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Partial nucleotide sequencing and phylogenetic analyses of Newcastle disease virus and infectious bursal disease virus isolated in South Korea

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Abstract

The present study was conducted to investigate the genetic profile of two prevalent avian pathogens in Korea namely, Newcastle disease virus (NDV) and infectious bursal disease virus (IBDV). Two farms located in Yeongi-gun, Chungnam were selected for this study. The two viruses were isolated from various organs (spleen, trachea, bursa of Fabricius) of deceased chickens that showed clinical symptoms of Newcastle Disease or Infectious bursal disease like swelling and congestion of the F bursa, facial edema, lacrimation, greenish yellow diarrhea as well as pathological signs like airsacculitis, haemorrhages in the intestines and so on. For analysis of NDV and IBDV, a 466 and 435 base pair fragments corresponding to the HN and VP2 regions which are highly conserved among related strains of NDV and IBDV, respectively, were amplified by RT-PCR and analyzed by sequencing. Comparison of the VP2 region showed a 99.3% homology between the Korean IBDV isolate and the BJ836-attenuated vaccine strain. In contrast, the HN region of the Korean NDV isolate only has an 83 to 84% homology with the vaccine strains LaSota, B1 and VGGA. Our findings reveal that the prevalent NDV strain in Korea is genetically different from the vaccine strains and may explain the recent outbreaks of Newcastle disease in the region.

Key words : NDV, IBDV, RT-PCR, Nucleotide sequencing, Homology

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Introduction

Newcastle disease is one of the leading causes of avian disease in commercial poultry in Korea. The causative agent, *Newcastle disease virus,* is classified under the family *Paramyxoviridae*, genus *Avulavirus*[1,2]. This enveloped virus has a negative-sense single-stranded RNA genome of approximately 15kb[3]. The genome of NDV codes for six major proteins, an RNA-directed RNA polymerase (L) associated with the nucleocapsid, haemagglutinin-neuraminidase (HN), fusion (F), matrix (M), phosphoprotein (P) and nucleocapsid (NP)[1,3]. HN and F play an important function in viral infectivity and pathogenicity[4]. Consequently, these proteins are also involved in inducing protective immunity.

Among the three membrane associated glycoproteins (HN, F, M), HN has a significant role in viral infectivity[5]. This multi-functional, type II membrane glycoprotein forms a tetrameric spike structure, consisting of a long, membrane-proximal stalk region that supports a terminal globular domain. HN has three main functions. First, it recognizes sialic acid-containing receptors on cell surfaces. Second, it promotes the fusion activity of F which facilitates penetration of the cell surface by converting it to its active form, and third, it acts as a neuraminidase, removing sialic acids from progeny virions that prevents viral self-agglutination[6].

Different strains of NDV cause various types of diseases. At present, they are broadly classified into 4 pathotypes. *Avirulent* strains are those that do not show any symptoms. *Lentogenic* strains induce only mild respiratory infections. *Mesogenic* strains cause acute respiratory symptoms with mild nervous disorders. Lastly the *velogenic* strains, considered the most virulent and highly fatal, are grouped into 2: *viscerotropic velogenic* strains induce hemorrhagic intestinal lesions, while the *neurotropic velogenic* strains cause acute respiratory symptoms accompanied by severe nervous disorders[1,3,5,7].

Different vaccines have been developed to prevent the spread of Newcastle disease. Currently, two types of vaccines are commercially distributed. These are the live, attenuated vaccines and killed vaccines[8,9]. For detection of NDV, hemagglutination assay (HA) and reverse transcription-polymerase chain reaction (RT-PCR) are being used to screen for the presence of the virus in biological samples such as mucus secretions and fecal specimens while the hemagglutination-inhibition (HI) test is being conducted to detect presence of NDV antibodies[2,3,10,11].

Another important avian disease in Korea is the *infectious bursal disease* (IBD). IBD is a common disease of chickens caused by a double-stranded RNA virus belonging to the *Birnaviridae* family[12]. IBDV replicates specifically in developing B-lymphoid cells, resulting in destruction of the precursors of antibody-producing B cells located at the bursa of Fabricius and immuno-suppression[13,14].

