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Site-directed mutagenesis of the C-terminal of the Newcastle disease virus V protein

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Summary. – Newcastle disease virus (NDV) is a paramyxovirus that is highly pathogenic to poultry causing severe economic loss worldwide. The non-structural V protein is one of the virulence factors of the virus. It antagonises the interferon of the host innate immunity in order to allow successful virus replication in the host cells. However, detailed investigation of recombinant NDV expressing mutated V protein is scarce. In this study, a mesogenic recombinant NDV expressing GFP (rAF-GFP) was used to investigate the relation of V protein mutation on virus pathogenicity. Site-directed mutagenesis was performed using overlapping PCR to introduce four premature stop codons 456G>T, 537G>T, 624C>T and 642G>T in the V gene reading frame. The virus was then rescued and propagated in embryonated chicken eggs. However, instead of the substituted thymine, this nucleotide was mutated into cytosine in three rescued mutants, while 537G>T mutant could not be rescued. As a result, the premature stop codon was substituted with other amino acid and the V protein was expressed in full length. The pathogenicity type of the rAF (456G>T>C), rAF (624C>T>C), and rAF (642G>T>C) mutants remained to be as in mesogenic strains, suggesting that substituted amino acids were functionally interchangeable with the original amino acids present in V protein. It appears that an intact V protein is important for the virus survival. This study explored the possibility of V protein mutation in NDV through exploiting genetic engineering and warrants a further investigation on modifying mutations on a conserved protein in NDV or other paramyxoviruses.

Keywords: Paramyxoviridae; Newcastle disease virus; V protein; C terminal; virulence factor

Introduction

Newcastle disease (ND) is a highly contagious viral disease among avian species that can cause severe economic loss to the poultry industry worldwide. It is caused by the Newcastle disease virus (NDV), an avian paramyxovirus serotype 1, APMV-1, that belongs to a newly classified genus *Orthoavulavirus* within the subfamily *Avulavirinae*, the family *Paramyxoviridae*, and the order *Mononegavirales* (Ganar *et al*., 2014; Amarasinghe *et al*., 2019). The genomic constituent of NDV is single-stranded, negative-sense ribonucleic acid (RNA) which consists of six genes encoding six proteins, namely, nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase protein (HN), and large protein (L). NDV isolates are categorised into three groups based on the severity of disease they cause.

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Abbreviations: GFP = green fluorescent protein; ICPI = intracerebral pathogenicity index; IFN = interferon; MDT = mean death time; MDA-5 = melanoma differentiation-associated gene 5; ND = Newcastle disease; NDV = Newcastle disease virus; NP = nucleocapsid protein; PIV-5 = parainfluenza virus 5; SPF = specific pathogen free; STAT = signal transducer and activator of transcription

Lentogenic strains such as LaSota and Hitchner B1 that are non-virulent are used as live vaccines worldwide to protect poultry against ND (Hitchner and Johnson, 1948; De Leeuw and Peeters, 1999). Mesogenic strains have intermediate virulence whereas viruses that cause the highest mortality rate in poultry are the velogenic strains (Huang *et al.*, 2003). The pathogenicity of NDV can be quantitated through mean death time (MDT), intravenous pathogenicity index (IVPI) and intracerebral pathogenicity index (ICPI) (Cattoli *et al.*, 2011).

AF2240-I is a local viscerotropic velogenic strain that has a total genome length of 15,192 nucleotides and normally used as a vaccine challenge virus in Malaysia (Murulitharan *et al.*, 2013) with MDT of 48, ICPI of 1.90 and IVPI of 2.56 (Lai and Ibrahim, 1987; Lee *et al.*, 2006). The pathogenicity of this virus is contributed by the presence of multi-basic cleavage site of fusion (F) protein, which is also a recognition site for cellular furin protease (Lamb and Parks, 2007). In addition, the virulence of the virus is also contributed by the V protein, an interferon (IFN) antagonist that degrades phosphorylated signal transducer and activator of transcription (STAT) 1 protein instead of unphosphorylated STAT1 protein. STAT 2 protein, however, is not degraded in NDV infected cells (Mebatsion *et al.*, 2001; Huang *et al.*, 2003; Park *et al.*, 2003ab; Qiu *et al.*, 2016). In this study, the transcription vector pOLTV5 bearing the antigenome of a velogenic NDV strain AF2240-I, known as rAF-GFP, was used as a template to introduce the mutations. Presence of foreign gene such as green fluorescent protein (GFP) gene in between the M and F genes had reduced the pathogenicity of AF2240-I from velogenic to mesogenic. Similar case has been shown when introducing a foreign gene such as chloramphenicol acetyltransferase (CAT) into mesogenic NDV, that caused an increased MDT and decreased ICPI (Krishnamurthy *et al.*, 2000).

