Indian Journal of Experimental Biology
Vol. 56, December 2018, pp. 875-882

Recombinant CFP-10 as antigen for diagnosis and quantification of IFN-γ expression by real-time PCR in guinea pigs sensitized with *Mycobacterium bovis*

Tista Mondal[1], Rishendra Verma[1]*, Sangram P Ramane[1] & Soumendu Chakravarti[2]

[1]Mycobacteria Laboratory, Division of Bacteriology & Mycology; [2]Division of Biological Products, ICAR-Indian Veterinary Research Institute (IVRI), Izatnagar-243122, Uttar Pradesh, India

Received 06 June 2017; *revised 05 January 2018*

Culture filtrate protein (CFP-10) is a low molecular weight protein and is an early secretory protein in *Mycobacterium tuberculosis* culture filtrate and plays key roles in tuberculosis pathogenesis and in the stimulation of immunity. Keeping in view the important role of CFP10, we investigated the CFP10 gene in Indian *Mycobacterium bovis* (*M. bovis*) (3/86Rv) and its expression in suitable prokaryotic host for its diagnostics potential by single intradermal test (SID). Real-time PCR was used to quantify mRNA interferon-γ expression levels. The study concluded that the expression of IFN-γ mRNA in blood was found up to 19.962, 20.795, 9.633, 34.511 and 1.688 times in rCFP10, bovine PPD (purified protein derivative), avian PPD, conA and PBS stimulated groups, respectively as compared to healthy control group. The mRNA expression level of bovine PPD and avian PPD stimulated group was found statistically significant (at P <0.05) and among conA and rCFP10 stimulated group, it was highly significant (at P <0.01) in comparison to 'without antigen stimulated' group.

Keywords: Interferon-γ, PPD skin Real-time PCR, Tuberculosis (TB)

A diagnostic antigen with sensitivity and specificity for detection of *Mycobacterium bovis* infection is needed to overcome the limitations of Purified Protein Derivative (PPD) and diagnosis of the infection based on specific Mycobacterial antigens would be appropriate[1]. Southern blotting of genomic DNA has shown that both ESAT-6 and CFP-10 genes are present in *M. tuberculosis* complex (MTBC) and these genes are absent in any BCG vaccine strains and in the non-tuberculous mycobacteria (NTM), with a few exceptions (*M. flavescens*, *M. gastri*, *M. gordonae*, *M. kansasii*, *M. marinum* and *M. szulgai*)[2]. CFP10 is a major antigen recognized by *M. tuberculosis*-specific human T and B cells[2] and this antigen is recognized *in vivo* and *in vitro* based on the induction of DTH responses and the ability to induce gamma interferon production by lymphocytes, respectively[3]. CFP-10 and ESAT-6 have been extensively investigated as *M. tuberculosis* and *M. bovis* specific antigens for the diagnosis of tuberculosis in humans[4] and bovine tuberculosis (BTB) in cattle[5].

IFN-γ assay is based on whole blood or isolated peripheral blood mononuclear cells (PBMCs) which are stimulated in the presence of mycobacterial antigens (avian or bovine PPD or other more specific antigens of *Mycobacterium* sp.), that induce previously sensitized T-cells to produce IFN-γ. Quantification of this IFN-γ is performed by sandwich enzyme-linked immunosorbent assay [ELISA] or enzyme-linked Immunospot assay (ELISPOT)[6]. Using a quantitative real-time PCR (qRT-PCR) method for detection of IFN-γ at the mRNA level is an alternative method of demonstrating IFN-γ induction as a measurement of the cell-mediated response to mycobacterial antigens. There are reports on the use of qRT-PCR for detection of IFN-γ in human TB patients[7], European badgers[8], in elk and red deer[9].

Considering the importance of CFP10 in T-cell recognition and protection, in this study, we investigated the presence of CFP-10 gene in Indian *M. bovis* (3/86Rv) strain and its expression in suitable prokaryotic host followed by evaluating its diagnostic potential by single intradermal test (SIDT) and qRT-PCR based interferon gamma (IFN-γ) assay.

