DISCUSSION

The current diagnostic tests for TB include sputum smear microscopy and culture target detection of causative agent; these are mostly used in all over the world and in India. Although sputum smears microscopy is a rapid diagnostic method but it has major limitations too. This test needs a minimum load of –bacilli in sputum and this test cannot be used in pediatric population as it is difficult to get sputum from children. Culture of *M. tuberculosis* on Lowenstein Jensen (LJ) media is still a gold standard technique to diagnose TB but the long generation time (18-24 hrs) (Cole et al., 1998) makes this technique time consuming. Some diagnostic tests for TB have also been developed taking into consideration of host immune response. The Tuberculin / Mantoux test is not as successful in India as compared to some developed countries as a large proportion of healthy population may have had subclinical infection, and may have healed of their own or with therapy. Misleading results may also be sometimes due to PPD containing antigens widely shared with different species of Mycobacteria such as environment mycobacteria and BCG strains (Andersen et al., 2000). On the other hand tuberculin / preparations from strains such as H37RV may not sensitize human populations to the extent clinical isolates might do. Various antigens of *M. tuberculosis* have also been tried all over the globe, but there is no commercial available serological test with acceptable specificity and sensitivity for implementing in routine laboratory use. A major breakthrough in diagnosis of latent TB was achieved when test based on IFN-γ production by T cells in response to some *M. tuberculosis* specific antigens such as ESAT-6 and CFP-10 was developed (Lalvani et al., 2001; Chapmam et al., 2002). The QuantiFERON-TB, QuantiFERON-TB gold are based on IFN-γ production which got approval for diagnosis from FDA, USA in 2001(CDC). These tests have some drawbacks for implementing into third world country such as India for practical and routine laboratory diagnosis, as these tests need well equipped laboratory, high skilled manpower and are not cost effective. Vaccine could work as a major tool to control any disease. No one can neglect the power of effective vaccine. The eradication of smallpox from the globe and declaration of polio free India is major example of vaccine strength. However, there is no major successful vaccine available to combat TB except BCG since its discovery. The BCG is given to more people than any other vaccine and side effects of
this vaccine are tolerable. BCG can prevent miliary and meningeal TB in young children (Kaufmann et al., 2000). However, BCG has major limitation as it fails to protect TB in adults. Data shows the protective efficacy of BCG in adults ranges from 0% in South India to 80% in UK (Garg et al., 2003). Hence there is an urgent need of effective vaccine for TB and simple, further there is need for cost effective and diagnostic test with acceptable sensitivity and specificity for effective management and control of TB.

*M. tuberculosis* is an intracellular pathogen. After infection with *M. tuberculosis* the host interacts with several proteins expressed or released by bacteria and these proteins may work as a good candidate in reference to diagnosis and vaccine. Exhaustive research has been done to find out new antigen using current laboratory standard H37Rv. H37Rv was isolated in 1905 (Cole et al., 1998) and has been subpassaged and distributed. It suggested that in comparison to H37Rv, clinical isolates isolated may express new/differentially/over-expressed proteins. The first evidence of expression of differentially/over-expressed proteins at intracellular level was shown in genomic studies. The complete gene profile of *M. tuberculosis* in mouse macrophage (Schnappinger et al., 2003), human macrophage (Dubnau et al., 2002) and human macrophage cell line THP-1 (Fontan et al., 2008) were analyzed and showed differential mRNA expression. However, gene based studies do not show that these mRNA products could convert in protein product. Clinical isolates of *M. tuberculosis* recently isolated from host (human) could work as a possible study material to know the differential proteomic profile in human stress as compared to H37Rv. Siddiqui et al., 2000; Betts et al., 2000; Rajavelu and Das 2003; Pheiffer et al., 2005; Shin et al., 2008; Kumar et al., 2012 also demonstrated in comparison H37Rv clinical isolates may have better chance to express new protein/antigen.

India is considered as a high burden country for tuberculosis. Molecular typing of *M. tuberculosis* strain has proven to be valuable tool for TB control in terms of tracking transmission chain, detecting suspected outbreaks and identification of successful clone (Barne and Cave, 2003). Several genotype of *M. tuberculosis* have been identified by spoligotyping and MIRU-VNTR which are found prevalent in India and globally (Sharma et al., 2008; Singh et al., 2004; Glynn et al., 2002). The *M. tuberculosis*
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genotype CAS1_Del (ST-26/25) and EAI3_Ind (ST-11) are reported predominant in different regions of India. Other genotype such as X-1, Haarlem and T-1 are also reported in India. The Beijing strain genotype is predominant in China and also reported its presence all over the world (Glynn et al., 2002).

In present study we have taken clinical isolates belonging to prevalent genotype (CAS, EAI, T-1, X-1, Haarlem and Beijing family). Main aim of the study is to find out differentially/over-expressed proteins in these strains than H37Rv and to understand the immune reactivity of these proteins. To achieve above target we divided whole study in four objectives which were achieved in four successive steps; first, we identified and selected recent clinical isolates showing higher immune response than H37Rv; second, we analyzed differentially/ over-expressed proteins in clinical isolates in comparison to H37Rv and tried to find immune-reactive proteins by 2 D gel electrophoresis and western blot; third, differentially/over-expressed and sero-reactive proteins were identified by MALDI- TOF mass spectrometry and lastly fourth, we analyzed the immune response to identified proteins in reference to adaptive immunity using their commercially synthesized peptides having T and B cell epitopes predicted by bioinformatics analysis to know its diagnostic and vaccine efficacy.

To achieve above objective one (Identification and selection of Indian M. tuberculosis strains showing higher immune response than the H37Rv) we used laboratory strain H37Rv, H37Rv- recently isolated form an animal, M. bovis – a BCG vaccine strain, PPD as control and 75 clinical isolates of M. tuberculosis from Mycobacterial repository Centre, NJIL&OMD, Agra. Out of 75 clinical isolates nineteen clinical isolates of M. tuberculosis were selected after their reconfirmation of family, sensitivity profile and growth profile. Out of nineteen selected clinical isolates, five (C2, C11, C19, C21, C23) were belonging to CAS1_Del, five (C5, C6, C26, C60, C62) belonging to EAI3_IND, five (C68, C69, C70, C71, 73) belonging to Haarlem family, two (C73, C74) belonging to T-1 family, one (C16) belonging to X-1 family and last one (C78) belonging to Beijing family.

