Pathotypical Characterization and Molecular Epidemiology of Newcastle Disease Virus Isolates from Different Hosts in China from 1996 to 2005[†]

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Thirty Newcastle disease virus (NDV) strains isolated from outbreaks in China during 1996 to 2005 were characterized pathotypically and genotypically. All strains except one were velogenic. An analysis of the variable region (nucleotides 47 to 420) of the F gene indicated that 6 isolates belonged to genotype II, 3 to genotype III, 1 (isolated from a pigeon) to genotype VI, and 20 to genotype VII. Isolates belonging to genotype VII were further divided into five subtypes, VIIa, VIIb, VIIc, VIId, and VIIe, and subtype VIId was made up of VIId1 to VIId5. These results showed that genotype VII isolates might have been the most prevalent in China during the past two decades. Genotype VII isolates shared high homology, but the homology was less than that between genotype VII viruses and the vaccine virus LaSota. Among these NDV isolates, 25 isolates had the velogenic motif ¹¹²R/K-R-Q-K/R-R-F¹¹⁷ that is consistent with results of the biological tests. However, four of five LaSota-type isolates that contained the lentogenic motif ¹¹²G-R-Q-G-R-L¹¹⁷ were velogenic, except SY/03, in the view of the biological test. The majority of genotype VII isolates had lost one or two N-glycosylation sites. Finally, a cross-protection experiment in which specific-pathogen-free chickens vaccinated with LaSota were challenged by six NDV isolates showed that more than three isolates were antigenic variants that could be responsible for recent outbreaks of Newcastle disease.

Newcastle disease (ND) is one of the most serious infectious diseases affecting birds, particularly poultry, worldwide and has been the cause of serious economic losses (1, 3). The etiological agent of ND, Newcastle disease virus (NDV) or avian paramyxovirus type 1, belongs to the *Avulavirus* genus, *Paramyxoviridae* family, *Mononegavirales* order and has a negative-sense single-stranded RNA genome of approximately 15,186, 15,192, or 15,198 nucleotides (nt) that encodes six proteins: nucleocapsid protein, phosphoprotein, matrix protein, fusion (F) protein, hemagglutinin-neuraminidase (HN), and a large RNA-directed RNA polymerase (6, 12).

NDV isolates are characterized according to the results of index in vivo pathogenicity tests and/or molecular determinants of the F protein cleavage site. Generally those NDV isolates resulting in severe outbreaks all had an intracerebral pathogenicity indices (ICPI) of 0.70 or greater in day-old chickens and intravenous pathogenicity indices (IVPI) of 1.40 or greater in 6-week-old chickens (2, 28). Previous studies of the F₀ precursor amino acid sequence of NDV that varied in virulence for chickens showed that the virulent isolate had the motif ¹¹²R/K-R-Q-K/R-R¹¹⁶ at the C terminus of the F₂ protein and a phenylalanine at residue 117 located at the N terminus of the F₁ protein, while weakly virulent viruses had the motif ¹¹²G/E-K/R-Q-G/E-R¹¹⁶ at the C terminus of the F₂ protein and a leucine at residue 117 (11). Therefore, the F

protein cleavage site sequence usually is used as a virulence criterion (8).

In the past three decades, due to a strict vaccination policy, outbreaks of ND were mild and sporadic, resulting in reduced deaths in chicken flocks throughout China. The sporadic cases, which showed few of the typical clinical and pathological manifestations of ND, such as acute diarrhea or dyspnea and hemorrhagic enteritis or tracheitis (29), were named atypical ND. The atypical ND cases were serious to the hens in the peak of production and have the potential to cause severe losses. Since 2001, ND has become increasingly common in broiler parents in the peak of production in Shandong, Jiangsu, Tianjin, Guangdong, and Hebei. Atypical ND differs from classical ND because of the higher hemagglutination inhibition titer antibody levels (log₂ 2⁸ to 2¹¹) in affected flocks (19). In this paper, 30 NDV isolates recovered from different hosts in China from 1996 to 2005 were characterized biologically and molecularly. The epidemiology of ND was evaluated by molecular analyses of the nucleotide sequence and deduced amino acid sequence of the F protein gene. The study provides a more detailed understanding of NDV that may help prevent future outbreaks of ND.

MATERIALS AND METHODS

Viruses. Thirty NDV isolates were recovered from different hosts, including chickens, broilers, geese, a duck, a pigeon, and a penguin from several regions of China during 1996 to 2005 (Table 1). Mortality during the outbreak varied from 90% in young chickens to less than 2% in hens; however, egg production dropped from 90 to 40%. Clinical signs of the disease were similar among most poultry farms. Filtrates of processed tissues from the trachea, oviduct, brain, and spleen from different hosts were used to inoculate specific-pathogen-free (SPF) eggs (Institute of Shandong Poultry Science) as previously reported (9, 16, 20, 25). All

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TABLE 1. Pathogenicity and phylogenetic analysis of the NDV isolates from China

