

Characterization and comparison of the pathogenicity of viscerotropic velogenic Newcastle disease virus isolates in Korea

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Abstract : A total of 18 Newcastle disease virus (NDV) isolates that were recovered from 1949 through 1997 were characterized and pathotyped. All viruses were highly virulent as determined by intracerebral pathogenicity indices ≥ 1.81 in day-old. These pathotypes are typical for viscerotropic velogenic NDV (VVNDV) pathotype viruses. Some differences were observed for the chicken red blood cell elution rate and thermostability of the hemagglutinin at 56°C. Three antigenic groups were identified by a hemagglutination-inhibition assay using NDV monoclonal antibodies. And the predominant gross lesions were as follows: discharge from the nasal cavity, tracheal mucus, petechial hemorrhage in the heart fat, kidney urates and hemorrhage with or without necrosis in the gastrointestinal tract. Severe hemorrhagic or necrotic lesions were also noted in the lymphoid organs and were localized primarily in the spleen and cecal tonsil. However, differences in the occurrence and frequency of the gross lesions were observed between the virus strains. Among them, NDV strains that induced neurological symptoms belonged only to genotype VI. This strain had spread throughout Korea during the late 1980s to the 1990s, which suggests that specific VVNDVs genotypes might result in neurological symptoms.

Keywords : avian paramyxovirus, Newcastle disease, pathogenicity, velogenic neurotropic, viscerotropic velogenic

Introduction

Newcastle disease (ND) is a major disease of poultry that results in severe economic losses in the poultry industry worldwide. Ever since it was first reported in 1927, panzootic viscerotropic velogenic ND (VVND) has been observed throughout Asia, Africa and Central and South America [2, 4]. Since 1991, an increase in frequency of velogenic ND epidemics in European countries has also been a serious problem [3]. Outbreaks of ND, primarily in pigeons and doves, caused by a variant ND virus (the 'pigeon paramyxovirus type 1 (PMV-1) variant') have been detected and observed to have spread throughout Europe [3, 11, 12].

Periodic VVND epizootics in Korea have occurred continually every 3 to 5 years [19, 30] ever since the first outbreak that was reported in 1927. VVND was known to be present in the country as early as 1924 [4].

According to the severity of clinical signs that manifest in affected birds, ND virus (NDV) has been grouped into the following five pathotypes: viscerotropic velogenic NDV (VVNDV), neurotropic velogenic NDV (NVNDV), mesogenic

NDV, lentogenic NDV and asymptomatic enteric NDV [6]. Clinical manifestations of NDV vary based on the viral strain, host species, age of host, route of exposure to infection and the environment [4, 8, 28]. As such, many attempts have been performed to characterize and differentiate NDV isolates [3, 5, 16, 17, 21, 22, 27] and to determine differences in pathology among different NDV pathotypes [7, 8, 24, 25]. However, few studies have compared the pathogenicity of various VVNDV isolates based on pathological differences between the strains [7, 18].

VVNDV strains usually cause hemorrhagic ulceration with necrosis in the intestinal wall [18, 25], lymphoreticular necrosis [8, 9, 13] and respiratory lesions [8, 9]. Furthermore, there is thought to be considerable variation in pathogenicity depending on the VVNDV strain.

This study was conducted to characterize Korean NDV isolates and to compare the pathological differences of various VVNDV isolates that have been isolated in Korea since the 1980s.

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Materials and Methods

Viruses

Working stocks of 18 NDV isolates and reference strains, including vaccine strains, were prepared using 9- to 10-day-old embryonated specific-pathogen-free (SPF) chicken eggs. The NDV isolates were isolated from commercial chicken flocks in Korea, primarily from chickens that had clinical signs of ND. The Korean (Kr)-D/84 and Kr-M/88 were isolated from clinical cases of ND infection in a peafowl and a quail, respectively.

Of the total 18 isolates, the following 11 were isolated at the National Veterinary Research Institute, Korea: Kr-48/82, Kr-1129/83, Kr-K/84, Kr-D/84, Kr-M/88, Kr-12A/89, Kr-163/90, Kr-9/91, Kr-104/92, Kr-147/97 and Kr-KJW/49. Kr-KJW/49 was isolated in 1949 and used as a reference challenge strain in Korea [26]. The following 7 isolates were isolated at the College of Veterinary Medicine, Seoul National University: 92-76A, 92-76B, 93-58GG, 93-58GS, 94-44, 95-119 and 95-132. The 92-76A and 92-76B isolates originated from the same isolate, and each viral isolate was propagated in SPF eggs after the clones were selected. This was also true for the 93-58GG and 93-58GS isolates. The years of virus isolation have been indicated in a previous paper [26].

