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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

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Summary. Twenty-nine strains of *Newcastle disease virus* (NDV) isolated from outbreaks in chicken and goose flocks in several regions of China during 1985–2001 were characterized pathotypically and genotypically. All except one of these strains were velogenic. For genotyping, phylogenetic tree analysis (nt 47–420), restriction site mapping (nt 334–1682) and residue substitution analysis (residues 4–124) of the F gene were performed using sequences of our isolates and sequences of selected NDV strains from GenBank. The results revealed that most of these newly characterized strains belonged to six novel genetic groups that were designated as VIf, VIg, VIIc, VIId, VIIe and IX. The genotype IX viruses, to which the China challenge strain F48E8 used for vaccine evaluation belonged, were found only in China and still induced sporadic infections in certain areas. Isolates belonging to group VIf and VIg were distinct from previously reported members of genotype VI, with genetic distance from 2.5 to 12.1%. Subgenotype VIIc, VIId and VIIe viruses, which were distributed in clusters in the phylogenetic tree distinct from members of subgenotypes VIIa and VIIb, were responsible for disease outbreaks in chicken and goose flocks and circulated predominantly in southern China in recent years. Finally, cross-protective testing showed that specific-pathogen free (SPF) chickens vaccinated with La Sota vaccines can be fully protected against challenge by strains from genetic groups VIb, VIg, VIId and IX, indicating that the antigenic differences between strains of various genotypes are insufficient to change the cross-protection conferred by the commonly used vaccine.

Introduction

Newcastle disease (ND) is regarded worldwide as one of the most devastating diseases of poultry [3, 5]. Its causative agent, *Newcastle disease virus* (NDV), is classified as a member of the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Rubulavirus* [21]. Recent determination of the sequence of the entire genome of NDV suggested that NDV and other avian paramyxoviruses are sufficiently different from other Rubulaviruses to warrant placing them in a separate genus [9, 11, 29]. NDV has a negative-sense, single-stranded RNA genome of 15 kb that contains six genes in the order of 3′-NP-P-M-F-HN-L-5′, encoding six proteins (nucleoprotein, phosphorprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase and large protein respectively) [20]. The F glycoprotein that mediates fusion of viral and cellular membranes is synthesized as an inactive precursor, F_0, containing 553 amino acids. The precursor is proteolytically cleaved at the peptide bond between residues 116 and 117, to generate active polypeptides F_1 and F_2, which are linked by disulphide bonds. Studies comparing the deduced amino acid sequence of the F_0 precursor of NDV strains varying in virulence for chickens showed that viruses that are virulent for chickens have the amino acid motif of ^{112}R/K-R-Q-K/R-R-F^{112} at the cleavage site whereas viruses of low virulence have sequence of ^{112}G/E-K/R-Q-G/E-R-L^{117} in the same region [1, 22]. Mutagenic experiments using infectious cDNA clones recently confirmed that the cleavability of F_0 protein is a major determinant for virulence [26].

Monoclonal antibody (mAb) reactivity has proven to be very useful in the antigenic analysis and epidemiological study of NDV strains from different outbreaks [2, 6, 28], however, molecular-based techniques for identifying and characterizing the NDV strains and for tracing their origins and spread is more definitive [1]. NDV strains isolated during three main pandemics and from endemic activity worldwide in more recent years have been classified into different genetic lineages and sublineages by restriction site mapping and sequence analysis of the F gene: genotype I contains avirulent viruses primarily from water fowls but also from chickens; genotypes II, III and IV contain viruses responsible for the first pandemic which started in the mid-1920s and subsided in the late 1950s; two new genotypes, V and VI, emerged during the second pandemic between the 1960s and 1970s; subtype VIb viruses were responsible for third pandemic of pigeon origin during the 1980s; novel genotypes and subtypes VIIa, VIIb and VIII appeared in late 1980s and 1990s in the Far East , Europe and South Africa [7, 13–16, 19, 32].

ND was first described in China in village chicken flocks in 1946 [17] and it has been regarded as an endemic disease in some regions of the country since that time. In the past two decades, an intensive vaccination program against ND has been practiced in both large-scale poultry operations and village poultry farming. Disease outbreaks occur infrequently in some vaccinated flocks, however, epizootic infections of velogenic ND in both chicken and goose flocks in some village poultry farming areas in the southern part of China have been more

frequently reported since the late 1990s [18, 23, 27, 35]. In the current study, we carried out pathotypical and genotypical characterization of NDV strains derived from different regions of China during the period of 1985–2001, to determine the phylogenetic relationships of these viruses and epidemiological relationships of ND outbreaks. We were particularly interested in obtaining information on NDV isolates that caused epizootics in both chickens and geese in recent years. Cross-protective testing was also done to determine whether the emergence of new genotypes or subgenotypes could be responsible for ND outbreaks in vaccinated flocks.

Materials and methods

Viruses

Twenty-nine virulent NDV strains derived from field outbreaks together with the standard challenge strain used in China, F48E8, are listed in Table 1 along with the year of isolation and host of origin. All the viruses were plaque-purified three times on primary chicken embryo fibroblasts [12] and grown in 9–10 day old specific-pathogen-free (SPF) chicken embryos (from Nanjing Bioproducts Firm) as previously described [12]. These virus stocks grown in allantoic fluids were stored at −70 °C until use. Pathotyping was performed using standard procedures to determine mean death time (MDT) of embryonated chicken eggs and intracerebral pathogenicity index (ICPI) in day-old chickens [4].