The humoral (antibody) immune response of healthy (PPD-ve & +ve), healthy house hold contacts (HHC) and pulmonary TB patients (PTB) were analyzed to whole
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cell lysate antigen of these *M. tuberculosis* isolates belonging to different prevalent genotypic family. Significant higher antibody response was noted in PTB group in comparison to healthy group to controls (*H*37*Rv*, *H*37*Rv* isolated from animal and PPD). Further, significant lower antibody response to PPD was noted in HHC individuals. Patients showed higher antibody response to *M. bovis* BCG strain but no significant difference was observed between any groups. Antibody response to whole cell antigen of clinical isolates was measured using strains belonging to CAS, EAI, Haarlem, T-1, X-1 and Beijing family. Out of 19 clinical isolates patients group show significant higher antibody response to whole cell antigens of seven clinical isolates in comparison to healthy group. Out of seven, two clinical isolates were belonging to CAS (C11, C23), two clinical isolates were belonging to EAI (C6, C26), one clinical isolates belonging to T-1 (C73), one clinical isolates were belonging to X-1 (C16) and last one were belonging to Beijing (C78) family. Significant higher antibody response in patients than HHC was noted to whole cell antigens of nine clinical isolates. Out of nine clinical isolates, two clinical isolates belonging to CAS (C11, C23) four clinical isolates belonging to EAI (C6, C26, C60, C62), one clinical isolates belonging to T-1 (C73), one clinical isolates belonging to X-1 (C16) and last one clinical isolates belonging to Beijing family (C78). HHC showed significant higher antibody response to WCL antigen of Beijing (C78) in comparison to healthy individuals. No difference in the antibody response between any groups was noted against WCL of clinical isolates belonging to Haarlem family. We observed that several clinical isolates belonging to CAS, EAI genotypes and Beijing strain could differentiate PTB from healthy group and further PTB from HHC group when their antibody reactivity was assessed. While antibody response to *H*37*Rv*, *H*37*Rv* animal and PPD can only differentiate PTB from healthy group. Further, antibody response to *M. bovis* BCG strain not showed any differentiate between groups. This observation suggests that *H*37*Rv* and clinical isolates have some proteins which have higher antibody reactivity in patients as compared to healthy but clinical isolates may have some up-regulated (differentially/over-expressed) proteins which will able to differentiate patients group to HHC group and further HHC group to healthy group which was not noted with *H*37*Rv*.
DISCUSSION

Cell mediated immune response to 19 clinical isolates belonging to different genotypic family and to controls (H₃₇Rv, H₃₇Rv animal, M. bovis BCG, PPD) was also measured using WCL antigen in PBMCs of healthy individuals by lymphocyte transformation test (LTT). Antigen showing Stimulation Index (S.I.) two or more than 2 was considered as positive stimulator. Results of LTT showed that H₃₇Rv, H₃₇Rv isolated from animal, M. bovis, PPD and clinical isolates belonging to prevalent genotype (expect C11) showed positive stimulation i.e. S.I. 2 or more than two. We noticed an interesting observation that H₃₇Rv which was isolated from animal (provided by experiment facility) showed higher S.I. value in comparison to laboratory passaged H₃₇Rv and M. bovis BCG. This suggests that H₃₇Rv isolated from animal expressed some differentially/over-expressed proteins which could be responsible for increased lymphoproliferation. Lower S.I. value of M. bovis BCG suggests that adults may not have long term memory to BCG immunization. Further, higher stimulation to PPD may be due to cross reactive proteins which may be present in PPD (Anderson et al., 2000). Further, out of 19 clinical isolates, 13 clinical isolates belonging to prevalent genotype showed higher S.I. value than H₃₇Rv isolated from animal. Out of 13 clinical isolates, three clinical isolates belonging to CAS (C2, C19, C23), three clinical isolates belonging to EAI (C5, C26, C60), five clinical isolates belonging to Haarlem (C68, C 69, C70, C71, C72), one clinical isolates belonging to T-1 (C73) and last one belonging to Beijing family (C78). This observation suggests that these clinical isolates could have some differentially /over-expressed proteins which could be responsible for higher lymphoproliferation than H₃₇Rv animal, H₃₇Rv and M. bovis BCG. After these observations one may need an explanation, why clinical isolates showed higher S.I. values than H₃₇Rv isolated from animal while both have recently faced in-vivo stress? The possible explanation could be behind the difference in physiological and immunological environment of clinical isolates isolated from human and H₃₇Rv isolated from animal. This further gave us lead to work on recent clinical isolates of M. tuberculosis isolated from humans for finding new diagnostic and vaccine candidate(s). Different reports also support our observations on BCG, PPD and clinical isolates such as; Fine et al. in 1995; Kaufmann, in 2000, suggested that protective efficacy of BCG in adults range from zero, in South India to 80% in UK. Chaparas et al. in 1970 and Anderson et al. in 1994 showed that PPD have low
specificity because it contains some proteins which are present in various mycobacterial species and in various unrelated species of bacteria. *Rajavelu and Das, 2003* and our group, *Kumar et al., 2012* also reported that CFP and WCL protein antigens of clinical isolates evoke better CMI and IFN-γ in comparison to laboratory strain H₃₇Rv but in comparison to these studies we analyzed clinical isolates six prevalent genotypic family simultaneously in comparisons to H₃₇Rv. Further, in present study we used total sensitive clinical isolates of *M. tuberculosis* for analysis while in previous studied sensitivity profile was not clear. Because H37Rv is a total sensitive strain so for comparison we have included fully sensitive clinical isolates of *M. tuberculosis* for better comparison at immune reactivity and proteomic level.

After the preliminary study (ELISA and LTT) we selected nine clinical isolates out of 19 clinical isolates belonging to prevalent family for one-dimensional gel electrophoresis. Out of nine clinical isolates, two clinical isolates from each belonging to CAS (C19 ‘on CMI basis’, C23 ‘on Ab & CMI basis’), EAI (C5 ‘on CMI basis’, C26 ‘on Ab & CMI basis’), Haarlem (C68, C71 ‘both on CMI basis’) and one clinical isolates belonging to X-1 (C16 ‘on Ab basis’), T-1 (C73 ‘on Ab & CMI basis’) and Beijing (C78 ‘on Ab & CMI basis’) prevalent genotypic family were selected on the basis of their higher antibody reactivity to patient sera in comparison to healthy group or higher S.I. values than H₃₇Rv (cell mediated immune response) (Objective 1).

