

**Biobarcode Nanoassay for rapid identification and detection of rifampicin-resistant
Mycobacterium tuberculosis in sputum samples**

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Abstract:

Tuberculosis (TB) is a devastating disease with approx. 8.6 million incident cases in 2012 and 1.3 million deaths among which half a million are children. About 320,000 deaths have been reported among HIV-infected persons worldwide (WHO, 2012). Pulmonary tuberculosis is caused by *Mycobacterium tuberculosis*, an obligate pathogenic bacteria that belong to the family *Mycobacteriaceae*. Pulmonary disease is the most common manifestation of TB. TB typically affect the lungs but it can also affect any other organ of the body. TB is treated usually with a regimen of drugs taken for six months to two years based on the type of infection. TB cannot be controlled unless it is detected at an early stage. The most crucial difficulties in the treatment of TB is the identification and discrimination of *MTB* from Non-Tuberculous Mycobacteria (NTM). In addition, simultaneous identification of rifampicin-resistance along with *MTB* detection is also highly essential so as to provide accurate diagnosis and treatment of MDR-TB. In this proposal, we aim to develop a novel DNA bio-barcode technique to identify rifampicin-resistant *MTB* strains and NTM.

Introduction:

India has been considered to be the country with highest burden of TB. World Health Organization (WHO) statistics for 2014 has reported an incidence figure of 2.2 million cases of TB in India among the global incidence of 9 million. The estimated TB prevalence figure is given as 2.5 million[1-6]. It is estimated that about 40% of the Indian population is infected with TB bacteria, among which majority of them have latent rather than active TB. Sputum smear microscopy is the most common TB test used to check whether the suspected TB patients show sputum smear positive. The people with both TB and HIV, children with TB may be paucibacillary and may show sputum smear negative[7-11].

Tuberculosis is caused by bacteria (*Mycobacterium tuberculosis*) most often affect lungs. Tuberculosis spread from person to person through air. When people with respiratory infection in TB cough, sneeze or spit[12-16], they spread the germs into the air. Only a small amount of pathogens are needed to infect another person. About 10% of infected cases have a lifetime risk for developing the disease. But people with compromised immune system such as people with HIV, malnutrition or diabetes and those who continuously use tobacco have a higher risk to develop the disease. TB is a silent contagion that causes devastating impact on life. If the people have a strong resistance to TB disease, these bacteria becomes dormant[17-21].

Mycobacterium tuberculosis (MTB) is a pathogenic bacterial species that belongs to the genus *Mycobacterium* and is the causative agent of most cases of tuberculosis. The bacterium was first discovered by Robert Koch in the year 1882[22-26]. The cell surface comprises of an unusual waxy coating that makes the cells impervious to Grams stain, while acid-fast stain are used[27-31]. The physiology of *M.tuberculosis* is highly aerobic and requires high levels of oxygen. The bacilli requires oxygen to grow and it does not retain any commonly used bacteriological stain due to high lipid content in the wall. Therefore[32-36], it is neither Gram positive nor Gram negative and hence Ziehl-Neelson staining or acid-fast staining is used. *M. tuberculosis* divides every 15-20 hours, which is extremely slow compared to other bacteria, which tend to have division times measured in minutes (*Escherichia coli* can divide roughly every 20 minutes). Its unusual cell wall, rich in lipids (e.g., mycolic acid), is likely responsible for this resistance and is a key virulence factor. When in the lungs, *M. tuberculosis* is taken up by alveolar macrophages, but they are unable to digest the bacterium. Its cell wall prevents the fusion of the phagosome with a lysosome. Specifically, *M. tuberculosis* blocks the bridging molecule, early endosomal autoantigen 1 (EEA1); however, this blockade does not prevent fusion of vesicles filled with nutrients. Consequently, the bacteria multiply unchecked within the macrophage[37-42].