Viral RNA purification

The virus preparation was purified and viral RNA was extracted as previously described [34]. Briefly, 1.5 ml of allantoic fluid was clarified in an Eppendorf tube at 8000 rpm for 5 min; after the supernatant was transferred to another tube, polyethylene glycol (PEG; 6000) and NaCl were added and dissolved to make final concentrations of 10%(w/v) and 2%(w/v), respectively. Then the mixture was stored at 4 °C for 2 h, clarified at 8000 rpm for 5 min, and the supernatant was centrifuged at 14000 rpm for 30 min. The pellet was resuspended in 0.5 ml STE buffer (10 mM Tris-HCl, pH7.4; 100 mM NaCl; 5 mM EDTA) which was then mixed with 0.5 ml 10% sodium dodecyl sulfate (SDS) and 0.5 ml phenol followed by gentle shaking for 5 min in an ice bath. The suspension was centrifuged at 4000 rpm for 10 min and the supernatant was recovered and extracted twice more with phenol. The viral RNA was obtained by precipitation with ethanol containing 0.4 M LiCl and 0.1 M NaAc, and resuspended in 50 μl of diethylpyrocarbonate (DEPC)-treated water and stored at −70 °C until use.

Reverse transcription-polymerase chain reaction (RT-PCR)

Primers for the RT-PCR amplification of the F protein genes were designed on the basis of published NDV nucleotide sequence data with the aid of Primer Premier software (Premier Biosoft International, Canada). For PCR amplification of a 970 base pair (bp) fragment spanning nt 1161 of the M gene through nt 889 of F gene, the pair of primers 5′-GCCGAATTCCCGAATCATCACGACGCTTAA-3′(sense) and 5′-GTGAAGCTTGAGT CTGTGAGTCGTAC-3′ (antisense) was used, whereas for amplifying the 839 bp fragment covering nts 862 through 1700 of F gene, the primer pair of 5′-CACCGGTACCCCTATTCT GATCG-3′ (sense) and 5′-TTAAGCTTGTAGTGGCTCTCATCTGATC-3′ (antisense) was used. Reverse transcription was carried out in a 25 μl reaction mixture as indicated by the

Table 1. Data of NDV strains and nucleotide sequences

NDV strains	Year of isolation	Host	MDT	ICPI	Accession numbers[b]	Genetic grouping[a]
F48E8	1948	fowl	48.0	1.99		IX
ZhJ-1/85	1985	fowl	46.8	1.96	AF458023	IX
FJ-1/85	1985	fowl	52.8	1.89	AF458009	IX
ZhJ-2/86	1986	fowl	55.2	1.66	AF458016	VIg
XJ-1/91	1991	fowl	50.4	1.89	AF458020	VIf
JX-1/94	1994	fowl	49.2	1.67	AF458021	VIf
PB9601	1996	pigeon	88.8	1.44		VIb
JS-1/97	1997	fowl	51.6	1.90	AF458022	IX
XJ-2/97	1997	fowl	57.6	1.94	AF458011	VIId
Sh-1/97	1997	fowl	48.0	1.86	AF458018	VIg
ZhJ-3/97	1997	fowl	52.8	1.64	AF458015	VIg
XJ-3/97	1997	fowl	56.4	1.81	AF458019	VIf
Sh-2/98	1998	fowl	50.4	1.79	AF458017	VIg
JS-2/98	1998	fowl	50.4	1.88	AF458013	VIIe
FJ-2/99	1999	fowl	45.6	1.94	AF458012	VIId
JX-2/99	1999	fowl	48.0	1.91	AF458014	VIIe
JS-3/00	2000	fowl	49.2	1.74	AF458010	VIIc
JS-4/01	2001	fowl	56.4	1.83		VIId
JS-5/01	2001	fowl	55.2	1.81		VIId
ShD-1/01	2001	fowl	52.0	1.79		VIId
JS-1/97(Go)	1997	goose	60.0	1.84	AF456435	VIId
JS-2/98(Go)	1998	goose	52.8	1.80	AF456439	VIb
JS-3/98(Go)	1998	goose	52.8	1.85	AF456436	VIId
GD-1/98(Go)	1998	goose	59.2	1.80	AF456437	VIId
ZJ-1/00(Go)	2000	goose	51.6	1.89	AF456438	VIId
JS-4/01(Go)	2001	goose	52.8	1.91	AF456440	VIId
JS-5/01(Go)	2001	goose	55.2	1.88	AF456442	VIId
JS-6/01(Go)	2001	goose	51.6	1.86	AF456441	VIId
JS-7/01(Go)	2001	goose	56.4	1.90	AF456444	VIId
JS-9/01(Go)	2001	goose	54.0	1.89	AF456443	VIId

[a]For grouping see Fig. 1

[b]Sequence data of F48E8, see reference 34; sequence data of JS-4/01, JS-5/01, ShD-1/01 have not been delivered to GenBank yet

manufacturer (Promega, M5101). Briefly, 12 μl of RNA template (100 ng) was mixed with 1 μl of reverse transcription primer (sense, 50 pmol) and denatured at 70 °C for 5 min and chilled immediately at 0 °C for 5 min; then 5 μl of 5 × RT buffer, 3 μl of dNTPs (10 mM), 3 μl AMV reverse transcriptase (10 u/μl) and 1 μl Rnasin (40 u/μl) were added, mixed gently and incubated at 42 °C for 1 h. PCR was carried out in a 50 μl reaction volume containing 10 × PCR buffer (5 μl), 15 mM $MgCl_2$ (3 μl), dNTPs (10 mM, 1 μl) , upstream primer (1 μl; 50 pmol), downstream primer (1 μl; 50 pmol), cDNA (5 μl), water (33 μl) and high-fidelity polymerase (Roche, 2140314; 0.5 μl). The PCR protocol was as follows: 94 °C for 30 s, 52 °C for 45 s and 72 °C for 1.5 min (adding 5 seconds per cycle) for 30 cycles, followed by 72 °C for 7 min.

