ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 8 (12): 1137-1150, 2009
ISSN 1682-8356

Phylogenetic Analysis of the Nucleoprotein Gene of Newcastle Disease Vaccine Viruses in India

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Abstract: The Nucleoprotein (NP) gene of four different vaccine viruses of NDV commonly used in India namely D58, LaSota, F and K were characterized to find out variations if any. The NP gene was observed to have 1747 nucleotides with a coding sequence of 1470 nucleotides coding for 489 amino acids in all four vaccine viruses irrespective of their virulence to chickens. The variations at the coding sequence were restricted mostly to positions 401-489 and positions between 1 and 400, irrespective of pathogenicity to chickens remain conserved. The epitopes in nucleoprotein gene was also predicted. Among the 20 different epitopes predicted in the NP gene, one epitope with sequence ^{447}FLDLMRA^{453} was found conserved in all vaccine viruses and was also found to be immunodominant.

Key words: Newcastle disease, avian paramyxovirus 1, genome, nucleoprotein gene, immunodominant epitope

INTRODUCTION

Of the many poultry diseases that cause economic losses, Newcastle Disease (ND) is considered as one of the most important infections globally, particularly in India. Since its first report in India between 1928 and 1930 at Ranikhet (Edwards, 1928) and Madras-Chennai (Kylasam Aier, 1930), the infection continues to be a major threat to the Indian poultry industry. The Newcastle Disease Virus (NDV) still remains endemic in India and outbreaks are reported regularly. While the disease is mostly reported with noticeable clinical signs in many outbreaks, good numbers of outbreaks go unnoticed, since in such outbreaks the infection occurs in asymptomatic form causing only a mild drop in egg production. Such asymptomatic form and the role of other common birds like village (desi) chicken (Ananth *et al*., 2008), crows (Sulochana *et al*., 1981), sparrows, pigeon (Gurkipal Singh *et al*., 1989) in the pathogenesis keeps the NDV endemic making the control process very difficult. As a result, the virus remains in the environment for a long time leading to regular outbreaks despite vaccination. Despite the extensive use of live and inactivated vaccines coupled with regular sero-monitoring to control ND in India, one of the major reasons identified for the persistent problem of ND outbreaks is the lack of techniques for Differentiating Vaccinated and Infected (DIVA) birds. In other words DIVA vaccines referred as marker vaccines are not available. This creates a situation in which during an outbreak of ND, it becomes difficult to differentiate vaccinated birds from infected birds. Hence, a strategy in which the differentiation of vaccinated and infected birds becomes possible is essential, so that proper remedial measures could be initiated in time and because of such measures, over a period of time the virus could be removed from the environment. Thus, DIVA vaccines with a Companion Discriminatory Test (CDT) are the needs of the hour. It has already been established that of the six genes of NDV other than Haemagglutinin-neuraminidase (HN) and Fusion Protein (F) genes, Nucleocapsid Protein (NP) gene has also been reported to be immunogenic (Errington *et al*., 1995). Further NP based immuno assays have already been reported to be useful in differentiation of vaccinated and infected birds in conjunction with subunit vaccines (Makkay *et al*., 1999). The Nucleoprotein gene (NP) has also been identified for modification in the development of marker vaccine against ND (Mebatsion *et al*., 2002). To the extent possible to us we could not come across any data on the NP gene of ND vaccine viruses commonly used in India namely lentogenic strains/isolates LaSota (Winterfield *et al*., 1957) F (Aspiln, 1952) and D58 isolate (John Kirubaharan and Palaniswami, 2003) and the mesogenic strain K (Komarov and Goldsmith, 1946). Hence, this study was carried out to characterize the NP gene including the identification of epitopes in NP gene that could be modified later in the development of a marker in the development of marker vaccine against ND.

MATERIALS AND METHODS

Viruses-selection, propagation and purification: The Newcastle Disease Virus (NDV) vaccine strains viz., F, K and LaSota, which are commonly used in this part of the country and a thermostabilized low Intracerebral Pathogenicity Index (ICPI) (0.14) isolate of NDV (D58) maintained at this laboratory were used in this study. All the four viruses used in this study were maintained by propagating them in Embryonated Chicken Eggs (ECE) as per the standard procedures mentioned in the Terrestrial Manual of OIE (2004).

Plaque purification of viruses: The viruses were plaque purified as per European Communities Standards (2004) and Harper (1989) using Chicken Embryo Fibroblasts (CEF) that was prepared as prescribed by Freshney (2000) using cell culture medium-MEM (Invitrogen, USA, Cat # 1109099). Agar overlay over the CEF was made using 2% agarose (AB gene, cat # AG-r 200/a). The confluent monolayer of primary CEF cells in 25 cm^2 flasks (M/s Tarson, Cat # 910010) and 6 well tissue culture plates (Nunc, cat # 140675) were infected with viruses at a titre of log $10^{8.5}$ EID_{50}/ ml and incubated at 37°C for 1 h for viral adsorption. The unadsorbed viruses were thoroughly washed off with MEM medium. A 2 x concentration of MEM without phenol red was mixed with 2% agarose in equal volumes and allowed to cool to 30°C with a final concentration of medium as 1 x and agarose at 1%. One and half ml quantities of this mixture was added to each well of a 6 well tissue culture plate (Nunc Cat # 150229) and incubated at 37°C (CO_2 Water Jacketed Incubator, Series 11, Forma Scientific, Inc.) for 48 hours at 5% CO_2 level. The cells were examined under 100 x magnification, the individual plaques were isolated and the gel was removed by centrifugation at 1000 rpm for 1 min. The supernatant constituted the plaque purified NDV and that was passaged two times in CEF as per Kumanan (1989) followed by six times in nine days old ECE as per Terrestrial Manual of OIE (2004). The HA activity was checked after each passage as per Terrestrial Manual of OIE (2004). The HA activity was confirmed as specific to NDV by neutralizing it with specific reference positive serum (Veterinary Laboratories Agency, U.KCode PAO 155) as per the standard procedure of Terrestrial Manual of OIE (2004). The EID_{50} of plaque-purified viruses was estimated as per Reed and Muench (1938) and stored at -76°C for further study.

Amplification of NP gene: The genomic RNA was extracted from plaque-purified viruses using TRIZOL LS reagent (Invitrogen, USA, cat # 10001 96-010) as per manufacturer's instructions with minor modification in RNA pellet washing technique. The RNA pellet was washed thrice with 75% ethanol instead of one time. The Thermoscript two-step RT–PCR kit with platinum *Taq* polymerase (M/s. Invitrogen, USA, cat # 1146-032) was used for the reverse transcription and subsequent polymerase chain reaction. Reverse transcription was carried out on 1 µg of RNA using Thermoscript RT at 50°C for 50 min using gene specific sense primer. The cDNA was amplified by XL PCR using sense primer -5' GAA GGT GTG AAT CTC GAG TGC G 3' and anti sense primer 5' GCT CGT CGA TCT CCG CAT CTG T 3' (Krishnamurthy and Samal, 1998). The XL PCR was carried out with an initial denaturation at 94°C for 2 min. The first 12 cycles of 94°C for 30 sec, 57°C for 30 sec, 72°C for 90 sec was followed by sec 28 cycles of 94°C for 30 sec, 57°C for 40 sec and 72°C for 90 sec with an elongation cycle of five seconds for each cycle and final extension of 72°C for 10 min.

Nucleotide sequencing and sequence analysis: The PCR product of NP gene was purified using Auprep PCR product purification kit (Life technologies, India Cat # Gx28-704 LT) following manufacturer's instruction. The sequencing of NP gene of vaccine viruses under study was carried out in an automated sequencer by big dye termination chemistry method in an automated sequencer (ABI Prism, version 3, Applied Biosystems, USA) by primer walking using the sense primer (*sequence already provided)* and three additional primers namely 47 BG - (5' GGA TCC TCT CTA TCC AGG C 3') 59 BG - 5' (CAT CAG CCC TTG CAC TTA GTA G 3') and 80 BG - (5' GGC TCA GGG AAG TAG CAT TAA C 3'). The sequences were analyzed using the Bio-Edit software (provided by the North Carolina State University, USA) for multiple sequence alignment and to generate sequence analysis data. The phylogenetic tree was developed using Neighbour Joining (NJ) algorithm using bootstrap values and distance using Mega 3.1 software.

