

## SEQUENCE AND PHYLOGENETIC ANALYSIS OF NEWCASTLE DISEASE VIRUS GENOTYPE VII ISOLATED IN MALAYSIA DURING 1999-2012

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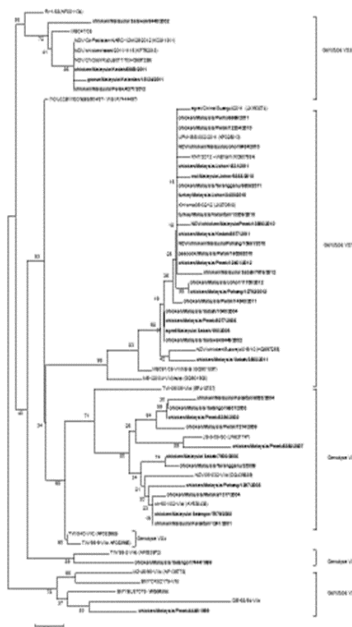
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### Graphical abstract



### Abstract

Newcastle disease virus (NDV) is a contagious viral disease of many avian species particularly domestic poultry, and is responsible for causing significant economic losses to the poultry industry in Southeast Asia including Malaysia. Here we report the sequence and phylogenetic analysis of NDV that has been circulating in Malaysia. A total of 151 NDV isolates were selected during 1999-2012 throughout Malaysia and were characterized phylogenetically. The partial region of matrix (M) and fusion (F) protein of NDV was amplified by reverse transcriptase PCR, directly sequenced and compared genetically to the published sequences obtained from GenBank. The deduced amino acid sequence of the F protein cleavage site revealed the presence of three different motifs; <sup>112</sup>RRRKR<sup>F117</sup>, <sup>112</sup>RRQKR<sup>F117</sup> typical for velogenic strains while <sup>112</sup>GKQGR<sup>L117</sup> indicates it is from avirulent strain or lentogenic strain. The phylogenetic analysis revealed that 13 isolates belonged to genotype I, 2 to genotype III, 6 to genotype VI, 1 to genotype VIII and 129 to genotype VII. Isolates belonging to genotype VII were further divided into five subgenotypes; VIIa, VIIb, VIIc, VIId and VIIh. Based on the phylogenetic tree and geographical data, it is found that NDV genotype VIIb and VIIc were isolated in 1999 while in year 2000 to 2009, most of the NDV isolates were NDV genotype VIId originated from China. No subgenotype VIId viruses were recovered after 2009 in Malaysia. In 2010-2012, NDV outbreaks were caused by subgenotypes VIIa and VIIh in Peninsular Malaysia. Interestingly, these subgenotypes have been isolated in East Malaysia since 2002 but did not cause major outbreak. These information points to the existence of multiple genotypes of NDV in Malaysia especially genotype VII and these findings emphasize the importance of continuous surveillance of NDV in Malaysia.

**Keywords:** Newcastle Disease Virus, NDV, genotype VII, Malaysia, virulent, poultry, phylogenetic tree

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## 1.0 INTRODUCTION

Newcastle Disease (ND) is a highly contagious viral disease of poultry. The disease is present worldwide and affects many species of birds causing severe losses in the poultry industry [1]. ND is caused by avian paramyxovirus serotype-1 (APMV-1), which belongs to the genus Avulavirus in the family of Paramyxoviridae [1]. The genome of APMV-1 is always either 15186, 15192 or 15198 nucleotides in length [2]. This enveloped virus has a negative-sense, single-stranded RNA which encodes six proteins, including the nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and RNA-directed RNA polymerase (L) [3, 4].

NDV strains can be categorized into highly virulent (velogenic), intermediate (mesogenic) or nonvirulent (lentogenic) based on their pathogenicity in chickens by using Intracerebral Pathogenicity Index (ICPI) [1]. Alternatively, pathogenicity or the virulence of NDV can also be evaluated by amino acid sequence analysis of the fusion (F) protein. NDV that are virulent for chickens have a multibasic amino acid sequence  $^{112}R/K-R-Q-K/R-R^{116}$  at the C-terminus of the F2 protein and F (phenylalanine) at residue 117, which is the N-terminus of the F1 protein, whereas the viruses of low virulence have a monobasic amino acid sequences in the same region of  $^{112}G/E-K/R-Q-G/E-R^{116}$  and L (leucine) at residue 117 [5, 8].

