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Confirmation of B-Cell Epitope of Nucleoprotein of Newcastle Disease Viruses by Dot-ELISA and Peptide ELISA

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Abstract: A conserved B-cell epitope of nucleoprotein with the amino acid sequence "FLDLMRA" was found to be located in the conserved region between ^{443}GETQFLDLMRAVANS^{457} of nucleoprotein of all four vaccine viruses used in South India. The conserved region incorporating the epitope ^{443}GETQFLDLMRAVANS^{457} has been synthesized as a custom peptide. The immunodominance of the epitope was confirmed by dot-ELISA and peptide ELISA. Peptide ELISA developed using the entire conserved region was found to produce a consistent result with HI and Whole Virus Protein (WVP) ELISA. The immunodominance and immunogenicity was identified and confirmed by significant positive correlation ($p \leq 0.01$) between peptide ELISA with HI and with WVP ELISA and also by Probability Plot (PP). Based on PP it could be appreciated that all points cluttered towards the expected line confirming the closeness of the results which is further strengthened by observation from ROC curves. The empirical ROC values for HI Vs Peptide ELISA and WVP ELISA VS Peptide ELISA were 0.97 and 1.00 respectively which are more than the standard value 0.8 confirming the closeness of the results proving immunodominance of the epitope and utility of the epitope to serve as a marker in DIVA vaccine.

Key words: Newcastle disease virus, NP gene, B cell epitopes, immunodominance, HI- ELISA, marker

INTRODUCTION

Newcastle disease caused by Newcastle Disease Virus (NDV)(Alexander, 1997) has accounted for significant economic loss to the poultry industry in many developing countries (Alexander, 2001). The NDV has been classified as a member of the order Mononegavirales in the family Paramyxoviridae subfamily Paramyxovirinae and under the genus Avula virus (VIII report of the International Committee on taxonomy of viruses). There are nine species under the genus Avulavirus and of which Avian Paramyxovirus-1 (APMV-1) with an Intracerebral Pathogenicity Index (ICPI) value of more than 0.7 causes clinical disease with respiratory distress, diarrhoea, increased morbidity and mortality (Alexander, 2003; Terrestrial Manual of OIE, 2004). Of the six proteins of NDV, other than HN and F, NP has been reported to be immunogenic (Errington *et al*., 1995). The potential of the internal viral components to function as the basis for a diagnostic system has focused interest towards the nucleoprotein of negative sense RNA viruses. The NP of many paramyxoviruses has been reported to be highly immunogenic in nature and used to produce diagnostic ELISA, which include disease like Mumps (Linde *et al*., 1987), Measles (Hummel *et al*., 1992; Fooks *et al*., 1993) and PPR (Dechamma *et al*., 2006). The conserved multifunctional NP of NDV has been reported to be efficient in inducing NDV specific antibody in chickens (Makkay *et al*., 1999; Reynolds and Maraqa, 2000; Ward *et al*., 2000; Mebatsion *et al*., 2002). The ELISA system developed by using NP expressed in Baculo virus system was found to be reliable and did not produce any false positive or false negative in comparison with whole virus protein (Errington *et al*., 1995). It has also been reported that in comparison to commercially available ELISA, a better correlation was observed between the recombinant NP ELISA with neutralizing antibody levels than with commercial ELISAs (Hummel *et al*., 1992; Errington *et al*., 1995). Further NP-based ELISA has been developed to discriminate between the antibody response to a recombinant fowl pox virus vaccine in which HN gene of NDV was expressed by a recombinant fowl pox virus (Makkay *et al*., 1999). A 18-mer NP-peptide based ELISA was reported to differentiate vaccinated birds from infected in association with a marker vaccine in which all sera from birds immunized with conventional live or inactivated vaccines were positive with NP peptide ELISA indicating sensitivity and specificity of the test under laboratory condition (Mebatsion *et al*., 2002).

Dot-ELISA for the detection of antibodies against NDV has also been reported (John Kirubaharan, 1996; Folitse *et al*., 1999; Manoharan *et al*., 2004; Farhid Hemmatzadeh and Alisharif Zadeh, 2006). Further it was reported to be more sensitive than HI test in detecting

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antibodies to NDV following infection and 90% of the infected chickens were diagnosed by Dot-ELISA as early as 4 days after infection (Folitse *et al*., 1999). A quantitative Dot immunoassay techniques for the detection of NDV antibodies has been reported and the results of this technique correlated to statistically significant level with HI, ELISA, Serum Neutralization (SN) and Leucocytic Migration Inhibition (LMI) assay values (John Kirubaharan *et al*., 2000). Immunocomb based Dot-ELISA were reported to be developed to measure the antibody status for the three major poultry diseases simultaneously Newcastle Disease (ND), Infecctious Bursal Disease (IBD) and Avian Infectious Bronchitis (IB) (Manoharan *et al*., 2004) and ND and IB (John Kirubaharan, 2004).

Development of DIVA vaccine is essential to evolve a strategy to eradicate ND. In the present study, base line data essential for development of DIVA (Marker) vaccine for ND was generated. An attempt was made to identify conserved B-cell epitopes of NP of ND vaccine viruses and confirmation of its immunodominance by Dot-ELISA (Qualitative) and peptide ELISA (Quantitative) and compared with Whole virus protein ELISA and HI and the utility of the epitope to serve as a marker in DIVA vaccine.

MATERIALS AND METHODS

Viruses: The Newcastle Disease Virus (NDV) vaccine strains viz., F, K and LaSota, which are commonly used in this part of the country and a thermo stabilized low Intracerebral Pathogenicity Index (ICPI) (0.14) isolate of NDV (D58) were used in this study. The vaccine strains were obtained from Institute of Veterinary Preventive Medicine (IVPM), Ranipet, Tamil Nadu and D58 isolate was provided by the courtesy ICAR scheme on Preparation and standardization of oral pellet vaccine against Newcastle Disease (ND) at the Department of Veterinary Microbiology, Madras Veterinary College, Chennai - 600 007, S.India.

Immunologicals: The different types of serum/ monoclonal antibodies used in this study were Reference positive serum against NDV (Veterinary Laboratories Agency, U.KCode PAO 155), Hyper immune serum against NDV and Negative serum against NDV (Department of Veterinary Microbiology, Madras Veterinary College, Chennai), Monoclonal antibodies against Infectious bronchitis (Dr. Syed Naqi, Professor (Avian Medicine), Cornell University, Ithaca, NY 18053, USA. Common reagents like Phosphate Buffered Saline (PBS) pH 7.2, normal saline, Alserver's solution and Tris Acetate EDTA (TAE) buffer were used in different procedures as per the standard composition.

