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FULL PAPER

Potentially virulent Newcastle disease viruses are maintained in migratory waterfowl populations

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Abstract

Forty-seven Newcastle disease virus (NDV) strains isolated from fecal samples of waterfowls in Alaska and Siberia from 1991 to 1996 were analyzed for their virulence. None of the viruses formed plaques on MDBK cells in the absence of trypsin. Of these, 29 strains showed virulent character by the mean death time with the minimum lethal dose in chicken embryos comparable to velogenic NDV strains. Of the 29 strains, 11 were sequenced for their fusion protein (F) gene. The results showed that 5 of them contained a pair of dibasic amino acids at the cleavage site of the F, which is of a virulent type. The present results suggest that potentially virulent strains of NDV are maintained in migratory waterfowl populations in nature, and that some of those may be transmitted to domestic poultry and acquire pathogenicity during passages in chicken population.

Key words: Chick Embryo, Newcastle Disease Virus, Viral Fusion Proteins, Virulence, Phylogeny

Introduction

Newcastle disease virus (NDV), the prototype of the genus *Paramyxovirus*, comprises a variety of strains which differ in pathogenicity for chicken, such as velogenic (acute, lethal infection of chickens of all ages), mesogenic (less pathogenic, only lethal in young birds), and lentogenic (mild or inapparent infections) forms of the disease[3]. NDV bears two types of glycoprotein, hemagglutinin-neuraminidase (HN) and fusion (F) proteins, on the surface of the particle[8,11]. The HN is responsible for attachment of the virus to the host cell receptor and receptor destroying activity. The F mediates fusion between the viral envelope and the host cell membrane, thereby enabling the penetration of viral genome into the cytoplasm of the host cell[22].

The F is initially synthesized as a precursor polypeptide F_0, and then proteolytically cleaved to two polypeptides, F_1 and F_2 to acquire biological activities[23–25]. It is generally accepted that the cleavability of the F is a critical determinant of the pathogenicity of NDV. Namely, the F of virulent strains are cleaved in a variety of cells, and virulent viruses, therefore, can be disseminated throughout their host and cause extensive disease. In contrast, the F of avirulent strains is cleaved only in limited types of

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cells (e.g., epithelia in intestinal, or respiratory tract)[13,26]. The cleavage site of the F of virulent strains consists of a pair of dibasic amino acid residues (RRQRR or RRQKR), while the corresponding region of avirulent strains is monobasic (GRQGR)[14,28].

Alexander[1] considered that three panzootics of Newcastle disease (ND) had occurred since the first recognition of the disease. The first outbreaks of this disease appear to have arisen in Southeast Asia and the disease spread worldwide and was still important in most countries in the early 1960s. The second panzootic appears to have begun in the Middle East in the late 1960s and to have reached most countries by 1973. The more rapid spread of the second panzootic was considered to be due to international trade in poultry industry and import of psittacine species[12]. The third panzootic of ND apparently arose in pigeons and doves in the Middle East in the late 1970s[16]. By 1981 it had reached Europe[7] and then spread rapidly to all parts of the world. However, it is not clear how NDVs are maintained in nature.

NDV has been isolated from a variety of wild, domestic, and cage birds around the world[2]. The majority of NDV strains isolated from waterfowls such as ducks and geese are lentogenic, causing no obvious disease, similarly to influenza viruses in waterfowls[21]. However, little is known how those viruses are maintained in nature. Waterfowls in the northern hemisphere migrate to south in autumn, back to north in spring, and breed in summer in the nesting places in northern territories such as Alaska, Canada, or Siberia. To provide information how NDV is maintained in duck population in their breeding places, virulence of isolates was assessed by pathogenicity test and phylogenetic analyses of the genes of the isolates were carried out. The present study revealed that potentially virulent strains are circulating among waterfowl populations, which may be implicated in outbreak of Newcastle disease in domestic poultry.

