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Expression and characterization of immunodominant region of fusion protein of peste des petits ruminants virus in *E. coli*

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ABSTRACT

The present study envisages expression of immunodominant ectodomain of peste des petits ruminants virus (PPRV) fusion (F) protein in *Escherichia coli* BL21 (DE3) and its characterization to assess its immunoreactivity. The ectodomain gene sequences corresponding to 222 amino acids, was amplified from PPR vaccine virus, cloned into pET33b vector and expressed in *E. coli* at an optimal temperature of 37 °C with 1 mM IPTG for 5 h. The expressed and Ni-NTA purified PPRV F protein (31 kDa) was characterized by SDS-PAGE and Western blot using anti-his-tagged-conjugate, anti-serum raised against recombinant PPRV F protein, hyper immune serum against whole PPRV and convalescent sera from sheep and goats. The expressed protein was assessed for its immunoreactivity by ELISA and immunoblotting. The antibody response mounted against the recombinant PPRV F protein in immunized rabbits was detected by recombinant PPRV F antigen based indirect ELISA, and whole virus antigen based indirect ELISA, which indicating the native confirmation of the expressed protein in *E. coli*. Indirect ELISA was optimized using known true positive and negative sera with respect to PPRV antibodies in order to assess the reactivity of the PPRV F protein in detecting PPRV F antibodies in small ruminants. The *E. coli* expressed recombinant ectodomain of PPRV F protein exhibits immunoreactivity and was able to specifically detect PPRV antibodies in response to both vaccination and disease in natural host.

1. Introduction

Peste des petits ruminants (PPR) or "goat plague" is a highly contagious transboundary disease of small ruminants and characterized by pyrexia, necrotic stomatitis, catarrhal inflammation of the ocular and nasal mucosa, enteritis and bronchopneumonia. It constitutes a serious constraint in developing small ruminant production in Africa, Asia and the Middle East where it is widespread and endemic in nature (Meyer and Diallo, 1995). Small ruminants are the main natural hosts of PPR virus (PPRV) although some other artiodactyls including camels and wildlife small-ruminants are also susceptible (Banyard et al., 2010).

The PPRV was classified in the *Morbillivirus* genus of family *Paramyxoviridae* and the viruses are known to be conserved across the genus with different species sharing similar characteristics (Parida et al., 2015). The PPRV genome is a single stranded negative sense RNA composed of 15,948 nucleotides, the longest of all *Morbillivirus* genomes sequenced so far (Bailey et al., 2005). It possesses six transcriptional units encoding eight proteins in the order of 3′_N-P/V/C-M-F-H-L_5′ and each gene is separated by inter-genic regions. Haemagglutinin (H) and fusion (F) are two surface glycoproteins and H glycoprotein interacts with a cellular receptor, whereas F glycoprotein allows the penetration of the virus into the cell. Based on their positions and functions, both F and H are very vital for the induction of protective immune response against the virus and most of the neutralizing antibodies are directed against these proteins.

Fusion protein gene of PPRV composed of 2405 nucleotides including the poly-(A) tail consists of 546 amino acids with a

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predicted molecular weight (MW) of 59.137 kDa (Dhar et al., 2006). F gene is considered as most conserved (Bailey et al., 2005; Dhar et al., 2006) and immunogenic, conferring protective immunity (Diallo et al., 2007). The paramyxovirus fusion protein normally synthesized as precursor F_0 and gets cleaved in to two subunits, F_1 and F_2 linked by a disulfide bond (Lamb and Kolakofsky, 2001). The cleavage is necessary for the virus to become fusogenic and infective (Kovamees et al., 1991). Of these, F_1 forms a membrane-anchoring subunit. This subunit has several conserved motifs among paramyxoviruses of which four are well studied: (1) a fusion peptide (FP) at newly generated N-terminus, (2) heptad repeat 1 (HR1), (3) another heptad repeat (HR2), and (4) the transmembrane (TM) domain. Of these conserved motifs, FP, HR1, and HR2 mediate fusion of the viral envelope and host cell membrane (Rahaman et al., 2003).

Several PPRV nucleocapsid (N), matrix (M) and H proteins were expressed in different heterologous system for assessing its immunoreactivity and evaluated (Yadav et al., 2009; Ismail et al., 1995; Choi et al., 2005; Liu et al., 2013; Balamurugan et al., 2006). Bacterial expression is perhaps the most economical and commonly employed expression vehicle for the production of recombinant proteins. It is relatively simple to manipulate, inexpensive to culture and less time consuming (Ian Hunt, 2005). Recently, Wang et al. (2013) expressed the F protein of PPRV without N-terminal signal peptide and transmembrane domain in *E. coli*, and assessed the immunoreactivity of expressed recombinant protein in western blot with rabbit polyclonal antibodies which were raised against the recombinant F protein. In the present study, F protein (lacking N-terminal fusion peptide, HR region 1 and C-terminal transmembrane domain), a highly immunogenic small region of ectodomain of PPR vaccine virus lineage IV (Sungri 96 strain) was selected to express it in *E. coli*. and the expressed F protein was characterized and assessed for its immunoreactivity in western blot and ELISA and showed specific detection of PPRV antibodies in sheep and goats.