Chemotherapy using various anti-viral drugs are normally used for the treatment of tuberculosis. The existence of numerous strains of diverse drug-susceptible *Mycobacteria*, detection and identification of causative bacterium is most crucial for the effective treatment. Chest X-ray examination and sputum test are commonly used for the diagnosis of tuberculosis. But Chest X-ray may mis-detect inactive tuberculosis or other types of chest diseases as active tuberculosis. Sputum test are generally performed by two methods namely (i) Sputum smear microscopy and (ii) culture methods. In sputum smear microscopy[43-45], sputum of a patient is thinly spread on

the slide and bacteria is stained for microscopic observation whereas in culture method the bacteria are cultured under physiologic conditions for visual observation. Sputum smear microscopy was considered to be relatively simple and rapid technique but fails to discriminate *Mycobacterium* from other MTB complexes. The detection and identification of *Mycobacterium tuberculosis* have been done by the culture method but results can be obtained at least 6-8 weeks due to slow growth rate of the bacteria. At this period of detection, use of inappropriate drug combinations have resulted in therapeutic failure and drug-resistance. In fact, increasing numbers of strains have been determined to be resistant to primary anti-tuberculosis drugs such as isoniazid (INH), rifampin (RIF), streptomycin (STR), ethambutol (EMB) and pyrazinamide (PZA). Even though molecular techniques such as PCR produced results within a relatively short time, many false positive or false negative results have been obtained. Therefore, for an effective control and treatment of tuberculosis, there is a necessity for rapid identification of the causative bacterium, tuberculosis and its drug resistant patterns.

Rationale of the study supported by cited literature (b) Hypothesis (c) Key questions.

MTB and NTM:

The genus *Mycobacterium* consists of the members of the *Mycobacterium tuberculosis* complex and >80 species of nontuberculous mycobacteria, including pathogenic, opportunistic, and nonpathogenic species. The most important species of this genus is *M. tuberculosis*, the causative agent of tuberculosis (TB). TB is one of the leading infectious diseases in the world and is responsible for more than 2 million deaths and 8 million new cases annually (Dye et al, JAMA 1999;282:677–86). *M. avium* is the most common nontuberculous mycobacterium found in clinical specimens. Infections caused by this species can be clinically significant, especially when associated with AIDS (Horsburgh et al, N Engl J Med 1991;324:1332–8.). The major difference between TB and other mycobacterial infections is that *M. tuberculosis* is transmitted from person to person. Thus it is particularly important to diagnose TB as rapidly as possible. In addition, antibiotic treatment varies according to the species of mycobacterium. Although the initial diagnosis of mycobacterial disease often is based on clinical data, definitive diagnosis usually involves the isolation and identification of the infecting organism in the laboratory. The usual laboratory procedure for clinical specimens involves decontamination and digestion of the

specimen, microscopic examination for the presence of acid-fast bacilli (AFB), isolation of the organism by culture, and identification and drug susceptibility testing of the recovered organism. Because of the slow growth rate of mycobacteria, isolation, identification, and drug susceptibility testing can take several weeks or longer (Soini and Musser, Clinical Chemistry 47:5).

Mycobacterial isolates have traditionally been identified to the species level based on their reactions in a series of phenotypic and biochemical tests. However, the biochemical reactions of isolates of the same species may vary from each other and from time to time, and in many cases no definitive identification is obtained. Because biochemical testing is slow, cumbersome, and may yield ambiguous results, laboratories are increasingly using molecular methods for species identification (Soini and Musser, Clinical Chemistry 47:5). In 1882, in a remarkable feat of microbiology, Robert Koch isolated *M. tuberculosis* for the first time, and conclusively demonstrated in the guinea pig that this slow-growing mycobacterium was the agent of a human disease (Koch, 1882). Together with other highly related bacteria, *M. tuberculosis* forms a tightly knit complex, a single species as defined by DNA/DNA hybridization studies (Imaeda, 1985), which is characterized by a singular lack of diversity in the bulk of its genes (Sreevatsan et al., 1997). The *M. tuberculosis* complex comprises six members : *M. tuberculosis*, the causative agent in the vast majority of human tuberculosis cases; *Mycobacterium africanum*, an agent of human tuberculosis in sub-Saharan Africa; *Mycobacterium microti*, the agent of tuberculosis in voles; *Mycobacterium bovis*, which infects a very wide variety of mammalian species including humans, and BCG (bacille Calmette-Guerin), an attenuated variant of *M. bovis*; and *Mycobacterium canettii*, a smooth variant that is very rarely encountered but causes human disease. Prior to the introduction of pasteurization of milk, *M. bovis* was responsible for about 6% of total tuberculosis deaths in humans in Europe. BCG was derived by Calmette and Guerin from a virulent *M. bovis* isolate by 230 serial passages in a broth containing glycerol, potato-extract and bile salts (Calmette, 1927). During the course of these passages the *M. bovis* strain progressively lost its virulence for animals and was first shown to be harmless and protective in a child in 1921. Since that time BCG has been used extensively as a live vaccine against tuberculosis and also protects humans against leprosy (Anon, 1996). Three billion doses have now been administered with negligible side effects and this is strong testimony to the safety of the vaccine (Bloom & Fine, 1994). The attenuation process undergone by BCG probably involved the serial loss of genetic material, rendering reversion to virulence impossible. *M.*