Based on phylogenetic analysis of the nucleotide sequence, it can be classified into class I strains which are avirulent in chickens, and class II strains which carry lentogenic, mesogenic and velogenic strains of NDV. Class II viruses can then be divided into 11 genotypes (I–XI) based on the partial sequence of the F gene. Genotypes VI and VII, which are genetically diverse are further classified into eight (a–h) and 5 (a–e) subgenotypes, respectively [6–8]. NDV is considered as endemic in Malaysia and NDVs outbreaks reported are caused predominantly by genotype VII viruses in recent years [9]. In 2010, Malaysia experienced several outbreaks not only in backyard poultry (village chickens) but also in vaccinated commercial poultry which affected the major poultry producing states. It was reported that the virus responsible for the outbreak was caused by genotype VII strain, similar to the strain isolated in 2004 [10]. The aim of this study is to characterize NDV isolates in Malaysia during 1999–2012 using molecular and phylogenetic analysis based on partial fusion gene.

## 2.0 EXPERIMENTAL

Hundred-and-fifty-one (151) NDV isolates were propagated into 9–10 day old Specific Pathogen Free (SPF) embryonated chicken eggs and the harvested allantoic fluid was used according to previous OIE method [1].

Viral RNA was extracted using Trizol LS (Invitrogen) according to manufacturer's instructions. One-step RT-PCR was carried out using SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen). A pair of primers (MV1/B2) was used to amplify 557bp which covers partially M and F genes [7]. After completing RT-PCR, the reaction mixture was loaded into 1.5% agarose gel containing SyBr Safe (Invitrogen) for electrophoresis and visualized by UV transilluminator.

PCR products were cut from agarose gel and purified using QIAQuick Gel Extraction Kit (Qiagen) prior to sequencing. The sequences were assembled using Seqman (DNASTar Lasergene, USA). Nucleotide sequences were then analyzed using BioEdit program version 7.0. Phylogenetic tree was constructed with MEGA v6.06 using neighbour joining Kimura 2 parameter model [11]. All isolates in this study together with other NDV sequences from the GenBank until March 2015; representative of different genotypes were included for phylogenetic analysis (GenBank, NCBI). Phylogenetic analysis of the NDV isolates was generated based on partial F gene (372bp) from nucleotide 47 to 418.

## 3.0 RESULTS AND DISCUSSION

Hundred-and-fifty-one 151 isolates that were recovered during 1999–2012 were sequenced based on partial fusion gene which covers the fusion protein cleavage site.

Based on fusion protein amino acid cleavage site, 3 types of motifs have been identified which are;  $^{112}R-R-Q-K-R-F-^{117}$ ,  $^{112}R-R-R-K-R-F-^{117}$  and  $^{112}G-K-Q-G-R-L-^{117}$ . The first two motifs have phenylalanine, F at position 117 which showed that both are considered as virulent strain while the latter showed Leucine (L) at position 117 indicating avirulent strain or lentogenic strain.

For the phylogenetic analysis, out of 151 isolates sequenced, 129 isolates were grouped under genotype VII. While the rest were clustered under genotype I (13 isolates), genotype III (2 isolates), genotype VI (6 isolates) and VIII (1 isolate). Genotype VII were then further divided into subgenotype VIIa, VIIb, VIIc, VIId, VIIe and VIIh (Table 1). Subgenotype VIIb and VIIc were isolated in 1999 and during 2000–2009, most of the isolates were clustered under subgenotype VIId which is closely related or similar to strain from China. For most of the isolates under genotype VIIh, they were recovered between 2010 to 2012 but this genotype has been isolated in East Malaysia since 2002. While for subgenotype VIIa, it was also first identified in East Malaysia in 2002 but mostly isolated in Peninsular Malaysia between 2011–2012 (Figure 1).

Genotype I, II and III strains are considered as "early genotypes" as they emerged between 1930 to 1960. Genotype I and II are low virulence and are used as vaccines worldwide such as La Sota, V4 and B1 [12] while genotype III is mesogenic strain and also

used as commercial vaccine strains such as Mukteswar [1]. (Figure 2).