The carboxyl terminal domain of V protein was found to contribute to the anti-IFN activity of NDV (Mebatsion *et al.*, 2001; Huang *et al.*, 2003; Park *et al.*, 2003a,b; Qiu *et al.*, 2016). It has one invariant histidine and seven conserved cysteines that bind to two atoms of zinc (Ramachandran and Horvath, 2010; Wang *et al.*, 2019). The V protein of NDV together with those from other paramyxoviruses such as parainfluenza virus type 5, Sendai virus and mumps virus are able to interact with the helicase domain of cellular RNA helicase, melanoma differentiation-associated gene 5 (MDA-5) through their highly conserved C-terminal domains (Childs *et al.*, 2007, 2009). This interaction causes the failure of MDA-5 to activate interferon regulatory factor 3 (IRF-3) and nuclear factor kappa B (NF-κB) in response to synthetic double-stranded RNA poly(I-C) resulting in the failure of IFN-β gene transcription (Childs *et al.*, 2007). Qiu *et al.* (2016) demonstrated that cells infected by wildtype NDV with an intact V protein has lower transcription level of IFN responsive genes when compared to cells infected by mutant NDV lacking the C-terminal domain of the V protein. This has further proven that the integrity of the C-terminal of the V protein is important in antagonising the IFN activity.

To our knowledge, there is no report on the introduction of mutations on different locations at the C-terminal of the V protein from a mesogenic NDV. Therefore, this research aimed to introduce several mutations at the C-terminal of V gene reading frame on the pOLTV5 (rAF-GFP), full-length NDV antigenome of strain AF2240-I expressing GFP to study the virulence of mutant NDV.

Materials and Methods

Construction of plasmids with NDV V protein mutation. The full-length sequence of NDV AF2240-I was cloned into a low-copy-number transcription vector pOLTV5 (Murulitharan *et al.*, 2013). This plasmid, known as pOLTV5 (rAF-GFP) is the antigenomic cDNA for AF2240-I and it was used as the template to introduce the mutation into V protein gene. The plasmid and its glycerol stock were provided by Virology Laboratory, Department of Microbiology, Faculty Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. A total of four mutations were designed, namely 456G>T, 537G>T, 624C>T and 642G>T (Fig. 1). The mutations were designed to introduce a stop codon into the V protein. The selection of a number of mutations was to ensure an unbiased study on the correlation of a mutation and its consequences in the absence of detailed information from other sources or studies. The numbering before the nucleotide indicates the position of the nucleotide, counting from the start codon of P gene. The insert was generated using overlapping PCR that combines two fragments with mutated nucleotide at the complementary end. Primers used for introducing mutation are shown in Table 1. All of the mutated fragments

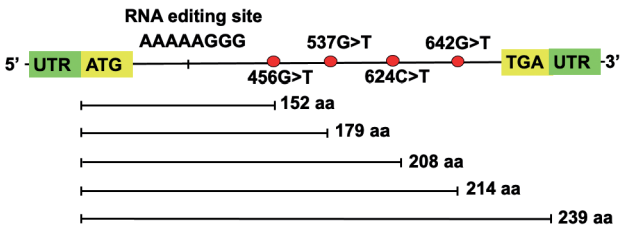

Fig. 1

Schematic diagram of mutation location and the length of V protein generated

The original length of V protein consists of 239 amino acids whereas the mutation generates a stop codon which results in different length of V protein.

Table 1. Sequences of primers used in overlapping PCR for substitution of nucleotides

Primer	Primer sequence	T_m
P1_456G>T	5'-^{2334}ACCCAACAACATGG**T**GATCAGCCGA^{2358}-3'	62.7°C
P2_456G>T	5'-^{2358}TCGGCTGATC**A**CCATGTT GTTGGGT^{2334}-3'	
P3_537G>T	5'-^{2418}GGCACAGACG C**T**AGCACAGCATAT^{2441}-3'	61.3°C
P4_537G>T	5'-^{2441}ATATGCTGTGCT**A**GCGTCTGTGCC^{2418}-3'	
P5_624C>T	5'-^{2504}GCAGAGCCAAGA**T**AGTACTCCTGTACC^{2530} -3'	58°C
P6_624C>T	5'-^{2530}GGTACAGGAGTACT**A**TCTTGGCTCTGC^{2504}-3'	
P7_642G>T	5'-^{2522}TCCTGTACCTGT**T**GATCATGTCCA^{2545}-3'	57.6°C
P8_642G>T	5'-^{2545}TGGACATGATC**A**ACAGGTACAGGA^{2522}-3'	

The underlined and bold nucleotide is the substituted nucleotide. The substituted nucleotide normally locates 10–15 bases away from the 5' terminus of the primer. P1 to P8 were used for introduction of stop codon by substituting guanine (456G>T, 537G>T, 642G>T) or cytosine (624C>T) into thymine.

