Chapter 4
Summary & Conclusions

An insertion like repeat sequence (TRC4) has been cloned, sequenced and characterized previously in our lab. TRC4 repeats 4 times in the genome of *M. tuberculosis* as evidenced by southern blot of *M. tuberculosis* DNA restriction digested with Bgl II enzyme which does not have a site in the *M. tuberculosis* genome. TRC4 is a conserved sequence which has been confirmed by RFLP studies carried out on 200 clinical isolates of *M. tuberculosis* (Sujatha Narayanan *et al.*, 1997).

In this study TRC4 has been used for development of an indigenous in house PCR. Four sets of primers were designed for this 2.0 kb sequence PCR was standardized. Out of the 4 sets of primers one set of primers (pri 1 & pri 2) amplified 173 base pair fragment consistently in all positive samples tested. Hence these primer pairs were used for further evaluation. Pulmonary tuberculosis has been detected by x-ray smear microscopy and culture which is the gold standard. Extra pulmonary tuberculosis are not detected easily because the culture, which is considered gold standard often gives a negative result due to pauci bacillary nature of the specimen. Hence there is an urgent need for a test to detect extra pulmonary tuberculosis. PCR has been evaluated on extra pulmonary tuberculosis like TB lymphadenitis, TB pleuritis and TB meningitis.

TB lymphadenitis

Results of TRC4 PCR and IS6110 PCR of 101 tuberculous lymphadenitis samples were compared with conventional methods like smear, culture and histopathology. The sensitivity of PCR using both probes is higher than the conventional methods out of the 101 samples analysed (49 fresh and 52 fixed specimens).

PCR using IS6110 and TRC4 primers were positive in 64 and 70 sampels respectively whereas culture and histopathology were positive only in 49 and 58 samples respectively. The problem of false negativity of IS6110 due to the absence of IS6110 copy in four *M. tuberculosis* isolates was overcome by using TRC4 primers. The results indicate with improvement in PCR techniques, PCR using both probes IS6110 and TRC4 can be a rapid and sensitive adjunct to other conventional techniques for the diagnosis of tuberculous lymphadenitis.

TB pleuritis

The results of PCR on 50 pleural effusion samples were compared with the results of conventional methods like smear, culture and adenosine deaminase activity. Thirty six specimens were positive and 14 were negative by PCR. Among the 36 samples, 33 were from patients with clinical evidence of tuberculosis including response to antituberculosis therapy. Only six samples were positive by the gold standard which is culture and three were positive by smear. The measurement of adenosine deaminase activity classified 19 samples as positives. The over all sensitivity and specificity of PCR was 100 and 85 percent respectively. PCR using IS6110 and TRC4 primers is a sensitive test as compared to conventional tests for detection of *M. tuberculosis* from pleural fluid samples of patients with tubercular pleuritis.

TB Meningitis

The TRC4 primers were evaluated in comparison with IS6110 primers commonly used in PCR to detect tuberculous meningitis among children. The levels of concordance between the results of IS6110 PCR and TRC4 PCR with cerebrospinal fluid specimens from patients with clinically confirmed tuberculous

meningitis were 80 and 86% respectively. Results from the two primer sets were concordant for 55 positive and 22 negative specimens (n=98). The sensitivity of PCR can be increased by using both IS6110 and TRC4 primers.

In this study, nucleotide sequence of TRC4 was analysed for any putative orfs. TRC4 had a 356bp ORF supposedly thought to be coding for a protein with a DNA binding signature sequence. Comparison of this protein sequence with a DNA database showed this to be partial protein. So primers were designed for the full length protein for further experiments. Amplification and cloning of the above quoted full-length sequence generated a new plasmid, pSNOXYR.

The DNA sequence of pSNOXYR showed partial homology with *oxyR* gene of *M. tuberculosis* implicated in stress response. The deduced amino acid sequence of pSNOXYR shows the presence of signature sequence for DNA binding, which is characteristic of LysR-type transcriptional regulators. The predicted amino acid sequence of pSNOXYR showed homology to close atypical mycobacteria *M.avium* and *M.xenopi* as well as pathogenic *M.leprae*. This protein also shares homology with enteropathogens like Salmonella and *E.coli*.

In this study, seroreactivity of this purified protein with endemic normals and tuberculosis patients was tested. The cross-reactivity of pSNOXYR peptide may be due to the presence of conformational B cell epitope or due to the priming of non-tuberculous patients to environmental mycobacteria as well as to BCG.

The PSNOXYR peptide when cleaved by Cyanogen Bromide for epitope mapping the smaller peptides were also recognized by normal and tuberculosis patients sera. But a 20 kDa peptide (incompletely digested peptide) has been recognized only by tuberculosis sera and not by volunteers sera. This has to be extended as a large scale study.

CONCLUSIONS

PCR has been proved to be a very sensitive and specific test. It is a versatile tool for both basic science as well as applied science. In the field of diagnosis of tuberculosis the PCR has been exploited for its ability to detect *M. tuberculosis* from clinical specimens. We have also shown from our results that PCR is highly sensitive compared to conventional tests like smear and culture in detecting extra pulmonary tuberculosis cases which are mostly pauci bacillary. At present, PCR can be used only in specialized lab in the developing countries. Before it can be used as a routine test PCR technique has to be simplified. The two drawbacks of PCR, false positivity and false negativity have to be removed.

Among the various targets used for detecting tuberculosis by PCR IS6110 is widely used. Our indigenous probe TRC4 is more sensitive than IS6110. Hence if PCR as a technique is simplified and made ready to be used in routine laboratory, TRC4 will be of great utility.

The oxyR gene is a proven transcriptional activator in the enterobacteriaceae which is responsible for the adaptation to conditions of oxidative stress. Part of the TRC4 fragment had homology of oxyR gene. The full gene has been amplified by PCR cloning and has been expressed in PR set B vector and purified by "IMAC" column. The diagnostic potentiality of this protein has been explored and only a small peptide resulting from CNBr digestion has differential recognition of sera from TB patients and healthy volunteers. The test has been done on a small number of samples. This finding has to be confirmed on a large no. of serum samples from TB patients and healthy volunteers. The oxyR gene of *M. tuberculosis* has a number of deletions and Deretic (1997) has predicted that it could be a pseudo gene. We have expressed the oxyR gene. The future studies should be targeted at finding out whether this oxyR gene is functional. Knockout studies have to be planned to see whether this function is essential for the organism to survive in the hostile environment.