Sequencing of the PCR products

The PCR products were analysed by gel electrophoresis; the DNA bands were recovered from the gel by using an agarose gel DNA extraction kit (Roche, 1696505). The purified PCR products were sequenced in both directions by using ABI-377-based (Applied Biosystems Inc.) fluorescent cycle sequencing technology (TaKaRa Biotechnology Co., Ltd. Dalian, China). The nucleotide sequences of the F genes (nt 1–1700) of NDV isolates in this study were deposited in GenBank as a single sense-strand contiguous sequence for each isolate under accession numbers: AF458009-AF458023 and AF456435-AF456444.

Analysis of sequence data

Nucleotide sequence editing, analysis, prediction of amino acid sequences and alignments were conducted using the MegAlign program (Windows 32, MegAlign 4.00) in the Lasergene package (DNASTAR Inc. Madison, WI 53715, USA). Phylogenetic trees were constructed with the Jotun Hein Method of the Program by comparison of the nucleotide sequences of F gene from nt 47 to 420. In addition to 30 strains in this study, 40 previously reported NDV sequences representative of different genotypes were also included for comparison. The designation of these NDV strains and their F gene sequence accession numbers can be found in references [7, 13, 15, 16, 19]. The restriction sites distribution analysis was based on sequence data of F gene from nt 334 to 1682 by using MapDraw 4.00 program.

Cross-protection experiment

Five NDV strains (PB9601, ZJ-2/86, XJ-2/97, JS-1/97/(Go) and F48E8) representing different genotypes or subtypes (VIb, VIg, VIId, and IX) and vaccine strain La Sota were used to prepare monovalent oil-emulsion vaccines as described previously [30]. Live vaccine of the La Sota strain from a commercial source (Nanjing Bioproducts, Ltd., Nanjing) was also used. Two weeks old SPF chicks were vaccinated with oil-emulsion vaccines by the subcutaneous route (0.2 ml/bird) or with live vaccine by eye-drop (following the manufacturer's instruction), while control birds were injected with phosphate-buffered saline. Three weeks later, the birds were challenged by eye-drop and intra-nasal routes with live PB9601, ZJ-2/86, XJ-2/97, JS-1/97/(Go) and F48E8 (10^4 $TCID_{50}$, 0.1 ml/bird). The birds were kept in isolators in the laboratory animal facility at Yangzhou University and observed for signs of disease and death for 14 days postchallenge.

Results

Virus pathotyping

As shown in Table 1, all except one of the 29 isolates pathotyped in this study were characterized by mean death times (MDT) in embryonated chicken eggs of 45.6–60.0 h and intracerebral pathogenicity indices (ICPI) of 1.64 to 1.96 in day-old chicks and therefore were velogenic. Strain PB9601 of pigeon origin induced an MDT of 88.8 h and an ICPI of 1.44 was a mesogenic virus. Further testing in ten-day old chickens demonstrated that all of the velogenic viruses killed infected birds within 4 days postinfection (data not shown). The deduced amino acid sequence of the F protein cleavage site of all of these velogenic strains was the ^{112}R-R-Q-K/R-R-F^{117} motif (Table 3) characteristic of virulent NDV strains [1, 22].

Phylogenetic relationships among NDV isolates

Phylogenetic analysis of the NDV strains characterized in this study and representative strains from the literature was performed using the variable region of the F gene (nt 47–420) (Fig. 1); the representative strains included viruses from genetic groups I to VIII and strains isolated recently in mainland China (Ch/2000, Ch/98-3, Ch/99, Ch/98-1, CHA7/96, Ch-BD3, ShX-2/99, GS-2/98, QH-4/85 and QH-2/84) and Taiwan (TW/2000) [7, 13, 15, 16, 19]. The phylogenetic tree showed that the twenty-nine isolates and one reference strain (F48E8) from this study and forty representative strains from the literature were distributed in nine distinct clusters corresponding to the different genotypes of NDV. Among these genotypes, eight groups (genotype I to VIII) have been reported previously, whereas a novel group recognized as genotype IX was reported more recently in China [8, 23]. The five genotype IX viruses (JS-1/97, ZhJ-1/85, FJ-1/85, Ch-DB3 and F48E8), which were isolated exclusively in mainland China, had a high nucleotide homology (99.5%–100%), whereas the genetic distance of this cluster from other major genotypes (I to VIII) was 9.2%–20.2%. Nine strains characterized in this study were assigned to genotype VI, within which two strains (JS-2/98/go and PB9601) belonged to the established subgroup VIb and other 7 strains fell into two subgroups, which we designated VIf and VIg. Seventeen strains, including most of goose isolates and more recent chicken isolates, were assigned to genotype VII, within which fifteen of these strains belonged to the established subgroups VIIc and VIId, while two strains (JS-2/98 and JX-2/99) which diverged from VIIc and VIId viruses by 2.5–6.8% were assigned to a novel subgroup VIIe.

Restriction site mapping

Restriction site mapping of thirty strains from this study for *Hin*fI, *Bst*OI and *Rsa*I across a region consisting of 75% of the F gene (nt 334–1682) to confirm the novel genotypic subtypes revealed by phylogenetic analysis. The distribution of *Hin*fI, *Bst*OI and *Rsa*I sites in this region is shown in Table 2. Genotype IX strains shared a pattern of restriction site distribution characterized by the presence of *Rsa*I site at nt 540 and the absence of an *Hin*fI site at nt 1198, a *Bst*OI site at nt 1478, and an *Rsa*I site at nt 1625, which was unique from genotype I to VIII viruses. Viruses in subgenotypes VIf and VIg not only had the restriction site pattern common to genotype VI of an *Hin*fI site at nt 883, a *Bst*OI site at nt 752 and an *Rsa*I site at nt 1625 but also had an additional *Rsa*I site at nt 872 which is absent for all other subgroups of genotype VI, with the exception of a few strains in subtype VIb. Compared with VIIa and VIIb, subgenotypes VIIc, VIId and VIIe shared the

