MOLECULAR PHYLOGENETICS OF NEWCASTLE DISEASE VIRUS ISOLATED FROM CHICKENS IN 2019

SYAMSIAH AINI, S.*, LEOW, B.L., FAIZUL FIKRI, M.Y., MUHAMMAD REDZWAN, S., AND FAIZAH HANIM M.S.

Veterinary Research Institute, 59 Jalan Sultan Azlan Shah, 31400 Ipoh, Perak
**Corresponding author*: syamsiah@dvs.gov.my

ABSTRACT. Newcastle disease (ND) is an economically important, contagious poultry viral disease reported across the globe. No recent reports on ND circulating in Malaysia. Therefore, the aim of the study is to characterize 16 Newcastle disease viruses (NDVs) isolated from chickens in Malaysia in the year of 2019. All isolates were genotypically analyzed using reverse transcription-polymerase chain reaction (RT-PCR) with primers specific to the viral fusion (F) protein gene. Analysis of the F protein cleavage site's deduced amino acid sequences revealed that from the Newcastle disease virus (NDV) isolates, three of them were virulent with two different motifs of 112RRQKRF117 and 12RRRKRF117 while other isolates were avirulent. Phylogenetic analysis demonstrated that three isolates were grouped in genotype VII, five in genotype I while eight in genotype II. All genotype VII isolates were clustered under sub-genotype VII.2 (VIIh and VIIi) which is the same strain causing previous outbreaks in Malaysia. Therefore, findings in this study demonstrated that there is no new introduction of NDV genotypes in Malaysia. However, farms should implement biosecurity measures at strict level as well as executing continuous monitoring and surveillance of the disease as these implementations would help them to conduct proper preventive measures and control of panzootic viruses in future.

Keywords: NDV, AOAV-1, VII.2, chickens, Malaysia

INTRODUCTION

Newcastle Disease is one of the most highly contagious viral diseases that causes severe economic losses to the poultry industry worldwide (Nagy *et al*., 2020). This disease is caused by virulent strains of avian paramyxovirus-1(APMV-1) viruses or commonly referred to as Newcastle disease virus (NDV). Of recent, NDV has been classified to Genus *Avian Orthoavulavirus*, family *Paramyxoviridae* which is known as *Avian Orthoavulavirus -1* (*AOAV-1*) (Abd El Hamid *et al*., 2020; ICTV, 2019; Amarasinge *et al*., 2018). In this research, the common term of NDV denomination is maintained. NDV is a negative, non-segmented, and single-stranded RNA virus of approximately 15kb composed of six major structural proteins encoding for fusion (F), matrix (M), nucleoprotein (NP), RNA polymerase (L), phosphoprotein (P), and hemagglutination–neuraminidase (HN) (Hussain *et al*., 2020; Nagy *et al*., 2020). Additionally, two non-structural proteins known as V and W are produced as the result of RNA editing of the P gene (Ganar *et al.,* 2014).

According to de Leeuw *et al*. (2005), among the proteins, HN and F protein are NDV surface proteins that play major roles in infections caused by NDV as well as pathogenicity and antigenicity. It was found from the study of Peeters and Koch (2019) that the F protein is the cause of fusion between host cell membrane and the virus which is required for viral entry as well as replication in host cells. The cleavage site of the *F* gene protein is known to be a major determinant for virulence of NDV (Ganar *et al*., 2014). World Organisation

for Animal (OIE) mentioned that virulent NDV strains are found to have multiple basic amino acids at the C terminus of the F2 protein and phenylalanine at residue 117, which is the N terminus of the F1 protein. Based on previous studies (Dimitrov *et al*., 2016; Ganar *et al*., 2014), the term "multiple basic amino acids" means the presence of at least three arginines (R) or lysine (K) residues from positions 113 through 116. In contrast, NDV with low virulence usually has sequences in the same region of 112G/E-K/R-Q-G/E-R116 and L(leucine) at residue 117 (OIE, 2018; Peeters *et al.,* 1999).

