SEQUENCE ANALYSIS OF THE FUSION (F) PROTEIN CLEAVAGE SITE
OF FOUR NEWCASTLE DISEASE VIRUS ISOLATES

Ramanujam, P.[1], Tan, W.S.[2,3], Nathan, S.[4] & Yusoff, K.[2,3*]

[1] Department of Biotechnology, Faculty of Applied Sciences, Asian Institute of Medicine,
Science and Technology, Sungai Petani, Kedah, Malaysia
[2] Department of Microbiology, Faculty Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[3] Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[4] Centre for Gene Analysis and Technology, Faculty of Science and Technology,
Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia.

SUMMARY

The amino acid sequence of the fusion (F) protein of Newcastle disease virus (NDV) is of particular interest as its virulence is highly dependent on susceptibility to proteolytic cleavage. The F protein precursor, F_o, can be proteolytically cleaved to form disulfide-linked F_1 and F_2 chains. The F_o cleavage is a prerequisite for producing infectious particles whereby the nature of the cleavage site correlates with virulence of the virus. In this study we determined the F protein gene cleavage site nucleotide sequence of NDV local isolates 2641/91 P2, 5953/89 P3, 1266/89 P3 and 8820/92 P3. Their sequences were also compared with other NDV strains and isolates, such as AF2240, 00/IKS, 01/TM, 01/C, 3410/92 P2, 6385/90 P3, V4 Que and F that had previously been determined. The deduced amino acid sequence of this site revealed the pathogenecity of these strains whereby 2641/91 P2 and 5953/89 P3 were classified as virulent isolates whilst 1266/89 P3 and 8820/92 P3 were avirulent isolates.

Keywords: newcastle disease virus, F cleavage site, virulence

INTRODUCTION

Newcastle disease virus (NDV) is a highly contagious avian virus that has a considerable impact on global economics. The severity of the disease caused by this virus is highly pathotype-dependent. The velogenic strains cause severe disease with very high mortality rates, the mesogenic has moderate effect and the lentogenic strains cause mild infection (Jordan, 1990). The velogenic and mesogenic strains are virulent whilst the lentogenic strains are considered to be avirulent. The virus strains can be divided into two groups that correlate to its virulence: virulent strains that have F proteins with multibasic residue at the F_o cleavage site and avirulent strains with a single basic residue at the cleavage site (Choppin and Scheid, 1980).

The mRNA sequence of the F protein contains 540-580 amino acid residues (Chambers *et al.*, 1986; Yusoff and Tan, 2001) and a calculated molecular weight of about 55 kDa (Salih *et al.*, 2000). The antigenic structure of F protein is highly conserved and conformational in nature (Yusoff *et al.*, 1989). It is involved in the viral fusion to the host cell and is synthesized as a precursor F_o that can be proteolytically cleaved at the peptide bond of amino acid residues at positions 116 and 117 to form disulfide-linked F_1 and F_2 chains (Gotoh *et al.*, 1992). The cleavage is accomplished by host cell proteases (Lamb and Kolakofsky, 1996). F proteins encoded by the virulent

NDV strains have amino acids at the cleavage site that are substrates for furin family proteases and are cleaved intracellularly in the trans-Golgi membranes. F proteins of avirulent strains do not have these sequence motifs but rather encode single basic residues that are cleaved by extracellular enzymes (Lamb and Kolakofsky, 1996).

In this study, the F cleavage site sequence of four local NDV isolates namely, 2641/91 P2, 5953/89 P3, 1266/89 P3 and 8820/92 P3 were performed to determine their pathotypes.

METHODOLOGY

Propagation and purification of NDV strains

NDV isolates 2641/91 P2, 5953/89 P3, 1266/89 P3, 8820/92 P3, AF2240 and V4 Que were propagated in embryonated chicken eggs and purified as previously described by Yusoff *et al.* (1996).