After completion of objective one, objective two (*To analyze the comparative proteomic profile of Indian prevalent strains and H₃₇Rv*) was devoted to analyze comparative proteomic profiles of selected clinical isolates to H₃₇Rv by 1-DGE and 2-DGE. First we did one dimensional gel electrophoresis or (SDS-PAGE) using while cell lysate of nine clinical isolates belonging to prevalent genotype and H₃₇Rv, H₃₇Rv animal and *M. bovis* BCG. H₃₇Rv, H₃₇Rv animal and *M. bovis* BCG were used as control for comparative one dimensional gel electrophoresis. In SDS-PAGE majority of clinical isolates belonging to CAS, EAI, X-1, T-1 Beijing and Haarlem showed over-expressed proteins in region of 41-59 kDa, 35 kDa, and at 15-21 kDa region in comparison to H₃₇Rv.
DISCUSSION

Western blots were also performed after SDS-PAGE using WCL of controls and clinical isolates belonging to prevalent genotype. Western blot were developed using pooled serum of PPD-ve, PPD+ve, HHC and PTB. Sera of TB patients showed high reactivity with H\textsubscript{37}Rv and H\textsubscript{37}Rv isolated from animal and with all clinical isolates than the sera obtained from PPD-ve, PPD+ve and HHCs. Further, though low reactivity of HHC with most of the clinical isolates was noted but they showed reactivity with clinical isolates C23, C26 and C78 at some regions. Sera of PPD-ve and PPD+ve healthy showed heterogeneous sero-reactivity to clinical isolates. We noted an interesting observation that WCL protein of \textit{M. bovis} BCG showed very low reactivity with patients sera in comparison to clinical isolates. Further, \textit{M. bovis} BCG showed very less reactivity with sera of HHC. Sera of PPD-ve and PPD+ve healthy showed reactivity with \textit{M. bovis} BCG proteins, however we noted difference in region of reactivity. Low reactivity of \textit{M. bovis} BCG to patients’ sera also supports the fact that adults TB patients have low memory to BCG vaccine. Same may be true with HHC also as they also show low reactivity with \textit{M. bovis} BCG. Reactivity of PPD+ve healthy with BCG could be due to the cross reactive antigens of \textit{M. tuberculosis} or environmental mycobacteria. We noted similar sero-reactivity of patients with H\textsubscript{37}Rv, H\textsubscript{37}Rv animal and clinical isolates in most of the regions. This suggests SDS-PAGE does not show a single protein unit so a band of protein in SDS-PAGE act as cocktail of antigen (pool of different proteins or antigen). Pooled antigens have high reactivity to patients. Various studies suggest that a single antigen shows variation in sero-reactivity from patients to patients (Abebe et al., 2007; Lyashchenko et al., 1998). Kumar et al. in 2008, suggested that cocktail of antigen may minimize the possible heterogeneity. In a study of Rajavelu et al. in 2006 difference in proteins expression in SDS-PAGE between clinical isolates and H\textsubscript{37}Rv has also been reported. They have also noted some difference in western blot pattern between healthy PPD-ve and patients. However, they have not shown sero reactivity status of HHC and PPD+ve healthy with either BCG or clinical isolates which we shoed in this study.

Proteomics is a global study of proteins that are translated in a given physiological state. Proteome of an organism implies not only an inventory of its gene products but also the translation rate and the post-translational events that occur in the organism. Classical study of proteomics involves two-dimensional gel electrophoresis.
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(2-DGE) in which protein(s) first separated by isoelectric point (pI) than by molecular weight (MW) (O’farrell, 1975). Every spot in 2-DGE is an individual protein and every spot of protein can be isolated, hydrolyzed and subjected to identification by MALDI-TOF mass spectrometry (Webster and Oxley, 2012; Zhang et al., 2014). By the help of western blot after 2-DGE it is possible to identify individual sero-reactive protein too. Taking these advantage over SDS-PAGE, 2-DGE and western blot were performed to identify differentially/over-expressed and sero-reactive proteins in clinical isolates in comparison to H$_{37}$Rv. Initially six clinical isolates (C23, C26, C78, C71, C73 and C16 belonging to CAS, EAI, Beijing, H, T1 and X1 family respectively) prevalent in our area and H$_{37}$Rv (as control) were selected for doing two-dimensional gel electrophoresis (2-DGE) after analysis of SDS-PAGE pattern, western blot and preliminary study of ELISA and LTT. However due to some technical problem with PDQuest software three clinical isolates (C23, C26 and C78 belonging to CAS, EAI and Beijing family respectively) and H$_{37}$Rv were finally selected for comparative proteomic analysis and for doing western blot coupled with 2-DGE.

2-DGE profile of H$_{37}$Rv and clinical isolates (C23, C26 and C78) showed that large area of separation exhibit similarly expressed proteins. 2-D gels of H$_{37}$Rv and clinical isolates were comparatively analyzed by PDQuest software as well as manually. After comparative proteomic analysis between clinical isolates and H$_{37}$Rv we found 32 differentially/over-expressed proteins spots in clinical isolates. Out of 32 protein spots, 13 proteins spots were differentially expressed i.e. present only in clinical isolates while absent in H$_{37}$Rv and 19 protein spots were over-expressed in clinical isolates i.e. present in higher intensity in clinical isolates in comparison to H$_{37}$Rv (objective two). Out of 32 differentially/ over-expressed proteins 18 proteins spots (4 proteins spot were differentially expressed and 14 were over-expressed in clinical isolates) were identified by MALDI-TOF mass spectrometry. In western after 2-DGE several proteins spots showed reactivity to TB patient sera in comparison to healthy individual’s sera. In these sero-reactive proteins spots, 13 spots were identified by MALDI-TOF mass spectrometry. By pooling the identified proteins spots of differentially/over-expressed and sero-reactive spots total 20 different proteins (4 differentially expressed, 12 over-expressed in clinical isolates 2 sero-reactive to TB patients sera in clinical isolates but expressed in lower
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intensity and 2 more protein sero-reactive to TB patients as well over-expressed in 
H_{37}Rv) were identified. (Objective 2)