Materials and Methods

Bacteria, PPDs, vectors, primer

M. bovis (3/86Rv) was isolated from tuberculosis positive cattle mediastinal lymph node in 1986, subsequently characterized and maintained at

*Correspondence:
Phone: +91 9359117376
E-mail: rishendra_verma@yahoo.com

Mycobacteria Laboratory, Division of Bacteriology and Mycology, IVRI, Izatnagar. PPD was prepared from the same *M. bovis* (3/86Rv)[10]. For expression construct, pET32b plasmid vector was used while *Escherichia coli* BL21 (pLysS) competent cells were used for inducing the over expression harboring the plasmid construct. All the primers were custom synthesized.

Animals

Apparently healthy adult guinea pigs of either sex weighing about 200-300 g were reared in pathogen free environment at Animal Shed, CADRAD, IVRI. The experiment had approval of the Institute Animal Ethics Clearance (IAEC).

Construction of recombinant CFP10-pET32b clone

Construction of rCFP10-pET32b was done by amplification of full length sequence encoding the CFP-10 protein from *M. bovis* (3/86Rv) using a primer set based on the reference sequence available at Gen Bank. The primers were designed using primer designer software in GeneTool Lite 1.0 software. The sequence of the oligonucleotides with added restriction sites (italics and underlined) for *BamH*I and *Hind*III at 5' end of forward and reverse primer along with tags (small letter), respectively, were CFP10F: 5'-cagca*GGATCC* CATGGCAGAGATGAAGACC-3' and CFP10R: 5'-gccc *AAGCTT*GAAGCCCATTTGCGAGG-3' (in which the stop codon present in the CFP-10 gene was not included). Chromosomal DNA was purified from *M. bovis* (3/86Rv) strain using standard chloroform: phenol procedures and was used as a template for the amplification of the CFP-10 gene. The PCR was performed in thin walled 0.2 mL PCR tubes containing approximately 100 ng of bacterial genomic DNA, 5 µL 10X PCR buffer, 2 mM $MgSO_4$, 1 µL of 10 mM dNTPs, 10 µM of the primer sets (CFP10F and CFP10R), 2.5U of Pfu DNA Polymerase (Thermo Scientific, USA) and the volume was made up to 50 µL with Nuclease Free water (NFW). The PCR amplification was performed in a thermo-cycler and consisted of initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min and extension at 70°C for 1 min, followed by a final extension at 70°C for 10 min. A non-template control was run in all the PCR experiments to rule out the possibility of contamination in the samples. The PCR amplified products were resolved on 2% agarose gel in Tris acetate EDTA (TAE) buffer (1X). The agarose gel was stained with ethidium bromide and documented under UV light in a gel documentation system. The PCR amplified product (50 µg) was digested with 10U of *BamH*I and *Hind*III and ligated in to pET32b vector. The recombinant rCFP10-pET32b was transformed into chemically competent *E. coli*BL21 (pLysS) cells. The transformed rCFP10-pET32b was selected on Luria-Bertani (LB) agar plate containing ampicillin (50 µg/mL) and chloramphenicol (34 µg/mL). The colonies on the LB agar plates were screened by colony PCR using gene specific primers and the plasmid was isolated from the colonies containing the CFP-10 insert using GeneJet™ Plasmid Miniprep Kit. The isolated plasmids were digested with *BamH*I and *Hind*III to release the specific insert. The isolated plasmid was assessed for quality and quantity; subsequently sequenced using the Big dye terminator v3.1 Cycle Sequencing Kit following the manufacturer's instructions on an automated DNA sequencer. The sequence chromatogram was annotated with BioEdit Sequence Alignment Editor Softwares 7.0.5. The annotated sequences were identified based on being the closest match to the sequences submitted in the NCBI database on BLAST analysis. The sequence analyzed in this study was submitted to the NCBI GenBank with the following Accession number KJ206083.