Viral RNA extraction

Total RNA of the NDV strains was extracted using TRIZOL LS Reagent (Gibco BRL, USA) and the procedure used was as described in the supplier's manual. Briefly, the infected allantoic fluid (250 µl) was mixed with TRIZOL LS Reagent (750 µl) and incubated at room temperature for 5 min. After incubation, 1-bromo-3-

chloropropane (200 μl) was added and left at room temperature for 5 min for phase separation. The RNA was then precipitated by adding isopropanol (500 μl) to the aqueous phase and washed with 70% (v/v) diethyl pyrocarbonate (DEPC) (Sigma, USA) treated ethanol. The RNA pellet was air dried and resuspended in DEPC treated water.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Universal primers BK1 (5' GGGAGGCATACAACA GGACA 3') and BK2 (5' TGGTTGCAGCAATGCTCTC 3') (Yusoff et al., 1993) were purchased from Clontech Laboratories, USA. These primers are located within the highly conserved regions of the F gene at nucleotides 289–307 and 512–530, respectively (Toyoda et al., 1989) and the fragment flanked by these primers contains the F cleavage site.

For the first strand cDNA synthesis, the RNA was reverse transcribed by adding the extracted RNA (5 μl) to the RT mix [RT buffer (10X; 0.05 M KCl, 0.02 M Tris-HCl, 3 μl); MgCl$_2$ (2.5 mM 3 μl); dNTPs (2.5 mM, 1 μl); RNAsin (10 U, 0.5 μl); AMV reverse transcriptase (2 U, 0.5 μl); primer BK1(10 μM, 2 μl); primer BK2 (10 μM, 2 μl); DEPC treated water, 13 μl;)] to a final volume of 30 μl. The mixture was incubated at 45°C for 15 min followed by a further incubation at 94°C for 5 min in a thermal cycler (Mastercycler 5330, Eppendorf) and then immediately cooled on ice for 5 min.

For PCR, the mixture containing PCR buffer (0.05 M KCl, 0.02 M Tris-HCl), MgCl$_2$ (2.5 mM), Taq DNA Polymerase (2.5 U) and distilled water was added to the first strand cDNA of a final volume of 50 μl. Amplification of the cDNA was performed in the thermal cycler and the cycling profile used was denaturation (94°C, 30 s), annealing (55°C, 30 s) and primer extension (72°C, 1 min) for a total of 40 cycles. A further incubation at 72°C for 10 min was performed after the last cycle to complete the elongation of DNA molecules.

Purification of RT-PCR products

The PCR products were analysed on a 2% (w/v) agarose gel. The gel was stained with ethidium bromide (1 μg/ml) and visualized under a UV transilluminator. The PCR products were purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim, Germany) according to the supplier's manual. Briefly, binding buffer (3 M guanidine thiocynate, 10 mM Tris-HCl, 5% ethanol; pH 6.6; 500 μl) was added to the PCR product (100 μl). The mixture was filtered through a glass fibre filter by centrifugation at 13,000 xg for 30 s. Bound DNA was washed 2X with the wash buffer (20 mM NaCl, 2 mM Tris-HCl; pH 7.5), eluted with ddH$_2$O (50 μl) and was used for sequencing.

Sequencing of PCR products

Sequencing of the purified RT-PCR product was performed using the ABI PRISM 377 Automated DNA Sequencer system (Applied Biosystems) at the Centre for Gene Analysis and Technology, U.K.M. Primers used were BK1 and BK2.

RESULTS AND DISCUSSION

The extracted viral RNA was reverse transcribed and amplified using primers BK1 and BK2. Analysis of the RT-PCR products through a 2% agarose gel electrophoresis showed the presence of sharp DNA bands with the predicted size of 242 bp for all the isolates (Fig 1). Table 1 and 2 respectively, show the nucleotide sequence and the deduced amino acid sequences for isolates 2641/91 P2, 5953/89 P3, 1266/89 P3 and 8820/92 P3. Sequences from strains AF2240 (Salih et al., 2000), 00/IKS, 01/TM and 01/C (Tan et al., 2001), 3410/92 P2, 6385/90 P3 and F (Kho, 1997) and V4 Que (Alexander, 1990) are also shown for the purpose of comparison. From the data obtained, it can be strongly suggested that isolates 2641/91 P2 and 5953/89 P3 are virulent whilst isolates 1266/89 P3 and 8820/92 P3 are avirulent.
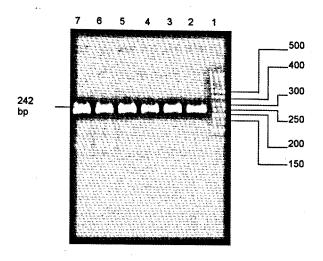

