

Identification and detection of rifampicin-Biobarcode Nanoassay for rapid in sputum samples

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Abstract:

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 Tuberculosis. 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) 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). 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:

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. It is estimated that about 40% of the Indian population is infected with TB bacteria [1-6], among which majority of them have latent rather than active TB. India has been considered to be the country with highest burden of TB. World Health Organization (WHO) statistics for 2014 has reported an incidence 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 [7-11], children with TB may be paucibacillary and may show sputum smear negative.

Tuberculosis is caused by bacteria (*Mycobacterium tuberculosis*) most often affect lungs. Tuberculosis spread from person to person through air [12-19]. When people with respiratory

infection in TB cough, sneeze or spit, 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[20-26].

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, 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. *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. The cell surface comprises of an unusual waxy coating that makes the cells impervious to Grams stain, while acid-fast stain are used. The physiology of *M. tuberculosis* is highly aerobic and requires high levels of oxygen. 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[27-32].

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[33-39], 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)[40-45], 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.

Gold and magnetic nanoparticle synthesis

It would employ the primers specific to Rv3618, IS6110 and rpoB sequences conjugated on the surface of the gold nanoparticles and magnetic nanoparticles. The project involves some nano-based approaches like conjugation chemistry, gold and magnetic nanoparticle synthesis which relates to the thrust area of PI which is nanobiotechnology (Dharanivasan et al, 2013; Dharanivasan et al, 2014; Michael et al, 2015; Dharanivasan et al, 2016). The Co-PI's laboratory is well-established in the management of sputum samples, isolation of mycobacterial DNA and detection of tuberculosis from clinical specimens (Balaji et al, 2014; Alamelu et al, 2006; Alamelu et al, 2004; Uma Devi et al, 2003; Ramalingam et al, 2003; Uma Devi et al, 2002)

Material and methods :

The PI's laboratory has been working on the diagnosis of geminiviruses, an ssDNA virus that infect commercial vegetable crops through nanobased techniques. The magnetic nanoparticles have been used for the bioseparation of icosahedral twin-shaped geminivirus particles from the crude extract of infected plant samples (Michael et al, 2015). Furthermore, we have used gold

nanoparticles for the detection of dsDNA targets through hybridization assay with the self-assembled monolayer (SAM) of bifunctional probe (Dharanivasan et al, 2016). We have good experience and knowledge in the immobilization of biomolecules on the surface of gold nanoparticles and magnetic nanoparticles. The Co-PI's laboratory has been working on the diagnosis of *Mycobacterium tuberculosis* in the clinical specimens. Specific antibody response in childhood tuberculosis and in pulmonary tuberculosis with HIV has been studied. The incidence of TB disease in respiratory and extra-pulmonary specimens in pediatric population has been diagnosed (Neeraj et al, 2015). So far, the sputum specimens have been collected, processed, *Mycobacterial* DNA isolated and diagnosed using conventional techniques.

Specific objectives (should be written in bulleted form, a short paragraph indicating the methods to be followed for achieving the objective and verifiable indicators of progress should follow each specific objective)

- 1) To isolate the *Mycobacterium tuberculosis* bacilli from the sputum samples using antibody conjugated magnetic nanoparticles, observation under microscope and extract the genomic DNA.
- 2) To identify the rifampicin-resistant *Mycobacterium tuberculosis* (*Mtb*) strains among the isolated bacilli.

Work Plan: should not exceed 3-4 pages (the section can be divided according to the specific aims and under each specific aim, the following should be stated clearly as sub headings)

Work plan (methodology/experimental design to accomplish the stated aim).

a. Segregation and Collection of sputum samples from infected patients

The sputum specimens will be collected under the direction of trained health care professional. The sputum will be induced with hypertonic saline to obtain specimens. The specimens will be collected in sterile, clear, plastic and leak-proof containers, for instance, a 50 mL screw cap centrifuge tube. The specimens will be brought to the

laboratory within 24 hrs of collection. The specimens will be refrigerated for long time storage to avoid growth of contaminating endogenous respiratory organisms. The specimens will be segregated based on patient's duration of incidence, age-wise and gender-wise.

b. Synthesis and characterization of gold nanoparticles and magnetic nanoparticles.