Overexpression, purification and Western blot of rCFP-10

*E.coli*BL21 (pLysS) cells harboring recombinant plasmid (rCFP10-pET32b) were grown at 37°C in 1 L LB broth containing appropriate antibiotics and induced with 1 mM IPTG. Fractionation was done at 2 h interval up to 8 h to check the expression level of rCFP-10 induced within the cells. The harvested cells were solubilized in a Lysis buffer and the protein was purified under native condition by affinity chromatography using Ni-NTA superflow cartridges as per standard procedures. The rCFP-10 was dialyzed in a chilled environment using a dialysis bag, concentrated and stored at −80°C until further use. The concentrated rCFP-10 was quantified by NanoDrop Spectrophotometer. For confirmation, purified rCFP-10 was run on SDS-PAGE to confirm the expected molecular weight, transferred onto nitrocellulose membrane using semidry immunoblot system and detected using anti *M. bovis* (3/86Rv) polyclonal hyperimmune guinea pig serum and goat anti-guinea pig IgG-HRPO conjugate as primary and secondary antibodies, respectively. The blot was incubated in substrate solution (10 mg DAB) in dark for colour development.

Guinea pigs immunization trials and evaluation of skin test using PPDs and rCFP10

A total of 24 guinea pigs of either sex were divided into two groups of 12 animals each. The first group (Group A) was inoculated intra-muscularly with 0.5 mL suspension containing 4 mg/mL of heat-killed *M. bovis* (3/86) bacilli in liquid paraffin as per standard procedures recommended by OIE. The other group (Group B) containing 12 animals were kept as control. Six guinea pigs from each group on 35th day of inoculation were injected different concentrations of purified rCFP-10 protein (0.5, 1.0, 1.5 and 2 µg) by intra-dermal route in the right flank; while bovine PPD (10U) and PBS were injected in left flank as positive and negative control, respectively. The injection sites were checked for erythema after 24, 48 and 72 h to measure any inflammatory responses/skin erythema. The diameter of both axis of skin erythema were independently measured and recorded.

Real-time PCR to quantify mRNA interferon-γ expression levels

Blood culture

Blood samples were collected from saphenous vein of six guinea pigs from each group in BD®Vacutainer (containing heparin as anticoagulant) and transported to the laboratory maintaining cold chain during transit. The whole heparinized blood was mixed with equal proportion of RPMI 1640 medium and 1.5 mL of the mixture was added to each well of the 24-well culture plate in aseptic conditions. The medium was supplemented with antibiotics (penicillin 50 U/mL and streptomycin 50 µg/mL) to reduce the risk of contamination. One of five antigens consisting of bovine PPD (30 µg/mL), *avium* PPD (30 µg/mL), concavillin A (5 µg/mL), rCFP10 (5µg/mL) and PBS (pH 7.4, control) was added in culture plates in triplicate under aseptic conditions. The culture plates were gently swirled on a smooth flat surface and incubated statically at 37°C in 5% humidified CO_2 tension for 6 h.

RNA isolation and cDNA synthesis

After 6 h of incubation, the cultured cells were pelleted on centrifugation at 2000 rpm for 10 min. In a fresh 1.5 mL microcentrifuge tube, 200 µL sedimented cells of blood culture was mixed with 1 mL of the RiboZol reagent and RNA was extracted following manufacturer's instructions. The RNA was extracted with Nuclease Free Water to a final volume of 20 µL. To remove any possible contamination by genomic DNA, the samples were submitted to digestion with DNase. The isolated RNA was assessed for quality and quantity using Nanodrop Spectrophotometer and stored at −80ºC until further use. Reverse transcription of total RNA (2 µg) was carried out using a RevertAidcDNA synthesis kit as per the manufacturer's recommendations employing 200U of RevertAid reverse transcriptase enzyme and oligodT (0.5 µg) during synthesis. The cDNAproduct was stored at −20°C.