The genome of IBDV is composed of two segments of double-stranded RNA[15].

The larger segment A contains two partially overlapping open reading frames (ORFs). The first, smaller ORF encodes a nonstructural protein VP5; whereas the second ORF encodes a precursor polyprotein, which is subsequently cleaved into VP2, VP4, and VP3. VP2 and VP3 are the major capsid proteins of IBDV, constituting 51% and 40% of the viral proteins, respectively[16]. The smaller segment B encodes VP1, is a 90 kDa RNA polymerase[17]. The VP2 protein has been identified as the major host-protective immunogen of IBDV and contains major epitopes responsible for eliciting neutralizing antibodies[18~21]. Passive antibodies to VP2 were found to protect chickens[16]. Many attempts have been made to express the structural proteins of IBDV as subunit vaccines for the control of this disease[22]. It has been demonstrated that the recombinant VP2 protein expressed in different expression systems provided significant protection against the disease. However, those efforts have not been translated for practical use due to limitation of the delivery systems[19,20].

Vaccination is the principal method used for the control of infectious bursal disease in chickens. However, several problems have been encountered. One, even though IBDV strains of different antigenic types have been incorporated into vaccines, the immunity induced by these strains do not confer sufficient protective immunity. Two, presence of maternal antibodies in young chickens decreases the efficacy of live IBDV vaccines. Three, in many cases, live IBDV vaccines also cause various degree of bursal atrophy that may contribute to the emergence of antigenic variants[14,22]. The apparent inability to control IBDV infection through current vaccination methods implies the need to develop alternate IBDV vaccine strategies.

The present study was conducted to characterize the genetic profile of NDV and IBDV isolates derived from two farms located in South Korea and determine epidemiological relationships of ND and IBD outbreaks. A phylogenetic analyses of partial sequence of NDV HN and IBDV VP2 genes were done to determine the molecular epidemiology of NDV and IBDV isolated in Korea.

Materials and Methods

Sample Collection

In 2004, NDV and IBDV were isolated from chickens showing clinical signs of Newcastle or infectious bursal disease from farms located in Yeongi-gun, Chungnam. Previously, it was reported that a farm located in Jonui-myun, Yeongi-gun, which was raising about 42,000 chickens, was experiencing a significant number of mortality among their livestock numbering about 600 deaths per week. These fowls exhibited clinical signs of classical nephritic infectious bursal disease (IBD) prior to death. Another farm located in Jochiwon, Yeongi-gun, which was raising 80,000 chickens, reported a weekly mortality rate of 700 chickens. It was observed that prior to death, the fowls showed clinical signs of IBD like enlargement and

congestion of the Fabricius bursa, huddling and ruffling of the feather. In addition, other symptoms like torticollis, hemorrhage of the glandular stomach, facial edema, lacrimation and whitish yellow diarrhea which is commonly associated with newcastle disease were observed. The spleen, trachea, fabricius bursa were collected from some of the recently diseased fowls. These organs were homogenized and placed in conical tubes containing 10 ㎖ Eagle's minimum essential medium (E.MEM) and centrifuged at 10,000×g for 10 min, 4℃. The supernatants were transferred to sterile tubes and stored at -80℃.

Viral RNA isolation

Viral RNA isolation was performed using QIAamp Viral RNA Mini Spin Column (QIAGEN). Briefly, 140 ㎕ of the clarified supernatant was mixed with 560 ㎕ AVL buffer containing carrier RNA and incubated at room temperature for 10 min to induce lysis of the viral particles. Absolute ethanol of equal volume with the AVL buffer was added to the mixture and vortexed vigorously for 15 sec. The mixture was loaded on QIAamp spin column and centrifuged at 6000×g for 1 min. The column was washed with 500 ㎕ AW1 buffer followed by 500 ㎕ AW2 buffer, spinning the column at 6000×g for 1 min during each washing step. Prior to elution of the viral RNA, the column was spin dried at 8,000×g for 2 min and transferred to a clean 1.5 ㎖ microcentrifuge tube. Finally, 60 ㎕ AVE buffer was placed over the column for 1 min at room temperature and centrifuged at 6000×g for 1 min. The eluate was used as template for RT-PCR.

