"STUDIES ON EARLY DETECTION OF *MYCOBACTERIUM TUBERCULOSIS (MTB)* USING NANOTECHNOLOGY"

A THESIS SUBMITTED
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SUBMITTED BY

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APRIL 2019

DECLARATION

I hereby declare that the work presented in this thesis entitled, **"Studies on early detection of *Mycobacterium tuberculosis (MTB)* using nanotechnology"** is entirely original and was carried out by me independently in the D. Y. Patil Education Society (Deemed to be University), Kolhapur under the supervision of Prof. (Dr.) S. H. Pawar, Emeritus scientist (CSIR) and Distinguished Professor, D. Y. Patil Education Society, Kolhapur. I further declare that it has not formed the basis for the award of any degree, diploma, fellowship or associate ship of similar title of any University or Institution. The extent of information derived from the existing literature has been indicated in the body of the thesis at appropriate places giving the references.

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on early detection of *Mycobacterium tuberculosis (MTB)* using nanotechnology"** is submitted herewith for the degree of Doctor of Philosophy in Clinical Microbiology under the faculty of Medical Science to D. Y. Patil Education Society, Kolhapur by **Mr. Deepak Vitthal Sawant** is absolutely based upon his own work under my supervision. Neither this thesis nor any part of it has been submitted elsewhere for any degree or diploma or any other academic award anywhere before.

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Deepak Vitthal Sawant

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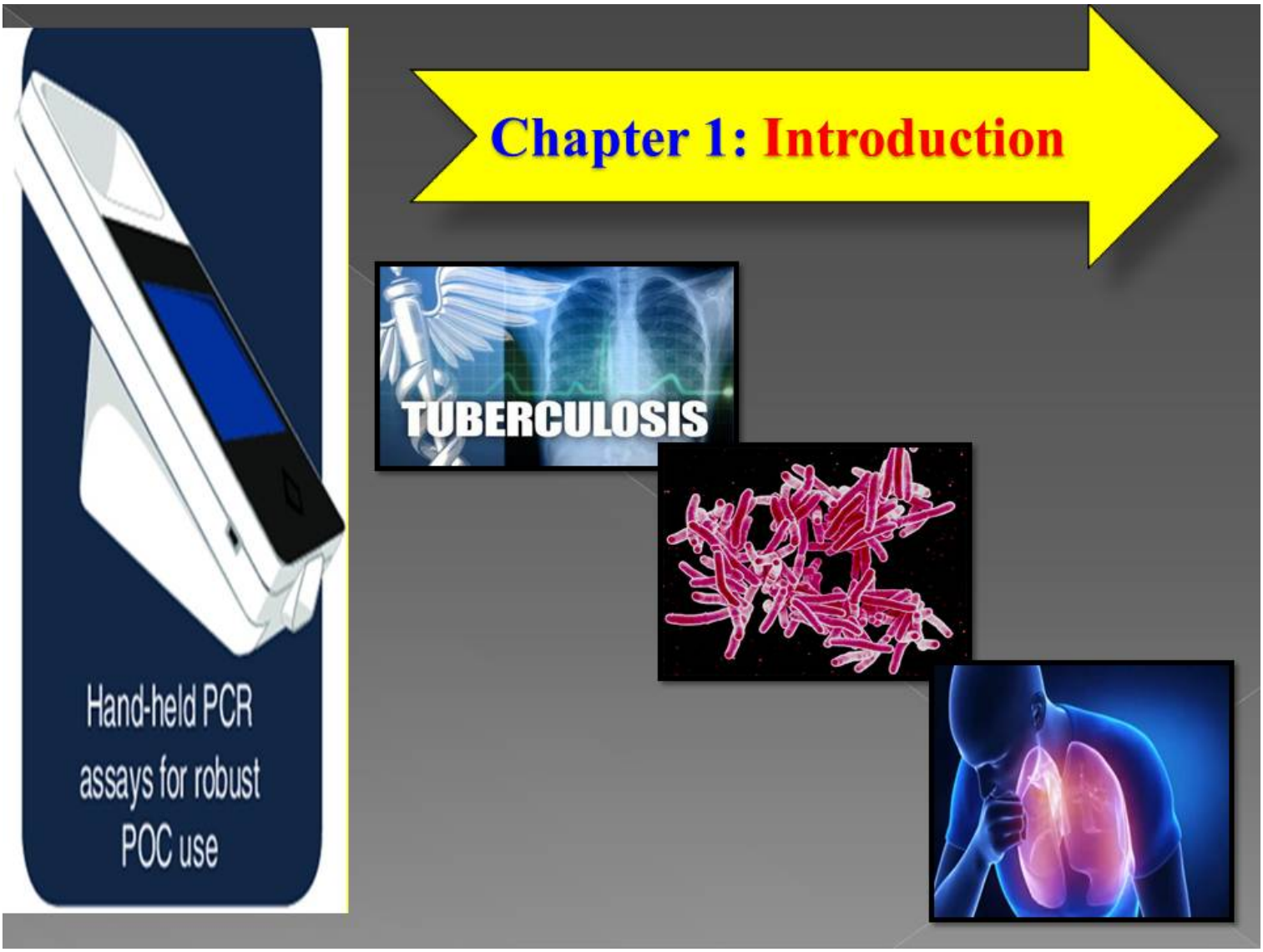
Chapter 1: Introduction
TUBERCULOSIS
Hand-held PCR assays for robust POC use

1.1 Introduction

Tuberculosis (TB) is the disease caused by *Mycobacterium tuberculosis bacilli (MTB)*. TB has existed for millennia and remains a major global health problem [1]. According to the latest global TB report an estimated 10.4 million people developed TB and 1.9 million died from the disease in 2017 alone. The toll comprised 890,000 male, 480000 female and 140,000 children. Globally 6.3 million new cases of TB were reported to world health organization (WHO), fewer than 61% of the estimated incidence of 10.4 million [2]. Which means globally 39% of latest cases have been either undiagnosed or were not reported. India has a high burden of TB cases; 15 million people suffer from TB in India of which over 3 million are infectious cases [3].

About 64 % of drug-sensitive TB patients were newly diagnosed and notified to national TB control programs. Still, this leaves about 3 million TB cases undiagnosed. An estimated 22 million lives saved through the use of directly observed treatment (DOTS) and the 'Stop TB strategy' recommended by WHO [4]. Most of the conventional methods used for detecting *Mycobacterium tuberculosis* bacilli (MTB) depend on microscopic sputum smear examination and culture techniques, which involve tedious processes, requires quality of the pulmonary sputum smear and skill of the pathologist and takes more time to produce the results varying from several days to months [5].

The present MTB detection methods involve smear microscopy, culture and molecular techniques. Ziehl-Neelsen (ZN) sputum staining relies upon on the ability of MTB to resist acid de-colorization for its excessive mycolic acid content. It depends on visual microscopic examination. This method cannot detect some early and mid-infections. The gold standard culture for MTB detection method taken into consideration with the same old approach. However, it takes 6 to 8 weeks to confirm TB on (L J) Lowenstein Jensen solid media [6].

Further, these conventional methods are less sensitive which can detect only half of the active MTB. The conventional method for detection of MTB still depends on acid fast bacilli (AFB) staining method and requires 5-10 x 10^3 bacilli/ml for detection of MTB. In addition, phenotypic identification such as culture and biochemical study, include, colony morphology, pigment production, urease test, niacin test, nitrate reduction test, catalase activity, pyrazinamide test and growth in the presence of p-Nitrobenzoic acid [6]. Although molecular detection methods show

high sensitivity and specificity, they are expensive, time consuming, require sophisticated laboratory infrastructure and highly trained personnel. None of the commercially available TB tests are not qualified as a perfect identification in MTB diagnostic methods [7].

Stop TB program identifies two key challenges to address TB diagnosis. The first challenge is improvement of current detection methods with a focus on developing sensitive and specific point of care testing (POCT) devices which could significantly contribute to TB detection. These devices are easily used, rapid, affordable, non-instrument dependent and require small volumes of reagents and samples. Diagnostics in patient's local vicinity renders patient compliance [8].

In this scenario, a new approach for rapid, safe and reproducible identification of MTB infection is real-time polymerase chain reaction (RT-PCR). The success of final amplification and detection of Nucleic Acid Amplification Test (NAAT) which depends on successful DNA extraction from pulmonary sputum samples [9].

We report a superparamagnetic iron oxide nanoparticle (SPION) mediated MTB DNA extraction from positive sputum samples. It proves to be a rapid, cheap and done in a single test tube, without any toxic chemicals and enzyme substances. Magnetic adsorption technique is used for isolation of DNA from pulmonary sputum samples. The extracted MTB DNA samples from this technique are worthy for real-time PCR detection. Thus development of higher and extensive accessible diagnostic test would greatly contribute to the management of the many infectious diseases, notably in limited-resource settings [10, 11].

Another goal is the need for rule out the complex tests. "Rule out diagnosis" is required for screening and epidemiology purposes and reducing number of patients who require complex investigations, thus leading to best allocation of medical resources. The best diagnostic approach for detection of the MTB must be quick results with a low detection limit. It should detect MTB in early infection stages. It needs to be specific to detect the *M. tuberculosis* complex species (MTBC). Besides, it should generate fast results to allow prompt initiation of treatment. The assay should not involve complicated procedures and expensive equipment. In our lab we have developed colorimetric gold nanobiosensor for early detection of TB [12].

The unmodified spherical gold nanoparticle (AuNPs) were used to detect *Mycobacterium tuberculosis* complex (MTBC) 16s rDNA gene sequence amplified from pulmonary sputum sample [13, 14]. The first step MTB PCR product is detected

and in second optimized version was used to directly detect genomic DNA in clinical specimens. The assay employs an oligonucleotides-target series in a conserved region in the 16s rRNA gene of TB genome [15]. TB diagnosis performance of the assay such as sensitivity, specificity and detection limit were calculated by RT-PCR [16].

