INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease caused by the acid-fast bacilli *Mycobacterium tuberculosis* (*M. tuberculosis*) and is one of the leading causes of mortality all over the world (WHO, 2014). It primarily affects lungs and causes pulmonary tuberculosis (PTB). It can also affect intestine, meninges, bones and joints, lymph glands, skin and other body tissue of the body and known as extrapulmonary tuberculosis (EPTB). The World Health Organization (WHO) declared TB as a global public health emergency in 1993 (Ozdemir et al., 2007, WHO, 2014). It is presumed that the genus *Mycobacterium* originated more than 150 million years ago (Daniel, 2006). The modern members of *M. tuberculosis* complex (MTBC) seem to have originated from a common progenitor about 15,000 - 35,000 years ago (Gutierrez et al., 2005). TB was documented in Egypt, India, and China as early as 5,000, 3,300 and 2,300 years ago, respectively (Daniel et al., 2006). Robert Koch (1843-1910) discovered the *M. tuberculosis* and proofed it as causative agent for TB.

TB is a major global health problem. Worldwide in 2014 there were an estimated 9.6 million incident cases of TB (fig. 1.1) and 1.5 million people died. Out of 1.5 million deaths 1.1 million among people who were HIV-negative and 0.4 million people were HIV-positive (WHO, 2015). Further, in 2013 Extensively Drug Resistant-tuberculosis (XDR-TB) has been reported by about 100 countries it is estimated that 9% of people with MDR-TB converted in XDR-TB (WHO, 2014). Globally the mortality, prevalence and incidence of TB rate per 100,000 populations were 16, 174 and 133 respectively in year 2014. India characterized as high TB, high HIV, high MDR-TB burden country, the mortality, prevalence, and incidence of TB rate per 100,000 populations was 17, 195 and 167 respectively in year 2014 (WHO, 2015).
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![Figure 1.1: The global coverage of incidence of TB in 2014 (WHO, 2015).](image)

*M. tuberculosis* is a typically slender, slight or slightly curved rod shaped strongly AFB (fig. 1.2 A). They are obligate aerobic, non-motile, non-spore bacilli having the dimension of 2-3µm x 0.2-0.4 µm arranged singly or in group (fig. 1.2 B). It has slow growth rate with generation time *in-vitro* 18-24 hrs. Colonies appears in about 14 days and sometimes take up to 56 days on Lowenstein Jensen (LJ) media. Optimum temperature is 37°C does not grow below 25°C or above 40°C. On solid media such as on LJ media *M. tuberculosis* form dry, rough, raised, irregular, wrinkled surface colonies (fig. 1.3 A). In liquid media with dispersing agents the growth begins at bottom, creeps up the sides. The cell envelope contains a polypeptide layer, a peptidoglycan layer and free lipids. Cell wall contains characteristically mycolic acid.
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The genome of H$_{37}$Rv was sequenced by Cole et al. in 1998 and they identified 3974 genes (3924 encodes proteins and 50 encodes stable RNA) and 44115296 nucleotides in it. As per tuberculist updates now there are 4,411,532 bp of DNA sequence representing the whole $M$. tuberculosis chromosome (strain H$_{37}$Rv) in which 4,018 are protein coding genes (mRNA), 13 are pseudogenes, 45 are tRNA genes, 3 are rRNA genes, 30 are ncRNA genes and 2 are miscRNA genes (fig 1.3) (http://tuberculist.epfl.ch/).

Figure 1.2: (A) Arrow shows $M$. tuberculosis in red color (the acid-fast character). (B) Scanning electron micrograph of $M$. tuberculosis. Scale shows the size of $M$. tuberculosis. (http://textbookofbacteriology.net/tuberculosis.html)

Figure 1.3: Distribution of functional categories of $M$. tuberculosis genome (as per information from Tuberculist, http://tuberculist.epfl.ch/). The red pie denotes hypothetical proteins (category 10) which contains more than 25% of the encoding genes in $M$. tuberculosis.
PTB is transmitted by inhalation of aerosol droplets containing *M. tuberculosis* bacilli. People in close contact with TB patients are at high risk of becoming infected (Ahmed and Hasnian, 2011). A person with active but untreated TB may infect 10–15 (or more) other people per year (WHO-Tuberculosis Fact Sheet, 2011). After inhalation of the droplets, the bacteria travel to the terminal bronchioles and alveoli where they are engulfed by alveolar macrophages (or by dendritic cells). Many of these bacilli are killed in the phagosomes after these fuse with lysosomes. *M. tuberculosis* is an intracellular pathogen and can efficiently inhibit phagosome-lysosome fusion. Some of the invading *M. tuberculosis* bacilli survive these initial host defenses and lyse the macrophages and spill out into the host tissue. In resistant individuals, released bacterial products stimulate strong cell-mediated immunity through Th1 signaling with IFN-γ, IL-2, and IL-12. Activated macrophages that can kill *M. tuberculosis*, as well as T cells, are recruited to the periphery of the infectious focus. The bacteria and cellular debris are contained in a tissue structure called “a caseous granuloma”. In sensitive individuals, cell-mediated immunity is weak and bacilli continue to multiply. Macrophages arrive to engulf the bacilli while T cells also accumulate. Because the macrophages are inadequately activated, the *M. tuberculosis* bacilli also parasitize the recruited macrophages. Cytotoxic T (Tc) cells produce toxic substances, which results in damage of host tissues. This cycle is repeated so that the granuloma enlarges. Eventually, tissue damage may result in cavity formation within the lung as well as in liquefaction of the granuloma. Most humans seem to be naturally resistant to *M. tuberculosis*; these individuals harbor the bacilli and develop only a latent infection with no symptom other than a positive reaction to the administration of purified protein derivative (PPD) of *M. tuberculosis*, indicating immune memory of *M. tuberculosis* infection.

