Veterinary Research Communications, 31 (2007) 371–383
DOI: 10.1007/s11259-006-3502-2

Adaptation of a Velogenic Newcastle Disease Virus to Vero Cells: Assessing the Molecular Changes Before and After Adaptation

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ABSTRACT

A velogenic Newcastle disease virus isolate was passaged 50 times in Vero cell culture and the virus was assessed for the molecular changes associated with the passaging. At every 10th passage, the virus was characterized conventionally by mean death time (MDT) analysis, intracerebral pathogenicity index (ICPI) and virus titration. At increasing passage levels, a gradual reduction in the virulence of the virus was observed. Molecular characterization of the virus included cloning and sequencing of a portion of the fusion gene (1349 bp) encompassing the fusion protein cleavage site (FPCS), which was previously amplified by reverse transcription–polymerase chain reaction. Sequence analysis revealed a total of 135 nucleotide substitutions which resulted in the change of 42 amino acids between the velogenic virus and the 50th passage virus. The predicted amino acid motif present at the cleavage site of the virulent virus was ^{109}SRRRRQRRFVG^{119} and the corresponding region of the adapted adapted virus was ^{109}SGGRRQKRFIG^{119}. Pathogenicity studies conducted in 20-week-old seronegative birds revealed gross lesions such as petechial haemorrhages in the trachea, proventricular junction and intestines, and histopathological changes such as depletion and necrosis of the lymphocytes in thymus, spleen, bursa and caecal tonsils in the birds injected with the velogenic virus and absence of the lesions in birds injected with the adapted virus.The 50th-passage cell culture virus was back-passaged five times in susceptible chickens and subjected to virulence attribute analysis and sequence analysis of the FPCS region, with minor difference found between them.

Keywords: Newcastle disease virus, Vero cells, fusion gene, sequencing studies, pathogenicity testing

Abbreviations: AAF, amnioallantoic fluid; CPE(s), cytopathic effect(s); dNTPs, deoxynucleotide triphosphate; ECE, embryonated chicken eggs; FPCS, fusion protein cleavage site; HA, haemagglutination; HI, haemagglutination inhibition; ICPI, intracerebral pathogenicity index; MDT, mean death time; MuMLV, Moloney murine leukemia virus; ND, Newcastle disease; NDV, Newcastle disease virus; p.i. post inoculation; RT-PCR, reverse transcription-polymerase chain reaction; $TCID_{50}$, tissue culture infective dose 50%

INTRODUCTION

Newcastle disease is a globally distributed avian disease that causes severe economic losses in commercial poultry. The causative agent of the disease Newcastle disease virus (NDV) is a non-segmented, single-stranded negative-sense RNA virus belonging to the family *Paramyxoviridae*, sub-family *Paramyxovirinae*, genus *Avulavirus* (Mayo, 2002). The 15 kb genomic RNA encodes six virus proteins whose genes are located on the genome in the order 3′-NP-P-M-F0-HN-L-5′ (Alexander, 2001). The two glycoproteins haemagglutinin-neuraminidase (HN) and fusion protein (F) are exposed on the surface of the virion envelope

and are required for initiation of viral infection. The HN protein is responsible for attachment of virus particles to sialic acid-containing receptors on host cells. The F protein mediates the subsequent fusion of viral and cellular membranes during penetration. It is also required for fusion between infected and adjacent non-infected cells (Nagai *et al*., 1989).

F protein is an important determinant of NDV pathogenicity (Russell *et al*., 1990). It is synthesized as a precursor, F0, that is proteolytically cleaved to yield F1 and F2 polypeptides. Efficiency of proteolytic cleavage is dependent on the host cell and the virus strain (Nagai *et al*., 1979). F protein of highly virulent virus strains are characterized by the presence of two pairs of dibasic amino acid residues near the cleavage site, which are recognized by ubiquitous host cell proteases (Alexander, 2001). These stains exhibit a velogenic pathotype. In contrast, F protein of lentogenic NDV strains is cleavable in only a restricted number of cell types, which correlates with the presence of only two single basic amino acids at the cleavage site and concomitant restriction in proteolytic activation. Sequences of mesogenic strains of intermediate virulence for chickens contain two pairs of basic amino acid residues or a single arginine and a lysine–arginine pair (Collins *et al*., 1996). Huang and colleagues 2004 recently reported that the haemagglutinin neuraminidase protein of NDV determines the tropism and virulence of the virus. In a recent study we have found that a velogenic virus isolate, when adapted to chicken embryo fibroblast cell culture system, did not produce any change in the amino acid motif of the fusion protein cleavage site even after 50 passages with marked reduction in the biological virulence of the virus (Madhan Mohan *et al*., 2005). With this background, the present study was undertaken to assess at gene level the changes taking place in a virulent field isolate of NDV pertaining to the fusion protein gene when serially passaged to Vero cells, and to then correlate these changes with both biological and pathogenicity studies.