Identification of B cell epitopes: The B cell epitopes of Nucleoprotein (NP) of NDV were predicted in a web based B cell epitope prediction software "Antigenic" available at the web site (http://bioinfo.bgu.ac.il/bsu/immunology/epitope_pred/index.htm). The immunodominance of the epitope identified among predicted B cell epitopes was confirmed by dot ELISA. To confirm immunodominance, the epitope was synthesized as custom peptide and used as an antigen. This was compared with the whole virus proteins of NDV (purification of NDV proteins not provided). The optimum concentration of custom peptide and whole virus protein as coating antigen for dot ELISA was estimated by checkerboard titration (Rose *et al*., 1997). The concentration of whole virus protein and custom peptide was found to be 150 ng/µl and 500 ng/µl respectively. The optimum concentration of anti chicken IgG peroxidase conjugate was found to be 1 in 1000. One µl of custom peptide (500 ng/ µl) and one µl of whole virus protein (150 ng/ µl) were spotted on to Immuno comb (MDI, Ambala) squares made up of Nitrocellulose membrane. The top layer of immuno comb was dotted with peptide antigen and lower layer with whole virus protein antigen. The immuno comb was air dried and then incubated at 37°C for 30 min. Subsequent steps were carried out by dipping the immuno comb in a flat bottom plate filled with 400 µl of the reaction solution. The unbound sites on the membrane were blocked with blocking buffer (5% skim milk powder) for 1 h at 37°C.

The immuno comb was washed three times with wash solution (PBS+1%Tween 20) for two minutes in each well. The immuno comb was allowed to react with the 1 in 50 diluted NDV reference sera sample (Veterinary Laboratories Agency, U.KCode PAO 155) in blocking buffer and incubated at 37°C for 1 h. The immuno comb was washed three times in wash buffer. The immuno comb was allowed to react with the anti chicken IgG peroxidase conjugate (Sigma-Aldrich, USA (Cat≠ A9046) (1:1000) and incubated at 37°C for half an hour. The immuno comb was washed three times with wash buffer. The immuno comb was allowed to react with DAB substrate (Sigma, USA (Cat≠D8001) for 10 min in darkness and the reaction was stopped by washing the immuno comb in running tap water, air dried and the results were observed.

RESULTS

Viruses-selection, propagation and purification: Three different Newcastle disease viruses belonging to the lentogenic category used in this study namely LaSota, F and D58 did not cause death of the embryo. The mesogenic strain K caused death of the embryo that occurred between 48-72 h in the present study. The Amnioallantoic Fluid (AAF) was collected after chilling the eggs for 24 h. The viruses were plaque purified using CEF in which multiple plaques were noticed in CEF cell culture at dilutions 10^1-10^3 for D58 and LaSota, at 10^1-10^5 for F and at 10^1-10^4 for K. The plaques were small and similar in size. The virus was extracted from individual plaques and passaged two times in CEF and six times in ECE. The presence of virus after every passage was confirmed by HA test.

Amplification and sequencing of Nucleoprotein gene: The NP gene comprising of both coding sequence and Untranslatable Regions (UTRs) of all four vaccine viruses were amplified by RT-PCR using NP gene specific primers. In all the four different vaccine viruses, the length of NP gene was observed to be of 1747 nucleotides with a coding sequence (cds) of 1470 nucleotides with UTRs at 5' and 3' ends numbering 66 and 211 nucleotides respectively. No difference was observed between the different strains of NDV used as vaccine viruses in India as for as number of nucleotides are concerned. The sequences generated from all four vaccine viruses were compared with sequences of NP gene of strains and isolates of NDV available with GenBank. The details of analysis for the coding sequences (cds) of NP gene are provided as amino acid sequence identity plot in Fig. 1 and as amino acid sequence identity matrix in Table 1. The Phylogenetic tree constructed by Neighbour Joining (NJ) algorithm with bootstrap value and distance for cds and entropy [H (x)] plot are provided as (Fig. 2 and 3) respectively.

The sequence of Untranslatable Region (UTR) at the 3' end generated from all four vaccine viruses were also compared with sequences of 3' UTR of strains and isolates of NDV available with GenBank. The comparison is provided as nucleotide sequence identity plot in Fig. 4 and as nucleotide sequence identity matrix in Table 2. The Phylogenetic tree developed by Neighbour Joining (NJ) algorithm with bootstrap value and distance for cds and entropy [H (x)] plot are provided in (Fig. 5 and 6) respectively.

B cell epitopes: The details of epitopes predicted and their position in ORF of NP gene are provided in (Table 3). In total 20 B cell epitopes were predicted. Of the 20 epitopes identified, 17 epitopes were located between 1-385 aminoacids and three epitopes were located between 386 and 469 aminoacids. Between amino acids 401-489 three B cell epitopes could be located at positions 389-399 having amino acid sequence "RRGLAAAAQRV", 410-419 having amino acid sequence "TQQVGVLTGL" and 447-453 having amino acid sequence reading "FLDLMRA". The epitope identified at position 410-419 was not selected for further study since this epitope was not found to be conserved among vaccine viruses of NDV used in South India in which the amino acid at position 417 Thrionine (T) has been replaced by Isoleucine (I) in strain K. In the same manner the epitope located at positions 389-399 was also not selected since it was not found to be conserved among ND viruses reported in India. In that epitope the amino acid at position 399 Valine (V) was replaced by Alanine (A). Whereas, the epitope identified between 447 and 453 was found to be conserved not only among all vaccine viruses used in South India but also in all NDV strains/isolates used in the sequence analysis. The epitope selected had a sequence of ^{447}FLDLMRA^{453} with a length of seven amino acids. This was found in the conserved region of NP gene between amino acids ^{443}GEQFLDLMRAVANS^{457}. The entire conserved region along with epitope was chemically synthesized in a protein synthesis facility and was used as custom peptide for confirmation of its immunodominance.

Immunodominance of epitope identified: The results of dot ELISA are provided in Fig. 7. The intensity of dots produced by both epitope as custom peptide and Whole Virus Protein (WVP) were found to be same. Dots were produced in all custom peptide coated combs as in WVP coated combs confirming the immunodominance of the epitope.

DISCUSSION

In the present study, the sequencing of NP gene was carried out by primer walking followed by analysis of all four vaccine viruses substantiated the facts on NP gene that the total length of NP gene is 1747 nucleotides, the cds comprises of 1470 nucleotides that code for 489 amino acids and the Untranslatable Regions (UTRs) at