2. Materials and methods

2.1. Construction of expression cassette in pET33b vector

The viral RNA was extracted from partially purified PPR vaccine virus (Sungri 96) using RNAeasy kit (RNeasy®Minikit Qiagen Inc, Valencia, CA, USA) and RT–PCR was carried out with random hexa primers (Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit). The F gene fragment was amplified from the generated cDNA, by PCR using *Pfu* DNA polymerase (MBI, Fermentas, MD, USA) and PPRV F gene-specific primers PPRVFF (*EcoRI*): 5′ CACC**GAATTC**CATG***CACCATCACCATCACCAT***GTCACATATGTGGAC ACAAGAG 3′ and PPRVFR (*Not I*) (His): 5′ CACC**GCGGCCGC*ATGGTGATGGTGATG GTG***TACCCCTTTAACAGTCTTCAG 3'modified from earlier reported primer (Rahman et al., 2003) which was designed based on published sequence (Accession # GQ452015.1). The cycling conditions include, preheating at 94 °C for 3 min followed by 10 cycles of 94 °C for 45 s, 50 °C for 1 min with an increment of 1 °C/cycle and 72 °C for 3 min then 25 cycles of 94 °C for 45 s, 60 °C for 1 min and 72 °C for 3 min with a final extension at 72 °C for 20 min. The PCR products were analyzed by electrophoresis and stained with ethidium bromide for documentation. The amplicons were purified using Gene JET™ Gel extraction kit (Fermentas, Life sciences, New Delhi, India) and were cloned directionally into pET33b vector (Novagen, Madison, WI, USA) at the *EcoRI* and *Not I* restriction sites. The recombinants were selected on LB agar with kanamycin (50 μg/ml). Later, the recombinant plasmid containing the target gene sequences were verified by colony PCR, restriction enzyme analysis and sequencing. The PPRV F gene sequences on the backbone of the pET vector were designated as pETPPRVF.

2.2. Expression of PPRV F immunodominant ectodomain

E. coli, BL21 (DE3) cells were transformed by heat-shock method with pETPPRVF recombinant plasmid and grown at 37 °C overnight on LB agar plates containing kanamycin (50 μg/ml). The transformed BL21 colonies were then screened for the presence of PPRV F gene specific sequences by PCR. The positive individual colonies were grown at 37 °C until the culture reached mid-log phase (OD600 nm of 0.4–0.5). The expression was induced at 37 °C by using 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG, Fermentas, USA) for 5 h. Samples were collected and were analyzed by SDS-PAGE for gene expression.

2.3. Optimization of expression conditions

The temperature, IPTG concentration and induction time for the expression of foreign proteins in *E. coli* BL21 (DE3) were optimized as per the procedures described by Liu et al. (2013). In brief, when OD of the culture reached 0.5 at 600 nm, after a vigorous shaking (200 rpm) at 37 °C, the culture was aliquoted and induced with IPTG under different temperature conditions (25, 27, 30, 35 and 37 °C) for an additional 5 h for optimization of temperature. Similarly, IPTG was added at different concentrations (0, 0.5, 1, 1.5, 2 mM) to each of the fresh culture (OD600 nm = 0.5) and was incubated for 5 h for optimization of IPTG concentration. For optimizing the induction time, cultures (OD600 nm = 0.5) were also incubated at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 h under different concentrations of IPTG and different induction temperatures. All induced cultures were pelleted, mixed with sample loading buffer (v/v: 1/1, Sigma) heated at 100 °C for 10 min for the analysis of expressed protein in 15% SDS–PAGE followed by Coomassie brilliant blue staining to identify the desired expressed protein.

2.4. Solubility testing

In order to test solubility of recombinant protein expressed, *E. coli* pellets obtained from 100 ml of cell cultures were resuspended in 5 ml lysis buffer of pH 8 (50 mM Tris HCl pH8, 300 mM NaCl, glycerol 10%, 5 mM β ME, 0.01% Triton X-100) containing 100 mM PMSF (Phenylmethylsulfonyl fluoride) and lysozyme (0.5 mg/ml). Soluble fraction was collected and the insoluble fraction was resuspended in 4 ml of solubilization buffer (20 mM Tris HCl pH8, 500 mM NaCl, 8 M urea, 5 mM β ME, 0.5% Triton X-100, 1 mM EDTA) and mixture was centrifuged at 10,000 rpm for 15 min to separate the insoluble fraction. The clear supernatant (soluble fraction) and insoluble fractions were collected and analyzed on 15% SDS–PAGE. Similar protocol was followed for pET 33b vector control.

2.5. Purification and refolding of PPRV F protein

The insoluble fraction after solubelization was mixed with 1 ml of Nickel NTA resin (Qiagen, Germany) which was equilibrated with 3 column volumes of binding buffer (Solubilization buffer). After washing the Ni-NTA bed thrice with wash buffer (Solubilization buffer), the bound recombinant His-tagged protein was eluted with imidazole containing elution buffer (Solubilization buffer with different concentration of imidazole-100, 300 and 500 mM imidazole) in 0.5 ml fractions. Purified his-tagged PPRV F protein fractions revealing single band were pooled together and dialyzed for renaturation and removal of imidazole in dialysis buffer (25 mM Tris HCl pH 8, 1 mM EDTA, 1 mM DTT, 5% glycerol) containing 8 M, 6 M, 4 M, 2 M, 1 M, 0.5 M, 0 M urea until the renaturation of protein. Further dialysis without urea was carried out to produce refolded protein

in soluble form for its use as immunogenic protein in ELISA. The final concentration of purified, dialyzed and solubilized protein was estimated calorimetrically using BSA standards and Bradford dye (Thermo Scientific), at 595 nm using spectrophotometer (Thermo Scientific).

