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Isolation and Molecular Characterization of Newcastle Disease Virus from Imported Birds at an Animal Quarantine Station in Malaysia

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ABSTRACT

Five Newcastle disease viruses (NDVs) were isolated from imported birds at an animal quarantine station in Malaysia from 2012 to 2017. Analysis of the deduced amino acid sequences of the fusion (F) protein cleavage site showed that all the Newcastle disease virus (NDV) isolates were virulent with the ^{112}RRQ/KKRF^{117} motif. Phylogenetic analysis of the F gene revealed that four isolates were grouped in genotype VIa while one was in genotype VIIi. Among the four VIa viruses, three were clustered together with the Belgium strain and one with the United States strain. Meanwhile, the VIIi virus was highly similar to the Pakistan strain. VIa viruses in Malaysia were mostly detected from imported avian and there are no currently reported outbreaks caused by this virus. Whereas NDV VIIi viruses caused outbreaks in poultry in Malaysia in 2011 to 2012. There were only slight differences between the F gene of the imported and local existing VIIi viruses. This study revealed the isolation of different genotypes of virulent NDV of different origin from imported birds. As captive birds can transmit NDV across international boundaries and viral transmission between birds and poultry may lead to outbreaks, hence, screening imported birds for diseases is the most crucial step in preventing the spread of this disease. NDV can cause significant economic losses to the poultry industries worldwide. With the increase in international trade of birds and poultry, strict quarantine systems and good laboratory diagnostic capabilities that can characterize and differentiate imported viral strains from existing circulating strains in

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the country are important in attempts to prevent the entry of foreign viral NDV strains.

Keywords: Genotype VI, genotype VII, imported birds, Newcastle disease

INTRODUCTION

Newcastle disease (ND) is a highly contagious disease in chicken where outbreaks can cause flock mortality up to 100% and has been one of the major causes of economic losses in the poultry industry (Aldous & Alexander, 2001). ND is caused by avian paramyxovirus serotype 1 (APMV–1) viruses, a member of the genus Rubulavirus, sub-family *Paramyxovirinae* and family *Paramyxoviridae* (Aldous & Alexander, 2001). NDV is an enveloped, non-segmented, negative-sense RNA virus. The genome encodes for the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase (HN) and large protein (L) (Aldous & Alexander, 2001; Dortmans et al., 2011b). V and W proteins are expressed by RNA editing during P gene transcription (Dortmans et al., 2011b; Guo et al., 2013). NDV is divided into class I viruses that are mainly isolated from shorebirds and waterfowl; and class II is detected mainly in poultry and multiple wild birds where most viruses in this class are virulent (Diel et al., 2012). Based on the phylogenetic analysis of the partial or complete F gene, the NDV can be further classified into genotypes (Aldous et al., 2003; Diel et al., 2012; Kim et al., 2008).

The first panzootic of ND had started in Indonesia and England in the mid-1920s, the second was in the Far East and had spread to Europe in the 1960s (Lomniczi et al., 1998). In the 1980s, the APMV-1 infection had spread worldwide among racing, show and feral pigeons which caused the third ND panzootic (Aldous et al., 2004; Guo et al., 2013; Kim et al., 2008; Liu et al., 2013; Lomniczi et al., 1998). These APMV-1 viruses can be differentiated by monoclonal antibodies (mAb) and were termed as pigeon paramyxovirus type 1 (PPMV-1) and they also form a special sublineage (VIb / 4b) in the phylogenetic analysis (Aldous et al., 2004; Lomniczi et al., 1998).

Wild birds are a natural reservoir of NDV and different genotypes of NDV have been identified (Liu et al., 2013). Viral transmission can occur between wild birds and poultry that may lead to outbreaks and losses in poultry industries (Liu et al., 2013). Hence, screening of birds especially those for import/export purposes is very important. Therefore, the aim of this study is to molecularly characterize NDV strains isolated from imported birds at an animal quarantine station in Malaysia based on the partial F gene of the NDV.

MATERIALS AND METHODS

Virus Isolates

Samples such as cloacal swabs and tracheal swabs were collected from the birds upon arrival at the quarantine station. The samples were then sent to Veterinary Research Institute for Avian Influenza and ND

screening. From year 2012 to 2017, five NDVs were isolated from live imported birds at an animal quarantine station. The isolates were designated as VRI 286-2012, VRI 2344-2012, VRI 9681-2012, VRI 10951-2012 and VRI 2164-2016 and were used in the present study. The viruses were propagated in 9 to 11 days old Specific Pathogen Free (SPF) embryonated chicken eggs via the intra-allantoic route. The isolates were confirmed as NDV by the Hemagglutination-Inhibition (HI) test using a specific antiserum against ND (World Organisation for Animal Health [OIE], 2012).