The NVNDV reference strain Texas-GB and vaccine strains B1, La Sota and Ulster 2C were obtained from the Southeast Poultry Research Laboratory (SEPR), ARS, USDA, USA [22].

Eggs and chickens

Embryonated SPF eggs for virus propagation and titration were obtained from the SPF white leghorn flock from the SEPR. Day-old SPF white leghorn chicks for the intracerebral pathogenicity index (ICPI) test and 4-week-old SPF white leghorn chicks for the pathogenicity test were hatched from SPF eggs and housed in negative-pressure isolators in the SEPR [20].

Monoclonal antibodies and antiserum

The AVS [31], B79, 10D11, and 15C4 [23] monoclonal antibodies (MAbs) were obtained from NVSL and used to differentiate the NDV strains by a hemagglutination-inhibition (HI) test. The HI reactivity of the MAbs is as follows: AVS only reacts with NDV lentogens, particularly B1 and La Sota [31], 10D11 reacts mostly with NDV mesogens and neurotropic velogens from U.S. poultry, 79 is broadly reactive with avian PMV-1 strains, including most pigeon NDV, and 15C4 is broadly reactive with PMV-1 strains, except for pigeon NDV isolates [23]. Four additional mouse MAbs (P10B8, P11C9, P15D7 and P3A11) were generated at the SEPR by immunization using the VVNDV strain CA 1083 (Fontana). A polyclonal chicken anti-NDV serum was prepared by immunization of SPF white rock chickens with two doses of inactivated NDV La Sota virus. The polyclonal chicken anti-NDV serum was used as a positive serum in the HI test.

Hemagglutination and HI assays

The hemagglutination (HA) and HI tests were performed using conventional microtiter methods. For HI tests, serial twofold serum or MAb dilutions were made in phosphate-buffered saline (PBS), pH 7.2. Four HA units of test antigen were added to each dilution, and the test was then incubated at room temperature for 30 min. An equal volume of 0.5% chicken erythrocytes in PBS was added as a test indicator. The HI endpoint was determined as the last dilution that maintained complete inhibition of HA activity. An aliquot of each viral isolate was used as a live virus antigen to differentiate strains by the HI assay. MAbs were originally diluted 1/10 in PBS. Therefore, HI titers that were less than 1 : 20 were considered negative, titers of 1 : 40 were indeterminate and titers equal to or greater than 1 : 80 were considered positive. Indeterminate reactions were considered negative if no increase in the titer was determined after a retest.

A La Sota virus that was inactivated with β -propiolacton was used as the antigen in HI test for chicken sera that were collected from the survivors of the ICPI and pathogenicity test.

Elution rate and HA thermostability

The stability of the virus attachment to chicken erythrocytes was determined the elution rate at 4°C. The thermostability of the virus hemagglutinin at 56°C was determined by standard procedures [14]. The HA titer was assayed after 0, 5, 10, 15, 30, 60 and 120 min of incubation at 56°C. The thermostability time was determined as the longest treatment period that the sample maintained a residual HA titer equal to or greater than 1 : 8.

ICPI test

The ICPI test in one-day-old white leghorn chicks was performed as described previously [1]. Hatchmates that were inoculated with virus diluent by the same route of infection served as negative controls. NDV infection of survivors after the ICPI assay was confirmed by an HI test of chicken sera that was collected from all birds at the end of the 8-day observation period after inoculation.

Pathogenicity test

Clinical signs, mortality and gross pathological findings after inoculation with a NDV isolate by eye drop method were measured in 4-week-old SPF white leghorn and white rock chickens. For two weeks, chickens were observed daily for clinical signs of depression, conjunctivitis and eyelid swelling, respiratory symptoms, diarrhea and neurological symptoms, including incoordination, tremor, torticollis and paralysis. All birds that died during the experiment or that were euthanized at the end of the experimentation time frame were necropsied for the evaluation of pathological lesion. NDV infection of the chickens that survived was confirmed by a HI test of the sera collected from all birds at the end of the 14 day observation period after inoculation.

