

Research Article

Coexpression of PPE 34.9 Antigen of *Mycobacterium avium* subsp. *Paratuberculosis* with Murine Interferon Gamma in HeLa Cell Line and Study of Their Immunogenicity in Murine Model

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Mycobacterium avium subsp. *paratuberculosis* (Map) is the causative agent of John's disease whose immunopathology mainly depends on cell mediated immuneresponse. Genome sequencing revealed various PPE (Proline-Proline-Glutamic acid) protein family of Map which are immunologically importance candidate genes In present study we have developed a bicistronic construct pIR PPE/IFN containing a 34.9 kDa PPE protein (PPE 34.9) of Map along with a cytokine gene encoding murine gamma Interferon gene (IFN γ) and a monocistronic construct pIR PPE using a mammalian vector system pIRES 6.1. The construct were transfected in HeLa cell line and expression were studied by Western blot as well as Immunofluorescent assay using recombinant sera. Further we have compared the immunoreactivity of these two constructs in murine model by means of DTH study, LTT, NO assay and ELISA. DTH response was higher in pIR PPE/IFN than pIR PPE group of mice, similar finding also observed in case of LTT and NO production assay . ELISA titer of the pIR PPE/IFN was less than that with PPE only. These preliminary finding can revealed a CMI response of this PPE protein of Map and IFN γ having synergistic effect on this PPE protein to elicit a T cell based immunity in mice.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is an intracellular pathogen, the causative organism of John's disease (paratuberculosis), a debilitating chronic enteritis in ruminants and has been implicated in Crohn's disease in humans characterised by hepatic granulomas in HIV-negative, nonimmunosuppressed patients [1]. This pathogen can multiply inside mononuclear phagocytes due to presence of various virulence determinants on their surface [2], and it is evident that cell-mediated immune response controls the resulting pathology.

The name PPE is derived from the motifs Pro-Pro-Glu, found in conserved domains near the N termini of these proteins having 180 amino acid sequences [3]. PPE proteins are thought to be expressed on the cell surface [4, 5] and

have been found to be immunodominant antigens [6]. Some of the PPE proteins of *Mycobacterium* species have been reported to be potent T cell and or B cell antigens [7–14].

Although studies on various secretory proteins of mycobacterial species have shown that they are potential immunogens and can be used as subunit vaccine, using efficient immune adjuvants can enhance the performance of the DNA vaccine. Various cytokines especially IFN γ , IL-2, IL-6, IL-12, and IL-1 play a key role in immunity against mycobacterial infections [15] and have been shown to increase the protectivity while used for coimmunization with DNA vaccines. The essential task of IFN γ in the resistance of mice to mycobacterial infections has been make clear by reports that knockout of IFN γ gene from the mice cannot control or inhibit different mycobacterial infections [16]. Recently, a recombinant PPE protein, Map41, which has been

reported as one of the IFN- γ -inducing antigens of MAP, also strongly induced IL-10 from macrophages obtained from infected calves [14].

Bicistronic vectors have been used to design DNA vaccine against HIV infection, which contained gp120 and GM-CSF gene [17], bicistronic DNA vaccine containing apical membrane antigen 1 and merozoite surface protein 4/5 can prime humoral and cellular immune responses and partially protect mice against virulent plasmodium chabaudi adami DS malaria [18], and a bicistronic woodchuck hepatitis virus core and gamma interferon DNA vaccine can protect from hepatitis [19]. Recently from our laboratory, Kadam et al. [20], have reported that coexpression of IFN γ with a 16.8 kDa gene of MAP can enhance immunogenicity of DNA vaccine using the same protein. In the present study, we have used a similar approach to clone a 34.9 kDa PPE (PPE34.9) antigen of MAP in the A frame of the bicistronic vector pIRES 6.1 having IFN γ gene in the frame B used by Kadam et al. [20]. Further, we have studied the coexpression of these two antigens in HeLa cell line. We have also preliminary attempted to elucidate the immunogenic effect of PPE 34.9 antigen of MAP on murine model and the role of IFN γ 's adjuvant properties.

2. Materials and Methods

2.1. Mycobacterial Strains. Solid and liquid cultures of MAP 316F were obtained from Central Diengenees Kunding Tieh Institute, Lelystad, the Netherlands and maintained at Biological Products Division of IVRI, Izatnagar, and later maintained at Gene Expression Laboratory, Division of Animal Biotechnology, IVRI, Izatnagar.

2.2. Plasmid Vectors and Host Strain Used. pTZ57R/T Cloning vector and host strain DH5 α of *E. coli* were supplied by MBI Fermentas, Germany. Bicistronic vector pIRES 6.1 was supplied from Clontech, USA.

2.3. Laboratory Animals. Swiss albino mice and New Zealand white rabbits were obtained from Laboratory Animal Resource Section, IVRI, Izatnagar. Standard prescribed guidelines for care and use of laboratory animals were followed during the experimentation with these animals.

2.4. Culture and Growth of MAP. MAP organisms were grown on Middlebrook 7H10 agar enriched with 0.1% glycerol v/v and 10% OADC with additional supplementation of Mycobactin J (2mg/L) and were maintained at 37°C.

2.5. Isolation of Genomic DNA Form MAP. The genomic DNA from MAP was isolated by following the published method [21].

2.6. Oligonucleotide Primers. A set of primers were designed for the specific amplification of the 1080 bp PPE34.9 gene of MAP based on the sequence information of MAP str.k10, complete genome Gene Bank Accession no. AE016958.

