INTRODUCTION
1. INTRODUCTION

*Mycobacterium tuberculosis* (MTB), the causative of tuberculosis (TB) disease, continues to be a great threat to mankind. TB in humans is caused by the family of *Mycobacterium tuberculosis* complex which include *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium africanum*, *Mycobacterium caprae*, *Mycobacterium microti*, *Mycobacterium pinnipedii* and *Mycobacterium canettii*. Though the exact pathological and anatomical descriptions of the disease came up in 17th century, recent studies on DNA of the bacilli genome from the skeletal remains in Southern Peru suggested that human tuberculosis is less than 6,000 years old. Further, in the 18th century, the bacillus was found to be contagious among humans and also can be passed from humans to cattle to rabbit. Robert Koch in 1882 discovered a staining technique which enabled him to isolate the organism. Soon after effective research was conducted to diagnose and treat the disease. Invention of X-Ray by Rontgen has made the diagnosis of disease easier through radiation (chest X-Ray) and the discovery of BCG vaccine has decreased the burdensome of spread of the disease though ineffective in adults but still widespread even today.

In 2015, the world has estimated with 10.4 million new tuberculosis cases. In this context, TB is among the top three diseases along with malaria and HIV. All the three leading diseases cause death from single infectious agent. Though there are different forms of TB, pulmonary TB is the most common form of TB which is highly contagious and life-threatening infection. Along with India, 6 countries account for nearly 80% TB burden worldwide. India has been depicted as TB endemic country which accounts for nearly one quarter of world’s TB burden. WHO has aimed for “End TB” rather than “Stop TB” by 2030 targeting to reduce deaths due to TB by 90% and TB incidence rates by 80% compared with 2015 [WHO, 2017]. However, the new “End TB” strategy by WHO depends upon how the countries infected fares in the upcoming years.

People infected with the droplets of bacilli showing symptoms of the active disease act as carrier of pathogen and are capable of spreading the disease by sneezing and coughing. Once inhaled, the infected droplet reaches the alveoli and alveolar sac and the macrophages
and other innate immune cells engulf the bacilli. In order to recruit other immune cells for fighting against the pathogen, the macrophages invade the subtending epithelial layer and cause a local inflammatory response. The incoming cells initiate the formation of granuloma, the hallmark of the TB disease pathology [Orme, 2014]. With the infiltration of lymphocytes and development of acquired immune response, the granuloma acquires a more organized and stratified structure which prevents the further growth and replication of bacilli.

Infection may lead to active TB disease or may reside inside the host for years as a dormant bacilli- the survival of the fittest strategy- depending upon the immunological tolerance of the host. The pathogen’s smart play inside the host immune cells has made it difficult to eradicate since many decades.

Tuberculosis, especially in India, is found very challenging to treat due to several reasons:

a. The detection of TB cases is poor as it takes about 6 months or more to detect the resistant strains
b. Co-infection with HIV/AIDS
c. Due to the presence of over crowdedness in slums, hospitals and prisons, there is possibility of enhanced transmission of the disease
d. Malnutrition, poverty and poor healthcare system
e. Poor patient compliance and
f. Absence of biomarker to monitor the success of treatment and complete eradication of the disease.

It is estimated that one third of the world’s population harbor TB as latent tuberculosis infection (LTBI) and each case of LTBI have chance of 5-10% life time risk of reactivation to active disease [Milburn et al., 2007]. This suggests that the persons with LTBI are the largest reservoir of infection which is the future source of active TB and also transmittable. In India, co-infection with HIV, immunosuppressive treatment, malnutrition, stress and age factor also play an important role in reactivation of LTBI into active disease [Horsburgh, 2003; Radhakrishna et al., 2003].
Thus, accurate diagnosis and treatment of both active and latent TB are the crucial elements for global control of TB. Lack of a simple accurate test for TB which can be used for rapid diagnosis at the primary care level and lack of a biomarker(s) which can identify the LTBI cases form the major gaps in the existing diagnostic tools. It is studied that every individual with active TB left untreated have probability of infecting 10-15 healthy individuals in average per year [WHO, 2015]. Thus it is important to establish efficient detection and treatment strategies to combat the infection in both endemic and non-endemic countries. Traditionally, the diagnosis of active TB disease has relied on one of three approaches: microscopic visualization of \textit{M. tuberculosis}, culturing of tuberculous bacilli and amplification and detection of \textit{M. tuberculosis} nucleic acids (NAAT). Among these, microscopy and culture test are considered as gold standard tests for active TB diagnosis.