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

The viral RNA was extracted from the infected allantoic fluid by the phenol chloroform method using TRIzol Reagent (Invitrogen) based on manufacturer's instruction. RT-PCR was carried out using the SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen). A primer set, MV1: 5'- CCY RAA TCA YYR YGR YRC YRG ATA A -3' and B2: 5'- KCR GCR TTY TGK KTG GCT KGT AT -3' (Herczeg et al., 1999) was used to amplify 557 bp which covered the partial matrix and F gene of NDV. In brief, the RT was carried out at 48°C for 30 min. The reaction mix was then subjected to 94°C for 5 min for initial denaturation, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 68°C for 1 min with a final extension for 10 min at 68°C. The amplicons were then analysed by electrophoresis on 1.5% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen).

Sequencing and Phylogenetic Analysis

The amplified PCR products were cut from the gel and sent for sequencing (First Base Laboratories Sdn Bhd). Nucleotide sequences were assembled using SeqMan Pro software (DNAStar Lasergene, USA). Fifty nine NDVs retrieved from GenBank representing each genotype were included in the study (Table 1). The nucleotide and deduced amino acid sequences were aligned and compared using *BioEdit* Sequence Alignment Editor (version 7.1.9). Subsequent phylogenetic analysis was carried out using MEGA version 6.06 by neighbor-joining statistical method with Kimura 2-parameter model and setting bootstrap 1000 replicates. A segment of 372 bp from the start codon of the F gene and ending just downstream from the cleavage activation site (from nucleotide 47 to 418) was used in constructing the phylogenetic tree.

RESULTS

The details of the NDV isolates are shown in Table 2. All five isolates were positive for NDV by *HI test using specific antiserum against ND and generated* PCR product of 557bp. Analysis of the F protein cleavage site showed all isolates were virulent with the presence of multiple basic amino acid sequence at position 112 to 116 and phenylalanine (F) at residue 117. Four isolates had the RRQKRF and one had the

RRKKRF motifs respectively. Phylogenetic analysis based on the 372 nucleotides of the F gene showed that four isolates i.e., the VRI 286-2012, VRI 9681-2012, VRI 10951-2012 and VRI 2164-2016 belonged to genotype VIa while VRI 2344-2012 was grouped in genotype VIIi (Figures 1 and 2).

Table 1
Details of NDVs retrieved from GenBank representing each genotype

Strain	Year isolated	Country	Host	Genetic group	Accession number
chicken/Australia/I-2/2005	2005	Australia	Chicken	I	AY935499.2
chicken/N. Ireland/Ulster/67	1967	Northern Ireland	Chicken	I	AY562991.1
V4	1966	Australia	Chicken	I	JX524203.1
chicken/US/LaSota/1946	1946	USA	Chicken	II	AF077761.1
turkey/US/VG/GA/1987	1987	USA	Turkey	II	EU289028.1
B1	NA[a]	NA	NA	II	AF309418.1
chicken/JS/7/05	2005	China	Chicken	III	FJ430159.1
goose/JS/9/05	2005	China	Goose	III	FJ430160.1
India/Mukteswar	NA	China	NA	III	EF201805.1
fowl/UK/Herts/33	NA	UK	Chicken	IV	AY741404.1
Italien	1944	Italy	NA	IV	EU293914.1
anhinga/USA(FL)/44083/93	1993	USA	Anhinga	V	AY288989.1
cormorant/ Canada/95DC2345/1995	1995	Canada	Cormorant	V	FJ705461.1
Rosella/Belgium/4940/08	2008	USA	Rosella	VIa	JN872162.1
PPMV-1/Belgium/11-09620/2011	2011	Belgium	Pigeon	VIa	JX901124.1
pigeon/Italy/1166/00	2000	Italy	Pigeon	VIa	AY288996.1
pigeon/US/RI166/2000	2000	USA	Pigeon	VIa	EU477189.1
WX-10-07-Pi	2007	China	Pigeon	VIa	GQ281086.1
YZ-23-07-Pi	2007	China	Pigeon	VIa	GQ281088.1
W4	2005	China	White-breasted water hen	VIa	HM063423.1
GB 1168/84	1984	Great Britain	NA	VIb	AF109885.1
Pigeon/Argentina/Tigre 6/99	1999	Argentina	Pigeon	VIb	AY734535.1
IT-227/82 Italy	1982	Italy	Pigeon	VIb	AJ880277.1
PPMV-1/New York/1984	1984	USA	Pigeon	VIb	FJ410145.1
PPMV-1 s-1 China	2002	China	Pigeon	VIb	FJ865434.1
Japan/Ibaraki/85	1985	Japan	Chicken	VIc	AB465606.1
Sh-1/97	1997	China	Chicken	VIc	AF458018.1
ZhJ-3/97	1997	China	Chicken	VIc	FJ766529.1
DK-1/95	1995	Denmark	Fowl	VId	AF001129.1
DK-6/95	1995	Denmark	Ostrich	VId	AF001130.1