2.6. SDS-PAGE and western blotting

Harvested expressed samples along with vector control, host cell control and eluted fractions were mixed separately with SDS sample buffer and resolved by polyacrylamide gel (5% stacking and 15% resolving). The separated proteins by SDS-PAGE were transferred on to a nitrocellulose membrane (NCM) (Hybond-C, Amersham Pharmacia, NJ, USA) following the method described by Burnette (1981) and the membrane was blocked with 5 per cent skim milk powder (SMP) in PBS overnight at 4 °C. The expressed recombinant proteins on the blot were detected as the case may be by incubation with convalescent serum (PPRV infected and recovered animal serum) from goat or sheep (1:25) or Anti-Histag antibody conjugate (1:15000, Sigma) for 1 h at room temperature. Then the blot was incubated with an anti-Sheep/Goat horseradish peroxidase (HRPO) conjugate (Sigma–Aldrich, St. Louis, MO, USA) with diamino benzidine (DAB) as a chromogen for development.

2.7. Immunoreactivity of the expressed protein

The expressed protein was tested for its suitability as immunogen or antigen in indirect ELISA (I-ELISA). I-ELISA was standardized using the purified recombinant protein for serological detection of PPRV antibodies following the earlier described protocols (Balamurugan et al., 2007) with some modifications.

2.7.1. PPRV antigen preparation

Antigen was prepared as per the method described by Singh et al. (2004a). Briefly, Vero cells infected with PPRV (Sungri-96) showing >80% CPE were harvested and TNE Buffer (Tris-HCl- 50 mM, NaCl- 150 mM, EDTA- 1 mM) was added and freeze thawed thrice. The harvested samples were sonicated at 20 amplitude with 30 s. interval for 6 min and were centrifuged at 2000 rpm for 5 min. The clarified supernatant was subjected to ultra centrifugation at 100,000*g* for 2 h at 4 °C over 60% and 30% sucrose discontinuous gradient. After centrifugation, the complete and incomplete virus particles were collected with the help of Pasteur pipette and the pooled viruses were pelleted in TNE buffer by centrifugation at 100,000*g* for 2 h. The partially purified PPRV collected was dissolved in Tris-EDTA buffer (pH 8) and used as whole virus antigen in ELISA.

2.7.2. Antigenicity of the recombinant PPRV F protein

In order to assess the antigenicity of the expressed protein, the protein was used as a coating antigen in indirect ELISA for optimization using standard PPRV negative and positive serum samples from sheep and goats.

A checker board titration was performed for working dilution of antigen and serum (antibodies) as per standard protocols. Antigen concentrations of 0.5 μg to 16 μg (0.5, 1, 2, 4, 8, 16 μg/well) and serum dilutions of 5 – 40 (5, 10, 20 and 40) were performed. The antigen and serum dilutions that gave maximum difference in absorbance at 492 nm between positive and negative (P/N) were selected.

Standardization of rPPRVFP indirect ELISA was carried out using the serum sample from animals of varied immunological status (pre-vaccinated, post-vaccinated, infected or recovered animals) collected during field visit and outbreaks investigation for detecting PPRV specific antibodies in sheep and goats. These samples were screened initially by PPR c-ELISA kit (Singh et al., 2004b) and PPR indirect ELISA (Balamurugan et al., 2007) for the selection of the true positive and true negative serum samples with respect to PPRV antibodies in order to determine cut-off value for the recombinant antigen (rPPRVFP) based indirect ELISA for sheep and goats.

ELISA was carried out according to Balamurugan et al. (2007) with some modifications. Briefly, purified F protein (4 μg/50 μl/well) was coated in a flat bottomed 96 well plate (Maxisorb plate, Nunc, Denmark). After incubation at 4 °C for overnight, the wells were washed thrice with wash buffer PBS-T (0.002 mol/L diluted PBS containing 0.1% Tween-20) and blocked with 100 μl of blocking buffer (PBS-T with 5% SMP and 3% LAH-lactalbumin hydrolysate) for 1 h at 37 °C in a shaker incubator. All control sera (strong positive and negative) and test sera were diluted in 1:10 dilution in blocking buffer and added in 50 μl volume/well. After incubation at 37 °C for 1 h, the plate were washed thrice with wash buffer. The antigen–antibody reaction was followed by incubation with anti-Sheep/Goat HRPO conjugate (1:3000 dilution) and detected by colour development with the chromogen, orthophenyl diamine (OPD) and H_2O_2 as the substrate. The reaction was stopped after 10 min by adding 50 μl of 1 M H_2SO_4. The absorbance values were measured at wavelength of 492 nm. The results were interpreted by calculating Percentage Positivity (PP) values using formula

$$PP = \frac{(\text{OD of the test sample} - \text{OD of negative control})}{(\text{OD of positive control} - \text{OD of negative control})} \times 100$$

The assay included standard controls (conjugate, strong positive, and negative) to assess the quality and accuracy. Cut of value was derived from percent positivity (PP) by two sided contingency table from Receiver Operating Characteristics (ROC) curve. The sensitivity and specificity of standardized ELISA were determined using the statistical formula given by Thrusfield (2007) using open source Win Episcope software version 2.