Genotype VI emerged in the 1960s and has been isolated from multiple avian species [5, 13]. This is in agreement with our study as most of the viruses originated from genotype VI are pigeons and exotic birds (data not shown). This genotype has the consequent risk for introduction to poultry flocks but fortunately till date, no outbreaks were reported due to this genotype in Malaysia.

Genotype VIII viruses have been circulating in South Africa since 1960s [14] and continue to circulate in Southeast Asia including Malaysia in 1990s [9].

Based on this study, it is revealed that genotype VII is the genotype most frequently associated with outbreaks of ND in Malaysia. This is in agreement with Miller *et al.* [12], where genotype VII has been the predominant strain in Asia since 1990s till now. Tan *et al.* [9], and Shohaimi *et al.* [10], also reported that genotype VII has caused outbreaks in Malaysia between 2000 to 2010.

In this study, phylogenetic analysis revealed that subgenotype VIIb and VIIe were isolated in 1999. Interestingly, subgenotype VIIb has also been discovered in Malaysia in 1990 [6]. Therefore the virus that was isolated in the later year was only re-isolation. Meanwhile, subgenotype VIIe isolate chicken/Malaysia/Selangor/7444/1999 from Selangor layer chicken has 99% similarity with Taiwan isolate (refer Figure 1). This is in agreement with Lien *et al.* [15] who reported that one of the genotypes that was active in Taiwan during 1995-2000 was identified as subgenotype VIIe.

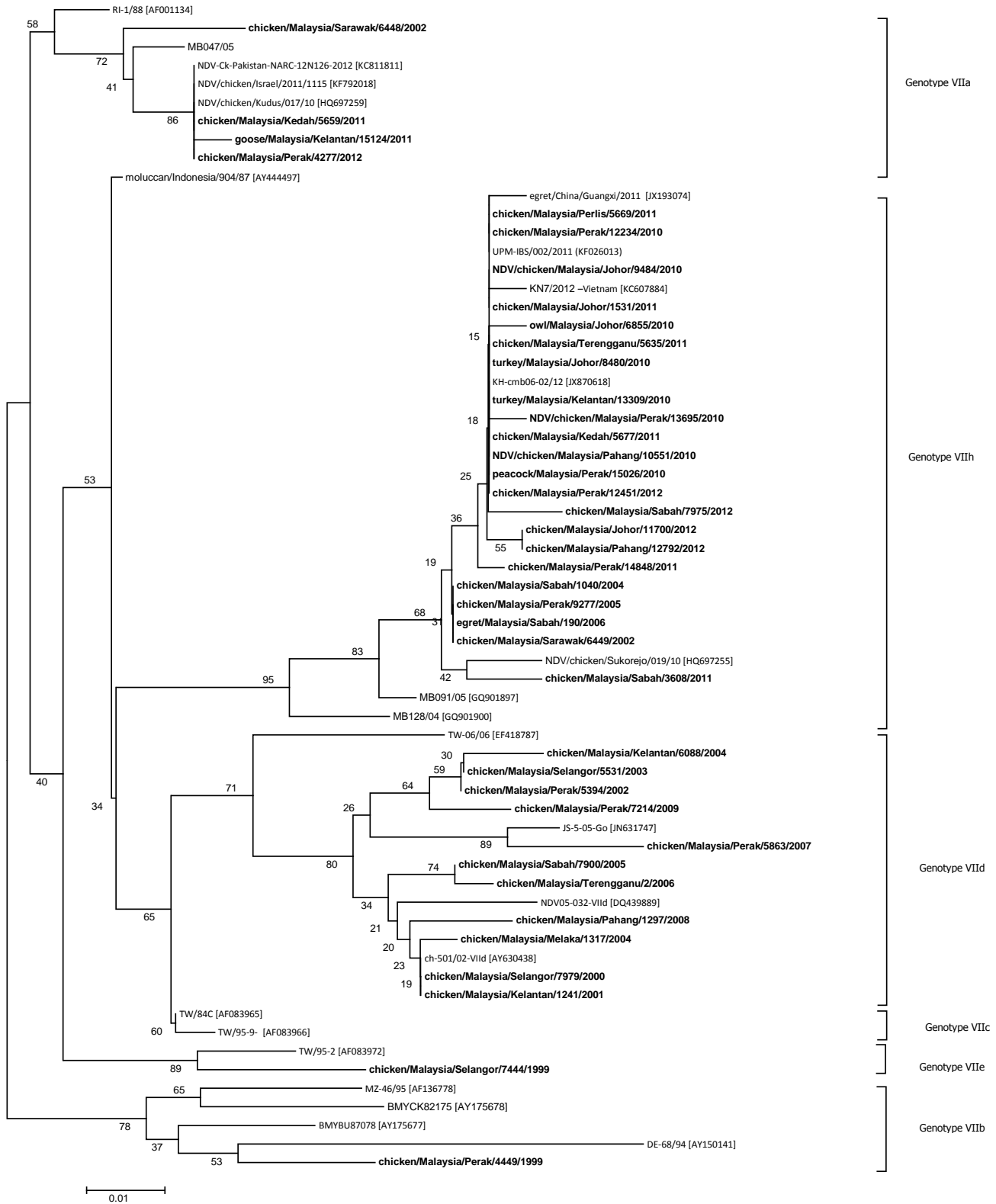
Malaysia faced major outbreaks in vaccinated birds during 2000- 2001 which was from subgenotype VIId [9, 16]. Due to that, this subgenotype was identified to be circulating in Malaysia between the year of 2000-2009. This subgenotype not only infects village chicken, but also commercial chickens

