DEVELOPMENT OF RECOMBINANT ANTIGEN FABRICATED SURFACE FOR DETECTING *PESTE DES PETITS RUMINANTS* VIRUS SPECIFIC ANTIBODIES

Thesis

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Master of Veterinary Science

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Dedicated to
my family

DIVISION OF VETERINARY BIOTECHNOLOGY
ICAR-INDIAN VETERINARY RESEARCH INSTITUTE
Izatnagar, Bareilly (U.P.) INDIA- 243 122

Dated: 05/07/2016

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Certificate

This is to certify that the research work embodied in this thesis entitled "Development of Recombinant Antigen Fabricated Surface for Detecting *Peste des Petits Ruminants* Virus Specific Antibodies" *submitted by* Dr. Resma K. Vasu, Roll No. 5448, *for the award of* Master of Veterinary Science *degree in* Animal Biotechnology *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate herself under my supervision and guidance.*

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We the undersigned Members of Advisory Committee of **Dr. Resma K. Vasu, Roll No. 5448,** a candidate for the degree of **Master of Veterinary Science** with the major discipline in **Animal Biotechnology** agree that the thesis entitled " **Development of Recombinant Antigen Fabricated Surface for detecting *Peste des Petits Ruminants* Virus Specific Antibodies**" may be submitted in partial fulfillment of the requirement for the degree.

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SIGNATURE OF THE EXTERNAL EXAMINER
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Izatnagar (Resma K. Vasu)

Abbreviations

$	Dollar
%	Percentage
^{0}C	Degree Centigrade
Ab	Antibody
AGID	Agar Gel Immuno-Diffusion test
APS	Ammonium persulphate
APTES/APTS	3-aminopropyl triethoxy silane
*BamH*1	*Bacillus amyloliquefaciens*
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
cELISA	Competitve ELISA
CIE	Counter-immunoelectrophoresis
COC	Cyclic Olefin Copolymers
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DW	Distilled water
EDC	Dimethylaminopropyl-N'Ethylcarbodiimide N-3-Hydrochloride
EDTA	Ethylene Diamine Tetra Acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EtBr	Ethidium bromide
F	Fusion protein
FITC	Fluorescein Isothiocyanate
FLISA	Fluorescent Linked Immunosorbent Assay
GA	Gluteraldehyde
GOPS	3-glycidoxypropyltrimethoxysilane
H/HN	Hemagglutinin-Neuraminidase protein

H_2O_2	Hydrogen Peroxide
H_2SO_4	Sulphuric acid
HCl	Hydrchloric acid
Hind III	*Heamophilus influenza*
HRPO	Horseradish peroxidase
ICE	Immunocapture ELISA
IFAT	Indirect immunofluorescence test
IPT	Immunoperoxidase test
IPTG	Isopropyl-1-thio-β-D-galactosidase
LB	Lurea Bertani
M	Molar
MAb	Monoclonal Antibody
MPTES/MPTS	Mercapto triethoxy silane
MUA	11-Mercapto undecanoic acid
MW	Molecular weight
N	Normal
NFW	Nuclease Free Water
NHS	N-Hydroxy Succinimide
Ni-NTA	Nicckel-nitrilotriacetic acid
OD	Optical density
OPD	O-Phenylenediamine
P	Phosphoprotein
P/N ratio	Positive/ Negative ratio
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PDMS	Poly(dimethylsiloxane)
PE	poly(ethylene)
pH	Hydrogen ion concentration
PI value	Percent inhibition value
PMMA	Poly(methylmethacrylate)

PP	Poly(propylene)
PPR	Peste des Petits Ruminants
PPRV	Peste des Petits Ruminants Virus
PS	Polystyrene
RE	Restriction Endonuclease
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPV	Rinderpest virus
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
SAM	Self assembled monolayer
SDEV	Standard deviation
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulphate
SMP	Skim Milk Powder
SPR	Surface Plasmon Resonance
TAE	Tris acetate EDTA
TEMED	N,N,N',N'-tetramethylethylene diamine
Tm	Annealing temperature
V/cm	Volts per centimeter
v/v	Volume/volume
VERO	African green monkey kidney cell line
VNT	Virus Neutralization Test
Δ	Truncated
ΔF1	Truncated fusion protein 1
ΔF2	Truncated fusion protein 2
ΔH1	Truncated Hemagglutinin-Neuraminidase protein 1
ΔH2	Truncated Hemagglutinin-Neuraminidase protein 1
ΔM	Truncated matrix protein
ΔN	Truncated nucleocaspid protein

Units of Measurement

µg	Microgram
µl	Microlitre
µm	Micrometer
bp	Base pair
gm	Gram
h	Hours
Kb	Kilobase (103 bases or base pairs)
kDa	Kilo dalton
M	Molar
mA	Milliampere
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
N	Normal
ng	Nanogram
nm	Nanometer
pg	Picogram
pmol	Picomol
S	Second
U	Units

List of Tables

List of Figures

Contents

Introduction

1 INTRODUCTION

Animal Husbandry is making a significant contribution to the national economy and socio-economic development of the country. As per 2012 livestock census the total livestock population in the country is 512.05 million numbers among which 39.10% is small ruminant including 65.06 million sheep and 135.17 million goats (DAHD, 2012). Small ruminants are a major source of income for the underprivileged families and they contribute about 17 to 24% of income to the family and livestock are central to their livelihoods and culture cost (ILRI, 2006).

India stands at second place with respect to goat population and at third place with respect to sheep population. Even though we are rich in small ruminant population, there is a decrease in their population when compared to the previous census which may be attributed to various health and management problems. Economic impact caused by PPR (*Peste des petits ruminants*) strike at the heart of vulnerable livelihoods as well as national and regional livestock production. Many countries have experienced cumulative losses ranging from tens to hundreds of millions of US dollars annually due to this highly spreading disease. Eradicating PPR will help improve food security, nutrition, income and livelihood resilience of millions of poor farmers around the world (OIE, 2015). PPR is an acute and highly contagious viral disease of small ruminants, characterised by high fever, ocular and nasal discharge, pneumonia, necrosis and ulceration of the mucous membrane and inflammation of the gastro-intestinal tract causing severe diarrhea (Abraham *et al.,* 2005). PPR virus belongs to the morbillivirus group of paramyxovirus family. It has close resemblance to the rinderpest virus of cattle and buffaloes, the measles virus of humans, the distemper virus of dogs and some wild carnivores, and the

morbilliviruses of aquatic mammals (FAO, 1999). PPR virions are enveloped pleomorphic particles with the genome size of 15,948 nucleotides and encodes sequentially for the nucleocapsid (N) protein, the phosphoprotein (P), the matrix protein (M), the fusion (F) and the hemagglutinin–neuraminidase (HN) membrane glycoproteins, and the large (L) protein (Munir, 2013). The serological value of these proteins have been exploited for diagnostic and vaccine development studies by various researchers (Libeau *et al.,*1995; Sinnathamby *et al.,* 2001; Seth and Shaila, 2001b; Mahapatra *et al.,* 2006; Sodjo *et al.,* 2008; Yadav *et al.,* 2009; Liu *et al.,* 2013; and Wang *et al.,* 2013).

PPR is one of the World Organization for Animal Health (WHO) notifiable diseases. Significant research has been directed toward developing improved vaccine, diagnosis, and epidemiology of the virus in recent years on account of rapid increase in disease spectrum and distributions patterns (Munir, 2013). Conventional techniques for diagnosis of PPR include agar gel immunodiffusion test, counter-immunoelectrophoresis and indirect-ELISA. These techniques were popular in earlier days but could not differentiate PPR infections from rinderpest infections. Therefore to overcome such disadvantages, the sophisticated techniques like cDNA hybridization, RT-PCR technique, virus neutralization test and monoclonal antibody based ELISAs have been used. RT-PCR technique and cDNA hybridization being the two sensitive means to diagnose the PPR virus infection, but the disadvantages are that they are time consuming and inconvenient for routine diagnosis with a large sample size. On the contrary, virus isolation is tedious, which may not be possible always with specimens under deteriorated conditions. Although, monoclonal antibody based immuno-capture ELISA has been efficacious for the diagnosis of PPR infection, for effective and extensive clinical surveillance of the disease, a simple, convenient, sensitive and cost effective diagnostic technique is required (Singh *et al.,* 2004a).

Immunoassay is a technique that exploits the sensitivity and specificity of antibody–antigen interactions for the detection of relevant analytes in samples. Immunoassays are used for the quantification of proteins and small molecules in a number of different fields such as medical diagnostics, proteomics, pharmaceutical research and biological researches. It can be classified into main two types: heterogeneous and homogeneous assays. In heterogeneous

immunoassays, antibodies/antigens are immobilized on a solid support and interact with the antigen/antibody at the boundary layer. In this format, unbound antibodies/antigens and other reagents can be easily removed by washing with proper reagents. In homogenous immunoassays, antibodies interact with antigens in solution. In this case, the bound and unbound antibodies are parted based on physical or chemical changes arising from the binding event. Though conventional immunoassays have many disadvantages, it can be circumvented by miniaturization in microfluidic systems. Microfluidic immunoassays offer at least three advantages over conventional methods like increased surface area to volume ratios to hasten antibody–antigen reactions, smaller dimensions significantly reduce the consumption of expensive reagents and precious samples and lastly automated fluid handling can improve reproducibility and throughput. These advantages can potentially improve the performance and reduce the operating cost of conventional immunoassays (Ng *et al.,* 2010; Lee *et al.,* 2011). Fluorescence based immunoassays have gained much popularity in medical and clinical diagnostics fields. Commonly used fluorescence assays are based on simple binding of labeled antigens or a sandwich format where second antibodies (Abs) have been labeled with a fluorescent tag (Soini and Hemmila 1979; Matveeva *et al.,* 2004). In fluorescence, a single photon of light is absorbed by a molecule (excitation) and re-emitted at a slightly lower energy. In theory, this should be the most sensitive detection method when compared with absorption, chemiluminescence or radioisotopic decay. Sufficiently sensitive detection of fluorescence emission permits even single molecule detection making it highly useful in immunoassays (Dickson *et al.,* 1995). Arrays of proteins covalently bound to solid surfaces like glass, plastics, silicon hydrogels were shown to retain their ability to interact specifically with other proteins or with small molecules in solution. Development of protein microarrays to determine the presence of specific antibodies directed against parasite *Toxoplasma gondii* and viral antigens, cytomegalovirus (CMV), rubella virus, and herpes simplex virus (HSV) types 1 and 2 in human sera have been reported (Mezzasoma *et al.,* 2002).

There are many articles and papers focusing on new technologies (including new materials such as paper-based chips, new fabrication processes, and novel uses), new architectures and integration with sensing technologies to improve controllability, throughput, and reliability, and a large range of diagnostic applications of microfluidics (Rivet *et al.,* 2011;

Ren *et al.,* 2012). First and foremost thing to be accounted before development of microassays include fabrication costs and material compatibility (Rivet *et al.,* 2011). The surfaces that have been used for fabrication of bioactive molecules include polymers, elastomers, glass, silicon, plastics, paper, hydrogels microbeads etc (Ren *et al.,* 2012; Kim and Herr, 2013). Current method for functionalization of solid surfaces are based on physisorption, covalent immobilization to chemically activated surfaces, or on the biosynthetic expression of a polypeptide 'tag' that mediates immobilization on an appropriately derivatized surface (Sun *et al.,*2006). Considerable effort has been made in the last years to improve immobilization of proteins on modified glass and other solid surfaces using a number of different strategies. The covalent mechanism of attachment of antigen/antibody requires the presence of reactive groups on the support (usually electrophilic groups such as epoxides, aldehydes, succinimidyl esters/ isothiocyanate functionalities) able to react with nucleophilic groups (amino, thiol, hydroxyl) on the ligand molecules. The functional groups on self-assembled monolayers (SAM) or polymers grafted surfaces are introduced by surface modification with organosilanes such as 3-glycidoxypropyltrimethoxysilane (GOPS) or 3-aminopropyltriethoxysilane (APTES) or 3-mercaptopropyl triethoxysilane (MPTS). Organosilanes can directly provide the functional groups for ligand attachment or react with a bifunctional ligand like glutaraldehyde bearing the desired reactive group (Bhatia *et al.,* 1989; Guilleaume *et al.,* 2005; Cretich *et al.,* 2006). Cost effective reagents can be used for the modification of surfaces so that they can be used for the fabrication of antigens/antibodies to be used for immunoassays, for example, chromic acid has been used to introduce carbonyl and carboxylic acid groups on poly(ethylene) (PE) and poly(propylene) (PP), potassium permanganate/sulfuric acid solutions provide similar functionalities to PE surfaces, sulfuric acid itself introduces sulfonate groups on PE (Terlingen, 2004). Covalent binding is the preferred method of attaching an antibody to a surface due to the strong, stable bond that is formed (Bhatia *et al.,* 1989).This type of immobilization of the protein is preferred when the coated substrate is subjected to flow or extended times in solution to prevent leaching of the protein from the surface and covalent immobilization chemistries have demonstrated to preserve functional capability of the immobilized protein for up to 2 years (Klibanov, 1979). Keeping this in view, the present study is envisioned with following objectives:

1. To produce and characterize recombinant proteins corresponding to immunodominant regions in PPRV
2. To develop recombinant antigen fabricated surface for detecting PPRV specific antibodies

Review of Literature

2 REVIEW OF LITERATURE

2.1 *Peste des petits ruminants* (PPR)

2.1.1 Introduction

Peste des petits ruminants is a highly contagious, fatal and economically important disease affecting domestic and wild small ruminants and camels (Munir, 2013). The disease, which is also known by many names like ovine rinderpest, goat plague, plague of small ruminants or Kata, but commonly used by the French acronym PPR across the globe (Kumar *et al.,* 2014), has great importance in Asia and Africa, where the small ruminants form an important component of agricultural food production (FAO, 1999). PPR was included in the OIE (Office International des Epizooties) list of notifiable terrestrial animal diseases owing to high morbidity and mortality (Munir, 2013).

2.1.2 History

The disease was first described by Gargadennec and Lalanne in the Ivory Coast, West Africa in 1942 (Gargadennec and Lalanne, 1942) and was later reported from different parts of sub-Saharan Africa, the Arabian Peninsula, the Middle East, and different parts of Asia (Balamurugan *et al.,* 2012). In India, PPR was first recorded in 1987 from the southern state of Tamil Nadu and northern India evidenced an epidemic outbreak of this disease during the year 1994(Nanda *et al.,* 1996). At present, PPR is enzootic in India and outbreaks occur regularly among small ruminants throughout the country incurring significant economic losses in terms of morbidity, mortality, and loss of productivity due to trade restriction (Dhar *et al.,* 2002).

2.1.3 Details of Causative Agent

Gargadennec and Lalanne who first described this disease, found many similarities between PPR and Rinderpest, but not transmissible to cattle. Later Mornet and colleagues shown that the PPR virus (PPRV) and the rinderpest virus (RPV) are antigenically related. In 1979, PPRV was classified as a Morbillivirus under the family *Paramyxoviridae* and the order *Mononegavirales* (Gibbs *et al.,* 1979). PPR virions are enveloped, pleomorphic particles having a diameter ranging from 400-500 nm. Genome is single-stranded RNA with negative polarity. The length of the entire genome of PPRV is 15,948 nucleotides, which makes it the second longest among all morbilliviruses, the first being a recently characterized feline morbillivirus (Munir, 2013). PPRV genome encodes six structural proteins namely the nucleocapsid protein (N), the phosphoprotein (P), the fusion protein (F), the matrix protein (M), the hemagglutinin-neuraminidase protein (HN), the large protein (L) and two non-structural proteins (C and V). These proteins are arranged in the genome as 3'-N-P/C/V-M-F-HN-L-5' (Bailey *et al.,* 2005). The C and V are two non structural proteins translated from the P gene open reading frame (Bailey *et al.,* 2005). Based on the partial sequence analysis of the F gene of PPR virus, four lineages have been identified (FAO, 1999; Balamurugan *et al.,* 2012), of which, strains I to III are from Africa and IV from Asia (Balamurugan *et al.,* 2012). Lineage III is also found in Southern India (Dhar *et al.,* 2002).

2.2 Disease Details

2.2.1 Clinical signs and symptoms

PPRV gains entry through the epithelial lining of the oral cavity, digestive and respiratory tract of the host. Clinical outcome can be peracute, acute, subacute depending on the physiological status, age and type of the host. Sheep and goat shows acute form of the disease, wherein, the incubation period ranges from 3-4 days (Munir, 2013). The clinical disease resembles rinderpest in cattle and characterised by pyrexia, serous ocular and nasal discharges, erosive lesions on different mucous membranes particularly in the mouth, diarrhoea and pneumonia (OIE, 2013). Secondary bacterial infections like *Pasteurella spp.*, *Escherichia coli* and *Mycoplasma spp.* further aggravates the condition (Kumar *et al.,* 2014). Peracute form is seen in kids and lambs and subacute in unnatural hosts like buffalo (Munir, 2013).

2.2.2 Host

PPR is a disease primarily affecting small ruminants (FAO, 1999). There has been a contradiction in severity of disease in sheep and goats. In general, goats are more severely affected than sheep, but some reports have shown cases of high mortality in sheep within mixed small ruminant flocks (Kumar *et al.,* 2014). Wild ruminants are also susceptible host, but PPR has been diagnosed only in captive wild ungulates from families of *Gazellinae* (Dorcas gazelle), *Caprinae* (Nubian ibex and Laristan sheep), *Hippotraginae* (gemsbok) to date (Sen *et al.,* 2010), Springbuck, Arabian gazelles, Barbary sheep, Impala, Afghan Markhor goat, Defassa waterbuck, Kobs, Arabian oryx (Banyard *et al.,* 2010). Epidemiological surveillance study revealed PPRV seroprevalence in cattle, buffalo and camel, which accounts upto 41% and 67% seroprevalence in buffalo and cattle. Cattle and buffaloes are considered as potential dead-end hosts for PPRV (Kumar *et al.,* 2014).

2.2.3 Prevalence

The disease once thought to be restricted to West Africa, is now found in most African countries situated in a wide belt between the Sahara and Equator, the Middle East (Arabian Peninsula, Israel, Syria, Iraq, Jordan), and reaching eastwards through Western and South Asia (Abubakar *et al.*, 2009). Even though the disease was first reported in 1987 in India, it is thought to be prevalent before that. The disease was misdiagnosed as pasteurellosis and rinderpest for a long time (Taylor *et al.,* 2002). Outbreaks of PPR are now known to be common in India, Nepal, Bangladesh, Pakistan and Afghanistan (FAO, 1999).

2.2.4 Economic importance

PPR is an important disease which curtails sheep and goat production in countries where it is endemic (Diallo *et al.,* 2007). This influences the daily earning of poor farmers who are the main owners and decelerate the poverty alleviation programs. According to the FAO estimates, the morbidity, mortality, production losses and treatment cost of PPR altogether are likely to cause an economic loss of $2,972.5 million/year during 2012-2017 in SAARC region. About 1.8 billion, 62.5 % of small ruminant population is at risk for PPR globally (Felix, 2013). In India annual losses due to this disease have been estimated at approximately INR 1,800 million (US$ 39 million) and about 200 million small ruminants are at risk (Balamurugan *et al.,* 2012).

2.3 Diagnosis of PPR

PPR can be tentatively diagnosed by clinical observations, symptoms, epidemiology, post-mortem lesions (Balamurugan *et al.,* 2014). In order to differentially diagnose PPR from various other diseases showing similar symptoms some laboratory tests should be carried out. These tests are for detecting the virus itself, detection of virus antigen or virus genetic material or detection of antibodies found in serum (FAO, 1999). However, conventional techniques such as agar gel immuno-diffusion test (AGID), counter-immunoelectrophoresis (CIE) and indirect ELISA which were once popular in earlier days, were replaced by new techniques, because they cannot differentiate PPR and RP infections. To overcome such disadvantages, the specific techniques like cDNA hybridization RT-PCR technique, virus neutralization test and monoclonal antibody based ELISAs have been used. (Singh *et al.,*2004b). In general, virus isolation, AGID/AGPT, CIE, nucleic acid hybridization, hemagglutination using piglet or chicken red blood cells, immuno-histochemical detection, serum or virus neutralization test (VNT) and ELISA using PPRV-specific monoclonal antibodies (MAbs) are used for diagnosis of PPR (Balamurugan *et al.,* 2014). Currently, the diagnosis of PPRV is made based on detection of `antibodies; because once an animal is infected with PPRV it carries antibodies for life, with the development of a sustained antibody response (Munir, 2013).

2.3.1 Antigen detection

PPRV antigen can be detected using a variety of tests like immuno-capture ELISA (ICE), counter immunoelectrophoresis (CIE) and agar gel immunodiffusion or agar gel immuno-precipitation test (AGID/AGPT) (Banyard *et al.,* 2010). AGID/AGPT was commonly used for PPRV antigen detection from clinical and post-mortem samples, but cannot differentiate between PPRV and RPV infection. CIE is more sensitive and rapid than AGID, and can also distinguish between PPR and RP infections (Banyard *et al.,* 2010; Balamurugan *et al.,* 2014). ICE and sandwich ELISA can efficiently confirm the PPRV antigen the secretions and tissues of the PPRV infected animals. Monoclonal antibodies (MAb) directed against the N protein of the PPRV are used for these assays (Singh *et al.,* 2004a; Munir, 2013). Indirect immunofluorescence test (IFAT) and Immunoperoxidase test (IPT) utilizing polyclonal and MAb provides rapid detection of virus antigen in situ in the infected cells or tissues samples.

IPT could be a test of choice for localization of PPRV antigen in samples where antigen has not been reported previously (Balamurugan *et al.,* 2014). Haemagglutination tests can also relied to detect PPRV antigen, it is, unlike AGID can differentiate PPRV and RP infections, since RPV has no haemagglutinating properties (Osman *et al.,* 2008). For conjunctival smears and post-mortem samples immunofluorescence and immunochemistry can be used (Banyard *et al.,* 2010). The lateral flow and the flow through (immunofiltration test) offers themselves as the tests of choice for development of field based kits. Though the lateral flow tests is a single step test and are conventionally used for antigen detection, it requires the availability of high affinity MAbs against two distinct epitopes on the antigen to be detected (Raj *et al.,* 2008). The development of immunochromatographic strip test using mAbs for detecting PPRV antigen has been reported by Hussain *et al.,* (2003) and Shrivastava (2006). The gold standard for the diagnosis of the PPRV is virus isolation. Blood collected at the peak of the temperature serve this purpose. Several primary bovine and sheep cells and also established cell lines like Vero (African green monkey kidney cells) and B95a (Marmoset B-lymphoblastoid cells) can be used for the propagation of the virus (Sreenivasa *et al.,* 2006).

2.3.2 Antibody detection

Virus Neutralization Tests (VNT) and competitive ELISA (cELISA) are the serological tests used for the detection of PPRV antibodies. VNTs are prescribed for international trade, but the major drawback is the need for interpretation of results. For this Vero cells are used but also can be grown in lamb kidney cells. Several cELISAs have been developed which are based on the use of monoclonal antibodies (MAbs) that recognize two viral proteins- HN and N proteins (OIE, 2013, Munir, 2013). Singh *et al.* (2004b) and Sharawi *et al.* 2010 has reported high relative specificity (98.4%) and sensitivity (92.4%) of cELISA based on the HN protein, that is comparable to the VNTs. Khan *et al.* (2008) also reported this ELISA for the diagnosis of PPRV antibody in wild animals. Muthuchelvan *et al.* (2015) reviewed a polyclonal antibody based indirect ELISA was developed for detection of antibodies against PPRV in the serum samples. cELISA has replaced VNT now a days (FAO, 1999). Synthetic peptide antigens have been developed for serodiagnosis of PPR and an immunocomb assay using synthetic peptide antigens have been reported for the rapid field diagnosis of PPR (Shrivastava, 2006).

2.3.3 Genome detection

Genome based detection techniques like reverse transcription polymerase chain reaction (RT-PCR) and nucleic acid hybridization are being used now a days for confirmatory diagnosis by virtue of their high sensitivity and specificity (Raj *et al.,* 2008). It is now being used in reference centres together with ELISA, as it is rapid, accurate and highly sensitive, despite of its high cost and expertise (FAO, 1999; Sharawi *et al.,* 2010). Combining RT-PCR and nucleotide sequencing, provides better chance to characterize the different lineages of the virus and helps in epidemiological studies (FAO, 1999). RT-PCR has been used for detection and differential diagnosis of RP and PPR viruses by targeting the N and F gene. To detect the PPRV from clinical samples N-Gene based RT-PCR-ELISA has developed at IVRI, Mukteswar. This RT-PCR ELISA is ten times more sensitive than the classical RT-PCR (OIE, 2013). Recently, a novel non-amplification technique was developed to detect nucleic acids of PPRV, wherein two probes complementary to the target sequences - one conjugated to magnetic microparticles and the second to gold nanoparticles labelled with horseradish peroxidase was used. The assay has great potential due to quick performance (45 min) and not requiring expensive instrumentations (Tao *et al.,* 2013). RT-PCR techniques based on the F genes have also been developed. A multiplex RT-PCR targeting fragment of N protein and M protein genes was also developed (OIE, 2013). Standard RT-PCR has been superseded by real-time RT-PCR assays specific for PPRV and loop-mediated isothermal amplification techniques, for molecular detection (Banyard *et al.,* 2010). M gene-based hydrolysis probe (TaqMan), SYBR Green I based real-time RT-PCR assays targeting the M gene of PPRV are in use for quantification and diagnosis of PPRV (Abera and Thangavelu, 2014).

2.3.4 Recombinant antigens in diagnosis of PPR.

Ismail *et al.,* (1995) reported the cloning and expression of full N gene of PPRV in Baculovirus to be used for serological diagnosis. Seth and Shaila (2001a) studied the biological activities of HN protein of PPRV, after expressing PPRV HN protein in eukaryotic cells. This was achieved by employing the transient expression of transfected cDNA construct in a eukaryotic expression vector under CMV promoter. Many articles reported the expression of various proteins of PPRV in eukaryotic expression systems for diagnosis, vaccine purposes and

for mapping of the proteins to determine the different domains (Libeau *et al.,*1995; Seth and Shaila, 2001b, Mahapatra *et al.,* 2006, Sodjo *et al.,* 2008, Khandelwala *et al.,* 2011). Prokaryotic expression of F, M, N and HN proteins were reported by Wang *et al.,* (2013), Liu *et al.,* (2013), Yadav *et al.,* (2009) and Sinnathamby *et al.,* (2001) respectively.

2.4 Functionalizing Solid Surfaces

A wide range of protein based technologies are available today. Among which solid phase based assays are gaining more attention (Sun *et al.,* 2006; Chan *et al.,* 2007). Microarrays, microbeads and biosensors are some of the examples for such assays. These assays helps in characterizing the interaction and function of biomolecules, purification schemes and development of diagnostics (Sun *et al.,* 2006). These assays require attaching the proteins to a solid surface without affecting its biological properties. Solid surfaces used for immobilization of proteins include polymers, glass, PDMS (poly (dimethylsiloxane)), plastics surfaces, metal beads, and supraparamagnetic beads etc (Chan *et al.,* 2007; Camarero, 2007; Kim and Herr, 2013). Current functionalization of solid surfaces are based on adsorption, covalent immobilization to chemically activated surfaces, or on the biosynthetic expression of a polypeptide 'tag' that mediates immobilization on an appropriately derivatized surface (Sun *et al.,* 2006).

2.4.1 Silicon

Silicon was used in the microfluidic system right from the late 1970s. It was employed for the gas analysis system which has been developed on the basis of gas chromatography (Terry *et al.,* 1979). Fine fluidic channels and microfluidic components have been created on the silicon substrate using high resolution microfabrication technique. As reported by Wang and coworkers, oxides of silicon whether natural or artificially produced are efficient for protein immobilization based on silanol chemistry (Wang *et al.,* 2006). Opaqueness of silicon, incompatibility of silicon with electrokinetic methods and the high cost of sophisticated microfabrication techniques used to microfabricate silicon limits its application in electrochemical analysis and Surface Plasmon Resonance (SPR) (Kim and Herr, 2013).

2.4.2 Glass

Other commonly used substrates for microfluidic devices are glass and quartz. Glass,

being resistant to acids and bases forms better substrates for this. Owing to the negligible autofluorescence of glass, it is well suited for the fluorescence based microfliudic assays (Zhu and Snyder, 2003; Kim and Herr, 2013). Glass was initially used in microfluidic electrophoresis systems (Kim and Herr, 2013). Glass remains as the most commonly used substrate for protein arrays though various other solid surfaces also comes up in market due to its readily availability and cost concern. Glass slides are highly sensitive and owing to its transparency, it is highly useful in detection analysis on microarrays (Jang and Liu, 2009). Many studies have been conducted to increase the efficiency of the glass to be used as the solid surface during the last years. Clean glass surfaces or beads should be used for the immobilization purposes. Different articles reported various methods to make the surface free of any organic impurities. McGovern *et al.,* (1994) reported the effectiveness of 5% NaOH and Rogers *et al.,* (1999) used 25% NH_4OH for cleaning the surfaces. The most commonly used method for removing organic impurities from the glass surfaces were acid wash and the use of piranha solution. Hydrochloric acid, nitric acid and sulfuric acid were the acids commonly used for the surface cleaning. The concentrations and incubation time of these acids for cleaning should be optimized based on the available resources (Cras *et al.,* 1999; Walsh *et al.,* 2001). Piranha solution is a strong oxidizing solution which is frequently used to remove organic impurities from the glass surfaces. It is a highly aggressive mixture of 30% H_2O_2 and 70% H_2SO_4. The incubation time should be optimized for each protocol being used. Many articles suggested 20-30 minutes of incubation in piranha solution. After incubation in piranha solution it can be washed with toluene, choloroform, acetone or ethanol. A further washing with ample quantity of clean distilled should be done (Zammatteo *et al.,* 2000; Arslan *et al.,* 2006; Labit *et al.,* 2008; Vistas *et al.,* 2013).The presence of reactive groups on the surface which is usually electrophilic groups such as epoxides, aldehydes, succinimidyl esters/isothiocyanate groups should be able to bond with the nucleophilic groups on the ligand molecules for covalent attachment. Glass has been fabricated using various silanol agents for immobilization or they can be inserted on Self assembling monolayers (SAM) or polymers grafted to the surface (Guilleaume *et al.,* 2005; Cretich *et al.,* 2006). Walsh *et al.,* (2001) used glass beads of 100 μm for immobilizing the DNA using APTS chemistry. Immunoassays on slide surfaces using fluorophores was also reported. Myoglobin fluorescence immunoassay was studied by Matveeva *et al.,* (2005) in

which they used Rhodamine red-X as fuorophore. A similar study was conducted by Staiano *et al.,* (2009) to detect gliadin, responsible for celiac disease of small intestine.

2.4.3 Plastics

Polymers have replaced silicon and glass in microarrays due to their reduced cost and they can be channelized by molding and embossing where as glass and silicon soild surfaces have to be etched for fabrication. Microfluidic devices made of polymers can be sealed by thermally or by using adhesives (McDonald *et al.,* 2000).Microfluidic chips fabricated from plastics such as poly(methylmethacrylate) (PMMA), cyclic olefin copolymers (COC), polycarbonate (PC), polyester, and polystyrene (PS) are extremely cheap to produce when using mold-based techniques like injection molding or hot embossing (Becker and Gartner, 2000; Nunes *et al.,* 2010). Poly(styrene) plates have been used for immobilization of antigen or antibody in ELISA procedures (Bora *et al.,* 2002). Moreover, resistance of plastic to solvents and acids/bases makes it more relevant. Plastic is rigid but not fragile, common in the market place, and optically transparent (Becker and Gartner, 2000). Plastics can contain a number of additives that impact their processing and shelf life and these may include fillers (e.g. mica, talc, calcium carbonate), plasticizers (e.g. dioctyl phthalate in PVC), heat stabilizers (e.g. organo-tin compounds in PVC), antioxidants (e.g. phenols, amines), and UV stabilizers (e.g. benzophenones, salicylates). Hydrophobic nature of these materials makes it inaccessible to the aquous phase based microarrays (Becker and Locascio, 2002).

