Analysis of the Fusion Protein Gene of Newcastle Disease Viruses Isolated in Japan

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ABSTRACT. The complete nucleotide sequences of the fusion (F) protein gene of Newcastle disease viruses (NDV) isolated in Japan from 1930 to 2007 (45 strains total) were determined and genetically analyzed. In the deduced amino acid sequences of fusion protein, the 5 potential asparagine-linked glycosylation sites and 10 cysteine residues were all conserved in the NDV examined in this study. The major epitopes involved in virus neutralization are conserved in most of the NDV strains isolated in Japan except a few strains. By virus neutralization test, no major antigenic differences were observed among representative strains of each genotype in Japan. All chickens vaccinated with the B1 strain survived without clinical signs after challenge with 2 NDV strains isolated in Japan (velogenic strains, JP/Ibaraki/2000 and JP/Kagoshima/91), which possess amino acid substitutions involved in virus neutralization in the F protein gene.

KEY WORDS: antigenicity, B1 vaccine, fusion protein, Newcastle disease virus, phylogenetic analysis.

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Newcastle disease is one of the most serious diseases in the poultry industry. The causative agent is the Newcastle disease virus (NDV), also designated avian paramyxovirus type 1 (APMV-1), which belongs to the genus *Avulavirus* within the family *Paramyxoviridae* [3].

Paramyxovirus infection is initiated by the action of two envelope glycoproteins [20]. One of them mediates attachment of the virus to the host cell receptor and is designated hemagglutinin-neuraminidase (HN) for the paramyxovirus. The other glycoprotein, designated fusion (F), is responsible for virus penetration into the host cell and virus-induced cell fusion and hemolysis [20]. These 2 surface glycoproteins are important as the targets of the host immune response. One characteristic feature in the host immune response to paramyxovirus infection is that although antibodies to either glycoprotein can neutralize infectivity in *in vitro* tests, antibodies to F appear to be predominantly necessary and important for preventing infection and spread of the virus *in vivo* [31]. In passive immunization in chicks, monoclonal antibodies directed to 2 antigenic sites of the F protein completely suppressed virus growth and prevented the death of chickens [41]. Therefore, the F protein gene of NDV was selected to construct a candidate of the DNA vaccine or recombinant vaccine expressing this gene and proved to efficiently protect against challenge with the virulent NDV strains [22, 38, 39]. The 5 major epitopes were mapped on the F protein of NDV, which was involved in fusion inhibition and neutralization [40, 43]. Toyoda *et al.* and Yusoff *et al.* found that the stretch of amino acids from residues 157–171 and individual residues 72, 78, 79 and 343 were critical for both the structures and functions of these epitopes.

To date, phylogenetic analysis based on the genomic

sequences have revealed that the strains of NDV could be divided into 2 distinct clades, class I and class II, and that each clade contains nine genotypes (1–9 and I–IX, respectively) [11, 17, 18]. Among them, class II NDV strains have been responsible for most of the outbreaks of ND reported throughout the world; in particular, strains of genotype VII (class II) are regarded as the major pathogen responsible for the recent outbreaks in eastern Asia, including Japan [19, 21, 23–25, 27, 28]. On the other hand, most of the class I isolates have been obtained from waterfowl, shorebirds or poultry from live bird markets in Asia or America [17, 18].

Previously, based on phylogenetic analysis of the above-mentioned F protein genes, NDV strains isolated in Japan could be classified into 6 genotypes of class II (I–III, VI, VII and VIII) [27, 28], suggesting that the NDV strains in Japan are genetically diverse. However, a full analysis of the amino acids related to these important epitopes located in the F protein gene involved in fusion inhibition and neutralization has not been performed. Therefore, we determined the full length of the F protein gene of NDV strains in Japan and mainly examined the amino acid residues of epitopes involved in neutralization of the NDV. Additionally, the efficiency of protection of live vaccine strain B1 was evaluated against isolated NDV strains from chickens in Japan.

MATERIALS AND METHODS

Viruses: A total of 45 Japanese NDV strains isolated from 1930 to 2007 were used and described previously (all viruses belonged to class II) [27, 28] (Table 1). The allantoic cavities of specific-pathogen-free (SPF) embryonated eggs were used for virus propagation, and the allantoic fluids were then stored at –80°C until use.

