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Peptide based Indirect ELISA for Detection of Antibodies against *Peste des petits ruminants virus*

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ABSTRACT

Background: Infectious virus antigen is not recommended for disease monitoring in global *Peste des petits ruminants* eradication strategies. Native virus antigens are gradually being replaced with recombinant or synthetic peptide antigens. The focus of the present study is to optimize and develop peptide-based immunoassay for the detection of antibodies to PPRV N and H proteins.

Methods: Epitopes of PPRV H and N proteins were selected based on prediction with bioinformatics tools and from previous studies. Two peptides each were synthesized for N and H proteins and peptide ELISA developed. The peptide ELISA's sensitivity and specificity were tested with sera samples collected at different time intervals of vaccination (goat =73, sheep= 62) and 88 random serum samples (goat =47, sheep=41). The collected sera were screened using cELISA before proceeding to peptide ELISA.

Result: In competitive ELISA, 106 goat serum samples and 96 sheep serum samples were found to be positive. Fourteen goat serum samples and seven sheep serum sample were shown to be negative. Among120 goat serum samples tested, 114 were found to be positive by peptide ELISA. Similarly, out of 103 sheep serum samples analyzed, 96 were found to be positive with peptide ELISA. The peptide ELISA based on the highly conserved and antigenic N and H epitope detected antibodies to PPRV in precise manner. This study demonstrated the effective use of synthetic peptides as an antigen in the detection of antibodies to PPRV.

Key words: B cell epitopes, cELISA, H protein, N protein, peptide antigen, *Peste des petits ruminants virus*.

INTRODUCTION

Peste des petits ruminants (PPR), also known as "small ruminant plague" or "Kata," is a highly infectious and economically significant transboundary animal disease (TAD) affecting sheep and goats. The World Organization for Animal Health has listed it as a notifiable terrestrial animal disease (OIE 2012). Small ruminant morbilli virus (SRMV), also known as PPR virus, is a member of the genus *Morbillivirus*, which belongs to the family *Paramyxoviridae* (http://ictvonline.org/virusTaxonomy.asp) (Gibbs *et al.* 1979). The PPRV genome is made up of six transcriptional units that code for six proteins: 3' N (Nucleocapsid protein), P (Phosphoprotein), M (Matrix protein), F (Fusion protein), H (Haemagglutinin) and L (Large protein) 5' (Diallo, 1990).

Among the structural proteins of PPRV, N protein is the most antigenically conserved among morbilli viruses and, considering its internal position, is highly immunogenic (Choi *et al*., 2005, Libeau *et al*., 1995). In morbilli virus-infected cells, the N protein is expressed at a very high level (Diallo *et al*., 1990).

Morbilliviruses have four distinct regions in their N protein, conserved regions I (aa 1-120) and III (aa 146-420), as well as variable regions II (aa 121-145) and IV (aa 421-525). (Munir *et al*., 2013). The C-terminal region of PPRV N (aa 454-472) were found to be highly selective for the virus. (Dechamma *et al*. 2006). The H and F, two surface glycoproteins, either together or separately, induce neutralising and defensive antibody synthesis in the host (Libeau, 2015 and Liu *et al*., 2014). B cell epitope (BCE) regions of H protein have neutralizing antibody binding capacities from 263 to 368 aa and 538 to 609 aa (Renukaradhya *et al*., 2002).

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The identification of PPRV N (Yu *et al.,* 2015) and H (Yu *et al.,* 2017) BCEs shows the antigenic properties of the N and H proteins, laying the groundwork for developing epitope-based diagnostic assays for PPRV infections.

The viral neutralization test (VNT), competitive ELISA (C-ELISA) and indirect ELISA are the antibody detection tests used in the diagnosis of PPR. VNT is difficult to perform for routine sero-surveillance or sero-monitoring due to the need for cell culture facilities and sterile serum, particularly when a large number of samples need to be screened (Singh *et al*., 2004).

Competitive ELISA was developed for the detection of antibodies to PPRV using a monoclonal antibody to a

neutralizing epitope of H protein. The efficacy of c-ELISA compared favourably to VNT, with high specificity (98.4%) and sensitivity (95.4%) and it can replace the viral neutralization test (Singh *et al*., 2004). Indirect ELISA, on the other hand, may be a good substitute for the time consuming and technically demanding viral neutralization test (VNT) and expensive competitive ELISA. When compared to cELISA, the indirect ELISA had a relative diagnostic specificity of 95.09 percent and a sensitivity of 90.81 per cent for PPR antibody detection (Balamurugan *et al*., 2007).