microti, the vole bacillus (Wells, 1937), is naturally attenuated for humans and has also been used successfully to protect against tuberculosis (Hart & Sutherland, 1977). Mycobacterium w, named Mycobacterium indicus pranii isolated from the sputum of patients, shared several antigens with Mycobacterium tuberculosis and Mycobacterium leprae (Ahmed et al, 2007; Tuberculosis 2007: 87: 123-129). When mice was vaccinated with the killed M.w, the animal was protected from subsequent tuberculosis (Talwar et al, 1990: Vaccine 8: 121-129; Singh et al, 1991: Vaccine 9: 10-14). BCG-non-responsive strains of mice was protected from TB infection when immunized with M.w followed by MTB infection (Talwar, 1992: Indian J. Tuberculosis 29:67-69). Immunotherapy with M.w has also minimized pulmonary TB cases (Patel et al, 2002: J. Indian. Med. Association 100: 191-193).

Identification of Antibiotic resistance-associated mutations:

Drug-resistant M. tuberculosis isolates are a serious threat to TB control because only a few effective drugs are available for treatment of this disease. M. tuberculosis acquires drug resistance by antibiotic selection of mutations that occur randomly at chromosomal loci. No plasmids or transposable elements (horizontal gene transfer) are involved in this process. Individual nucleotide changes (point mutations) confer resistance to single drugs, and the stepwise accumulation of these mutations leads to multidrug-resistant TB (Azar Dokht Khosravi et al, 2012; Braz J Infect Diseases). Drug-resistant strains emerge when chemotherapy is intermittent or otherwise inadequate. After the primary culture results are available, conventional drug susceptibility testing performed on solid medium takes 2–4 weeks. When the radiometric BACTEC drug susceptibility testing system is used, several weeks are needed to obtain results (WHO, 2008). Advances in molecular biology have made it possible to investigate the genetic mechanisms of drug resistance in M. tuberculosis and to develop methods for rapid detection of mutations associated with resistance. The currently available methods have mostly been developed for detection of rifampin (RIF) resistance because the genetic basis of RIF resistance in M. tuberculosis is fairly simple and well characterized, whereas the molecular basis of resistance to other first-line TB drugs is much more complex. Moreover, resistance to RIF can often be used as a marker of multi-drug resistant TB (Zhang and Telenti, 2000; Washington DC,

