3.1: Diagnosis of tuberculosis-

3.1.1: Conventional tests for diagnosis of tuberculosis-

3.1.1a: Microscopy-

Microscopy is used to examine clinical specimens or cultures for the presence of AFB. Ziehl–Neelsen (ZN) and Auramine-O fluorescent staining are commonly used to stain AFB such as MTBC. Microscopy is not very accurate, but remains the most rapid technique and indicates only the presence of AFB. The main limitation of the ZN method is its varying sensitivity (60-80%). It has been estimated using meta analysis that fluorescence microscopy (FM) has approximately 10% higher sensitivity than ZN staining in detecting AFB in comparison to ZN staining in clinical specimens (NTI, 2014).

3.1.1b: Culture-based techniques-

*M. tuberculosis* can grow on media based on egg (LJ media) or on agar-based (Middlebrook 7H9, 7H10 and 7H11). Culture is the most sensitive of currently available tests (sensitivity 98%). Further, culture is more expensive than microscopy and requires a high standard of technical competence and takes longer time.

3.1.2: New tests for diagnosis of active tuberculosis-

3.1.2a: Rapid liquid culture systems-

Faster culture of mycobacterial isolates could be achieved by mycobacterial growth indicator tube (MGIT) used in BACTEC 460 TB, BACTEC 9000MB and MGIT 960 systems. These systems measure changes in gas pressure, carbon dioxide production or oxygen consumption fluorimetrically or colorimetrically.

3.1.2b: Nucleic acid amplification tests-

Nucleic acid amplification tests (NAATs) are molecular systems which are able to detect small amounts of genetic material (DNA or RNA target sequences) from the micro-organism, and based on repetitive amplification of target sequences. If the target organism is not present in the sample, no amplification will occur. Polymerase chain reaction and LPA are common amplification method.
3.1.3: Tests for the detection of latent tuberculosis Infection (LTBI)-

3.1.3a: Tuberculin skin test-

Latent TB infection is traditionally diagnosed by performing a tuberculin skin test (TST) using PPD. PPD contains various antigens widely shared among different species of mycobacteria such as *M. tuberculosis*, *M. bovis* (Andersen *et al.*, 2000). Various studies have demonstrated that the PPD does not safely distinguish individuals vaccinated with BCG from those exposed to environmental mycobacteria or infected with *M. tuberculosis* (Fine *et al.*, 1994).

3.1.3b: Immune-based blood tests for the rapid identification of latent tuberculosis infection: the interferon-γ assays-

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 (Sociendade Brasileria, 2004). Two tests based on the IFN-γ production by T-cells in culture, using antigens expressed by the genes present in RD-1, are commercially available- first, ELISpot or T-SPOT.TB and second, ELISA (I –the QuantiFERON-TB, II- the QuantiFERON-TB Gold).

3.2: Major antigens of *M. tuberculosis*-

3.2.1: 38 kDa antigen-

The 38-kDa antigen (antigen 5 or antigen 78, Rv0934) is a surface-exposed lipoprotein, a phosphate transport protein. A study indicates that antibody responses to the 38-kDa antigen were associated with active disease (Silva *et al.*, 2003). The sensitivity of the assays using this antigen have been reported to range from 15% to 94%, largely depending on the sputum smear status of the patient and the patient population used in the studies. This antigen has been used in the development of several commercial assays for the detection of TB (Harboe and Wiker, 1992). This antigen in a microtitre plate ELISA has been evaluated in different laboratories. In patients with culture positive results, the sensitivity varies from 40% to 89% (Cole *et al.*, 1996) and the specificity from 44% to 100% (Chaudhary *et al.*, 2005). Serodiagnostic tests based on the 38-kDa antigen have been used to detect specific antibodies in different types of TB, bone and
joint TB (Demkow et al., 2002), tuberculous pleuritis (Chierakul et al., 2001) and
tuberculous meningitis (Patil et al., 1996; Chandramuki et al., 1989). Using an ELISA
for the detection of specific antibodies to this antigen in CSF, Patil et al. in 1996 reported
a sensitivity of 41.7%. In another study, a sensitivity of 80% was reported in culture-
positive patients (Kadival et al., 1994). A study on HIV-positive TB patients conducted
in the UK, a sensitivity of 72.4% and a specificity of 94.9% (Wilkinson et al., 1997) was
reported. Ramalingam et al. in 2003 have reported sensitivities of 38%, 43% and 7% for
IgG, IgA and IgM to the 38-kDa antigen respectively in HIV-positive TB patients.

3.2.2: 19 kDa antigen-
The 19-kDa antigen (TB23, Rv3763) is a surface-exposed glycolipoprotein that
causes host signaling events by interacting with the toll-like receptor-2 when M.
tuberculosis enters macrophages (Noss et al., 2001; Thoma-Uszynski et al., 2001). The
19-kDa protein was shown to be an immunodominant antigen that is recognized by sera
from TB patients (Greenaway et al., 2005; Bothamley et al., 1992a; Bothamley et al.,
1992b; Jackett et al., 1988). Jackett et al. in 1988 have shown a sensitivity of 62% in
sputum smear-negative TB patients. A Gambia in which RD-1 antigens were compared
with other antigens for serodiagnostic potential, the 19-kDa antigen showed a sensitivity
of 24% and a specificity of 75% in sputum smear-positive TB patients (Bothamley et al.,
1992a).

3.2.3: 16 kDa antigen-
The 16-kDa antigen is a cytosolic regulatory protein (virulence factor), specific to
MTBC and is essential for the survival of the bacilli in the hostile environment of the
host, particularly during latency (Wayne, 1994). Using ELISA tests, Raja et al. in 2002
showed sensitivities of 62%, 52% and 11%, and specificities of 100%, 97% and 95% for
IgG, IgA and IgM in sputum smear negative and culture positive children. In another
study Imaz et al. in 2001 showed sensitivities of 34%, 19% and 3% for IgG, IgA and
IgM antibody isotypes respectively using the recombinant 16-kDa antigen. Using
recombinant antigens of the 38-kDa and 16-kDa antigens sensitivities of 59% (culture
positive) and 54% (culture negative) and a specificity of 98% were obtained in patients
with pulmonary TB. In patients with EPTB, sensitivities of 56%, 65%, 40% and 43%
were obtained for bone and joint, kidney, lymph node and pleural TB respectively. Two
studies suggest that antibody responses to the 16-kDa antigen may be important for the detection of latent TB (Beck et al., 2005; Demissie et al., 2006).

