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Genetic comparisons between lentogenic Newcastle disease virus isolated from waterfowl and velogenic variants

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Abstract Avirulent Newcastle disease viruses (NDV) harbored by waterfowl have the potential to become virulent after transmission to and circulation within chicken populations. In order to investigate how virulent viruses are selected from an avirulent background, we compared the complete sequences of the avirulent NDV isolate Goose/Alaska/415/91 and its virulent variant strain 9a5b, which was obtained by nine and five passages in the chick air sac and brain, respectively. Seven amino acid substitutions were detected in the M, F, and HN proteins. Two were detected between variants 9a3b and 9a5b (128P to H and 495E to K in HN protein) that were passed through the brain. Pathogenicity determined by the MDT and IVPI tests also differed between 9a3b and 9a5b. These results suggest that in addition to the F cleavage site sequence, these two amino acids in HN protein are also related to the pathogenicity of NDV in chickens.

Keywords Newcastle disease virus · Hemagglutinin–neuraminidase protein · Waterfowl · Chicken · Virulence

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Newcastle disease virus (NDV) is the causative agent of a serious avian disease that can result in significant economic losses to the poultry industry [1]. It is a negative-stranded enveloped RNA virus of the genus *Avulavirus*, family *Paramyxoviridae* in the order *Mononegavirales* [2]. The approximately 15.2-kb viral genome [3, 4] contains six genes encoding the major polypeptides, nucleocapsid protein, phosphoprotein, matrix protein, fusion (F) protein, hemagglutinin–neuraminidase (HN), and large RNA-dependent polymerase protein [5].

The pathotypes of NDV isolates are classified, from the most to the least virulent, as velogenic, mesogenic, or lentogenic. The primary molecular determinant for the pathogenicity of this virus comprises the amino acids of the cleavage site in the F protein [6–8].

Ecological studies suggest that the natural reservoir of NDV is wild waterfowl [9, 10]. Shengqing et al. [11] experimentally demonstrated that the lentogenic NDV isolates circulating among wild waterfowl have the potential to become velogenic in chickens as a result of amino acid substitutions in the F protein cleavage site. However, other factors such as the HN protein might also affect NDV pathogenicity in chickens. Here, we compared the entire viral genome sequences of a lentogenic NDV isolate from a waterfowl with that of a velogenic variant obtained by passage in chicks to determine the mechanism of acquired pathogenicity.

The avirulent NDV strain Goose/Alaska/415/91 was isolated from a wild waterfowl that had migrated to Alaska [12]. The virus becomes highly virulent in chickens after inoculation and nine consecutive passages in the chicken air sac, followed by five passages in the chicken brain [11]. Virulent isolates were identified by passage number and the organ through which they were passaged. For example, 9a5b indicates that the virus was passaged nine times in the

