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Sequence Analysis and Expression of the Phosphoprotein (P) Gene of a less Virulent Newcastle Disease Virus (D58) Isolated from an Unvaccinated Village Chicken

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Abstract: The P gene of a less virulent Newcastle disease virus isolated from unvaccinated village chicken was studied in detail. This D58 isolate had a coding sequence of 1188 nucleotides coding for 395 amino acids. The site for RNA editing for the evolution of the V and W proteins was found to be at positions 402 at the nucleotide and 134 at amino acid levels. The P gene has two N-Glycosylation sites at positions 126 and 288; one glycosaminoglycan attachment site at position 307, six protein kinase C phosphorylation sites at positions 72, 125, 203, 235, 345 and 369 and ten casein kinase II phosphorylation sites at positions 3, 18, 83, 91, 138, 229, 239, 291, 374 and 381. The D58 isolate was grouped with the other lentogenic strain B1 in the phylogentic analysis. On comparison with 23 other NDV strains/isolates, the P gene sequences of NDV was observed to be evolved with same pattern of substitutions and there is not much evolutionary divergence. The mean evolutionary diversity for entire population of NDV strains/isolates was estimated to be only 0.147. The phosphoprotein gene was expressed in a prokaryotic vector and strong antibody response against recombinant phosphoprotein was reported in the serum of vaccinated birds in ELISA.

Key words: Newcastle disease, P gene, sequence analysis, phylogenetic analysis, epitope prediction, expression, ELISA

INTRODUCTION

The Office International des Epizooties (OIE) has defined Newcastle disease as an "infection of birds caused by Avian Paramyxovirus 1 (APMV-1) with an ICPI value of more than 0.7, possessing three arginine (R) or lysine (K) residues between position 113 and 116 of F gene and phenylalanine (F) at position 117". The disease was earlier classified as a list A infection and now reclassified as one of the notifiable avian diseases by the OIE. The disease has also been reported to cause severe production and economic losses. The economic impact of ND and its effect on trade in commercial poultry was found to be more important and serious (Alexander, 2001a,b). The NDV virus particles were observed to be pleomorphic with variation in size ranging from 150-400 nm and an envelope covered with spike glycoproteins measuring 8-12 nm in diameter. The genome was also observed to be typical of Baltimore group V-single stranded RNA of negative sense with a molecular weight of 5.2-5.7 x 10^6 Daltons (Alexander, 1997) having 15,186 nucleotides (Krishnamurthy and Samal, 1998; Phillips *et al*., 1998; De Leeuw and Peeters, 1999) adhering to the "rule of six theory" (Calain and Roux, 1993) specific for the members of the family *Paramyxoviridae*. The genomic RNA has been reported to encode for six proteins namely-Nucleocapsid (NP) protein, Phosphoprotein (P), Matrix Protein (M), Fusion glycoprotein (F), Haemagglutinin Neuraminidase Protein (HN) and Large Protein (L), (Peeples, 1988; Steward *et al*., 1993). The other proteins V and W were reported to be formed as a result of mRNA editing at P gene (Steward *et al*., 1993; Locke *et al*., 2000). The genomic RNA was found to be associated with the NP, P and L proteins forming the Ribonucleoprotein Complex (RNP) that serves as a template for RNA synthesis and transcription (Hamaguchi *et al*., 1983; Horikami *et al*., 1992; Yusoff and Tan, 2001). The negative sense RNA genome encapsidated by about 2,200-2,800 molecules of NP was reported to form the nucleocapsid core (Lamb *et al*., 1960) along with auxiliary nucleocapsid proteins P and L (Kingsbury, 1974; Bhella *et al*., 2002). Analysis of mRNAs produced from the P gene showed that 68% were P- encoding mRNA, 29% were V-encoding mRNA and 2% were W-encoding mRNA (Mebatsion *et al*., 2001). The NDV edits its P gene mRNA by the insertion of G residues at 402nd nucleotide. When one G is inserted it results in the production of V protein, which plays a direct role in virus replication besides serving as virulence factor. In the same gene, when two G residues are inserted in 402 position another protein W is produced. Of the two additional proteins V and W reported, V was reported to function as an alpha interferon antagonist (Huang *et al*., 2003) but the role of W protein remains uncertain (Cattaneo *et al*., 1989). The P protein was also reported to be a modulator component during RNA synthesis besides acting as a chaperone to prevent the uncontrolled encapsulation of non-viral RNA by the unassembled NP protein (Errington and Emmerson, 1997). The gene sequence data on NDV isolates obtained from India are minimal. Of the six genes of NDV, most of the data available for NDV