MATERIALS AND METHODS

Virus

The velogenic NDV isolate used in this study was isolated from a 20-week-old layer bird from Namakkal, Tamil Nadu, India. The virulence characteristics of the virus have been assessed previously and were typed to belong to group C1 by monoclonal antibody typing (Kumanan *et al*., 1992).

Eggs and chicken

Embryonated chicken eggs (ECE) (9 to 11 days old) were obtained from the Poultry Research Station, Nandanam, Chennai, Tamil Nadu, India and used for virus isolation and pathogenicity assay. One hundred, day-old, White Leghorn male chicks were obtained from a single commercial hatchery at Chennai and were reared in isolation at the Department of Animal Biotechnology, Madras Veterinary College, Chennai to 20 weeks of age. They were shown to be serologically negative to ND by the haemagglutination inhibition (HI) test.

Preparation of confluent Vero cells

Vero cells available at the Central Tissue Culture Laboratory, Department of Animal Biotechnology, Madras Veterinary College, were sub-cultured and used for the study. The split ratio for Vero cells was 1:3. To the prescription bottle containing Vero monolayer was added 1 ml of trypsin versene glucose TVG (sodium chloride (Sigma, USA), 0.8 g; potassium chloride (Sigma) 0.02 g; disodium hydrogenphosphate (Merck, Germany), 0.115 g; potassium dihydrogenphosphate (Merck) 0.02 g; glucose (Sigma) 0.05 g; EDTA (Sigma) 0.20 g; trypsin 1: 250 (Difco, USA), 0.25 g; phenol red (Sigma), 0.001 g; distilled water to 100 ml). The TVG was spread over the monolayer by gently moving the bottle manually. The TVG was removed after 1–2 min and the bottle was left in the incubator for 2–3 min. After proper rounding of the cells, which can be judged by viewing under the inverted microscope, growth medium was added to the cells and dispensed into fresh prescription or milk dilution bottles and the cells were incubated at 37°C overnight to allow the monolayer to develop.

Propagation of NDV in Vero culture

The velogenic NDV isolate was inoculated into 9-day-old ECE and the amnioallantoic fluid (AAF) was collected following death of the embryo. Confluent Vero culture was inoculated with 1 ml of AAF having haemagglutination (HA) property. Virus adsorption was performed by incubating the cells with the inoculated virus at 37°C for 1 h. The unadsorbed virus was removed after an hour fresh medium (Life Technologies, USA) was added, and the cells were incubated for 4–5 days at 37°C. Regular microscopic examination was carried for development of characteristic cytopathic effects (CPEs). The virus was harvested at the height of CPE by subjecting the cultures to three cycles of alternate freezing and thawing. The harvested virus thus obtained was used for subsequent passaging.

Virus titration and pathotyping of the virulent and adapted viruses at every 10th passage

Virus titration was carried out in 96-well flat-bottom microtitre plates (Nunc, Denmark) as described elsewhere (Kumanan and Venkatesan, 1994). The virulence of the velogenic NDV and the adapted virus at every 10th passage was evaluated by standard pathogenicity tests (Alexander, 1997). These include the mean death time (MDT) and the intracerebral pathogenicity index (ICPI) tests. The MDT test was conducted as described elsewhere (Alexander, 1997) with the exception that the diluent used was the maintenance medium used for cell culture work. Candling was done at 6-hourly intervals to determine the time of embryo death. The ICPI, test was done and scored in the standard manner (Alexander, 1997).

Viral RNA extraction and RT-PCR

These were carried out according per standard procedures (Chomczynski and Saachi, 1987). Briefly at the height of CPE, the medium from the prescription bottle was discarded and the

cells were frozen. The cells were thawed once and 1 ml of solution D was added directly to the cell monolayer. The solution was gently mixed by pipetting and transferred onto a sterile microcentrifuge tube, followed by the addition of 100 µl of 2 mol/L sodium acetate and 1 ml of water-saturated phenol. After thorough mixing, 22 µl of choloroform–isoamyl alcohol mixture (49:1v/v) was added and vortexed for complete mixing. The mixture was kept in ice for 15 min before centrifugation at 10 000*g* for 20 min in a refrigerated centrifuge. Aqueous supernatant was drawn out carefully and to this an equal amount of isopropanol was added to precipitate the RNA. The mixture was kept at −20°C overnight and centrifuged at 12 000*g* for 20 min to pellet the RNA. Subsequently, the RNA pellet was washed with 70% ethanol two to three times. The pellet was partially air dried and used as template in RT-PCR reaction.