Hatchmates that were inoculated with the NVNDV Texas GB strain by the same route served as a positive control. Hatchmates infected with a NDV B1 strain and non-inoculated birds served as negative controls for the pathogenicity evaluations.

Reverse transcription-polymerase chain reaction and gene sequencing

Viral genomic RNA was extracted from infective allantoic fluid using the Viral Gene-spin kit (iNtRON biotechnology, Korea), according to the manufacturer's instructions. Complementary DNA fragments from the N-terminal region of the F gene, including the fusion protein cleavage site and the matrix protein gene, were amplified using the AccuPower Reverse transcription-polymerase chain reaction (RT/PCR) PreMix kit (Bioneer, Korea) and a previously described primer set (M1055 and F508) [26]. The amplified DNA fragments

were purified using the Gel purification kit (iNtRON biotechnology, Korea). The DNA sequence was determined using the Dye Terminator Cycle sequencing method and analyzed using the ABI 377 Autosequencer (PE Applied Biosystems, USA).

Sequence analysis

Assembly of sequencing contigs and translation of the nucleotide sequence into protein sequence were performed using the VectorNTI suite 10 program (Invitrogen, USA). Using the CLUSTAL W multiple alignment algorithm, sequence data were aligned by the MegAlign program in the Lasergene package (DNASTAR, USA). Sequences were aligned using Clustal-X [32]. Phylogenetic trees were constructed using the neighbor-joining method within Clustal X with 1,000 bootstrapping replicates. TreeView [29] was used to display the phylogenetic trees.

Table 1. Biological and physical characteristics of NDV isolates

NDVs	Elution rate*	Thermo-stability†	ICPI	HI pattern with a battery of MAbs‡							
				B79	AVS	10D11	15C4	P10B8	P11C9	P15D7	P3A11
Kr-48/82	R	10	1.89	+§	–	±	–	+	–	–	–
Kr-1129/83	R	15	1.88	+	±	±	–	+	–	–	–
93-58GG	R	120	1.81	+	±	–	–	+	–	–	–
92-76A	R	0	1.94	+	–	–	–	+	–	–	–
93-58GS	R	0	1.91	+	–	–	–	+	–	–	–
94-44	R	90	1.89	+	–	–	–	+	–	–	–
Kr-147/97	R	>120	1.9	+	–	–	–	+	–	–	–
Kr-KJW/49	R	15	1.99	+	–	–	+	–	+	+	+
Kr-K/84	R	30	1.88	+	–	–	+	±	+	+	+
Kr-12A/89	R	30	1.88	+	±	–	+	–	+	+	+
Kr-M/88	R	>120	1.85	+	±	–	+	+	+	+	+
Kr-163/90	S	>120	1.89	+	–	–	+	+	+	+	+
Kr-9/91	S	>120	1.88	+	–	–	+	+	+	+	+
Kr-104/92	R	60	1.9	+	–	–	+	+	+	+	+
95-119	R	30	1.9	+	–	–	+	+	+	+	+
95-132	R	60	1.91	+	–	–	+	+	+	+	+
92-76B	R	30	1.88	+	–	–	+	+	+	+	+
Kr-D/84	I	90	1.88	+	–	–	+	+	+	+	+
(76A+76B)¶	n.d.¶	n.d.	1.88	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
(58GS+GG)¶	n.d.	n.d.	1.88	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
B1	R	n.d.	0.15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
La Sota	S	5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ulster 2C	n.d.	>120	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Texas GB	n.d.	n.d.	1.85	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

NDV: Newcastle disease virus, ICPI: intracerebral pathogenicity index, HI: hemagglutination-inhibition, MAb: monoclonal antibody. *Elution rate given as rapid (R), intermediate (I), or slow (S). †Thermostability of hemagglutinin at 56°C given in min. ‡Reactivity of MAbs; B79: all paramyxovirus type 1 (PMV-1) including most pigeon PMVs, AVS: lentogens, 10D11: most mesogens and neurotropic velogens from U.S. poultry, 15C4: all PMV-1 except pigeon PMV. §±: HI titer 2 log₂, +: HI titer higher than 3 log₂. MAbs were originally diluted in 1/10, therefore, HI titer lower or less than 1 indicates a negative reaction (–). ¶76A+76B and 58GS+GG mean combined subclones with 92-76A and 92-76B, and 93-58GS and 93-58GG, respectively, in which each combination were mixed with an equal volume of the individual subclone. ¶not done.