2.8. Immunogenicity of recombinant PPRV F protein

2.8.1. Production of polyclonal antibody against PPRV F protein

Three six months old New Zealand white female rabbits were inoculated intramuscularly with 100 micrograms of purified protein emulsified with an equal volume of Montanide ISA-206 (Seppic S.A., Paris La Défense, France). All rabbits were boostered on 14th and 28th days with same dose of protein and were bled on 0th, 14th, 21st, 28th, 35th, 45th and 60th day post-inoculation (dpi). Sera samples collected on zero day were maintained as negative controls.

2.8.2. Raising hyper immune sera in rabbits

Two Russian gray giant rabbits of 4–6 months old were inoculated intramuscularly with 1 ml of purified whole PPR virus (200 μg/rabbit) emulsified with an equal volume of Montanide ISA 206 (Seppic S.A., Paris La Defense, France) along with two controls which received the PBS with adjuvants. These animals were further boostered on 14 days post inoculation (dpi) and blood was collected on days 0, 21, 28, 35 and 45th dpi.

Further, Rabbit hyperimmune serum raised against HR2 region of PPRV F protein (Seth and Shaila, 2001) was obtained from Prof. M.S. Shaila, Department of Microbiology and Cell Biology, IISc, Bangalore, India was also used as strong positive reference serum for western blot.

2.8.3. Titer determination of PPRV F antibodies

Recombinant PPRV F antigen based indirect ELISA and whole PPRV ELISA were used to determine the titer of the obtained F protein polyclonal antibodies in rabbits as per (Balamurugan et al., 2007) with a little modification. ELISA micro titer plates (Maxisorp®, Nunc) were coated with 50 μl (1 μg protein/well) of purified recombinant PPRV F protein or 1:100 PPRV sungri in PBS (pH 7.2), incubated overnight at 4 °C. Plates were washed thrice

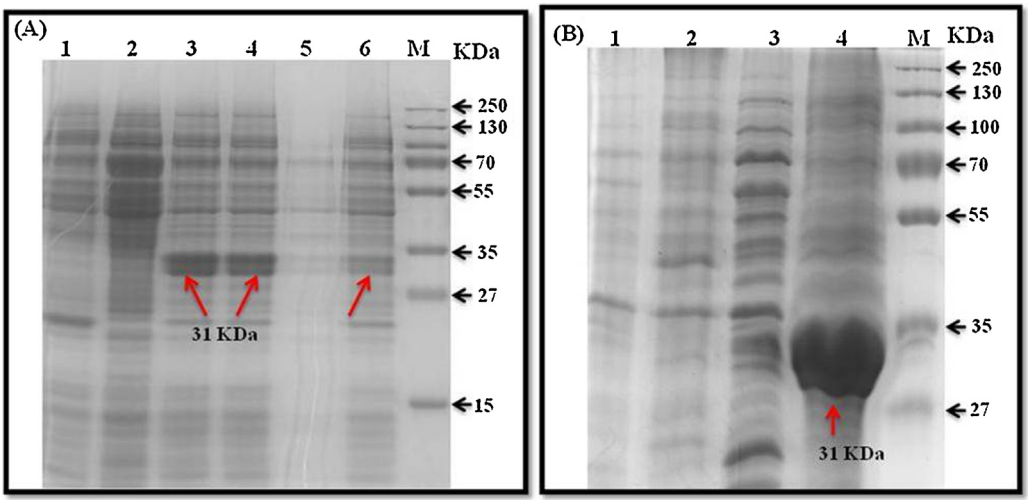

Fig. 1. SDS–PAGE analysis of expressed recombinant PPRV F protein (A) Preliminary expression of His-tagged PPRV Fusion protein. Lane1: BL21 (DE3)/Host Control; Lane 2: pET33b/vector control; Lane 3–6: pET33b + PPRVF clones; Lane M: Prestained protein ladder plus (MBI Fermentas, USA). (B) Solubility testing of recombinant PPRVF protein. Lane1: pET 33b (Soluble fraction); Lane 2: pET 33b (Insoluble fraction); Lane 3: pET 33b + PPRVF (Soluble fraction); Lane 4: pET 33b + PPRVF (Insoluble fraction).

with washing buffer (PBS-T, 0.1% Tween-20) and were blocked with 100 μl/well blocking buffer {5% (w/v) SMP, 3% (w/v) LAH in PBS-T} for 1 h at 37 °C. Rabbit serum was diluted 2 folds in negative serum (1:2, 1:4, 1:8, 1:16, 1:32) and tested at 1:20 dilution in dilution buffer (3% (w/v) SMP, 1% (w/v) LAH in PBS-T) as per protocols. Fifty microliters of diluted serum was added to the wells and the plates were incubated for 1 h at 37 °C. After washing thrice, the plates were incubated with 50 μl of HRPO conjugated anti-rabbit secondary antibody (1:10,000) at 37 °C for 1 h. Plates were incubated at 37 °C for 5–10 min in darkness after the addition of 50 μl of OPD with 3% H_2O_2. The reaction was quenched by the addition of 50 μl of 1 M H_2SO_4, and absorbance was read at 492 nm in ELISA reader (Biorad). Regression analysis was carries out to measure antibody titre.

2.8.4. Immunoblot analysis

The electroblotting was carried out as mentioned earlier and the blot was incubated with respective primary antibody as the case may be {antibody raised against HR2 region of PPRVF (1:1000)/rabbit hyper immune sera against PPRV vaccine virus (Sungri) (1:500)/immune sera raised against F protein (1:50)} for 1 h at room temperature. Then the blot was incubated with an anti-rabbit HRPO conjugate (1:12000) with diamino benzidine (DAB) as a chromogen for development.