Based on updated classification by Dimitrov *et al*. (2019), complete *F* gene nucleotide sequencing classified NDVs into two different classes which are Class I and Class II. Viruses from Class I belong to a single genotype, while 20 genotypes have been identified for Class II viruses. Mainly, Class I includes avirulent isolates such as wild waterfowl that occasionally spill over into poultry. The majority of NDV strains, however, includes both of virulent and non-pathogenic strains that belong to Class II (Alexander *et al*., 2012; Aldous *et al*., 2003). As at current, Class II NDVs are identified in ND outbreaks in many parts of Asian countries which cause high mortality in poultry (Hussain *et al*., 2020).

ND is considered as endemic in Malaysia where it faced major outbreaks in vaccinated birds during the year 2000 to 2001 that were caused by sub-genotype VIId (Tan *et al*., 2010; Berhanu *et al*., 2010). Shohaimi *et al*. (2015) reported that during the year of 2010 to 2012, major outbreaks occurred not only in village chickens but also in vaccinated chickens caused by sub-genotype VIIh and VIIa. Miller *et al*. (2015) reclassified sub-genotype VIIa as VIIi. Sub-genotype VIIi was also identified from isolates in Malaysia in the year 2014 and 2015 (Aljumaili *et al*., 2017). Since then, there are no reports on NDV circulating in Malaysia. Therefore, this study aims to characterize the NDV isolates in 2019 using molecular and phylogenetic analysis based on the *F* gene and compare the fusion cleavage site and nucleotide sequences with previously published strains.

MATERIALS AND METHOD

Virus Isolates

Sixteen NDV isolates were detected positive by real-time Reverse Transcription Polymerase Chain Reaction (qRT-PCR) and isolated by egg inoculation in the year 2019 at the Avian Virology Section, Veterinary Research Institute (VRI) in Ipoh, Perak, Malaysia. The samples were received in the form of allantoic fluid from Regional Veterinary Laboratories around Malaysia (Table 1). The viruses were propagated in the allantoic cavity of 9 to 11-day-old specific-pathogen-free (SPF) embryonated chicken eggs. The isolates were confirmed as NDV by the Hemagglutination-Inhibition (HI) test using a specific antiserum against ND (OIE, 2018). Infected allantoic fluid samples were centrifuged at 2500 × g for 10 minutes at 4°C and the supernatant was stored at -80 °C for later analysis.

Extraction of viral RNA and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total viral RNA was extracted from the infected allantoic fluid using the Indispin Pathogen kit (Indical Bioscience, Germany) in accordance with the manufacturer's instructions. RT-PCR was carried out using the SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen, USA). Three primer sets (NDV-4358F/NDV-5307R, NDV-4701F NDV-5849R and NDV-5724F/ NDV-7021R) were used which covered the complete *F* gene of NDV (Gould *et al*., 2003). RT was

conducted at 48°C for 30 min. The reaction mixture was then subjected to 94°C for 5 min for initial denaturation, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 68°C for 2 min with a final extension for 10 min at 68°C. The amplicons were loaded into 1.0% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen, USA) for gel electrophoresis and visualized by UV transilluminator (Aplegen, USA).

Sequencing, Analysis of Nucleotide and Amino Acid Sequences and Phylogenetic Analysis

PCR products were excised from agarose gel prior to Sanger sequencing. Sequencing was performed by Apical Scientific (M) Sdn. Bhd. The primers used for sequence analysis were the same as those used for RT-PCR amplification. The raw sequences were manually edited and assembled using SeqMan Pro software (DNASTAR Lasergene, USA). The alignment of nucleotide and deduced amino acid sequences were done with another 55 NDVs that represent each genotype which were retrieved from GenBank, NCBI database. Comparison of the sequences were done with BioEdit Sequence Alignment Editor (version 7.1.9) (Hall, 1999). Subsequently, phylogenetic analysis was conducted with MEGA version 6.06 using neighbour-joining statistical method (Kimura 2-parameter model) as well as setting 1000 bootstrap replicates (Tamura *et al.*, 2013). The phylogenetic tree was generated based on the F gene from nucleotide 1 to 1662.

RESULTS

All sixteen isolates were successfully amplified and sequenced. Table 1 shows the list and details of isolates. In 2019, the 16 positive NDV isolates were from Sabah (3), Sarawak (5), Johor (3), Pahang (4) and Selangor (1), respectively. Based on Table 1, the BLAST results of the nucleotide sequences revealed that four isolates from Sarawak had 100% nucleotide homology with VG/GA-AVINEW vaccine strain (Gen Accession No: KC906188). Eight (8) isolates from Sabah, Johor and Pahang had 100% nucleotide homology with LaSota vaccine strain. Meanwhile, two isolates from Sabah revealed 97.54% nucleotide identity with Indonesia strain (chicken/Kudus/018/10). Lastly, one isolate from Selangor was 100% identical with Malaysia strain (IBS002/11) isolated in the year 2011.