USA: ASM Press, pp 235-54). For the direct detection of MTB strain from the sputum samples certain tests like Enhanced Mycobacterium tuberculosis Direct Test (E-MTD; Gen-Probe, San Diego, CA) and the Amplicor Mycobacterium tuberculosis Test (Amplicor; Roche Diagnostic Systems, Inc., Branchburg, NJ) is being used. The accuracy of the GeneXpert MTB/RIF (Cepheid Corporation) in the diagnosis of tuberculosis in patients have been carried out (Nguyen et al, 2014: Journal of Clinical Microbiology 52 (1):226-233). These methods are based on the nucleic acid amplification tests (NAAT) that is used for the detection of mycobacterial DNA or RNA. The sensitivities of Xpert, smear, and MGIT culture among patients diagnosed with MTB were 59.3% (108/182 [95% confidence interval {CI}, 51.8 to 66.5%]), 78.6% (143/182 [95% CI, 71.9 to 84.3%]) and 66.5% (121/182 [95% CI, 59.1 to 73.3%]), respectively. One false-positive Xpert MTB/RIF test (99.5% specificity) have been reported. Four cases of RIF resistance (4/109; 3.7%) were also identified by Xpert, of which 3 were confirmed to be multidrug-resistant (MDR) TBM and one was culture negative. In order to aid specific identification of mycobacterial strains *M.tuberculosis* complex, *M. avium*, *M. intracellulare*, *M. avium* complex, *M. kansasii*, and *M. gordonae*, species specific probes have been used. (Evans et al, J Clin Microbiol 1992;30:2427–31). PCR-based sequencing and DNA microarrays have been used for the identification of Mycobacterial species (Springer et al, J Clin Microbiol. 1993;31:3083–3089). Further, the antibiotic resistant mycobacterial strains have been identified using primary culture of mycobacterial cells in the presence of suspected antibiotic for instance, rifampicin. The genetic basis of such resistance to first line drugs involves the mutations in the *rpoB* gene which is responsible for the β -subunit of RNA polymerase. Therefore, mutations in the *rpoB* gene creates impact on the conformation of the β -subunit of RNA polymerase thereby preventing the RIF antibiotic binding to the polymerase and inhibits transcription. Line probe assay have also been used for the identification of rif-resistant mycobacterial strains (Soini and Musser, Clinical Chemistry 47:5, 809–814, 2001). In spite of its sensitivity certain methods fails to reserve their specificity and if they are highly specific they lose their sensitivity. Therefore, alternative methods should be developed for the identification of drug resistant tuberculosis with high sensitivity and specificity. The proposed technique would produce good sensitivity and specificity in the identification of rifampicin-resistant and sensitive tuberculosis.

Current status of research and development in the subject (both international and national status)**International status :**

Sputum smear microscopy is the mainstay for TB diagnostics in endemic developing countries (Perkins, 2009). However, this technology has found to be less sensitive as it can only detect half of all active cases of TB if used properly – in people with co-infections and in children the sensitivity is even much lower. Even though, microscopy has been considered to be simple technology in the routine diagnosis of TB, it requires diligence of the technician and multiple examinations. As a result, it takes several weeks for the examination to get completed wherein many patients drop out during the diagnostic process. There are several diagnostic approaches have shown incremental improvements in the direct detection, species identification and drug susceptibility testing of Mycobacteria, thereby reducing the time of experiment from weeks to a few several days (Barnard et al., 2008; D'Amato et al., 1995; De Beenhouwer et al., 1995; Griffith et al., 2007; Hillemann et al., 2005; Hirano et al., 1999; Moore et al., 2006; Park et al., 2002; Rossau et al., 1997; Sharma et al., 2003; Traore et al., 2000). Most of these approaches for direct detection of TB and drug susceptibility from clinical specimens, including several commercial tests, rely on complex and expensive DNA amplification based procedures (e.g. PCR), whereas the need is for affordable, simple and high-throughput systems with the possibility to use small amounts of sample (Cheng et al., 2005; Das et al., 2010; Shamputa et al., 2004; Watterson et al., 1998).

Nanotechnology has brought up new paradigms in support of molecular diagnostics through nanodiagnostic approaches with increased sensitivity, specificity, speed and reduced cost. There serve as an alternative for the existing conventional techniques (Azzazy et al., 2006). The standards for molecular diagnostics have been redefined by nanodiagnostics, adopting new and radical approaches in the recognition of biomolecules including nucleic acids, antibodies and proteins. These promising approaches have been triggered using nanoparticles (NPs), nanotubes, nanopores and nanocantilever technologies (Baptista et al., 2008; Branton et al., 2008; Jain, 2007; Rosi & Mirkin, 2005; Wang et al., 2009). The occurrence of one-to-one interactions between the analytes and signal-generating nanostructures plays a vital role in the increase of

sensitivity and specificity at lower costs. There are different nanodiagnostics systems available for the molecular diagnostics of TB. Apart from the use of nanoscale systems for biomolecular assays in general (e.g. electromechanical, electrochemical) (Azzazy et al., 2006; Das et al., 2010; Jain, 2007), nanoparticle based systems, such as gold, silver, silica and quantum dots (QDs), have been the most widely used for TB diagnostics due to their unique physicochemical properties, that offer greater sensitivity than conventional reporter molecules and can be easily tuned and functionalized by simple chemistry modulation and derivatization (Azzazy, 2009).

