

PHYLOGENETIC ANALYSIS AND VIRULENCE-CODING GENES CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS ISOLATED FROM LAYING HEN BRAIN WITH TORTICOLLIS SYMPTOM

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ABSTRACT

The purpose of this study was to identify Newcastle disease (ND) viruses in the brain of chickens with torticollis symptoms to analyze its phylogenetic and to characterize its virulence genetic code. Samples used were 12 dead chickens with historically had torticollis symptoms, obtained from poultry farms at several areas in West Java and Banten. Chicken brains were prepared for reverse transcriptase-polymerase chain reaction (RT-PCR) test. All positive samples were sequenced to obtain its nucleotide sequences from some of Fusion (F) genes to analyze its phylogenetic by comparing with Indonesian ND isolate virus from GenBank using Mega X software. The results of RT-PCR test showed that only one sample (Virus MSL.03) contained genes of ND virus. Based on homology tests and phylogenetic analysis, the virus belonged to subgenotype VIIIh with an identical level of 95.34-95.86% when compared to several isolates from Indonesia. The MSL.03 ND virus has 112RRRKRF117 pattern in F0 which indicated its virulent category.

Key words: brain, Newcastle disease, RT-PCR, sequencing, torticollis

ABSTRAK

Tujuan dari penelitian ini untuk mendeteksi keberadaan virus Newcastle disease (ND) pada otak ayam yang mengalami gejala tortikolis, serta menganalisis secara filogenetik dan mengkaraktisasi gen penyandi sifat virulen dari virus ND. Sebanyak 12 ekor ayam mati dengan gejala tortikolis didapat dari peternakan ayam petelur di beberapa wilayah di Jawa Barat dan Banten. Otak ayam dipreparasi untuk dilanjutkan dengan uji reverse transcriptase-polymerase chain reaction (RT-PCR). Sampel positif kemudian dilanjutkan dengan pengurutan untuk mendapatkan urutan nukleotida dari sebagian gen Fusion (F) dan dianalisis secara filogenetik dengan virus ND isolat Indonesia yang tersedia di GenBank menggunakan software Mega X. Hasil uji RT-PCR menyatakan bahwa hanya terdapat satu sampel (virus MSL.03) yang positif mengandung gen virus ND. Berdasarkan uji homologi dan analisis filogenetik, virus penelitian tersebut masuk dalam subgenotipe VIIIh dengan tingkat identik sebesar 95,34-95,86% bila dibandingkan dengan dengan beberapa isolat asal Indonesia. Virus MSL.03 memiliki motif 112RRRKRF117 pada bagian F0 yang mengindikasikan bahwa virus ini termasuk dalam kategori virulen.

Kata kunci: otak, Newcastle disease, RT-PCR, pengurutan, tortikolis

INTRODUCTION

Newcastle disease (ND) or known as *tetelo* in Indonesia is an acute viral infection in poultry (Kencana *et al.*, 2012; Oni *et al.*, 2016). It has high mortality and morbidity (80-100%) in the infected population. Therefore, ND is one of disease to be feared by farmers as well as avian influenza (Narayanan *et al.*, 2010; Ashraf and Shah, 2014). ND virus belong to the family of Paramyxoviridae, genus Avulavirus, *Avian paramyxovirus* species, serogroup Avian paramyxovirus Type I (Abdisa and Tagesu, 2017).

ND is divided into five strains which are classified based on the level of each viral pathogenicity and the symptoms (Alexander *et al.*, 2004). The first strain is commonly referred to as neurotropic velogenic, which attacks the nervous system of infected birds. The symptoms caused respiratory abnormalities accompanied by head-twisting behavior or commonly called torticollis symptoms as the effect of virus penetration to the brain barrier (Dimitrov *et al.*, 2016). The second strain is commonly referred to as

viscerotropic velogenic, a type of ND virus that replicates in the visceral organs of infected poultry. In general, there are no torticollis symptoms observed in this infection, thus initial detection is confirmed through necropsy. The anatomical pathology examination found swelling of the Peyer's patch accompanied by hemorrhage in other organs such as small intestine and proventriculus (Lee *et al.*, 2016). The third strain is mesogenic strain and lentogenic strain. Both of these strains are characterized by respiratory disorders, additionally with neurological disorders in mesogenic strain. When compared to the other two previous strains, infection of these strains can be worsened by the presence of secondary infection from bacteria and will trigger significant losses (Dey *et al.*, 2014). The last strain is the asymptomatic one with subclinical symptom (Ashraf and Shah, 2014).