3.2.4: Antigen 85 complex-

The Mycobacterium Ag85 complex (30/32 Mycolyl transferase complex) is involved in biological processes such as cell wall biosynthesis and immunogenic responses (Tang et al., 2012; Baldwin et al., 1999; Langermans et al., 2005). This complex compromises three variant Ag85 proteins, A, B and C, which are encoded by separate genes. They are generally expressed in 3:2:1 ratio of B:A:C but this ratio varies in response to changes in the environment. Although B has the greatest expression level, C is 8 times more biologically active than B. The Ag85 proteins are secreted and can be found in the phagosome, the external layer of the bacterium’s cell wall, and the blood (Ronning et al., 2000; Anderson et al., 2001). Ag85 expression is essential for the intracellular survival of M. tuberculosis within the macrophage (Armitige et al., 2000) and the protein complex possess mycolyl transferase activity required for the synthesis of cord factor. Rinke de Wit et al. in 1993 reported that Ag85 complex cross-reactive with other mycobacteria. Sanchez-Rodriguez et al. in 2002 reported that Ag85 complex shows 72% sensitivity and 100% specificity for pulmonary TB. Malen et al. in 2008 reported immuno-dominant nature of Ag85 complex. Our group has also reported sensitivity of 84.0% and specificity of 85.2% (Kumar et al., 2010). Our group has also detected antibody reactivity to Ag85A (a component of Antigen 85 complex) and noted 44.68% sensitivity and 55.66% specificity for TB patients (Kumar et al., 2010). Antibody response to different components of the Ag85 complex was also reported earlier (Lim et al., 1999; Samanich et al., 2000; Van Vooren et al., 1992). Van Vooren et al., 1992 suggested that Ag85B (Rv1886c) was the most useful component of the Ag85 complex for the serodiagnosis of the active form of TB (Van Vooren et al., 1991). This was further supported by Lim et al., 1999, who showed that the antibody responses to Ag85B and Ag85A in patients were significantly greater than those to the Ag85C protein (Lim et al., 1999). Ag85B (Rv1886c) was reported to be 41–94% sensitive in various studies (Raja et al., 2002, Raja et al., 2004; Steingart et al., 2009; Uma Devi et al., 2003; Vikerfors et al., 1993). We reported that Ag85B component has sensitivity of 34%
and specificity of 74.1% for TB patients. Ag85c has Sensitivity of 80.9% and specificity of 40.7% (Kumar et al., 2010).

3.2.5: 27kDa antigen-

27kDa antigen is considered as fourth protein of the antigen 85 complex (Nagai et al., 1991) and is also known as MPT51, Ag85D, Rv3803c. MPT51 is known to exhibit more than 60% sequence similarity in its N-terminal region with the antigen 85 complex proteins (Nagai et al., 1991; Ramalingam et al., 2004). Epitopes common to this protein and the proteins of the antigen 85 complex have also been identified (Wiker and Harboe, 1992). In ELISA tests the antigen has yielded a sensitivity of 71% and a specificity of 95% (Ramalingam et al., 2004).

3.2.6: 14-kDa antigen-

The 14-kDa antigen (Rv0455c) is a culture filtrate protein that has shown great potential in detecting TB infection in a population for which TST is unreliable (Jackett et al., 1988). In general, the results indicate that antibody responses to the 14-kDa antigen are associated with chronic exposure rather than active disease (Jackett et al., 1988; Lyashchenko et al., 1998). A study by Jackett et al., in 1988 showed that responses to this antigen were higher in household contacts than among patients with active disease. In another study, healthy subjects with heavy occupational exposure to TB were found to have higher responses than community controls (Bothamley et al., 1992b). Silva et al. in 2003 compared the diagnostic potential of the 38-kDa, 19-kDa and 14-kDa antigens and indicated that responses to the 14-kDa antigen were associated with latent TB that had increased risk of progressing into active disease (Silva et al., 2003).

3.2.7: MPT-32-

MPT32 or DPEP is a culture filtrate protein and actively secreted by M. tuberculosis (Nagai et al., 1991). Few studies indicate that MPT-32 is secreted by bacilli early during course of infection. Samanich et al. in 1998 reported that MPT-32 is recognized by sera from patients with early non-cavitary or cavitary disease. Samanich et al. in 2000 evaluated the reactivity of three antigens, the 88-kDa protein, Ag85c and MPT-32 and results show that MPT-32 detected 69% in sputum positive cases and 33% in sputum negative cases.
3.2.8: Antigen 60-

Antigen 60 is a heat stable component of PPD and *M. tuberculosis* culture filtrate ([Wiker et al., 1988; Harboe et al., 1977; Harboe, 1981]). The antigen is immunodominant and is strongly recognized by sera from *M. tuberculosis* infected individuals ([Harboe et al., 1977]). A60 is one of the most studied *M. tuberculosis* antigens and a commercial ELISA kit specific for this antigen has been developed. The sensitivity and specificity of IgG against A60 in pulmonary TB cases varied from 36% to 91% and 68–98% respectively ([Garg et al., 2003; Chan et al., 2000]). One study indicated that A60 can differentiate active TB from both infection and BCG vaccination. The major drawback for this antigen is that it is not specific for mycobacteria because it is also present in Nocardia and Corynebacterium.

3.2.9: TB9.7, TB15.3, TB16.3 and TB51-

TB9.7 (Rv3354), TB15.3 (Rv1636), TB16.3 (2185c) and TB51 (2462c) have been identified as novel antigens that have equal diagnostic performances to the 38-kDa antigen. After cloning and expression of 35 *M. tuberculosis* recombinant proteins in *E. coli*, [Weldingh et al. in 2005](#) analyzed the serodiagnostic potentials of these antigens on panels of sera from sputum smear-positive and sputum smear negative TB patients from TB endemic and non-endemic areas. The sensitivities of antigens ranged from 31% to 93% and with a specificity of at least 97%. The TB16.3 was the single-most important antigen with a sensitivity of 46–98% and a specificity of 97%.

3.2.10: MPT-64-

MPT64 (also known as Mpb64 and Rv1980c) is a secreted, immunogenic protein of *M. tuberculosis* ([Cole et al., 1998; Martin et al., 2011]). It is highly specific for the MTBC including *M. tuberculosis*, *M. africanum*, virulent *M. bovis*, and some sub-strains of the *M. bovis* BCG. Most importantly, it is not found in mycobacteria other than tuberculosis (MOTT) ([Roche et al., 1996; Abe et al., 1999]). Mpt64 falls within 1 of the 11 regions of differences (RDs) absent in certain BCG strains ([Behr, 2002; Wang et al., 2007; Weldingh and Andersen, 1999; Kozak et al., 2011]). Various molecular weights for Mpt64 have been reported in the literature: 22.3 kDa ([Protein Data Bank, 2012]); 23 kDa ([Kumar et al., 2011]). The proteins secreted by mycobacteria are important antigens recognized early in the host response to infection; they stimulate T-cell proliferation and
interferon-γ (IFN-γ) release from peripheral blood mononuclear cells (PBMCs) (Chaves et al., 2010). In patients with early M. tuberculosis infections, T-cells recognize several MPT64 epitopes. MPT64 is being investigated as a candidate vaccine, both alone and as a fusion protein with ESAT-6, a potent T-cell antigen (Bai et al., 2008; Brodin et al., 2004) and ubiquitin (Wang et al., 2012).

### 3.2.11: CFP-10/ESAT-6 family proteins-

CFP-10 and ESAT-6 are early secreted proteins with molecular weights of 10- and 6-kDa, respectively. In culture, they are some of the most abundant M. tuberculosis antigens. They are not found in M. bovis BCG or most MOTT but are present in M. kansasii, M. africanum M. szulgai, M. marinum, and M. riyadhense (Harboe et al., 1996; Dillon et al., 2000; Nguyen et al., 2012). These proteins fall within RD1, which plays a role in virulence and are missing in BCG. CFP-10 and ESAT-6 are members of a protein family encoded by genes aligned in pairs within the mycobacteria genome, and their secretion is an active process involving a specialized transport system, itself the product of several flanking genes (Renshaw et al., 2005; Lightbody et al., 2008). The genes encoding these proteins are co-transcribed and form a tight 1:1 complex. The C terminus of CFP-10 forms a long flexible arm, which plays an important role in cell surface attachment (Renshaw et al., 2005). These proteins are important stimulators of T cells and are used in interferon-γ (IFN-γ) releasing assays (IGRAs). Members of the CFP family other than CFP-10 namely CFP-25, CFP-20.5 and CFP-3 are highly immunogenic as well. Our group have also evaluated the efficiency of CFP-10 and ESAT-6 separately, and reported that EAST-6 had 64.5% sensitivity and 88.9% specificity while CFP-10 had 66% sensitivity and 85.2% specificity (Kumar et al., 2010).

