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

Safira Iqlima Sarah, Christian Marco Hadi Nugroho, Ryan Septa Kurnia, Dhandy Koesoemo Wardhana, and Heni Puspitasari

1Master Program, Diseases and Veterinary Public Health, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia
2Biomedical Doctoral Program, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia
3Research and Development Unit, PT. Medika Satwa Laboratoris, Bogor, Indonesia
4Department of Veterinary Public Health Sciences, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia
5Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia
6Corresponding author: christianmarco3085@gmail.com

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 VIIh 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

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 payer’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 VIIb and VIII 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 micropaste 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 PlatinumTM Taq DNA Polymerase (Invitrogen) using forward primer: 5'- ATGGGCTCCAGACCTTCTACCA-3' and reverse primer: 5'-CTGCCACCTGCTAGTTGTGATAATCC-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

<table>
<thead>
<tr>
<th>No.</th>
<th>Code sample</th>
<th>Origin</th>
<th>Organ</th>
<th>Age (Weeks)</th>
<th>Symptom</th>
<th>ND vaccination record</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MSL.01</td>
<td>Sukabumi</td>
<td>Brain</td>
<td>26</td>
<td>Torticollis</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>MSL.02</td>
<td>Subang</td>
<td>Brain</td>
<td>35</td>
<td>Torticollis, Respiratory disorder</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>MSL.03</td>
<td>Tangerang</td>
<td>Brain</td>
<td>42</td>
<td>Torticollis, Respiratory disorder, Lime stools</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>MSL.04</td>
<td>Tangerang</td>
<td>Brain</td>
<td>33</td>
<td>Torticollis, Respiratory disorder</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>MSL.05</td>
<td>Sukabumi</td>
<td>Brain</td>
<td>40</td>
<td>Torticollis, Respiratory disorder, Bluish wattle</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>MSL.06</td>
<td>Sukabumi</td>
<td>Brain</td>
<td>40</td>
<td>Torticollis</td>
<td>Yes</td>
</tr>
<tr>
<td>7</td>
<td>MSL.07</td>
<td>Bogor</td>
<td>Brain</td>
<td>24</td>
<td>Torticollis, Respiratory disorder</td>
<td>Yes</td>
</tr>
<tr>
<td>8</td>
<td>MSL.08</td>
<td>Subang</td>
<td>Brain</td>
<td>33</td>
<td>Torticollis, Respiratory disorder, Lime stools</td>
<td>Yes</td>
</tr>
<tr>
<td>9</td>
<td>MSL.09</td>
<td>Bogor</td>
<td>Brain</td>
<td>27</td>
<td>Torticollis, Respiratory disorder, Bluish wattle</td>
<td>Yes</td>
</tr>
<tr>
<td>10</td>
<td>MSL.10</td>
<td>Subang</td>
<td>Brain</td>
<td>56</td>
<td>Torticollis, Respiratory disorder, Bluish wattle</td>
<td>Yes</td>
</tr>
<tr>
<td>11</td>
<td>MSL.11</td>
<td>Subang</td>
<td>Brain</td>
<td>43</td>
<td>Torticollis, Respiratory disorder</td>
<td>Yes</td>
</tr>
<tr>
<td>12</td>
<td>MSL.12</td>
<td>Subang</td>
<td>Brain</td>
<td>45</td>
<td>Torticollis, Respiratory disorder, Bluish wattle</td>
<td>Yes</td>
</tr>
</tbody>
</table>
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. Bioline 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 (Berhana 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. 2018.

<table>
<thead>
<tr>
<th>Isolat</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 HQ697256.1_chicken/Makassar/003/09</td>
<td>91.32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 HQ697257.1_chicken/Gianyar/013/10</td>
<td>91.32</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 HQ697259.1_chicken/Kudus/017/10</td>
<td>91.32</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 HQ697258.1_chicken/Sragen/014/10</td>
<td>91.32</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 HQ697260.1_chicken/Kudus/018/10</td>
<td>98.56</td>
<td>90.80</td>
<td>90.80</td>
<td>90.80</td>
<td>90.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 HQ697261.1_chicken/Bali/020/10</td>
<td>98.56</td>
<td>91.34</td>
<td>91.34</td>
<td>91.34</td>
<td>91.34</td>
<td>98.57</td>
<td></td>
</tr>
<tr>
<td>7 HQ697255.1_chicken/Sukorejo/019/10</td>
<td>95.34</td>
<td>90.28</td>
<td>90.28</td>
<td>90.28</td>
<td>98.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 MSL.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**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 VIIh was mostly found in West Java areas with dense poultry farms. Although, it has been widely circulated in Indonesia, the pathogenicity differences between subgenotype VIIh 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 112RRRKRFF117 pattern at the virulent determinant position (Figure 3). Bello et al. (2018) showed that some ND isolates from several regions in Nigeria had RRRKRFF 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 RRRKRFF, KRQKRF, and RRQRKF 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 Tanggerang. The ND virus in this study belongs to subgenotype VIIh and is classified as virulent ND virus based on the 112RRRKRFF117 pattern found in the cleavage site (F0) of the Fusion gene.

**ACKNOWLEDGEMENTS**

Thank you to 1st base Malaysia for the sequencing services and Immunology Laboratory, Department of Animal Disease and Health Sciences, Faculty of Veterinary Medicine, Bogor Agricultural University for the confirmation test.

**REFERENCES**


