SEQUENCE ANALYSIS OF THE FUSION (F) AND MATRIX (M) GENES OF RECENTLY ISOLATED NEWCASTLE DISEASE VIRUS FROM MALAYSIA

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SUMMARY

The partial amino acid sequences of fusion (F) and matrix (M) genes encompassing the F cleavage site and the nuclear localization signals, respectively, of 4 recent Newcastle disease virus (NDV) isolates obtained from the field outbreaks between 2000 and 2001 were characterised. Isolates 01/C, 01/TM and 01/GNS shared the same amino acid sequences at the F cleavage site ^{111}GRRQKRF^{117}. However, isolate 00/IKS has ^{111}ERRQKRF^{117} motif at the F cleavage site. Isolate 01/TM also has the highest number of amino acid substitutions at the F gene. Both isolates 00/IKS and 01/GNS shared 100% amino acid sequence identity at the M gene whilst isolates 01/C and 01/TM have 3 and 1 unique amino acid substitutions. All the 4 isolates also have 2 unique amino acid substitutions (R200RS and E213D). However, no particularly distinguishing sequence variations were identified among the different NDV pathotypes. All the 4 NDV isolates were grouped into genotype VII with bootstrap value of 90%. The genotype VII comprises the recent NDV strains recovered during the outbreaks between years 1995 and 2001 which have been reported in many countries including China, Taiwan, South Africa and Europe. As the 4 NDV isolates were found phylogenetically related to goose isolate ZJI and pigeon isolate 1307/US/75, they were placed in the same group with bootstrap value of 78% based on the M gene sequence analysis.

Keywords: Newcastle disease virus, fusion protein, matrix protein, phylogenetic analysis

INTRODUCTION

Newcastle disease (ND) is a highly contagious viral disease of many avian species that can lead to substantial losses in the poultry industry worldwide. ND is included in the Office Internationale de Epizootics (OIE) List A disease with enforced statutory control measures in the event of outbreaks of the disease (Aldous and Alexander, 2001). ND is caused by Newcastle disease virus (NDV) which is classified as a member of the order *Mononegavirales*, family *Paramyxoviridae* and genus *Avulavirus* (Mayo, 2002a; 2002b). NDV has a negative sense, non-segmented single stranded RNA genome that consists of 15,586 nucleotides (Philips *et al.*, 1998). Isolates of NDV are categorised into three pathotypes, namely velogenic (high virulence), mesogenic (intermediate virulence) and lentogenic (low virulence), depending on the severity of disease caused by an isolate (Alexander, 1997).

Pathotype prediction initially involves NDV inoculation of embryonated eggs to determine mean death time of the embryo (MDT). Further testing entails inoculation of chickens to determine the intracerebral pathogenicity index (ICPI) and the intravenous pathogenicity index (IVPI). NDV pathogenicity has been shown to be dependent on the amino acid sequence motif present at the protease cleavage site of the precursor fusion (F_0) protein and the ability of cellular proteases to cleave the F_0 protein of different pathotypes (Ogasawara *et al.*, 1992; Collins *et al.*, 1993; 1994). Hence, the F cleavage site of NDV of different pathotypes has been well characterised based on sequence analysis (Aldous *et al.*, 2001; Yusoff and Tan, 2001). The matrix (M) protein is believed to play an important role in the assembly of the virus by interacting with the nucleocapsid, the lipid bilayer and also the regions of the surface glycoproteins that are exposed on the inner surface of the membrane (Peeples *et al.*, 1992). The M protein is considered to be relatively conserved among paramyxoviruses and this is substantiated by the low number of non-synonymous base substitutions (Seal *et al.*, 2000).

Outbreaks of velogenic NDV in vaccinated flocks have been reported in various states in Peninsular Malaysia in the years 2000 to 2001. In this study, we describe the partial sequence analysis of the F and M genes of highly virulent NDV isolates isolated from the recent outbreaks of ND from vaccinated flocks in Peninsular Malaysia.

METHODOLOGY

Virus isolation

Chicken tissue specimens were collected from 4 separate highly virulent ND cases from different states of Peninsular Malaysia during ND outbreaks between years 2000 to 2001. The NDV isolates were propagated in the allantoic cavity of 9-10 day-old specific-pathogen-free (SPF) embryonated chicken eggs according to Blaskovic and Styk (1967).

Mean death time test

The procedure for determining the mean death time (MDT) of the virus was carried out according to Alexander *et al.* (1988). Briefly, virus-infective allantoic fluid was diluted 10-fold in 1x phosphate buffer saline (PBS) from 10^{-1} to 10^{-10}. For each dilution, ten 10-day-old SPF embryonated chicken eggs were used. One hundred µl of the diluted virus was inoculated into each SPF embryonated eggs via allantoic cavity and incubated at 37°C. After 24 hours, the eggs were examined for mortality at 8 hour intervals for 96 hours. The MDT is the mean time in hours for the lowest dilution to kill all eggs of the dilution.