The peptide based immunoassays are being developed as DIVA assays for several viruses (Shen *et al*., 1999). Peptide ELISA for influenza subtypes showed promising results compared to HI, Immunofluorescence assay and immune-dot blot assay which had high cross reactivity (Velumani *et al.* 2011). Peptide ELISA was also found to be a good choice for the detection of antibodies against Infectious bronchitis virus (IBV) and the evaluation of IBV vaccines when compared to immunofluorescence assay (Yin *et al*., 2021). Peptide ELISA was also employed for detecting the immunogenicity and immunoreactivity of the protein compared to whole virus protein (WVP) based ELISA for Newcastle disease and it showed a positive correlation with HI. (Ramani Pushpa *et al*., 2012). Synthetic peptide based competitive ELISA was also developed for PPR antibody detection (Shrivastava, 2006). However, it has not been validated for diagnostic use with field samples. An epitope based competitive ELISA was developed by using hyperimmune serum prepared against epitope peptides of N protein of PPRV. In this assay recombinant N protein was used as the coating antigen. The sensitivity and specificity of the assay were 96.18 and 91.29%, respectively, in comparison to commercial cELISA kit (Zhang *et al*.2013).

In this study, we developed ELISA for PPRV antibody detection by employing PPRV N and H peptides and compared them with cELISA.

MATERIALS AND METHODS

Synthetic peptide and serum samples

Epitope prediction and peptide synthesis

Using the abcPRED2.0 and Bepipred 2.0 software, the Bcell epitopes of N and H protein for the Sungri 96 virus sequence (GenBank accession no: AY560591.3 Sungri 96) were predicted. Based on prediction and epitopes mapped in previous studies (Yu *et al*., 2015; Yu *et al*., 2017), 8 mer antigenic peptide sequences of PPRV N and H proteins were selected. Two peptides each for N (N1 and N2) and H (H1 and H2) were custom synthesized (M/S GenScript, USA) by linking two 8 mer peptides with GGGS linker added in between.

Serum samples

Serum samples were collected randomly from sheep and goats vaccinated with live attenuated PPRV vaccine. Serum samples were also collected at specific intervals by following the schedule of pre-vaccination, 0, 3, 7, 10, 14, 17, 35 days of post vaccination from sheep and goats. A total of hundred and thirty-five serum samples (Goat =73, sheep= 62) were obtained by collection on the specific intervals mentioned above from Instructional Livestock Farm Complex (ILFC), TANUVAS.

Eighty eight random serum samples were collected (Goat =47, sheep= 41) from sheep and goats vaccinated with live attenuated PPRV vaccine from PGRIAS, Kattuppakkam and ILFC, TANUVAS. The serum samples were collected sixty days post vaccination.

Confirmation of immunoreactivity of the peptides by using indirect ELISA

Checkerboard titration

Checkerboard titration method as per Rose *et al* .(1997) was employed to get the optimum concentrations of peptide antigen and serum samples to get the results.

Different dilutions of peptide antigen (25ng/100µl, 50ng/100µl, 100ng/100µl, 200ng/100µl and 400ng/100µl), serum (1:100, 1:200, 1:400, 1:800, 1:1600 and 1:13200) and conjugate (1:2000, 1:4000, 1:8000, 1:16000 and 1:32,000) were used and titrated.

Standardization and optimization of indirect peptide ELISA procedure

Peptide ELISA for detection of PPRV antibodies was designed for PPRV N and H peptides according to Ramani Pushpa *et al*. (2012). Wells of flat bottomed 96 well Maxisorp plates were coated overnight at 4°C with 100 ng of PPRV N and H peptide antigen in 100 µL of 0.1 M Sodium Carbonate bi-carbonate buffer (pH 9.6).The wells were washed thrice with PBST and then blocked with blocking buffer and incubated for one hour at 37°C. After blocking, the wells were washed thrice with PBST. Test sera were added at a dilution of 1:100 and incubated for one hour at 37°C, followed by washing. The plate was then incubated for one hour at 37°C with a 1 in 20,000 anti-goat (Sigma) IgG whole molecule peroxidase conjugate or a 1 in 6000 dilution of anti-sheep (Sigma) IgG whole molecule peroxidase conjugate. The wells of the plate were then washed again and a 3,3',5,5' - Tetramethylbenzidine (TMB) substrate solution containing 2µL H_2O_2 in 100 µL of 0.05M sodium citrate buffer (pH4.2) was applied, followed by incubation in room temperature for 10 minutes in the dark. The reaction was stopped with 1% H_2SO_4. After blanking, OD values were taken at 450 nm in an automated ELISA reader.

cELISA

The samples which were screened by peptide ELISA as per above method, same were also screened by cELISA. The cELISA kits were made avaible from IVRI, Mukteswar . The protocol outlined in the manufacturer's manual was followed.