1.2 History of *Mycobacterium tuberculosis*

Egyptian mummies of four thousand years old revealed that TB was a common infection in ancient Egypt. In ancient India the first references to tuberculosis in non- European civilization is found in the *Vedas*. The oldest of them (*Rigveda*, 1500 BC) calls the disease *yaksma* (Zysk 1998). It was also described by our ancestor, such as Assyrian and the Greeks.

MTB is believed to be a soil-living microorganism infecting humans after serial mutations. It is proposed that the spread of TB to Europe was caused by the animals of travelling merchants. In 1882, Prussian health practitioner Robert Koch utilized a new staining approach and applied it to the sputum of TB patients, revealing for the first time the causative agent of the MTB disease [17]. As a result of his ground breaking research on tuberculosis Koch received the Nobel Prize in 1905.

1.2.1 *Mycobacterium tuberculosis* bacteriology

MTB and other mycobacterium are biologically part of Gram positive group. MTB is an acid fast bacillus, straight or slightly curved rod of 0.2 to 0.8 microns in size occurring singly, in pairs, clumps or chains [18]. The cell wall of MTB consist lipid layer composed with mycolic acid, polysaccharides, peptidoglycan, plasma-membrane, lipoarabinomannan, phosphatidylinositol and mannoside. The MTB is comprised by a remarkably protein rich cell wall. The peptidoglycan complex of MTB is crucial and it maintains basal structure associated with outer layer of Myco-membrane; Lipoarabinomannan helps to link peptidoglycan with the external mycolic acid shown in Fig 1.1.

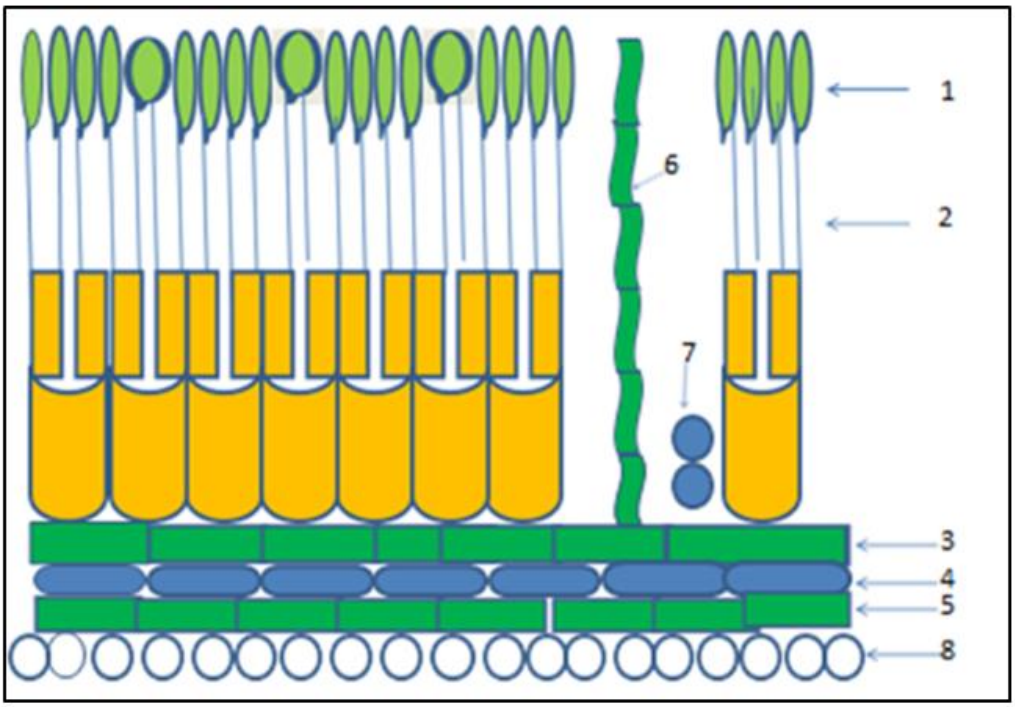

Fig.1.1 Schematic representation of *M. Tuberculosis* Cell wall: 1-Outer lipids, 2-Mycolic acid, 3- Polysaccharides, 4-Peptidoglycan, 5-Plasma membrane, 6-Lipoarabinomannan, 7-Phosphatidylinositol mannoside, 8-Cell membrane

1.2.2 Pathophysiology of *Mycobacterium tuberculosis*

TB is caused by the bacteria which is spread from person to person through the air. However, HIV reduces the sensitivity of TB microscopy because of immediate killing of TB bacilli by alveolar macrophages. This is achieved using different bactericidal mechanisms such as reactive nitrogen species and oxygen intermediates [19, 20]. However, the bacilli may survive in 25-50% of the infected individuals and continue to divide within macrophage cytoplasm [21]. The macrophages then present the Mycobacterial antigen to CD^{4+} lymphocytes, which become activated and initiate a cell-mediated response. The sensitized lymphocytes produce various cytokines such as TNF-α, IFN-γ, IL-6 and IL-12 which attract and activate more macrophages, enhancing their ability to kill the MTB [22].

Such activated macrophages become enlarged and differentiate into what are known as epitheliod macrophages to form a granuloma. The granuloma is a compact aggregate of epitheliod cells populated with many other cell types such as neutrophils, dendritic cells, natural killer cells and fibroblasts [23]. It is generally believed that the main purpose of the granuloma is to 'wall off' the bacteria in the host resulting in

containment or cure in 90% of individuals [24]. However, this view has recently been revisited and the granuloma is now thought to have a role in the dissemination of TB infection [25]. Considering that the granuloma is mainly a protective structure in the host, and that the CD $^{4+}$ lymphocyte plays an important role in the formation of the granuloma [26]. This results in poor containment of the MTB resulting in uncontrolled spread of the bacilli in the lung and elsewhere in the body. In addition, the poorly formed granuloma results into less cavitatory disease in the lungs. This results in less numbers of bacilli in the expectorated sputum. These numbers are often below the detection limit of MTB [27]. The pathophysiology of MTB schematic representation as shown is Fig.1.2

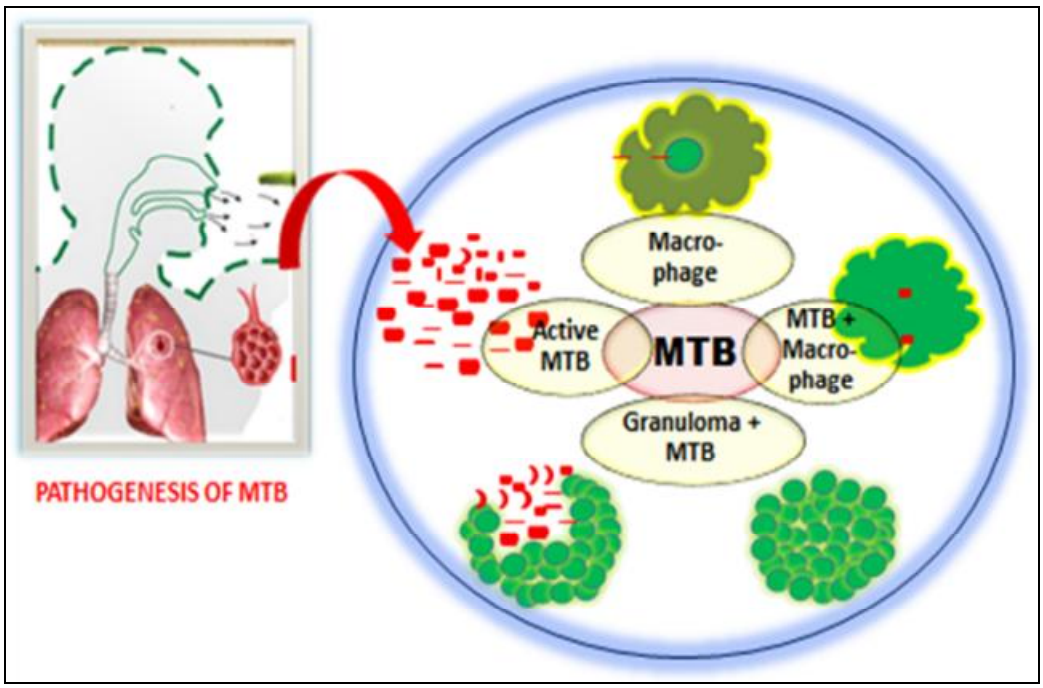

Fig. 1.2 Pathogenesis of *Mycobacterium tuberculosis*

1.2.3 *Mycobacterium tuberculosis* vaccine

Bacillus Calmette–Guerin (BCG) vaccine is largely used against MTB. The genetic differences in the populations, changes in environment, exposure to other bacterial infections, and lab culture conditions may affect the viability of tuberculosis vaccine. It also include genetic variations between the cell lines being cultured and the choice of growth medium [27].

1.2.4 *Mycobacterium tuberculosis* treatments

Anti-tuberculosis Drugs are the keystone of tuberculosis management. However TB drug therapy is long term as compared to other types of microbial infections. Anti TB therapy for active tuberculosis requires at least six to nine months. The precise TB drugs and timespan of TB management depend on age group and body location. Nationwide recommendation for anti TB treatment involves two stages- an initial stage and a second prolongation stage. The total period of anti TB management is 6-9 months [28]. Generally, for active TB drug resistant strain will be required multiple drugs at single time. Mainly Isoniazid, Rifampin, Ethambutol and Pyrazinamide are commonly used for anti TB therapy. In case of multidrug resistant TB infections fluoroquinolones injections such as amikacin is commonly used. New anti TB drugs with additional combination of Bedaquiline and linezolid may be used. Sputum Acid Fast Bacilli (AFB) microscopy is done recurrently to screen the anti TB drug response [29].The complete anti TB course of therapy exactly as prescribed by physician is important in this approach. Directly Observed Therapy (DOT), Centre is recommended by WHO.

