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Sequence analysis of the fusion protein gene of Newcastle disease virus isolated from breeder ducks in Korea

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Abstract

Newcastle disease (ND) is an infectious poultry disease that caused high mortality and reduced egg production. NDVs are regularly present in the domestic duck population. And ducks play a possible role in the maintenance and transmission of NDVs. While we were monitoring the Avian Influenza, NDVs were isolated from field samples by accident. So we analysed the biological and genetic characteristics of these viruses. Lentogenic NDVs were isolated from two farms among twenty breeder duck farms. The ages of ducks were 39 weeks old in the 'A' farm and 3～72 weeks old in the 'B' farm. And they were not inoculated with the NDVs vaccine. In the biological characteristics, the both viruses which separated from the farm 'A' and 'B' were thermostable. The amino acid sequence of a site from 112 to 119 in the fusion (F) protein was 'GKQGRLIG' which has monobasic motif in the samples of both farms. And this means the separated NDVs are lentogenic. Phylogenetic analysis was performed by entire nucleotide sequence of F protein. The virus strains from the A farm (MN095239) and the B farm (MN095240) belonged to class II genotype I. Using the analysis of whole F protein nucleic acid sequence, the MN095239 (GenBank) had homology with Ulster strain about 99.95% and the MN095239 (GenBank) had homology with KR/CK/KU_LBM255/09 strain about 99.89%. NDV surveillance is needed to investigate epidemiological relationship of domestic breeder duck isolates in Korea.

Key words : Newcastle disease virus, Fusion protein, Phylogenetic tree

INTRODUCTION

Newcastle disease virus (NDV) is a nonsegmented negative-sense singlestranded RNA virus of the species Avian avulavirus 1 (AAvV-1) of the genus Orthoavulavirus of the Paramyxoviridae family (ICTV, 2018). ND was an infectious poultry disease that caused substantial economic losses to the Korean poultry industry until the early 2,000s, and is a disease with high mortality and reduced egg production (Lee et al, 2004). The disease has decreased since the mandatory vaccination policies of chickens and has not been reported since June 2010 (Lee et al, 2009a). But, according to surveillance data for duck NDVs in China, NDVs are commonly present in the domestic duck population and have a isolation rate of 3.24%. The ducks are not vaccinated because they show little clinical symptom even if the duck are infected with Newcastle disease. Despite not getting vaccinated, Vaccine-like NDVs are isolated from ducks without symptoms. This suggests that ducks play a possible role in the maintenance and transmission of NDVs (Liu et al, 2009).

NDVs are classified into class I and II based on the full sequence of the F protein gene (Dimitrov et al, 2019). The gene is a hypervariable region and is associated with virus pathogenicity. The sequence of the F

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protein is used for genetic diversity analysis. Class I viruses have low genetic diversity, lentogenic and they are typically isolated from wild waterfowl, live-bird markets (Seal et al, 2005; Dimitrov et al, 2016). Class II can be further divided into 18 genotypes multiple subgenotypes (Bello et al, 2018). The class II strains generally isolated from wild and domestic birds and contained virulent and avirulent isolates (Kabilov et al, 2016). Among these, genotypes I and II are not pathogenic for chickens, thus some isolates belonging to these genotypes have been a target for development of live attenuated vaccine strain (Lee et al, 2009a).

Some studies have reported to play an important role in the epidemiology of NDVs (Dai et al, 2014). Therefore, the analysis of phylogenetic relationship of isolated duck NDVs and the comparison with reference strains are necessary for the investigation into epidemiological relationship of NDVs.

The aim of this study was to analyse the biological and genetic characteristics of these NDVs that were detected in two duck farms.

MATERIALS AND METHODS

Virus isolation and identification

NDVs were inspected in healthy domestic breeder duck farms. Twenty pieces of feces per cage were collected from twenty duck farms every month for the avian influenza monitoring. NDVs were isolated from two farms in Eumseong-gun.

All fecal samples were inoculated in embryonated chicken eggs (9～11 days) via Allantoic Cavity route and incubated at 37℃ during 5 days (Brauer et al, 2015). The allantoic fluid, which was collected from eggs, was performed by hemagglutination (HA) assay according to standard procedures (Anonymous, 2006) and confirmed by reverse transcription polymerase chain reaction (RT-PCR), i.e. LiliF^{TM} NDV Real-time RT-PCR Kit (iNtRON Biotech, Korea).

Hemagglutination (HA) activity test (heat treatment)

In order to determine the thermostability of NDV strains, virus samples were heat-treated at 56℃ for 30 min and then the HA titers were compared with those of untreated virus. The HA titer is a reciprocal number of the highest dilution factor that produced a positive results. If the HA titer is less than 4 after heat treatment, the virus has not thermostability.