### 3.2.12: PE and PPE antigens-

PE/PPE proteins are unique proteins found exclusively in pathogenic mycobacteria. There are genes for 99 PE proteins and 68 PPE proteins in the genome of M. tuberculosis, constituting 10% of its coding capacity (Cole et al., 1998). They are characterized by the presence of proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) motifs near their N-termini (Sampson, 2011). Many PE/PPE proteins are localized to the bacterial cell surface and many are secreted, making them readily accessible to the immune system (Abdallah et al., 2009). Indeed, PE/PPE proteins are
known to induce strong humoral and cellular immune responses based on studies that used traditional immunological assays (Akhter et al., 2012).

3.2.13: Glycolipid antigens-

Lipoarabinomannan (LAM) is a polysaccharide antigen that constitutes 40% of the cell wall of mycobacteria. When IgG against LAM was measured by an ELISA in sera from 66 patients with TB, a sensitivity of 72% and a specificity of 91% were obtained. However, a low degree of sensitivity was obtained when sera from HIV positive persons, suspected of having TB were tested, using the commercially available serologic assay, Myco-Dot TM (DynaGen, Inc., Cambridge, MA, USA). In addition to LAM, the acylated trehalose family has been the most frequently investigated group of glycolipids. They are 2,3-diacyltrehalose (DAT), 2,3,6- triacyltrehalose (TAT), 2,3,6,6-tetraacyltrehalose 2'-sulfate (sulfolipid-I, SL-I) and trehalose 6,6'-dimycolate (cord factor). Results of studies conducted using these antigens in ELISA tests showed extensive variability in antibody detection (Julian et al., 2002). Test sensitivities varied from 11% to 88% for the DAT and 51–93% for the TAT antigen.

3.3: Vaccine for tuberculosis-

3.3.1: BCG vaccine-

BCG is the only vaccine available against TB and is still the only vaccine licensed to prevent TB all over the world. It is a live attenuated strain of \textit{M. bovis} obtained by Albert Calmette and Camille Guerin through 230 in-vitro passages over 13 year period (Liu et al., 2009). BCG is widely used in TB endemic countries where newborn are immunized as soon as possible after birth with a single intra-dermal dose (Hesseling et al., 2009). To date it is estimated that BCG has been administered over four billion times and 120 billion children receive BCG every year globally (Dalmia and Ramsay, 2012). Meta analyses of published studies have clearly reported that BCG prevent against meningeal TB and disseminated from in children (Colditz et al., 1995; Trunz et al., 2006). However various clinical trial report that protection against pulmonary TB vary from nil to 80 percent such as nil in South India and 80% in UK. (Behr, 2002; Colditz et al., 1994; Fine, 2001). Various hypotheses have put to explain to these discrepancies by BCG. Researchers believe that difference in the geography, operation and demography of the trials along with different study designs were responsible for the variability in
protective efficacy. The most commonly used strains for preparation of BCG vaccine are Denmark, Japan and Bulgaria. Many developing countries use several BCG vaccine strain. In India a BCG vaccine laboratory was established in 1948. BCG Danish strain 1331 is being used for vaccine production. Immunological studies in both animal and human have also shown strain specific protection variation in BCG. The BCG-Pasteur-Denmark and Glaxo strain showed better protection in mice while BCG- Denmark showed better protection in guinea pig. A study by Castillo-Rodal et al. in 2006 showed that when they immunized mice with ten different BCG strain and after few week mice were challenged with M. tuberculosis H37Rv. After 2-4 months their lung pathology showed each strain has a specific cytokine profile. On humans, Aguirre-Blanco et al. in 2007 showed that the commonly used BCG vaccine namely Danish, Glaxo and Pasteur elicit different in vitro magnitudes of T-cell activation and IFN-γ in healthy BCG vaccinated individuals. The major problem with BCG is that the memory generated by BCG disappears in adults. BCG must be presented to MHC class II molecules to activate CD4+ cells and MHC class I molecule to activate CD8+cells however BCG fails to activate CD8+cells sufficiently (Grode et al., 2005).

3.3.2: Development of vaccine candidate other then BCG-

BCG vaccination is not having significant impact on TB in adults and shows non targeted protection in children. Numerous new approaches to vaccine development are pursued and tested in animal models. The new technique for preparing vaccine includes development of modified BCGs, live attenuated stains of mycobacteria and DNA vaccine as well as subunit vaccine. Several studies showed that the M. tuberculosis antigens recognized by T-cells and stimulating IFN-γ secretion are considered as promising candidate for development of new vaccine against TB.

3.3.2a: Recombinant BCG-

For the development of TB vaccine candidate, genetically modified variant of the live BCG vaccine are in progressive phase, which express protective antigen(s) or cytokine(s), which will boost its protective efficacy (Murray et al., 1996). Bao et al. in 2003 constructed two rBCG strains expressing ESAT-6 but found not significantly better than BCG in animal model. Dhar et al. in 2004 used rBCG with Ag85A which showed increased humoral response compared to wild type BCG in BALB/c mice. Sugawara et
al. in 2007 showed that rBCG with Ag85A was better than Ag85A DNA in term of protective efficacy against *M. tuberculosis*. Beside that they concluded that peptide boosting is important for the induction of higher protective efficacy by rBCG. Wang *et al.* in 2009 used recombinant BCG expressing Ag85B and PPE protein Rv3425 from *M. tuberculosis* (rBCG:Ag85B-Rv3425) which has been advocated as a strong vaccine candidate. rBCG30 with over-expressed Ag85B proved good in phase I clinical trial (Horwitz and Harth, 2003; da Costa *et al.*, 2014).

### 3.3.2b: Live mycobacterial vaccine-

Many alternative living and non-living mycobacteria were explored as possible TB vaccine. Live vaccine includes less virulent, naturally attenuated mycobacteria such as auxotrophic *M. tuberculosis*. Naturally attenuated strains of mycobacteria such as *M. vaccae*, *M. microti*, and *M. Habana* can serve as potential vaccines for TB. Recombinant *M. vaccae* and *M. smegmatis* that express *M. tuberculosis* epitopes are also reported. However, none of them performed better than BCG in the experimental models (de Bruyn and Garner, 2003; Abou-Zeid *et al.*, 1997; Orme, 1997). Further, live auxotrophic *M. tuberculosis* strains are also interesting vaccine candidates. Knocking out certain genes or inserting one or more mutation produce auxotrophic mutants of *M. tuberculosis*, generated to produce improved live vaccine for TB (Hernandez Pando *et al.*, 2006; Sambandamurthy and Jacob, 2005; Smith *et al.*, 2001) but few of them conferred better protection than the standard BCG in animal model. In the wrath of the growing HIV pandemic, a double- deletion mutant of *M. tuberculosis* was developed by deleting the RD1 region and two gene required for pantothenate synthesis by Sambandamurthy *et al.* in 2005 which was proposed to be considered as a human vaccine candidate for protecting both healthy and HIV-infected individuals against TB. Gupta *et al.* in 2009 used *Mycobacterium w* in both live and killed form as a vaccine and they reported that Mw activates macrophages as well as lymphocytes. Mw immunization by both the parenteral route and aerosol administration gave higher protection than BCG.