Fig. 1. Phylogenetic tree of the nucleotide sequences of NDV strains based on a variable portion (nt 47–420) of the F gene. Sequences of unboxed strains were taken from the GenBank (Strain designation and Accession numbers derived from references [7, 13, 15, 16, 19]). Accession numbers of most boxed strains in this study were listed in Table 1

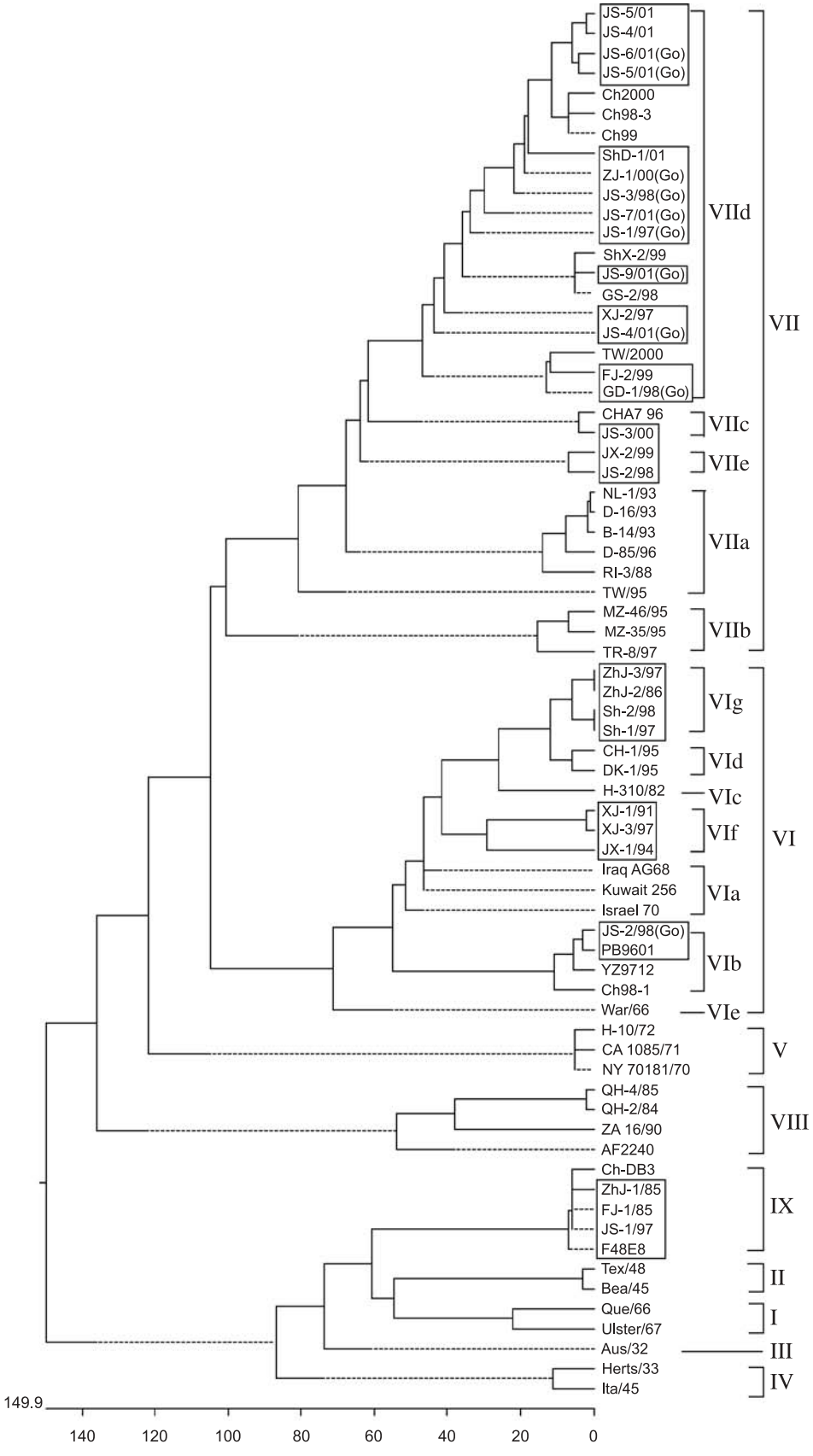
JS-5/01
JS-4/01
JS-6/01(Go)
JS-5/01(Go)
Ch2000
Ch98-3
Ch99
ShD-1/01
ZJ-1/00(Go)
JS-3/98(Go)
JS-7/01(Go)
JS-1/97(Go)
ShX-2/99
JS-9/01(Go)
GS-2/98
XJ-2/97
JS-4/01(Go)
TW/2000
FJ-2/99
GD-1/98(Go)
CHA7 96
JS-3/00
JX-2/99
JS-2/98
NL-1/93
D-16/93
B-14/93
D-85/96
RI-3/88
TW/95
MZ-46/95
MZ-35/95
TR-8/97
ZhJ-3/97
ZhJ-2/86
Sh-2/98
Sh-1/97
CH-1/95
DK-1/95
H-310/82
XJ-1/91
XJ-3/97
JX-1/94
Iraq AG68
Kuwait 256
Israel 70
JS-2/98(Go)
PB9601
YZ9712
Ch98-1
War/66
H-10/72
CA 1085/71
NY 70181/70
QH-4/85
QH-2/84
ZA 16/90
AF2240
Ch-DB3
ZhJ-1/85
FJ-1/85
JS-1/97
F48E8
Tex/48
Bea/45
Que/66
Ulster/67
Aus/32
Herts/33
Ita/45
VIId
VIIc
VIIe
VIIa
VIIb
VII
VIg
VId
VIc
VIf
VIa
VIb
VIe
VI
V
VIII
IX
II
I
III
IV
149.9
140
120
100
80
60
40
20
0