Results

Elution rate and thermostability of NDV isolates

Of the 18 total isolates, a rapid elution rate was observed for 15 isolates, a slow rate for 2 isolates and an intermediate rate was observed for one isolate. The reference NDV vaccine strains La Sota and B1 were observed to have a slow and rapid elution rate, respectively (Table 1).

The thermostability of the hemagglutinin of the NDV isolates varied from 0 to 120 min. Five of the isolates that had been isolated since 1988 were observed to have a thermostability in excess of 120 min, which is similar to the thermostable vaccine strain Ulster 2C. Differences in the thermostability were evident between the 93-58GS (0 min) and the 93-58GG (120 min) NDV isolates, which were clone-picked from the same isolate.

Reactivity pattern to MAb

After analysis by the HI test, the 18 NDV isolates were observed to have three distinct binding patterns against 8 MAbs. All of the isolates were inhibited by the MAb B79 but not by the MAbs AVS or 10D11.

For the remaining 5 MAbs, 7 isolates were inhibited by only MAb P10B8, but these isolates were not inhibited by MAb 15C4. The MAb 15C4 reacted with all of the PMV-1 isolates except for the pigeon PMV. Three isolates, including the reference challenge virus in Korea Kr-KJW/49, were not inhibited by MAb 15C4 alone. The remaining 8 isolates were observed to have a strong inhibition reaction with the 5 MAbs (Table 1).

Pathotyping

All of the 18 isolates were of the velogenic pathotype. The isolates had a high pathogenicity, as indicated by an ICPI that ranged from 1.99 (Kr-KJW/49) to 1.81 (93-58GG). The ICPI of the B1 strain and the NVNDV Texas-GB reference strain were 0.15 and 1.85, respectively (Table 1).

Clinical symptoms

A majority of the 4-week-old white leghorn chicken groups that were inoculated with the viral isolates had indications of severe depression, conjunctivitis and eyelid swelling with watery eye within 2 to 4 days post-inoculation. The incubation period ranged from 2 to 4 days. Infection with 92-

Table 2. Clinical symptoms and mortality following eye-drop inoculation of NDV isolates in 4-week-old SPF Leghorn chicks

NDVs*	Genotype	No. of the inoculated	No. of the dead/ Day of death [†]	Clinical symptoms [‡]		
				Conjunctivitis	Depression	Neurological sign
Kr-48/82	<u>V</u> [§]	5	5/6, 7	5	5	0
Kr-1129/83	<u>V</u>	5	5/4, 5	5	5	0
Kr-K/84	<u>V</u>	5	5/4, 5	5	5	0
Kr-D/84	<u>VII</u>	5	5/3, 4	5	5	0
Kr-M/88	<u>VI</u>	5	5/7, 8	5	5	0
Kr-12A/89	<u>VI</u>	5	5/5, 6	5	5	1/5
Kr-163/90	<u>VI</u>	5	5/4	5	5	0
Kr-9/91	<u>VI</u>	5	5/4, 6	5	5	0
Kr-104/92	<u>VI</u>	5	5/6, 7	5	5	1/5
92-76A	VI	5	5/4, 5	5	0	2/3,5
92-76B	VI	5	3/5, 6	5	2	2/5,13
93-58GS	VI	5	5/4, 5	5	5	3/3,4
93-58GG	VI	5	0	5	5	1/11
94-44	VI	5	5/5	5	5	2/4
95-119	VII	5	5/5-7	5	5	0
95-132	VII	5	5/5, 6	5	5	0
Kr-147/97	VI	5	5/5, 6	5	5	2/5
Kr-KJW/49	III	5	5/4, 5	5	5	0
B1	II	10	0	0	1	0
Texas GB	II	10	10/5, 6	10	0	9/4,5
Control	—	10	0	0	0	0

NDV: Newcastle disease virus, SPF: specific-pathogen-free. *Each group was inoculated with 10^5 EID₅₀ of a NDV isolate by eye drop method and observed for 2 weeks. [†]Days post-inoculation when death occurred. [‡]Conjunctivitis included swelling of the eyelid. Neurological signs were incoordination, tremor, paralysis of the leg or torticollis of the neck (No. of chicks/days post-inoculation when signs appeared). [§]Underlined genotypes were described in the previous paper [26]. ^{||}All survived chicks were seroconverted 2 weeks post-inoculation except a control group.