Materials and Methods

Virus isolation

Each fecal sample was put into phosphate-buffered saline (PBS ; pH 7.2) containing antibiotics (10,000 units of penicillin, 10 mg of streptomycin, 600 μg of gentamycin per ml) to give 10–20% suspension. The suspension was centrifuged at 2,500 rpm for 20 minutes. One tenth ml of the supernatant was inoculated into allantoic cavities of 10-day-old fertile hen's eggs. The eggs were incubated at 35°C for 2 to 3 days unless death of the embryo was detected. At the end of the incubation period or upon embryo death, the allantoic fluids were taken and tested for hemagglutinating activity. Each hemagglutinating agents was identified in hemagglutination-inhibition test using specific antiserum to NDV strain Miyadera[20].

Plaque assay

Plaque assay was performed using MDBK cells[24]. MDBK cells were grown in Eagle's minimum essential medium (EMEM) with 10% calf serum, L-glutamine, and antibiotics. After 1 hr adsorption of virus, the inoculum was removed and the cells were then overlaid with EMEM containing 1% Bacto-Agar (Difco) in the absence or presence trypsin (5 μg/ml). After incubation at 35°C or 41.5°C for 2 days in a 5% CO_2 atmosphere, cells were overlaid again with EMEM containing 1% Bacto-Agar and 0.005% neutral red, followed by plaque count.

Sequencing

Viral RNA of each virus was isolated by treatment of allantoic fluid containing virus with proteinase K (Boehringer Mannheim) and sodium dodecyl sulfate, followed by extraction with phenol-chloroform (1:1)[6]. cDNA was synthesized from the viral RNA template by using Moloney murine leukemia virus reverse transcriptase (Gibco BRL) and random hexamer primers[19]. Polymerase chain reaction (PCR)

Table 1. Isolation of NDV from fecal samples of waterfowls in Alaska and Siberia in 1991–1996

Location	Species	Number of NDV isolated in the following year					
		1991	1992	1993	1994	1995	1996
Alaska							
Lake Spenard	Duck	1	2				
Lake Hood	Duck	1		2			
Lake Cheney	Duck	1					
Potter Marsh	Gull	1					
	Goose	7					
Delta Junction	Duck	1					
Big Minto Lake	Duck	1		1	1		
Fairbanks	Duck	2			1		
Creamer's Filed	Duck	1	1				
Mallard Lake	Duck		1	9			
Nenana	Duck		1				
Chena	Duck		1				
Heart Lake	Duck			2	1		
Russia							
Khabarovsk	Duck					6	
Lena River	Duck						2
		16	6	14	3	6	2

Table 2. Pathogenicity of NDV isolates for chicken embryos

Strain	MDT/MLD[a]	Strain	MDT/MLD[a]
Duck/Alaska/260/91	>120	Duck/Alaska/3024/93	>120
Duck/Alaska/300/91	64	Duck/Alaska/3026/93	>120
Duck/Alaska/320/91	48	Duck/Alaska/3028/93	>120
Gull/Alaska/325/91	64	Duck/Alaska/3034/93	48
Goose/Alaska/356/91	48	Duck/Alaska/3037/93	60
Goose/Alaska/363/91	48	Duck/Alaska/3039/93	56
Goose/Alaska/375/91	48	Duck/Alaska/3049/93	64
Goose/Alaska/382/91	>120	Duck/Alaska/3062/93	64
Goose/Alaska/415/91	48	Duck/Alaska/3084/93	48
Goose/Alaska/418/91	48	Duck/Alaska/3228/93	48
Goose/Alaska/421/91	48	Duck/Alaska/3365/93	64
Duck/Alaska/428/91	48	Duck/Alaska/3366/93	48
Duck/Alaska/465/91	>120	Duck/Alaska/5051/94	64
Duck/Alaska/485/91	>120	Duck/Alaska/5091/94	60
Duck/Alaska/685/91	>120	Duck/Alaska/5156/94	48
Duck/Alaska/817/91	>120	Duck/Siberia/160/95	56
Duck/Alaska/2347/92	64	Duck/Siberia/166/95	60
Duck/Alaska/2492/92	>120	Duck/Siberia/219/95	60
Duck/Alaska/2580/92	72	Duck/Siberia/223/95	64
Duck/Alaska/2608/92	>120	Duck/Siberia/224/95	60
Duck/Alaska/2702/92	>120	Duck/Siberia/229/95	>120
Duck/Alaska/2728/92	>120	Duck/Siberia/363/96	>120
Duck/Alaska/3020/93	>120	Duck/Siberia/411/96	>120
Duck/Alaska/3021/93	>120		

a MDT/MLD=mean death time (hr) for chicken embryos infected with one minimum lethal dose of virus.