The first application of AuNPs for the molecular diagnostics of *M. tuberculosis* was introduced by Baptista et al. (Baptista et al., 2006).

This approach of Baptista et al has identified the presence of different DNA targets by the differential stabilization of gold nanoprobe. The gold nanoprobe solution colour remains red in the presence of complementary targets as they prevent the nanoprobe against particle aggregation while non-complementary/mismatched targets favors the gold nanoprobe aggregation thereby changing the color of the solution to blue. Thiol-modified oligonucleotides harboring a sequence derived from *M.tuberculosis* RNA polymerase β -subunit gene sequence were functionalized on the gold nanoprobe. High efficiency was demonstrated with this methodology in clinical samples when combined with an initial round of PCR for target amplification (Baptista et al., 2006).

The attained results have shown a 100% concordance with the available commercial molecular TB diagnostics test INNO-LiPA Rif.TB. Following optimization towards detection of single base mismatches (Doria et al., 2010), this strategy was applied to the rapid detection of NTM strains and simultaneous characterization of the presence of mutations associated with rifampicin resistance (Veigas et al., 2010). This low-complexity assay enabled the detection of mutations D516V and S531L from NTM clinical specimens with remarkable sensitivity in just a few hours. Based on the molecular signatures of MTB complex members and the most common mutations associated with RIF resistance in *M. tuberculosis*, a two-step approach based on the PCR amplification of a fragment of *rpoB* gene and subsequent hybridization with specific nanoprobe, namely a probe for the *rpoB* locus shared by all the members of the MTB complex and a probe specific to MTB complex members, was developed. Three additional sets of probes specific for

the most common point mutations associated with RIF resistance (D516V; H526D; S531L) were also designed and synthesized. Each set composed of two probes: one complementary to the wild-type sequence and the other complementary to the mutation. A limit of detection could be set at 75 nM, however, for robust single base mismatch determination, 117 nM of DNA target were used per assay. This non-cross-linking approach correctly detected the presence of DNA from members of the MTB complex in 83.3% of all samples, when compared to the INNO-LiPA Rif.TB assay. By means of a set of two probes for each mutation associated to RIF resistance to be screened (mutations in codons 516, 526 and 531 of the *rpoB* gene), it was possible to correctly score the presence of at least one of the mutations in 81% of all samples also screened via the INNO-LiPA Rif.TB assay. Following PCR amplification, the method takes only 90 min to yield a colorimetric result which, through the use of a suitable photodetector (e.g. UV/visible spectrophotometer, microplate reader, etc.), may be used in medium throughput analysis at a peripheral laboratory or point-of-care. Fast and reliable identification of MTB complex members and mutations within the *rpoB* gene is of great advantage as it is a secondary marker for isoniazid resistance allowing to predict, with a high degree of confidence, whether the strain is indeed a multidrug-resistant TB (Hillemann et al., 2005).

Towards a point-of-care application, Baptista and co-workers further integrated the noncross-linking nanoprobe-based method in an innovative optoelectronic platform that allows an analytical measurement of the colorimetric changes, hence to detect a target without the need of experienced personnel. The device integrates an amorphous/nanocrystalline biosensor and a light emission source with the non-cross-linking method for specific DNA detection. This low cost, fast and simple optoelectronic platform was optimized for the specific identification of MTB complex members and the consequent improvement of the laboratorial diagnostics algorithms of TB (Bernacka-Wojcik, et al., 2010; Silva et al., 2008, 2010). The integration of these technologies together with the possibility of miniaturization are of utmost importance for the development of an integrated biosensor suitable for peripheral laboratories and/or point-of-care diagnostics, providing a new tool in the fight against TB (Veigas and Baptista, 2012; Dr. Pere-Joan Cardona (Ed.), InTech,).

