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Isolation and Characterization of Genotype VII Newcastle Disease Virus from NDV Vaccinated Farms in Malaysia

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ABSTRACT

Molecular analysis, particularly sub-genotype classification, and study on the relationship of recent Malaysian NDVs with other isolates from around the world are lacking. Therefore, in the present study, a molecular epidemiological investigation was conducted to characterise six Newcastle disease viruses (NDV) isolated between 2014 and 2015 from vaccinated commercial poultry flocks. Partial Fusion (F) and Hemagglutinin-neuraminidase (HN) genes were amplified from IBS046/2014, IBS060/2014, IBS061/2014, IBS074/2014, IBS160/2015, and IBS162A/2015 isolates using one-step reverse transcription polymerase chain reaction (RT-PCR), sequenced and phylogenetically analysed. Sequence and phylogenetic analysis revealed that all the recently isolated strains of NDV belonged to sub-genotype VIIa and lineage 5a. Moreover, deduced amino acid sequence at the F protein cleavage site of the isolates revealed either ^{112}RRQKRF^{117} or ^{112}KRRKRF^{117} consistent with the motif found in velogenic pathotypes. The study concluded that the genotype VIIa was the causative agent of recent ND outbreaks in vaccinated broiler flocks from Malaysia. Interestingly, five out of the six isolates characterised in this study had a unique F0 protein cleavage site (^{112}KRRKRF^{117}). Further studies are required to determine the role of these motifs on the virulent potential of the isolates.

Keywords: Genotype VII Newcastle disease virus, F protein cleavage site, F and HN phylogenetic analysis

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INTRODUCTION

Newcastle Disease Virus (NDV) is similar to avian paramyxovirus-1(APMV 1), which is a member of the Avulavirus genus in the Paramyxoviridae family, including a different collection of single-stranded, non-segmented, negative-sense enveloped

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RNA viruses that are about 15.2 kb. NDV has a wide range of hosts and is known for its ability to infect more than 200 different species of bird (Fauquet & Fargette, 2005). The genome of NDV encodes for six main essential proteins: phosphoprotein (P), hemagglutinin-neuraminidase (HN), matrix (M), nucleocapsid (NP), fusion (F), the RNA dependent RNA polymerase (large structural protein) (L), and also for a seventh protein (V), which is resulted through frame shifts that are bordered by the P coding region (Chambers & Samson, 1982; Collins et al., 1982). Viruses with low virulence are habitually exploited as vaccines and characteristically cause mild diseases that are associated with respiratory organs or the digestive system. The clinical signs of Newcastle Disease (ND) are moderate and sometimes, the disease may present only mild respiratory symptoms with sporadic nervous signs and sometimes, death may occur. Severe forms of ND are classical in animal organs that are affected by NDV. The viscerotropic form of ND causes widespread hemorrhaging in several organs of the gastrointestinal tract with slight nervous signs, while the neurotropic form principally affects the central nervous system with little or no additional gross injuries or lesions (Alexander, 2000).

NDV of low virulence has monobasic fusion cleavage location motifs at amino acid (aa) positions 112-113 and 115-116 and a leucine (L) at position 117 of the F protein (Glickman et al., 1988) that will only cleave through trypsin-like enzymes that are within the intestinal and respiratory tracts, limiting their duplication in these systems (Aldous & Alexander, 2001). The virulent ND viruses have various basic amino acids in the fusion cleavage site namely, ^{112}R-K/R-Q-K/R-R-F^{117}. Additionally, the length of HN genes has often been used to classify NDV into virulent and avirulent strains. In the case of the virulent strains, the length of the HN protein is 571aa (Munir et al., 2012; Wang et al., 2013) while most lentogenic strains, including the conventional LaSota vaccine strains and other genotype 1 strains have either 577 or 616 amino acids (Tirumurugaan et al., 2011; Yuan et al., 2012). Analysis of the HN gene sequence shows several enteric NDV strains having low virulence possess an open-reading frame (ORF) that is large (616 amino acids) with extra 45 aa at the C-terminus comparing to some virulent and some low virulent NDV strains (571 and 577 aa). Presently, molecular epidemiological studies are not available on the outbreak causes of ND among commercial poultry farms vaccinated against ND in Malaysia. Molecular investigation of the sub-genotype of NDVs and also analysis of the relationship between recent Malaysian NDVs and other isolates from different parts of the world, in particular, are not available. In view of the unavailability of data showing the relationship between the recent Malaysian NDV strain and other isolates from different region of the globe, the aim of this present study was to determine the molecular epidemiology and to characterise six Newcastle Disease

Viruses (NDV) isolated between 2014 and 2015 from vaccinated commercial poultry flocks in Malaysia.