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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                          10         20         30         40         50         60         70
D58              MSSVFDEYEQ LLAAQTRPNG AHGGGEKGST LKVDVPVFTL NSDDPEDRWS FVVFCLRIAV SEDANKPLRQ
LaSota           .......... .......... .......... .......... .......... .......... ..........
F                .......... .......... .......... .......... .........N .A........ ..........
K                .......... .......... .......... .......... .........N .A........ ..........
Mukteswar        .......... .......... .......... ...E...... .........N .A........ ..........
Clone 30         .......... .......... .......... .......... .......... .......... ..........
I2               .......... .......... .......... .......... .......... .......... ..........
Herts-33         .......... .......... .......... ...E...... .N.......N .A........ ..........
LaSota/C         .......... .......... .......... .......... .......... .......... ..........
Ulster           .......... .......... .L........ ...E...... .........N .S........ ..........
V4               .......... .......... .......... .......... .......... .......... ..........
B1               .......... .......... .......... .......... .......... .......... ..........
Sterna           .......... .......... T......... ...E...... .........N .A........ ..........
Malysia          .......... .......... ......R... .R.E...... .........N .A........ ..........
PHY-LMV          .......... .......... .......... ...E...... .........N .T........ ..........
NA-1             .......... F......... T......... ...E....A. .........N .A........ ..........
DE-R49/99        .......... .......... S......... ...E...... .........N .A........ ..........
KBNP-4152        .......... .......... T......... ...E...... .........N .A........ ..........
NDV_1            .......... .......... .......... .......... .......... .......... ..........
NDV_2            .......... .......... .......... ...E...... .........N .S........ ..........
NDV_3            ......D... .......... T......... ...E...... .........N .A........ ..........
NDV_4            .......... .......... .......... .......... .........N .A........ ..........
NDV_5            .......... .......... T......... ...E...... .........N .A........ ..........
NDV_6            .......... .......... T......... ...E...... .........N .A........ ..........
NDV_7            .......... .......... .......... ...E...... .........N .A........ ..........
NDV_8            .......... .......... .L........ ...E...... .........N .S........ ..........
GUANGXI 1        .......... .......... T......... ...E...... .........N .A........ ..........
GUANGXI 2        .......... .......... T......... ...EI..... .........N .A........ ..........
GUANGXI-3        .......... .......... T......... ...E...... .........N .A........ ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                          80         90        100        110        120        130        140
D58              GALISLLCSH SQVMRNHVAL AGKQNEATLA VLEIDGFANG TPQFNNRSGV SEERAQRFAM IAGSLPRACS
LaSota           .......... .......... .......... .......... .......... .......... ..........
F                .......... .......... .......... ..........M......... .......... ..........
K                .......... .......... .......... ..........M......... .......... ..........
Mukteswar        .......... .......... .......... .......T.S V......... .........L. ..........
Clone 30         .......... .......... .......... .......... .......... .......... ..........
I2               .......... .......... .......... .......... .......... .......... ..........
Herts-33         .......... .......... .......... .......T.S V......... .........L. ..........
LaSota/C         .......... .......... .......... .......... .......... .......... ..........
Ulster           .......... .......... .......... .......T.. V......... .........M. ..........
V4               .......... .......... .......... .......... .......... .......... ..........
B1               .......... .......... .......... .......... .......... .......... ..........
Sterna           .......... .......... .......... .......TDS V......... .........MV ..........
Malysia          .......... .......... .........T .......TSS V......... .........MV .........ST
PHY-LMV          .......... .......... .......... .......T.D V......... .........M. ..R.......
NA-1             .......... .......... .......... .........S V......... .........MV ..........
DE-R49/99        .......... .......... ....S..... I......S.. M......... .........M. ..........
KBNP-4152        .......... .......... .......... .........S V......... .........MV ..........
NDV_1            .......... .......... .......... .......... .......... .......... ..........
NDV_2            .......... .......... .......... .......T.S V......... .........MV ..........
NDV_3            .......... .......... .......... .....S..DS V......... .........MV ..........
NDV_4            .......... .......... .......... .......T.. V......... .........M. ..........
NDV_5            .......... .......... .......... .....S..DS V......... .........MV ..........
NDV_6            .......... .......... .......... .....S.T.S V......... .........MV ..E.......
NDV_7            .......... .......... .......... .......T.S V......... .........MV ..........
NDV_8            .......... .......... .......... .......T.. V......... .........M. ..........
GUANGXI 1        .......... .......... .......... .........N V......... .........MV ..........
GUANGXI 2        .......... .......... .......... .....S..DS V......... .........MV ..........
GUANGXI-3        .......... .......... .......... .........S V......... .........MV ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        150        160        170        180        190        200        210
D58              NGTPFVTAGA EDDAPEDITD TLERILSIQA QVWVTVAKAM TAYETADESE TRRINKYMQQ GRVQKKYILY
LaSota           .......... .......... .......... .......... .......... .......... ..........
F                .......... .......... .......... .......... .......... .......... ..........
K                .......... ......N... .......... .......... .......... .......... ..........
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Fig. 1: Continued

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Mukteswar     .....T...V ..........  .....I....  ..........  ..........  ..........  .........H
Clone 30      ..........  ..........  ..........  ..........  ..........  ..........  ..........
I2            ..........  ..........  ..........  ..........  ..........  ..........  ..........
Herts-33      .........V  ..........  ..........  ..........  ..........  ..........  .........H
LaSota/C      ..........  ..........  ..........  ..........  ..........  ..........  ..........

                       150         160         170         180         190         200         210
Ulster        .........V  ..........  ..........  ..........  ..........  ..........  .........H
V4            ..........  ..........  ..........  ..........  ..........  ..........  ..........
B1            ..........  ..........  ..........  ..........  ..........  ..........  ..........
Sterna        .........V  ..........  .......V..  ...I......  ..........  ..........  ..I......H
Malysia       .........V  ..........  ..........  ..........  ..........  ..........  .........H
PHY-LMV       .........V  ..........  ..........  ..........  ..........  ..........  .........H
NA-1          .........V  ..........  .........V  ........T.  ..........  .....R....  .........H
DE-R49/99     .........V  ..........  .........V  ..........  ..........  ..........  .....R...H
KBNP-4152     .........V  ..........  .........V  ..........  ..........  ..........  .........H
NDV_1         ..........  ..........  ..........  ..........  ..........  ..........  ..........
NDV_2         .........V  ..........  ..........  ..........  ..........  ..........  .........H
NDV_3         .........V  ..........  ..........  ..........  ..........  ..........  .........H
NDV_4         .........V  ..........  ..........  ..........  ..........  ..........  .........H
NDV_5         .........V  ..........  ..........  ..........  ..........  ..........  .........H
NDV_6         .........V  ..........  ..........  ..........  ..........  ..........  .........H
NDV_7         .........V  ..........  ..........  ..........  ..........  ..........  .........H
NDV_8         .........V  ..........  ..........  ..........  ..........  ..........  .........H
GUANGXI 1     .........V  ..........  .........V  ..........  ..........  ..........  ..........
GUANGXI 2     .........V  ..........  .........V  ..........  ..........  ..........  .........H
GUANGXI-3     .........V  ..........  .........V  ..........  ..........  ..........  .........H

                       220         230         240         250         260         270         280
D58           PVCRSTIQLT IRQSLAVRIF  LVSELKRGRN  TAGGTSTYYN  LVGDVDSYIR  NTGLTAFFLT  LKYGINTKTS
LaSota        ..........  ..........  ..........  ..........  ..........  ..........  ..........
F             ..........  ..........  ..........  ..........  ..........  ..........  ..........
K             ..........  ..........  ..........  ..........  ..........  ..........  ..........
Mukteswar     .....A....  ..........  ..........  ....S....S  ....I.....  ..........  ..........
Clone 30      ..........  ..........  ..........  ..........  ..........  ..........  ..........
I2            ..........  ..........  ..........  ..........  ..........  ..........  ..........
Herts-33      .....A....  ..H.......  ..........  ....S.....  ..........  ..........  ..........
LaSota/C      ..........  ..........  ..........  ..........  ..........  ..........  ..........
Ulster        .....A....  ..........  .......A..  ..........  ..........  ..........  ..........
V4            ..........  ..........  ..........  ..........  .........K  ......L...  ..........
B1            ..........  ..........  ..........  ..........  ..........  ..........  ..........
Sterna        .....A....  ..H.......  ..........  ....S.....  ..........  ..........  ..........
Malysia       .....A....  ..H.......  ..........  ....S.....  ..........  ..........  ..........
PHY-LMV       .....A....  ..........  ..........  ..........  ..........  ..........  ..........
NA-1          .....A....  ..H.......  ..........  ....S.....  ..........  ..........  ..........
DE-R49/99     .....A....  ..........  ..........  ..........  ..........  ..........  ..........
KBNP-4152     .....A....  ..H.......  ..........  ....S.....  ..........  ..........  ..........
NDV_1         ..........  ..........  ..........  ..........  ..........  ..........  ..........
NDV_2         .....A....  ..H.......  ..........  ....S.....  ..........  ..........  ..........
NDV_3         .....A....  ..H.......  ..........  ....S.....  ..........  ..........  ..........
NDV_4         .....A....  ..........  ..........  ..........  ..........  ..........  ..........
NDV_5         .....A....  ..H.......  ..........  ....S.....  ..........  ..........  ..........
NDV_6         .....A....  ..H.......  ..........  ....S.....  ..........  ..........  ..........
NDV_7         .....A....  ..........  ..........  ....S.....  ..........  ..........  ..........
NDV_8         .....A....  ..........  .......A..  ..........  ..........  ..........  ..........
GUANGXI 1     .....A....  ..H.......  ..........  ....S.....  ..........  ..........  ..........
GUANGXI 2     .....A....  ..H.......  ..........  ....S.....  ..........  ..........  ..........
GUANGXI-3     .....A....  ..H.......  ..........  ....S.....  ..........  ..........  ..........