Similarly, one set of primers was designed for the amplification of murine interferon gamma gene based on sequence information (Gene bank Accession no. NM_008337). The primers were synthesized by Integrated DNA Technologies, USA. The nucleotide sequences of these primers were as follows (Table 1).

2.7. Polymerase Chain Reaction and Amplification of PPE34.9 Gene Fragment. Specific amplification of the PPE gene from the genomic DNA of *M. a. paratuberculosis* was carried out using the above-mentioned primers pIRES MAP PPE F and pIRES MAP PPE R. The PCR was carried out in 25 μ L reaction volume using 1 μ L of genomic DNA (10 ng) as template, 2.5 μ L of PCR buffer, 1 μ L of MgCl₂ (1.5 mM), 1 μ L (25 μ M) of each primers, 1 μ L of dNTP mix (200 μ M of each dNTP), and 1 U of Taq DNA polymerase. The volume was made up to 25 μ L by adding DNase-free water. The thermal cycling steps were carried out in PTC-200 thermocycler MJ Research Inc., USA with initial denaturation at 94°C for 5 min followed by 30 cycles with denaturation at 94°C for 1 min, annealing at 55.0°C for 1 min, extension at 72°C for 30 seconds, and final extension at 72°C for 10 min. Size of the amplified product was confirmed by using DNA molecular weight marker in a 1% agarose gel and quantified by spectrophotometric analysis.

2.8. Cloning of PPE34.9 Gene of MAP in pTZ57R/T Cloning Vector. 2 μ L (100 ng) of eluted PCR product, 1 μ L of pTZ57R/T (55 ng), 2 μ L of 5X ligation buffer, 1 μ L of T4 DNA ligase (5 units) were mixed in a sterile microcentrifuge tube and the volume was made up to 10 μ L with nuclease free water. The ligation mixture was kept at 22°C overnight and stored at -20°C. Competent *E. coli* DH5 α cells were prepared and transformed with 10 μ L of ligation mixture as stated above. The transformed cells were spread on LB agar plate containing ampicillin (100 μ g/mL), X-GAL (25 μ g/mL) and IPTG (25 μ g/mL). Appropriate positive and negative controls were processed simultaneously. Plates were incubated at 37°C overnight and later stored at 4°C. Ten white colonies were picked up and grown in LB broth containing ampicillin and incubated at 37°C overnight in a shaker incubator at 180 rpm. Plasmid DNA was extracted by miniprep plasmid isolation method [22]. Identification of positive colonies was done by Colony PCR and subsequently confirmed by RE analysis and designated as pTZ PPE.

2.9. Cloning of PPE Gene of MAP in a Mammalian Bicistronic Expression Vector pIRES and Plasmid Construct pIR IFN. The insert from the positive clone pTZ PPE (containing the appropriate restriction sites *Nhe*I and *Eco*RI specific for frame A of pIRES vector) was released by digesting with the enzymes *Nhe*I and *Eco*RI. The digested product was then ligated in the frame A after digestion of the vector with *Nhe*I and *Eco*RI to prepare monocistronic construct pIR PPE. The ligation mixture was transformed in *E. coli* competent DH5 α cells. Further, to prepare bicistronic construct pIR PPE/IFN, pIR IFN [20] was used and same strategy was adapted to insert the PPE 34.9 in the frame A.

TABLE 1: List of specific primers used for the present study.

Primers	Oligonucleotide sequences
pIRES MPPPE F(Sense)	5' GCCGCTAGCATGTGGGTCCAGGCCGCCAC 3'-29 mers
pIRES MPPPE R(Anti sense)	5' GCCGAATTCTTACTCGGTTCCAGCGTTGC 3'-29 mers
IFN F(Sense)	5' GCCTCTAGAATGAACGCTACACACTGC 3'-27 mers
IFN F(Anti sense)	5' CCGCGGCCGCTCAGCAGCGACTCCTTTT 3'-28 mers

2.10. Preparation of Transfection Grade Plasmid. Large scale purification of the plasmid constructs pIR PPE and pIR PPE/IFN was done using endotoxin-free QIAGEN mega kit according to the manufacturer's instructions (Qiagen, Inc., Valencia, California).

2.11. Transfection of Plasmid Constructs in HeLa Cell line. The purified recombinant plasmids pIR PPE and pIR PPE/IFN were transfected to 60–70% confluent HeLa cells using SuperFect transfection reagent kit, following manufacturer's instructions (QIAGEN, Germany). Briefly, HeLa cell monolayer was subcultured and the cells were seeded in 25 cm² tissue culture flask. When 60–70% monolayer was achieved, the cells were used for transfection. 5 µg of each DNA in 20 µL TE (pH 7.5) was diluted separately in optiMEM. Then 30 µL of superfect transfection reagent was added to the DNA solution. Afterwards, growth medium was aspirated from the dish and cells were washed with two mL DMEM (without serum and antibiotic). Then, 0.8 mL of the same DMEM were added to the reaction tube containing the transfection complexes and mixed properly. The mixtures thus prepared were layered separately over the cells and incubated for 6 hrs at 37°C followed by addition of DMEM with 10% FCS, and incubation was continued in a humidified CO₂ incubator. Cells transfected with the respective plasmid constructs were harvested after 72 hrs of incubation by adding about 80 µL of 2X SDS-PAGE loading buffer, and the expressed proteins were resolved on SDS-PAGE and western blotting using hyperimmune sera raised in rabbit against recombinant PPE 34.9 protein (1 : 200 in PBS).