MATERIALS AND METHOD

Isolation of NDV

Viral RNA extraction. The Laboratory of Vaccine and Immunotherapeutics, Institute of Bioscience (IBS)/UPM received tissue samples of suspected cases of NDV outbreaks between 2014 and 2015 for diagnosis of NDV. The isolates were from different parts of Malaysia: IBS 160/2015 and IBS 074/2014 were from Johor; IBS 162A/2015 was from Melaka; IBS 060/2014 and IBS 046/2014 were from Perak; and IBS 061/2014 was from Penang. All the isolates were obtained from vaccinated flocks. Viral RNA was extracted using TRIzol LS® (Life Technologies, USA) via phenol and guanidine thiocyanate system with some modification. The processed sample (250 µL) was mixed with 750 µL TRIzol LS® in a 1.5-mL micro-centrifuge tube and incubated at room temperature for 5 min. Chloroform (200 µL) was added and the tube was vigorously shaken to mix the contents well and then incubated at room temperature for another 10 min. After that, the tubes were centrifuged at 12,000 xg for 20 min at 4°C. The colourless aqueous top layer was collected and mixed with 800 µL isopropanol. After 15 min of incubation at room temperature, all the samples were centrifuged at 12,000 xg for 20 min at 4°C. Then, the supernatants were discarded and the pellet was washed twice with 75% of ethanol (800 µL) and absolute ethanol (1000 µL), respectively. Finally, the RNA pellets were air-dried in laminar flow, dissolved with RNase-free water (Promega, USA) and stored in a -70°C freezer for future use. RNA concentration and purity were measured using a UV/Visible spectrophotometer (Ultraspect 3000 Pro-Biochorm, UK) based on the method described by Wilkinson (Martin et al., 2001). The optical density of each sample was measured at both 260 nm and 280 nm wavelength. Concentration and 260/280 absorbance ratio were recorded. Only RNA with the ratio of 1.8 to 2.0 was used in the following study.

F and HN gene RT-PCR amplification

Reverse transcriptase-polymerase chain reaction (RT-PCR) PCR amplification, primers and sequencing were achieved through the use of degenerative primers 5'-ATGGGC(C/T) CCAGA(C/T) CTTCTAC-3' (sense) and 5'-CTGCCACTGCTAGTTGTGATAATC C-3' (antisense), which are precise to fusion (F) protein gene (Yang et al., 1999)1984, and 1995. The sequences 5'-ATATCCCGCAGTCGCATAAC-3'(sense) and 5'-TTTTTCTTAATCA AGTGACT-3' (antisense) were specific to the HN protein gene (Peroulis-Kourtis et al., 2002) (Table 3). This primer produced an estimated size of band of the amplicon of 535 bp (nt 47-535) fragment covering from nucleotides 47 to 581 of the fusion protein that comprised the F0 cleavage position and 320 bp products, demonstrating fragmentd

inside HN protein gene, separately. Standard RT-PCR was implemented by means of the SuperScript®III One-Step RT-PCR kit (Invitrogen, USA) in 25 µl reaction volume. The cycling parameters of F and HN genes' specific primers were 50°C for 30 min at reverse transcription (RT), followed by an initial denaturation of 2 min at 94°C; 40 cycles of 15 s of denaturation at 94°C; 30 s of annealing at 59°C (48°C for HN); and 1 min (30 s for HN) of extension at 68°C and finally, extension at 68°C for 5 min using C1000 Touch™ thermo-cycler (Bio-Rad, USA). A percentage of 1.5% agarose gel was used to separate the amplicons by electrophoresis, and the gel eas then stained in ethidium bromide before final visualisation under ultraviolet light using gel doc (Bio-Rad, USA).

Partial NDV F and HN Gene Sequencing

Samples that were positive for NDV amplification were analysed by sequence analysis (ABI PRISM® 377 DNA Sequencer). Gel purified RT-PCR products for both partial F and HN genes from each of the samples were sent to 1st Base™ Sdn Bhd (Kuala Lumpur, Malaysia).

Sequence Alignment, Analysis and Phylogenetic Study

The Basic Local Alignment Search Tool (BLAST) was used to analyse the raw sequence data and the sequence were compared with other sequences in GenBank NCBI (Johnson et al., 2008). The evolutionary relationship of F and HN genes of NDV isolates in our study was compared with other NDV isolates of different genotypes recorded in other studies (39 isolates for F gene and 31 isolates for HN gene) (Table 1).

Analysis of sequences and phylogeny of the partial F and HN genes was done using the ClustalW multiple alignment method of the Molecular Evolutionary Genetics Analysis Version 6 (MEGA 6) software (Tamura et al., 2011) that employed 1000 bootstrap replications. Construction of the phylogenetic trees was done using the maximum likelihood method (Zhang & Sun, 2008).