Molecular characterization of vaccine viruses: The pathogenicity of all the vaccine viruses used in this study was confirmed by analyzing the amino acid sequence of the fusion protein cleavage site of F gene after amplifying the region by RT-PCR using Sense Primer - CCTTGG TGA'ITC TAT CCG IAG and Antisense primer - CTG CCA CTG CTA GTT GIG ATA ATC C (Seal *et al*., 1995).

Sequence analysis of NP gene: The sequencing of NP gene of vaccine viruses under study was done by primer walking using four primers that were synthesized Sense primer - 5' GAA GGT GTG AAT CTC GAG TGC G 3', 47 BG - 5' GGA TCC TCT CTA TCC AGG C 3', 59 BG - 5' CAT CAG CCC TTG CAC TTA GTA G 3', 80 BG - 5' GGC TCA GGG AAG TAG CAT TAA C 3'. The sequences were analyzed using the Bio-Edit software for multiple sequence alignment supplied by North Carolina State University, USA to generate sequence analysis data. The parameters used for sequence analysis were multiple alignment (Clustal W), sequence identify plotter and sequence identity matrix for both nucleotide and amino acids.

Epitope prediction: The B cell epitopes of NP protein were predicted in a computer algorithm using 3D JIGSAW web server based software - http://www.bmm.icnet.uk/~3djigsaw/ following the algorithm developed by Bates and Sternberg (1999); Bates *et al*. (2001); Contreras-Moreira and Bates (2002). The epitope predicted was synthesized as custom peptide in protein synthesis laboratory and used in NP peptide ELISA.

Tests to confirm immunodominance

Indirect ELISA for IgG with whole virus protein (WVP ELISA): The antibodies against NDV were estimated using single serum dilution ELISA kit supplied by the Department of Veterinary Microbiology, as per the manufacturer's instruction. The procedure for preparation of antigen for indirect ELISA as specified by the manufacturer of the kit. The D58 field isolate of NDV was used as ELISA antigen. The virus was propagated in the allantoic route in the 9-10 days old ECE. The infected allantoic fluid was clarified by centrifugation at 7,000 g for 30 min at 4°C (Hettich, universal 30 RF). The virus was palette by centrifuging at 35,000 rpm for three hours in a Beckman (L7-80) ultracentrifuge in 70 Ti rotor at 4°C. The pellet obtained was suspended in 1x TE buffer and again centrifuged at 35,000 rpm for three hours in an ultracentrifuge at 4°C in sucrose gradients of 35% and 55%. Protein was estimated by measuring the optical density values in a spectrophotometer (Bio-Tek Instruments, Inc, µ Quant) at 260 and 280 nm wavelengths and the concentration was estimated by applying the formula as provided by Wilson and Walker (2006). The dynamic working range with regard to antigen and conjugate was determined by repeated checkerboard titration described as per Rose *et al*. (1997). Coating antigen at a concentration of 5 ng/ul and

conjugate at concentration of 1 in 2000 was found to be ideal.

ELISA procedure: The reaction volume for the entire assay was 100 µl /well. After each step, the ELISA plate was washed six times in an automatic plate washer (Bio-Rad, Model # 1575, USA) utilizing 400 µl of wash solution. The microplate (Immunoplate) was sensitized with 500 ng of NDV whole virus protein antigen in 100 µl 0.1 M Sodium Carbonate bi carbonate buffer (pH 9.6) overnight at 4°C. After washing, the plate was blocked with blocking buffer and incubated for one hour at 37°C. After another washing, the plate was incubated with 1: 1000 dilutions of test sera in duplicate for optimum results and the plate was incubated for 90 min at 37°C in humidified atmosphere. After another washing cycle, anti chicken peroxidase conjugate at 1:2000 dilution in blocking buffer was added to the washed plate and incubated for 45 min at 37°C. The enzyme substrate solution (ABTS) was added to the microplate and incubated for 10 min in the dark. The reaction was stopped by adding one per cent Sodium Dodecyl Sulfate (SDS). Optical Density (OD) values were measured at 405 nm in an automated ELISA reader (Bio-Rad, model ≠ 550, CA, USA). The log ELISA titres were arrived by applying the formula provided by the manufacturer Log10 titre = (Corrected absorbance +0.4208)/0.1838. Corrected absorbance was obtained by taking the mean value of two replicates of a sample and subtracting it with conjugate control.

Peptide (NP) ELISA

Synthesis and coupling of peptide: An 15-mer synthetic peptide comprising the NP aminoacid sequence GETQFLDLMRAVANS was synthesized (M/S GenScript, USA) and used as antigen. The synthesized peptide was coupled to BSA as per the method described by Mebatsion *et al.* (2002). The synthesized peptide (0.5 mg/ml) was coupled to BSA (0.4 mg/ml) in PBS by adding 2.5% glutaraldehyde solution (final concentration 0.067 M). After adding glutaraldehyde, the reaction mixture was incubated at 25°C in darkness. The coupling reaction was stopped by the addition of glycine to a final concentration of 100 mM. Peptide-BSA complexes were stored frozen at -20°C. BSA treated alone in the above method was used as a control. The dynamic working range with regard to antigen and conjugate was determined by repeated checkerboard titration as per Rose *et al.* (1997). Coating antigen at a concentration of 5 ng/ ul and conjugate at concentration of 1 in 1000 was found to be ideal.

Estimation of PNT line: The positive negative threshold line to find out the titre was carried out following the procedure of Snyder *et al.* (1983). Briefly, ten serum samples, which had HI titer of less than 2, were selected. Serial ten fold dilutions of the serum were carried out and ELISA was performed with the samples. The resultant OD values were plotted on Y-axis against dilutions in X-axis. The resultant line is referred as PNT line and used for finding out the Observed Titre (OT).

Estimation of Observed Titre (OT): The OT of 160 serum samples was calculated using the subtraction method (OD value for sample - OD value of conjugate control) as specified by Snyder *et al.* (1983) using the PNT line. Serial ten fold dilutions of the serum samples were carried out and ELISA was performed with the samples. The resultant OD values were plotted on Y-axis against dilutions in X-axis. The point where the sample line cuts the PNT line is taken as titre of the sample.

Development of titre calculation formula: The formula to find out the titre from single serum dilution instead of serial logarithmic dilution was developed by applying the principle of linear regression. The OD values obtained for every logarithmic dilution was compared with OT and correlation coefficient was obtained. The dilution that gives maximum positive correlation was selected for predicting the titre from that dilution. The other parameters like slope and intercept are arrived by plotting a scatter chart with OD values in y axis and OT in x axis. The linear regression formula of y=ax+b is reversed as x=(y-b)/a to find out the log titre and antilog of titre is referred as predicted titre of the sample.