                       290         300         310         320         330         340         350
D58           ALALSSLSGD  IQKMKQLMRL  YRMKGDNAPY  MTLLGDSDQM  SFAPAEYAQL  YSFAMGMASV  LDKGTGKYQF
LaSota        ..........  ..........  ..........  ..........  ......N...  ..........  ..........
F             ..........  ..........  ..........  ..........  ..........  ..........  ..........
K             ..........  ..........  ..........  ..........  ..........  ..........  ..........
Mukteswar     .......A..  ..........  ..........  ..........  ..........  ..........  ..........
Clone 30      ..........  ..........  ..........  ..........  ..........  ..........  ..........
I2            ..........  ..........  ..........  ..........  ..........  ..........  ..........
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Fig. 1: Continued

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Herts-33     .......T.. .......... .....E.... .......... .......... .......... ..........
LaSota/C     .......... .......... .......... .......... .......... .......... ..........
Ulster       .......... .......... .......... .......... .......... .......... ..........
V4           ........S. .......... .......... .......... .......... .......... ..........
B1           .......... .......... .......... .......... .......... .......... ..........
Sterna       .......T.. .......... .....E.... .......... .......... .......... ..........
Malysia      .......T.. .......... .....E.... .......... .......... .......... ..........
PHY-LMV      .......... .......... .......... .......... .......... .......... ..........
NA-1         .......T.. .......... .....E.... .......... .......... .......... ..........
DE-R49/99    .......... .......... .....E.... .......... .......... .......... .....V....
KBNP-4152    .......T.. .......... .....E.... .......... .......... .......... ..........
NDV_1        .......... .......... .......... .......... .......... .......... ..........
NDV_2        .......T.. .......... .....E.... .......... .......... .......... ..........
NDV_3        .......T.. .......... .....E.... .......... .......... .......... ..........
NDV_4        .......... .......... .......... .......... .......... .......... .....S....
NDV_5        .......T.. .......... .....E.... .......... .......... .......... ..........
NDV_6        .......T.. .......... .....E.... .......... .......... .......... ..........

             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    290        300        310        320        330        340        350
NDV_7        .......A.. .......... .....E.... .......... .......... .......... ..........
NDV_8        .......... .......... .......... .......... .......... .......... ..........
GUANGXI 1    .......T.. .......... .....E.... .......... .......... .......... ..........
GUANGXI 2    .......T.. .......... .....E.... .......... .......... .......... ..........
GUANGXI-3    .......T.. .......... .....E.... .......... .......... .......... ..........

             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    360        370        380        390        400        410        420
D58          ARDFMSTSFW RLGVEYAQAQ GSSINEDMAA ELKLTPAARR GLAAAAQRVS EETSSIDMPT QQVGVLTGLS
LaSota       .......... .......... .......... .......... .......... .......... ..........
F            ........I. .......... .......... .......... .......... .......... ..........
K            .......... .......... .......... .......... .......... ..N...N... ......I..H
Mukteswar    .......... .......R.. .......... .......... ........A. ...G.M.I.. ..A.......
Clone 30     .......... .......... .......... .......... .......... .......... ..........
I2           .......... .......... .......... .......... .......... .......... ..........
Herts-33     .......... .......... .......... .......... .......... ...G.M.I.. ..A.......
LaSota/C     .......... .......... .......... .......... .......... .......... ..........
Ulster       .......... .......... .......... .......... .......... .....M.... ..A.......
V4           .......... .......... .......... .......... .......... .......... ..........
B1           .K........ .......... .......... .......... .......... .V........ ..........
Sterna       .......... .......... .......... .......... .......... ..IG.V.I.. ..A.......
Malysia      .......... .......... ......H... .X........ .......... ...G.V.I.. ..A.......
PHY-LMV      .......... .......... .......... .......... .......... .....ME... ..A.......
NA-1         .......... .......... .......... .......... .......... ..IG.M.I.. ..A.......
DE-R49/99    .......... .......... .......... .......... .......... .DA.NM.L.. ..A.......
KBNP-4152    .......... .......... .......... .......... .......... ..I..M.I.. ..A.......
NDV_1        .......... .......... .......... .......... .......... .V........ ..........
NDV_2        .......... .......... .......... ..D....... .......... ..IG.M.I.. ..A.......
NDV_3        .......... .......... .......... ..D....... .......... ..IG.M.I.. ..A.......
NDV_4        .......... .......... .......... .......... .......... .....M.... ..A......G
NDV_5        .......... .......... .......... .......... .......... ..IG.M.I.. ..A.......
NDV_6        .......... .......... .......... .......... .......... ..IG.M.I.. ..A.......
NDV_7        .......... .......... .......... ..D....... .......... ..IG.M.I.. ..A.......
NDV_8        .......... .......... .......... .......... .......... .....M.... ..A.......
GUANGXI 1    .......... .......... .......... .......... .......... ..IG.V.I.. ..A.......
GUANGXI 2    .......... .......... .......... .......... .......... ..IG.M.I.. .EA.......
GUANGXI-3    .......... .......... .......... .......... .......... ..IG.M.I.. ..A.......

             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    430        440        450        460        470        480        490
D58          EGGSQALQGG SNRSQGQPEA GDGETQFLDL MRAVANSMRE APNSAQGTPQ SGPPPTPGPS QDNDTDWGY*
LaSota       .......... .......... .......... .......... .....K.... .....N.... .........*
F            .......... .......... .......... .......... .......... .......... .........*
K            .......... .......... .......... .......... .......... .......... ...N.N...*
Mukteswar    D..P..P... L.......D. .......... .......... ....V.N.T. QE..S..... .........*
Clone 30     .......... .......... .......... .......... .......... .......... .........*
I2           .......... .......... .......... .......... .......... .......... .........*
Herts-33     D.DPR.P... ...P....D. .......... .......... T.....S.T. PE..S..... .G.......*
LaSota/C     .......... .......... .......... .......... .......... .......... .........*
Ulster       D.....P... ........DT .......... .......... S......... P......... .........*
```

Fig. 1: Continued