Fig. 1. RT-PCR product of the F gene of NDV isolates. Lane 1- DNA markers (Gene Ruler™ 50 bp ladder), 2- strain AF2240, 3- strain 2641/91 P2, 4- strain 5953/89 P3, 5- strain V4 Que, 6- strain 8820/92 P3 and 7- strain 1266/89 P3.

The deduced amino acid sequence data (Table 2) demonstrate that velogenic strains AF2240, 00/IKS, 01/TM, 01/C and 3410/92 P2 carry the peptide sequence [112]Arg-Arg-Gln-Lys/Arg-Arg-Phe[117]. The mesogenic strains 2641/91 P2, 5953/89 P3 and 6385/90 P3 harbour [112]Arg/Gly-Arg-Gln-Lys/Arg-Arg-Phe[117] motif while the lentogenic strains V4 Que, F, 8820/92 P3 and 1266/89 P3 possess [112]Gly/Arg-Lys/Arg-Gln-Gly-Arg-Leu[117]

Table 1: Nucleotide sequence of F cleavage site. Comparison of the nucleotide sequence of the F_0 cleavage site among velogenic, mesogenic and lentogenic strains from positions 374 to 415. The underlined sequence represents the cleavage site. The blank spaces indicate nucleotide homology with strain AF2240. The arrow indicates the cleavage site on F protein.

NDV strains	374						↓						415
Velogenic													
AF2240	GGA	GGG	*AGG*	*AGA*	*CAG*	*AAA*	*CGC*	TTT	ATA	GGT	ATT	ATC	GGC
00/IKS	CAG												
01/TM													
01/C													
3410/92 P2		A			CGC					G	T	T	
Mesogenic													
2641/91 P2													
5953/89 P3			GGC										
6385/90 P3		A			CGG							T	
Lentogenic													
F		G		GGG	C							T	
V4 Que		GA		GG	C								
8820/92 P3		G		GGC	C C								
1266/89 P3				GGC	C C								

Table 2: Amino acid sequence of F cleavage site. The deduced amino acid sequences for velogenic, mesogenic and lentogenic strains that correspond to positions 110 to 120. The arrow indicates the cleavage site.

NDV strains	Deduced amino acid sequence										
	110	111	112	113	114	115	116	117 ↓	118	119	120
Velogenic											
AF2240	Gly	Gly	Arg	Arg	Gln	Lys	Arg	Phe	Ile	Gly	Ala
00/IKS	Gly	Gln	Arg	Arg	Gln	Lys	Arg	Phe	Ile	Gly	Ala
01/TM	Gly	Gly	Arg	Arg	Gln	Lys	Arg	Phe	Ile	Gly	Ala
01/C	Gly	Gly	Arg	Arg	Gln	Lys	Arg	Phe	Ile	Gly	Ala
3410/92 P2	Gly	Gly	Arg	Arg	Gln	Arg	Arg	Phe	Ile	Gly	Ala
Mesogenic											
2641/91 P2	Gly	Gly	Arg	Arg	Gln	Lys	Arg	Phe	Ile	Gly	Ala
5953/89 P3	Gly	Gly	Gly	Arg	Gln	Lys	Arg	Phe	Ile	Gly	Ala
6385/90 P3	Gly	Gly	Arg	Arg	Gln	Arg	Arg	Phe	Ile	Gly	Ala
Lentogenic											
F	Gly	Gly	Gly	Lys	Gln	Gly	Arg	Leu	Ile	Gly	Ala
V4 Que	Gly	Gly	Gly	Arg	Gln	Gly	Arg	Leu	Ile	Gly	Ala
8820/92 P3	Gly	Gly	Gly	Arg	Gln	Gly	Arg	Leu	Ile	Gly	Ala
1266/89 P3	Gly	Gly	Arg	Arg	Gln	Gly	Arg	Leu	Ile	Gly	Ala