2.4.4 PDMS

The polymers commonly used for microfluidic solid surfaces include poly(styrene), poly(propylene), poly(vinyl chloride) (PVC), poly(carbonate), poly(methyl methacrylate) (PMMA) and poly(dimethylsiloxane) (PDMS) (Zhou *et al.,* 2010). Among these polymers, PDMS, owing to rapid design fabricate test cycle and low cost, is frequently used and studied substrates for microfluidics. PDMS is a transparent elastomer (Kim and Herr, 2013). The main advantages of PDMS biocompatibility, gas permeability, optical transparency, ease of molding into (sub)micrometer features, ease of bonding to itself and glass, relatively high chemical inertia, and low manufacturing costs (McDonald *et al.,* 2000; Zhou *et al.,* 2010). Different methods for surface modification of PDMS surface are plasma treatment, silanization, chemical

vapor deposition (CVD), layer-by-layer (LBL) deposition, surfactant treatment, protein adsorption, graft polymer coating and hydrosilylation-based PDMS surface modification (Zhou *et al.,* 2012). For binding to glass it must be treated with oxygen plasma. They can be irreversibly sealed with plastic substrates, glass or PDMS slabs forming enclosed channels (Kim and Herr, 2013). Hydrophobicity is the main disadvantage of the PDMS which makes introducing aqueous solutions into the microchannels of PDMS-based devices difficult (Yu *et al.,* 2007; Zhou *et al.,* 2010; Viefhues *et al.,* 2011).

2.4.5 Metals

Metal films of gold, silver and platinum has been deposited on the silicon or glass surfaces for protein immobilization. This type of immobilization helps in detection of proteins by methods other than fluorescence and colorimetric methods (Kim and Herr, 2013). Zhu and Synder (2003) reported thio-alkylene based functionalisation of gold surface. Detection methods such as surface plasmon resonance (SPR), Raman spectroscopy, and electrochemical analysis invariably depend on the immobilization of proteins on the metal films (Kim and Herr, 2013). Silicon, metals (mainly Au and Ag), and semiconductor (i.e., Ag2S, CdS, and CdSe) based substrates are among the most common materials used to immobilize proteins in micro and nanobiotechnology (Camarero, 2007).

2.4.6 Paper

Paper is a highly porous matrix made of cellulose. When certain areas of paper are hyrophobically modified, aqueous solution can be applied to the paper, which will be precisely guided by the capillary effect through the hydrophilic region of the paper (Martinez *et al.,* 2007). Applying polymer solution to the paper using lithographic methods makes it expensive but shows high resolution. In contrast, printing methods to generate hydrophobic region is less costly since it requires only simpler equipments. When coupled with colorimetric detection methods, paper-based microfluidic devices realize a platform for inexpensive, power free, portable bioassays (Ren *et al.,* 2014).

2.4.7 Three dimensional materials

Compared to planar immobilization surfaces, three-dimensional (3-D) surfaces have many advantages. Common formats include micro/nanostructures created using microposts, microbeads, hydrogels, sol-gels, polymer monoliths and membranes. Selection of 3-D

structures stems from the increased surface-area-to-volume ratio offered. The increased effective surface area possessed by the 3-D material offers a larger number of immobilization sites, as compared to channel wall (2-D) systems (Kim and Herr, 2013). Bead-based microfluidic immunoassays have an edge over normal fluidic systems. The main advantages in the use of these microbeads include the surface to volume ratio is greatly increased even in a microfluidic device, the analytes attached onto the beads can be easily transported in a fluidic system using pressure-driven flow or electric fields and there are a variety of surface modifications available on these microbeads, which can introduce multiple functionalities to a single microfluidic design (Lim and Zhang, 2007). Paramagnetic beads can be used for immobilization of proteins or other bioactive substances on them (Arnau *et al.,* 2006). This magnetic properties help them for localization and further separation from the surrounding fluids (Pamme N. 2006). Sista *et al* (2008) performed and optimized a sandwich immunoassays based on magnetic beads. Hydrogels are 3D networks of hydrophilic polymer chains that span in aqueous medium and have almost 99% water content. Hydrogels, resembling the extracellular matrix, have been widely used to engraft cells for various applications; microchannels can be built in the hydrogels for delivery of solutions, cells, and other substances. Owing to the high porosity, they allow small molecules or even bioparticles to diffuse through. The combination of aqueous nature and high permeability makes hydrogels suitable for encapsulating cells for 3D culture (Ren *et al.,* 2012). Kuan *et al.,* (2008) studied the immobilization of D-amino oxidase from *Rhodotorula gracilis* onto Ni2+-chelated nitrilotriacetic acid (Ni-NTA) magnetic agarose beads.Synthetic PEGDA (PEG diacrylate) gel and polyacrylamide gel are popular with natural gels like chitosan or agarose gels (Kim and Herr, 2013).

2.5 Methods for Functionalizing Solid Surfaces

The main aim of surface modification, for fabricating protein chips, is to provide good accessibility for proteins and other biomolecules for immobilization. The functionalized surface must also reduce the uncontrolled non-specific adsorption of proteins and provide stability to the protein bound interface (Jang and Liu, 2009). Most of the methods suitable for the chemoselective attachment of proteins to surfaces are based on ligation methods originally developed for the synthesis, semisynthesis, and selective derivatization of proteins by chemical

or biochemical means. These methods involve derivatizing a protein with a chemical group at a defined position, which will later react chemoselectively with a complementary group previously introduced into the surface. This will provide stable linkage of the ligand on the surface. (Camarero, 2007).

2.5.1 Fabrication of silicon substrate

Silicon and glass were the solid surfaces used in first generation microfluidic devices. These were modified using photolithography and wet –etching in a clean room (Han *et al.,* 2013). Derivatised trialkoxysilanes like (3-aminopropyl)- trialkoxysilane or (3-mercaptopropyl) -trialkoxysilane, which are able to introduce an amino or thiol group respectively are commonly used for functionalization of silicon surfaces. Long chain alkyl-trichlorosilanes are better agent for fabricating the silicon surfaces (Camarero, 2007). One study compared different strategies for covalent attachment of antibodies and bioaffinity proteins (protein A or G) on silicon substrates. This study found that covalent attachment with long flexible linkers such as polyethyleneimine (PEI) and dextran (DEX) were more favourable than shorter linkers such as 3-aminopropyltriethoxysilane (APTES). These long flexible linkers outwit problems associated with antibody accessibility and steric hinderance (Ng *et al.,* 2010). Yakovleva *et al.* (2002) compared the efficacy of branched PEI and linear PEI on the silicon surfaces. Sung *et al.* (2012) demonstrated that rationally designed random copolymers that has a surface anchoring moiety like dodecyl, benzyl, or trimethoxysilanyl, an antibiofouling polyethylene glycol (PEG) component, and an NHS ester functional group for conjugation could be used to modify the surfaces of plastic or Si/SiO2 wafer surfaces, and they would form stable polymer layers at low pH on which biomolecules could be selectively immobilized.

2.5.2 Fabrication of glass substrate

The fabrication of glass substrates by silanization can be done by immersing the clean dried glass slides in a solution of chlorodimethyloctadecylsilane (0.02 M) in an anhydrous toluene solution overnight. This silanization should be carried out in dry box under nitrogen. After silanization, washing with toluene and later with methanol removes any non-bonded chlorodimethyloctadecylsilane from the glass slides and then dried in an oven **(Inerowicz *et al.***,

2002). Development of antibody/antigen microarrays on poly-L-lysine-coated glass slides using fluorescent labeling of antigens or antibodies respectively was also reported. Epoxy-silanization as well as mercapto-silanization with maleimido-succinimidyl crosslinker (aminoreactive NHS-surface) or amino silanisation with maleimido-succinimidyl crosslinker (thiolreactive maleimide- surface) proved to be simple and cheap derivatization strategies. These surface derivatising agents found to be three to four times better signal-to-background ratios compared with poly-L-lysine slides as well as high signal and also reported that epoxysilanized slides possess the highest sensitivity for this type of surface modification (Kusnezow and Hoheisel, 2003). Slides that have been treated with an aldehyde-containing silane reagent are another type of fabrication. Aldehydes react readily with primary amines on the proteins to form a Schiff's base linkage. Slides can also be fabricated using BSA NHS (Bovine Serum Albumin-*N*-hydroxysuccinimide) by attaching a molecular layer of BSA to the surface of glass slides and then activating the BSA with *N*,*N* 9-disuccinimidyl carbonate. This activates lysine, aspartate, and glutamate residues on the BSA which then react readily with surface amines on the printed proteins to form covalent urea or amide linkages. In contrast to the aldehyde derivatised slides, proteins or peptides printed on BSA-NHS slides are displayed on top of the BSA monolayer, making them accessible to macromolecules in solution (Macbeath and Schreiber, 2000).Among the various surface modification techniques, the deposition of a self-assembled monolayer (SAM) or multilayer of organosilane on a glass slide is very versatile. This technique has many advantages over other approaches. Silanization of solid surfaces with APTES produce amino functional groups for immobilization of biomolecules. These amine groups are highly reactive toward various functional groups that can be incorporated into SAMs. The SAM of APTES compounds on glass surfaces can be used to immobilize a variety of functional groups like hydroxyl groups and can support the formation of monolayer coverage under carefully controlled conditions. Further activation with 10% gluteraldehyde in PBS render the surface to be aldehyde terminated (Jang and Liu, 2009). APTES plays a significant role in the surface modification of bioanalytical surfaces and the immobilization of biomolecules on modified surfaces. It is a colorless liquid with density of 0.946 g/mL at 25°C, and melting and boiling points of -70°C and 217°C, respectively. It has been widely used for the development of invitro diagnostics, particularly those based on enzyme-linked immunosorbent assays (ELISA), microgravimetry, surface plasmon resonance (SPR), surface acoustic wave (SAW) and

nanomaterials (Vashisht S. K. 2014). Arslan *et al.,* (2006) reported the silanization of glass surfaces using 1% APTES in anhydrous toluene for 24 h and then washed with toluene, deionised water and ethanol to remove any physisorbed APTES. APTS fabrication of glass surface followed by EDC- NHS activation was studied for protein immobilization on a dextran layer by Akkoyun and Bilitewski (2002).Vistas *et al.,* (2013) investigated and optimized the conditions for generating high quality layer with well oriented thiol groups in glass chips using 3-mercaptopropyltrimethoxysilane (MPTS). They studied effect of different concentrations of MPTS and time of incubation. Glass coverslips can be silanized using MPTS by immersing into the 2% (v/v) MPTS solution in dry toluene for 2h under inert conditions as reported by Bhatia *et al.,* (1989). As acconted by Zammatteo *et al.,* (2000), 30 minute incubation in 3% (v/v) APTES in 90% methanol/water can also be used for silanization of glass slides. Sensitive flow-through immunoassay lab-on-a-chip based on a zinc oxide *(ZnO)* nanorod decorated glass capillary for quantitative detection of tumor biomarkers was developed by Hu and coworkers in 2013. In this, biomarker-targeted antibody is covalently attachcd on the ZnO nanorods tethered on its interior surface, which allows for convenient flow-through detection (Hu *et al.,* 2013). Lee *et al.* (2012) reported the use of Titanium sputtering on glass plate to fabricate hydrophobic SAM –DDMS, and hydrophilic SAM –APTES, to create hydrophobic/hydrophilic patterned microchip.

2.5.3 Fabrication of polymer substrate

Polymer surfaces can be modified by plasma treatment, UV radiation and UV/ ozone to make them hydrophobic or by chemical treatment (Sui *et al.,* 2006). Polymers can be chemically modified by liquid of gas phase derivatization reactions for example, the use of strong oxidizing acids have been used to introduce carbonyl and carboxylic acid groups at polyolefinic surfaces in order to improve adhesive binding. Chromic acid has been used to introduce carbonyl and carboxylic acid groups on poly(ethylene) (PE) and poly(propylene) (PP). By applying potassium permanganate / sulfuric acid solutions to PE surfaces, similar derivatisation was obtained. Sulfuric acid itself introduces sulfonate groups on PE. For perfluorinated polymers more rigorous treatments are required e.g., reactions with sodium/ liquid ammonia, have to be applied to introduce functional groups like carbonyl and carboxylic acid groups (Terlingen, 2004). Polystyrene surfaces can be activated by a rapid and simple

method using 1-fluoro-2-nitro-4-azidobenzene (FNAB) in a photochemical reaction (Bora *et al.,* 2002). Wen *et al.* (2009) used biotin-poly(L-lysine)-graft-poly(ethylene glycol) (biotin-PLL-g-PEG) was used as a surface linker to minimize the repulsive force between antibody and PMMA surface. PMMA surface was activated by plasma oxidation followed UV graft polymerization using poly(acrylic acid) (PAA) to add functional carboxyl groups.

2.5.4 Fabrication of PDMS substrate

The conventional method of making the PDMS surface hydrophilic is by oxidation through UV exposure, oxygen plasma treatment and CO2 pulsed laser which makes the rotation of the Si–OH polar group rotates back into the bulk polymer to minimize the surface energy (Inerowicz *et al.,* 2002; Henares *et al.,* 2008). Yu *et al.* (2007) developed a robust method of derivatising the PDMS surface by chemical functionalization with the sequential introduction of aminopropyltriethoxysilane, glutaraldehyde, polyvinyl alcohol and glutaraldehyde for getting uniform surface for IgG immobilization. Sui *et al.* (2006) reported efficacy of solution based oxidation of PDMS surface using H_2O_2 and HCl to impart hydrophilicity on the surface. The PDMS can be modified by using 7-octenyltrichlorosilane to obtain a silane layer with vinyl terminated layer. It is then oxidized and fabricated using NHS and EDC to immobilize biotin which can be further used to immobilize biomolecules by avidin –biotin linkage (Jang *et al.,* 2006).

2.5.5 Fabrication on metal substrates

Thiol SAMs modification of gold films were prepared by immersing the thin gold film into thiol solutions for atleast 18 h. Aldehyde terminated SAMs can be used for the immobilization which will form a Schiff's base with the primary amines of the protein(s)/ peptide(s) to be immobilized (Wadu-Mesthrige 2000). Alkanethiols as well as alkyl disulfides can also be used for the derivatisation of gold films. Both give rises to similar levels of surface coverage on clean gold surface, but thiols react faster than disulfides (Camarero, 2007). Wang *et al.,* (2005) conducted an extensive study on –COOH and $–NH_2$ SAM on gold surface using 11-mercapto undecanoic acid (MUA) and 11-amino-1-undecanethiol respectively.

2.6 Methods for Immobilizing Protein(s)/Peptide(s)

Novel methods for immobilization of bioactive molecules, including antibodies,

enzymes, peptides, polysaccharides, and nucleic acids, on solid materials have been attracting considerable attention in the field of biosensors and biochips (Sung *et al.,* 2012). Highly efficient antibody immobilization without affecting its activity is a crucial step in the development of microscale immunoassay systems. The random immobilization of antibody molecules on the substrate results in a great reduction of the specificity of antibody to bind antigen, so to increase the binding efficiency the controlling the orientation of antibody should be done(Wen *et al.,* 2009). A specific immobilization method relies on a variety of factors including immobilization surface, sample matrix, protein property, buffer constituents etc. Active sites for antibody binding or enzymatic conversion should be accessible to reaction partners (i.e., proteins face away from the immobilization surface to mitigate steric hindrance and are not sterically blocked by neighboring immobilized proteins). After immobilization, protein conformation should be intact ensuring that their functions are retained for a high performance, reproducible assay (Kim and Herr, 2013). Immobilization of bioactive molecules can be by electrostatic forces, by physisorption methods or by covalent attachments (Goddard and Hotchkiss, 2007).

2.6.1 Immobilizing via amine terminal of peptides/proteins

The most widely used route to conjugate biomolecules to solid supports makes use of reactions involving primary amino groups (-NH2). The utility of amines arises from their high nucleophilic character and the existence of different variety of amine base coupling chemistry suitable for use in aqueous conditions. The lysine residues of proteins have amino groups in ε-position (Dugas *et al.,* 2010). In this strategy, the highly abundant amine group of the lysine (Lys) side chain plays a crucial role. It can readily react with an interface support bearing activated esters such 1-ethyl-3-[3- dimethylaminopropyl]carbodiimide hydro chloride (EDC or EDAC) and Nhydroxysuccinimide (NHS) simultaneously or sequentially, or with surfaces terminated with aldehyde groups, or with epoxide functionalized surfaces (Tan *et al.,* 2012). The water-soluble EDC-mediated reaction converts the carboxyl group termini on the surface into the unstable O-acylisourea intermediate that readily reacts with the amine group, resulting in covalent amide bonds between biomolecules and solid surfaces. Then the bioactive can be immobilized on the carboxylate terminated monolayers via EDC and N - hydroxysulfosuccinimide (Sulfo-NHS) (Walsh *et al.,* 2001). The surfaces terminated with

aldehyde, isothiocyanate, imidoester can also form stable bonds with the amine termini under suitable conditions (Dugas *et al.,* 2010).

2.6.2 Immobilizing via carboxyl terminal of peptides/proteins

The highly abundant aspartic acid (Asp) and glutamatic acid (Glu) residues on the cytoplasmic face of proteins have also been commonly used for chemical grafting of proteins (Tan *et al.,* 2012). Fernandex -Lafuente *et al.,* (1993) proposed the conversion of the carboxyl groups of the protein by reaction with carbodiimide to activate the –COOH group, or their conversion to activated esters in-situ using a carbodiimide coupling reagent in conjunction with an auxiliary nucleophile, such as NHS.

2.6.3 Immobilizing via thioethers or disulfides

Thiolates owing to their nucleophilic character in aqueous solutions they are found to be a potent candidate to exploit for use in grafting of proteins via the thiolate group of a cysteine residue (Kalia and Raines, 2010). Covalent coupling strategies using thiol groups allow site-specific reactions. The sulfur atom of cysteine belongs to form a sulfhydryl (or thiol) group (Dugas *et al.,* 2010). As cysteine is the second least common amino acid in natural proteins, site -specific conjugation can be performed at a unique cysteine residue. Typical thiol-reactive functional groups include iodoacetamides, maleimides, and disulfides (Kalia and Raines, 2010). Strategies use either cysteine residue bonding by reaction with surface epoxides or through conjugating to unsaturated carboxyl groups, such as maleimides, to form stable thioester bonds, have been established. One of the advantages of the thioester bond formation is that it favors bioconjugation of proteins almost near to physiological conditions, for example, in a pH range of 6.5 to 7.5 (Tan *et al.,* 2012).

Materials and Methods

3 MATERIALS AND METHODS

The present study was conducted in Genetic Engineering of Virus lab and Bioassays and Bio-sensor lab, Division of Veterinary Biotechnology, ICAR- IVRI, Izatnagar. To accomplish the objectives the study was conducted in three major phases as described below:

Phase 1: Production and purification of recombinant proteins of *Peste des petits ruminants virus* antigens namely C terminal F (ΔF1), N terminal F (ΔF2), C terminal H (ΔH1), N terminal H (ΔH2) and C terminal M (ΔM)

Phase 2: Fabrication of purified recombinant antigen(s) on suitable surfaces using appropriate strategies and assessing their immunoreactivity potential

Phase 3: Comparison of developed methods with the existing diagnostic methods to determine diagnostic effectiveness parameters

Different steps performed in each phase of study are enlisted below:

3.1 Cloning and expression of recombinant proteins corresponding to immunodominant regions in PPRV antigens namely ΔF1, ΔF2, ΔH1, ΔH2 and ΔM

3.1.1 Procurement of Vero cell infected PPRV

3.1.2 Isolation of RNA from cell culture adapted PPRV

3.1.3 Preparation cDNA

3.1.4 Amplification of immunodominant regions (ΔF1, ΔF2, ΔH1, ΔH2, ΔM) in different genes of PPRV

3.1.5 Cloning of selected immunodominant regions in prokaryotic expression vector

3.16 Screening and characterization of recombinant clones by PCR, RE digestion

3.17 Optimizing conditions for expression of recombinant proteins such as concentration of IPTG, induction temperature, time etc.

3.1.8 Characterization of recombinant proteins by western/dot blot

3.1.9 Purification of recombinant proteins using Ni-NTA affinity chromatography

3.1.10 Up-scaling and purification of expressed recombinant proteins

3.1.11 Assessing the suitability of the recombinant antigens in detecting PPRV antibodies by immunoassay

3.1.12 Dialysis of recombinant proteins in suitable buffers as per requirements of surface fabrication

3.2 Optimization of conditions for fabrication of recombinant antigens on suitable solid surface(s) to be used for detecting PPRV specific antibodies

3.2.1 Selection of suitable surface(s) for fabricating recombinant antigens

3.2.2 Cleaning surfaces using appropriate solution(s) to make the surface(s) free off any organic residues

3.2.3 Activating the surface(s) for coupling recombinant antigens using various agents

3.2.4 Optimizing conditions for coupling antigen on the modified surface(s)

3.2.5 Assessing the reactivity of antigen bound surface(s)

3.3 Comparative evaluation of developed method(s) with existing diagnostic methods to determine diagnostic effectiveness parameters

3.3.1 Optimizing the conditions like antigen concentrations, blocking agents etc to be adopted for using the recombinant antigen coated surface(s) for detecting PPRV specific antibodies

3.3.2 Screening of a panel of PPRV positive and negative serum samples using the recombinant antigen coated surface(s)

3.3.3 Comparison of developed methods with the existing diagnostic methods to determine diagnostic effectiveness parameters

MATERIALS

Chemicals

All chemicals used in this study were of molecular biology grade purchased from Sigma-Aldrich (USA), Promega (USA), Kapa Biosystems (USA), Hi media (India), Qiagen (Netherland), MBI Fermentas (Maryland), Invitrogen (USA), New England Biolabs (U.K.), Bangalore Genei (Bangalore, India), Merck India Ltd. (Mumbai, India), MP biomedicals (USA).

FITC and HRPO conjugates

Anti-goat HRPO, anti-goat FITC conjugates, Ni-NTA conjugate (Sigma Aldrich, USA) were used for western/dot blot, indirect ELISA, FLISA.

Target Genes

Table 1: PPRV antigens selected for the study

Sl. No	PPRV antigens	Abbreviations
1	Fusion protein	F
2	Nucleocaspid protein	N
3	Matrix protein	M
4	Hemagglutinin-Neuraminidase protein	H/HN

Primers

Table 2: **The primers used for amplification of target genes**

Sl. No.	Target Gene	Immunodominant regions	Amplicon Size (bp)
1	ΔF	ΔF1	366
		ΔF2	644
2	ΔH	ΔH1	600
		ΔH2	810
3	ΔM	ΔM	503

Host bacterial strains and vectors

Escherichia coli DH5α (Promega, Madison) strain for gene cloning experiments and *Escherichia coli* BL21 DE3 (Invitrogen, USA) strain for expression of immunodominant regions of ΔF1, ΔF2, ΔH1, ΔH2, and ΔM were used. For expression of heterologous proteins in *E.coli*, prokaryotic expression vector pET32b (Merck Millipore, USA) was used.

Markers and Ladders

The molecular markers used in this study included 1kb DNA ladder (Promega, Madison, USA) and 100bp DNA ladder (Fermentas, Waltham USA), unstained protein molecular weight marker for SDS PAGE and prestained protein molecular weight marker for western blotting (Bio-Rad Laboratories, USA).

Solid surface for protein coupling:

Glass beads were procured from Sigma-Aldrich (USA).

Media, buffers and reagents:

The details of the media, buffers and other reagents used in the study are given in appendix.

Plastic glassware and consumables:

Plastic wares and other consumables used in this study were purchased from Axygen (USA), Nunc (Denmark), Merck Millipore (USA), Thermoscientific (USA), Tarsons (India), Corning (USA). Glasswares used were from Borosil (India) and Schott-Duran (Germany).

METHODS

3.1 Cloning and expressions of immunodominant regions of PPRV antigens namely ΔF1, ΔF2, ΔH1, ΔH2 and ΔM

3.1.1 Isolation of RNA from Vero cell infected PPRV (Sungri strain)

The supernatant of PPRV infected Vero cell culture was obtained from I. V. R. I. Mukteswar. RNA was isolated manually using TRIZOL. 0.5 ml of cell suspension was taken into RNase/DNase free 2 ml eppendorf and to this 1 ml TRIZOL was added. This mixture was incubated at room temperature (RT) for 5 min. After incubation 200 µl of chloroform was added. The mixture was centrifuged at 12000 g for 15 min at 4^0C following 5 min incubation at

RT and centrifugation results in separation of solution into upper aqueous phase and lower organic phase. RNA containing upper aqueous phase was collected in a new RNase/DNase free 1.5 ml eppendorf and pelletted by adding 500 µl of isopropanol after 5 min incubation at RT. The pellet was washed in 75% ethanol in DEPC treated water by centrifuging at 12000 g for 10 min at 4^0C. Pellet was air dried partially and resuspended in 20 µl of nuclease free water (NFW).

3.1.2 Preparation of cDNA using isolated RNA

RNA isolated from the virus infected cell suspension was used as a template to prepare cDNA using Revert Aid cDNA synthesis kit (Fermentas, USA) as per protocol provided by the manufacturer. Reaction was carried out in two steps.

Table 3: Reaction I of Revert Aid cDNA synthesis

RNA	11 µl
OligodT primer	1 µl
Total	**12 µl**

This reaction mixture was incubated at 65^0C for 5 min and then chilled to 4^0C.

Reaction II

Master Mix was added to Reaction I making up the final volume 20 µl. This reaction mixture was incubated at 42°C for 1 h, followed by inactivation of enzyme at 72°C for 5 minutes.

Table 4: Master Mix of Revert Aid cDNA synthesis

Reaction Components	**Quantity**
5X RT buffer	4 µl
Ribolock	1 µl
dNTP mix (10 mM each)	2 µl
Reverse Transcriptase enzyme	1 µl

3.1.3 Amplification of immunodominant regions of PPRV antigens: ΔF1, ΔF2, ΔH1, ΔH2 and ΔM

Primers were designed for the immunodominant regions of (ΔF1, ΔF2, ΔH1, ΔH2 and ΔM) in PPRV. Annealing temperatures of the primer sets was optimized using gradient PCR.

The PCR reaction mixture consisted of the following components:-

Table 5: PCR reaction components for amplification of target genes

2X Kappa PCR master mix	25 µl
Forward primer (20pmol)	1 µl
Reverse primer (20pmol)	1 µl
Template cDNA	3 µl
NFW	20 µl
Total	**50** µl

Reaction mixture was divided in 5 tubes of 10 µL each in PCR tubes, which were subjected to different annealing temperatures in a gradient thermal cycler (Veriti™ 96 well thermal cycler, Applied Biosystems). The reaction components were subjected to following cycling conditions:-

Table: 6 Stages for optimizing the annealing temperature of primer sets

Cycle No.	Description	Temperature (^{0}C)	Time	No. Of Cycles
Cycle 1	Initial denatura-tion	95	3 min	1
Cycle 2	Denaturation	98	.20 seconds	30
	Annealing	52-64	.30 seconds	
	Extension	72	.40 seconds	
Cycle 3	Final Extension	72	5 min	1

To optimize the annealing temperature, different temperatures ranging between 52-64℃ were set in different blocks of gradient PCR machine. Rest of the reaction temperatures were same in all the blocks. After the reaction, PCR products were analyzed by agarose gel electrophoresis. performed as per the method of Sambrook and Russel (2001). Appropriate quantity of analytical grade of agarose was boiled with 1X TAE buffer to get uniform molten agarose of desired gel percentage. Molten agarose was allowed to cool to 45°C-50°C and 0.5µg/ml ethidium bromide (EtBr) was added. This was casted in a suitable gel casting tray fitted with acrylic comb and left for setting. The acrylic comb was carefully removed after the gel was perfectly set. The tray with the gel was submerged in an electrophoresis tank containing 1X TAE buffer. DNA to be analyzed was mixed with appropriate volume of 6X

DNA loading dye and loaded into wells along with DNA ladder. Electrophoresis was carried out at 5V/cm. The DNA bands were then visualized under UV illumination and documented. The molecular sizes of the amplicons were analyzed in relation to molecular weight marker. The annealing temperatures at which best yield of amplicons was observed with no non-specific amplification were selected for the each primer sets.

The annealing temperature selected for amplification of different genes are shown below:

Table 7: Optimized annealing temperature for different primer sets

Sl. No.	Amplified Immunodominant regions	Annealing temperature (^{0}C)
1	F1	60
2	F2	52
3	H1	60
4	H2	52
5	ΔM	60

3.1.4 Cloning of predicted immunodominant regions of PPRV antigens ΔF1, ΔF2, ΔH1, ΔH2 and ΔM

3.1.4.1 Isolation of pET32b plasmid for cloning of recombinant antigens

The pET32b plasmid isolation was done as per the alkaline lysis method (Birnboim and Dolly, 1979). In short, plasmid culture was inoculted in 5 ml of LB broth containing 100 µg/ml of ampicillin and incubated overnight at 37^{0}C and 180 rpm in orbital shaker. The culture was pelleted next day at 8000 rpm for 10 min at 4^{0}C. The pellet was resuspended in 300 µl of P1 buffer and mixed properly. 300 µl of buffer P2 was also added and mixed by inverting 4-5 times. Then 300 µl P3 solution was added and incubated in ice for 5 min followed by centrifugation at 12000 rpm for 10 min at 4^{0}C. The supernatant was transferred to fresh microfuge gently. Then equal volume (1:1 v/v) of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the supernatant and mixed vigorously. Properly mixed solution was centrifuged at 12,000 rpm for 15 min resulting in separation of upper aqueous phase and lower organic phase with an intermediate protein phase. The upper aqueous phase containing plasmid DNA was collected and precipitated with 0.8 volume of isopropanol. The pellet obtained by centrifugation at 12,000 rpm for 10 min was washed with 70% ethanol and air dried. Pellet was resuspended in 20 µl of NFW and stored in -20^{0}C until further used.

3.1.4.2 Linearization of pET32b vector by RE digestion with *Bam HI* and *Hind III*

Isolated pET32b plasmid was digested with *Bam HI* and *Hind III* restriction endonucleases. The composition of reaction mixture was as follows:-

Table 8: RE digestion of pET32b vector

Reaction Components	Quantity
pET32b Plasmid	50 µl
Bam HI	5 µl
Hind III	5 µl
10X buffer	8 µl
NFW	12 µl
Total	**80 µl**

The reaction mixture was kept at 37^0C in a circulating water bath for 2 h.

3.1.4. 3 Purification of PCR amplified products of PPRV antigens

PCR amplified products of ΔF1, ΔF2, ΔH1, ΔH2, and ΔM genes were purified using Minelute PCR purification kit (Qaigen) as per the manufacturer's protocol. Briefly, 225 µl of PB buffer was added to 45 µl of PCR reaction mix and mixed well. This mixture was added to Minelute column and centrifuged at 13000 rpm for 1 min at 20^0C. After discarding the flow through, 700 µl of PE buffer was added to the column followed by centrifugation at 13000 rpm for 1 min at 20^0C. Product was eluted in 40 µl of elution buffer (EB) after placing the column in properly labeled 1.5 ml eppendorf provided, by centrifuging at 13000 rpm for 1 min. PCR purified DNA products were further proceeded for RE digestion as follows:

Table 9: RE digestion of PCR purified DNA products

Reaction Components	Quantity
pET32b Plasmid	40 µl
*BamH*I	2 µl
*Hind*III	2 µl
10X buffer	5 µl
NFW	1 µl
Total	**50 µl**

Reaction mixture was kept at 37^0C water bath for 2 h.

RE digested PCR products and pET32b plasmids were cleaned up using MinElute

Reaction cleanup kit as per the manufacturer's protocol. The procedure in brief was: Added 300 µl of buffer ERC to the RE digested products, mixed well and applied to the columns provided after proper labeling followed by centrifugation at 13000 rpm for 1 min at 20^0C. 750 µl of buffer PE was added to the column after discarding the flow through. It was then centrifuged at the same speed and temperature as mentioned above. The product was eluted from the column using buffer EB by centrifugation.

3.1.4.4 Ligation of *BamH*I and *Hind*III digested plasmid and DNA

The RE digested products were ligated using T4 DNA ligase. The reaction was set up as follows:

Table 10: Ligation reaction for cloning of PPRV genes fragment in pET32b vector

Reaction components	**Quantity (µl)**
2X ligation buffer	5
T4 DNA ligase enzyme (Promega)	0.5
*BamH*I & *Hind*III linearized pET32b vector	3
*BamH*I & *Hind*III digested PPRV genes fragment	1
NFW	0.5
Total	**10**

The ligation mixture was kept at 8^0C overnight in the cold chamber.