isolates from India are for the F gene particularly for the Fusion Protein Cleavage Site (FPCS). The reason being that the F gene is one of the virulence determinants of NDV. Of late, it has been reported that apart from F gene, HN and P genes also have role in the virulence. However, to the extent possible to us we could not come across information on the sequence of P gene of any of the Indian isolates of NDV. Hence, it was decided to study the P gene thoroughly in a NDV isolate named D58 available at the Veterinary Microbiology Department of the Madras Veterinary College. The D58 isolate of NDV was obtained from an apparently normal village chicken, which has never been vaccinated. Further this isolate was found to have a ICPI value of 0.14 possessing the amino acids GGQGRL between positions 112 and 117 in the F gene (Ananth *et al*., 2008). It is being used as live vaccine. In this work the Phosphoprotein (P) gene of D58 isolate of NDV was amplified by RT-PCR, sequenced and expressed in prokaryotic system.

MATERIALS AND METHODS

Virus: The thermostabilized low intracerebral pathogenicity index (ICPI-0.14) isolate of NDV (D58) obtained from apparently normal village chicken was used in this study (Ananth *et al*., 2008). This virus was maintained in the laboratory by propagating it in the Embryonated Chicken Eggs (ECE) as per the standard procedures mentioned in the Terrestrial Manual of OIE (2004). The virus was cultivated in the allantoic cavity of embryonated chicken eggs and the infected Amnioallantoic Fluid (AAF) collected after 96 hrs of infection was used for RNA extraction.

Viral RNA extraction and RT-PCR: The RNA was extracted from plaque purified D58 isolate of NDV using TRIZOL®LS reagent as per manufacturer's instructions with minor modifications at RNA pellet washing stage. The RNA pellet was washed thrice with 75% ethanol by centrifuging at 7,500 g for 10 min instead of one time as suggested by the manufacturer and the RNA pellet was subsequently air-dried. The primers for P gene were designed using FastPCR software and validated using softwares Oligoanalyser and Genetool. The sense (5'CAG AGG CAC AAT GCG GCT CAC TA3') and anti sense (5'CCT GTG TCT TGT AGG ACG ATC GGA A3') primers bind at nucleotide positions 1745-1767 and 3358-3382 respectively in the whole genome of NDV. Two-step RT-PCR was carried out using Thermoscript RT-PCR kit (Invitrogen, USA, cat # 1146-032). Reverse transcription was carried out at 50°C for 50 min using P gene specific sense primer and the amplification of cDNA was carried out at an initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 57°C for 50 sec, 72°C for 1 min and final extension of 72°C for 10 min.

DNA sequencing and phylogenetic analysis: The P gene amplicons were purified by using PureLink™ Quick Gel Extraction Kit (Life technologies, India Cat # K2100-12) following manufacturer's instruction. The sequencing of purified P gene of ND vaccine virus under study was carried out by big dye termination chemistry method in an automated sequencer (ABI Prism, version 3, Applied Biosystems, USA). The multiple sequence alignment of P genes was carried out using the Bio-Edit software (Hall, 1999) to generate sequence analysis data. The phylogenetic tree was developed using Neighbour Joining (NJ) and maximum parsimony algorithms using bootstrap values and distance in Mega 4.1 software (Tamura *et al*., 2007). The B cell epitopes of P gene was predicted in a computer algorithm using web server based software http://bioinfo.bug.ac.il/bus/immunology/epitope_pred/index.htm following the algorithm developed by Bates and Sternberg (1999), Bates *et al*. (2001), Contreras-Moreira and Bates (2002). The secondary structure was predicted using predict protein algorithm of Rost *et al*. (2004) using web based protein predeiction software http://www.predictprotein.org/newwebsite. The secondary structures predicted included identification of domains, glycosylation and phosphorylation sites.