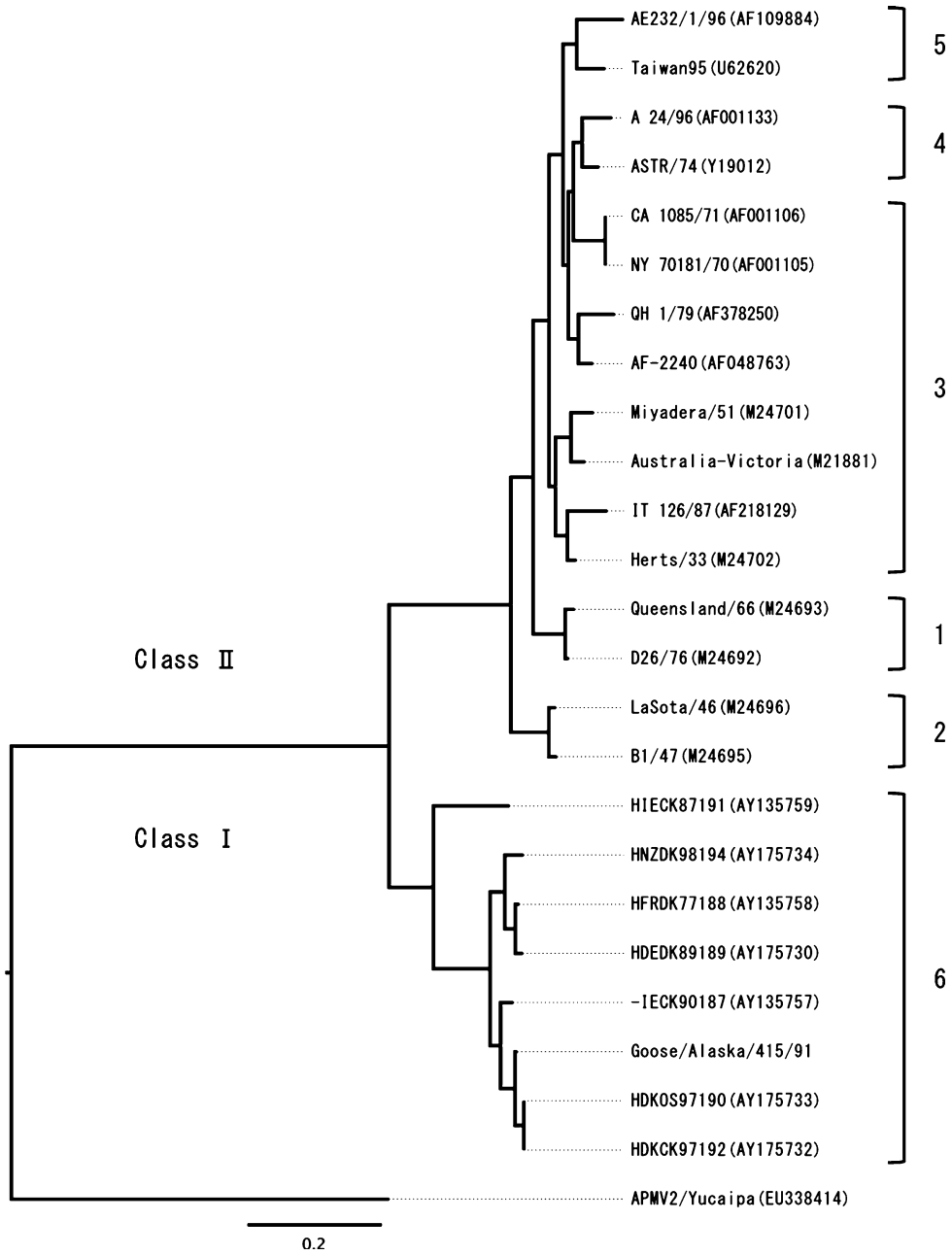

Fig. 1 Phylogenetic analysis based on first 375 bp of NDV F gene coding region. Tree was generated by neighbor-joining with 1,000 bootstrap replicates. *Numbers* on right indicate genetic lineages designated by Aldous et al. [14]. *Numbers* in parentheses indicate GenBank accession numbers. *Scale* indicates branch length based on number of nucleotide substitutions per site

air sac and five times in the brain. Viruses were propagated in the allantoic cavities of 10-day-old embryonated chicken eggs for 48 h at 37°C and then the allantoic fluid was harvested and stored at −80°C.

Viral RNA was extracted directly from infected allantoic fluid using the QIAamp Viral RNA Minikit (Qiagen, CA, USA) according to the manufacturer's protocol, and the reverse transcriptase (RT) reaction proceeded using Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech Inc., Uppsala, Sweden) with random 6-mers. Genes were amplified by PCR with this cDNA and gene-specific oligonucleotide primers. The oligonucleotide sequences used as primers for PCR are available on request.

The recovered PCR products were directly sequenced using the BigDye Terminater Cycle Sequencing Reaction (Applied Biosystems, CA, USA) and the Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech Inc., Uppsala, Sweden) according to the manufacturer's protocol.

We applied single-strand ligation to single-stranded cDNA (SLIC) [13] to determine the 3′ end of viral genome RNA. Viral RNA was incubated at 95°C for 5 min and then on ice for 2 min. The V5 anchor oligomer (5′-AATAGGA GACGTGTGTGAGGGAGGTCGACATTGCC) was ligated to the 3′ end of viral RNA at 16°C overnight using T4 RNA ligase. The ligation product was purified using the

RNeasy Mini Kit (Qiagen, CA, USA) according to the manufacturer's protocol and used for RT–PCR. The primer sets used for RT–PCR were as follows: RT, universal amplification primer, 5′-GGCAATGTCGACCTCCCTC ACAC; 1st PCR, universal amplification primer and ND816R (5′-ACTAGGAAGATTCTGACAGC); 2nd PCR, V5 amplification primer (5′-CTCCCTCACACACGTCTC CTATT), and ND185R (5′-GAGATCCATTAGGGCGT GTC). The recovered PCR product was sequenced as described above.