(broiler, layer, breeder) and ducks. Ducks can be considered as a natural reservoir for NDV as they can be infected and spread the virus isolates without causing clinical sign [1]. This factor may contribute on why this particular subgenotype circulated for quite a period of time in Malaysia (2000-2009). Based on this study, no subgenotype VIId isolates were recovered after 2009 as another new genotype has become the new causative strain of outbreaks in Malaysia. The same situation has been faced in Vietnam where this strain has not been isolated since 2011 as it was replaced by a new circulating strain in Vietnam [8]. Therefore it is suggested that VIId are no longer considered the major genotype VII responsible for outbreaks in Malaysia. In 2010, Malaysia had major outbreaks in states with high density of poultry population as reported by Shohaimi *et al.* [10]. The finding is consistent with the present study showing that many isolates recovered during 2010-2011 caused by the new genotype known as subgenotype VIIh. This genotype caused outbreak in Indonesia in 2009 and 2010 [17], in Vietnam and Cambodia during 2011-2012 [8]. It is worth to mention that Malaysia also reported the similar strain but Tan *et al.* [9] and Berhanu *et al.* [16] reported the strain was VIId. It was after the outbreaks in 2010, the strain was named as genotype VIIh by Choi *et al.* [8].

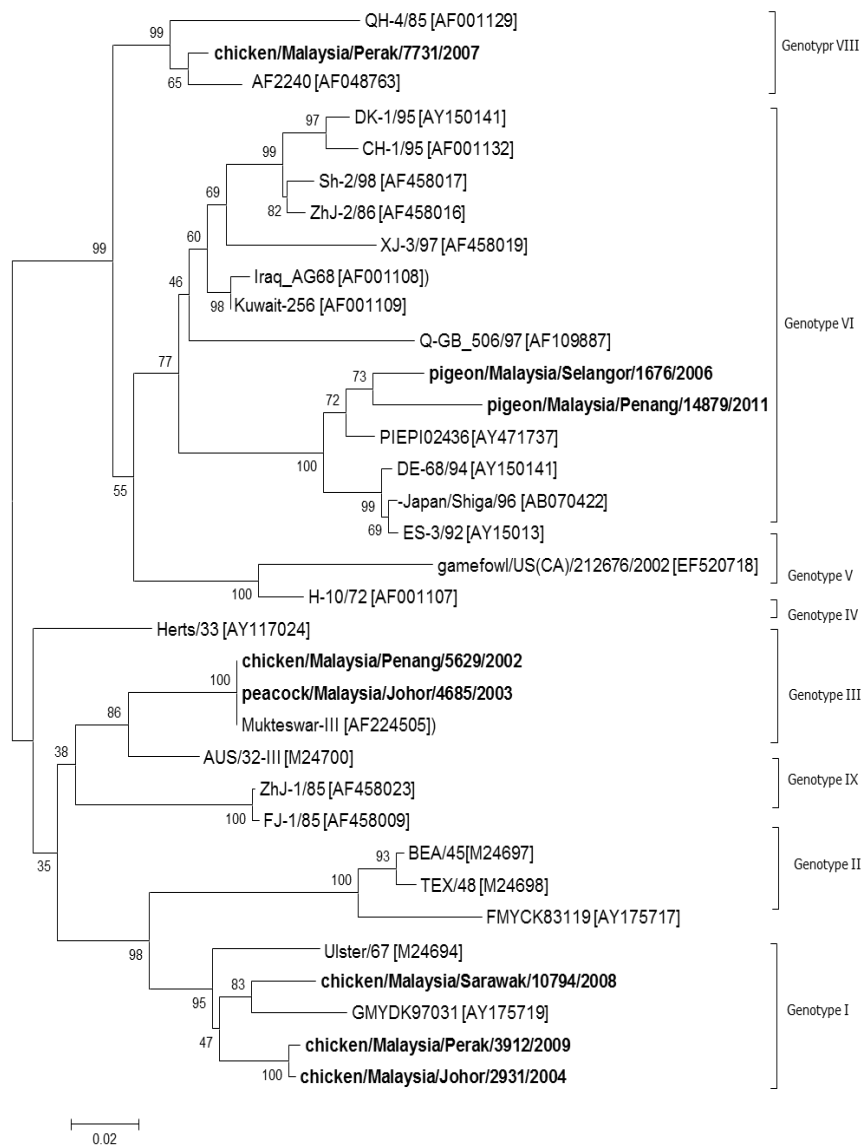
Lastly, subgenotype VIIa caused outbreaks during end of 2011 to 2012 where this is the same strain that causing outbreaks in Indonesia [17] and Pakistan [18]. Interestingly, based on this study it has been discovered that two subgenotypes i.e. VIIh and VIIa strains have been in Malaysia since 2002 in Sarawak and Sabah since 2004 but no major outbreaks were reported. Conclusively, based on this study, genotype VII strain may have increased its virulence in poultry over the years and these viruses are spreading to other locations around the world [19].