Cloning and expression of P gene: The P gene amplicons were purified by using PureLink™ Quick Gel Extraction Kit (Life technologies, India Cat # K2100-12) following manufacturer's instruction. The PCR product of P gene of D58 virus was cloned into pTrcHis2-TOPO cloning vector. (Cat # 4400-01 of Invitrogen USA), which contained the vector and one shot chemically competent TOP 10 *E. coli* cells. The LB broth (Himedia, India, cat # M1245), LB agar (Himedia, India, cat # M1151), Glycerol (Merck, cat # 17863), Ampicillin sodium salt (cat # RM 645 4218) at a concentration of 50 µg/ml of medium and Glucose (SRL, cat # 074013) at a final concentration of 0.5% of the medium were the other reagents used. Cloning of P gene into pTrcHis2-TOPO vector and transformation into chemically competent Top 10 *E. coli* cells and analysis of the transformants were carried out as per the supplier's instructions. Further, scaling-up of expression and storage of clones for longer duration was also carried out as per the supplier's instruction. Plasmids were extracted from transformed cells by alkaline lysis method as suggested by Sambrook and Russell (2001) using the standard reagents and the plasmids were confirmed by electrophoresis at 70V for 35 min in 1% agarose gel. After identification of the positive clones, expression of the P gene insert was induced by adding 1mM IPTG (Isopropylthio-beta-D-galactoside) (Sigma-Aldrich # 15502) as per the manufacturer's instruction and time point samples were collected for subsequent analysis by PAGE.

Confirmation of viral proteins

Sodium Dodecyl Sulphate (SDS)-Poly Acrylamide Gel Electrophoresis (PAGE): The presence of recombinant protein was confirmed by SDS-PAGE following the procedure of Laemmli (1970). The proteins were solubilized with equal volume of sample buffer and heated at 100°C for 2 min. Samples were loaded into wells in a 5% acrylamide stacking gel over a 12% acrylamide separating gel. The gels that were meant for Western blotting were not stained with Coomassie brilliant blue, whereas other gels were stained for four hours in Coomassie brilliant blue and destained with destaining solution until the gel background became clear.

Western blotting: The proteins were also confirmed by Western blotting following the procedure of Reynolds Maraqa (2000) in Nitrocellulose membrane (NC) with pore size of 0.45 (Sigma-Aldrich cat # N 8267), Skim milk powder (Himedia cat # RM1254) at a concentration of 5%, Anti-chicken IgG peroxidase conjugate (Sigma-Aldrich, USA cat # A9046) at a dilution rate of 1:2000 and Sigma Fast 3,3 Diaminobenzidine (DAB) tablet (Sigma-Aldrich cat # D-4291) were the other reagents/items used in Western blotting.

Purification and quantification of recombinant protein: The recombinant proteins were purified by expanded bed adsorption chromatography using Nickel-Cl agarose column (M/s Genei cat# PC-137) after dialysis following manufacturer's instruction. The concentration of recombinant protein was estimated by measuring the optical density values in a spectrophotometer (Inc, µ Quant) at 260 and 280 nm wavelength, then the concentration was estimated by applying the formula as specified by Sambrook and Russell (2001).

Confirmation of reactivity of recombinant P protein: The reactivity of recombinant P protein was checked by Indirect ELISA following the procedure of John Kirubaharan *et al.* (2008). The indirect ELISA was carried out with four positive serum samples (received from birds that have received two lentogenic and one mesogenic vaccine) and one negative serum sample obtained from unvaccinated chicken. The reactivity of recombinant P protein to these samples was also compared with whole virus protein, recombinant F, HN and NP proteins. The recombinant F, HN and NP proteins are already available in the department were used.

RESULTS AND DISCUSSION

RT-PCR: The P gene of the D58 virus was amplified by RT-PCR. The PCR products of P gene was purified and sequenced. The complete coding sequence (CDs) of P gene sequence generated for D58 isolate of NDV was submitted to GenBank and the accession number EU419882 was obtained.

Structure of P gene: The P gene of the D58 isolate of NDV was found to have 1443 number of nucleotides in total with a coding sequence of 1188 nucleotides coding for 395 amino acids. The 5' and 3' UTR's was found to have 75 and 180 nucleotides respectively. The site for RNA editing in the CDs was found at nucleotide position 402 and at amino acid position 134 as shown in the Fig. 1 and 2 respectively. Based on the prediction of secondary structures the P gene was found to have two N-Glycosylation sites at positions 126 and 288; one glycosaminoglycan attachment site at position 307, six protein kinase C phosphorylation sites at positions 72, 125, 203, 235, 345 and 369 and ten casein kinase II phosphorylation sites at positions 3, 18, 83, 91, 138, 229, 239, 291, 374 and 381.