Mean death time (MDT)

Allantoic fluid containing NDVs was serially diluted 10-fold (10^{-5} to 10^{-9}) and each diluted fluid was inoculated into 10～11 day old five SPF chicken eggs. The mean death time (MDT) was measured at the highest virus dilution that all inoculated eggs were died. The virulence of the virus is judged on the basis of their MDT, i.e. Velogenic - MDT less than 60 hours; Mesogenic: MDT 60 to 90 hours; Lentogenic: MDT greater; Avirulent: does not cause disease (Alexander et al, 1988).

Sequencing of the F protein

The F protein gene of the NDV strains was amplified by RT-PCR for sequence analysis. Viral nucleic acid

Table 1. RT-PCR primers used for F protein gene amplification

	Sequence 5'-3'	ORF (nt)	Nucleotide length (nt)	Reference
ND1	GCTGATCATGAGGTTACCTC AGTCGGAGGATGTTGGCAGC	M1055-F508	695	(Lee et al, 2009a)
ND2	AACCGCTGCACAGATAACAG CTTCTATCACGGAACCGACC	F390-F994	605	In this study
ND3	AAAGGATTTGCCTCAGCAC CGAGTTGTTGACATTCCCAA	F934-F1419	466	In this study
ND4	GTGACAGGCAATCTYGATA GCTGTCAGACTTTACACACA	F1369-5'UTR57	331	In this study

was extracted using the Patho Gene-spin Plus Extraction kit (iNtRON Biotech, Korea) according to the manual method and amplified by Maxime^{TM} RT-PCR PreMix (iNtRON Biotech, Korea). 4 pairs of primers were designed base on the NDV sequences registered in NCBI, the primers amplify 1,662 nt ORF of F protein gene by overlapping the amplification sites (Table 1). Nucleotide sequencing was performed with a BigDye Terminator v3.1 Cycle Sequencing Kit and products were analysed on the ABI 3,500 xL genetic analyzer (Applied Biosystems, USA). Sequence alignments, editing and amino acid sequence translation performed using basic local alignment searh tool (BLAST). The pathogenicity of NDVs was investigated by analysis of the amino acid sequences corresponding to the positions from 112 to 119 of cleavage site of F protein (Liu et al, 2009).

Phylogenetic analysis of F protein

Phylogenetic analysis that based on F protein gene was performed with the MEGA X program using the neighboring joining method with 1,000 bootstrapping replicates (Kim et al, 2012). The sequences used for phylogenetic analysis were from NDV strains isolated from the this study (CB1904151 and CB1904152), vaccine strain(V4, Ulster 2C, Amori and LaSota) and reference strains that were registered in GeneBank (NCBI). The accession numbers are included in phylogenetic tree (Wang et al, 2016).

RESULTS

Genetic & biological characteristics

NDVs were isolated from two farms among seven breeder duck farms. The age of ducks were 39 weeks old in the 'A' farm and 3～72 weeks old in the 'B' farm. And they were not inoculated with the NDV vaccine. In the biological characteristics, both viruses that isolated in the 'A, B' farm had thermostability. The HA titer was not less than 4 after heat treatment. Viruses isolated from farm A and B had a mean death time more than 120 hours. The amino acid sequence of site from 112 to 119 in the F protein was 'GKQGRLIG' which has monobasic motif in the samples of both farms. The isolated strains in this study were classified as avirulent NDVs according to the results of Mean Death Time (MDT) and amino acid sequence of F protein (Table 2).

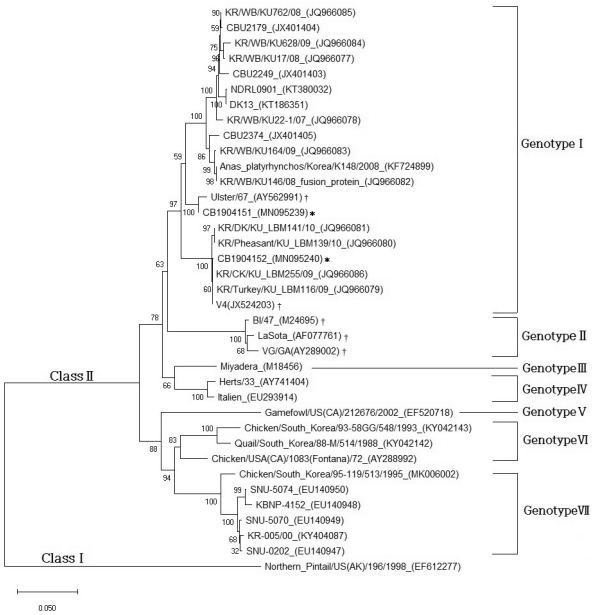

Fig. 1. Phylogenetic analysis of complete F protein gene sequence (1,662 nt). Sequence of reference strains that downloaded from National Center for Biotechnology Information (NCBI). The accession numbers of reference strains were included in parenthesis. The scale length is equal to 0.05 nucleotide substitutions per site. *In this study. †Vaccine strains.