share the same forward (5'-^{18218}TTACCGCCTTTGAGTGAGCT GATACCG^{18244}-3') and reverse primer (5'-^{3080}TCAACCATTCAGCG CAAGGCGTTTGAT^{3053}-3') which are upstream and downstream of *BsiW*I and *Pac*I sites, respectively. Taking mutation 456G>T as an example, the first fragment was amplified using primer containing *BsiW*I site and P2 whereas the second fragment was amplified using P1 and primer containing *Pac*I site from the template pOLTV5 (rAF-GFP). The cycling parameters were 1 cycle at 95°C/1 min, 30 cycles of 95°C/15 s, 60°C/15 s, 72°C/1 min 30 s followed by 72°C/2 min for final extension. Both fragments were fused through overlapping PCR with profile of 98°C/30 s, 30 cycles of 98°C/10 s, 68°C/15 s, and 72°C/1 min 45 s followed by a final extension step at 72°C for 5 min. The fragments and pOLTV (rAF-GFP) were double-digested with *BsiW*I and *Pac*I

Table 2. Schematic representation of P mRNA and V mRNA for mutant NDV
(addition of one guanine frameshifts the P mRNA into V mRNA)

				456G>T				
P mRNA	CCU	CCG	ACC	CAA	CAA	CAU	GG***u***	GAU
	P	P	T	Q	Q	H	G	D
V mRNA	ACC	UCC	GAC	CCA	ACA	ACA	UGG	***u***GA
	T	S	D	P	T	T	W	***stop***
				537G>T				
P mRNA	GGA	AGC	CGG	GGC	ACA	GAC	GC***u***	AGC
	G	S	R	G	T	D	A	S
V mRNA	UGG	AAG	CCG	GGG	CAC	AGA	CGC	***u***AG
	W	K	P	G	H	R	R	***stop***
				624C>T				
P mRNA	CAA	UCA	GGG	CAG	AGC	CAA	GA***u***	AGU
	Q	S	G	Q	S	Q	D	S
V mRNA	CCA	AUC	AGG	GCA	GAG	CCA	AGA	***u***AG
	P	I	R	A	E	P	R	***stop***
				642G>T				
P mRNA	GAC	AGU	ACU	CCU	GUA	CCU	GU***u***	GAU
	D	S	T	P	V	P	V	D
V mRNA	AGA	CAG	UAC	UCC	UGU	ACC	UGU	***u***GA
	R	Q	Y	S	C	T	C	***stop***

The amino acids encoded by the nucleotides are indicated below the nucleotide sequence. The mutated nucleotide is in bold and italics whereas the location is shown above.

and ligated at 4°C overnight. The resulting recombinant plasmids were transformed and confirmed by sequencing. Positive clones were designated as pOLTV5 (rAF 456G>T GFP), pOLTV5 (rAF 537G>T GFP), pOLTV5 (rAF 624C>T GFP) and pOLTV5 (rAF 642G>T GFP). The mutation in V mRNA (italic lower case) did not change the amino acid content in P reading frame as indicated in Table 2. The project has been approved by the Institutional Biosafety Committee for genetic modification organism research prior to its start.

Cells and viruses. DF1 (continuous cell line of chicken embryo fibroblasts, CRL-12203), HEK293 (human embryonic kidney, CRL-1573), HT29 (human colorectal adenocarcinoma cell line, HTB-38) and Vero cells (kidney epithelial cells from African green monkey, CCL-81) were obtained from Virology Lab 1, Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. These cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, USA) supplemented with 10% foetal bovine serum (Gibco, UK). BSR T7/5 (baby hamster kidney derived cell line stably expressing T7 RNA polymerase, created by Buchholz *et al.* (1999) was maintained in Glasgow's Minimum Essential Medium (GMEM) (Sigma-Aldrich, USA) supplemented with 10% new born calf serum (Himedia, India), 1% (v/v) tryptose phosphate and 1% (v/v) Minimum Essential Medium (MEM) nonessential amino acids (Buchholz *et al.*, 1999). The cells were incubated at 37°C with 5% CO_2. The recovered mutant NDVs from transfection supernatant were propagated in 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs.

Transfection and recovery of mutant viruses. BSR T7/5 cell line with phage T7 RNA polymerase expression was seeded at concentration of 5.05×10^5 cells/well in a 6 well plate to achieve 80% confluency on the day of transfection. The verified plasmid with respective mutation (1 µg) was co-transfected with 0.4 µg of pCI-neo NP, 0.2 µg of pCI-neo P, and 0.2 µg of pCI-neo L (Peeters *et al.*, 1999; Mebatsion *et al.*, 2001; Cheow *et al.*, 2019) using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's instructions. Transfected cells were incubated at 37°C with 5% CO_2 for several days until fluorescence could be observed under fluorescence microscope (NIKON, Japan). Transfection supernatant was harvested and inoculated into the allantoic cavity of 9-day-old specific pathogen free (SPF) embryonated eggs for virus propagation and amplification.

Reverse transcription-PCR and sequence analysis. When the embryos died, 300 µl of allantoic fluid was harvested and subjected for RNA extraction using TRIzol (Ambion, USA). The extracted RNA was reverse transcribed into complementary DNA (cDNA) using a SensiFAST cDNA Synthesis kit (Bioline, Australia) according to the manufacturer's protocol. Using cDNA as template, the whole P gene region was amplified using forward primer 5'-ATGGCTACCTTTACAGATGCGGAG-3' and reverse primer 5'-TCAACCATTCAGCGCAAGGCGTTT-3' that flank the P gene region. The PCR product was purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Netherlands) and sent for sequencing using the aforementioned forward and reverse primer to determine the sequence at the site of mutation.