Reverse transcription-polymerase chain reaction (RT-PCR)

For detection of NDV and IBDV RNA, we performed reverse transcription-polymerase chain reaction using the ONE-STEP RT-PCR PreMix kit (Intron Biotech) following manufacturer's instructions. For NDV, we used the NDV HN sense primer (5′-*GGATCC*ACCGCCCAACACAGTCACACTCA-3′) and the NDV HN antisense primer (5′-*GGTA CC*AATGCTGAGACAATAGGTCTTATTAG-3′) to detect the NDV HN gene while for IBDV, we used the IBDV VP2 sense (5′-*GGATCC*TACACCATAACTGCAGCCGACGATTAC-3′) and IBDV VP2 antisense (5′-*GGTACC*TGTGACGGGACGGAGGGCCCCTGGATAGTT-3′) to detect the IBDV VP2 gene. In a 200 ㎕ reaction tube, we mixed 3 ㎕ of template RNA with 2 ㎕ of sense and antisense primers (50 nM each), 5 ㎕ of RNase-free water and 8 ㎕ of Intron ONE-STEP PreMix composed of AMV Reverse transcriptase, RT-PCR buffer (10×), dNTPs, Taq DNA polymerase and stabilizing buffer. Thermocycling condition was carried out as follows: reverse transcription at 45℃ for 35 min, initial denaturing at 94℃ for 15 min, then 40 cycles of 94℃ for 50 sec, 54℃ for 50 sec, 72℃ for 50 sec and a final extension of 72℃ for 10 min. The resulting amplicons were analyzed by agarose gel electrophoresis (1% agarose gel) and ethidium bromide staining.

Cloning of amplified viral gene fragments

A portion of the NDV HN gene and IBDV VP2 gene among samples positive for the viral RNA were cloned in pGEM-T Easy plasmid vector (Promega) in preparation for sequence analysis. The amplified fragments detected by RT-PCR were purified using PCRquick-spin™ PCR Product Purification kit (Intron Biotech) following manufacturer's instructions. Briefly, 20 ㎕ of the amplicon was mixed with 500 ㎕ binding buffer for 1 minute then placed on the PCRquick-spin™ column and centrifuged at 6000×g for 1 min.

The column was washed with 750 ㎕ of washing buffer and centrifuged at 8,000×g for 1 min then spin dried at 8,000×g for 1 min. The bound amplicon was eluted using 50 ㎕ distilled water and centrifuged at 8,000× g for 1 min. Purified amplicons were ligated to pGEM-T Easy and transformed in *E coli* JM83. Positive clones were screened using blue-white colony selection on LB/ampicillin/ IPTG/X-Gal plate and cultured in 3 ㎖ LB Amp (0.1 ㎎/㎖) at 37℃ for 18-20 hours. The plasmid constructs were recovered by plasmid mini-prep isolation (Bioneer) and confirmed by restriction endonuclease digestion using *Bam*HI/*Kpn*I double digestion. Correct plasmid constructs were kept at -20℃.

Sequence analyses of NDV HN and IBDV VP2 gene fragments

NDV HN and IBDV VP2 gene sequencing was done using Big dye terminator cycle sequencing ready reacton kit (Perkin-Elmer). Cycle sequencing reaction was carried out using 25 cycles of 96℃ for 10 sec, 50℃ for 5 sec, 60℃ for 4 min and analyzed using ABI 310 genetic analyzer (Perkin Elmer). The resulting nucleotide sequence was translated using DNAStar 5.0 software. The corresponding amino acid sequence were compared with known NDV and IBDV sequence by pBLAST and phylogenetic analysis was done using Clustal W method. A phylogenetic tree was constructed with TreeView v.1.5 for Windows using neighbor-joining algorithm.