```
V4          .......... .......... .......... .......... ....R..... .......... .........*
B1          .......... .......... .......... .......... .......... .......... .........*
Sterna      DE.PR.P..R ..KP....D. ....A..... .......... ..SP..S.AH PE.......P .........*
Malysia     D..PR.S... ..K.....D. .......... .......... ......S.TH PE........ .........*
PHY-LMV     D.....P... L.......DT .......... .......... .......... P...Q..... .........*
NA-1        DE.PRTP... ..KP....D. .........F .......... ...P..S.TH PE......A. .........*
DE-R49/99   DSTPS.QPS. PSKP.SSADG N......... .........D .......S.. PA......GG .........*
KBNP-4152   DE.PRIP... ..KP....D. .........F .......... ...P..N.TH PE......V. .........*
NDV_1       .......... .......... .......... .......... .......... .......... .........*
NDV_2       DE.PR.P... PS......D. .......... .......... ......S.TH PE........ .........*
NDV_3       DE.PR.P... PS......D. .......... .......... ......S.TH PE.L..H... .........*
NDV_4       D.....P..A ........D. .......... .......... ........S. P......... .........*
NDV_5       DE.PR.P... ..KP....D. .......... .......... ......S.TH PE........ .........*
NDV_6       DE.PR.P... ..KP....D. .......... .......... ...P..S.TH PE........ P........*
NDV_7       D..PR.P... .S......D. .......... .......... ......S.SH PE........ .........*
NDV_8       D.....P... ........DT .......... .......... S......... P......... .........*
GUANGXI 1   DENPRTP... ..KP....D. .........F .......... ...P..S.TH PE......T. .........*
GUANGXI 2   DE.PRTS... ..KP....D. .........F ......N... ...P..N.TH PE..Q...A. .........*
GUANGXI-3   DE.PRTS... ..KP....D. .........F ......N... ...P..N.TH PE..Q...A. .........*
```

Fig. 1: Amino acid sequence arrangement at cds of NP gene-Vaccine viruses compared with standard strains and isolates

Abbreviations used for NDV strains/isolates	GenBank Accession No.
D58	DQ839549
LaSota	EF442113
K	EF442114
F	EF442115
Mukteswar	EF201805
Clone 30	DD306025
I2	AY935499
Herts 33	AY741404
LaSota/C	AY845400
Ulster	AY562991
V4	AY225110
B1	AF309418
Sterna	AY865562
Malaysian	AF284646
PHY-LMV	DQ097394
NA-1	DQ659677
DE-R 49/99	DQ097393
KBNP-4152	DQ839937
NDV_1	AJ306301
NDV_2	AB124598
NDV_3	AB124599
NDV_4	AB124600
NDV_5	AB124601
NDV_6	AB124602
NDV_7	AB124603
NDV_8	AB124604
Guangxi 1	DQ485239
Guangxi 2	DQ485238
Guangxi 3	DQ485237

3' and 5' end of NP gene comprises of 55 and 211 nucleotides respectively, which correlates with data on NP gene of other NDV strains like like D_{26} (Ishida *et al*., 1986), Beaudette C (Krishnamurthy and Samal, 1998), LaSota (De Leeuw and Ben Peeters, 1999), Ulster 2C and Texas GB (Ward *et al*., 2000). No difference could be observed between the number of nucleotides at both cds and UTRs in NP gene among vaccine viruses in South India. This assumes significance since isolates obtained from geese have been reported to have extra six nucleotides insert at the 3' UTR of NP gene between nucleotide positions 1646 and 1647 (Huang *et al*., 2003). Further, in another report, which discusses on evolutionary relationship between ND viruses based on NP gene sequences also confirms this variation (Czegledi *et al*., 2006). In the same report it has also been reported about the existence of yet another of NDV with different number of nucelotides, in which the extra 12 nucleotide insert has been observed in both Phosphoprotein (P) and NP genes. The sequences of cds of NP gene of these viruses were compared with other NDV strains/isolates belonging to apathogenic,

Table 1: Amino acid nucleotide sequence identity matrix for cds of NP gene - Vaccine viruses with standard strains and isolates

																			NDV_								GUANGXI		
Seq->	D58	LaSota	F	K	Mukteswar	Clone30	I2	Herts-33	LaSota/C	Ulster	V4	B1	Stema	Malysia	PHY-LMV	NA-1	DE-R49/99	KBNP-4152	1	2	3	4	5	6	7	8	1	2	3
D58		**99**	**99**	**98**	**93**	**100**	**100**	**93**	**100**	**96**	**99**	**100**	**91**	**92**	**96**	**91**	**91**	**92**	**100**	**93**	**92**	**96**	**93**	**92**	**94**	**96**	**92**	**91**	**92**
LaSota	100		**99**	**97**	**93**	**99**	**99**	**93**	**99**	**96**	**99**	**99**	**91**	**92**	**95**	**91**	**90**	**92**	**99**	**92**	**91**	**96**	**92**	**92**	**93**	**96**	**92**	**90**	**91**
F	98	98		**98**	**93**	**99**	**99**	**93**	**99**	**96**	**98**	**99**	**91**	**92**	**96**	**92**	**91**	**93**	**99**	**93**	**92**	**97**	**93**	**92**	**94**	**96**	**92**	**91**	**92**
K	97	97	98		**92**	**98**	**98**	**92**	**98**	**95**	**97**	**98**	**90**	**91**	**95**	**91**	**90**	**92**	**98**	**92**	**91**	**96**	**92**	**91**	**93**	**95**	**91**	**90**	**91**
Mukteswar	88	88	89	87		**93**	**93**	**96**	**93**	**95**	**93**	**93**	**93**	**94**	**95**	**93**	**90**	**94**	**93**	**95**	**94**	**95**	**95**	**94**	**96**	**95**	**93**	**93**	**94**
Clone30	100	100	98	97	88		**100**	**93**	**100**	**96**	**99**	**100**	**91**	**92**	**96**	**91**	**91**	**92**	**100**	**93**	**92**	**96**	**93**	**92**	**94**	**96**	**92**	**91**	**92**
I2	100	100	98	97	88	100		**93**	**100**	**96**	**99**	**100**	**91**	**92**	**96**	**91**	**91**	**92**	**100**	**93**	**92**	**96**	**93**	**92**	**94**	**96**	**92**	**91**	**92**
Herts-33	89	88	89	88	92	89	89		**93**	**95**	**92**	**93**	**95**	**96**	**95**	**96**	**91**	**96**	**93**	**97**	**96**	**95**	**97**	**97**	**97**	**95**	**96**	**95**	**96**
LaSota/C	100	100	98	97	88	100	100	89		**96**	**99**	**100**	**91**	**92**	**96**	**91**	**91**	**92**	**100**	**93**	**92**	**96**	**93**	**92**	**94**	**96**	**92**	**91**	**92**
Ulster	91	90	91	90	92	91	91	92	91		**95**	**96**	**93**	**94**	**98**	**93**	**92**	**94**	**96**	**95**	**94**	**98**	**94**	**94**	**96**	**100**	**93**	**92**	**93**
V4	100	99	98	97	88	100	100	88	100	90		**99**	**90**	**91**	**95**	**91**	**90**	**92**	**99**	**92**	**91**	**96**	**92**	**91**	**93**	**95**	**91**	**90**	**91**
B1	99	99	98	97	88	99	99	88	99	90	99		**91**	**92**	**95**	**91**	**90**	**92**	**100**	**93**	**92**	**96**	**92**	**92**	**93**	**96**	**92**	**91**	**91**
Stema	85	85	86	86	88	85	85	90	85	88	85	85		**96**	**93**	**96**	**90**	**97**	**91**	**96**	**96**	**93**	**98**	**97**	**96**	**93**	**97**	**96**	**96**
Malysia	86	86	87	86	90	86	86	92	86	90	86	86	91		**94**	**95**	**90**	**96**	**92**	**97**	**96**	**94**	**97**	**96**	**97**	**94**	**96**	**95**	**96**
PHY-LMV	90	90	90	90	91	90	90	91	90	96	90	90	86	88		**93**	**92**	**94**	**96**	**95**	**94**	**98**	**94**	**94**	**96**	**98**	**93**	**93**	**93**
NA-1	85	85	86	86	88	85	85	91	85	88	85	85	93	90	87		**91**	**98**	**91**	**97**	**96**	**93**	**98**	**98**	**96**	**93**	**98**	**98**	**98**
DE-R49/99	77	77	77	77	77	77	77	77	77	79	77	77	77	77	78	77		**92**	**91**	**92**	**91**	**92**	**92**	**91**	**92**	**92**	**91**	**90**	**91**