Continuous passaging in five cell lines. The same transfection supernatant containing mutant virus that was used to infect the embryonated eggs was further passaged in BSR T7/5, DF1, HEK293, HT29 and Vero cells. The five cell lines mentioned above were seeded onto 24-well plates. Different cell density was seeded based on the size and the growth rate of the cell lines. The cell seeding density for BSR T7/5 and HEK293 was 2.5×10^5 cells/well whereas for DF1 was 2×10^5 cells/well. On the other hand, the cell seeding density for HT29 and Vero cells was 3.5×10^5 cells/well and 1.2×10^5 cells/well, respectively in 400 µl of media in each well. After overnight incubation for cell attachment, the cell monolayers were infected with 100 µl of transfection supernatant. The cells were incubated at 37°C for 4 days. The supernatant was collected on day 4 after centrifugation at 1000 rpm for 5 min to remove cell debris. This is considered as the first passage infection supernatant. The infection was repeated until passage 10 by taking 100 µl of the infected supernatant from the previous passage and re-infected in the subsequent passages. This is because the mutant viruses might require more than two cell culture passages in order to produce cytopathic effects due to the abolishment of the V protein expression that delays the viral replication time (Mebatsion *et al.*, 2001). RNA extraction was performed using infection supernatant from 10^{th} passage and subjected to reverse transcription-PCR (RT-PCR) amplification.

Determination of mean death time (MDT). Determination of MDT was conducted as described previously (Alexander, 1989). Fresh allantoic fluid harvested from infected embryonated eggs was diluted in sterile phosphate buffer saline in order to generate a 10-fold dilution series between 10^{-3} and 10^{-12}. Up to 100 µl of diluted allantoic fluid was inoculated into allantoic cavity of each of five 9-day-old embryonated SPF chicken eggs and the eggs were incubated at 37°C. The remaining virus dilutions were kept at 4°C. After 8 h, 100 µl of the diluted allantoic fluid was inoculated into another five eggs and placed at 37°C. This is to produce a batch of 10 eggs with 8 h intervals. Each egg was examined twice daily for 7 days and the times of any embryo death was recorded. The minimum lethal dose is the highest virus dilution that causes all the embryos inoculated with that dilution to die. The MDT is the mean time in hours for the minimum lethal dose to kill embryos.

Results

Incubation period and the relative total of fluorescent cells vary in different NDV mutants

Four mutations on V protein (456G>T, 537G>T, 624C>T and 642G>T) were carried out on the plasmid containing

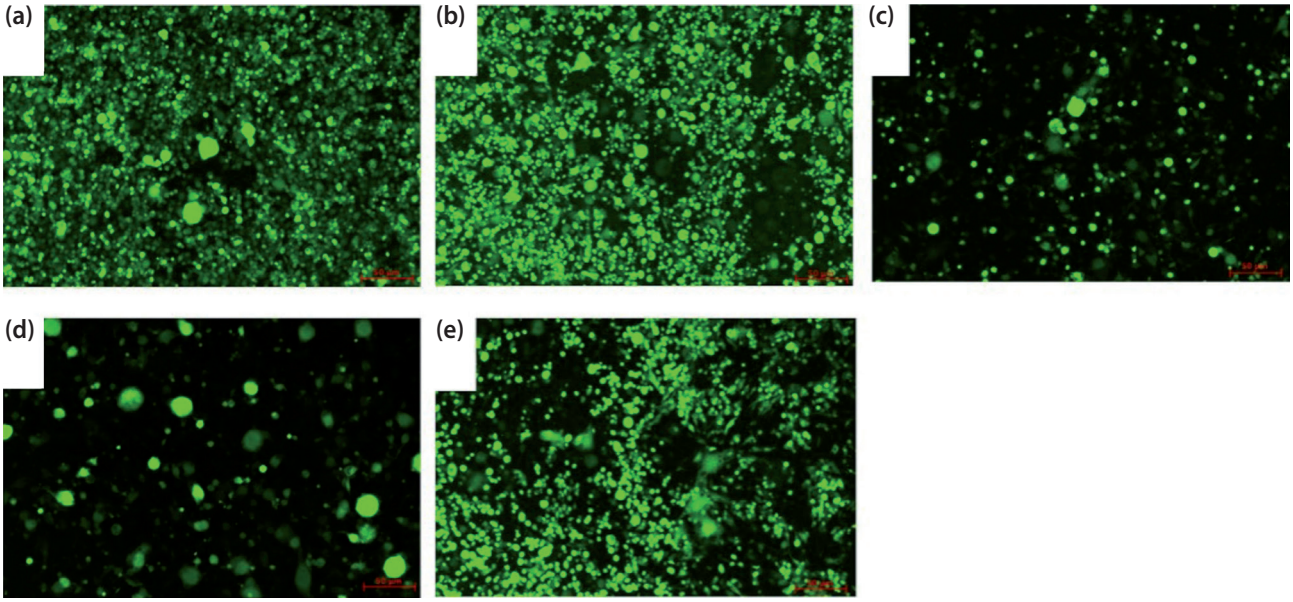

Fig. 2

Fluorescence micrographs of rescued viruses
BSR-T7/5 cells were co-transfected with three helper plasmids: pCI neo-NP, pCI neo-P, pCI neo-L together with the mutated recombinant plasmid. The relative total of fluorescent cells of **(a)** rAF-GFP, **(b)** rAF (456G>T), and **(e)** rAF (642G>T) were greater than **(c)** rAF (537G>T) and **(d)** rAF (624C>T). Magnification, 100x.