F gene GenBank accession no.	Strain	Abbreviation	F ₀ cleavage site (aa ^a 112–117)	ICPI ^b	IVPI ^c	Virulence ^c	Genotype
DQ227248	Broiler/Shandong/SGM/01	SGM/01	RROKRF	1.78	2.41	V	VIId
DQ363531	Goose/Jiangsu/JS04/04	JS04/04	RROKRF	1.95	2.67	V	VIId
DQ363535	Layer/Shandong/SF/02	SF/02	RROKRF	1.81	2.69	V	VIId
DQ363536	Layer/Tianjin/TJ05/05	TJ05/05	RROKRF	1.47	2.30	V	VIId
DQ227251	Broiler/Shandong/SKY/03	SKY/03	RROKRF	1.89	2.67	V	VIId
DQ417110	Goose/Jiangsu/JS01/01	JS01/01	RROKRF	1.91	2.56	V	VIId
DQ363534	Goose/Jiangsu/JS03/03	JS03/03	RROKRF	1.60	0.75	V	VIId
DQ227253	Broiler/Shandong/SPY/03	SPY/03	RROKRF	1.92	2.11	V	VIId
DQ228922	Broiler/Shandong/SL/03	SL/03	RROKRF	1.94	2.36	V	VIId
DQ227247	Chicken/Shandong/SDD/01	SDD/01	RROKRF	2.00	2.63	V	VIId
DQ227249	Broiler/Shandong/SQD/04	SQD/04	RROKRF	1.96	2.80	V	VIId
DQ363533	Broiler/Shandong/SCL/03	SCL/03	RROKRF	1.91	2.66	V	VIId
DQ363530	Layer/Shandong/WHZ/03	WHZ/03	RROKRF	1.94	2.33	V	VIId
DQ368683	Broiler/Guangdong/GD/05	GD/05	RROKRF	1.23	1.93	V	VIId
DQ417111	Chicken/Shandong/SSX/03	SSX/03	RROKRF	1.95	2.66	V	VIId
DQ363538	Broiler/Shandong/Lye/01	Lye/01	RROKRF	1.88	2.70	V	VIId
DQ363537	Broiler/Shandong/Jlan/04	Jlan/04	RROKRF	1.88	2.16	V	VIId
DQ227254	Broiler/Shandong/SWS/03	SWS/03	RROKRF	1.91	2.76	V	VIId
DQ227246	Goose/Jiangsu/JS02/99	JS02/99	RROKRF	1.91	2.67	V	VIId
DQ858357	Goose/Jiangsu/YG/03	YG/03	RROKRF	1.85	2.64	V	VIId
DQ417113	Pigeon/Beijing/PB01/96	PB01/96	KROKRF	1.65	ND ^d	V	VII
DQ227252	Broiler/Shandong/SBD/02	SBD/02	RROKRF	1.70	2.70	V	VII
DQ227244	Broiler/Tianjin/TJ03/03	TJ03/03	RROKRF	1.60	0.75	V	VII
DQ858356	Goose/Jiangsu/JS06/03	JS06/03	RROKRF	1.91	2.57	V	VII
DQ417112	Chicken/Shandong/SRZ/03	SRZ/03	RROKRF	1.70	1.91	V	II
DQ227245	Chicken/Shandong/SBZ/02	SBZ/02	GROGRL	1.86	2.49	V	II
DQ228923	Duck/Shandong/SY/03	SY/03	GROGRL	0.46	0.46	L	II
DQ228922	Chicken/Shandong/SQZ/04	SQZ/04	GROGRL	2.00	2.80	V	II
DQ227250	Penguin/Peking/QE01/99	QE01/99	GROGRL	1.81	2.24	V	II
DQ363532	Goose/Jiangsu/JS05/03	JS05/03	GROGRL	1.75	2.58	V	II

^a aa, amino acid.^b A strain with an ICPI below 0.7 was considered to be lentogenic, while those with an ICPI equal to or greater than 1.60 were considered to be velogenic, and those with ICPI values between 0.7 and 1.60 were considered to be mesogenic. The ICPI of avirulent viruses were near 0.00.^c A strain with an IVPI below 1.40 was considered to be lentogenic, while those with an IVPI equal to or greater than 1.40 were considered to be velogenic, and those with ICPI values between 1.40 and 1.60 were considered to be mesogenic. The IVPI of avirulent viruses were near 0.00.^d ND, not done.^e V, velogenic; L, lentogenic.

viruses were purified three times using the virus plaque technique before being propagated in SPF embryonated eggs. Virus stocks grown in allantoic fluids were stored at -70°C until being used.

Biological characterization. The initial characterization of the isolates was performed using the hemagglutination inhibition test with NDV-specific polyclonal antisera (4). Pathotyping was performed using standard procedures to determine the ICPI of day-old chickens and the IVPI of 6-week-old chickens according to the Office International des Epizooties manual of standards (6, 7).

Viral RNA extraction and reverse transcription-PCR (RT-PCR). Viral RNA was extracted from infective allantoic fluid using the RNeasy total RNA isolation system (Promega, Madison, WI) according to the manufacturer's instructions.