Extraction of viral RNA

Viral RNA of the NDV isolates were extracted using Tri Reagent® (Life Technologies, USA) according to the manufacturer's instructions.

Primers and reverse transcription-polymerase chain reaction (RT-PCR)

The forward primer BK1 (5'-GGGAGGCATACAACAGGACA-3') and reverse primer BK2 (5'-TGGTTGCAGCAATGCTCTC-3') were used to amplify F gene encompassing the cleavage site (Kianizadeh *et al.*, 2002) whilst forward primer M1 (5'-TCGAGICTGTACAATCTTGC-3') and reverse primer M2 (5'-GTCCGAGCACATCA CTGAGC-3') were used to amplify M gene region encoding the nuclear localisation signal (Seal *et al.*, 1995).

A two-step reverse transcription polymerase chain reaction (RT-PCR) was carried out using reverse transcription system (Promega, USA). The reaction was performed in Peltier Thermal Cycler, PTC-200 (MJ Research, USA). The first strand cDNA was synthesised in 20 µl mixture containing 1x RT-buffer [10 mM Tris-HCl, 50 mM KCl, 0.1% Triton® x-100, pH 8.8], 0.5 µM of each primer, 50-100 ng template RNA, 10 U recombinant RNasin ribonuclease inhibitor, 25 mM $MgCl_2$, 1 mM dNTPs and 2 U AMV reverse transcriptase. The reaction mixture was incubated at 42°C for 45 minutes followed by inactivation at 99°C for 5 minutes before being chilled immediately on ice for 5 minutes. During the incubation on ice, a PCR mixture containing 1x RT Buffer [10 mM Tris-HCl, 50 mM KCl, 0.1% Triton® x-100, pH 8.8], 2.0 mM $MgCl_2$, 0.5 µM each primer, 2.5 U of Taq DNA polymerase (Promega, USA) and 10 µl of cDNA mixture was prepared to a final volume of 50 µl. The final RT-PCR mixture was then subjected to 35 amplification cycles; denaturation at 94°C for 1 minute, primer annealing at 55°C for 1 minute and extension at 68°C for 1.5 minutes. A final extension was done at 68°C for 10 minutes.

Sequencing of PCR amplified DNA

The PCR products were subjected to agarose gel electrophoresis and purified using GENECLEAN III Kit (BIO 101, CA) according to the manufacturer's instructions. The purified PCR products were directly sequenced using automated DNA sequencing (ABI PRISM 377, Perkin Elmer). The primer sets BK1/BK2 and M1/M2 which were used earlier in F and M gene amplification, respectively, were used in the DNA sequencing. Each PCR product was sent for sequencing at least twice in order to get the complete sequence. The nucleotides and deduced amino acid sequences of the NDV isolates were assembled and analysed using BLAST (www.ncbi.nlm.nih.gov/BLAST/) and BioEdit™ version 7.0.4 (Hall, 1999). The partial sequence of F and M genes, respectively, were submitted to GenBank and their accession numbers are listed in Tables 2 and 3, respectively.

Sequence alignment and phylogenetic analysis

Multiple sequence alignments were generated using the Clustal X™ version 1.83 (Thompson *et al.*, 1997). Phylogenetic analysis was done using the NJplot program of the Clustal X™ software version 1.83. Datasets were bootstrapped 1000 times and subsequently analyzed using neighbor-joining method. The phylogenetic trees were edited and visualised using TreeView (Page, 1996).

RESULTS

Virus isolation and MDT

All four isolates exhibited embryo lethality with MDT ranging from 50 to 55 hours (Table 1). This indicated that all the NDV isolates were velogenic strains.

Alignment of the predicted amino acid sequences of F and M genes

Using specific primers, amplification from coding sequences including the F cleavage site resulted in a 242 bp product, while amplification products from the region surrounding nucleotide sequences encoding the M protein nuclear localisation signal were 232 bp long (data not shown). Figures 1 and 2, respectively, show 53 and 77 residues of amino acid of the F and M proteins of the 4 NDV isolates aligned with other published NDV strains. In Figure 1, isolate 01/TM shows the highest number of amino acid substitutions (P94N, L95S, D97N and R101N) among the NDV isolates compared with the consensus sequence meanwhile isolates, 00/IKS and 01/GNS, each of which had 2 amino acid substitutions S107A and G111E and S107A and V125G, respectively. Isolate 01/C had only one substitution at position 95 from

Table 1: Comparison of mean death time and F cleavage site of NDV isolates.