Table 2. Distribution pattern of restriction enzyme cleavage sites on F genes of NDV strains assigned to different genetic groups

Genetic groups or representative strains	*Hinf* I**				*BstO* I					*Rsa* I					
	736	883	1198	1350	752	953	1116	1478	1601	540	683	872	973	1249	1625
I*	−	+	−/+	−	+	−/+	+	−	+	−	−/+	+	+	−	+
II	−	−/+	−	−	−	−	+	−	−/+	−	−/+	−/+	−	−	−/+
III	−	+	+	−	−/+	−/+	+	−	−/+	+	−/+	+	−	−	−/+
IV	−/+	+	−	−/+	+	−	+	−	−/+	−/+	−/+	+	−	−	+
V	−	−	−	−	+	−	+	+	+	−/+	−/+	−	−	−	−
VIa	−	+	−	−	+	−	+	+	+	−	−	−	−	−	+
VIb	−	+	−	−	+	−	+	+	−	−	−	−/+	−	−	+
JS-2/98(Go)	−	+	−	−	−	−	+	+	−	−	−	+	+	−	+
PB9601†	−	+	−	−	−	−	+	+	−	−	−	−	+	−	+
VIc	−	+	−	−	+	−	+	−	+	−	−	−	−	−	+
VId	−	+	−	−	+	−	+	+	+	−	−	−	−	−	+
VIe	−	−	−	−	+	−	+	+	+	−	−	−	−	−	+
VIf	−	−/+	−	−	+	−	+	−/+	+	−	−	+	−	−	+
XJ-1/91	−	−	−	−	+	−	+	−	+	−	−	+	−	−	+
JX-1/94	−	+	−	−	+	−	+	+	+	−	−	+	−	−	+
XJ-3/97	−	+	−	−	+	−	+	+	+	−	−	+	−	−	+
VIg	−	−/+	−	−	+	−	+	−/+	−/+	−	−	+	−	−/+	+
ZhJ-2/86	−	−	−	−	+	−	+	+	−	−	−	+	−	−	+
ZhJ-3/97	−	+	−	−	+	−	+	−	+	−	−	+	−	−	+
Sh-1/97	−	+	−	−	+	−	+	+	+	−	−	+	−	−	+
Sh-2/98	−	+	−	−	+	−	+	−	+	−	−	+	−	+	+
VIIa	−	+	−	+	−	−	+	−	−	−	−	−	−	−	+
VIIb	+	+	−	−	+	−	+	−	+	−	−	−	−	−	−
VIIc	−	+	−	−	+	−	+	−	+	−	−	+	−	−	−
JS-3/00	−	+	−	−	+	−	+	−	+	−	−	+	−	−	−
VIId	−	+	−	−	+	−	+	−	−/+	−/+	−/+	+	−/+	−/+	−/+
ShD-1/01	−	+	−	−	+	−	+	−	−	−	−	+	+	+	+
XJ-2/97	−	+	−	−	+	−	+	−	+	−	−	+	−	−	−
FJ-2/99	−	+	−	−	+	−	+	−	+	+	+	+	−	−	−
JS-1/97(Go)	−	+	−	−	+	−	+	−	+	+	+	+	+	+	+
ZJ-1/00(Go)	−	+	−	−	+	−	+	−	−	−	−	+	+	+	+
JS-5/01(Go)	−	+	−	−	+	−	+	−	−	−	−	+	+	+	+
VIIe	−	+	−	−	+	−	+	−	−/+	−	−	+	+	+	+
JS-2/98	−	+	−	−	+	−	+	−	−	−	−	+	+	+	+
JX-2/99	−	+	−	−	+	−	+	−	+	−	−	+	+	+	+
VIII	+	+	−	−	+	+	−	−	−	−	+	−	−	−	+
IX	−	+	−	−	+	−	+	−	+	+	+	+	−	−	−
F48E8	−	+	−	−	+	−	+	−	+	+	+	+	−	−	−
FJ-1/85	−	+	−	−	+	−	+	−	+	+	+	+	−	−	−
JS-1/97	−	+	−	−	+	−	+	−	+	+	+	+	−	−	−

*Data for genotypes I, II, III, IV, V, VI (VIa, VIb, VIc, VId, VIe), VII (VIIa, VIIb) and VIII were derived from references [7, 13, 19]; data for genetic groups VIf, VIg, VIIc, VIId, VIIe and IX were from this study

†Representative strains in this study, the restriction sites distribution was analyzed by using MapDraw 4.00 program in the Lasergene package (DNASTAR Inc. Madison, WI 53715, USA)

** + sign means that the genetic group or representative strain bears the assigned restriction site; − sign means that the genetic group or representative strain does not bear the assigned restriction site; −/+ sign means that some strains in the given genetic group bear the assigned restriction site, but others does not

Table 3. Deduced amino acids of the F gene variable region used for differentiation of genetic groups or subgroups