Receiver operating characteristic (ROC) curves were generated as cELISA vs N, H and cocktail peptide for sheep and goat separately.

RESULTS AND DISCUSSION

Epitope prediction and peptide synthesis

abcPRED2.0 and Bepipred 2.0 were used for predicting

epitopes in PPRV N and H proteins. Epitope prediction results of Bepipred 2.0 for PPRV N and H proteins are shown in (Fig1-2). In addition to the prediction, B cell epitopes of PPRV N and H proteins fine mapped by previous studies (Yu *et al*. 2015; Yu *et al*. 2017) were also used to select the 8 mer antigenic peptides. The peptides were found to be conserved among PPRV by BLASP analysis. Four peptides namely N1, N2, H1 and H2 custom synthesized by linking two 8 mer antigenic peptides with GGGS linker are shown in Table 1. The GGGS was used to link the peptides to increase the antigenicity and to improve sequence flexibility of the peptide without affecting the protein function (Chichili *et al.* 2013).

Confirmation of immunoreactivity of peptides

The immunoreactivity of peptides was confirmed by peptide ELISA. All the four selected peptides reacted with PPRV antisera. Among them, N1 and H2 gave highest OD values and positive-negative ratio of OD values. Based on immunoreactivity, the N1, H2 and a cocktail of N1 and H2 was selected as antigen for peptide ELISA to detect antibodies of PPRV.

Table 1: Synthesized peptide sequences of *Peste des petits ruminants virus* N and H protein.

Peptides id	Peptide sequences
N1	RGETPGQL (460-467) -GGGS-YNDKDLLG (518-525)
N2	VSFLQHQT (417-424) - GGGS- DRKQTRPG (450-457)
H1	ESIDHQTK (82-89) - GGGS - LVEACKTR (382-389)
H2	EVMPHILT (477-484) – GGGS - TDEEVHTR (590-597)

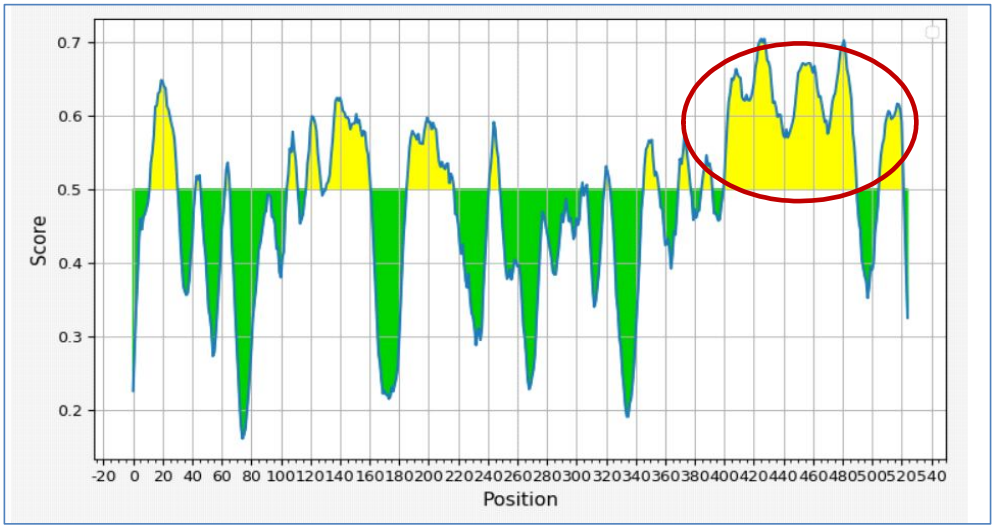

Fig 1: B cell epitope prediction of N of *Peste des petits ruminants* virus by BepiPRED 2.0 or N protein. The red circles depicted the predicted epitope sequences of PPRV N protein.