Isolate	MDT (hr)	Pathotype	F cleavage site[c]	Accession no.	References
00/IKS	55.2	Velogenic	^{111}ERRQKRF^{117}	AY839237	This study
01/C	51.2	Velogenic	^{111}GRRQKRF^{117}	AY839238	This study
01/TM	52.0	Velogenic	^{111}GRRQKRF^{117}	AY839239	This study
01/GNS	50.4	Velogenic	^{111}GRRQKRF^{117}	AY839240	This study
ShX-2/99	55.8	Velogenic	^{111}GRRQKRF^{117}	AF378245	Liang *et al.*, 2002
ShX-3/99	55.6	Velogenic	^{111}GRRQKRF^{117}	AF378246	Liang *et al.*, 2002
Ch/98-3	48.0	Velogenic	^{111}GRRQKRF^{117}	AF364835	Yu *et al.*, 2001
Ch/98-1	80.0	Mesogenic	^{111}GKRQKRF^{117}	AF358785	Yu *et al.*, 2001
AF2240	53.0 [a]	Velogenic	^{111}GRRQKRF^{117}	AF048763	Salih *et al.*, 2000
B1/46	120.0 [b]	Lentogenic	^{111}GGRQGRL^{117}	U22266	Seal *et al.*, 1995
LaSota/47	108.0 [b]	Lentogenic	^{111}GGRQGRL^{117}	U22266	Seal *et al.*, 1995

[a] MDT determined by Lai (1985)
[b] MDT determined by Toyoda *et al.* (1989)
[c] Position of amino acid is numbered according to Toyoda *et al.* (1989). The amino acid residues which differed from the conserved motif (^{111}GRRQKRF^{117}) were bold.

leucine to isoleucine. Isolates 01/C, 01/TM and 01/GNS share the same amino acid sequences at the F cleavage site ^{111}GRRQKRF^{117} (Table 1 and Figure 1). However, isolate 00/IKS had an unique substitution from glycine to glutamic acid at position 111, resulting in an ^{111}ERRQKRF^{117} motif at the F cleavage site. The cleavage site of velogenic NDV strains contained two pairs of dibasic amino acids [arginine (R) or lysine (K)] at positions 112, 113, 115 and 116 separated by a single glutamine (Q) residue ^{112}K/RRQK/RR^{116} in the C-terminus of the F_2 protein and phenylalanine (F) at position 117, the N-terminus of the F_1 protein. In contrast, the lentogenic strains possessed single pairs of basic amino acids ^{112}GR/KQGR^{116} and has a leucine (L) at position 117 (Table 1 and Figure 1).

NDV strains can be divided into eight genotypes (I to VIII) by restriction site mapping and amino acid sequence analysis of the F gene (Ballagi-Pardony *et al.*, 1996; Yu *et al.*, 2001). Based on the amino acid substitutions isolates 00/IKS, 01/C and 01/GNS possessed amino acid lysine at position 101 and valine at position 121, a characteristic of genotype VII viruses (Figure 1). Isolate 01/TM shared the same residue at position 121, but had a substitution at position 101 from arginine to asparagines that was unique when compared with other NDV strains.

Figure 2 shows amino acid sequences of the M gene from residues 199 to 275 that include the nuclear localisation signal. Both isolates 00/IKS and 01/GNS shared 100% amino acid sequence identity. There were 3 unique amino acid substitutions (D215A, K217T, F236L) in isolate 01/C compared to other local NDV isolates. However, isolate 01/TM had only one amino acid substitution at position 244 from threonine to isoleucine. All the 4 isolates had 3 unique amino acid substitutions from arginine to serine at position 200, glutamic acid to aspartic acid at position 213 and leucine to isoleucine at position 257. Residue 259, located within the nuclear localization signal, appeared to be highly variable with primarily charged amino acids located at that position. All of the 4 NDV isolates possessed a glutamic acid residue at the position 259. However, no particularly distinguishing sequence variations were identified among the lentogenic viruses in comparison with the mesogenic and velogenic pathotypes.

Phylogenetic analysis

In Figure 3, a phylogenetic tree was constructed based on the partial sequences of the F gene of 38 NDV strains. The NDV strains were separated into eight genotypes (I to VIII). All the 4 NDV isolates in this study were grouped into genotype VII with a bootstrap value of 90% (Figure 3). Most of the genotype VII viruses were found originating from Asia countries, included Malaysia, China, Taiwan, India, Indonesia and United Arab Emirates. All the lentogenic strains (LaSota, D26/76, Queensland/V4 and Ulster) were grouped into the ancient genotypes I and II. The classical velogenic Malaysian isolate, AF2240, which was isolated in the 1960s was placed in genotype VIII together with isolates from China QH-2/84 and QH-1/79 that were isolated in the early 1980s (Figure 3).

The phylogenetic relationship among the NDV strains based on the partial sequences of the M gene is shown in Figure 4. All the low virulence viruses (mesogenic and lentogenic) were placed in the same group and separated from the velogenic viruses.