Procedure (single serum dilution): Flat bottomed 96 well Maxisorp plates were sensitized with 500 ng of NDV NP peptide antigen in 100 µl 0.1 M Sodium Carbonate bi-carbonate buffer (pH 9.6) overnight at 4°C. After washing with PBST in automatic micro plate washer the wells were blocked with one per cent BSA fraction V and incubated for one hour at 37°C. Then the plate was washed as done previously and incubated with the test sera diluted in 1:100 and incubated for 45 min at 37°C, followed by washing. Then plates were incubated with anti chicken IgG whole molecule peroxidase conjugate at 1 in 1000 dilution. The plates were again washed and 2,2'-Azino -bis (3-ethylbenz- Thiosoline-6- Sulfonic acid) containing 6 µl H_2O_2 in 10 ml of 0.05 M sodium citrate buffer (pH4.2) was added as substrate. The plates were left undisturbed in the dark for 10 min. The reaction was stopped with 1% SDS. Optical density values were taken in automatic ELISA reader (Bio- Rad, model ≠ 550, CA, USA) at 405 nm after blanking. The log ELISA titres were arrived by applying the formula Log10 titre = (Corrected absorbance +0.11)/0.12. The SP ratio was also estimated the formula of SP ratio = (OD of sample - OD of Negative control)/(OD of positive control - OD of negative control).

Dot ELISA: The optimum dilution of whole virus protein, custom peptide and conjugate was estimated by checkerboard titration described as per Rose *et al.* (1997). Coating whole virus protein antigen at a concentration of 150ng/µl, Peptide antigen at a rate of 500 ng/µl and conjugate at concentration of 1 in 1000 was found to be ideal. The procedure of John Kirubaharan (2004) was followed with slight modifications. One µl of custom peptide (500 ng/µl) and 1 µl of whole virus protein (150 ng/µl) were spotted on to Immuno comb squares. The top layer of immuno comb was dotted with peptide antigen and lower layer with whole virus protein antigen. The immunocomb was air dried and then incubated at 37°C for half an hour. 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 for one hour at 37°C. The immuno comb was washed three times with PBST for two minutes in each well. The immuno comb was allowed to react with the 1 in 50 diluted sera samples to be tested in blocking buffer and incubated at 37°C for 1 hr. The immuno comb was washed three times with PBST. The immuno comb was allowed to react with the anti chicken IgG peroxidase conjugate (1:1000) and incubated at 37°C for half an hour. The immuno comb was washed three times with PBST. The immuno comb was allowed to react with DAB substrate for 10 min in darkness and the reaction was stopped by washing in tap water and air dried and the results were observed.

Statistical validation: The results of three tests obtained to confirm the immunodominance of the epitope were validated following the guidelines of Jacobson (1998) and Terrestrial Manual of OIE (2004). The Receiver Operating Curves (ROC) were drawn to confirm the cut-offs fixed in this test correlating sensitivity and specificity were drawn using ROC fit web based software Johns Hopkins University, Baltimore, USA (www.jrocfit.org). The probability plots (PP plot) were drawn using SPSS package Ver 10.1. The estimation of correlation coefficient and all other graphs were carried out in MS-Excel.

The data format followed for generation of ROC matrix is briefly provided. Each line represents one sample. On each line, two numbers were written. The first number is either "0" or "1", depending on whether the sample is truly positive ("1") or truly negative ("0"). The second number is an integer (1, 2, 3, 4, 5) representing the confidence rating for each sample in a 6-point rating scale.

Sequence analysis of epitope: The repetition of epitope by in chance in any other protein of NDV viz., P, M. F, HN and L was checked in BioEdit software by performing a simple Clustal W alignment.

Table 1: B Cell epitopes of NP gene

Epitope			
Num.	From	To	Amino acid sequence
1	50	62	SFVVFCLRIAVSE
2	200	235	QGRVQKKYILYPVCRSTIQLTIRQSLAVRIFLVSEL
3	69	82	RQGALISLLCSHSQ
4	30	40	TLKVDVPVFTL
5	163	180	ERILSIQAQVWVTVAKAM
6	410	419	TQQVGVLTGL
7	280	290	SALALSSLSGD
8	98	105	TLAVLEID
9	324	333	PAEYAQLYSF
10	248	258	YYNLVGDVDSY
11	131	139	IAGSLPRAC
12	265	274	TAFFLTLKYG
13	85	91	RNHVALA
14	4	15	VFDEYEQLLAAQ
15	361	371	RLGVEYAQAQG
16	381	387	ELKLTPA
17	389	399	RRGLAAAAQRV
18	447	453	FLDLMRA
19	141	149	NGTPFVTAG
20	337	343	MASVLDK

• Epitope identified for further studies is indicated in bold letters.
• Amino acids are indicated by IUPAC codes.
A-Alanine; R-Arginine; N-Asparagine; D-Aspartic acid; B-Asparagine or Aspartic acid; C-Cysteine; Q-Glutamine; E-Glutamic acid; Z-Glutamine/Glutamic acid; G-Glycine; H-Histidine; I-Isoleucine; L-Leucine; K-Lycine; M-Methionine; F-Phenyl alanine; P-Proline; S-Serine; T-Threonine; W-Tryptophan; Y-Tyrosine; V-Valine; Num. = Number

RESULTS

B cell epitopes in NP gene: The identification of B cell epitopes in NP gene was carried out with 3D JIGSAW software. This software predicts B cell epitopes from amino acid sequences. The details of epitopes predicted and their position in ORF of NP gene are provided in Table 1. In total 20 B cell epitopes were predicted by the software. One B cell epitope after 375th amino acid at positon 447-453 with amino acid sequence 'FLDLMRA' was selected for further analysis since amino acid from 1 to 375 in NP gene are essential for herring bone structure of NP gene and are indispensable.

The three dimensional structure of epitope identified was generated by molecular modeling using BallView software Version. 1.1.1. The molecular structure were generated in the following four models viz., ball and stick, Van- Der-Vals (VDV), cartoon and Solvent Excluded Surface (SES). While generating the molecular models, the drawing molecule was kept as solid and each amino acid residue in the epitope was taken in to consideration.

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 and 457. The entire conserved region along with epitope was chemically synthesized in a protein synthesis facility and was used as custom peptide for further work.

