

Phylogenetic Analysis of Newcastle Disease Virus Isolated from Vaccinated Chicken in West Java, Indonesia

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Abstract

Newcastle disease (ND) is a highly contagious disease of poultry worldwide, caused by Newcastle Disease virus (NDV), also known as Avian Paramyxovirus Type1. Newcastle disease virus is responsible for causing significant economic losses to the poultry industry in Southeast Asia including Indonesia. Despite vaccination, outbreaks of ND in vaccinated chicken flocks in Indonesia have been regularly reported. Although the current vaccines are substantially effective, they do not completely prevent infection, virus shedding and disease. The emergence of new virulent genotypes implies that distinct genotypes of NDV are simultaneously evolving at different geographic locations across the globe. The genomic diversity of NDV increases the possibility of diagnostic failures, resulting in unidentified infections. Our study aim to determine the genotype of isolates and to find out phylogeny relationship among NDV isolates compared with other NDV published on the GeneBank. Four characterized isolates used in this study were obtained from the repository of the Laboratory of Immunology, Faculty of Veterinary Medicine, Bogor Agricultural University. Two isolates belong to virulent NDV strain and other two isolates belong to avirulent NDV strain. A set of primer NDV-F-Forward 5'- ATG GGC TCC AAA CCT TCT AC- 3' and NDV-F- Reverse 5'- TTG TAG TGG CTC TCA TC – 3' were used to generate an amplicon targeting 1662 bp. Phylogenetic analyses of the F gene revealed that NDV/Ck/BGR/011 and NDV/Ck/GS/014 belong to genotype VII (sub) genotype (VIIh and VIIi) and NDV/Ck/CJR/015 and NDV/Ck/BGR/015 belong to genotype II. Our study revealed that NDV virulent strains which belong to genotype VII (sub) genotype (VIIh and VIIi) were circulating among vaccinated chicken flocks in West Java, Indonesia. ND outbreaks among vaccinated flocks, suggesting that vaccination strategies not effective in controlling the virus yet.

Keywords: Newcastle disease, genotype, phylogenetic tree, cleavage site, F gene.

1. INTRODUCTION

Newcastle disease (ND) also known as Avian Paramyxovirus Typelis a highly contagious infection which affects more than 250 species of birds, and causes high economic losses in the poultry industry worldwide, especially in chickens (A-Garib *et al.* 2003). The disease is still one of the most important diseases in poultry production and classified as a list A contagious disease of poultry by the Office International des Epizooties (OIE) (OIE 2012). According to the severity of the disease in chicken, NDV strain are generally grouped as velogenic (high virulence), mesogenic (intermediate virulence) and lentogenic (avirulence) pathotypes (Alexander and Senne2008).

Based on genome length and sequence of the F gene, NDV strains have two major subdivisions: class I and II (Czegledi *et al.* 2006; Miller *et al.* 2010). Class I NDV was divided into nine genotypes (Miller *et al.* 2010) and has been recovered from waterfowl and shorebirds and mostly avirulent to chickens (Czegledi *et al.* 2006), whereas class II NDV were mainly isolated from poultry, pet, and wild birds. Class II NDV are further categorized into eleven genotypes I to X (Diel *et al.* 2012). Genotypes V, VI, and VII of virulent viruses are the predominant genotypes circulating worldwide (Miller *et al.* 2009, 2010). In 1990s, two novel NDV genotypes, VII and VIII, were reported in Asia, South Africa, and several European countries (Liu *et al.*, 2003). Genotypes I, II, VI and VII being further divided in sub-genotypes such as; Ia and Ib, II and IIa, VIa - VIh, and VIIa - VIIh (Miller *et al.* 2010). Lately, genotype VII NDV is responsible to the most recent outbreaks ND in Asia, Africa, and the Middle East (Wang *et al.* 2006).

Intensive vaccination programs have been implemented in all commercial chicken in Indonesia, however ND continues to be a major problem for the poultry industry (Samal 2011). In 2009 and 2010, outbreaks of ND occurred in commercial chickens in Indonesia, causing up to 70% to 80% mortality (Xiao *et al.* 2012; OIE 2012). Recently, NDV infection of genotype VII has been reported to cause outbreaks in several commercial poultry farm in Indonesia (Xiao *et al.* 2012; Dharmayanti *et al.* 2014). West Java is one of the provinces in Indonesia have experienced recurrent outbreak of ND because high density of poultry population in these areas. Reports about the disease from some farm in West Java show evidence for the existence of virulent NDV strains. Understanding the pathotypic and genotypic character of NDV isolated from clinical outbreaks were important to control the diseases in West Java, however such information is limited. Our study aim to determine the genotype of isolates and to find out phylogeny relationship among NDV isolates compared with other NDV published on the GeneBank