Real-time PCR

The primers for guinea pig IFN-γ was designed using online Primer Quest software based on the guinea pig IFN-γ reference sequence available at GenBank while the primers for endogenous housekeeping gene β-actin were designed based on the published sequence[11]. The primer sequences have been enumerated. Real Time PCR was carried out in MX3000P Real Time PCR System using 2 µL of cDNA, 10 µL 2X Maxima SYBR Green Master Mix, 0.3X ROX as a passive reference dye, 5 pmol of forward and reverse primer and the volume was made up to 20 µL using Nuclease Free water. Cycling parameters for the real time PCR was initial denaturation for 10 min, followed by 40 cycles at 95°C for 30 s, 56°C for 30 s and 72°C for 30 s. Melting curve analysis was performed for each sample to verify the specificity of each products.The real-time data obtained was analyzed by MxPro^{TM}QPCR Software version 4.10. Intra-assay and inter-assay variability were determined for each target gene and each sample was run in triplicate.

Statistical analysis

Data generated during the skin test guinea pig experimentation was expressed as means of diameters (in mm) of erythema±SEM using SPSS 16.0 software. The change in the level of expression of IFN-γ mRNA in the antigen-stimulated blood cultures relative to that in the control cultures was calculated using the $2^{-\Delta\Delta Ct}$ method[12]. The statistical significance of differences in mRNA expressions of the examined factors was assessed by Paired t-test using SPSS 16.0 software. Differences were considered significant if $P < 0.05$.

Results

***M. bovis* CFP-10 sequence analysis and construction of rCFP10-pET32b clone**

The full length CFP-10 gene of *M.bovis*(3/86Rv) consists of 303 bp ORF which encodes 100 aa of 10.8 kDa. The stop codon of the gene was not included in the primer sequence so as to rCFP-10 protein would consist of N and C terminal T7 tag of the vector pET32b (Fig. 1, Panel A). The vector

pRT23b provides only one tail of histidine repeats, and that is the carboxy terminal. The amino terminal portion of pET23 provides a T7 tag. The PCR amplified product gave an expected size of 322 bp on 2% agarose gel electrophoresis. The product was eluted from the gel, digested with BamHI and HindIII, ligated to predigested pET32b vector, transformed to *E. coli*BL21 (pLysS). The recombinant clones were screened by colony PCR with specific primers which gave an amplified product of 322 bp in the positive samples (Fig. 1 Panel-B Lane-1). Plasmid isolation was done for a positive sample, sequenced with vector specific primers in both the strands. The sequence (300 bp as the stop codon was not included in the primer sequence) revealed 100% sequence identity at the nucleotide level with other CFP-10 gene of other *M. bovis* strains. The sequence had 63.67% GC content; while the encoded polypeptide (100 aa) consisted of 9 basic, 14 acidic, 34 hydrophobic and 32 polar aa with an isoelectric point of 4.479. CFP-10 protein has a high antigenic index, hydrophilicity and surface probability plots as shown by PROTEAN in the Lasergene Software package (Fig. 2 Panel A). Protein secondary structure prediction by online PSIPRED v3.3 revealed two alpha-helix (H1:11-38 aa and H2: 44-84 aa) having a connecting loop (39-43 aa) between the two helices (Fig. 2 Panel-B). The polypeptide sequence matched with cfp10-esat6 complex from *M. tuberculosis* (PDB id: 3fav) in PDBsum in the database.

Expression, purification and Western blot analysis of rCFP10

IPTG induction of the *E. coli* BL21 (pLysS) cells resulted in over-expression of rCFP10 which accounted for a molecular weight of approximately 30 kDa, of which rCFP10 was approximately 10.8 kDa (100 aa) and 19 kDa was the coding region of the vector pET32b containing the one his tags at both the N and the C terminal. The un-induced, induced and the purified rCFP10 were run on 12% SDS-PAGE; the purified rCFP10 migrated as a single protein of approximately 30 kDa (Fig. 1 Panel-C Lane-3; Fig. 1 Panel-D Lane-1). The 30kDa protein was formed by 11 kDa of the CFP-10 protein and 19kDa from the vector sequence. The rCFP10 was also detected and confirmed by immunoblot assay using hyper immune serum raised against heat killed *M. bovis* (3/86Rv) in guinea pigs (Fig. 1 Panel-E Lane-1).