Comparison of P gene: The details of sequence difference count and different matrices at nucleotide and amino acid levels are provided as Table 1. The sequence difference matrix was calculated at both nucleotide and amino acid level. The percentage of variation between D58 and other isolates range from 0-22%. The average percentage of variation between D58

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       370        380        390        400        410        420
P PROTEIN       CTTGACAAGC TCAGCAATAA ATCGTCCAAT GCTAAAAAGG GCCCATGGTC GAGCCCCCAA
V PROTEIN       CTTGACAAGC TCAGCAATAA ATCGTCCAAT GCTAAAAAGG GGCCCATGGT CGAGCCCCCA
W PROTEIN       CTTGACAAGC TCAGCAATAA ATCGTCCAAT GCTAAAAAGG GGGCCCATGG TCGAGCCCCC
```

Fig. 1: RNA editing at nucleotide position 402-evolution of sub proteins V and W

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      130        140        150        160        170        180
P PROTEIN       LDKLSNKSSN AKKGPWSSPQ EGNHQRPTQQ QGSQPSRGNS QERPQNQVKA APGNQGTDVN
V PROTEIN       LDKLSNKSSN AKKGPMVEPP RGESPTSDST AGESTQSRKQ SGKTAEPSQG RPWKPGHRRE
W PROTEIN       LDKLSNKSSN AKKGAHGRAP KRGITNVRLN SRGVNPVAET VRKDRRTKSR PPLETRAQT*
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Fig. 2: RNA editing at amino acid position 134-evolution of sub proteins V and W

Table 1: Comparison of P gene of D58 with other strains/isolates of NDV

	Parameters						
	SDM-AA (in %)	SDM-Nt (in %)	SDC-AA (in Nos.)	SDC-Nt (in Nos.)	Substitution pattern	Base Composition bias	Disparity index
D58	ID	ID	ID	ID	ID	ID	ID
LaSOTA	4.9	3.8	19	44	1	0.004	0
CLONE 30	5.1	3.8	20	45	1	0.001	0
B1/48	8.9	5.9	35	69	0.258	0.076	0.018
Queensland V4/66	14.2	15	56	178	1	0.091	0
I-2	15	16.3	59	193	0.32	0.194	0.032
I-1 progenitor	15	16.3	59	193	0.276	0.194	0.032
HB92/V4	4.6	3.6	18	42	1	0.003	0
Beaudette C	6.9	5.6	27	66	1	0.001	0
Anhinga	23.8	21.8	94	258	1	0.019	0
Mukteswar	14.5	15.5	57	183	1	0.044	0
Roakin/48	6.4	5.3	25	62	1	0.011	0
Texas GB	10.4	7.9	41	93	1	0.026	0
Herts/33	11.2	13.3	44	157	1	0.035	0
Sterna	21.3	19.7	84	233	1	0.103	0
GPMV	20	20.2	79	239	1	0.077	0
Italien	13.7	15.1	54	179	1	0.104	0
AF2240	17	17.1	67	203	1	0.008	0
ZJ1	20	20.4	79	242	1	0.04	0
NA-1	20	19.4	79	230	1	0.04	0
PHY-LMV42	11.2	12.8	44	151	0.156	0.215	0.088
Wfan/2/00	20.3	19.8	80	235	1	0.173	0
PPVM	5.4	4.3	21	50	1	0.009	0
