Review of Literature
The existence of immunity to tuberculosis was pointed out as early as 1886 by Marfan, who noted that pulmonary tuberculosis (PTB) was rare among those with healed lupus or healed tuberculous adenitis. Calmette and Guerin in 1921, first introduced vaccination against tuberculosis i.e. BCG (Guerin, 1957). BCG was first administered orally to infants by Weill- Halle in 1921. In the Indian sub-continent, BCG was introduced by Dr. Ukil in 1948, but mass vaccination programme was started in full swing in the year 1951 (Baily, 1981). Kraus (1931) first of all doubted the protective efficacy of BCG against *M. tuberculosis* infection. To test the efficacy of BCG, eight scientifically valid controlled field trials were organised in different part of the world on diverse populations during 1935-1955. BCG has shown variable protection against tuberculosis ranging from 0 to 80%. A brief summary of the 8 trials is as follows.

In view of these conflicting results a large scale BCG trial was planned in India and the protective effect of BCG vaccination evaluated in a controlled, double blind, community trial near Madras in South Indian population of about 3,60,000 persons. This study revealed that BCG, over 7 & ½ years did not show any protection against the development of pulmonary tuberculosis. This unexpected finding added fuel to the controversy of whether BCG vaccination affords protection against tuberculosis (Tuberculosis Prevention Trail, 1980). Scientists working in this field thought for an alternative to BCG. Youman and co-workers (1961) showed that prior infection with *M. avium*, *M. kansasii* and *M. intracelluar* resulted in significant reduction in mortality when mice were subsequently challenged with virulent strains of *M. tuberculosis*. 
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Trial Area</th>
<th>Population and age group</th>
<th>Trial period and duration of follow-up</th>
<th>Percent protection</th>
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<tr>
<td>1.</td>
<td>North America</td>
<td>Native Indians 0-20 years</td>
<td>1935-1938, 9-11 years</td>
<td>80%</td>
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<tr>
<td>2.</td>
<td>Chicago High Risk Area</td>
<td>Infants under 3 months</td>
<td>1937-1948, 12-13 years</td>
<td>75%</td>
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<td>3.</td>
<td>Georgia, USA</td>
<td>School Children 6-17 years</td>
<td>1947, 20 years</td>
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<td>4.</td>
<td>Puerto Rico</td>
<td>General population</td>
<td>1949-1951, 5 ½ years</td>
<td>31%</td>
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<td>5.</td>
<td>Georgia and Alabama</td>
<td>General Population, 5 years and above</td>
<td>1950, 14 years</td>
<td>14%</td>
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<tr>
<td>6.</td>
<td>Great Britain</td>
<td>Urban Population, 14-15 ½ years</td>
<td>1950-1952, 15 years</td>
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<tr>
<td>7.</td>
<td>Madanapalli South India</td>
<td>Rural population, All age</td>
<td>1950-1955, 9-14 years</td>
<td>31%</td>
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<td>8.</td>
<td>Chingleput South India</td>
<td>General population, over one month</td>
<td>From 1968, 7 ½ years</td>
<td>Nill</td>
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After the Palmer and Long's (1966) hypothesis that environmental mycobacteria may be responsible for the suppression and/or addition of the protective efficacy of BCG, the search for an atypical mycobacteria which may replace BCG was extensified. Arcy Hart (1967) reported that four strains of Vole bacillus (M. microtii) vaccine gave 74%, 91%, 75% and 86% protection against TB. Medical Research Council's 4th report (1972) suggested an overall 73% protection against TB by Vole bacillus vaccine. Earlier in 1961,
Youman et al. reported that mice of strain vaccinated with representative cultures of unclassified mycobacteria belonging to Group I, II, III and IV, developed a high degree of immunity to infection with *M. tuberculosis* in animals vaccinated with Group I (*M. kansasii*) and Group III (Battery) strains.