2. MATERIALS AND METHODS

Four characterized isolates used in this research were obtained from the repository of the Laboratory of Immunology, Faculty of Veterinary Medicine, Bogor Agricultural University. Two isolates belong to virulent NDV strain and other two isolates belong to avirulent NDV strain. RNAs of the viruses were extracted from allantoic fluids using QIAamp® Viral RNA Mini Kit (Qiagen, Germany) according to manufacturer instruction. RT-PCR was performed using One-step RT-PCR kit (Qiagen, Germany) according to manufacturer instruction. RT-PCR reaction mixture of each sample consisted of 2 µL of dNTPs mix (10 mM), 2µL of forward primer (10 pM), 2µL of reverse primer (10 pM), 2µL of purified template RNA, 10µL of 5x Onestep RT-PCR Qiagen buffer, 30µL of RNase free water, and 2µL Onestep RT-PCR enzyme mixed in a final volume of 50µL. Amplification for F gene was setup as 50°C for 40 min

followed by initial denaturation at 94°C for 2 min and 35 cycles of denaturation at 94°C for 60 s, annealing at 52°C for 60 s, extension at 72°C for 60 s and final extension at 72 °C for 10 min (Yuan *et al.* 2012). A set of primer NDV-F-Forward 5'- ATG GGC TCC AAA CCT TCT AC-3' and NDV-F- Reverse 5'- TTG TAG TGG CTC TCA TC – 3' were used to generate an amplicon targeting 1662 bp (Yuan *et al.* 2012).

Purified PCR products were sequenced by First Base Company (Malaysia) with the primer (NDV-F-Forward and NDV-F- Reverse)based on a variable portion (nt 1–1662) covering the complete F gene. The positive results of PCR products were sequenced using BigDye® Terminator v3.1 cycle sequencing Kit (Thermo Fisher Scientific, USA) according to manufacturer instruction. The phylogenetic tree was constructed by Neighbor-Joining Kimura 2 parameter model with 1000 bootstrapped replications. Numenclatur of genotypes and sub-genotypes were assigned based on Diel *et al*'s classification (Diel *et al.* 2012; Miller *et al.* 2015).

3. RESULTS AND DISCUSSION

Examination of the amplified PCR products revealed the expected sizes 1662 bp of amplicons for all NDV isolates (Fig. 1).Comparison of nucleotide sequences of F gene demonstrated NDV/Ck/BGR/011 isolate have similarity sequence of nucleotides 90.30%, 82.12% and 81.25% with NDV/Ck/GS/014, NDV/Ck/CJR/015 and NDV/Ck/BGR/015 respectively. NDV/Ck/GS/014 isolate have similarity sequence of nucleotides 81.45% and 80.57% with NDV/Ck/CJR/015 and NDV/Ck/BGR/015 respectively. The nucleotide sequence of NDV/Ck/CJR/015 and NDV/Ck/BGR/015 isolates showed 98.95 % nucleotides were similar. Comparison of nucleotide sequences of F gene between the virulent ND isolates and the earlier Indonesia ND isolates (GeneBank database) shown NDV/Ck/BGR/011 isolate have similarity sequence of nucleotides 91.64 – 99.62% with earlier Indonesia NDV isolates, and the isolate have closely related to NDV/Ck/Banjarmasin-010/10, NDV/Ck/Gianyar-013/10, NDV/Ck/Sragen-014/10, NDV/Ck/Kudus-017/10 and NDV/Ck/Kudus-018/10. The NDV/Ck/GS/014 isolate showed 90.64 – 97.27% homologous nucleotide with earlier Indonesia NDV isolates and closely related to NDV/Ck/Makasar-003/10, NDV/Ck/Sukerejo-019/10 and NDV/Ck/Bali-020/10.

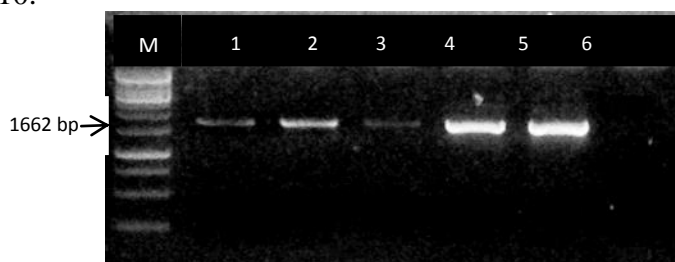


FIGURE 1. Fusion gene amplification results. RT-PCR amplification of the Newcastle disease virus F gene targeting 1662 bp. Lanes: M, molecular size marker; Lane 1 NDV/Ck/BGR/, lane 2 NDV/Ck/GS/14, Lane 3 NDV/Ck/CJR/15, Lane 4 NDV/Ck/BGR/15, Lane 5 is Sato (used as positive control) and Lane 6 is H₂O (used as negative control)