Table 1 *(continue)*

Strain	Year isolated	Country	Host	Genetic group	Accession number
S-1/95	1995	Sweden	Fowl	VId	AF001131.1
CH-1/95	1995	Switzerland	Fowl	VId	AF001132.1
A-24/96	NA	Austria	Fowl	VId	AF001133.1
NDV BBGPI95039 Bulgaria	1995	Bulgaria	Pigeon	VId	AY135742.1
JS/2/98/Go	1998	China	Goose	VIe	AF456439.1
PPMV-1 YN-P1	NA	NA	Pigeon	VIe	AY325798.1
pigeon STP96 China	NA	China	Pigeon	VIe	DQ417113.1
PG/CH/JS/1/05	2005	China	Pigeon	VIe	FJ480825.1
NDV05-029	2005	China	Pigeon	VIe	FJ766528.1
Pigeon/Indiana/18002/1991	1991	USA	Pigeon	VIf	JN872186.1
Chicken/Texas/309968/2004	2004	USA	Chicken	VIf	JN942022.1
dove/Nigeria/VRD07-163/2007	2007	Nigeria	Dove	VIg	JQ039385.1
pigeon/Nigeria/VRD07-173/2007	2007	Nigeria	Pigeon	VIg	JQ039395.1
Pigeon/Argentina/Capital 3/97	1997	Argentina	Pigeon	VIh	AY734536.1
pigeon/Nigeria/VRD08-37BRpe(7-9)/2008	2008	Nigeria	Pigeon	VIh	JQ039387.1
pigeon/Nigeria/VRD07-231/2007	2007	Nigeria	Pigeon	VIh	JQ039391.1
NDV/DOVE/IT/11RS98_102VIR/2011	2011	Italy	Dove	VIi	JN638234.1
NDV/DOVE/IT/11RS100_104VIR/2011	2011	Italy	Dove	VIi	JN638235.1
TW/2000	2000	Taiwan	Fowl	VII	AF358786.1
JS-3/00	2000	China	Chicken	VII	AF458010.1
chicken/Sukorejo/019/10	2010	Indonesia	Chicken	VII	HQ697255.1
MB128/04-Malaysia	2005	Malaysia	Chicken	VII	GQ901900.1
chicken/China/QH1/1979	1979	China	Chicken	VIII	FJ751918.1
chicken/China/QH4/1985	1985	China	Chicken	VIII	FJ751919.1
AF2240 Malaysia	1960s	Malaysia	NA[a]	VIII	AF048763.1
duck/JS/1/02	2002	China	Duck	IX	FJ436306.1
China/F48E9	NA	China	NA	IX	AF163440.1
DE-R49/99	NA	Germany	Duck	Class I	DQ097393.1
Canada goose/US(OH)/87-78/1987	1987	USA	Canada goose	Class I	EF564833.1