The open reading frame (ORF) of the F gene, consisting of a 1,662-bp fragment containing the variable region and the cleavage site sequence (19), was amplified by RT-PCR (21) using Expand RT and the Expand high-fidelity PCR system (Boehringer Mannheim, Germany). RT was performed in a final reaction volume of 20 μl containing 7 μl RNA template, 4.5 μl random primer (5'-ACGGGTAGAA-3'), 2 μl 10-mmol/liter deoxynucleoside triphosphates, 4 μl 5 $\times$ RT buffer, 0.5 μl 40-U/ μl RNasin, and 2 μl 10-U/ μl avian myeloblastosis virus reverse transcriptase. The reaction mixture was incubated at 37°C for 60 min. PCR was performed using the following primers designed according to the alignment of the F gene sequences from GenBank: forward primer, 5'-ATGGGCTCCAAACCTTCTAC-3'; reverse primer, 5'-TTGTAGTGGCTCTCATC-3'. Each PCR was performed with a final reaction volume of 50 μl . The following program was used for PCR amplification: 32 cycles of denaturation (94°C for 1 min), annealing (52°C for 1 min), and extension (72°C for 2 min), followed by a final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis in a 1% agarose gel stained with ethidium bromide.

Nucleotide sequencing and phylogenetic analysis. PCR products were purified using the advantage PCR-Pure kit (Clontech), ligated into pGEM-T Easy vector (system I kit; Clontech), and used to transform *Escherichia coli* DH5 α . The F gene (nt 1 to 1,662) clones were sequenced by Biotech (Shanghai, China).

Nucleotide sequence editing, analysis, the prediction of amino acid sequences, and alignments were performed using the MegAlign program in the Lasergene package (DNASTar Inc., Madison, WI) (14). Phylogenetic trees were constructed by the neighbor-joining method of MEGA 3.1 (15) by a comparison of the nucleotide sequences of the F gene from nt 47 to 420, the so-called nonvariable portion of F gene from nt 421 to 1,662, the complete F gene from nt 1 to 1,662, and the F gene deduced amino acid sequences (23). In addition to the 30 strains described in this study, 38 previously reported reference strains representative of different genotypes also were included for comparison. The deduced F protein amino acid sequences of the cleavage site, the N-glycosylation sites, and the cysteine sites of each strain were analyzed.

Cross-protective test. Three-week-old SPF chicks were vaccinated using live vaccine of the LaSota strain from a commercial source (Shandong Qilu Bioproducts, Ltd., Jinan, China) (3.4×10^6 50% egg infectious doses [EID₅₀]; 0.1 ml/bird) by eye drop, while the control group was injected with phosphate-buffered saline (PBS). Three weeks later, the birds were challenged with the six NDV strains SGM/01, SCL/03, SKY/03, SSX/03, SRZ/03, and F48E9, representing different genotypes (genotype II, one isolate; VII, four isolates; and genotype III, one isolate) (10^8 EID₅₀; 0.1 ml/bird) by the intramuscular route. The birds were kept in isolators in the laboratory animal facility at the Institute of Shandong Poultry Science and observed for signs of disease or death for 14 days postchallenge.

Accession numbers for previously sequenced isolates used in sequence analysis. The GenBank accession numbers of some of the NDV strains utilized here (16, 22, 25, 29) include the following: strain Ch/99, AF358787; Ch/2000, AF358788; GPMV/QY97-1, AF192406; TW/2000, AF358786; JX-2/99, AF458014; JS-2/98, AF458013; CH62/96, AF109880; CH-A7/96, AY028995; Cockatoo/Indonesia/14698/90, AY562985; Taiwan95, U62620; Sterna/Astr/2755/2001, AY865652; ZA 360/95, AF109876; ZW 3422/95, AF109877; Chicken/U.S.(CA)/1083(Fontana)/72, AY562988; ASTR/74, Y18728; Dove/Italy/2736/00, AY562989; Chicken/Italy/3286/00, AY288994; Chicken/Kenya/139/90, AY288997; IT-227/82, AJ880277; GB 1168/84, AF109885; Ch/98-1, AF358785; JS/2/98, AF456439; AF2240, AF048763; Anhinga/U.S.(FI)/44083/93, AY562986; Turkey/USA(ND)/43084/92, AY289001; Mixed species/USA(FL)/Largo/71, AY288987; Chicken/Mexico/37821/96, AY288999; Gamefowl/U.S.(CA)/211472/02, AY562987; F48E9, AY508514; MIY/51, M24701; Australia-Victoria, M21881; Herts/33, AY741404; Queensland/V4, AF217084; Chicken/N.Ireland/Ulster/67, AY562991; TEX/48, M24698; Chicken/USA/Roakin/48, AY289000; B1, AF309418; and LaSota, AF077761.

Nucleotide sequence accession numbers. The GenBank accession numbers for F gene sequences determined in the course of this work are presented in Table 1. Accession numbers for HN genes determined in the course of this work are the following: strain Broiler/ShandongSCL/03, DQ228932; Goose/JiangsuJS01/01, DQ228927; Goose/JiangsuJS03/03, DQ228935; Broiler/ShandongSWS/03, DQ234588; Broiler/ShandongSGM/01, DQ234592; Goose/JiangsuJS02/99, DQ228928; Broiler/ShandongSRZ/03, DQ234584; Broiler/ShandongSKY/03, DQ234583; Broiler/ShandongSSX/03, DQ234581; F48E9, AY997298; and LaSota, AF077761.