Genetic analysis of F protein gene: Viral RNA was extracted from the infected allantoic fluids using a kit (ISOGEN-LS, Nippon Gene, Tokyo, Japan). A reverse

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Table 1. NDV strains used in this study

Virus	Abbreviation	Isolation year	Genotype
APMV1/chicken/Japan/Sato/30	JP/Sato/30	1930	III
APMV1/chicken/Japan/Miyadera/51	JP/Miyadera/51	1951	II
APMV1/chicken/Japan/Ishii/62	JP/Ishii/62	1962	I
APMV1/Japanese blue magpie/Japan/Narashino/68	JP/Narashino/68	1968	VI
APMV1/chicken/Japan/Chiba/69	JP/Chiba/69	1969	VI
APMV1/pigeon/Japan/Ibaraki/84	JP/Ibaraki-pg/84	1984	VI
APMV1/chicken/Japan/Ibaraki/85	JP/Ibaraki/85	1985	VI
APMV1/pheasant/Japan/Tochigi/85	JP/Tochigi-ph/85	1985	VI
APMV1/chicken/Japan/TY-1/85	JP/TY-1/85	1985	VI
APMV1/pheasant/Gunma/85	JP/Gunma-ph/85	1985	VII
APMV1/chicken/Japan/Hyogo/85	JP/Hyogo/85	1985	VII
APMV1/chicken/Japan/Niigata/85	JP/Niigata/85	1985	VII
APMV1/chicken/Japan/Saitama/85	JP/Saitama/85	1985	VII
APMV1/chicken/Japan/Shizuoka/85	JP/Shizuoka/85	1985	VII
APMV1/chicken/Japan/Wakayama/85	JP/Wakayama/85	1985	VII
APMV1/pigeon/Japan/Tochigi/86	JP/Tochigi-pg/86	1986	VI
APMV1/chicken/Japan/Chiba/86	JP/Chiba/86	1986	VII
APMV1/chicken/Japan/Chiba/87	JP/Chiba/87	1987	VII
APMV1/pigeon/Japan/Niigata/88	JP/Niigata-pg/88	1988	VI
APMV1/chicken/Japan/Nara/89	JP/Nara/89	1989	VII
APMV1/chicken/Japan/Niigata/89	JP/Niigata/89	1989	VII
APMV1/pigeon/Japan/Kushiro/91	JP/Kushiro-pg/91	1991	VI
APMV1/chicken/Japan/Kagoshima/91	JP/Kagoshima/91	1991	VIII
APMV1/chicken/Japan/Okinawa/91	JP/Okinawa/91	1991	VIII
APMV1/chicken/Japan/MET/95	JP/MET/95	1995	II
APMV1/pigeon/Japan/Kumamoto/95	JP/Kumamoto-pg/95	1995	VI
APMV1/pigeon/Japan/Tochigi/95	JP/Tochigi-pg/95	1995	VI
APMV1/pigeon/Japan/Shiga/96	JP/Shiga-pg/96	1996	VI
APMV1/parakeet/Japan/Chiba/97	JP/Chiba-pa/97	1997	VI
APMV1/pheasant/Ibaraki/97	JP/Ibaraki-ph/97	1997	VII
APMV1/chicken/Japan/Ibaraki/1/99	JP/Ibaraki-1/99	1999	VII
APMV1/pigeon/Japan/Gunma/2000	JP/Gunma-pg/2000	2000	VI
APMV1/chicken/Japan/Ibaraki/2000	JP/Ibaraki/2000	2000	VII
APMV1/pigeon/Japan/Kumamoto/2001	JP/Kumamoto-pg/2001	2001	VI
APMV1/chicken/Japan/Ibaraki/254/2001	JP/Ibaraki-254/2001	2001	VII
APMV1/chicken/Japan/Nagano/2001	JP/Nagano/2001	2001	VII
APMV1/chicken/Japan/Ibaraki/258/2001	JP/Ibaraki-258/2001	2001	VII
APMV1/duck/Japan/Hyogo/2002	JP/Hyogo-dk/2002	2002	I
APMV1/pigeon/Japan/Shizuoka/2002	JP/Shizuoka-pg/2002	2002	VI
APMV1/chicken/Japan/Okayama/1/2001	JP/Okayama-1/2002	2002	VII
APMV1/pigeon/Japan/Saitama/2003	JP/Saitama-pg/2003	2003	VI
APMV1/chicken/Japan/Fukuoka/1/2004	JP/Fukuoka/1/2004	2004	VII
APMV1/laughing kookaburra/Japan/Nara/2005	JP/Nara-lk/2005	2005	VI
APMV1/cormorant/Japan/Gifu-Ibaraki/2005	JP/Gifu-Ibaraki/cor/2005	2005	VII
APMV1/pigeon/Japan/Niigata/2007	JP/Niigata-pg/2007	2007	VI