[a] Not Available

Table 2
Details of NDV isolates used in this study

Isolates	Year isolated	Host	Origin Country of Import	Specimen submitted	BLAST Analysis			F0 cleavage site motif[a]	Virulence[b]	Sub-genotype[c]
					Significant alignment	Query Covery (%)	Identity (%)			
VRI-286-2012	Jan 2012	Bird	*Philippines*	Cloacal swabs	NDV/Pigeon/PA/USA	100	97	RRKKRF	Virulent	VIa
VRI-2344-2012	Mac 2012	Bird	Pakistan	Cloacal swabs	chicken/Pakistan/ Sheikhupura/12A/994/2015	100	99	RRQKRF	Virulent	VIIi
VRI-9681-2012	Aug 2012	Bird	Belgium	Cloacal swabs	PPMV-1/ Belgium/11-09620/2011	100	99	RRQKRF	Virulent	VIa
VRI-10951-2012	Sep 2012	Pigeon	Bulgaria	Tracheal swabs	PPMV-1/ Belgium/11-09620/2011	99	97	RRQKRF	Virulent	VIa
VRI-2164-2016	Mac 2016	Avian	Belgium	Cloacal swabs	PPMV-1/ Belgium/11-09620/2011	99	97	RRQKRF	Virulent	VIa

[a]Cleavage site motif corresponds to amino acids 112 to 117 of the fusion protein
[b]OIE (2012) defines virulent ND viruses as those with a phenylalanine (F) residue at position 117 and at least three basic amino acids (R or K) between residues 113 and 116
[c]Genotypes within class II of NDV

Table 3
Comparison of fusion amino acid in this study using Lomniczi's study (1998) as reference

Genotype	4	5	9	10	13	16	17	19	23	28	63	93	101	104	106	107	109	112	113	114	115	116	117	118	121	124[a]
VIa[c]	K	P	I	P	L	I	T	I	L	L	V/I	T	R	G	V	S	S	R	R	Q	K	R	F	I	I	S[b]
VIb[c]	-[d]	-	-	-	-	-	-	T	-	P	V	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-
VRI-286-2012	-	-	-	L	-	-	-	-	-	-	V	-	-	-	-	-	P	-	-	K	-	-	-	-	-	-
VRI-9681-2012	-	-	-	-	-	-	-	-	-	S	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VRI-10951-2012	-	-	-	-	-	-	-	-	-	S	V	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-
VRI-2164-2016	-	-	-	-	-	-	-	-	-	S	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PPMV-1/Belgium/11-09620/2011	-	-	-	-	-	-	-	-	-	S	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VII[c] (Indonesia)	K	P	I	P	L	I	T	I	L	L	V	T	K	G	V	S	S	R	R	Q	K	R	F	I	V	S
VRI-2344-2012	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-

[a] Position of residues in the amino acids of the fusion protein
[b] Amino acid of the fusion protein at the corresponding position (Lomniczi et al., 1998)
[c] Amino acid of genotypes and sub-genotypes within class II of NDV (Lomniczi et al., 1998)
[d] (-) Indicate identical amino acid

