# Molecular Analysis of *Newcastle Disease Virus* Isolated from A Vaccinated Layer Farm in Indonesia

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**Abstract**. Newcastle disease (ND) was first identified in Bogor, Java, Indonesia in 1926 and became major infectious in global commercial poultry which has morbidity and mortality rate up to 100%. Vaccination is one of prevention measures to control *Newcastle disease virus* (NDV) infection in layer poultry; however, cases of NDV infection are still reported in well vaccinated farms around the world. Molecular determination was aimed to examine genomic variation that might contribute to better control NDV infection. A Virulent NDV was isolated by egg inoculation of sample originated from a vaccinated layer farm suffering significant irreversible egg drop production. The NDV isolate was detected using rRT-PCR targeting region of matrix (M) and fusion (F) gene. Confirmation of virulence was determined by sequence analysis of partial F and hemagglutinin-neuraminidase (HN) genes. Phylogenetic analysis shows the isolate clustered within sub genotype VIIh with Indonesia NDV isolates. Identification of polybasic motif of $^{112}$RRRKR/F$^{117}$ in the cleavage site of fusion protein confirms its virulence. Analysis of putative amino acid sequence highlighted E347K substitution in HN region, which has been reported to affect the capability of NDV in infecting vaccinated poultry. This finding indicates the urgent need of NDV molecular surveillance in vaccinated farms in larger area.
**Keywords:** cleavage site, fusion, hemagglutinin-neuraminidase, phylogenetic, substitution

## 1. Introduction

Newcastle disease (ND) is one of the most important infectious diseases of poultry causes high morbidity and mortality. The disease is caused by *Newcastle disease virus* (NDV) or avian paramyxovirus-1 (APMV-1) that belongs to the genus *Avulavirus* of *Paramyxoviridae* family. The case of NDV infection was first reported in 1926 in Bogor, West Java Indonesia and in 1927 an outbreak of NDV was occurred in Newcastle-upon-Tyne, England [1] . Newcastle disease is very contagious, causes economic losses and become a global problem in poultry farms.

*Newcastle disease virus* is a single stranded negative sense viral RNA, enveloped with diameter ±150 nm. The RNA genome has 6 open reading frame (ORF), namely the nucleoprotein (NP), phosphoprotein (P), protein matrix (M), fusion protein (M), hemagglutinin-neuraminidase (HN), and RNA dependent RNA polymerase (L). There are 2 major divisions of NDVs, class I and class II [2]. Class I strains are mostly avirulent and mainly isolated from wild birds and waterfowl birds, whereas

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class II contains low virulent (lentogenic), intermediate virulent (mesogenic), and high virulent (velogenic) strains [3,4]. Class II is further devided into 18 genotypes, I–XVIII [5,6]. Since 1984, genotype VII has a role in ND outbreak in Asia and Europe with mortality rates about 40–60%. Genotype VII was divided in 9 subgenotypes (VIIa-VIIi) based on the diversity of complete F gene sequence. There are 2 subgenotypes of NDV genotype VII that circulating in Indonesia, a.i VIIi and VIIh[5].

Vaccination is one of essential measures to control ND and has been routinely practiced in commercial poultry farms worldwide. In Indonesia, diverse array of ND vaccines are available in the market, which contain low virulent virus vaccines strains of LaSota, V6/GA, Ulster, VH, HB1, V4 and Hitchner-B1, intermediate virulent strains of Komarov and Mukteswar, and high virulent strains of local isolates ITA and G7 [7]. Cho et al. [8]revealed that mortality due to NDV infection can be suppressed by vaccination, but infection in vaccinated birds can still lead to a decrease in egg production. Since 2011, NDV infection in China has been dominated by genotype VIId with amino acid (aa) E347K variation in HN protein. Isolates with E/G347K/R variation was successfully isolated from vaccinated poultry farms and widely reported in China, Taiwan and Korea [8–12]. This residual E347K variation is thought to increase the risk of vaccination failure to stop viral shedding despite the absence of clinical symptoms and deaths due to NDV infection. This phenomenon is mainly due to antigenic variation between the vaccine and E347K strains as demonstrated by Cho et al.[8] and Zhu et al.[12].Information related to NDV infection in vaccinated chickens in Indonesia has been reported Xiao et al.[13], but the E347K variation is not detected. Further molecular study needs to be investigated to determine the diversity of circulating NDV strains in Indonesia, which can be used as a consideration to prepare (generate) optimal vaccination.