**Table 1** Distribution of genotypes of Newcastle Disease Virus circulating in Malaysia between 1999-2012

Year	Genotype VII					Genotype VIII	Genotype VI	Genotype III	Genotype I	No. of isolates
	A	b	d	e	h					
1999	-	1	-	1	-	-	-	-	-	2
2000	-	-	1	-	-	-	-	-	-	1
2001	-	-	12	-	-	-	-	-	-	12
2002	1	-	11	-	2	-	-	1	-	15
2003	-	-	4	-	-	-	-	1	-	5
2004	1	-	8	-	1	-	-	-	1	11
2005	-	-	3	-	2	-	-	-	-	5
2006	-	-	9	-	5	-	1	-	5	20
2007	-	-	2	-	-	1	-	-	2	5
2008	-	-	4	-	-	-	-	-	2	6
2009	-	-	3	-	-	-	1	-	1	5
2010	-	-	-	-	21	-	-	-	-	21
2011	4	-	-	-	22	-	3	-	1	30
2012	7	-	-	-	4	-	1	-	1	13
Total	13	1	57	1	57	1	6	2	13	151



**Figure 1** Phylogenetic tree of the NDV isolates from Malaysia belonging to genotype VII in class II based on the comparison of the partial F gene sequence (nt 47–418) of the NDV strains. Tree construction was done using the neighbor joining method with 1,000 replicates. The NDV isolates obtained in the present study are in bold. Accession numbers of the sequences from GenBank are shown in parenthesis



**Figure 2** Phylogenetic tree of the NDV isolates from Malaysia belonging to other than genotype VII in class II based on the comparison of partial F gene sequence (nt 47–418). Tree construction was done using the neighbor joining method with 1,000 replicates. The NDV isolates obtained in the present study are in bold. Accession numbers of the sequences from GenBank are shown in parenthesis

## 4.0 CONCLUSION

In summary, this information points to the existence of multiple genotypes of NDV in Malaysia especially genotype VII. It is suggested that no new introduction of the virus occurred during 1999 – 2012 except for genotype VII. These findings emphasize the importance of continuous surveillance of NDV in Malaysia and more studies should be conducted for better intervention strategies and deeper molecular knowledge.

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