Objective three (To identify differentially expressed protein in Indian prevalent 
strains of M. tuberculosis) was devoted to identification of differentially/over-expressed 
proteins and sero-reactive proteins. MALDI-TOF mass spectrometry was done for 
identification of differentially/over-expressed proteins and sero-reactive proteins spots.
Out of 32 differentially/over-expressed proteins in clinical isolates belonging to 
prevalent genotypes 18 proteins were identified. Out of 18 spots, three spots [spot nos. 4 
(Rv0148), 7 (Rv0635) and 8 (Rv2901c)] were found differentially or uniquely expressed 
in all clinical isolates [CAS (C23), EAI (C26), Beijing (C78)] compared to H_{37}Rv; three 
spots [spot nos. 3 (Rv1656), 5 (Rv0632c) and 12 (Rv0733)] were found over-expressed 
in all clinical isolates [CAS (C23), EAI (C26), Beijing (C78)] in comparison to H_{37}Rv; 
one spot no 16 (Rv0951) was over-expressed in CAS (C23) as well as in Beijing (C78) 
genotype as compared to H_{37}Rv; two spots Spot 13 (Rv2031c) and 17 (Rv3248c) were 
over-expressed in EAI (C26) as well as in Beijing (C78) genotype in comparison to 
H_{37}Rv; five spots [spot nos. 1 (Rv2973c), 2 (Rv0512), 11 (Rv2140c), 14 (Rv2623), 15 
(Rv1654)] were found over-expressed only in C26 (EAI) clinical isolate when compared 
with H_{37}Rv; two Spot nos. 6 (Rv2628) and 9 (Rv2031c) were over-expressed in CAS 
only when compared with H_{37}Rv; two spots, spot nos. 10 (2445c) and 18 (Rv3248c) were 
over-expressed only in Beijing genotype as compared to H_{37}Rv. In Mass spectrometric 
identification protein spot 3 and 13 were identified as same proteins (Rv2031c) by 
MALDI-TOF, further protein spot 17 and 18 were also identified as same proteins 
(3248c) by MALDI-TOF hence it may be expressed in subunit.

After two-dimensional gel electrophoresis western blot was performed using 
pooled sera of PPD-ve, PPD+ve healthy and TB patients sera to measure IgG antibody 
reactivity to WCL proteins of H_{37}Rv and three clinical isolates (C23, C26, C78 belonging 
to CAS, EAI and Beijing genotype respectively). We noted differential reactivity of 
patients sera to same protein expressed in these strains which also confirms heterogeneity 
of antibody reactivity of patients. Total 11 sero-reactive proteins from different clinical 
isolates were selected on the basis of their sero-reactivity to patient’s sera in comparison
to pooled sera of PPD-ve and PPD+ve subjects or in comparison to sero-reactivity with H\textsubscript{37}Rv. Further, MALDI-TOF mass spectrometry was done for identification of these protein spots. In western blot sera of different group (TB patient group, PPD-ve group and PPD+ve group) showed heterogeneous antibody response to sero-reactive proteins when present in different clinical isolates (CAS, EAI and Beijing) and laboratory strain (H\textsubscript{37}Rv). Pulmonary TB patients sera showed distinct higher sero-reactivity in comparison to PPD+ve sera to following proteins spots to Rv2623, Rv0951, Rv3248 (when present in CAS), Rv 2031c, Rv0632c, Rv0733, Rv2623, Rv1654, Rv0951, Rv0896 (when present in EAI), Rv2031c (when present in Beijing), Rv2031c and Rv0363c (when present in H\textsubscript{37}Rv). Further, Pulmonary TB patient’s sera showed higher sero-reactivity in comparison to PPD-ve sera to following proteins Rv2623, Rv1654 Rv3248c (when present in CAS), Rv2031c, Rv2623, Rv0363c, Rv0951, Rv0896 (when present in EAI), Rv2031c, Rv1654, 3248 (when present in Beijing), Rv2031c, Rv3248c (when present in H\textsubscript{37}Rv).

By pooling the identified proteins spots of differentially/over-expressed and sero-reactivity, total 20 different proteins (4 differentially expressed, 12 over-expressed in clinical isolates 2 sero-reactive to TB patients in clinical isolates but expressed in lower intensity and 2 more protein sero-reactive to TB patients as well over-expressed in H\textsubscript{37}Rv) were identified as Rv2973c, Rv0512, Rv1656, Rv0148, Rv0632c, Rv2428, Rv0635, Rv2901c, Rv2031c, Rv2445c, Rv2140c, Rv0733, Rv2623, Rv1654, Rv0951, Rv3248c, Rv0363c, Rv0896,Rv1373, Rv1843c (Objective three).

Out of 20 identified proteins, seven proteins (Rv0632c, Rv2031c, Rv0733, Rv2623, Rv1654, Rv0951 and Rv3248c) were found sero-reactive as well as over-expressed in clinical isolates. In these seven proteins three proteins (Rv0733, Rv2623 and Rv1654) were found first time sero-reactive as well over-expressed in our study using this approach.

Further, the functional category of these proteins was investigated using TubercuList (genolist.pasteur.fr/TubercuList/) server hosted by Pasteur Institute, Paris. Out of these 20 proteins, 13 (Rv0512, Rv1656, Rv0148, Rv0635, Rv2445c, Rv0733, Rv1654, Rv0951, Rv3248c, Rv0363c, Rv0896, Rv1373, Rv1843c) were belonging to.

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functional category of intermediary metabolism and respiration. Further, three proteins (Rv2428, Rv2031c, Rv2623) out of 20 identified were belonging to functional category of virulence, detoxification, adaptation. Out of 20, two proteins (Rv2901c and Rv2140c) were belonging to conserved hypothetical functional category. Out of 20 proteins, one protein (Rv0632c) was belonging to lipid metabolism, and one protein Rv2973c was belonging to information pathway functional category.