## 2. Materials and methods

### *2.1. Virus isolation and propagation*

Samples of respiratory and alimentary organs were collected from six ND vaccinated layer farms suffering decreased egg production and high feed conversion rate. Samples were the collection of PT IPB Shigeta Pharmaceuticals, which collected from some areas in Java Island, i.e. Kediri and Sukabumi in 2013; Serang, Kuningan, Bogor, and Cianjur in 2016. Virus was propagated in 9–10 day-old specific-pathogen-free (SPF) embryonated chicken egg (ECE) [14]. The eggs were incubated at 37 °C for 7 days and observed every 8 hours. The dead embryo was stored in the refrigerator and the allantois fluid was collected and tested by hemagglutination test (HA).

### *2.2. Viral RNA purification and rRT-PCR*

Viral RNA was extracted from allantois fluid using Viral Extraction Kit III (Geneaid) following the protocol recommended by the company. Viral RNA was stored at -80 °C until use. The viral RNA amplification was used real-time reverse transcriptase polymerase chain reaction (rRT-PCR) method, which is used primer and probe set to detect matrix gene [15]. The viral RNA was confirmed through using forward primer M+4100 (5'-AGTGATGTGCTCGGAC CTTC-3'), reverse primer M-4220 (5'-CCTGAGGAGAGGCATTTGCTA-3'), and probe M+4169 (5'-[FAM] TTCTCTAGCAGTGGGACAGCCTGC [TAMRA]-3'). The rRT-PCR was performed using Verso RT-PCR Kit with Thermoprime Taq (Thermo Scientific) with Applied Biosystems 7500 Real Time PCR System. The complementary DNA (cDNA) was synthesize in 45 °C for 10 minutes followed by an initial denaturation 94 °C for 10 minutes and 40 amplification cycles of 94 °C for 10 seconds, 56 °C for 30 seconds, and extension 72 °C 10 seconds.

### *2.3. RT-PCR and sequencing of the F and HN genes*

Amplification of the fusion (F) and hemagglutinin-Neuraminidase (HN) gene was performed with One-Step RT-PCR Kit Transcriptor (Roche Life Science) using the M+4100 primer (5'-CCTGAGGAGAGGCATTTGCTA-3') [15] and HN-8847 (5'-ATGATCTGGTGCTCTGCCCTT-3')

[12]. The RT-PCR-thermal cycler was set 50 ° C for 30 minutes and 94 ºC for 7 minutes for initial denaturation, followed by 35 amplification cycle begins denaturation stage at 94ºC for 15 seconds followed by primers annealing stage at 55 ºC for 30 seconds, and extension at 68 ºC for 2 minutes. The final extension stage was set at 68 ºC for 5 minutes. The RT-PCR product was visualized with electrophoresis on 1% agarose gel (w/v) with 5% ethidium bromide. The RT-PCR product was possessing the expected length was purified and sequenced (1st Base, Malaysia).

*2.4. Sequence and phylogenetic analyses*

The nucleotide (nt) sequence of F and HN genes were blasted using BLASTN tool (NCBI) to determine the sequence identity and homology to other isolates submitted to GenBank database. Published Indonesian NDV sequences [13,16] were incuded in phylogenetic analysis and LaSota strain was used as outgroup. Sequences analysis was performed using BioEdit version 7.2.5. package (http://www.mbio.ncsu.edu/bioedit/bioedit.html), CLC Sequence Viewer 7.7. (Qiagen Bioinformatics). Phylogenetic tree was constructed by maximum likelihood method using MEGA 7 software version 7.0.14 (MEGA, PA, USA) [17].

