
Phylogenetic characterization of Newcastle disease viruses isolated in Thailand during 2006–2007

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Abstract

A total of 18 Thai NDV isolates from cloacal swabs of chicken and duck were obtained from processing plants prior to slaughtering and submitted to National Institute of Animal Health for isolation of Newcastle disease virus (NDV). Further characterization of the viral fusion (F) protein genes was carried out using reverse-transcription polymerase chain reaction (RT-PCR) with specific primers. Phylogenetic analysis of the nucleotide sequences (389 nucleotides) and their deduced amino acid sequences was performed using the previously reported sequences for comparison. The results showed that all of the Thai isolates used in this study belonged to the Genotype I. In addition, these isolates carried the 112-GKQGRL-117 motif, which was assigned as the lentogenic type. It can be implied from this study that these Thai isolates shared similarity with vaccine virus and not found virulent strain in 2006 to 2007.

Keywords : Newcastle disease virus, fusion (F) protein, phylogenetic analysis

Introduction

Newcastle disease (ND) is an important disease of poultry as it is highly contagious and responsible for a high mortality in susceptible birds. (Alexander, 2004) It is caused by Newcastle disease virus (NDV), also known as avian Paramyxovirus type I, a member of the genus Avulavirus in the family Paramyxoviridae. The genome of approximately 15 Kb long is single stranded and negatively-sense, which contains 3'-NP-P-M-F-HN-L-5' and encodes nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large protein (L), respectively. (de Leeuw and Peeters, 1999; Mayo, 2002) NDV has a worldwide distribution and severely affect to many species of domestic and wild birds. Classification according to the pathogenicity has put NDV into highly pathogenic (velogenic), intermediate (mesogenic), or apathogenic (lentogenic) categories. (Handon et al., 1980). NDV outbreaks vary in clinical signs, severity, transmissibility and can be highly lethal (Alexander, 1991) depending on virus strain. The clinical signs include for example respiratory distress, diarrhea, circulatory disturbances and impairment of the central nervous system. Although NDV has a single serotype, 8 genotypes of the NDV strains were described (Ballagi-Pordany et al., 1996; Herczeg et al., 1999; Kwon et al., 2003; Liu et al., 2003; Lomniczi et al., 1998). The primary molecular determinant for NDV pathogenicity is the F protein cleavage site (de Leeuw et al., 2005; Kianizadeh et al., 2002; Panda et al., 2004) which mediates fusion between the virus and cellular membranes, which is synthesized as an inactive precursor, F0, consisting of 553 amino acids. The precursor is proteolytically cleaved at the peptide bond between residues 116 and 117, to generate active polypeptides, F1 and F2, that are disulphide linked (Panda et al., 2004). Thus, analyzing the sequence of the F protein cleavage site can aid in pathogenicity prediction and diagnosis of NDV virulence. (OIE, 2004) In this study we collected 18 strains of NDV that were isolated from processing plants prior to slaughtering and submitted to National Institute of Animal Health for isolation of NDV during 2006 to 2007 and conducted sequence analysis compared with previously published sequences.

Materials and methods

Virus

During 2006-2007, a total of 18 Thai NDV isolates (Table 2) from poultry of surveillance cases were obtained from processing plants and grown in 9-11 day - old embryonated eggs using standard procedures (Alexander, 1989 ; OIE, 2004) and identified with the standard

hemagglutination inhibition (HI) test. Inoculated allantoic fluids from different isolates were harvested and stored at -70°C for PCR amplification.

Reverse transcription-polymerase chain reaction

Viral RNA was extracted from infected allantoic fluids using the Qiagen RNA viral kit (Qiagen, Germany). The viral RNA was resuspended in DEPC-treated water and stored at -70°C. Then, the RNA samples were reverse transcribed and amplified in a one-step nested RT-PCR assay and the following primers of the fusion (F) protein which includes important structures such as the cleavage site (Collins et al., 1993; Seal et al., 1995; Gould et al., 2001). The sequences of primers are described in Table 1. (Mase et al., 2002) Briefly, 5 µL of RNA were added in a 50 µL RT-PCR reaction containing 25 µL of 2x reaction buffer (Invitrogen), 1 µM of NDV-M1, 1 µM of NDV-F1, 1 µL of SuperScript III/Platinum Taq High Fidelity enzyme mix (Invitrogen) and 17 µL of DEPC-treated water. Reactions were incubated at 50°C for 30 min, 94°C for 2 min, 40 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1.5 min, followed by 72°C for 10 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with 0.5 µg/mL ethidium bromide (EtBr) and visualized under ultraviolet transilluminator. In the first step of amplification, a 921 bp primary product was obtained by using external primers. If insufficient amounts of products were obtained, then a second amplification was performed as the second step. In this case, a 766 bp product was obtained using the internal primers.