Results

RT-PCR and molecular genetic diagnosis of the samples

Field samples were reported as either NDV or IBDV positive based on the presence of correctly amplified gene fragments. For NDV positive samples, an amplicon size of 478 bp were observed while for the IBDV positive samples, the amplicon size was 447 bp (Fig 1). Among the 11 samples tested from both farms, one sample from farm A (extracted from Fabricius bursa) was positive for the presence of the IBDV RNA while two tracheal samples taken from farm B were positive for NDV RNA. Clinical and

molecular diagnosis of the specimens were confirmed using the results of RT-PCR, and observed clinical and pathological symptoms.

Cloning and sequence analyses of NDV HN and IBDV VP2 gene fragments

Plasmid constructs with the correct inserts of NDV HN (pGEM : NDV-HN) and IBDV VP2 (pGEM : IBDV-VP2) were confirmed by restriction endonuclease digestion before being sequenced (Fig 2). Five plasmid constructs of pGEM : NDV-HN and pGEM : IBDV-VP2 showing bands of similar size with the original HN and VP2 amplicons and the 3.1kb pGEM-T vector were selected for sequencing. After determining the consensus sequence among 5 individual clones, it was observed that the 435 nt VP2 fragment of the single isolate of IBDV from farm A (IBDV No.1) showed high sequence similarity with very virulent IBDV (vvIBDV) strains. Sequence comparison with BJ836-attenuated vaccine strain, Indian strain and kal2001-Egypt strain, both of which are classified under very virulent (vv) phenotype, and Tri-Bio strain showed 99.3% homology and a slightly lower sequence homology of 99.1% with the china strain (Table 1). In the case of the NDV strains isolated from farm B, the 466 nt HN fragments of NDV No.8 and No.11 isolates showed 98.8% similarity. In contrast with the findings on IBDV, the NDV isolates showed significantly lower homology with known vaccine strains. Compared with the HN sequence of known vaccine strains (B1, Lasota and VGGA), both strains revealed less than 86% homology. When compared with the wild type strains, these new isolates showed sequence similarities between 85% and 98% (Table 2).

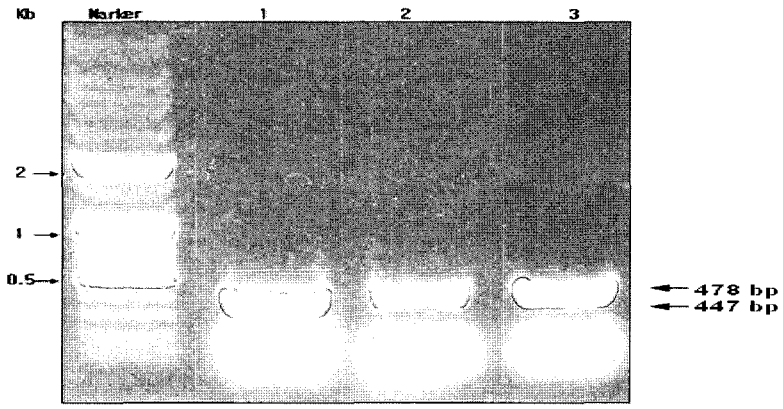

Fig 1. RT-PCR amplicons of NDV HN and IBDV VP2 fragments. The expected size for HN is 478 bp, while for IBDV VP2 it is 447 bp. Size includes the added BamHI and KpnI sites flanking the gene fragment. (Marker; 100 bp plus DNA ladder, lane 1; VP2 of IBDV No.1, lane 2; HN of NDV No.8, lane 3; HN of NDV No.11)