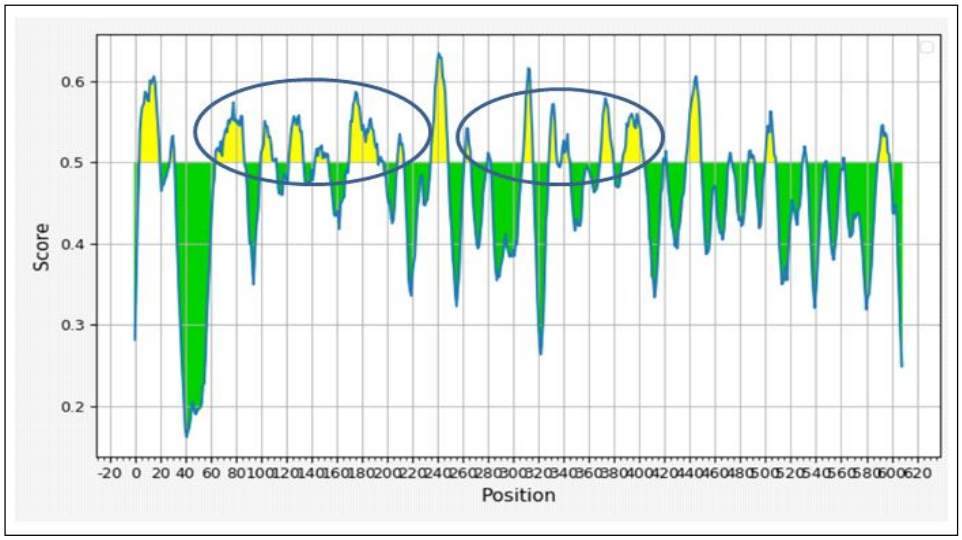

Fig 2: B cell epitope prediction of H protein of *Peste des petits Ruminants virus* by BepiPRED 2.0. The blue circle denotes the predicted epitope sequences of H protein of PPRV.

Development and standardization of peptide ELISA

Checker board titration

Different concentration of peptides, serum dilutions and conjugate dilutions were tested by checker board titration for standardization of ELISA procedure. The optimum antigen concentration for the peptide ELISA was found to be 100ng/100ul which concurred with Ramani Pushpa *et al.*, 2012 (Fig 3).The serum dilution of 1:200 (Fig 4) was optimized in this assay. This dilution showed better positive-negative difference value compared to other dilutions. This is in concurrence with the report by Balamurugan *et al.,* 2007. The optimal dilutions for the conjugates were 1:20,000 and 1:6000 for goat and sheep respectively. In this peptide assay specific conjugate for goat and sheep were optimized to ensure more specific binding unlike the previous reports using same secondary conjugate for both species (Balamurugan *et al.,* 2007, Sumi *et al.,* 2019).

Comparison of peptide ELISA with competitive ELISA

In the present study, cELISA was used for comparing and correlating the peptide ELISA. Out of 120 goat and 103 sheep serum samples, 106 goat and 96 sheep serum samples were found to be positive. Fourteen goat serum samples and seven sheep serum sample were shown to be negative (Table 2 and 3).

In this study, 120 goat serum samples were tested and 114 of them were found to be positive by peptide ELISA. N, H and cocktail of N and H Peptide ELISAs positively

Table 2: Results of cELISA for goat serum samples.

	Strong positive	Weak positive	Negative
Random (n=47)	28	18	1
Prevaccination (n= 8)	1	6	1
Zero day (n=10)	0	5	5
Post vaccination 3rd day (n=10)	0	8	2
7th day (n=10)	5	4	1
10 th day (n=10)	5	3	2
14th day (n=10)	6	2	2
17th day (n=10)	3	7	0
35th day (n=5)	0	5	0
	48	58	14