Recently, Liandris et al. have developed a non-cross-linking approach to the detection of TB without the need of target amplification (Liandris et al., 2009). The method relied on the same

non-cross-linking hybridization approach of Baptista and co-workers, whereas the aggregation of the gold nanoprobe was induced by an increasing acid concentration instead of salt. Only a very small number of these new nanodiagnostics platforms have been translated to the clinical setting for TB molecular diagnostics.

National Status:

India contributes the highest number of new cases, accounting for 20% of the global burden, of which approximately 1.2 % occur in persons with HIV (WHO, 2009). In India, pulmonary TB is the most common form of the disease; however, extra-pulmonary TB comprises 20% of cases (Arora and Chopra, 2007). The majority of molecular tests have been focused on: (i) detection of nucleic acids both DNA and RNA, which are specific to *Mycobacterium tuberculosis*, by amplification techniques such as polymerase chain reaction (PCR) focusing on detection and molecular epidemiology of *M. tuberculosis*; and (ii) detection of mutations in the genes which are associated with resistance to anti-tuberculosis drugs by sequencing or nucleic acid hybridization. The development and use of rapid diagnostic tools become increasingly important in addressing the emergence and treatment of multi-drug resistant (MDR) and extreme-drug (XDR) resistant *M. tuberculosis* strains. Molecular tests such as polymerase chain reaction (PCR), which are considerably faster than culture, often have a high turnaround time as specimens are often sent to distant laboratories. PCR have been used for the amplification of IS6110 insertion sequence that belongs to the IS3 family found in almost all members of *M.tuberculosis* complex (Anamika and Kashyap, 2012; Interdisciplinary Perspectives on Infectious Diseases).

Presently, about one third of world's population is infected with *Mycobacterium tuberculosis*. Currently, the number of people dying of tuberculosis is more than any other infectious diseases. Death from tuberculosis comprises 25% of all avoidable deaths in developing countries (Ramachandran and Parmasivan 2003). Nearly 95% of all tuberculosis cases and 98% of deaths due to tuberculosis are in developing countries and 75% of tuberculosis cases are in the economically productive age group. Currently, more people die of tuberculosis than from any other infectious disease. In India, out of a total population of more than 1 billion, approximately 2 million develop active disease and up to half a million die of tuberculosis. It also imparts a

financial burden on the economy in terms of out-put losses because of premature deaths and ill health. To add to the existing cost burden, the cumulative effect is seen because of ever increasing number of new TB cases associated with HIV patients and about 1.8 million of these are co-infected with TB (Ramachandran and Paramasivan, 2003).

Early diagnosis of the tuberculosis in the patients is a challenging task especially in the paucibacillary and extra-pulmonary forms. The conventional methods that are still the mainstay of the diagnosis of TB like Tuberculin test/ Mantoux test, radiological examination and other imaging methods and sputum smear microscopy have their own limitations. Sputum smear microscopy requires 10,000 to 1,00,000 organisms/ ml and acid fast bacilli could be any pathogenic or saprophytic mycobacteria. Although smear microscopy may be made more convenient by using various fluorochromes (auramine, rhodamine, FITC etc.) but the scarce presence of tuber bacilli in the sputum has its own disadvantage. The smear positivity has to be supplemented with the culture positivity that has its own limitations because of failure of bacilli to grow or often become contaminated with other microbes. The slow growth of the tubercle bacilli on medium lingers on the confirmation of the causative organism. Histopathology is characteristic but there could be problems to get representative specimen, and non-specific features. Immunoassay based approaches are doubtful as the antibodies and the antigens may persist for some time after control of the clinical or sub-clinical disease. Thus Acid Fast Bacilli (AFB) staining of clinical material followed by smear microscopy remains the most cost effective, frequently used microbiological test for detection of TB. The major drawback of sputum smear microscopy is its poor sensitivity, especially to be ~70% in a recent review (Steingart et al., 2006). Although the AFB staining is easy to perform in the field settings especially in the poor third world countries as well in the developing countries but the sensitivity of sputum smear microscopy is clearly less in many settings and may be sometimes as low as ~35% in some situations with high rates of TB and HIV co-infection (Khatri and Frieden, 2002). Compounding the poor test sensitivity is inadequate or absent test quality assurance in some resource-constrained settings, further cut down the overall yield of the microscopy, driving up the laboratory workload as more sputum tests per patient are performed in an effort to reach a diagnosis, and increasing delay in diagnosis and patient's compliance to repeated follow-up (Dorman 2010). Moreover drug-susceptibility status cannot be determined from the smear microscopy. Unfortunately, the world's largest democracy India has over 1.2 billion people and this overpopulated country also has the highest burden of