*M. tuberculosis* may spread to any part of the body, but most commonly occurs in the lungs (known as PTB). EPTB occurs when *M. tuberculosis* develops outside the lungs, although EPTB may coexist with PTB as well. General signs and symptoms include fever, chills, night sweats, loss of appetite, weight loss and fatigue.

Attenuated *Mycobacterium bovis* (*M. bovis*) Bacillus Calmette-Guérin (BCG) is the only available vaccine against TB (Shin et al., 2008). Its side effects are tolerable, and it can prevent miliary and meningeal TB in young children to an appreciable degree.
In striking contrast, BCG fails to protect against the most prevalent disease form, pulmonary TB in adults (Kaufmann, 2000).

The diagnostic methods currently used, such as sputum smear microscopy, microbiological culture, chest X-rays, and the intradermal injection of purified protein derivative (PPD) test, or tuberculin skin test (TST) have not been as successful as expected. An immunological diagnostic test is directly related to the immune response of patient therefore the advantage of an immunological test lies in its capacity to demonstrate that the patient has been previously sensitized to the mycobacterium and confirms an infection without the need to detect the bacillus in sputum or any other biological sample of the patient. To develop serological method for the diagnosis of TB, different mycobacterial antigens have been evaluated but there are no commercially available sero-diagnostic tests for TB with acceptable sensitivity and specificity for routine laboratory use. Immunological tests related to IFN–γ production by T-cells, in response to antigens present in *M. tuberculosis* but absent in *M. bovis* (BCG), such as ESAT-6 and CFP-10 have been developed in an attempt to replace the PPD skin test. Two tests based on the IFN-γ production by T-cells in culture, using antigens expressed by the genes presents in RD-1, are commercially available-first ELISpot or T-SPOT.TB and second ELISA (I-the QuantiFERON-TB, II- the Quant FERON-TB Gold). But due to high cost and need of well developed laboratory, these tests are not applicable in routine screening for TB in resource limited country. Control of TB depends on early identification and proper treatment of individuals with active disease and the lack of an accurate diagnostic technique has contributed to the emergence of TB as a threat to global health. To date, there is no simple, rapid, cost effective and specific test that can differentiate active TB from latent infection and slowly progressive TB. Lack of proper diagnostic tools remains the most critical problem of TB control.

Many studies demonstrate that the clinical isolate have better chance to predict the new antigen and virulence factors in comparison to the laboratory strain by the proteomic approach. For identification and immunological characterization of differentially expressed antigenic proteins the two-dimensional gel electrophoresis (2-DGE) along with western blot and identification of individual spot (differentially expressed) by mass
spectrometry is the useful immuno-proteomic approach. The current laboratory strain H37Rv was isolated on 1905 (Cole et al., 1998) and maintained in the laboratories from a long time by in-vitro passages for more than 100 years in all over the world. Its protein profile is unknown in human stress and therefore, appears to have lost its virulent factor and may be also have some immunodominant antigens (Kumar et al., 2012). India being a high incidence country of M. tuberculosis infection there are better chances to identify new immune reactive proteins from clinical isolates which were isolated recently in comparison to H37Rv. Molecular typing of M. tuberculosis strain has proven to be valuable tool for TB control in term of tracking transmission chain detecting suspected outbreaks and identifying successful clone (Barnes and Cave, 2003). Several genotypes have been identified by spoligotyping and MIRUs, which are found prevalent globally and in India (Sharma et al., 2008; Singh et al., 2004; Glynn et al., 2002). In north India CAS1_Del (ST-26) genotype is predominant. It is also found in other countries such as Iran and Pakistan (Hasan et al., 2006). The second most prevalent clinical isolates belong to EAI (ST-11) predominant in south India (Sharma et al., 2008) Beijing (ST-1) genotype family is reported most prevalent worldwide (Glynn et al., 2002). 90-92% of M. tuberculosis strains belong to this family in china (Qian et al., 2002; van Soolingen et al., 1995). So Beijing stain may be transmitted to neighboring countries. Identification of individual differentially/over-expressed proteins from these isolates will be helpful if reference to diagnosis or vaccine candidates.

To obtain protein in large quantity from clinical isolates for assaying its immunological characterization recombinant protein will be required which could be produced by recombinant DNA technology (RDT). In RDT, heterologous hosts like Escherichia coli (E. coli) and the purification of recombinant protein free of E. coli component are needed. Further, RDT is still a challenging task with high cost and needs human application. In addition, the expression of mycobacterial antigens is usually quite difficult in this system (Mustafa et al., 2002). To overcome the problems associated with the expression and purification of recombinant protein, use of synthetic peptides can be an alternative (Mustafa et al., 2005). Further, it is well known that the presentation of antigen to T-cell requires their processing into small peptides by antigen presenting cells and associate with MHC molecules. The length of T-cell epitopes ranges usually from 8-
10 amino acids (Oftung et al., 1997) which could easily be synthesized by using standard chemistries to overcome the problem associated with obtaining full length recombinant protein. The use of synthetic peptide in TB has been successfully demonstrated for diagnosis application in human and cattle (Ravn et al., 1999; Arend et al., 2000; Vordermeier et al., 2001).

The present study has been designed to identify and immunologically characterize differentially/over-expressed proteins in M. tuberculosis clinical isolates belonging to prevalent genotypes. T-cell and B-cell peptides of differentially expressed proteins will be used for immunological characterization after prediction by bioinformatics tools and these peptides will be synthesized commercially.