Genotypes or representative strains	4	5	9	10	13	16	17	19	23	28	63	93	101	104	106	107	109	112	113	114	115	116	117	118	121
	K	S	I	P	L	I	T	I	L	L	V	T	R	G	V	S	S	R	R	Q	K	R	F	I	I
I*	R	–	–	–	–	T	V	–/V	–	P	I	–	–	E	–	T	–	G	K	–	G	–	L	–	–
II(Le)	R	–	–	–	–	T	I	V	–	P	–	–	–	E	–	T	–	G	–	–	G	–	L	–	–
II(Me/Ve)	R	P	N	–	M	T	V	V	–	P	–	–	–	E	–	T	–	–	–	–	–	–	–	–	–
III	R	–	–	–	–	T	I	–	–	–	–/I	–	–	E	–	T	–	–	–	–	–/R	–	–	–	–
IV	R	–	–	–	–/P	–	I	–	–	–	–	–	–	E	–	T	–	–	–	–	R	–	–	–	–
V	–	P	–	S	–	–	–	–	–	–	–	–	–	–	A	T	–	–	–	–	–	–	–	V	–
VIa	–	P	–	–	–	–	–	–	–	–	–/I	–	–	–	–	–	–	–	–	–	–	–	–	–	–
VIb	–/R	–/P	–	–	–/P	–	–	–/T	–	P	–	–	–	–	–	–	–	G/K	–	–	–	–	–	–	–
PB9601†	R	P	–	–	P	–	–	–	–	P	–	–	–	–	–	–	–	K	–	–	–	–	–	–	–
JS-2/98(Go)	–	–	–	–	P	–	–	–	–	P	–	–	–	–	–	–	–	K	–	–	–	–	–	–	–
VIc	–	P	V	S	–	–	–	–	–	–	I	S	–	–	–	–	–	–	–	–	–	–	–	–	–
VId	–	P	–/V	S	P	–	–	–	–	–	I	S	–	–	–	–	–	–	–	–	–	–	–	–	–
VIf	–	P	–/T	–	P	–	–	–	–	–/Q	–	–	–/K	–	–	–	–	–	–	–/R	–	–	–	–/V	–
XJ-1/91	–	P	T	–	P	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	V	–
JX-1/94	–	P	–	–	P	–	–	–	–	Q	–	–	K	–	–	–	–	–	–	R	–	–	–	–	–
XJ-3/97	–	P	T	–	P	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	V	–
VIg	–	P	–	–	–	–	–	–	–	–	I	S	–	–	–	–	–	–	–	–	–	–	–	–	–
ZhJ-2/86	–	P	–	–	–	–	–	–	–	–	I	S	–	–	–	–	–	–	–	–	–	–	–	–	–
ZhJ-3/97	–	P	–	–	–	–	–	–	–	–	I	S	–	–	–	–	–	–	–	–	–	–	–	–	–
Sh-1/97	–	P	–	–	–	–	–	–	–	–	I	S	–	–	–	–	–	–	–	–	–	–	–	–	–
Sh-2/98	–	P	–	–	–	–	–	–	–	–	I	S	–	–	–	–	–	–	–	–	–	–	–	–	–
VIIa	–	P	T	–	–	–	–	–	S	–	–	–	K	–	–	–	P	–	–	–	–/R	–	–	–	V
VIIb	–	P	–	–	–	–	–	V	–	S	–	–	–	–	–	–	–	–/K	–	–	–	–	–	–	V
VIIc	–	P	–	–	P	–	–	–	–	–	–	–	K	–	–	–	–	K	–	–	–	–	–	–	V
JS-3/00	–	P	–	–	P	–	–	–	–	–	–	–	K	–	–	–	–	–	–	–	–	–	–	–	V
VIId	–	P	–	–	–	–	–	–	–	–/P	–	–	K	–	–	–	–	–	–	–	–	–	–	–	V
ShD-1/01	–	P	–	–	–	–	–	–	–	–	–	–	K	–	–	–	–	–	–	–	–	–	–	–	V
XJ-2/97	–	P	–	–	–	–	–	–	–	–	–	–	K	–	–	–	–	–	–	–	–	–	–	–	V
FJ-2/99	–	P	–	–	–	–	–	–	–	–	–	–	K	–	–	–	–	–	–	–	–	–	–	–	V
JS-1/97(Go)	–	P	–	–	–	–	–	–	–	–	–	–	K	–	–	–	–	–	–	–	–	–	–	–	V

(*continued*)

Table 3 (*continued*)

Genotypes or representative strains	4 K	5 S	9 I	10 P	13 L	16 I	17 T	19 I	23 L	28 L	63 V	93 T	101 R	104 G	106 V	107 S	109 S	112 R	113 R	114 Q	115 K	116 R	117 F	118 I	121 I
ZJ-1/00(Go)	–	P	–	–	–	–	–	–	–	P	–	–	K	–	–	–	–	–	–	–	–	–	–	–	V
JS-5/01(Go)	–	P	–	–	–	–	–	–	–	–	–	–	K	–	–	–	–	–	–	–	–	–	–	–	V
VIIe	–	P	–	–	–	–	–	–	–	–	–	–	K	–	–	–	–	–	–	–/R	–	–	–	–	V
JS-2/98	–	P	–	–	–	–	–	–	–	–	–	–	K	–	–	–	–	–	–	–	–	–	–	–	V
JX-2/99	–	P	–	–	–	–	–	–	–	–	–	–	K	–	–	–	–	–	–	R	–	–	–	–	V
VIII	–	–	–/F	S/L	–/S	–/V	–	–	–	–/P	–	–	–	–	–	T	–	–	–	–/R	–	–	–	–	V
IX	–	–	V	–	–	T	V	–	–	–	–	–	–	E	A	T	–	–	–	–	R	–	–	–	–
F48E8	–	–	V	–	–	T	V	–	–	–	–	–	–	E	A	T	–	–	–	–	R	–	–	–	–
FJ-1/85	–	–	V	–	–	T	V	–	–	–	–	–	–	E	A	T	–	–	–	–	R	–	–	–	–
JS-1/97	–	–	V	–	–	T	V	–	–	–	–	–	–	E	A	T	–	–	–	–	R	–	–	–	–

*Data for genotypes I, II, III, IV, V, VI (VIa, VIb, VIc, VId), VII (VIIa, VIIb) and VIII were derived from references [7, 13, 19]; data for genetic groups VIf, VIg, VIIc, VIId, VIIe and IX were from this study