Treatment regimens for Tuberculosis:

1. Intensive phased continuation phase: New smear positive, TB new smear negative, Pulmonary TB, New extra pulmonary TB. Seriously ill patients are treated with Ethambutol-Rifampicin, Isoniazid, Pyrazinamide 2 (EHRZ), 3 - (24 dose), 4 (HR) 3 (54 dose).
2. Sputum smear positive pulmonary relapses sputum smear positive treatment, failure cases sputum smear positive, cases treatment after default 2 (5 HER 2) 3+19 EHRZ) 3 (24+12 doses), 5 (HRE) 3 (66 doses).
3. New smear negative, pulmonary TB 2 (3HRZ2) 3+ (24 dose) 4 (HR) 3 (54 dose).

1.3 Overview of conventional MTB detection methods

All the traditional methods are less sensitive and require more sample volume. According to WHO, serological blood tests are not recommended due to false positive and false negative percentage of MTB detection tests. Culture methods are still considered gold standard but they take 3 to 4 weeks to identify TB [30]. The schematic representation of diagnostic methods for MTB detection is shown in Fig.1.3

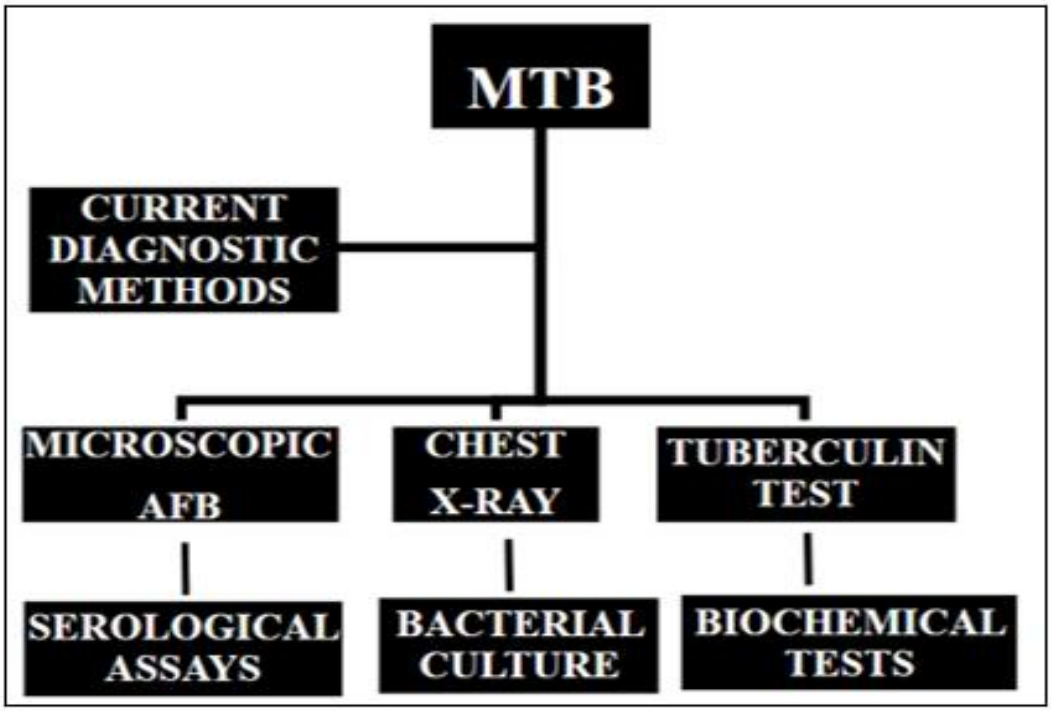

Fig.1.3 Schematic representation of MTB detection by conventional methods

1.3.1 Direct sputum smears Microscopy

Acid fast sputum microscopy was developed more than 100 years ago. This technique requires the examination of fresh 2-4 ml pulmonary sputum samples. The thick smear is prepared and stained by Ziehl Neelson method (ZN). This method requires trained medical technician for observation of acid fast bacilli under microscopic examination. The presence of red colour rod shape bacteria results Acid Fast (AF) positive sputum [30]. Currently, as per definitions endorsed by WHO, this test is still used for detection of smear-positive pulmonary TB and it is depicted in Fig 1.4.

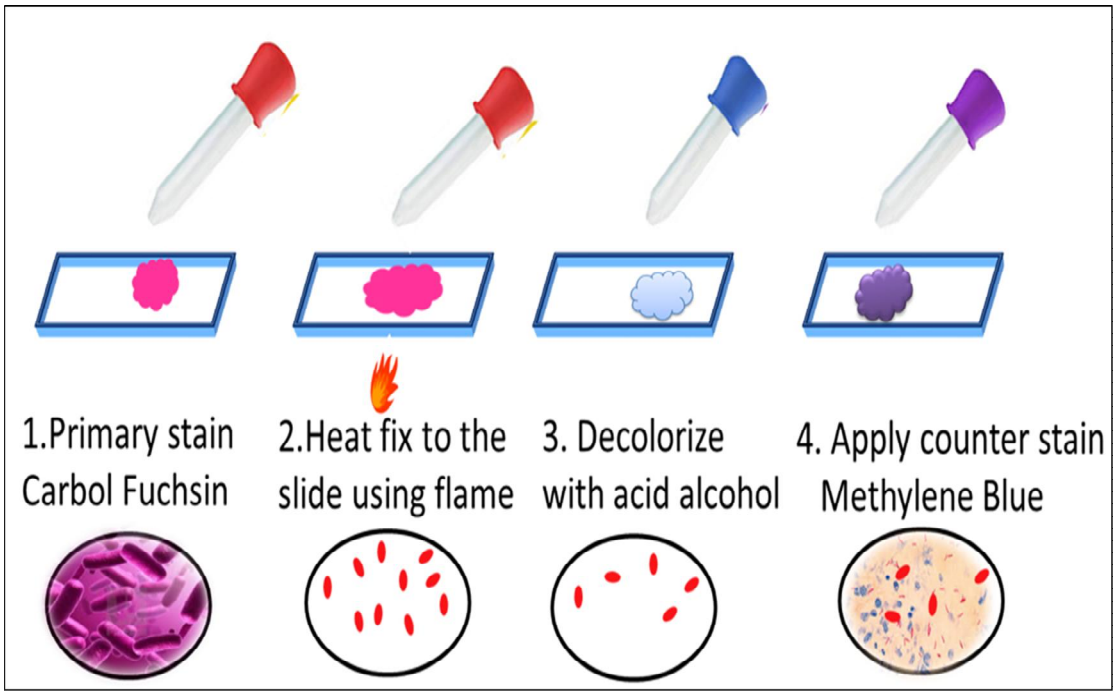

Fig. 1.4 Schematic representation of Acid fast staining microscopy of *Mycobacterium tuberculosis*

1.3.2 Culture-based methods

The culture method is considered 'the most accurate test' due to high sensitivity and specificity. It is labor-intensive and slow. The clinical laboratories hold cultures for 6 to 8 weeks to achieve maximum sensitivity on solid Lowenstein Jensen (L.J.) media. The liquid culture (BACTEC MGIT 960) is the most sensitive culture technique for recovery of MTB from clinical samples. The liquid culture is not currently utilized by all laboratories, particularly in low income countries, because of limited funds, inadequate number of trained or qualified personnel and proper biosafety management and availability of equipment. The existing reference standard methods require more advanced laboratory and can take up to 12 weeks to disburse reports [31].

1.4 Role of Nanotechnology in MTB detection

Nanotechnology is the control of matter at dimensions between 1 and 100 nm. This field is expected to play an important role in molecular diagnostics by enabling new methods based on the unique properties of nanometer scale materials. Nanomaterials display enhanced optical, thermal, magnetic, electrical and chemical behavior compared to bulk materials and single atoms or molecules of similar

composition. In addition, they interact differently with molecules in the same size range, such as proteins and nucleic acids. Thus nanomaterials may improve assay sensitivity, specificity, speed, affordability and portability [32]

1.4.1 Nanomaterials in MTB detection

The materials produced by nanotechnology include nanoparticles, bio-barcodes, dendrimers, nanowires, nano-cantilevers, nano pores and nanofluidic devices. Many of these structures can be functionalized with biorecognition molecules to create specific diagnostic probes. Nanoparticles such as quantum dots and gold nanoparticles produce distinct fluorescent or light scattering patterns, which are controlled by varying particle size. Thus, Nanomaterials are likely to play an important role in the development of early detection of MTB infection [33].

The wide range of nanoscale systems are being used for biomolecule assays like electrochemical and electromechanical nanoparticles based system, such as gold, silver, silica and quantum dots for MTB diagnosis. Because of their unique physical and chemical properties these nanomaterials offer greater sensitivity than conventional methods and can be easily functionalized by simple chemistry modulation and derivation. Many types of nanodevices have been developed for the MTB detection with combination of molecular biology. The different types of MTB diagnosis methods are shown in Fig 1.5.