Oligonucleotide primers were designed to amplify regions of the fusion protein gene encompassing the fusion protein cleavage site of velogenic and 50th-passage Vero-adapted virus (Ballagi Pordany *et al*., 1996). The nucleotide sequences of the primers used were

P1: 5′ TCACTCTATCCGTAGGATACAAGAGTCTG 3′

P2: 5′ GATCTAGGGTATTATTCCCAAGCCA 3′

A two step RT-PCR was done using MuMLV reverse transcriptase (MBI Fermentas, Germany) and Supratherm Taq DNA polymerase (Genecraft, Germany). The PCR-amplified products were run on 1% agarose gel stained with ethidium bromide and visualized using a UV transilluminator.

Cloning of PCR products

To elucidate the degree of genetic variation, the PCR products were cloned and sequenced. The purified PCR products were treated with T4 DNA polymerase and subcloned into the *Sma*I site of dephosphorylated plasmid pBluecscriptII KS(+) (Stratagene, USA) (Sambrook and Russell, 2001). The recombinant plasmids were used to transform the DH5α strain of *E. coli*-competent cells. DNA minipreps using an alkaline lysis method were performed on white colonies suspected of containing an insert (blue/white selection). Colonies with the correct sizes were further cultured for preparation of recombinant plasmids. Recombinant plasmids were then purified using a purification kit (Qiagen GmBH, Germany) and sequenced on an ABI Prism fluorescent sequencing instrument.

Analysis of nucleotide and deduced amino acid sequences

Nucleotide and deduced amino acid sequences were aligned using the MEGA 1.02 software of Gene Tool. The deduced amino acid sequences of the F protein between the virulent and the Vero-adapted viruses were aligned, corresponding to amino acid residues 97–545.

Nucleotide sequence accession numbers

The fusion protein gene nucleotide sequences have been assigned the following GenBank accession numbers: virulent virus AY528452, and Vero-adapted virus AY528454.

Pathogenicity studies of the velogenic and vero adapted virus in sero negative birds

Velogenic and Vero-adapted viruses were inoculated to NDV-seronegative birds by the oculo-nasal route. For the velogenic virus 10^7 virus particles were given and for the adapted virus 10^8 virus particles were given. Two groups 20-week-old birds ($n = 18$ per group) were used for the study. After inoculation of the viruses until the 18th day post inoculation, one bird from each group was sacrificed humanely. Tissues from organs such as spleen, caecal tonsils, trachea and intestine were used for virus isolation studies in ECE as described elsewhere (Allan *et al*., 1978). Tissues such as thymus, trachea, lungs, spleen, intestine, caecal tonsils and bursa were routinely processed into paraffin and 3 μm sections were cut for haematoxylin and eosin (HE) staining.

Back-passage studies of the adapted virus

Tissue samples (500 μl) that were positive by virus isolation studies were injected into seronegative birds. The birds were kept for 7 days and sacrificed humanely. Tissue samples such as spleen, trachea, intestine and caecal tonsils were collected and virus isolation was done (Allan *et al*., 1978). The inoculum was passaged 5 times in the same way and at the end of 5th back-passage the virus was assessed for virulence by MDT and ICPI analysis. Sequence analysis of the 254 bp FPCS was also done (Seal *et al*., 1995).

RESULTS

Serial passage of velogenic NDV and cytopathic effects of the virus in Vero culture

The velogenic NDV was serially passaged 50 times in Vero culture and the cytopathic effects (CPEs) for the initial three passages appeared 120 h post inoculation (p.i.) with rounding and grouping of cells. The CPEs were consistent as the passage level increased and the presence of characteristic CPEs were observed at 72–96 h p.i. from the 8th passage onwards. At 24 h p.i. initial grouping of cells tended to occur, followed by vacuolation with marked grouping at 48 h. Syncytia formation with multinucleated giant cells, marked vacuolation of the cytoplasm and cytoplasmic anastomosis appeared between 72 and 96 h, with the presence of acidophilic intracytoplasmic inclusion bodies as compared to uninfected controls.

The infectivity titre of the virus was found to increase gradually from the 10th to the 30th passage. The $TCID_{50}$ titre was 9.6 after 40 passages. The titre stabilized up to 40 passages, and after 50 passages a slight drop in $TCID_{50}$ titre (8.5) was observed (Table I).

TABLE I
Conventional characterization of virulent and Vero-adapted viruses

Virus category and passage level	MDT (h)	ICPI	Virus titre $TCID_{50}$/50 µl
Virulent-unadapted	46	1.62	–
Vero 10	62	1.00	8.7
Vero 20	84	0.87	9.0
Vero 30	96	0.68	9.0
Vero 40	120	0.2	9.6
Vero 50	120	0.1	8.5

Pathotyping of the virulent and adapted viruses

The MDT of the virulent virus was 46 h. The MDT of the Vero-adapted viruses ranged from 62 h to 120 h when the MDT reading was taken at every 10th passage up to the 50th passage. A gradual increase in MDT was noted as the passage level increased (Table I). The ICPI value of the virulent virus was 1.62. The ICPI values of the Vero-adapted viruses ranged between 1.00 and 0.1 (Table I). It was noted that the ICPI values decreased as the passage level increased.