3. Results

3.1. Cloning of F gene sequences in pET 33b vector

The immunodominant ectodomain of PPRVF gene sequences was amplified by RT-PCR, which resulted in specific product of 728 bp. The amplified PCR product was ligated into pET33b vector at *EcoR I* and *Not I* sites. Ligated mixture was transformed to *E. coli* TOP 10F' cells. The efficiency of transformation was 2×10^4/μg of ligated DNA product. Amplification of the gene specific product with virus specific primers, confirmed the presence of the insert in five out of seven colonies screened. The positive colonies were grown and plasmid DNA was purified and subjected to RE digestion. The release of gene specific fragment could be seen in clones with insert. The sequence of the insert in the recombinant clone was confirmed by sequence analysis (data not shown). In order to express the F protein, the cloned DNA was transformed into *E. coli* BL21 (DE3) cells by heat-shock method along with negative control of the pET33b vector. The BL21 clones were screened for the presence of the target insert by PCR using insert specific primers, which resulted in specific amplification in five out of five colonies.

3.2. Expression of immunodominant ectodomain of PPRV F protein

PCR positive BL21 clones were induced to express the F protein with IPTG. The expressed protein was analyzed by SDS-PAGE (Fig. 1A). A single intense band of 31 kDa could be seen (Fig. 1A, lanes 3, 4 and 6) corresponding to the expressed immunodominant ectodomain F protein, whereas no such protein bands were seen in the controls (Fig. 1A, lanes 1, 2). The observed, predicted and calculated molecular weight of the expressed recombinant ectodomain of PPRV F protein along with the fused His-tagged protein is approximately 31 kDa.

A single intense band of 31 kDa F protein was distributed in insoluble fraction, whereas very light protein band was seen in the soluble fractions (Fig. 1B, Lanes 4 & 3) but no such bands were observed in vector control.

3.3. Purification and characterization of PPRV F protein

PPRVF protein was better expressed at 37 °C and 30 °C in *E. coli* BL21 (DE3) cells. An intense band was seen at 1 mM IPTG concentration than at 1.5 and 2 mM IPTG and expression was observed up to 10 h of induced period. For subsequent expression, the optimized duration of 5 h induction with 1 mM IPTG concentration at 37 °C incubation was chosen for harvest and subsequent downstream processing of the protein.

As the target proteins were found in the inclusion body fraction (Fig. 1B), protein purification was performed under denaturing conditions at room temperature. Nickel NTA resin was used for purification of the recombinant protein by affinity chromatography. Histidine residues in the His tag bind to the vacant positions in the coordination sphere of the immobilized nickel ions with high specificity and affinity. His-tagged proteins are bound, and other proteins pass through the matrix. After the removal of unbound proteins, His-tagged F protein was recovered by elution with different concentration of imidazole in the elution buffer. The SDS–PAGE analysis showed that relatively high-purity proteins (Fig. 2 lane 8 and 9) in the preparation and 500 mM imidazole was found to be optimum with elution of specific purified protein band and

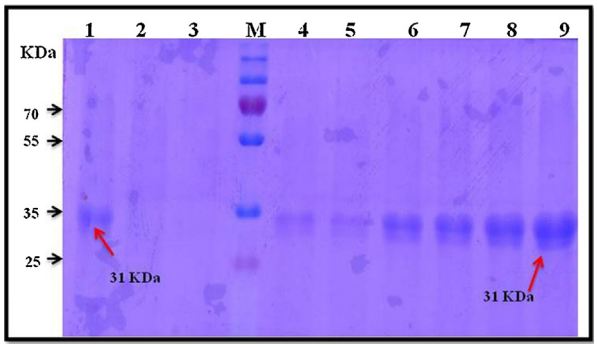

Fig. 2. SDS-PAGE analysis of His-tag purified recombinant PPRVF. Lane1: Insoluble fraction of pET PPRV F (Before passing via column); Lane 2: flow though 3; Lane 3: Wash 3; Lane M: Prestained protein ladder plus (Thermo scientific PIERCE marker); Lane 4 & 5: Elution 1 & 2 (100 mM imidazole); Lane 6 & 7: Elution 3 & 4 (300 mM imidazole); Lane 8 & 9: Elution 5 & 6 (500 mM imidazole).

Table 1
Two sided contingency table of tested samples.

Assays		Whole virus antigen based I ELISA		Total
		Positive	Negative	
Recombinant F protein based I-ELISA	Positive	102	6	108
	Negative	24	68	92
Total		126	74	200

was thus used subsequently. Different eluted fractions containing expected single His-tagged purified PPRV F protein was pooled and dialyzed for renaturation at 4 °C in 8 M, 6 M, 4 M, 2 M, 1 M, 0.5 M urea. Further, dialysis was carried out without urea to produce refolded protein in soluble form for its suitability in the ELISA. The final concentration of purified and dialyzed solubilized protein was 3 mg/ml and the yield was around 12 mg/100 ml of the bacterial induced pellet of 5 h.

3.4. Antigenicity of the recombinant PPRV F protein

Immunoblot analysis has shown that the expressed crude protein, His-tagged purified and/or dialyzed ectodomain PPRV F protein reacted with Anti-his-tagged antibody conjugate/convalescent serum from goat and sheep (Fig. 3A–C). The vector protein did not react with any of the above said sera in the western blot. Further, the expressed protein was not recognized by healthy Goat/Sheep serum (Fig. not shown).