RESULTS

Pathogenicity of NDV isolates. The initial biological characterizations of 30 NDV isolates, including the ICPI and IVPI, are presented in Table 1. Twenty-nine isolates were velogenic or mesogenic, having an ICPI ranging from 1.23 to 2.00. One isolate, SY/03 (from a duck), was lentogenic, with an ICPI of 0.46. The majority of ICPI corresponded with the IVPI, with the exception of those for strains TJ05/05, JS03/03, TJ03/03, and GD/05.

Phylogenetic relationship and analysis. The phylogenetic analyses of the 30 NDV isolates characterized in this paper and the 38 reference NDV strains from GenBank were performed using the variable region of the F gene (nt 47 to 420) (Fig. 1). In addition, the so-called nonvariable portion of the F gene (nt 421 to 1662) (Fig. 2), the entire F gene coding region (nt 1 to 1,662) (Fig. 3), and the complete amino acid sequence (residues 1 to 553) of the F protein (Fig. 4) of 61 NDV strains were analyzed.

The 68 NDV strains were divided into eight genotypes (I to VIII) (16, 29). Thirty NDV isolates were individually assigned to four different genotypes: II, III, VI, and VII. Of these strains, 20 (66.7%) belonged to genotype VII, 6 (20.0%) belonged to genotype II, and 3 (10.0%) belonged to genotype III. Only one isolate belonged to genotype VI, and it was a pigeon paramyxovirus type 1-type virus from a pigeon. The results indicated that isolates of genotype VII were the most prevalent in China in the past decade. There were two novel subtypes, including 3 isolates belonging to genotype VIIc (SWS/03, JS02/99, and YG/03) and 17 isolates belonging to VIId, which was made up of the five subtypes VIId1 to VIId5, including the most recent enzootic strains from ND outbreaks in different regions of China in the past decade (Fig. 1). The results indicated that VIId isolates were the subtype most responsible for the most newly emerging strain of virulent NDV, which differed from Taiwan95 (VIIa) and Sterna/Astr/2755/2001 (VIIB) as well as CH62/96 and CZ3898/96

(29). Five genotype II isolates belonged to the LaSota type that corresponded to the poultry vaccines used in particular regions, and one belonged to the TEX/48 type that corresponded to the virulent NDV that emerged in the United States in 1948. For genotype III, there was a better relationship between the three isolates and the typical virulent strain F48E9 found in China in 1946 (30) than between the three isolates and strains with the same genotype as that of Australia-Victoria (Australia) or MIY/51 (Japan).

The results shown in the first four figures are similar, particularly for comparisons of the so-called nonvariable portion of the F gene (nt 421 to 1662), the entire nucleotide coding region, and amino acid sequence, which indicated that the mutations of nucleotides were stochastic. However, based on the variable region of the F gene (nt 47 to 420), SRZ/03 may be a variant isolate belonging to genotype II. On the other hand, based on the nonvariable portion (nt 421 to 1662), the entire coding region, and the complete amino acid sequence of the F gene, SRZ/03 probably belongs to genotype VII (Fig. 2 to 4). This may be a result of recombinant events in SRZ/03. Similar events may have occurred in Chicken/Italy/3286/00 and Chicken/U.S.(CA)/1083(Fontana)/72.

Homology analysis of the nucleotide sequence of and amino acid sequence encoded by the F gene. A comparison of nucleotide and amino acid sequences of the 30 isolates and 31 reference strains showed higher homology among strains having the same genotype. For example, genotype VII isolates showed 96.2 to 99.1% nucleotide homology and 95.5% to 98.7% amino acid sequence homology. In genotype III isolates, the nucleotide homology ranged from 99.2 to 99.6%, and the amino acid sequence homology ranged from 98.7 to 99.6%. These results are similar to those for comparisons of genotypes Australia-Victoria and MIY/51, which share 92.3 to 94.3% nucleotide and 93.9 to 95.7% amino acid sequence homology. For genotype II strains, including all LaSota-type isolates except SRZ/03, the nucleotide homology was 96.3 to 99.3% and homology was 96.4 to 99.6% at the amino acid level.

The homologies of nucleotide and amino acid sequences were relatively low between different genotypes. For instance, the nucleotide and amino acid sequence homologies between the LaSota isolate and various genotypes were the following: genotype VII, 84.0 to 85.7% (nucleotide) and 88.2 to 89.5% (amino acid); genotype III, 88.9% to 89.4% (nucleotide) and 92.2% to 92.4% (amino acid); and genotype VI, isolate PB01/96, 86.4% (nucleotide) and 89.2% (amino acid).

As we have noted, the SRZ/03 isolate had nucleotide homologies of 89.5, 90.9 and 87.3% and amino acid homologies of 93.3, 94.6, and 91.3% with LaSota, TEX/48, and F48E9, respectively. The SRZ/03 homologies with genotype VII isolates were 92.6 to 94.4% (nucleotide) and 92.0 to 93.1% (amino acid). These results indicated that the SRZ/03 isolate is more closely related to genotype VII isolates on the basis of the entire coding regions (nt 1 to 1,662), even though it was classified as genotype II according to the nt 47 to 420 fragment of the F gene (Fig. 1).