RT-PCR and sequence analysis

Using a set of PCR primers we were able to amplify a fragment with the expected size of 1349 bp from the F gene. Recombinant colonies harbouring the 1349 bp F gene product were further cultured and the plasmids were purified and sequenced. The deduced amino acid alignment between the virulent and the adapted viruses is given in Figure 1. A total of 135 nucleotide differences were found between the virulent and cell culture-adapted virus, which resulted in 42 amino acid changes when conceptually translated. The predicted amino acid motif present at the fusion protein cleavage site (FPCS) of the virulent virus was ^{109}SRRRRQRRFVG^{119} and the corresponding region of the 50th-passage adapted virus was ^{109}SGGRRQKRFIG^{119}. The overall non-synonymous to synonymous nucleotide substitution ratio of the gene encoding specific amino acid substitutions are as follows: A→C, 16; A→G, 55; A→T, 10; C→G, 8; C→T, 42; and G→T, 4.

Pathogenicity trials of virulent and adapted viruses

Clinical symptoms. The clinical symptoms shown by the birds infected with the virulent virus included severe oculonasal discharge from the nostrils, greenish white diarrhoea, depression and anorexia with torticollis from day 2 p.i. Eight of the 18 birds of this group died on the day 3 p.i. and the remaining 10 birds died on day 4 p.i. There were no apparent clinical symptoms in the birds administered with the adapted virus during the entire observation period of 18 days.

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LCN2   ESIRRIQGSV SASRRRRQRR FVGAVIGSVT LGVATAGQIT AVAALIQASQ NAANILRIKE
Vero50 D......E.. TT.GG...K. .I..I..G.A ......A... .A......K. .......L..

LCN2   SIASTNEHQL EVTKGLSQLA VAVGKMQQFA NDQFNKTAQE LDCIKIAQQV GVELNLYLTE
Vero50 ...A...AVH ...D...... .........V .........K .G..R..... ..........

LCN2   LTTVFGPQIT SPALNKLTIQ ALYNLAGGNM DYLLTKLGIG NNQLSSLIGS GLITGNPILY
Vero50 .......... .......... .......... ...V....V. .......... ..........

LCN2   DSQTQLLGIQ VTLPSVGNLN NMRATYLETL SVSTTRGFAS ALVPKVVTQV GSVIEELDTS
Vero50 .......... .......... .......... .......... .......... ..........

LCN2   YCIETDLDLY CTRIVTFPMS LGIYSCLSGN TSACMYSKTE GALTTPYMAL KGSVIANCKI
Vero50 .......... .......... P......... .......... ........TI .........M

LCN2   TTCRCADPPG IISQNYGEAV SLIDRHSCNV LSLDGITLRL SGEFDATYQK NISILDSQVI
Vero50 .....VN..P .......... ....KQ.... .......... ...L...... ....Q.....

LCN2   VTGNLDISTE LGNVNNSVSN ALDKLAESNS KLDKVNVRLT STSALITYIV LTVISLVFGI
Vero50 I......... .......I.. ..N..E.... .......K.. .......... ..I.......

LCN2   LSLILACYLM YKQKAQQKTL LWLGNNTLD
Vero50 ...V...... .......... .........
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Figure 1. Amino acid differences of the 1349 bp fusion protein gene between the velogenic and adapted virus analysed by MEGA. Fusion protein cleavage site is indicated in bold type. LCN2, Velogenic virus amino acid sequence; Vero50, Vero-adapted 50th-passage virus amino acid sequence.

Gross pathology. Gross pathological lesions noted in the birds inoculated with virulent virus included petechial haemorrhages in the trachea, proventricular junction and intestines, congestion of the lungs, spleen and kidney, and distended caecal tonsils. There were no gross pathological lesions present in the birds injected with the adapted virus.

Histopathological studies

Virulent virus group. In birds administered the virulent virus, on day 1 p.i. there was depletion of lymphocytes from regions of the thymus, marked congestion of the lungs with parabronchial and interstitial lymphoid cell accumulation, mild congestion and oedema with a few heterophilic infiltrations in the tracheal mucosa. On day 2 p.i. splenic congestion with an increase in the goblet cell activity together with loss of brush border from the intestines were the other characteristic changes observed.