2.12. RT-PCR (Reverse Transcription Polymerase Chain Reaction) for Conformation of Expression of IFN. One mL of trizol was layered on the transfected monolayer and the cells were lysed. Total RNA was isolated from the cells and amplified by RT-PCR. The amplified product was checked on 1.5% agarose. One mL trizol was added on the transfected monolayer and homogenized by passing the lysate 10 times through a sterile 20 G needle fitted to a syringe and transferred to a sterile 1.5 mL eppendorf. Further, the sample was kept at room temperature for five minutes. 200 µL of chloroform was added to the sample and mixed by vortexing. It was allowed to stand at room temperature for 10 minutes. The sample was then centrifuged at 12000 rpm for 20 minutes. The aqueous phase was collected in a separate

vial to which 500 µL of isopropanol was added, mixed gently, and kept at room temperature. RNA pellet was washed with 500 µL 70% ethanol and dissolved in nuclease-free water. The yield of total RNA was determined spectrophotometrically using the formula

$$OD_{260} \times 33 \times 200 \times 10^{-3} = \mu\text{g}/\mu\text{L}. \quad (1)$$

RT-PCR was carried out using RT PCR kit (MBI Fermentas, Germany). In a sterile microfuge tube, 2 µg of total RNA from pIR PPE/IFN were carried out to which 1 µL of random hexamer primer was added and incubated at 70°C for 5 min. Then, mixture, 1 µL RNase inhibitor, 2 µL of DNTP, and 4 µL of 5x RT buffer were added and tube was incubated at 37°C for 5 min. Thereafter, 1 µL of m MLV reverse transcriptase was added and the volume was made up to 20 µL by adding nuclease free water. The sample was incubated at 42°C for 1 hr, followed by incubation at 72°C for 10 min. The cDNA was stored at -20°C, until used. Amplification of IFN γ specific fragment from the cDNA preparation was prepared containing 4 µL of the cDNA sample, 2.5 µL of 10x PCR buffer, 200 µM of each Dntp, and 50 pMol each primers IFNpIR F and IFNpIR R. IFN γ amplification mixture was subjected to 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 45 sec), and extension (72°C, 1 min) with a further final cycle for primer extension (72°C, 5 min).

2.13. Indirect Immunofluorescence Assay (IFA). HeLa cells were seeded in 24 well plates and when a 60–70% confluent monolayer was achieved, two wells each were transfected with pIR PPE, pIR PPE/IFN, and pIRES (mock) plasmid. After incubation for 72 hr the medium was aspirated from all the wells, and the cells were permeabilized by adding 250 µL of 80% acetone for 30 min. Then, acetone was aspirated and the plate was dried at RT for 1 hr. Blocking was done using 1% BSA for 2 h at 37°C. Primary antibody (hyperimmune sera) was added at 1 : 50 dilution and kept for one hr at 37°C. This was followed by three gentle washes with PBS. FITC-labeled antirabbit conjugate was added at 1 : 200 dilution and kept for 1 hr at 37°C followed by washing with PBS and mounted in 50% PBS-glycerol. Cells were examined under fluorescent microscope.

2.14. Immunization of Animals with Plasmid Constructs. Swiss albino mice supplied by Laboratory Animal Section, IVRI, Izatnagar were maintained on ration comprising wheat dalia 62%, maize 30%, wheat bran 7%, salt 1%, and mineral mixture 25 ppm with 5 mL milk per mouse. The animals were divided into four groups, namely, A, B, C, and D each containing ten mice. They were vaccinated with the purified recombinant plasmid as shown in the Table 2.

Mice (three numbers) from each group were bled on the 21st and the 42nd days for serum separation which were stored at -20°C and used in the determination of antibody titres by ELISA.

2.15. Collection of Macrophages and Splenocytes from Plasmid-Immunized mice. On the 42nd day after immunization of mice, four mice from each group were selected randomly.

TABLE 2: Mice Immunization schedules.

S. no.	Mice group	Plasmid construct used	First dose (0 day)	Booster dose (35th day)
1	A	TE buffer control	100 μ g	100 μ g
2	B	pIRES mock control	100 μ g	100 μ g
3	C	pIRESPPE	100 μ g	100 μ g
4	D	pIRESPPE /IFN	100 μ g	100 μ g

About 5 mL of sterile RPMI 1640 medium were injected into the peritoneal cavity of each mouse, gently massaged, and the mice were left in the cage for 5 min. Then, the mice were sacrificed by CO₂ asphyxiation, and the peritoneal fluid was aspirated. The macrophages in the aspirated fluid were collected by centrifugation and resuspension of the obtained pellet in RPMI 1640 medium and subsequently used for nitric oxide (NO) estimation. After aspiration of peritoneal fluid, abdominal cavity was cut open. Spleens were harvested from sacrificed mice and made into a single cell suspension. The cells suspended in RPMI-1640 were layered over Ficoll-Paque PLUS, and mononuclear splenocytes were isolated by density gradient centrifugation at 1350 \times g for 30 min. Splenocytes thus obtained were used for LTT and RNA isolation.