Table 2: Immunodominance of Epitope identified-comparison of results between HI, Whole Virus Protein (WVP) ELISA, Peptide ELISA and Dot ELISA

Sl. No	Sample details	HI tire Log 10	SP Ratio		Dot
			WVP	Peptide	ELISA
1	PNT	0	0.0	0.1	NEGATIVE
2	MAB IB	0	0.0	0.0	NEGATIVE
3	PNT	0	0.1	0.0	NEGATIVE
4	PNT	0	0.1	0.1	NEGATIVE
5	PNT	0	0.1	0.1	NEGATIVE
6	MAB IB	0	0.1	0.1	NEGATIVE
7	MAB IB	0	0.1	0.1	NEGATIVE
8	15(12)	0.3	0.1	0.1	POSITIVE
9	60509	0.3	0.2	0.2	POSITIVE
10	658T7	0.6	0.4	0.3	POSITIVE
11	8(12)	1.2	0.5	0.6	POSITIVE
12	8(4)	1.2	0.5	0.6	POSITIVE
13	10(3)	1.2	0.6	0.6	POSITIVE
14	12B(6)	1.2	0.6	0.6	POSITIVE
15	12B5	1.51	0.8	0.9	POSITIVE
16	8(5)	1.81	0.1	0.1	POSITIVE
17	18(5)	1.81	0.8	0.8	POSITIVE
18	8(1)	2.11	0.5	0.6	POSITIVE
19	8(10)	2.11	0.8	0.8	POSITIVE
20	12B14	2.11	0.8	1.0	POSITIVE
21	15(2)	2.11	0.9	1.0	POSITIVE
22	12B21	2.11	1.4	1.6	POSITIVE
23	8(3)	2.41	1.2	1.4	POSITIVE

Immunodominance of the epitope: The imunogenicity and immunodominance of the epitope was confirmed by two tests viz., dot- ELISA and indirect peptide- ELISA. The former was used as a qualitative test and the later as a semi quantitative test.

Dot ELISA: The concentration of whole virus protein and peptide to be used for coating were arrived by checker board and was found to be 150 ng/µl and 500 ng/µl respectively. The dilution of conjugate to be used was also arrived by checker board and was found to be 1:1000 for both. The dilution of serum to be used was arrived by checker board and was found to be 1:100. The results of the dot- ELISA were compared with HI, Peptide ELISA and indirect Whole Virus Protein (WVP) ELISA. The details are provided in Table 2. The intensity of dots produced by both custom peptide and WVP were found to be same. Dots were produced in all custom peptide coated combs as in WVP coated combs confirming the immunodominance of epitope.

Development and standardization of peptide ELISA: The concentration of peptide antigen used for coating the ELISA plates (5 ng/µl of coating buffer), the concentration of conjugate (1 in 2000) were arrived by checker board titration method.The indirect peptide ELISA was standardized to predict titre from single serum dilution, instead of using multiple serum dilutions. The prediction was made through a formula developed following the principles of linear regression. For developing the formula to predict the titre, 47 numbers of sera samples obtained from controlled field trail were used. The ELISA titres of the sample are referred as observed titre and this was calculated by' subtraction method. To find out the Observed Titre (OT), Positive Negative Threshold (PNT) line was developed with 10 negative samples. The dilution at which a sample cut PNT line was taken as titre and referred as Observed Titre (OT). The PNT line generated is provided as Fig. 1. The OT thus arrived was compared with OD values of sample taken at different serum dilutions viz., 100, 1000, 2000, 5000, 10000, 100000 and correlation coefficient was arrived for each comparison. The correlation coefficient at 1: 100 was 0.92 and which was more than that of all other dilutions, Hence 1:100 was preferred to predict the titre. The two other constants required to predict titres from serum dilution of 1:100 viz., slope (A) and intercept (B) were estimated by drawing a scatter chart. The scatter chart thus developed is provided as Fig. 2. The slope and intercept were 0.12 and 0.11 respectively. Using these two constants, by applying the linear regression equation (y=ax + b), the formula for calculating titre was obtained which is as read as follows x = (y-b)/a. log10 titre (x) = (Absorbance-intercept)/slope. Further, the sample: positive ratio was also calculated by applying the formula already mentioned in materials and methods. A copy of the model work sheet is provided as Table 3. The table comprises of statistical parameters of measures of dispersion like Standard Deviation (SD) and Coefficient

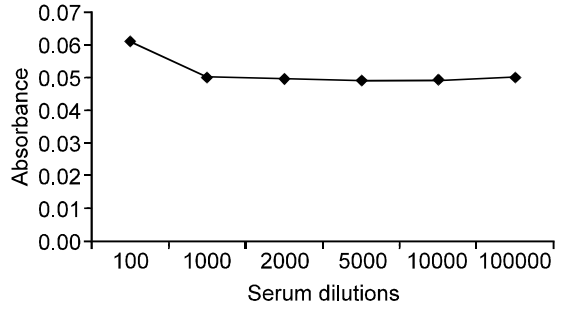

Fig. 1: Positive negative threashold line (PNT line) for peptide ELISA

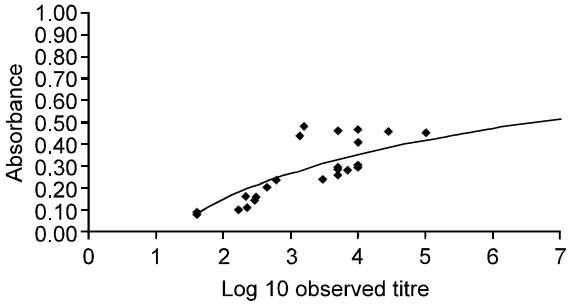

Fig. 2: Scatter chart for prediction of titre

of Variation (COV). The reliability of results developed by single serum peptide ELISA was compared with results of another single serum dilution indirect ELISA wherein Whole Virus Protein (WVP) was used as antigen and with HI test. The results of comparison in the form of Log10 values for HI and SP ratio for peptide ELISA and WVP ELISA are provided in Table 4 and graphically