The 4 NDV isolates used in this study were grouped together with goose isolate ZJI and pigeon isolate 1307/US/75 with a bootstrap value of 78%. This group was further divided into two lineages: isolates 01/C and 01/TM were related to goose isolate ZJI with a bootstrap value of 86%, however isolates 00/IKS, 01/GNS and pigeon isolate 1307/US/75 formed the second lineages within the group with a bootstrap value of 57%.

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                      ....|....| ....|....| ....|....| ....|....| ....|....| ...
                               97        107        117        127        137
Consensus             LTTLLTPLGD SIRRIQGSVS TSGGRRQKRF IGAVIGSVAL GVATAAQITA AAA
00/IKS                .......... ...K.....A ...E...... .......... .......... ...
01/C                  .......I.. ...K...... .......... .......... .......... ...
01/TM                 ......NS.N ...N...... .......... .......... .......... ...
01/GNS                .......... ...K.....A .......... .......G.. .......... ...
AF2240                .......... .........T .......... .......... .......... ...
QH-1/79               .......... .........T .......... .......... .......... ...
QH-2/84               .......... .........T .......... .......... .......... ...
CH 62/96              .......... ...K...... .......... .......... .......... ...
DE R143/95            .......... ...K...... .P........ .......... .......... ...
ZA C1868/95           .......... .......... .......... .......... .......... ...
CZ 3898/96            .......... ...K...... .......... .......... .......... ...
ZA 360/95             .......... .......... .......... .......... .......... ...
ZW 3422/95            .......... .......... .......... .......... .......... ...
WB1-94                .......... .......... .......R.. .......... .......... ...
Cockatoo/14698/90     .......... ...K...... .......... .......... .......... ...
ShD-1/99              .......... ...K...... .......... .......... .......... ...
ShX-2/99              .......... ...K...... .......... .......... .......... ...
ShX-3/99              .......... ...K...... .......... .......... .......... ...
ShX-6/99              .......... ...K...... .......... .......... .......... ...
Ch/98-3               .......... ...K...... .......... .......... .......... ...
Ch-A7/96              .......... ...K...... ....K..... .......... .......... ...
TW/95-9               .......... ...K...... .......... .......... .......... ...
TW/2000               .......... ...K...... .......... .......... .......... ...
AE 232/1/96           .......... ......E... ....K..R.. .......... .......... ...
ASTR/74               .......... .......... ..R....... ...I...... ....S..... ...
Largo/71              .......... ........AT .......... V..I...... .......... ...
Italien/45            .......... ......E..T .......R.. ...I...... ....P..... .S.
Herts33               .......... ....K.E..T .......R.. ...I...... .......... .S.
Miyadera/51           .......... ......E..T .......R.. ...I...... .......... .S.
Victoria              .......... ......E..T .......R.. ...I...... .......... .S.
GB 1168/84            .......... .......... ....G..... ...I...... ....S..... ...
Anhinga/44083/93      .......... ........AT ..R....... V..I...I.. .......V.. ...
Ch/98-1               .......... .......... ...EK..... ...I...... ....S..... ...
Beaudette C           .......... ......E..T .......... ...I..G... .......... ...
LaSota/46             .......... ......E..T ....G..G.L ...I..G... .......... ...
D26/76                .......... ......E..T ....GK.G.L ...I..G... .......... .S.
Queensland/V4         .......... ......E..T ....GK.G.L ...I..G... .......... .S.
Ulster                .......... ......E..T ....GK.G.L ...I..GA.. .......... .S.
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Fig. 1: Deduced amino acid sequences of the F gene of NDV isolates from residues 88 to 140 (numbering of Toyada *et al.*, 1989) compared with the consensus sequences. A dot indicated position where the sequence is identical to that of the consensus. The position of F cleavage site is underlined and bold.

DISCUSSION

Conventional biological characterisation of NDV isolates includes MDT of embryonated chicken eggs, ICPI in 1-day-old chicks and IVPI in 6-week-old chickens (Beard and Hanson, 1984). However, these methods are time consuming and not applicable for rapid screening analysis. It has been shown that the primary molecular determinants for NDV pathogenicity are the amino acids of the F cleavage site (Toyoda *et al.*, 1989; Peeters *et al.*, 1999). All the 4 NDV isolates in this study showed a good relation between MDT and F cleavage site sequences confirming the isolates were velogenic strains. Typically lentogenic, mesogenic and velogenic strains have MDT of more than 100 hours, 60 to 90 hours and less than 60 hours, respectively, to kill embryos following allantoic cavity inoculation (Alexander, 1988). However, Yu *et al.* (2001) have indicated that the virulence of NDV strains can be qualified rather than quantified by the analysis of cleavage site motifs and that pathogenicity test such as MDT cannot be replaced by analysis of F cleavage site to precisely determine the virulence of NDV strains. Molecular techniques offer a rapid alternative method for pathotyping of NDV. Recently, Pham *et al.* (2004) successfully differentiated the virulent NDV strains from the avirulent strains by using restriction enzyme analysis on nucleocapsid (NP) protein.