### 3.3.2c: Subunit vaccine-

Instead of using whole protein as vaccine candidate, the T-cell epitope will be recognize directly by the receptors on T-cells and may be used in the development of vaccine. Investigators are trying to find out the peptide which could elicit an immune
response against *M. tuberculosis*. In order to develop an effective peptide based vaccine for TB, the specific antigens must be identified and its ability in protective immunity may be evaluated. Various antigens of *M. tuberculosis* used for development of subunit vaccines are Antigen 85 complex, ESAT-6, 38kDa, 19kDa and MPT64. Further, there are three components in Ag85 complex (Ag85A, Ag85B and Ag85C) and Ag85A and Ag85B reported ideal vaccine candidate (Lozes et al., 1997; Mustafa et al., 2000).

3.3.2d: DNA vaccine-

The nucleic acid or DNA vaccines are also an innovative approach currently applied in the search of a BCG replacement. DNA vaccines are used as either naked DNA encoding antigens and transfect cells *in-vivo* or a viral vector coding for specific disease antigens. DNA vaccine may work as promising alternative to BCG. It is constructed to code for very few antigens which can be selected as not to interfere with skin sensitivity tests, further it is easily produced as stable vaccine and can be used worldwide for universal vaccination program. Various mycobacterial antigen DNA sequence used as DNA vaccine after inserting in plasmid such as the mycolyltransferase family Ag85 complex (Baldwin et al., 1998; Kamath et al., 1999; Ulmer et al., 1997), the phosphate binding protein family Pst-3 (Tanghe et al., 1999), the 38 kDa protein (Fonseca et al., 2001), ESAT-6 (Brandt et al., 2000), and heat-shock protein 60, 65 and 70 (Ferraz et al., 2004; Johansen et al., 2003), PPE protein:Rv2770c (Romano et al., 2008). Further, some studies showed that DNA vaccine encoding more than one antigen are more effective than those encoding single antigen (Tian et al., 2004; Tian et al., 2005). There are two major advantages with DNA vaccine, first it induced strong Th1 as well as CD8+ T cell mediated cytotoxic response which play major role to combat *M. tuberculosis* infection and second is that DNA vaccine can be designed together with immune-stimulatory DNA sequence such as the CpG (Iwasaki et al., 1997) and cytokine to enhance their immunogenicity. Further, table 3.1 summarizes some selected advanced types of TB vaccine under clinical trials (Ottenhoff and Kaufmann, 2012; Leunda et al.,).
Table 3.1: Most advanced TB vaccine candidates in clinical trials.

<table>
<thead>
<tr>
<th>Type</th>
<th>Candidate</th>
<th>Description</th>
<th>Clinical Trial Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant BCG for pre-exposure prime vaccination</td>
<td>VPM 1002</td>
<td>rBCG-expressing listeriolysin and urease deletion</td>
<td>Phase IIa ongoing</td>
</tr>
<tr>
<td></td>
<td>rBCG30</td>
<td>rBCG-expressing Ag85B</td>
<td>Phase I completed/on hold</td>
</tr>
<tr>
<td></td>
<td>Aeras-422</td>
<td>rBCG-expressing perfringolysin and Ag85A, 85B, Rv3407</td>
<td>Phase I terminated due to side effects</td>
</tr>
<tr>
<td>Whole bacterial vaccine for therapeutic vaccination</td>
<td>RUTI</td>
<td>Detoxified <em>M. tuberculosis</em> in liposomes</td>
<td>Phase IIa ongoing</td>
</tr>
<tr>
<td></td>
<td>M. vaccae</td>
<td>Inactivated <em>M. vaccae</em></td>
<td>Phase III completed</td>
</tr>
<tr>
<td>Fusion protein in adjuvant for pre-exposure booster vaccination</td>
<td>Hybrid 1+CAF01</td>
<td>Fusion of Ag85B and ESAT-6 in adjuvant CAF01</td>
<td>Phase I ongoing</td>
</tr>
<tr>
<td></td>
<td>Hybrid 1+IC31</td>
<td>Fusion of Ag85B and ESAT-6 in adjuvant IC31</td>
<td>Phase I, soon entering IIa</td>
</tr>
<tr>
<td>Viral-vector for pre-exposure booster Vaccination</td>
<td>Oxford MVA85A/Aeras-485</td>
<td>Modified vaccinia Ankara-expressing Ag85A</td>
<td>Phase IIb ongoing</td>
</tr>
<tr>
<td></td>
<td>Crucell Ad35/Aeras-402</td>
<td>Replication-deficient adenovirus 35-expressing Ag85A, Ag85B, TB10.4</td>
<td>Phase IIb ongoing</td>
</tr>
</tbody>
</table>

3.4: Immunity against *M. tuberculosis*-

*M. tuberculosis* is an obligatory aerobic intracellular pathogen. The tubercle bacilli enter in the body via the respiratory route and spread in body via lymphatic or blood vascular systems. The EPTB occurs in about 15% cases. Host immune response against *M. tuberculosis* can be divided in two arms, innate immunity and adaptive immune.

3.4.1: Innate immunity against *M. tuberculosis*-

3.4.1a: Macrophage-

In body phagocytosis of *M. tuberculosis* by alveolar macrophages is the first major interaction event between host and *M. tuberculosis*. Within 2-6 weeks cell mediated immune response develops and activated lymphocyte and activated macrophages migrate to lesion resulting granuloma formation. Within granuloma the *M. tuberculosis* remains forever or get re-activated later or get discharged into the airways after enormous increases in number, necrosis of bronchi and cavitation. Reactive oxygen
intermediates (ROI), reactive nitrogen intermediates (RNI) and apoptosis or programmed cell death are also defense mechanisms found inside the macrophages. IFN-γ and TNF-α mediated antimycobacterial effects have also been reported (Vishwanath et al., 1998).

3.4.1b: Dendritic cells-

Dendritic cells are clearly involved in the protective immune response to *M. tuberculosis* infection. Dendritic cells recognize, capture and process antigens thus being able to present them in the context of major histocompatibility complex (MHC) as well as through CD1 molecules (Banchereau and Steinman, 1998; Gumperz and Brenner, 2001). In a protective immune response, dendritic cells induce maturation of T cells towards a T-helper 1 (Th1) profile by secreting cytokines, such as IL-12, IL-18, IL-23, and probably IFN-α and β but not IFN-γ (Wozniak et al., 2006; Kadowaki et al., 2001; Kalinski et al., 1999; Thurnher et al., 1997).

3.4.1c: Natural resistance Associated Macrophage Protein (Nramp)- Nramp is crucial in transporting nitrite from intracellular compartments (cytosol) to phagolysosome (acidic environment) where it can be converted to NO. Defects in Nramp production increase susceptibility to mycobacteria (Newport et al., 1995).

3.4.1d: Neutrophils-

The accumulation of neutrophils in granuloma and increased chemotaxis suggests its role in mycobacterial diseases (Edwards and Kirkpatrick, 1986). At the site of multiplication of bacilli, first neutrophils arrive followed by NK cells, γδ cells and αβ cells. The granulocyte-macrophage-colony stimulating factor (GM-CSF) enhances phagocytosis of bacteria by neutrophils (Fleishmann et al., 1986). Human studies show that neutrophils provide defensins (Ogata et al., 1992). Majeed et al., in 1998 showed that neutrophils have a role in killing of *M. tuberculosis* in the presence of calcium under *in-vivo* conditions.