transcriptase (RT) reaction was carried out with Superscript III (Life Technologies, Gaithersburg, MD, U.S.A.) using random 9-mers. The primers for the PCRs used for this study are listed in Table 2. PCR was performed using 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. Each PCR product was purified with Montage (Millipore, Billerica, MA, U.S.A.) according to the manufacturer's instructions. The purified PCR products were used as a template for sequencing on an Applied Biosystems 3100 automated DNA sequencer using dye terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA, U.S.A.). The purified PCR products were sequenced from both directions. The derived nucleotide sequences

were analyzed using GENETYX-Mac ver. 13.0 (Software Development Corp., Tokyo, Japan). Sequences of the NDV strains were aligned with the ClustalX program, and phylogenetic trees were constructed by the neighbor-joining method as described previously [27]. To assess the genetic diversity, including the possibility of recombination in this gene [35, 36], among the NDV strains, phylogenetic trees were constructed with two portions (nt 47 to 751 and nt 752 to 1708) of the F protein gene (Fig. 1a and 1b).

Virus neutralization test (VNT): To understand the differences of antigenicity among NDV strains belonging to each genotype, a virus neutralization test using three antisera against NDV [12] was performed by microneutralization

Table 2. List of RT-PCR and sequencing primers

Name	Primer sequence (5'-3')	Genomic site in B1/47 (Acc No. AF309418)
ND-19F	TTCTCTAGCAGTGGGACAGC	4,169-4,188
ND-26F	TGGAGCCAAACCGCGCACCTGCGG	4,235-4,258
ND-14F	TTAATGGCAGGCCTCTTGC	4,638-4,656
ND-10F	CTTTGCTCACCCCTTGG	4,812-4,830
ND-15R	GGAGGATGTTGGCAGCATT	5,000-4,982
ND-11R	CATCTTCCCAACTGCCACTG	5,089-5,070
ND-1F	AACCCTATTCTGTACGACTCAC	5,357-5,378
ND-23F	AGGCTCAGTGGGAATTTGATG	5,846-5,867
ND-4R	AACGATATAGGTGATGAGAGC	6,061-6,041
ND-25R	ATCCGAAAACCAGGCGCCATGT	6,491-6,469

Fig. 1a. Phylogenetic tree of NDV isolates based on nucleotide sequences from a portion (nt 47 to 751) of the F gene. The horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences.

methods with 48-well plates. Briefly, the constant virus and varying serum technique was employed in which two hundred 50% tissue culture-infected doses (TCID₅₀) of virus isolates and twofold dilutions of antisera, starting from 1:4, were used. Two wells were used per virus-serum mixture. The neutralization titers were read one week after inoculation, and the reciprocal of the highest dilution of the serum showing a 50% endpoint of neutralization was used.

Cross-protection experiments using the B1 strain against isolated NDV from chickens in Japan: The B1 strain is mainly used for commercial poultry flocks in Japan. The effect of the B1 strain used as a live vaccine strain was eval-

uated against NDV isolates in Japan according to method reported previously [12]. Two NDV isolates (JP/Ibaraki/2000 and JP/Kagoshima/91, which were classified into genotypes VII and VIII, respectively) were used in this experiment because both strains possessed amino acid substitutions involved in virus neutralization as described later. Four-week-old specific pathogen free chicks (line M, Nisseiken, Hokuto, Japan) were vaccinated with the B1 strain orally (10⁶ EID₅₀, 0.2 ml/bird). Two weeks later, the birds were challenged by the intratracheal route with live JP/Ibaraki/2000 and JP/Kagoshima/91 (10⁴ EID₅₀, 0.1 ml/bird). The birds were placed in a negatively pressured house with

Fig. 1b. Phylogenetic tree of NDV isolates based on nucleotide sequences from a portion (nt 752 to 1708) of the F gene. The horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences.

high efficiency particulate air-filtered intakes and exhaust. All chicks were observed daily for 14 days, and overt clinical signs (edema, muscular tremors, torticollis and paralysis of wings and legs) and/or deaths were recorded. Tracheal and cloacal swabs were taken from each chicken at 3 days post-challenge for virus isolation to examine excretion of the virus. The swabs were frozen at -80°C and later thawed for ease of handling. Solid debris was pelleted by centrifugation, and the supernatants were titrated for virus infectivity in primary chicken kidney cell cultures (CKCs). CKCs were cultured as described previously [13]. Serum samples for a hemagglutination inhibition (HI) assay to detect antibodies to NDV were taken by wing bleed before and after the challenge. All experimental procedures and animal care were performed in compliance with the guidelines of the National Institute of Animal Health for the humane use of laboratory animals.