Table 1
Newcastle disease virus (ndv) strains used in this study for phylogenetic analysis of the fusion protein gene and Hemagglutinin-Neuraminidase Gene

F Gene	Genotype	HN Gene	Genotype
HM125898_China_I_2004	I	HM063422 isolate D3 I	I
DQ097394__Hungary_I	I	JX401404 isolate CBU2179 I	I
JX401404_Korea_I_2007	I	FJ939313 NDV/Chicken/Egypt/1/2005 II	II
JX524203_Australia_V4_I_1966	I	AF07761 LaSota 578aa II	II
HM063422_D_3_China_I	I	JX193082 duck/China/Guangxi21/2010 II	II
AF077761 B1_USA__II_1947	II	HQ902590 NDV2K17/Quail/Chennai/ India/1998 II	II
Y18898_Clone_30_USA__II	II	Y18898 clone 30 II	II

HQ902590_India_II_1998	II	FJ430160 isolate JS/9/05/Go III	III
JF950510_LaSota_USA_ II_1946	II	FJ430159 isolate JS/7/05/Ch III	III
FJ939313_Egypt_II_2005	II	KM056353 isolate NDV55/TN/Namakwa IV	IV
JX193082_China_II_2010	II	KF915807 strain NDV-BJ IX	IX
JF950509_Mukteswar_China_III	III	FJ436302 strain F48E8 IX	IX
FJ430159 isolate JS/7/05/Ch III	III	KC246549 HBNU/LSRC/F3 IX	IX
FJ430160 isolate JS/9/05/Go III	III	HM117720 isolate NDV-P05 V	V
EU293914_Italiano_italy_IV_1944	IV	KJ577136 strain Chimalhuacan V	V
FJ986192 isolate 2K3/Chennai/Tamil Nadu IV	IV	AY562990 isolate mixed species/U.S./ Largo/71 V	V
HM117720_Mexico_2005_V	V	HQ839733 Chicken_Sweden_95 VI	VI
AY562990_Largo_USA_V_1971	V	FJ766527 isolate JS/07/16/Pi VI	VI
AY562988_Fontana_USA_VI_1972	VI	AY562988 isolate chicken/ U.S.(CA)/1083(Fontana)/72 VI	VI
FJ766529 isolate ZhJ-3/97 VI	VI	JN618349 strain JS-3-05-Ch VII 571aa	VII
HQ839733 strain Chicken_Sweden_95 VI	VI	KC542893 isolate Chicken/China/ Liaoning/02/2005VIId 571aa	VII
AY562985_cockato_Indonesia_VII 1990	VII	KF188408 UPM-IBS/002/2011 VII 571aa	VII
JN986837_Netherlands_VII_1993	VII	KM670337strain chicken/Pak/Quality Operations Lab/SFR-611/13 VIIi 571aa	VII
GU585905_Sweden_97_VIIb	VII	HQ697254 strain chicken/Banjarmasin/010/10 VII	VII
JN618348_VII_China_1997	VII	AB605247 strain: NDV/Bali-1/07 VIIa 571aa	VII
GQ901895 strain MB047/05 VII	VII	KC542892 isolate Chicken/China/ Liaoning/01/2005 VIId 571aa	VII
JN800306_Peru_VII_2008	VII	HQ697256 strain chicken/Makassar/003/09 VII 571aa	VII
JX390609_Togo_Peru_VII_2009	VII	HQ697260 strain chicken/Kudus/018/10 VII 571aa	VII
HQ697254_benjamasin_Indonesia_VII_2011	VII	KP776462 strain chicken/NDV/Pak/AW-14 VIIi 571aa	VII
KF026013 IBS_002 Malaysia 2011	VII	FJ751918 isolate QH1 VIII	VIII
HQ697255_sukarjo_Indonesia_VII_2011	VII	FJ751919 isolate QH4 VIII	VIII
JQ823260 IBS005 Malaysia 2011	VII		
JX532092_Pakistan_VII_2012	VII		
FJ751918_west_China_VIII	VIII		
JX012096_AF2240__malaysia_VIII_2010	VIII		
FJ751919_West China_VIII	VIII		
KF915807_China_IX_2013	IX		
FJ436302_F48E9_China_IX	IX		
KC246549_China_IX_2012	IX		

RESULTS AND DISCUSSION

From the phylogenetic analysis using the maximum likelihood method, a phylogenetic tree was constructed based on the partial length F and HN genes nucleotide sequences of six isolates and 39 previously published NDVs. Genotypes I to IX of Class II of NDV were used for the analysis. Furthermore, the phylogenetic relationship of the partial F gene nucleotide sequence of NDV represented by sub-genotype VII for nine sub-genotypes (a b, c, d, e, f, g, h and i) was studied. The results of the analysis revealed that six NDV isolates were grouped as genotype VII (Figure 1) and sub-genotype (VIIa) (Figure 2) and the partial HN gene sequence for the same six NDV isolates was grouped as genotype VII (Figure 3). They were phylogenetically close to previous NDV isolates from Malaysia and Indonesia.