3.1.4.5 Transformation of ligated products in DH5α competent cells

For preparing DH5α competent cells, the bacterial cells from glycerol stock of *E. coli* DH5α were streaked onto LB agar plate and incubated overnight at 37°C in incubator. Fifty milliliter of LB broth was inoculated from overnight grown culture in a 250 ml flask and incubated in a shaker incubator at 37°C until OD at 600 nm reached 0.25-0.35. The bacterial cells were pelleted at 8000 rpm for 10 minutes at 4°C. The pellet was resuspended in $1/10^{th}$ volume of TSS and kept on ice for 1 hour. The competent cells were then dispensed in aliquots of 200 µl into 2 ml microfuge tubes and stored at -80°C until further use. The ligation reaction mixtures were added to 200 µl of *E. coli* competent cells. The bacterial cells were gently mixed with the DNA and kept on ice for 60 min. After incubation, heat shock was given at 42°C for 45 seconds and the cells were again incubated on ice for 5 minutes. 600 µl of SOC medium was

added to each tube and the tubes were kept in orbital shaker incubator at 37°C for 2 h. Transformed bacteria were plated on LB agar plates containing 100µg/ml ampicillin and incubated at 37°C overnight. When bacterial colonies were visible on plates, the colonies were cultured in LB broth and plasmids were isolated using alkaline lysis method as mentioned before.

3.1.5 Screening and Characterization of recombinant plasmids pET32bΔF1, pET32bΔF2, pET32bΔH1, pET32bΔH2 and pET32bΔM

3.1.5.1 Confirmation of recombinant colonies by PCR amplification

Colonies were screened for recombinants plasmids, pET32bΔF1, pET32bΔF2, pET32bΔH1, pET32bΔH2 and pET32bΔM by colony PCR (Gupta *et al*., 2002). Few colonies from each plate were picked and dissolved in 5 µl of NFW. 0.5 µl of cell suspensions were used as source of template DNA for PCR amplification. PCR was carried out using gene specific primers as described previously. After amplification, 5 µl of PCR product was subjected to agarose gel electrophoreses along with DNA molecular weight marker. The analysis of PCR product was done in 1.5% agarose gel at 50 volts for 1-2 hours. The colonies which gave amplicons of expected size were considered as recombinant. The recombinant colonies were selected for all 5 target genes. The colonies were revived overnight in LB broth containing 100µg/ml ampicillin and from this culture plasmid DNA was isolated using alkaline lysis method (Sambrook and Russel, 2001). The plasmids were analyzed for PCR amplification of target genes using gene specific forward and reverse primers. The PCR was carried out at standardized conditions optimized earlier for annealing temperatures. After amplification, 10µl of PCR products were analyzed along with DNA molecular weight marker for amplicons of expected size on agarose gel electrophoresis. The plasmids which gave amplicons of expected sizes were considered as recombinant for the target genes.

3.1.5.2 Confirmation of recombinant plasmids by RE digestion

The plasmids isolated from PCR positive colonies were characterized by restriction digestion using suitable restriction enzymes. *BamH*I and *Hind*III enzymes were used for the release of insert from the recombinant pET32b-ΔF1, pET32b-ΔF2, pET32b-ΔH1, pET32b-ΔH2 and pET32b-ΔM clones.

The reaction mixtures for RE digestion were prepared as follows:

Table 11: RE digestion of recombinant pET32b plasmids with *BamH*I and *Hind*III restriction endonucleases

Reaction components	**Quantity (µl)**
Plasmid DNA	15
10X buffer	2
*Hind*III	1
*BamH*I	1
NFW	1
Total	**20**

The reaction was set up in a microfuge tube and incubated at 37℃ for 4h. The digested products were analyzed by agarose gel electrophoresis.

3.1.6 Transformation of recombinant plasmids in expression strain of *E. coli* strain BL21 DE3 and optimization of expressing conditions

3.1.6.1 Transformation of recombinant plasmids in expression strain of *E. coli*-BL21DE3

Recombinant plasmids namely pET32b-ΔF1, pET32b-ΔF2, pET32b-ΔH1, pET32b-ΔH2 and pET32b-ΔM were transformed in E. *coli* BL21 DE3 competent cells. The transformed cells were plated on LB agar plate containing 100ug/ml ampicillin and the plates were incubated at 37^0 C overnight.

3.1.6.2 Optimization of conditions for expression of target proteins

Conditions such as induction temperature, concentration of IPTG, induction time etc., were optimized for expression of recombinant proteins. General procedures for optimization of expression, confirmation of protein expression and large scale purification of proteins were similar for all the proteins. All the recombinant plasmids in the expression strain of *E. coli* were cultured in LB broth containing 100 µg/ml of ampicillin at 37^0C overnight. Subsequently 50 µl of those were inoculated in 50 ml LB broth and incubated for 2-3 hrs at 37^0C till an OD of 0.4-0.5 has eached. Once the cultures attained the desired OD 10 ml of each culture was

aliquoted in different tubes. One of each culture was kept uninduced and and the rest 4 cultures were induced by adding 0.5mM, .08mM, 1mM and 1.2mM of IPTG. All the cultures were inoculated for 6 h in orbital shaker incubator at 37^0C and 180 rpm shaking. At the end of the incubation, the cultures were pelleted at 8000 rpm for 10 min at 4^0C in refrigerated centrifuge.

3.1.6.3 Characterization of recombinant proteins by SDS PAGE analysis

The pelleted cultures were subjected to sonication under denaturing conditions. For this 8M urea solution was used for lysing the cells along with lysozyme at the concentration of 10 mg/ ml of culture. The lysate was incubated in an ice box for 1 h and then sonicated with 6 pulses of 12 seconds each. The sonicated products were centrifuged in refrigerated microcentrifuge at 13000 rpm for 30 min. The supernatant was collected and 20 µl of induced and uninduced cell supernatants were analyzed by SDS PAGE for expression of recombinant protein along with SDS PAGE unstained protein marker (Biorad, USA). The gel was run initially at 60 volts in stacking gel and 80 volts in resolving gel for 2-3hrs. The gel was stained with coomassie brilliant blue (Sigma-Aldrich, USA) for 30 min and then destained using gel destaining solution.

3.1.7 Confirmation of recombinant proteins by western/ dot blot

The expression of recombinant protein was further confirmed by dot blot/western blot. In dot blot 10 µl of each proteins were coated on a nitrocellulose membrane of 0.45 µ pore size and air dried. Then membrane was kept for blocking in 5% skimmed milk powder (SMP) in PBS overnight after complete drying. Next day, membrane was washed 2 times in 0.05% PBST and 1: 10 diluted PPRV positive sera was added to the blot. The dilution was made in 2.5 % SMP in PBST. Membrane in diluted serum was incubated at room temperature (RT) for 1 h on end to end rocking. Subsequently the membrane was washed in PBST thrice and anti-goat HRPO conjugate at the dilution of 1:4000 was added to the membrane. Thereafter membrane was incubated at (RT) for 1 h on shaker followed by washing in PBST 4 times. Then membrane was incubated with chromogenic substrate, 3,3'-Diaminobenzidine (DAB) solution in 100mM Tris buffer and 1µl/ml of H_2O_2 was added. The membrane was observed for colour development. The reaction was stopped immediately once the color was developed by washing in running water. Western blot analysis for characterization of recombinant protein was done by reacting with using Ni-NTA conjugate. In western blot recombinant proteins were first resolved by SDS PAGE along with a low range prestained marker (BioRad, USA). Then the proteins along with marker bands were transferred to a nitrocellulose membrane of 0.45 µ

size by placing the gel over the nitrocellulose and supporting by stacks of filter paper over and below nitrocellulose membrane and gel assembly. This assembled stack after equilibrating in transfer buffer was placed on a cassette provided with the western blot apparatus (Atto, Japan) and placed on the transfer apparatus with membrane oriented towards anode and gel facing towards cathode. The apparatus was connected to power pack and run at 400 mA for 1 h. After transfer membrane was blocked in 5% SMP. After blocking, membrane was washed 3 times in PBST and kept for incubation of 1 h at RT in Ni-NTA conjugate at the dilution of 1:4000. Once incubation was completed, membrane was washed and chromogen substrate DAB was added the and incubated for color development. Reaction was stopped immediately in running water.

3.1.8 Large scale expression and purification of recombinant proteins

The colonies which showed highest expression of recombinant proteins were revived by overnight culture in LB broth. Next day 500 µl of overnight culture was inoculated in 100 ml LB broth and the culture was incubated for 2-3 hrs till the desired OD of 0.5 was achieved. The culture was induced by adding IPTG concentration that was optimized earlier for different recombinant proteins. The culture was pelleted after about 6 h incubation at 37^0C. The culture pellet was dissolved in lysis buffer containing 8M Urea and 40µl of 10mg/ml lysozyme. The lysate was kept in an ice box for 1 hr. Thereafter samples were sonicated as described earlier. The sonicated samples were centrifuged in a refrigerated microcentrifuge at 4 ℃ for 30 min at 13,000 rpm. The supernatants were loaded onto column packed with 500 µl of Ni-NTA agarose (Qiagen) and incubated for 2h in rocker in refrigerated conditions. After 2 h it was washed 5 times with wash buffer (pH-6.3) and finally the recombinant protein was eluted in elution buffer of pH 4.5. Ten elutes of 500ul each were collected and were further analyzed on SDS PAGE. ΔN proteins has been already cloned and expressed in the GEV lab. ΔN protein was upscaled and expressed as per the conditions previously optimized. Since ΔN was a soluble protein, it was purified under native conditions. The culture pellet was resuspended in 10 mM imidazole buffer (pH 8.0) and loaded onto Ni-NTA column for 2 h. Then washed the column with 50 mM imidazole buffer (pH 8.0) and protein was eluted by 500 mM of imidazole buffer (pH 8.0).

3.1.9 Assessing the suitability of the recombinant antigens in detecting PPRV antibodies by indirect ELISA

Indirect ELISA was standardized for detection of antibodies to PPRV antigens. Indirect ELISA was optimized for the amount of antigen coated per well. For this ΔF1, ΔF2, ΔH1, ΔH2 and ΔM were coated in 5 plates as follows.

			Antigen concentration →											
			1	2	3	4	5	6	7	8	9	10	11	12
			500 ng	400 ng	300 ng	200 ng	100 ng	50 ng	500 ng	400 ng	300 ng	200 ng	100 ng	50 ng
Positive serum	A	1:50	„	„	„	„	„	„	„	„	„	„	„	„
	B	1:100	„	„	„	„	„	„	„	„	„	„	„	„
	C	1:200	„	„	„	„	„	„	„	„	„	„	„	„
	D	1:400	„	„	„	„	„	„	„	„	„	„	„	„
Negative serum	E	1:50	„	„	„	„	„	„	„	„	„	„	„	„
	F	1:100	„	„	„	„	„	„	„	„	„	„	„	„
	G	1:200	„	„	„	„	„	„	„	„	„	„	„	„
	H	1:400	„	„	„	„	„	„	„	„	„	„	„	„

The plates were kept overnight at 4 °C. Next day after two washings with PBST, blocking was done at 37^0 C with 5% SMP for 2 h. The plates were then washed thrice with PBST. PPRV positive and negative serum was diluted in 2.5% SMP. 100 µl of positive serum after dilution was added as in the format in the wells from A-D and 100 µl of PPRV negative serum in the wells designated E-H. The plate was incubated for 1 hr at 37°C and then washed four times with PBST. Then 100µl of 1:15,000 diluted anti-goat HRPO conjugate (Sigma) was added to the plates and plates were incubated further at 37°C for 1hr. The dilution was made in 2.5% SMP. The ELISA plates were washed3+- 5 times with PBST and 100 µl of OPD substrate in phosphate citrate buffer was added. The plates were kept in dark and observed for colour development. The reaction was stopped by adding 100 µl of 1M H_2SO_4 and OD was taken at 492 nm. After optimizing the antigen, serum samples were screened.

3.1.10 Dialysis and refolding of recombinant proteins under native conditions

The recombinant proteins were refolded back through process of dialysis in dialysis membrane (Snake skin, Thermo Scientific). Buffer exchange was done with decreasing molarity of urea from 8 molar to 2 molar and then two changes of PBS (pH 7.4) were done. After dialysis recombinant protein was analyzed for integrity and concentration on SDS PAGE. The concentration of protein was measured using Qubit fluorometer (Life Technologies, USA). The recombinant proteins were stored at -80℃ till further use.

3.2 Optimization of the conditions for coating of recombinant antigens on suitable surface(s) to be used for detecting the PPRV specific antibodies

3.2.1 Selection of surface(s) for fabricating recombinant proteins

Glass beads (Sigma) of diameter 425-600 µm was selected for covalent coupling of recombinant proteins.

3.2.2 Cleaning glass beads surface for making it free off organic residues

Glass beads were washed in piranha solution (H_2SO_4 and H_2O_2 in 3:1 ratio). Weighed 500 mg of glass beads in an empty glass vial and added 3 ml of 30% H_2O_2 and 9 ml of concentrated H_2SO_4 drop by drop in fumehood carefully and incubated for 30 min. The beads were agitated occasionally. The piranha solution was carefully discarded in plenty of running water. The glass beads were washed in distilled water twice and transferred to a new vial for further washing. Then the glass beads were subjected to alternate washing in DW (distilled water) and absolute ethanol. After a final washing in ethanol the beads were dried in a vacuum desiccator overnight.

3.2.3 Activating the surface for coupling the recombinant proteins using appropriate agents

3.2.3.1 APTS (Aminopropyl triethoxy silane) fabrication of glass beads

APTS (Thermo scientific) was used to make the glass surfaces amine terminated. 10% v/v of APTS in absolute ethanol was prepared by taking 150 µl of APTS in 1350 µl of ethanol. This APTS solution was added to 250 mg of dried glass beads in an eppendorf. The eppendorf after properly protected from light was kept on end to end rocking for 10 min at RT. The beads

were washed after incubation in ethanolic APTS solution successively with DW and ethanol three times and cleaned in an ultrasonic bath for 10 min. Beads were dried in a desiccator after giving a final washing in ethanol. Amine terminated beads were further processed with gluteraldehyde for making it aldehyde terminated surfaces. For gluteraldehyde activation dried beads were washed with PBS thrice and then added 10% v/v gluteraldehyde solution in PBS by taking 100 µl of gluterldehyde (Sigma) and 900 µl of PBS. This was kept for overnight shaking. Discarded the supernatant once the incubation was over and washed in PBS thrice. The beads were kept in PBS until further processed.

3.2.3.2 MPTS (Mercaptopropyl triethoxysilane) fabrication of glass beads

MPTS functionalization was done to make thiol terminated glass surfaces. 2% of MPTS (Sigma) solution was made in absolute ethanol by taking 30 µl of MPTS and 1470 µl of ethanol. This MPTS solution was added to 250 mg of dried glass beads and incubated for 2 h in end to end rocking. Care was taken to protect MPTS solution from light. After 2 h the beads were washed alternatively in ethanol and water thrice. Beads were cleaned in an ultrasonic bath for 5 min and washed in ethanol. Finally the beads were dried in a desiccator. Thereafter beads were treated with MUA (11-mercaptoundecanoic acid) overnight to incorporate –COOH groups on glass surface. The beads were washed alternatively with ethanol and DW to remove excess MUA and finally dried.

3.2.4 Optimizing the conditions for coupling the recombinant antigen on the modified glass beads' surface

3.2.4.1 Covalent coupling of recombinant proteins in APTS functionalized beads

Gluteraldehyde treated beads were mixed with 500 µl of recombinant ΔN protein dialysed in PBS. Before coupling the protein was concentrated to have a concentration of 500 µg/ml by keeping the protein in a refrigerated concentrator (Labconco. USA). The gluteraldehyde treated beads were incubated in concentrated protein solution under refrigerated conditions for 5 h on end to end rocking. Following the incubation the supernatant was collected and the beads were washed in PBS thrice. Then the beads were treated with 1ml of 1M ethanolamine for 30 min to quench unreacted groups. The beads were washed in PBS thrice after quenching and kept in PBS until further used at 4^0C.

3.2.4.2 Covalent coupling of recombinant ΔN protein in MPTS functionalized beads

Following MUA incubation MPTS functionalized beads were washed alternatively in water and ethanol for 3 times. Then the beads were equilibriated in 10 mM sodium acetate buffer by washing the beads using sodium acetate buffer thrice. 100 mM NHS (N-hydroxy succinimide) and prepared 400 mM of EDC was prepared. A 1:1 EDC: NHS solution was prepared and added to beads after decanting sodium acetate buffer and kept for incubation at RT for 10 min with proper mixing. 100 µg/ml concentration of ΔN protein was prepared in sodium acetate buffer and kept at 4^0C for 10 min to ensure the equilibration of PBS dialyzed protein in sodium acetate buffer. The beads were washed in sodium acetate buffer thrice at the earliest after incubation in EDC: NHS solution. 500 µl of ΔN protein in sodium acetate buffer was added to the beads and incubated at RT for 25 min with end to end rocking. Once the incubation is finished, collected supernatant and the beads were washed in sodium acetate buffer thrice followed by quenching in 1M ethanolamine as mentioned in APTS functionalized beads. The beads were washed twice in sodium acetate buffer and then in PBS and kept in PBS at 4^0C until further processed. 25 µg of ΔN, ΔH1, ΔH2, ΔF1, ΔF2 and ΔM proteins were added to 500 µl of sodium acetate buffer. This cocktail of proteins was used for coating 100mg of MPTS functionalized beads.

3.2.5 Assessing the efficacy with which the antigen bound to the surface

3.2.5.1 Measuring the concentration of protein before and after coupling on the beads

Concentration of protein in sodium acetate buffer was measured before and after coupling on the beads using Qubit fluorometer to determine the amount of protein bound to each bead.

3.2.5.2 Assessing the reactivity of PPRV positive serum and negative serum to protein coupled on beads

Assessing the reactivity of PPRV positive serum and negative serum to protein coupled on beads by colorimetric assay

5 beads were aliquoted in two vials in duplicates. In two vials added 100 µl of a known PPR positive sera and in other two vials known negative sera was added at the dilution of 1: 50

prepared in 1% BSA (Bovine Serum Albumin). Beads were incubated in diluted serum at RT on a vortex for 1h. after the incubation ,beads were washed in PBST. The beads were shifted to new vials and added 100 µl of anti-goat HRPO conjugate at the dilution of 1:5000 prepared in 1% BSA and incubated for 1 h on a vortex to ensure proper mixing. After incubation, the beads were washed in PBST thrice and shifted the beads to new eppendorfs. Added 50 µl of colored chromogen substrate OPD prepared in PCB and added with 0.4 µl/ml of H_2O_2 to the beads and waited for the color development. Once the color started developing, supernatant was taken out and 1M H_2SO_4 was added. The reactivity was measured by taking OD at 492 nm.

Assessing the reactivity of PPRV positive serum and negative serum to protein coupled on beads by fluorometric assay

The reactivity of antigen coated beads to the serum was also analyzed using anti-goat FITC labeled antibodies. In this assay, 50 µl of 1: 10 diluted positive and negative serum was added to the eppendorf containing 10 ΔN protein coupled beads in duplicates. All eppendorfs were incubated at RT for 1h on a vortex. After that washed thrice in PBST and then added 50 µl of 1:1000 diluted anti-goat FITC antibody (Sigma) prepared in 1% BSA to the beads. Incubated for 1h and then washed in PBST thrice and immediately viewed the beads kept on a slide in fluorescence microcope (Olymbus, Bx 53, USA). Other recombinant proteins- ΔF1, ΔF2, ΔH1, ΔH2 and Δ M were also coupled individually to the beads using MPTS chemistry, since it found to be more reactive and satisfactory than proteins coupled by APTS chemistry.

3.3 Comparative evaluation of developed method(s) with existing diagnostic methods to determine diagnostic effectiveness parameters.

3.3.1 Optimizing the conditions like antibody dilutions, blocking agents etc to be adopted for using the recombinant antigen coated glass beads surface to detect PPRV specific antibodies.

3.3.1.1 Optimizing conditions for colorimetric assay

For optimization of serum dilutions, 10 beads were aliquoted in different 0.6ml microfuge and positive and negative serum was added at different dilutions starting from 1:10 to 1:100. Thereafter the immunoassay was performed as described earlier. The anti-goat HRPO conjugate was used at 1:5000 dilution.

Next step was optimizing anti-goat HRPO conjugate. For this, 10 ΔN coated beads were dispensed in 6 microfuge tubes. To three microfuge added positive serum at the dilution of 1: 50 and next three negative sera at the same dilutions. To first positive-negative set added anti-goat HRPO conjugate at the dilution of 1:5000, next set added with 1:7500 and last with 1:10000 dilution of conjugate. Other steps were carried out as mentioned previously.

Number of beads that should be taken per microfuge was also optimized by taking 5 beads in one eppendorf, 10 beads in other, 15, 20, 25, 30, 35, 40, 45 beads per tube after properly labelling. Positive serum was added to these 9 eppendorfs and negative sera to the next set of 9 eppendorfs similarly aliquoted. Further steps were similar as mentioned previously. 10 beads were dispensed in 16 microfuge tubes and colorimetric assay was performed with hyperimmune and preimmune serum (raised in rabbit against ΔN recombinant protein) at the dilutions of 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800. Anti-rabbit HRPO conjugate dilution was 1:5000.

3.3.1.2 Optimizing the conditions for fluorometric assay with hyperimmune and preimmune serum

10 beads were aliquoted on 16 microfuge tubes and fluorometric (FLISA-Fluorescent Linked Immunosorbent assay) assay was performed with hyperimmune and preimmune serum (raised in rabbit against ΔN recombinant protein) at the dilutions of 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800. Anti-rabbit FITC labeled antibody at a dilution of 1:1000 was used in the assay. The fluorescent intensity was calculated using Imagej software and plotted as average intensity of several beads.

Based on this optimization results, serum samples were screened by FLISA with ΔN and cocktail of protein coated beads. Fluorometric assay was done using ΔF1, ΔF2, ΔH1, ΔH2 ΔM, ΔN and cocktail of protein coated beads to compare the assay reactivity.

3.3.2 cELISA of the serum samples to ensure the efficacy of the glass beads assay

Competitive ELISA (cELISA) was done to compare the results of developed assays since it is recommended as diagnostic method for detecting PPRV antibodies by OIE. The cELISA kit was obtained from IVRI, Mukteswar. The procedure of cELISA is as follows:

Lyophilized PPRV antigen was reconstituted in 1ml of DW. This reconstituted stock antigen

was diluted in a sterile 50 ml tube at the rate of 1:100 in PBS. For 1 plate 60 µl of stock antigen was diluted in 6 ml of PBS. 50 µl of this diluted antigen was added to all the wells of a 96 well ELISA plate ((NuncMaxisorp, Denmark)). Plates were incubated for 1 h at 37^0C with occasional mixing. After the incubation, discarded the antigen and washed three times using wash buffer which is prepared by diluting the PBS 4 times with DW. Blocking buffer was prepared in 20 ml of PBS to which 20 µl of tween-20 (T-20) and 40 µl of negative serum was added. Negative serum was also supplied in lyophilized form which has to be reconstituted in 1 ml of DW. Monoclonal antibody (MAb) provided in the kit was reconstituted in 1 ml of sterile DW. Strong positive serum, weak positive serum and negative serum were also reconstituted in 1 ml of sterile DW. Following reagents were added carefully to the plates in the order mentioned.

- 40 µl of blocking buffer added in all the wells
- 20 µl of additional blocking buffer was added to monoclonal antibody control (Cm) wells.
- 60 µl of additional blocking buffer to each of the conjugate control (Cc) wells.
- 20 µl of test serum sample was added per well in a set of two wells using separate tip for each sample.
- 20 µl of strong positive serum control (C++) was added in each of the four designated wells in the plate.
- 20 µl of weak positive serum control (C+) added in each of the four designated wells in the plate.
- 20 µl of negative serum control (C-) added in each of the two designated wells in the plate.

40 µl of diluted MAb in each well of the plate except the conjugate control wells (Cc) was added. MAb was diluted in blocking buffer. 50 µl of MAb was diluted in 5 ml of blocking buffer for 1 plate.

Mixed the contents in the well and incubated at 37^0C for 1 h. Following the incubation discarded the plates and washed thrice with wash buffer. 50 µl of diluted anti-mouse conjugate was added in all the wells. For dilution of the anti-mouse conjugate 6 µl of conjugate was diluted in 6 ml of blocking buffer. The plate was incubated at 37^0C for 1 h. Plate was washed thrice with the wash buffer after incubation. Chromogen substrate OPD was prepared using the

OPD tablet provided in the kit . One tablet was dissolved in 75 ml of DW and stored as 12 ml aliquots in 15 ml tubes at -20^{0}C. 50 µl of OPD was added per well after the addition of 4 µl/ml of the H_2O_2. The plates were incubated for 20 min at 37^{0}C for 20 min after which the reaction was stopped by adding 50 µl of 1M H_2SO_4. Reading was taken in multifunction plate reader (Enspire multimode plate reader, Perkin Elmer, USA) at 492 nm. The reading was analyzed using PI (Percent Inhibition) which was calculated as per the following formula.

PI = 100 - {(OD of test sample/ OD of Cm) x 100}

The format of the plate is as follows:

Control set		Test samples									
Cc	Cc	1	5	9	13	17	21	25	29	33	37
C++	C++	1	5	9	13	17	21	25	29	33	37
C++	C++	2	6	10	14	18	22	26	30	34	38
C+	C+	2	6	10	14	18	22	26	30	34	38
C+	C+	3	7	11	15	19	23	27	31	35	39
Cm	Cm	3	7	11	15	19	23	27	31	35	39
Cm	Cm	4	8	12	16	20	24	28	32	36	40
C-	C-	4	8	12	16	20	24	28	32	36	40

3.3.3 Statistical analysis of cELISA results, fluorometric and colorimetric assay results

The result of cELISA was compared with the glass beads' assay done colorimetrically and fluorometrically to arrive at the efficacy of these assays. ROC curve, area under curve (AUC), as well as, sensitivity and specificity of the assay were determined with MedCalc 9.2 software. The method of DeLong *et al*. (1988) was used for calculation of standard error of the AUC and an exact binomial confidence interval for the AUC was calculated. Statistical analysis of assay data was done using Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA) software.

Results

4 RESULTS

4.1. Amplification of Immunodominant Regions of F, H and M Genes

Immunodominant regions in different genes of PPRV (ΔF1, ΔF2, ΔH1, ΔH2 and ΔM) were amplified after standardizing the annealing temperature for each primer set using gradient PCR as per the procedure mentioned. These PCR products were run in 1.5% agarose gel along with 100 bp ladder. Based on the agarose gel result, as shown in the **Fig. 1a-1e,** the annealing temperatures were optimized. The optimized annealing temperatures and the amplicon size are given in the table 12.

Table 12. Amplicon size obtained for different primer sets at optimized annealing temperatures

Sl. No.	Immunodominant regions	Annealing temperature (^{0}C)	Amplicon Size (bp)
1	ΔF1	60	366
2	ΔF2	52	644
3	ΔH1	60	600
4	ΔH2	52	810
5	ΔM	60	503

4.2. Cloning of immunodominant regions-ΔF1, ΔF2, ΔH1, ΔH2 and ΔM in pET32b vector and confirmation by plasmid PCR and RE digestion

Selected regions ΔF1, ΔF2, ΔH1, ΔH2 and ΔM were amplified in 50 µl of reaction in

PCR. These PCR products were used for RE enzyme digestion using *BamH*1 and *Hind*III and ligation into *BamH*1 and *Hind*III digested pET32b vector. The recombinant plasmids were transformed into DH5α competent cells using the protocol mentioned previously. Transformed competent cells were plated on LB agar containing 100 μg/ml of ampicillin and incubated overnight. The colonies formed on the next day were analyzed by plasmid PCR and RE digestion by *BamH*1 and *Hind*III after isolating the plasmid by alkaline lysis method. PCR products were resolved in 1.5% agarose gel with 100 bp ladder **(Fig. 2a-2e)**. Bands were obtained in the gel at the place of expected size compared to DNA ladder. The RE digested products were resolved in 1% agarose gel with 1kb ladder **(Fig. 3a-3e)**. Size of the digested products obtained was same as the size of the insert in RE digestion in comparison with DNA ladder. Table 13 shows the size of the PCR product obtained in plasmid PCR and the size of digested product obtained in RE digestion. The positive colonies were cultured for plasmid isolation for transformation into BL21 DE3 competent cells. Transformed competent cells were plated on LB agar and incubated overnight.

Table 13: Size of gene products obtained in plasmid PCR and RE digestion

Recombinant Clone	Size of PCR product (bp)	Size of digested product (bp)
ΔF1	366	366
ΔF2	644	644
ΔH1	600	600
ΔH2	810	810
ΔM	503	503

4.3. Expression and Purification of Recombinant Proteins after Optimizing the IPTG Concentration, Induction Time and Temperature.

Colonies from the LB agar plate were cultured in LB broth for analyzing the expression of recombinant proteins. The proteins were expressed using varying concentration of IPTG at 37^0C for 6.5 h. The cultures were analyzed after lysing and sonicating the culture in 8M urea buffer and running in SDS PAGE. Expression of various proteins **(Fig. 4a-4e)** was optimized as shown in table14. The thick bands were present in the resolved SDS PAGE gel corresponding to the expected size of fusion proteins compared to the uninduced processed culture ran along.