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                        ....|....| ....|....| ....|....| ....|....| ....|....|
                              208        218        228        238        248
Consensus               SRLYNLALNV TIDVEVDPKS PLVKSLSKSD SGYYANLFLH IGLMSTVDKK
00/IKS                  .S........ ....D..... .... ..... .......... ..........
01/C                    .S........ ....D.A.T. .......... .......L.. ..........
01/TM                   .S........ ....D..... .......... .......... .....I....
01/GNS                  .S........ ....D..... .......... .......... ..........
ZJI                     .S........ ....D..... .......... .......... ..........
99MB-135                .S........ ........R. .......... N......... ....T...RR
Layer chicken/2/87      .......... .......... R......... .......... ..........
Pigeon/1307/US/75       .......... ....D..... .......... .......... ..........
Chicken/37821-2/96      .......... .......... .......... .......... ..........
Parakeet/11592/91       .......... .......... .......... .......... .........R
Partridge/37182/90      .......... .......... ..A...A.P. ..S....... ...V......
Cockatoo/14698/90       .......... .......... .......... .......... .........R
Florida                 .......... .......S.. .......... .......... ......I..R
Fontana                 .......... .......... .......... .......... ..........
Largo                   .......... .......... .......... .......... ..........
AF2240                  .......... .......... .........I V...I..... ..VW...ER.
Beaudette C             .......... ..N...SR. .......... .......... ....T...RR
Herts33                 .......... .......... .......... .......... ..........
Kimber                  .......... ........R. .......... .......... ....T...RR
26407/chicken/1995      .S....V.. ..N.....R. .......... .......... ....T...R.
VGGA                    .......... ........R. .......... .......... ....T...R.
Queensland/V4           ........D. .......... .......... .......... ..........
Ulster                  .......... ........R. .......... .......... ....T...RR
B1                      .S........ ..N.....R. .......... .......... ....T...R.
LaSota                  .......... ........R. ............. .........T ...R.

                        ....|....| ....|....| ....|..
                              258        268
Consensus               GKKVTFDKLE EKIRRLNLSV GLSDVLG
00/IKS                  ........I. .......... .......
01/C                    ........I. .......... .......
01/TM                   ........I. .......... .......
01/GNS                  ........I. .......... .......
ZJI                     ........I. .......... .......
99MB-135                .......... R...S.D... .......
Layer chicken/2/87      .........I .......... .......
Pigeon/1307/US/75       .........I .......... .......
Chicken/37821-2/96      .......... K......... .......
Parakeet/11592/91       ........I. .......... .......
Partridge/37182/90      .......... R......... .......
Cockatoo/14698/90       .........I .......... .......
Florida                 .......... R......... .......
Fontana                 ........I. G......... .......
Largo                   .......... R......... .......
AF2240                  .....LT... G......... ..RI...
Beaudette C             .......... K...S.D... .......
Herts33                 .......... K.....D... ......E
Kimber                  .......... K...S.D... .......
26407/chicken/1995      .......... K...S.D... .......
VGGA                    .......... K...S.D... .......
Queensland/V4           .......... R.....D..E .......
Ulster                  .......... K...S.D... .......
B1                      .......... K...S.D... .......
LaSota                  .......... K...S.D... .......
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Fig. 2. Deduced amino acid sequences of the M gene of NDV isolates from residues 199 to 275 (numbering of Seal *et al.*, 1998) compared with the consensus sequences. A dot indicated position where the sequence is identical to that of the consensus. The M protein nuclear localization signal from position 247 to position 263 is underlined and bold.

Table 2: NDV isolates used for phylogenetic analysis based on F gene.