Based on phylogenetic analysis, the ND virus genetic relation is divided into class I and II. Only Class II is known to infect poultry throughout the world to date (Kim *et al.*, 2008). In addition, class II is divided into several genotypes. There are genotypes I

to XVIII which are often used in the classification of ND viruses (Li *et al.*, 2019). However, there are two genotypes that are more “popular” in Indonesia, Genotype VII which is common in Indonesia and Genotype II especially LaSota strain which is widely used as a seed in live vaccines (Wulanjati *et al.*, 2018). As vaccination widely spread, it will allow the ND virus which is an RNA virus to mutate rapidly, resulting in the emergence of new subgenotype. Subgenotypes VIIIh and VIIIi are the most frequent cause of infections at poultry farms in Indonesia (Shofa *et al.*, 2018).

ND virus genome consists of several genes such as genes F, P, M, HN, and L that encode several proteins, each with its own role (Wise *et al.*, 2004; Jindal *et al.*, 2009). F gene is a part of ND virus that influences pathogenicity level of the virus. Amino acid residues sequences at the cleavage site (F0) position in the F gene become the molecular determinant in classification for pathogenicity level. The gene is also used to define class and genotype classification of ND viruses, although some other genes can also be used in molecular identification of ND disease (Choi *et al.*, 2010).

First ND case was reported in Java in 1926, followed by reports in 1927 in the UK, until now several cases has been reported at multiple area in Indonesia (Indriani and Dharmayanti, 2016). In the past decade, diagnoses based on clinical symptoms accompanied by pathological analysis of ND disease are considered ineffective. This is mainly because of similar avian viral diseases such as Avian Influenza (AI) share similar clinical symptoms and pathological abnormalities.

Therefore, molecular diagnosis is a practical choice in detecting ND virus infections. Shofa *et al.*, (2018) proved that molecular diagnosis through reverse transcriptase-polymerase chain reaction (RT-PCR) followed by sequencing can provide accurate results about ND viruses along with its genotypes and even subgenotypes to determine appropriate virus seed for vaccination program. The purpose of this study were to detect the presence of ND viruses in the brains of laying hens that displays torticollis symptoms prior to death and to conduct phylogenetic analysis and characterization of the virulent genes of the ND virus based on its F gene.

MATERIALS AND METHODS

Twelve laying hen brains were collected from Research and Development unit of PT. Medika Animal Laboratories (Bogor), stored at -20° C during June 2019. Samples were collected from various animal farm areas in West Java and Banten Provinces. The purposive sampling was implemented to select the brain from a dead laying hen with history of torticollis symptom. Sample information can be seen in Table 1.

Sample Preparation and RNA Isolation

Hen brain was suspended with a sterile phosphate buffer saline (PBS) with the volume ratio of the brain and PBS was 2 : 3, then mixed in a 1.5 mL microtube using a micropastle provided by Tissue Total RNA Mini Kit (Geneaid) according to the procedure. The suspension of organ is mixed vigorously and then centrifuged at a speed of 12000 rpm for 3 minutes. Supernatants that contain viruses are extracted using Total Tissue RNA Mini Kit (Geneaid). The results of RNA isolation were stored at -20° C before further testing. ND strain LaSota virus vaccine was used as positive control.

Detection and Sequencing of Partial Gene Fusion (F)

Amplification of partial Fusion (F) gene was conducted using Superscript III One Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen) using forward primer: 5'-ATGGGCTCCAGACCTTCTACCA-3' and reverse primer: 5'-CTGCCACTGCTAGTTGTGATAATCC-3' with target band size 535 bp (Radwan *et al.*, 2013). Each microtube PCR consists of 25 µL 2× reaction mix, 2 µL Superscript III reverse transcriptase-Platinum Taq high-fidelity polymerase enzyme mix, 9 µL sterile H₂O, each 1 µL primer, and 4 µL RNA template. SimpliAmp thermal cycler (Applied Biosystems) machine was used for RT-PCR with the following stages: cDNA synthesis and pre-denaturation at 50° C for 20 minutes and 94° C for 2 minutes. Then, 40 cycles consisting of denaturation at 94° C for 15 seconds, annealing at 52° C for 30 seconds, and extending at 68° C for 30 seconds were performed. Amplification process was terminated with final extension at 68° C for 5 minutes.