76A or the NVNDV Texas GB strain resulted in no conjunctivitis or eyelid swelling. Eight isolates that have been isolated since 1989 induced neurological symptoms of disease, including incoordination, head tremor, paralysis of the leg or torticollis of the neck. Among these 8 isolates, the 93-58GS was observed to have more dramatic neurotropic characteristics (Table 2).

With the exception of 92-76B and 93-58GG, all of the isolates induced 100 % mortality within 3 to 8 days post-inoculation. However, the day of death after inoculation varied considerably depending on the isolate. Chicks that were inoculated with Kr-D/84 all died within 3 to 4 days after inoculation, but chickens infected with isolate Kr-M/88 died within 7 to 8 days after inoculation. A sharp cough and greenish diarrhea were frequently observed for chickens in several of the experimental groups.

For the group that was inoculated with the NVNDV Texas GB, 10 birds died within 5 to 6 days after inoculation and presented with neurological symptoms within 4 to 5 days after inoculation. Although there appeared to be an increase in the number of birds with neurological symptoms, this increase was a result of the lower mortality rate. Thus, as a consequence, there was an increase in the number of birds suffering from convalescent torticollis or paralysis.

Gross pathological findings

As shown in Table 3, the primary gross findings in the internal organs in almost all of the white leghorn birds inoculated with the 18 NDV isolates were mucous nasal discharge, mucus in the trachea, petechial hemorrhage in the heart fat and kidney urates. The Texas GB strain also caused petechial hemorrhage in the heart fat.

In the respiratory tract, nasal discharge and tracheal mucus were commonly present in all of experimental groups. Tracheal mucus without nasal discharge was found in all of the birds inoculated with the NVNDV Texas GB. Tracheal reddening, petechia, or necrosis along the tracheal ring was rarely observed. Hemorrhage in the lungs was frequently observed in birds that were inoculated with 4 isolates (Kr-163/90, Kr-9/91, 92-76A and Kr-147/97). The air sac was observed to have a normal appearance after inoculation.

In the lymphoid organs, small, discrete necrotic foci in the spleen were observed. This was particularly evident for the Kr-163/90, Kr-9/91 and 95-119 isolates. Hemorrhage in the thymus was also a common lesion. However, the number of birds with thymus lesions was not as numerous as those that were observed to have spleen necrosis. Hemorrhage in the spleen and bursal edema was also observed on occasion.

Table 3. Gross findings in the internal organs in SPF Leghorn chicks inoculated with NDV isolates at 4 weeks of age

NDV*	Trachea [†]	Lung [‡]	Hemorrhage [§]					Spleen		Kidney urate	Edema	
			L	H	P	M	T	HE	N		BF	Subcue
Kr-48/82	5	0	1	4	0	0	0	1	0	5	0	0
Kr-1129/83	5	0	5	5	0	0	2	0	1	5	0	0
Kr-K/84	5	1	2	4	0	0	2	0	2	5	0	0
Kr-D/84	5	0	1	2	0	0	0	0	4	1	0	0
Kr-M/88	2	0	0	2	0	0	1	0	0	4	0	0
Kr-12A/89	4	0	0	3	0	0	2	1	3	5	0	0
Kr-163/90	5	3	0	5	1	0	0	0	5	5	0	0
Kr-9/91	4	3	0	2	0	0	0	0	5	5	0	0
Kr-104/92	5	1	0	3	0	0	1	0	1	5	0	0
92-76A	4	3	0	2	1	0	1	0	1	5	0	0
92-76B	2	0	0	1	0	0	0	0	0	1	0	0
93-58GS	3	0	0	0	1	4	1	1	0	3	0	0
93-58GG	0	1	0	4 [¶]	0	0	0	2	0	0	0	0
94-44	5	1	2	3	1	0	1	1	4	5	1	3
95-119	5	0	1	4	2	0	1	0	5	5	4	0
95-132	5	0	0	3	0	0	1	0	1	5	1	0
Kr-147/97	5	3	2	3	1	1	2	0	1	4	0	0
Kr-KJW/49	5	1	0	2	0	0	0	0	0	5	0	0
Texas GB	9	0	0	4	0	0	0	0	0	0	0	0