RESULTS

Nucleotide sequencing and sequence analysis: The amplified F protein gene coding sequence for each strain was 1662 nucleotides, directing the synthesis of a protein predicted to be 553 amino acids in length. The 5 potential asparagine-linked glycosylation sites (positions 85, 191, 366, 447 and 471) and 10 cysteine residues (positions 76, 199, 338, 347, 362, 370, 394, 399, 401 and 424) [6, 30] were

all conserved in NDV strains in Japan.

The stretch of amino acids from residues 157–171 and individual residues D72, K78, A79 and L343, which are involved in virus neutralization, have been identified in previous studies [33, 40, 43]. These amino acids are highly conserved among the NDV strains used in this study with some exceptions. Some of the 2000–2001 isolates (JP/Ibaraki/2000, JP/Ibaraki-254/2001 and JP/Nagano/2001 strains, which were classified into genotype VII) possessed an amino acid substitution at position 78 (K to R). The genotype VIII strains (JP/Kagoshima/91 and JP/Okinawa/91) strains possessed an amino acid substitution at position 79 (A to T) in addition to 78 (K to R). Some isolates from pigeons, which were classified into genotype VI, possessed an amino acid substitution at position 170 (D to N; Table 3).

Phylogenetic analysis: The NDV strains are displayed in the 2 trees as belonging to the same genotypes, showing that each of the 2 regions in the F protein gene can be used to determine phylogenetic relatedness among NDV strains in Japan. Both trees share a very similar topology, indicating that no recombinant events occurred between F protein genes to generate strains of different lineage.

Virus neutralization test: The results of the cross-neutralization tests are shown in Table 4. No major antigenic differences were observed among representative strains of each genotype in Japan.

Cross-protection experiments: It has been suggested that

Table 3. Amino acids involved in neutralization sites of representative genotypes NDV in Japan

Virus	Genotype	Amino acids position at																		
		72	78	79	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	343
		D	K	A	S	I	A	A	T	N	E	A	V	H	E	V	T	D	G	L
JP/Hyogo-dk/2002	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JP/Miyadera/51	II	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JP/Sato/30	III	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JP/Narashino/68	VI	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JP/Ibaraki-pg/84	VI	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	
JP/Nara-lk/2005	VI	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JP/Niigata-pg/2007	VI	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	
JP/Shizuoka/85	VII	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JP/Ibaraki/2000	VII	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JP/Fukuoka/1/2004	VII	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
JP/Kagoshima/91	VIII	.	R	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	

Table 4. Results of the cross-neutralization test among NDV strains

Viruses	Genotype	Antiserum against		
		B1/47	JP/Sato/30	JP/Shizuoka/85
JP/Hyogo-dk/2002	I	64	1024	2048
JP/Miyadera/51	II	128	1024	2048
JP/Sato/30	III	64	2048	1024
JP/Narashino/68	VI	64	1024	2048
JP/Ibaraki-pg/84	VI	32	256	2048
JP/Nara-lk/2005	VI	64	256	256
JP/Niigata-pg/2007	VI	16	128	512
JP/Shizuoka/85	VII	64	1024	4096
JP/Ibaraki/2000	VII	64	128	512
JP/Fukuoka/1/2004	VII	64	256	256
JP/Kagoshima/91	VIII	32	512	2048

Table 5. Results of vaccinating chickens with B1 and challenging them with JP/Ibaraki/2000 and JP/Kagoshima/91

Group	Immunity	Challenge	Number of chickens		HI titers		Recovery of virus at PID3 (No. of chickens with virus/No. tested ($\log_{10}$ TCID ₅₀ /ml))	
			Sick	Dead	Pre-challenge	Post-challenge	Tracheal	Cloaca
1	+	JP/Ibaraki/2000	0/5	0/5	2–6 (4 ^b)	6–12 (10.8)	3/5 (2.7 ± 2.0) ^c	1/5 (0.75)
2	–	JP/Ibaraki/2000	5/5	5/5 (5.2 ^a)	< 2	NT	5/5 (3.3 ± 0.8)	4/5 (2.3 ± 0.6)
3	+	JP/Kagoshima/91	0/5	0/5	2–5 (3.6)	12–13 (12.4)	5/5 (2.9 ± 1.4)	1/5 (2.0)
4	–	JP/Kagoshima/91	5/5	5/5 (5.4)	< 2	NT	5/5 (4.1 ± 1.6)	3/5 (2.7 ± 0.9)

a) Mean death time in days.

b) Geometric mean HI titers.

HI titers are expressed as $\log_2$ of the reciprocal of the highest dilution completely inhibiting 4 HAU of virus.

HI titers were recorded using the JP/Ishii/62 strain as the antigen.

c) Mean ± SD for the number of virus-positive chickens.