sequences. All the virulent strains (velogenic and mesogenic) with the exception of 3410/92 P2 and 6385/90 P3 had a Lys residue at position 115. All the strains analyzed had an Arg residue at position 113 except for the F strain which has the Lys residue. The most striking characteristic from the sequence analysis is that the lentogenic strains have Gly and Leu at positions 115 and 117 respectively, whereas the velogenic and mesogenic strains have residues Lys/Arg and Phe at these positions.

Cleavage activation of the F protein precursor, F_0 to F_2 and F_1 is a prerequisite for viral infectivity and the expression of virulence (Peeters et al., 1999). The

cleavage of F_o results in a conformational change which exposes the hydrophobic region at the N terminus of F_1, thus promoting virus-host cell fusion (Millar *et al.*, 1988). The F_o cleavage site of virulent viruses (velogenic and mesogenic strains) is characterized by one pair (Collins *et al.*, 1994), two pairs (Collins *et al.*, 1993) or less frequently, a run (Oberdorfer and Werner, 1998) of basic amino acids and Phe at position 117. Viruses with these motifs are apparently more susceptible to cleavage by ubiquitous proteases that occur in a wide range of cell types (Nagai, 1995). Owing to this, some NDV strains are more virulent, some are less virulent and others are avirulent. In comparison, only single basic amino acids and Leu occur at the cleavage site of less virulent or the lentogenic strains (Toyoda *et al.*, 1989). Proteases that can process precursors with these motifs are found in only a few cell types and thus, the infection is therefore restricted. Cleavage usually takes place between Arg[116] and Phe/Leu[117] although for some isolates, cleavage might also take place between Lys/Arg[113] and Gln[114] (Gorman *et al.*, 1990).

We had previously isolated a fusion phage carrying the peptide sequence CTLTTKLYC from a phage display peptide library biopanned against NDV strain AF2240 (Ramanujam *et al.*, 2002). Using this isolated fusion phage as a pathotyping reagent, isolates 2641/91 P2, 5953/89 P3, 1266/89 P3 and 8820/92 P3 were found to have clustered together in a group represented by mesogens and lentogens (Ramanujam *et al.*, 2004). This result suggests that the fusion phage could be used to do a preliminary delineation of isolates into groups and then virulence can be verified by sequence analysis.

ACKNOWLEDGEMENTS

REFERENCES

Alexander, D.J. (1990). Avian Paramyxoviridae-recent developments. In: Advances in Veterinary Virology. Edwards, S. and Pastoret, P.P. (Eds). Elsevier, New York. pp. 103-114.

Chambers, P., Millar, N.E. and Emmerson, P.T. (1986). Nucleotide sequence of the gene encoding the fusion glycoprotein of Newcastle disease virus. *J Gen Virol.* 67: 2685-2694.

Choppin, P.W. and Scheid, A. (1980). The role of viral glycoproteins in adsorption, penetration and pathogenicity of viruses. *Rev Infect Dis.* 2: 40-61.

Collins, M.S., Bashiruddin, J.B. and Alexander, D.J. (1993). Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. *Arch Virol.* 128: 363-370.

Collins, M.S., Strong, I. and Alexander, D.J. (1994). Evaluation of the molecular basis of pathogenecity of the variant Newcastle disease viruses termed "pigeon PMV-1 viruses". *Arch Virol.* 134: 403-411.

Gorman, W.L., Gill, D.S., Scroggs, R.A. and Potner, A. (1990). The haemagglutinin-neuraminidase glycoproteins of human parainfluenza virus type 1 and Sendai virus have high structure-function similarity with limited antigenic relatedness. *Virol.* 175: 211-221.

Gotoh, B., Ohnishi, Y., Innocencio, M., Esaki, E., Nakayama, K., Barr, P.J., Thomas, G. and Nagai, Y. (1992). Mammalian subtilisin-related proteinases in cleavage activation of the paramyxoviruses fusion glycoprotein: superiority of furin/PACE to PC2 or PC1/PC3. *J Virol.* 66: 6391-6397.