Weiszfeiler and Karasseva (1981) reported that *M. simiae* remarkably depressed the immunogenic effect of BCG vaccination in guinea pigs. Extensive studies of Orme and Collins (1983, 1984) subsequently confirmed that tuberculosis resistance in prior infected mice seem to depend upon the ability of atypical mycobacteria to survive *in vivo*. Baldwin in 1911, first reported that mycobacteria retain their antigenicity after killing. Petroff (1923), and Freund and Opie (1938) found that killed organisms injected subcutaneously were as effective as live BCG. Wilson et al. (1940) working on guinea pigs with various degrees of heat killed organisms were required to induce immunity and the process of killing virtually had no effect on the antigenicity of the strain. Rook (1980) observed that *M. tuberculosis*, autoclaved at 15 psi was effective in producing tuberculin test as live *M. tuberculosis* in guinea pigs. One common factor with all these killed mycobacteria was that large dosage or repeated injections or both were needed.

Inactivated vaccines are safe as they do not produce disease, but the antigenic stimulus they provide in the body is short lived and frequent boosting with the antigen is required to evoke and maintain the immunity to a level to provide protection. The fear that is endowed in live vaccines prevent their initial mass scale use unless confirmed for their non-convertible nature of virulence. Larson and Wicht (1964) reported that strain H37Ra of *M. tuberculosis* was capable of multiplying and conferring immunity in mice but the strain could not be used as vaccine for the fear of reversion, since it was an avirulent variety of a very virulent strain of *M. tuberculosis*.

An atypical strain *Mycobacterium habana* TMC 5135 developed by CDRI generated strong protectivity in mice against *M. tuberculosis* (Gupta, et al., 1979) and *M. lepra* challenges (Singh, et al., 1985, 1989; Gupta, et al., 1987). *M. habana* was injected intravenously into mice and monkeys, and subcutaneously in guinea pigs to assess its pathogenicity. It was found to be non-pathogenic in all the three animal species. Collins et al. (1975) too found *M. habana* to survive in pathogen free CD-1 mice upto 60 days without causing mortality, when injected intravenously. It's immunogenicity is accompanied with non-allergenicity and non-pathogenic character. *M. habana* was also found to give significant protection against *M. ulcerans* in mice which cause a local ulcerative disease.
of humans (Singh, et al., 1981) and Leishmania donovani in hamsters (Anuradha, et al., 1995). There has been a considerable degree of in vivo cross reactivity between M. habana, L. donovani and M. bovis BCG antigens in developing delayed hypersensitivity reactions in mice (Anuradha, et al., 1995; Singh and Sinha, 1985). It has been studied for its antigenic cross-reactivity with delayed type of hypersensitivity responses in guinea pigs. Guinea pigs sensitized with M. habana, M. leprae and M. tuberculosis when challenged with habanin, lepromin and tuberculin in cris-cross fashion have demonstrated strong cross reactivity with each other, which was demonstrated by Singh, et al. (1988a,b). M. habana possesses some immunodominant proteins such as 65, 35, 23 and 18 kDa, which have been identified by monoclonal antibodies of M. tuberculosis and M. leprae (Lamb, et al., 1990). The most interesting observation was found by Lamb, et al. (1990) is that 18 kDa protein is only present in M. habana and M. leprae, which signifies that, this protein can play important role in diagnosing M. leprae (Lamb, et al., 1990). Bisht, et al. (1996) reported that 23 kDa immunodominant protein present in M. leprae and M. tuberculosis is also abundantly present in M. habana and is a superoxide dismutase (SOD).


The mycobacterial cell contains a large number of different proteins which are classified into major group with common features for example: in physical and chemical features, function or localization as exemplified by lipoproteins, heat shock proteins, cytoplasmic, membrane bound, excreted and secreted proteins, respectively (Wiker and Harboe, 1992).