†Representative strains in this study, deduced amino acid sequences and alignments were conducted by using MegAlign program (Windows 32, MegAlign 4.00) in the Lasorgene package (DNASTAR Inc. Madison, WI 53715, USA)

common feature of lacking a *Bst*OI site at nt 1478, however these subgenotypes also had an *Rsa*I site at nt 872 that distinguishes them from genotypes VIIa, VIIb and VIII. Similar to subgenotype VIIb, The VIIc, VIId, and VIIe viruses also lacked a *Hin*fI site at nt 1350 that is present in VIIa and contained a *Bst*OI site at nt 752 which is absent in VIIa. In addition, *Rsa*I sites at nt 973 and 1249 appeared in some strains assigned to subgroups VIId and VIIe. The presence of *Rsa*I sites at nt 872, 973 and 1249 is a characteristic common to most strains of goose origin in subgroup VIId.

Comparison of amino acid sequences in the variable region of fusion protein

When deduced amino acid sequences (residues 1–130) of the F genes of different strains were compared, several characteristic amino acid substitutions were identified for specific genotypes or subgenotypes (Table 3). Genotype IX strains displayed simultaneous substitutions of $I^{9} \rightarrow V$ and $V^{106} \rightarrow A$ that distinguished

Table 4. Cross-protective efficacy in chicken immunized with homologous vaccine or La Sota vaccines when challenged against virulent strains of different genetic groups

Group	Challenge strain (genogroup)	Vaccine[a]	D/T	Protective efficacy (%)
1	ZJ-2/86 (VIg)	La Sota (L)	0/25	100
	ZJ-2/86 (VIg)	La Sota (K)	0/25	100
	ZJ-2/86 (VIg)	ZJ-2/86 (K)	0/25	100
	ZJ-2/86 (VIg)	no	20/20	0
2	PB9601 (VIb)	La Sota (L)	0/25	100
	PB9601 (VIb)	La Sota (K)	0/25	100
	PB9601 (VIb)	PB9601 (K)	0/25	100
	PB9601 (VIb)	no	20/20	0
3	XJ-2/97 (VIId)	La Sota (L)	0/25	100
	XJ-2/97 (VIId)	La Sota (K)	0/25	100
	XJ-2/97 (VIId)	XJ-2/97 (K)	0/25	100
	XJ-2/97 (VIId)	no	20/20	0
4	JS-1/97/(Go) (VIId)	La Sota (L)	0/25	100
	JS-1/97/(Go) (VIId)	La Sota (K)	1/25	96
	JS-1/97/(Go) (VIId)	JS-1/97/(Go) (K)	0/25	100
	JS-1/97/(Go) (VIId)	no	20/20	0
5	F48E8 (IX)	La Sota (L)	0/25	100
	F48E8 (IX)	La Sota (K)	0/25	100
	F48E8 (IX)	F48E8 (K)	0/25	100
	F48E8 (IX)	no	20/20	0

[a] (L) = Live vaccine; (K) = Killed oil-emulsion vaccine

[b] D/T = number of birds died during 10 days postchallenge/number of birds tested

them from all other genotypes. Subgenotype VIf was differentiated from the other members in genotype VI by the simultaneous presence of $L^{13} \rightarrow P$ and absence of the $V^{63} \rightarrow I$ and $T^{93} \rightarrow S$ substitutions, whereas the unique features of subgenotype VIg were the presence of $V^{63} \rightarrow I$ and $T^{93} \rightarrow S$ and lack of $I^{9} \rightarrow V$ or T and $L^{13} \rightarrow P$ substitutions. Substitutions of $R^{101} \rightarrow K$ and $I^{121} \rightarrow V$, which are unique to subgenotype VIIa, were shared by subgroups VIIc, VIId and VIIe.

Cross protection

In order to answer the question whether of field NDV strains belonging to novel genotypes or subtypes could cause ND outbreaks in vaccinated flocks, cross protection tests were conducted; the results are shown in Table 4. Chickens vaccinated with either live or fixed (oil-emulsion) La Sota vaccine or oil-emulsion vaccines derived from the virulent field strains were fully protected from disease and death against challenge by PB9601, XJ-2/97, JS-1/97/(Go) and F48E8 respectively. No control birds were protected from disease and death by any of the challenge strains.

Discussion

Recently, Li et al. [15] and Liang et al. [16] reported the characterization of NDV strains isolated from outbreaks in several regions in China. The genetic classification of NDV isolates in this study is essentially in good agreement with their results with minor discrepancies. In Li et al. [15], strain Ch-DB3 is classified as a member of genotype III, however in our classification, Ch-DB3 together with four other strains (ZJ-1/85, FJ-1/85, JS-1/97, and F48E8) were placed in the novel genotype IX. In combination with the position of these strains on the phylogenetic tree (Fig. 1), the restriction site distribution (Table 2), deduced amino acid residue substitutions (Table 3), and genetic distance from other established genotypes, these strains are sufficiently different from other NDV strains to warrant placing them in a separate genotype. These data [especially the shared amino acid substitutions T^{16} for I, V^{17} for T, E^{104} for G and T^{107} for S (Table 3)] showed that genotype IX is closer to genotypes II, III and IV, responsible for the first pandemic of ND, than to genotypes V and VI, responsible for the second and third pandemics, and the recently recognized genotype VII. This coincided with the fact that strain F48E8, the Chinese standard challenge strain for vaccine evaluation assigned to this genotype, was isolated in 1940s during the first pandemic.