Table 1 Primers use in this study

Name	sequences (5->3)	Target gene	Position	Expected size (bp)	Remark
NDV_M1	TTCTCTAgCAGTgggACAgC	M protein	914-933	921	external primer
NDV_F1	CATCTTCCCAACTgCCACTg	F protein	592-573		external primer
NDV_M2	TggAgCCAAACCCgCACCTgCGg	M protein	980-1003	766	internal primer
NDV_F2	ggAggATgTTggCAgCATT	F protein	503-485		internal primer
M13F	TgCAAggCgATTAAgTT	Sequence			(Promega, U.S.A.)
M13R	gAAACAgCTATgACCAT	Sequence			(Promega, U.S.A.)

Sequencing and phylogenetic analysis

The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia Calif.), and the concentration of transcripts was determined using an ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, USA) at 260 nm, ligated with a pGEM[®]-T vector system (Promega, USA) and transferred into DH5 α Escherichia coli by using Gene Pulser (Bio-RAD, USA). The clones were determined using gene specific primers and Bigdye[®] Terminator version 3.1 chemistry (Applied Biosystems, USA), according to the manufacturer's instructions. Reactions were run on a 3100 DNA analyser (Applied Biosystems, USA). Sequencing was performed from both directions of M13 primers (pGEM[®]-T vector) in the PCR amplification, assembly of sequences, removal of low quality sequence data by using SeqScape[®] software version 2.5 (Applied Biosystems, USA), nucleotide sequence translation into protein sequence, additional multiple sequence alignments and processing were performed with the Bioedit software version 7.0.4.1 with an engine based on the Clustal W algorithm (Thompson, 1994). The phylogenetic analysis of the partial genome nucleotide and the deduced amino acid sequences were constructed with Molecular Evolutionary Genetics Analysis (MEGA, version 4) (Tamura et al., 2007) using neighbor joining tree inference analysis with the Tamura-Nei model, with 1000 bootstrap replications. The phylogenetic analysis placed 18 NDV isolates from Thailand in 2006 to 2007 comparison of the nucleotide sequences with M24692, M24707, M24696.1, AY630409.1, AF001129.1, AF001130.1, AY630413.1, AF001109, AY630412.1, AY630420.1, AY630434.1, AY630437.1, AF378245, U62620, AF136771, AF001107, AY648305, AF136773.1, AF378251, AF136786, AF525366, M24702, M24700, M24701, AY170137, AY170138, DQ195265, AF309418, EU547760, EU419321, ZA37401, AY175774, AY210503 and AF217084 isolates that have been published in GenBank.

Results

Proteolytic cleavage site analysis of F0 protein and sequence analysis

The amplified products were sequenced; the nucleotide sequences determined in this study are available in GenBank, accession numbers EU847292 – EU847309. The predicted amino acid sequences of the NDV isolates were compared. Prediction of the pathogenicity of the 18 isolates suggested that they were members of the lentogenic group as indicated by the 112G-K-Q-G-R-L117 motif. (Table 2)

Phylogenetic analysis

Phylogenetic analysis of NDV isolates obtained in Thailand and phylogenetic relationships with other representative NDV isolates were performed based on sequence analysis of the regions of the F gene (nt. 1 to 389). The results of the phylogenetic analysis placed 18 NDV isolates from Thailand in 2006 to 2007 belonged to the Genotype I. (Figure 1)

Table 2 GenBank accession number and fusion protein connecting peptide sequence of virus isolated in Thailand.

No.	Isolate	Accession number	Virulence	Cleavage site	Genotype
1.	AMP1/dk/Mahasarakham/407410/07	EU847292	L	112G-K-Q-G-R-L117	I
2.	AMP1/ck/Prachuapkhirkhan/16385/06	EU847293	L	112G-K-Q-G-R-L117	I
3.	AMP1/ck/Nakhonsawan/16884/06	EU847294	L	112G-K-Q-G-R-L117	I
4.	AMP1/dk/Sakaeo/5903/06	EU847295	L	112G-K-Q-G-R-L117	I
5.	AMP1/ck/Ratchaburi/12429/06	EU847296	L	112G-K-Q-G-R-L117	I
6.	AMP1/ck/Phrachinburi/17148/06	EU847297	L	112G-K-Q-G-R-L117	I
7.	AMP1/ck/Nakhonphatom/15293/06	EU847298	L	112G-K-Q-G-R-L117	I
8.	AMP1/ck/Phetchaburi/15655/06	EU847299	L	112G-K-Q-G-R-L117	I
9.	AMP1/ck/Ratchaburi/8191/06	EU847300	L	112G-K-Q-G-R-L117	I
10.	AMP1/ck/Buriram/16803/06	EU847301	L	112G-K-Q-G-R-L117	I
11.	AMP1/ck/Lopburi/11508/06	EU847302	L	112G-K-Q-G-R-L117	I
12.	AMP1 ck/Prachuapkhirkhan/17041/06	EU847303	L	112G-K-Q-G-R-L117	I
13.	AMP1/ck/Ratchaburi/11608/07	EU847304	L	112G-K-Q-G-R-L117	I
14.	AMP1/ck/Sakaeo/11624/07	EU847305	L	112G-K-Q-G-R-L117	I
15.	AMP1/ck/Lopburi/11582/07	EU847306	L	112G-K-Q-G-R-L117	I
16.	AMP1/ck/Nakhonratchasima/9833/07	EU847307	L	112G-K-Q-G-R-L117	I
17.	AMP1/ck/Lopburi/8598/07	EU847308	L	112G-K-Q-G-R-L117	I
18.	AMP1/ck/Chonburi/10939/07	EU847309	L	112G-K-Q-G-R-L117	I