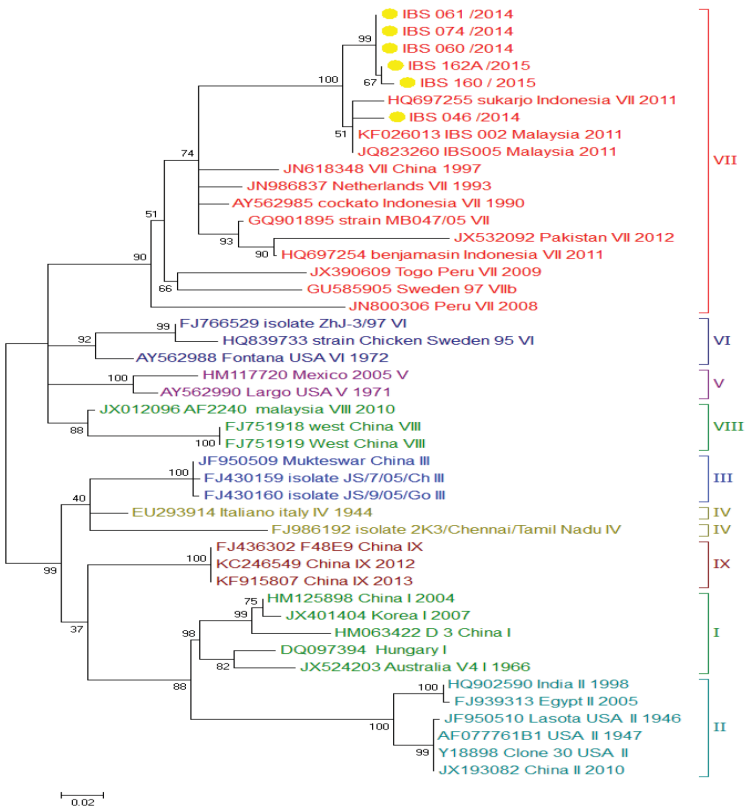

Figure 1. The phylogenetic analysis of Malaysian NDV isolates based on partial sequence of the F protein gene is given here. Viruses highlighted with the coloured circle (●) were characterised in this study. The phylogenetic tree was constructed by the maximum likelihood method as implemented in MEGA 6. Numbers at the nodes indicate a confidence level of bootstrap analysis with 1000 replications as a percentage value. The isolates were grouped under genotype VII

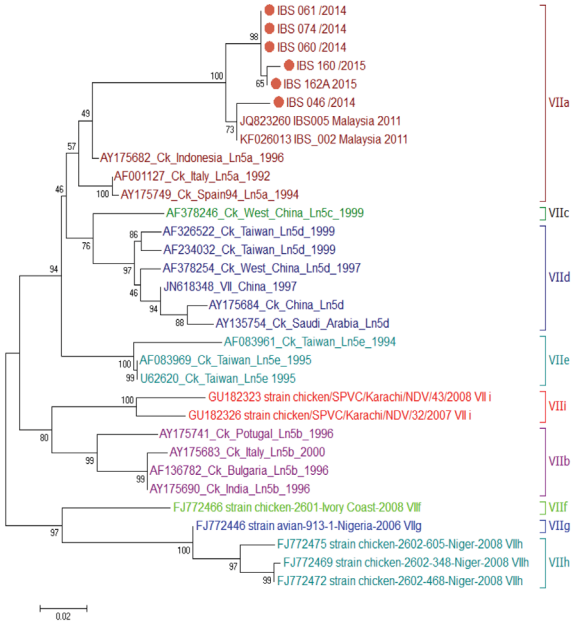

Figure 2. The phylogenetic analysis of Malaysian NDV isolates based on partial sequence of the F protein gene is given here. Viruses highlighted with the coloured circle (●) were characterised in this study. The phylogenetic tree was constructed using the maximum likelihood method as implemented in MEGA 6. Numbers at the nodes indicate a confidence level of bootstrap analysis with 1000 replications as a percentage value. The isolates were grouped under sub-genotype VIIa