KBNP-4152	85	84	85	85	87	85	85	90	85	87	84	85	92	90	86	96	77		**92**	**97**	**97**	**94**	**98**	**98**	**97**	**94**	**98**	**98**	**99**
NDV_1	99	99	98	97	88	99	99	89	99	90	99	100	86	87	90	85	77	85		**93**	**92**	**96**	**93**	**92**	**93**	**96**	**92**	**91**	**92**
NDV_2	87	87	88	88	91	87	87	94	87	91	87	87	92	94	89	93	78	92	87		**98**	**95**	**98**	**98**	**99**	**95**	**97**	**96**	**97**
NDV_3	86	86	86	86	89	86	86	92	86	89	86	86	92	92	88	95	77	94	86	96		**94**	**98**	**97**	**98**	**94**	**96**	**96**	**96**
NDV_4	93	92	93	92	92	93	93	93	93	96	92	93	89	90	95	89	79	88	93	92	90		**95**	**94**	**96**	**98**	**93**	**93**	**93**
NDV_5	87	87	87	87	89	87	87	92	87	89	87	87	93	92	88	97	78	96	87	94	97	90		**99**	**98**	**94**	**98**	**98**	**98**
NDV_6	88	87	88	88	91	88	88	94	88	91	87	88	94	93	89	95	78	94	88	96	95	92	96		**97**	**94**	**98**	**97**	**98**
NDV_7	89	88	89	89	92	89	89	94	89	92	89	89	92	94	91	93	78	92	89	96	95	93	95	95		**96**	**96**	**95**	**96**
NDV_8	91	90	91	90	92	91	91	92	91	100	90	90	88	90	96	88	79	87	90	91	89	96	89	91	92		**93**	**92**	**93**
GUANGXI 1	85	85	86	86	87	85	85	90	85	88	85	85	92	90	86	97	77	96	85	92	95	88	96	94	92	88		**97**	**98**
GUANGXI 2	85	85	86	85	88	85	85	90	85	88	85	85	92	90	86	97	77	96	85	92	95	89	97	95	92	88	97		**99**
GUANGXI-3	86	85	86	86	88	86	86	91	86	88	85	86	92	91	87	97	77	97	86	93	95	89	97	95	93	88	98	99	

*Amino acid identities in percentage are given in bold letters and nucleotide identifies are provided in ordinary letters

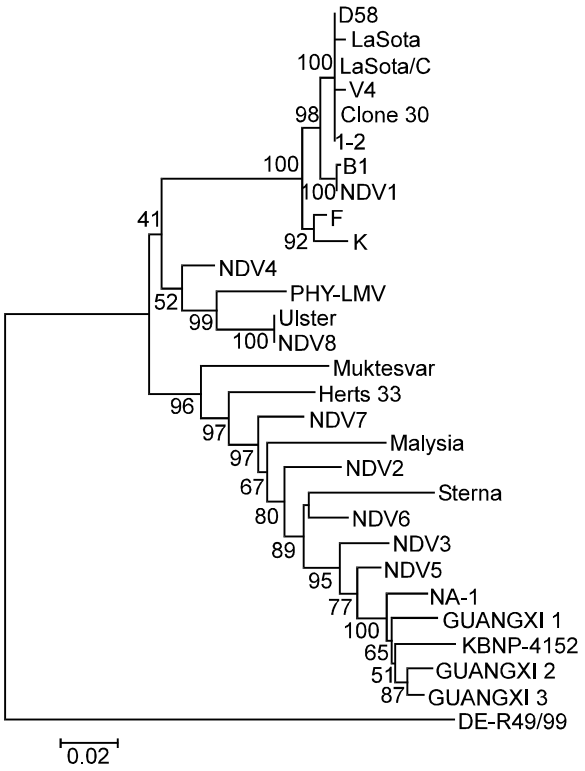

Fig. 2: Phylogenetic tree (NJ) with distance and bootstrap value-ORF of NP gene of vaccine viruses with standard strains and isolates

Table 2: B Cell epitopes of NP gene

Epitope		
From	To	Amino acid sequence
50	62	SFVVFCLRIAVSE
200	235	QGRVQKKYILYPVCRSTIQLTIRQSLAVRIFLVSEL
69	82	RQGALISLLCSHSQ
30	40	TLKVDVPVFTL
163	180	ERILSIQAQVWVTVAKAM
410	419	TQQVGVLTGL
280	290	SALALSSLSGD
98	105	TLAVLEID
324	333	PAEYAQLYSF
248	258	YYNLVGDVDSY
131	139	IAGSLPRAC
265	274	TAFFLTLKYG
85	91	RNHVALA
4	15	VFDEYEQLLAAQ
361	371	RLGVEYAQAQG
381	387	ELKLTPA
389	399	RRGLAAAAQRV
447	453	FLDLMRA
141	149	NGTPFVTAG
337	343	MASVLDK

- Epitope identified for further studies is indicated in bold letters
- Amino acids are indicated by IUPAC codes

lentogenic, mesogenic and velogenic namely Ulster2C, V4, I2, B1, Clone 30, LaSota,/C, Mukteswar, Herts-33, Sterna, Malaysian, PHY-LMV, NA-1, DE-R 49/99, KBNP-4152, Guangxi 100/2003, Guangxi 8/2002, Guangxi 6/2002 and virulent NDV isolates 1-6. Based on the sequence identify matrix at amino acid level, the variation at cds of NP gene was observed to be 1% among lentogenic strains/isolates (D58, LaSota, F, B_1, Clone 30

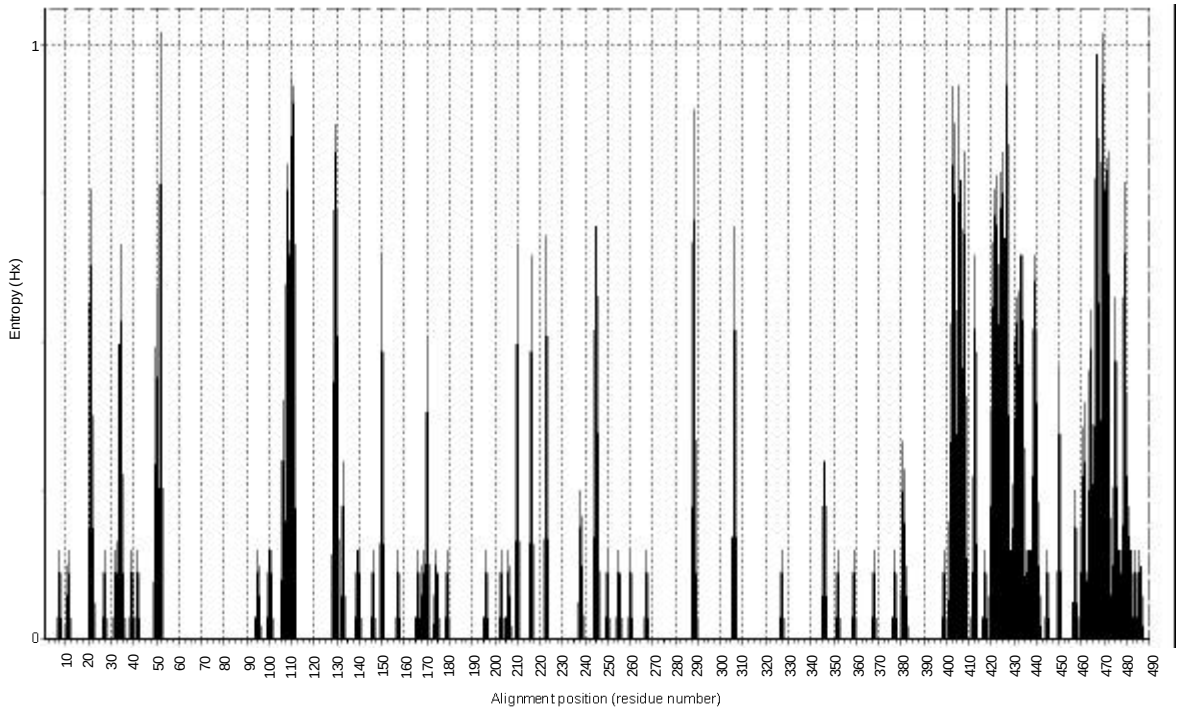

Fig. 3: Entropy (H (x)) plot for amino acids of cds of NP gene Vaccine viruses with standard strains and isolates

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50         60
D58             TGGACAAAAC CCAGCCTGCC TCCACAAAAT CATCCCAATG CCCTCACCCG TAGTCGACCC
LaSota          .......... .........T .........A .......... .......... ..........
F               .T........ .....T...T .......... .......... .......... ..........
K               .T..T.T.TT .TCC.T..TT .......... AGGGGGGCGC G..CGC.... ..A.A..A..
Clone 30        .......... .........T .........A .......... .......... ..........
I2              CCA...GC.. .......... .AT.TG...C ...T....CC .TTC.GT.T. C.CCTC...A
LaSota/C        .AA....... .........T .........A .......... .......... ..........
V4              .......... .........T .........A .......... .......... ..........
B1              .T........ .........T ..T....G.A .......... .T........ ..........
Ulster          CCA....C.. .....T.... ..T.TG.... ........CT ...C.G.T.. C.CC.C....
Herts 33        CC.....C.. ....T..... ......GG.C ..C.....CC ..TCTG.... C.CC.C....
Mukteswar       CC.....C.. T......... ....TCG... ....T.G..T .TTCTG..T. ..AC.T.A..

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        70         80         90        100        110        120
D58             CTCAATTTGC GGCTCTATAT GACCACACCC TCAAACAAAC ATCCCCCTCT TTCCTCCCTC
LaSota          ...G...... .......... .......... .......... .......... ..........
F               ...G...... .......... .......... .......... .......... ..........
K               .C.G..C... ...C...... .......A.. .......... ........A. ..........
Clone 30        ...G...... .......... .......... .......... .......... ..........
I2              .C....CC.. AATC.CGC.. AG...A.... A..G.GGG.. CC....GGT. CC.TC.TT..
LaSota/C        ...G...... .......... .......... .......... .......... ..........
V4              ...G...... .......... .......... .......... .......... ..........
B1              ...G...... .......... .......... .......... .......... ..........
Ulster          TC..G.CC.. A.TC.C.C.C .G...A.... A.....G... CC..T.A... CC.TC.....
Herts 33        .CTG..CC.. A..C.CGC.. .G...A.... A...CAG... CC....A... C......TC.
Mukteswar       .CTGG.CCA. A.TCTC.C.C .G...A.... G.....G.G. CC........ C.......C.

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       130        140        150        160        170        180
D58             CCCCTGCTGT ACAACTCCGC ACGCCCTAGA TACCACAGGC ACAATGCGGC TCATTAACAA
LaSota          .......... .......... .......... .......... .......... ...C......
F               .......... .......... .........G .......... .......... ...C......
K               .........A .......... .........G .......... .......... ...C......
Clone 30        .......... .......... .......... .......... .......... ...C......
I2              ..T....CTC .....C..A. CT....A..G C.A..T...T ........A. ...C...TG.
LaSota/C        .......... .......... .......... .......... .......... ...C......
V4              .......... .........CG .......... .......... .......... ...C......
B1              .......... .......... .........G C.A..G.... .......... ...C......
Ulster          ....A..CCC .......TA. CT....A..G C.A....... ........A. C..CC.....
Herts 33        .T..A....C ..G..C..A. C.....A..G ..A....... ...GCC.AA. ...CC.....
Mukteswar       .T..A..CAC ..GG....AA C.....A.A. C.A...G... ....CT..A. ...C.C.T..

                ....|....| ....|....| ....|....| .
                       190        200        210
D58             TCAAAACAGA GCCGAGGGAA TTAGAAAAAA A
LaSota          .......... .......... .......... .
F               .......... .......A.. .......... .
K               .......... .......A.. .......... .
Clone 30        .......... .......... .......... -
I2              ....T....G ...A.A.A.. .......... G
LaSota/C        .......... .......... .......... G
V4              .......... .......... .......... G
B1              .......... .......... .......... G
Ulster          ....T..... ...A.A.A.. .......... G
Herts 33        ..T.T..... ...A.A.AT. .......... .
Mukteswar       ..T.C..... .....A.ATC .......... .
```