Table 3: Model work sheet for single serum dilution peptide ELISA

Sample	Absorbance values									Titre		SP
No	Value 1	Value 2	Mean	Negative	Positive	Corrected	SD	COV	Status	Log 10	Numerical	ratio
1	0.291	0.278	0.285	0.016	0.844	0.269	0.009	3.231	TRUE	3.154	1426	0.3
2	0.579	0.581	0.580	0.016	0.844	0.564	0.001	0.244	TRUE	5.617	413682	0.7
3	0.506	0.459	0.483	0.016	0.844	0.467	0.033	6.888	TRUE	4.804	63704	0.6
4	0.389	0.486	0.438	0.016	0.844	0.422	0.069	15.678	TRUE	4.429	26864	0.5
5	0.465	0.580	0.523	0.016	0.844	0.507	0.081	15.563	TRUE	5.138	137246	0.6
6	0.407	0.453	0.430	0.016	0.844	0.414	0.033	7.564	TRUE	4.367	23263	0.5
7	0.223	0.225	0.224	0.016	0.844	0.208	0.001	0.631	TRUE	2.650	447	0.3
8	0.116	0.105	0.111	0.016	0.844	0.095	0.008	7.039	TRUE	1.704	51	0.1
9	0.398	0.335	0.367	0.016	0.844	0.351	0.045	12.155	TRUE	3.838	6879	0.4
10	0.110	0.123	0.117	0.016	0.844	0.101	0.009	7.890	TRUE	1.754	57	0.1
11	0.300	0.300	0.300	0.016	0.844	0.284	0.000	0.000	TRUE	3.283	1920	0.3
12	0.317	0.337	0.327	0.016	0.844	0.311	0.014	4.325	TRUE	3.508	3224	0.4
13	0.218	0.180	0.199	0.016	0.844	0.183	0.027	13.503	TRUE	2.442	276	0.2
14	0.199	0.248	0.224	0.016	0.844	0.208	0.035	15.503	TRUE	2.646	442	0.3
15	0.335	0.335	0.335	0.016	0.844	0.319	0.012	4.315	TRUE	3.575	3758	0.4
16	0.167	0.172	0.170	0.016	0.844	0.154	0.004	2.086	TRUE	2.196	157	0.2
17	0.221	0.205	0.213	0.016	0.844	0.197	0.011	5.312	TRUE	2.558	362	0.2
18	0.111	0.106	0.109	0.016	0.844	0.093	0.004	3.259	TRUE	1.688	49	0.1
19	0.080	0.097	0.089	0.016	0.844	0.073	0.012	13.583	TRUE	1.521	33	0.1
20	0.356	0.341	0.349	0.016	0.844	0.333	0.011	3.044	TRUE	3.688	4870	0.4
21	0.326	0.349	0.338	0.016	0.844	0.322	0.016	4.819	TRUE	3.596	3943	0.4
22	0.159	0.156	0.158	0.016	0.844	0.142	0.002	1.347	TRUE	2.096	125	0.2
23	0.061	0.082	0.072	0.016	0.844	0.056	0.015	20.768	FALSE	1.379	24	0.1
24	0.316	0.188	0.252	0.016	0.844	0.236	0.091	35.917	FALSE	2.883	764	0.3
25	0.363	0.310	0.337	0.016	0.844	0.321	0.037	11.137	TRUE	3.588	3868	0.4
26	0.475	0.400	0.438	0.016	0.844	0.422	0.053	12.122	TRUE	4.429	26864	0.5
27	0.347	0.279	0.313	0.016	0.844	0.297	0.048	15.362	TRUE	3.392	2464	0.4
28	0.343	0.327	0.335	0.016	0.844	0.319	0.011	3.377	TRUE	3.575	3758	0.4
29	0.390	0.415	0.403	0.016	0.844	0.387	0.018	4.392	TRUE	4.138	13725	0.5
30	0.399	0.358	0.379	0.016	0.844	0.363	0.029	7.660	TRUE	3.938	8660	0.4
31	0.342	0.381	0.362	0.016	0.844	0.346	0.028	7.629	TRUE	3.796	6249	0.4
32	0.190	0.145	0.168	0.016	0.844	0.152	0.032	18.997	TRUE	2.179	151	0.2
33	0.177	0.123	0.150	0.016	0.844	0.134	0.038	25.456	FALSE	2.033	108	0.2
34	0.227	0.106	0.167	0.016	0.844	0.151	0.086	51.387	FALSE	2.171	148	0.2
35	0.136	0.079	0.108	0.016	0.844	0.092	0.040	37.493	FALSE	1.679	48	0.1
36	0.329	0.247	0.288	0.016	0.844	0.272	0.058	20.133	FALSE	3.183	1525	0.3
37	0.317	0.263	0.290	0.016	0.844	0.274	0.038	13.167	TRUE	3.200	1585	0.3
38	0.267	0.252	0.260	0.016	0.844	0.244	0.011	4.087	TRUE	2.946	883	0.3
39	0.413	0.393	0.403	0.016	0.844	0.387	0.014	3.509	TRUE	4.142	13857	0.5

Table 4: Immunodominance of Epitope-Titre values for HI, Whole virus protein ELISA and Peptide ELISA

Sample No	HI titre Log 10 value	ELISA SP ratio WVP	ELISA SP ratio Peptide
1	0.90	0.71	0.71
2	2.11	0.80	0.77
3	1.20	0.55	0.60
4	2.11	0.96	0.99
5	0.90	0.17	0.15
6	0.90	0.26	0.26
7	1.20	0.36	0.35
8	2.11	0.60	0.58
9	0.90	0.18	0.18
10	2.11	0.56	0.55
11	2.11	0.86	1.00
12	2.11	0.83	0.96
13	2.11	0.71	0.82
14	2.11	0.80	1.00
15	2.11	1.35	1.60
16	1.51	1.20	1.43
17	2.41	0.95	1.13
18	1.20	0.78	0.88
19	1.81	0.76	0.83
20	1.20	0.52	0.56
21	1.20	0.52	0.55
22	1.51	0.80	0.92
23	1.20	0.57	0.59
24	2.11	1.03	1.22
25	1.20	0.64	0.71
26	0.90	0.42	0.43
27	0.30	0.13	0.07
28	0.00	0.10	0.02
29	0.30	0.07	-0.03
30	0.60	0.13	0.06
31	0.30	0.14	0.08
32	0.60	0.30	0.24
33	0.60	0.37	0.34
34	2.11	1.30	0.66
35	2.41	1.44	0.76
36	2.11	1.24	0.62
37	1.20	1.07	0.55
38	0.90	0.82	0.43
39	2.11	0.98	0.54
40	0.60	0.31	0.15
41	2.11	0.86	0.39
42	1.20	1.20	0.62
43	1.51	1.24	0.64

Table 5: Correlation coefficient values between WVP ELISA, peptide ELISA and HI

	WVP	PEPTIDE	HI
WVP		0.91*	0.75
Ppetide	0.91*		0.65
HI	0.75*	0.65	

N = 43; Table Value for 42df at 1% = 0.38 ($p<0.01$)

presented in Fig. 3. Correlation coefficient was estimated between HI, peptide ELISA and WVP ELISA. The correlation coefficient values are provided in Table 5. The correlation coefficient values were more than table value at 1% ($p<0.01$) indicating a good dependence between these three tests. The details of PP plot drawn for HI, peptide ELISA and WVP ELISA are provided as Fig. 4. The green line at the middle indicate the probable values and the red dots indicate the values that are obtained. The ROC curves drawn between HI Vs. peptide ELISA and WVP ELISA Vs. peptide ELISA are provided as Fig. 5. In the graph validation parameters like accuracy, sensitivity and specificity are also provided. The empirical ROC values for HI Vs. peptide ELISA and WVP ELISA Vs. peptide ELISA were 0.97 and 1.00, which are more than the standard value of 0.8.