full-length cDNA of NDV AF2240-I, pOLTV5 (rAF-GFP) as shown in Table 2. All of the sequences of transformants were aligned with pOLTV5 (rAF-GFP) as a control to determine the nucleotide substitution using MEGA 7. From the sequencing result, there is only a single peak under each base position indicating the absence of any contamination of the unmutated wild type. After verification of each of the mutations, each of the recombinant plasmids together with three helper plasmids encoding NP, P and L genes were transfected into BSR T7/5 to rescue the mutant virus. Due to the presence of the GFP gene between the M and F genes, the BSR T7/5 cells exhibited green fluorescence a few days post-transfection. In transfection, there were two main observations that could be compared in each mutant virus; the period of incubation and the relative total of fluorescent cells. In terms of the incubation period, different mutant NDVs required different periods of incubation until fluorescence could be observed. For example, rAF (537G>T) and rAF (624C>T) required 14 days of incubation whereas bright fluorescence can be observed within 7 days in rAF (456G>T) and rAF (642G>T) mutant. Although the incubation period for rAF (537G>T) and rAF (624C>T) was longer than in rAF (456G>T) and rAF (642G>T), the relative total of fluorescent cells remained lower. Therefore, the incubation period did not have any correlation with the relative total of fluorescent cells. Besides, the relative total of fluorescent cells of rAF (456G>T) and rAF (642G>T) were comparable to the positive control wildtype rAF-GFP whereas both of these mutants had twice the number of relative totals of fluorescent cells than rAF (537G>T) and rAF (624C>T) (Fig. 2).

Three out of four NDVs with V protein mutations were successfully rescued

After 7 day incubation of rAF-GFP, rAF (456G>T), rAF (642G>T) and 14 day incubation of rAF (537G>T) and rAF (624C>T), the transfection supernatants containing the recovered mutant virus were harvested and inoculated into 9-day-old embryonated eggs for virus propagation. Upon the embryo death, allantoic fluid was harvested for viral genomic RNA extraction using Trizol (Ambion, USA). RT-PCR was conducted to amplify the fragment spanning the P gene. However, no RT-PCR products were obtained from rAF (537G>T). These results indicated the failure of mutant NDV recovery for mutation 537G>T although fluorescence was observed during transfection. Our results imply that fluorescence of transfected cells is not an indicator of successful virus recovery. Since the development of innate immune system is corresponding to the age of the embryonated egg, we have tried to propagate the mutant NDV in 6-day-old embryonated SPF eggs (Sekellick *et al.*, 1990). Still, both 6-day-old and 10-day-old

Table 3. Summary of mutation changes from plasmid to mutant NDV recovered from transfection

Mutation	P protein			V protein			Virus recovery
	Original	Mutated (plasmid)	Mutant virus	Original	Mutated (plasmid)	Mutant virus	
456G>T	GG**G** (Gly)	GG**T** (Gly)	GG**C** (Gly)	**G**GA (Gly)	**T**GA (Stop)	**C**GA (Arg)	Yes
537G>T	GC**G** (Ala)	GC**T** (Ala)	GC**C** (Ala)	**G**AG (Glu)	**T**AG (Stop)	-	No
624C>T	GA**C** (Asp)	GA**T** (Asp)	GA**C** (Asp)	**C**AG (Gln)	**T**AG (Stop)	**C**AG (Gln)	Yes
642G>T	GT**G** (Val)	GT**T** (Val)	GT**C** (Val)	**G**GA (Gly)	**T**GA (Stop)	**C**GA (Arg)	Yes

The substituted nucleotide in the plasmid is from guanine or cytosine to thymine. However, the substituted thymine is mutated to cytosine uniformly in recombinant viruses. The substitution of thymine to cytosine maintains the amino acids in P reading frame but not in V protein. Only three out of four mutant NDVs with V protein mutations were successfully rescued.

embryonated eggs were unable to recover rAF (537G>T) (data not shown).