Overall 2-DGE and western blot revealed that clinical isolate have many proteins which were over/differentially-expressed in comparison to H$_{37}$Rv. Many of these proteins were also sero-reactive. Other investigators have also compared protein profile of clinical isolates with H$_{37}$Rv. Betts et al. in 2000 compared the proteins of *M. tuberculosis* H$_{37}$Rv with clinical isolates CDC 1551. Both of these are virulent strain and their gene sequence is now available. Total cell lysate of H$_{37}$Rv and CDC 1551 were analyzed by 2-DGE. They showed that 7 proteins were unique to CDC 1551 and 3 proteins were unique for H$_{37}$Rv. They also suggested that protein profile of two strains was very similar. We also observed same finding in our study, that protein profile of clinical isolates and H$_{37}$Rv is very similar. Pheiffer et al. in 2005 showed that protein profile of Beijing strain is different form H$_{37}$Rv as well as another clinical isolate belongs to 23 strain lineage. They use 2-DGE, liquid chromatography tandem mass spectrometry and western blot. Most importantly Beijing stain showed increased expression of $\alpha$-crystallin and decreased expression of 47kDa, Hsp65, and PstS1 in comparison to other clinical isolate and H$_{37}$Rv. Further, they found difference in western blot and patient to patient variation in antibody reactivity to these antigens. Our study also showed the same observation in reference to antibody reactivity with some protein (antigenic spot) in western blot. Shin et al. in 2008 compared the culture filtrate protein of H$_{37}$Rv and *M. tuberculosis* K strain, a dominant clinical isolate in Korea. They found that ESAT-6, Hsp-x and CFP-10 were abundantly expressed in strain K. They also analyzed the humoral response to these proteins using ELISA. Further, other researcher found difference between H$_{37}$Rv and *M. bovis* BCG and some other showed differential protein expression by *M. tuberculosis* at different physiological condition or intracellular environmental condition. Jungblut et al. in 1999, compared the proteomic profile of two *M. tuberculosis* virulent strains (H$_{37}$Rv and Erdman) with two non-virulent vaccine strain *M. bovis* BCG (Chicago and
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Copenhagen). 16 proteins differ in intensity or position between *M. tuberculosis* H$_{37}$Rv and Erdman. Further, 25 proteins differed between *M. bovis* BCG Chicago and H$_{37}$Rv. **Mattows et al. in 2001** also compared the virulent strain of *M. tuberculosis* to *M. bovis* BCG strain and found 96 variable spots. Out of 96 spots, 56 proteins were unique to virulent strains and out of 56, 32 were identified by mass spectrometry. Further, 40 protein form 96 proteins were uniquely belonging to attenuated strains and 9 out of these 40 protein spots were identified. **Monahan et al. in 2001** showed six differentially expressed proteins in *M. bovis* BCG phagocytosed by macrophage cell line THP-1, which suggests that these proteins expressed only in intra-cellular condition. This study supports our finding i.e., *in-vivo* stress of human may be responsible for differential/over-expression of proteins in recently isolated clinical strains of *M. tuberculosis* from humans. **Starck et al. in 2004** found 50 proteins were differentially expressed in *M. tuberculosis* in anaerobic condition in comparison to aerobic condition. Out of 50 proteins they identified 16 proteins MALDI-TOF mass spectrometry. This work attracted attention because in chronic infection such as tuberculosis, *M. tuberculosis* forms granuloma which is characterized by low level of oxygen (Via et al., 2008). Such physiological condition in human body may be responsible for differential expression of proteins in recent clinical isolates. Further, some studies demonstrated differential gene expression of *M. tuberculosis* after *in-vitro* infection in macrophages (Dubnau et al., 2002; Fontan et al., 2008). **Gao et al. in 2005** also observed differential gene expression among *M. tuberculosis*10 clinical isolates as well as H$_{37}$Rv and H$_{37}$Ra. They identified 527 genes (15% of those tested) that are variably expressed among the isolates studied. The remaining genes were divided into three categories based on their expression levels: unexpressed (38%), low to undetectable expression (31%) and consistently expressed (16%). They concluded that even though the clinical isolates grow in same condition their gene expression varies from strain to strain. This study support our observation that some proteins were differentially expressed in all genotype of clinical isolates and some proteins were only expressed in one or more than one clinical isolates.

Proteins we identified in our study were also reported by some authors. **de Souza et al. in 2011** identified 19 proteins, in culture filtrate, membrane fraction (Malen et al., 2011) and in whole cell lysate by except Rv1373 which was sero-reactive and
differentially expressed in H37Rv in our study. Further, these studies were done at proteomic level. However, apart from proteomic analysis in our study we have also seen immune reactivity of differentially expressed proteins by western blot. Further, gene of four proteins (Rv2428, Rv0896, Rv0635, Rv2031c; which we also identified in this study) are also reported to be over-expressed during M. tuberculosis infection of THP-1 cell line (Fontan et al., 2008). Rv0632c, Rv2031c, Rv2140c, Rv0951, Rv3248c and Rv0896 identified in our study were also reported by Kunnath-Velayudhan et al. in 2010. They reported that these proteins were reactive with active TB patient’s sera. Seven proteins (Rv2445c, Rv3248c, Rv0363c, Rv0632c, Rv0733, Rv2031c and Rv2140c) identified in our study were also identified by Deenadayalan et al. in 2010. They reported their discrimination ability between healthy house hold contacts to active TB patients on the basis of IFN-γ release. Further, they reported that Rv0632c, Rv0733 and Rv2031c were contact specific. These studies also support our finding that clinical isolates belonging to prevalent strain have differentially/over-expressed proteins in comparison to H37Rv. However, detailed immune profiling of proteins, Rv2901c, Rv0512, Rv0635 (for CMI and humoral), Rv2428, Rv1843 (for humoral) were analyzed first time in our study. Further, four more proteins were selected form protein database of M. tuberculosis and were also analyzed for their immune reactivity; Rv0036c, Rv1791, Rv2588c (for CMI and humoral); Rv3020c (for CMI) in the present study.

Individual protein eliciting antibody response in crude sample could be identified by using western blot after 2-DGE and mass spectrometry. Identifying an individual protein which shows CMI response in crude sample is quite difficult. T cell immunity plays a major role in protection against M. tuberculosis infection. For characterization of proteins eliciting CMI response, we extracted proteins by electro-elution from SDS-PAGE gel selected on the basis of their expression and sero-reactivity. Electro-eluted proteins from bands were then used to perform LLT. All three protein bands which we eluted showed positive stimulation index i.e. more than 2. However, due to limitation of SDS-PAGE we were only able to show CMI response to whole mixture of proteins present in the band, not a single protein. Further, we tried to elute protein from spot of proteins separated by 2-DGE but we were not able to elute protein. Hence an alternative approach was adapted. Ten protein spots, which were identified by mass spectrometry,
were selected after 2-DGE and western blot on the basis of their intensity in nitrocellulose membrane; then antigen bearing particles were prepared as described by Abu-Zeid et al. in 1987. After antigen bearing particle preparation, we stimulated PBMCs, using these particles. Out of ten spots, eight spots (Rv2031c, Rv0632c, Rv0363c, Rv2901c, Rv0512, Rv0733, Rv0635 and Rv1843) show positive stimulation i.e. stimulation index 2 or more than two.