Excreted proteins are produced in large quantities by mycobacteria during the first few days of culture (Andersen, et al., 1991a). Live mycobacterial vaccine induces higher levels of antituberculous resistance in experimental animals than either whole dead bacilli or their cell components, even when presented in a suitable adjuvant (Collins, 1984). Several protein antigen present in the extracts of M. bovis, BCG or M. tuberculosis have been recognized by Ivanyi, et al. (1985) using monoclonal antibodies developed against killed M. tuberculosis or M. leprae; however, it is not clear how many of these antigens (if any) are involved in the development of cell-mediated immunity by the actively infected host (Emmrich, et al., 1986; Young, et al., 1986); These so called protective antigens
(Ivanyi, et al., 1985) must be released by the actively growing mycobacteria (live vaccine) within the infected tissues, but they may be rendered far less effective if presented to the relevant T-cells in the presence of large amounts of dead or metabolically inactive bacteria i.e. in fairly old culture (Lagrange, et al., 1976). Such sensitizing antigens may also be produced by mycobacteria as they multiply, in artificial culture media (De Bryn, et al., 1987), and in fact, mycobacterial sensitins have long been known to be present in aging cultures of BCG (Nagai, et al., 1981) and M. tuberculosis (Turcotte, 1969). However, such preparations are heavily contaminated with irrelevant polysaccharide and autolytic cell products (Turcotte and Des Ormeaux, 1972). They accumulate in large quantities in the medium but are present only in trace amounts in the intact bacilli. Secreted proteins of the outer cell wall are gradually released during growth of the bacilli. The concentration of these proteins increases steadily during the late logarithmic growth phase. The appearance of these proteins correlates well with the massive release of isocitrate dehydrogenase (ICD), which indicates that substantial autolysis has occurred (Andersen, et al., 1991a). In an effort to avoid this problem, Collins, et al. (1988) harvested virulent M. tuberculosis cultures during the mid-logarithmic growth phase, where the smaller yields of protein antigen being offset by the absence of contaminating lytic products. The immunological activity of the culture filtrate proteins both in vivo and in vitro, indicates that they can be recognized by those T-cells responsible for the expression of tuberculosis sensitivity but not by monoclonal antibodies directed against heat killed whole M. tuberculosis (Collins et al., 1988). The secreted proteins have been suggested as protective antigens responsible for rapid recognition of bacilli by host lymphocytes (Abou-Zeid, et al., 1988b). This hypothesis was supported by the findings that whereas immunization with live bacilli efficiently generated protective T-lymphocytes, killed preparations did not (Orme, 1988). Thus group of secreted proteins is of great current interest in relation to the immune response to infection since these proteins are candidates of particular importance for development of protective immunity as well as clinical symptoms and complications of the disease (Pal and Horwitz, 1995).

Mycobacteria have been shown to secrete a number of proteins to the surrounding medium (Harboe and Nagai, 1984; Wiker, et al., 1986a,1986b, 1991; Abou-Zeid, et al., 1988a, 1988b; Andersen, et al., 1991a) and these proteins have been demonstrated to be extremely potent in generating a cellular immune response in mice infected with live M. tuberculosis (Andersen, et al., 1991b, Orme, et al., 1992).
It is almost a dogma that it is not possible to vaccinate against tuberculosis by means of killed bacteria. One explanation for this may be for the secreted proteins, that after administration of a live vaccine strain, the protein synthesis machinery of the bacteria is still working and proteins are continuously being secreted into the surrounding medium. T-lymphocytes directed towards these proteins may be responsible for the recognition of the infected macrophage leading to an efficient control of the infection at a very early stage. This hypothesis is supported by several researchers who have provided experimental evidence strengthening the relevance of secreted proteins in relation to protective immunity in tuberculosis (Rook, et al., 1986; Orme, 1988, Andersen, et al., 1991b; Hubbard, et al., 1992; Orme, et al., 1992). Collins, et al. (1988) isolated *M. tuberculosis* culture filtrate (MTCF) protein antigens from mid-logarithmic phase culture grown in liquid medium and examined by high pressure liquid Chromatography and Western blot (immunoblot) analysis. A major protein band with a molecular mass of about 68 kDa and several fainter bands in the 38 and 24 kDa range were observed. The MTCF protein produced a significant delayed footpad hypersensitivity response in *M. bovis* BCG-vaccinated C57 BL/6 mice, comparable to that observed with standard purified protein derivative (PPD). The same proteins induced a blastogenic response in tuberculin sensitive human peripheral blood monocytes and in T-cell clones developed from these cells. The proliferative responses to the MTCF antigens were equivalent to those observed following stimulation with PPD or *M. tuberculosis* sonic extracts. However, the MTCF sensitins were not recognized by five monoclonal antibodies directed against killed *M. tuberculosis* antigens in an enzyme immunoassay, although some response was seen with a monoclonal antibody (ML34) directed against *M. leprae* antigens. The ability of the MTCF to stimulate T-cell responses both in vivo and in vitro while not being recognized by antibodies directed against dead mycobacterial antigens suggests that they may be of interest as potential protective immunogen (Collins, et al., 1988).

