

## Molecular differentiation of Korean Newcastle disease virus (NDV) by restriction enzyme analysis and pathotype-specific RT-PCR

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**Abstract :** Newcastle disease virus (NDV) is a single-stranded negative sense RNA virus, which has been classified as a member of the *Avulavirus* genus of the *Paramyxoviridae* family. It is also one of the most important pathogens in the poultry industry. The glycoproteins, fusion (F) and hemagglutinin-neuraminidase (HN), determine the virulence of NDV, and the relevant molecular structures have already been determined. NDV isolates differ in terms of virulence, and at least 2 of 9 genotypes (I-IX) have been shown to co-circulate. Therefore, it is clearly important to differentiate between vaccine strains and field isolates. *In vivo* pathogenicity tests have been the standard protocol for some time, but molecular methods appear preferable in terms of the rapidity of diagnosis, as well as animal welfare concerns. In this study, we have designed primer sets from HN gene for phylogenetic analysis and restriction enzyme analysis, and from F gene for pathotype-specific RT-PCR. Via the combination of 2 methods, 106 Korean NDV isolates obtained from 1980 to 2005 were differentiated into vaccine strains, and virulent genotypes VI and VII. The genotype VI viruses were only rarely isolated after 1999, and genotype VII, after it was initially isolated from poultry in 1995, recurred in 2000, and then became the main NDV constituting a threat to the Korean poultry industry.

**Key words :** molecular differentiation, Newcastle disease virus, pathotype-specific RT-PCR

### Introduction

Newcastle disease (ND), which is caused by the ND virus (NDV), poses a disastrous risk to the poultry industry. NDV is an enveloped, single-stranded, negative-sense RNA virus, which belongs to the *Avulavirus* genus of the *Paramyxoviridae* family [18, 19]. NDV has been classified into 3 pathotypes; lentogenic, mesogenic and velogenic types on the basis of conventional *in vivo* pathogenicity indices such as mean death time (MDT) of chicken embryo and intracerebral pathogenicity index (ICPI) [3]. On the envelope, 2 biologically important glycoproteins, fusion (F) protein and hemagglutinin-neuraminidase (HN), have been detected. These proteins coordinate in order to invade host cells, and play a major role in the

determination of viral virulence. The multi-basic amino acids at the proteolytic cleavage site in F are relevant to systemic replication, and HN determines the tropisms of NDV [9, 11].

ND outbreaks can be characterized by the co-circulation of genetically distinct virus lineages [23]. Strains of NDV have been classified into 10 provisional genetic groups (I-X) and epizootics in western Europe (VIIa and VIId), Taiwan (VIIa and III), southern Africa (VIIb and VIII), and Korea (VIIf and VIIa) during the first half of 1990s were determined to have been caused by 2 distinct strains of NDV [10, 13, 16, 17, 28, 29]. Therefore, it is clearly important that some protocol be developed which would allow for differentiation between virulent field isolates, as well as between vaccine strains and field isolates.

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In this study, we have amplified a small fragment of HN via RT-PCR, and phylogenetic analysis and restriction enzyme analysis (REA) were conducted for NDV differentiation. In order to rapidly differentiate between the vaccine and virulent NDV strains, we also developed an F gene-based pathotype-specific RT-PCR technique.

## Materials and Methods

### Viruses

During 1980-2005 106 NDV isolates have been

isolated from diseased birds in the Laboratory of Avian Diseases of Seoul National University via standard procedures [2]. The virulence of these isolates were classified into velogenic and lentogenic types on the basis of the mean death time of the chicken embryo (MDT) and the intracerebral pathogenicity indices (ICPI), as well as the multi-basic amino acids located at the F protein cleavage site [2, 8, 13, 20, 25]. The NDV isolates used in this study are listed in Table 1.

