Summary and Conclusion
Tuberculosis (TB) an air borne communicable disease and one of the three World Health Organization (WHO) priority diseases is caused by transmission of aerosolized droplets of *M. tuberculosis*. It kills about 2 million people annually and the epidemic is spreading globally, assuming alarming proportion. Around 8 million people become infected with TB every year. Diagnosis of tuberculosis is primarily based on X-ray examination and / or sometimes on symptoms that are often confusing and misleading. While infectious cases are frequently missed, some people are mistakenly diagnosed with TB and inappropriately treated.

Further the prevention of disease presently is made through BCG vaccine administered at the time of birth. However this vaccine also has several limitations and its efficacy has been reported to vary from 0-80%. Hence a new safe and immunogenic vaccine against tuberculosis is highly desirable. In past, several attempts have been made to develop killed and live vaccines to protect against the disease however, till date there is no alternative to the BCG vaccine.

Hence, there is an urgent need to develop identifying the immunoprotective and immunodiagnostic antigens of *M. tuberculosis* for the development of effective vaccine and / or diagnostic tool.

The present study was designed to isolate and screen low molecular weight secretory proteins possessing potentials to activate the cellular immune
response and those having ability to induce high titer antibodies for the
development of prophylactic and diagnostic tool.

Secretory protein(s) of *Mycobacterium* are synthesized by the actively growing
*M. tuberculosis* cells and induce the significant immune response. These
proteins have been demonstrated are recognized by T-cells isolated from
human (Tuberculosis) TB patients as well as mice and cattle experimentally
infected with tuberculosis. Experimental work in animal models suggests that
these proteins activate both CD4+ and CD8+ T-cells.

Our efforts in the present study were intended to focus on secretory protein
antigen, which can be suitable for developing a reliable diagnostic test for
tuberculosis and utilization of subunit vaccine. Such antigens, if found, can be
easily incorporated into Enzyme Linked Immunoassays or all those related
assays which utilize antibody antigen interactions as a marker of diagnosis, as
has been utilized by others. The ELISA technique has the advantage of being
a semi-automated procedure with the capacity to analyze multiple samples
and the equipment needed is available in many hospitals even in the
developing countries of the world. As opposed to other infections, the use of
ELISA in the diagnosis of tuberculosis has never been widely implemented
due to problems with both sensitivity as well as specificity. For developing
such assays, it was desirable to identify and purify Secretory protein (CFP)
antigens, which may specifically diagnose active tuberculosis sufferers.
Purification of protein(s)

1. The Secretory proteins were isolated from logarithmic phase culture (5 week old) of Mycobacterium tuberculosis H_{37}Rv without shaking at 37 °C.

2. Protein secretion was monitored through the protein estimation of culture medium by at A_{280} and the profile of proteins released into the culture supernatants of the tubercle bacilli by SDS-PAGE.

3. The major secretory proteins were purified with reference to the Nagai et al (1991) using DEAE sepharose CL-6b anion exchange column.

4. Five proteins (6.0, 26.0, 30.0, 38.0 and 64.0kDa) were obtained in purified form by column chromatography and SDS-PAGE. These proteins were selected for serology and cellular reactivity.

Humoral response

For measuring the humoral response and detection of antigens, indirect ELISA was used for this, the conditions for ELISA were optimized.

All five proteins were assayed by using sera from tuberculosis positive patients. These proteins demonstrated presence of antibodies in the serum however the quantity of some was lesser compared to the other as observed by western blot band intensity. The reactivity profiles showed is similar to that observed by several other investigators.
The humoral response against the five proteins individual and as a cocktail of five antigens was studied in sera of mice immunized with total culture filtrate proteins.

Linear increase in the antibody titer was observed up to four weeks of immunization. Out of the five antigens two antigens 64 kDa, and 38 kDa demonstrated significant higher level of antibodies against them in the sera of immunized animals.

Since low titer antibodies were also seen against other three proteins we prepared a cocktail of these five proteins and used them in combination for testing the presence of antibodies against them in sera of tuberculosis patients.

The results of serology by ELISA demonstrated that the present cocktail effectively detects the antibodies in the patient sample with increased sensitivity.