Proteolytic cleavage site of the F₀ protein and virulence. The cleavage site motifs and initial biological characterization of the 30 NDV isolates are depicted in Table 1. The results of virulence tests, as determined by the ICPI and IVPI tests, were, for the most part, in accordance with those determined by the

FIG. 1. Phylogenetic relationships of the nucleotide sequences of 68 NDV strains based on a variable portion (nt 47 to 420) of the F gene. Sequences previously published in GenBank are listed in Materials and Methods and Table 1. The phylogram was generated by the neighbor-joining method using MEGA 3.1 software.

sequence of the F protein cleavage site (residues 112 to 117). Of these, 25 virulent isolates shared the cleavage site motif $^{112}\text{R/K-R-Q-K/R-R-F}^{117}$, which is a molecular characteristic of virulent NDV strains. Five isolates contained the $^{112}\text{G-R-Q-G-R-L}^{117}$ motif, which is the same as the motif in LaSota and

is the molecular characteristic of avirulent or low-virulence strains. However, ICPI and IVPI tests showed that isolates SBZ/02, SQZ/04, QE01/99, and JS05/03 were velogenic, which was inconsistent with the results of the phylogenetic analysis. These results indicated that the cleavage site motif of the F_0

FIG. 2. Phylogenetic relationships of the nucleotide sequences of 61 NDV strains based on the so-called nonvariable portion (nt 421 to 1,662) of the F gene. Sequences previously published in GenBank are listed in Materials and Methods and Table 1. The phylogram was generated by the neighbor-joining method using MEGA 3.1 software.

protein is not the only factor that determines the virulence of NDV isolates.

Analysis of the deduced amino acid sequences for F genes. The predicted amino acid sequence of the F protein was 553 residues in length for 30 NDV isolates, with the first 30 residues being the most variable. This hypervariable portion represents a highly antigenic region of the protein that is predicted to be hydrophilic for the first 10 residues.

The predicted N-glycosylation sites of 30 NDV isolates were conserved, and a major transmembrane region was predicted to exist from amino acids 495 to 526. The majority of the isolates had six potential N-glycosylation sites, Asn-X-Ser/Thr (N-X-S/T), located at positions 85 to 87, 191 to 193, 366 to 368,

447 to 449, 471 to 473, and 541 to 543. However, isolates JS01/01, JS02/99, JS03/03, YG/03, SKY/03, and SWS/03 contained an N-to-A alteration at position 543 that resulted in the loss of the N-glycosylation site at positions 541 to 543. Interestingly, four of these six isolates were from geese, suggesting that the NDV mutation is related to water birds.

Cysteine residues were conserved in most NDV isolates. There were 12 cysteine residues located at positions 25, 76, 199, 338, 347, 362, 370, 394, 399, 401, 424, and 523 in the F protein. Isolates SBZ/02 and SY/03 contained a C-to-Y substitution at residue 25, the SBZ/02 isolate contained a C-to-Y substitution at residue 199, and the SQZ/04 isolate contained a C-to-Y substitution at residue 374; these iso-

FIG. 3. Phylogenetic relationships of the nucleotide sequences of 61 NDV strains based on the entire ORF (nt 1 to 1,662) of the F gene. Sequences previously published in GenBank are listed in Materials and Methods and Table 1. The phylogram was generated by the neighbor-joining method using MEGA 3.1 software.

lates all contained a C-to-R substitution and belonged to genotype II. It is unclear why these isolates are velogenic, as all of them contain the lentogenic motif $^{112}\text{G-R-Q-G-R-L}^{117}$ at the cleavage site.

Neutralizing epitopes and antigenic variants of NDV isolates. At least seven neutralizing epitopes, positioned at residues 72, 74, 75, 78, 79, 157 to 171, and 343 of the F protein, have been identified (18, 24, 30). An analysis of the amino acid sequences showed that there were neutralizing epitope variants among NDV strains, including JS04/04 in genotype VIIId (K^{78} for I), AF2240 in genotype VII (A^{79} for P), and Taiwan95 in genotype VIIId (A^{79} for T).

Unique or genotype-specific residue substitutions. Genotype- and subtype-specific residue substitutions in the deduced F protein sequences are listed in Table 2. The genotype II isolates shared unique N^9 -for-I, V^{22} -for-I, I^{32} -for-L, and D^{82} -for-E substitutions with LaSota and TEX/48. Isolate SRZ/03 had an amino acid sequence similar to that of TEX/48 up to residue 272, but after residue 288 the sequence of SRZ/03 was similar to that of subtype VIIc isolates.