tuberculosis in the world. India accounts for about 20% incidence of tuberculosis besides a high incidence of global occurrence of multi drug resistant (MDR) tuberculosis. The poor sanitation conditions, thickly populated urban and rural area, scanty medical services in villages and rural area, malnourishment, insensitivity of private sector towards quick diagnosis, treatment and management of TB positive patients and higher cost of non-standard methods of diagnosis of TB are some of the important reasons of concern. It is obvious that any global effort towards control and eradication of TB and fast emerging MDR-TB is invariably dependent upon success of concerted efforts to contain the spread of this contagious disease. In India, the National TB Programme (NTP) was initiated in 1962. However, the poor infrastructure, inadequate funding, administrative lack-luster approach, irregular drug supply, non-standard and multiple anti-tuberculosis drug therapy, irregularity or non-compliance of patients to the clinician and a long treatment period had little effect on containing the spread of TB and controlling the emerging MRD-TB strains. The management and control of TB was further compounded and complicated with low rates of TB case detection, compliance of treatment regimen (30%), high rate of default (40-60%) and continuing high mortality (1: 2000) the NTP programme had little success rate. To overcome the deficiencies of NTP a Revised National Tuberculosis Control Programme (RNTCP) was launched by the Government of India in 1997, based on the global DOTS (Directly Observed Treatment, Short Course) approach that was used to exert an epidemiological impact by achieving 70% case detection and 85% cure rate. It was an encouraging sign that by 2002, 100% of the Indian population was covered by the India's own drug-delivery model - the DOTS programme, making this extended coverage as India's most significant public health accomplishment. The RNTCP thus achieved a pronounced success in cure rates (>80% in new infectious cases), substantial decline in mortality with low rate (<10%) of default (Khatri and Frieden 2002; TB India 2009; Bhargava et al., 2011).

The overall pattern of drug resistance to first line anti TB therapy is similar in HIV positive and negative patients; however, MDR-TB is marginally higher (3-4%) in HIV positive patients with newly diagnosed tuberculosis status (Swaminathan 2005). Rifampicin monoresistance is more common in HIV infected patients and arises independently from mutations in drug susceptible strains. Treatment of MDR-TB should employ at least 3-4 new drugs. The regimen should include an aminoglycoside and be given under direct observation. Extensively drug resistant (XDR) TB strains have emerged and have been reported from India (Singh et al., 2007; Thomas

et al., 2007). Such strains appeared to be as an outcome of the mismanagement of TB. It seems that XDR-TB is practically untreatable and thus an attempt may be made to limit its spread by strengthening the TB control programme. Presently, there is no national policy regarding TB preventive therapy for HIV positive patients in India (Swaminathan and Narendran 2008). A clinical trial conducted at the Tuberculosis Research Center, Chennai investigated two different regimens; a 6-month regimen of ethambutol and isoniazid vs. a 3-year regimen of isoniazid alone, in order to establish ideal duration of therapy. In a TB-endemic country like India, consideration shall be given to provide preventive therapy to HIV-infected persons. Line probe assays, a family of novel DNA strip-based tests use PCR and reverse hybridization methods for the rapid detection of mutations associated with drug resistance. These kits [INNO-LiPA Rif TB kit, Innogenetics NV, Gent, Belgium; GenoType MTBDR assay; Hain Life-science GmbH, Nehren, Germany] are not currently FDA approved for use in USA. Line-probe assays are designed to identify *M. tuberculosis* complex and simultaneously detect mutations associated with drug resistance. In June 2008, WHO announced a new policy statement endorsing the use of line probe assays for rapid screening of patients at risk of MRD-TB (<http://www.who.int/tb/en/>). However, the line probe assays are not recommended as a complete replacement for conventional culture and drug susceptibility testing. Culture is still required for smear-negative specimens and conventional drug susceptibility testing is still necessary to confirm XDR-TB.