### Primers

To distinguish virulent genotypes VI and VII viruses

**Table 1.** Phylogenetic differentiation of Korean NDV isolates used in this study

Year of isolation	No. of isoates	Designation of NDV isolates (pathotype <sup>a</sup> /genotype)
1980	3	SNU8050(v/VI), SNU80108(l/I), SNU80143(v/VI)
1984	4	SNU8419(l/II), SNU8426(l/II), SNU8465(v/VI), SNU8490(v/VI)
1985	5	SNU8550(v/VI), SNU8557(v/VI), SNU8559(v/VI), SNU8566(v/VI), SNU85135(v/VI)
1988	6	SNU8861(v/VI), SNU8871 <sup>b</sup> (v/VI), SNU8876(v/VI), SNU8887(v/VI), SNU88139 <sup>b</sup> (v/VI), SNU88147(v/VI)
1990	2	SNU9033 <sup>b</sup> (v/VI), SNU90111 <sup>b</sup> (v/VI)
1991	1	SNU9139 <sup>b</sup> (v/VI)
1992	11	SNU9213 <sup>b</sup> (v/VI), SNU9222 <sup>b</sup> (v/VI), SNU9227 <sup>b</sup> (v/VI), SNU9229 <sup>b</sup> (v/VI), SNU9257 <sup>b</sup> (v/VI), SNU9275 <sup>b</sup> (v/VI), SNU9280 <sup>b</sup> (v/VI), SNU9282 <sup>b</sup> (v/VI), SNU9283 <sup>b</sup> (v/VI), SNU9284 <sup>b</sup> (v/VI), SNU92105 <sup>b</sup> (v/VI)
1993	1	SNU9358GG <sup>b</sup> (v/VI)
1994	1	SNU9444 <sup>b</sup> (v/VI)
1995	11	SNU9512 <sup>b</sup> (v/VII), SNU9515 <sup>b</sup> (l/-), SNU9550 <sup>b</sup> (v/VII), SNU9553 <sup>b</sup> (v/VII), SNU9575 <sup>b</sup> (v/VII), SNU9586 <sup>b</sup> (v/VII), SNU9598 <sup>b</sup> (v/VI), SNU95107 <sup>b</sup> (v/VII), SNU95119 <sup>b</sup> (v/VII), SNU95124 <sup>b</sup> (v/VII), SNU95132 <sup>b</sup> (v/VII)
1999	8	SNU9969(v/VI), SNU9970(v/VI), SNU9971(v/VI), SNU9983(l/-), SNU9991(l/II), SNU99105(v/VI), SNU99111(l/II), SNU99123(l/I)
1996	1	SNU96130(l/-)
2000	15	SNU0021(v/VII), SNU0036(v/VII), SNU0079(v/VII), SNU0086(v/VII), SNU0105(v/VII), SNU0117(v/VII), SNU0118(v/VII), SNU0119(v/VII), SNU0123(v/VII), SNU0125(v/VII), SNU0129(v/VII), SNU0164(v/VII), SNU0165(v/VII), SNU0169(v/VII), SNU0202(v/VII)
2001	1	SNU1024(v/VII)
2002	19	SNU2001(v/VII), SNU2009(v/VII), SNU2017(v/VII), SNU2026(v/VII), SNU2028(v/VII), SNU2048(v/VII), SNU2049(v/VII), SNU2063(v/VII), SNU2064(v/VII), SNU2078(v/VII), SNU2080(v/VII), SNU2083(v/VII), SNU2084(v/VII), SNU2091(v/VII), SNU2100(v/VII), SNU2102(v/VII), SNU2108(v/VII), SNU2117(v/VII), SNU2124(v/VII)
2003	1	SNU3058(v/VII)
2004	1	SNU4152(v/VII)
2005	15	SNU5005(v/VII), SNU5009(v/VII), SNU5047(v/VII), SNU5062(v/VII), SNU5063(v/VII), SNU5064(v/VII), SNU5065(v/VII), SNU5070(v/VII), SNU5074(v/VII), SNU5076(v/VII), SNU5079(v/VII), SNU5081(v/VII), SNU5084(v/VII), SNU5085(v/VII), SNU5100(v/VII)

<sup>a</sup> v, velogen; l, lentogen; -, not determined.

<sup>b</sup> Previously identified [13] and reconfirmed in the present study.

Pt	AGG	AGA	CRR	AAA	CGC	T	
B1	G--	---	-AG	GGT	---	C	
La Sota	G--	---	-AG	GGT	---	C	
Ulster	G--	-A-	-AG	GGT	---	C	
SNU88139	---	---	-GG	---	---	-	
SNU9280	---	---	-GA	---	---	-	
SNU9222	---	---	-AA	---	---	-	
SNU9512	---	---	-AG	---	---	-	
I-1N	TGC	CAC	TGM	TAG	TTG	YGA	TA
B1	---	---	---	---	---	---	---
La Sota	---	---	---	---	---	T--	--
Ulster	---	---	---	---	---	T--	--
SNU88139	---	---	---	---	---	---	---
SNU9280	---	---	---	---	---	---	---
SNU9222	---	---	---	---	---	---	---
SNU9512	---	---	-A	---	---	T--	--

**Fig. 1.** Comparison of the nucleotide sequences of the pathotyping primer set. The homologous nucleotides are shown with dashes.

from commercial vaccine strains the nucleotide sequences of the F protein cleavage site were compared, and a virulent NDV-specific forward primer [Pt(334-349): 5'-AGGAGACRRAAACGYT-3'] and a reverse primer [I-1N(534-515): 5'-TGCCACTGMTAGTTGYGATA-3'] common to NDV were designed (pathotyping primer set) (Fig. 1). The HN genes of the NDV strains registered in the GenBank and of the Korean isolates were compared, and a primer set that was conserved in the majority of NDV strains and generated the smallest amplicon was selected (common primer set; numbering from the start codon): ComHNF (638-657) 5'-CATCTG CAACAGGGAGGGTA-3'; ComHNR (757-737) 5'-TMG AGCACAG CATATCACAAC-3'. The specificity and primer sequence conservation among the NDVs were determined via BLAST searches [4].