## 3. Results

*3.1. NDV Isolation and Identification*

Hemaggutinating activity was detected in one allantoic fluid harvest of ECE that died following 36 hours of inoculation by sample collected from a layer farm in Cianjur, West Java. The NDV identity was confirmed by rRT-PCR (table 1).

**Table 1.** Result of identification of NDV by using rRT-PCR with target gene M.

| Sample | Year | Ct | Result |
|---|---|---|---|
| Layer/Kediri-1/013 | 2013 | Undet | Negative |
| Layer/Sukabumi-1/013 | 2013 | Undet | Negative |
| Layer/Kuningan-1/016 | 2016 | Undet | Negative |
| Layer/Serang-1/016 | 2016 | Undet | Negative |
| Layer/Bogor-1/016 | 2016 | Undet | Negative |
| Layer/Cianjur-1/016 | 2016 | 15.9225 | Positive |

*3.2. Sequence and phylogenetic analysis*

Sequence analyses showed that Layer/Cianjur-1/016 isolate was highly similar to Indonesian isolates Chicken/Makasar/003/09, Chicken/Bali/020/10, Chicken/Sukorejo/019/10 and NDV/Bali-1/07 [13,16] with 93–98% nt and 91–97% aa identities, and 93–98% nt and 96–98% aa identities for partial F and HN genes, respectively (table 2). Lower similarity was observed with other Indonesian isolates Chicken/Banjarmasin/010/10, Chicken/Giayar/013/10, Chicken/Sragen/014/10, Chicken/Kudus/017/10, and Chicken/Kudus/018/10 [13] with 90% nt and 92% aa identities for both F and HN genes.

Putative aa analysis of cleavage site in the F protein identified $^{112}$RRRKRF$^{117}$ motif. Amino acid substitutions were observed at conserved region F2, i.e. R8G, R18Q, G46R, and M69I. Meanwhile, aa substitutions S76L, T232N, and E347K were noted in the HN protein.

Phylogenetic trees of both partial F and HN genes demonstrated branching consistency as shown in figure 1 where Layer/Cianjur-1/016 clustered with Chicken/Makasar/003/09, Chicken/Bali/020/10, Chicken/Sukorejo/019/10 and NDV/Bali-1/07 identified as subgenotype VIIh, whereas Chicken/Banjarmasin/010/10, Chicken/Giayar/013/10, Chicken/Sragen/014/10, Chicken/Kudus/017/10, and Chicken/Kudus/018/10 clustered in separate branch identified as subgenotype VIIi.