The expressed recombinant PPRV F protein was tested as coating antigen instead of the PPRV antigen in ELISA for its reactivity. The mean OD values of conjugate, strong positive and negative serum samples were 0.063, 0.442, and 0.0737 with recombinant PPRV F protein respectively. Further out of the 200 selected sera, 126 were positive and 74 were negative for PPRV antibodies (Table 1). Sensitivity and specificity were calculated for 20 different cut-off values (5–100) by two sided contingency table. Using Receiver operative curve cut off ≥30PP was arrived. The relative sensitivity and specificity of the recombinant assay with whole virus I-ELISA was 80.95% and 91.89% respectively. An excellent area under curve of 0.9548 and good agreement of 0.694 kappa value was obtained.

3.5. Immunological response to recombinant F protein

Immunogenicity of expressed protein was further confirmed by immunoblotting using different rabbit sera. The results revealed the presence of 31 kDa protein corresponded to recombinant PPRVF protein (Fig. 4A–C); whereas, vector protein failed to react with above said sera except crude lysate which showed a little reactivity. This indicates that the expressed protein is indeed of PPRV specific epitope and there was no cross reaction with vector protein. Further, the expressed protein was not recognized by healthy rabbit sera (fig. not shown).

The collected rabbit sera at different dpi were tested for the presence of PPRV specific antibodies by recombinant PPRV F antigen and whole virus antigen based indirect ELISA as described earlier. Regression analysis showed that sera collected on 33rd day had peak OD compared to other days (Fig. 5A) when tested with PPRV F protein as reflected by average OD values from three rabbits. In whole virus antigen based indirect ELISA; Regression analysis showed that sera collected on 43rd dpi had peak OD compared to other days (Fig. 5B).

4. Discussion

Agriculture and Animal husbandry becoming a major occupation, perhaps attaining a status of industry in developing countries like India. Among the animal husbandry practices, sheep and goats are largely reared by low income, landless and marginal farmers as their main source of income. India has approximately 200 millions small ruminants (www.dadf.nic.in) which are plagued by many infectious diseases, of which PPR is a major transboundary disease. Generally whole PPR virus based ELISA and Serum neutralization test (SNT) are employed to detect PPRV neutralizing antibodies after vaccination. Neutralizing antibodies are mounted against H and F protein of PPRV. Fusion Proteins of morbilliviruses are highly immunogenic and confer protective immunity (Diallo et al., 2007). Antibodies to F protein are predominantly necessary for preventing infection and spread of the virus *in vivo* (Merz et al., 1980). Further compared to the H protein of PPRV, the immunogenic properties of F protein have not been studied in detail. To contribute in developing a recombinant antigen based approaches in future or to further explore the biological function of PPRV F, the present work was undertaken for the first time to express the immunogenic ectodomain portion of PPRV F protein in *E. coli* and its characterization to assess its immunoreactivity and also to detect PPRV specific antibodies in natural host. Although many alternative organisms and expression systems are now available for recombinant protein production, bacteria such as *E. coli,* system continue to be the most attractive host for the production of heterologous proteins (Khow and Suntrarachun, 2012). In this system, the desired product can be up to 50% of the total cell protein in few hours post-induction (Studier et al., 1990). Therefore, in the present study, expression of the ectodomain of F protein of PPRV without the translational stop codon (encoded by nt 793 to nt 1458 encompassing amino acids 265–486) containing two B cell epitopes, one of which is immunodominant and protective, whose epitope sequences are conserved across morbilliviruses, and the other, a T helper epitope originally identified in measles virus F protein and conserved in PPRV (Atabani et al., 1997; Partidos and Steward, 1990) was expressed as 31 kDa recombinant protein *in E. coli* and its immunogenic potentials has been evaluated.

The predicted size of the ectodomain of F protein from amino acid sequences is about 24 kDa. The calculated size as per amino acid composition along with the fused His-tag protein was observed by its mobility in SDS-PAGE which is in agreement with the reported size indicating that the 31 kDa protein was the product from cloned gene product. Optimizations of conditions are very crucial for the expression of heterologous proteins. In the present study, comparatively thicker bands were observed at 30 °C and 37 °C than at 25 °C and 28 °C. It is very common that high-level expression of recombinant proteins in *E. coli,* results in the formation of inclusion bodies (Singh and Panda, 2005; Burgess, 2009).