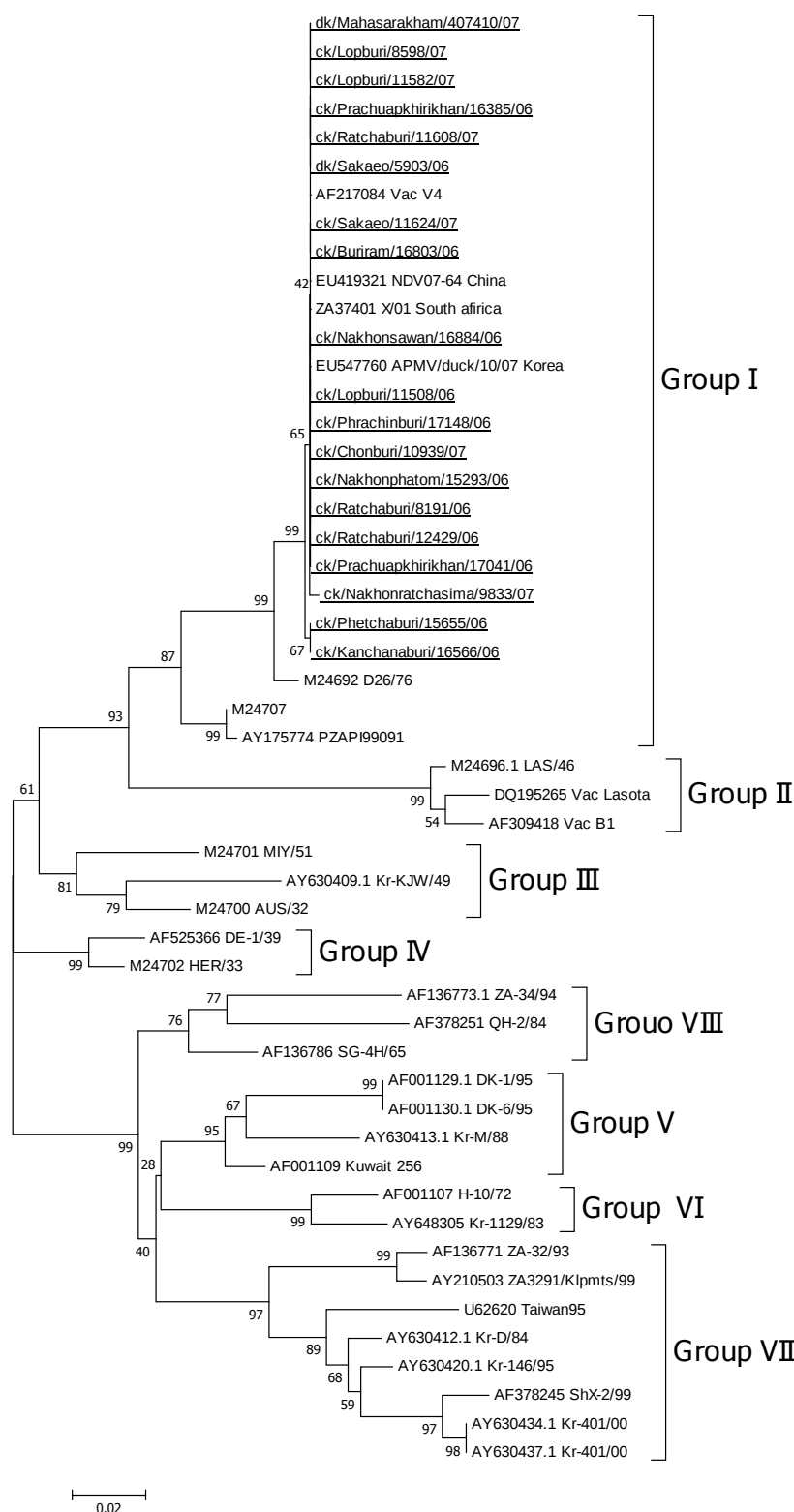


Figure 1 Phylogenetic relationships of the fusion (F) protein of representative Newcastle disease viruses isolated in Thailand compared with other NDV sequences in GenBank. Tree was generated by using MEGA 4 program (neighbor-joining analysis method and with Tamura-Nei model). Numbers below branches indicate bootstrap values from 1000 replicates. Analysis was based on nucleotides 1 to 389 (389 bp).