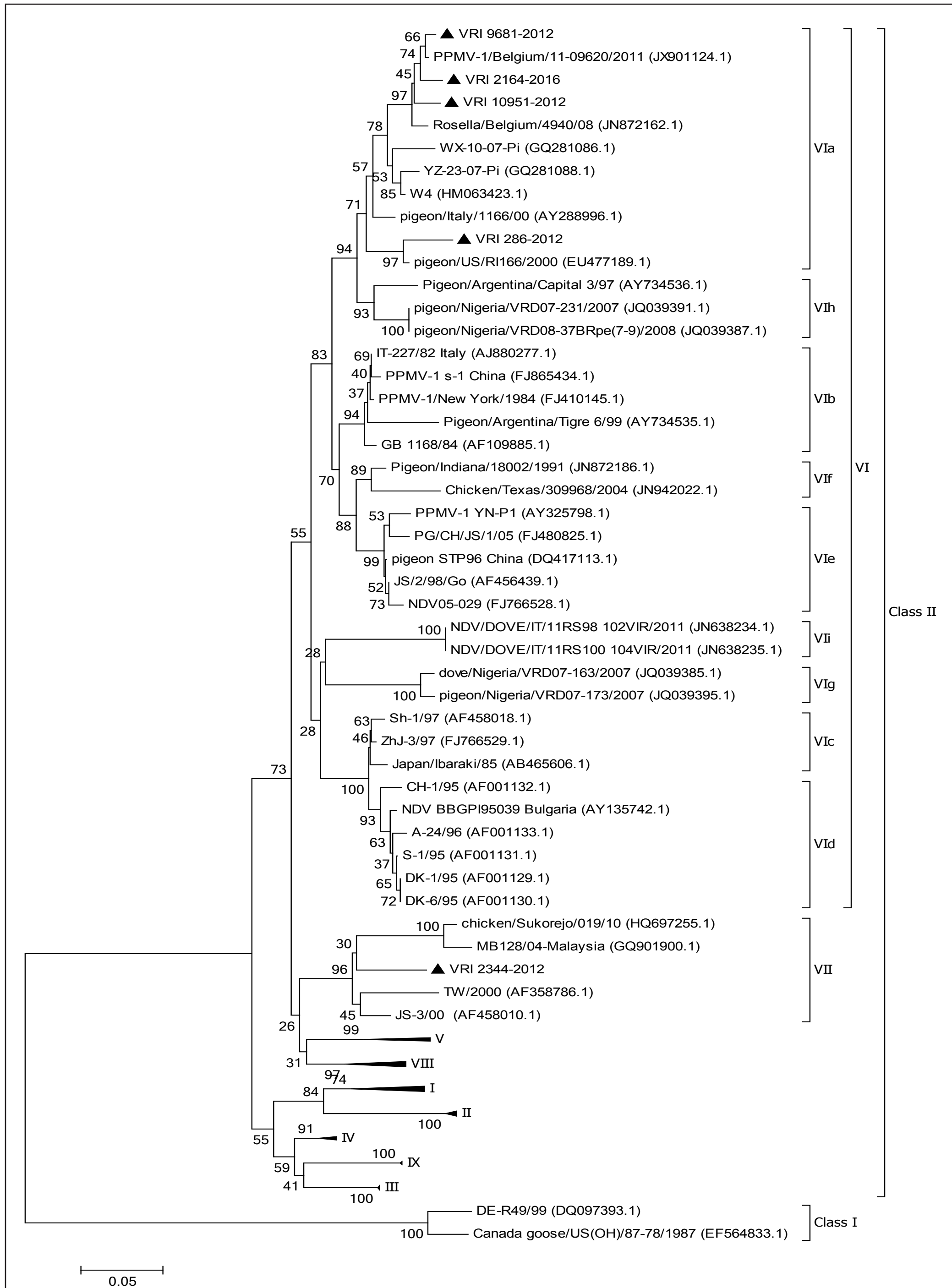

Figure 1. Phylogenetic tree of NDV isolates from quarantine station based on partial fusion gene (nuleotide 47-418) of NDV. Tree was constructed using MEGA version 6.06 by Neighbor-Joining statistical method with Kimura 2-parameter model and setting bootstrap 1000 replicates. GenBank accession numbers are shown in parenthesis