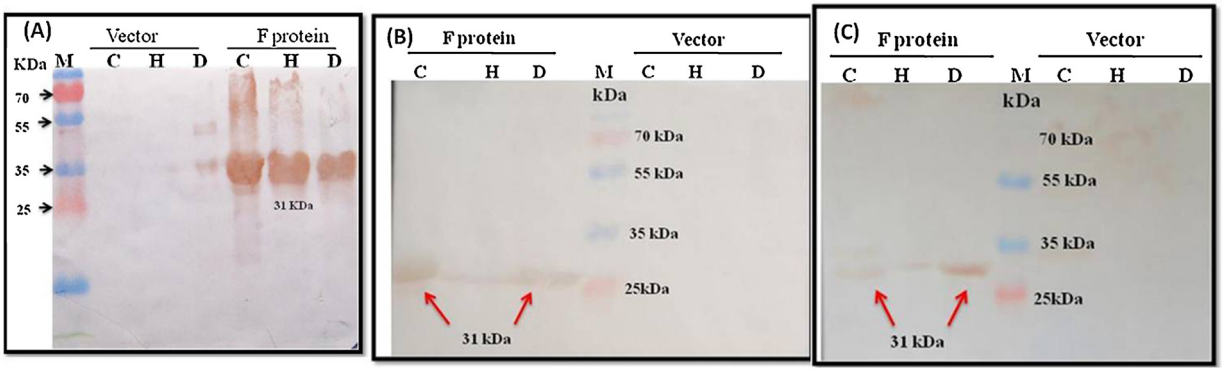

Fig. 3. Western blot analysis of expressed recombinant PPRV F protein. A. Anti histag conjugate at 1:15000 dilution; B: Goat convalescent serum; C: Sheep convalscent serum. Lane M- Prestained protein ladder plus (Thermo scientific PIERCE marker), Lane C- Crude protein, Lane H-His-tag purified protein, Lane D- Dialysed protein.

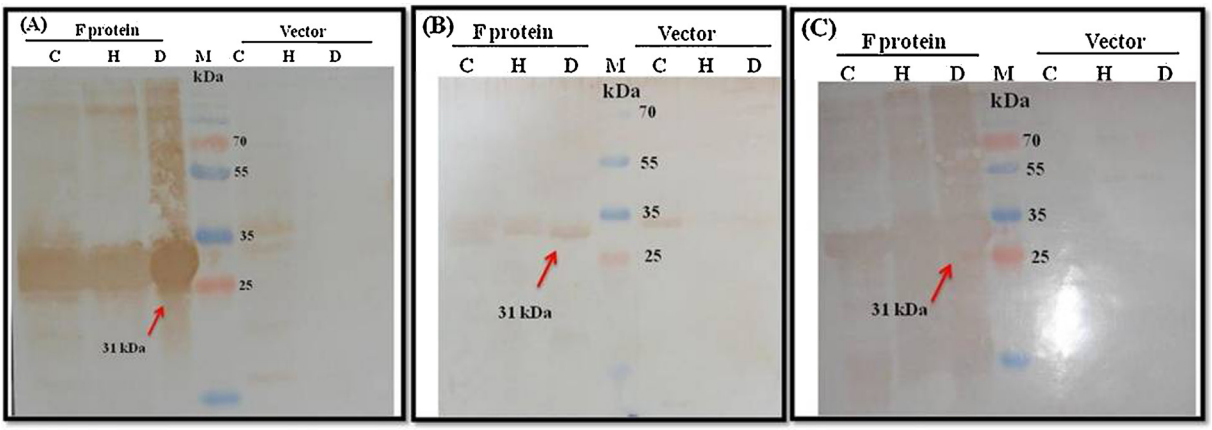

Fig. 4. Immunoblot analysis of recombinant PPRV F protein using rabbit hyperimmune sera. A- Rabbit HR2 specific PPRV F serum; B. Rabbit hyper immune serum against PPRV vaccine virus (Sungri 96); C- Rabbit hyper immune serum against rPPRV F protein. Lane M- Prestained protein ladder plus (Thermo scientific PIERCE marker) Lane C- Crude protein, Lane H-His-tag purified protein, Lane D- Dialysed protein.

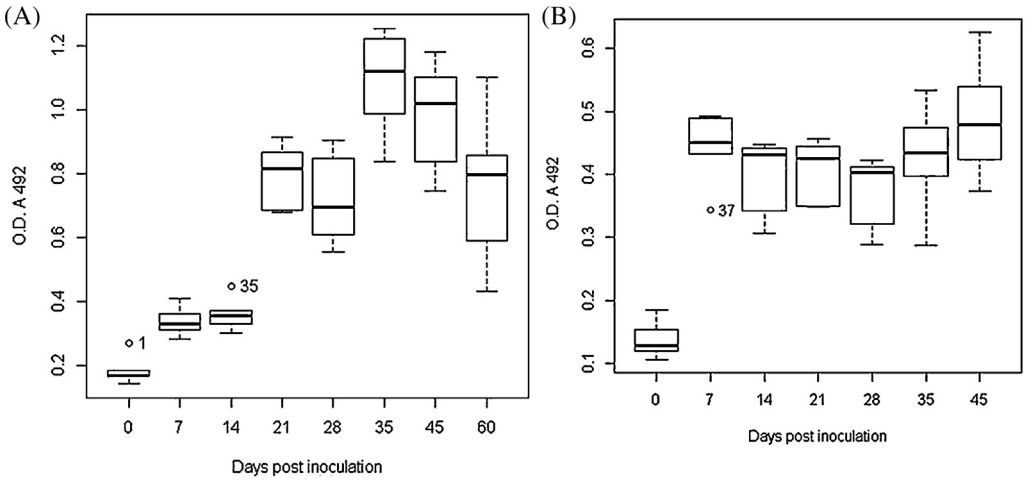

Fig. 5. Reactivity of immune sera raised against rPPRV F protein A. Recombinant F antigen based indirect ELISA; B. Whole PPRV antigen based indirect ELISA.