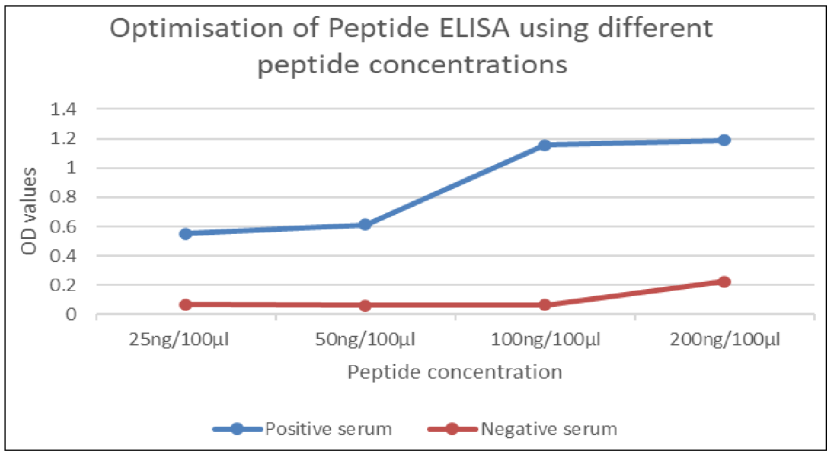

Fig 3: Optimization of peptide concentrations for peptide ELISA.
100 ng/100µL concentrations of peptide showed high positive negative difference.

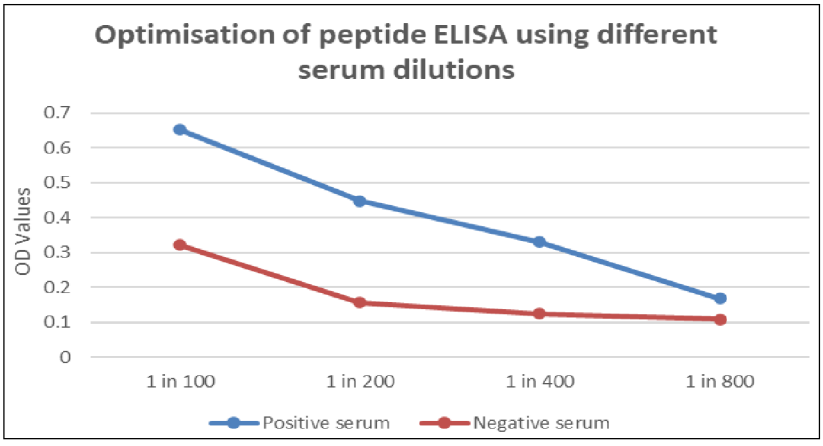

Fig 4: Optimization of serum dilutions for peptide ELISA.
The picture shows 1:200 dilution of serum had a high positive negative ratio than other dilutions.

Table 3: Results of cELISA for sheep serum samples.

	Strong positive	Weak positive	Negative
Random (n=41)	27	8	6
Prevaccination (n=7)	2	5	0
Zero day (n=10)	4	6	0
Post vaccination 3rd day (n=10)	6	4	0
7th day (n=10)	1	9	0
14th day (n=10)	7	3	0
17th day (n=10)	5	5	0
35th day (n=5)	0	5	0
	52	45	6

correlated with competitive ELISA method. For goat sera tested by the peptide ELISA, the correlation coefficients were N(0.78638), H(0.8309) and cocktail (0.82412) (Table 4). Similarly, out of 103 sheep serum samples analyzed, 96 were found to be positive with peptide ELISA with a correlation values for N (0.809), H (0.770) and cocktail (0.772) (Table 5). The correlation was highly significant. These findings were further strengthened by statistical analysis using ROC curve. ROC curves were generated as cELISA vs N, H and cocktail peptide for sheep and goat separately. Out of the 120 goat samples tested, the accuracy, sensitivity and specificity were found to 94.2%, 98% and 72.2% for N, 92.5%, 99% and 55.6% for H, 92.5, 98% and