Duck/1/05	20.6	20.6	81	244	1	0.031	0

SDM-Sequence Difference Matrix in %; SDC-Sequence Difference Column in numbers; AA-Amino Acids; Nt-Nucleotides; ID-Identical

and other lentogenic starins like LaSota, Clone 30 and B1/48 was 6.3%; with apathogenic starins like HB92/V4, Quensland V4/66, I-2 and I-2 Progenitor it was 12.2 %; with mesogenic strains like Beaudette C, Anhinga, Mukteswar and Roakin/48 it was 12.9 %; with velogenic strains like TEXAS GB, Herts/33, Stema, GPVM, Italien, AF2240, ZJ1, NA-1, PHY-LMV42 and Wfan/2/00 it was 16.51% and with the other strains like PPVM and Duck/1/05 it was calculated to be 13%.

The amino acid sequence of P gene was compared with the P gene sequences of 23 other NDV strains/isolates. The substitution pattern homogeneity between D58 and other 23 NDV strains/isolates as disparity index are presented in Table 1. These values represent the extent of differences in base composition biases between sequences on the basis of Monte Carlo test with 1000 replicates. The disparity index values, when reported to be less than 0.05 were considered significant, meaning that sequences have not been evolved with same pattern of substitution. However, in the present study the p values obtained between D58 and other strains/isolates of NDV was found to be not significant and were more than 0.05 suggesting that the sequences have evolved with same pattern of substitutions. In an earlier study by Locke *et al.* (2000) the synonymous substitution rates (0.2978) were found to be more than the non-synonymous substitutions (0.0733). Our findings correlated with this earlier report. Further, when the net base composition bias disparity between D58 and other 23 strains/isolates of NDV was carried out, the composition bias was observed to be more than '0' between D58 and B1 (0.018) D58 and I2 and I2 progenitor (0.032) and D58 and PHY-LAM (0.088). Whereas, it was '0' in all other comparison suggesting that there is not much difference in evolutionary divergence at the P gene of NDV. This fact is further strengthened by positive correlation between the net base composition and sequence difference count (r = + 0.73; n = 24) minimal average evolutionary divergence value of 0.158 and a low Tajima test statistic value of 0.098694 obtained in this study. The