2.16. Measurement of DTH Reaction. Six mice from each group were selected for DTH study. On the 42nd day after first immunization. All the mice were injected intradermally with 10 μ g of Johnin in right hind foot pad and 10 μ g of purified PPE 34.9 recombinant protein in the left hind foot pad. The results of the local skin reactions (DTH) were observed after 48 h by measuring the two transverse diameters of erythema using Vernier calipers with a minimum measurable increment of 0.01 mm. Data was statistically analyzed using Student's *t*-test at a significant level of $P < .05$.

2.17. Lymphocyte Transformation Test (LTT). The mononuclear splenocytes (5×10^5 cells per well) from four mice were placed in 96 well plates (Nunc, Denmark) in complete RPMI-1640 (phenol red free) medium containing 10% heat-inactivated foetal calf serum, 2 mM L-glutamine, 100 U of penicillin, and 100 mg streptomycin per ml. Respective Ni NTA column-purified recombinant PPE 34.9 [23] and ConA (positive control) were added at the final concentration of 40 μ g/mL and 10 μ g/ml, respectively. RPMI was used as the negative control. The total volume per well was 200 μ L. The plates were kept in a humidified CO₂ incubator at 37°C for 72 h. At the end of the incubation, 20 μ L of the yellow tetrazolium salt MTT (5 mg/mL) were added and incubated at 37°C for 4 h. In the presence of living cells, MTT is transformed to purple formazan [24]. Subsequently, 100 μ L of 0.04 N HCl in isopropanol were added and allowed to react for 30 min to stop the colour development reaction and dissolve the formazan crystals. The absorbance (OD) of the samples was measured in an ELISA reader at 570 nm (and 650 nm as reference) wavelength. Assays were conducted in triplicates, and the results expressed as Mean \pm SE. Stimulation index (SI) was calculated using the formula

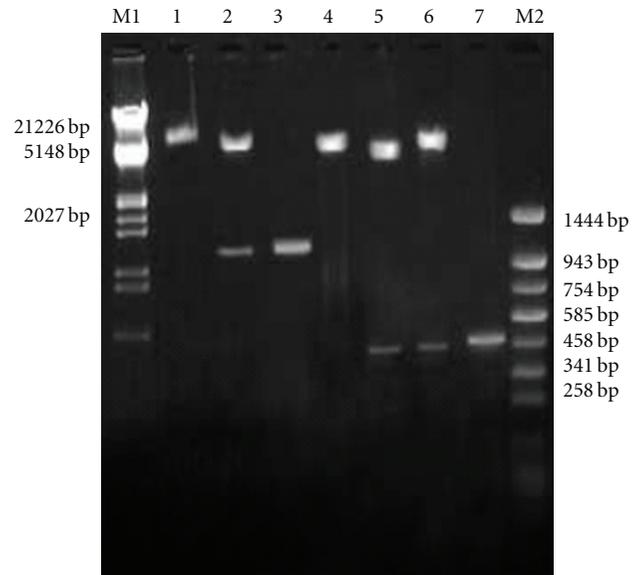


FIGURE 1: Agarose gel electrophoresis (1%) of the cloned fragment of the gene encoding PPE protein in pIRES IFN PPE mammalian vector. Lane M1: DNA molecular weight marker Lambda DNA/EcoRI/Hind III. Lane 1: pIRES IFN PPE linearised with EcoRI. Lane 2: released insert of 1080 bp after *Nhe*I and *Eco*RI digestion of recombinant pIRES IFN PPE recombinant plasmid DNA. Lane 3: PCR amplified fragment encoding PPE protein of *Mycobacterium avium* subsp. *paratuberculosis*. Lane 4: pIR IFN linearised with *Xba* I. Lane 5: release IFN fragment (467 bp) by digestion with *Xba* I and *Not* I from pIR IFN vector DNA. Lane 6: release IFN fragment (467 bp) by digestion with *Xba* I and *Not* I from pIR IFN PPE vector DNA. Lane 7: PCR amplified fragment of IFN (467 bp). Lane M2: DNA molecular weight marker pUC18/*Sau*3AI-pUC18/*Taq*I digest.

SI = OD of stimulated culture \div OD of unstimulated culture. SI value of >1.2 (i.e., 33% more than the control) was considered for lymphocyte proliferation. Data was analysed for significance between mock pIR and pIR PPE as well as pIR PPE and pIR PPE/IFN constructs by Student's *t*-test, and differences with $P < .05$ were considered within the level of significant.

2.18. NO Production Assay. The RPMI 1640 complete medium was supplemented with 5 mM of L-arginine for this assay. 100 μ L of the cell suspension containing 2×10^5 peritoneal macrophages from four mice from each group were plated in triplicate in 96 well plates. Respective antigen Ni NTA column-purified recombinant PPE 34.9 [23] and LPS (positive control) in RPMI 1640 medium (100 μ L) were added at the final concentration of 40 μ g/mL and 2 μ g/mL, respectively. RPMI was used as the negative control. The total volume per well was 200 μ L. The plates were incubated at 37°C in a humidified CO₂ (5%) incubator for 48h. Supernatants were collected from all the wells and stored at -20°C until NO estimation. For NO estimation NaNO₂ (sodium nitrite) in different concentrations was used as standard. In a 96-well ELISA plate to 50 μ L of the cell culture supernatant or standard, 60 μ L of Griess reagent (1% sulfanilamide in 1.2 N HCl) (Sigma) was added, and the plates were incubated at 37°C for 30 min, A550 reading was



FIGURE 2: Western blot assay of 72-hour culture of HeLa cell transfected with pIRPPE and pIRPPE/IFN showing expressed 34.9 kDa PPE protein. Lane M: prestained protein molecular weight marker. Lane 1: whole cell lysate of HeLa cell transfected with pIRES (mock control). Lane 2: whole cell lysate of HeLa cell transfected with pIRES PPE. Lane 3: whole cell lysate of HeLa cell transfected with pIRES PPE/IFN.

taken on a microplate ELISA reader. By using the standard curve (NaNO_2 concentration versus A550) the NO levels in the samples were estimated. Data was analysed by Student's *t*-test, and differences with $P < .05$ were considered significant.