Vaccination against TB with live, attenuated BCG originally derived from \textit{Mycobacterium bovis} has helped in reducing the incidence of the disease. But the endurance of the vaccine is less as it becomes ineffective with age and also provides variable protection in case of pulmonary tuberculosis which is supposed to be the greatest burden for global mortality. Thus, the world is in urgent need of diagnostic kits, vaccines and drug targets and drugs which have capability to cure the symptoms in shorter duration, improved treatment outcomes and patient adherence, less side-effect, effective against drug resistant strains and also appropriate to administer along with any other co-infection.

The primary aim of TB treatment is to eliminate the pathogen from the body, control transmission of infection and also to decrease the incidence of drug resistance. The current drug regimen for active TB recommended by WHO namely DOTS therapy includes four first line drugs (isoniazid, rifampicin, pyrazinamide and ethambutol- called the first line drugs). Both sensitive as well as non-resistant strains of MTB can be treated effectively using the above regimen. These four drugs are effective on bacteria that grow actively and also lay in the low metabolic activity state. Since 1995, nearly 41 million people have been successfully treated and approximately 6 million lives were saved through this therapy.
The effectiveness of the drug relies mainly on the patient compliance of ingesting medicines. It is studied that isoniazid kills over 90% of the bacilli in the initial days of treatment during which the bacilli will be in the multiplying phase. But in order to achieve killing of the remaining bacilli, persistent intake of combination of rifampicin and isoniazid for 6-9 months is needed. When the bacterial load inside the granuloma becomes high or whenever the nutrients available for growth of the bacilli become limited, the bacilli enter a persistent phase where they stop multiplying and undergo hibernation. This achievement of latency aid the bacilli to survive for a long time as there is no diagnostic test for the detection of this form of bacteria.

Even after completion of the proposed treatment regimen, there are chances of relapse of persistant form of bacilli to active form or re-infection of new bacilli if the patient becomes immunocompromised. This may even develop into a more complicated form of tuberculosis called the drug-resistant tuberculosis. Since there is no biomarker available to detect the complete absence of dormant form of the bacilli in the host [Keshavjee and Farmer, 2012], every individual after their treatment are prone to relapse and thus continuous monitoring of the treated individuals for another 6 months to 1 year is needed.

Besides these factors, mismanagement of antitubercular drugs may also lead to the development of drug resistant form of bacilli. According to DOTS therapy, the time period for complete cure of disease is 6-9 months. During the intensive phase of the treatment, most of the bacilli are killed and thus the patients show reduced or no symptoms of the disease. Hence, patients may discontinue the treatment and this becomes the major cause for the development of drug resistance. On the other hand, some patients may not take all the four prescribed drugs or may delay the regular administration of the drugs which are also the causes for drug resistance.

The multi drug resistant tuberculosis (MDR-TB) individuals become non responsive to the two most effective first line drugs. The use of second line drugs and injectibles like streptomycin, amikacin, kanamycin and other oral fluoroquinolones like moxifloxacin, gatifloxacin as an alternate therapy may aid in decrease the intensity of infection but these
drugs are very toxic, less efficacious and very expensive. Moreover, the duration of treatment may extend up to 18-24 months instead of 6-9 months.

Tuberculosis research in order to achieve the WHO “STOP TB” goal should be fastened up but the barriers in the discovery of new drugs make it less possible. Factors like limited knowledge about the basic etiology of \textit{M. tuberculosis}, the compatibility of new drugs with the existing ones, and unavailability of biomarkers and tools to evaluate the efficacy of new drugs act as hurdle in drug discovery for tuberculosis disease. Thus, the new research on exploring drug targets and drug discovery should come out of the barriers and act in an effective way by properly using the sources and resources available.

Increasing the knowledge of various mycobacterial virulence genes and reviewing and updating the critical new information on the entire genome of \textit{MTB} may greatly promote the identification of genes that code for new drug targets. These findings may be used for designing drugs using \textit{in silico}/genomics/proteomics and drug development using quantitative structure-activity relationships. The validation of drugs using bioinformatic tools may aid in testing the quality and effectiveness of the drug in the site of action. In addition to these factors, development of new types of drug administration systems using drug vehicles may enable effective drug delivery to the target \textit{in vivo}.