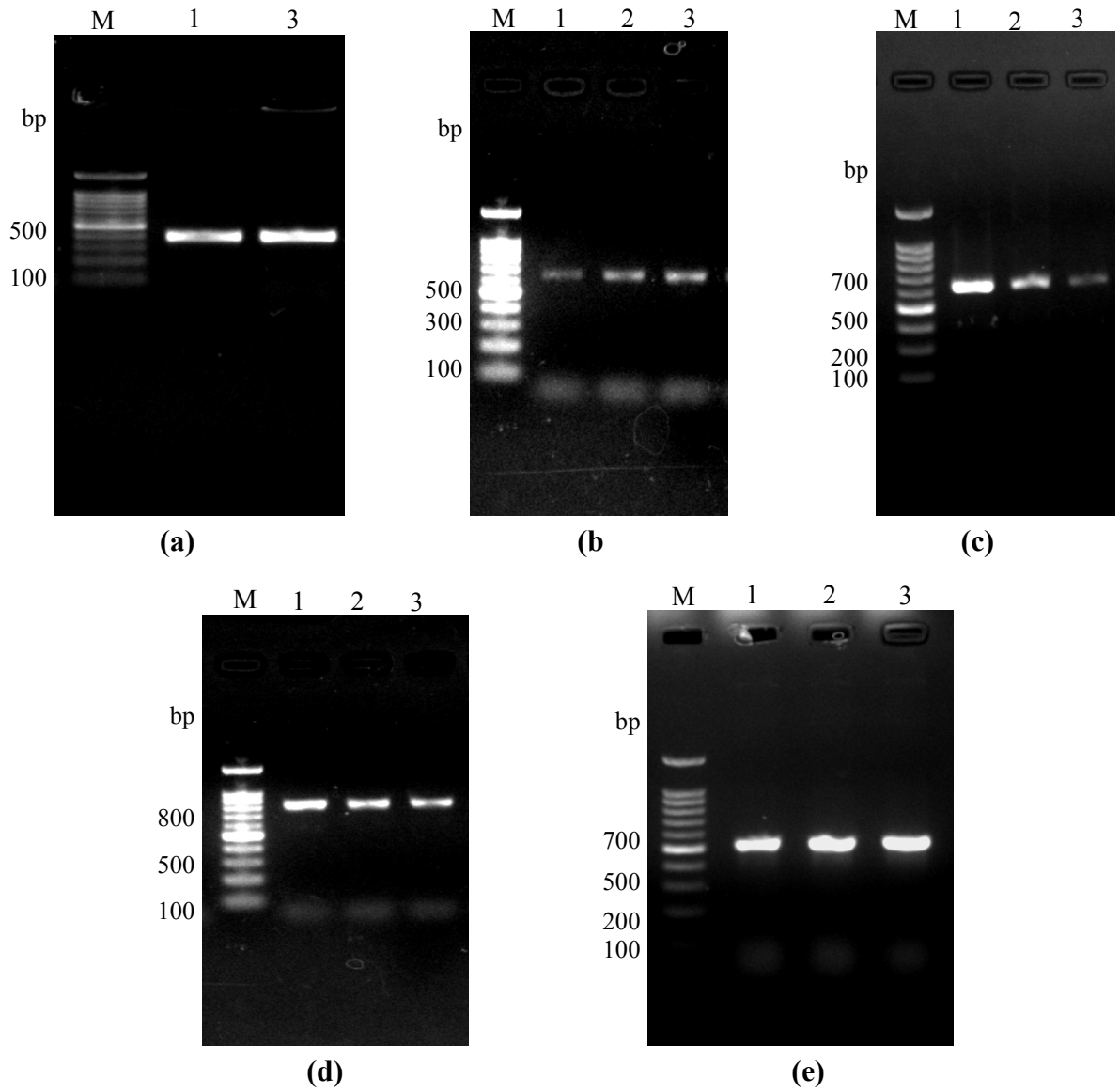


Fig. 1: Agarose gel electrophoresis: The selected immunodominant regions in different genes of PPRV (F, H and M) were amplified by PCR using specific primers and amplified PCR products were resolved in 1.5 % agarose gel and visualized under UV light after addition of EtBr. Shown in the figures above are the amplification of different target genes [**(a)** ΔF1; **(b)** ΔF2; **(c)** ΔH1; **(d)** ΔH2; **(e)** ΔM]

Lane M: 100 bp DNA ladder (Fermentas, USA)

Lane 1 –3: PCR amplified products

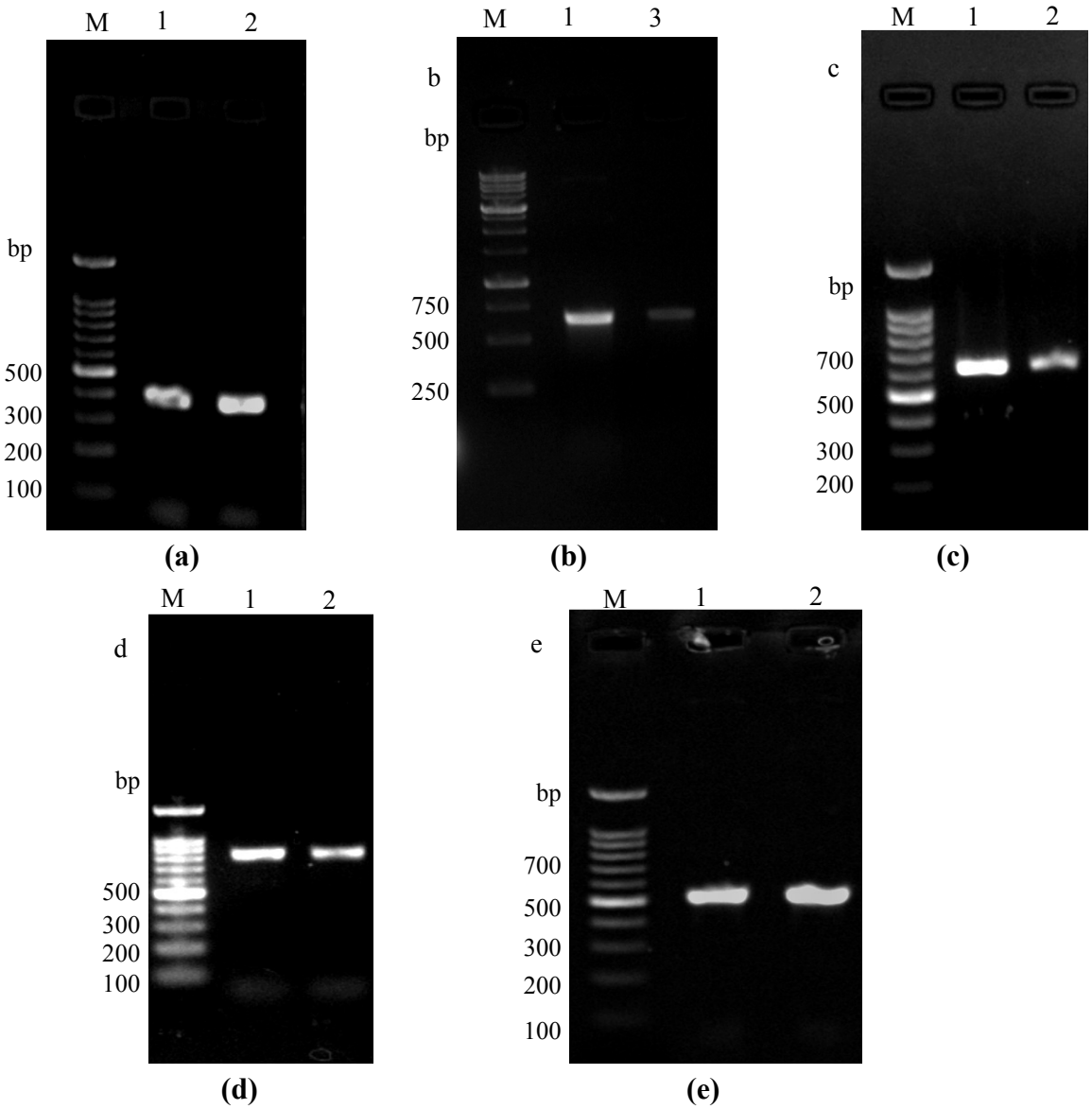


Fig. 2: Confirmation of recombinant clones by plasmid PCR: Immunodominant regions in F, H and M gene of PPR virus were cloned in pET32b vector confirmed by plasmid PCR. The PCR products were resolved in 1.5 % agarose gel containing 0.5 µg/ml EtBr, to confirm size of recombinant clone. Resolved PCR products [**(a)** ΔF1; **(b)** ΔF2; **(c)** ΔH1; **(d)** ΔH2; **(e)** ΔM] are shown in the above figure

Lane M: 100 bp DNA ladder for (a), (c), (d), (e) (Fermentas, USA) and 1 kb DNA ladder for (b) (Promega USA); **Lane 1 and 2**: PCR product

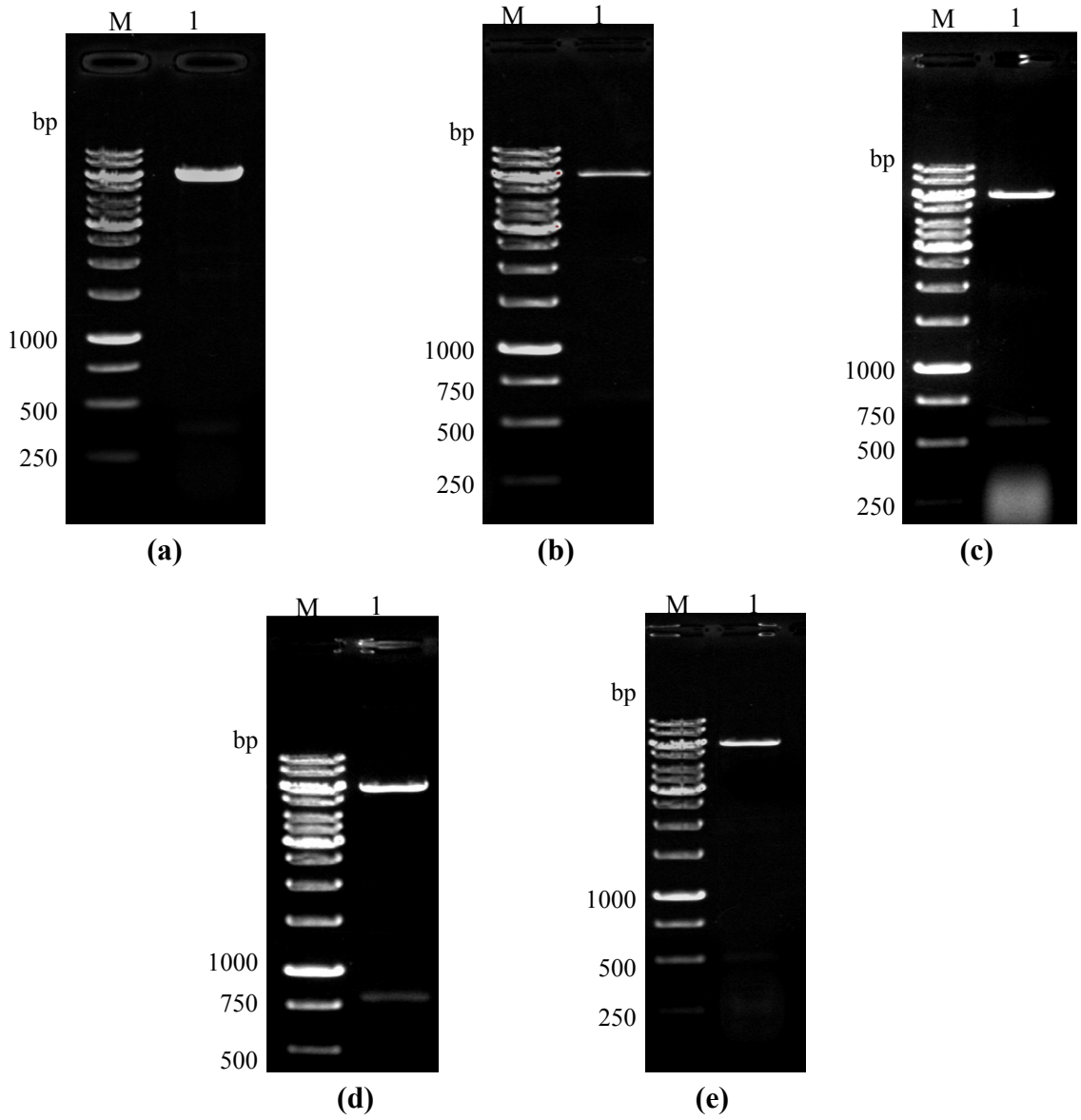


Fig. 3: Confirmation of cloned immunodominant regions by RE digestion: PPRV immunodominant regions [ΔF1, ΔF2, ΔH1, ΔH2 and ΔM] cloned in pET32b vector were digested using RE enzymes *BamH*1 and *Hind*III. The product obtained after RE digestion was resolved in 1 % agarose gel and documented. Shown in the figures above are the RE digestion of recombinant pET32b vector cloned with target genes [**(a)** ΔF1; **(b)** ΔF2; **(c)** ΔH1; **(d)** ΔH2; **(e)** ΔM]
Lane M: 1 kb Ladder (Promega, USA)
Lane 1: RE digested product

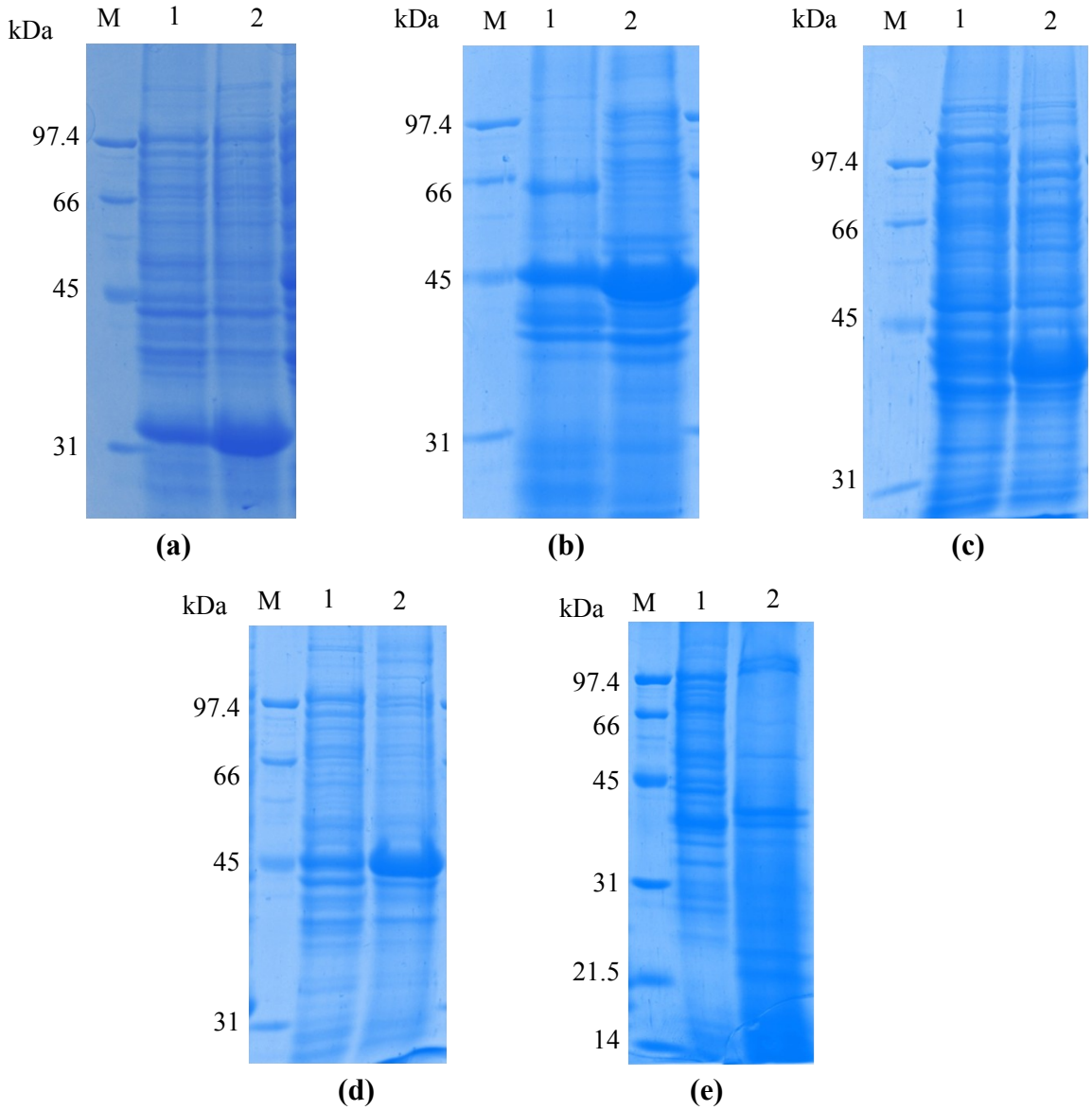


Fig. 4: SDS PAGE analysis of expressed recombinant proteins: Recombinant proteins expressed in prokaryotic expression system were induced by 1 mM IPTG at 37^0C. Induced and uninduced cultures were lysed in denaturing buffer and sonicated. Both induced and uninduced processed cultures were resolved in 12 % SDS PAGE along with unstained low range protein marker.

The above figures show the expression of recombinant proteins [**(a)** ΔF1; **(b)** ΔF2; **(c)** ΔH1; **(d)** ΔH2; **(e)** ΔM]

SDS PAGE gels showing figures (a), (b), (c) and (d)

Lane M: Unstained protein marker, Low range (Biorad, USA); **Lane 1**: Uninduced culture; **Lane 2**: Culture induced with 1 mM IPTG

SDS PAGE gel shown in Fig. 4e

Lane M: Unstained protein marker, Low range (Biorad, USA); **Lane 1**: Culture induced with 1 mM IPTG; **Lane 2**: Uninduced culture

Table 14: Optimized conditions for the expression of ΔF1, ΔF2, ΔH1, ΔH2 and ΔM proteins

Recombinant protein	IPTG concentration (mM)	Induction Time (h)	Induction Temperature (^{0}C)
ΔF1	1	6.5	37
ΔF2	1	6.5	37
ΔH1	1	6	37
ΔH2	1	6.5	37
ΔM	1	6	37

The cultures which showed better expressions were induced in 100 ml LB broth. The induced cultures were lysed and sonicated in 8M urea buffer and supernatant was collected after centrifuging as mentioned earlier. The supernatant was used to purify the protein using Ni-NTA agarose beads. The purified proteins were analyzed in SDSPAGE **(Fig. 5a-5e)**. Bands obtained in gel were of expected size of fusion protein as shown in table 15. Purified ΔN protein already cloned and expressed in the lab was also purified and run in SDS PAGE **(Fig. 6)**. Size of the protein obtained was 36 kDa in the gel.

Table 15: Size of purified recombinant protein obtained in SDS PAGE

Recombinant protein	Size of recombinant protein (kDa)
ΔF1	32.5
ΔF2	39.7
ΔH1	42.9
ΔH2	49
ΔM	38.6
ΔN	36

4.4. Confirmation of Recombinant Proteins by Western Blot and Dot Blot.

The recombinant proteins after running in SDS PAGE along with a prestained marker, were transferred to nitrocellulose membrane and probed for presence of His tag in recombinant protein using Ni-NTA conjugate **(Fig 7a-7e)**. The blot showed brown sharp bands on the nitrocellulose membrane of expected size compared to prestained marker confirming presence

of recombinant protein with His tag. A dot blot after blotting the proteins in nitrocellulose membrane as explained earlier was also used to test serological activity of purified recombinant proteins **(Fig. 8)**. On addition of chromagen substrate, dark brown circular dots at blotted place were obtained corresponding to each recombinant protein. Degree of darkness indicates degree of reactivity of protein to serum. The dot blot indicates that truncated N protein show more reactivity than other proteins as evident from darker circular spot.

4.5. Coating of Purified Recombinant Antigens on Glass Surfaces.

Glass beads after washing in piranha solution were functionalized using APTS and MPTS. Both APTS and MPTS functionalized beads were bound with ΔN recombinant proteins as per the protocol mentioned earlier **(Fig. 9 and 10)**. These beads were analyzed for the reactivity towards PPRV positive and negative serum by taking a representative 5 beads from each lot (APTS and MPTS functionalized lot). The reactivity was measured as OD at 492 nm after the completion of the assay. The result in table 16 and figure 11 shows the reactivity of ΔN coupled beads having APTS and MPTS SAM layers. ΔN fabricated on APTS SAM layer showed 0.27 ± 0.063 to positive serum and .098 to negative serum while ΔN fabricated on MPTS SAM layer showed 0.269 ± 0.032 to positive serum and 0.0705 ± 0.002 to negative serum. P/N ratio of ΔN fabricated on APTS SAM layer showed 2.76 and ΔN fabricated on MPTS SAM layer showed 3.81 indicating that protein coated on MPTS SAM is better than ΔN fabricated on APTS SAM layer.

Table 16: Comparison of assay reactivity of protein functionalized on APTS and MPTS SAM layers

ΔN on APTS SAM		**P/N Value**	**ΔN on MPTS SAM**		**P/N Value**
Positive	**Negative**		**Positive**	**Negative**	
0.226	0.098	2.36	0.246	0.069	3.56
0.315	0.098	3.21	0.292	0.072	4.05

4.6. Assessing the Binding Capacity of ΔN on MPTS fabricated beads.

200 µg of ΔN protein of concentration 1mg/ml was added to the 800 µl of coupling buffer, sodium acetate. This solution was added to the 500 mg of MPTS functionalized beads for coupling. After the incubation supernatant was collected and analyzed for the concentration of protein using Qubit fluorometer. The concentration of protein was 72.1 µg/ml. This indicates

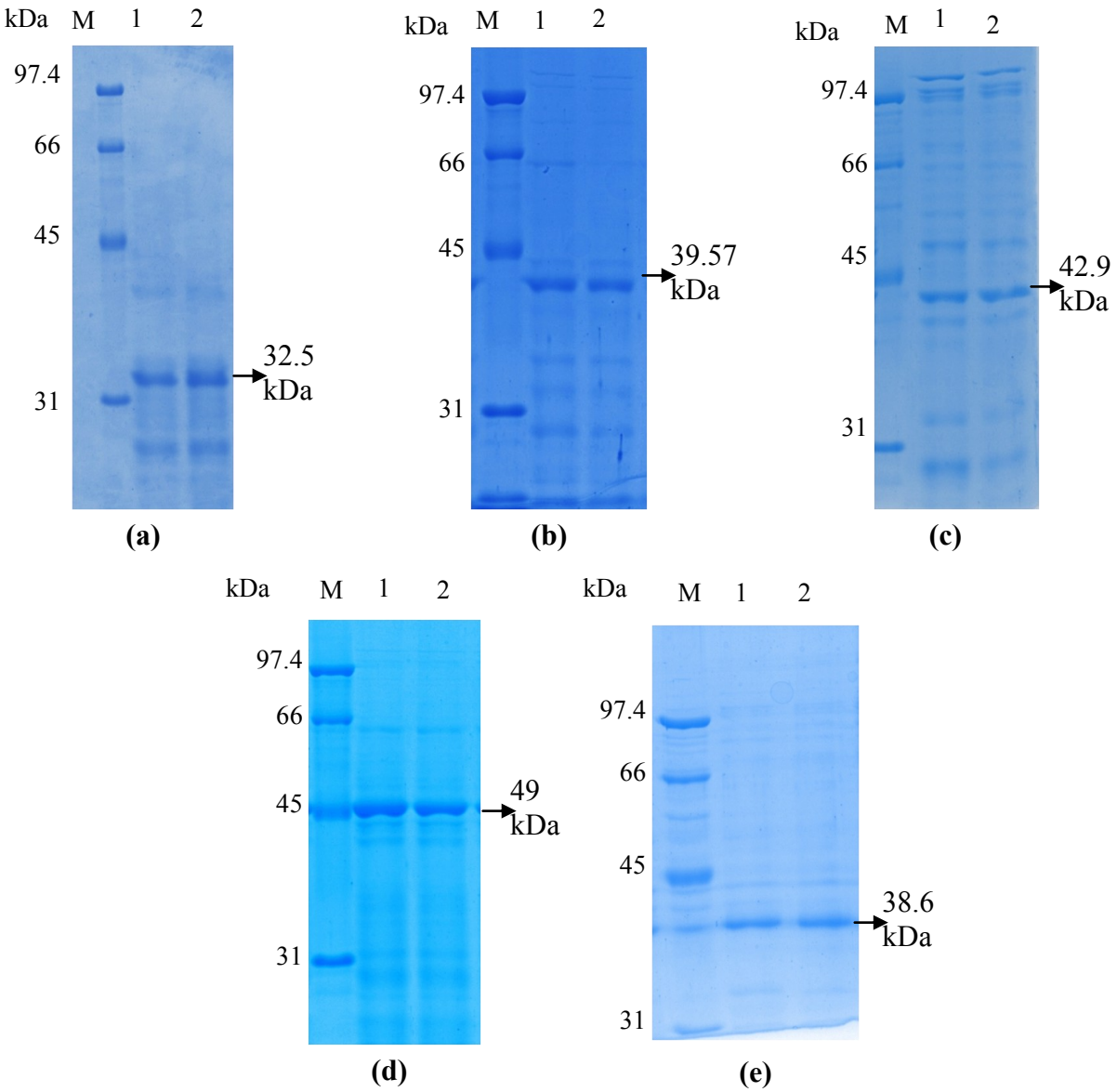


Fig. 5: SDS PAGE analysis of purified recombinant proteins: Recombinant proteins were expressed as His tagged proteins and purified under denaturing conditions using Ni-NTA affinity chromatography. These purified proteins were characterized for molecular mass by resolving in SDS PAGE along with protein marker. Figures given above shows the molecular sizes of purified fusion proteins [**(a)** ΔF1; **(b)** ΔF2; **(c)** ΔH1; **(d)** ΔH2; **(e)** ΔM]

Lane M: Unstained protein marker, Low range (Biorad, USA)

Lane 1 and 2: Purified proteins. The arrow indicates purified protein resolved on gel

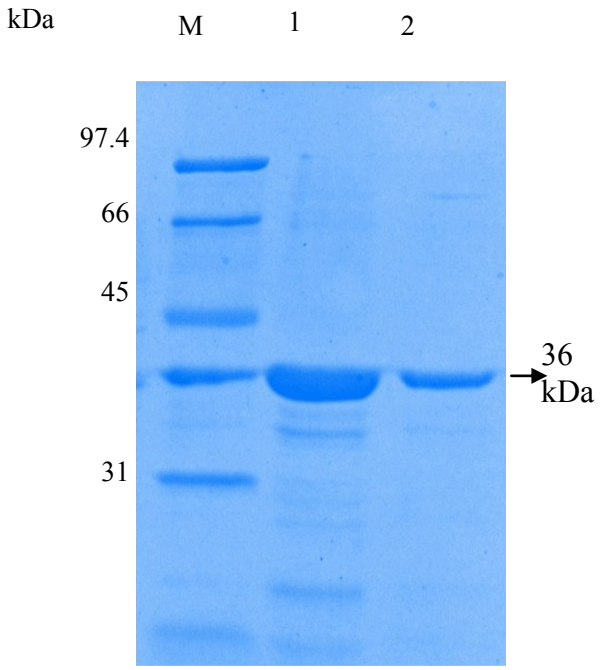


Fig. 6: SDS PAGE analysis of purified ΔN: Cloned and expressed ΔN –His tag fusion protein was purified in native condition using Ni-NTA affinity chromatography. To asses the purity of ΔN protein, the purified products were resolved in SDS PAGE
Lane M: Unstained protein marker, Low range (Biorad, USA)
Lane 1 and 2: Purified protein. The arrow indicates purified protein resolved on gel

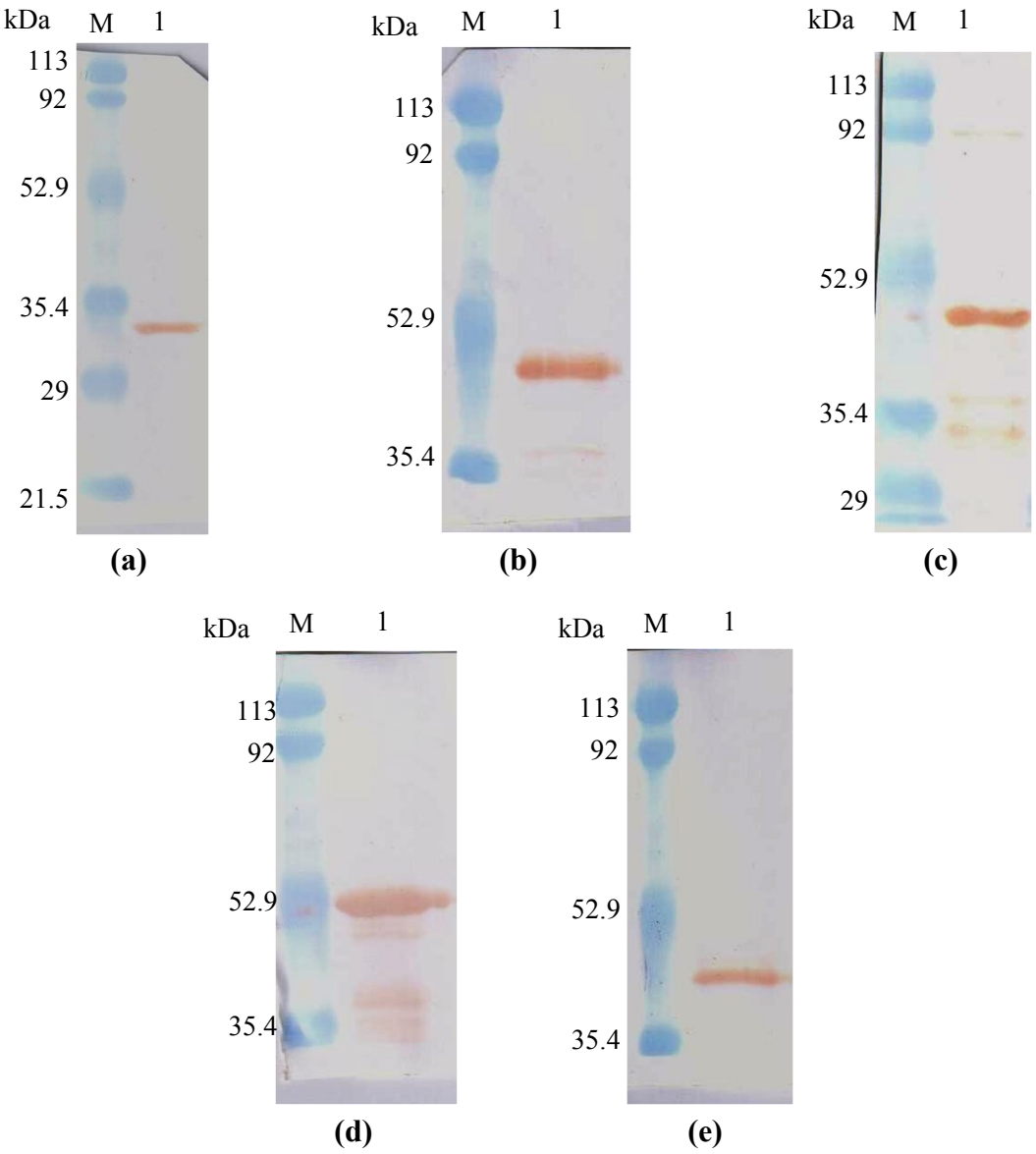


Fig. 7: Characterization of recombinant proteins by Western Blot using Ni-NTA conjugate: Recombinant proteins after resolving on SDS PAGE along with prestained protein marker were transferred to nitrocellulose membrane. Blotted nitrocellulose membrane was probed for the presence of His tag using Ni-NTA conjugate (Sigma, USA) at dilution of 1:4000. Shown above are the reactivity of purified recombinant proteins with Ni-NTA conjugate [**(a)** ΔF1; **(b)** ΔF2; **(c)** ΔH1; **(d)** ΔH2; **(e)** ΔM]
Lane M: Prestained marker, low range (Biorad, USA)
Lane 1: Reactivity of purified recombinant proteins with Ni-NTA conjugate

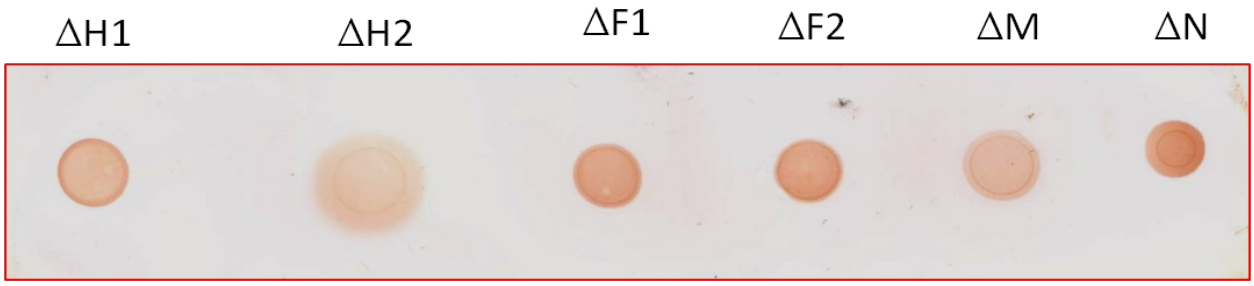


Fig. 8: Confirmation of reactivity of purified recombinant proteins by Dot blot: Purified proteins were dot blotted on nitrocellulose membrane and air dried. The membrane was processed for confirming the serological activity of recombinant proteins using pooled PPRV positive goat serum. The antigen-antibody complex was detected using anti-goat HRPO conjugate and color was developed using DAB. Given above are the reactivity of different proteins with PPRV positive serum showing distinct brown colored dots

