	A	.	.	.	.	.	.	N	
IT-227/82 (AJ880277)	VI	.	G	.	E	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	
NA-1 (DQ659677)	VII	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	
Kyr/1-16 (MK423875)	VII	.	K	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	

[a] The consensus amino acid sequence was derived from 180 velogenic, mesogenic and lentogenic NDV strains from GenBank.
[b] Same as above sequence.

responsible for HA and thirteen cysteine residues. Furthermore, no aa substitutions were found in the receptor binding site and the neuraminidase activity site of HN protein in Kyr/1-16[6,8]. Residues 193–201 (LSGCRDHSH) and ten others (R^{263}, D^{287}, K^{321}, $^{332}GR^{333}$, $^{346}DEQDYQIR^{353}$, K^{356}, N^{481}, D^{494}, $^{513}RITRVSSSS^{521}$, and G/D^{569}) have been previously described as neutralizing epitopes of the HN proteins[20,25]. Among these residues, four substitutions R^{263}→K, R^{333}→K, E^{347}→K, and I^{514}→V were found in Kyr/1-16 (Table 3). Moreover, analysis of HN sequence showed aa substitutions at residues L^{133}→I and A^{140}→L located on the globular head of the protein.

Discussion

NDV remains one of the most devastating diseases in the poultry industry in many Asian countries. Although hundreds of NDV gene sequences are currently available, limited data is accessible regarding the genomic characteristics of NDVs isolated from the poultry industry in Kyrgyzstan. In this study, the Kyr/1-16 strain was sequenced and found to be clustered into genotype VII in class II NDV. The term of multiple basic aa (at least three arginine (R) or lysine (K)) within the cleavage site of the F protein is determined as virulent strains[26]. Furthermore, the cleavage site of the F protein has the motif ^{112}R-R-Q-K-R-F^{117}, which was previously reported as a typical motif for virulent NDV strains[30]. Therefore, the Kyr/1-16 strain was designated as a virulent strain.

Historically, based on phylogenetic analyses, there are four main ND panzootics that have occurred in poultry since 1926. It is worth noting that genotype VII was responsible for the fourth panzootic outbreaks which spread in Asia, Europe, the Far East and South Africa since the

1980 and is still a currently circulating virulent strain worldwide[19,28]. Phylogenetic analyses of full-length F and HN genes revealed that the Kry/1-16 strain was classified in genotype VII. For identifying the sub-genotype, the F gene nucleotide sequence, with particular emphasis given on the variable region (47–421 nucleotide position), is considered as standard criterion for genotyping[29]. Our phylogenetic analysis of the F variable region indicated that Kyr/1-16 belongs to the sub-genotype VIId, which is the predominant genotype causing NDV outbreaks across many Asian countries, including Kyrgyzstan[4,18,25]. Complementary to this, another report found that K^{101} and V^{121} are typical features of the F protein of sub-genotype VIId. Our study confirmed that the current NDV isolate belongs to subgenotype VIId. Moreover, the results of phylogenetic analysis suggested that Kyr/1-16 is closely related to the Chinese NDV isolate from 2005 (KC542893). In addition, the previous NDV isolates from Kyrgyzstan also clustered with the Chinese NDV isolates in subgenotype VIId, suggesting that the current and previous isolates could have been introduced from China to Kyrgyzstan. Geographically, Kyrgyzstan shares a long border with China. In addition, informal trade of live poultry has been reported between China and its neighboring countries[11,41]. Possible factors related to NDV introduction into Kyrgyzstan may involve the movement of contaminated materials and illegal infected poultry-related products originating from other countries across international borders[4]. Another factor could be that wild birds play a role in the circulation of NDV[35]. To investigate this issue, thorough surveillance of wild birds for NDV is a fundamental requirement for establishing the epidemiology of the virus.

In general, the length of HN protein in class II NDVs may be related to genotype. The HN protein consists of six types based on length (571 aa, 577 aa, 578 aa, 580 aa, 585 aa, and 616 aa)[34]. Among those, the most common lengths are 571 aa (genotypes III–IX and XI), 577 aa (genotype II), and 616 aa (genotype I). The HN length of Kyr/1-16 (571 aa) fits into one of the common protein types. Comparison of the aa sequences of the functional domains and neutralizing epitopes of the HN proteins between Kyr/1-16 and other velogenic, mesogenic, and lentogenic NDVs identified several substitutions (Table 3 and data not shown). It was previously reported that the aa substitution at position L^{133} replaced by I or position A^{140} replaced by L in the global head of HN protein could cause enhanced or decreased virus attachment activity[13]. Additionally, another research group found that a E^{347} to K/G point substitution caused 2–3 fold decreases in viral neutralizing titers of field NDV strains[35]. Other studies also showed that aa substitution in the neutralizing epitopes generated neutralizing escape variants[5,15,29]. In the present study, these substitutions were observed in the HN protein of the Kyr/1-16 isolate. It can be postulated that these modifications may affect the formation of antigenic epitopes of the NDV isolate; however, these speculations need to be clarified. To address this question, future studies will include the production of a monoclonal antibody of this field NDV strain.

In summary, the gene and protein level analyses of the Kyr/1-16 isolate revealed that subgenotype VIId viruses of class II continue to circulate in the poultry industry of Kyrgyzstan. This study also provides the first report on the molecular characterization of coding regions of the genome of an NDV strain isolated from Kyrgyzstan. Furthermore, the present NDV strain was isolated from an unvaccinated flock; thus, our study has provided essential information regarding the genetic nature of circulating NDV, which may be used for disease diagnosis and control.

Conflict of interest

The authors declare no conflict of interest.

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