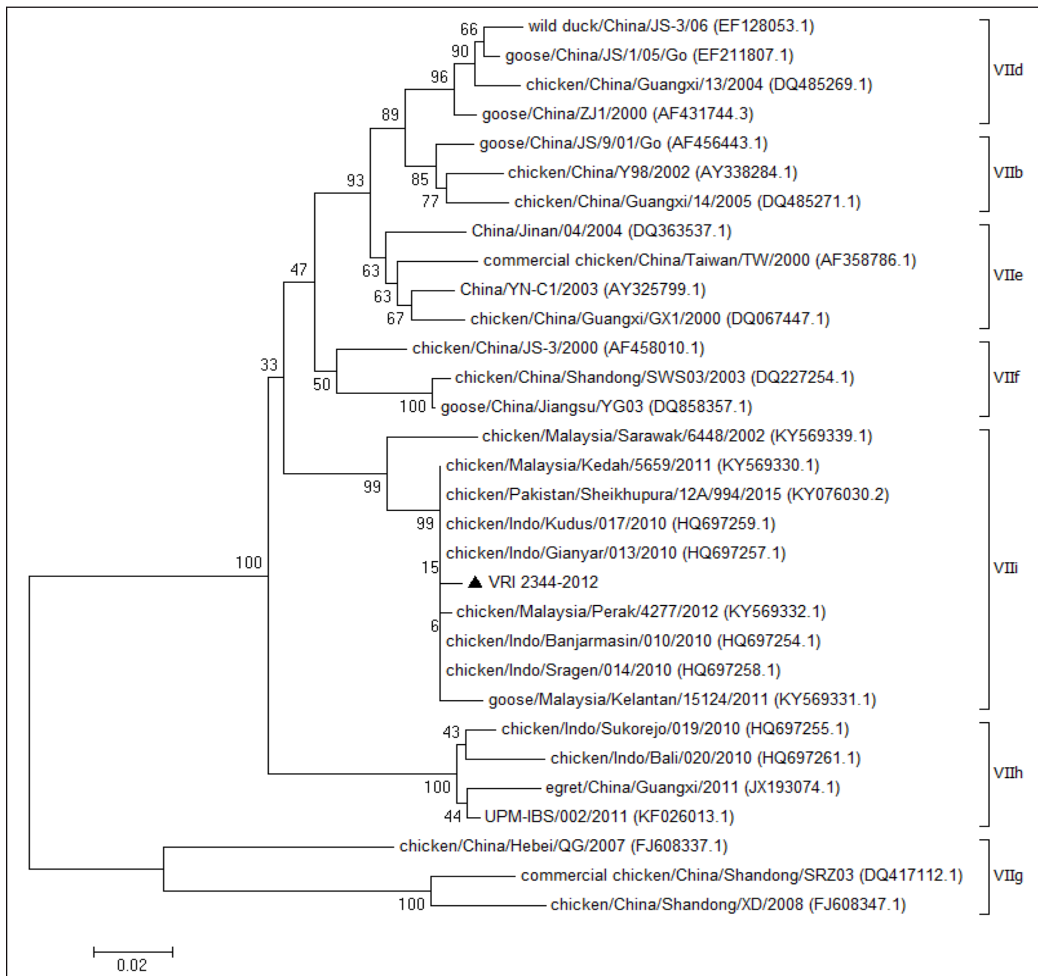

Figure 2. Phylogenetic tree of NDV isolate genotype VII isolated from birds in quarantine station based on partial fusion gene (nucleotide 47-418) of NDV. Tree was constructed using MEGA version 6.06 by Neighbor-Joining statistical method with Kimura 2-parameter model and setting bootstrap 1000 replicates. GenBank accession numbers areshown in parenthesis

DISCUSSION

Phylogenetic analysis of the F gene revealed that four NDVs isolated from imported birds belonged to genotype VIa and one was to genotype VIIi. Isolation of ND genotype VI and VII from imported birds at quarantine stations is common in Malaysia. Muhammad et al. (2012) had reported similar findings where ND genotype VIa, VIb and VIId were isolated from imported pigeons while genotype VIa was also retrieved from imported chicken.

Genotype VI had emerged since the 1960's (Lomnizci et al., 1998) and could be further divided into nine (a-i) subgenotypes (Liu et al., 2013). Genotype VI was often detected in the *Columbidae* species and had become a threat to the poultry industry (Diel et al., 2012). The genotype VIa includes mainly ancient isolates originating from

the Middle East (Aldous et al., 2003) and have been found mainly in *Columbidae* birds in Asia, Europe, the Middle East and the United States since the 1990s (Guo et al., 2013; Kim et al., 2008; Snoeck et al., 2013b). This is consistent with the finding in this study where the VIa viruses were retrieved from Asia and Europe (Table 2). To our knowledge, VIa viruses in Malaysia were mostly detected from imported avian (Mohamad et al., 2012) and to date there is no reported outbreak by this virus. However, the importance of VIa viruses cannot be neglected as two outbreaks caused by genotype VIa have been reported in South Africa (Snoeck et al., 2013a).

Genotype VII is a very large and genetically diverse group of viruses and always associated with ND outbreak in the Middle East and Asia currently (Diel et al., 2012). Genotype VII viruses can be subdivided into VIIb, VIId, VIIe, VIIf, VIIg, VIIh and VIIi (Miller et al., 2015). This virus is related to the fourth ND panzootic, where it started in Southeast Asia in the year 1985, and has been spreading from Asia, Europe, Africa and in South America (Miller et al., 2015) until today. In this study, the isolate VRI-2344-2012 was grouped together with the Pakistan and Indonesian strains in genotype VIIi. The Indonesian strains such as chicken/Indo/Kudus/017/2010, chicken/Indo/Gianyar/013/2010, chicken/Indo/Banjarmasin/010/2010 and chicken/Indo/Sragen/014/2010 were initially grouped as genotype VIIa by Choi et al. (2014). It was later re-classified by Miller et al., (2015) as genotype VIIi as Miller and colleagues found that this new sub-genotype VIIi is associated with the Indonesian NDV isolated from wild birds since the 1980s. The grouping of VRI-2344-2012 together with the Indonesian strains is in agreement with Miller et al. (2015) who stated that VIIi viruses isolated from poultry and infrequently from pet birds in Pakistan were associated with the current Indonesian strains. From the BLAST analysis, this isolate was 99% similar with the chicken/Pakistan/Sheikhupura/12A/994/2015 strain which corresponded to the origin of the import country, Pakistan. Again, Wajid et al. (2017) reported that sub-genotype VIIi was frequently isolated from poultry and various non-poultry avian species from year 2011 to 2016 in Pakistan. In Pakistan, the majority of ND outbreaks during the winter season from October 2011 to March 2012 was caused by VIIi viruses (Miller et al., 2015) which coincides with the isolation of VRI-2344-2012 in March 2012.