3.4.1e: Natural killer cells-

NK cells are also the effector cells of innate immunity which can lyse the pathogens or infected monocytes. NK cells produce IFN-γ and can lyse mycobacterium pulsed target cells at early infection. These cells can activate phagocytic cells at the site of infection.
3.4.1f: CD1d-restricted natural killer T cells-
These are a unique subset of human natural killer T cells characterized by the expression of an invariant V alpha 24 T cell receptor that recognizes the nonclassical antigen-presenting molecule CD1d. Once activated by alpha-galactosylceramide CD1d-restricted natural killer T cells contribute to human host defense against *M. tuberculosis* infection. Functions of these cells include IFN-γ secretion, proliferation, lytic activity, and anti-mycobacterial activity; latter via the antimicrobial peptide granulysin which damages the mycobacterial surface.

3.4.1g: The Toll-like receptors (TLR)-
Mycobacterial components may interact with different members of the TLR family. However, *M. tuberculosis* activates cells via TLR2 or TLR4 in a CD independent manner. TLR8 and TLR9 are known to play a pivotal role in pulmonary TB via modulating sensor expression and/or effector responses (Bharti *et al.*, 2014).

3.4.2: Acquired immune response against *M. tuberculosis*-

3.4.2a: Humoral immune response-
Since *M. tuberculosis* is an intracellular pathogen serum components may not get access and may not play any protective role but a study by Bosio *et al.* in 2000 suggests their role in TB. Studies of B cell-deficient mice (Vordermeier *et al.*, 1996), severe combined immunodeficiency (SCID) mice (Guirado *et al.*, 2006), passive immunization using monoclonal antibody and polyclonal antibodies and immune responses against specific mycobacterial antigens in experimental animals reveal that in addition to a significant immunomodulatory effect on CMI antibodies play an essential protective role against mycobacterial infections (Abebe and Bjune, 2009). Various proteins from *M. tuberculosis* have been evaluated to show is utility in diagnosis of infection (Raja *et al.*, 2001; Kumar *et al.*, 2010).

3.4.2b: Cellular immune response-
3.4.2b.I: T- cells- *M. tuberculosis* is a classic example of a pathogen for which the protective response relies on cell mediated immunity. The study on mouse models shows that after one week infection the number of activated CD4+ and CD8+ T cells in the lung draining lymphnodes increases (Feng *et al.*, 1999). Between 2 and 4 week post-infection, both CD4+ and CD8+ T cells migrate to the lungs and demonstrate an effector/memory
phenotype (CD4^hi^CD45^lo^CD62L^-); approximately 50 per cent of these cells are CD69+. This indicates that activated T cells migrate to the site of infection and are interacting with APCs. The tuberculous granulomas contain both CD4+ and CD8+ T cells (Randhawa, 1990) that contains the infection within the granuloma and prevent reactivation.

3.4.2b.II: T-helper (CD4+) cells- CD4+T cells are most important in the protective response against *M. tuberculosis*. Various studies with antibody depletion of CD4+T cells (Muller *et al.*, 1987), adoptive transfer (Orme and Collins, 1984), or the use of gene-disrupted mice (Caruso *et al.*, 1999) have shown that the CD4+ T cell subset is required for control of infection. Humans infected with HIV demonstrated that the loss of CD4+ T cells thus shows increase susceptibility to both acute and re-activation TB (Selwyn *et al.*, 1989). The production of IFN-γ and possibly other cytokines (primary effector function of CD4+ T cells) is sufficient to activate macrophages. Apoptosis or lysis of infected cells by CD4+ T cells may also play a role in controlling infection (Keane *et al.*, 1997).

3.4.2b.III: T-cytotoxic (CD8+) cells- CD8+ cells are also capable of secreting cytokines such as IFN-γ and IL-4 and thus may play a role in regulating the balance of Th1 and Th2 cells in the lungs of patients with pulmonary TB. The mechanism by which mycobacterial proteins gain access to the MHC class I molecules is not fully understood. Further, Teitelbaum *et al.* in 1999 indicated that mycobacteria induced pore or break in the vesicular membrane which covers the bacilli resulting mycobacterial antigen to enter the cytoplasm of the infected cell and finally binds to MHC molecules. Yu *et al.* in 1995 analyzed CD4 and CD8 T cell populations from patients with rapid, slow or intermediate regression of disease while receiving therapy and found that slow regression was associated with an increase in CD8+ cells in the BAL. Lysis of infected human dendritic cells and macrophages by CD1- and MHC class I-restricted CD8+ T cells specific for *M. tuberculosis* antigens reduced intracellular bacterial numbers (Stenger *et al.*, 1997). The IFN-γ production in the lungs by the CD8 T cell subset increased at least four-fold in the perforin deficient mice, suggesting that a compensatory effect protects from acute infection (Matloubian *et al.*, 1999). Studies defining antigens recognized by CD8+ T cells from infected hosts without active TB provide attractive vaccine candidates and
support the notion that CD8+ T cell responses as well as CD4+ T cell responses must be stimulated to provide protective immunity.

3.4.2b.IV: \(\gamma/\delta\) T-cells in tuberculosis- \(\gamma/\delta\) T cells are large granular lymphocytes that can develop a dendritic morphology in lymphoid tissues. In general, \(\gamma/\delta\) T cells are felt to be non-MHC restricted and they function largely as cytotoxic T cells. Studies on animals suggest that \(\gamma/\delta\) T cells play a significant role in the host response to TB in mice and in other species (Izzo and North, 1992) including humans. \(M.\) \textit{tuberculosis} reactive \(\gamma/\delta\) T cells can be found in the peripheral blood of tuberculin positive healthy subjects and these cells are cytotoxic for monocytes pulsed with mycobacterial antigens and secrete cytokines that may be involved in granuloma formation (Munk et al., 1990). Studies (Ueta et al., 1994; Tazi et al., 1992) demonstrated that \(\gamma/\delta\) T cells were relatively more common (25 to 30% of the total) in patients with protective immunity as compared to patients with ineffective immunity. The childhood TB patients showed that the proportion of T cells expressing the \(\gamma/\delta\) T cell receptor was similar in TB patients and controls (Swaminathan et al., 2000). These studies on \(\gamma/\delta\) T cells show their role in early immune response against TB and is an important part of the protective immunity in patients with latent infection (Ladel et al., 1995).

3.4.2b.V: Th1 and Th2 dichotomy in tuberculosis- Two broad possibly overlapping categories of T cells have been described based on the pattern of cytokines they secrete upon antigen stimulation. Th1 cells secrete IL-2, IFN-\(\gamma\) and play a protective role in intracellular infections. Th2 type cells secrete IL-4, IL-5 and IL-10 and are either irrelevant or exert a negative influence on the immune response. The balance between the two types of response is reflected in the resultant host resistance against infection. The type of Th0 cells shows an intermediate cytokine secretion pattern. The differentiation of Th1 and Th2 from these precursor cells may be under the control of cytokines such as IL-12. In mice infected with virulent strain of \(M.\) \textit{tuberculosis}, initially Th1 like and later Th2 like response has been demonstrated (Orme et al., 1993). It has been reported that PBMC from TB patients, when stimulated \textit{in vitro} with PPD release lower levels of IFN-\(\gamma\) and IL-2 as compared to tuberculin positive healthy subjects (Huygen et al., 1988). Other studies have also reported reduced IFN-\(\gamma\) (Vilcek et al., 1986) and increased IL-4 secretion (Sanchez et al., 1994) or increased number of IL-4 secreting cells (Surcel et
al., 1994). Zhang et al. in 1994 studied cytokine production in pleural fluid and found high levels of IL-12 after stimulation of pleural fluid cells with M. tuberculosis. IL-12 is known to induce a Th1-type response in undifferentiated CD4+ cells and hence there is a Th1 response at the actual site of disease. The same group (Lin et al., 1996) observed that TB patients showed evidence of high IFN-γ production and no IL-4 secretion by the lymphocytes in the lymph nodes. There was no enhancement of Th2 responses at the site of disease in human TB. Robinson et al. in 1994 found increased levels of IFN-γ mRNA in situ in BAL cells from patients with active pulmonary TB.