SPF: specific-pathogen-free, NDV: Newcastle disease virus. *Five chicks were inoculated in each experimental group with 10^5 EID₅₀ of a NDV isolate by eye drop method and were observed for necropsy for 2 weeks. For the Texas-GB group 10 chicks were used. [†]Tracheal lesions included mucus, reddening and/or necrosis. [‡]Lesions in lungs included reddening and hemorrhage. [§]Hemorrhage (HE) and/or necrosis (N) in the larynx (L), heart fat (H), pancreas (P), rib or thigh muscle (M), thymus (T). ^{||}BF: bursa of fabricious, Subcue: subcutaneous tissue of the neck. [¶]One chick showed hemorrhage only in the heart muscle, and the other three revealed flaccid heart muscles without hemorrhage.

Table 4. Gross findings in the gastrointestinal tract in SPF Leghorn chicks inoculated with NDV isolates at 4 weeks of age

NDV*	HE in proventriculus and/or gizzard	Sub-total	HE, N and/or D of small intestines									HE in cecal tonsil	HE and/or N in large intestines			
			Duodenum			Jejunum			Ileum				Colon		Cloaca	HE
			HE	N	D	HE	N	D	HE	N	D		HE	N		
Kr-48/82	4 (2) [†]	2 [*]	0	1	0	0	0	0	0	0	1	0	0	0	0	0
Kr-1129/83	5	5	4	2	2	0	0	1	1	0	3	4	0	0	0	0
Kr-K/84	5	5	5	3	0	0	0	1	5	0	3	1	0	1	0	1
Kr-D/84	4	5	4	2	1	2	1	3	2	1	5	5	0	0	0	0
Kr-M/88	3	3	2	1	1	0	0	1	1	0	3	0	0	0	0	0
Kr-12A/89	5	5	3	5	1	2	0	2	3	0	5	2	0	0	0	0
Kr-163/90	5 (1)	5	2	5	1	0	1	1	3	1	5	2	0	0	0	0
Kr-9/91	5 (1)	5	4	5	0	1	1	2	5	1	5	1	2	0	0	0
Kr-104/92	3 (2)	3	1	2	0	0	1	2	2	1	2	1	0	1	0	1
92-76A	4	5	4	1	2	1	0	2	1	0	4	1	0	1	0	1
92-76B	2	1	0	0	0	0	1	1	1	1	2	1	0	3	0	3
93-58GS	2 (1)	4	4	0	2	0	0	3	0	0	5	1	0	0	0	0
93-58GG	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
94-44	5 (2)	5	5	3	0	0	0	5	3	0	5	3	0	1	0	1
95-119	5	4	3	3	1	1	1	2	2	1	3	4	1	1	0	1
95-132	5 (2)	4	2	2	0	0	0	1	4	0	3	1	1	1	0	1
Kr-147/97	5 (2)	4	4	4	2	2	0	3	4	0	5	3	1	2	0	2
Kr-KJW/49	5	5	5	0	0	0	2	3	2	0	3	1	0	0	0	0
Texas GB	3 [§]	3	3	0	0	0	0	0	0	0	0	1	0	0	0	0

SPF: specific-pathogen-free, NDV: Newcastle disease virus. HE: hemorrhage, N: necrosis, D: distention. ^{*}Five chicks were inoculated in each experimental group with 10⁵ EID₅₀ of a NDV isolate by eye drop method and were observed for necropsy for 2 weeks. For the Texas-GB group, 10 chicks were used. [†]No. of chicks with hemorrhage (No. of the gizzard hemorrhage in the parenthesis). ^{*}No. of chicks showed any pathological lesions in the small intestines. [§]Slight focal reddening.

However, the appearance of gross lesions in certain organs varied based on the NDV isolate. Hemorrhage in the larynx (Kr-1129/83), hemorrhage in the rib or thigh muscle (93-58GS), severe necrotic foci in the spleen (Kr-163/90, Kr-9/91 and 95-119), BF edema (95-119) and subcutaneous edema in the neck (94-44) were clearly present when chickens were infected with certain isolates. Unusually flaccid hearts (3/5) were observed in only one of the groups. This group was inoculated with the 93-58GG isolate that did not develop significant lesions in any other organs. Small discrete hemorrhages in the pancreas were occasionally observed, and thymus atrophy was rarely found in 3 birds from 3 different groups (Kr-1129/83, 92-76B and 93-58GG).