Objective four [To analyze the immune response of identified protein in reference to adaptive immunity (humoral and cell mediated immunity)] was devoted to analyze the immune response to identified proteins. Two alternative approaches are used to obtain a protein, first production of desired protein using gene expression vector and second commercial synthesis of peptides. Because commercial synthesis of peptides containing T-cell epitopes have some benefits over whole proteins synthesis such as, reproducibility, easy to perform obtain and cost effectiveness so we used synthetic peptides. Hence to know the practical application of identified proteins we uses commercially synthesized peptides and analyses their efficacy in diagnosis and other immune parameters by performing LTT, cytokine ELISA, Flow cytometry and peptide ELISA.

By using different bioinformatics servers, T-cell restricted to MHC-II and MHC-I and B-cell peptides could be predicted. Various servers such as Propred 2, HLAPred, HLA-DR-4 Pred (predict T cell peptides restricted to MHC-II), Propred 1, nHLAPred, HLAPred, (predict T cell peptides restricted to MHC-I) were used for T-cell peptide prediction. BeePred and ABCpred servers were used For B-cell epitope prediction.

Twenty proteins identified in this study and four more additional proteins which were reported earlier to be present in M. tuberculosis and three out of four proteins absent in M. bovis BCG in different published studies [Rv0036c expressed in only H37Rv not expressed in M. bovis BCG (Mattow et al. 2001); Rv1791 and Rv3020c are PE/PPE family proteins (Cole et al., 1998); Rv2588c a CFP proteins] were used to run in of predict T-cell and B-cell epitopes. Overall eight T-cell peptides [T-PEP-1 (Rv2901c), T-PEP-2 (Rv0635), T-PEP-3 (Rv0512), T-PEP-4 (Rv1843c), T-PEP-5 (Rv0036c), T-PEP-6 (Rv1791), T-PEP-7 (Rv3020c) T-PEP-8 (Rv2588c)] sequences (on basis the binding rank to different HLA alleles) and nine B-cell peptides [B-PEP-1 (Rv2901c), B-PEP-2
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(Rv0635), B-PEP-3 (Rv0632c), B-PEP-4 (Rv2428), B-PEP-5 (Rv0512), B-PEP-6 (Rv0896), B-PEP-7 (Rv0036c), B-PEP-8 (Rv1791), B-PEP-9 (Rv2588c)] sequences were selected for commercial synthesis. All B-cell peptides were labeled with Biotin using linker Aminohexanoic (Ahx) to increase binding.

After commercial synthesis of T-cell peptides we analyzed CMI response using these peptide in TB patients, household contacts, PPD+ve and PPD-ve healthy individuals. We performed lymphocyte transformation test and Th1 and Th2 response was measured by cytokine IFN-γ (Th1) and IL-4 (Th2) estimation at two different time point (48 hrs and 5 days). PPD and ESAT-6 was used as control antigens. PPD is used in montoux test and ESAT-6 is one of the components used in quantiferon diagnostic test based on IFN-γ release (Lalvani and Millington, 2008; Dorman et al., 2014). PPD showed positive stimulation in all study subjects (PTB, HHC, PPD+ve and PPD-ve) and ESAT-6 showed positive stimulation only in HHC and TB patients. This indicates and support earlier finding that PPD have cross reactive proteins and it could not distinguish between any groups. Further, ESAT-6 distinguishes between PTB and HHC to PPD+ve and PPD-ve individuals. Out of eight T-cell synthetic peptides, seven peptides (except T-pep-5) show positive stimulation in PTB. Positive stimulation was seen in HHCs to four T-cell peptides (T-pep-3, 5, 7, 8), PPD+ve showed positive stimulation to four T-cell peptides (T-pep -1, 2, 6, 7) and PPD-ve shows positive stimulation to T-pep -3 and T-pep-4. Further, on the basis of LTT, T-PEP-8 distinguishes PTB from PPD+ve and PPD-ve groups. It also distinguishes HHC from PPD+ve and PPD-ve group which may be due to high exposure of this group to patients. T-pep-7 distinguishes PTB, HHCs and PPD+ve group to PPD-ve group. T-pep-6 can distinguish PTB group from HHCs, PPD+ve and PPD-ve group may work in diagnosis of TB infection. T-pep-1 and T-pep-2 show strong stimulation in PTB and PPD+ve group.

The Th1 and Th2 type response was measured using cytokine ELISA for IFN-γ and IL-4. The detection of IFN-γ in cell supernatant at 48 hrs time point shows that ESAT-6 stimulate secretion of IFN-γ significantly in PPD+ve individuals than HHCs and PPD-ve group. Further, three T-cell peptides (T-pep-3, 4, 6) stimulate higher IFN-γ secretion in HHCs than different groups. Further, T-pep-4 stimulates higher IFN-γ...
secretion in PTB group than PPD+ve group. At 5 day time point no significant difference in IFN-\(\gamma\) secretion was observed between any groups. The detection of IL-4 in cell supernatant at 48 hrs time point shows that PPD, T-pep-3, 5, 6 stimulate significantly higher IL-4 production in PPD-ve than other different groups. T-pep-5 stimulates strong IL-4 production in PTB than PPD+ve group, T-pep-6 stimulates strong IL-4 production in PPD-ve than PTB individuals. T-pep-8 stimulates strong IL-4 production in HHCs than PTB individuals. At 5days time point T-pep-4 stimulates high IL-4 production in PTB than PPD+ve and in PPD-ve than HHC group. Th1 and Th2 response in PBMCs elicited by different T-cell peptides in different groups was combined. T-pep-3 and 6 generate Th1 response in predominantly HHCs while Th-2 response in PPD-ve group. Further, at 48hrs time point T-pep-4 elicited Th1 type response predominantly in HHCs and PTB group and T-pep-5 elicited only Th-2 response in PPD-ve. Higher IFN-\(\gamma\) and low IL-4 response shown by T-pep-4 in HHC suggests its utility in inducing protective response and its efficacy for vaccine purpose needs to be evaluated. Further, only T-pep-4 shows Th1 type response at 5 days time point predominately in PPD-ve and PTB.