For genotype VII, most NDV isolates belonged to subtypes VIIc and VIId, the latter of which was divided into five small clusters, including VIId1, VIId2, VIId3, VIId4, and VIId5 (Fig. 1). As shown in Table 2, the genotype VII isolates shared some

FIG. 4. Phylogenetic relationships of 61 NDV strains base on the deduced amino acid sequence (residues 1 to 553) of the F protein. The nucleotide sequences previously published in GenBank are listed in Materials and Methods and Table 1. The phylogram was generated using the neighbor-joining method of MEGA 3.1 software.

unique amino acid substitutions despite being grouped in different subtypes. The VIIc and VIId isolates shared unique VI⁵²-for-I, K¹⁰¹-for-R, S¹⁷⁶-for-A, and Y³¹⁴-for-F substitutions. Genotype VII, including subtypes VIIa, VIIb, VIIc, and VIId, had four unique and conserved residues at positions 341, 385, 396, and 482. In addition, subtypes VIIa, VIIc, and VIId shared many special residue substitutions, such as K¹⁰¹ for R and S¹⁷⁶ for A. Among genotype VII strains, most residues of the F protein were conserved, with the following exceptions: subtype VIIa, L²⁸-for-P, K¹⁰¹-for-R, and S¹⁷⁶-for-A substitutions; VIIb, S²⁸-for-P, H²⁷²-for-Y, and T²⁸⁸-for-N substitu-

tions; VIIc, K¹⁰¹-for-R, S¹⁷⁶-for-A, and Y³¹⁴-for-F substitutions; and VIId, V⁵²-for-I, K¹⁰¹-for-R, S¹⁷⁶-for-A, and Y³¹⁴-for-F substitutions.

A comparison of the deduced F protein amino acid sequences indicated that there was a closer relationship between genotype II strains (except SRZ/03) and genotype VII strains due to the following substitutions (Table 2): A²⁰ for M, E¹⁰⁴ for G, T¹⁰⁷ for S, I¹²¹ for V, K¹⁹² for N, Q¹⁹⁵ for R, N²⁷² for Y, T²⁸⁸ for N, T³⁴¹ for S, T³⁸⁵ for A, M³⁹⁶ for I, E⁴⁸² for A, and K⁴⁹⁴ for A, as well as the unique substitutions L⁶⁹ for M and G¹²⁴ for S. Genotypes III, IV, and V had similar genetic fea-

TABLE 2. Genotype and subtype-specific residue substitutions in the deduced F protein sequence

Genotype, subtype, and strain(s)	Subtype-specific residue substitution in the deduced F protein sequence for consensus residue:																													
	4	9	16	17	20	22	28	32	52	69	82	101	104	107	121	124	176	192	195	272	288	314	341	385	396	482	494	516	520	
I ^a	R	L ^b	T	V	A	E	-	-	-	L	-	-	E	T	-	G	-	K	Q	N	T	-	T	T	T	M	E	K	I	-
II	R/-	N	A/T	I/V	A	V	-	I	-	L	D	-	E	T	-	G	-	K	Q	N	T	-	T	T	T	M	E	K	I/V	I/-
SBZ/02	-	N	A	I	A	V	-	I	-	L	D	-	E	T	-	G	-	K	Q	N	T	-	T	T	T	M	E	K	I	I
SY/03	R	N	A	I	A	V	-	I	-	L	D	-	E	T	-	G	-	K	Q	N	T	-	T	T	T	M	E	K	I	I
JS05/03	-	N	T	I	A	V	-	I	-	L	D	-	E	T	-	G	-	K	Q	N	T	-	T	T	T	M	E	K	V	I
TEX/48	R	N	T	V	A	V	-	I	-	L	D	-	E	T	-	G	-	K	Q	N	T	-	T	T	T	M	E	K	I	I
SRZ/03	R	N	T	V	A	V	-	I	-	L	D	-	E	T	-	G	-	K	Q	N	-	Y	-	-	-	-	-	-	-	-
VII ^d	R/-	V/-	T	V/I	A	A	L	-	-	-	-	-	E	T	-	-	-	-	Q	N	T	-	T	T	T	M	E	K	I	-
Aus-Vic ^e	R	-	T	I	A	A	L	-	-	-	-	-	E	T	-	-	-	-	Q	N	T	-	T	T	T	M	E	K	I	-
SBD/02	-	V	T	V	A	A	L	-	-	-	-	-	E	T	-	-	-	-	Q	N	T	-	T	T	T	M	E	K	I	-
IV	R	-	-	I	V	T	L	-	-	-	-	-	E	T	-	-	-	-	Q	N	T	-	T	-	-	M	E	-	V	-
V	R/-	L/N/-	T/L/-	I/-	T/A/-	T/-	L/-	-	-	-	-	-	-	T	-	-	-	-	Q/-	N	-	-	-	-	-	M	E/-	K/N	V/L	I/-
VII	-	-	-	-	T	-	L	-	-	-	-	-	-	-	V	-	-	-	-	N	-	-	-	-	-	M	-	-	V	-
VI	T/-	-	-	-	T/-	-	L/-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	L/-	T/-	-	-	M/-	-	K/-	T/-	I/-
PB01/96	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	T	-	-	-	-	-	-	I
VIIa	E/-	V/-	-	-	-	-	L	-	-	-	-	K	-	-	V	-	S	-	-	-	-	-	-	-	-	-	-	K/-	-	-
VIIb	-	-	-	-	-	-	S	-	-	-	-	-	-	-	V	-	-	-	-	H	T	-	-	-	-	-	-	-	-	-
VIIc	R/T/-	-	-	-	I/-	-	L/-	-	V/-	-	K	-	-	-	V	-	S	-	-	-	-	Y	-	-	-	-	-	-	-	-
SWS/03	-	-	-	-	I	-	-	-	-	-	-	K	-	-	V	-	S	-	-	-	-	Y	-	-	-	-	-	-	-	-
VIIId	T/-	-	V/-	-	-	M/-	S/L/-	-	V	-	-	K	-	-	I/V	-	S/-	K/-	Q/-	N/-	-	Y	-	-	-	-	-	K/G/-	-	G/-
SGM/01	-	-	-	-	-	-	-	-	V	-	-	K	-	-	V	-	S	-	-	-	-	Y	-	-	-	-	-	-	-	G