Correlation of epitope with other proteins of NDV and other Important avian viruses: The amino acid sequence of the eptope in the conserved region was compared with other proteins of NDV Viz., P, M, F, HN and L to check whether the sequences are repeated and it has been found that the epitope sequence were not reported in any other proteins of NDV and it was found to be unique in NP gene of NDV. Further, the epitope was also found to be vary from these proteins by 99.8,100, 99.1 and 100% respectively.

The epitope sequence was also compared with whole genome sequence of IB and IBD viruses. The epitope sequences were not repeated and the variation was found to be 100%. The custom peptide did not react with monoclonal antibodies against S1 protein of Arkansas, Connecticut and Massachusetts serotype of IBV and also against S2 and M proteins of IBV.

DISCUSSION

Newcastle disease identified as one of the notifiable avian disease by the OIE caused by Newcastle disease virus of the genus Avulavirus in the family Paramyxoviridae. In as much as NDV is responsible for one of the most devastating diseases of poultry with negative economic impact over the poultry industry, development of improved vaccines and rapid, reliable, sensitive and specific diagnostic tests are greatly craved by the poultry industry. In most of the countries, the control strategy against ND is aimed at regular vaccination of flocks followed by periodic sero-monitoring by haemagglutination inhibition (HI) test or by Enzyme Linked Immunosorbant Assay (ELISA). Though this system is effective to an assured extent, an eradication programme could not be developed due to the inability of this system to differentiate between vaccinated and infected chickens (DIVA) (Mebatsion *et al*., 2002). As a consequence to this, birds suffering from mild infection go undetected and they act as reservoirs spreading virus to other birds besides retaining the virus in the environment. Hence, it becomes crucial to have a vaccine, which will help in differentiation of infected birds from vaccinated birds. Number of vaccines like live attenuated, inactivated and subunit vaccines prepared from different APMV-1 strains/isolates are available to control ND. However, almost none of these vaccines

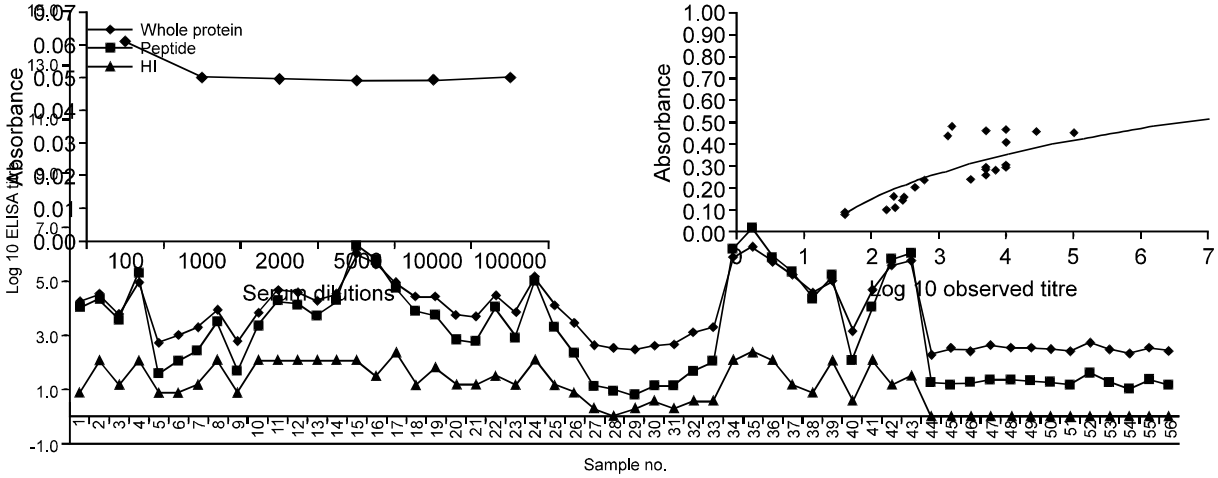

Fig. 3: Comparison of results - Peptide ELISA vs. Whole protein ELISA vs. HI test