Accordingly, F protein was also expressed as inclusion bodies, as quantity of protein was very high. In general, cultivation of *E. coli* at low temperature causes expression of soluble foreign proteins: but even at these low level temperatures, soluble protein was not formed. Whereas high concentration of IPTG increases toxicity in *E. coli* (Onodera et al., 1996) and thereby inhibits cell growth to some extent particularly at 1.5 and 2 mM IPTG compared to 1 mM IPTG. Besides, prolonged expression may result in the degradation of foreign proteins in *E. coli*. Upon comparison of protein profiles of the recombinant clones at different time intervals, 31 kDa protein was observed as early as 1–2 h post induction (hpi). The intensity of the bands increased gradually up to 8 hpi. In zero hour cultures, such specific bands were not observed, which indicate the importance of IPTG to induce the expression of cloned gene under promoter. The optimum time of harvest was 5 hpi with no significant change up to 8 hpi and decreasing gradually thereafter. This

reduction could be attributed to autolysis of bacterial cells (Pathak et al., 2008). Thus, the optimization of three expression parameters led us to carry out the recombinant protein induction at 37 °C for 5 h with 1 mM IPTG. The results indicated that protein yields were not much affected by temperature, IPTG concentration and time interval.

Further, to assess the utility of the expressed proteins, His-tagged protein was purified using Ni-NTA affinity columns to their homogeneity. Generally the N-terminal His-tag is not preferred due to several disadvantages reported elsewhere, especially in purification (Svensson et al., 2006). In contrast to these, presence of N and C-terminal His-tag, both in cloned gene product and expression vector facilitated the easy and efficient purification. It was found that 500 mM imidazole concentration was optimum for elution of recombinant protein. Immunoblotting of PPRV F protein using Anti His-tagged conjugate, goat and sheep convalescent sera (Fig. 3A–C) revealed specific band of 31 kDa PPRV F protein. This indicated that the bands observed in SDS-PAGE are intact His-tagged and virus specific.

Though other immuno-assays are used, ELISA is one of the most sensitive and extensively applied methods to evaluate expressed proteins (Choi et al., 2005; Pathak et al., 2008; Yadav et al., 2009). The results indicated that the expressed F protein reacted well with PPR serum in ELISA making it evident that the epitopes present in expressed protein are well recognized by the antibody. For determination of cut-off value in I-ELSIA, OD values were converted to percent positivity (PP) values to minimize the variation in OD values. A cut off value (PP) of 30 per cent was fixed upon comparison with I-ELISA by two sided contingency table which showed 80.95 per cent sensitivity and 91.89 per cent specificity which gave a better separation of positive and negative animals sear samples. However in the present study, low sensitivity and specificity might be due to less number of samples screened, lack of knowledge on PPRV antibodies (H and F or N) kinetics in infected or vaccinated animals as these samples were collected at different time of PPR infection during outbreaks. However, this assay needs further systematic evaluation by using more number of serum samples with respect to PPRV antibodies in vaccinated and infected animals prior to samples testing in recombinant PPRV F antigen based ELISA and its comparison with SNT.

The polyclonal antibodies produced in rabbits from purified recombinant F protein showed PPRV F protein specific antibody response when tested in indirect ELISA (Fig. 5A). This indicated that the expressed PPRV F protein in *E. coli* retained the conformational epitopes and displayed immunogenic potential in rabbits. Specific antibody response to the recombinant protein was also detected in I- ELISA based on whole PPRV antigen (Fig. 5B). It indicates that recombinant ectodomain of PPRV F protein expressed in *E. coli* retained its immunodominant epitope and elicited antibody response which was able to recognize whole virus particles in the PPRV antigen based ELISA (Balamurugan et al., 2007). Further, Specificity of the anti-F protein polyclonal antibodies was checked by western blot (Fig. 4). An intense band of 31 kDa was observed in dialyzed protein compared to crude and his-tag purified protein with HR2 specific serum indicating the presence of immunodominant epitope in expressed protein. Protein was also reacted with rabbit hyper immune sera against whole virus (Fig. 4B); hence rPPRV F protein can be used prophylactically as subunit vaccine in combination with H glycoprotein to elicit protective immune response against infection. Further when probed with rabbit immune serum raised against PPRVF, an intense band but with a smearing in the lane was observed (Figs. 4C). This could be due to some antibodies produced in rabbits against *E. coli* proteins along with recombinant F protein. Crude lysate of vector protein also showed thin bands and this could be due to presence of *E. coli* antibodies in rabbits. Further, purified and dialyzed vector protein did not show any reactivity. However, probing with negative rabbit sera, did not reveal any bands (Fig. not shown), thus ruling out any nonspecific reactions.

Perusal of available literature revealed not many reports on expression of PPRV F protein and its antibody kinetics. Further, the reports by Rahman et al. (2003), Lotfi et al. (2007) and Wang et al. (2013) on PPRVF protein revealed no immune response studies in natural hosts against the expressed PPRV F proteins. To the best of our knowledge this is the first attempt on detection of PPRV F antibodies from the natural host using recombinant expressed F protein.

5. Conclusion

The immunodominant ectodomain of PPRV F gene coding sequences was expressed in prokaryotic expression (*E. coli*) system at very high level without toxicity. The expressed PPRV F protein can be purified under denaturing condition followed by renaturation methods to obtain the protein in solubilization form. The *E. coli* expressed PPRV F protein exhibits and retained its antigenicity and immunogenicity and can be used in ELISA for detection of PPRVF specific antibodies in sheep and goats. Systematic evaluation by comparing with SNT to detect or know the presence of neutralizing PPRVF antibodies during infection or vaccination to be assessed.

Conflict of interest

No conflicts of interests are declared by authors for the contents in the manuscript.

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