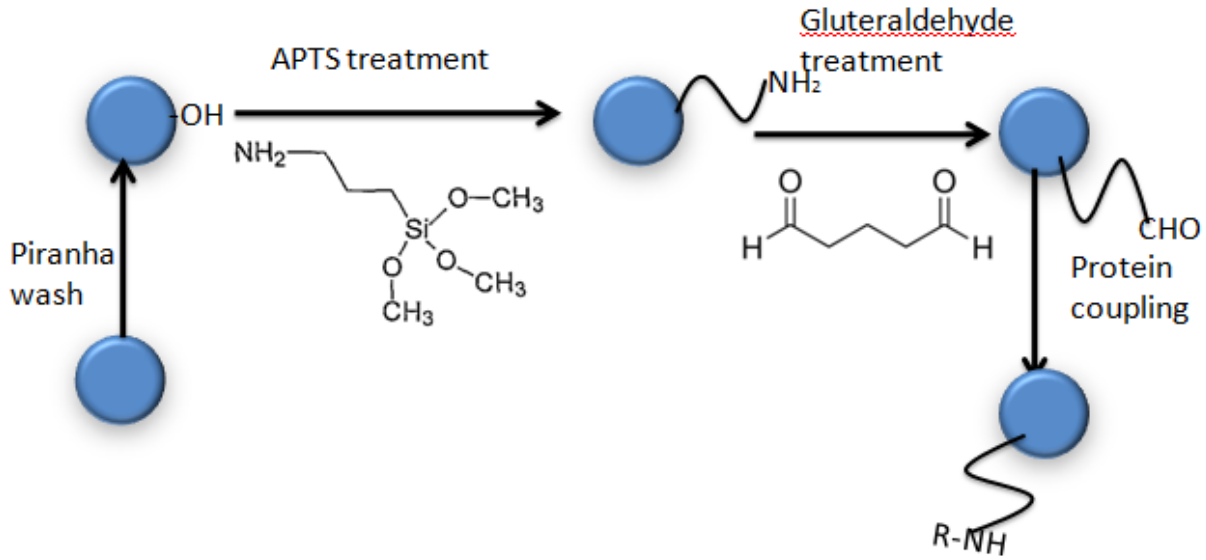


Fig. 9: Flow diagram showing covalent coupling of proteins on glass beads using APTS chemistry: Piranha washed glass beads are modified by 10 % ethanolic solution of APTS followed by gluteraldehyde treatment and protein coupling. Blue colored circle represents single glass bead of diameter 425– 600 µm. Chemical formula of the chemical substances are shown below the arrow mark

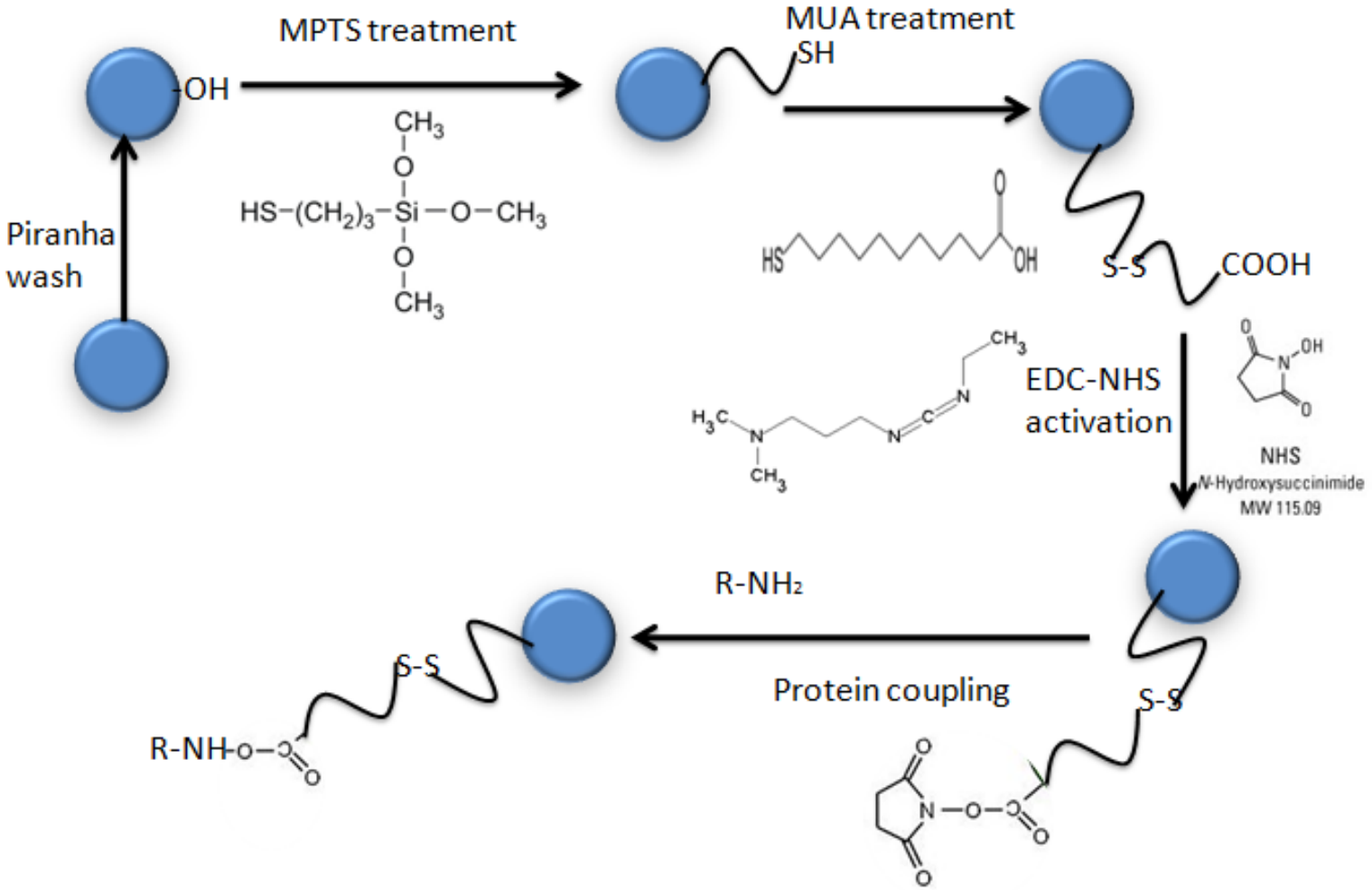


Fig. 10: Flow diagram showing covalent coupling of proteins on glass beads with MPTS: 2 % ethanolic solution of MPTS was used to modify piranha washed glass beads followed by MUA, EDC- NHS activation and protein coupling. Blue colored circle represents single glass bead of diameter 425– 600 µm. Chemical formula of the chemical substances are shown below the arrow mark

that 127 µg of protein has been coupled on to 500 mg of the beads. Approximately 2500 beads are present in 500 mg which means that 60 ng of protein is coated per bead.

4.7. Optimizing the conditions for APTS and MPTS chemistry

Recombinant protein, ΔN coated in MPTS and APTS functionalized beads were dispensed as 5 beads per tube, 10 beads per tube, 15, 20, 25, 30, 35, 40 and 45 beads per tube. These were analyzed with positive serum and negative serum to test the effect of number of beads on reactivity. The results **(Table 17, Fig. 12, Fig. 13)** indicate that ΔN coated on MPTS SAM layers show more reactivity than on APTS SAM layers. The P/N result **(Fig. 14)** shows that number of beads shows negligible effect on reactivity. Though there is slight increase in fold change when number of beads are increased per tube, for 10 beads showed a good fold change (positive /negative ratio) of 3.4. This study concluded the use of MPTS functionalized 10 beads per tube to optimize the immunoassay and screen samples. Reusability of the beads was also analyzed after regenerating the beads in 50 mM NaOH. On regeneration the OD at 492 nm of ΔN coated on MPTS functionalized beads decreased to 0.253 from 0.485 and for ΔN coated on APTS functionalized beads decreased to 0.215 from 0.421. P/N ratio showed a decrease to 2.13 from 4.17 for ΔN coated on MPTS functionalized beads and for ΔN coated on APTS functionalized beads a decrease to 1.9 from 2.53. On regeneration, though it shows difference in positive serum and negative serum, P/N value is not as in the first use of the beads **(Fig. 15).**

Table 17: Reactivity of PPRV positive serum to the ΔN coated on MPTS and APTS

No of Beads per	MPTS		P/N Value	APTS		P/N Value
5	0.446	0.153	2.91503	0.328	0.143	2.29371
10	0.485	0.142	3.41549	0.421	0.139	3.02878
15	0.464	0.132	3.51515	0.486	0.129	3.76744
20	0.538	0.153	3.51634	0.531	0.142	3.73944
25	0.598	0.166	3.60241	0.615	0.167	3.68263
30	0.761	0.205	3.7122	0.829	0.159	5.21384
35	0.746	0.182	4.0989	0.839	0.181	4.63536
40	1.03	0.264	3.90152	0.636	0.173	3.6763
45	0.82	0.19	4.31579	0.545	0.139	3.92086
50	0.9	0.173	5.20231	0	0	0

For optimizing the serum dilutions, ΔN coated on MPTS functionalized beads were aliquoted in 20 tubes (10 beads per tube) and analyzed for positive and negative serum starting from 1:10 dilution to 1:100 dilution. P/N ratio of 1:10 dilution was 3.50. The results **(Table 18, Fig. 16)** show that 1:10 dilution shows better reactivity. Similarly study of conjugate dilutions optimized 1:5000 dilutions of anti- goat HRPO conjugates **(Table 19, Fig. 17)**. 4.38 P/N ratio was obtained in 1:5000 dilution in 20 min of incubation after adding the chromagen substrate without any background.

Table 18: Optimization of serum dilutions

Dilutions	Positive	Negative	P/N Value
1:10	0.298	0.085	3.505
1:20	0.215	0.079	2.721
1:50	0.190	0.072	1.406
1:30	0.180	0.128	1.425
1:40	0.124	0.087	2.639
1:60	0.111	0.091	1.219
1:70	0.088	0.066	1.333
1:80	0.088	0.091	0.967
1:90	0.119	0.086	1.384
1:100	0.102	0.076	1.342

Table 19: Optimization of HRPO conjugate dilutions

Dilutions		Avg. (OD_{492} nm)	P/N Value
1:5000	Positive	0.3355	4.3856
	Negative	0.0765	
1:7500	Positive	0.2810	3.771812
	Negative	0.0745	
1:10000	Positive	0.2650	3.35443
	Negative	0.0790	

The beads coated with ΔN were also analyzed using anti-goat FITC conjugate in fluorometric assay. Beads treated with positive serum samples showed bright fluorescence where as negative serum sample showed negligible fluorescence or were visible as black beads

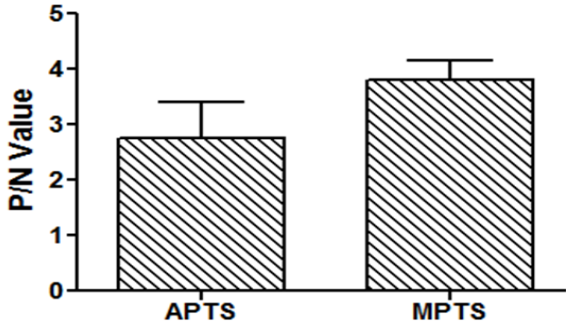


Fig. 11: Comparison of reactivity of APTS and MPTS functionalized beads: APTS and MPTS modified beads were coated with ΔN protein. These ΔN coated beads were assessed for serological activity using immunoassay. The assay was conducted using pooled positive and negative PPRV serum at the dilution of 1:50. The reactivity was measured as OD at 492 nm and P/N value is plotted as bar diagram

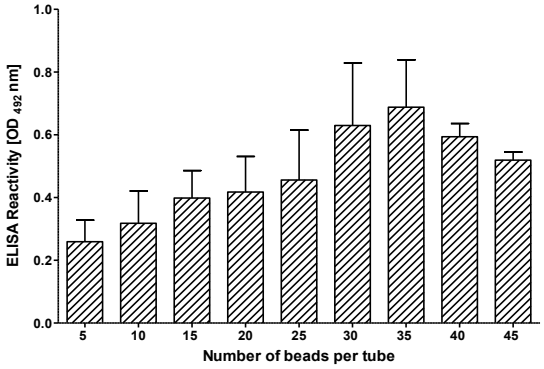


Fig. 12: Studying effect of number of beads for immunoassay (APTS modified): Reactivity of PPRV positive serum to ΔN coated APTS functionalized beads with increasing number of beads per tube (starting from 5 beads per tube to 45 beads per tube). The reactivity was measured as OD at 492 nm and plotted as bar diagram. Number of taken per tube is shown on X– axis and corresponding average OD_{492} nm ±SEM on Y– axis. The primary and secondary antibody concentration were kept constant

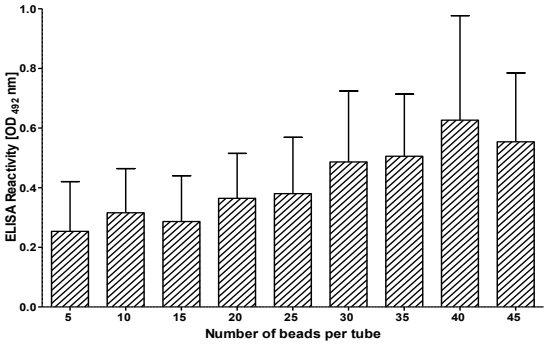


Fig. 13: Studying effect of number of beads for immunoassay (MPTS modified): Reactivity of PPRV positive serum to ΔN coated MPTS functionalized beads with increasing number of beads per tube (starting from 5 beads per tube to 50 beads per tube). The reactivity was measured as OD at 492 nm. Number of beads taken per tube is shown on X-axis and corresponding OD at 492 nm ± SEM on Y-axis. The primary and secondary antibody concentration were kept constant

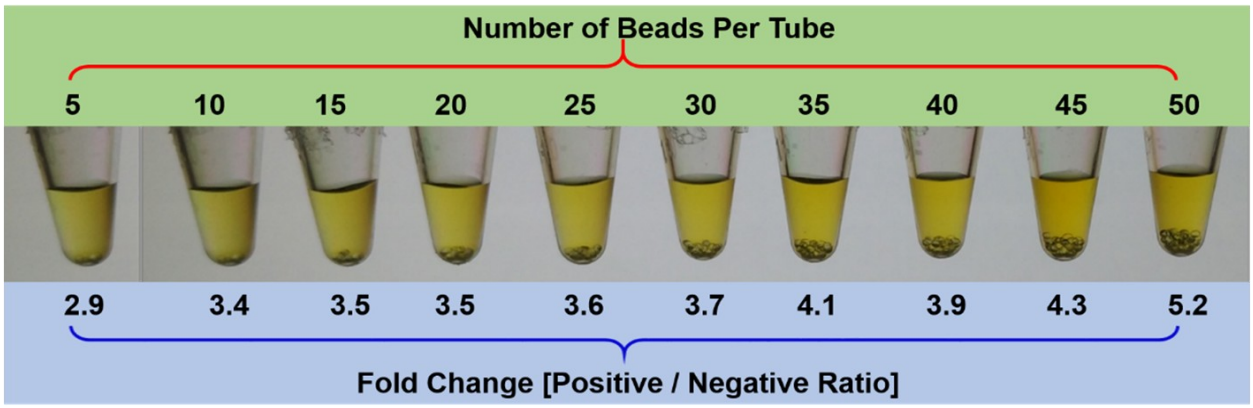


Fig. 14: Positive/Negative ratio of MPTS modified beads: MPTS functionalized beads dispensed in 10 tubes starting from 5 beads per tube to 50 beads per tube as two sets. One set was reacted with a known PPRV serum and other with a PPRV negative serum. Figure above shows the reactivity of positive serum to ΔN coated MPTS functionalized beads. The positive/negative ratio (fold change) is presented below each tube

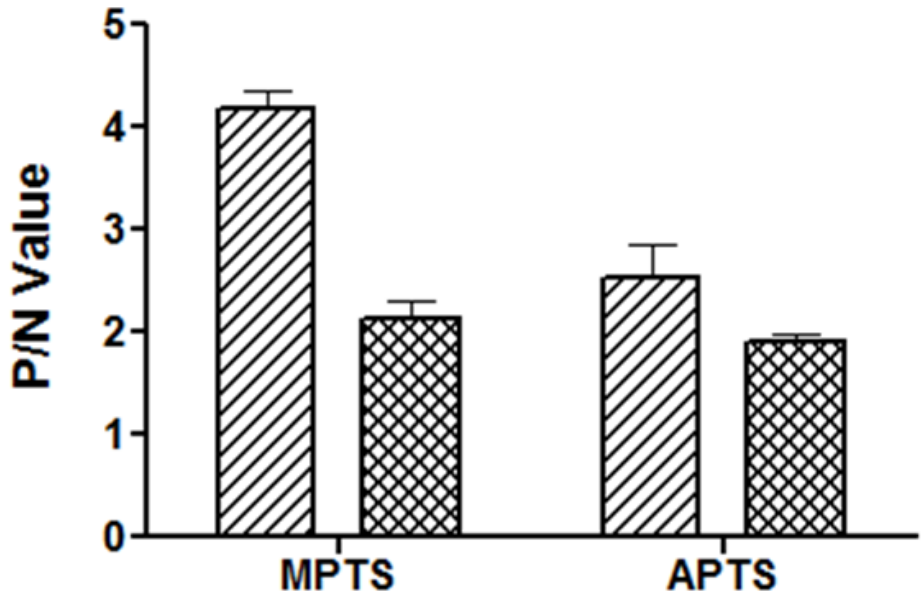


Fig. 15: Reusability of APTS and MPTS functionalized beads after regeneration: Beads used once in immunoassay were regenerated in 50 mM NaOH and reacted again with positive and negative sera. The bar diagram denotes reactivity of protein coated beads before and after regeneration. The P/N ratio is shown on Y-axis

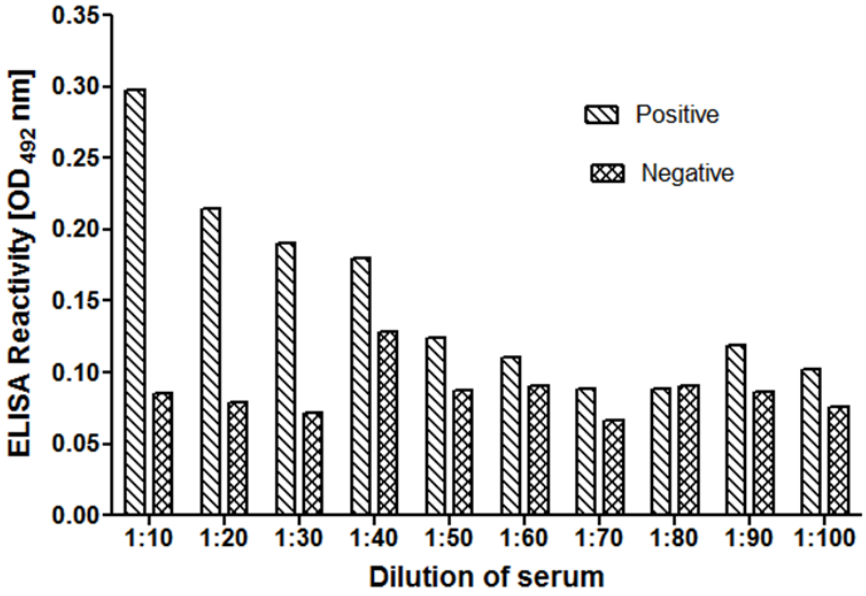


Fig. 16: Optimization of serum dilution in colorimetric assay: ΔN coated MPTS functionalized beads were dispensed as 10 beads per tube and analyzed in a checkerboard titration. The known positive and negative PPRV serum at different dilution (starting from 1:10 to 1:100) was tested by colorimetric immunoassay. Result obtained as OD at 492 nm are shown as bar diagram. Serum dilutions is shown in X-axis and OD at 492 nm on Y-axis

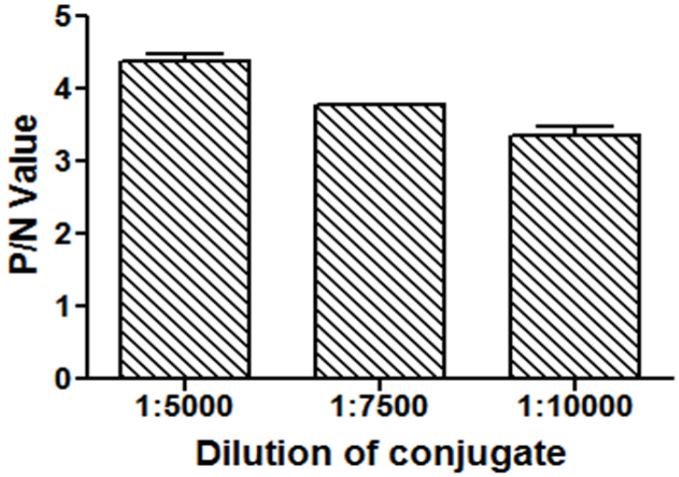


Fig. 17: Optimization of anti-goat HRPO conjugate: Colorimetric assay using ΔN coupled on MPTS functionalized beads was standardized for anti-goat HRPO conjugate (Sigma, USA) dilution starting from 1:5000 -1:10000. Optimization was done using a known positive and negative PPRV serum and assay reactivity was measured as OD at 492 nm. Conjugate dilution was shown on X-axis and OD at 492 nm on Y-axis

(Fig. 18). ΔN coated beads were also analyzed both colorimetrically and fluorometrically with hyperimmune serum and preimmune serum and even in 1:12800 dilutions of hyperimmune serum, beads were showing reactivity **(Table 20, Fig. 19a-19d)**. The fluorescence intensity was measured using Imagej software (Schindelin *et al.,* 2015). Regression curve analysis of the result of colorimetric and fluorometric assay with hyperimmune and preimmune serum showed almost a linear regression with r^2 0.9080 and 0.7775 for colorimetric and fluorometric assay. Since for both assay r^2 value is higher, the result datas are close to the fitted regression line **(Fig 20e and 20f).**

Table 20: Colorimetric and fluorometric assay with ΔN coated beads using hyperimmune serum and preimmune serum

Dilution	Colorimetric assay (OD_{492} nm)		P/N Value	Fluorometric assay (Fluorescence intensity)		P/N Value
	Positive	Negative		Positive	Negative	
1:100	0.915	0.176	5.2	26.08 x10^6	1.91 x10^6	13.63
1:200	0.896	0.167	5.36	25.05 x10^6	1.91 x10^6	13.11
1:400	0.829	0.116	7.14	24.87 x10^6	1.74 x10^6	14.24
1:800	0.735	0.126	5.83	18.34 x10^6	1.91 x10^6	9.60
1:1600	0.688	0.126	5.46	15.19 x10^6	1.83 x10^6	8.29
1:3200	0.666	0.121	5.50	14.84 x10^6	1.94 x10^6	7.62
1:6400	0.533	0.119	4.48	13.78 x10^6	1.74 x10^6	7.9
1:12800	0.411	0.096	4.28	5.04 x10^6	1.65 x10^6	3.04

The recombinant proteins, ΔM, ΔF1, ΔF2, ΔH1, ΔH2, ΔN and a cocktail of all these proteins mixed in equal concentration were coated on MPTS functionalized beads separately. Table 21 and figure 21 presents the reactivity data based on the fluorometric assay. It is evident from the result that the cocktail of proteins and ΔN coated beads give more reactivity than when single proteins are considered. The fluorescent intensity measurement of cocktail and ΔN coated beads were 7978187 and 8175566 respectively (which show almost similar reactivity), indicating their increased reactivity towards pooled positive serum than other proteins. Hence for further screening of samples, both with colorimetric and fluorometric assay, pooled protein coated and ΔN coated beads were used.

Table 21: Fluorescence intensity obtained when 6 proteins and cocktail of protein was coated

	Fluorescence intensity (Positive samples)	Fluorescence intensity (Negative samples)
Cocktail of proteins	7.97 x10^6 ±1.63	1.21 x10^6 ±0.262
ΔN	8.17 x10^6 ± 1.21	0.639 x10^6 ±0.021
F1	4.06 x10^6 ±1.0.393	1.15 x10^6 ±0.074
F2	4.97 x10^6 ± 0.545	0.798 x10^6 ±0.053
H1	5.89 x10^6 ±1.17	0.582 x10^6 ±0.069
H2	4.71 x10^6 ±0.684	0.725 x10^6 ±0.054
ΔM	6.73 x10^6 ±1.088	1.62 x10^6 ±0.235

ΔN and cocktail of proteins coated beads were also used for the analysis of serum collected from vaccinated, unvaccinated and challenged animals on 3rd day, 7th day, 14th day and 21st day to study the antibody response pattern. With ΔN coated beads there was little fluorescence till day 14 after vaccination and serum collected on 21st day showed distinct fluorescence, and for cocktail of protein coated beads a similar trend was shown. 21st day serum showed more fluorescence than serum collected from other days. In case of serum collected from challenged animals, antibody against N protein was not developed till 13th day but 19th day showed an increased fluorescence. Serum collected from challenged animals, fluorometric assay showed an increase in fluorescence from 13th day onwards with cocktail protein coated beads and in 19th day serum showed more fluorescence. Table 21 and figure 22 presents this data.

4.8. Screening of serum samples using cELISA

Serum samples available in the lab and the serum samples collected from PPRV infected goat and sheep from IVRI farms, Izatnagar campus were used for screening by cELISA. 234 samples were screened using the cELISA kit obtained from IVRI, Mukteswar; out of which 77 samples were positive and 157 were detected as negative **(Fig. 23)**. The positive samples were again categorized into 7 groups based on the PI value **(Fig. 24).** These panels of positive and negative serum samples were used for further tests.

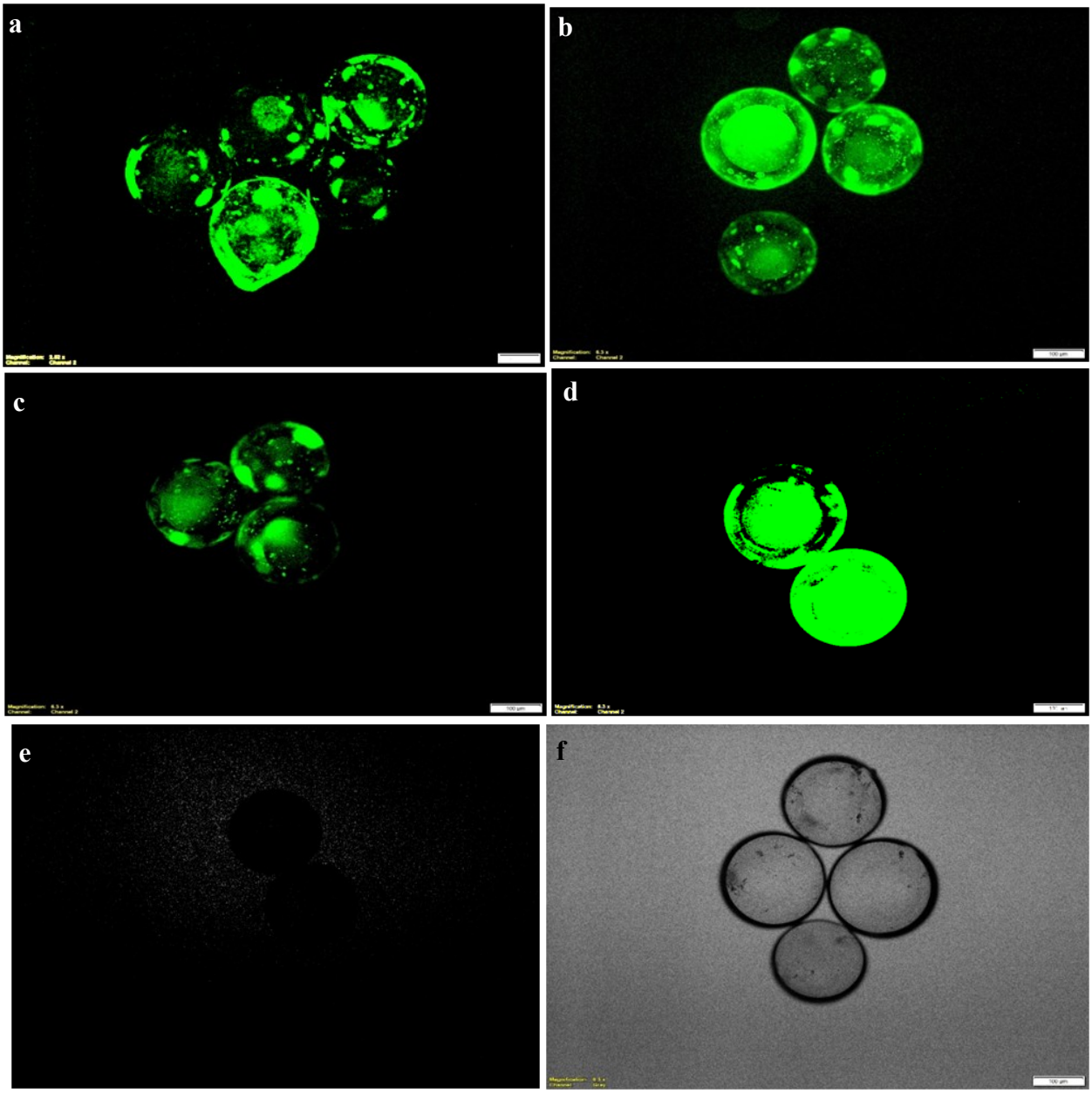
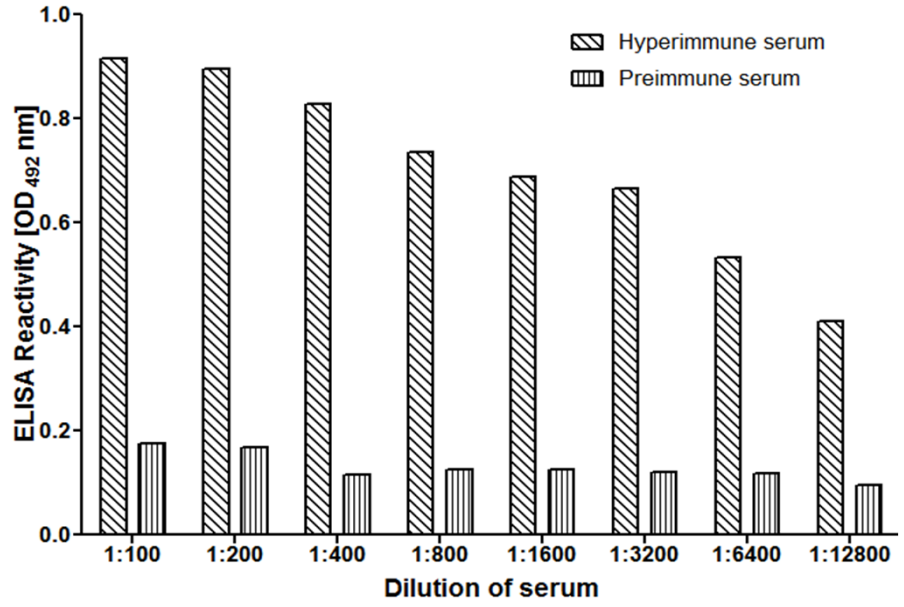
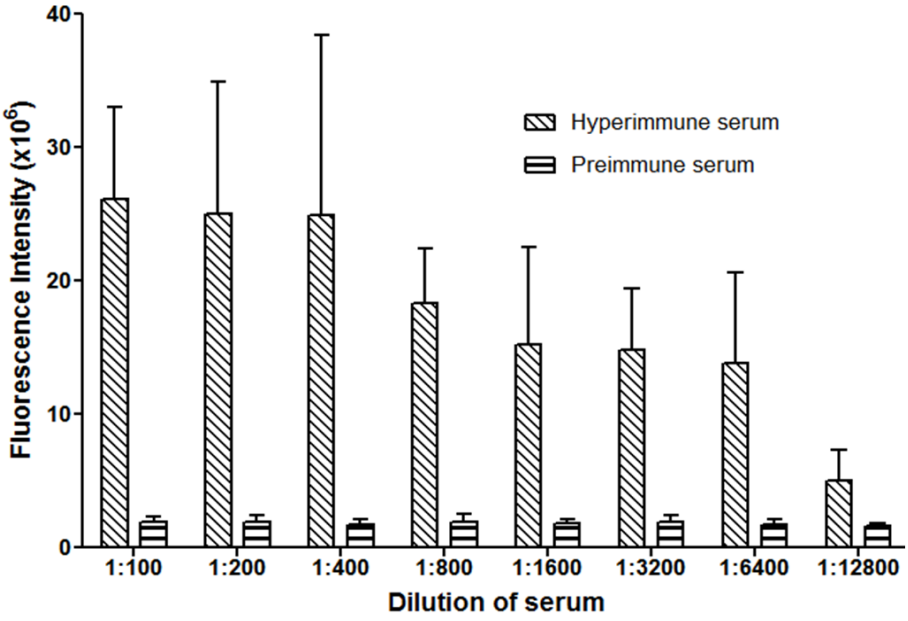


Fig. 18: Fluorometric immunoassay using ΔN coupled on MPTS functionalized beads: Fluorometric immunoassay was optimized by taking serum dilution 1:10 and FITC conjugate 1:1000. Fluorescence emission was visualized under fluorescence microscope (Olympus Bx 53) at 4X magnification. ΔN coated beads tested with negative serum showed negligible fluorescence. Reactivity of PPRV serum sample with different number of ΔN coated beads is shown in figure above [**(a)** 5 beads; **(b)** 4 beads; **(c)** 3 beads; **(d)** 2 beads; **(e)** Reactivity of PPRV negative sample with ΔN coated beads; **(f)** Beads coated with ΔN observed under bright field (4X)]



(a)



(b)

Fig. 19: Checkerboard titration showing reactivity of ΔN protein coated beads with anti-ΔN protein hyperimmune and preimmune sera raised in rabbits: The Colorimetric and fluorometric assay was performed with different dilutions of sera (from 1:100-1:12800) keeping the number of beads constant (10 beads) in each tube and reading with same concentration of secondary conjugate (either anti-rabbit HRPO or anti-rabbit FITC)

(a) Colorimetric assay of ΔN coated beads with hyperimmune and preimmune rabbit serum. Reactivity measured as OD at 492 nm is shown on Y-axis.