Strain	Country of Origin	Genotype	Pathotype[a]	Accession no.
00/IKS	Malaysia	VII	V	AY839237
01/C	Malaysia	VII	V	AY839238
01/TM	Malaysia	VII	V	AY839239
01/GNS	Malaysia	VII	V	AY839240
AF2240	Malaysia	VIII	V	AF048763
QH-1/79	China	VIII	V	AF378250
QH-2/84	China	VIII	V	AF378251
CH 62/96	Switzerland	VII	V	AF109880
DE R143/95	Germany	VII	V	AF109881
ZA C1868/95	South Africa	VII	NA	AF109882
CZ 3898/96	Czech Republic	VII	V	AF109883
ZA 360/95	South Africa	VII	NA	AF109876
ZW 3422/95	Zimbabwe	VII	NA	AF109877
W81-94	India	VII	NA	AJ249530
Cockatoo/Indonesia/ 14698/90	Indonesia	VII	V	AY288998
ShD-1/99	China	VII	V	AF378260
ShX-2/99	China	VII	V	AF378245
ShX-3/99	China	VII	V	AF378246
ShX-6/99	China	VII	V	AF378247
Ch/98-3	China	VII	V	AF364835
Ch-A7/96	China	VII	V	AY028895
TW/2000	Taiwan	VII	V	AF358786
TW/95-9	Taiwan	VII	V	AF083966
AE 232/1/96	United Arab Emirates	VII	NA	AF109884
ASTR/74	Russia	VI	NA	Y18728
GB 1168/84	Untied Kingdom	VI	M	AF109885
Ch/98-1	China	VI	M	AF358785
Largo/71	United States	V	V	AY288987
Anhinga/US/44083/93	United States	V	M	AY288989
Italian/45	Italy	IV	V	M17710
Herts/33	United Kingdom	IV	V	M24702
Miyadara/51	Japan	III	V	M18456
Victoria/32	Australia	III	V	M21881
Beaudette C/45	United States	II	M	X04719
LaSota/46	United States	II	L	AF077761
D26/76	Japan	I	L	M24692
Queensland/V4	Australia	I	L	AF217084
Ulster	Northern Ireland	I	L	D00243

[a]V, velogenic; M, mesogenic; L, lentogenic; NA, not available.

The phylogenetic tree constructed based on the partial sequences of the F gene is similar to the previous studies (Lomniczi *et al.*, 1998; Herczeg *et al.*, 1999; Yu *et al.*, 2001; Liang *et al.*, 2002). All the 4 NDV isolates recovered from different states of Peninsular Malaysia between 2000 and 2001 belonged to genotype VII based on the sequence analysis of F gene. Genotype VII viruses first appeared in Taiwan and Indonesia in 1980s and then caused outbreaks in Europe, Southern Africa, Middle East and Asia in 1990s, constituting the fourth panzootic of ND (Lomniczi *et al.*, 1998, Yu *et al.*, 2001).

Genotype VII viruses can be further divided into four subgenotypes (VIIa to VIId). In this study, the viruses could not be further divided into the subgenotypes after phylogenetic analysis was performed. This is because only partial sequence of F gene was used in the phylogenetic assessment. Each subgenotype contains their own subgenotype-specific residue substitution (Yu *et al.*, 2001). Residue K101R is specific for subgenotypes VIIa, VIIc and VIId meanwhile residues V52I and Y314F are specific for subgenotype VIId only. In order to determine the subgenotypes among the genotype VII viruses, more information on the amino acid sequence or full length of F gene should be obtained.

The phylogenetic analysis based on M gene showed that the 4 NDV isolates in this study were related to the pigeon isolate1307/US/75 and goose ZJ1, respectively.

Table 3: NDV isolates used for phylogenetic analysis based on M gene.

Isolate	Year of isolation	Country of Origin	Pathotype[a]	Accession no.
00/IKS	2000	Malaysia	V	AY839237
01/C	2001	Malaysia	V	AY839238
01/TM	2001	Malaysia	V	AY839239
01/GNS	2001	Malaysia	V	AY839240
ZJ1	2000	China	NA	AF431744
99MB-135	2000	Canada	NA	AY029227
Layer chicken/Namakkal/India/NDV/2/198	1998	India	NA	AF204741
Pigeon/1307/US/75	1975	United States	NA	AY008318
Chicken/Mexico/37821/550/2/96	1996	Mexico	V	AF015520
Parakeet/Myanmar/11592/91	1991	Myanmar	V	AF015507
Partridge/Singapore/37182/90	1990	Singapore	V	AF015517
Cockatoo/Indonesia/14698/90	1990	Indonesia	V	AF015508
Florida	1980	United States	V	U22291
Fontana	1971	United States	V	U22274
Largo	1971	United States	V	U22279
AF2240	1960	Malaysia	V	AF060563
Beaudette C	1959	United States	V	X04687
Herts 33	1933	United Kingdom	V	U22275
Kimber	1947	United States	M	U22278
26407/chicken/NY/1995	1995	United States	L	U79552
VGGA	1989	United States	L	U22273
Queensland/V4	1966	Australia	L	U22283
Ulster	1964	Northern Ireland	L	U22290
B1	1948	United States	L	U22266
LaSota	1946	United States	L	U22292

[a] V, velogenic; M, mesogenic; L, lentogenic; NA, not available.