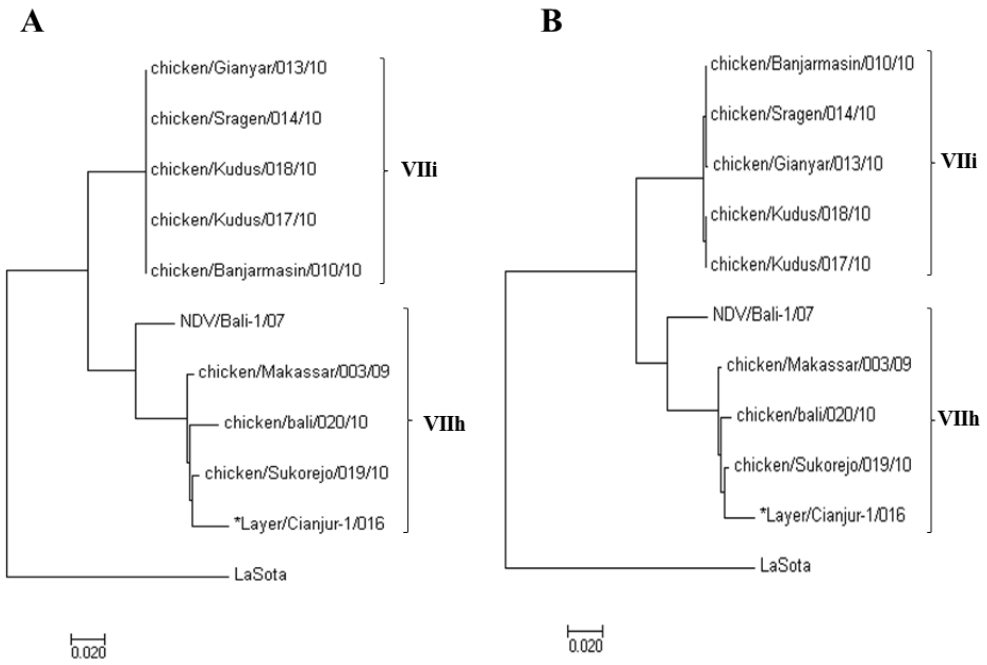



**Figure 1.** The phylogenetic tree NDV isolates Indonesia. A: based on nt sequence partial F (A) and HN (B) gene using Maximum Likelihood method.

**Table 2.** Comparison of aa substitution in the 347 residue of HN protein.

| NDV Strain | Subgenotype | 345-353 aa residue | GenBank Acc. No. |
|---|---|---|---|
| Chicken/Banjarmasin/010/10 | VIIi | PDEQDYQR | HQ697254.1 |
| Chicken/Giayar/013/10 | VIIi | PDEQDYQR | HQ697257.1 |
| Chicken/Sragen/014/10 | VIIi | PDEQDYQR | HQ697258.1 |
| Chicken/Kudus/017/10 | VIIi | PDEQDYQR | HQ697259.1 |
| Chicken/Kudus/018/10 | VIIi | PDEQDYQR | HQ697260.1 |
| NDV/Bali-1/07 | VIIh | PDEQDYQR | AB605247.1 |
| Chicken/Makasar/003/09 | VIIh | PDEQDYQR | HQ697256.1 |
| Chicken/Bali/020/10 | VIIh | PDEQDYQR | HQ697261.1 |
| Chicken/Sukorejo/019/10 | VIIh | PDEQDYQR | HQ697255.1 |
| Layer/Cianjur-1/016 | VIIh | PD**K**QDYQR | This study |

## 4. Discussion

Decrease in production is a major problem in the poultry business, both decreased feed conversion and decreased egg production. Samples were collected from farm with decreased feed conversion cases at 5 weeks old chickens, while egg production decline cases occurred at 27–30 weeks old chickens. One of the infectious agents that cause decrease in production is NDV. The vaccination system applied to the farm has been well scheduled and monitored. Information from the farm revealed that the antibody titer against NDV was uniform and reached a protective figure of $\geq 5$ $\log_2$, but a decrease in production was still occurring. Vaccination using heterologous virus genotypes with field virus can still stimulate high antibody titers, but the risk of viral shedding by infected poultry can still occur [12,18,19].

Necropsy examination of chickens where the studied samples originated from reported that the majority of pathological lesions include oovoritis, salphingitis, gastrointestinal and brain hemorrhage. Only one out of six samples was NDV positive; however, the possibility of other infectious agent

involvement for the necropsy findings cannot be ruled out in this study. It is known that NDV is one of the infectious agents that can lead to decreased production and egg shell abnormalities; in addition, virulent NDV infections may cause hemorrhagic lesions of the gastrointestinal, respiratory or central nervous systems [14].