direct sequencing of the F gene was carried out using an autosequencer (Applied Biosystems Inc., CA). Primers were designed based on comparison of published NDV F gene nucleotide sequences as follows: forward primer (F314) 5'-ACTTTGCTCACCCCCCTTGGGTG-3' and reverse primer (F689) 5'-CTGTTGCTTTCC-TCTAACTT-3'.

Pathogenicity tests

Mean death time (hr) at minimum lethal dose (MDT/MLD) of chicken embryos, intracerebral pathogenicity index (ICPI) in 1-day-old chicks, and intravenous pathogenicity index (IVPI) in 6-week-old chickens were measured to assess the virulence of each NDV strain[5]. ICPI represents the mean score of 10 chicks per observation; maximum index 2.00=all chicks dead within 24 hr, 0.00=no clinical sign in any chick over eight days. IVPI represents the mean score of 8 chickens per observation; maximum index 3.00 =all chickens dead within 24 hr, 0.00=no clinical sign in any chick over 10 days. White Leghorn chickens were hatched and raised exclusively in our laboratory and used.

Phylogenetic analysis of the F genes of virus isolates

Nucleotide sequences of the F genes (positions 355–580) of NDV isolates were analyzed together with those from Genbank, by unweighted-pair group method with arithmetic means (UPGMA)[27], using a computer software, ODEN version 1.1.1 (Yasuo Ina, National Institute of Genetics, Misima, Japan).

Results

Isolation of Newcastle disease viruses from fecal samples of waterfowls

We collected fecal samples of birds in different areas in Alaska, USA from 1991 to 1994 and Siberia, Russia from 1995 to 1996. These samples were inoculated into allantoic cavities of hen's eggs and incubated. From a total of 4,196 samples 244 hemaggulutinating agents were isolated. Of them, 47 agents were serologically identified as NDV by hemagglutination-inhibition test. Of these, 39 were isolated from ducks, 7 were from geese and 1 from gull (Table 1).

Plaque forming ability of NDV isolates

The F of virulent NDV strains is cleaved by cellular protease in cultured cells, while that of avirulent strains require extracellular protease, such as trypsin for the F to be cleaved so that their multiple replication takes place. Therefore, virulent strains form plaques on cultured cells, but avirulent strains do not in the absence of trypsin. We examined the ability of 47 virus isolates to form plaques in the absence of trypsin on MDBK cells. None of the isolates formed plaques in the absence of trypsin, though all of those formed plaques in the presence of trypsin. The results suggest that the F of these viruses was of avirulent type which is not cleavable by intracellular proteases.

Pathogenicity of virus isolates

Virulence of virus isolates was assessed by pathogenicity tests with chicken embryos and chickens (Table 2). MDT/MLD was determined with 9-day-old chicken embryos. Those of 29 out of 47 isolates were 48 to 70 hr, which ranged within those of virulent strains. On the other hand, other virus isolates exhibited more than 120 hr, which were typical for avirulent viruses.

Pathogenicity of these viruses for 1-day-old chicks by intracerebral inoculation was then tested. None of chicks died (ICPI=0.00), indicating that these NDV strains are not neurotropic. Each isolate was inoculated intravenously into 6-week-old chickens. None of chickens intravenously inoculated with each isolate showed clinical signs within 10 observation days (IVPI= 0.00). The results indicate that 29 of these isolates were virulent for chicken embryos, however none of them were virulent for chickens.