The viruses were placed in the same group of the phylogenetic tree. This suggests that the recent isolates might have originated from domestic and free-living birds. This further supports the epidemiological information from previous studies implicating that highly virulent NDV continues to circulate among birds other than chickens, threatening commercial poultry worldwide (Yang *et al.*, 1999; Seal *et al.*, 1995, 1998). Hence, the importance of NDV isolates from free-living birds and water birds in the emergence of new velogenic strains of NDV in Malaysia remains to be studied. In addition, the susceptibility to NDV varies among species and water birds have been recognised as being the most resistant (Alexander, 1997; Seal *et al.*, 2000). Recently, it has been shown that avirulent NDV isolates from waterfowl become highly velogenic in chickens after passages in chicken populations (Shengqing *et al.*, 2002).

Despite routine vaccination, outbreaks of velogenic NDV have been reported in vaccinated flocks in Malaysia. The reasons for this is largely unknown but has been postulated to be due to several factors such as improper use of vaccines and vaccination schedule and presence of immunosuppressive agents (Sharifah *et al.*, 2001). In that study they also indicated that the commercial vaccines can induce 100% protection against challenge with other recent NDV isolates isolated in years 2000 to 2001. Nevertheless, the role of water fowls and free-living birds in the emergence of fresh outbreak of velogeinc NDV and characterisation of the complete genome sequences of the recent NDV isolates merit further investigations.

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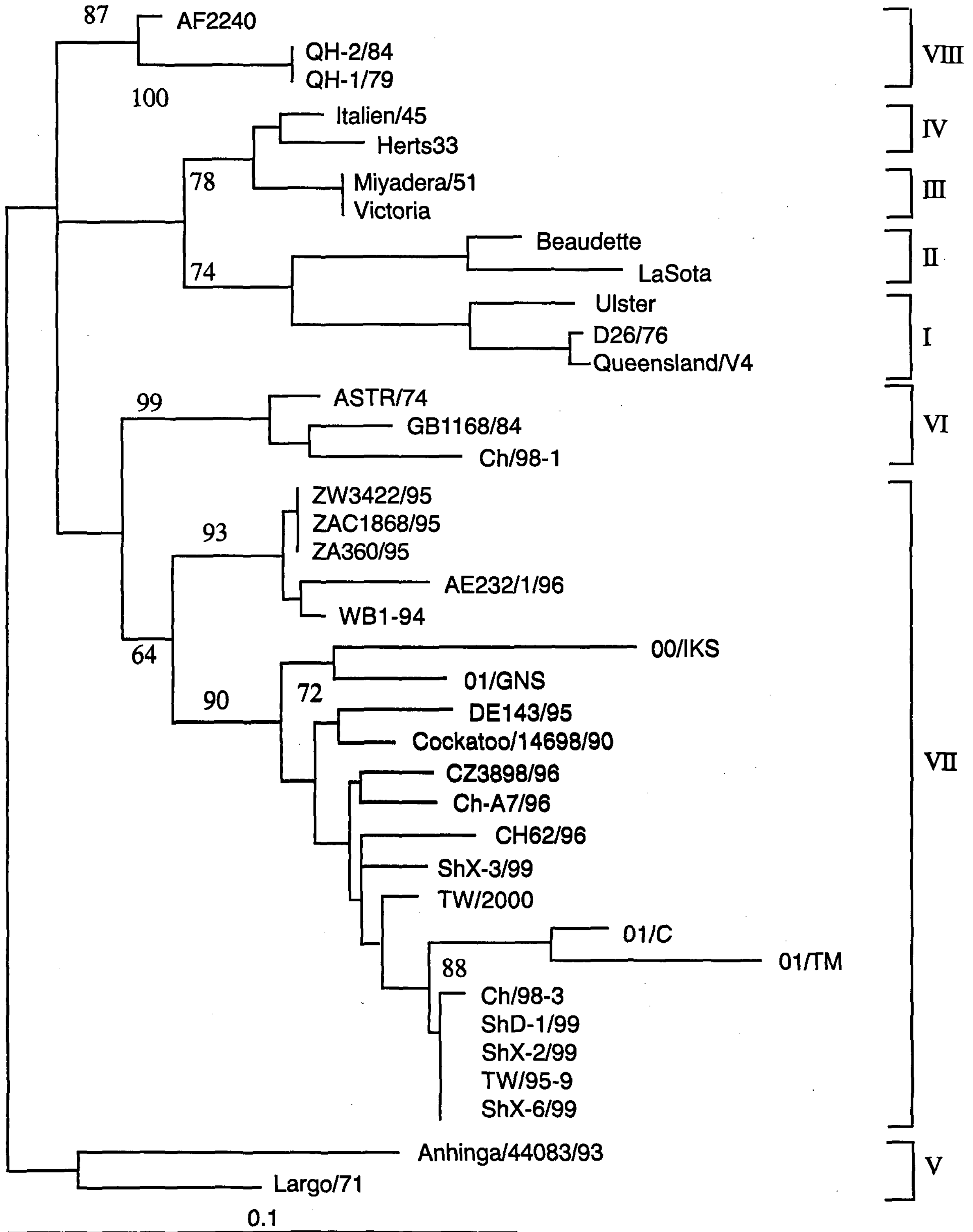

Fig. 3. Phylogenetic analysis of NDV isolates based on partial sequences of F gene (nucleotide position from 308 to 468).