Table 1. Sample information

No.	Code sample	Origin	Organ	Age (Weeks)	Symptom	ND vaccination record	
						Killed	Live
1	MSL.01	Sukabumi	Brain	26	Torticollis	Yes	Yes
2	MSL.02	Subang	Brain	35	Torticollis, Respiratory disorder	Yes	Yes
3	MSL.03	Tangerang	Brain	42	Torticollis, Respiratory disorder, Lime stools	Yes	Yes
4	MSL.04	Tangerang	Brain	33	Torticollis, Respiratory disorder	Yes	Yes
5	MSL.05	Sukabumi	Brain	40	Torticollis, Respiratory disorder, Bluish wattle	Yes	Yes
6	MSL.06	Sukabumi	Brain	40	Torticollis	Yes	Yes
7	MSL.07	Bogor	Brain	24	Torticollis, Respiratory disorder	Yes	Yes
8	MSL.08	Subang	Brain	33	Torticollis, Respiratory disorder, Lime stools	Yes	Yes
9	MSL.09	Bogor	Brain	27	Torticollis, Respiratory disorder, Bluish wattle	Yes	Yes
10	MSL.10	Subang	Brain	56	Torticollis, Respiratory disorder, Bluish wattle	Yes	Yes
11	MSL.11	Subang	Brain	43	Torticollis, Respiratory disorder	Yes	Yes
12	MSL.12	Subang	Brain	45	Torticollis, Respiratory disorder, Bluish wattle	Yes	Yes

Finally the PCR product was visualized by electrophoresis in 1% (w/v) agarose gel with the addition of 5% ethidium bromide. Positive sample showing the 535 bp band was shipped for purification and sequencing by 1st Base sequencing services in Malaysia.

Data Analysis

Nucleotide of F gene from sequencing was analyzed using BLASTN (NCBI) by matching similar genes with other Indonesian ND isolates available at GenBank. Homology comparison and phylogenetic tree were analyzed using MEGA X software version 10.1.

RESULTS AND DISCUSSION

Appropriate and accurate diagnosis is still a challenge in the field of veterinary medicine in detecting the presence of a causative agent of a disease in advanced technology era. Reverse transcriptase-polymerase chain reaction is one of the accurate and fast methods commonly used to determine the presence or absence of disease agents including viruses in a single disease outbreak. Almost all types of viruses can be detected with this method.

According to Yi and Liu (2011) one of the diseases in the veterinary world that require rapid diagnosis is newcastle disease due to its high mortality and morbidity rates. Several studies have been conducted regarding diagnosing ND by RT-PCR. This study used partial detection test of the F gene from the ND virus in laying hens brains that showed clinical symptoms of torticollis prior to death, and conducted phylogenetic analysis and characterization of the genes coding for the virulent nature of the virus.

Partial Detection Results of Gene F using RT-PCR

Visualization of PCR product on electrophoresis gel from a partial ND fusion virus gene in this study

produced an amplicon approximately 535 bp. The results showed that from twelve samples, only one sample was detected contain the F gene of ND virus (Figure 1). The sample with MSL.03 code came from laying hens in Legok, Tangerang, Banten Province. The other brain samples were negative for the ND virus based on the RT-PCR test.

These positive results indicate that in the last few years, Newcastle disease virus (NDV) is circulating throughout the Tangerang area which is dense with poultry farm. A study conducted by Emilia *et al.* (2015) found that purebred and native chickens traded in traditional markets in Tangerang region contained ND viruses through samples from cloaca swabs. Detection of the virus in the cloaca swab indicates shedding of the virus which can cause increase in ND virus circulation at a certain area. Chickens are susceptible to ND because of its simple transmission through inhaled air containing virus. Shofa *et al.* (2018) added that the ND virus able to infect vaccinated poultry. Vaccination failure results in the shedding of the ND virus. Imbalance between the amount of virus and antibodies is the main cause of ND virus discharge through feces. Indriani and Dharmayanti (2016) stated that in laboratory testing, the results of ND vaccination on several strains of representative genotype showed the same high protection results of 100% but there were differences in shedding of the virus through feces. Vaccination with certain genotypes still causes shedding when challenged with genotype seed vaccines from different strains.

Comparison of Homology and Phylogenetic Analysis of MSL.03 Virus

Based on homology comparison analysis, MSL.03 virus has similarity about 90.28-95.86% with seven isolates from Indonesia found in Genbank (Table 2). MSL.03 virus nucleotide shares 90.28% identical to the four ND isolates available at GenBank,