In Malaysia, genotype VII had caused several outbreaks from2000 to 2001 (Tan et al., 2010) and in 2010 (Shohaimi et al., 2015). Previously, genotype VIIa (it has been now reclassified as VIIi by Miller et al., 2015) had only been reported in East Malaysia (Sabah since 2004 and Sarawak since 2002) but it had not caused any outbreaks (Shohaimi et al., 2015). However, Shohaimi et al., (2015) found out that the same genotype had caused outbreaks not only in East Malaysia but also in Peninsular Malaysia from 2011 to 2012 and the same virus had also caused outbreaks in Pakistan and Indonesia. The rapid spreading of the VIIi virus and the ability of this

virus to infect various avian species may cause a panzootic if the disease is not kept under control (Miller et al., 2015). It is important for Malaysia and other countries in Southeast Asia which practise multiple farming practices such as commercial and backyard farming to control this virus as it may lead to the emergence of a virulent NDV as a new sub-genotype due to this production system (Miller et al., 2015).

The BLAST search showed that three VRI isolates, the VRI 9681-2012, VRI 10951-2012 and VRI 2164-2016 were 97-99% similar to the PPMV-1/Belgium/11-09620/2011 strain and were grouped in genotype VIa. Nevertheless, it was in contrast with the study conducted by Wang et al., (2015) who reported that the PPMV-1 Belgium strain belonged to genotype VIb. Lomniczi et al. (1998) reported that the deduced amino acid of the F gene at a certain position can be used to differentiate between a genetic group or sub-group. By comparing the amino acid we identified that the PPMV-1 Belgium strain has the same amino acid at all positions described by Lomniczi et al. (1998) for genotype VIa except at position 28 where Lomniczi et al. showed that Leucine (L) was specific for this position whereas the Belgium strain was observed to have Serine (S) (Table 3 and Figure 3). Lomniczi et al. (1998) also mentioned that the amino acid to differentiate genotype VIa and VIb was located at position 19, 28 and 112 where VIa had I^{19}, L^{28} and R^{112} while VIb has T^{19}, P^{28} and G^{112} (Table 3). Since the Belgium strain did not possess T^{19}, P^{28} and G^{112} as required for genotype VIb and more importantly, the strain was grouped under VIa in the phylogenetic analysis; therefore, we strongly suggest that this Belgium strain belongs to the genotype VIa group.

Although the four isolates belonged to genotype VIa, there were discrepancies among the isolates. As for VRI 286-2012, it did not fall in the same cluster as others. It is clustered with pigeon US strain which also corresponds with the BLAST search. The discrepancies among the four VIa isolates can be further explained from the aspect of amino acid sequences (Table 3).

Figure 3. Alignments of fusion amino acid sequences of imported genotype VIa NDVs compared with reference strain. A *dot* indicates an amino acid identical to that of PPMV-1 Belgium strain. The position of amino acids start with methionine that encodes the start codon of the F protein

Three isolates that were similar to the PPMV Belgium strain have the same amino acids when compared to the Lomniczi's study (1998) except for position 28 where the three isolates have S (serine) instead of L (leucine). Among these, VRI-10951-2012, has V (Valine) at position 118 which is different from others. On the other hand, VRI-286-2012 has more differences in amino acid as compared to the Lomniczi's study and the other three VIa isolates. It has L^{10}, P^{109} and K^{114} instead of P^{10}, S^{109} and Q^{114}. It is noteworthy that the pigeon US strain also has the same amino acid of L^{10} and K^{114} respectively as compared to the VRI-286-2012 isolate (Figure 3). Whereas the S at position 109 is in contrast with VRI-286-2012 and is in agreement with Lomniczi's study.