Tajima test was carried out to test nucleotide mutation hypothesis by DNA polymorphism. The average evolutionary distance over the sequences were estimated with in groups and between groups. For this purpose the strains were grouped in to five groups namely lentogenic (D58, LaSota, Clone 30 and B1/48), apathogenic (HB92/V4, Quensland V4/66, I-2 and I-2 Progenitor) mesogenic (Beaudette C, Anhinga, Mukteswar and Roakin/48) velogenic (TEXAS GB, Herts/33, Stema, GPVM, Italien, AF2240, ZJ1, NA-1, PHY-LMV42 and Wfan/2/0 and others (PPVM and Duck/1/05). The average evolutionary distance over the sequences within and between groups were tabulated and shown in the Table 2. The mean evolutionary diversity within subpopulation was found to be 0.127, the mean evolutionary diversity for entire population was 0.147 and the mean inter population diversity was 0.020.

The B-cell epitopes of the P gene were predicted and it was found to have 14 epitopes, whose sequences and positions in the P gene are provided in Table 3. To the best of our knowledge we could not any other

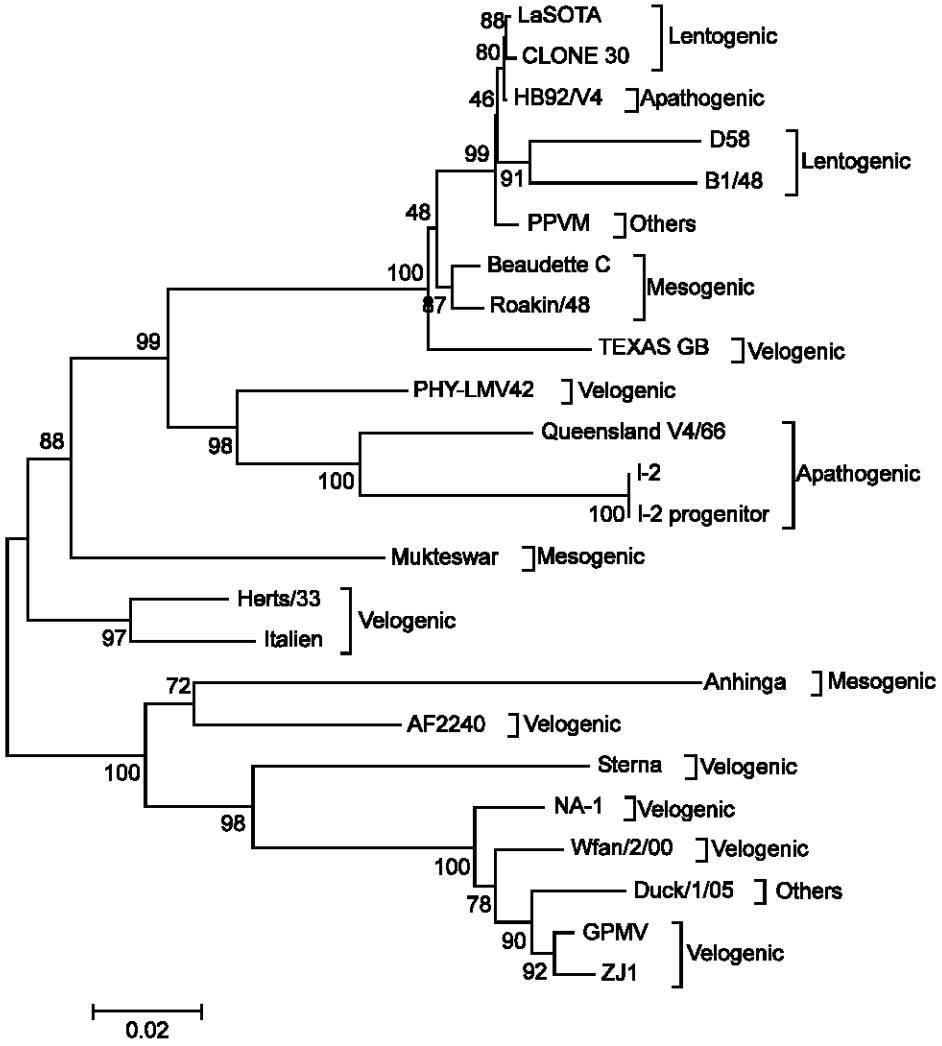

Fig. 3: Phylogenetic tree (Neighbour joining) with distance and bootstrap value-phosphoprotein gene of newcastle disease viruses

Table 2: Average evolutionary distance within and between groups among NDV strains/isolates using P gene

Groups	Lentogenic	Apathogenic	Mesogenic	Velogenic	Others
Lentogenic	0.038	0.099	0.155	0.135	0.209
Apathogenic	0.127				
Medsogenic	0.120	0.147			
Velogenic	0.176	0.168	0.160		
Others	0.127	0.159	0.148	0.136	

The average evolutionary distance values within group are provided in bold letters

publication dealing with the epitopes in the P gene. The phylogenetic tree was developed using Neighbour Joining (NJ) and Maximum Parsimony (MP) and are provided in Fig. 3 and 4 respectively. In the phylogenetic tree, major clades comprise of all the pathotypes. However, at the subgroup level the group formation is purely based on the pathotype with bootstrap values for number of subgroups close to 90. The D58 isolate was found to be in a subgroup along with B1 with boot strap values as high as 91. In an another study, where phylogeneic tree was drawn with sequences of nucleocapsid protein gene of D58 isolate with other strains/isolates of NDV, clades