Immunization trials in guinea pigs and evaluation of skin test using PPDs and rCFP10

Different doses of rCFP10 protein (0.5, 1.0, 1.5 and 2.0 µg), 10IU bovine PPD (positive control) and 100 µL of PBS (negative control) were tested for

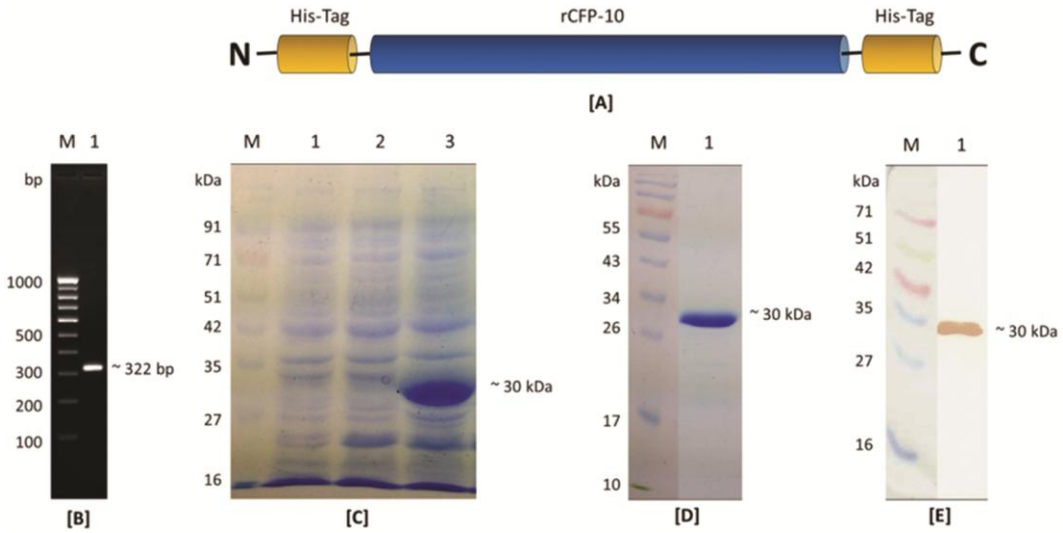

Fig. 1 — Schematic representations of rCFP-10 construct amplification, expression, purification and immune blot detection. Panel A: Mature rCFP-10 with both N- and C-terminus hexa-histidine tags; Panel B. PCR Amplification of *cfp10* gene from *M. bovis* (Strain 3/86), Lane M: 100 bp DNA ladder (Thermo Scientific, USA); Lane 1. Amplified *cfp10* gene product (~322 bp); Panel C. Overexpression of rCFP-10 in *E. coli* BL21-pLysS, Lane M: Four colour pre-stained protein ladder (Puregene, USA); Lane 1. *E. coli* cell lysate without rCFP10-pET32b plasmid; Lane 2. Un-induced *E. coli* cell lysate; Lane 3. Induced *E. coli* cell lysate with 1mM IPTG; Panel D**:** Purified rCFP-10; Lane M. Pre-stained protein ladder (Thermo Scientific, USA); Lane 1. Purified rCFP-10 (~30 kDa); Panel E:Immunoblot of rCFP-10; Lane M. Four colour pre--stained protein ladder (Puregene, USA); Lane 1. Development of positive reaction of rCFP-10 with guinea pig hyper immune serum against *M. bovis*.