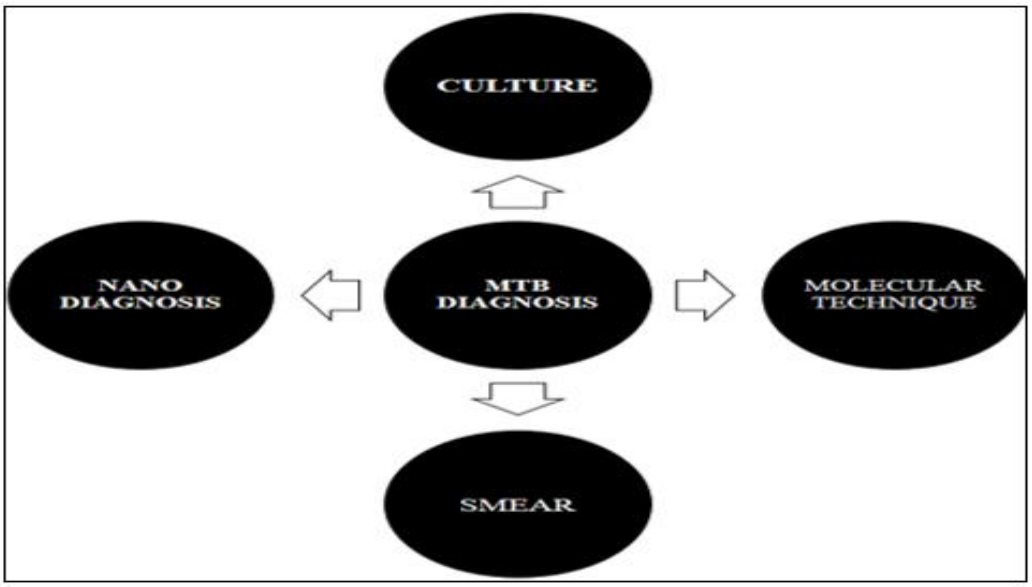

Fig.1.5 Different types of MTB detection methods

1.4.2 SPION mediated MTB detection

Iron oxide nanoparticles based MTB DNA extraction technique is applied for magnetic adsorption and detection of MTB. The SPIONs (super paramagnetic iron oxide nanoparticles) have emerged as promising candidate for various biomedical applications, such as contrast agent for MRI, targeted drug delivery, imaging hyperthermia, gene therapy, tracking molecule of stem cells, cellular tracking, magnetic separation technologies, rapid DNA sequencing. Further SPIONs are used for the early detection of infectious diseases, such as cancer, diabetes and atherosclerosis diagnosis [34]. The main advantages of SPIONs are easy to synthesize, provides a large surface area and do not interfere the structure and properties of DNA. Owing to their ultrafine size, biocompatibility and magnetic properties, SPIONs are helpful in magnetic DNA extraction and detection of MTB. SPIONs are practically used as a core surrounded by hydrophilic and biocompatible coating usually made up of polymer for magnetic adsorption of DNA [35]. A very similar MTB detection methodology is applied where microorganism are detected with the help of magnetic NPs with a giant Fe -core and a thin ferrite shell NPs, focused into a microfluidic chamber, and detected via nuclear magnetic resonance [36].The clinical utility of this diagnostic platform is evaluated by detecting TB bacilli are shown in Fig 1.6..

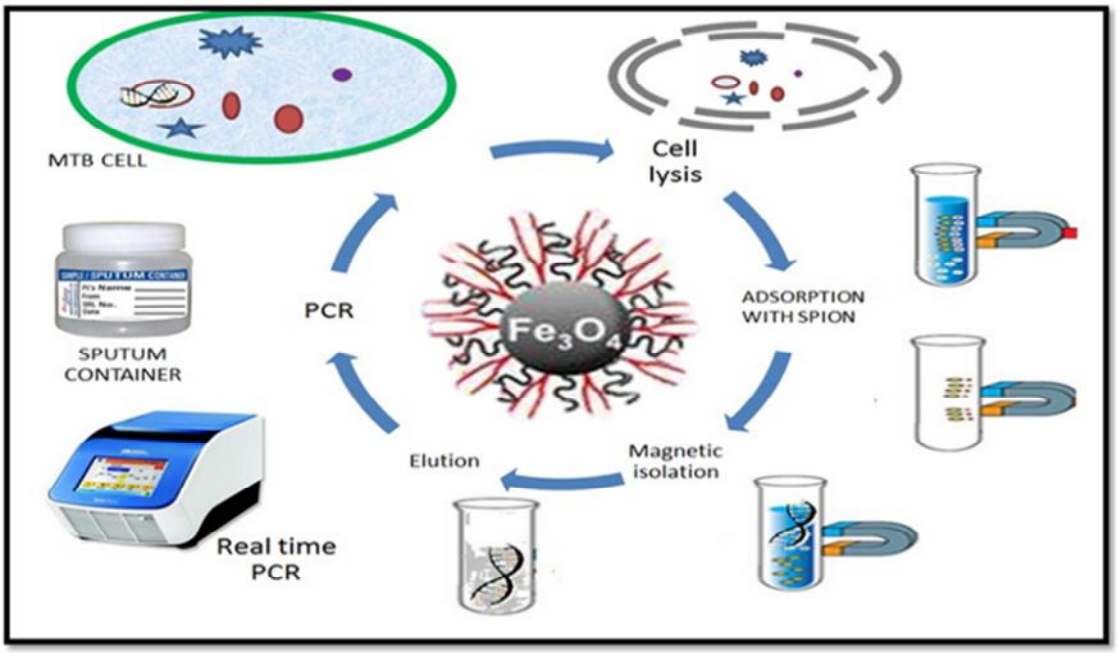

Fig.1.6 Schematic representation MTB detection by MNP methods.

1.4.3 Biosensors for MTB detection

Bioassays are the methods by which one may determine the absolute concentration of a specific biomolecule in a sample; the target molecule is called analyte. The device converts biological activity into quantifiable signal for MTB target detection. The biomolecules consist of a vast array of species molecules that exist within organisms including nucleic acids, proteins and fatty acids. Each species serves as specific purpose in an organism and interact with other biomolecules. A biosensor can be defined an independent analytical device, which is capable of providing specific detection of quantitative analytical data employing a biorecognition constituents like enzymes, DNA and antibodies [36].

Based on different sensing elements, the biosensors can be divided into immuno-sensor, DNA sensor, cell-based biosensor, aptasensor, enzyme-based sensor, and other combinations. DNA based biosensors typically depend on the immobilization of a single-strand DNA (ssDNA) probe onto a surface near its complementary DNA target sequence by using hybridization. Transduction of the DNA hybridization can be measured electronically, optically, electrochemically or by using mass-sensitive devices. Gold Nanoprobe for species specific detection of *Mycobacterium tuberculosis* complex using synthetic oligoprobes GP-1 and GP-2 from *IS6100* are designed specifically hybridize with DNA of MTBC and MTB strains. Some biosensors use mechanical forces and motion to report the amount of analyze present in a sample related to micro cantilevers. Microcantilevers (MEMS) sensors, give rise to charactertics mechanical vibration to recognize MTB [37].

'Magnetic nanoparticle with antibodies coupled detectors' for MTB bio-sensing are used for signal amplification in resolution for sandwich assays. A major advantage of solution phase assays is significantly faster assay times as compared with diffusion dependent assay. The diagnosis of MTB by magnetic resonance, by capture and detection of both agents are present in the solution and linked to magnetic particles for target MTB detection [38].

The optical transducers are widely used due to their high sensitivity with several well-established optical phenomena such as surface plasmon changes, scattering and interferometry. Surface plasmon resonance (SPR) is the excitation of an electro-magnetic wave propagating along the interface of two media with dielectric constants of opposite signs, such as metal and sample buffer, by a specific angle of incident light beam. The signal is based on total internal reflection that results in a

reduced intensity of the reflected light. The angle at which the resonance occurs is sensitive to any change at the interface, such as changes in refractive index or formation of a nanoscale film thickness due to surface molecular interactions Therefore, these optical colour changes can be measured colorimetric method for MTB diagnosis [39].

1.5 Statement of problem

Despite the advent of scientific advancements in recent years, tuberculosis still remains a public healthcare threat. The WHO has decided to eliminate the global TB burden by the year 2035.

To achieve this goal it is believed that nanomaterrials will have an increasingly promising role to develop better MTB diagnostics, prevention and treatments. Nanomaterials (e.g., gold nanoparticles, quantum dots, and magnetic nanoparticles) have proven to be sensitive and accurate for diagnostics with short turnaround time. However, these technologies rely on DNA extraction from sputum samples, which are difficult to be obtained from patients with improved symptoms.

In addition, the nanomaterials are still unable to provide quantitative diagnostic result, and thus they cannot be used to monitor the progress of MTB treatments.

A quantitative MTB sputum-based detection system without compromised sensitivity and accuracy would be a future direction for the development of nanomaterial- based effective diagnostics. The assay analyte should be meticulously selected respective to genome, proteome and phylogeny analysis of pathogenic MTBC members to guarantee assay convenience.

There is a rigorous need for large scale multi center testing in different epidemiologic contexts to evaluate reliability of assay performance in different regions. Sensitivity of MTB nanodiagnostic ought to be exploited more using non-sputum samples (e.g., blood, urine) where target biomarkers are too scanty to be detected by conventional diagnostics. There are number of limitations in detecting MTB like:

- MTB diagnostic landscape still lacks the ideal detection method.
- TB diagnostic test are not affordable in poor resource settings.
- MTB nanodiagnostics (nanobiosensor) detect DNA, antigen, antibody, interferon, or volatile organic compounds.

- Preliminary clinical performance characteristics of the developed nanoassays qualify them for a new era in MTB detection.
- We have planned to develop magnetic nanoparticle based MTB DNA extraction method for downstream application of RT-PCR.

In an attempt to improve these shortcomings, we have planned to develop novel SPION mediated MTB DNA extraction and detection method for early detection of *Mycobacterium tuberculosis*.

Furthermore, MTB DNA based colorimetric nanobiosensor is planned to fabricate using gold nanoparticle. This innovative colorimetric device shows better results in tertiary care centre. The positive facets of gold nanoprobe are excellent biocompatibility and chemical inertness for early detection of MTB DNA target.

The current work is subjected to following objectives:

¤ Synthesis of Fe_3O_4 NPs by Co-precipitation method

¤ Characterization of Fe_3O_4 NPs.

¤ SPION mediated MTB DNA extraction and detection with real time PCR.

¤ Development of colorimetric MTB DNA nanobiosensor.

¤ The colorimetric MTB nanobiosensor with quantitative analysis by UV-visible spectroscopy and cyclic voltammetry for early detection of MTB.

Taking into account the above mentioned objectives, the plan for fabrication of colorimetric gold nanobiosensor was accomplished via two means: colorimetric detection of MTB DNA target *IS-6100* from pulmonary sputum samples and quantitative analysis of MTB DNA with UV-visible spectroscopy and cyclic voltammetry.