We used 5′RACE to determine the 5′ end of the viral genomic RNA using the 5-Full RACE Core Set (TaKaRa, Shiga, Japan) kit according to the manufacturer's protocol. First strand cDNA was synthesized by reverse transcription using the 5′-phosphorylated ND13905F primer (5′-*p*-CAG AGGAAAGTGACCTGACCTC) and then incubated with RNase H for 1 h at 30°C. The mixture was purified by ethanol precipitation and incubated with T4 RNA ligase at 15°C overnight. The ligation product was included in PCR with the following primer sets: first, ND14355F (5′-ATGAGATG GCATTGATGGCG) and ND14231R (5′-CCCTACAGG CATACCCGTTG); second, ND14757F (5′-TGGGAGAC ATCATAAGCTTG), and NDR14185 (5′-CGGGGTGAA AAGATTCACAAG). The recovered PCR product was sequenced as described above.

Sequences were phylogenetically analyzed as described by Aldous et al. [14]. The sequences of the first 375 bp nucleotides of the coding region of the F gene were aligned using Clustal W version 1.83 software. A phylogenetic tree was generated by neighbor-joining using FigTree version 1.2.2 software (Andrew Rambaut, Edinburgh, UK). All sequences used for the phylogenetic analysis were obtained from GenBank except for that of strain 415/91.

The complete sequence of the lentogenic NDV isolated from waterfowl strain 415/91 genome comprised 15,198 nt (GenBank accession number: AB524405). Phylogenetic analysis of the first 375 bp of the coding region of the F gene resulted in 415/91 strain being classified into Class I lineage 6 (Fig. 1). NDVs of this genome size, which is 12-nt longer than most chicken NDV isolates, were confirmed among waterfowl isolates in Class I [3], which includes viruses from wild waterfowl and US live bird markets [15].

We compared the sequences of the 415/91 and velogenic variant 9a5b genomes (GenBank accession number: AB524406). We identified 11 mutations between them, of which 7 led to amino acid substitutions (Table 1). Three mutations in the cleavage site coding region of the F gene caused changes from the lentogenic sequence (^{112}E–R–Q–E–R–L^{117}) to the typical velogenic sequence (^{112}K–R–G–K–R–F^{117}) as described [11]. These mutations clearly contribute to the increased viral pathogenicity of this virus in chickens [7]. Four other mutations leading to amino acid substitutions on the M and HN proteins were identified after passage through the chicken brain (Table 2). Two of these occurred between brain-passaged variants 9a3b and 9a5b (128P to H and 495E to K in HN protein). The MDT and IVPI tests also identified different pathogenicity between 9a3b and 9a5b. These results suggest that these two amino acid substitutions in HN protein are related to the pathogenicity of NDV in chickens in addition to the F cleavage site sequence.

Table 1 Mutations and amino acid substitutions between 415/91 and 9a5b strains

No. of nucleotides (no. of amino acids)	Gene	Codon (deduced amino acids)	
		415/91	9a5b
1325–1327 (402)	NP	GAT (Asp)	GAC (Asp)
2937–2939 (351)	P	GAT (Asp)	GAC (Asp)
4316–4318 (339)	M	GTG (Val)	GCG (Ala)
4889–4891 (112)	F	GAA (Glu)	AAA (Lys)
4898–4900 (115)		GAG (Glu)	AAG (Lys)
4904–4906 (117)		TTG (Leu)	TTC (Phe)
6601–6603 (60)	HN	CCA (Pro)	CCG (Pro)
6805–6807 (128)		CCT (Pro)	CAT (His)
7906–7908 (495)		GAG (Glu)	AAG (Lys)
8140–8142 (573)		GAG (Glu)	TAG (Stop)
14225–14227 (1945)	L	AAC (Asn)	AAT (Asn)

Mutations of F gene have been published by Shengqing et al. [11]

The HN protein has at least three functions: neuraminidase (NA) activity, receptor binding, and fusion promotion [16–18]. Gavel and Morrison [19] reported that the region from residue 124 to 152 is involved in fusion promotion. Thus, it is possible that 128P to H substitution, which was found between 9a3b and 9a5b, may affect the fusion promotion activity. It was also reported that the 495E to A substitution increased hemadsorption (HAd) and NA activities [18]. Consequently, another amino acid substitution, 495E to K, may act on the HAd and NA activities. Further investigation using protein expression is required to clarify this notion.

Each variant virus stock contains mixed sub-populations. In this study, therefore, the major population in each uncloned virus was sequenced by PCR direct sequencing method. However, it is also important to compare the sequences of cloned viruses to clarify the heterogeneity of the variant viruses. Sequencing of the biologically cloned viruses is now undergoing.