Amino acid sequence at the cleavage site of the F gene of the isolates

Amino acid sequence at the cleavage site of

Table 3. Comparison of amino acid sequences at the cleavage site of the F protein of field isolates

Strain	Amino acid sequence[a)]	Pathotype[b)]
Duck/Alaska/300/91	^{109}SGG**RRQKR**-FVG^{119}	virulent
Goose/Alaska/415/91	SGGG**RQGR**-LIG	avirulent
Goose/Alaska/418/91	SGG**RRQRR**-FIG	virulent
Duck/Alaska/685/91	SGGE**RQER**-LVG	avirulent
Duck/Alaska/817/91	SGGG**KQGR**-LIG	avirulent
Duck/Alaska/2580/92	SGG**RRQRR**-FIG	virulent
Duck/Alaska/2728/92	SGGG**KQGR**-LIG	avirulent
Duck/Alaska/3228/93	SGG**RRQRR**-FIG	virulent
Duck/Alaska/5091/94	SGG**RRQRR**-FIG	virulent
Duck/Alaska/5156/94	SGGG**KQGR**-LIG	avirulent
Duck/Siberia/160/95	SGGG**KQGR**-LIG	avirulent
Duck/Siberia/219/95	SEGG**KQGR**-LIG	avirulent
Duck/Siberia/223/95	SEGG**KQGR**-LIG	avirulent
Duck/Siberia/224/95	SEGG**KQGR**-LIG	avirulent
Duck/Siberia/229/95	SEGG**KQGR**-LIG	avirulent

a) Basic amino acids are bolded.

b) Virulent indicate that strains consists of a pair of dibasic amino acid residues at the cleavage site. Avirulent indicate that strains consists of a pair of monobasic amino acid residues.

the F was deduced from the nucleotide sequence of the corresponding gene of the 15 isolates from ducks and geese in Alaska and Siberia from 1991 to 1996. As shown in Table 3, nine of these possess ^{112}GR(K)QGR-LI^{118} at the fusion cleavage site which is typical for avirulent viruses. That of Duck/Alaska/685/91 had unique sequence, ^{112}ERQER-LV^{118} at the cleavage site which is of avirulent type. The sequences of the remaining 5 isolates at the cleavage site were ^{112}RRQR(K)R-FI(V)^{118}, which is of virulent type.

Phylogenetic analysis of the F gene of NDV isolates

Fig. 1 shows an evolutionary tree constructed on the basis of partial sequences of the F genes together with those from Genbank. The NDV isolates were divided into four lineages by the phylogenetic analysis. Lineage A contains isolates such as lentogenic vaccine strains, Queesland/66 and Ulster/64. Lineage B includes velogenic Herts/33 and Australia/Victoria/32. Lineage C includes velogenic Texas/GB/48, and lentogenic vaccine strains, B1/48 and La Sota/46. Lineage D contains velogenic Chicken/Ireland/34/90 and lentogenic Shelduck/France/MC110/77 strains.

All isolates from Siberia in lineage A consisting of lentogenic NDV strains, possessed avirulent type of the cleavage site. Virus isolates from Alaska were scattered through all lineages, A, B, C, and D. Five isolates with avirulent type F from Alaska belonged to the lineage A, except for 2 isolates. Duck/Alaska/415/91 was included in the lineage C. Duck/Alaska/685/91 was found in the lineage D. Five isolates with virulent type F from Alaska belonged to the lineage B or C.

Discussion

In the present study, 47 NDV strains were isolated from fecal samples of birds in Alaska and Siberia. ICPI and IVPI tests suggested that these isolates were avirulent for chickens. Although MDT/MLDs of 29 strains were 48 to 70 hr, which ranged within that of virulent strains, none of the NDV isolates formed plaques on MDBK cells. It was noted that 5 out of these strains contained a pair of dibasic amino acid residues at the cleavage site of the F, which is typical for virulent strains.

