SUMMARY AND CONCLUSIONS

The quest for identifying biomarkers for TB diagnosis has lead to detailed study of the *Mtb* and host components by many researchers. TB is a complex disease and has wide spectra of clinical outcomes which make the identification of biological markers difficult. Sputum smear microscopy and bacterial culture still remain the gold standard tests for TB diagnosis. However, the major limitation of these assays or any other antigen based serodiagnostic assay is their inability to differentiate between active and latent TB. The need of the hour is to identify antigens which when used alone or in combination with the known antigens can aid in the accurate diagnosis and differentiation of TB into different classes.

In this study, attempts were made to; (i) characterize Cut5b in *Mtb* H37Rv, and infected macrophages, (ii) antigenically differentiate *M. simiae* from *Mtb* and, (iii) evaluate Cut5b for its potential as biomarker of TB in serodiagnosis and nucleic acid based assay.

I. Characterization of Cut5b in *Mtb* H37Rv

- RT-PCR analysis demonstrated the presence of ORF splitting in *cut5* gene of *Mtb* H37Rv. Transcript for *cut5b* was detected while, under the experimental conditions used, no transcripts for *cut5a* and *cut5* could be detected. This indicates that full length *cut5* is not transcribed in *Mtb* H37Rv but it undergoes ORF splitting into *cut5a* and *cut5b*.
- Clustal omega analysis of *cut5b* sequences revealed the presence of the single nucleotide 'T' insertion in *Mtb* CDC1551, KZN1435 and F11 which indicated that this insertion might not have been be the sole reason for ORF splitting because in CDC1551 and F11 only *cut5b* is present whereas in separate genes for *cut5a* and *cut5b* are present in KZN1435. This observation suggests that the 'T' insertion in the *cut5b* gene may be a molecular signature that could be exploited as a biomarker for species specific identification of mycobacteria. Moreover, this study also indicates the presence of three different *cut5* organizations in *Mtb*;

(i) overlapping genes as in *Mtb* H37Rv, (ii) presence of only *cut5b* e.g. in CDC1551 & F11 and, (iii) presence of two separate genes for *cut5a* and *cut5b* as in KZN1435.

- Immunoblotting studies using the anti-Cut5b antisera (*Mtb* Cut5b sera) revealed that Cut5b (a ~24 kDa protein) was primarily in the cell wall and membrane of *Mtb* H37Rv. It was also present to a lesser extent in the cytosolic fraction. These observations were consistent with the results obtained in immunoelectron microscopy experiment where Cut5b was seen as majorly being associated with the *Mtb* H37Rv cell surface and to a lesser extent in the cytosol.
- Expression of Cut5b was studied in the extract and cell wall fractions prepared from *Mtb* H37Rv cells harvested at early exponential, late exponential and stationary growth phase cultures. Immunoblotting studies revealed that the expression of Cut5b was increased in the late exponential and stationary phases indicating the possible role of Cut5b in *Mtb* physiology during intracellular survival.
- Expression of Cut5b was found downregulated under acid stress, nitrosative stress and nutrient stress as compared to that in *Mtb* H37Rv grown under normal conditions. But the expression of Cut5b was unaffected under the oxidative stress condition. Further, it was observed that the expression of Cut5b, under oxidative stress, did not change in the cell wall and membranes but it was upregulated in the cytosolic fraction of *Mtb*.
- Cut5b was also found in intracellular *Mtb* H37Rv (in the THP-1 cells). Interestingly, when checked in *Mtb* H37Rv infected mouse model, presence of Cut5b could be observed in infected lung tissue sections prepared even after 28 days post infection.
- The rCut5b was able to hydrolyze p-nitrophenyl butyrate efficiently and could hydrolyze p-nitrophenyl decanoate to some extent. Its lipolytic activity decreased for substrates with increasing C chain length, unlike that of *C. rugosa* lipase, which suggested that Cut5b was an esterase. The observations obtained in p-nitrophenyl ester hydrolysis assays were found to be consistent with the molecular docking studies where Cut5b structure obtained in homology based modeling was used to dock p-nitrophenyl butyrate and palmitate structures at the active site residues (Ser80, Asp155, His167). The binding energies for both the substrates deduced from the docking study indicated that Cut5b has higher preference for short carbon chain

substrate p-nitrophenyl butyrate as compared to long carbon chain containing substrate p-nitrophenyl palmitate thus indicating the fact that Cut5b may function as an esterase in *Mtb*. Docking studies, performed with triacylglycerol (TAG) as substrate, indicated that TAG may not be a suitable substrate for Cut5b.