Note: The tree was constructed using neighbor-joining method. Numbers show bootstrap percentage values. Only pertinent values are shown. The recent NDV isolates 00/IKS, 01/C, 01/TM and 01/GNS that used in this study are shaded in grey.

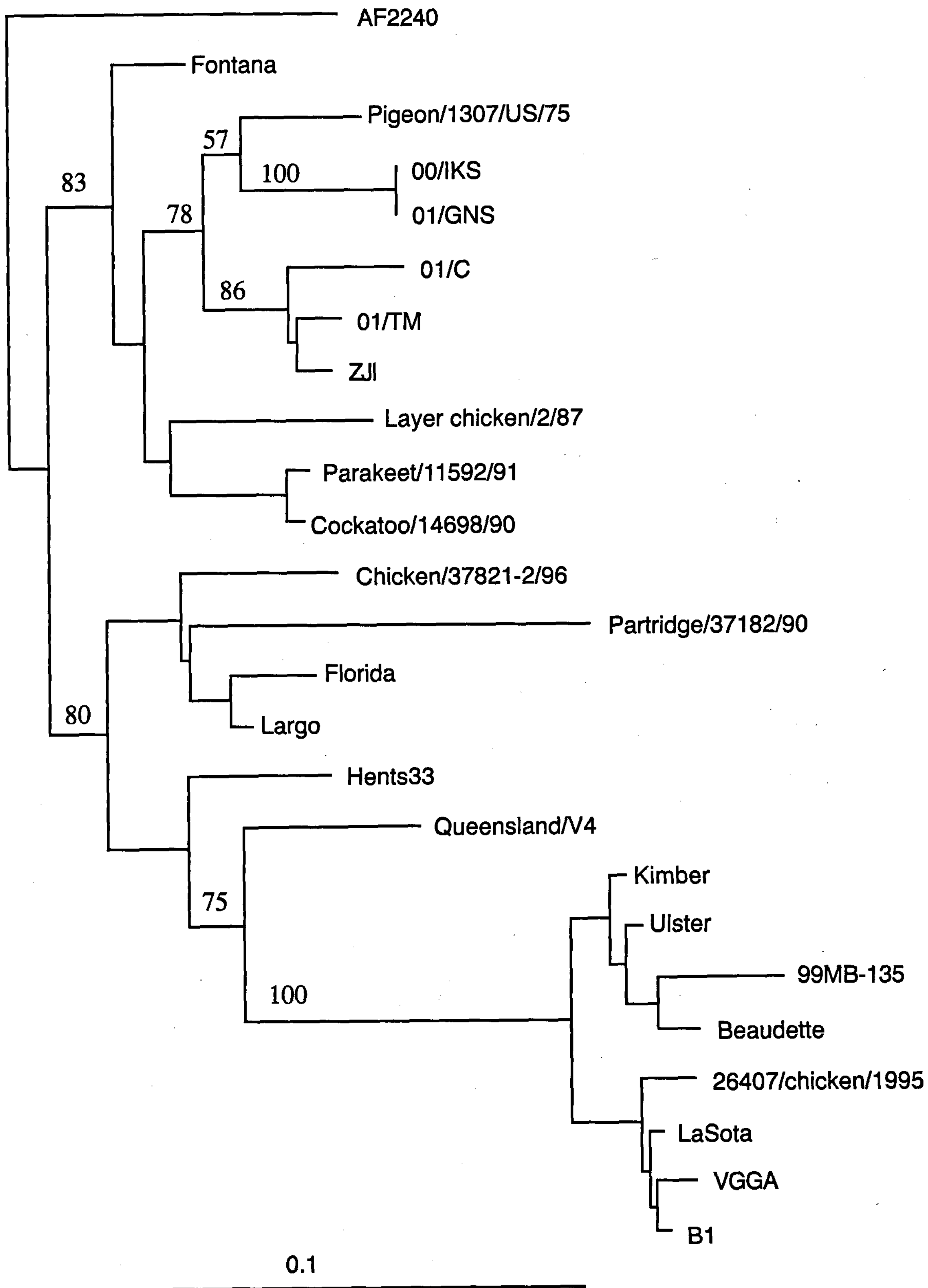

Fig. 4. Phylogenetic analysis of NDV isolates based on partial sequences of M gene (nucleotide position from 604 to 836).

Note: The tree was constructed using neighbor-joining method. Numbers show bootstrap percentage values. Only pertinent values are shown. The recent NDV isolates 00/IKS, 01/C, 01/TM and 01/GNS that used in this study are shaded in grey.

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