Though there are number of drugs available for TB treatment, most of them are not designed based on the specificity of drugs to the target. Drug targets obtained from comprehensive \textit{in silico} analysis and characterization of the same may aid in proper validation of the use of the protein as a drug target. The target antigens can be segregated according to its function namely cellular metabolism, virulence, detoxification, adaptation, cell and cell wall processes, regulatory proteins etc [Raman \textit{et al.}, 2008]. In this respect, DNA binding proteins, categorized under regulatory proteins, play a major role in growth, survival, and pathogenesis of the bacilli. Thus, the study was initiated with exploring two such DNA binding proteins: Rv3716c- found to be associated with latent tuberculosis diagnosis [Prabhavathi \textit{et al.}, 2015] and Rv3405c- a candidate drug target from \textit{in silico} predictions [Raman \textit{et al.}, 2008].
Previously in our laboratory, the gene product of rRv3716c, along with the existing QFT-GIT kit, was shown to be a probable surrogate biomarker for detecting LTBI [Prabhavathi et al., 2015]. rRv3716c, a hypothetical protein annotated to be a DNA binding protein, is placed under the YbaB family of DNA binding proteins. Proteins of this family are co-transcribed (operonic) with RecR, which is one of the proteins involved in replication recovery following DNA damage [Yeung et al., 1990; Alonso et al., 1990]. The genome becomes more sensitive to DNA damaging agents upon deletion of this operon [Peláez et al., 2001]. It has been shown that the YbaB protein from *Haemophilus influenza* has a “tweezer” like opening which is suitable for binding a double stranded DNA [Lim et al., 2003]. An extensive study on the DNA binding ability of *Borrelia burgdorferi* EbfC, a homolog of YbaB family of proteins, revealed that the protein binds to the operator region of *erp* gene containing a consensus palindromic DNA sequence (GTnAC, where “n” can be any nucleotide). Binding of EbfC to the palindromic region results in bending of DNA [Riley et al., 2009].

Although the rRv3716c was proposed to be a biomarker, the function of the protein is not fully established. We present biophysical characterization of rRv3716c along with its three-dimensional structure obtained by X-ray diffraction techniques. The recombinant full length protein was overexpressed in *E. coli* and purified to near homogeneity by Ni-NTA affinity chromatography. The qualitative gel retardation assays showed that rRv3716c is indeed a DNA binding protein. The structure reveals an interesting case of metal ion induced (Cd$^{2+}$) crystal packing. Comparative structural analysis with other homologs of YbaB family proteins highlights structural features of rRv3716c not previously observed in other homologs.

Currently, the application of broad spectrum antibiotics for treating infectious diseases has become less due to the emergence of wide-spread resistance mechanisms [Peláez et al., 2001]. The resistance is achieved by dynamic changes that occur inside the pathogens leading to an alteration in gene expression patterns and an exchange of gene segments through transposons or plasmids. This adaptation to the environmental changes aids in the easy survival of the organism inside a host.
Tetracycline resistance is common among Gram negative prokaryotes where the drug is actively transported out of the cell. The resistance mechanism is based on the expression of tetA gene which indeed is regulated by the Tet repressor (tetR). TetR family of transcriptional regulators (TFTRs) function as homodimers which contain a conserved N-terminal helix-turn-helix (HTH) motif which acts as a DNA binding domain and a variable regulatory domain in its C-terminus which is involved in ligand binding and dimerization [Balhana et al., 2015]. The binding of the ligand (TCN) causes conformational changes in the N-terminal region of the protein and thus the protein loses its affinity for DNA [Sogi et al., 2013]. Though TFTRs are involved in antibiotic effluxing, they do engage in different functions like activator or repressor of gene activity, peptide ligand binding and regulation of enzymatic activity. Analysis by Balhana et al., 2015 showed that in mycobacteria, majority of the TFTRs aid in the regulation of enzymes involved in energy and cellular metabolism which indicates that these proteins are involved in cellular adaptation. Thus, targeting TFTRs may pave the way in disrupting the adaptation strategy of the pathogen and aid in its clearance inside the host.

The second part of the work was thus focused on the characterization of Rv3405c; a member of TFTRs. Rv3405c has a typical HTH motif in its N terminal domain and a C-terminal ligand binding domain. Rv3405c protein is divergently oriented to its adjacent gene Rv3406 [Balhana et al., 2015], an iron and α-ketoglutarate dependent sulphur ester dioxygenase, which is involved in scavenging sulphur from medium-chain alkyl sulphates particularly 2-ethylhexyl sulphate (2-EHS) [Sogi et al., 2013]. Binding of Rv3405c protein to the palindromic inverted repeat region present upstream of Rv3406 causes repression of Rv3406 gene expression thereby disruption of sulphur scavenging mechanism. As tetR proteins, the most abundant transcription factors in mycobacteria, play an important role in antibiotic resistance and regulation of efflux mechanism [Lin et al., 2005; Gristwood et al., 2008; Guazzaroni et al., 2005], studying the biophysical and biochemical function of one such protein may give clue about its role in pathogenesis and survival inside host.