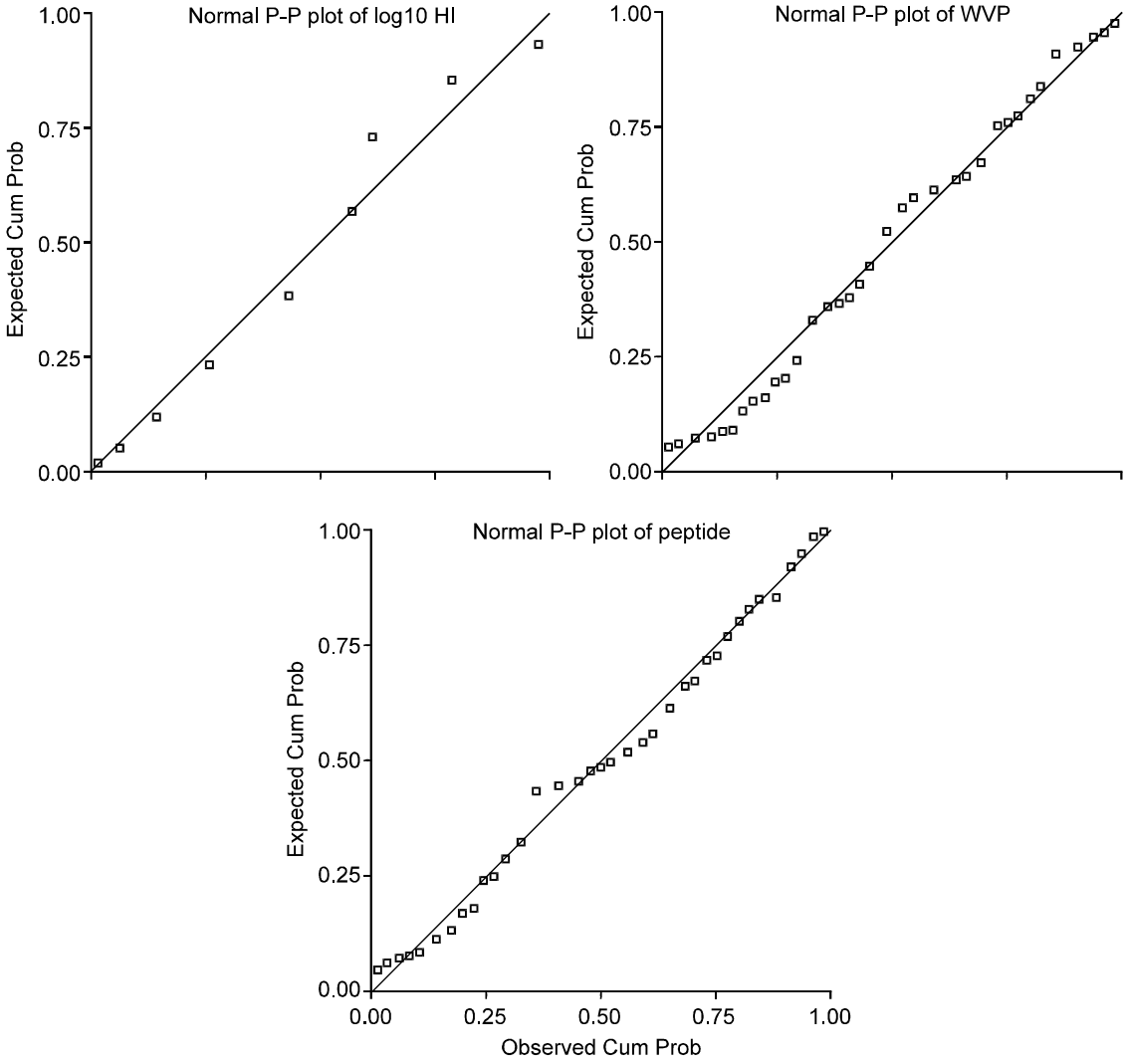

Fig. 4: Probability plot charts for HI, WVP ELISA and peptide ELISA

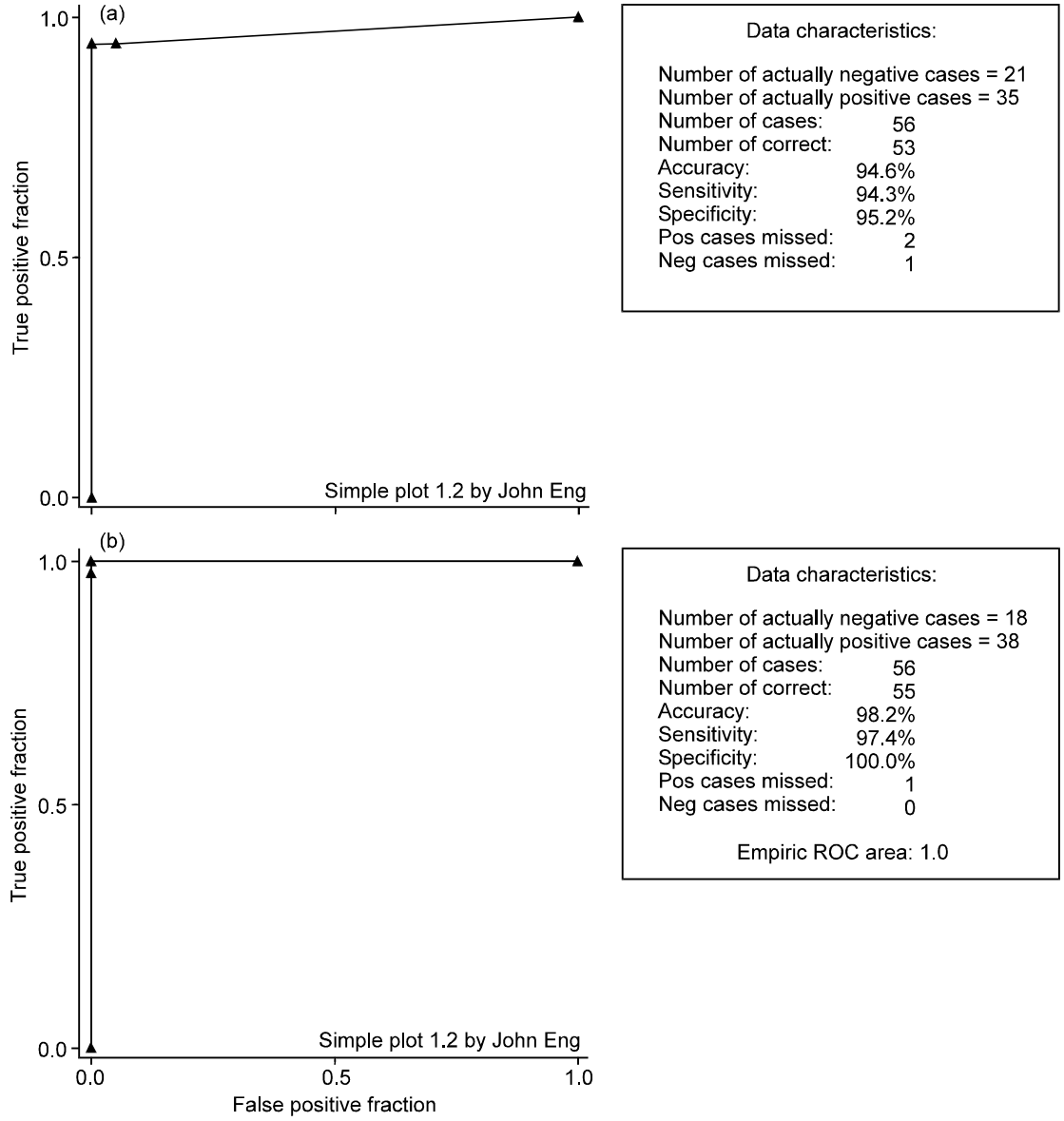

Fig. 5: Receiver operating characteristic curves. (a) HI vs Peptide ELISA, (b) WVP ELISA vs Peptide ELISA

help in differentiation of infected from vaccinated, as a result the outbreaks of ND are not uncommon and still ND remains as a great threat especially in the countries in the continents of Asia, Africa and America (Aldous and Alexander, 2001). While the subunit vaccines offer little scope in the area of differentiation of infected from vaccinated, these vaccines are less effective than whole virus vaccines and do not protect more than 90 percent vaccinated chickens upon virulent virus challenge. This further emphasizes the need to develop an alternative strategy to control and eradicate ND by using DIVA (marker) vaccines. Marker vaccines are unique vaccines that either lacks a characteristic peptide or posses a novel peptide that is not present in the wild virus and in conjunction with a diagnostic test, referred as companion discriminatory test, enables serological differentiation of vaccinated animal from infected animal. An animal diagnosed as positive for the presence of field infection has to be either eliminated or other suitable alternatives need to be carried out irrespective of prior vaccination. Hence, vaccination with marker vaccine in a designated area surrounding an outbreak significantly reduce the number of outbreaks and duration of an epizootic, which drastically limit economic damage to the industry in the area. The present strategies that are followed in development of marker vaccines include gene deletion, addition of a novel peptide or insertion of a foreign immunogenic gene

(Babuik, 1999). One of the major reasons identified for a poor control strategy against ND in India is the lack of techniques for differentiating between vaccinated and infected birds. In other words DIVA vaccines commonly referred as marker vaccines with a Companion Discriminatory Test (CDT) are the need of the hour. Development of a DIVA vaccine (marker vaccine) with CDT voluminous base line data need to be generated which include identification of vaccine strains, confirmation of their pathogenicity by molecular methods, identification of a suitable gene for inserting/ deleting/modifying part or entire length, sequencing the gene, analyzing the sequences to establish phylogenetic relationship, epitope identification and confirmation of immunogenicity and immunodominance of the epitope to be used as a marker. Once these data are generated, research on reverse genetics could be initiated.