Gold nanoparticles will be synthesized using Turkevich method with sodium citrate as reducing and capping agents. Magnetic nanoparticles were synthesized with modifications on the previously demonstrated protocols through co-precipitation of ferrous and ferric salts under the presence of N₂ gas with the help of NaOH solution. The salts of FeCl₃ and FeCl₂ will be dissolved in 200 ml of distilled water with an Fe²⁺/Fe³⁺ ratio of 2:1 in the solution. The nitrogen gas provides an inert atmosphere, thereby preventing the oxidative conversion of magnetite to maghemite particles. The solution will be stirred for 1 h at 30°C. The co-precipitation reaction will occur by raising the pH of the solution to 12 by adding 2M NaOH drop-wise into the reaction system at 70°C. The reaction was sustained for 3 hours at 70°C with the continuous supply of nitrogen gas. The synthesized gold nanoparticles and magnetic nanoparticles will be characterized using standard physicochemical analytical techniques.

c. Isolation of Mycobacterial strains from sputum samples using anti-LAM conjugated magnetic nanoparticles.

The anti-LAM, Anti-Ag85 and anti-HBHA antibodies will be conjugated on the magnetic nanoparticles based on the following protocol. The antibodies will be conjugated to the magnetic nanoparticles based on the dilution factor provided commercially. 10 mM EDC and 5 mM NHS will be prepared in 0.1 M MES buffer at pH 5 and incubated with anti-LAM, Anti-Ag85 and anti-HBHA antibody in a humid chamber for 1 h. Then EDC–NHS activated antibody solution was added to MNP solution. Following which phosphate buffer was added to the reaction solution. The reaction mixture was incubated for

overnight at 30°C in vortex mixer. Antibody-conjugated MNPs were separated by a magnet. The pellet will be collected and re-suspended in PBS for washing. Upon subsequent washing steps the magnetic immunoprobe was suspended in PBS. The antibody conjugated magnetic nanoparticles will be added to the decontaminated sputum samples and the bacilli will be separated out using a magnet.

d. Isolation and processing of genomic DNA from the *Mycobacterium tuberculosis* (Mtb) and *Mycobacterium tuberculosis* complex (Mtb complex).

The sputum will be suspended in decontamination solution mixture containing 4% (w/v) NaOH, 1% (w/v) N-acetyl-L-cysteine. The sample will be then added in the extraction buffer. 50 mg/mL of lysozyme was added to the sample tube. 10X proteinase K buffer containing Tris-HCl, EDTA and SDS will be added. Then buffer-saturated phenol will be gently warmed to 68°C into which hydroxyquinoline of 0.1% will be added. An equal volume of Tris-HCl will be added. The solution will be stirred for at least 10 mins and allow phase separation. Tris-HCl will be added along with mercaptoethanol. Phenol/Chloroform/isoamyl alcohol will be used to precipitate DNA from the solution.

e. Functionalization of IS6110 primers on the surface of magnetic nanoparticles.

The capture DNA (IS6110 primers) will be conjugated to the surface of magnetic nanoparticles based on the EDC activation in Imidazole buffer according to Miao et al (2008). The amine modified magnetic nanoparticles will be washed with imidazole buffer and the capture DNA will be immobilized on the surface of activated MNPs followed by incubation for 1 hr at 37°C. The forward and reverse primers specific to IS6110 sequences are given below

INS1: 5'-CGTGAGGGCATCGAGGTGGC-3'

INS2: 5'-GCGTAGGCGTCGGTGACAAA-3'

CP DNA1: 5'-A10-CACCCATCGTCTGGAGTGG-3'

CP DNA2: 5'-CCAAGCGGATGCACCGG-A10-3' (Po-Chi et al, 2009).

IS6110 insertion sequence is an important target for the identification of *Mycobacterium tuberculosis*. The insertion sequence of IS6110 is a mobile genetic element and has universal acceptance for the identification of strains in the *Mycobacterium tuberculosis* complex (Gunisha et al, 2001; Indian J. Pathol. Microbiol. 44(2): 97-102).

f. Functionalization of Rv3618 and poly(A) primers on the surface of gold nanoparticles as oligoprobe 1.

