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NOTE

Avian Pathology

Hemagglutinin-neuraminidase gene of genotype VII Newcastle disease virus strains isolated in Japan

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ABSTRACT. Recently, genotype VII of Newcastle disease virus (NDV) has become the most prevalent NDV genotype in Asia. Here the hemagglutinin-neuraminidase (HN) gene of genotype VII NDV strains isolated in Japan was analyzed. Notably, point amino acid substitutions in the HN protein at position 347, which is located on the major linear epitope of the HN protein, were found in two strains. However, by a hemagglutination inhibition assay, major antigenic differences did not exist between the studied strains. Additionally, chickens vaccinated with the B1 strain did not exhibit clinical effects after challenge with variants possessing the substitution at position 347 (E to K), whereas all unvaccinated chickens subjected to this challenge died within 5 days.

KEY WORDS: B1 vaccine, E347K, hemagglutinin-neuraminidase, Newcastle disease virus

Newcastle disease (ND) is a serious contagious viral disease that affects the poultry industry worldwide [14]. ND is caused by specific strains of avian orthoavulavirus 1 (AOAV-1), which belongs to the genus *Avulavirus* in the family *Paramyxoviridae* [16] and is synonymous with avian paramyxovirus type-1 or Newcastle disease virus (NDV). NDV can be grouped into three pathotypes depending on the virulence of the isolate: lentogenic, mesogenic, and velogenic [14]. Strains of the latter two NDV pathotypes are considered virulent; such strains have frequently been identified as the causative agents of ND outbreaks in many countries [14].

The 15-kb NDV genome is composed of six genes that encode six structural proteins [14]. Of these proteins, the cleavability of the fusion (F) glycoprotein is the main determinant of NDV pathogenicity in chickens. The molecular basis of this pathogenicity seems to be primarily determined by the amino acid sequence motif present at the cleavage site of the precursor fusion protein (F0) and the cleavability of the F0 protein by proteases [15]. Another surface glycoprotein, namely hemagglutinin-neuraminidase (HN), is also known to play a critical role in the viral infectivity of NDV and to produce virus-neutralizing antibody responses [1, 2, 4]. HN protein recognizes and attaches to sialic acid receptors on the surface of permissive cells and mediates the fusion activity of the F protein at the cell membrane leading to the release of the nucleocapsid complex into the cytoplasm [14].

Genetically, NDV can be separated into two distinct classes of one serotype, i.e., class I and II viruses, based on phylogenetic analysis [5]. Typically, ND outbreaks are caused by class II viruses. Among the class II NDVs, genotype VII has become a predominant genotype in poultry and is responsible for recent ND outbreaks in Asian countries including Japan [5].

Notably, among genotype VII isolates, an NDV variant has emerged that possesses an amino acid substitution at position 347 of the HN protein; this variant is frequently reported in vaccinated chickens [7, 20]. It seems that, compared with other antigenic sites, position 347 may be vulnerable to substitutions driven by immune pressure from vaccine immunity [3, 22]. Furthermore, this unique amino acid substitution is located on the major linear epitope of the HN protein, which indicates the possibility of antigenic variation [3, 22]. Therefore, it is important that researchers confirm the existence of this variant in poultry and determine how it is affected by current vaccines. To date, analysis of the HN protein gene of genotype VII NDV strains has yet to be completed in Japan; thus, the epidemiology of such variants in Japan remains unclear. In this study, the author analyzed the HN protein gene of genotype VII isolates from Japan as well as isolates from chicken materials imported from China to determine important amino acid substitutions. Additionally, vaccine efficacy tests were conducted using the B1 strain (the major vaccine strain in Japan) against the challenge posed by variants possessing an amino acid substitution at position 347 (E to K) of the HN protein.

The NDV strains examined in this study are shown in Table 1. In previous studies, these strains have been classified into genotype VII in class II [6, 11, 12]. The names of these strains have been abbreviated in the tables and figure presented here. All strains were derived from chickens except for JP/Gifu-Ibaraki/cor/2005. Allantoic cavities of embryonated eggs were used for virus

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Table 1. Amino acids constituting neutralizing epitopes of hemagglutinin-neuraminidase (HN) protein