3.4.2c: Cytokines-

Various cytokines play important role in TB.

3.4.2c.I: Interleukin-12- Bacteria, bacterial products and parasites act as inducer for IL-12 secreted by peripheral lymphocyte. IL-12 induced macrophages and dendritic cells for phagocytosis (Ladel et al., 1997) which leads to development of Th1 response with production of IFN-γ. Mice deficient with IL-12p40 gene was found susceptible to infection and reduced IFN-γ production could be responsible for decreased survival of these mice (Cooper et al., 1997). Humans with mutation in IL-12p40 or IL-12R genes show reduced IFN-γ production from T-cells (Ottenhof et al., 1998). Lowrie et al. in 1999 indicated that DNA administration reduce bacterial number in mice with a chronic M. tuberculosis infection. McDyer et al. in 1997 found that stimulated PBMC from MDR-TB patients had less secretion of IL-2 and IFN-γ than did cells from healthy control subjects. IFN-γ production could be restored if PBMC were supplemented with IL-12 prior to stimulation and antibodies to IL-12 caused a further decrease in IFN-γ upon stimulation. Taha et al. in 1997 demonstrated that in patients with drug susceptible active TB both IFN-γ and IL-12 producing BAL cells were abundant as compared with BAL cells from patients with in active TB.

3.4.2c.II: Interferon-γ- IFN-γ, a key cytokine in control of M. tuberculosis infection is produced by both CD4+ and CD8+ T cells as well as by NK cells. IFN-γ might increase antigen presentation leading to recruitment of CD4+ T-lymphocytes and/or cytotoxic T-lymphocytes which might participate in mycobacterial killing. Although IFN-γ production alone is insufficient to control M. tuberculosis infection it is required for the protective response to this pathogen. IFN-γ gene knockout mice are most susceptible to
virulent *M. tuberculosis* (Cooper et al., 1993). Humans defective in genes for IFN-γ or the IFN-γ receptor are prone to serious mycobacterial infections including *M. tuberculosis* (Jouanguy et al., 1996). Although IFN-γ production may vary among subjects some studies suggest that IFN-γ levels are depressed in patients with active TB (Lin et al., 1996; Zhang et al., 1995). IFN-γ production was most severely depressed in patients with moderately advanced and far advanced pulmonary disease and in malnourished patients. Production of IL-12, IL-4 and IL-10 was similar in TB patients and healthy tuberculin reactors. These results indicate that the initial immune response to *M. tuberculosis* is associated with diminished IFN-γ production which is not due to reduced production of IL-12 or enhanced production of IL-4 or IL-10.

3.4.2c.III: Tumor necrosis factor (TNF-α)- TNF-α is believed to play multiple roles in immune and pathologic responses in TB. *M. tuberculosis* induces TNF-α secretion by macrophages, dendritic cells and T cells. *M. tuberculosis* infection resulted in higher bacterial burden and rapid death of the mice deficient in TNF-α or the TNF receptor in comparison to control mice (Flynn et al., 1995). This cytokine play a role in granuloma formation. Further, it affects cell migration and localization of *M. tuberculosis* in tissue. TNF-α influences expression of adhesion molecules, chemokines and chemokines receptors.

3.4.2c.IV: Interleukin -1- IL-1 along with TNF-α plays an important role in the acute phase response such as fever and cachexia prominent in TB. The major antigens of mycobacteria triggering IL-1 release and TNF-α have been identified (Wallis et al., 1990).

3.4.2c.V: Interleukin -2- This cytokine induces an expansion of the lymphocytes specific for an antigen. CD4 Th1 cells secrete this cytokine and can influence the mycobacterial infection either alone or in combination with other cytokines (Blanchard et al., 1989).

3.4.2c.VI: Interleukin -4- Various human studies show strong Th1 response observed in PBMC from TB patients than Th2 response (Lin et al., 1996; Zhang et al., 1995). In a study of cytokine gene expression in the granuloma of patients with advanced TB by in-situ hybridization, IL-4 was detected in 3 of 5 patients but never in the absence of IFN-γ expression (Fenhalls et al., 2000).
3.4.2c.VII: **Interleukin -10**- IL-10 is considered as an anti-inflammatory cytokine. This cytokine produced by macrophages and T cells during *M. tuberculosis* infection possesses macrophage-deactivating properties including down regulation of IL-12 production which in turn decreases IFN-γ production by T cells. IL-10 directly inhibits CD4+ T cell responses by inhibiting APC functions mycobacteria infected cells (Rojas et al., 1999).

3.4.2c.VIII: **TGF-β**- TGF-β is present in the granulomatous lesions of TB patients and is produced by human monocytes after stimulation with *M. tuberculosis* (Toossi et al., 1995) or LAM (Dahl et al., 1996). TGF-β has important anti-inflammatory effects, including deactivation of macrophage production of ROI and RNI (Ding et al., 1990), inhibition of T cell proliferation (Rojas et al., 1999), interference with NK and CTL function and down regulation of IFN-γ, TNF-α and IL-1 release (Ruscetti et al., 1993). Toossi et al., in 1995 have shown that when TGF-β is added to co-cultures of mononuclear phagocytes and *M. tuberculosis* both phagocytosis and growth inhibition were inhibited in a dose-dependent manner.

3.5: **Comparative genomics of *M. tuberculosis* after infection**-

During lung infection *M. tuberculosis* resides in macrophages and subverts the bactericidal mechanisms of these professional phagocytes. This host-pathogen relationship is fundamental for the development of new therapies to cure and prevent TB. Fontan et al. in 2008 analyzed the transcriptional profile of *M. tuberculosis* infecting human macrophage-like THP-1 cells in order to identify putative bacterial pathogenic factors that can be relevant for the intracellular survival of *M. tuberculosis*. They compared the gene expression profile of *M. tuberculosis* H₃₇Rv by DNA microarray technology after 4 hrs and 24 hrs of infection of human macrophage-like THP-1 cells with the gene expression profile of the strain growing exponentially in broth cultures. They found 585 genes expressed differentially by intracellular *M. tuberculosis*.

The complete gene expression profile of *M. tuberculosis* growing in mouse macrophages was defined by Schnappinger et al. in 2003 and by Rachman et al. in 2006a. These analyses indicate that inside the mouse macrophage phagosome *M. tuberculosis* has to face a DNA- and cell envelope-damaging environment that is rich in fatty acid and deficient in iron. The transcriptional profile of *M. tuberculosis* infecting
human lungs indicates that the bacteria regulate genes involved in the evasion of the immune system (Rachman et al., 2006b). A similar analysis of M. tuberculosis in human monocyte-derived macrophages after 7 days of infection suggested the relevance of bacterial genes involved in transcriptional regulation (Volpe et al., 2006). In addition, M. tuberculosis genes that are essential for the survival of bacteria in mouse macrophages and in mouse lungs have been identified by using the transposon site hybridization technique (Rengarajan et al., 2005) and designer arrays for defined mutant analysis (Lamichhane et al., 2005).