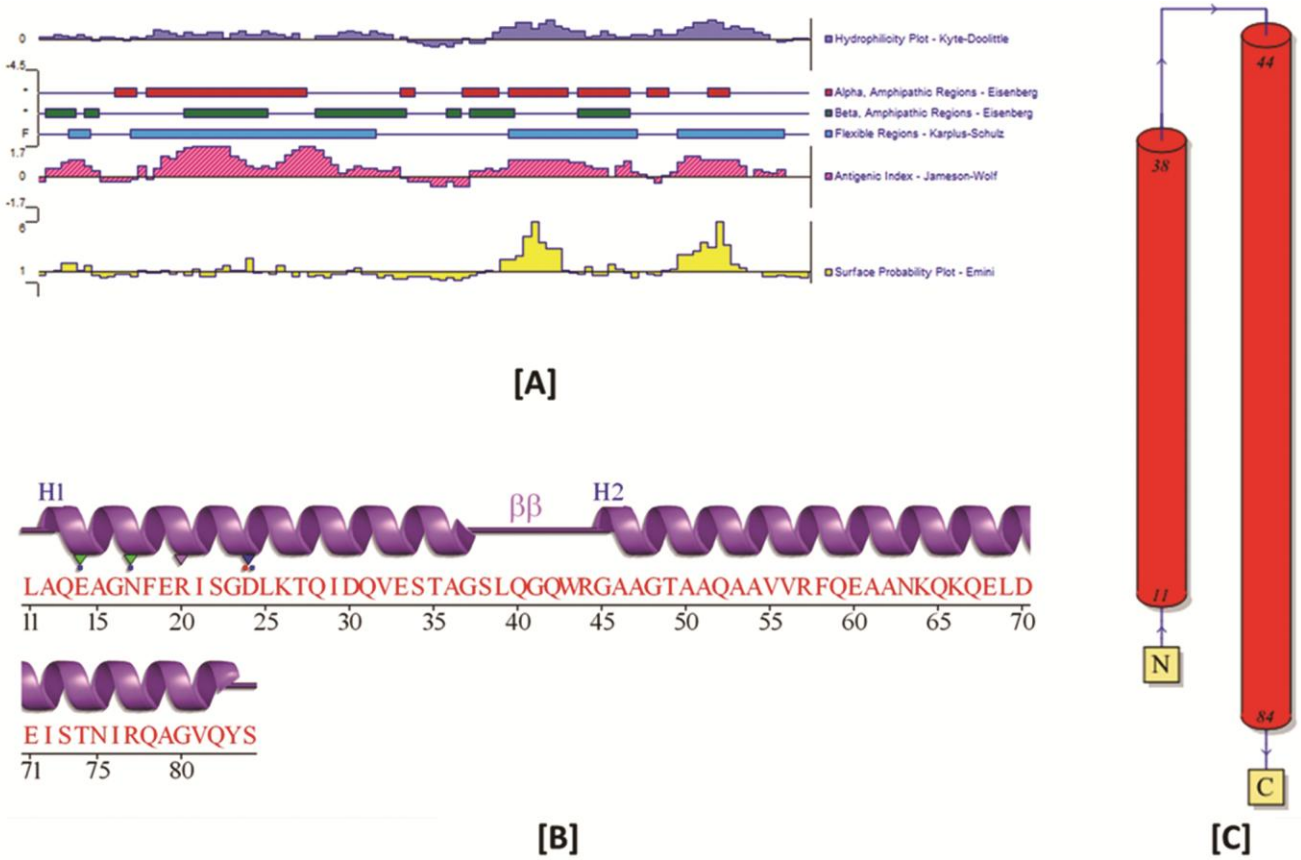

Fig.2 — Predicted characterization of CFP-10 protein of *M. bovis*. Panel A: Schematic representation of CFP-10 depicting hydrophobicity plot, alpha amphipathic regions, beta amphipathic regions, flexible regions, antigenic index and surface probability plot by PROTEAN; Panel B**:** Predicted secondary structure of CFP-10 by PSIPRED v3.3 of ExPasy depicting two alpha helices (H1 and H2) joined by connecting loop; Panel C: Predicted secondary structure of CFP-10 indication as predicted by GenTHREADER start and end amino acid of two helices.

their ability to produce DTH response in guinea pigs sensitized with heat killed *M. bovis*. All the four doses of rCFP10 elicited positive DTH response at 24 h and 72 h after injection; as evident by skin fold thickness of greater than 5 mm in diameter in most of the groups. The higher dose of rCFP10 (1.5 and 2.0 µg) elicited stronger response than at lower doses. The positive response measured after 24 h was higher in all the groups, which gradually reduced after 48 and 72 h. A skin fold thickness of less than 5 mm was considered as negative.