#### RNA isolation, RT-PCR and REA

RNA isolation and RT-PCR were conducted as previously described with little modifications [13]. Briefly 100  $\mu$ l of allantoic fluid was added to 1 ml of easy Blue reagent (iNtRON Biotechnology, Korea) and followed the manufacturer's protocol. RNA pellet was resolved in 50  $\mu$ l of DEPC (0.1% diethyl pyrocarbonate)-treated distilled water (DW). cDNA was synthesized by Power cDNA kit as manufacturer's protocol (iNtRON Biotechnology, Korea). cDNA was diluted 5-fold and 1  $\mu$ l of cDNA was added to the PCR reaction [1  $\mu$ l of 10  $\times$  PCR buffer, 0.2  $\mu$ l of 2.5 mM dNTPs, 0.2  $\mu$ l

of each primer, Taq polymerase (1U/ $\mu$ l; iNtRON Biotechnology, Korea), and 7.2  $\mu$ l of DW]. After denaturation at 94°C for 3 min, consecutive reactions of 94°C for 30 sec, 48°C for 15 sec, 65°C for 20 sec were repeated for 35 times and final elongation was performed at 72°C for 7 min. For the multiplex RT-PCR the quantity of the pathotyping primer set was twice that of the common primer sets. On the basis of the sequence data, we predicted the restriction enzyme sites (MacDNAsis Ver. 3.1) and selected 2 enzymes (*Hinf* I and *Pst* I) for REA. The digested fragments were separated on polyacrylamide gel, and visualized via silver staining [6]. For the sake of simplicity, we also utilized 2.5% agarose gel and ethidium bromide.

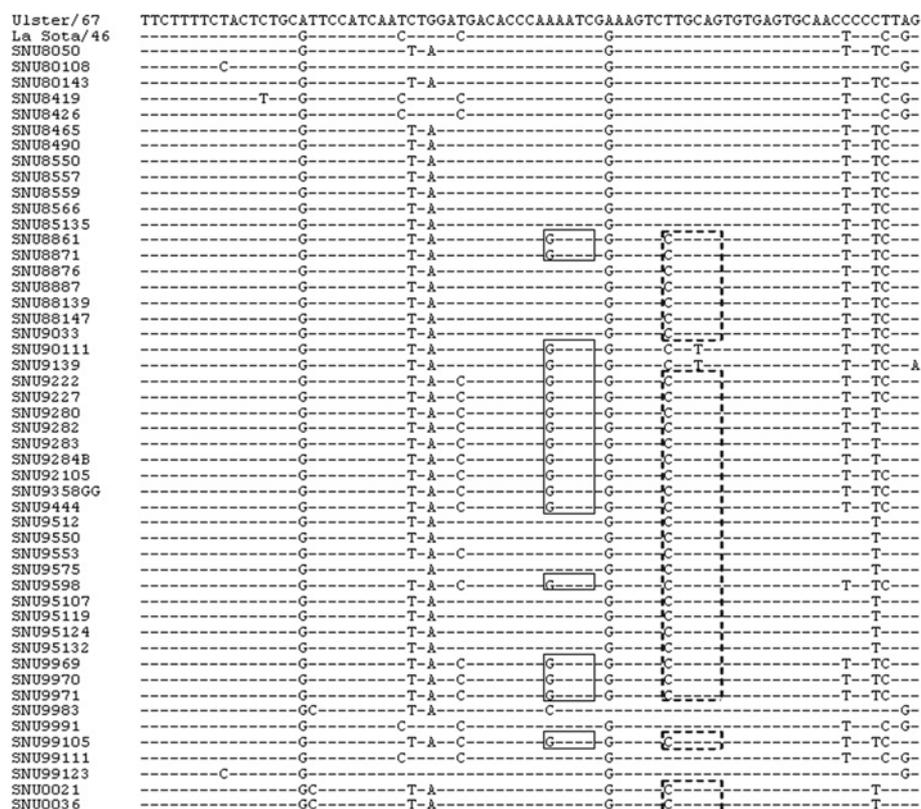
#### Sequencing and Sequence analysis

The comHN of the HN gene was sequenced using an ABI 377 automatic DNA sequencer and a Dye Terminator kit (Perkin Elmer, USA), as reported previously [14]. Nucleotide sequences were aligned via the multiple alignment algorithm contained in the MegAlign package (Windows version 3.12e; DNASTAR, USA), and a phylogenetic tree was constructed via neighbor-joining method.