The LTT and cytokine ELISA to IFN-\(\gamma\) and IL-4 are very good techniques to know the ability of antigen for cell proliferation and Th1 and Th2 response. However, these techniques are not able to show the cell type which are stimulated, proliferate and secrete cytokine. Hence we preformed flow cytometry using T-cell peptides. Using flow cytometry we analyzed percentage of T-helper or Th (CD4+ cells) and T-cytotoxic (Tc) cells in response to all eight synthetic T-cell peptides in PTB, HHC, PPD+ve and PPD-ve groups. Further, percentage of T-helper (Th) and T-cytotoxic (Tc) cells producing IFN-\(\gamma\), IL-2, IL-4 and IL-10 cytokines were also analyzed. The percentages of Th cells were found higher in PPD+ve individuals compared to HHCs after stimulation with T-PEP-8. The percentage of IFN-\(\gamma\) producing Th cells were found higher in PTB compared to PPD-ve group in response to ESAT-6, T-pep-1, 3, 6 and 7. Further, PTB group showed higher IFN-\(\gamma\) producing cells compared to HHC after stimulation with T-pep-3 and 8. In unstimulated condition high IFN-\(\gamma\) level was found in HHCs in comparison to PPD-ve individual. The percentage of IL-2 producing Th cells were found higher in PTB compared to PPD-ve individual in response to ESAT-6, T-pep-1, 2, 6, 7. Further, high percentage of IL-2 producing cells was found in HHCs than PPD-ve in response to T-
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pep-1 and 6. ESAT-6 stimulates higher percentage of Th cells producing IL-2 in PPD+ve than PPD-ve group. In unstimulated condition higher Th cells producing IL-2 was found in PTB than PPD-ve and also these cells were also higher in HHCs than PPD+ve and PPD-ve group. The percentage of IL-4 producing Th cells was found higher in PTB group in comparison to PPD-ve after stimulation with PPD, ESAT-6, T-pep-2 and 7. ESAT-6, T-pep-7 stimulates higher percentage of Th cells producing IL-4 in PTB than HHC. T-PEP-2 stimulates higher IL-4 production by Th cells by PPD+ve than PPD-ve. Unstimulated cells shows high percentage of IL-4 producing Th cells in PTB than PPD-ve and HHC. Further, HHC shows high IL-4 producing Th cells than PPD-ve group in unstimulated condition. PPD, ESAT-6 and T-pep-1, 4, 5, 6, 7 and 8 stimulate high percentage IL-10 producing Th cells in PTB than PPD-ve. IL-10 expressing Th cell were found high in PTB and PPD+ve after stimulation with PPD and T-pep-5. ESAT-6, PPD, T-pep-1, 2, 3, 4, 7 stimulated higher percentage of Th cells expressing IL-10 in PTB than HHC. The production of IL-10 by peptides/antigen was not taken into consideration because IL-10 is produced by many cells which play a role in innate immunity, such as dendritic cells, macrophages, natural killer cells and T cells in response to various pathogens in mycobacterial infection (Larsen et al., 2007). Further, India belongs to high load country of M. tuberculosis and environmental mycobacterial in India M. bovis BCG is regularly injected in childhood to protect children against TB. It may possible that BCG induce the production of IL-10 by dendritic cells (Larsen et al., 2007) which we noted after stimulations with ESAT-6, PPD, and T cell peptides. Mustafa et al. in 2011 also reported that many RD regions (specific regions for M. tuberculosis absent in M. bovis BCG) also induce high and low production of IL-10. Several other authors have also reported that IL-10 production is stimulated by large number of M. tuberculosis antigens (Mustafa et al., 2011; Mustafa et al., 2012; Kassa et al., 2012). This is well known that Th-1 response (IFN-γ and IL-2) play a significant role in protection against TB and the role of Th-2 type response (IL-4, except IL-10) in protection against TB is still questionable (Mustafa et al., 2012). Further, various authors reported that antigen which produces vigorous Th-1 response may be preferred as vaccine or diagnostic candidates. ESAT-6, T-pep-2 (Rv0635) and 7 (Rv3020c) induced Th-1 and Th-2 type cytokine in PTB. Further, four peptides T-pep-1 (Rv2901c), T-pep-3 (Rv0512), T-pep-6
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(Rv1791), T-pep-8 (Rv2588c) which were potent inducer of Th-1 type response in PTB in our study may work as diagnostics antigen targeting CD3+CD4+ cells producing IFN-γ or IL-2 or both. Further, Th-1 response elicited in HHCs in response to T-pep-1 (Rv2901c) and T-pep-6 (Rv1791) in our study may work as vaccine candidate targeting CD3+CD4+ cells producing IFN-γ or IL-2 or both.