(b) Fluorometric assay of ΔN coated beads with hyperimmune and preimmune rabbit serum measured using Imagej software.The fluorescence intensity is shown on Y-axis

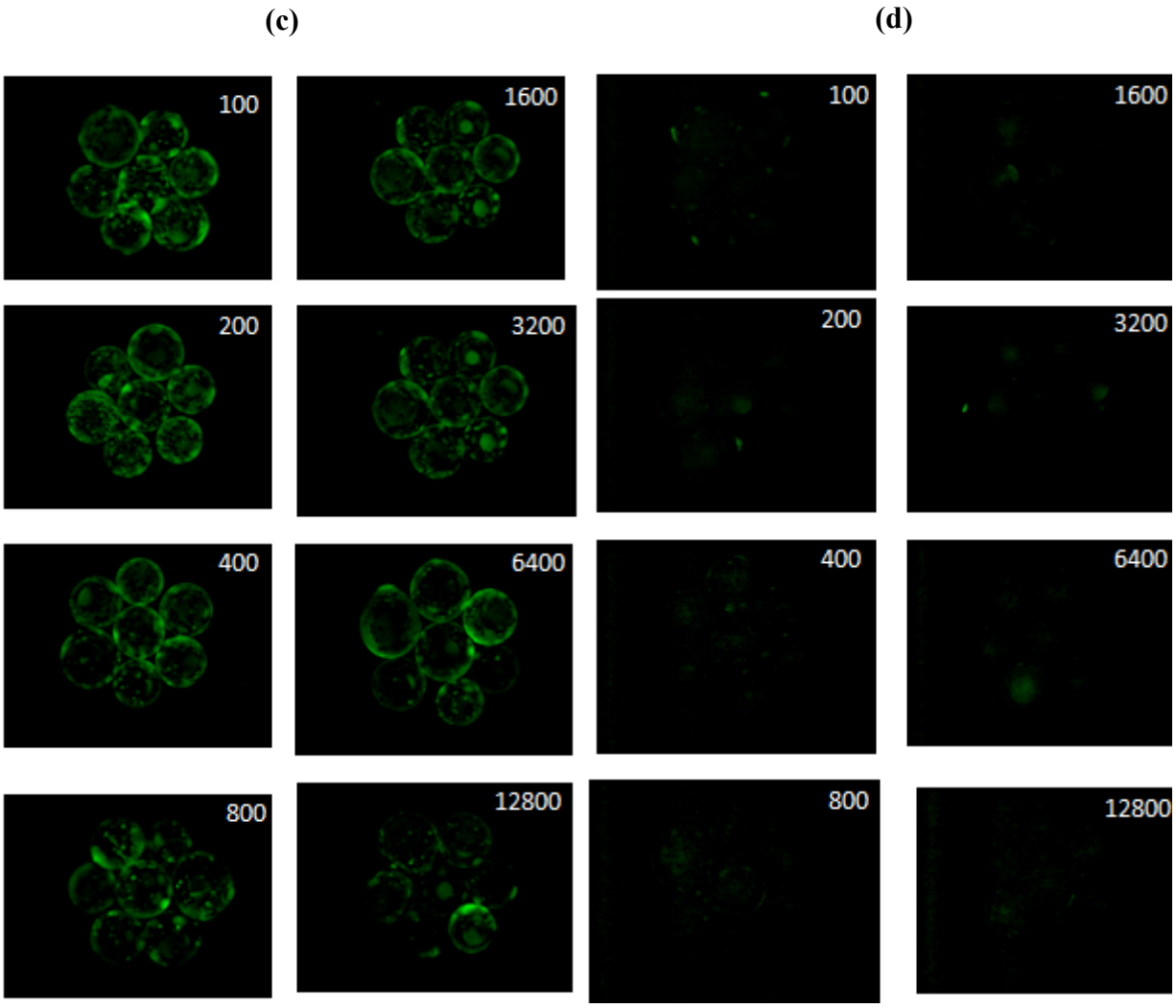


Fig. 19: Checkerboard titration showing reactivity of ΔN protein coated beads with anti-ΔN protein hyperimmune and preimmune sera raised in rabbits: The Colorimetric and fluorometric assay was performed with different dilutions of sera (from 1:100-1:12800) keeping the number of beads constant (10 beads) in each tube and reading with same concentration of secondary conjugate (either anti-rabbit HRPO or anti-rabbit FITC)

(c) Reactivity of beads with different dilutions of hyperimmune serum viewed under fluorescence microscope. Dilutions of serum are labeled on the top right side of each figure.

(d) Reactivity of beads with different dilutions of preimmune serum viewed under fluorescence microscope. Dilutions of serum are labeled on the top right side of each figure

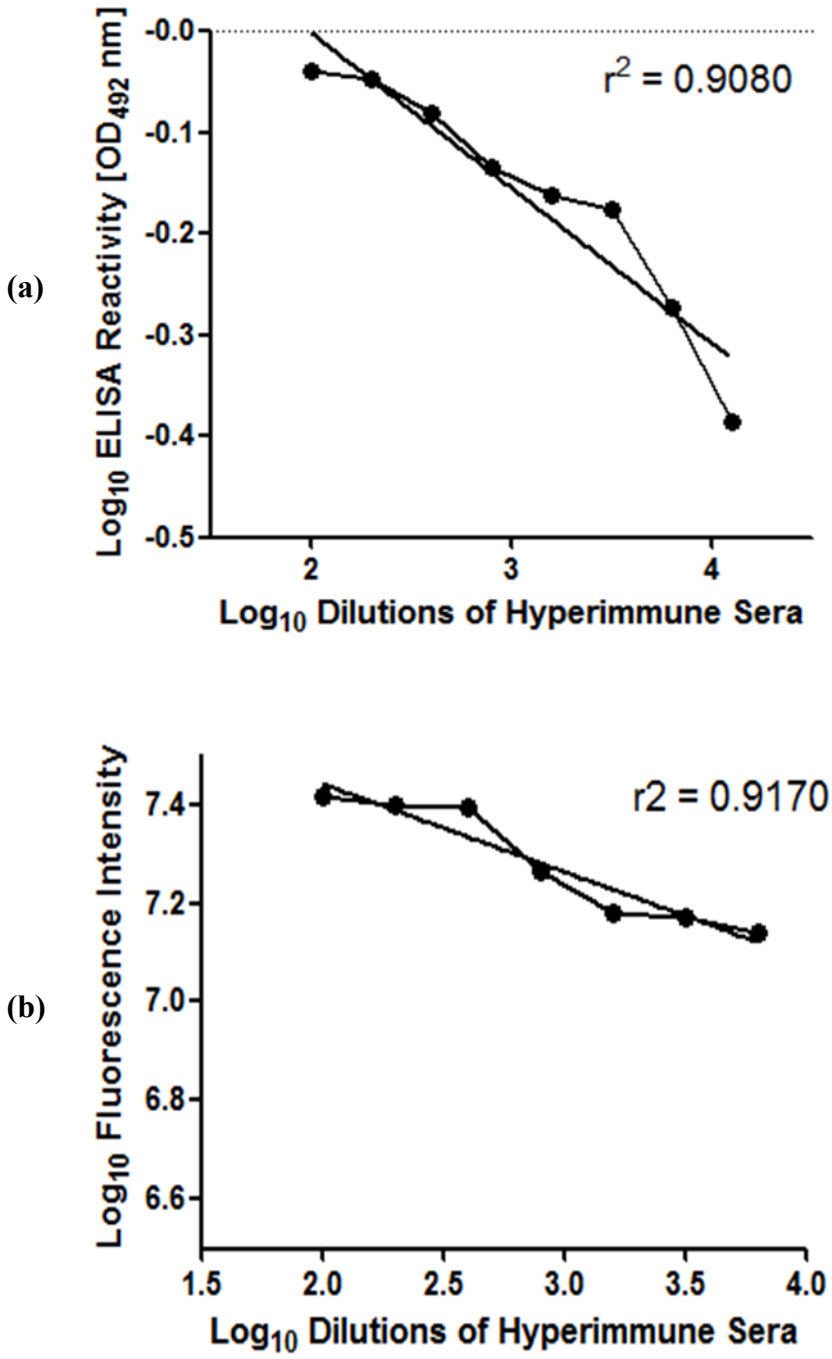


Fig. 20: Calibration curve showing reactivity of different dilution of rabbit anti ΔN protein hyperimmune serum with ΔN coated beads (10 beads in each tube):
(a) Calibration curve of colorimetric assay showing goodness of fit (r^2) = 0.9080
(b) Calibration curve of fluorometric assay showing goodness of fit (r^2) = 0.9170

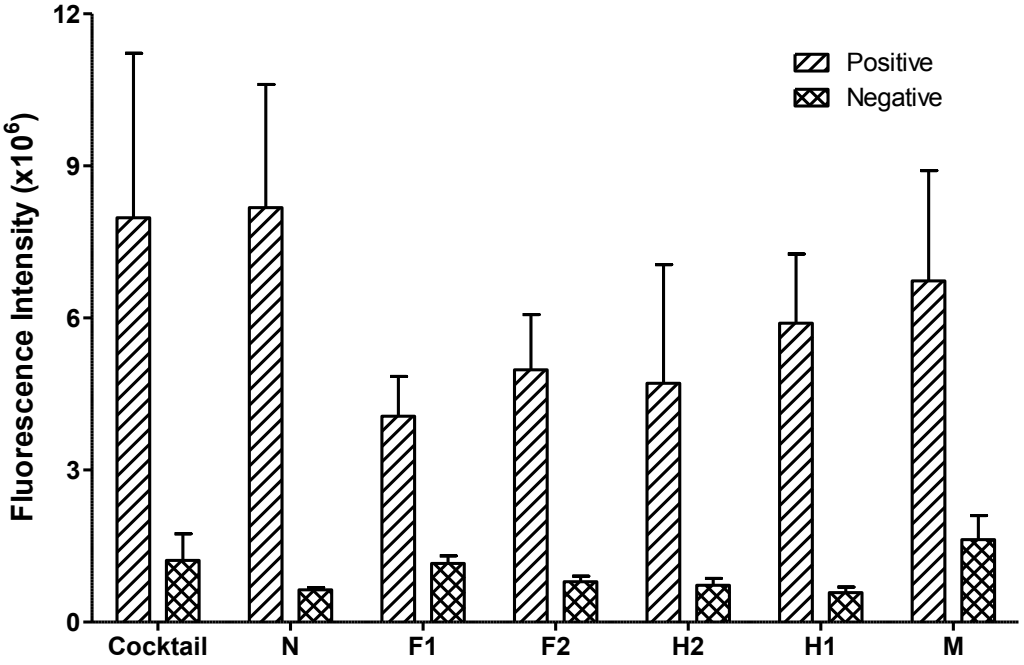


Fig. 21: Fluorometric assay using different recombinant proteins coated beads: Purified recombinant proteins ΔN, ΔF1, ΔF2, ΔH1, ΔH2, ΔM and a cocktail of protein mixed in equal concentration were coated on MPTS modified beads. These beads were tested by reacting with pooled PPR positive and negative serum. The average fluorescence intensity was measured using Imagej software is presented as bars

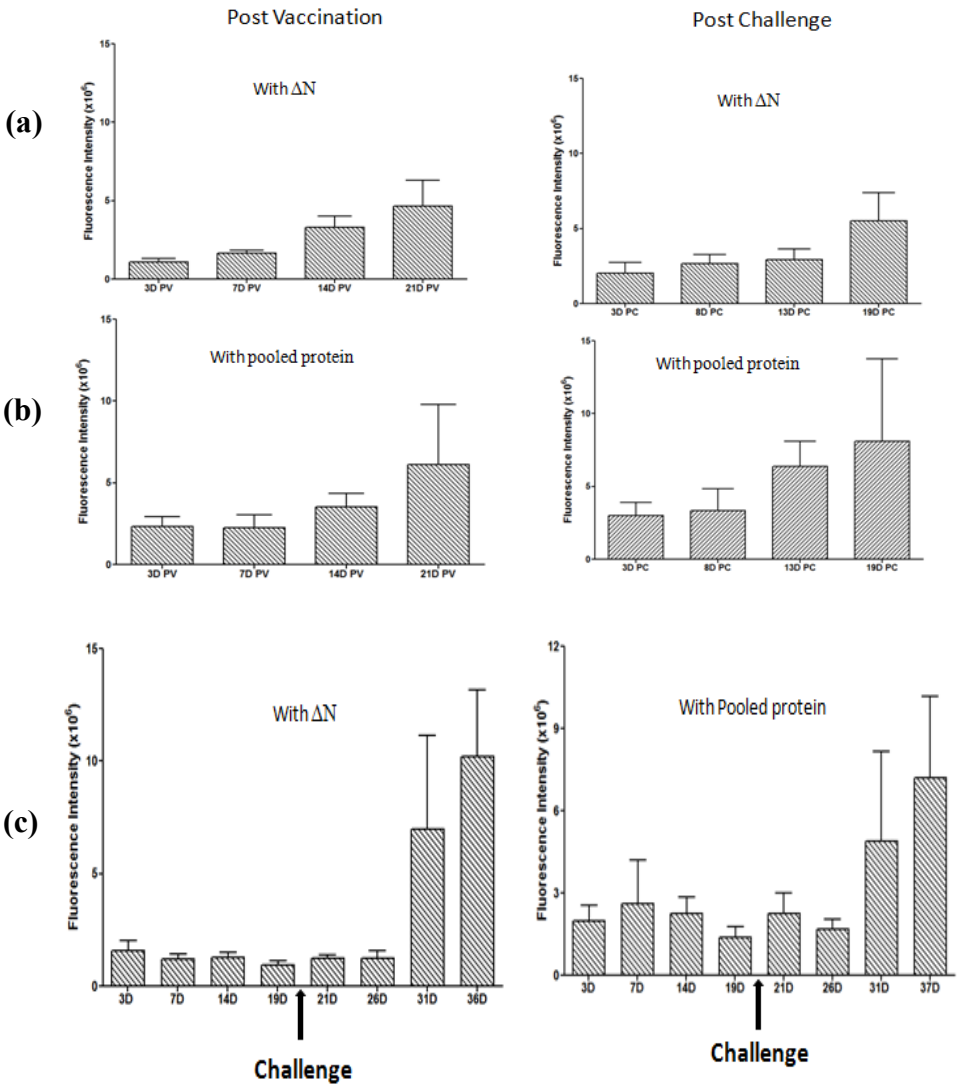


Fig. 22: Analysis of antibody response in vaccinated and challenged goats: ΔN and cocktail of protein coated beads were used to study the antibody kinetics in vaccinated and challenged goats at different time interval.

(a) Fluorometric assay using ΔN coated beads showing reactivity with sera from vaccinated and challenged goat

(b) Fluorometric assay using cocktail of proteins coated beads showing reactivity with sera from vaccinated and challenged goat

(c) Fluorometric assay ΔN and cocktail of protein coated beads showing reactivity at different time intervals before and after challenge (at 19th day)

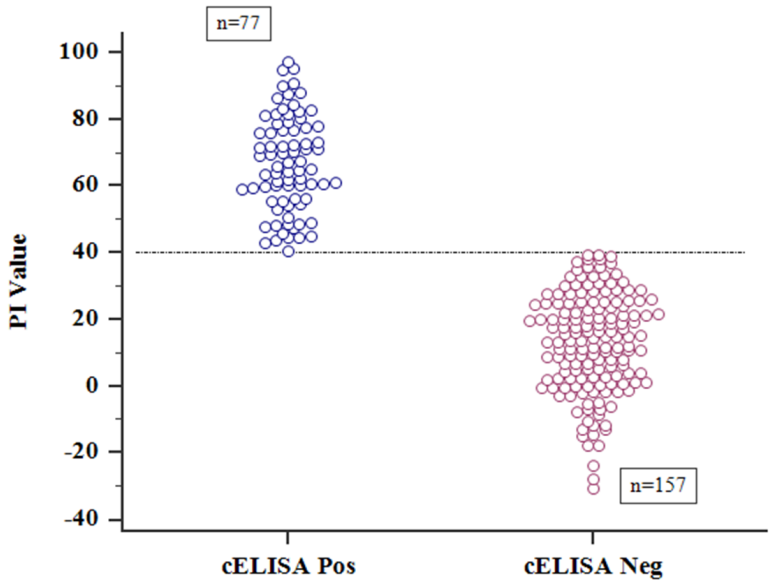


Fig. 23: Reactivity of goat sera tested by cELISA: Out of 234 serum samples tested, 77 were positive and 157 were negative. Blue dotted line denote 40 % cut off value for differentiating positive and negative samples. The percent inhibition (PI) value is plotted on Y-axis

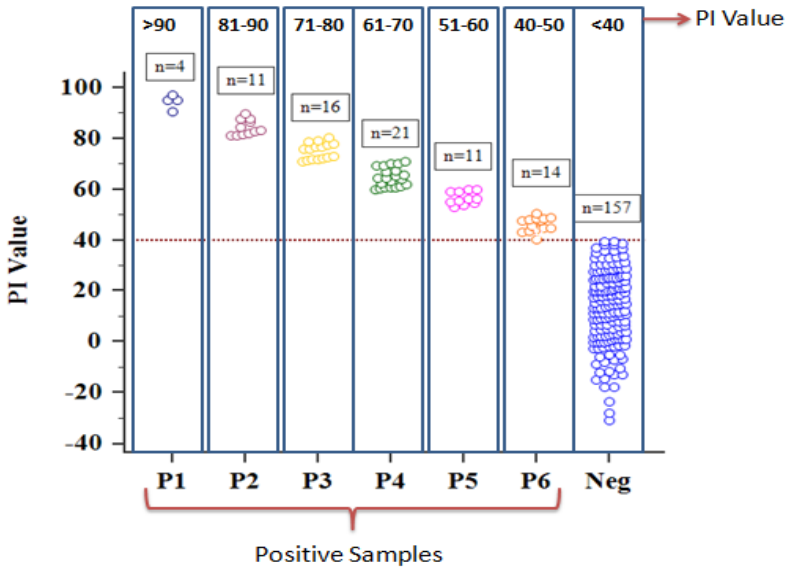


Fig. 24: Distribution of different sera samples based on PI value in cELISA: 77 positive and 157 negative serum samples were distributed using a scattered diagram based on PI value obtained in cELISA. Red dotted line indicates the 40 % cut off value for differentiating positive and negative samples. The number of serum samples in each category is given in corresponding box. The PI value is plotted on Y-axis

Table 22: Comparison of reactivity of ΔN and cocktail of protein coated beads with serum from vaccinated, unvaccinated and challenged animals

	Days	ΔN (Fluorescence intensity x 10^6)	Cocktail of protein (Fluorescence intensity x 10^6)
Post vaccination	3	1.09 ±0.108	2.34±0.304
	7	1.66 ±0.091	2.26±0.391
	14	3.29 ±0.357	3.51±0.415
	21	4.64 ±0.832	4.79±1.83
Post challenge	3	2.04 ±0.355	2.99± 0.447
	8	2.65±0.313	3.35±0.75
	13	2.92±0.365	6.36±0.867
	19	5.51±0.946	8.10±2.830
Without vaccination	3	1.58±0.230	1.99±0.278
	7	1.20±0.117	2.62±0.789
	14	1.28±0.107	2.26±0.292
	19	0.95±0.084	1.38±0.191
Post challenge	21	1.25±0.073	2.26±0.372
	26	1.23±0.178	1.7±0.171
	31	6.98±2.076	4.9±1.62
	36	10.19±1.479	7.2±1.48

4.9. Assessing the suitability of the recombinant antigens in detecting PPRV antibodies by indirect ELISA

Indirect ELISA procedure was optimized for coating concentration, serum dilutions etc. for recombinant proteins -ΔM, ΔF1, ΔF2, ΔH1 and ΔH2. From the optimization results **(Table 23a-23e, Fig. 25a-25e)**, 1:50 dilution of serum dilution with coating concentration of 300 ng per well was standardized and used for screening the samples. 60 samples were screened using indirect ELISA as per optimized conditions. Cut off value for differentiating positive and negative serum samples for each protein based indirect ELISA was obtained by taking average of negative serum samples (negative in cELISA also) plus standard deviation. Table 24 shows the standard deviation and cut off value of each protein in indirect ELISA. Table 25 and figure 26 presents data obtained by the screening of samples.

Table 23 : Optimization of conditions for indirect ELISA with different proteins

(a) ΔF1

		Protein concentration (ng)					
		500	400	300	200	100	50
Serum dilution	50	2.69	2.71	2.39	2.29	1.86	1.64
	100	2.22	2.09	1.94	1.86	1.58	1.49
	200	1.86	1.83	1.76	1.47	1.44	1.4
	400	1.53	1.52	1.91	1.45	1.31	1.24

(b) ΔF2

		Protein concentration (ng)					
		500	400	300	200	100	50
Serum Dilution	50	3.7	3.75	3.64	3.06	2.86	1.87
	100	2.6	2.54	2.51	2.3	2.74	1.58
	200	2.11	2.09	2.1	1.88	2.47	1.36
	400	1.49	1.51	1.61	1.4	2.07	1.21

(c) ΔH1

		Protein concentration (ng)					
		500	400	300	200	100	50
Serum Dilution	50	1.92	1.96	1.95	1.89	1.68	1.63
	100	1.65	1.67	1.67	1.53	1.43	1.4
	200	1.63	1.6	1.59	1.49	1.38	1.25
	400	1.62	1.45	1.44	1.35	1.39	1.16

(d) ΔH2

		Protein concentration (ng)					
		500	400	300	200	100	50
Serum Dilution	50	1.81	1.56	1.67	1.52	1.62	1.47
	100	1.64	1.37	1.41	1.28	1.39	1.31
	200	1.37	1.34	1.47	1.1	1.26	1.26
	400	1.26	1.19	1.18	1.15	1.17	1.1

(e) ΔM

		Protein concentration (ng)					
		500	400	300	200	100	50
Serum Dilution	50	3.15	3.07	3.28	3.56	2.97	2.18
	100	2.61	2.61	2.63	2.56	2.35	1.84
	200	2.37	2.25	2.25	2.03	1.94	1.65
	400	1.8	1.82	1.8	1.72	1.55	1.41

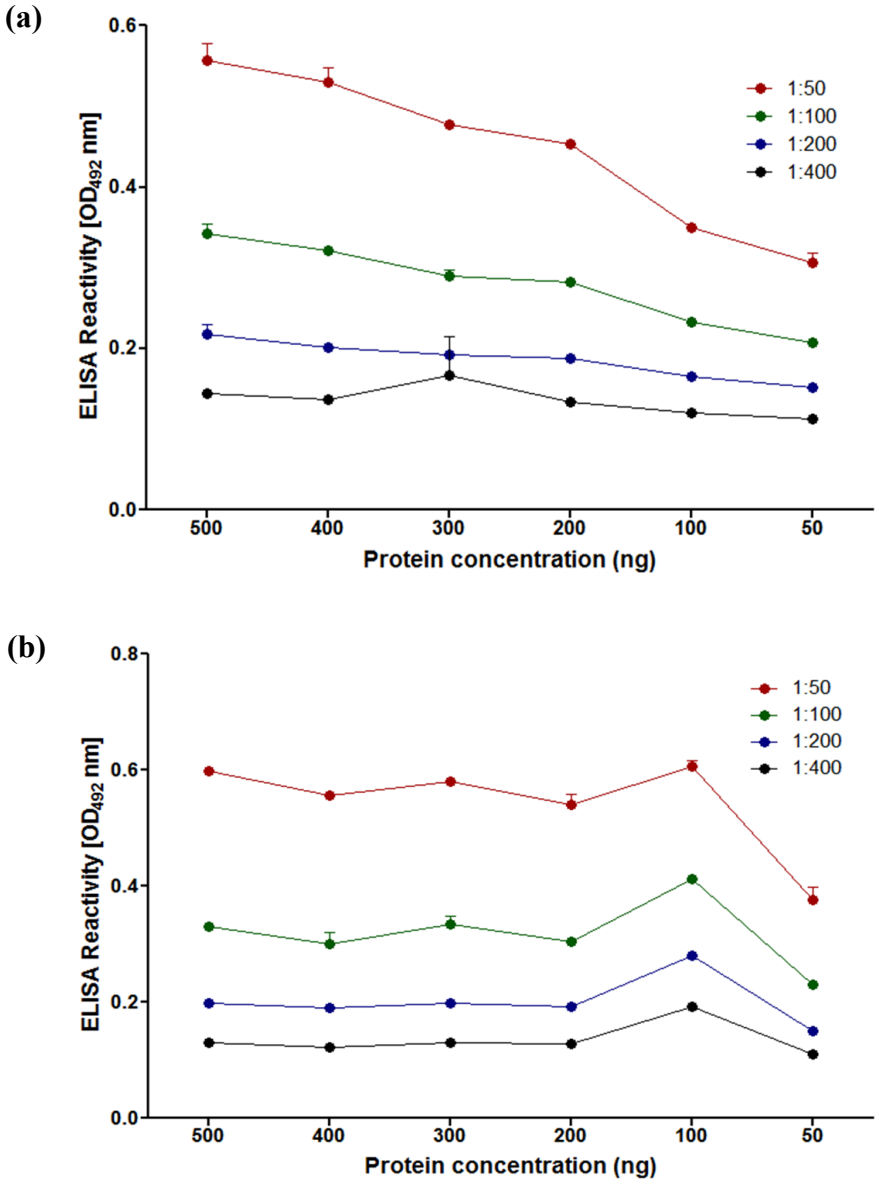
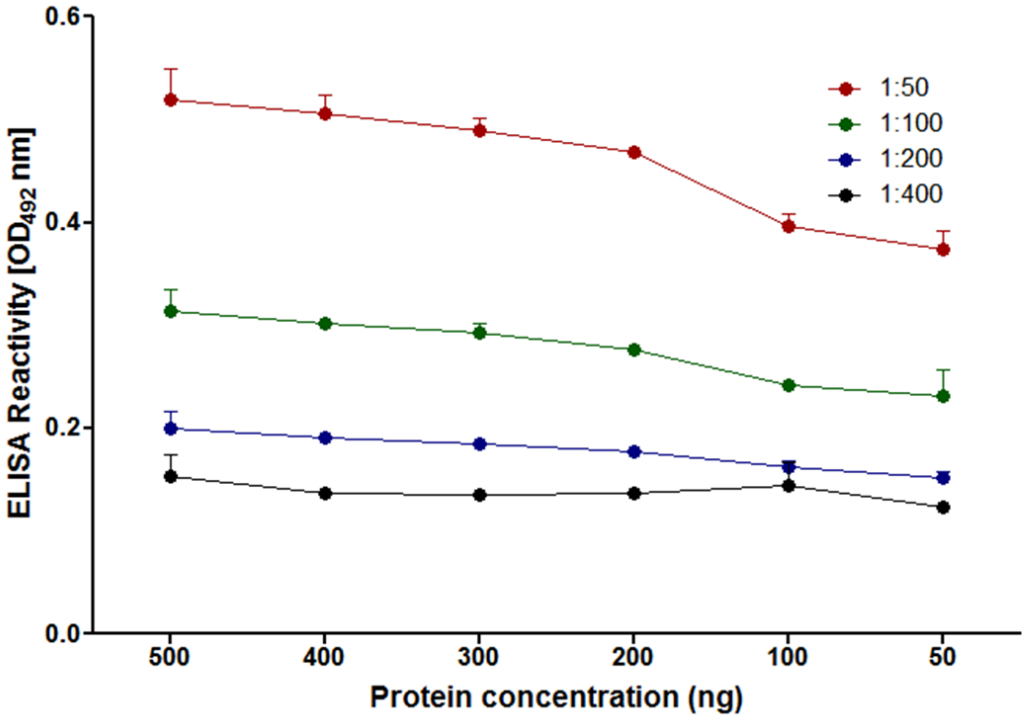


Fig. 25: Checkerboard titration for optimizing indirect ELISA conditions using different recombinant proteins: Indirect ELISA based immunoassay was standardized by optimizing coating concentration per well for each recombinant protein and various serum dilutions. Checkerboard titration at different coating concentration (500, 400, 300, 200,100, 50 ng/well) and serial dilution of serum starting from 1:50-1:400 was studied. The Y- axis shows OD at 492 nm and X-axis indicates protein concentration per well. Graphs given figure 25 show the result of optimization study for different proteins [**(a)** ΔF1; **(b)** ΔF2; **(c)** ΔH1; **(d)** ΔH2; **(e)** ΔM]

(c)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0.4
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ELISA Reactivity [OD492 nm]
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1:100
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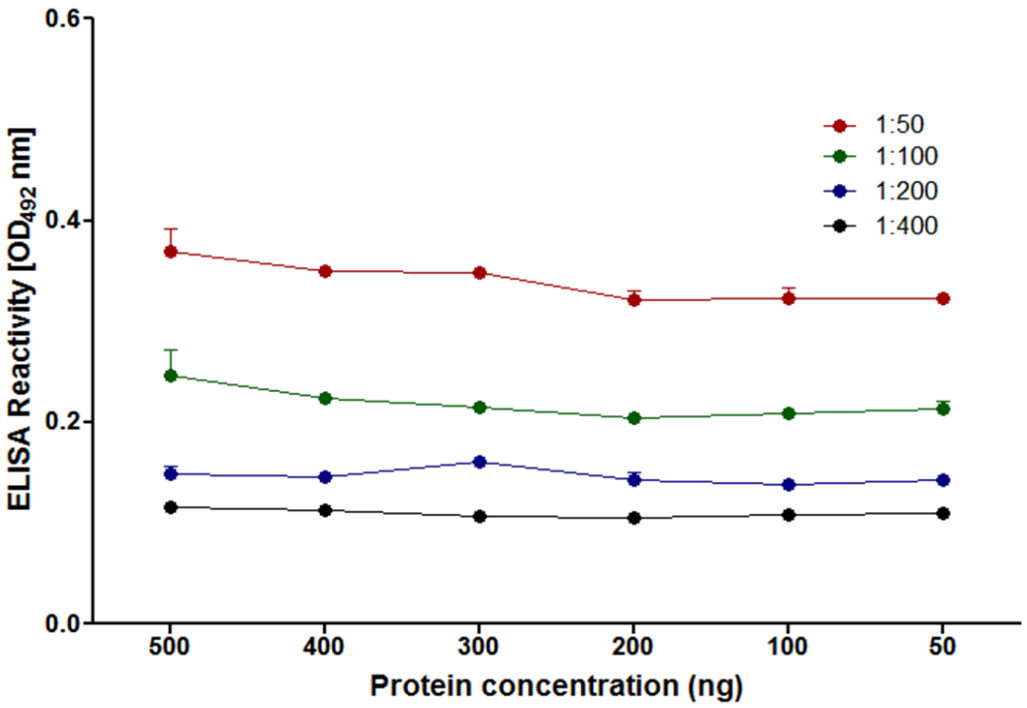
50
Protein concentration (ng)

(d)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ELISA Reactivity [OD492 nm]
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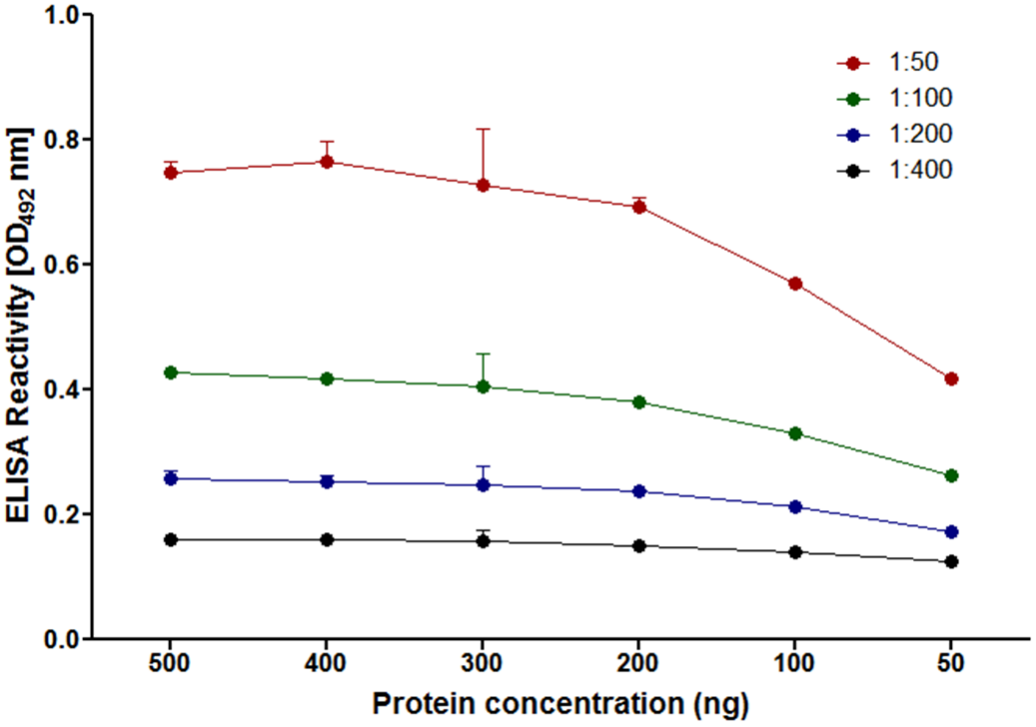
Protein concentration (ng)

(e)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ELISA Reactivity [OD492 nm]
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1:400
500
400
300
200
100
50
Protein concentration (ng)

Table 24: Standard deviation and cut off value of each protein in indirect ELISA

Recombinant Protein	Standard deviation	Cut off value
ΔF1	0.034	0.206 (Avg+1.5* SDEV)
ΔF2	0.0309	0.200 (Avg+1.5* SDEV)
ΔH1	0.0319	0.205 (Avg+1.5* SDEV)
ΔH2	0.0248	0.169 (Avg+1.5* SDEV)
ΔM	0.0503	0.236 (Avg+1.5*SDEV)
ΔN	0.0681	0.489 (Avg+3*SDEV)

Table 25: Result of Indirect ELISA tested with recombinant protein

Protein	Positive	Negative	Total
ΔN	28	32	60
ΔM	20	40	60
ΔF1	25	35	60
ΔF2	26	34	60
ΔH1	29	31	60
ΔH2	21	39	60

4.10. Screening of Serum Samples using ΔN and Cocktail of Protein Coated Beads by Colorimetric Assay

142 samples were screened using ΔN coated beads by colorimetric assay; of which 65 samples were positive and 77 samples were negative **(Fig. 27)**. Cut off value for determining positive and negative was obtained by taking the average reading of negative serum samples and adding 1.5 times standard deviation of those samples to the average value obtained. Cut off value obtained was 0.119. With cocktail of protein coated beads 49 samples were screened and in this 22 samples were positive and 27 samples were negative **(Fig. 28)**. Cut off value was obtained by adding standard deviation and value obtained was 0.199.