Fig. 4: Nucleotide sequence arrangement at Untranslatable Region (UTR) at the end of NP gene-Vaccine viruses compared with strains

and I2) and the variation between lentogenic and apathogenic strain (V4) was found to be upto 2%. The lentogenic strains were also compared with mesogenic strains like Komarov and Mukteswar, the percentage of variation was found to be upto 7% and the variation between lentogenic and velogenic strains/isolates (Herts 33, Guangxi 8/2002, 100/2003, 6/2000) was upto 10%. In the present study, an isolate from Hungary DE-R49/99 (Czegledi *et al*., 2006) had shown 23% of nucleotide sequence variation with lentogenic

Table 3: Nucleotide sequence Identity matrix for untranslatable region IUTR) at the end of NP gene - Vaccine viruses with other strains

Seq->	D58	LaSota	F	K	Clone 30	I2	LaSota/ C	V4	B1	Ulster	Herts 33	Muktes war
D58		98	97	82	98	63	97	97	94	71	70	67
LaSota	98		98	83	100	63	99	99	96	70	71	67
F	97	98		85	97	63	97	96	95	72	71	67
K	82	83	85		82	57	82	82	81	64	64	61
Clone 30	98	100	97	82		63	99	99	96	70	70	66
I2	63	63	63	57	63		64	63	65	81	74	67
LaSota/C	97	99	97	82	99	64		98	96	71	70	66
V4	97	99	96	82	99	63	98		95	70	70	66
B1	94	96	95	81	96	65	96	95		71	70	66
Ulster	71	70	72	64	70	81	71	70	71		79	75
Herts 33	70	71	71	64	70	74	70	70	70	79		78
Mukteswar	67	67	67	61	66	67	66	66	66	75	78	

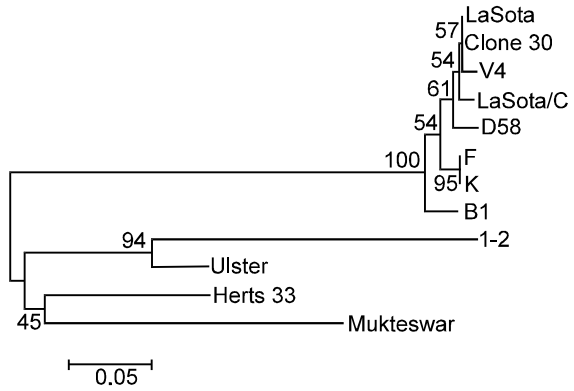

Fig. 5: Phylogenetic tree (NJ) with distance and bootstrap value-Untranslatable Region (UTR) at the end of NP gene of vaccine viruses other strains

mesogenic and velogenic strains and the deduced amino acid sequence between this isolate and other strains of NDV was also observed to be upto 10%.

In a previous study comparison that has been carried out at the cds of NP gene between velogenic strain Beaudette C with the lentogenic strains D_{26} and Ulster 2C revealed 4% variation at amino acid level and between lentogenic strains it was observed to be 1% (Krishnamurthy and Samal, 1998). In another report the variation between lentogenic and velogenic strains was reported to be up to 3% (Huang *et al*., 2003). In the present study a higher variation was observed between lentogenic and velogenic strains (up to 10%). To the best of our knowledge we could not come across any other published information of NP sequence comparison between lentogenic and mesogenic or lentogenic and apathogenic and we report a variation of 7% and 2% respectively for the above categories based on our findings. In the present study, we are also reporting a higher variation at amino acid level between lentogenic and velogenic strains at cds of NP gene. This could not be considered as unusual since in the earlier comparisons (Krishnamurthy and Samal, 1998; Huang *et al*., 2003) only Beaudette C and Texas GB strains were used, where as in the present study apart from velogenic strains, we have also included velogenic isolates in comparison. The higher variation we report could be due to the availability of more numbers of velogenic strains/isolates and most of them have been deposited with GenBank after the two works mentioned here.

The phylogenetic tree was constructed by neighbour joining algorithm with bootstrap values along with distance. Based on the findings of phylogenietic tree, it could be concluded that except the Hungarian isolate DE-R49/99 all other strains/isolates of NDV including those used in the present study cluttered into one group. The bootstrap values for numbers of inner sub groups are more than 95% confirming the topology and stability of inner branches. The sub group comprising of vaccine strains assumes lot of significance in that the boot strap value are very high between 92 and 100 confirming a stable topology. This proves the conserved nature NP gene of NDV.