On the other hand, for the recovered viruses, confirmation of the introduced mutations was obtained by sequencing. A total of eight independent rescue attempts from the transfection in cells until injection in embryonated eggs was conducted. Surprisingly, all the thymine introduced in the mutations were changed into cytosine uniformly in every mutant virus, 456G>T>C, 624C>T>C and 642G>T>C in every rescue attempt (Fig. 3). The substitution of thymine to cytosine retained the amino acid in P protein but the stop codon was mutated into amino acid in V protein (Table 3). In rAF (456G>T) and rAF (642G>T), the amino acid changes from wildtype glycine to stop codon after mutation and to arginine upon virus recovery. However, the amino acid of rAF (624C>T) mutant changed from stop codon to glutamine which is the same as in the wildtype. This is because the nucleotide change after recovery was cytosine which is the same as before mutation. In short, we suspect that the mutation of thymine into cytosine is the reason for the successful virus recovery.

Mutation of thymine into cytosine in mutant NDVs occurred also in cell cultures

In order to determine whether the occurrence of this nucleotide change phenomenon was either host-dependent or host-independent, repeated passages in cell cultures were conducted. In order to maintain the consistency, same source of transfection supernatant containing recovered virus was used for both injection in embryonated eggs as well as passaging in BSR T7/5, DF1, HEK293, HT29 and Vero cell lines. BSR T7/5 cell line was used for transfection whereas DF1, a chicken embryo fibroblast cell line, was used as host for NDV. HEK293 cells are normal human kidney cells, while HT29 cells are human colorectal cancer cells. HEK293 and HT29 cell lines were not used as host cells for NDV, but to determine the effects of human cells on NDV replication. In addition, the Vero cell line was chosen because it is defective in IFN production (Desmyter *et al.*, 1968).

Infectious supernatant from the cell culture was collected at the 10th passage and used for RNA extraction in order to check the mutated sequence. The converted cDNA was used as a template for amplification of P gene which is similar to the downstream procedure after extracting RNA from allantoic fluid. Remarkably, the mutant NDVs that could be detected in all of the cell lines were rAF (456G>T), rAF (624C>T), and rAF (642G>T), which all had the initial mutated thymine changed into cytosine. These results were consistent with the results obtained from mutant NDVs propagated in allantoic fluid. The growth rate of mutant NDV and wildtype NDV was not much different in terms of the relative total of fluorescent cells even in Vero cells. Similar observations were also made in other cell lines such as BSR-T7/5 cells, DF1 cells, HEK293 cells and HT29 cells. In conclusion, mutant NDVs that were successfully recovered during transfection can further infect various cell lines whereas the mutant NDVs that could not be rescued remain undetectable in any of the cell line supernatant.

The pathotypes of rescued mutant NDVs are mesogenic

Mean death time (MDT) was carried out to determine the pathogenicity of the mutant NDVs by calculating the mean time of death of the virus-inoculated egg. The wildtype AF2240-I and rAF-GFP were set as the positive controls. The minimum lethal dose is the highest virus dilution where all the embryonated eggs inoculated within