Analysis of the F protein cleavage site demonstrated that three isolates were virulent while the remaining 13 isolates were avirulent (Table 1). The virulent isolates showed the presence of multiple basic amino acid sequences at position 112 to 116 and phenylalanine (F) at residue 117 with two different motifs of ^{112}RRQKRF^{117} and ^{112}RRRKRF^{117}. Likewise, the avirulent isolates also had two different motifs of ^{112}GRQGRL^{117} and ^{112}GKQGRL^{117} where they had monobasic amino acid at position 112 to 116 and Leucine (L) at residue 117. Phylogenetic analysis based on the *F* gene (Figure 1) showed that NDV can be divided into two classes i.e., Class I and Class II. All isolates in this study were clustered under Class II. Based on the phylogenetic tree constructed, these 16 isolates were divided into three genotypes (VII, I and II).

Thirteen isolates under genotypes I and II were closely related to vaccine strains VG/GA-AVINEW and LaSota respectively. Meanwhile, for isolates under genotype VII, based on the classification by Diel *et al.* (2012), two isolates from Sabah (chicken/ Sabah/1502/2019 and chicken/ Sabah/9457/2019) fell under sub-genotype VIIi while one isolate (chicken/ Selangor/8857/2019) was clustered under sub-genotype VIIh. Under new classification suggested by Dimitrov *et al*. (2019), all these three isolates fell under sub-genotype VII.2.

Table 1. List of isolates used in this study including the F0 cleavage motif, and BLAST results Legend; F: Phenylalanine; G: Glycine, K: Lysine; L: Leucine; R: Arginine; Q: Glutamine; n/a: not applicable

	DI No	Date Received	State	Species	Breed	BLAST Analysis		F0 Cleavage site motif	Virulence
						Highest Nucleotide Homology	% Nucleotide Identity		
1	1502/2019	26/2/2019	Sabah	Chicken	kampung	chicken/ Kudus/018/10	97.54	RRQKRF	Virulent
2	2996/2019	10/4/2019	Sarawak	Chicken	Arbor Acres	VG/GA-AVINEW	100	GKQGRL	Avirulent
3	5796/2019	25/7/2019	Pahang	Chicken	kampung	LaSota	100	GRQGRL	Avirulent
4	5797/2019	25/7/2019	Pahang	Chicken	Ross	LaSota	100	GRQGRL	Avirulent
5	5798/2019	25/7/2019	Pahang	Chicken	Cobb	LaSota	100	GRQGRL	Avirulent
6	5799/2019	25/7/2019	Pahang	Chicken	Cobb	LaSota	100	GRQGRL	Avirulent
7	6458/2019	19/8/2019	Johor	Chicken	Ross	LaSota	100	GRQGRL	Avirulent
8	6798/2019	29/8/2019	Johor	Chicken	Ross	LaSota	100	GRQGRL	Avirulent
9	7073/2019	12/9/2019	Sarawak	Chicken	Ross	VG/GA-AVINEW	100	GKQGRL	Avirulent
10	7074/2019	12/9/2019	Sarawak	Chicken	Ross	VG/GA-AVINEW	100	GKQGRL	Avirulent
11	7357/2019	24/9/2019	Sabah	Chicken	n/a	LaSota	100	GRQGRL	Avirulent
12	8097/2019	17/10/2019	Johor	Chicken	Cobb	LaSota	100	GRQGRL	Avirulent
13	8551/2019	30/10/2019	Sarawak	Chicken	Cobb	VG/GA-AVINEW	100	GKQGRL	Avirulent
14	8857/2019	11/11/2019	Selangor	Chicken	Ross	IBS002/11	100	RRRKRF	Virulent
15	9457/2019	3/12/2019	Sabah	Chicken	Fighting cock	chicken/ Kudus/018/10	97.54	RRQKRF	Virulent
16	9682/2019	11/12/2019	Sarawak	Chicken	Ross	VG/GA-AVINEW	100	GKQGRL	Avirulent