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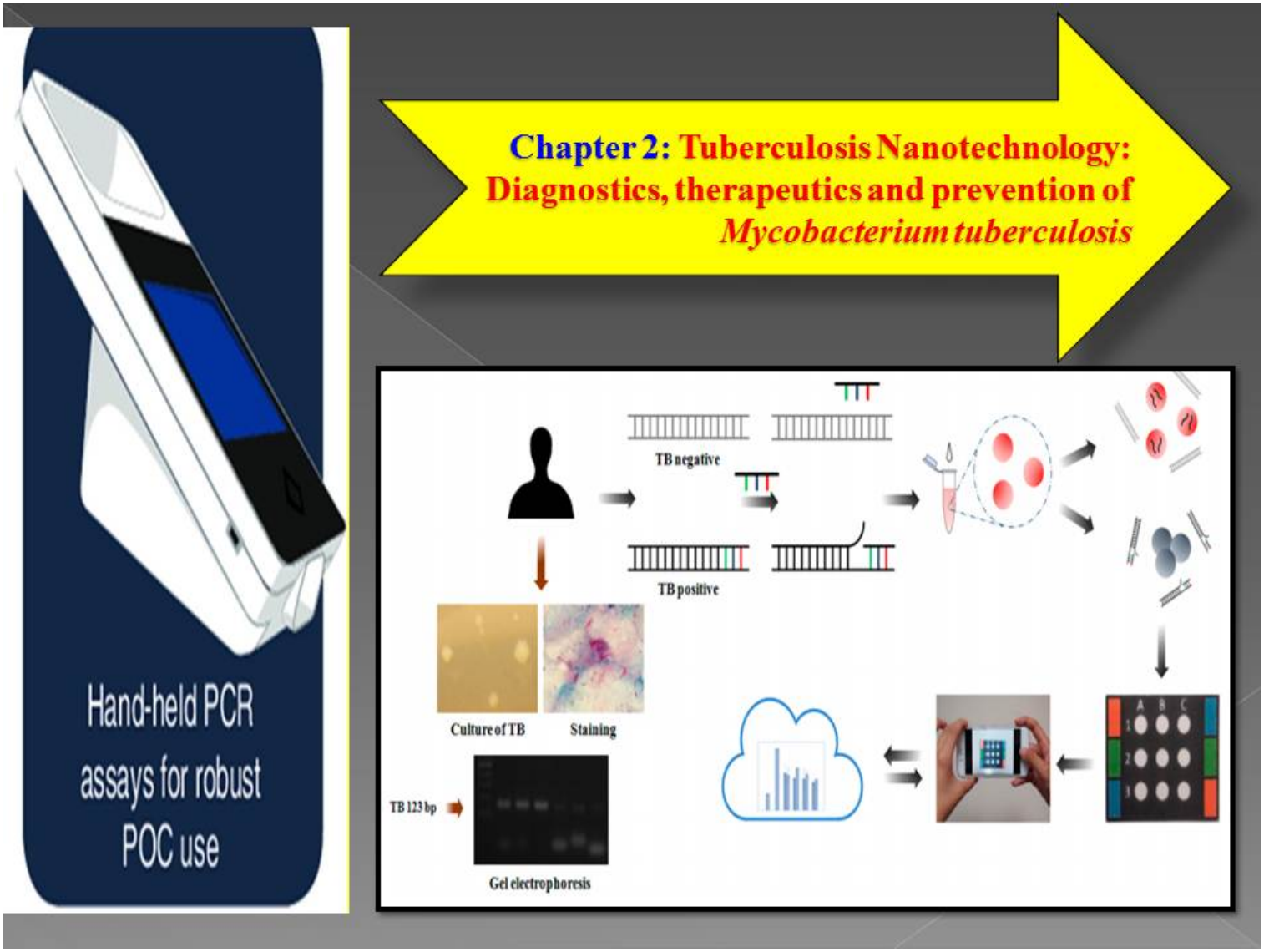
Hand-held PCR assays for robust POC use
Chapter 2: Tuberculosis Nanotechnology: Diagnostics, therapeutics and prevention of Mycobacterium tuberculosis
TB negative
TB positive
Culture of TB
Staining
TB 123 bp
Gel electrophoresis

2. 1 Introduction

According to World Health Organization report (WHO) 2017, 10 million people (range, 9.0–11.1 million) developed TB disease in which 5.8 million were men, 3.2 million women and 1.0 million children. This is considerably a large number attributed to the fact that delay in diagnosis is due to conventional method implementation. This means more the chances of spreading TB infection from person to person via the airborne route. When an untreated individual with active TB coughs, droplet nuclei containing the TB bacillus are expelled into the air, mostly inhaled by others in close proximity. Nontuberculosis mycobacterial infections (NTM) have increased in recent years, particularly in patients with chronic pulmonary diseases. In this study, the diagnostic microbiological methods of TB are reviewed [1].

The innovative nanodiagnostic technologies like the nanoscale visual image, nanoparticle biolabels, biochips, microarrays and nanoparticle primarily based on macromolecule and nano-proteomics medicine are recently used. The Bio-barcode assay, nanopore technology, DNA nanobiosensor, nanomachines and nanoparticle constructed bioassay unit are extremely popular within the field of medicine [2]. In addition, they interact differently with molecules in the same size range, such as proteins and nucleic acids. Thus nanomaterials may improve assay sensitivity, specificity, speed, affordability, portability and they are easy to use [3].

The mainstay of diagnosis has been microscopy and X-ray, both these tests have limitations which is a tedious process and required skilled persons to handle the test. It is fast and used heavily in poor-resource countries for TB diagnosis and monitoring the patients receiving anti-tuberculosis antibiotics. X-ray allows detection of MTB in resources limited settings and are recommended by the WHO. Chest X-ray (CXR) is the commonly used method to find TB, but it is done in conjunction with tuberculin skin test (TST). Latest study results have shown that chest radiographs are the ideal methodology for finding pediatric tuberculosis. Bacterial culture and drug sensitivity test method is still a gold standard method for diagnosis of MTB. Several solid culture media are used for isolation, identification, species differentiation and drug-sensitive test (DST) as Lowenstein-Jensen (L J), middle brook 7H10 and blood agar. The MTB is slow growing bacteria, it requires minimum 2 and 6 weeks for growth in liquid and solid culture medium [4].

Molecular-based diagnosis techniques are sensitive to detect MTB but it requires a large infrastructure and a big investment. Identification with Line Probe Assay methods (LPA) are based on the specific amplification and subsequent hybridization in solid phase with immobilized genetic probes on nitrocellulose strips [5]. Hybridization becomes evident using an enzymatic process. Different commercial tests available in market are Inno-Lipa Mycobacteria (Innogenetics, Ghent, Belgium) and GT Mycobacterium (HAIN Life science, Nehren, Germany) [6]. The former test carries out amplification of the intergenic16S-23S space and then hybridizes on a nitrocellulose strip which has 22 probes to enable the identification of the MTBC [7] and another 16 NTM, of clinical relevance. DNA microarrays depends on determining sequences within the 16s rRNA by fluorescence-labeled probes. The intensity of the fluorescence is proportional to the hybridization pattern and is measured by fluorescent microscopy and thus the results are made available within a few hours. The traditional TB diagnosis methods are less sensitive, delay in TB diagnosis and it requires Microbiologist [8]. The traditional TB diagnosis methods are listed in table 2.1.

Table No.2.1 Traditional TB Diagnosis methods

Test	Method	Description	Main strengths	Main weaknesses	Ref.
Immuno assays	Tuberculin skin Test (Montoux)	Immunological skin response to tuberculin	Extensive practical and published experience	Low sensitivity with immune compromised cross-reaction with BCG vaccine	84
Inter-feron-γ	Detection of released Interferon-γ to identify active MTBC infection	Highly specific	Relatively fast	Moderate training, consumables dependent imperfect sensitivity	85
Digital Radio-graph	X-ray Chest Digital radiography	Detection of abnormalities in pulmonary radiographies	Indications and use not restricted to TB	Low specificity; low sensitivity; requires equipment and trained interpreter	86
Smear micro-	Ziehl-Neelsen	Detect TB bacteria by	Rapid	low consumables	87

scope	(AFB)	common Fluorescent microscopy			
Culture	Solid culture	Detect TB bacteria (manual or automated) and screen drug susceptibility	Good sensitivity; low equipment	Slow	88
Culture	Liquid culture	High sensitivity; faster than solid culture	Slow;	infrastructure and consumables dependent	89
NAAT	Line probe assays	Strip tests, detect MTBC and assess INH and RIF resistance	Rapid	Moderate training expensive	89
Auto Systems	Automated processing, detect MTBC and assess RIF resistance	Rapid	No training required	Expensive; equipment and consumables dependent	90

In this chapter, our main aim is to provide an overview of the various nanomaterials that have been used in TB diagnosis which include Nano-biosensors. It is a growing field of interest and upgraded techniques become available to meet the demands for increased sensitivity and for analysis of low quantities of biological material. Nanostructure materials reveal exciting properties such as a large surface to-volume ratio, high surface reaction activity, high catalytic efficiency and strong adsorption ability that make them potential applicant resources to play a vital role in the fabrication of a nano-biosensor. The combination of nanomaterials which conjugate mainly gold, silver, magnetic nanoparticles and Quantum dots accelerates signal transduction, which can be amplified by several orders of magnitude. The increase in sensitivity and flexibility offer many benefits over conventional TB diagnostic techniques. A dormant state seems to be a critical component of the MTBC life cycle and a frequent reason for treatment failure or relapse, thus presenting an important challenge for improving TB therapeutic, and prevention. The future of nanomaterials-based TB research will also be discussed as we seek to develop better

diagnostic, therapeutic and prevention tools as arsenals in our fight against this global TB epidemic.

2.2 Tuberculosis Nanotechnology: MTB detection

Nanotechnology in diagnostic applications has been appreciably studied to fulfill the requirements of medical diagnostics with high sensitivity and earlier detection of various infections. The beauty of nanodiagnostic is it requires very less amount of volumes of clinical samples from patients [9]. Nanoparticles offer the opportunity to detect MTB infection in patients quickly and at an affordable cost. Nanotechnology-based TB diagnostic commercial kits, which is now in the clinical trials stage, these commercial kits don't need expert technicians. However, the most promising application of nanotechnology will be in the field of point- of- care (POC) diagnostics, which will qualify the primary care physician and patients to perform assay at their respective settings. Presently, nanotechnology-based tuberculosis diagnosis is highlighted to reduce the gap between conventional and novel nanoparticle-based methods by Wang [10, 11].