TABLE II
Virus isolation details from various organs of birds administered velogenic and adapted virus in embryonated chicken eggs[a]

Virus category and days post inoculation	Time or death of the embryo (hours) with corresponding haemagglutination titres of AAF			
	Spleen	Intestine	Caecal tonsil	Trachea
Velogenic				
1	48 (64)[b]	48 (64)[b]	36 (64)[b]	48 (64)[b]
2	42 (64)[b]	48 (64)[b]	42 (128)[b]	48 (32)[b]
3	36 (128)[b]	36 (64)[b]	36 (128)[b]	36 (32)[b]
4	54 (128)[b]	48 (128)[b]	48 (256)[b]	36 (32)[b]
Vero				
1	36 (16)[b]	96 (8)	96 (−)	96 (−)
2	96 (−)	96 (8)	96 (−)	96 (−)
3	96 (−)	96 (8)	96 (−)	96 (−)
7	96 (64)	96 (4)	96 (−)	96 (−)
10	96 (−)	96 (−)	48 (32)[b]	96 (−)
11	96 (−)	96 (−)	96 (−)	72 (32)[b]
12	96 (−)	96 (−)	96 (−)	96 (16)
15	96 (−)	96 (−)	96 (64)	96 (−)
16	96 (64)	96 (16)	72 (−)	72 (64)[b]

[a] The data include only those days post inoculation when a significant HA titre was observed in the Vero-adapted virus category. During other days there were no HA titres in the allantoic fluid of ECE from any of the organs used for viral isolation.
[b] AAF collected from dead embryos.

On day 3 p.i., the bursa had an indistinct cortex and medulla, moderate depletion of lymphoid cells in the spleen, and mild lymphoid depletion in the thymus with virtual absence of germinal centres from the spleen. On day 4 p.i., there was marked necrosis and depletion of lymphocytes from the bursa, thymus, spleen and caecal tonsils, deciliation of tracheal mucosa and reticulum cell hyperplasia of the spleen.

Adapted virus group. In birds administered with the adapted virus, all the organs studied (trachea, thymus, bursa, spleen, caecal tonsils, intestine and lungs) were generally normal during the entire period of the study. An increase in the number of germinal centres of the spleen and caecal tonsils was observed.

Virus re-isolation studies

Attempts were made to re-isolate the virus from the experimentally infected birds. All the birds inoculated with the unadapted virus died within 4 days. All the birds administered with the adapted viruses survived.

The viruses were re-isolated from the birds administered the virulent and the adapted virus as shown in Table II. Virulent NDV was isolated from all the tissues under study (spleen, intestine, caecal tonsils and trachea) from birds administered the virulent virus. The inoculum prepared with these tissues, when inoculated into embryonated eggs, killed the embryos within 36–54 h. Virus recovery was possible up to 4 days following inoculation.

Except for a few Vero-adapted viruses, the majority did not kill the embryos when recovered from birds and inoculated into ECE. In birds inoculated with the adapted virus, the spleen was the major organ that yielded the virus, followed by caecal tonsils and intestines. No virus could be isolated from the trachea.

Back-passage of the adapted virus

Vero-adapted virus was back-passaged five times in seronegative birds. The MDT and ICPI values were 120 h and 0.1 respectively. There were no amino acid changes between the adapted and the back-passaged viruses encompassing the FPCS corresponding to amino acids 97–179 (Figure 2).

DISCUSSION

A velogenic NDV isolate was adapted to grow satisfactorily in Vero cell culture, with increase in infectivity and reduction in virulence on serial passage. Tissue culture-adapted NDV showed an increase in MDT values, and a reduction in ICPI values, indicating a gradual loss of virulence as the passage level increased. These findings concurred with the results of Kumanan and colleagues (Kumanan and Venkatesan, 1994; Kumanan et al., 1993). However, in a few instances an elevated ICPI following cell culture passage of lentogenic and mesogenic strains was noted, for which no explanation has been proposed (Slosaris *et al*., 1989). The MDT and ICPI values (120 h and 0.1) of the 50th-passage virus indicate that the virus has become lentogenic biologically.

A small amino acid motif in the fusion protein precursor (F0), termed the cleavage site, has been identified as a pathotype determinant. Thus, viruses with multibasic amino acids at the cleavage site are considered virulent, and those characterized by monobasic amino acids at the cleavage sites are avirulent (Collins *et al*., 1993, 1994). Cleavage of F0 polypeptides into F2 and F1 is essential for a virus particle to become infectious (Nagai, 1995). In chickens, the F protein of pathogenic NDV is cleaved by ubiquitous intracellular furin-like proteases, and the F protein of nonpathogenic viruses is cleaved only by trypsin–like proteases secreted from a limited number of tissues (Fujii *et al*., 1999). Thus, virulent viruses can spread throughout the host and cause the disease. In comparison, the avirulent motif can be cleaved only in the respiratory tract and gut, where trypsin-like proteases are available. The replication of these viruses is therefore restricted to these organs. In the present study involving the 1349 bp sequence of the fusion gene encompassing the FPCS region of virulent and adapted virus, a total of 134 nucleotide substitutions have occurred. No insertions or deletions were found in the tested region. The predicted amino acid motif present at the cleavage site of the virulent virus was ^{109}SRRRRQRRFVG^{119}, in which three pairs of dibasic amino acids were present. In adapted virus, the amino acid motif for the