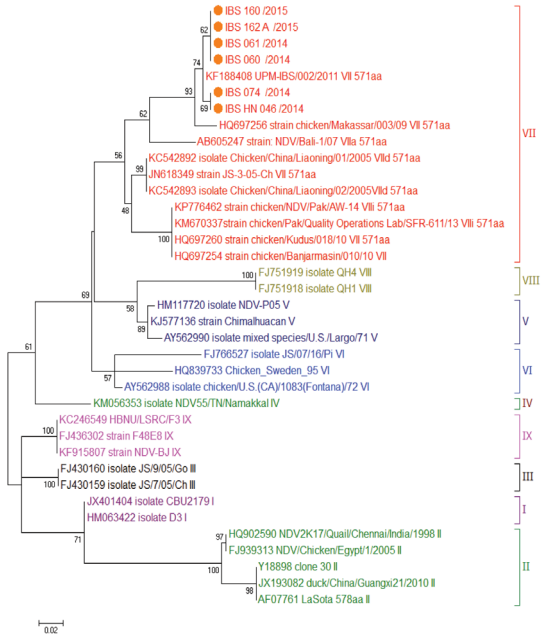

Figure 3. The phylogenetic analysis of Malaysian NDV isolates based on partial sequence of the HN protein gene of NDV isolates is given here. Viruses highlighted with the coloured circle (●) were characterized in this study. The phylogenetic tree was constructed by maximum likelihood method as implemented in MEGA 6. Numbers at the nodes indicate a confidence level of bootstrap analysis with 1000 replications as a percentage value

Detection of NDV and Virus Isolation

A total of six suspected cases had positive RT-PCR. These isolates were designated as IBS 046/2014, IBS 060/2014, IBS 061/2014, IBS 074 /2014, IBS 160/2015 and IBS 162A/2015. All the isolates possessed a multiple basic amino acid motif at the F cleavage site of either ^{112}RRQKRF^{117} or ^{112}KRRKRF^{117} (Table 2).

Table 2
Cleavage site of F Gene of Malaysian NDV isolates

Isolate	Cleavage site
IBS 160 /2015	^{112}KRRKR↓F^{117}
IBS 162A /2015	^{112}KRRKR↓F^{117}
IBS 060 /2014	^{112}KRRKR↓F^{117}
IBS 046 /2014	^{112}RRQKR↓F^{117}
IBS 061/ 2014	^{112}KRRKR↓F^{117}
IBS 074 /2014	^{112}KRRKR↓F^{117}

It is believed that the HN gene can recognise the genotypes of NDV and may expect the pathogenicity of the isolates in light of the fact that the length of the HN protein differs and the cleavage site is not the single criterion for pathogenicity. Along these lines, the phylogenetic examination was directed to utilise the complete coding locale of the HN gene. As a rule, a similar topology of the tree was observed with the Bayesian tree of F quality investigation. IBS 046/2014, IBS 060/2014, IBS061/2014, IBS 074/2014, IBS 160/2015 and IBS 162A/2015 were grouped together under genotype VII in connection to its HN protein length of 571 aa (Table 3).

Table 3
Analysis of C-terminus extension length of HN gene protein

Isolate	Deduce Amino Acid Sequence	C-Terminus Amino Acid Extension Length	HN Length **
IBS046/2014	KDDRV*	0	571
IBS074/2014	KDDRV*	0	571
IBS060/2014	KDDRV*	0	571
IBS061/2014	KDDRV*	0	571
IBS2A/2015	KDDRV*	0	571
IBS 160/2015	KDDRV*	0	571
HQ697256 strain chicken/ Makassar/003/09	KDDRV*	0	571
Y18898 clone 30 II	KDDGVREARSG*	6	577
AF07761 LaSota 578aa II	KDDGVREARSG*	6	577
AY562989 isolate dove/Italy/2736/00	KDDGVREARSG*	6	577
JX524203 strain NDV V4	KDDGVREARSSRLSQLR EGWKDDIVSPIFCDAKN QTEYRRELESYAASWP*	45	616
HM125898 WDK/ JX/7793/2004	KDDGVREARSGRLSQLQ EGWKDDIVSPIFCDIKNQ TEYRRGLESYAASWP*S	45	616
GQ922501 strain MB091/05	KDDRV*	0	571
JX193082 duck/China/ Guangxi21/2010	KDDGVREARSG*	6	577
HQ902590 NDV2K17/ Quail/Chennai/ India/1998	KNDGVREARSG*	6	577
AY562985 isolate cockatoo/ Indonesia/14698/90	KDDRV*	0	571
HM063422 isolate D3 I	KDDGVRKARSG*	6	577
DQ097394 strain PHY-LMV42	KDDGVREARSGRLSQLR EGWKDDIVLPIFCDAKN QTEYRSMLESYAASWP*	45	616
GQ922500 strain MB043/06	KDDRV*	0	571

* Indicates the stop codon
** Predicted number of amino acid based on ORF analysis of the gene nucleotide sequence

The distance matrix analysis results of the F and HN genes compared to other isolates (from genotype I to genotype IX) are shown in (Table 4). All the isolates showed a higher genetic variation with genotype II and lowest distance to genotype VII. A maximum distance of 24.1% nucleotide variation was observed between the F genes of IBS/046/2014 and genotype II. The same pattern was observed for the HN gene in which the maximum distance was detected between the group, IBS/061/2014, IBS 060/2014 and IBS 160/2015, and genotype II, with 20.7% nucleotide variation (Table 5).