A comparison of mesogenic and lentogenic NDV using chimeric viruses has suggested that the HN protein is related to tropism and virulence [20]. This finding and ours suggest that the HN protein might be another factor that is involved in increasing the pathogenicity of NDV in chickens.

Another mutation led to an amino acid substitution in the M gene; however, we could not determine the relationship between the M protein and NDV virulence.

Table 2 Amino acid substitutions and pathogenicity tests of viruses passaged in chickens

Virus	Amino acid substitution					Pathogenicity test[a]		
	M protein	F protein cleavage site[a]	HN protein					
	339[b]	112–117	128	495	573	MDT[c]	ICPI[d]	IVPI[e]
415/91	V	E–R–Q–E–R–L	P	E	E	>120	0	0
9a1b	A	K–R–Q–K–R–F	P	E	Stop	75	1.27	1.70
9a3b	A	K–R–Q–K–R–F	P	E	Stop	72	1.75	1.88
9a5b	A	K–R–Q–K–R–F	H	K	Stop	56	1.88	2.67

[a] Obtained from Shengqing et al. [11]

[b] Amino acid position

[c] Mean time to death (h) of chicken embryos infected with single minimal lethal dose of virus

[d] Intracerebral pathogenicity index in day-old chicks

[e] Intravenous pathogenicity index in 6-week-old chickens

Because the M protein is necessary for NDV budding [21], a substitution might affect budding efficiency in different host cells.

References

1. D.J. Alexander (ed.), *Newcastle Disease* (Kluwer Academic Publishers, Dordrecht, 1988), pp. 147–160
2. M.A. Mayo, Arch. Virol. **147**, 1071–1076 (2002)
3. A. Czeglédi, D. Ujvari, E. Somogyi, E. Wehmann, O. Werner, B. Lomniczi, Virus Res. **120**, 36–48 (2006)
4. O. de Leeuw, B. Peeters, J. Gen. Virol. **80**, 131–136 (1999)
5. N.S. Millar, P. Chambers, P.T. Emmerson, J. Gen. Virol. **69**, 613–620 (1988)
6. B. Gotoh, Y. Ohnishi, N.M. Inocencio, E. Esaki, K. Nakayama, P.J. Barr, G. Thomas, Y. Nagai, J. Virol. **66**, 6391–6397 (1992)
7. O. de Leeuw, L. Hartog, G. Koch, B.P.H. Peeters, J. Gen. Virol. **84**, 475–484 (2003)
8. Y. Nagai, H.D. Klenk, R. Rott, Virology **72**, 494–508 (1976)
9. C.J. Kelleher, D.A. Halvorson, J.A. Newman, D.A. Senne, Avian Dis. **29**, 400–407 (1985)
10. J. Spalatin, R.P. Hanson, Avian Dis. **19**, 573–582 (1975)
11. Y. Shengqing, N. Kishida, H. Ito, H. Kida, K. Otsuki, Y. Kawaoka, T. Ito, Virology **301**, 206–211 (2002)
12. H. Takakuwa, T. Ito, A. Takada, K. Okazaki, H. Kida, Jpn. J. Vet. Res. **45**, 207–215 (1998)
13. A. Apte, P. Siebert, Biotechniques **15**, 890–893 (1993)
14. E.W. Aldous, J.K. Mynn, J. Banks, D.J. Alexander, Avian Pathol. **32**, 239–257 (2003)
15. B.S. Seal, M.G. Wise, J.C. Pedersen, D.A. Senne, R. Alvarez, M.S. Scott, D.J. King, Q. Yu, D.R. Kapczynski, Vet. Microbiol. **106**, 7–16 (2005)
16. R.A. Lamb, Virology **197**, 1–11 (1993)
17. R.A. Lamb, D. Kolakofsky, in *Fields Virology*, ed. by D.M. Knipe, P.M. Howley (Lippincott Williams and Wilkins, Philadelphia, 2001), pp. 1305–1340
18. T. Takimoto, L.G. Taylor, C.H. Connaris, J.S. Crennell, A. Portner, J. Virol. **76**, 13028–13033 (2002)
19. K.A. Gravel, T.G. Morrison, J. Virol. **77**, 11040–11049 (2003)
20. Z. Huang, A. Panda, S. Elankumaran, D. Govindarajan, D.D. Rockemann, S.K. Samal, J. Virol. **78**, 4176–4184 (2004)
21. H.D. Pantua, L.W. McGinnes, M.E. Peeples, T.G. Morrison, J. Virol. **80**, 11062–11073 (2006)