Measurement of mRNA IFN-γ levels by Real-time PCR

The linearity and the efficiency of the real-time PCR primers for guinea pig IFN-γ and β-actin was calculated based on the serial dilution of recombinant plasmid containing the insert. Both the primers had high regression coefficient (R^2 of 0.99 and 0.98) with an efficiency of 99 and 98% of IFN-γ and β-actin, respectively. The Ct values of the samples from different groups (stimulated with rCFP10, bovine PPD, *avium* PPD and PBS as negative control) was recorded in triplicate and was calculated based on the adaptive baseline threshold method for each sample and the melting temperature based on the Melting curve analysis in the Segment 3 of the program was analyzed using MxPro^{TM}QPCR Software version 4.10. The inter-assay and the intra-assay was not statistically significant at P <0.001. The mRNA expression fold change was calculated using the Livak method using β-actin mRNA to normalize the expression within the groups. IFN-γ gene was significantly up regulated in antigen stimulated groups in comparison to unstimulated control group. The expression of IFN-γ mRNA in blood 5 h post-stimulation was found up to 19.962, 20.795, 9.633, 34.511 and 1.688 times in rCFP10, bovine PPD, avian PPD, conA and PBS stimulated groups, respectively as compared to healthy control group. The mRNA expression level of bovine PPD

and avian PPD stimulated group was found statistically significant (at P <0.05) and among conA and rCFP10 stimulated group it was highly significant (at P <0.01) in comparison without antigen stimulated group.

Discussion

There are number of proteins which are actively secreted in the culture filtrate of mycobacteria and identification of these secreted proteins has received considerable attention because of their possible immunodominant role in protective immunity[13]. Many proteins like ESAT-6, CFP-10, TB10.4 have been exploited for the diagnosis of BTB. Recombinant CFP-10 protein induced DTH reaction in guinea pig have been shown to be a viable alternative for tuberculin skin test (TST)[14]. Upon infection with *M. tuberculosis*, CFP-10 may contribute specifically to neutrophil recruitment and activation during *M. tuberculosis* infection, representing a novel biological role for CFP-10 in the ESAT-6:CFP-10 complex[15]. It has been reported that ESAT6-CFP10 could support the survival of *M. tuberculosis* in the host through altering the host immune response[16].

We cloned and expressed CFP-10 gene of *M. bovis* (3/86Rv) in *E. coli*. For this purpose CFP-10 gene was PCR amplified, cloned and sub cloned into expression vectors and expressed as a~30 KDa fusion protein, termed as recombinant CFP-10 protein (rCFP-10). The identity of the expressed protein was confirmed by sequence analysis and Western blotting with anti-His antibodies. PCR was standardized from the genomic DNA of *M. bovis* (3/86Rv) and obtained specific product (322 bp). Sequence analysis revealed similar sequences as reported earlier; where an insert corresponding to a 300 bp open reading frame encoding CFP-10, a 100 amino acids polypeptide with an average molecular mass of 10.7 kDa[17]. The sequence cfp-10 gene of *M. bovis* (3/86Rv) (Gene Bank Accession number KJ206083) was found to be having 100% sequence homology with most of the members of MTB complex and corroborates the previous findings which indicated that CFP-10 gene is highly conserved in most of the members of MTB[14]. There are some reports that described the cloning and expression of the CFP-10 gene in different expression vectors such as, pET28b and pQE60[1], pET28a[18], pET102/D[17]. We expressed CFP-10 protein from the recombinant pET vector as a ~30 kDa fusion protein including vector sequence and His tag. The 30 kDa protein was formed by 11 kDa of the CFP-10 protein and 19 kDa from the vector sequence. Successful expression was achieved when the culture was induced by 1 mM IPTG concentration and incubated 2 h at 37°C with shaking. The expression increased gradually and optimum expression was obtained when the culture was incubated 4 h at 37°C with shaking and then reduced on further incubation, which is may be due to the autolysis of bacterial cells. Early expression of CFP-10 protein may be due to smaller size of protein. The result of our study was quite similar to Wu *et al*.[19] who also got highest concentration when induction (1 mM IPTG) took place for 4 h and also observed that the induction time and temperature affected the expression level significantly.