Jordan, F.T.W. (1990). Newcastle Disease. In: Poultry Diseases. Baillieve Tindall, London. pp. 123-132.

Kho, C.L. (1997). Nucleotide sequence analysis of the F cleavage sites in velogenic NDV isolates. B.Sc (Hons) Thesis, Universiti Putra Malaysia.

Lamb, R.A. and Kolakofsky, D. (1996). Paramyxoviridae: the viruses and their replication. In: Fields Virology. Fields, B.N., Knipe, D.M. and Howley, P.M. (Eds.). Philadelphia, PA: Lippincott-Raven. pp. 1177-1203.

Millar, N.S., Chambers, P. and Emmerson, P.T. (1988). Nucleotide sequence of the fusion and haemagglutinin-neuraminidase glycoprotein genes of Newcastle disease virus strain Ulster: molecular basis for variations in pathogenicity between strains. *J Gen Virol,* 69: 613-620.

Nagai, Y. (1995). Virus activation by host proteinases. A pivotal role in the spread of infection, tissue tropism and pathogenicity. *Microbiol Immunol.* 39: 1-9.

Oberdorfer, A. and Werner, O. (1998). Newcastle disease virus: detection and characterization by PCR of recent German isolates differing in pathogenecity. *Avian Pathol.* 27: 237-243.

Peeters, B.P., de Leeuw, O.S., Koch, G. and Gielkins, A.J.L. (1999). Rescue of Newcastle disease virus from cloned cDNA: evidence that cleavability of the fusion protein is a major determinant for virulence. *J Virol.* 73: 5001-5009.

Ramanujam, P., Tan, W.S., Nathan, S. and Yusoff, K. (2002). Novel peptides that inhibit the propagation of Newcastle disease virus. *Archives of Virology*. **147**: 981-993.

Ramanujam, P., Tan, W.S., Nathan, S. and Yusoff, K. (2004). Pathotyping of Newcastle disease virus with a filamentous bacteriophage. *BioTechniques*. **36**: 296-300.

Salih, O., Omar, A.R., Ali, A.M. and Yusoff, K. (2000). Nucleotide sequence analysis of the F protein gene of a Malaysian velogenic NDV strain AF2240. *J Mol Biol Biochem Biophy*. **4**: 51-57.

Tan, S.W., Aini, I., Omar, A.R., Yusoff, K. and Tan, W.S., (2001). Phylogenetic analysis of recently isolated NDV. In: Proceedings of the 13th National Biotechnology, Penang Malaysia. pp. 79-81.

Toyoda, T., Sakaguchi, T., Hirota, H., Gotoh, B., Kuma, K., Miyata, T. and Nagai, Y. (1989). Newcastle disease virus evolution II. Lack of gene recombinant in generating virulent and avirulent strains. *Virol*. **169**: 273-282.

Yusoff, K., Nesbit, M., McCartney, H., Meulemans, G., Alexander, D.J., Collins, M.S., Emmerson, P.T. and Samson, A.C.R. (1989). Location of neutralizing epitopes on the fusion protein of Newcastle disease virus strain Beaudette C. *J Gen Virol*. **70**: 3105-3109.

Yusoff, K., Ng, B.K., Tan, W.S., Lau, C.H. and Ibrahim, A.L. (1993). Detection of Malaysian field isolates of Newcastle disease virus in allantoic fluid by RNA-polymerase chain reaction. *Asia-Pacific J Mol Biol Biotechnol*. **1**: 108-112.

Yusoff, K. and Tan, W.S. (2001). Newcastle disease virus: macromolecules and opportunities. *Avian Path*. **30**: 439-455.

Yusoff, K., Tan, W.S., Lau, C.H., Ng, B.K. and Ibrahim, A.L. (1996). Sequence of the haemagglutinin-neuraminidase gene of Newcastle disease virus oral vaccine strain V4 (UPM). *Avian Path*. **25**: 837-844.