Table 2. Genetic and biological characteristics of NDVs isolated from breeder duck farms

Farm (NDV isolate)	NDV RT-PCR		Genotyping		F0 motif sequence	Heat treatment		Mean death time
	Common	Pathotype	Class	Genotype		Before	After	
A (MN095239)	Positive	Negative	II	I	GKQGRLIG	256	64	>120
B (N095240)	Positive	Negative	II	I	GKQGRLIG	512	128	>120

Phylogenetic analysis

Phylogenetic analysis was performed by entire nucleotide sequence of F protein (1,662 nt). The virus from A farm (MN095239) and B farm (MN095240) belonged to class II genotype I. The MN095239 had homology with Ulster strain about 99.94% and the MN095240 homology with KR/CK/KU_LBM255/09 strain about 99.94% (Fig. 1). The amino acid sequence of the F protein gene was analysed. MN095239 and MN095240 had 100% amino acid sequence homology to the strain mentioned above.

DISCUSSION

This study is a biological and genetic characteristics analysis of NDVs that were detected from breeder duck farms. Once a month, fecal samples were collected from all breeder duck farms in Chungcheongbuk-do for Avian influenza monitoring. In monitoring, NDVs were isolated from the A and B farms in April 2019. The ducks from both farms were not vaccinated with NDVs and no other poultry was bred together.

The genetic diversity was analysed to investigate the epidemiological relationship of isolated NDVs. The full genetic sequence of the F protein is used for phylogenetic analysis because it is well characterized and has variable regions (Kim et al, 2013). The partial sequence of the F protein gene does not accurately classify NDVs at the sub-genotype level compared to the full sequence of the F protein gene (Almeida et al, 2013; Snoeck et al, 2013). NDV strains were divided into two classes I and II based on the full sequence of the F protein gene. Class I is divided into 1 genotypes 3 subgenotypes and class II is divided into 18 genotypes multiple subgenotypes (Bello et al, 2018). The strains isolated in this study were accurately delineated using the full sequence of the F protein gene. Both strains (MN095239, MN095240) belong to class II genotype I that is regularly isolated from various domestic poultry (Aldous et al, 2003; Seal, 2004; Kim et al, 2007). MN095239 is most closely related to Ulster strain which is commonly used for vaccinating chickens in Korea. This means that the MN095239 was originated from a Ulster vaccine strain rather field viruses of other poultry origin. But, breeder ducks of 'A' farm were not vaccinated against NDVs. They were infected by indirect exposure that mechanical transmission, i.e. feed and veterinary drugs vehicle that contact virus carring poultry. Therefore, it is possible that the virus invade the duck farm by the route of chicken to duck before the virus was adapted to the duck population (Lee et al, 2009b).

A close phylogenetic relationship was found between breeder duck farm isolate (MN095240) and chicken LBM isolate (JQ966086). This means that strains isolated from LBM are likely to exchange NDVs with poultry farms. Previous studies have demonstrated that LBM is a place where many poultry viruses are mixed and can be transmitted to new hosts. These places have appropriate conditions for increase viral evolution and spread (Webster, 2004; Guan et al, 2005; Lee et al, 2010). Currently, for the sale of poultry at LBM, only AIV monitoring is conducted, not NDV monitoring. Therefore, the surveillance of NDV evolutions between LBM and poultry farms is needed.

MN095239 and MN095240 were isolated from breeder ducks. Embryonated-duck eggs are contaminated by feces of breeder ducks that were infected with NDVs. So, hatched ducklings can easily be infected NDVs. In addition, NDVs evolve from low to high virulence with only a few point mutations (Kattenbelt et al, 2006; Westbury, 2001). There is a possibility of outbreaks of ND on broiler duck farms if virus mutations were occurred in the process of transmission from breeder ducks. Therefore, breeder duck farms should be thoroughly prevented from introducing NDVs.

In conclusion, our results reveal that surveillance of relatedness of NDV isolates between domestic duck farms and LBMs would be to understand epidemiological relationship and route of propagation. And, investigation of NDVs isolated from breeder duck farms that can easily transfer virus through contaminated eggs is needed. These data can be applied to the ND Control System.

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