2.2.1 Nanoparticle-based MTB detection

The next generation of TB diagnostics need to be more precise, quick and economical; nanomaterials has the potential to navigate this line of research in the right direction [12, 13]. Functional nanomaterials has recently been developed as promising tools for sensitive, fast and accurate detection of various toxins and diseases-related analytes [14]. The materials produced by nanotechnology includes nanoparticles, bio-barcodes, dendrimers, nanowires, nano-cantilevers, nanopores and nanofluidic devices [15, 16]. Many of these structures can be functionalized with biorecognition of molecules to create specific diagnostic probes. Nanoparticles such as quantum dots and gold nanoparticles produce distinct fluorescent or light scattering patterns, which can be controlled by varying particle size. Thus the nanomaterials are likely to play an increasing role in early disease detection. The wide range of nanoscale systems being used for biomolecule assays. Nanoparticle-based electrochemical system, such as gold, silver, silica and quantum dots (QDS) has been most widely used for MTB diagnosis. Owing to their unique physical and chemical properties, that offer greater sensitivity than conventional methods reported [16]. The nanomaterials-based TB diagnosis methods are listed in Table No. 2.2. In addition, these can be easily tuned and functioned by simple chemistry of modulation and derivation.

Table No. 2.2 Nanomaterials based MTB diagnosis

Nanomaterials	Applications	Detection limit	Description	Ref.
Noble metal NPs (AuNPs)	*M. tuberculosis complex*, *M. avium complex, M. avium subsp. paratuberculosis, M. bovis*	20 pmol were prepared from PCR	Detection relies on the evaluation of SPR change upon aggregation	37
Quantum Dots	*M. tuberculosis and M. avium subsp. paratuberculosis*	detection limit of the assay was defined to 10^4 bacteria/ml	Conjugation of streptavidin-coated QDs	31
Silica NPs	Tuberculosis	detection of as low as 3.5×10^3 and 3.0×10^4 cells ml	fluorescent silica NPs and SYBR Green	91
Electrochemical sensor	*M. Tuberculosis complex MTBC*	1.25 ng/Ml.	Dual probe SAM/ITO electrode	16
Portable OLED-based SPR biosensor	*M. Tuberculosis Complex MTBC*	63 pg/mL. PCR product	light-emitting diode-(OLED-) based surface Plasmon resonance (SPR) biosensor	45
Reduced graphene oxide–gold nanoparticles (rGO–AuNPs)	*M. Tuberculosis MTB*	1.0×10^{-15} and 1.0×10^{-9} M.	DNA based biosensor	92
Voltammetry biosensor CHIT-IO-GR/FTO electrode	MTB DNA	0.9 $fg{\cdot}mL^{-1}$	biotinylated DNA aptasensor	80
Paper base device using gold Nanoprobe	MTB DNA	1.95×10^{-2} ng/mL	DNA based label-free oligonucleotide	20
ITO electrode-gold nanoparticle modified streptavidin (SA)	MTB DNA	7.0 F.mol.	DNA based biosensor	93
DNA/CHIT-NanoZrO2/ITO bio-electrode	MTB DNA	0.00078 μM	DNA based biosensor	31

Gold nanoparticle Spots on paper Colorimetric detection	MTB DNA	2.6 nM	Paper base device	14
Probe–based colorimetric assay using gold nanoparticles	MTB DNA	0.75 µg	Paper base device	37
Plastic-chip-based magnetophoretic immunoassay using magnetic and gold nanoparticles	MTB ANTIBODIES	1.8 pg/ml	Plastic chip based immunoassay	65

2.2.2 Innovative techniques using MNPs for MTB detection

Magnetic nanoparticles (MNPs) have been utilized for magnetic capture of pathogens in clinical sample preparation giving aid to detection and identification [17], and are promising candidates for specific interaction usually capping with bio-recognition molecules such as antibody, aptamers and oligonucleotide probes, which allow for species-specific recognition and targeting. Due to their ultrafine size, magnetic properties and biocompatibility, superparamagnetic iron oxide nanoparticles (SPION) have emerged as promising candidate for various biomedical applications such as enhanced resolution contrast agent for MRI, targeted drug delivery and imaging hyperthermia, gene therapy, stem-cells tracking molecules, cellular tracking and magnetic separation technologies [18]. The bacteria which are nanometer-sized particles of magnetite or iron sulfide enclosed within the bacterial cells. Owing to their unique properties, super paramagnetic nanoparticles have been intensively developed and have found numerous applications in biomedical, optical and electronic fields. The rapid DNA sequencing, early detection of TB infection, cancer, diabetic, atherosclerosis, immunoassay and tissue repair are the best examples of magnetic nanoparticles. Iron oxide nanoparticles are suitably used as a core surrounded by a hydrophilic and biocompatible coating usually made up of the polymer [19].

Recently, Lee *et al.* developed similar methodology using magnetic NPs with a giant Fe -core and a thin ferrite shell NPs, focused into a microfluidic chamber, and detected micro-organisms via nuclear magnetic resonance [20, 21]. The clinical utility

of this diagnostic platform was evaluated by detecting TB using the Bacillus Calmette-Guérin (BCG) as a surrogate for *M. tuberculosis.* Since biomolecules and cellular organelles lie in the nano-size range, NPs can be modified with various biomolecules e.g., antibodies [22], nucleic acid [23], and peptides [24], to detect specific analytical functions: as their applications in biosensors. The schematic representation of the magnetic separation of MTB DNA is shown in Fig 2.1.

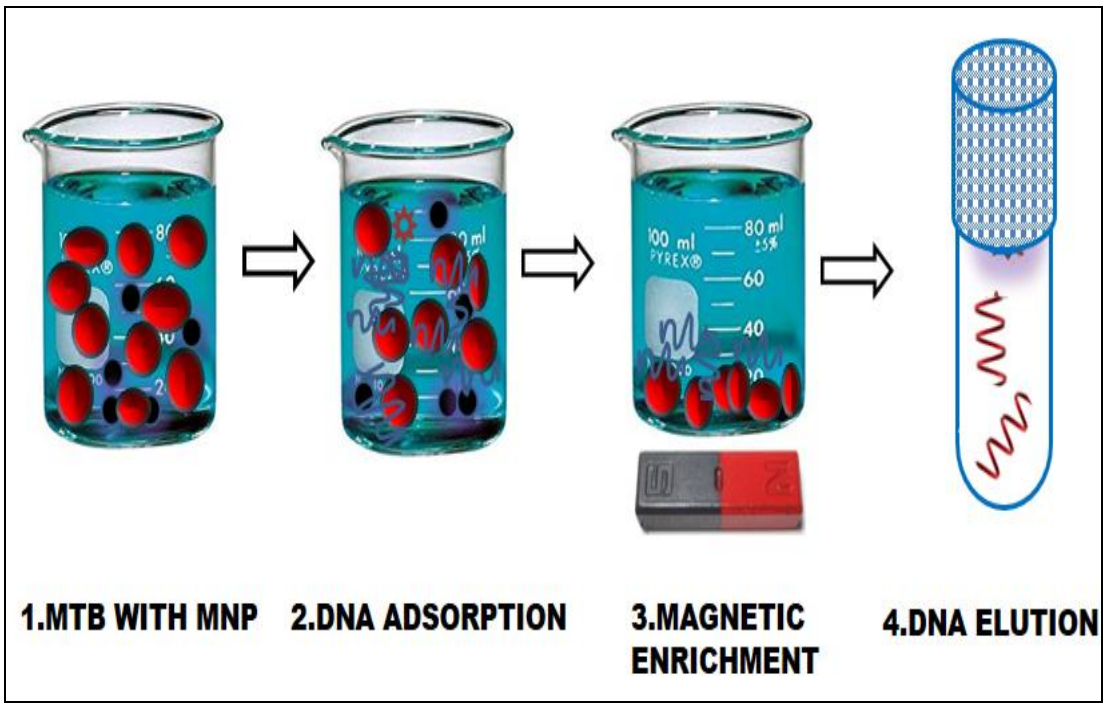

Fig.2.1 Schematic representation of the magnetic separation of MTB DNA

2.2.3 Silica nanoparticles for MTB detection

The capability of bioconjugated RuBpy-doped silica nanoparticles detect MTB from pulmonary sputum sample. Silica nanoparticle functionalized with ruby-doped, mouse monoclonal anti-*M. Tuberculosis* fluorescent antibody was conjugated to the agents in the sputum specimen. The primary amine group of the *M. tuberculosis* monoclonal antibody was reacted with the activated fluorescent silica nanoparticle, forming antibody-conjugated FSNP by Alireza Ekrami *et.al*. [24], through their study, showed that bio-conjugated silica nanoparticle gives the best result in detecting TB compared with conventional methods. The test results showed 97.1% sensitivity and 91.35% specificity in 152 sputum samples analyzed. These results are far superior to the AFB (acid-fast bacilli) test results (86% sensitivity and 84.9% specificity) and

nested PCR which gives only 86.9% sensitivity and 88.6% specificity. Also variable bacterial dilutions specified that 100 cfu units/ml of MTB detected by this method.