4.11. Screening of Serum Samples using ΔN and Cocktail of Protein Coated Beads by Fluorometric Assay

49 samples were screened using ΔN coated and cocktail of protein coated beads.

Fluorescence intensity above $1.467x10^6$ was taken as negative for ΔN coated fluorometric assay. This cut off value was obtained by adding 1.5 times standard deviation to the average of negative samples' reading. Based on the cut off value obtained 20 samples were positive and 29 samples were negative in fluorometric assay with ΔN coated beads **(Fig. 29)**. In fluorometric assay using cocktail of protein coated beads, a cut off value for determining the positive and negative samples were obtained by adding 3 times standard deviation with the average fluorescence intensity value obtained for negative samples and value was $2.17x10^6$. In the cocktail of protein based fluorometric assay 29 positive and 20 negative samples were recorded **(Fig. 30)**.

4.12. Comparison of Beads' Assay Results with cELISA Results to determine the Diagnostic Effectiveness Parameters.

In colorimetric assay with ΔN coated beads, 65 samples were positive and 77 were negative; when serum samples were tested with cELISA positive samples were 66 and negative were 76 **(Fig. 31)**. Out of 49 samples tested with cocktail of protein coated beads in colorimetric assay 22 positive and 27 negative samples were recorded; out of these cELISA positive samples were 23 and 26 samples were found as negative in cELISA**(Fig. 32)**.

In fluorometric assay, ΔN coated beads yielded 20 positive and 29 negative (n=49), and in this cELISA positive serum samples were 23 and negative 26 **(Fig. 33)**. Sensitivity and specificity of this test was 65% and 65.5% respectively **(Table 26)**. Cocktail of protein based fluorometric assay showed 29 positive and 20 negative samples (n=49); in cELISA 23 samples were positive and 26 samples were negative **(Fig. 34)**. Specificity and sensitivity calculated was 95% and 79.3 % respectively **(Table 27)**. The ROC curve analysis of the developed fluorometric immunoassay using ΔN and cocktail of protein coated beads is presented in the figure **35a** and **35b**. Table 28 shows the ROC curve analysis table of fluorometric assay using ΔN coated beads. Standard error was determined based on studies of DeLong *et al.,* 1988. ROC curve analysis table of fluorometric assay using cocktail of protein coated beads is shown in table 29. The P value of fluorometric assay using ΔN coated beads is 0.1677 and that of cocktail of protein coated beads is <0.0001 indicating high significance for fluorometric assay using cocktail of protein coated beads.

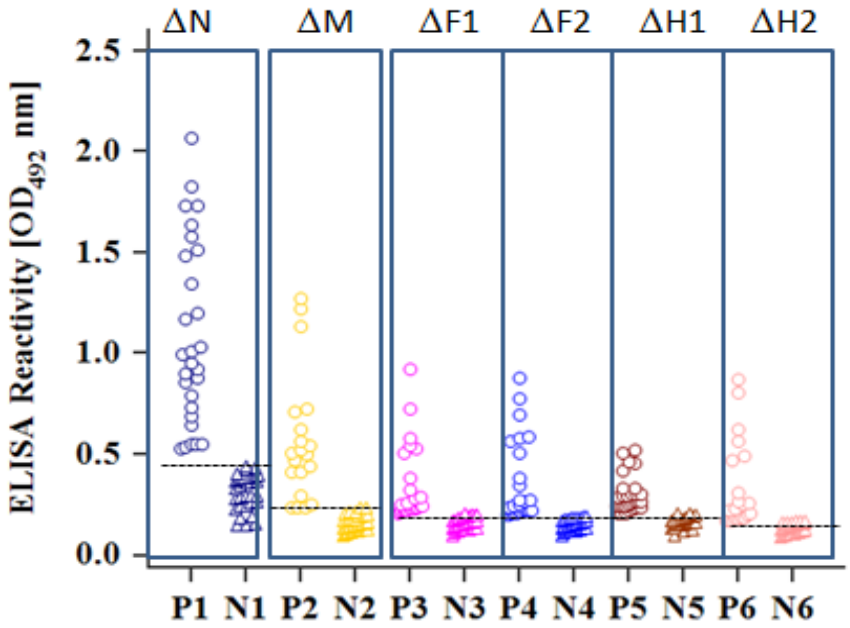


Fig. 26: Distribution plot of serum tested by indirect ELISA: 300 ng of recombinant proteins were coated on each well of polystyrene ELISA plates. Goat serum samples were tested in indirect ELISA at the dilution of 1:50 using different recombinant proteins. The reactivity of positive (P) and negative (N) serum samples are shown. The dotted line denotes cut off value for positive and negative serum which was taken as average OD of negative (N) serum samples ± standard deviation (SD)

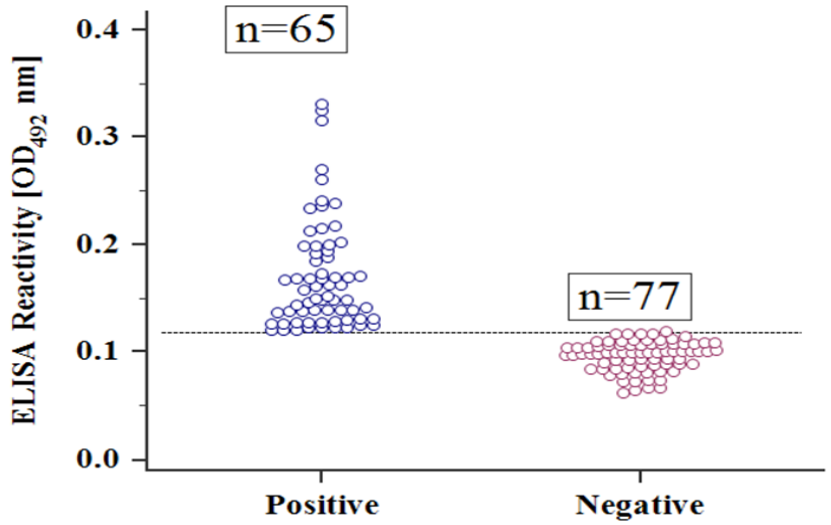


Fig. 27: Colorimetric assay using ΔN coated beads: A total of 142 samples were tested colorimetrically using ΔN coated beads, out of which 65 were positive and 77 were negative. The dotted line denotes cut off value (OD_{492} = 0.119) for differentiating positive and negative serum samples

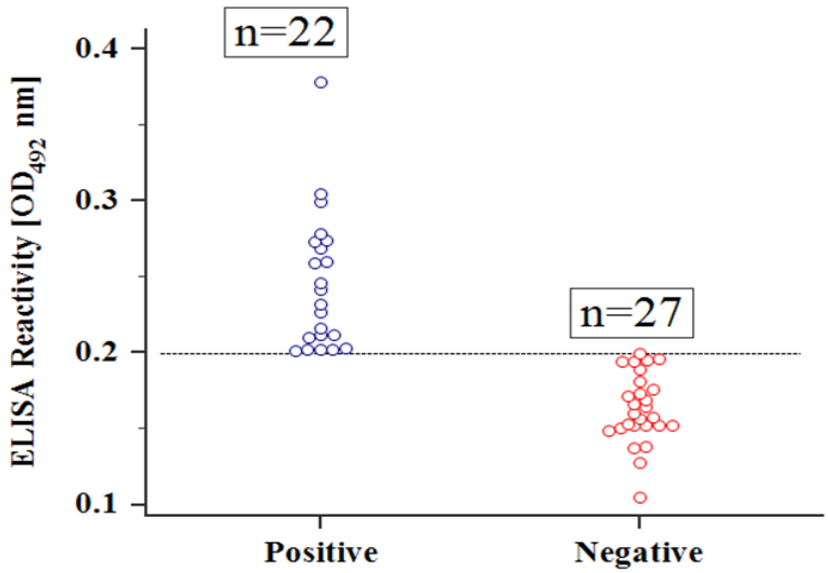


Fig. 28: Colorimetric assay using cocktail of protein coupled beads: A total of 49 samples were screened of which 22 were positive and 27 were negative. Dotted line denotes cut off value (OD_{492} = 0.199) for differentiating positive and negative serum samples

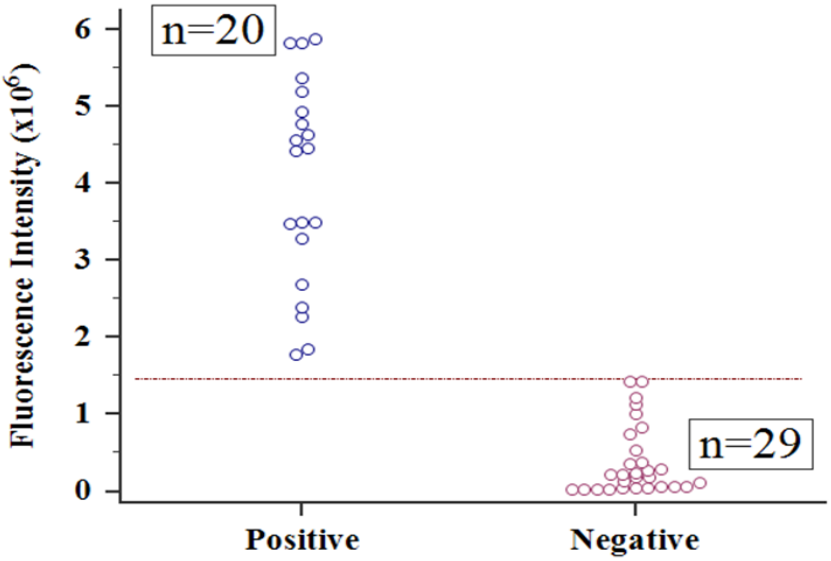


Fig. 29: Screening serum samples using ΔN coated beads by fluorometric assay: A total of 49 samples were screened of which 20 were positive and 29 were negative. The Y axis shows fluorescence intensity measured by Imagej software. The dotted line denotes cut off value (1.4 x10^6) for differentiating positive and negative serum samples

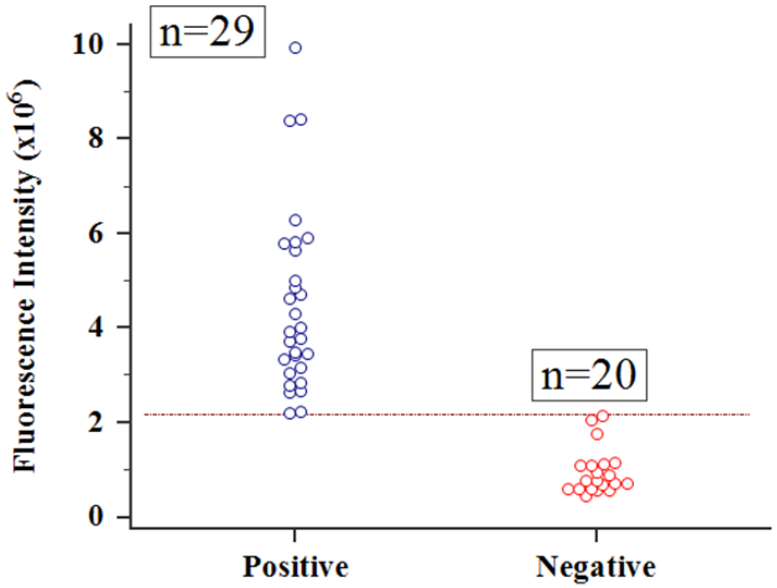


Fig. 30: Screening serum samples using cocktail of proteins coated beads by fluorometric assay: A total of 49 samples were screened of which 29 were positive and 20 were negative. The Y axis shows fluorescence intensity measured by Imagej software. The dotted line denotes cut off value (2.1 x10^6) for differentiating positive and negative serum samples

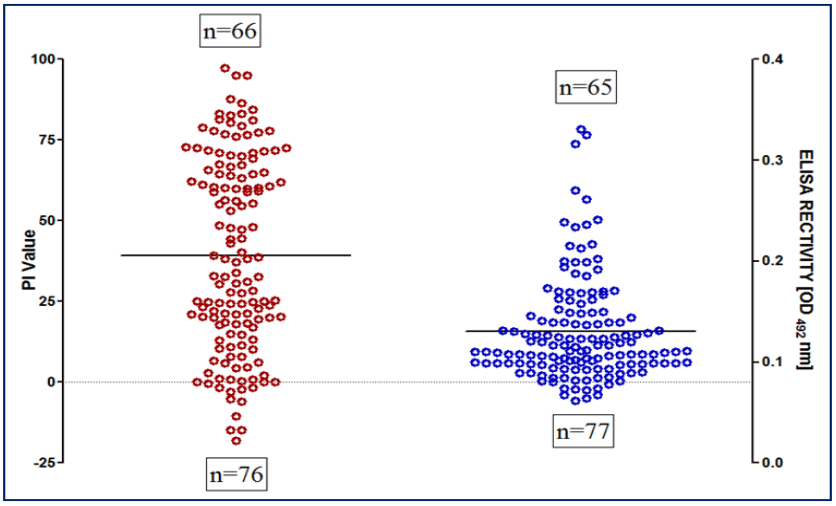


Fig. 31: Comparison of reactivity of goat serum analyzed by cELISA and ΔN coated beads colorimetric assay: 142 samples were tested by ΔN coated beads in colorimetric assay. The red dots are the cELISA results scattered diagram and blue dots indicates colorimetric assay. Black horizontal line indicates cut off value for corresponding tests

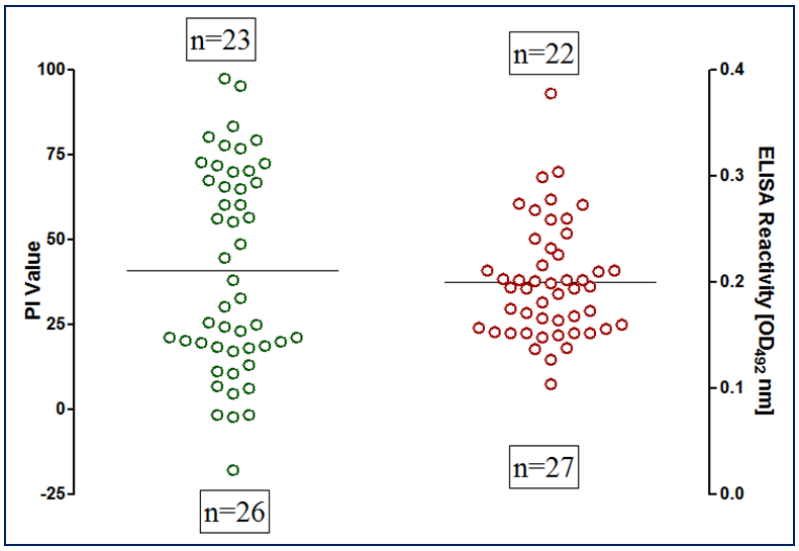


Fig. 32: Comparison of reactivity in serum analyzed by cELISA and cocktail of protein coated beads colorimetric assay: 49 samples were tested by colorimetric assay using cocktail of protein coated beads. Green dots in scatter diagram are the cELISA results and red dots indicates colorimetric assay. Black line indicates cut off value for corresponding tests

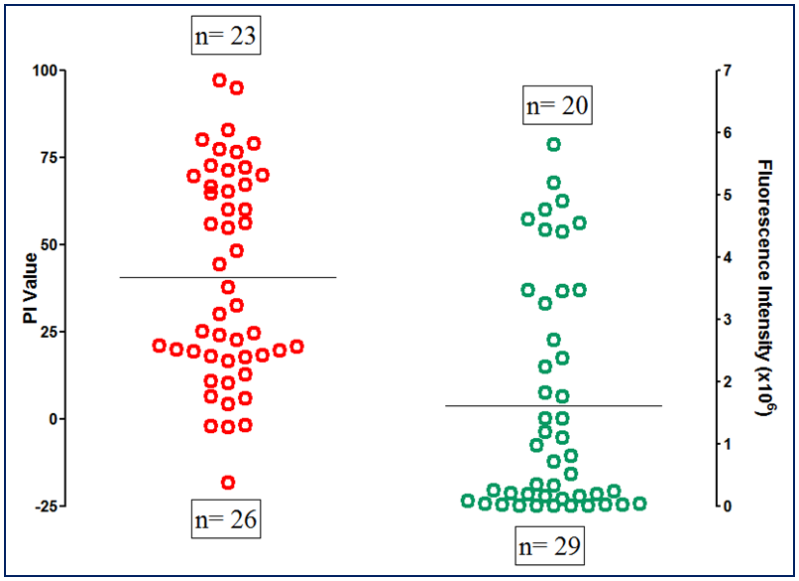


Fig. 33: Comparison of reactivity of serum analyzed by cELISA and ΔN coated beads in fluorometric assay: 49 samples were tested by fluorometric assay using ΔN coated beads. Red dots in scatter diagram are the cELISA results and green dots indicates fluorometric assay. Black line indicates cut off value for corresponding tests

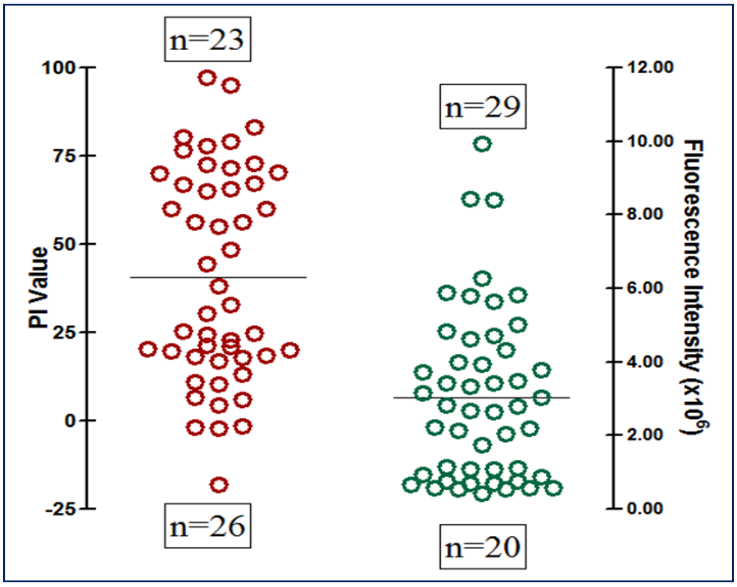
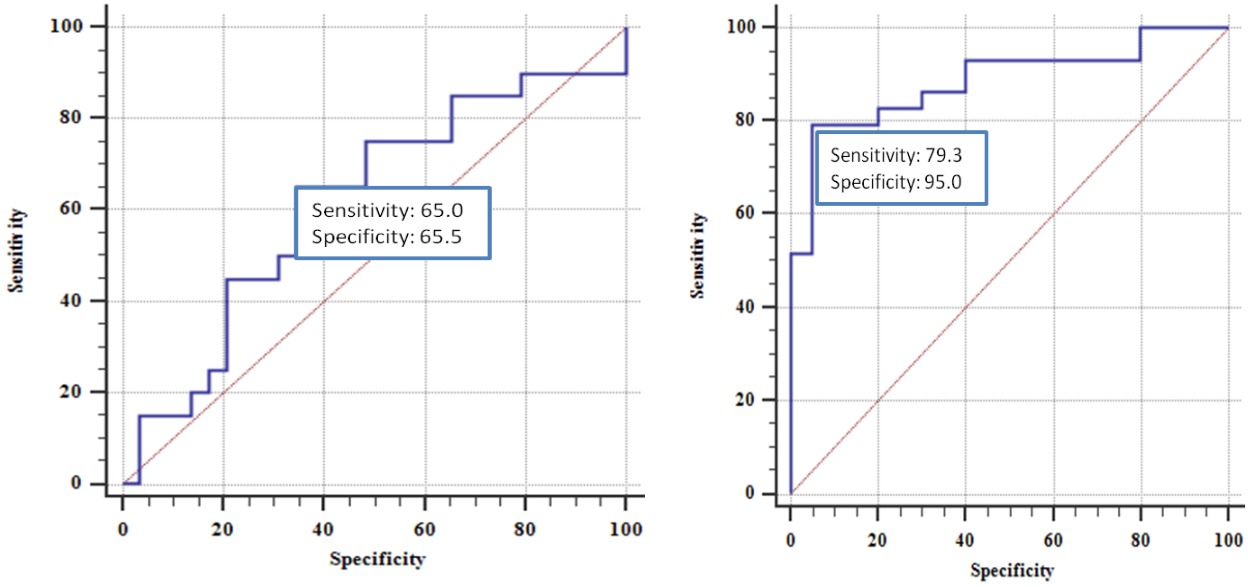


Fig. 34: Comparison of reactivity of serum analyzed by cELISA and cocktail of protein coated beads fluorometric assay: 49 samples were tested by fluorometric assay using cocktail of protein coupled beads. Red dots in scatter diagram are the cELISA results and green dots indicates fluorometric assay



(a) **(b)**

Fig. 35: ROC curve analysis of fluorometric assay: ROC curve was prepared using Medcalc software based on the data obtained in fluorometric assay using ΔN and cocktail of protein coupled beads. Graphs shown above are the ROC curve analysis of **(a)** fluorometric assay using ΔN coated beads; **(b)** fluorometric assay using cocktail of protein coupled beads. Sensitivity and specificity in relation to cELISA is shown in the rectangle boxes

Table 26: Diagnostic effectiveness parameters of ΔN based fluorometric assay

True positives	13/49=0.265
True negatives	19/49=0.388
False positives	10/49=0.204
False negatives	7/49=0.143
Accuracy	(13+19)/49=0.653
Sensitivity	13/20=0.65 (65%)
Specificity	19/29=0.655 (65.5%)

Table 27: Diagnostic effectiveness parameters of cocktail of proteins based fluorometric assay

True positives	23/49=0.469
True negatives	19/49=0.388
False positives	1/49=0.02
False negatives	6/49=0.122
Accuracy	(23+19)/49=0.857
Sensitivity	23/29=0.793 (79.3%)
Specificity	19/20=0.95 (95%)

Table 28: ROC curve analysis of fluorometric assay using ΔN coated beads

Area under the ROC curve (AUC	0.617
Standard Error	0.0850
95% Confidence interval	0.467 to 0.752
z statistic	1.380
Significance level P (Area=0.5)	0.1677

Table 29: ROC curve analysis of fluorometric assay using cocktail of protein coated beads

Area under the ROC curve (AUC)	0.886
Standard Error	0.0482
95% Confidence interval	0.763 to 0.959
z statistic	8.020
Significance level P (Area=0.5)	<0.0001

Independent T test was conducted using SPSS software (version 16) to know level of significance of fluorometric test developed in this study. Table 30 indicates T test results obtained for fluorometric assay using ΔN coated beads and table 31 shows result for fluorometric assay using cocktail of protein coated beads. In fluorometric assay using ΔN coated beads 2-tailed significance 0.037 and 0.56 for positive serum samples and negative serum samples respectively was observed which means that it is less significant, but in fluorometric assay using cocktail of protein coated beads 2-tailed significance 0 for both indicating high significance level. (2-tailed significance value should be <0.05 for a test to be significant).

Table 31: T-test result of fluorometric assay using ΔN coated beads

		Levene's test for equality of variances		t-test for equality of means		
		F	Sig	t	df	Sig. (2-tailed)
N FLISA	Positive	15.942	0.000	2.147	47	0.037
	Negative			2.019	22.72	0.056
cELISA	Positive	0.031	0.862	14.729	47	0.000
	Negative			14.676	45.481	0.000

Table 32: T-test result of fluorometric assay using cocktail of protein coated beads

		Levene's test for equality of variances		t-test for equality of means		
		F	sig	t	df	Sig. (2 -tailed)
Cock FLISA	Positive	0.583	0.449	5.104	47	0.000
	Negative			5.062	44.097	0.000
cELISA	Positive	0.31	0.862	14.729	47	0.000
	Negative			14.676	45.481	0.000

Discussion

5 DISCUSSION

A number of infectious diseases affecting sheep and goat imperil their optimal productivity. Among them, *Peste des petits ruminants* (PPR) is of prime importance disease. PPR is an acute, highly contagious viral disease and included in the notifiable terrestrial animal diseases of OIE (Office International Des Epizooties), the World Organization for Animal Health. The methods for detection, prevention and control of PPRV vary widely depending on local facilities and techniques used. Researchers are working towards the development of diagnostics for early and specific diagnosis of PPR. The recommended specimens from live animals are swabs of conjunctival discharges, nasal secretions, buccal and rectal mucosae, and anticoagulant-treated blood for antigen detection, serum samples for detection of antibodies (**OIE, 2013**). In this study, an attempt was made to develop recombinant antigen fabricated surface for detecting *Peste des petits ruminants* virus specific antibodies.

PPRV is an enveloped virus and has helical nucleocapsid. The nucleocapsid have characteristic "herring-bone appearance". The PPRV genome is organized into six transcriptional units encoding six structural proteins viz., the nucleocapsid (N) protein, the matrix (M) protein, the polymerase or large (L) protein the phosphoprotein (P), two envelope proteins [haemagglutin (H) and fusion (F) protein] and two nonstructural proteins (V, C). The gene arrangement from 3' to 5' on the genome is N-P-M-F-H-L separated by intergenic region (Baron and Barret, 1995; Diallo 1989). Many authors have reported the expression and purification of PPRV proteins to be used for vaccine development and for diagnostic purposes. Production and characterization of PPRV proteins immunodominant regions were undertaken in this study. Immunodominant regions in F, H and M genes PPRV genome were identified using

bioinformatics tools and based on previous data available in lab and cloned into pET32b vector. pET series vectors are commonly used vectors for expression of proteins and these vectors are of low copy number since they have pMB1 origin (Bolivar *et al*., 1977). For expression of these proteins BL21 DE3 strain of *E. coli* was used. Two truncated F proteins were expressed in BL21 DE3 strain. Among all the morbilivirus, the F protein (59.137 kDA) is one of the highly conserved proteins (Taylor and Abegunde, 1979). Wang and colleagues (2013) expressed and purified two regions of F gene in pET30 (a) vector. They used 0.4 mM IPTG for the expression of these recombinant proteins with an induction period of 6 h at 37^0C. They assessed the reactivity of polyclonal antibody raised against F antigen in rabbit by indirect ELISA and Western Blot. In our experiment, induction with 1 mM IPTG concentration showed optimum expression in pET32b plasmid. H protein was also expressed in the present study as two truncated proteins namely H1 (N terminal) and H2 (C terminal) upon induction with 1 mM IPTG for 6h at 37^0C. Since the protein was expressed in insoluble fraction of cell lysate, the protein was solubilized using 8 M urea. Seth and Shaila (2001) studied the expression of H protein in mammalian expression system using pCMX vector. Sinnathampy *et al*., (2001) cloned the H proteins in pRSETC vector and expressed in BL21 DE3 *E.coli* strains as his tag fusion proteins. They have expressed the fusion protein at 37^0C incubation temperature for 4 h with IPTG concentration 0.4mM. The recombinant protein was expressed in insoluble fraction of cell lysate and hence purification of recombinant proteins was under denaturing conditions. Immunodominant region of M gene was also expressed using conditions similar to that of H1 and H2. Expression of two truncated proteins of M gene was expressed in BL21 DE3 after cloning in pET30 (+) vector by Liu *et al.,* (2013). The expression conditions used were 28^0C, 12 h and concentration of IPTG was 0.2mM and purification was under denaturing conditions. Owing to the insolubility of the proteins, the expressed proteins, F1, F2, H1, H2 and ΔM proteins were purified as his tag protein under denaturing conditions. In order to confirm the immunoreactivity of recombinant proteins, dot blot using PPR positive sera from different goats was pooled and used as primary antibody. Presence of distinct brown dot indicated reactivity with each protein. The immunoreactivity of the proteins was further tested and confirmed by indirect ELISA using PPRV positive sera. Recombinant ΔN protein, which was expressed previously in the lab, was also purified. The ΔN protein, was found in soluble fraction and was

purified under native condition. Similar expression and purification study was observed by Yadav *et al.,* (2009). These proteins along with N proteins were dialyzed in PBS to refold the protein.

Coupling of purified recombinant proteins to a solid surface for use in detecting PPRV specific antibodies was the next challenge. Many authors have reported the efficacy of various surfaces for binding bioactive molecules for different purposes. Physical adsorption of antibodies is the most simple and probably one of the most commonly used immobilization methods; however, as noted in a number of publications, a low surface activity was detected for antibodies physically adsorbed on silanized surfaces, of which <15% of antibodies were active (Chang *et al.,* 1995). Seo *et al.,* (2011) also reported that while using covalent chemistry of acrylate/thiol modified surfaces higher density of molecule can be assembled in comparison with physisorption methods. Zammatteo *et al.,* (1997) studied the efficacy of binding capture DNA probes on magnetic beads and PS microwell plate for detecting human cytomegalovirus DNA amplicons. The comparative study revealed that high amounts of DNA was found to couple the beads than the plates; on account of increased surface area. This indicated the use of beads for coupling of recombinant proteins. Glass beads were selected as the surface for coupling of the recombinant proteins produced in the lab. Glass beads of size 425-600 μm were used for coupling. Glass beads were cleaned using piranha solution (H_2SO_4: 30 % H_2O_2 in 3:1 ratio). The glass beads were immersed in piranha solution for 30 min. Then beads were washed successively in distilled water and ethanol. Zammatteo *et al.,* (2000) described the washing of glass slide in piranha solution for 30 min and the washing in DW and boiled DW for making the surface organic free and dried. Arslan *et al.,* (2006) used piranha solution for cleaning glass beads surface for 1h and then rinsed in high purity Millipore water. 20 min protocol for cleaning the glass chips using piranha solution was reported by Vistas *et al.,* (2013). Cleaning of glass slide in piranha solution for 30 min and then rinsing in ethanol and DW was also used by Jang and Liu (2009). Thus there are several reports on the use of piranha solution for cleaning glass surfaces. The glass beads used in our study were cleaned using this procedure. The boiling piranha solution generates reactive groups which cleans the surfaces and makes them organic impurity free.