Table 4
Distance matrix analysis of Malaysian NDV isolates based on F Gene

Isolates	Genotypes								
	I	II	III	IV	V	VI	VII	VIII	IX
IBS 160 /2015	23.7%	24.0%	21.5%	18.5%	17.8%	16.5%	10.7%	16.1%	19.8%
IBS 162A /2015	22.8%	23.1%	20.7%	17.7%	17.0%	15.7%	10.0%	15.4%	19.0%
IBS 061 /2014	23.2%	23.5%	21.1%	18.1%	17.4%	16.1%	9.9%	15.2%	19.4%
IBS 046 /2014	23.2%	24.1%	20.3%	19.9%	16.6%	16.1%	9.8%	15.5%	21.0%
IBS 060 /2014	23.2%	23.5%	21.1%	18.1%	17.4%	16.1%	9.9%	15.2%	19.4%
IBS 074 /2014	23.2%	23.5%	21.1%	18.1%	17.4%	16.1%	9.9%	15.2%	19.4%

Table 5
Distance Matrix Analysis of Malaysian NDV Isolates Based on HN Gene

Isolates	Genotypes								
	I	II	III	IV	V	VI	VII	VIII	IX
IBS 160 /2015	12.3%	20.7%	14.8%	14.8%	8.5%	13.8%	7.1%	14.8%	15.6%
IBS 162A /2015	11.7%	20.0%	14.1%	14.1%	8.5%	13.4%	6.5%	14.1%	14.9%
IBS 061 /2014	12.3%	20.7%	14.8%	14.8%	8.5%	13.8%	7.1%	14.8%	15.6%
IBS 046 /2014	11.6%	19.8%	14.0%	14.0%	10.6%	14.1%	6.7%	15.6%	14.8%
IBS 060 /2014	12.3%	20.7%	14.8%	14.8%	8.5%	13.8%	7.1%	14.8%	15.6%
IBS 074 /2014	11.6%	19.8%	14.0%	14.0%	10.6%	14.1%	6.7%	15.6%	14.8%

Similarly, the distance analysis results of sub-genotype VII (from a to i) based on F gene sequencing showed that all the isolates had the highest genetic distance to the sub-genotype VIIh and the lowest genetic distance to sub-genotype VIIa. A maximum distance of 20% nucleotide variation was observed between the F genes of IBS/160/2015 and the sub-genotype VIIh (Table 6).

Table 6
Distance matrix analysis of sub-genotype VII of Malaysian NDV isolates

Isolates	Genotypes								
	VIIa	VIIb	VIIc	VIId	VIIe	VIIf	VIIg	VIIh	VIIi
IBS 160 /2015	6.9	16.3	11.2	12.0	13.6	17.6	16.8	20.0	14.9
IBS 162A /2015	6.3	15.6	10.5	11.3	12.9	16.8	16.8	20.0	14.1
IBS 061 /2014	6.0	15.2	10.8	11.7	13.3	17.2	16.4	19.5	14.1
IBS 046 /2014	5.6	15.0	10.8	11.7	12.5	17.2	16.4	19.0	14.9
IBS 060 /2014	6.0	15.2	10.8	11.7	13.3	17.2	16.4	19.5	14.1
IBS 074 /2014	6.0	15.2	10.8	11.7	13.3	17.2	16.4	19.5	14.1

The causative agent of ND outbreaks in vaccinated broiler flocks from Malaysia between 2014 and 2015 was isolated and characterised. Sequencing of the F cleavage site of the isolated viruses showed the presence of the polybasic amino acid motif ^{112}KRRKRF^{117} and ^{112}RRQKRF^{117}, indicating that all the NDV isolates analysed in this study (IBS 046/2014, IBS 060/2014, IBS 061/2014, IBS 074 /2014, IBS 160/2015 and IBS 162A/2015) were classified as a velogenic NDV. It is widely accepted that the number of basic amino acids immediately upstream to the F0 protein cleavage site determines viral pathogenicity, which is clearly described by the World Organisation for Animal Health (OIE, 2013). The presence of these characteristic patterns of amino acid demonstrated that the isolates could be considered as virulent. It has been observed that F proteins of virulent NDV strains contain lysine (K) and arginine (R) at their cleavage site (^{112}R-R-Q-R/K-R^{116}) and a phenylalanine at position 117 of F_1. It is of utmost importance to note that the F0 cleavage site of isolates (IBS 060/2014, IBS 061/2014, IBS 074/2014, IBS 160/2015 and IBS 162A/2015) isolated from Malaysia, was unusual, containing a lysine (K) arginine (R) substitution at residue 112-114, unlike the results of a previous study by Roohani et al (2015) that indicated that genotype VII viruses isolated from Malaysia in 2011 had different motifs at the F cleavage site. However, there is no major sequence difference between our five isolates and the NDV isolates of 2011 other than in the cleavage site. The IBS 046/2014 isolate had motifs similar to the MB047/05 isolate at the F cleavage site as described by Berhanu et al. (2010). However, in recent years, similar results have been reported in South African genotype VIII viruses (Abolnik et al., 2004) and in Taiwan (Tsai et al., 2004) as well as in Eurasian collard dove and pigeon isolates containing a ^{112}R-R-K-K-R^{116}, ^{112}R-R-Q-K-R^{116} and ^{112}R-R-R-K-R^{116} motif (Huovilainen et al., 2001; Terregino et al., 2003). Even though the contribution of arginine (R) at amino acid 114 in our isolates needs further study, other studies have indicated that arginine residue at different positions 113, 115 and