We also analyzed percentage of T-cytotoxic cells (Tc or CD3+ CD8+ cells), Tc producing IFN-γ, IL-2, IL-4 and IL-10 after stimulation with ESAT-6, PPD, and eight synthetic T-cell peptides. HHC showed higher percentage of Tc cells than PPD-ve and PTB group in unstimulated condition. The percentage of Tc cells producing IFN-γ were found higher in PTB and PPD+ve than PPD-ve after stimulation with T-pep-3. This peptide also stimulated higher percentage of Tc cells producing IFN-γ in PPD+ve than HHC and PPD-ve. T-pep-2 stimulated higher percentage of Tc producing IFN-γ in PPD+ve than PPD-ve, T-pep-6 stimulated higher percentage of Tc producing IFN-γ in HHC than PPD-ve. ESAT-6 stimulated higher percentage of Tc cells producing IFN-γ in PTB and HHC than PPD-ve. The percentage of Tc cells producing IL-2 was found higher in HHC than PPD-ve after stimulation of PPD, ESAT-6 and T-pep-1. ESAT-6 stimulated higher percentage of Tc cells producing IL-2 in PTB than HHC, PPD+ve and PPD-ve. Further, percentage of Tc cells producing IL-4 were higher in PTB and HHC than PPD-ve after stimulation with PPD. IL -4 was also higher in PTB and PPD+ve than PPD-ve after stimulation with ESAT-6. ESAT-6, T-PEP-1, 2, 4, 7 and 8 stimulated higher percentage of Tc cells producing IL-10 in PTB than different groups. ESAT-6 was found to stimulate Tc cells to produce IFN-γ, IL-2, IL-4, IL-10 in PTB group. In PTB Th-1 type response elicited by T-pep-3 and ESAT-6 elicited Th1 and Th-2 response in PTB, while PPD elicited Th-2 type response in PTB. Hence T-pep-3 (Rv0512) may work in diagnostics by inducing CD3+CD8+ cells. Th-1 type was noted in HHCs after stimulation with ESAT-6, T-pep-1 (Rv2901c) and 6 (Rv1791). Protective role that is Th-1 type response was also elicited in PPD+ve individual after stimulation with ESAT-6, T-pep-1 (Rv2901c) and 6 (Rv1791). PPD also elicited Th-1 and Th-2 type response in HHCs by activating CD3+CD8+ cells.
Finally we conclude that T-pep-1 (Rv2901c), 3 (Rv0512), 6 (Rv1791) and 8 (Rv2588c) selectively eliciting Th-1 response in PTB by stimulating T helper cells additionally T-pep-3 also elicited Th1 response by stimulating T cytotoxic cells so these peptides could work as diagnostic candidate for TB (Objective 4). Further, T-pep-1 and 6 selectively elicited Th-1 type responses in HHCs by eliciting T helper and T cytotoxic cells which could work in protection (Objective 4). IFN-γ/IL-2 and IL-4 production was noted by T helper and cytotoxic cells after stimulation with ESAT-6.

Antibody detection-based diagnostic assays have been successfully devised for several infectious diseases but efforts to develop a sero-diagnostic test for TB have yielded disappointing results for several decades and no currently available commercial immunodiagnostic tests for TB provide high sensitivity and specificity. Studies also demonstrated the heterogeneous sero-reactivity to different purified antigens such as 38kDa, 14kDa, 19kDa, Ag85B, ESAT-6, KatG etc (Lyashchenko et al., 1998). Further, for the evaluation whole antigen is needed but the reproducible production of same protein in high quality with minimum batch variation is difficult and expensive. To escape the limitation of using whole antigen we used synthetic peptides. We analyzed sero-diagnostic potential of nine synthetic B-cell peptides predicted by bioinformatics [B-PEP-1 (Rv2901c), B-PEP-2 (Rv0635), B-PEP-3 (Rv0632c), B-PEP-4 (Rv2428), B-PEP-5 (Rv0512), B-PEP-6 (Rv0896), B-PEP-7 (Rv0036c), B-PEP-8 (Rv1791), B-PEP-9 (Rv2588c)] is this study. We used Biotin- Ahx linker- B-cell peptides. Ahx increases the solubility and flexibility of peptide chains which might keep peptide chains open to react with antibody during reaction and biotins increases peptides binding to ELISA plate. The isotypic restriction of antibodies is correlated with biochemical nature of antigen; most antibodies against protein are of IgG1 and IgG3 isotype (Sousa et al., 1998). Hussain et al. in 1995 found IgG1 and IgG3 specific against *M. leprae* sonicate. Subclasses IgG1 and IgG3 have also been reported to viral protein (Skvaril, 1986). Sousa et al. in 1998 reported that in serum of TB patients about 48% had elevated total IgG level mainly due to IgG1 and IgG3. Gupta et al. in 2005 also reported elevated level of IgG1 and IgG3 to mycobacterial serine protease in active tuberculosis and added it as a possible marker for diagnosis of tuberculosis. In our study we analyzed the reactivity of IgG, IgG1 and IgG3 subclasses to B-cell peptides (Biotin-Ahx-N terminal to C terminal) in PTB, HHCs,
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PPD+ve and PPD-ve individuals by ELISA to know which subclass of antibody shows maximum reactivity to peptides. Out of nine B-cells peptides, B-PEP-1 showed best sero-reactivity to IgG, IgG1 and IgG3 subclasses. Further, B-PEP- 2, 8 and 9 showed higher sero-reactivity to IgG3 subclass too. B-pep-1 showed highest AUC (0.815), specificity (72%), sensitivity (72%), PPV (94.74%) and NPV (26.87%) in comparison to B-pep-2, 3, 4, 5, 6, 7, 8 and 9 for IgG. B-pep-1 further showed highest AUC (0.576), specificity (73.33), sensitivity (51.16), PPV (84.62%) and NPV (34.38%) in comparison to B-pep-2, 3, 4, 5, 6, 7, 8 and 9 for IgG1. B-pep-1 also showed highest AUC (0.714), specificity (80.00%), sensitivity (69.77%), PPV (90.91%) and NPV (48.00%) in comparison to B- pep-2, 3, 4, 5, 6, 7, 8 and 9 for IgG3. B-pep-8 showed best sero-reactivity to IgG3 after B-pep-1 with AUC (0.672), specificity (86.67%), sensitivity (65.12%), PPV (93.33%) and NPV (46.43%). B-pep-9 and B-pep-2 also showed best reactivity to IgG3. B-pep-9 showed AUC (0.679), specificity (80.00%) sensitivity (65.12%), PPV (90.32%) and NPV (44.14%) while B-pep 2 showed AUC (0.679), specificity (73.33%), sensitivity (65.12%), PPV (87.50%) and NPV (42.31%). Overall B-pep-1 (Rv2901c) showed best sero-reactivity with IgG, IgG1 and IgG3. Further, B-pep-8 (Rv1791), 9 (Rv2588c) and 2 (Rv0635) showed better sero-reactivity to IgG3 in comparison to rest of the peptides (objective 4).

Overall results of flow cytometry showed the T-cell peptides predicted from proteins Rv2901c, Rv0635, Rv0512, Rv1791, Rv3020c and Rv2588c induced *M. tuberculosis* specific Th1 type of immune response in pulmonary TB patients and could be used as diagnostic candidates. Further, T-cell peptide of two proteins (Rv2901c and Rv1791) showed better Th1 type of immune response in healthy household contacts and could be used as possible subunit vaccine candidates. B-cell peptides of four proteins (Rv2901c, Rv0635, Rv1791, and Rv2588c) showed better antibody reactivity with pulmonary TB patient’s sera and could be investigated for their sero-diagnostic potential. Hence utility of these peptides needs to be evaluated in larger samples.