2.2.4 Metallic (gold) Nanoparticles for MTB detection

AuNPs are widely used due to their surface plasmon resonance (SPR) which results from the collective oscillation of the electrons of the conduction band [25]. This gives the solution red color and the absorption peak is at 520 nm for approximately 20 nm diameter of AuNPs. These methods depend on the change in the color and aggregation of AuNps [26]. Bio-conjugation of AuNps with thiol groups were tailored because of thiol bonding (-SH group), electrostatic interaction or covalent linkage [27, 28]. The diagrammatic representation *IS6110* MTB target sequence detection process of the SPR biosensor is shown in Fig. 2.2

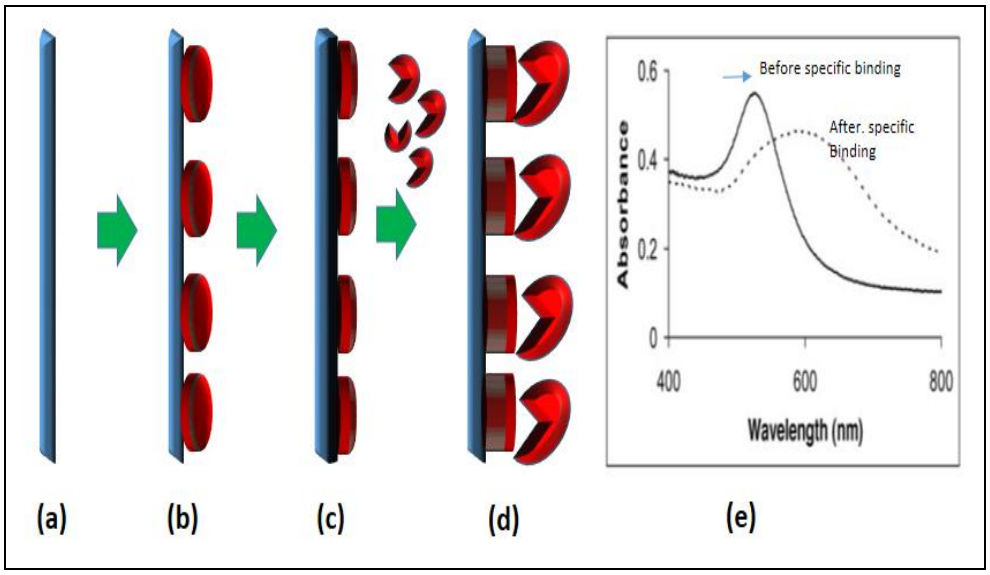

Fig 2.2 Diagrammatic representation of the MTB target sequence detection process of the SPR biosensor. (a) The bare gold nanoparticle, (b) Alkyl-thiol modified Oligo probe GP-1 and GP-2 (c) Gold nanoparticle bio-conjugation and (d) MTB *IS-6110* target detection and (e) UV-Vis spectra before and after specific bonding

The modified AuNPs were used to detect MTB and MTBC. If the target is present in the sample, the complementary sequences will anneal and AuNPs become free and aggregate in the presence of NaCl and change color from red to purple [29]. The AuNPs based assay detect MTB target of *IS6110* with amplicons of nested PCR. MTB DNA from clinical samples target rpoB region detect 0.75 µg according to Costa *et al.* [30]. Unamplified DNA was detected with a detection limit of 18.75 ng. by Das *et al.* [31] AuNPs based biosensor by depositing zirconium oxide linked to an

MTB with specific probe on a gold surface. This method detects 0.065 ng/µl of MTB DNA target. The schematic diagram of the proposed TB diagnosis methods illustrated below in Fig. 2.3.

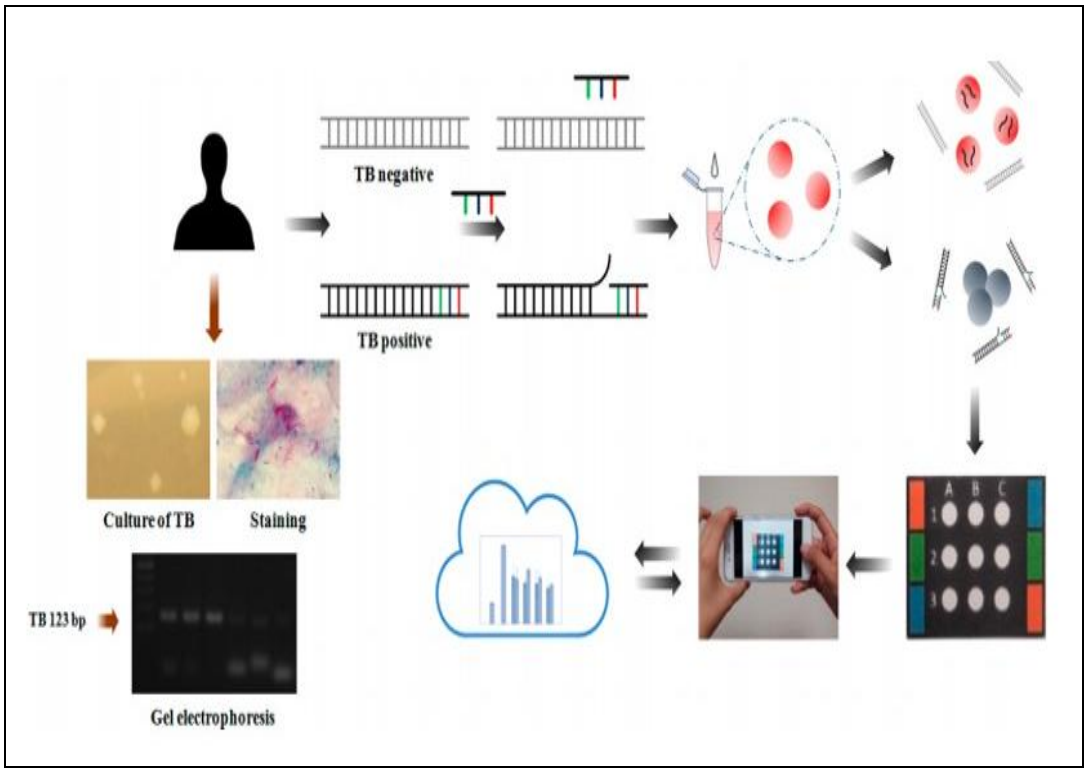

Fig. 2.3 Schematic illustration of the proposed TB Diagnostic Method, [Reprinted with permission of ACS, American Chemical Society, K. Sing *et al.* 2018]

2.2.5 Semiconductor Quantum dots for MTB detection

The semiconductor nanocrystals less than 10 nanometers were prepared for MTB detection by different researchers. The semiconductor nanocrystals are known as the quantum dots (QDs) which possess unique optical and physical property making them biocompatible. Functionalization of QDs with a wide range of biomolecules made them eligible for imaging, diagnostic and therapeutic purpose. QDs are good imaging agents provide additional information pertaining to the physiology and function, which enables more accurate and early disease diagnosis, with highly sensitive detection of early stage TB. Liandris *et al.* formulated a methodology to detect pathogenic TB using functionalized QDs with immunomagnetic separation [32].

2.3 Nano-biosensors for MTB detection

Nano-biosensors give speedy results with investigative yield. Biosensors is a device by which one may determine the absolute or relative concentration of a specific biomolecules in the sample. The target molecule is called analyze. The surface of the sensor may be bioconjugated with a targeting species that will interact only with analyze. Different types of nanomaterials, such as gold nanoparticles, carbon nanotube (CNTs), magnetic nanoparticles and quantum dots are being progressively use for biosensor because of their unique physical, chemical, mechanical and optical properties and distinctly improve the sensitivity and specificity of MTB detection. They have permitted the real-time multiplex detection of many diseases biomarkers at a very early stage [33]. The schematic illustration of functioning of nano-biosensor is shown in Fig 2.4.

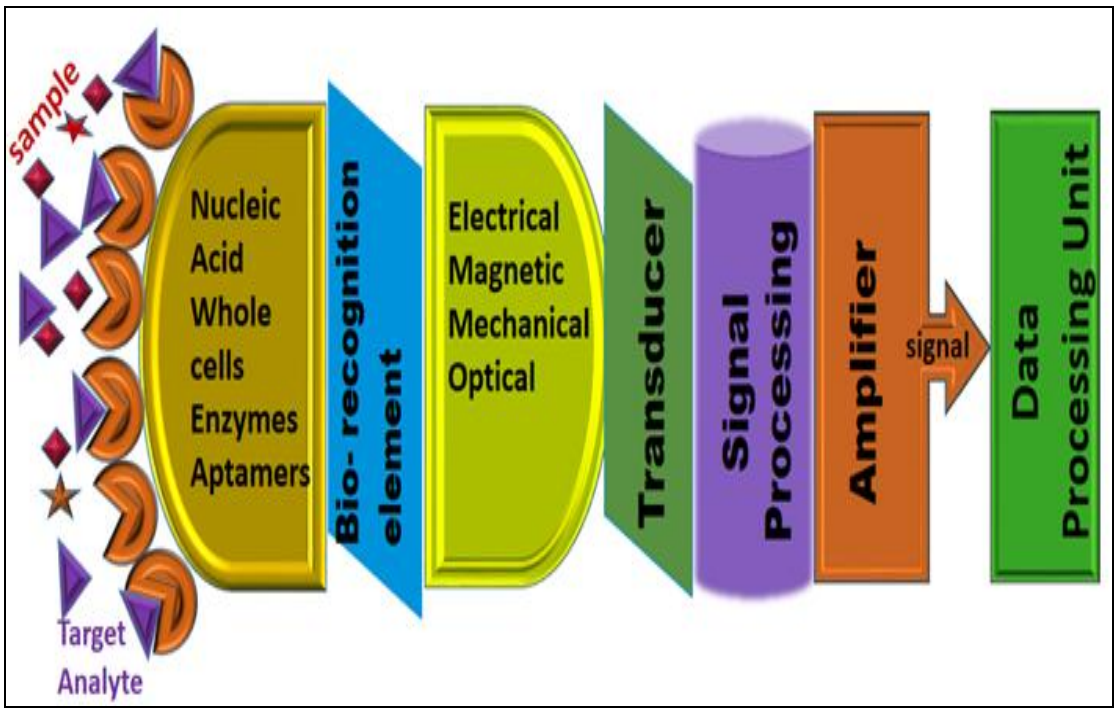

Fig 2.4 Schematic representation: functioning of nano-biosensor

2.3.1 Mechanical biosensor:

Some biosensors use mechanical forces and motion to report the amount of analyze present in a sample. The best example is Microcantilevers. The change in shape give rise to charactertic mechanical vibrations for detection of bacteria. The MEMS biosensor detect MTB use microcantilever sensor for enhanced biosensing of

MTB [34]. Measurement of mechanical forces produced on the cellular scale, such as movement and mass changes, provides essential information in biological systems at the nanoscale and micro. Mechanical biosensors have been broadly used in biosensing and binding studies, with measuring antibody-antigen interactions of MTB. Mechanical sensing strategies using cantilever-based and quartz crystal microbalance (QCM) based sensors detect MTB and related diseases

2.3.2 Electrical biosensor

Biosensor that measures electrical properties of a system change due to proximity or contact with analyzing have become widespread [35]. The gold Nanoprobe for species-specific detection of *Mycobacterium tuberculosis* complex developed by Po chi Soo *et al* [29]. GP-1 and GP-2 for *IS6100* and GP-3 and GP-4 for Rv3618 were designed to specifically hybridize with DNA of MTBC and MTB strains.