Discussion

In this study, the F protein of 18 Thai isolates have 99.7 to 100% for nucleotide and amino acid homology with their strains. No insertion or deletion were found on these sequences. Sequence analysis of the F protein cleavage site can be used to predict potential pathogenicity of NDV which is consistent with the conventional methods such as mean death time (MDT) and intracerebral pathogenic index tests (ICPI). (Panda et al., 2004) Low pathogenic viruses can only be cleaved by trypsin like proteases, secreted in the respiratory and intestinal tract, causing localised infection. In contrast the virulence of NDV is known to be associated with different amino acid sequence surrounding the post-translational cleavage site of the F0 protein, which is needed to be cleaved by the host cell proteases in order for the virus to become infectious and not dependent upon trypsin for cleavage but can be cleaved by furin which recognizes the multibasic cleavage site. Most viruses that are virulent for chickens have the amino acid sequence 112R/KR-Q-K/R-R116 at the C-terminus of the F2 protein and F (phenylalanine) at residue 117, the N-terminus of the F1 protein. In contrast, viruses of low virulence have sequences in the same region of 112G/E-K/R-Q-G/E-R116 and L (leucine) at residue 117 according to the definition of OIE. (OIE, 2004) Characterization of the cleavage site is important because it is directly related to the virulence of NDV strains. The phylogenetic analysis demonstrated that NDV isolates in this study distributed in eight distinct clusters corresponding to the different genotypes, namely genotypes I–VIII. (Fig. 1) The vaccine strains used in Thailand are the Lasota (Genotype II) and V4 (Genotype I). The 18 Thai isolates showed the high similarities to V4 at 99.7 to 100% for nucleotide and amino acid where as the similarities to Lasota were 86.6%. Based on our phylogenetic analysis, it can be postulated that 18 Thai isolates were similar with V4 vaccine strain. This vaccine strain was isolated from digestive tract and developed protective immune response to oral vaccine. (Rehmani et al., 1995; Spradbrow, 1993/94) Although NDV has single serotype, the commercial vaccine is available to provide adequate protection when the birds are challenged with heterologous strains of different genotypes. (Liu et al., 2003) But Yu et al. (2001) reported the inconsistent result and suggested that new vaccine against the Genotypes VII and VIII of NDV should be developed to prevent and control ND more effectively. Further investigation on the molecular epidemiology of NDV and its vaccines is needed.

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การศึกษาคุณสมบัติทางด้านชีวโมเลกุลของเชื้อไวรัสนิวคาสเซิล ที่แยกในประเทศไทยระหว่างปี 2549-2550

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บทคัดย่อ

ศึกษาเชื้อไวรัสนิวคาสเซิล จำนวน 18 ตัวอย่างที่แยกได้จาก cloacal swab ของ ไก่ และ เป็ด ก่อนเข้าโรงฆ่า ที่ส่งมาตรวจ ที่สถาบันสุขภาพสัตว์แห่งชาติ โดยดูลำดับเบสของสารพันธุกรรม (DNA sequencing) ที่ตำแหน่ง 1 ถึง 389 นิวคลีโอไทด์ของ Fusion (F) protein พบว่าตัวอย่างทั้งหมด มี multiple basic amino acids of cleavage site เป็น 112G-K-Q-G-R-L117 บ่งบอกว่าเป็นสายพันธุ์ที่ไม่รุนแรง (Lentogenic strain) และเปรียบเทียบกับสายพันธุ์ที่มีการรายงานตามภูมิภาคต่างๆของโลกก่อนหน้านี้ใน GenBank โดยวิธี Phylogenetic analysis พบว่าเชื้อไวรัสนิวคาสเซิลจำนวน 18 ตัวอย่าง จัดอยู่ในกลุ่ม 1 (Genotype I) ซึ่งเป็นกลุ่มเดียวกับวัคซีนสายพันธุ์ V4 มีความเหมือนของลำดับเบสของสารพันธุกรรม (Sequence homology) 99.7 ถึง 100% และผลของการศึกษานี้ ไม่พบเชื้อไวรัสนิวคาสเซิล ที่มีความรุนแรง ในช่วงปี พ.ศ. 2549 ถึง 2550

คำสำคัญ : เชื้อไวรัสนิวคาสเซิล fusion (F) protein phylogenetic analysis