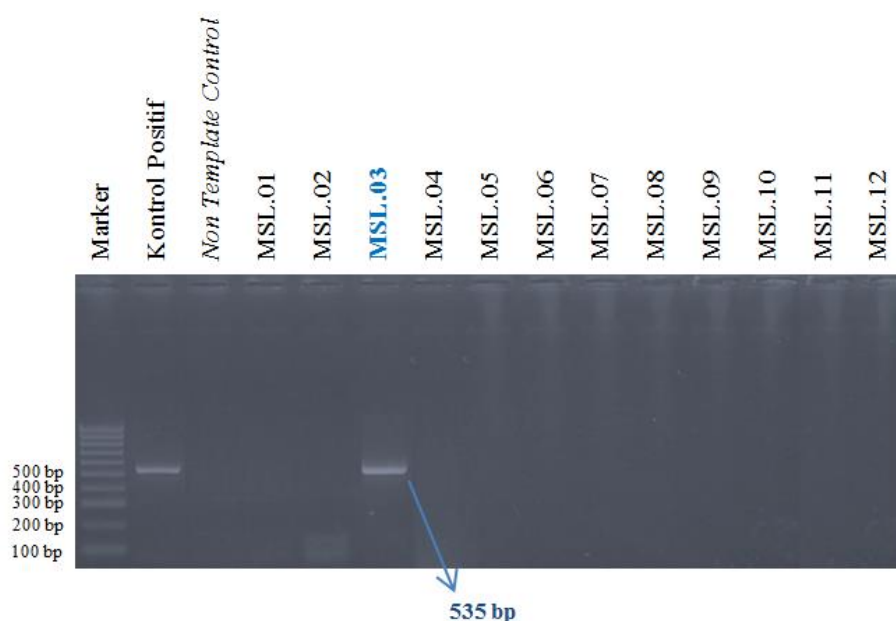


Figure 1. RT-PCR test results visualized on the gel electrophoresis showed positive results on MSL.03 samples with an amplicon of approximately 535 bp. Biline 100 bp was used as marker

chicken/Gianyar/013/10 (Access code HQ697257), chicken/Kudus/017/10 (Access code HQ697259), chicken/Sragen/014/10 (Access code HQ697258) and chicken/Kudus/018/10 (Access code HQ697260). Shofa *et al.* (2018) stated that the four isolates were included in subgenotype VIIi. On the other hand, when compared to Indonesian isolates in genotype VIIh, MSL.03 virus nucleotides showed higher identical by 95.34% to chicken/Makassar/003/09 (Access code HQ697256), 95.35% to chicken/Bali/020/10 (Access code HQ697261), and 95.86% to chicken/Sukorejo/019/10 (HQ697255). These results were supported by phylogenetic analysis through phylogenetic trees which proved that the MSL.03 virus belongs to the ND virus subgenotype VIIh branch as well as isolates chicken/Makassar/003/09, chicken/Bali/020/10 and chicken/Sukorejo/019/10 (Figure 2).

Outbreaks caused by ND virus genotype VII have been widely reported since the 1990s and are widely circulating in several regions around globe such as Europe, China, Middle East and South Africa (Herczeg *et al.*, 1999; Wang *et al.*, 2006; Bogoyavlenskiy *et al.*, 2009). The development of researches in molecular biology shows that genotype VII is endemic in Southeast Asia region such as Malaysia (Berhanu *et al.*, 2010). In Indonesia, genotype VII of the ND virus is already widely spread, as supported by a research conducted by Indriani and Dharmayanti (2016) who characterized and developed vaccines with seeds belonged to genotype VII.

Miller *et al.* (2015) stated that there are two subgenotypes VII which has spread widely at poultry farms throughout Indonesia, they are subgenotypes VIIh and VIIi. Putri *et al.* (2017) and Shofa *et al.*

Tabel 2. Virus MSL.03 homology comparison of nucleotide with Indonesia isolate from Genbank (%)

Isolat	1	2	3	4	5	6	7
1 HQ697256.1_chicken/Makassar/003/09							
2 HQ697257.1_chicken/Gianyar/013/10	91,32						
3 HQ697259.1_chicken/Kudus/017/10	91.32	100					
4 HQ697258.1_chicken/Sragen/014/10	91.32	100	100				
5 HQ697260.1_chicken/Kudus/018/10	91.32	100	100	100			
6 HQ697261.1_chicken/Bali/020/10	98.56	90.80	90.80	90.80	90.80		
7 HQ697255.1_chicken/Sukorejo/019/10	98.56	91.34	91.34	91.34	91.34	98.57	
8 MSL.03	95.34	90.28	90.28	90.28	90.28	95.35	95.86