2.19. Characterization of PPE Specific Antibodies in Mice Groups Immunized with Plasmid Constructs by ELISA. The optimum concentration of Ni NTA column-purified recombinant PPE 34.9 [23] antigen and conjugate were determined using block titration as described by Engwal and Pearlman [25]. The wells of ELISA plates (Nunc, Denmark) were coated with 200 ng/well of antigen diluted in carbonate bicarbonate buffer, and the plates were incubated at 4°C overnight. The plates were washed thrice with PBS-Tween 20 (PBS-T) and blocked with 5% skim milk powder in PBS-T for 2 h at 37°C. Then, 1 : 200 dilution of serum in 100 μL volume of PBS-T were added in duplicate and incubated at 37°C for one hour. The plates were washed thrice with PBS-T for 3 min at each wash. Conjugate antimouse IgG HRPO at dilution of 1: 10,000 in 100 μL volume was added to each well, and the plates were incubated for 1hr at 37°C. The plates were then washed three times with PBS-T, and colour was developed with 100 μL of 10 mg/mL OPD with 10 μL of 30% H_2O_2 in substrate buffer. After sufficient colour development, the reaction was stopped by the addition of 50 μL 1N H_2SO_4 , and the plates were read at 490 nm in an ELISA reader (Tecan, Austria).

3. Results

3.1. Construction and Characterization of Plasmids pIR PPE and pIR PPE /IFN. The bicistronic constructs were generated after cloning PPE34.9 gene fragment from *Nhe* I and *Eco* RI digested PCR product into frame A of *Nhe* I and *Eco* RI digested plasmid vector and *Xba* I- *Not* I digested IFN γ into frame B of the same vector. The positive colonies from the construct was identified by obtaining desired size products using colony PCR and subsequently confirmed by the release of identical size inserts on RE analysis (Figure 1).

TABLE 3: DTH responses in mice immunized with plasmid constructs expressed as mean differences (mm) upon recall with 10 microgram of PPE34.9.

Groups	PBS	PPE 34.9
Group A (TE buffer as control)	0.082 \pm 0.001	0.235 \pm 0.018
Group B pIRES (mock)	0.082 \pm 0.0007	0.111 \pm 0.011
Group C pIRES PPE	0.211 \pm 0.007	2.516 \pm 0.132
Group D pIRES PPE	0.215 \pm 0.0104	3.115 \pm 0.005

The plasmid constructs (monocistronic and bicistronic) were transfected into 60–70% confluent HeLa cell line, and the expressed PPE34.9 protein was detected from 72-hour posttransfected cell lysate in western blot using polyclonal serum raised in rabbit against recombinant PPE34.9. No such band was observed in cell lysate transfected with mock plasmid (Figure 2). The 72-hour posttransfected HeLa cells with plasmid constructs pIR PPE and pIR PPE/IFN on IFA using FITC-labeled conjugate exhibited fluorescence under fluorescent microscope, indicating the expression of the PPE34.9 protein (Figures 3(a), 3(b), 3(c), and 3(d)). The monoclonal antibodies against murine IFN γ could bind with HeLa cell expressed IFN protein to reconfirm IFN γ expression from the construct pIRPPE/IFN, RT-PCR was done for the cDNA obtained from total RNA of a 72-hour posttransfected HeLa cell lysate using specific primers of murine IFN γ . At 55°C, annealing temperature gave the amplified product of 467 bp (Figure 4).

3.2. Induction of DTH Response. DTH response was measured with Vernier Calipers 72 hrs after injection of PPE34.9 protein in the foot pad of Plasmid-immunized groups of mice. Skin reactions to recombinant PPE34.9 protein was studied. Both the control groups showed no significant reaction to antigen. Mice group immunized with pIR PPE and pIR PPE/IFN evoked visible skin reactions in the form of necrosis and erythema. However, more significant (3.115 \pm 0.005) erythematous lesions were observed in mice immunized with pIR PPE/IFN in comparison to pIR PPE-immunized groups (2.516 \pm 0.132). Whereas, mice immunized with plasmid pIRES (mock) showed no significant swelling when compared to the injection of PBS (Table 3). Statistical analysis with Student's *t*-test showed significant difference ($P < .05$) between pIR PPE/IFN and pIR PPE alone.

3.3. LTT-Based In Vitro Lymphocyte Proliferation Test. The purified recombinant PPE34.9 protein stimulated a significant proliferation of mononuclear splenocytes from mice groups immunized with constructs pIR PPE and pIR PPE/IFN. Higher proliferation was obtained with construct pIR PPE/IFN (1.38 \pm 0.079) followed by group pIR PPE (1.21 \pm 0.076) upon stimulation with purified PPE34.9 protein. (Table 4). Statistical analysis with student's *t*-test showed significant difference ($P < .05$) between pIR (mock) and pIR PPE as well as pIR PPE and pIR PPE/IFN groups.