Our study demonstrated that rCFP-10 from *M. bovis* (3/86Rv) was purified in soluble form by Ni-NTA chromatography using the principle that the protein carrying an exposed histidine tag bind to resin charged with divalent nickel ions. By this process, contaminating proteins could be removed by appropriate washings and the protein of interest could then be eluted. The finding contradicted with those who reported that rCFP-10 proteins of *M. tuberculosis* were expressed mostly in insoluble form and purified in low-yield after a time consuming and laborious process[17,18] was able to express CFP-10 from *M. tuberculosis* in soluble form using Champion pET Directional TOPO kit and pET102/D expression vector having a thioredoxin stretch in the N-terminus that makes the expression of protein in the soluble phase. The soluble rCFP-10 could be easily purified by metal affinity chromatography and used for establishing a sensitive and specific test as an alternative to traditional diagnostic procedures. The same gene form *M. tuberculosis* have been previously cloned, expressed (induction temperature 30°C) and purified in soluble and the amount of rCFP-10 protein obtained ranged from 35-50% of the total soluble proteins[15]. Different doses of rCFP-10 protein were given to check for the ability to produce delayed type hypersensitivity (DTH) responses in guinea pigs sensitized with killed *M. bovis* (3/86Rv). As low as 0.5 µg of rCFP-10 produced positive skin reaction. Specifically, 2 µg of purified. rCFP-10 protein of *M. bovis* (3/6Rv) produced DTH response and produced up to 7 mm diameter of erythema where as 10 IU of bovine PPD produced 11 mm of diameter of erythema in guinea pigs. The DTH response produced by rCFP-10 was weaker than that of PPD because PPD contain a large numbers of proteins. The peak DTH response in guinea pig was observed at 24 h. The responses to rCFP-10 and PPD used in the

experiment reduced gradually at 48 and 72 h, respectively. This observation in guinea pig was consistent with the studies of Wu *et al*.[19]. Our result was quite similar with previous findings but the diameter of erythema in both the cases for PPD and rCFP-10 were greater/bigger than our study[3,20]. The probable reason of increase in diameter of erythema in their studies may be due to the sensitization of animals by live culture; whereas in our study we used killed culture of *M. bovis* (3/86Rv).

In guinea pig sensitized with heat killed *M. bovis* (3/86Rv) IFN-γ mRNA quantitative real-time PCR method was standardized. In case of cattle, IFN-γ mRNA expression was found to increase 12.796, 16.808, 7.840 and 25.524 fold in CFP-10, bovine PPD, avian PPD and ConA stimulated whole blood respectively as compared to whole blood of healthy control animal. The IFN-γ gene response was significantly upregulated in response to avian PPD and ConA but a specific elevated IFN-γ response to bovine PPD was only observed after 12 hour post stimulation (40.1-fold upregulation)[21]. After that, the expression of IFN-γ mRNA due to bovine PPD stimulation was decreased and this evidence was supported by Blanco *et al*.[19] who reported that PBMC from infected animals responded to bovine PPD stimulation, expressing 12.8 fold more IFN-γ mRNA than cells from control animals[22]. The IFN-γ assays are replacing the traditional tuberculin skin testing in diagnosis of tuberculosis both in humans and animals[23,24] as being reported in this study. With respect to our study, probably, there is no contemporary literature published for guinea pig. The significance of CFP-10 reported in one of the study reported in 58 *M. bovis* isolates confirmed the high conservation CFP-10 along with ESAT-6 and EspC in local *M. bovis* field isolates and reinforced the use of these three antigens in the diagnosis of bovine tuberculosis[25]. In-house developed esxB (CFP-10) primer-probe for real-time PCR TaqMan assay has also reported its potential in detecting *M. bovis* in the blood and lymph node aspirates, hence used in the diagnosis of *M. bovis* in blood samples and lymph node aspirates of cattle and buffaloes[26].

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