The gross lesions in the gastrointestinal (GI) tract in white leghorn chicks were severe hemorrhage in the proventriculus, duodenum, ileum and cecal tonsil, and ulcerative necrosis associated with the Payer's patch. Lesions in the jejunum were rare (Table 4). Some isolates resulted in hemorrhage in the gizzard and cloaca, distended small intestines, and hemorrhage and necrotic foci in the colon. There were no gross lesions in birds that were inoculated with isolate 93-58GG,

except for hemorrhage in the cecal tonsil. The birds that were inoculated with Kr-48/82 presented only with hemorrhage in the proventriculus and gizzard without significant lesions in any other GI tract locations. The appearance of gross lesions in the GI tract varied considerably depending on the NDV isolate. With the Texas GB strain, there were slight hemorrhages in the proventriculus and duodenum (3/5) but not in the cecal tonsil.

Phylogenetic analysis

The sequence of the F gene was compared by phylogenetic analysis to genotype the following isolates: 92-76A and B, 93-58GS and GG, 94-44, 95-119, 95-132 and Kr-147/97. Based on the topology of the tree, the recent Kr isolates could be classified into the following 3 genotypes: V, VI and VII. The isolates that were isolated early in 1980s belonged to genotype V, those isolated in the late 1980s to the early 1990s belonged to genotype VI, and those that were isolated in 1995 belonged to genotype VII. However, the peafowl isolate Kr-D/84 was genotype VII (Table 2). The Korean reference challenge strain, Kr-KJW/49, was genotype III.

Discussion

Because infection with NDV in chickens appeared to vary from unapparent to peracute disease resulting in high mortality [4], it has recently been suggested that the epidemiology and pathogenicity of NDV is similar to that of an avian influenza virus [2].

The characterization and pathotyping of NDV have been essential and reliable tools for the establishment of control and eradication policies, and characterization depends primarily on the mean time of death, ICPI and intravenous pathogenicity test. Regardless of pathogenicity, there are several methods for characterizing the biological properties of NDV, such as the elution rate of agglutinated red blood cells, thermostability of the hemagglutinin and the ability to form a plaque [1, 4].

Almost all of 18 NDV isolates were observed to have a rapid elution rate. For the thermostability of hemagglutinin, thermostable isolates were more common than thermolabile isolates, which supports previous data where 13 out of 18 NDV isolates that were isolated from 1945 to the early 1960s were observed to have heat stable hemagglutinin (unpublished data, 1963). However, it has also been reported that heat stable hemagglutinin (≥ 30 min at 56°C) was only found in NDV strains that were isolated since 1988 in 16 isolates that were collected from 1984 to 1991 in Korea [10]. In this experiment, strong thermostability (≥ 120 min) was observed for all isolates that were collected after 1988. The increased frequency of NDV with strong thermostability might have been involved in the recent, sporadic cases of ND in the summer in Korea. ND had been very rare in the prior year before these sporadic cases were reported. Difference in the thermostability was found between the 2 subclones that were prepared by the clone-picking method: 93-58GG and 93-58GS. It may be important that different clones in the same MAb group had different pathogenicity profiles in 4-week-old chicks.

Two other subclones prepared by the same method, 92-76A and 92-76B, were observed to have markedly different HI pattern when compared against a battery of 8 MAbs. Moreover, the 92-76A and 93-58 GS subclones resulted in syncytial cell formation in chicken embryo fibroblast cell cultures, while the 92-76B and 93-58GG subclones resulted in degenerative cytopathic effect. This result confirmed that field NDV isolates consisting of various subpopulations may be significantly different from each other [15].

Rapid elution rates were observed for the NDV Kr-KJW/49 isolate, which is the reference challenge virus in use since 1950 in Korea.

As a result of the HI analysis using a battery of 8 MAbs, the 18 NDV isolates were divided into 3 distinct groups. There were no isolates that fell into lentogens, mesogens or neurotropic velogens. These data were in accordance with the pathotyping analysis and pathogenicity test, which demonstrated no reaction with AVS for lentogens or with 10D11 for

most mesogens and neurotropic velogens isolated from U.S. poultry [22, 23]. It was not thought that the weak HI reaction (\pm HI titer $2 \log_2$) observed for some isolates with AVS or 10D11 was a result of reacting against a common lentogen or mesogen antigen, respectively. These results may be the result of nonspecific HA inhibition. Seven of the NDVs that were isolated from chickens were observed to have no reaction with 15C4, which was observed to react with all PMV-1 isolates except for the pigeon PMV.