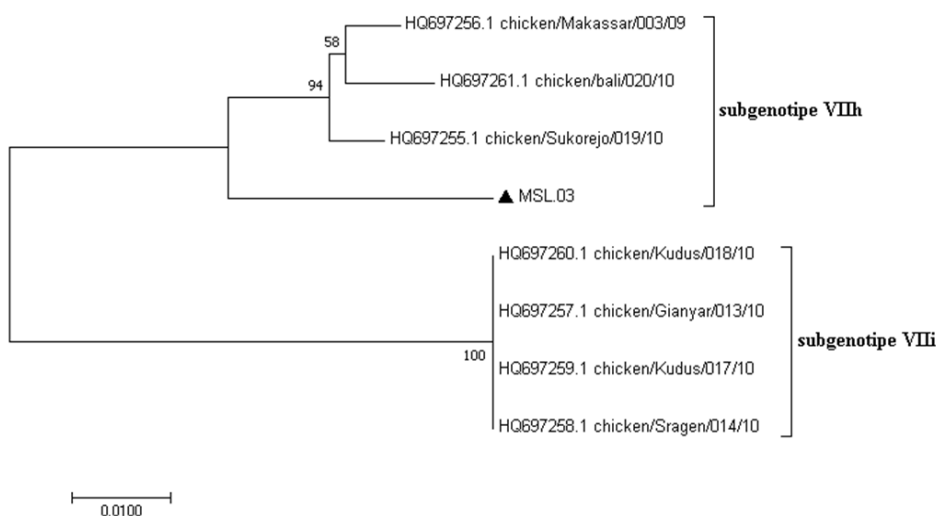


Figure 2. Phylogenetic relation of the partial Fusion (F) gene ND MSL.03 virus from brain torticollis symptom in Tangerang with Indonesian isolates representative from two subgenotypes available at GenBank

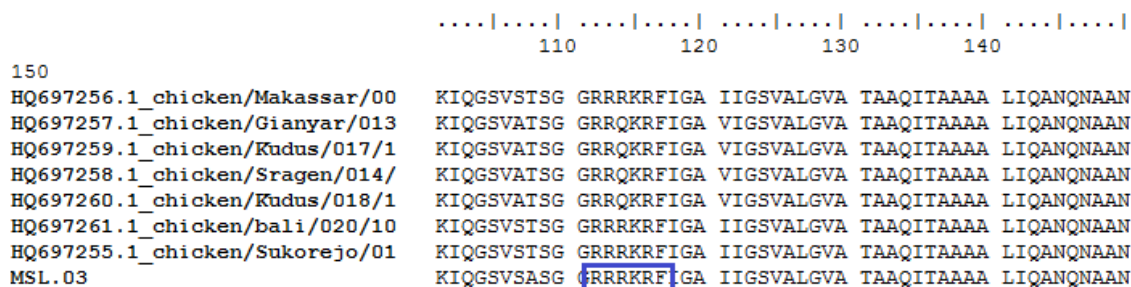


Figure 3. Comparison of amino acids in the cleavage site (F0) from the Fusion gene (F) of ND virus MSL.03 compared with Indonesian isolates from Genbank. Special line shows amino acid sequence from 112 to 117 of MSL.03 viruses that is RRRKRF

(2018) added that genotype VIIIh was mostly found in West Java areas with dense poultry farms. Although, it has been widely circulated in Indonesia, the pathogenicity differences between subgenotype VIIIh and other subgenotypes have not been studied adequately. According to Miller *et al.* (2015), the spreading of subgenotypes ND virus into an area is generally through transportation of reservoir animals. Some subgenotypes of ND viruses do not cause symptoms or death in certain species. This create potential new problem of virus shedding that will emerge a new subgenotypes.

Virulence Gene Analysis of MSL.03 Virus

Choi *et al.* (2010) mentioned that the virulence of ND virus has multibasic amino acids in the cleavage site (F0) of the ND virus Fusion gene. The amino acid sequence at positions 112 to 117 in the F gene could describe virulence level of the ND virus. In this study, the MSL.03 virus had 112RRRKRF117 pattern at the virulent determinant position (Figure 3). Bello *et al.* (2018) showed that some ND isolates from several regions in Nigeria had RRRKRF pattern at F0. Previously, Choi *et al.* (2013) found the same pattern in ND cases in Cambodia. According to Choi *et al.* (2010) the existence of several pattern such as RRRKRF, KRQKRF, and RRQRRF become a criterion of virulence of ND virus.

CONCLUSION

Partial Gene Fusion (F) from the NDV is successfully detected by RT-PCR from brain of laying hens with history of torticollis symptom in Tangerang. The ND virus in this study belongs to subgenotype VIIIh and is classified as virulent ND virus based on the 112RRRKRF117 pattern found in the cleavage site (F0) of the Fusion gene.

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