## Results

#### Molecular differentiation of Korean NDV isolates by phylogenetic analysis

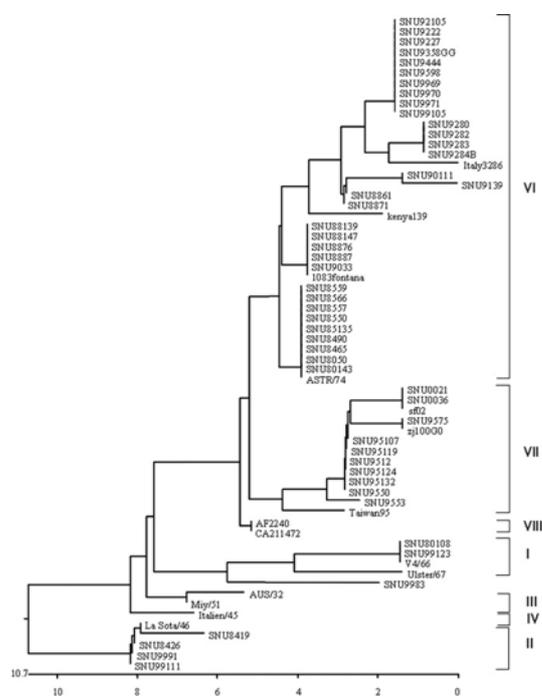
The PCR products amplified by the comHN primer set were directly sequenced. The nucleotide and amino acid similarity between the Korean and foreign isolates including the vaccine strains, were found to be 81.0%-100% and 92.3%-100%, respectively. Representative nucleotide sequences are summarized in Fig. 2. A phylogenetic tree was constructed on the basis of the nucleotide sequences. The clustering and branching patterns of the tree were similar to those of the tree constructed around the F gene [5]. SNU8050, SNU80143, SNU8465, SNU8490, SNU8550, SNU8557, SNU8559, SNU8566, SNU85135, SNU8861, SNU8871, SNU8876, SNU8887, SNU88139, SNU88147, SNU9033, SNU90111, SNU9139, SNU9222, SNU9227, SNU9280, SNU9282, SNU9283, SNU9284B, SNU92105, SNU9358GG, SNU 9444, SNU9598, SNU9969, SNU9970, SNU9971 and SNU99105 were classified into genotype VI (Table 1, Fig. 3). SNU9512, SNU9550, SNU9553, SNU9575, SNU 95107, SNU95119, SNU95124, SNU95132, SNU2083,



**Fig. 2.** Comparison of the nucleotide sequences of the common HN region (nucleotides 658-736) of Newcastle disease virus (NDV). Ulster/67 (M19478) and La Sota/46 (AF077761) were compared with Korean NDV isolates. Only representative sequences are listed. The homologous nucleotides were shown with dashes. The nucleotide sequences of the foreign NDV strains were taken from the GenBank databases, and their accession numbers are as follows. The *Hinf*I and *Pst*I recognition sequences are boxed in solid and broken lines, respectively. La Sota = SNU8426 = SNU9991 = SNU99111; SNU8050 = SNU80143 = SNU8465 = SNU8490 = SNU8550 = SNU8557 = SNU8559 = SNU8566 = SNU85135; SNU80108 = SNU99123 = V4; SNU8861 = SNU8871; SNU8876 = SNU8887 = SNU88139 = SNU88147 = SNU9033 = SNU9222 = SNU9227 = SNU92105 = SNU9358GG = SNU9444 = SNU9598 = SNU9969 = SNU9970 = SNU9971 = SNU99105; SNU9280; SNU9282 = SNU9283 = SNU9284B; SNU9512 = SNU9550 = SNU95107 = SNU95119 = SNU95124 = SNU95132; SNU0021 = SNU0036 = SNU0079 = SNU0086 = SNU0105 = SNU0117 = SNU0118 = SNU0119 = SNU0123 = SNU0125 = SNU0129 = SNU0164 = SNU0165 = SNU0169 = SNU1024 = SNU2009 = SNU2017 = SNU2026 = SNU2028 = SNU2048 = SNU2063 = SNU2064 = SNU2078 = SNU2080 = SNU2084 = SNU2091 = SNU2102 = SNU2117 = SNU2124 = SNU3058 = SNU5009 = SNU5047 = SNU5062 = SNU5063 = SNU5064 = SNU5065 = SNU5070 = SNU5074 = SNU5081; SNU0202 = SNU2001 = SNU2049 = SNU2100 = SNU5005 = SNU5076; SNU2108 = SNU4152 = SNU5079 = SNU5084 = SNU5085 = SNU5100.

SNU0021-like viruses (SNU0036, SNU0079, SNU0086, SNU0105, SNU0117, SNU0118, SNU0119, SNU0123, SNU0125, SNU0129, SNU0164, SNU0165, SNU0169, SNU1024, SNU2009, SNU2017, SNU2026, SNU2028, SNU2048, SNU2063, SNU2064, SNU2078, SNU2080, SNU2083, SNU2084, SNU2091, SNU2102, SNU2117, SNU2124, SNU3058, SNU5009, SNU5047, SNU5062, SNU5063, SNU5064, SNU5065, SNU5070, SNU5074, SNU5081), SNU0202-like viruses (SNU2001, SNU2049,

SNU2100, SNU5005 and SNU5076) and SNU2108-like viruses (SNU4152, SNU5079, SNU5084, SNU5085, SNU5100) were classified into genotype VII (Table 1, Fig. 2 & 3). SNU0021-like viruses and SNU9575 were found to be 100% similar to SF02 (AF473851) and ZJ/1/00/Go (AF456432), respectively, in China. SNU80108 and SNU99123 were V4-type, and SNU8419, SNU8426, SNU9991 and SNU99111 were La Sota-type vaccine viruses (Fig. 3). SNU9993 was clustered with the geno-