The MAb reaction profiles for the NDVs may not reveal the exact antigenicity patterns because the MAbs used in the present study do not cover all antigenic groups for NDV. Therefore, the study should be confirmed using a more varied MAb profile.

A majority of the 18 NDV isolates caused mortality accompanied by depression, conjunctivitis and swelling of the eyelid, as described previously [4, 13, 25]. The 93-58GG was determined to have an ICPI of 1.81 but caused no mortality or gastrointestinal lesions except for hemorrhage of the cecal tonsil. Because the chickens that were inoculated with this isolate lived for the duration of the experiment and were necropsied at the end of the 2-week observation period after inoculation, observable lesions may have become less severe or may have fully resolved at the time of necropsy. No relationship between the day of death and ICPI were observed; however, 2 isolates that had an ICPI less than 1.85 were observed to result in delayed mortality or no mortality.

Neurological symptoms, including incoordination, tremor, torticollis and paralysis [4], appeared in birds that were inoculated with 7 isolates. In addition, the neurological symptoms appeared in 2 distinct patterns according to the time after inoculation. Some isolates caused neurological symptoms that were accompanied with other symptoms prior to the death of the chicken. Other isolates caused convalescent torticollis of the neck and paralysis of the leg after 10 days of inoculation. Isolates that were observed to have low pathogenicity were included in this latter group. However, the VVNDV isolates that resulted in intestinal pathological lesions were easily classified as having neurological symptoms. Nevertheless, clinical symptoms and pathological lesions as a result of NDV were varied depending on the strains of virus, pathogenicity of the virus, the host species, susceptibility of the host and the environment [4, 8, 28].

The predominant gross lesions that were induced by all of the NDV isolates were conjunctivitis with hemorrhage in the inner eyelid, nasal discharge, tracheal mucus, kidney urates, severe necrosis in the spleen and hemorrhage of the heart fat, proventriculus, duodenum and cecal tonsil. In addition, bursal edema and subcutaneous edema in the neck were observed for the 95-119 and 94-44 isolates, respectively. However, the total numbers of cases that were observed to have lesions was significantly higher in cecal tonsil (64/90) and duodenum (53/90) when compared to other organs. As previously described [25], VVNDVs primarily developed hemorrhage with necrosis in the duodenum, ileum and cecum and hemor-

rhage in the mucosa and submucosa of the proventriculus and gizzard. NDVs have differences depending on the pathotypes and genotypes VVNDVs, although further study is needed to confirm this hypothesis. VVNDV has been known to cause predominant GI, respiratory tract and lymphoid tissue lesions [13, 18], and the spleen is more susceptible to the necrotic effects of NDV [13].

There were considerable differences in clinical symptoms and gross findings between NVNDV and VVNDV. This was especially true for neurological symptoms [4, 8]. Compared to the VVNDVs, NVNDVs induced severe neurological symptoms prior to death, no conjunctivitis, no kidney urate and no visible necrosis or hemorrhage in the GI tract, except in the proventriculus and duodenum. Unexpectedly, in the present study, it was observed that NVNDV induced slight focal reddening in the proventriculus as well as hemorrhaging in the duodenum. Because the presence of hemorrhagic lesions in the intestine of infected chickens has been a criteria to distinguish VVNDV from NVNDV [9, 16], hemorrhage in the proventriculus and duodenum could be eliminated from this classification scheme. All of the isolates that were tested in this experiment were classified as VVNDV. Nevertheless, mesogens, NVNDV and lentogens may still be circulating in poultry farms in Korean because all of the isolates were obtained from chickens that became ill.

The Korean NDV isolates were classified into three different genotypes by phylogenetic analysis of F gene, V, VI and VII, as described previously [26]. Each of the three genotypes may correspond to ND epidemics that occurred in the 1980s, late 1980s to early 1990s and after 1995, respectively. Although there was little direct correlation between the NDV tissue tropism and the genotype, all of the isolates that induced neurological symptoms belonged to genotype VI. This genotype had spread nationwide during the late 1980s through the 1990s, and these data support a previous report [19, 26]. Therefore, specific genotypes of VVNDVs may cause neurological symptoms in chickens.

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