3.6: Prevalent genotypes of M. tuberculosis-

Molecular typing of M. tuberculosis strains has proven to be a valuable tool for TB control in terms of tracking transmission chain, detecting suspected outbreaks and indentifying successful clone (Barnes and Cave, 2003).

3.6.1: The Central Asian-Delhi (CAS1_Del) (ST-26) genotype family or Delhi lineage-

The CAS1-Delhi family is essentially localized in the Middle-East and Central Asia more specifically in South-Asia (21.2%) and preferentially in India (75%) (Bhanu et al., 2002). It is also found in other countries of this region such as Iran and Pakistan (Gascoyne-Binzi et al., 2002; Hasan et al., 2006). It has also been found in several other regions (Africa, 5.3%; Central America, 0.1%; Europe, 3.3%; Far-East-Asia, 0.4%; North-America, 3.3%; Oceania, 4.8%). In Europe and Australia these strains were frequently found to be linked with immigrants from South Asia.

The presence in India of a specific lineage of the M. tuberculosis complex was concomitantly and independently reported by two different groups using IS6110 RFLP and spoligotyping respectively (Bhanu et al., 2002; Filliol et al., 2003). This lineage was also shown to be endemic in Sudan, other sub – Saharan countries and Pakistan (Brudey et al., 2006). This genotype family could be the ancestor of the Beijing family since it clusters close to Beijing when analyzed by a combination of MIRU, spoligotyping and VNTR (Sola et al., 2003). In India its frequency varies from one region to another: it is more prevalent in the North than in the South where the EAI family predominates (Suresh et al., 2006). An outbreak strain named CII was recently reported in Leicester, United Kingdom. It belonged to the CAS family and harbored a specific deletion.
(Rv1519). In broth media this strain was found to grow more slowly and to be less tolerant to acid and H$_2$O$_2$ than two laboratory strains, CDC1551 and H$_3$7Rv.

3.6.2: The East African – Indian (EAI) genotype (ST11)–

The East-African-Indian (EAI) family is also highly prevalent in these areas (33.8% in Far-East-Asia, 24.3% in the Middle East and Central Asia, and 22.9% in Oceania). The EAI lineage is more prevalent in South-East Asia, particularly in the Philippines (73%) (Douglas et al., 2003), in Myanmar and Malaysia (53%) (Phyu et al., 2003), in Vietnam and Thailand (32%) (Brudey et al., 2006), in India (Kulkarni et al., 2005). This lineage was first described in Guinea-Bissau (Kallenius et al., 1999) and was shown to be frequent in South-East Asia, India and East Africa (Kremer et al., 1999). This group of strains is characterized by a low number of IS6110. Predominant spoligotype prevalent in North India (Kanpur) belongs to Central Asian Delhi family (CAS1_Del) (37%), East African Indian family (11%), T1 family (8%) and Beijing (4%) family. This study reveals that CAS is the most predominant family in this rural area of Kanpur (Sharma et al., 2008). An early study carried out in Kerala shows that Majority of the isolates (64.28%) belonged to the ancestral East-African Indian (EAI) lineage (Joseph et al., 2013).

3.6.3: Beijing genotype-

The Beijing family is reportedly the most prevalent spoligotype worldwide and constitutes 90 to 92 % of $M. tuberculosis$ strains in china (van Soolingen et al., 1995). High rates of infection with Beijing strain in the countries neighboring china suggest that this particular strain may have radiated from Beijing to other regions. The prevalence of Beijing strain is 4 – 8 % in India (Sharma et al., 2008) and 31 % in a study in Dhaka (Banu et al., 2004). Two studies from India have reported 3% (Mistry et al., 2002) and nearly 10% (Suresh et al., 2006) Beijing type to be associated with MDR. Beijing strain is more virulent and elicits a non protective immune response compared to other genotypes during experimental disease in mice (Lopez et al., 2003). It is speculated that strains belonging to Beijing family have a genetic advantage to cause disease and that the wide dispersion of this family compared to other less prevalent clinical isolates may be
related to differential protein expression (Bifani et al., 2002). Pheiffer et al. in 2005 compared the protein expression and antigen recognition profiles of local Beijing strain with a less prevalent clinical isolates belonging to the family 23 strain lineages and the laboratory strain *M. tuberculosis* H37Rv. Using 2-DGE and western blot analysis Beijing strain showed increased expression of $\alpha$-crystalline and decreased expression of Hsp65, PstS1 (38kDa) and 47kDa protein compared to the other strains and H37Rv. The success of the Beijing family as a human pathogen may in part due to expression of Hsp65, PstS1 (38kDa) and 47kDa protein as previously it has been suggested that reduced expression of certain major antigens may allow strains to evade the host immune response (Stewart et al., 2001).

3.6.4: Other genotypic family of *M. tuberculosis*

Most of the studies from India show that CAS genotype is most prevalent genotype in country especially in north India (Bhanu et al., 2002; Singh et al., 2007), followed by the EAI genotype that is predominant in South India (Singh et al., 2007). However, some more families have also been reported such as the T1 family (ST 54) found most common family in Turkey (Oral Zeytinli and Köksal, 2012). Further, two Chinese groups found that T1 family is the second most prevalent genotype (Dong et al., 2010; Zhou et al., 2011). Sharma et al. in 2008 from India also reported this genotype in Kanpur region. The Haarlem family was found predominant in western world, about 25% of isolates in Europe, Central America and Caribbean belongs to this family (Cubillos-Ruiz et al., 2010, Schierloh et al., 2014). The X1 (ST-119) family is reported in Delhi, India, however its presence in this region could be linked to the past British history (Singh et al., 2004).

3.7: Proteomics and immune-proteomics *M. tuberculosis*

Cole et al. in 1998 published the complete DNA sequence of the *M. tuberculosis* strain H37Rv. Its genome has a total of 3924 individual genes. Very soon after the publication of its genome, bioinformatics tools were applied to predict the proteomic profile of *M. tuberculosis*. The bioinformatics-predicted proteome was compared to 2-DGE protein maps obtained from *M. tuberculosis* whole cell lysates. This work demonstrated that proteins with a molecular mass below 10 kDa were not predicted from
the genome sequence. By proteomic approach, Jungblut et al. in 2001 predicted 6 genes not predicted in the genome of M. tuberculosis H₃₇Rv.

Proteomics, the global study of proteins that are translated in a given physiological state by the proteome of an organism implies not only an inventory of its gene products but also the transduction rate and the post-translational events that occurs in the organism (Betts, 2002). Classical studies of proteomics involve two dimensional electrophoresis (2-DGE) in which proteins are first separated by the isoelectric point (pI) and then by the molecular weight (O’Farrell et al., 1975). Every spot of protein is then isolated, hydrolyzed and subjected to techniques of matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS) or Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/MS). Other proteomic approaches that do not involve the use of gels such as two dimensional liquid chromatography (LC) and the subsequent analysis by MS (2 DLC/MS) have been shown to be very efficient in the identification of hydrophobic and membrane proteins (Isobe et al., 1991). The comparative proteomic analysis using 2-DGE and MALDI/MS is applied for comparative protein profile between two strains.