Meanwhile, the VIIi isolate, VRI 2344-2012 has the same amino acid as defined by Lomniczi for genotype VII in all positions except at positions 5 and 107 (Table 3). This VRI isolate has L^5 and A^{107} instead of P^5 and S^{107} respectively. In order to understand the difference between the imported VIIi virus and the existing local VIIi viruses in Malaysia, the F protein's amino acid is compared (Figure 4). All the local VIIi viruses have P (proline) at position 5 while the imported VIIi virus has L (leucine). In general, the five local isolates do not differ much as compared to the imported virus except the Sarawak isolate which has W^{18}, V^{19}, C^{25}, N^{30} and I^{52} that was totally dissimilar from others. Perhaps these unique amino acids categorized this Sarawak isolate into a distinct lineage in the VIIi sub-genotype (Figure 2).

All isolates in this study were virulent NDV belonging to the VI and VII genotypes. These findings which are in agreement with Diel et al., (2012) who reported that genotype V, VI, VII and VIII contained only virulent viruses and were the predominant genotypes circulating in the world. Pathogenicity of the NDV is correlated with the F protein (Meulemans et al., 2002) and the amino acid sequence at the F cleavage site is a major determinant of virulence (Dortmans

Figure 4. Alignments of fusion amino acid sequences of imported genotype VIIi NDVs compared with local VIIi isolates. A *dot* indicates an amino acid identical to that of VRI-2344-2012 strain. The position of amino acids start with methionine that encodes the start codon of the F protein

et al., 2011a). In this study, four isolates have the ^{112}RRQKRF^{117} motif at the F cleavage site while VRI-286-2012 has ^{112}RRKKRF^{117}. Tan et al. (2010) pointed out that most of the published NDV had a Q (glutamine) at position 114 as a dominant motif. However, the presence of R (arginine) or K (lysine) in the same position has also been reported. Motif ^{112}RRKKRF^{117} has been reported in Japan (Mase et al., 2002) from pigeon and in Taiwan from chicken (Lien et al., 2007). A similar motif has also been reported in European countries in PPMV-1 (Huovilainen et al., 2001; Meulemans et al., 2002; Terregino et al., 2003; Werner et al., 1999) and also in North America (Kim et al., 2008).

Kaleta and Baldauf (1988) explained that NDV outbreaks could occur in captive birds in quarantine stations, zoos and bird parks. Factors such as the high population density of one or more species in the captive environment, uncleaned droppings, uncontrolled flow of air and water and human traffic, limited water containers, and the constant flow of arriving and departing birds can contribute to the newly captive birds acquiring NDV infection when they transit in the quarantine station (Kaleta & Baldauf, 1988). Unsatisfactory environmental conditions and lack of social stimuli may suppress the inherited behaviour of the birds to just survive (Kaleta & Baldauf, 1988). This may increase the susceptibility of the birds to disease and eventually cause the high incidence of ND in captive birds (Kaleta & Baldauf, 1988). Kaleta and Baldauf (1988) also highlighted that in many countries, birds for export purposes were frequently infected with the NDV from backyard chicken when both birds and chicken were kept nearby. It is believed that a panzootic ND in Europe and North America during 1969/73 was associated with imported NDV from parrots from South American countries where these parrots were kept with infected backyard chickens in their local collection centre (Kaleta & Baldauf, 1988). Hence, NDV recovered from birds in quarantine stations in this study is not uncommon.

As mentioned earlier, NDV has been isolated from imported pigeons and chicken at quarantine stations in Malaysia. Similar cases have also been reported where velogenic NDV has been detected in pet birds that were imported to Canada and the United States (Clavijo et al., 2000). Therefore, strict enforcement of the regulations for the importation of live birds, and import risk analysis to assess the biosecurity risk associated with importing birds/animals into Malaysia are important. Preventive measures such as satisfactory hygiene in quarantine stations, placing different batches of birds arriving and departing in separate rooms, and controlled movement of water, animal feed and human traffic can prevent the chances of viral transmission between the birds. At the same time, screening the birds for diseases before release from the quarantine station is the most crucial step to prevent introducing viruses from other countries.

CONCLUSION

In conclusion, NDVs isolated from imported birds at a quarantine station were virulent NDVs as characterized by the presence of the ^{112}RRQ/KKRF^{117} motif at the F cleavage site. These viruses belonged to genotype VIa and VIIi respectively. Imported birds can therefore be a source of new strains of NDVs introduced into the country. With the increase in international trade in birds and poultry, efficient quarantine systems especially, the establishment of rapid and accurate diagnostic capabilities that can identify virulent NDVs are crucial in preventing the entry and exit of foreign NDV strains via the import and export trade respectively.

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