DISCUSSION

In this study, 16 samples received from Regional Veterinary Laboratories around Malaysia were analysed. These cases were submitted to VRI to confirm for ND. Samples were received not only from vaccinated chickens but also from *ayam kampung* (village chicken) and fighting cock. Out of 16 isolates involved in this study, 13 were classified as avirulent based on cleavage site motifs of ^{112}GKQGRL^{117} and ^{112}GKQGRL^{117}. These isolates were clustered under two different genotypes: genotype I and genotype II. These genotypes are considered as "early genotypes" as they emerged between 1930 to 1960 and are commonly used as vaccines worldwide (Miller *et al*., 2010). This is in agreement with

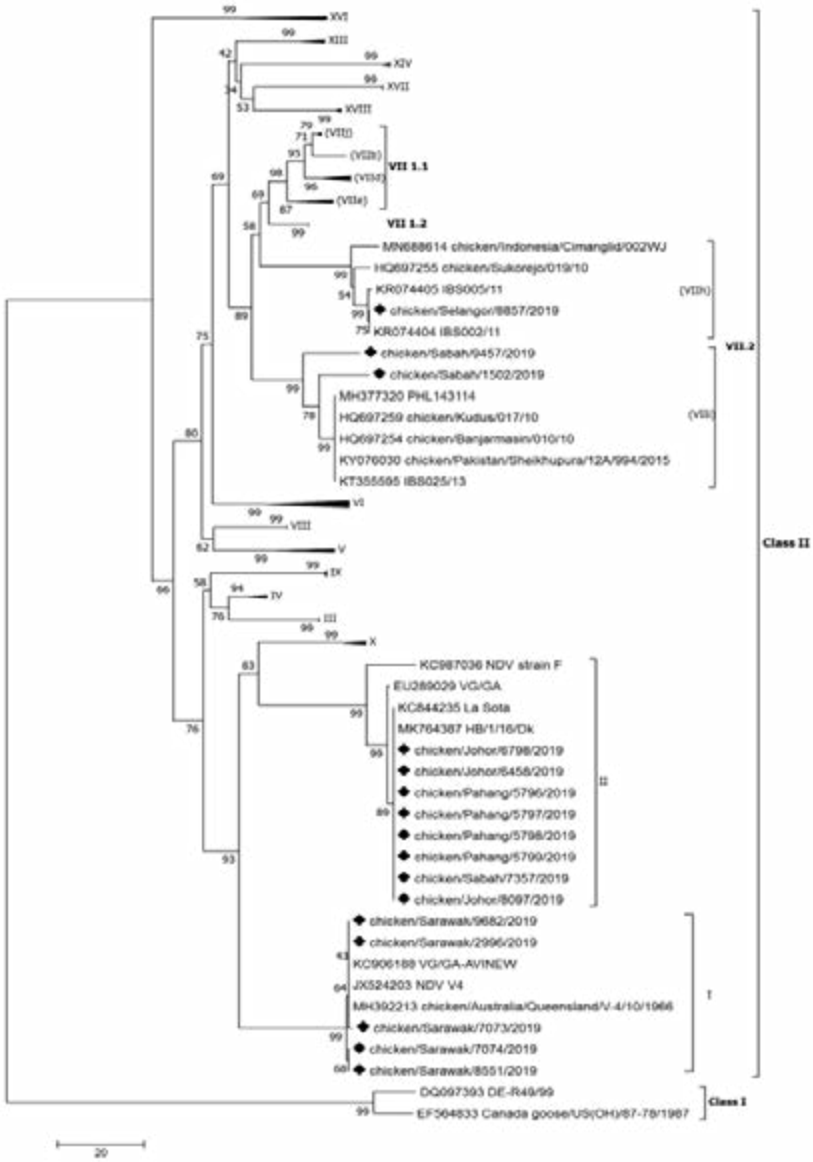

Figure 1. Phylogenetic tree constructed on the basis of complete fusion gene sequences of NDV isolates obtained from this study and other NDV isolates representing different genotypes and sub-genotypes of Class II and Class I. Genotypes are according to Dimitrov et al. (2019). Genotypes according to Diel et al. (2012) are shown in parenthesis. The 16 strains in this study are designated with a rotated square. Tree was constructed using MEGA version 6.06 by the Neighbour-Joining statistical method with the Kimura 2-parameter model and setting bootstrap 1000 replicates.

our finding that these isolates were identical to commercial vaccines such as LaSota and VG/GA-AVINEW strains. Based on the List of Approved Veterinary Vaccines in Malaysia (2018), these are the common live vaccine strains which are commercially available in Malaysia (DVS, 2018).