Jungblut et al. in 1999 compared proteomic profile of the two virulent M. tuberculosis strain (H₃₇Rv, Erdman) and two non virulent vaccines M. bovis BCG strain (Chicago, Copenhagen) to identify the protein virulent in these strains. They detected 31 variants, six identified proteins in H₃₇Rv were without any counterpart in BCG: L-alanine dehydrogenase (40kDA antigen, Rv2780), isopropyl malate synthase (Rv3710), nicotinate-nucleotide pyrophosphatase, (Rv1596), MPT64 (Rv1980c) and two conserved hypothetical (Rv2449c and Rv0036c). Andersen et al. in 1992 and Li et al. in 1993 also demonstrated the absence of L-alanine dehydrogenase and MPT64 in BCG. Comparison between M. tuberculosis Erdman and M. bovis Chicago reveals four mobility variants. Comparison of 2-DGE patterns from M. tuberculosis H₃₇Rv against Erdman revealed 18 variant proteins, 16 of which were identified. In the M. tuberculosis Erdman proteome, six protein species newly appeared to be increased in intensity, two protein species newly appeared, six were absent and two represented mobility variants. BCG Chicago and Copenhagen expressed highly similar 2-DGE patterns.
He et al. in 2003 have characterized the differences in protein expression between virulent *M. tuberculosis* H37Rv and attenuated H37Ra. Three spots were present in H37Rv but absent in H37Ra. Starck et al. in 2004 compared the proteome of *M. tuberculosis* grown under aerobic and anaerobic conditions, 2-DGE revealed 50 protein spots that were either unique to, or more abundant during anaerobic conditions and 16 of these were identified by MALDI-TOF.

Many studies demonstrate that the recent clinical isolate have better chance to predict the new antigen and virulence factors in comparison to the laboratory strain by the proteomic approach. *M. tuberculosis* H37Rv is the most commonly studied laboratory strain and was first isolated in 1905. Betts et al. in 2000 compared the two virulent strains of *M. tuberculosis* (H37Rv and CDC1551). Total cell lysates of the two strains were analyzed by 2DGE, out of a total 17 proteins spot differences seven were unique to CDC1551 and three to H37Rv. Pheiffer et al. in 2005 compared the protein expression and antigen recognition profiles of local Beijing strain with a less prevalent clinical isolate belonging to the family 23 strain lineage and the laboratory strain *M. tuberculosis* H37Rv. In this study Beijing strain showed increased expression of α-crystallin and decreased expression of Hsp65, PstS1 (38kDa) and 47kDa protein was found in Beijing strain in comparison to the other strain and H37Rv. In a study ESAT-6, HSP-X and CFP-10 were found to be abundantly expressed in the culture filtrate of Strain K which is dominant isolate in Korea when compared with those present in culture filtrates from *M. tuberculosis* H37Rv (Shin et al., 2008).

In addition to these proteomic studies, clinical isolates were used to measure the cell mediated immune response in comparison to *M. tuberculosis* H37Rv. Rajavelu and Das, in 2003 selected 7 most prevalent strain of south India and analyzed the T-cell proliferation and IFN-γ and IL-12 in 25 healthy individual. They showed two clinical isolates are potent inducers of T-cell proliferation and also IFN-γ secretion. Siddiqui et al. in 2000 compared the CFP preparation from two standard strain H37Rv and Erdman and four contemporary Indian isolates of *M. tuberculosis*. Two Indian isolates were significantly more potent than the CFP of the standard laboratory strain in inducing
activation of T-cells of normal human donors by T-cell proliferation and IFN-γ. These finding suggest that clinical isolates could have some immune-dominant T-cell epitopes.

3.8: Synthetic peptides and its use in mycobacterial diseases-

It is now possible to obtain the primary sequence of peptides or a protein with aid of whole genome sequence. On the basis of primary sequence now it is possible to synthesize the peptide commercially. Further, various bioinformatics tools are available which can predict B-cell and T-cell epitope in a primary amino acids sequence of a protein. In an immune reaction only the specific region of a particular protein actively react which is known as epitope. Commercial synthesis of peptide provides an alternative method for the production of antigenic peptide for immunoassays. These peptides are relatively simple to synthesize and are cheaper to produce than the production of whole protein (Gonzalez et al., 1997) by expression vector in suitable host. Further, synthetic peptides are advantageous for diagnostic application since they are well defined, easily produced in large amount, highly pure and often cost-saving. In general the use of synthetic peptide increases the specificity of immunoassays compared to crude antigen (Ferrer et al., 2003).

Various researchers used synthetic peptide in mycobacterial research and showed antigenic and immunogenic nature of the peptides. A study on bovine TB on cattle by Vordermeier et al. in 2001 showed that cocktail of five peptides derived from CFP-10 and five peptides derived from ESAT-6 can discriminate between M. bovis infection and BCG vaccination with high degree of sensitivity and specificity. Shams et al. in 2004 showed that peptide from CFP-10 is an excellent candidate for inclusion in a subunit anti-TB vaccine. They showed that 15 mer peptide from CFP-10 elicit IFN-γ production and cytotoxic T lymphocyte (CTL) activity by both CD4+ and CD8+ T-cell from person with latent TB infection. Two studies carried out by Mustafa et al. in 2005 and Mustafa et al. in 2006 successfully showed that peptide based vaccine candidate and diagnostic reagent may be useful in control of TB. Mustafa et al. in 2008 selected 220 synthetic peptide covering sequence of 12 open reading frame of RD1 and tested them as single pool with PBMCs obtained from pulmonary TB patients and M. bovis BCG vaccinated healthy subjects in Th1 cell assay that measure antigen induced proliferation and IFN-γ secretion. These results showed that RD1 pool induced strong response in both TB
patients and BCG vaccinated subjects. Shen et al. in 2009 showed that peptides from three proteins of *M. tuberculosis* (Ag85B, BfrB and TrxC) induce potent antibody immune response. In another study seventy five synthetic peptides from 25 *M. tuberculosis* proteins were found to be capable of inducing Th1 cell reactivity (Seghrouchni et al., 2009). Singh et al. in 2009 showed that four peptides from the proline-threonine repetitive protein (Rv0538) have diagnostic potential and can identify >80% sputum smear positive and >50% smear negative HIV- negative TB positive patients and >80% of HIV- positive TB positive patients. Christy et al. in 2012 used four T cell epitopes from four well defined *M. tuberculosis* antigens Ag85C, CFP-10, PPE68 and INV2, constructed epitope based recombinant BCG vaccine and found that it elicits specific Th1 polarized immune response in BALB/c mice. Salman et al. in 2012 showed that synthetic overlapping peptide mixture from ESAT-6 clearly distinguishes between patients and tuberculin skin test (TST) positive healthy subjects with sensitivity of 70.3% and specificity 96.9%. These studies suggest that peptides, by careful analysis on selective *M. tuberculosis* proteins can be used to develop as simple peptide based sero-diagnostic test for TB.

There is little information available regarding the proteomic and immunogenic profile associated with prevalent genotype of *M. tuberculosis* in India. Therefore, this study has been designed for immunological characterization of differentially expressed protein in whole cell lysate from prevalent Indian clinical isolates of *M. tuberculosis* CAS, EAI, Beijing family and others prevalent genotypic isolates in India in comparison to laboratory stain H$_3$Rv by doing 2-DGE, western blot and mass spectrometry. By the bioinformatics tools applying on identified protein, peptides sequence will be identified which will be contains T-cell or B-cell epitopes. These peptide will be synthesized commercially and will be used for evaluation of their role in diagnosis and protective immune response in TB.