ND outbreaks in vaccinated poultry flocks are due to the emergence of antigenic variants [16, 42]. To investigate this issue, we conducted the protection test using the B1 vaccine strain against two isolates (JP/Ibaraki/2000 and JP/Kagoshima/91). All chickens vaccinated with the B1 strain survived without clinical signs after challenge with 2 NDV isolates in Japan, whereas all unvaccinated control chickens challenged with the JP/Ibaraki/2000 and JP/Kagoshima/91 strains died within 6 days. However, vaccination did not prevent infection and excretion of either challenge virus (Table 5). When compared with unvaccinated chickens,

vaccination reduced the proportion of swabs that were positive and the titers of excreting virus.

DISCUSSION

Antibodies to epitopes on either the F or HN protein of NDV may result in neutralization of infectivity [1, 2, 7, 14, 32, 33, 37]. However, while single mAbs directed to sites on the F protein have shown strong neutralizing activity, neutralization by individual HN mAbs has been shown to be incomplete, requiring antibodies to 4 sites for complete neu-

tralization [14]. In passive immunization in chicks, the mAbs directed to the F protein had a protective effect against challenge with virulent NDV [32, 41]. Therefore, the importance of antibodies to F protein for vaccine protection against ND has been emphasized [29], and it is important to understand the genetic diversity of the F protein gene of field NDV strains to evaluate and develop an effective vaccine against NDV.

Here, we examined the NDV strains isolated from 1930 to 2007 in Japan on the basis of molecular analysis of the F protein gene. The amplified F protein gene coding sequence for all strains was 1662 nucleotides and encoded one long open reading frame of 553 amino acids in length. These results showed that deletions and insertions did not occur in the NDV strains used in this study. Furthermore, five potential asparagines-linked glycosylation sites and ten cysteine residues were conserved in NDV strains used in this study, suggesting that the F protein of NDV was highly conserved in length among all strains. These results were consistent with those of other previously analyzed strains of NDV [6, 16, 42].

On the other hand, a recombinant variant in the F protein gene of NDV was recently reported in China [35, 36]. Qin *et al.* described that the recombination was found in the F protein gene of the SRZ03 strain isolated in China. By analysis of complete nucleotide sequences of this strain, they revealed that the N-terminal of this strain's F protein gene originated from a genotype II NDV strain, whereas the C-terminus of the F protein gene and the rest of the genes originated from a genotype VII NDV strain. Previously, we reported that NDV strains in Japan were genetically close to foreign isolates, particularly those from Korea and China [27, 28]. Hence, we compared the two portions of the F protein genes of NDV. Our analysis showed no recombination occurred in the F protein gene, showing that such recombinant variant have not been introduced into Japan.

The 5 major epitopes on the F protein of NDV were previously mapped [40, 43]. The stretch of amino acids from residues 157–171 and individual residues 72, 78, 79 and 343 were critical for both the structures and functions of these epitopes [8]. With some exceptions, these amino acids are conserved in the NDV strains used in this study. This is in good agreement with the results of a VNT including anti-B1 strain (live vaccine strain) sera, suggesting that no major antigenic differences were observed among representative strains of each genotype in Japan.

On the other hand, point amino acid substitutions at residues 78 and 79 in the F protein were identified in the strains of NDV genotype VIII (JP/Kagoshima/91 and JP/Okinawa/91), and some reports have suggested that such amino acid substitutions have an influence on the antigenicity and effect of a vaccine [16, 42]. However, our cross-protection test showed that vaccination of chickens by using the B1 strain protected them against the challenge with viruses (JP/Ibaraki/2000 and JP/Kagoshima/91) with such point amino acid substitutions in the F protein. A previous cross-protection report [12] also demonstrated that vaccination of chickens

by using the B1 strain protected the chickens against challenge with viruses of genotype VI and VII strains isolated in 1985, which do not possess amino acid substitutions on the 5 epitopes involved in neutralization according to the analysis in the present study. Furthermore, vaccination of chickens using the B1 strain protects chickens against challenge with isolates from pigeons [10, 34]. These results and previously published reports [4, 15, 26] suggest that antigenic variants, which fail to be neutralized by antibodies induced by conventional vaccines, would be unlikely to arise easily as described by others [9] and suggest that analysis of the F protein gene is important in estimating the emergence of antigenic variants.

However, as previously reported [5, 15], vaccination did not prevent infection and excretion of either challenge virus. Protection against ND is dependent on the immune status of the vaccinated flocks [15], and therefore, it would be important to develop an effective vaccine that is able to produce and maintain a high antibody level in vaccinated flocks.

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