In the present work, the three strains of vaccine viruses used in South India viz., F, LaSota, K and a local thermostabilized NDisolate D58 used for live and oral pellet vaccine production have been identified and their pathogenicity was confirmed by e ed by a computer algorNPgene has been predicsequencing the FPCS region of F gene. The nucleoprotein gene of vaccine viruses under study has been sequenced and the B cell epitopes available in the computer algorithm. One B cell epitope located at the end of NP gene has been synthesized as custom peptide and its immunogenicity and immunodominance has been confirmed by enzyme immunoassays, thus its use as marker in differentiation of vaccinated from infected has been established. The data generated could be used for reverse genetics.

In the present study, twenty B cell epitopes were predicted from the amino acid sequence using JIGSAW software. It is clear from earlier publications and from the observations on NP gene from this study that amino acid sequences from 1-400 are conserved in NDV irrespective of the virulence. However, though 18 B cell epitopes were located in this region. Hence we did not focus on them since, it has already been established that the amino acids 1 to 375 in NP gene are essential for herring bone structure of NP gene and are indispensable (Kho *et al*., 2003). We also focused on B cell epitopes alone, since it has already been established that circulating neutralizing antibodies are essential for protection against virulent NDV (Reynolds and Maraqa, 2000; Varalakshmi, 2005).

Between amino acids 401-489 two B cell epitopes could be located at positions 410-419 having amino acid sequence reading “TQQVGVLTGL” and 447 to 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 the amino acid at position 417 Thrionine (T) has been replaced by Isoleucine (I) in strain K. Whereas, the epitope identified between 447-453 was found to be conserved not only among all vaccine viruses used in South India but also in all NDV strains.

Hence, the conserved area located between 443-457 containing the epitope was synthesized as a custom peptide to confirm its immunogenicity and immunodominance. Immunodominance is a very important character to be proved since if any epitope is not immunodominant it will not be useful in DIVA. The structure of the epitope was also drawn using molecular modeling software Ball View Ver 1.1.1. Four types of molecular structures viz., ball and stick, Van-Der-Vals (VDV), cartoon and Solvent Excluded Surface (SES) were generated.

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 to 489 of nucleoprotein were checked for immunodominance and a region at position 447-455 of the NP region was found to be immunogenic. This region was also found to be conserved reinforcing the significance of this as a robust diagnostic tool (Mebatsion *et al*., 2002). Our method of prediction of B cell epitope using computer algorithm correlated with pepscan analysis and our findings also correlated with those of 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 to 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 as qualitatively and as indirect peptide ELISA quantitatively.

In the present study it was observed that all the 16 samples found positive with the HI test were positive for dot ELISA with epitope and compared with whole virus protein, thus giving a sensitivity of 100% relative to HI. All seven samples found negative for HI, tested negative for dot ELISA as well, thus giving specificity 100% relative to HI (Table 2). These findings were inagreement with earlier work done in which dot ELISA was found to be more sensitive than the HI test in detecting antibodies to NDV (Folitse *et al*., 1999). Thus the results obtained with dot blot ELISA with epitope confirmed the immunogenicity and immuno dominance of epitope and hence indicated that the epitope predicted and synthesized from 443 to 457 conserved region of nucleoprotein may be useful for detection of NDV anthodies in chickens after infection. Dot ELISA with epitope was found to be as specific as the HI test because no false positive reactions were observed. The results obtained for dot blot ELISA were satisfactory for sensitivity, specificity and consistency.

In the present study a B cell epitope with the sequence 'FLDLMRA' was found to be located at the conserved region between 443 to 457. Peptide ELISA developed using the entire conserved region was found to produce a consistent result in comparison with HI and Whole Virus Protein (WVP) ELISA. Based on the PP plot, it could be appreciated that all the points cluttered towards the expected line confirming the closeness of the results. This fact generated in this study is further strengthened by the observation from ROC curve analysis. ROC curves were generated as HI vs peptide ELISA and WVP ELISA vs peptide ELISA. A total of 50 samples were tested. The accuracy, sensitivity and specificity between HI and peptide ELISA was found to be 94.6%, 94.3% and 95.2% respectively. The empiric ROC area was found to be 0.97. When ROC was generated by using WVP ELISA and peptide ELISA, accuracy, sensitivity and specificity was found to be 98.2%, 97.4% and 100% respectively. The slightly lower values for peptide ELISA vs HI could be due to the fact that in ELISA IgG is mainly identified where as HI could identify IgM and IgG. Further the HI and ELISA titre do not correlate at the early stage of post vaccination where in for development of IgG seven to 10 days are needed. It is a reported fact that (Jacobson, 1998) ROC values close to one were considered significant. In the present study when peptide ELISA results were compared with HI and WVP ELISA, the empirical ROC values are very close to one confirming the immunodominance of the epitope.

When the sequences of the epitope was checked with the other proteins of NDV and other common poultry viruses like avian infectious bronchitis virus and Infectious bursal disease virus, the epitope was not found as such in any of them. Further the variation was also found to be more than 99.9% in all the comparisons.

These observations confirmed the immunodominance of the epitope identified thus making it candidate epitope for serving as a marker in differentiation of vaccinated and infected birds. If this epitope could be replaced by a foreign epitope belonging to any other unrelated foreign virus epitope, the presence of antibodies against foreign virus epitope will indicate vaccinated status and antibodies against this epitope will indicate the infectious status. In other words the recombinant vaccine virus will not have this epitope and field virus alone will have this epitope.

In conclusion, the B cell epitope of nucleoprotein identified have been found to be conserved in all the vaccine viruses in the present study and the epitope identified has been found to be immunodominant and could serve candidate epitope for DIVA vaccine.

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