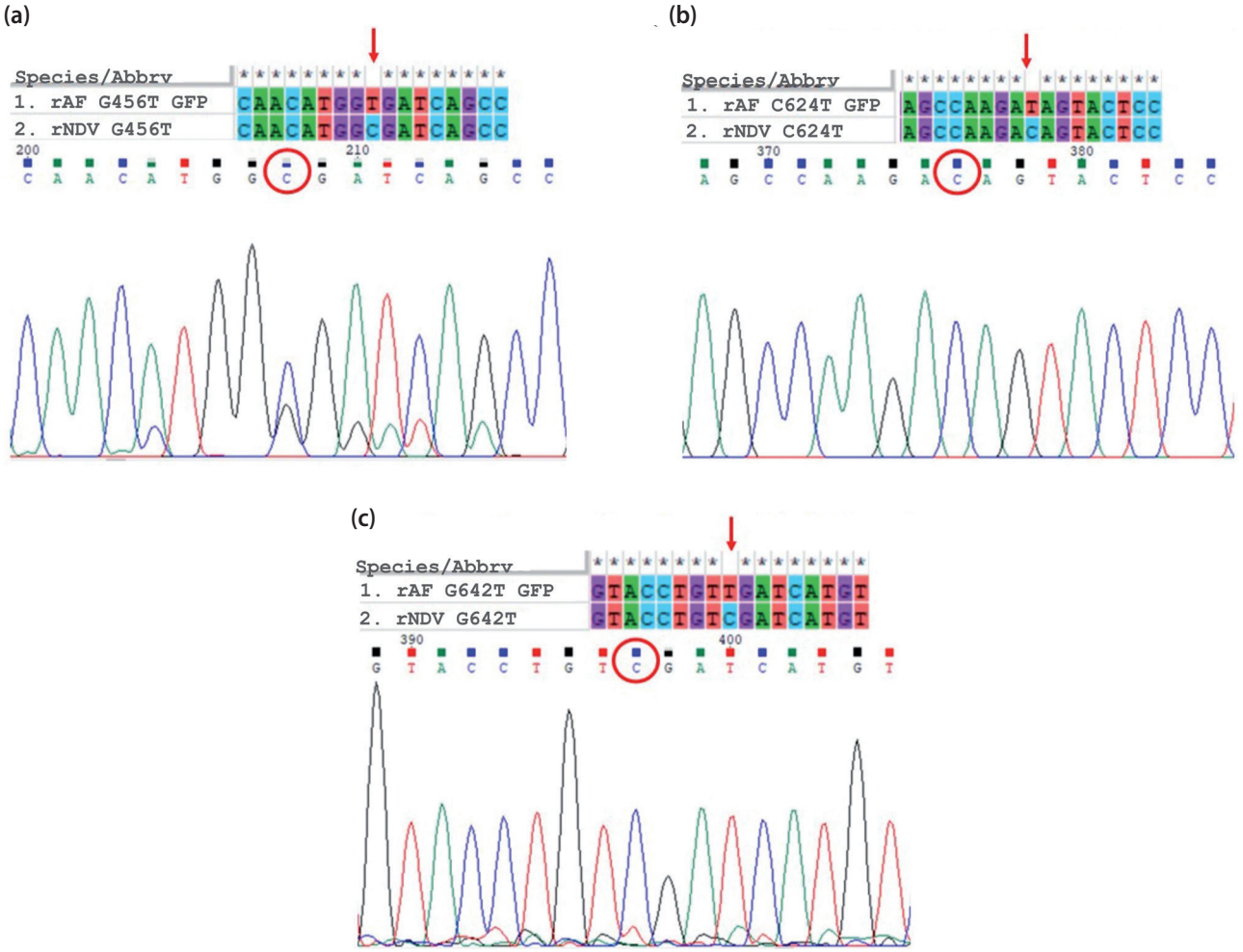

Fig. 3

Sequence alignment of V ORF

Sequence alignment of V ORF between the recombinant plasmid and mutant NDV mutation **(a)** 456G>T, **(b)** 624C>T and **(c)** 642G>T using MEGA 7. **(a)** The nucleotide substitution at position 456 (nucleotide with red circle) was mutated from thymine to cytosine. There were two peaks at position 456 which are guanine and cytosine. From the sequencing pattern, the peak at the bottom is almost the same as from the previous signal. This peak was suspected to generate from the V PCR products that is frameshifted from P gene. Therefore, the peak of guanine at the position 456 came from the previous nucleotide which is guanine. **(b)** The nucleotide substitution at position 624 (nucleotide with red circle) was mutated from thymine to cytosine. **(c)** The nucleotide substitution at position 642 (nucleotide with red circle) was mutated from thymine to cytosine.

Table 4. Summary of MDT (h) and pathotype of the wildtype and mutant viruses

Virus	MDT (h)	Pathotype
AF2240-I	59.2	Velogenic
rAF-GFP	75.2	Mesogenic
rAF (456G>T)	64.8	Mesogenic
rAF (624C>T)	74.4	Mesogenic
rAF (642G>T)	81.6	Mesogenic

Velogenic viruses need less than 60 h to kill the embryos whereas mesogenic NDVs need 60–90 h to kill the embryos (lentogenic strain do not kill the eggs even after 90 h).

the dilutions are dead. Based on the observation of embryo deaths for 7 days, rAF-GFP and the three mutant NDVs were mesogenic. The minimum lethal dose and MDT for each virus is presented in Table 4.

Discussion

The C-terminal of the V protein is known to possess IFN antagonistic activity, and mutant NDVs without the C-terminus of the V protein were unable to replicate in host cells, suggesting that intact V protein is important to

counteract host antiviral response pathways (Mebatsion *et al*., 2001; Huang *et al*., 2003; Park *et al*., 2003a,b; Rama chandran and Horvath, 2010). However, the precise domains that are involved in this effect are unknown. In this study, four mutations were introduced into the C-terminal of the P gene. During RNA editing, additional of one non-templated guanine at the conserved editing locus of P gene, AAAAAGGG (antigenome sense) leads to the frameshift mutation. Hence the P and V proteins share a common N-terminus but have different C-termini. Point-nonsense mutations were introduced in this study by substituting selected nucleotides with thymine without disturbing the P open reading frame. As the P protein forms part of the RNA-dependent RNA polymerase (RdRp) complex, it is important for viral replication. Nucleotide substitutions were made only to the third position of codons in the P protein, and therefore the first position of codons in the V protein. This was to better evaluate the pathogenicity of recombinant viruses with different mutations to the V protein without compounding structural or functional changes in other proteins.

In this study, three out of four mutant NDVs [rAF (456G>T), rAF (624C>T), and rAF (642G>T)] were successfully rescued. However, sequencing revealed that all substituted thymine in rAF (456G>T), rAF (624C>T), and rAF (642G>T) had mutated to cytosines, resulting in the non-stop mutations of the intended premature stop codons in the V protein (Table 3). These substitutions occurring in mutant viruses recovered from both allantoic fluid and cell supernatants strongly suggest that the nucleotide changed is host-independent and the intact V protein is required for viral replication. This phenomenon was also observed by He *et al.* (2002), where two premature stop codons introduced by simultaneous thymine substitution into the V protein of parainfluenza virus 5 (PIV-5) spontaneously mutated to cytosine after four to five passages in Vero cells. Compared to the wildtype PIV-5, this resulted in the introduction of a glutamine where there was originally a phenylalanine and reversion back to a conserved arginine in the V protein at the first and second location respectively. This latter reversion suggests strong selective pressure for a functional C-terminus of the V protein in viral replication. Mutant PIV-5 cannot drive STAT1 degradation and therefore fails to block IFN signalling. However, further studies are required to elucidate the mechanism behind the remarkable consistency of mutations from thymine to cytosine rather than the original nucleotides, particularly as oxidative deamination normally drives transition of the opposite mutation, from cytosine to thymine.