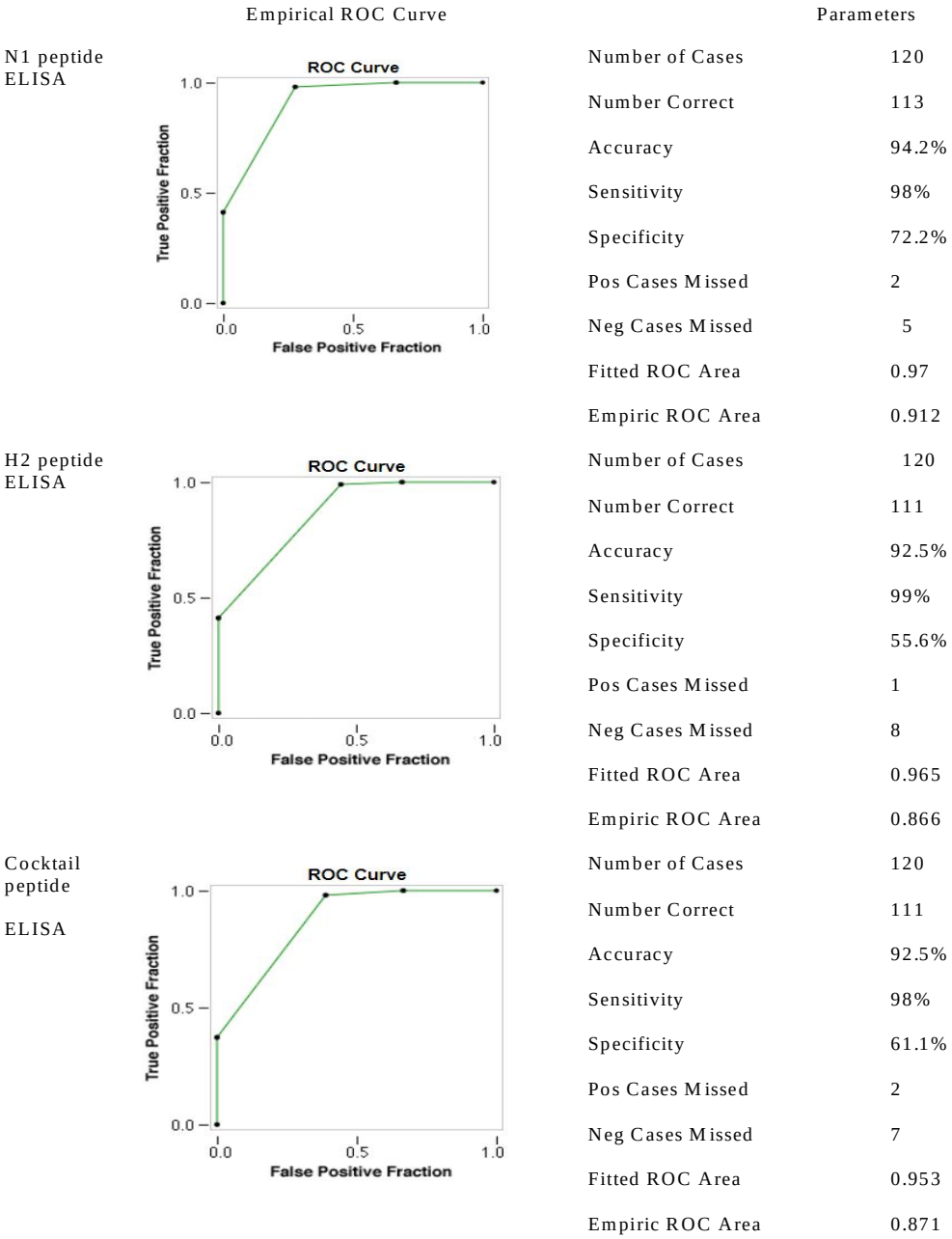

Fig 5: Receiver operating characteristics ROC curve analysis for goat samples

61.1% for cocktail ELISA respectively (Fig 5). Out of the 103 sheep samples tested, the accuracy, sensitivity and specificity were found to be 93.2%, 93.8% and 85.7% for N, 94.2%, 94.8% and 85.7% for H and cocktail peptide ELISA respectively (Fig 6). The empirical ROC values for competitive ELISA vs. peptide ELISA were more than the standard value of 0.8. The lower specificity in peptide ELISA may be due to testing of less number of negative serum samples and the higher sensitivity of the indirect ELISA format compared to cELISA. An indirect ELISA using purified PPRV as coating antigen had 90.81% sensitivity and 95.09% specificity for PPR antibody detection (Balamurugan *et al*., 2007). A competitive ELISA using recombinant N protein and hyper immune serum against N peptide was found to have 96.18% sensitivity and 91.29% specificity (Zhang *et al*., 2013). The peptide ELISAs developed in the present study have sensitivities similar to the above reports.