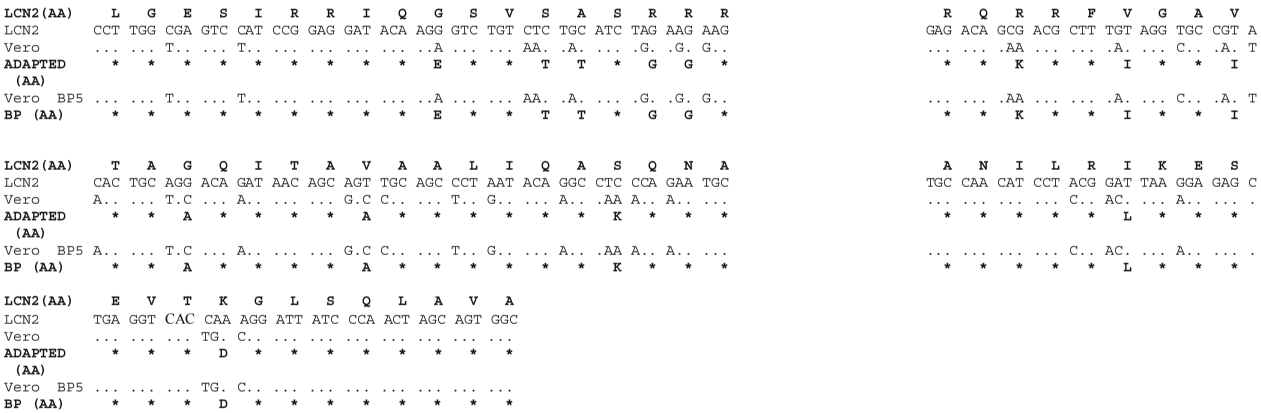

Figure 2. Nucleotide and amino acid differences of the FPCS region between the virulent and cell culture-adapted viruses analysed by MEGA. 1.CN2, velogenic virus nucleotide sequence; LCN2, velogenic virus nucleotide sequence; Vero, Vero-adapted 50th-passage virus nucleotide sequence; Adapted (AA), adapted virus amino acid sequence; Vero BP5, fifth back-passaged virus of Vero origin nucleotide sequence; BP (AA), amino acid sequence of back passaged virus.

corresponding region was ^{109}SGGRRQKRFIG^{119}, in which only two pairs of dibasic amino acids were present. One possible reason for the various nucleotide substitutions could be the selective pressure exerted by the cell culture system during adaptation coupled with a lack of proofreading and postreplication error-correction mechanisms that are inherent to some single-stranded RNA viruses (Koonin and Dolja, 1993).

There have been several reports of proteins such as HN, V, W and C proteins of NDV and other paramyxoviruses being responsible for virus virulence (Mebatison *et al*., 2001; Park *et al*., 2003). Many studies showing the requirement of the HN protein in fusion with the F protein suggest the importance of interaction of these two viral proteins in influencing viral infectivity. Another protein influencing viral virulence is the V protein (Takimoto *et al*., 2002), which suppresses interferon alpha and beta activity in host cells, thus helping virus survivability and spread. Therefore, the V protein may also be an important determinant of NDV virulence. Also, Huang and colleagues (2004) recently showed that the cleavage efficiency of F protein of NDV alone may not determine the neurovirulence phenotype differences among the NDV isolates, and that entry into the brain is necessary for neurovirulence. Even in our previous study involving the same virus adapting to chicken embryo fibroblast culture, we observed similar results, with the retention of the fusion protein cleavage site amino acid motif and a drastic reduction in the virulence of the virus after adaptation (Madhan Mohan *et al*., 2005).

Correlating the FPCS sequence with pathotype of the adapted virus, the MDT and ICPI values indicate the virus to belong to the lentogenic type, whereas the amino acid motif of the FPCS belongs to the mesogenic/velogenic type. This type of ambiguity in correlating the FPCS sequence with MDT and ICPI values in pathotyping NDV has been reported by some workers in recent studies. It was found that a chicken isolate from Germany had an ICPI of 1.44 but with a lentogenic FPCS amino acid motif, namely TTSGGGRQGRLT (Oberdorfer and Werner, 1998). Also, in isolation of ND viruses from outbreaks in Australia between 1998 and 2000, both the virulent and the avirulent NDV had the same FPCS amino acid motif as that of the progenitor (Peat's Ridge) virus, which was GGRRQGRL (Gould *et al*., 2001). These workers attributed an increase in virulence to two nucleotide substitutions (nt 4888G>A and nt4894C>T).

In the present study, the birds infected with virulent virus had the typical symptoms of NDV, in agreement with earlier studies (Hamid *et al*., 1991; Kommers et al., 2002). The death of all the birds on day 4 p.i. indicates that the virus was highly virulent. The birds infected with the Vero-adapted virus survived the entire 18-day period of the experiment, further confirming that the viruses had lost their virulence during serial passages in the cell culture.