This combination (cocktail of five proteins) thus can be used to develop diagnostic tool for detection of antibodies however, further work is needed to establish the sensitivity and specificity of this combination in selectively identifying the *M. tuberculosis* complex in the sample
Cellular immune response against the five proteins in mice was determined by immunizing the animals (mice) with total culture filtrate proteins (CFP) administered as CFP-IFA complexes. Immune response was determined by measuring T-cell proliferation by these antigens.

Significant T-cell proliferation was observed by all the five antigens however maximum response against antigens having molecular weight of 30 and 6kDa.

Maximum T-cell activity was observed in the third week post immunization for 6 and 30kDa secretory proteins.

Further studies were undertaken to test the immunogenic potential of 6.0 kDa secretory protein for this animals were immunized with three different concentrations i. e. 75µg, 37.5µg and 18.75µg.

An enhanced T-cell Proliferation was observed in all the groups of mice immunized with different concentrations of 6kDa antigen throughout the study period attaining its peak level 4\textsuperscript{th} week p. im.

Furthermore, the immune response in present study was found to be dose dependent, demonstrating higher activation of immune cells in animals immunized with highest concentration of antigen (75µg).

Furthermore, the immune response induced with 75µg concentration of the antigen was higher than observed in BCG vaccinated mice. Our observations
are in agreement with earlier reports that the immunization with pure protein or combination of proteins induces better immune responses compared to that induced by the whole organism.

Protective potential of 6.0kDa protein

The immunization of animals with different concentrations of 6kDa secretory protein complexes in IFA resulted in significantly higher percent survival against an intravenous challenge with LD$_{50}$ of $M. tuberculosis$ H$_{37}$Rv as compared to unimmunized animals.

At the end of 30 days observation, 75% survivals were animals immunized with 75μg concentration of 6kDa protein that was similar to survival (70%) afforded by BCG vaccine.

Moreover, the protection induced was found to be dose depended which was maximum (75%) in mice immunized with 75μg concentration of the antigen, again emphasizing the effect of dose the antigens used to mediate desired immune response and protection.

Further, the immunization with 6kDa antigen demonstrated a significant percent survival which was better than that observed in BCG vaccinated mice (70%) and 45% in control groups, challenged at the peak of immune response with LD$_{50}$ of $M. tuberculosis$ H$_{37}$Rv.
These findings are further substantiated by the earlier reports where in the protective effect of the secretory proteins has been demonstrated to be comparable to BCG vaccine.

Thus our results also support the emerging hypothesis that secretory proteins are the key immunoprotective molecules capable of generating a strong cell mediated immune response leading to an efficient acquired resistance against virulent *M. tuberculosis* H$_{37}$Rv and other intracellular pathogens.

Further, studies were conducted to evaluate the immunoprotective behavior of 6kDa secretory protein using liposome as adjuvant.

Immunization with 6kDa antigen encapsulated in liposomes, included significant humoral and cell mediated immune responses as determined by ELISA. The augmentation of T and B lymphocyte mediated immune response is in accordance with earlier reports in which liposomes have been used as adjuvant for *Mycobacterial* antigens.

The immune response (cellular and humoral) elicited by the 6kDa secretory protein entrapped in liposomes were similar to that elicited by 6 kDa-FIA and BCG vaccine, thus indicating that liposomes can be effectively used to generate strong immune response by the entrapped antigen.

Immunization with 6kDa secretory protein entrapped in liposomes resulted in increased resistance to intravenous challenge with LD$_{50}$ of *M. tuberculosis*
H₃₇Rv as immunized animals showed considerably higher (p<0.001) survival rates (76%) as a compared to control animals (32%) and BCG vaccinated animals (72%) on 30 days post challenge.

There was a generalized drop in the number of bacteria isolated from spleen, lungs and liver as compared to control mice indicated that immunization with 6kDa-Lip ensure better clearance of the microorganism from the three organs (liver, lungs and spleen).

Our findings that liposomes could be effectively used to elicit the desired immune response and confer significant protection in the immunized animals are similar to the observation of other workers who have demonstrated increased immune response with the liposomal encapsulated antigen.

Thus, the results of the present study strongly suggest that the secretory proteins of *M. tuberculosis* are immunogenic and induce strong humoral and cellular responses.

Further, it was also observed that the immune response induced by some of these antigens is strongly humoral and that by other antigens are cellular. A careful and selective selection of these antigens can by useful in development of an effective diagnostic and a prophylactic tool.