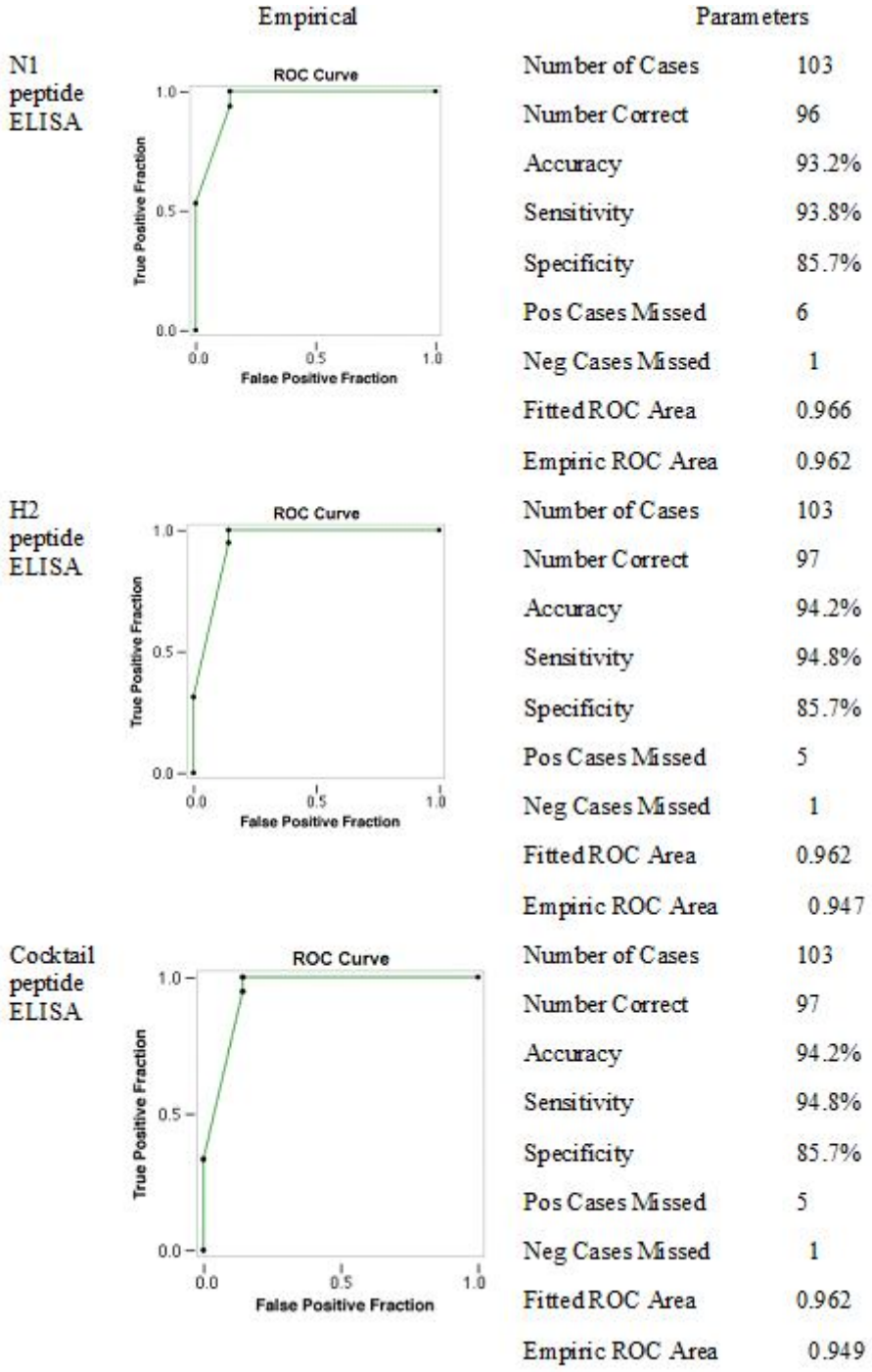

Fig 6: Receiver operating characteristics ROC curve analysis for sheep samples.

Table 4: Correlation between c ELISA Versus N, H and cocktail peptide ELISA - Goat samples.

	N peptide ELISA	H peptide ELISA	Cocktail of N and H peptide ELISA
cELISA	0.787**	0.858**	0.851**

N=120, Table value for 119df at 1% = 0.0001(p<0.01).

Table 5: Correlation between c ELISA Versus N, H and cocktail peptide ELISA – Sheep samples.

	N peptide ELISA	H peptide ELISA	Cocktail of N and H peptide ELISA
cELISA	0.809**	0.770**	0.772**

N=103, Table value for 102df at 1% = 0.0001(p<0.01).

CONCLUSION

Peptide ELISAs using N, H and cocktail of N and H peptides were found to be more sensitive for detection of PPRV antibodies. The peptide ELISAs correlated well with the cELISA. Peptide based immunoassay developed can form an alternative assay with sensitivity and specificity for sero-surveillence program without using live virus antigen. Repeatability and reproducibility will be better as exact concentration of antigen can be ensured when using the peptide ELISA assay.

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