^a Data for genotypes I, II, III, IV, V, VI, VII (VIIa and VIIb), and VIII were derived from references 15 and 28 and from this study.
^b A dash indicates genetic identity with the consensus residue.
^c The majority of the isolates or strains show V-for-I substitutions, while a few isolates or strains exhibit no change.
^d For the representative strains used in this study, the deduced amino acid sequences and alignments were conducted using the MegAlign program (MegAlign 4.00) in the Lasergene package (DNASStar Inc., Madison, WI).
^e NDV strain Australia-Victoria.

TABLE 3. Cross-protection efficacy in chickens immunized with the LaSota vaccine after challenge with virulent strains of different genotypes

Group	Challenge strain	Genotype	No. of chickens:			% Protective efficacy ^b
			Immunized	Affected	Dead	
1	SGM/01	VII	10	5	0	50
2	SCL/03	VII	10	0	0	100
3	SKY/03	VII	10	3	1	60
4	SRZ/03	II	10	1	0	90
5	SSX/03	VII	10	0	0	100
6	F48E9	III	10	0	0	100

^a Affected means that the chickens challenged with NDV were in poor spirits and exhibited dyspnea and diarrhea, but they were not dead.

^b The percent protective efficacy was determined with the following formula: $[1 - (\text{no. of affected chickens} + \text{no. of dead chickens}) / \text{no. of immunized chickens}] \times 100$.

tures and were closely related to genotype VII due to shared T¹⁰⁷-for-S, I¹²¹-for-V, Q¹⁹⁵-for-R, N²⁷²-for-Y, M³⁹⁶-for-I, and E⁴⁸²-for-A substitutions. Genotypes III, IV, V, VI, and VII had unique conserved residues at positions 32, 52, 69, 82, 124, 176, and 192. Residues 17 and 482 were conserved and unique to genotypes VI, VII, and VII.

Cross-protection. In view of the recent outbreaks of ND in populations of vaccinated poultry in China and the genetic features of the isolates, we conducted a laboratory investigation to determine whether the vaccine strain LaSota can fully protect against five isolates, including SGM/01, SRZ/03, SSX/03, SKY/03, and SCL/03, and the classical velogenic F48E9 strain. The results showed that strain LaSota could partly protect against SRZ/03, SGM/01, and SKY/03 and fully protect against SSX/03, SCL/03, and F48E9 (Table 3). However, the control chickens all died after being challenged with the NDV isolates. These results indicated that SGM/01, SRZ/03, and/or SKY/03 isolates are responsible for the recent outbreaks of ND in China during 1996 to 2005 as immune response-escaping antigenic variants.

DISCUSSION

In this study, 30 NDV isolates recovered from China during 1996 to 2005 were genotypically and pathotypically characterized. It is well known that NDVs exist as a single serotype based on the neutralizing test and cross-protective analysis (5). Phylogenetic analysis (Fig. 1 to 4) and unique residue substitution analysis (Table 2) suggested that most of these isolates are antigenic variants. To investigate the possibility of vaccination failure caused by antigenic variation, a cross-protective experiment was performed, and it showed that VIIc isolates SGM/01 and SKY/03 are immune response-escaping antigenic variants.

The VIIc and VIId virulent isolates were the newly emerging and primary NDVs in China that had been previously reported (16, 29). In those studies, SBD/02, TJ03/03, and JS06/03 belonged to genotype III and were closely related to the ancient velogenic strain F48E9 from China and strain Australia-Victoria, as well as to the relatively old genotypes II and IV. We suggest that the genotype III isolates were not eradicated and were a reason for the sporadic outbreaks of ND in China. The only pigeon-derived isolate, PB01/96, belonged to genotype

VI. The host-derived and worldwide geographic distribution of genotype VI suggested that PB01/96 should be classified as an exotic virus due to the migration of wild or commercial birds. Furthermore, six NDV isolates belonged to genotype II and were related to the extensively used vaccine strain LaSota, indicating that the genotype II isolates in China are variants resulting from the mutation of the vaccine strain (26). Strong immune pressure might contribute to the rate of evolution of NDV, helping these isolates escape the protective immune response of the vaccine strain LaSota.