Phylogenetic analyses of the F gene revealed that NDV/Ck/Bogor/011 and NDV/Ck/GS/014 belong to genotype VII (sub) genotype VIIh and VIIi, while NDV/Ck/CJR/015 and NDV/Ck/BGR/015 belong to genotype II. NDV/Ck/BGR/011 belongs to sub genotype VIIi

along with NDV/Ck/Banjarmasin-010/10, NDV/Ck/Gianyar-013/10, NDV/Ck/Sragen-014/10, NDV/Ck/Kudus-017/10 and NDV/Ck/Kudus-018/10 while NDV/Ck/GS/014 isolates belong to sub genotype VIIh along with NDV/Ck/Makassar-003/10, NDV/Ck/Sukerejo-019/10 and NDV/Ck/Bali-020/10. The two other avirulen NDV isolates belong to genotype II along with lasota vaccine. Phylogenetic tree of the isolates are presented in Fig. 2.

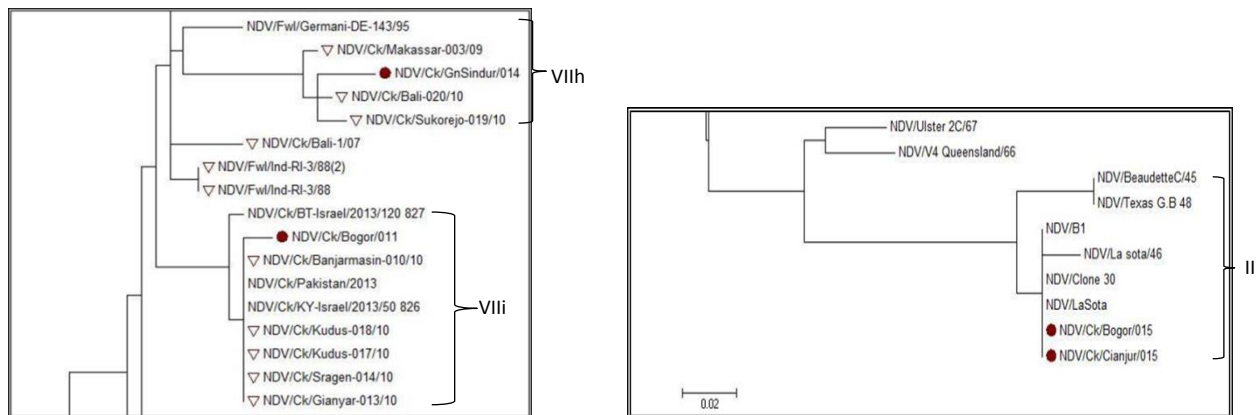


FIGURE 2. Phylogenetic tree of the isolates compared with earlier NDV isolates from GeneBank database. All studied isolates were marked in (●); All earlier Indonesia ND isolates (Genebank database) were marked in (▽)

Since the first outbreak reported of ND in Indonesia in 1926, ND spread and become endemic in all of Province in Indonesia. In 2009 and 2010, outbreaks of ND occurred in commercial chickens in Indonesia, causing up to 70% to 80% mortality and caused by genotype VII (Xiao *et al.* 2012; OIE. 2012). Genotype VII became more prevalent in this region, which is further divided into eight sub genotypes (VIIa–VIIh). Sub genotypes, VIIa–VIIe represent isolates from China, Malaysia, and Kazakhstan (Wang *et al.* 2006; Bogoyavlenskiy *et al.* 2009) and VIIf–VIIh represent African isolates (Snoeck *et al.* 2009). The sub-genotype type VIIh circulating from 2009 - 2012 in Indonesia, China, and Cambodia are most closely related to the Indonesia/Bali/01/2007 strain (Adi *et al.* 2010). The new sub-genotype VIIi closely related to earlier isolates from Indonesia and with isolates were collected in Pakistan and Israel (2013). The NDV sub genotype VIIi have been responsible for ND outbreaks in Pakistan since 2012. (Miller *et al.* 2015).

The avirulent NDV strain of isolates NDV/Ck/CJR/015 NDV/Ck/BGR/015 were closely related with live lasota vaccine in genotype II indicating the vaccine were used in this farm prevent disease but cannot stop viral shedding (Miller *et al.* 2009). Intensive vaccination programs (which provide selective immune pressures and may be executed improperly in developing countries), may contribute to the evolution of virulent viruses (Kapczynski *et al.* 2013). Result from current study suggested that virulent strain of ND viruses genotype VII (sub-genotype VIIhdanVIIi) were circulating among vaccinated flocks in West Java, Indonesia. ND

outbreaks among vaccinated flocks, suggesting that vaccination strategies not effective in controlling the virus yet.

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