were found to be more stable (data not provided) than P gene. This could be due to the more conserved nature of the nucleocapsid protein gene. The less conserved nature of P gene among NDV strains/isolates in comparison to NP gene was further strengthened in the entropy chart developed using the amino acid sequence of the P gene, which is provided as Fig. 5. It could be appreciated that the conserved areas in CDs are restricted to the terminal part of the gene starting from 294th amino acid only. Whereas, in NP gene the CDs is conserved upto 375th amino acid irrespective of the virulence (Krishnamurthy and Samal, 1998). The P gene despite being part of ribonucleoprotein complex is not found to be as conserved as the other member of the complex namely

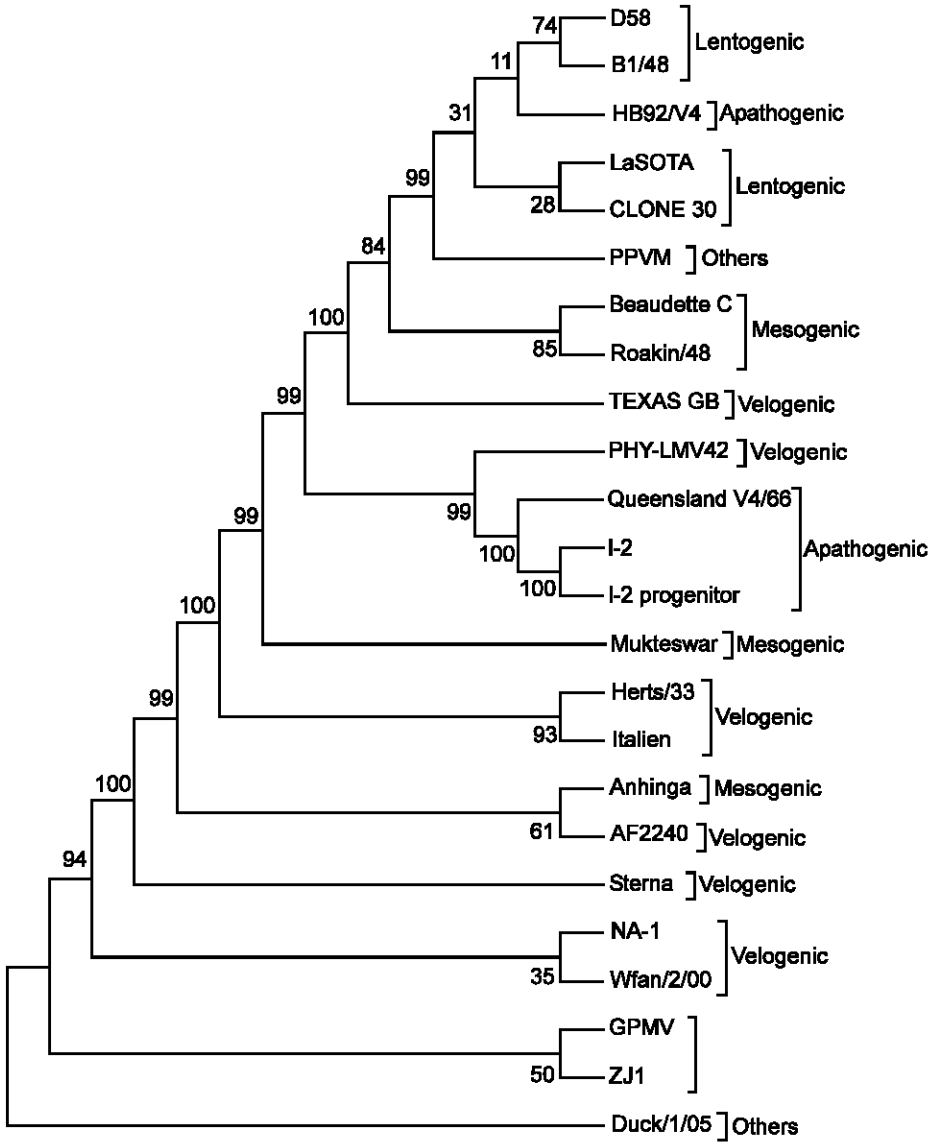

Fig. 4: Phylogenetic tree (Maximum parsimony) with bootstrap value-phosphoprotein gene of newcastle disease viruses

Table 3: B cell epitopes of P gene

Epitope Number	From	To	Amino acid sequence
1	283	309	DPGCANISSLSDLRAVARSHPVLVSGP
2	235	253	SQRVSKVDYQLDLVLKQTS
3	208	226	TIPLYLRIMSSHLLDFVQA
4	262	274	IQQLKTSVAVMEA
5	325	349	NKLSQPVPHPSELIKPATACGPDIG
6	115	122	NSLLLMLD
7	366	379	PSSSAKLLSKLDAA
8	43	50	KTKVLSAA
9	312	318	PSPYVTQ
10	355	361	VRALIMS
11	36	41	RSAIPQ
12	166	171	NQVKAA
13	56	61	SIQPPA
14	191	203	QLSAGATPHALRS

Amino acids are indicated by IUPAC code

the Nucleocapsid Protein (NP). No variation was reported as far as site of the RNA editing was concerned between this study and earlier studies.

Cloning and expression of P gene: Expression of P gene insert in pTrcHis2 vector was then induced by 1 mM IPTG. After pilot expression analysis of time point samples that have collected has been analyzed by running the SDS-PAGE gel and compared with a control sample containing TOP 10 *E. coli* cells alone without any insert. The details are provided as Plate 1. The protein separated by SDS-PAGE was transferred to nitrocellulose membrane and the recombinant P protein was confirmed by treating the membrane with ND positive serum available in the lab.

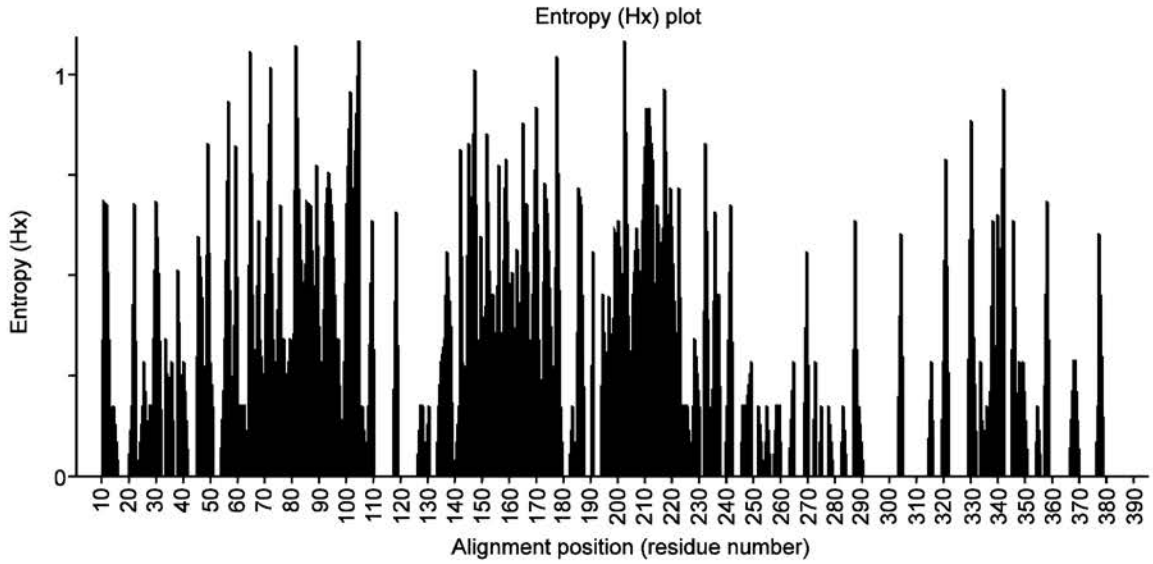

Fig. 5: Entropy (H(x)) plot for amino acid of Phosphoprotein gene of newcastle disease virus

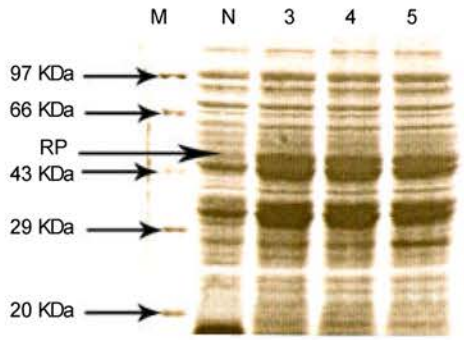