It has been established that cleavage of the F of NDV is a major determinant for pathogenicity of the virus[13,24,26]. The F of virulent NDV strains possesses a pair of dibasic amino acids at the cleavage site and is cleaved in a wide variety of cells. In the present study, none of the isolates formed plaques on MDBK cells in the absence of the trypsin, although some of the strains contained a pair of dibasic amino acid residues at the cleavage site of the F. It is possible that carbohydrate chain in the vicinity of the cleavage site interfere with access of cleavage enzyme, as was the case of the hemagglutinin of A/chicken/Pennsylvania/83 influenza virus[17,18]. However, glycosylation site was not found in the sequence data of the area covering vicinity of the cleavage site of the F of these isolates. Another explanation could be that alteration of amino acids in the F may affect the tertiary structure or that cleavage of the F was hampered by other viral protein, resulting in its resistance to cleavage enzymes.

Collins *et al.*[10] reported that 2 out of 15 pigeon NDV isolates had the virulent type amino acid sequence (RRQKR) at the cleavage site of the F. Pathogenicity index, on the other hand, showed that these isolates were of moderate and low virulence to chicken. In the present study, some isolates, which had the virulent type of the F, indicated low virulence to the chickens. These findings indicate that wide variety of pathogenicity of NDV strains for chickens are maintained in nature and that pathogenicity of NDV strains is not necessarily correlated to the amino acid sequence at the cleavage site of the F.

Alexander and Parsons[4] reported that virulence of NDV isolates from pigeons was increased after passage through chickens or embryonated chicken eggs. We reported that the virulence of an NDV isolate from quail increased by passages through chicken brain[15]. The original isolate for quail had a pair of dibasic amino acid residues at the cleavage site of the F but was low virulent to chicken (unpublished data). In the present study, the virulence of Duck/Alaska/418/91 isolate increased pathogenicity for chicks by the passage thought 14-day-old embryonated chicken eggs (data not shown). Therefore, it is of interest to investigate the increase of pathogenicity of field isolates during passages among bird populations or embryonated eggs and should provide additional information on the factors responsible for the pathogenicity of NDV for chickens.

Collins *et al.*[9] reported that a virulent NDV strain, Chicken/Ireland/34/90 had a close antigenic relationship and homologous nucleotide sequence to avirulent Shelduck/France/MC110/77, although a small part of the genome was studied (32 nucleotides near the cleavage site), suggesting that they may have arisen from the same gene pool. Virulent strains which cause outbreak in domestic poultry, thus may be derived from the population of NDVs circulating in migratory birds. In the present study, phylogenetic analysis of the F genes revealed that Duck/Alaska/685/91 isolate belonged to the lineage D, which had avirulent type of the F was most closely related to the Chicken/Ireland/34/90 and Shelduck/France/MC110/77 (Fig. 1), suggesting that these viruses originated from the same source.

In the present study, 5 viruses, which contained virulent type F, were isolated from duck population in their breeding places. Amino acid sequence (position 95 to 213) of the F of Duck/Alaska/300/91 isolate belonging to the lineage B was compared with that of virulent strains. Duck/Alaska/300/91 isolate was most closely related to virulent strains Herts/33 and Australia/Victoria/32, especially. Three amino acid differences were found between Duck/Alaska/300/91 and Australia/Victoria/32. Four amino acid differences were found between Duck/Alaska/300/91 and Herts/33. Since the lineage C includes strains of variety of virulence as