An experimental vaccine that was based on secreted proteins of *M. tuberculosis* was investigated by Andersen (1994) in a mouse model of tuberculosis. Short-term culture filtrate (ST-CF) containing proteins secreted from actively replicating bacteria grown under defined cultural conditions were used. The immunogenicity of the ST-CF was investigated in combination with different adjuvant and peak proliferative responses were observed when ST-CF was administered with the surface-active agent dimethyloctadecyle ammonium chloride. The immunity induced by this vaccine was dose dependent as in the
optimal concentration, the vaccine induced a potent T-helper-1 response which efficiently protected the animals against a subsequent challenge with virulent *M. tuberculosis*. Antigenic targets for the T-cells generated were mapped by employing narrow-molecular weight secreted antigens present in ST-CF. A vaccination with viable *M. bovis*, Bacillus Calmette Guerin (BCG), in contrast, induced a restricted T-cell reactivity directed to two secreted protein fractions with molecular masses of 5 to 12 kDa and 25 to 35 kDa. The protective efficacy of the ST-CF vaccine was compared with that of a BCG standard vaccine, and both induced a high significant protection of equal magnitude. The vaccination with SF-CF gave rise to a population of long lived CD4 cells, which could be isolated 22 weeks after vaccination and could adoptively transfer acquired resistance to T-cell deficient recipients. The results confirm the hypothesis that *M. tuberculosis* cells release protective antigens during growth.

Boesen *et al.* (1995) investigated the T-cell response of human donors to secreted antigen fractions of *M. tuberculosis*. The donors were divided into five groups, active pulmonary tuberculosis (TB) patients with minimal and with advanced disease, *M. bovis* BCG vaccinated donors with and without contact with TB patients, and nonvaccinated individuals. The patients with active minimal tuberculosis responded powerfully to secreted antigens contained in a short-term culture filtrate. The response to secreted antigens was mediated by CD4+Th-1-like lymphocytes and the gamma interferon released by these cells was markedly higher in patients with active minimal TB than in healthy BCG-vaccinated donor. Patients with active advanced disease exhibited depressed responses to all preparations tested. The specificity of the response to secreted antigens was investigated by stimulating lymphocytes with narrow molecular mass fractions of short-term culture filtrate obtained by the multi elution technique. Considerable heterogenicity was found within the donor groups. Patients with active minimal TB recognized multiple secreted targets, but interestingly, six of eight patients demonstrated a predominant recognition of a low mass (<10 kDa) protein fraction which induced high levels of gamma interferon release *in vitro*. Only a few of 12 previously characterized secreted antigens were recognized by T-cells isolated from TB patients, suggesting the existence of a number of as yet undefined antigenic targets among secreted antigens was reported by the authors (Boesen, et al., 1995).