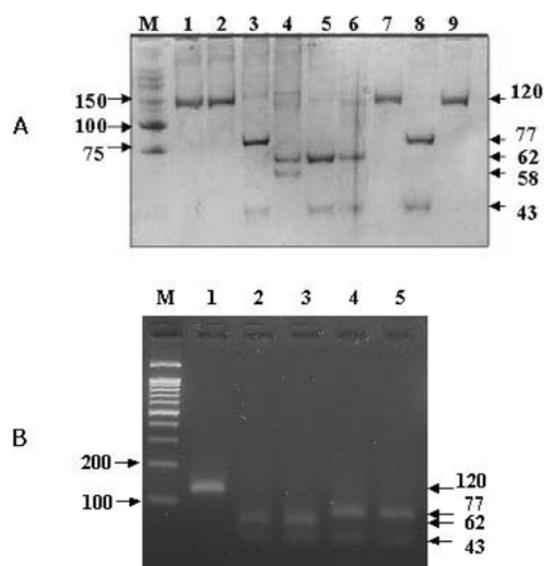


**Fig. 3.** Phylogenetic tree based on the nucleotide sequences of the common HN (nucleotides 658-736) via neighbor-joining. The nucleotide sequences of the foreign NDV strains were obtained from the GenBank database, and their accession numbers are as follows. Ulster/67 (M24694), La Sota/46 (AF077761), ASTR/74 (Y19016), AUS/32 (M24712), Italien/45 (M24715), Miyadera/51 (M24713), sf02 (AF473851), zj/1/00/Go (AF456432), Taiwan95 (U62620), AF2240 (X79092), CA211472 (AY246050), V4/66 (J03911), Ck/Kenya/139/90 (AY288997), Ck/USA (CA)/1083 (Fontana)/72 (AY288992), Ck/Italy/3286/00 (AY288994).

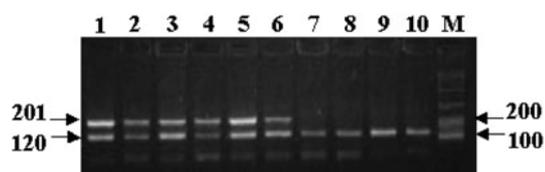
type I viruses, but exhibited only a 92.4% nucleotide similarity with Ulster and V4 (Fig. 3).

#### Differentiation of NDV by REA

In order to differentiate between vaccine strains and the recent genotype VI and VII field isolates, *Hinf* I and *Pst* I were selected and REA was conducted. 41 isolates and 2 reference vaccine strains, Ulster and La Sota, were employed for the REA. NDVs were grouped into 4 REA types, H-/P- (both enzyme recognition sites negative, 120 bp), H+/P- (*Hinf* I recognition site positive, 62 bp/58 bp), H-/P+ (*Pst* I recognition site positive, 77 bp/43 bp) and H+/P+ (both enzyme recognition sites positive, 62 bp/43 bp/15 bp) (Table 2, Fig. 2 & 4). Ulster, La Sota, SNU8050, SNU80143, SNU8419, SNU8426, SNU8465, SNU8490, SNU8550, SNU8557,



**Fig. 4.** Differentiation of NDV strains via REA analysis. Panel A (polyacrylamide gel electrophoresis and silver staining), M: size marker (25/100; Bioneer, Korea), lane 1: Ulster, 2: La Sota, 3: SNU88139, 4: SNU90111, 5: SNU 9222, 6: SNU9358GG, 7: SNU9515, 8: SNU95124, 9: SNU 96130. Panel B (agarose gel electrophoresis and ethidium bromide staining), M: size marker (100 bp; iNtRON Biotechnology, Korea), 1: Ulster, 2: SNU9969, 3: SNU9970, 4: SNU 95124, 5: SNU0021.



**Fig. 5.** Multiplex RT-PCR for differentiation of vaccine strains and pathogenic field isolates (velogens). Lanes 1: SNU92105, 2: SNU9550, 3: SNU9575, 4: SNU9586, 5: SNU9598, 6: SNU95124, 7: La Sota, 8: Ulster, 9: SNU 9515, 10: SNU96130, M: size marker (100 bp; iNtRON Biotechnology, Korea).

SNU8559, SNU8566, SNU85135, SNU9515, SNU96130 and SNU9983 were grouped into H-/P- (Table 2). SNU90111 was digested only by *Hinf* I (H+/P-) and SNU8876, SNU8887, SNU88139, SNU88147, SNU9033, SNU9550, SNU9575, SNU9586, SNU95107 and SNU 95124 were digested only by *Pst* I (H-/P+) (Table 2). SNU8861, SNU8871, SNU9222, SNU9227, SNU9229, SNU9257, SNU9275, SNU9280, SNU9282, SNU9283, SNU9284B, SNU92105, SNU9358GG, SNU9598, SNU 9969, SNU9970 and SNU9971 were grouped into H+/