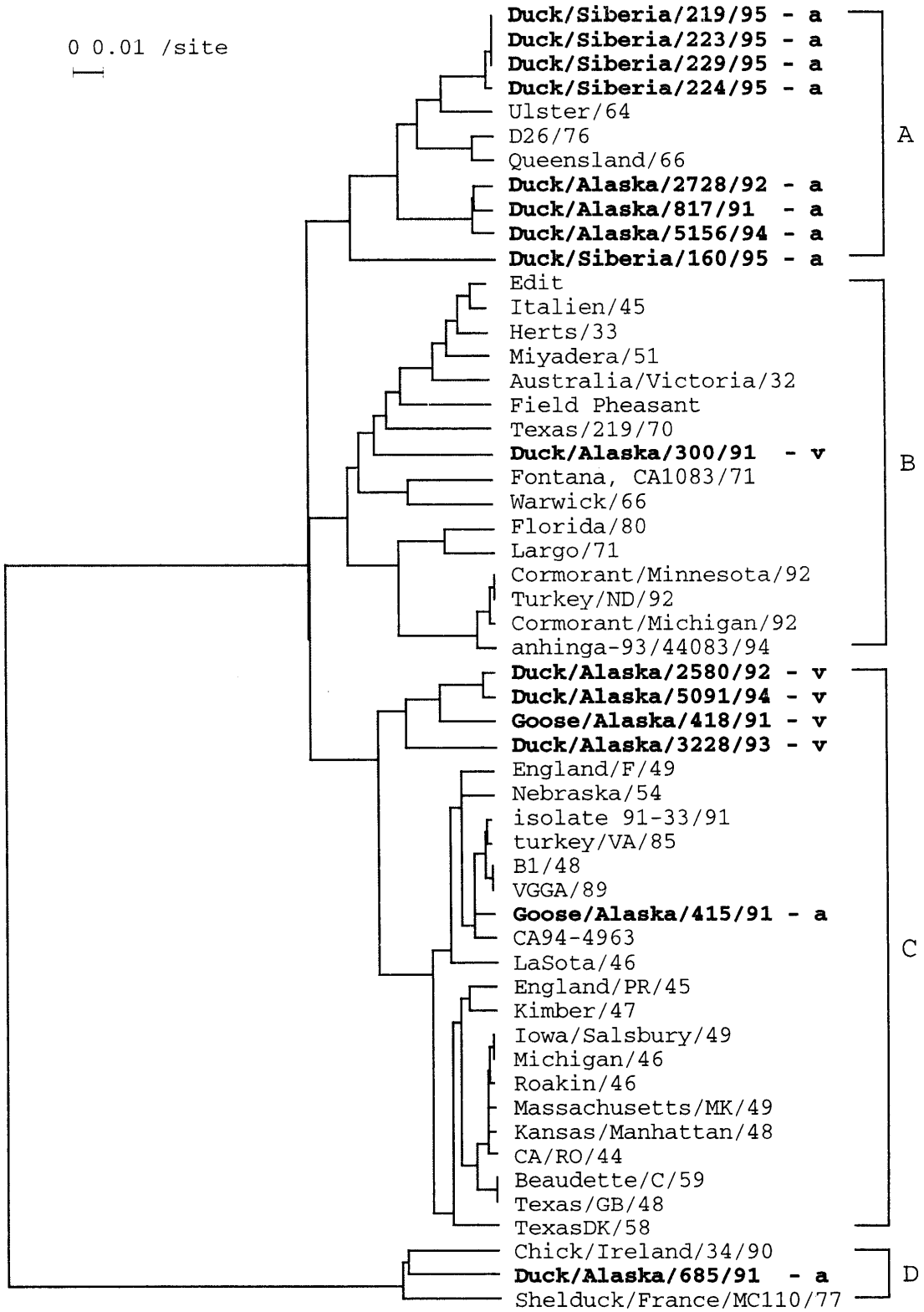

Fig. 1. Phylogenetic tree for NDV F genes. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Vertical lines are for spacing branches and labels. Viruses isolated from waterfowls in Alaska are in boldes. Abbreviations : **a**, avirulent type sequnce or **v**, virulent type amino acid sequnce at the cleavage site.

velogenic and lentogenic vaccine strains, the other isolates containing virulent type F, which belonged to the lineage C, were closely related to strains of each virulence. There results suggest that Duck/Alaska/300/91 may increase the pathogenicity for the chickens by passage through chickens or embryonated chicken eggs.

The major breeding areas of ducks in Alaska located near the arctic regions. This area is cold enough for viruses to survive for long period in autumn when ducks leave for migration to south. It is possible that NDVs are preserved during winter when ducks are absent and that ducks coming back from south in spring are infected with those viruses.

In the present study, phylogenetic analysis showed that a variety of NDV strains existed in duck population in their breeding places (Fig. 1), indicating that NDV are maintained in duck populations in breeding area. Furthermore, some strains were closely related to virulent strains and consisted with virulent type at the cleavage site of the F. This result suggests that potentially virulent NDVs may be maintained in migratory waterfowl populations.

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