- The activity of Cut5b, unlike that of *C. rugosa* lipase, was not affected when THL was used at a molar ratio of 1:200. This property differentiated Cut5b activity from those of Cut4 and Cut7 which have been reported to be inhibited by THL. Pairwise sequence alignment of Cut5b with those of Cut4 and Cut7, using CLUSTALW software, indicated that many amino acid stretches were missing in Cut5b as compared to Cut4 and Cut7 inspite of having 46% and 49% sequence similarity. It was thus hypothesized that these differences may also lead to differences in the structures of these proteins. Homology based modeling using *T. reesei* cutinase (4PSC) as a template revealed that the structure of Cut5b was very different from that of Cut4 and Cut7 which have been reported to be structurally related. Cut5b had a more open structure with two parallel beta sheets and seven alpha helices as compared to Cut4 and Cut7 which had five parallel beta sheets and seven alpha helices. Structural overlay analysis revealed the presence of several unaligned regions among Cut5b and Cut4 and Cut5b and Cut7. All these observations indicated that despite of having conserved active site Cut5b is structurally very different from Cut7 (an esterase) and Cut4 (a lipase/phospholipase).

II. Antigenic differences between *M. simiae* and *Mtb*

- Immunoblotting analysis and ELISA with MAbs against protein antigens [HYT6 (anti-LpqH), HYT28 (anti-PstS1/38 kDa lipoprotein), HAT1 (anti-DnaK), HAT5 (anti-Hsp65) and anti-Ag85B] and polysaccharide antigens [MAbs 24C5 (anti-glucan) and 9d8 (anti-LAM)] indicated antigenic similarities as well as differences between *M. simiae* and *Mtb* H37Rv. For example epitopes of some immunodominant antigens, like LpqH, Hsp65 and Ag85B were conserved in *M. simiae* while those of PstS1 and DnaK were lacking. However, epitopes of polysaccharide antigens (glucan and LAM) were found to be conserved in *M. simiae*. All these observations indicated that combination of these MAbs may be useful for differentiating infections caused by *M. simiae* and *Mtb* H37Rv.

- Reactivity patterns of MAbs C10B5 (generated in lab) and HYT6 (reference antibody) with *M. simiae*, *Mtb* H37Rv and pure rLpqH (19 kDa lipoprotein) indicated the antigenic similarity and differences in this protein of these two species of mycobacteria.
- The mycobacteria infected macrophage membrane antisera was found to recognize a ~53 kDa antigen in the *Mtb* H37Rv infected THP-1 membrane. This observed cross reactivity indicated the antigenic similarity on the membrane of macrophage infected with heterologous species of mycobacteria.

III. Evaluation of Cut5b as a biomarker of TB

- Sera from active TB patients classified as smear positive – culture positive [SS+] (n = 10) and smear negative-culture positive [S-C+] (n=5) TB patients and non-TB subjects (n=8) were tested in dot blot assay to detect antibodies against rCut5b and rLppI. For comparative analysis rAg85B and rLpqH were also used in the assay. Cut5b was recognized by all the SS+ and S-C+ sera tested but while variability was observed in the reactivities with other proteins. Also Cut5b, as compared to Ag85B and LpqH, showed very less cross reactivity with sera from non-TB subjects. Although, this observation needs validation taking more number of different samples, yet the results indicated that Cut5b alone or in combination with LppI/Ag85B/LpqH can be tested for detection of *Mtb* infection.
- Isolation of RNA from sputum samples of SS+ (n = 4) and S-C+ (n=2) TB patients and non-TB subjects (n=3), followed by a two step RT-PCR analysis, revealed that *cut5b* transcripts were present in all SS+ patient sputum samples while no transcript for *cut5* could be detected in the samples. Also, no transcripts were detected in non-TB samples. The absence of *cut5b* transcript in S-C+ samples may be owing to the less copy number of *cut5* mRNA because of low bacillary burden in these patients. However, more number of samples needs to be tested to substantiate these results.