Viruses	HN protein									
	193-201	263	287	321	332-333	346-353	356	494	513-521	569
Consensus[a]	LSGCRDHSH	N	D	K	GK	DEQDYQIR	K	G/D	RITRVSSSS	D
JP/Nara/89 [11]	-	K	-	-	-	-	-	-	I514V	-
JP/Ibaraki-ph/97 [11]	-	K	-	-	-	E347G	-	-	I514V	-
JP/Ibaraki-1/99 [11]	-	K	-	-	-	-	-	-	I514V	-
JP/Ibaraki/2000 [11]	-	K	-	-	-	E347G	-	-	I514V, S521N	-
JP/Okayama-1/2002 [12]	-	K	-	-	-	-	-	-	I514V	-
JP/Fukuoka-1/2004 [12]	-	K	-	-	-	E347K	-	-	S521N	-
JP/Gifu-Ibaraki/cor/2005 [12]	-	K	-	-	-	-	-	-	I514V, S521N	V
AQ-JP/Y-15/01 [6]	-	K	-	-	-	-	-	-	I514V	-
AQ-JP/O-26/01 [6]	-	K	-	-	-	-	-	-	I514V	-
AQ-JP/Y-43/01 [6]	-	K	-	-	-	-	-	-	I514V	-
AQ-JP/Y-75/01 [6]	-	K	-	-	-	-	-	-	I514V	-
AQ-JP/Y-14/02 [6]	-	K	-	-	-	E347K	-	-	I514V	-

[a] The consensus amino acid sequence was derived from Newcastle disease virus (NDV) vaccine strains (B1, LaSota).

Table 2. List of RT-PCR and sequencing primers

Name	Primer sequence (5′-3′)	Genomic site in B1/47 (Acc No. AF309418)
ND-28F	AGCAATACACGGGTAGAACG	6313–6332
ND-31R	GAGGGTATCCGAGTGCAACC	6941–6922
ND-30F	GTCACATCATTCTATCCTTCTGC	6853–6875
ND-33R	GAATTGGGTTTTAGCCCTCC	7382–7363
ND-32F	TTGACGACCGYGTATGGTTC	7334–7353
ND-29R	GTYGATGTYGTGTATGCTGC	8000–7981
ND-34F	GGGTCTTCGGGACAATGCTTGAT	7868–7890
ND-35R	GCTCGCCATGTCCTACCCGT	8389–8370

propagation. Viral ribonucleic acid (RNA) was extracted from the infected allantoic fluids using a commercial kit (QIAamp Viral RNA Mini Kit; QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription, polymerase chain reaction (PCR) amplification, and sequencing were performed as previously described [12]. Briefly, reverse transcription with SuperScript™ III (Life Technologies, Gaithersburg, MD, USA) was first conducted using random 9 mers, after which PCR was performed using Takara ExTaq (Takara, Tokyo, Japan) to amplify cDNAs. For PCR amplification, 35 cycles were performed at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec. The primer sequences and locations are shown in Table 2.

The sequenced fragments were assembled using ATGC-Mac ver.5 (GENETYX Corp., Tokyo, Japan). The derived nucleotide sequences and deduced amino acids were then analyzed using GENETYX-Mac ver. 18.0 (GENETYX Corp.). The nucleotide sequences of the complete HN protein coding region were used to construct a phylogenetic tree using MEGA7 [10]. All sequences obtained in this study were deposited in the GenBank database (accession numbers LC648229–LC648240).

The HN protein of all the strains studied here was composed of 571 amino acids, which is consistent with the length of virulent NDV. A cysteine residue and ten previously reported amino acids constituting the sialic acid binding site [4] were completely conserved in all studied strains. Analysis of the ten known neutralizing epitopes in the HN protein [1, 9] led to the identification of amino acid substitutions in each studied strain (Table 1). Notably, substitutions at position 347 (E347K or G), which was located on the major linear epitope of the HN protein suggesting the possibility of antigenic variation, were found in some strains (Table 1).

According to phylogenic analysis, the NDV strains isolated from Japanese poultry were genetically close to strains isolated in Korea (Fig. 1). As expected, all NDV strains isolated from chicken materials derived from animal quarantine in China were genetically close to Chinese strains.

By a hemagglutination inhibition (HI) assay with three antisera against NDV, which was performed according to a previously described procedure [8], it was possible to verify the influence on antigenicity of amino acid substitutions at position 347. Based on the results of this assay, major antigenic differences did not exist among the studied strains (Table 3).

Finally, cross-protection experiments in which the B1 strain was used against the E347K variant were performed because inactivated LaSota vaccine has been shown previously to provide incomplete protection against infection with E347K variants [22]. Notably, the B1 strain is mainly used for vaccination of commercial poultry flocks in Japan. Furthermore, the effect of the B1 vaccine on JP/Ibaraki/2000, which was identified to be an E347G variant in this study, was examined in an earlier study [13]. Two NDV strains (JP/Fukuoka-1/2004 and AQ-JP/Y-14/02) were used in the present experiments because both strains possess amino acid substitutions at position 347 (E to K). The cross-protection experiments were conducted according to previously reported