**Table 2.** Summary of restriction enzyme analysis (REA)

REA type (Fragment size)	No. of viruses	Vaccine strains and field isolates
H-/P- (120)	20	Ulster, La Sota, SNU8050, SNU80108 <sup>a</sup> , SNU80143, SNU8419, SNU8426, SNU8465, SNU8490, SNU8550, SNU8557, SNU8559, SNU8566, SNU85135, SNU9515, SNU96130, SNU9983, SNU9991 <sup>a</sup> , SNU99111 <sup>a</sup> , SNU99123 <sup>a</sup>
H+/P- (62/58)	2	SNU90111, SNU9139 <sup>a</sup>
H-/P+ (77/43)	66	SNU8876, SNU8887, SNU88139, SNU88147, SNU9033, SNU9512 <sup>a</sup> , SNU9550, SNU9553 <sup>a</sup> , SNU9575, SNU9586, SNU95107, SNU95119 <sup>a</sup> , SNU95124, SNU95132 <sup>a</sup> , SNU0021 <sup>a</sup> , SNU0036 <sup>a</sup> , SNU0079 <sup>a</sup> , SNU0086 <sup>a</sup> , SNU0105 <sup>a</sup> , SNU0117 <sup>a</sup> , SNU0118 <sup>a</sup> , SNU0119 <sup>a</sup> , SNU0123 <sup>a</sup> , SNU0125 <sup>a</sup> , SNU0129 <sup>a</sup> , SNU0164 <sup>a</sup> , SNU0165 <sup>a</sup> , SNU0169 <sup>a</sup> , SNU0202 <sup>a</sup> , SNU1024 <sup>a</sup> , SNU2001 <sup>a</sup> , SNU2009 <sup>a</sup> , SNU2017 <sup>a</sup> , SNU2026 <sup>a</sup> , SNU2028 <sup>a</sup> , SNU2048 <sup>a</sup> , SNU2049 <sup>a</sup> , SNU2063 <sup>a</sup> , SNU2064 <sup>a</sup> , SNU2078 <sup>a</sup> , SNU2080 <sup>a</sup> , SNU2083 <sup>a</sup> , SNU2084 <sup>a</sup> , SNU2091 <sup>a</sup> , SNU2100 <sup>a</sup> , SNU2102 <sup>a</sup> , SNU2108 <sup>a</sup> , SNU2117 <sup>a</sup> , SNU2124 <sup>a</sup> , SNU3058 <sup>a</sup> , SNU4152 <sup>a</sup> , SNU5005 <sup>a</sup> , SNU5009 <sup>a</sup> , SNU5047 <sup>a</sup> , SNU5062 <sup>a</sup> , SNU5063 <sup>a</sup> , SNU5064 <sup>a</sup> , SNU5065 <sup>a</sup> , SNU5070 <sup>a</sup> , SNU5074 <sup>a</sup> , SNU5076 <sup>a</sup> , SNU5079 <sup>a</sup> , SNU5081 <sup>a</sup> , SNU5084 <sup>a</sup> , SNU5085 <sup>a</sup> , SNU5100 <sup>a</sup>
H+/P+ (62/43/15)	19	SNU8861, SNU8871, SNU9222, SNU9227, SNU9229, SNU9257, SNU9275, SNU9280, SNU9282, SNU9283, SNU9284B, SNU92105, SNU9358GG, SNU9598, SNU9969, SNU9970, SNU9971, SNU9444 <sup>a</sup> , SNU99105 <sup>a</sup>

<sup>a</sup> Expected result based on nucleotide sequence.

P+ (Table 2). Based on the nucleotide sequences, SNU 80108, SNU9991, SNU99111 and SNU99123 (H-/P-), SNU9139 (H+/P-), SNU9512, SNU9553, SNU95119, SNU95132, SNU2083, and SNU0021-, SNU0202- and SNU2108-like viruses (H-/P+), and SNU9444 and SNU99105 (H+/P-) were grouped into each of the REA groups (Table 2).

#### Differentiation of NDV via pathotype-specific RT-PCR

The vaccine strains and field lentogens evidenced no bands on pathotype-specific RT-PCR, but the virulent field isolates did exhibit a specific band. All of the NDV isolates previously grouped into virulent genotypes VI and VII as velogens, as well as the new viruses in the present study, were identified as velogens and lentogens via pathotype-specific RT-PCR (Table 1). In addition, 2 primer sets were determined to be suitable for multiplex-RT-PCR (Fig. 5).

#### Discussion

NDV evolves only via nucleotide substitutions introduced by RNA-dependent RNA polymerase without recombination [23, 27]. The principal determinant of virulence is the proteolytic cleavability of the fusion

protein, but the results of a reverse genetic study have revealed that the HN gene is also relevant to virulence [11].

The presence of genotype VII dates back to 1984 and 1985, where it was identified in Taiwan and Japan, respectively [29]. The first genotype VII virus was isolated from imported peafowl in a zoo in 1984 [15], but the genotype VII viruses from chickens were initially reported in Korea, in 1995 [13]. Genotype VII, in addition to genotypes VI and IX, was identified in China [16]. Therefore, genotype VII viruses had already spread throughout the Far East in the 1990s [29]. According to the results of phylogenetic analyses with the HN gene, Korean NDV isolates obtained between 1980 and 2005 were grouped into the VI and VII genotypes. The genotype VI viruses were present from 1980 to 1999, but were rarely isolated after the beginning of 2000. Genotype VII first appeared in 1995 but since then, had never been isolated in poultry prior to 2000 [15]. They recurred in 2000, and have since become the only detectable genotype in Korea. The Korean government has been exerting a great deal of effort to eradicate Newcastle disease, via the vaccination of the majority of one-day old chicks. The fact that genotype VI is no longer isolated can be attributed to this massive vaccination effort, but it appears that