The successful recovery and subsequent mutation of recombinant viruses such as rAF (456G>T), rAF (624C>T), and rAF (642G>T) could be driven by several

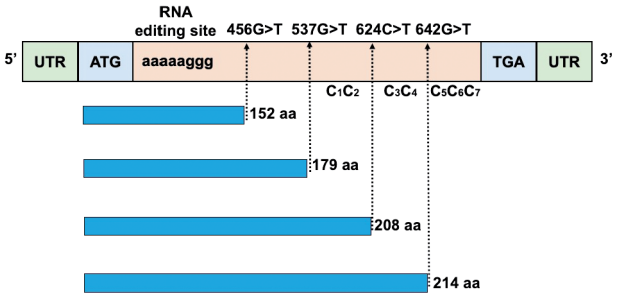

Fig. 4

Localisation of designed mutations and the seven conserved cysteine residues

Cysteine residues are indicated by C_1-C_7 at the carboxyl terminal of V protein. Mutation 456G>T and 537G>T removes all the cysteine residues whereas mutation 624C>T and 642G>T removes five and three cysteine residues, respectively. The sequence 'aaaaaggg' represents the RNA editing site.

factors. Firstly, most of the mutations occurred near the C-terminus of the V protein, which has seven conserved cysteine residues that contribute to pathogenicity and host interferon antagonist activity, as observed in other members of the *Paramyxoviridae* (Mebatsion *et al*., 2001; Huang *et al*., 2003; Park *et al*., 2003a,b; Qiu *et al*., 2016; Tham *et al*., 2019). For instance, mutation of second cysteine to a different amino acid in Nipah virus (NiV) and parainfluenza virus 5 (PIV-5) abolished MDA interference, and substitution of cysteine with alanine at position 189, 207, 214 in mumps virus reduced proteasome-mediated degradation of STAT1 (Kubota *et al*., 2002; Yokosawa *et al*., 2002; Ramachandran and Horvath, 2010). Our results suggest that the disruption to V protein function is even greater in the case of cysteine deletions, and potentially places selective pressure on the virus to accumulate non-stop mutations during virus recovery and restore V protein function. Notably, mutations 456G>T (resulting in a truncated protein of 152 amino acids) and 537G>T (179 amino acids) eliminated all cysteine residues, the first of which occurs at position 196 (Fig. 4). Furthermore, mutation 624C>T removed five cysteine residues whereas mutation 642G>T eliminated three cysteine residues. Interestingly, although both 456G>T and 537G>T involved the removal of all seven cysteines, only rAF (456G>T) was recovered, indicating a failure of rAF (537G>T) to force a non-stop mutation of thymine to cytosine at position 537. Similarly, the introduction of a stop codon after the RNA editing site in a mutant NDV generated by Mebatsion *et al.* (2001) resulted in the complete abrogation of replication in allantoic fluid. Likewise, Park *et al.* (2003a) observed that mutant NDV expressing truncated V protein without the C-terminus did not grow to measurable titres in chick em-

bryo fibroblasts (CEFs). Furthermore, Huang *et al.* (2003) found that the yield of mutant viruses with stop codons in the V protein decreased with the embryo gestational age. Our results and others suggest that truncation of the C-terminus of the V protein could affect the viral replication.

NDV V proteins with different pathotypes have varying levels of IFN antagonistic activity (Wang *et al.*, 2019). For example, the reduction of IFN-β expression by velogenic V protein is twice as strong as that induced by mesogenic V protein (Wang *et al.*, 2019). V protein from highly virulent NDV plays a much stronger contribution to viral replication and pathogenicity compared to lentogenic and mesogenic V proteins. These differences may also explain the variation in recovery rates of mutant NDVs after similar mutations.

Conclusion

This study is the first to report the introduction of a stop codon at different locations on the C-terminal of the NDV V proteins. Our data demonstrated that the successful recovery of mutant NDV was due to conversion of the intended nonsense mutations to non-stop mutations by thymine to cytosine transitions, restoring the C-terminus of the V protein and therefore pathogenicity of the mutant NDV. Since the pathogenicity of mutant NDV were not reduced, we can deduce that glycine at the location of 152 (456G>T) and 214 (642G>T) in V protein is functionally interchangeable with arginine. Future attenuation studies on the V protein should consider substitutions rather than truncations to obtain viable recombinant NDV because integrity of the V protein is crucial for host interferon antagonist activity. In conclusion, this study provides new insights into the role of the V protein, particularly its C-terminus, in recombinant NDV recovery.

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