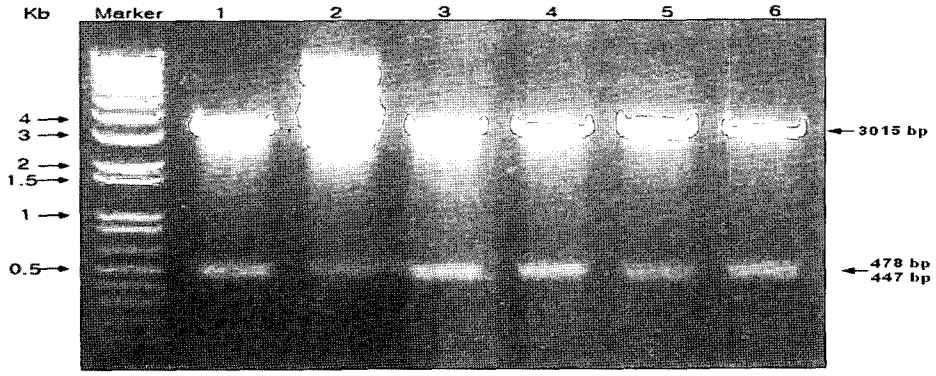

Fig 2. Restriction digest patterns of pGEM:IBDV-VP2 and pGEM : NDV-HN clones by BamHI/KpnI double digestion. Gene inserts were observed between the 400 bp and 500 bp mark. (M; 100bp plus DNA ladder, lanes 1 and 2; pGEM : IBDV-VP2 clones; lane 3, 4, 5, and 6: pGEM:NDV-HN clones)

Table 1. Percent similarity (upper diagonal) and percent divergence (lower diagonal) among known NDV HN sequence and newly isolated known NDV strains

	1	2	3	4	5	6	7	8	9	10	11		
1		98.8	85.3	85.0	84.6	91.0	98.2	85.9	94.1	90.1	97.7	1	8.SEQ
2	0.9		84.6	84.1	83.9	90.5	97.5	85.3	93.4	89.9	97.0	2	11.SEQ
3	15.5	16.2		97.9	98.8	85.6	84.6	83.4	87.1	87.3	84.6	3	B1-HN.seq
4	15.5	16.5	1.9		97.5	85.6	84.1	83.4	86.1	86.4	84.1	4	Lasota-HN.seq
5	16.4	17.1	1.2	2.6		84.9	83.9	82.9	86.4	86.6	83.9	5	VGGA.SEQ
6	9.6	9.6	15.1	15.0	15.9		90.7	89.8	92.5	93.4	91.4	6	Italy00.SEQ
7	1.9	2.4	16.1	16.4	17.0	9.6		86.6	94.6	90.4	99.1	7	JS-3-98-Go.SEQ
8	13.0	13.0	15.3	15.3	16.2	8.8	11.8		88.9	89.6	86.9	8	Russia91.SEQ
9	6.2	6.7	13.4	14.0	14.3	7.9	5.7	9.2		92.9	94.8	9	Taiwan95.SEQ
10	10.3	10.3	14.0	14.6	14.9	7.0	9.8	8.4	7.3		90.6	10	US72.SEQ
11	2.4	2.8	16.1	16.4	17.0	8.8	0.9	11.5	5.4	9.5		11	ZJ1-China.SEQ
	1	2	3	4	5	6	7	8	9	10	11		

Table 2. Percent similarity (upper diagonal) and percent divergence (lower diagonal) among known IBDV VP2 sequence and newly isolated Korean IBDV strain

	1	2	3	4	5	6		
1		99.3	99.1	99.3	99.3	99.3	1	IBD-Korean sample.SEQ
2	0.7		99.8	100.0	100.0	100.0	2	BJ836- attenuated vaccine.SEQ
3	0.9	0.2		99.8	99.8	99.8	3	China.SEQ
4	0.7	0.0	0.2		100.0	100.0	4	India.SEQ
5	0.7	0.0	0.2	0.0		100.0	5	Kal2001-Egypt.SEQ
6	0.7	0.0	0.2	0.0	0.0		6	Tri-Bio SEQ
	1	2	3	4	5	6		

Discussion

Newcastle disease (ND) and infectious bursal disease (IBD) are two of the leading causes of death among domesticated fowls in South Korea. ND is characterized by respiratory and nervous problems among fowls, as well as enteritis and hemorrhage[3,7]. In humans, the infection with NDV can cause conjunctivitis[23]. For IBD, the clinical symptoms associated are depression, watery diarrhea, anorexia, tremor and sever weakness in young chickens. In our study, two local farms in South Korea were investigated for the presence of NDV and IBDV. Chickens from these farms manifested clinical symptoms of ND and IBD. We performed confirmatory test by reverse transcription-polymerase chain reaction (RT-PCR) using NDV HN and IBDV VP2 specific primers. Further analysis by sequence comparison with known vaccine and wild type strains were done for molecular epidemiology of both diseases.