this measure has had no effect on genotype VII, as this genotype continues to be isolated. The conventional vaccines resulted in an attenuation of mortality due to the genotype VII subtypes [16] but there is currently a paucity of data regarding protection against viral shedding. Therefore, further studies will be required into the mechanisms underlying the prevalence of genotype VII among Korean poultry farms. Thus far, the origin and the manner of transmission of the genotype VII in 1995 and 2000 have remained unclear. However, the similarity of the first NDV (SNU9575) and the recurrent NDV (SNU0021-like viruses) with the Chinese isolates (SF02 and ZJ/1/00/Go), coupled with the presence of various genotype VII subtypes in China [16], militate toward further studies regarding the mechanisms underlying viral transmission between countries [15].

Under certain conditions, NDV vaccine strains appear to persist for an extended period in the vaccinated flocks, and the clustering of some field isolates with vaccine strains may originate from the inoculated vaccine strains [25]. Although it seemed that the nucleotide sequences for the phylogenetic analysis were too short the generated tree was exactly same in clustering patterns with previous reports [13, 15]. Therefore, the partial nucleotide sequence of HN was suitable for the phylogenetic analysis.

In order to differentiate between the field isolates and vaccine strains, a variety of molecular methods have been developed, including conventional PCR with labeled oligonucleotide probes, as well as real-time PCR techniques [1, 12, 22]. Due primarily to its inherent ease and simplicity in terms of laboratory applications, REA has become the method of choice for the identification and differentiation of NDV genotypes [10, 16, 17]. Genotype VI viruses isolated between 1980 and 1985 have been shown to be indistinguishable from vaccine strains, but vaccine strains and recent virulent field isolates, as well as the recently isolated VI and VII genotypes, have been differentiated via REA. The PCR products amplified by the common primer set and its digested fragments were quite small, but the REA patterns could be readily differentiated using 2.5% agarose gel.

For efficient amplification of the PCR products, homology between the primer and target sequence is essential. In cases in which a short oligonucleotide primer (16-mer) is involved, a single mismatch in the

center of the primer doesn't allow for annealing to the target sequence in most cases [24]. Furthermore, mismatches located at the 3'-ends of primers reduce DNA synthesis efficiency by 200-, 1,400-, and 2,500-fold, depending on whether the mismatch is a G-T, C-T, or T-T mismatch, respectively [21]. Cho [7] designed a degenerate primer set that annealed only to the F gene cleavage site of virulent NDV, and successfully differentiated the vaccine strains from the genotype VI viruses. However, nucleotide changes in genotype VII at the reverse primer-annealing region resulted in false negatives. Therefore, we improved the reverse primer via nucleotide degeneracy (I-1N). Previously reported virulent field isolates were classified into velogens using pathotype-specific RT-PCR [8, 13, 26], and this method was applied to the successful differentiation of avirulent and virulent field isolates. Some errors affecting RNA quality may result in false negatives, and additional RT-PCR for the evaluation of RNA quality is crucial. For this purpose, the comHN primer set also proved useful in both single RT-PCR and multiplex pathotype-specific RT-PCR.

## Conclusion

Both REA and pathotype-specific RT-PCR may facilitate the rapid and economic differentiation of field isolates. The genotype VI viruses were rarely isolated after 1999, and genotype VII, after its initial isolation in poultry in 1995, recurred in 2000 and then became the main NDV constituting a threat to the Korean poultry industry.

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## References

1. **Aldous EW, Alexander DJ.** Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). *Avian Pathol* 2001, **30**, 117-128.
2. **Alexander DJ.** Newcastle disease. In: Purchase HG, Arp LH, Domermuth CH, Pearson JE (eds.). *A Laboratory Manual for the Isolation and Identification of Avian Pathogens*. 3rd ed. pp. 114-120, The American Association of Avian Pathologists, Kennett Square,