Plate 1: Cloning of phosphoprotein (P) gene-analysis of time point samples for checking recombinant protein by SDS-PAGE; (M) Protein maker, (N) Negative cell lysate (RP) Recombinant phosphoprotein (3, 4, 5) Time point samples of 3rd, 4th and 5th hour

Confirmation of reactivity of recombinant P protein: The reactivity of recombinant Protein was confirmed by indirect ELISA. The result of ELISA for each sample as OD values after blanking is shown in the Table 4 and Fig. 6. The protein was found to react with serum obtained from vaccinated birds and it did not react with serum obtained from unvaccinated birds. While starting the study we were under the assumption that the antibody response against P protein may not be very strong since it is an internal protein and concerned only with the replication of viruses. But to our surprise we found that based on the OD values strong antibody response was observed in chicken vaccinated against NDV. We are not concluding that P gene protein could be an immunogenic protein, this need to be further studied

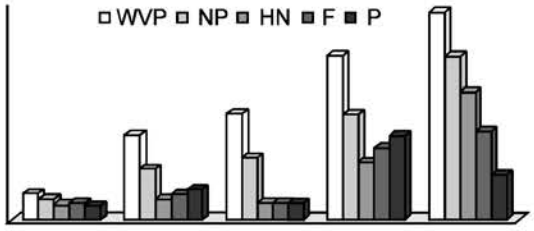

Fig. 6: Antibody response against different proteins of NDV in comparison with whole virus protein

Table 4: Antibody response against different proteins of NDV in comparison with whole virus protein

	Optical Density values at 405 nm				
Sample	WVP	rNP	rHN	rF	rP
Negative	0.367	0.286	0.219	0.229	0.211
Positive 1	1.178	0.711	0.294	0.366	0.432
Positive 2	1.474	0.857	0.236	0.237	0.234
Positive 3	2.260	1.454	0.793	0.988	1.158
Positive 4	2.860	2.243	1.747	1.211	0.612

WVP- Whole Virus Protein, rNP- recombinant Nucleocapsid Protein, rHN-recombinant Haemagglutinin Neuraminidase Protein, rF- recombinant Fusion Protein, rP-recombinant Phosphoprotein

since the role of Phosphoprotein in immunity has not been elucidated so far. To the best of our knowledge, we could not come across any other paper dealing with expression of P gene of NDV in prokaryotic system.

Conclusion: The P gene of NDV was found to be not as conserved as the other member of the ribo-nucleoprotein complex namely the nucleocapsid protein gene. There is not much difference in evolutionary divergence at the P gene of NDV. Further, strong

antibody response was detected in the serum of vaccinated birds against recombinant phosphoprotein.

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