116 contribute to intracellular cleavage of virulent NDV fusion proteins (Fujii et al., 1999).

Based on the regions we characterised, there were no changes in the neutralising epitopes, the cysteine residues and the N-linked glycosylation sites of the F0 protein. The changes were only in the cleavage site of five isolates as mentioned in the manuscript. It is, however, possible that other epitopes not located in the regions we studied might have mutated, thereby further facilitating the emergence of these virulence isolates. Further study is needed to confirm this.

Virulence was confirmed by the length of the HN protein. Analysis of the C-terminus extension length of the HN protein gene revealed that the six virulent NDV isolates shared 0 amino acid extension length with a total HN length of 571 amino acids regardless of their cleavage site sequence profile (terminating in the sequence KDDRV). Most lentogenic strains, including the conventional LaSota vaccine strains and other genotype I strains have either 577 or 616 amino acids and share either six or 45 amino acid extension length. Moreover, phylogenetic studies on the partial F and HN gene revealed that six NDV isolates were classified as a genotype VII NDV and clustered together with other genotype VII isolates from Indonesia (Xiao et al., 2012), Cambodia (Choi et al., 2013) and China (Xie et al., 2012). A phylogenetic relationship between the partial F gene nucleotide sequence of NDV in the genotype VII group for nine sub-genotypes (a, b, c, d, e, f, g, h, and i) revealed that six NDV isolates were classified under the sub-genotype, VIIa, contrary to what was reported by Berhanu et al. (2010), who indicated that genotype VII viruses isolated from Malaysia between 2004 and 2007 belonged to the sub-genotype, VIId. The distance matrix analysis of the length F and HN genes demonstrated that all six isolates had the highest amino acid variation compared to the genotype II (23.5%-24.1%). The same pattern was observed for the HN gene in which the maximum distance was detected between six NDV isolates and genotype II (19.8%-20.7%). It was expected that the F and HN amino acid sequence of the six NDV isolates would share a close similarity (6.7%-10.7%) with the genotype VII strains.

CONCLUSION

In conclusion, the causative agent of recent ND outbreaks in vaccinated broiler flocks from Malaysia was found to belong to the velogenic genotype VIIa. This strain is genetically close to other Malaysian genotype VII isolates obtained in the last decade. The deduced amino acid sequence of the F0 protein cleavage site showed a unique amino acid motif in five of the isolates incriminated for sporadic cases that occurred in different parts of the country.

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REFERENCES

Abolnik, C., Horner, R. F., Bisschop, S. P. R., Parker, M. E., Romito, M., & Viljoen, G. J. (2004). A phylogenetic study of South African Newcastle disease virus strains isolated between 1990 and 2002 suggests epidemiological origins in the Far East. *Archives of Virology*, *149*(3), 603–619.

Aldous, E. W., & Alexander, D. J. (2001). Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). *Avian Pathology, 30*, 117–128.

Alexander, D. J. (2000). Newcastle disease and other avian paramyxoviruses. *Revue scientifique et technique (International Office of Epizootics), 19*(2), 443-462.

Berhanu, A., Ideris, A., Omar, A. R., & Bejo, M. (2010). Molecular characterization of partial fusion gene and C-terminus extension length of haemagglutinin-neuraminidase gene of recently isolated Newcastle disease virus isolates in Malaysia. *Virology Journal*, *7*(1), 183-192.

Chambers, P., & Samson, A. C. R. (1982). Non-structural proteins in Newcastle disease virus-infected cells. *Journal General Virology, 58*(1), 1–12.

Choi, K. S., Kye, S. J., Kim, J. Y., Damasco, V. R., Sorn, S., Lee,Y. J.,… Lee, H. S. (2013). Molecular epidemiological investigation of velogenic Newcastle disease viruses from village chickens in Cambodia. *Virus Genes, 47*(2), 244–9.

Collins, P. L., Wertz, G. W., Ball, L. A., & Hightower, L. E. (1982). Coding assignments of the five smaller mRNAs of Newcastle disease virus. *Journal of Virology*, *43*(3), 1024–1031.

Fauquet, C. M., & Fargette, D. (2005). International Committee on Taxonomy of Viruses and the 3,142 unassigned species. *Virology Journal*, *2*(1), 64.