Collins (1988) reported that acquired anti-tuberculous resistance is mediated by subpopulation of T-cell able to recognize so-called protective antigens which are produced
by the intracellular pathogens as it multiplies within the tissues. Increased resistance was demonstrated by Collins and Mackness (1970) by comparing the growth of virulent *M. tuberculosis* in the spleen and lungs of vaccinated and non-vaccinated mice. Killed vaccines are non-protective unless suspended in Freund's complete adjuvant (FCA). Dead bacilli thus appears to possess the necessary protective antigens but their effectiveness seems to be diminished or masked by the large amounts of irrelevant antigen also present in this immunizing preparation (Collins, 1991). Thus the resulting acquired resistance is quantitatively inferior to that seen in mice vaccinated with the Bacilli Calmette-Guerin (BCG) live vaccine (Collins, 1984). Hubbard, *et al.* (1992) obtained culture filtrate proteins from *tuberculosis* cultures after 7 days growth in Proskauer and Beck medium. The protein yield increased substantially to peak about the time and the number of viable organisms reached its maximum level on day eight. Examination of the protein concentration by SDS-PAGE revealed the presence of at least 12 separated protein bands varying from 10 to 90 kD. Mice were injected subcutaneously with 20 μg of *M. tuberculosis* culture filtrate (MTCF) proteins suspended in saline or Freund's complete or incomplete adjuvant. The vaccinated mice were subjected to an aerogenic challenge with $10^3$ colony forming unit (CFU) of *M. tuberculosis* Erdman and a significant reduction in the number of viable organisms was observed in the spleen and lungs determined over a 21- day period compared with age matched normal controls. Mice immunized with the same culture filtrate proteins bound to nitrocellulose particles also showed some resistance to the virulent challenge, was also reported by the authors suggesting that individual antigens present in the culture filtrate were able to induce a protective T-cell mediated immune response in appropriately immunized mice. Pal and Horwitz (1992) found that immunization of guinea pigs with a selected fraction of the extracellular molecules induces cutaneous delayed- type hypersensitivity and splenic lymphocyte proliferation and a substantial level of protective immunity to challenge with aerosolized *M. tuberculosis*.

Daugelat, *et al.* (1992) have reported that *M. tuberculosis* lives and replicates inside mononuclear phagocytes and immunity crucially depends on T-lymphocytes. Thus potential vaccine antigen must be characterised at the T-cell level. So far, the potential of bacterial cell wall and cytoplasmic antigens to stimulate T-lymphocytes has been studied by Kaufmann (1992). Processing and presentation to T-cells of these antigens should depend on prior death and degradation of the microorganisms. However, at least at early stage of infection, such antigens would be available only to a limited degree. In
contrast, proteins secreted by bacteria persisting inside macrophages should be readily accessible to the antigen processing machinery. Hence, T-cell reactivity to proteins secreted by actively growing bacteria could serve as a model for an early stage of infection when pathogen still live within phagosome. Recently, a method has been developed that allows direct T-cell screening of hundreds of different protein fractions after two-dimensional separation (Gulle, et al., 1990). This technique was used to investigate the repertoire of T-cells from tuberculous patients and healthy contacts towards secreted antigens of *M. tuberculosis*. Little is known about T-cell antigens involved in immunity against *M. tuberculosis*. Most model systems used *in vitro* culture of human T-lymphocytes with bacterial lysate or secreted proteins as antigens. In this study, Daugelat, et al. (1992) separated proteins from 3-week old *M. tuberculosis* culture filtrates by two dimensional PAGE and subsequently transferred into soluble phase. The resulting 480 fractions were screened with T-lymphocytes from tuberculosis patients and healthy contacts. T-cells from all 9 patients and from 8 of 10 tuberculosis positive contacts preferentially responded to a cluster of acidic proteins with molecular masses of 30-100 KDa, although they also recognized a number of other fractions. In contrast, of 7 tuberculin-negative contacts, 4 were not and 3 were only weakly stimulated by this cluster region. Therefore, this distinct cluster of secreted proteins seems to comprise dominant T-cell antigens of *M. tuberculosis* was opined by the authors (Daugelat, et al., 1992).