The major histopathological changes that occurred in the birds infected with the virulent virus were depletion and neurosis of the lymphocytes in all the lymphoid organs tested such as thymus, spleen, bursa and caecal tonsils. The presence of mild congestion of the trachea with infiltration of heterophilic cells on day 1 p.i. of this study was similar to results of earlier studies in which it was shown that the tracheal mucosa had an intense lymphoid proliferation together with infiltration of cells on day 1 p.i. (Hamid *et al*., 1991). An increase in the number of germinal centres in spleen and caecal tonsils in birds infected with the adapted virus may be due to active trapping of the virus in these organs. This accords with earlier work in which the development of active germinal centres in birds with a higher level of immunity was observed (Jungherr *et al*., 1946).

Virus re-isolation from all the organs studied from birds infected with the virulent virus concurred with previous findings (Hamid *et al*., 1991; Parede and Young, 1990). In birds infected with the adapted virus, no virus could be isolated from the trachea. The presence of local antibody from bronchial-associated lymphoid tissue aggregates in respiratory secretions might be the main mechanism of resistance to re-infection via the respiratory route (Heuschelle and Easterday, 1970).

Live-attenuated viruses need to be back-passaged in a homologous host system for study of gain in virulence. Although an earlier report (Alexander and Parsons, 1984) demonstrated slight to moderate increase in virulence after sequential passages of some NDV isolates in chicks, no such increase was observed in this study even after five passages.

The findings of this study further support the hypothesis that the virulence of NDV is multigenic and that there is interplay of unknown virulence factors that play a role in the marked diversity of virulence and pathogenicity among cultured viruses that have similar virulence amino acid motifs at the F protein cleavage site.

ACKNOWLEDGEMENTS

The authors thank the Dean, Madras Veterinary College, Chennai for providing necessary facilities to carry out this work.

REFERENCES

Alexander, D.J., 2001. Newcastle disease. *British Poultry Science*, **42**, 5–22

Alexander, D.J. 1997. Newcastle disease and other paramyxoviruses. In: *Diseases of Poultry*, 10th ed (Iowa State University Press, Ames, IA), 541–570

Alexander, D.J. and Parsons, G., 1984. Avian paramyxovirus type I infection of racing pigeons: pathogenicity experiments in pigeons and chickens. *The Veterinary Record*, **114**, 466–469

Allan, W.H., Lancaster, J.E. and Toth, B., 1978. Newcastle disease vaccines, their production and use. *FAO Animal Production and Health Series* No. 10 (FAO, Rome)

Ballagi Pordany, A., Wehmann, E., Herczeg, J., Belak, S. and Lominiczi, B., 1996. Identification and grouping of Newcastle disease virus strains by restriction site analysis of a region from the F gene. *Archives of Virology*, **141**, 243–261

Collins, M.S., Bashiruddin, J.B. and Alexander D.J., 1993. Deduced aminoacid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. *Archives of Virology*, **128**, 363–370

Collins, M.S., Strong, I. and Alexander, D.J., 1994. Evaluation of the molecular basis of the pathogenicity of the variant Newcastle disease viruses termed pigeon PMV-1 viruses. *Archives of Virology*, **134**, 403–411

Collins, M.S., Strong, I. and Alexander, D.J., 1996. Pathogenicity and phylogenetic evaluation of the variant Newcastle disease viruses termed pigeon PMV-1 viruses based on the nucleotide sequence of the fusion protein gene. *Archives of Virology*, **141**, 635–647

Chomczynski, P. and Saachi, N., 1987. Single step method for RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry*, **162**, 156–159

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

Gould, A.R., Kattenbelt, J.A., Selleck, P., Hansson, E., Della-Porta, A. and Westbury, H.A., 2001. Virulent Newcastle disease in Australia: molecular epidemiological analysis of viruses isolated prior to and during the outbreaks of 1998–2000. *Virus Research*, **77**, 51–60

Hamid, H., Campbell, R.S.F. and Parede, L., 1991. Studies of the pathology of velogenic Newcastle disease: virus infection in non-immune and immune birds. *Avian Pathology*, **20**, 561–575

Heuschelle, W.P. and Easterday, B.C., 1970. Local immunity and persistence of virus in the trachea of chickens following infection with Newcastle disease virus. *Journal of Infectious Diseases*, **121**, 486–496

Huang, Z., Panda, A., Elankumaran, S., Govindarajan, D., Rockemann, D.D. and Samal, S.K., 2004. The haemagglutinin neuraminidase protein of Newcastle disease virus determines tropism and virulence. *Journal of Virology*, **78**, 4176–4184.