3.4. NO Production Assay. Peritoneal macrophages collected from various mice groups were stimulated with the respective

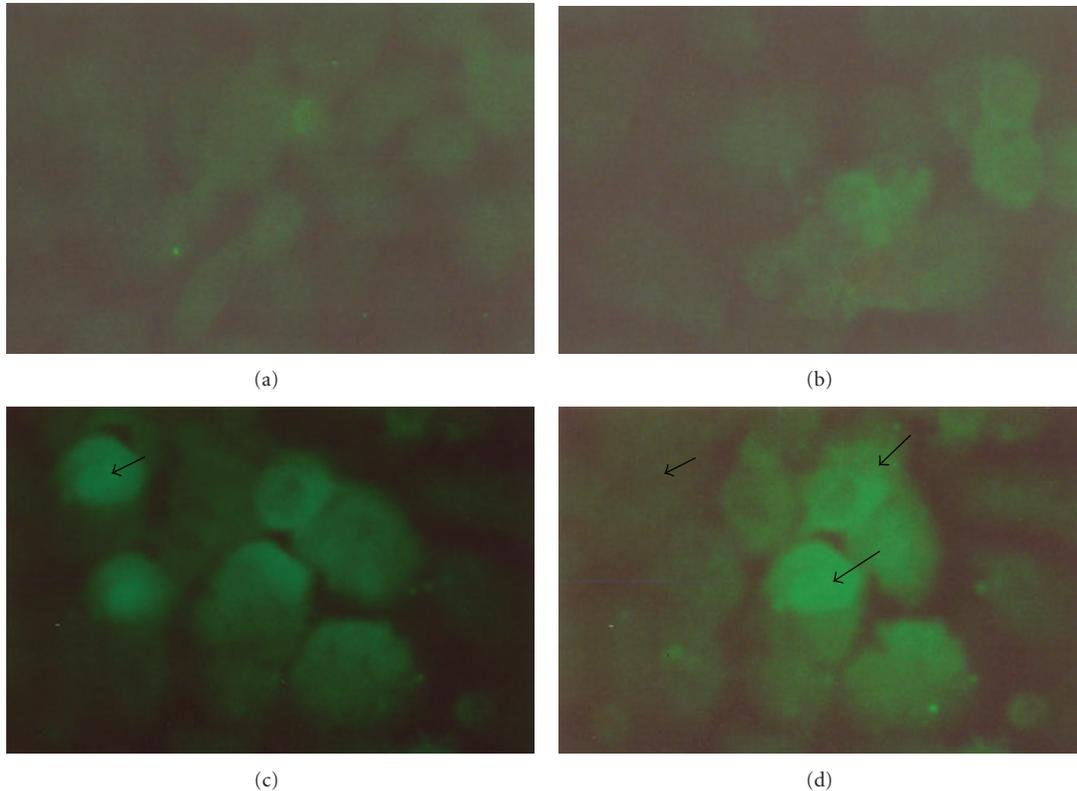


FIGURE 3: (a) Healthy HeLa cells (untransfected). (b) HeLa cells transfected with pIRES mock control. (c) HeLa cells transfected with pIRES PPE vector construct showing expression of PPE protein using a polyclonal sera raised in rabbit against PPE 34.9 react with FITC-labeled antirabbit conjugate. (d) HeLa cells transfected with pIRES PPE/IFN vector construct showing expression of PPE protein using a polyclonal sera raised in rabbit against PPE 34.9 react with FITC-labeled antirabbit conjugate.

TABLE 4: Lymphocyte transformation test for mice groups immunized with Plasmid constructs (SI = Mean \pm SEM).

Groups	ConA	PPE protein
A (TE buffer control)	1.03 \pm 0.05	0.96 \pm 0.042
B (pIRES mock control)	1.04 \pm 0.116	0.97 \pm 0.031
C (pIRES/PPE)	1.3 \pm 0.101	1.21 \pm 0.076
D (pIRES/PPE /IFN)	1.41 \pm 0.17	1.38 \pm 0.079

antigens to measure the amount of nitric oxide produced by the cells. The quantity of NO produced was estimated by comparing with known standards of sodium nitrite. LPS induced significant production of NO in all the groups. Among the immunized groups, NO production was found highest in group pIR PPE/IFN (Mean \pm SEM = 38.62 \pm 1.02 $\mu\text{m}/2 \times 10^5$ cells) followed by group pIR PPE (Mean \pm SEM = 26.19 \pm 0.53 $\mu\text{m}/2 \times 10^5$ cells) upon stimulation with recombinant PPE34.9 protein. (Table 5). Statistical analysis with student's *t*-test showed significant difference ($P < .05$) between pIR (mock) and pIR PPE as well as pIR PPE and pIR PPE/IFN groups.

3.5. Detection of Immune Response Induced by Various Plasmid Constructs in Mice by ELISA. To evaluate the humoral immune response induced by plasmid constructs in mice

TABLE 5: Nitric oxide production assay of peritoneal macrophages from mice groups immunized with Plasmid constructs (μm of NO/ 2×10^5 cells = Mean \pm SEM).