In recent years, RT-PCR has become a convenient tool as a confirmatory test for diagnosis of various diseases, including ND and IBD[7,10]. Antibodies to NDV may be detected in poultry sera by a variety of tests including agar gel precipitation, VN in chick embryos, hemagglutination-inhibition (HI) test and enzyme-linked immunosorbent assays (ELISA)[9,24]. But the presence of specific antibodies to NDV in the serum of a bird gives little information on the infecting strain of NDV and, therefore, has limited diagnostic value.

Antibodies directed against either of the functional surface glycopolypeptides, the HN and the F polypeptides, can neutralize NDVs[3,5,6]. The hemagglutinin-neuraminidase (HN) protein of NDV plays a crucial role in the process of infection. However, the exact contribution of HN to the pathogenesis of NDV is not known[25]. As for IBD, the ELISA procedure is presently the most commonly used serologic test for evaluation of IBDV antibodies in poultry flocks. The other method used for the detection of IBDV antibodies is the AGP test. These methods are based on the principle that IBDV proteins, particularly the VP2 protein, serve as the main host protective antigen that induce production of neutralizing antibodies[18,19]. The antigenic epitopes located within VP2 are conformation-dependent and able to elicit serologically-specific neutralizing antibodies [20,21]. Acute clinical outbreaks of IBD in fully susceptible flocks are easily recognized and a presumptive diagnosis can be readily made[13]. Confirmation of the diagnosis can be made at necropsy by examination for characteristic grossly visible changes in the cloacal bursa[26]. Other tests like nucleic acid probes and antigen-capture ELISA using monoclonal antibodies to detect IBDV directly in tissue is beneficial for rapid diagnosis of field viruses[27,28]. Also, reverse transcription-PCR is useful for detection of the virus.

Based on our study, the genetic profile of local isolates of NDV and IBDV was investigated to determine any correlation with recent outbreaks of ND and IBD. Based on our results, comparison on the VP2 sequence of korean IBDV with vaccine strains showed high homology (>99%). However, homology was also high with the very virulent IBDV

phenotypes (>99%). Based on the high similarities of nucleotide sequence between the Korean IBDV and foreign vvIBDV isolates (India, Egypt), it is strongly suggested that the Korean IBDV strain identified in this study, is categorized under the same group as the foreign vvIBDV isolates.

Control of IBD in young chickens have been primarily achieved by vaccination with a live attenuated strain of IBDV at the age of 0 to 5 weeks or by transferring high levels of maternal antibody induced by administration of live or killed IBD vaccines to breeder hens[16,29]. However, since the late 1980s, the emergence of very virulent IBDV (vvIBDV) has rendered IBD even more difficult to control[22, 30]. The present attenuated or inactivated vaccines used for preventing IBD in fowl, induce polyclonal antisera that do not recognize virus-specific neutralizing epitope. Therefore there is a need to develop vaccines that will induce production of neutralizing antibodies against theses specific epitopes.

In contrast to the findings on the local IBDV isolate, the Korean NDV isolates showed low nucleotide similarity less than 86% with vaccine strains B1, Lasota, and VGGA. while, there was high homology with the foreign strains which includes JS-3-98-Go, ZJ1-China (>97%) and Taiwan (approximately 94%). These data suggest that Korean NDV isolates share low homology with the lentogenic types (vaccine strains B1, Lasota and VGGA) and their similarity with wild type strains may explain the recent outbreaks observed. These ND outbreaks have caused large economic losses in the poultry industry in Korea. Because the present vaccines have been unable to control the persistence of this disease, there is a need to develop more effective vaccines to prevent the spread of newcastle disease.

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