- 1989.
3. **Alexander DJ**. Newcastle disease virus diagnosis. In: Alexander DJ (ed.). Newcastle Disease. pp. 151-153, Kluwer Academic, Boston, 1988.
  4. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ**. Basic local alignment search tool. *J Mol Biol* 1990, **215**, 403-410.
  5. **Ballagi-Pordány A, Wehmann E, Herczeg J, Belak S, Lomniczi B**. Identification and grouping of Newcastle disease virus strains by restriction site analysis of a region from the F gene. *Arch Virol* 1996, **141**, 243-261.
  6. **Bassam BJ, Caetano-Anollés G, Gresshoff PM**. Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal Biochem* 1991, **196**, 80-83.
  7. **Cho SH**. Nucleotide sequences of fusion proteins of Korean isolates of Newcastle disease virus and pathotyping by reverse transcriptase polymerase chain reaction. Master's Thesis. Seoul, Seoul National University, 1997.
  8. **Choi KS**. Characterization of Korean isolates of Newcastle disease virus. Master's Thesis. Seoul, Seoul National University, 1993.
  9. **Collins MS, Bashiruddin JB, Alexander DJ**. Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. *Arch Virol* 1993, **128**, 363-370.
  10. **Herczeg J, Wehmann E, Bragg RR, Travassos Dias PM, Hadjiev G, Werner O, Lomniczi B**. Two novel genetic groups (VIIb and VIII) responsible for recent Newcastle disease outbreaks in Southern Africa, one (VIIb) of which reached Southern Europe. *Arch Virol* 1999, **144**, 2087-2099.
  11. **Huang Z, Panda A, Elankumaran S, Govindarajan D, Rockemann DD, Samal SK**. The hemagglutinin-neuraminidase protein of Newcastle disease virus determines tropism and virulence. *J Virol* 2004, **78**, 4176-4184.
  12. **Jarecki-Black JC, King DJ**. An oligonucleotide probe that distinguishes isolates of low virulence from the more pathogenic strains of Newcastle disease virus. *Avian Dis* 1993, **37**, 724-730.
  13. **Kwon HJ, Cho SH, Ahn YJ, Seo SH, Choi KS, Kim SJ**. Molecular epidemiology of Newcastle disease in Republic of Korea. *Vet Microbiol* 2003, **95**, 39-48.
  14. **Kwon HJ, Park KY, Yoo HS, Park JY, Park YH, Kim SJ**. Differentiation of *Salmonella enterica* serotype *gallinarum* biotype pullorum from biotype *gallinarum* by analysis of phase I flagellin C gene (*fljC*). *J Microbiol Methods* 2000, **40**, 33-38.
  15. **Lee YJ, Sung HW, Choi JG, Kim JH, Song CS**. Molecular epidemiology of Newcastle disease viruses isolated in South Korea using sequencing of the fusion protein cleavage site region and phylogenetic relationships. *Avian Pathol* 2004, **33**, 482-491.
  16. **Liu XF, Wan HQ, Ni XX, Wu YT, Liu WB**. 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* 2003, **148**, 1387-1403.
  17. **Lomniczi B, Wehmann E, Herczeg J, Ballagi-Pordány A, Kaleta EF, Werner O, Meulemans G, Jorgensen PH, Manté AP, Gielkens ALJ, Capua I, Damoser J**. Newcastle disease outbreaks in recent years in western Europe were caused by an old (VI) and a novel genotype (VII). *Arch Virol* 1998, **143**, 49-64.
  18. **Mayo MA**. A summary of taxonomic changes recently approved by ICTV. *Arch Virol* 2002, **147**, 1655-1663.
  19. **Mayo MA**. Virus taxonomy-Houston 2002. *Arch Virol* 2002, **147**, 1071-1076.
  20. **Office International des Epizooties**. Newcastle disease. In: Manual of Standards for Diagnostic Tests and Vaccines. 4th ed. pp. 221-232, Office International des Epizooties, Paris, 2000.
  21. **Petruska J, Goodman MF, Boosalis MS, Sowers LC, Cheong C, Tinoco I Jr**. Comparison between DNA melting thermodynamics and DNA polymerase fidelity. *Proc Natl Acad Sci USA* 1988, **85**, 6252-6256.
  22. **Pham HM, Konnai S, Usui T, Chang KS, Murata S, Mase M, Ohashi K, Onuma M**. Rapid detection and differentiation of Newcastle disease virus by real-time PCR with melting-curve analysis. *Arch Virol* 2005, **150**, 2429-2438.
  23. **Sakaguchi T, Toyoda T, Gotoh B, Inocencio NM, Kuma K, Miyata T, Nagai Y**. Newcastle disease virus evolution. I. Multiple lineages defined by sequence variability of the hemagglutinin-neuraminidase gene. *Virology* 1989, **169**, 260-272.
  24. **Sambrook J, Russell DW**. Working with synthetic oligonucleotide probes. In: Molecular Cloning: A Laboratory Manual. 2nd ed. pp. 11.6-11.7, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989.
  25. **Samuel JL, Spradbrow PB**. Persistence of the V4

- strain of Newcastle disease virus in an open-range flock of chickens. *Vet Rec* 1989, **124**, 193-196.
26. **Seo SH**. Biological characteristics of local isolates of Newcastle disease virus and detection of the virus by an oligonucleotide probe. Master's Thesis. Seoul, Seoul National University, 1994.
27. **Toyoda T, Gotoh B, Sakaguchi T, Kida H, Nagai Y**. Identification of amino acids relevant to three antigenic determinants on the fusion protein of Newcastle disease virus that are involved in fusion inhibition and neutralization. *J Virol* 1988, **62**, 4427-4430.
28. **Tsai HJ, Chang KH, Tseng CH, Frost KM, Manvell RJ, Alexander DJ**. Antigenic and genotypical characterization of Newcastle disease viruses isolated in Taiwan between 1969 and 1996. *Vet Microbiol* 2004, **104**, 19-30.
29. **Yang CY, Chang PC, Hwang JM, Shieh HK**. Nucleotide sequence and phylogenetic analysis of Newcastle disease virus isolates from recent outbreaks in Taiwan. *Avian Dis* 1997, **41**, 365-373.