Groups	LPS	PPE protein
A (TE buffer control)	29.32 \pm 0.5042	7.85 \pm 0.2933
B (pIRES mock control)	30.77 \pm 0.6617	7.29 \pm 0.3199
C (pIRES/PPE)	33.43 \pm 1.9381	26.19 \pm 0.535
D (pIRES/PPE /IFN)	40.89 \pm 2.3452	38.62 \pm 1.020

groups, on the 21st day and the 42nd day postimmunization antibody titres were determined by indirect ELISA. Antibodies were detected in all the plasmid constructs immunized groups of mice (OD490 > 0.3 in the serum dilution range of 1:200). Whereas, insignificant titres were observed in control groups (Table 6).

4. Discussion

Presently, chemotherapy is unrewarding and economically not feasible to control the diseases. Effective control programmes for the disease are hampered due to lack of specific diagnostic tests to detect infection in the early stages of disease. Further the currently available immunodiagnostic tests have limited sensitivity [26] and specificity [27].

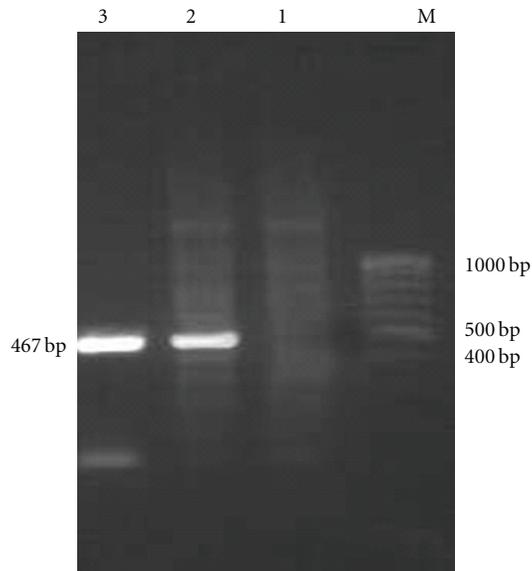


FIGURE 4: Agarose gel electrophoresis (1.5%) showing RT-PCR based expression of IFN γ ORF in pIR PPE IFN transfected HeLa cells. Lane M: prestained protein molecular weight marker (100 bp ladder). Lane 1: negative control mock plasmid transfected HeLa cells. Lane 2: IFN γ encoding gene from pIR PPE IFN transfected HeLa cells with super natanty. Lane 3: positive control (IFN γ) encoding gene from ConA induced mice splenocytes.

TABLE 6: ELISA titres of plasmid construct-injected mice groups (OD₄₉₀ = Mean \pm SEM).

Groups	21st day	42nd day
Blank	0.031 \pm 0.0035	0.044 \pm 0.0034
A (TE buffer control)	0.131 \pm 0.0076	0.136 \pm 0.012
B (pIRES mock control)	0.142 \pm 0.0371	0.156 \pm 0.012
C (pIRES/PPE)	0.312 \pm 0.0204	0.322 \pm 0.030
D (pIRES/PPE /IFN)	0.263 \pm 0.018	0.283 \pm 0.021

Conventional live attenuated vaccines are not completely protective [28, 29]. Studies have shown that CMI develops in early stages for clearing infection [30] whereas high serum antibody concentration is often seen in advanced clinical cases [31]. The cell-mediated immunity plays a pivotal role to control the spread of organisms within the host body [32]. DNA vaccines may open new horizons for effective vaccination against paratuberculosis as strong CMI responses including CTL and Th1 type cytokines are induced [20].

Expressions of T cell antigens in prokaryotic vector have failed to induce CTL and cytokine response. However, expression of T cell antigen in a mammalian vector for eliciting CD4⁺ T cell response and CD8⁺ cytotoxic T cell response to generate immunity have been reported in a number of animal models [33–36]. Cytokines also (mainly IFN γ , TNF α , IL10 etc.) play a major role in the protective immune response against mycobacterial diseases [14, 32]. Coexpression of T cell antigen with costimulatory molecules in a bicistronic eukaryotic system made the DNA vaccine

more effective [17–20]. Moreover, expression of two T cell antigens in eukaryotic bicistronic system may also be useful for enhancing protective immunity.

After the completion of the genome sequencing of MAP, the PPE protein family has been widely assumed to represent immunologically important antigens of the mycobacterial species. The present work envisaged keeping in view the role of a PPE antigen and the concept of bicistronic DNA constructs using an immunostimulatory molecule IFN γ is likely to potentiate immune response in mice. The use of cytokines as adjuvant is known to enhance immune responses when they were administered during the development of immune response against a particular antigen [37, 38]. IFN γ is the most extensively studied cytokine in mycobacterial infections. It is the defining cytokine of Th1 subset and activates macrophages for microbicidal activity. It induces IL12, which causes Th cells to differentiate into Th1 subset [39].

In the present study, the gene fragment encoding PPE34.9 protein was cloned into the frame A of the bicistronic vector pIRES6.1 containing IFN γ gene in frame B and also a monocistronic plasmid construct pIR PPE was made. The constructs were designated as pIR PPE/IFN and pIR PPE, respectively. Bicistronic vector pIRES6.1 contained immediate early CMV promoter for simultaneous expression of the two genes downstream to it as active protein. Expression of the PPE34.9 and IFN γ (17 kDa) proteins was confirmed by western blot and immunofluorescence assay in 72-hour posttransfected HeLa cell lysates using polyclonal sera. Size of mouse IFN γ gene is 1208 bp in length but coding sequence is 467 bp, which was used for IFN γ ORF expression. The results were in agreement with the eukaryotic bicistronic expression of 16.8 kDa antigen of MAP and murine IFN γ in a bicistronic vector [20], a glycoprotein C of pseudorabies virus [40] and an apical membrane antigen and merozoite surface protein of *Plasmodium chabaudi* DS malaria [18].