Just as viruses of genotypes VI and VIIb were responsible for outbreaks of ND in western Europe (17), viruses of genotype VII were responsible for outbreaks in southern Africa (13), and viruses of genotype VIIa were responsible for outbreaks in the Middle East, northern and eastern Europe, India, and Taiwan (29); the NDV isolates belonging to genotypes II, III, and VII were responsible for outbreaks of ND in China. Since the 1990s, genotype VII has been the most prevalent NDV isolate found throughout the world, and within regions these strains shared a common endemic characterization, suggesting the succession of NDV transition and variation.

It has been reported that each of the three F gene regions, including nt 47 to 420, nt 329 to 582, and the entire ORF, can be used to determine the phylogenetic relatedness of NDV strains (29). The nucleotide sequence of the F gene fragment (nt 47 to 420) is regarded as a standard criterion for genotyping. The 30 NDV isolates and 38 reference strains from GenBank were divided into genotypes I to VII. Meanwhile, three phylogenetic trees, based on the so-called nonvariable portion (nt 421 to 1,662), the entire ORF sequence (nt 1 to 1,662), and the deduced amino acid sequence (residues 1 to 553), were constructed. A phylogenetic analysis comparing the nt 47 to 420 sequences indicated similarity among strains, except for SRZ/03, which was grouped as genotype VII by the ORF and amino acid phylogenetic trees but as genotype II according to the nt 47 to 420 tree. SRZ/03 also had a velogenic motif at the cleavage site of the F protein that was similar to that of velogenic strain TEX/48 (preceding residue 272) and that was similar to that of genotype VIIc strains (after residues 288). Therefore, SRZ/03 was grouped as genotype VII. The results showed that recombinant events have occurred between F genes to generate strains of different lineages. However, the phylogenetic trees based on F gene fragments (nt 47 to 420) did not reveal the recombination events, indicating that the phylogenetic analyses should be based on the entire F gene coding sequence or the associated deduced amino acid sequences.

When the cleavage site ¹¹²G-R-Q-G-R-L¹¹⁷ was changed to ¹¹²R-R-Q-R-R-F¹¹⁷, the ICPI of modified strain LaSota increased from 0.00 to 1.28 (14). The genotype II isolates (except SY/03) containing the lentogenic motif ¹¹²G-R-Q-G-R-L¹¹⁷ were virulent. Although the homologies were very high between genotype II isolates and the reference strain LaSota, it was hypothesized that the cleavage site motifs of the F gene were not enough to distinguish between low-virulence and virulent NDV strains and that the other genes of the viral genome play important roles in determining virulence. Therefore, in order to correctly characterize NDVs, neither the biological tests nor the analyses of the F gene should be omitted.

FIG. 5. Phylogenetic relationship of 11 NDV strains based on the deduced amino acid sequence (residues 1 to 571) for the HN gene. The phylogram was generated using the neighbor-joining method of MEGA 3.1.

The molecular determinants responsible for the antigenic variant remain to be determined. In most of the NDV isolates, the neutralizing epitopes (30) were conserved and contained no deletions or insertions, except in the genotype VIIId isolate JS04/04 (with a K⁷⁸-for-I substitution). An analysis of cysteine residues (10) showed that the sites were relatively conserved except in isolates SBZ/02 and SY/03, which contained a C-to-Y change at position 25 and a C-to-R change at position 199 of SBZ/02 and position 374 of SQZ/04. Isolates JS01/01, JS02/99, JS03/03, YG/03, SKY/03, and SWS/03 all contained an N-to-A change at position 543 that caused a loss of the nt 541 to 543 N-glycosylation site. Interestingly, four of these NDV strains were isolated from water birds, which indicated that the mutation of virus is related to the host. All point mutations of the F gene might together contribute to the antigenic variant of NDV.

The analysis of unique residues showed that many viruses shared specific molecular characterizations. The subtype VIIc and VIIId isolates shared unique V⁵²-for-I, K¹⁰¹-for-R, S¹⁷⁶-for-A, and Y³¹⁴-for-F substitutions, while genotype VII, including subtypes VIIa, VIIb, VIIc, and VIIId, all had four unique and conserved residues at positions 341, 385, 396, and 482. The VIIa, VIIc, and VIIId subtypes all contained the special K¹⁰¹-for-R and S¹⁷⁶-for-A substitutions. For genotypes III, IV, V, VI, and VII, the amino acid residues at positions 32, 52, 69, 82, 124, 176, and 192 all were unique. Residues 17 and 482 were specific to genotype VI, VII, and VII strains. Therefore, the presence of unique residues at specific positions on the F protein sequence may be used for genotyping NDV strains.

It has been suggested that ND outbreaks in vaccinated poultry flocks are due to the emergence of antigenic variants. The F gene plays an important role in determining the virulence of NDV. In a recent study (21a), we also found that the HN gene of NDV, which is not protected by the vaccine virus LaSota, had an obvious genetic sequence variation (Fig. 5). Apparently, both the F and HN genes contribute to antigenic variants, which might be important reasons why the vaccine LaSota barely protects poultry flocks attacked by NDV. At present, it remains to be determined if there is a relationship between antigenicity and pathogenicity, and further work needs to be undertaken to understand the causes of frequently occurring vaccine failure.

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