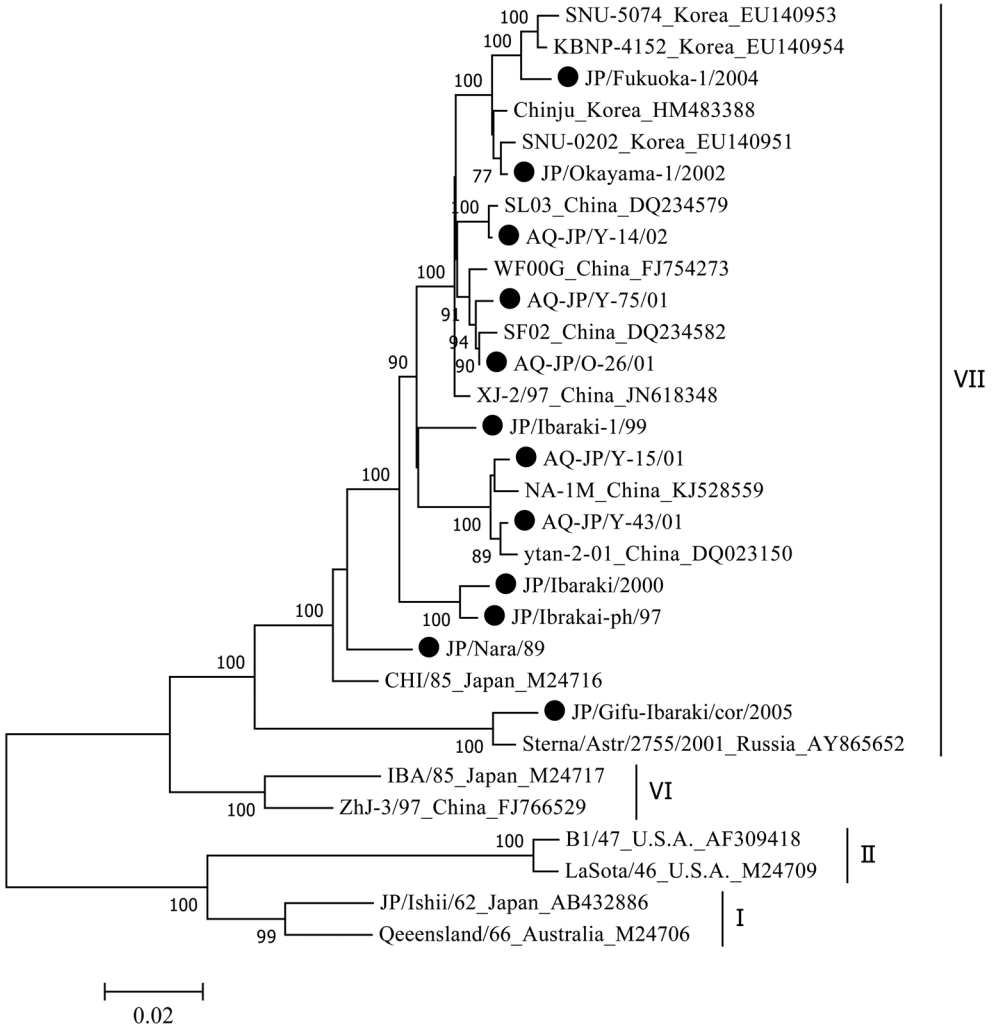

Fig. 1. Phylogenetic tree based on the complete. Hemagglutinin-neuraminidase (HN) gene of Newcastle disease virus (NDV). Nucleotides 6,412–8,145 (1,734 bases) of the HN gene of NDV B1/47 strain (GenBank Accession No. AF309418) were subjected to phylogenetic analysis. The phylogenetic tree was generated using the neighbor-joining method in MEGA 7 [10] with 1,000 bootstrap replications. All tools were run with their default parameters unless otherwise specified. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. The strains examined in this study are shown by black circles.

Table 3. Results of the hemagglutination inhibition tests

Viruses	Amino acid positon at 347	Antiserum against NDV strain		
		B1/47 (II)[a]	JP/Sato/30 (III)	JP/Shizuoka/85 (VII)
JP/Nara/89	E	64	1,024	4,096
JP/Ibaraki-ph/97	G	128	512	1,024
JP/Ibaraki-1/99	E	128	512	4,096
JP/Ibaraki/2000	G	64	512	2,048
JP/Okayama-1/2002	E	64	512	2,048
JP/Fukuoka-1/2004	K	64	256	2,048
JP/Gifu-Ibaraki/cor/2005	E	64	64	512
AQ-JP/Y-15/01	E	128	512	4,096
AQ-JP/O-26/01	E	32	256	2,048
AQ-JP/Y-43/01	E	16	256	1,024
AQ-JP/Y-75/01	E	32	512	2,048
AQ-JP/Y-14/02	K	64	256	1,024

[a] Genotype is shown in parentheses. NDV, Newcastle disease virus.