In the present study, we have cloned and coexpressed a 34.9 kDa protein-encoding PPE gene family antigen with IFN γ gene in HeLa cell line. Further, we have studied the immune responses of these plasmid constructs in mice. Elucidation of DTH response against recombinant P35 proteins and 16.8 kDa proteins of MAP has been studied by and Basagoudanavar et al. [41] and Kadam et al. [20], respectively. DTH-based immune response is an indicator of T-cell-based immunity. We have already elucidated the DTH response of purified recombinant PPE 34.9 protein in mice [23]. In the present study we have compare the effect of IFN as pIR PPE/IFN construct on PPE 34.9 as pIR PPE construct, which showed that a significant higher immune response of the first construct on the second one indicate the role of IFN γ to elicit a T cell based immune response.

Cell proliferation as a test has been used to assess DNA vaccines against mycobacterial infections [20, 41]. In the present study, mononuclear splenocytes from mice group immunized with pIR PPE/IFN showed higher cell proliferation than pIR PPE, which may indicate the effect of IFN γ . IL2 is known as the cytokine for cell proliferation, but IFN γ indirectly induces cell proliferation by activating macrophages and increasing antigen presentation which

induces IL2 receptors on T cell surface, thereby inducing cell proliferation. The results were in consensus as found by other workers who used cytokines as immunoadjuvant in bicistronic DNA vaccine. Chow et al. [42] have reported increased cell proliferation in group that received hepatitis B virus surface protein and IL2 as bicistronic DNA vaccine. Barouch et al. [17] found twofold augmentation of cell proliferation in bicistronic group which coexpressed gp120 gene of HIV and GM-CSF than in monocistronic gp120 immunized group. Kadam et al. [20], also found that bicistronic vector expressing a 16.8 kDa protein of MAP along with IFN γ gene induce higher proliferative response than the protein alone.

It is known that RNI (nitrogen intermediates), especially nitric oxide (NO), are most effective in direct killing of mycobacteria [15]. An increased production of NO-induced vaccine candidate genes may be one of the important causes of effective immune response against mycobacterial infection. As in our present study, NO production from cells of pIR PPE/IFN group was comparatively higher than PPE34.9 alone, it may again indicate the role of IFN γ in the induction/stimulation of macrophages to release RNI (NO). Recombinant protein PPE 34.9 was purified using single-step Nickel-NTA (pQE 30 UA containing His tag vector was used) affinity column chromatography [23], chance of LPS/endotoxin contamination is negligible. The results were in consensus as found by other workers who reported that it plays an important role in release of NO from monocytes [20, 43].

ELISA adopted to study the humoral immune response following DNA vaccination in mice for 22 kDa antigen of *M. bovis* [44] and MPT64, Ag85B, and ESAT-6 [45] antigens of *M. tuberculosis* showed significant increase in log titre of circulating antibodies. In the present study, antibody titer of the construct pIR PPE/IFN was less than that with PPE34.9 only. It may be possible that here IFN down regulating the IgG mediate humoral immunity induced by PPE34.9 protein which needs to be further confirmed in large number of animals. This result may be correlated to the groups who find that codelivery of IFN-gamma or IL-4 encoding EG95 protein of *Echinococcus granulosus*, the causative agent of hydatid appeared to reduce the ability of the DNA vaccine to prime an IgG antibody response demonstrated the efficacy of the codelivery of cytokines to modulate immune responses generated in a DNA prime-protein boost strategy [46].

Overall, the preliminary findings possibly revealed that the PPE34.9 antigen of MAP may be a T-cell-based immunogen. This is in agreement with the studies reported on PE antigen of *M. avium* by Parra et al. [47], antigen induced both cell-mediated [48] and humoral immune responses [49] which again was in corroboration with the earlier works.

Immune adjuvants plays an important role to enhance the protective efficacy of DNA vaccines [50]. IFN γ is a potent activator of macrophages and is the key cytokine in Th1-type immune response in paratuberculosis infection produced by both CD4+ and CD8+ cells [20, 51]. Hence for the development of an effective measure against paratuberculosis, it is necessary to apply those strategies that should enhance the T cell mediate response. From our preliminary observations,

we have also noticed that the monocistronic construct pIR PPE elicited a comparatively milder CMI response than pIR PPE/IFN. This may revealed that the presence of IFN γ synergized the T cell response of PPE34.9 protein.

These preliminary observations need further confirmation like in vitro study of the Th1 cytokine mediate response of the PPE34.9 and challenge studies in experimental as well as natural hosts for the development of an effective bicistronic DNA vaccine against paratuberculosis infection.

Abbreviations

OADC:	Oleic acid dextrose catalase
RPMI:	Roswell Park Memorial Institute
DMEM:	Dulbecco's modified eagle medium
FCS:	Fetal calf serum
rpm:	Revolutions per minute
DTH:	Delayed type hypersensitivity
MTT:	4,5-dimethyl thiazol-2-4 diphenyl tetrazolium bromide
NO:	Nitric oxide
ELISA:	Enzyme linked immunosorbent assay
RE:	Restriction enzyme.

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