From the results of this study, the three remaining isolates exhibited virulent NDV characteristics based on the appearance of multiple basic amino acids sequence of ^{112}RRQKRF^{117} and ^{112}RRRKRF^{117} at the F protein cleavage site. Previous studies on Malaysian isolates by Shohaimi *et al.* (2015) and Tan *et al.* (2010) also reported similar motifs. All of the three isolates were found to be clustered in genotype VII based on phylogenetic analysis. The emergence of viruses in genotype VII was revealed during the early 1990s in Europe and East Asia (Lomniczi *et al*., 1998). Since then, they became the dominant virulent viruses and the most frequently associated with outbreaks in commercial poultry farms and backyard chickens in Asia (Miller *et al*., 2015; Yi *et al*., 2011). Diel *et al.* (2012) had classified NDV genotypes based on complete *F* gene sequence. Genotype VII comprises highly diverse viruses, therefore this genotype has been further divided to sub-genotypes: VIIa to VIII. According to this classification, one isolate (chicken/Selangor/8857/2019) was categorized under sub-genotype VIIh, while the remaining 2 isolates (chicken/Sabah/1502/2019 and chicken/Sabah/9457/2019) were clustered under sub-genotype VIIi. These two sub-genotypes have been reported to cause major outbreaks in Malaysia during the year 2010 to 2012 and also been isolated in the year 2014 and 2015 (Shohaimi *et al*., 2015; Aljumaili *et al*., 2017). Therefore, findings from this study suggest that viruses from these sub-genotypes are still circulating in Malaysia.

Recently in 2019, an updated classification has been introduced where genotype VII was reclassified into only three sub-genotypes: VII.1.1 including previous sub-genotypes (b, d, e, j, l), VII.1.2 included previous sub-genotype (f) and VII.2 included the previous sub-genotypes (h, i, and k) (Dimitrov *et al*., 2019). Since ND was first discovered in 1926, four global panzootic of ND have been recognized (Liu *et al*., 2019). According to Miller *et al*. (2015), genotype VIIh and VIIi have the potential to be the fifth panzootic as these sub-genotypes have been endemic not only in Southeast Asia countries but also in Pakistan and Africa. In China, this sub-genotype has been detected in wild birds since 2011 but it was later detected in domestic poultry. It was suggested that these viruses might have formed a stable lineage in poultry during 2012-2016 and have the potential to cause enzootic in China (Liu *et al*., 2019). As a result of this, sub-genotypes involved in the fifth panzootic were merged together under single sub-genotype VII.2. Therefore, based on this new classification, the three virulent isolates in this study are reclassified under sub-genotype VII.2.

Malaysia has been an endemic country for ND, despite control strategies that have been done to eradicate the viruses (Miller *et al*., 2015). The transmission and spread of NDV continue to be a threat to the poultry sector. Since many years, the commercial poultry flocks' sector in Malaysia has been involved in intensive vaccination programs (Berhanu *et al*., 2010). Unfortunately, backyard chickens or non-commercial chickens in Malaysia are not properly vaccinated. This is in agreement with our findings where two virulent NDVs were isolated from *ayam kampung* (village chicken) and fighting cock. Even though these isolates do not cause any outbreak, it has been suggested that backyard chickens or non-poultry avian species are possible amplification host based

on the fact that they are prone to get the virulent strains due to insufficient biosecurity measures and vaccination (Hussain *et al*., 2020).

CONCLUSION

In conclusion, findings from this study confirmed that sub-genotype VII.2 (VIIh and VIIi) are still circulating in Malaysia. There is no new introduction of NDV genotypes into Malaysia. However, farm level should implement biosecurity measures strictly as well as continuously monitoring the condition. Apart from that, surveillance of the disease in commercial poultry, backyard flocks and different species of birds is important for proper preventive measures and control of panzootic viruses in future.

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