Eighteen of our isolates (62%) were classified as belonging to subgenotypes VIIc, VIId and VIIe, which have been predominant among NDV infections in recent years, accounting for 78% of the total strains isolated since 1996. When four strains (Ch/2000, Ch/98-3, Ch/99, and TW/2000) of subgenotype VIId from Li et al. [15] and two strains (ShX-2/99 and GS-2/98) of subgenotype VIIa from Liang et al. [16] were used for comparison in our phylogenetic analysis (Fig. 1), all of them segregated into subgenotype VIId. Unique residue substitutions among

Table 5. Unique residue substitutions of deduced amino acid sequences of F gene for NDV strains of different genotypes or subgenotypes[a]

Genotype or subgenotype	Residue substitutions							
	52	71	176	272	314	402	489	520
I	I	K	A	N	F	A	D	V
II								I[b]
III								I
IV								
V								
VI								I
VIIa				Y				
VIIb								
VIIc			S	Y	Y			
VIId	V	R	S	Y	Y	T/−[c]	E/−	G/−
VIIe			S	Y	−/Y[d]			
VIII				−/Y				
IX								

[a]Based on data of 30 NDV strains in this study and 21 other strains with full length of F gene sequences from GenBank (GB1168/84: AF109885; Taiwan95: U62620; CHA7/96: AY028995; Ch/98-1: AF358785; Ch/98-3: AF364835; Ch/99: AF358787; Ch/2000: AF358788; TW/2000: AF358786; JL-1/97: AF400614; JL-2/97: AF400615) and published work [33]

[b]Only residues different from that of genotype I were listed

[c]The majority of the isolates or strains shown A → T substitution

[d]A few isolates or strains exhibited F → Y substitution, while most others showed no change

viruses of this particular genetic group, which have been isolated exclusively from mainland China and Taiwan, were found when we aligned the full length F gene sequence data of 30 NDV strains in this study and 21 other strains from GenBank (Table 5). All subgenotype VIId viruses had unique $I^{52} \rightarrow V$ and $K^{71} \rightarrow R$ substitutions and most of them additionally shared $A^{402} \rightarrow T$, $D^{489} \rightarrow E$ and $V^{520} \rightarrow G$ substitutions. Furthermore, VIId viruses also shared substitutions $A^{176} \rightarrow S$ and $F^{314} \rightarrow Y$ with VIIc and VIIe viruses, and $N^{272} \rightarrow Y$ with VIIa, VIIc and VIIe viruses, respectively. More importantly, VIId viruses not only comprised the overwhelming majority of strains in genotype VII in this study but also were responsible for ND outbreaks in goose flocks in southern China in recent years, because all but one of the strains of goose origin fell into subgenotype VIId. Because the goose is usually considered to be resistant even to the most virulent strains of NDV for chickens [5], the novel disease entity of NDV infection in this species was described as "goose paramyxovirus infection" in earlier reports of Chinese literature [18, 35] although virulent NDV strains were invariably isolated from diseased geese in field outbreaks. Results in this study disclosed for the first time the epidemiological link of subgenotype VIId viruses with the novel NDV entity in the goose.

From our work in this study in conjunction with recent publications of other groups [8, 15, 16, 23], we can present a general picture of ND prevalence in China in the past two decades: sporadic infections in some regions were caused by strains in genotypes IX and VIII, which are closely related to "old" genotypes II, III, and IV; more frequent outbreaks were induced by strains of genotype VI (9 of 29 strains), including subgenotype VIb of pigeon origin and two newly assigned subgenotypes VIf and VIg; epizootic infections were predominantly caused by strains in genotype VII, including subgenotype VIId that are responsible for most outbreaks in both chicken and goose flocks in more recent years. The genetic divergence of NDV strains isolated in China in recent years may reflect the fact that village poultry farming has been flourishing extensively since 1990 while "stamping out" policy is difficult to be implemented and intensive vaccination is the major control strategy for ND in this country.

It has been suggested that ND outbreaks in vaccinated poultry flocks are due to the emergence of new genotypes or antigenic variants [15, 16, 24, 25]. Li et al. reported recently [15] that CHA7/96, a mainland China strain of NDV, was an antigenic variant responsible for recent outbreaks of ND in vaccinated flocks. In a cross-protection test, these authors reported that when CHA7/96 was used as the challenge virus, clinical protection conferred by the vaccine strain La Sota was only 40%. However, the results of our cross-protection experiment (Table 4) were that chickens vaccinated with either live vaccine (from commercial source) or oil-emulsion killed vaccine, both of the La Sota strain, were fully protected from disease and death against challenge with heterologous NDV strains of different genotypes and subtypes. This is in good agreement with data reported by Alexander et al. [6] who demonstrated that vaccination of birds by using the vaccine strain Hitchner B1 defends them against the challenge with a virus (strain 34/90) that differs from the vaccine strain in the most of the F and HN antigenic epitopes. Toyoda et al. [31] and Yusoff et al. [36] mapped the five major epitopes (A1 to A5) on the F protein of NDV that are involved in fusion inhibition and neutralization. They found that the stretch of amino acids from residues 157–171 and individual residues 72, 78, 79 and 343 were critical for both structures and functions of these epitopes. More recently, Chen et al. [10] determined the three-dimensional structure of the F protein of NDV and confirmed these findings. When we aligned the full length F gene sequence of 30 NDV strains in this study and 21 other strains from GenBank and previously published reports (Table 5), we found that residues^{157} SIAATNEAVHEVTG^{171} and residues D^{72}, K^{78}, A^{79} and L^{343} are highly conserved and shared by all of the 51 strains, including La Sota (data not shown). This fact may explain in part the cross-protection conferred by this vaccine strain against different challenge strains in experimental challenges. On the other hand, the antigenic differences between strains representing new genotypes or subgenotypes and the vaccine strain might influence the level of virus replication and shedding in vaccinated birds that could be an important contributing factor to vaccine break in the field. More work needs to be undertaken to understand the role played by the emergence of new genotypes or subtypes in frequently occurring vaccination failure.

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