Table 4. Results of vaccinating chickens with B1 and challenging them with JP/Fukuoka/1/2004 and AQ-JP/Y-14/02

Group	Immunity	Challenge	Number of chickens		Hemagglutination inhibition (HI) titers		Recovery of virus at post infection day 3 (No. of chickens with virus/No. tested (log_{10} EID_{50}/ml))	
			Sick	Dead	Pre-challenge	Post-challenge	Tracheal	Cloaca
1	+	JP/Fukuoka-1/2004	0/5	0/5	5–7 (5.4[b)])	6–9 (7.3)	0/5	0/5
2	-	JP/Fukuoka-1/2004	5/5	5/5 (4.6[a)])	<2	NT	5/5 (4.3 ± 1.0)[c)]	4/5 (2.7 ± 0.9)
3	+	AQ-JP/Y-14/02	0/5	0/5	6–8 (7.1)	7–8 (7.5)	1/5 (1.0)	1/5 (1.0)
4	-	AQ-JP/Y-14/02	5/5	5/5 (4.8)	<2	NT	5/5 (5.0 ± 0.9)	5/5 (4.1 ± 0.8)

[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 ± standard deviation for the number of virus-positive chickens.

methods [8, 13]. Specifically, four-week-old specific-pathogen-free chicks (line M; Nisseiken, Hokuto, Japan) were vaccinated orally with the B1 strain (10^6 EID_{50}; 0.2 ml /bird) at National Institute of Animal Health (NIAH). Two weeks later, the birds were challenged via an intratracheal route with JP/Fukuoka-1/2004 and AQ-JP/Y-14/02 (10^4 EID_{50}; 0.1 ml/bird). The birds were placed in a negatively pressured house with high efficiency particulate air-filtered intakes and exhausts in biosafety level 3 facility at NIAH. All chicks were observed daily for 14 days, and overt clinical signs and/or deaths were recorded. Tracheal and cloacal swabs were collected from each chicken at 3 days post-challenge to isolate and examine excretion of the virus. The virus infectivity in these swabs was titrated using chicken embryos. To conduct a HI assay for the detection of antibodies to NDV, serum samples were taken by wing bleed before and after the viral challenge. All experimental procedures and animal care were performed in compliance with the guidelines of the NIAH for the humane use of laboratory animals.

All chickens vaccinated with the B1 strain survived without clinical signs after challenge with the two NDV isolates. In contrast, all unvaccinated control chickens challenged with the JP/Fukuoka-1/2004 and AQ-JP/Y-14/02 strains died within 5 days (Table 4). In these experiments, viruses were barely recovered from the vaccinated chickens.

Antibodies to epitopes on either the F or HN proteins of NDV may result in neutralization of infectivity [9, 19]. Recently, point amino acid substitutions at residues 347 in the HN protein were identified in some strains; thus, previous reports have suggested that such amino acid substitutions can influence the antigenicity and effects of vaccines [20, 22]. The cross-protection tests performed here showed that vaccination of chickens with the B1 strain protected them against E347K variant viral challenge. In addition, previously conducted cross-protection tests [8] found that vaccination of chickens using the B1 strain provided protection against challenge with the IBA/85 strain [17], which possesses the E347K substitution and is classified into genotype VI. These findings indicate that the current vaccination system using live B1 strain is effective against the challenge posed by NDV variants such as those studied here; moreover, amino acid substitutions at position at 347 seem to have little effect on B1 vaccine efficacy.

Antibodies are reportedly detected in the trachea regardless of whether the B1 strain is inoculated via drinking water, nasal, intratracheal, or intramuscular routes and the titer is almost the same as the serum antibody titer. In contrast, intramuscular inactivated vaccines have lower tracheal antibody titers, even if the serum antibody titers are similar to those of the live vaccine [21]. Because inactivated LaSota vaccine has been suggested to provide incomplete protection against infection with the E347K variant of NDV [22], it seems that local immunity induction is particularly important for protection against challenge by virulent NDV and there is a need for an efficient induction method for local immunity in poultry. However, the live vaccine can be applied to a large number of chickens at one time, but the antibody titer of the applied chickens tends to be uneven due to the influence of the maternal antibody. In many commercial flocks, when the current live vaccine is used for vaccination, it is difficult to collect uniform antibody titers throughout the flock because of maternal antibodies [18]. Therefore, it remains necessary to develop an effective vaccination system that can produce and maintain high antibody levels in vaccinated flocks.

CONFLICTS OF INTEREST. The author declares no conflict of interest.

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