Following cleaning and drying the glass beads were subjected for the production of

SAM layers. For producing SAM layers APTS and MPTS chemistries were tried. For producing APTS SAM layer the glass beads were immersed in 10 % APTS solution in ethanol for 10 min with end to end rocking. Then washed in ethanol and DW and dried under vacuum. Arslan *et al.,* (2006) used 1 % APTS solution in anhydrous toluene for SAM layer preparation for 24 h. Following the incubation in APTS washed in anhydrous toluene, DW and then in ethanol and then dried in nitrogen atmosphere. Qin *et al.,* (2007) reported silanization of glass slides using 5% APTS in anhydrous toluene for 5 min and the washing in anhydrous toluene and acetone followed by drying. Taylor *et al.,* (2005) used 10% APTS solution in toluene to aminate the glass surface. Jang and Liu (2009) used 10 % APTS solution in ethanol to prepare silane layer on the glass slide. The treatment of glass which already has –OH groups, generates $–NH_2$ surface.

MPTS functionalization of glass beads were carried out by immersing the cleaned glass beads in 2 % MPTS solution in ethanol for 2 h with end to end rocking. Then the beads were washed successively in ethanol and DW and dried in vacuum. Vistas *et al.,* (2013) compared different concentration of MPTS in acetone-1 %, 2.5 % and 5 %- at different temperature and reported that 2.5 % MPTS for 2 h showed effective silanization of glass slides to generate thiol groups. Rogers *et al.,* (1999) studied silanization of glass slides in 1 % MPTS in ethanol and 16 mM acetic acid for 30 min. Similarly Bhatia *et al.,* (1989) used 2 % MPTS solution in toluene for silanization at room temperature for 2 h. The treatment of glass surface with MPTS effectively generates thiol groups.

Following the silanization of glass beads with APTS, many authors have tried different chemistries further for coupling of bioactive molecules. Some reports indicate, that short spacers, such as APTES, are not sufficient to keep the large antibody molecule away from the surface and overcome steric hindrance from the vicinity of the support. Atomic force microscopy (AFM) images of IgG immobilized using APTES-GA revealed the random packing of IgG aggregates, probably partially denatured (Weiping *et al.,* 1999). Stark and Holmberg (1989) used PEG spacer after APTS functionalization. Gluteraldehyde activation was tried in this study after APTS SAM layer formation. 10 % gluteraldehyde in PBS was used for treating the glass beads. Glass beads were kept in this solution overnight. Then washed with PBS to remove unbound gluteraldehyde. Jang and Liu (2009) used 10 % gluteradlehyde. Marquette *et al.,* (2002) has reported use of 25 % gluteraldehyde in PBS after APTS functionalization

for covalent immobilization. However, Wadu-Mesthrige *et al.,* (2000) reported that covalent coupling of protein on amine terminated surfaces using gluteraldehyde has a drawback that both ends of gluteraldehyde can be reacted with the amine surface.

In MPTS monolayered beads, which were thiol terminated, 11-mercaptoundecanoic acid was used to generate –COOH group on surface. 1mM ethanolic solution of MUA was added to the beads and incubated overnight to generate carboxyl groups. MUA treatment is commonly used in SPR (Surface Plasmon Resonance) protocols where the gold chips are carboxylated using MUA. Frey and Corn (1996) produced MUA layer on the gold chips silanized using MPTS. They reported the 1 mM concentration of MUA in ethanol for 24 h to make it carboxyl terminated whereas Yu and Irudayaraj (2007) used 20 mM ethanolic solution of MUA for 3 h. We achieved successful –COOH group generation on glass beads by reacting with MUA.

Next step in both chemistries was coupling of recombinant proteins. In aldehyde terminated surfaces which had been silanized using APTS, ΔN protein in PBS was added and the concentration of the protein was 500 μg/ml. This protein solution was added and incubated for 5 h under refrigerated conditions. Yakovleva *et al.,* (2002) used 1 mg/ml of protein in borate buffer (pH 5.0) for binding to the gluteraldehyde treated aminated surface. Although, concentration of 0.02 mg/ml of protein is sufficient for protein immobilization studies as reported by Cullen and Lowe (1994), Norde *et al.* (1995), Patel *et al.* (1997), Wadu -Mesthrige *et al.,* (2000). Jang and Liu (2009) has used 0.333 mg/ml of protein in PBS for immobilizing antibodies.We used slightly higher concentration (500 μg/ml) for coupling our protein on to beads

MPTS silanized beads with carboxyl termini after MUA treatment were treated with EDC-NHS for activating these terminal carboxyl groups. 100 mM NHS and 400 mM EDC was used for activating terminal carboxyl groups. Proteins at a concentration 100 μg/ml, equilibriated in sodium acetate buffer of pH 4.5 were used for coupling on modified surfaces. Wadu- Mesthrige (2000) studied the immobilization of BSA in sodium acetate buffer of pH 4.5 on MUA SAM layers and found to be effective method of coupling BSA to SAM layer at this pH. Ahmed and Moore (2012) reported 50 mM EDC and 50 mM NHS to activate carboxyl group on gold surfaces. Similarly, Krazinski *et al.,* (2011) reported 100 mM EDC and 50 mM NHS for activating carboxyl group on gold chip for use in SPR. However, Jang *et al.,* (2006) used 100 mM EDC and 200 mM NHS for protein coupling on polydimethoxy silane surface.

MES buffer was used for coupling of protein to EDC- NHS activated surface by Krazinski *et al.,* (2011) and Jang *et al.,* (2006). We could achieve protein coupling of approximately 50-60 ng per bead after treatment of MPTS functionalized beads with MUA and EDC-NHS activation.

The immunoassay used to test serological activity of antigen coated on beads were fluorometric assay and colorimetric assay. Fluorometric assay showed better result than the colorimetric assay using anti-goat HRPO conjugate. Many authors relied on the fluorometric assay for confirming the serological activity of proteins coated on solid surfaces (Inerowicz *et al.,* 2000; Qin *et al.,* 2007; Jang and Liu, 2009). Sufficiently sensitive detection of fluorescence emission permits even single molecule detection (Dickson *et al.,*1995) as it is evident from the fluorescence result even single antigen coated beads is sufficient for differentiating positive and negative serum samples. Alkaline phosphatase labeled secondary antibodies were used to determine the immunoreactivity of protein coated on surfaces (Lyashchenko *et al.,* 2000; Jang *et* al., 2006; Ahmad and Moore, 2012).

Many researches were oriented towards developing a diagnostic assay for PPR virus. cELISA is most commonly used assay for diagnosis of PPR now a days. cELISA test developed by Singh *et al.,* (2004a) reported a high specificity of 98.4 % and sensitivity of 92.2 %. Raj *et al.,* (2008) used antigen competition ELISA using truncated N proteins and immunofitration test to detect PPR antibodies. They reported sensitivity of 80% and specificity of 100% on comparison of the immunofiltration test with the antigen-competition ELISA. Libeau *et al.,* 1995 developed a competitive ELISA using recombinant N protein and sensitivity and specificity they got was 94.5 % and 99.4 % in relation to VNT. Fluorometric assay developed in this study showed more sensitivity and specificity. Sensitivity of single antigen coated assay (ΔN) coated fluorometric assay was 65 % and specificity was 65.5 %. On using the cocktail of proteins coated beads sensitivity of fluorometric assay increased to 79.3 % and specificity to 95 %. Multiantigen printed immunoassay for detecting different antigens of *Mycobacterium tuberculosis* was reported by Lyashchenko *et al.,* (2000). They have printed the cocktail of Mycobacterial antigens on a nitrocellulose membrane. Their study showed that the serological activity of multiple antigens were more than that showed in ELISA. On comparison to cELISA fluorometric assay could give the result in a span of 2 h and could be completed at room temperature. In our study, we used fluorescence microscopy for interpreting results. However,

the future course of action could be to use some simple laser light source which excites in visible region to give visible colour or some colorimetric insoluble substrate like ODN or chloronaphthol can be used to give visible results. Alternatively, the colloidal gold conjugate particles can be used to give visual signals. Therefore, we can conclude that the assays developed in our study using cocktail of proteins can be modified further to make them more user friendly and field applicable for providing rapid on-farm diagnosis of PPR especially in the light of National PPR Eradication programme launched by the Government of India.

Summary and Conclusions

6 SUMMARY AND CONCLUSIONS

India is one of the fastest growing economies in the world and the livestock industry contributes 4.36% of the total GDP. The small ruminant sector plays an important role in the sustainable livelihood of the poor and marginal farmers. Infectious diseases significantly affect the productivity of this industry. Among them, peste-des-petits-ruminants (PPR) is ranked on top and is one of the targets for global eradication by Food and Agricultural Organization (FAO) and World Animal Health Organization (OIE). Presently, PPR is mainly circulating in Western and Central Africa, the Arabian Peninsula and Southern Asia including India. Study in India on serosurveillance of PPR in sheep and goats reveals the 41.01% and 46.11% case respectively with an overall prevalence of 43.56 %. This indicates the increased and widespread infection with the virus in India that gives the endemic status for PPR. Significant research has been directed toward developing improved vaccine, diagnostics and epidemiology of the virus in recent years on account of rapid increase in disease spectrum and distributions patterns. Present study attempted to develop new generation improved diagnostic assay using recombinant protein fabricated surface for detecting antibodies against PPR.

Immunogenic regions of PPRV genes, fusion (F), heamagglutinin (H), matrix (M) proteins were identified and cloned and expressed pET32b vector. C terminal and N terminal immunodominant regions of H and F genes and C terminal region of M antigen were expressed using the expression strain of *E.coli* BL21 DE3. Conditions for optimal expression of the recombinant proteins were standardized. Ni-NTA affinity chromatography was used for purification of these recombinant proteins. Recombinant proteins expressed as His tag fusion protein are found to be insoluble, hence purified under denaturing conditions. The truncated N

protein of PPRV (already cloned and expressed in bacterial system, available in the lab) was also purified and scaled up. Purified recombinant proteins were confirmed by western blot with Ni-NTA HRPO conjugate. Serological activity of the purified proteins was also confirmed by dot blot using pooled PPRV positive serum. The recombinant proteins were dialyzed in PBS to remove urea and for further studies.

Glass remains as the most commonly used substrate for protein arrays, though various other solid surfaces like polydimethoxysilane, polyester, metal are also in market, due to its readily availability and cost concern. Glass sides are highly sensitive and owing to its transparency, it is highly useful in detection analysis on microarrays. Compared to glass slides, glass beads have increased surface area to volume ratio. Glass beads of size 425-600 μm was used for protein fabrication. Piranha solution was used for cleaning glass surface and also rendered the surface hydroxyl terminated. Two surface modification chemistries were studied on glass surface- 3-aminopropyltriehoxysilane (APTS) and 3-mercaptopropyltriethoxysilane (MPTS). Piranha washed beads were treated by ethanolic solution of MPTS and APTS to make surface thiol and amine terminated respectively. MPTS modified beads were further treated with MUA to make carboxyl terminated which was further activated using EDC-NHS solution for protein coupling. In case of APTS beads, gluteraldehyde treatment was done for protein coupling. Truncated N protein (ΔN) was used initially for optimizing the glass bead based immunoassay. Compared of immunoassay using ΔN functionalized on APTS, MPTS modified beads showed better reactivity. Optimization of serum and anti-goat HRPO conjugate dilution was analyzed using checkerboard titration studies. Fluorometric immunoassay was also optimized using ΔN functionalized MPTS modified beads. Fluorescence intensity was visualized using fluorescence microscope at 4X magnification and documented. The fluorescence intensity was measured using Imagej software. Serological reactivity of ΔN functionalized on MPTS modified beads were confirmed by hyperimmune serum and preimmnune serum raised in rabbit against ΔN. A panel of positive and negative goat serum sample was obtained by cELISA screening and this panel was used for testing the efficacy of developed colorimetric and fluorometric immunoassay. To increase the sensitivity of the test, a cocktail of recombinant proteins pooled in equal concentration was coupled on MPTS modified beads and different samples were tested.

A total of 234 serum samples were screened using cELISA to get a panel of positive and negative goat serum. Out of this 77 serum samples were found positive and 157 were negative. This panel of serum samples was used to test the diagnostic effectiveness of the immunoassay developed in this study. The result of the developed immunoassay using ΔN coated and cocktail of protein coated beads is as follows:

- 142 samples were tested by colorimetric assay using ΔN functionalized beads out of which 65 were positive and 77 were found to be negative.
- Out of 49 samples tested by colorimetric assay using cocktail of protein coated beads 22 samples were positive and 27 were negative.
- In fluorometric assay using ΔN coated beads 49 samples were tested. In this 20 samples were positive and 29 samples were negative.
- 49 samples tested using cocktail of protein coated beads via fluorometrically, out of which 29 samples were positive and 20 samples were found as negative.

Sensitivity of fluorometric assay using ΔN coupled beads was 65 % and specificity was 65.5 % which was increased to 79.3 % sensitivity and 95 % specificity when cocktail of protein coated beads were used. This immunoassay could be conducted at room temperature and result would be available in a span of 2 h in comparison to other immunoassays. Using the baseline data obtained in this experiment, the future course of action could be:

- Protein array using multiple recombinant proteins can be developed for detecting antibodies to different proteins of PPR virus
- Glass slide or nitrocellulose membrane or functionalized beads can be used to develop multiplexed assay
- Regeneration conditions can be optimized for reusing protein coated beads to economize the assay
- Studies on shelf life and stability of protein coated beads can be taken up
- Protein specific antibodies can be developed for detecting virus or viral antigens in immunoassay or immunohistochemistry
- Similar studies can be taken up with other recombinant proteins to develop a multiple disease detection system

Mini Abstract

7 MINI ABSTRACT

Sheep and goat contribute a major share to the livelihood of poor and marginal farmers in our country. Any disease affecting the performances of these small ruminants reflect on their day to day living. A number of infectious and non-infectious diseases affect small ruminants resulting in severe economic losses. Among the infectious diseases of small ruminants, Peste-des-petits-ruminants (PPR), an acute, highly contagious viral disease is of prime importance. Many countries have experienced cumulative losses ranging from tens to hundreds of millions of US dollars annually due to this highly spreading disease. OIE has emphasized that eradicating PPR will help improve food security, nutrition, incomes and livelihood resilience of millions of poor farmers around the world. Specific, unambiguous and rapid diagnosis of PPR is therefore, mandatory for early eradication of this highly contagious disease. Researchers are working towards developing newer methods for early and specific diagnosis of PPR. In this study an attempt was made to fabricate solid surface with immunodominant epitopic regions of PPRV antigens expressed in bacterial system as recombinant protein. Glass beads, owing to the low cost of procurement and easily amenable were selected as the surface of choice for covalent coupling of recombinant proteins. Epitopic regions of PPRV antigens namely fusion (F), heamagglutinin (H), matrix (M) and nucleocaspid (N) proteins were cloned and expressed in prokaryotic expression system and purified using Ni-NTA affinity chromatography. The recombinant proteins were characterized for reactivity by western blot and dot blot. 3-aminopropyl triethoxy silane (APTS) and 3-mercaptopropyl triethoxy silane (MPTS) chemistries were studied for functionalizing the glass beads. Immunoassay based on recombinant nucleocaspid protein coupled on glass beads revealed that MPTS chemistry showed better reactivity towards the coupled protein than APTS chemistry. Fluorometric and colorimetric immunoassays were optimized using the glass beads coupled with recombinant nucleocaspid protein. The developed assay could be performed in 2 h time period. Positive and negative panels of serum samples obtained screening a number serum samples by cELISA were further used to test the efficacy of the developed immunoassays. Fluorometric immunoassay showed better sensitivity and specificity than colorimetric assay. To increase the sensitivity and specificity of the immunoassay, a cocktail of recombinant protein pooled in equal concentration

was used to functionalize MPTS modified beads. Serum samples were screened by this cocktail of protein coated beads. A cocktail based serological assay helps in the diagnosis of infectious disease characterized by heterogenous antibody repertoire; (eg. PPRV) as indicated by the increased sensitivity and specificity when cocktail of proteins were used for screening samples. The sensitivity of truncated N based assay (65 %) was increased to 79.3 % and specificity reached upto 95 % from 65.5 % when a cocktail of proteins were used as antigen. The developed immunoassay offered many advantages like reduced labor, time and easiness in conducting the immunoassay. Therefore, the fluorometric assay developed in this study can be further applied for developing a multiple antigen protein assay/chip for multiplexed detection of antibodies directed against different protein of PPR. Further there is a scope for developing a multiple disease detection system using this methodology.

लघु सारांश

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हमारे देश में गरीब और सीमांत किसानों की आजीविका के लिए भेड एवं बकरी का प्रमुख योगदान है। इन लघु रोमन्थियों की उत्पादकता को प्रभावित करने वाली कोई भी बीमारी, किसानों की जीविका को प्रभावित करती है। विभिन्न प्रकार के सक्रामक एवं गैर–संक्रामक रोग इन पशुओं को प्रभावित कर आर्थिक नुकसान पहुंचाते हैं। लघु रोमन्थियों को प्रभावित करने वाले संक्रमण रोगों में Peste-des-petits-ruminants (पी.पी.आर.) एक तीव्र, अत्याधिक संक्रामक विषाणु जनित रोग है जोकि बहुत से देशों में कई अमरीकी डॉलर का सालाना नुकसान पहुंचाता है। OIE ने भी जोर दिया है कि पी.पी.आर. उन्मूलन दुनिया भर के किसानों की खाद्य सुरक्षा, बेहतर पोषण, आय एवं आजीविका में सुधार करने में मदद करेगा। पी.पी.आर. के उन्मूलन के लिए बीमारी का विशिष्ट, स्पष्ट एवं शीघ्र निदान अत्यंत आवश्यक है। इस अध्ययन में पी.पी.आर विषाणु के इम्युनो प्रमुख मिलान क्षेत्रों का पुनः संयोजक प्रतिजन तैयार किया गया एवं इसकी ठोस सतह पर गढना कर पी.पी.आर विशिष्ट एंटीबॉडी के परीक्षण के लिए एक परख का विकास किया गया। कांच के मोतियों को पुनः संयोजक प्रतिजन के सहसंयोजक युग्मन के लिए चयन किया गया। पी.पी.आर. विषाणु के प्रतिजनों अर्थात Fuson (F), haemagglutination (H), Matrix (M) एवं Nucleocapsid (N) के इम्युनो प्रमुख क्षेत्रों को क्लोन कर जीवाणु अभिव्यक्ति प्रणाली में व्यक्त किया गया एवं Ni-NTA आत्मीयता क्रोमेटोग्राफी का उपयोग कर शुद्ध किया गया। पुनः संयोजक प्रतिजन का परीक्षण वेस्टर्न एवं डॉट ब्लॉट तथा ELISA विधि द्वारा किया गया। इसके पश्चात कांच की सतह पर पुनः संयोजक प्रतिजन को APTS एवं MPTS रासायनिक विधि द्वारा युग्मित किया गया। इस अध्ययन में यह पता चला कि MPTS विधि द्वारा तैयार किये गये परख ने APTS विधि द्वारा तैयार परख की अपेक्षा बेहतर प्रर्दशन किया। कांच के मोतियां पर पुनः संयोजित (N) प्रतिजन गढ़कर फ्लोरोमेट्रिक एंव वर्णमिति–इम्युनो परख का विकास किया गया। इस परख से 2 घंटे की समय अवधि में प्रदर्शन किया जा सकता है। विकसित किये गये परखों की प्रभावकारिता की जांच हेतु बकरियों के सीरम नमूनों को c-ELISA द्वारा परीक्षण कर सकारात्मक एवं नकारात्मक सीरम पैनल तैयार किया गया। फ्लोरोमेट्रिक परख के वर्णमिति परख की तुलना में बेहतर संवेदनशीलता एवं विशिष्टता दिखायी। परख की संवेदनशीता एवं विशेषता को बढ़ाने के लिए, पुनः संयोजक प्रतिजनों को बराबर एकाग्रता में मिला कर MPTS विधि द्वारा तैयार कांच की मोतियों को गढ़ा गया एवं सीरम नमूनों की जांच की गई। जब N प्रतिजन लिपटे कांच के मोतियों द्वारा सीरम के नमूनों की जांच की गई तो संवेदनशीलता और विशिष्टता क्रमशः 65 प्रतिशत एवं 65.5 प्रतिशत पायी गई। जबकि F, H, M एवं N पुनः संयोजित प्रतिजनों के मिश्रण में लिपटे कांच के मोतियों द्वारा सीरम के नमूनों की जांच करने पर परख की संवेदनशीलता एवं विशिष्टता क्रमशः 79.3 एवं 95 प्रतिशत पाई गयी। इस अध्ययन में पी.पी.आर. की जांच के लिए विकसित फ्लोरोमेट्रिक प्रतिरक्षा परख सुगमता से कम समय एवं कम श्रम में परिणाम देने में सक्षम है। विकसित फ्लोरोमेट्रिक परख को आगे एक बहु पुनःसंयोजित प्रतिजन परख/लिप्लेक्स प्रोटीन चिप के रूप में पी.पी.आर. विषाणु के विभिन्न प्रोटीन के खिलाफ निर्देशित एंटीबाड़ी का पता लगाने के लिए विकास किया जा सकता है। भविष्य में इस अध्ययन में विकसित पद्धति पर आधारित विभिन्न रोगों के समकालिक निदान हेतु परख का विकास करने की संभावनाएं हैं।

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Appendix

Reagents used in Agarose Gel Electrophoresis

Tris -acetate-EDTA (TAE) buffer 50X

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Add DW to make final volume upto 1000 ml. A working solution of 1X is used.

Ethidium bromide stock solution (10 mg/ml)

Ethidium bromide	100 mg
DW	10 ml

The solution is mixed and stored in 4^0C. A concentration of 0.5 µg/ml is used in preparing agarose gel.

Reagents used for Bacteriological Procedures.

LB (Luria Bertani) broth

LB broth	2.5 g
DW	100 ml

Sterilize by autoclaving at 15 lbs for 15 min. Store at room temperature.

LB agar

LB agar	4 g
DW	100 ml

Sterilize by autoclaving at 15 lbs for 15 min. Store at room temperature.

SOC medium

SOC medium (powder)	3.41 g
DW	100 ml

Sterilize by autoclaving at 15 lbs for 15 min. Store at 4^0C.

Ampicillin (100 mg/ml)

Ampicillin	500 mg
DW	5 ml

Sterilize by filtration through 0.22 µ filter and store at -20^0C.

IPTG (100 mM)

IPTG	238 .3 mg

DW	10 ml

Sterilized by filteration through 0.22 µ filter and store at -20^{0}C.

Solution for Plasmid Isolation

P1 (Resuspension) buffer

Glucose	50 mM
TrisCl (pH 8.0)	25 mM
EDTA (pH 8.0)	10 mM

Solution is sterilized by autoclaving.

P2 (Lysis) buffer

NaOH	0.2 N
SDS	1 %

Freshly prepared.

P3 (Neutralization) buffer

5M Potassium acetate (pH 5.5)	60 ml
Glacial acetic acid	11.5 ml
Double DW	28.5 ml

Sterilized by 0.22 µ filter and stored at 4^{0}C.

Phenol: Chloroform: Isoamyl alcohol (25:24:1)

Tris-saturated phenol	25 ml
Choloroform	24 ml
Isoamyl alcohol	1 ml

Mix thoroughly and store at 4^{0}C in dark container.

Reagents for ELISA

Washing buffer (PBS-T)

PBS (pH 7.4)	100 ml
Tween- 20	50 µl

Coating buffer

Sodium carbonate (15 mM)	1.6 g
Sodium bicarbonate (35 mM)	2.9 g
Distilled water	1000 ml

Adjust pH to 9.5 and store at 4°C

Blocking buffer

Skimmed milk powder	5 g
Washing buffer (PBS-T)	100 ml

ELISA Substrate

1. Phophate Citrate buffer (pH 5.0)

Citric acid	0.52 g
Di-sodium hydrogen phosphate	1.78 g
Distilled water	100 ml

Adjust pH to 5.0 and store at 4°C

2. Substrate solution

Phosphate Citrate buffer (pH 5.0)	10 ml
H_2O_2 (30%)	4 µl
O-phenylene diamine dihydrochloride (OPD)	10 mg

Make fresh at the time of use.

Reagents for SDS PAGE and Western Blot

Acrylamide-bis-acrylamide solution (30%)

Acrylamide	29.8 g
N,N-bismethylene acrylamide	0.8 g

Make volume to 100 ml with distilled water, filter and store at 4°C in amber coloured bottle.

1.5 M Tris HCl, pH 8.8

Tris base	18.17 g
SDS	0.4 g

Adjust to pH 8.8 with 10 N HCl and make volume to 100 ml with distilled water. Store at 4°C.

1 M Tris HCl, pH 6.8

Tris base	3.025 g

Adjust to pH 6.8 with 10 N HCl and make volume to 100 ml with distilled water. Store at 4°C.

20% Ammonium persulphate (APS) (w/v)

Ammonium persulphate	2 g
Distilled water	10ml

Store at -20^0C for 1 week

Electrophoresis buffer (10x)

Tris base	30.0 g
Glycine	144.0 g
SDS	10.0 g
DW	900 ml

Adjust the pH to 8.3. Make volume to 1000 ml with distilled water.

2x Lamelli buffer

1 M Tris (pH 6.8)	1.7 ml
10% SDS	4.5 ml
Glycerol	1.00 ml
Bromophenol blue	2 mg

Make volume to 10 ml with distilled water and store at 4 °C

Separating Gel (12 %) (10 ml)

DW	3.3 ml
Acrylamide/bisacrylamide (30 %)	4 ml
1.5 M Tris (pH 8.8)	2.6 ml
10 % SDS	100 µl
20 % APS	100 µl
TEMED	5 µl

Stacking gel (5 %) (2 ml)

DW	1.3 ml
Acrylamide/bisacrylamide (30 %)	330 µl
1 M Tris (pH 6.8)	250 µl
10 % SDS	20 µl
20 % APS	20 µl
TEMED	2 µl

Staining Solution (0.25%)

Coomassie brilliant blue R-250	0.5 g
Methanol	90 ml
Glacial acetic acid	20 ml

DW	90 ml.

Filter through Whatman filter paper No. 1 and store at room temperature in amber colored bottle.

De-staining solution

Methanol	150 ml
Glacial acetic acid	50 ml
Distilled water	300 ml

Store at RT.

Transfer buffer (pH 8.3)

Tris base	8.7 g
Glycine	35.85 g
SDS	.555 g

Dissolve in 1000 ml of DW

Methanol	300 ml

Volume makes upto 1.5 L with DW.

DAB solution 100 mM Tri (pH 7.6)

100 mM Tris	1.21 g
DW	100 ml

Substrate Solution

3,3'-Diaminobenzidine	7.0 mg
100 mM Tris	10 ml
H_2O_2 (30 %)	10 µl

Buffers used for Purification under Denaturing Conditions

Lysis or Equilibriation buffer

100 mM Sodium dihydrogen phosphate	1.38 g
10 mM Tris HCl	0.12 g
8 M Urea	48.048 g

Final volume makes upto 100 ml with autoclaved double DW. Adjust the pH to 8.0 with NaOH.

Wash buffer

100 mM Sodium dihydrogen phosphate	1.38 g

10 mM Tris HCl	0.12 g
8 M Urea	48.048 g

Final volume makes upto 100 ml with autoclaved double DW. Adjust the pH to 6.3 with HCl.

Elution Buffer

100 mM Sodium dihydrogen phosphate	1.38 g
10 mM Tris HCl	0.12 g
8 M Urea	48.048 g

Final volume makes upto 100 ml with autoclaved double DW. Adjust the pH to 4.5 with HCl.

Buffers used for Purification under Native Conditions

Lysis or Equilibriation buffer

50 mM sodium dihydrogen phosphate	1.38 g
300 mM Sodium Chloride	3.508 g
10 mM Imidazole	0.136 g

Final volume makes upto 200 ml with autoclaved double DW. Adjust pH to 8.0 using NaOH.

Wash buffer

50 mM sodium dihydrogen phosphate	1.38 g
300 mM Sodium Chloride	3.508 g
50 mM Imidazole	0. 68 g

Final volume makes upto 200 ml with autoclaved double DW. Adjust pH to 8.0 using NaOH.

Elution buffer

50 mM sodium dihydrogen phosphate	1.38 g
300 mM Sodium Chloride	3.508 g
500 mM Imidazole	6.808 g

Final volume makes upto 200 ml with autoclaved double DW. Adjust pH to 8.0 using NaOH.

Dialysis Buffer

Phosphate buffered saline (PBS 1X) (10 mM)

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Disodium hydrogen phosphate	1.44 g
Potassium dihydrogen phosphate	0.24 g

DW	800 ml

Adjust pH to 7.4. Volume makes upto 1000 ml.

Reagents and Buffers used for Glass Bead Assays

Piranha solution

H_2O_2 (30 %)	2 ml
H_2SO_4	6 ml

MPTS (2 %)

MPTS	30 µl
Absolute ethanol	1470 µl

APTS (10 %)

APTS	150 µl
Absolute ethanol	1350 µl

Gluteraldehyde (10 %)

Gluteraldehyde	200 µl
1 X PBS	1800 µl

11-Mercaptoundecanoic acid (MUA) 1 mM

MUA	11 mg
Absolute ethanol	50 ml

Sodium acetate buffer (pH 4.5) 10 mM

10 mM Sodium acetate (anhydrous)	82.03 g
DW	95 ml

Adjust pH to 4.5 using acetic acid. Make final volume to 100 ml with DW.

Ethanolamine (pH 8.5) 1M

1 M Ethanolamine	600 µl
DW	10 ml

Adjust pH to 8.5 with HCl

EDC 400 mM

EDC	153.4 mg
DW	2 ml

NHS 100 mM

NHS	23 mg

DW	2 ml
PBST (0.05 %)	
IX PBS	100 ml
Tween 20	50 µl
Bovine Serum Albumin (BSA) (1 %)	
BSA	10 mg
PBST	1 ml
Assay Substrate	
1.Phophate Citrate buffer (pH 5.0)	
Citric acid	0.52 g
Di-sodium hydrogen phosphate	1.78 g
Distilled water	100 ml
Adjust pH to 5.0 and store at 4°C	
2. Substrate solution	
Phosphate Citrate buffer (pH 5.0)	10 ml
H_2O_2 (30%)	4 µl
O-phenylene diamine dihydrochloride (OPD)	10 mg
Make fresh at the time of use.	

Equipments used for study

Sl. No.	Equipments	Company	Model
1	Thermal cycler	Thermoscientific, USA	Veriti™
2	Shaker Incubator	Eppendorf, USA	New Brunswick Innova™ (42)
3	Bench top centrifuge	Thermoscientific, USA	Sorvall™ Legend™ XT/XF
4	pH Meter	Horiba, Japan	Laqua bnechtop pH meter
5	Upright microscope	Life sciences,USA	OlympusBX53
6	Multimode plate reader	Perkin Elmer, USA	Enspire®
7	Gel documentation systems	Bio-Rad, USA	ChemiDoc™ MP
8	Refrigerated vacuum concentrator	Labconco,USA	Centrivap vacuum concentrator
9	SDS PAGE apparatus plus power pack	Bio-Rad, USA	Mini protean™ tetra cell Powerpac™ HC
10	—20^0 C freezer	Elanpro, India	EFSV 340
11	—80^0 C freezer	Eppendorf, Germany	New Brunswick™ U700 premium

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