Jungherr, E.L., Tyzzer, E.E., Brandly, C.A. and Moses, H.E., 1946. The comparative pathology of Fowl plague and Newcastle disease. *American Journal of Veterinary Research*, **7**, 250–288

Kommers, G.D., King, D.J., Seal, B.S., Carmichael, K.P. and Brown, C.C., 2002. Pathogenesis of six pigeon-origin isolates of Newcastle disease virus for domestic chickens. *Veterinary Pathology*, **39**, 353–362

Koonin, E.V. and Dolja, V.V., 1993. Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. *Critical Reviews in Biochemistry and Molecular Biology*, **28**, 375–430

Kumanan, K. and Venkatesan, R.A., 1994. Adaptation of Newcastle disease virus strains to different cell culture. *Indian Journal of Comparitive Microbiology, Immunology and Infectious Diseases*, **15**, 39–41

Kumanan, K., Elankumaran, S., Vijayarani.,K., Palaniswamy, K.S., Padmanabhan, V.D., Manvell, R.J. and Alexander, D.J., 1992. Characterization of Newcastle disease virus isolated in India. *Journal of Veterinary Medicine B*, **39**, 383–387

Kumanan, K., Vijayarani, K. and Padmanabhan, V.D., 1993. Adaptation of a velogenic Newcastle disease virus violate to Vero cells. *The Indian Veterinary Journal*, **70**, 993–995

Madhan Mohan, C., Dey, S. and Kumanan, K. 2005. Molecular changes of the fusion protein gene of chicken embryo fibroblast adapted velogenic Newcastle disease virus: effect on its pathogenicity. *Avian Diseases*, **49**, 56–62.

Mayo, M.A., 2002. A summary of the changes recently approved by the ICTV. *Archives of Virology*, **147**, 1655–1656

Mebatison, T., Verstegen, S., de Vaan, L.T., Romer Oberdorfer, A. and Schrier, C.C., 2001. A recombinant Newcastle disease virus with low level V protein expression is immunogenic and lacks pathogenicity for chicken embryos. *Journal of Virology*, **75**, 420–428

Nagai, Y., 1995. Virus activation by host proteinases. A pivotal role in the spread of infection, tissue tropism and pathogenicity. *Microbiology and Immunology*, **39**, 1–9

Nagai, Y., Shimokata, K., Yoshida, T., Hamaguchi, M., Iinuma, M., Maeno, K., Matsumoto, T., Klenk, H.D. and Rott, R. 1979. The spread of a pathogenic and an apathogenic strain of Newcastle disease virus in the chick embryo as depending on the protease sensitivity of the virus glycoproteins. *Journal of General Virology*, **48**, 263–272

Nagai, Y., Hamaguchi, M. and Toyoda, T., 1989. Molecular biology of Newcastle disease virus. *Progress in Veterinary Microbiology and Immunology*, **5**, 16–64

Oberdorfer, A. and Werner, O., 1998. Newcastle disease virus detection and characterization by PCR of recent German isolates differing in pathogenicity. *Avian Pathology*, **27**, 237–243

Parede, L. and Young, P.L., 1990. The pathogenesis of velogenic Newcastle disease virus infection of chickens of differential ages and different levels of immunity. *Avian Diseases*, **34**, 803–808

Park, M.S., Shaw, M.L., Munoz Jordan, J., Cros, J.F., Nakaya, T., Bouvier, N., Palase, P., Garcia Sastre, A. and Basler, C.F., 2003. Newcastle disease virus based assay demonstrates interferon antagonistic activity for the NDV V protein and the Nipah virus V, W and C proteins. *Journal of Virology*, **77**, 1501–1511

Russell, P.H., Samson, A.C.R. and Alexander, D.J., 1990. Newcastle disease virus variations. *Applied Virology Research*, **2**, 177–195

Sambrook, J. and Russell, D.W., 2001. *Molecular Cloning—A Laboratory Manual*, 3rd edn (Cold Spring Harbour Laboratory Press, New York), 1.84–1.87.

Seal, B.S., King, D.J. and Benett, J.D., 1995. Characterization of Newcastle disease virus isolates by reverse transcription PCR coupled to direct nucleotide sequencing and development of sequence database for pathotype prediction and molecular epidemiological analysis. *Journal of Clinical Microbiology*, **33**, 2624–2630.

Slosaris, M., Levy, B., Katz, E., Levy, R. and Rones, Z.Z., 1989. Elevated virulence of Newcastle disease virus strains following serial passages in kidney cells *in vitro*. *Avian Diseases*, 33, 248–253.

Takimoto, T., Taylor, G.L., Connaris, H.C., Crennell, S.J. and Portner, A. 2002. A role of the haemagglutinin neuraminidase protein in the mechanism of paramyxo cell membrane fusion. *Journal of Virology*, **76**, 13028–13033.

(Accepted 12 November 2005; Published online: 23 December 2006)