To study the immunological activity of proteins secreted by *M. tuberculosis*, Haslov, et al. (1995) carried out comparative studies in guinea pigs infected intravenously with 2.5 X 10^3 CFU of this organism or with 2.5 X 10^3 CFU of *M. bovis* BCG. Groups of infected guinea pigs were skin tested with secreted proteins covering well-defined narrow-molecular-mass regions, and such fractions were used for lymphocyte stimulation experiments. Evidence was put forward in which lymphocyte stimulation experiments were found to show that the fraction containing proteins with molecular mass below 10kDa had a superior stimulating capacity in tuberculous guinea pigs, whereas the 24 to 30 kDa fraction gave significantly higher skin reactions as compared with BCG-vaccinated guinea pigs. Thus, the results obtained suggested that guinea pig T cells respond strongly to secreted antigens during tuberculous infection and that a secreted 24kDa protein termed MET 64 is a promising candidate for a specific diagnostic reagent.

Nagai, et al. (1991) purified five actively secreted proteins (MPT32, MPT45, MPT51, MPT53 and MPT 63) and the MPT 46 protein layer to homogeneity, from *M. tuberculosis*
culture fluid and compared with proteins previously purified. Antisera were obtained by immunization of rabbits with all of the newly isolated proteins identified to be immunogenic. Two dimensional electrophoresis of culture fluid obtained each week for two to ten weeks culturing of *M. tuberculosis* revealed characteristic changes, permitting identification of two distinct group of proteins being actively secreted from the mycobacterial cells or appearing later in the culture fluid as a result of the release of soluble proteins from cytosol after lysis of bacteria. The N-terminal amino acid sequences of five MPTs were shown to be identical to those of proteins previously isolated by other investigators and given different designations, and five new sequences were given. These sequences and the use of antisera were reported to serve to identify these proteins with mycobacterial constituents correspond to the C-components of the antigen 85 complex. The 27 kDa MPT51 protein was demonstrated to cross react with the three components of antigen 85 complex, and the N-terminal amino acids sequence of MPT51 and MPT59 showed 60% homology. This finding and the extensive cross-reactivity between the components of the antigen 85 complex may indicate that there is a family of closely related secreted proteins in mycobacteria.

Ten major antigens from *M. bovis* culture filtrate of 39, 32, 30, 25, 24, 22 (a and b forms), 19, 15, and 12 kDa have been purified and characterized by classical physicochemical methods by Fifis, et al. (1991). With monoclonal antibodies and/or N-terminal amino acid sequencing data, it was found that the antigen of 32, 30, 24, 22, 19 and 12 kDa are related to *M. bovis* and *M. tuberculosis* antigen P32, MPB 59, MPB 64, MPB 70, 19 kDa and 12 kDa, respectively. The 39-25,22 (b), and 19 kDa antigens showed concanavalin A-binding properties and were positive in a glycan detection test, suggesting that they are glycoprotein.

Nassau, et al. (1976) reported the application of ELISA for the detection of antibodies to *M. tuberculosis* they remarked that ELISA has practical advantages over the fluorescent antibody tests and radioimmunoassay in detecting positive cases of tuberculosis.

Excretory antigens of *M. tuberculosis* H37 Ra and PPD showed higher antibody titres in tuberculosis cases compared to phosphate buffer saline soluble antigen (PBS-SAg) and sodium dodecyl sulphate soluble antigen (SDS-SAg) of *M. tuberculosis* H37Ra. Thereafter, an extensive study was done by Kumar, et al., (1994) analysing higher number of sera in each group for the detection of tuberculous IgG antibodies using excretory antigens and PPD. The excretory antigen showed better sensitivity (87%) and specificity (85%)
compared to the sensitivity (73%) and specificity (78%) achieved with PPD. The excretory antigens also showed higher IgG antibody titre than PPD. This envisaged that excretory antigen has high diagnostic potential in tuberculosis (Kumar, et al., 1994).