It is an established fact that the amino acid 1-375 are essential for herringbone formation and negative single standard RNA is placed in the groove (Kho *et al*., 2003). Because of this reason the NP gene remains a conserved one. This is further confirmed in the study with a stable tree with high bootstrap values. All the vaccine strains are grouped in two sub groups with a common node with high bootstrap value. Hence, it could be concluded that NP gene in vaccine strains used in South India remains conserved. The Entropy plot for amino acid of cds of NP gene also confirms the conserved nature of cds of NP gene. It has been reported that Entropy is being used in sequence analysis to identify the variable region. The Entropy plot of NP gene clearly proves the conserved nature of 1-400 amino acid and variable nature of 401-489 amino acids. When the amino acids of cds at positions between 1-400 were compared between lentogenic strains the variation was observed to be only up to 1%, between lentogenic and mesogenic strains it was observed to be

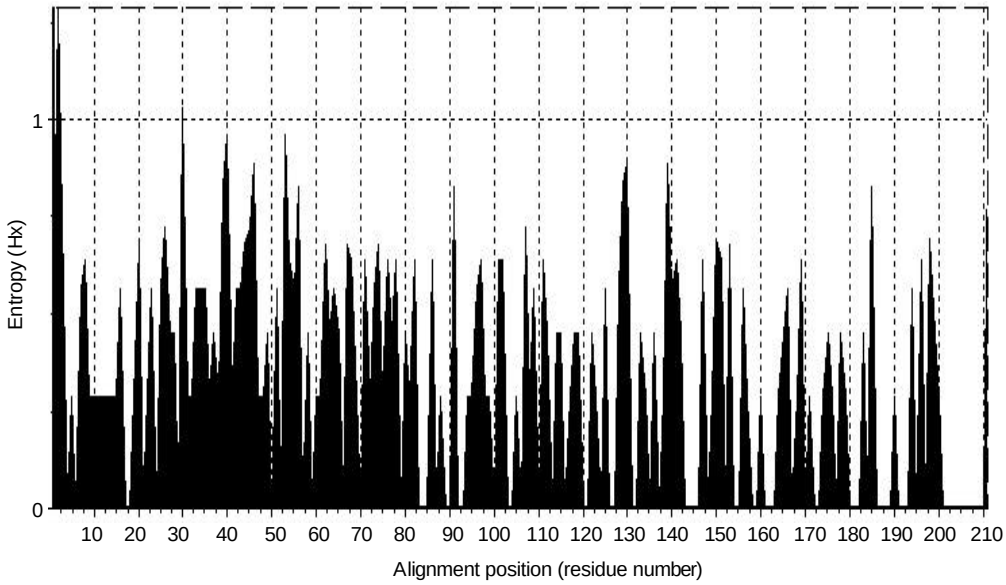

Fig. 6: Entropy (H (x)) plot for non-translatable region at the end of NP gene Vaccine viruses with other strains

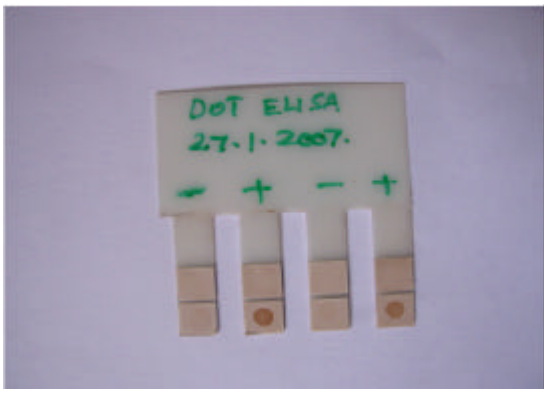

Fig. 7: Results of dot ELISA confirming the immunodominance of epitope identified

Whole virus protein		Epitope as custom peptide	
Negative Serum Blank No dot	Positive Serum Blank Dot	Negative Serum Blank No dot	Positive Serum Blank Dot

upto 4% and between lentogenic and velogenic strains the variation was observed to be upto 5% and it could be concluded that cds from 1-400 amino acids is conserved. Whereas, variation was observed to be relatively high between amino acids 401-489. This feature is characteristic of members of the family *Paramyxoviridae* where, the ORF is conserved from 1-400 amino acids and varied with very low level of identity in the last 125 amino acids (Rosenblatt *et al*., 1985; Sanchez *et al*., 1986). In the present study, between different strains/ isolates of NDV, the variations between 401-489 was found to be upto 2% between lentogenic strains and upto 13% between lentogenic and mesogenic. Whereas between lentogenic and velogenic the variations were as high as 27%. Same percentage of variation was also observed between mesogenic and velogenic strains.

Another interesting observation in the comparison among vaccine viruses used in this study is between D58, LaSota and F with K, where the variation was observed to be 9%. When the same comparison was made between D58, LaSota and F with Mukteswar (another mesogenic strain) the variation was observed to be 17%. Further, between K and Mukteswar the variation was observed to be 24%. In an observation by Krishnamurthy and Samal (1998) on variations in the C terminal region of NP gene of NDV was hypothesized on virulence characteristics of NDV. The findings of this study also agree with this observation that variations in the C region of NP could result in phenotypic change from extremely virulent to avirulent characteristics. However, further studies need to be carried out before equating it with molecular characterization method by sequencing the cleavage site of Fusion (F) protein gene (FPCS) region.

It has been reported that the 3' and 5' non-coding sequence of the NP gene shows considerable variation between the virulent and non-virulent strains. The NP mRNA sequence of strain Beaudette C was shown to have 31% variation at 3' region with lentogenic strain Ulster 2C and the variation in the level of identity at the C terminus was 92% (Krishnamurthy and Samal, 1998). In the present study, the sequence arrangement of vaccine viruses under study at 3' UTR of NP gene was compared with standard strains viz.,I2, Lasota/C, V_4, B_1,

Ulster, Herts 33, Mukteswar and Clone 30. Based on the sequence identity matrix at nucleotide level, the nucleotide sequence variation among lentogenic strains was found to be only 2%. The lentogenic strain when compared with mesogenic strain was observed to show variation upto 18% and with velogenic strains the same was observed to be 30%. The entropy plot also substantiates the variation in the region. In an earlier work comparison was made between lentogenic and velogenic alone. But in the present study based on the sequence data generated and comparison of the same with available sequence data in GenBank reveals grouping of viruses based on UTR sequences. But it cannot be correlated with phenotypic change from extremely virulent to avirulent characteristics, since it is a non-coding region and responsible only for terminating mRNA transcription from the preceding gene, before initiating transcription of the subsequent gene (Ishida *et al*., 1986; Millar *et al*., 1986; Yusoff *et al*., 1987; Philips *et al*., 1998). We did not investigate the UTR at 5' end since it is very minimal in length.

In an earlier published work, different attempt was used to identify immunodominant epitopes where, by pepscan analysis using 72 over lapping peptides covering the entire region coding 335-489 of nucleoprotein were checked for immunodominance and a region at position 447-455 of the NP region was found to be immunogenic. Our method of prediction of B cell epitope using computer algorithm correlated with pepscan analysis (Mebatsion *et al*., 2002). In the present study, the epitope predicted having length of seven mer has been found in the conserved region of 443-457 of nucleoprotein. Hence, a 15 mer peptide the minimum length required to identify NDV antibodies has been synthesized and its immunodominance was confirmed by dot-ELISA. We did not study any epitope predicted between positions one and 375, since, this region is essential for herringbone formation and negative single standard RNA is placed in the groove (Kho *et al*., 2003).

Conclusion: In conclusion, we have sequenced and analysed the NP gene of three lentogenic and one mesogenic vaccine viruses and found that the gene remains conserved and variations are mininimal with in viruses of lentogenic and mesogenic categories. We have also found that the method of prediction of B cell epitopes using computer algorithm useful and correlates with pepscan analysis. The sequence analysis data and epitope prediction will be useful with the work on reverse genetics in the development of marker vaccine against ND.

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