Ineffective TB diagnostics are a lucrative market in India. Patients seeking TB care in the private medical institutes are commonly subjected to diagnostic tests i.e. the anti-body based blood tests, including ELISA that are completely ineffective at detecting TB. This is because a large number of the world's population has anti-TB antibodies, though only 10% of them do develop the active form of the disease. Obviously if patients who do not have TB are misdiagnosed, they could undergo 6-months of nasty toxic anti-TB chemotherapy. If patients have active TB and the test misses it, the disease may worsen and they may continue to spread the disease in their community. According to a preliminary analysis of over 80 labs in India, it is estimated that patients undergo more than 1.5 million useless TB antibody tests each year (WHO recommends against inaccurate tuberculosis tests by Kelly Morris; www.thelancet.com vol 377 Jan 8, 2011). The absence of any regulatory mechanisms results in the import of these dubious diagnostics from France, UK, USA and other countries, where these tests are not approved for TB diagnosis. These tests generate at least US \$ 15 million. In a country that has ~100,000 labs this is probably

a fraction of the enormous total market. Accurate diagnose is critical to the control of tuberculosis in India, particularly in view of the fact that India has set new targets as a part of RNTCP, which includes early detection of 90% of all TB cases by year 2015.

In India various institutes working on TB have sufficient technical expertise and financial affordability to use molecular diagnostic methods for detection of tubercle bacilli in the samples. For a laboratory with good sample load and which is using rapid methods for early growth detection the additional cost of sample analysis shall not be significant. If 10-25 isolates/ growths are assessed for identity simultaneously, additional cost for each isolate using a non-radioactive detection system like digoxigenin (DIG) should not be more than 200-250 rupees (~US \$ 4-5). Similarly for a PCR system using primers, which are not patented, cost should be in similar range as prices of primers and reagents have considerably been reduced during the last couple of years (Katoch, 2004). The nucleic acid amplification tests (NAATs) are designed to amplify nucleic acid regions specific to the *Mycobacterium tuberculosis* complex. Such tests may be used directly on clinical samples/ sputum samples. Nucleic acid amplification test (NAAT) commercial kits are available under various brands like Amplified M. tuberculosis Direct Test (MTD; Gen-Probe Inc., CA, USA), the Amplicor M. tuberculosis (MTB) tests (Roche Molecular Diagnostics, CA, USA and the BD ProbeTec ET assay (BD Biosciences, MD, USA. Besides in-house lab developed PCR assays vary widely in their protocols and vary from lab to lab. In-house NAAT are cheap and are often used in research in developing countries where commercial NAATs are quite expensive to test large number of samples and thus in-house PCR technique/ protocol(s) provide a cheap option (Pai 2004). However, all the detection methods including the conventional AFB-staining, skin tuberculin test and new generation NAAT tests have some advantages as well as limitations. So far, in India nanobased detection and identification of rifampicin-resistant *Mycobacterium tuberculosis* is not yet reported and this is the first approach which can produce results with good sensitivity and specificity.

Conclusion:

The proposed project will lead to a remarkable achievement in the development of novel rapid and sensitive technique for the early detection of Mtb and discrimination of rifampicin-resistant from rif-sensitive tuberculus bacteria. The outcome of the project will significantly help the

clinical persons to diagnose TB at an early stage and aid in the appropriate administration of anti-TB drugs. The specificity of the primers immobilized on the surface and signal amplification of gold nanoparticles would increase the specificity and the sensitivity of the technique.

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