The primers specific to Rv3618 gene of *Mycobacterium tuberculosis* will be commercially obtained with functionalization of –SH groups on the 5' and 3' end of the primers. The poly(A) nucleotide will also be commercially obtained with –SH at the 3' end of the probe. The probes specific to Rv3618 and poly (A) tail will be added in 1:3 ratio. The following are the primer pair specific to Rv3618 gene sequences.

Rv3618-F: 5'-SH-ATTGCACATCCGCCCC-3'

Rv3618-R: 5'-GGACAAACCCTGCCGC-SH-3'

TB DNA1: 5'-SH-A10-AGCCATGATTTGCGCCATCG-3'

TB DNA2:5'-CTGGGCACGTCGCTGTT-A10-SH-3'

Poly(A) : 5'-AAAAAAAAAAAAAAAAAAAAAAAAAA-SH-3'.

The primer pairs Rv3618 is specific to *Mycobacterium tuberculosis* and helpful in the selective identification of MTB strains with high sensitivity and specificity. The poly(A) oligoprobes contribute in the amplification of signals and identification of Rv3618.

g. Functionalization of rpoB and poly(G) primers on the surface of gold nanoparticles as oligoprobe II.

The primers specific to *rpoB* gene of *Mycobacterium tuberculosis* will be commercially obtained with functionalization of –SH groups on the 5' and 3' end of the primers. The poly(G) oligonucleotide will also be commercially obtained with –SH at the 3' end of the probe. The probes specific to *rpoB* and poly(G) oligonucleotide will be added in 1:3 ratio. The following are the primer pair specific to *rpoB* gene sequences.

rpoB-F: 5'-SH-CGCGATCAAGGAGTTCTTC-3'

rpoB-R: 5'-TGACAGACCGCCGGGCCC-SH-3'

gyrA-F: 5'-SH-GGTGCTCTATGCAATGTTCG-3'

gyrA-R 5'-GCTTCGGTGTACCTCATCG-SH-3'

Poly(G) : 5'-GGGGGGGGGGGGGGGGGGGGGGGGGGGG-SH-3'.

The determination of resistance of *Mtb* to rifampicin (Rif) in approx. 95 percent of the cases is due to the mutations in the 81-bp rifampicin-resistance determining region of the *rpoB* gene corresponding to 507-533 codons that codes the β -subunit of RNA polymerase of *Mtb* (Mani et al, 2001: J Clin Microbiol 39:2987-2990). Therefore, *rpoB* primer sequences are helpful in the detection of Rif-resistant *Mtb*.

h. Hybridization of target DNA with the gold oligoprobes I and II.

The isolated bacterial genomic DNA will be digested to smaller fragments to achieve effective hybridization with the capture DNA and the signal gold oligoprobe. The hybridization will occur in a sandwich fashion involving dual hybridization event. The two target DNAs will be added to the hybridization buffer containing magnetic nanoparticles. After hybridization for about 1 hr at 37°C, the complex will be magnetically separated and suspended in DNA-AuNPs for 1 hr at 37°C. The hybridization occurs only in the presence of complementary target in the sample.

i. Release of barcode DNAs and dipurinization of barcode DNAs.

The sandwich complex will be separated using magnet and dispersed in 0.05M DTT with gentle shaking for 60 mins. Following which the supernatant will be transferred into a fresh tube and treated with H_2SO_4 along with heat to dry. An acetate buffer containing Cu^{2+} will be used to transfer the digested barcode DNA into the electrochemical cell.

j. Modification of electrode and measurement of redox potential of A and G nucleobases.

The glassy carbon electrode was modified as per the literature (Wang et al, 2002). The glassy carbon electrode will be oxidized under stirring in PBS and then reduced by cyclic sweep until a steady state curve was obtained in the electrolyte solution. Upon addition of the digested DNA, the accumulation of purine bases on the glassy carbon electrode will be measured. Differential pulse voltammetry will be performed to analyze the redox potential of the nucleobases A and G.

k. Statistical analysis and Interpretation of data

All the data will be compared with the standard and control. All the data will be analyzed by statistics using T-square test and finally published.

Connectivity of the participating institutions and investigators (in case of multi-institutional projects only)

The principal investigator is working in Nanobiotechnology for past five years, in the diagnosis and analysis of the whitefly-transmitted begomovirus in a multi-vegetable cropping system and weeds using gold nanoparticles and magnetic nanoparticles by conjugating biomolecules on their surface.

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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