Fujii, Y., Sakaguchi, T., Kiyotani, K., & Yoshida, T. (1999). Comparison of substrate specificities against the fusion glycoprotein of virulent Newcastle disease virus between a chick embryo fibroblast processing protease and mammalian subtilisin-like proteases. *Microbiology and Immunology*, *43*(2), 133–140.

Glickman, R. L., Syddall, R. J., Iorio, R. M., Sheehan, J. P., & Bratt, M. A. (1988). Quantitative basic residue requirements in the cleavage-activation site of the fusion glycoprotein as a determinant of virulence for Newcastle disease virus. *Journal of Virology*, *62*(1), 354–6.

Huovilainen, A., Ek-Kommonen, C., Manvell, R., & Kinnunen, L. (2001). Phylogenetic analysis of avian paramyxovirus 1 strains isolated in Finland. *Archives of Virology*, *146*(9), 1775–85.

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., & Madden, T. L. (2008). NCBI BLAST: A better web interface. *Nucleic Acids Research*, *36*(Web Server issue), W5–9. https://doi.org/10.1093/nar/gkn201

Martin, L. A., Smith, T. J., Obermoeller, D., Bruner, B., Kracklauer, M., & Dharmaraj, S. (2001). RNA purification. *Molecular Biology Problem Solver: A Laboratory Guide*, *7*, 197–224.

Munir, M., Cortey, M., Abbas, M., Afzal, F., Shabbir, M. Z., Khan, M. T., ... & Zohari, S. (2012). Biological characterization and phylogenetic analysis of a novel genetic group of Newcastle disease virus isolated from outbreaks in commercial poultry and from backyard poultry flocks in Pakistan. *Infection, Genetics and Evolution, 12*(5), 1010–1019.

OIE. (2013). Manual of diagnostic tests and vaccines for terrestrial animals. *World Organisation for Animal Health*, 1185–91.

Peroulis-Kourtis, I., O'Riley, K., Grix, D., Condron, R. J., & Ainsworth, C. (2002). Molecular characterisation of Victorian Newcastle disease virus isolates from 1976 to 1999. *Australian Veterinary Journal*, *80*(7), 422–4.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutioanry distance, and maximum parsimony methods. *Molecular Biology and Evolution*, *28*(10), 2731–9.

Terregino, C., Cattoli, G., Grossele, B., Bertoli, E., Tisato, E., & Capua, I. (2003). Characterization of Newcastle disease virus isolates obtained from Eurasian collared doves (Streptopelia decaocto) in Italy. *Avian Pathology*, *32*(1), 63–8.

Tirumurugaan, K. G., Vinupriya, M. K., Vijayarani, K., & Kumanan, K. (2011). Analysis of the fusion protein cleavage site of newcastle disease virus isolates from India reveals preliminary evidence for the existence of II, VI and VII genotypes. *Indian Journal of Virology*, *22*(2), 131–7.

Tsai, H. J., Chang, K. H., Tseng, C. H., Frost, K. M., Manvell, R. J., & Alexander, D. J. (2004). Antigenic and genotypical characterization of Newcastle disease viruses isolated in Taiwan between 1969 and 1996. *Veterinary Microbiology*, *104*(1–2), 19–30.

Wang, J., Liu, W., Ren, J., Tang, P., Wu, N., & Liu, H. (2013). Complete genome sequence of a newly emerging Newcastle disease virus. *Genome Announcements*, *1*(3), 3–4.

Xiao, S., Paldurai, A., Nayak, B., Samuel, A., Bharoto, E. E., Prajitno, T. Y., … & Samal, S. (2012). Complete genome sequences of Newcastle disease virus strains circulating in chicken populations of Indonesia. *Journal of Virology, 86*(10), 5969–70.

Xie, Z., Xie, L., Chen, A., Liu, J., Pang, Y., Deng, X., …. & Fan, Q. (2012). Complete genome sequence analysis of a Newcastle disease virus isolated from a wild egret. *Journal of Virology, 86*(24), 13854–5.

Yang, C., Shieh, H. K., Lin, Y. L., & Chang, P. (1999). Newcastle disease virus isolated from recent outbreaks in Taiwan phylogenetically related to viruses (genotype VII) from recent outbreaks in Western Europe. *Avian Diseases*, *43*(1), 125–30.

Yuan, P., Paterson, R. G., Leser, G. P., Lamb, R. A., & Jardetzky, T. S. (2012). Structure of the Ulster strain Newcastle disease virus hemagglutinin-neuraminidase reveals auto-inhibitory interactions associated with low virulence. *PLoS Pathogens*, *8*(8), 1–15.

Zhang, W., & Sun, Z. (2008). Random local neighbor joining: A new method for reconstructing phylogenetic trees. *Molecular Phylogenetics and Evolution*, *47*(1), 117–28.