Virulence of NDV strains is determined based on intracerebral pathogenicity index (ICPI) test as biological basis and cleavage site motif of F protein as molecular basis [14]. The F protein cleavage site sequence is a major determinant of virulence NDV. Non-virulent NDV strain (lentogenic) contains only monobasic aa at the cleavage site of F gene, whereas virulent NDV strains (mesogenic and velogenic) have multibasic motif. Analysis of partial F gene identified cleavage site motif $^{112}$RRRKRF$^{117}$ which indicates that Layer/Cianjur-1/016 isolate is categorized as a virulent strain. Virulent NDV gene has at least contain three basic aa (lysine/K and arginine/R) between positions 113 and 116 at the C terminus (cleavage site motif $^{112}$R/KRQR/K-F$^{117}$) and phenylalanine (F) at the N terminus cleavage at F0 area glycoprotein into F1 and F2 by protease that the virus needs a host cell to initiate infection. While F protein of non-virulent NDV cleaved by extracellular trypsin-like proteases that are limited to specific tissues of respiratory and gastrointestinal systems, F protein of virulent strain is broken down by ubiquitous subtilin-like endoprotease furin resulted in a systemic infection [20,21].

The phylogenic analysis of partial F gene showed that Layer/Cianjur-1/016 isolate clustered in subgenotype VIIh with other Indonesian isolates (figure 1 and figure 2). The clustering of Indonesian NDV strains into VIIh and VIIi demonstrated in figure 1 is consistence to the previous report by Miller *et al*. [5]. Strains of subgenotype VIIh were also reported circulating in Vietnam, Malaysia, and Cambodia [5,22].

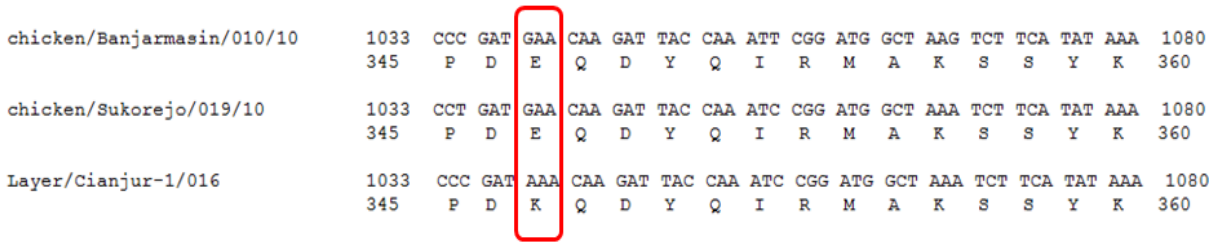



**Figure 2.** Comparison of nt and putative aa sequences of Layer/Cianjur-1/016 highlights G1039A mutation causes E347K substitution. Chicken/Banjarmasin/010/10 and Chicken/Sukorejo/019/10 represent subgenotypes VIIi and VIIh, respectively.

Hemagglutinin-neuraminidase is a glycoprotein that has an important role in viral infection. HN glycoproteins play a role in the attachment of viruses to sialic acid receptors on the host cell surface, contributing to the successful fusion between viruses and host cells, and preventing self-aggregation [4,23]. HN protein acid sequences encode at least 5 antigenic sites that are associated with epitopes on the HN protein so mutations in HN are most likely to affect the success of infection. E to K substitution at 347 aa position of HN protein due to G to A mutation at 1039 nt posision (G1039A) believed to have an effect on NDV infection in that vaccinated poultry[10]. Amino acids 345–355 has been suggested to be an important antigenic determinant that play a crucial role in viral attachment to host cell surface receptors [24,25]. Substitution of aa on antigenic sites may lead to alterations in the ability of antibodies to neutralize the virus [8,10–12] . Antigenicity alteration due to E347K mutation has been demonstrated using monoclonal antibodies [10] and cross-neutralization and cross-hemaggutination experiments [12]highlights the importance of the residue at position 347 of the HN protein.

## 5. Conclusions

In the present study, we reported the isolation of a distinct virulent NDV strain subgenotype VIIh Layer/Cianjur-1/016 with E347K substitution in linier epitope domain of HN protein which may indicate aselective pressure on surface antigens of field NDVs in vaccinated flocks. This finding prompts the need for a wider molecular surveillance of NDV circulating in vaccinated poultry farms to enable a better ND prevention strategy.

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