The potential of *M. tuberculosis* H37Ra, excreted secretory (ES) antigen in the diagnosis of pulmonary tuberculosis was explored by Basak, et al., (1991). IgG antibody to *M. tuberculosis* ES antigen was detected by indirect penicillinase ELISA; 56 cases of pulmonary tuberculosis (AFB positive and clinically diagnosed) were studied, of which 47 were positive (84%). The sensitivity of the test system was 84%, specificity 86%; positive predictive value 84% and negative predictive value 86%. These results are quite encouraging.

Espita, et al. (1989) identified a unique associated protein antigen of *M. tuberculosis* culture filtrate of 38 kDa, which was found to react with 57% of patients sera but with none of the controls. It was tested as ELISA reagent in the serodiagnosis of pulmonary tuberculosis. A specificity of 0.96 and sensitivity of 0.68 were obtained.

Thongkrajai and his associates (1989) recorded a 91% sensitivity with antigen 5 of culture filtrate of *M. tuberculosis* H37Ra an ELISA to detect anti-mycobacterial antibody in sera of patients with pulmonary tuberculosis. Verban et al. (1992) found 24 kDa and 12 kDa proteins of *M. tuberculosis* H37Rv reacted with most of the tuberculosis patients sera, and in western blots, all sera from tuberculosis patients reacted with 65, 61, 58, 30 and 24 kDa protein.

The findings reported by several authors (vide supra) provide enough basis and evidence for the recently emerging hypothesis that secreted proteins are the key protective antigens leading to an efficient acquired resistance to *M. tuberculosis* and has high diagnostic potential for tuberculosis.

Thus, it is interesting to look for the secretory proteins of vaccine strain *Mycobacterium habana* for all purposes seems to be a broad based vaccine candidate having enough potentiality to protect vaccinated mice against several mycobacterial infections, needs an exploration for its secretory proteins for affording protection against *M. tuberculosis* in mice, the status of cell mediated immune response generated in vaccinated animals, and detection of tuberculous antibodies.
Evaluation of Protective Immunity

To test the efficacy of a candidate vaccine against tuberculosis in the laboratory there are two tools: One is to test the overall protection conferred by the candidate vaccine against subsequent challenges with the infecting organism and the other is to test the cell mediated responses evoked by the candidate vaccine in sensitized animals. The protective efficacy is tested in animals and cell mediated immune responses may be tested in vivo or in vitro conditions. To assess the overall protection, vaccinated animals were examined for various features viz: survival time, weight loss, lesions in visceral organs such as lungs and spleen etc. Rene (1964) hypothesized that vaccination does not prevent the initial establishment of infection but it retards its course and limits the extent of dissemination. Youman and Youman (1965) used the survival time of vaccinated animals after challenge as a parameter to test the efficacy of an immunogenic agent. Earlier Lurie, et al. (1952) challenged BCG vaccinated and control rabbits with virulent M. tuberculosis and reported that there was a significant reduction in the number of lesions that are present in lungs of vaccinated group of rabbits over the controls. Middlebrook, et al. (1967) also reported similar observations. Gupta, et al. (1979) found survival time and necropsy score of mice after virulent challenge with M. tuberculosis as promising parameter in the study of experimental tuberculosis. They noted a trend of steep death rate in control mice after challenge with M. tuberculosis H₃7Rv. In their study all the control mice died with in 30 days of challenge thereby the survival time of the animals in vaccinated group was considered to be of significance in assessing the protective efficacy of vaccine strains.

Standard techniques for cell mediated immune responses like lymphocyte transformation test (LTT) and delayed type hypersensitivity (DTH) responses were followed. Other tests like Enzyme linked immunosorbant assay (ELISA), Ouchterlony (immunodiffusion), Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (western blotting) were done.