2.3.3 Optical biosensor

There are five types of biosensors having the principle of optical technology. These includes: Absorption [36], Reflection [37], Chemiluminescence [38], Fluorescence [39] and Phosphorescence [40]. The optical biosensors are a powerful alternatives to conventional analytical techniques because these are highly sensitive, reproducible, rapid and simple to operate [41]. The optical transducers are widely used due to their high sensitivity [42-44]. A highly sensitive optical biosensor based fiber-optic sensing technique has been presented by A. M. Pariwono *et al*. The processed sputum with pure DNA collection strategy has been explained with onsite portable diagnostic technique for MTB [46]. The optical sensor with SPR sensing technique detecting MTB with high sensitivity of 30 ng/ul.

2.3.4 Magnetic biosensor

Magnetic NPs-coupled detectors for bio-sensing can be used for signal amplification with the advantage that they are amenable to use in solution phase sandwich assays. A major advantage of solution phase assays is significantly faster assay times compared with diffusion-dependent surface structure-based assays. When an analyte of interest is present, the magnetic particles cluster as the antibodies bind the analyte [47]. Diagnostic Magnetic Resonance (DMR) based magnetic sensor has been described by Lee, Hakho for detecting the MTB cells in just thirty minutes from direct sputum sample with the detection limit of 20 cfu/ml [47-48].

2.4 Bacterial detection with the magnetic field

Rapid and real-time detection of bacteria using MNP with perfect and definitive bacterial identification and detection are essential for exact disease diagnosis, treatment of infection and trace-back of disease outbreaks associated with microbial infections. For this, innovative, rapid, sophisticated and highly sensitive detection methods are required [48]. Magneto response is a sense, which permits a bacterium to notice a magnetic field to perceive direction, altitude or location. This sensory modality is employed by a distance of animals for orientation and navigation, and as a technique for animals to develop regional maps. An unequivocal demonstration of the use of magnetic fields for orientation within an organism has been in a class of bacteria known as magnetotactic bacteria [49 50].

2.5 Tuberculosis nanotechnology: A therapeutic approach

The use of nanoparticles improves the bioavailability and pharmacokinetics of therapeutics. Nanocarriers take the drugs directly to the target sites of disease in the body by avoiding exposure of healthy tissues, which increases the availability of drugs at the target site and reduces the treatment dose. There are several novel diagnostic therapeutics and preventive measures being accessible to TB infection [51]. The use of the new TB drugs, Delaminid, Bedaquiline, Linezolid and Clofazimine are expected to play an important role in the treatment of tuberculosis.

Furthermore, the substantial requirement of new drugs is increased in this era due to the increase in the number of TB patients. As per WHO report published in August 2018, the pipeline for new anti-TB drugs is tabulated in Fig 2.5. The pipeline has expanded in recent months, and there are now 20 drugs in Phase I, II or III trials, compared with 17 in August 2017. Eleven new compounds (up by three since August 2017) Contezolid, Delpazolid, GSK-3036656, Macozinone, OPC-167832, Pretomanid, Q203, SQ109, Sutezolid, TBA-7371 and TBI-166.1 are at coming sooner in the market. Two other drugs (Bedaquiline and Delamanid) have already received accelerated or conditional regulatory approval based on Phase II B trial results [52-54]. Seven repurposed drugs are undergoing further testing: Clofazimine, Linezolid, Levofloxacin, Moxifloxacin, Nitazoxanide, Rifampicin (high dose) and Rifapentine [55].

Phase I a	Phase II a	Phase III a
■ Contezolid (MRX-1)b ■ GSK-303656b ■ Macozinone (PBTZ169)b ■ OPC-167832 ■ Q203b ■ TBA-7371b ■ TBI-166	■ Delpazolid (LCB01-0371) ■ SQ109 ■ Sutezolid (PNU-100480)b ■ Linezolid dose-ranging ■ Nitazoxanide ■ High dose rifampicin for DS-TB (PANACEA) ■ Bedaquiline and delamanid (ACTG A5343 DELIBERATE trial) ■ Bedaquiline – Pretomanid – Moxifloxacin – Pyrazinamide (BPaMZ) regimen ■ Bedaquiline and pretomanid with existing and re-purposed anti-TB drugs for MDR-TB (TB PRACTECAL Phase 2/3 trial) ■ Delamanid, linezolid, levofloxacin, and pyrazinamide for quinolone sensitive MDR-TB (MDR-END trial) ■ Levofloxacin with OBRc for MDR-TB (OPTI-Q)	■ Bedaquiline (TMC-207)b ■ Delamanid (OPC-67683)b ■ Pretomanid (PA-824) ■ Clofazimine ■ High dose rifampicin for treatment of DS-TB ■ Rifapentine for treatment of DS-TB ■ Bedaquiline – Pretomanid – Linezolid (NiX-TB trial) ■ Bedaquiline – Pretomanid – Linezolid (ZeNix trial) – Linezolid optimization ■ Bedaquiline with two optimised background regimens (oral, 9 months; with oral and injectables, 6 months) (STREAM trial) ■ Bedaquiline – Linezolid – Levofloxacin with OBRc for MDR-TB (NExT tiral) ■ Bedaquiline and delamanid with various existing regimens for MDR-TB and XDR-TB (endTB trial) ■ Pretomanid – Moxifloxacin – Pyrazinamide regimen (STAND trial) ■ Rifapentine – Moxifloxacin for treatment of DS-TB (TB Trial Consortium Study 31/A5349

Fig. 2.5 The global clinical development pipeline for new anti-TB drugs and regimens (WHO report 2018).

2.5.1 Tuberculosis: Current treatments and their limitations

Revised national tuberculosis control program (RNTCP) is an application of WHO recommended a strategy of directly observed therapy (DOTS). In India it is largely based on the research done at National tuberculosis institutes (NTI), Bangalore and Tuberculosis Research Centre, Chennai. National guideline treatment of tuberculosis consists of two-phase an initial phase and a second continuation phase. The total duration of treatment is 6-9 months. Early morning sputum AF- microscopy observation confirm to succeed 1st line anti-tuberculosis treatment with ethambutol-rifampicin, isoniazid and pyrazinamide [56, 59]. The second line drugs (WHO groups 2, 3 and 4) are only used to treat a disease that is resistant to first-line therapy, (i.e., for extensively drug-resistant tuberculosis (XDR-TB) or multidrug-resistant tuberculosis (MDR-TB). Third-line regimen contains rifabutin and macrolides, e.g., Clarithromycin (CLR), Linezolid (LZD), Thioacetazone (T), Thioridazine, Arginine, Vitamin D, and Bedaquiline [60, 63].

2.5.2 Nanomaterials in MTB drug delivery

Nanocarriers have been developed to address these deficiencies and improve TB drug delivery [64]. These nanoparticle-based drug delivery systems have been demonstrated to enhance *`in-vivo'* drug performance, with improved plasma membrane permeability, tissue selectivity [65] and even controlled drug release. The novel drugs by using nanocarrier may improve tuberculosis treatment [66]. Nanotechnology in the treatment of TB with better-quality sustained release profiles and bioavailability will increase compliance through reduced drug necessities and therein minimizing MDR-TB [67]. Chemotherapy of TB is complex due to the requirement of multidrug regimens that need to be administered over long periods [68, 69]. The underprivileged TB patient's compliance that the single commonest reason may be failure of TB therapy [70].

The microencapsulation of pharmaceutical substances in biodegradable polymers used in controlled drug delivery has seen as an evolving technology [71]. Transporter or carriage systems such as liposomes and microspheres have been developed for the sustained delivery of anti-TB drugs and have found better chemotherapeutic efficacy when investigated in animal models (e.g. mice). These carriers also can be intended to change controlled (sustained) drug release from the matrix [72-75]. Gelperina *et al.* summarize major data on nano-particulate formulations of the anti-TB drugs treatment. Sharma *et al*. conducted a study to explore lectin-functionalized polylactide-co-glycolide nanoparticles (PLG-NPs) as bio-adhesive drug carriers against tuberculosis (TB), in order to reduce the drug dosage frequency of anti-tubercular drugs and thus improve patient compliance in TB chemotherapy [76]. They conjointly determined that upon administration of uncoated PLG-NPs (oral/aerosolized) rifampicin was detectable in plasma for 4–6 days, whereas antibacterial drug and pyrazinamide were detectable for 8–9 days. Getting these results, they ended that WGA-functionalized PLG-NPs may be potential drug carriers for anti-tubercular medication through the aerosol route for effective TB management. Aerosolic nanoparticles are a suspension of nanoparticles in dry powdered aerosol form. They are used for the development of nanoparticles as prospective drug delivery molecules that can be inhaled and treat the successive pulmonary infections. TB, being one of such respiratory infections having multidrug resistance, can be therefore very efficiently be dispensed with by the use of

nanoparticles with much less systemic toxicity [77, 78]. The Fig 2.6 gives different nanotechnology-based approaches to target the MTB.

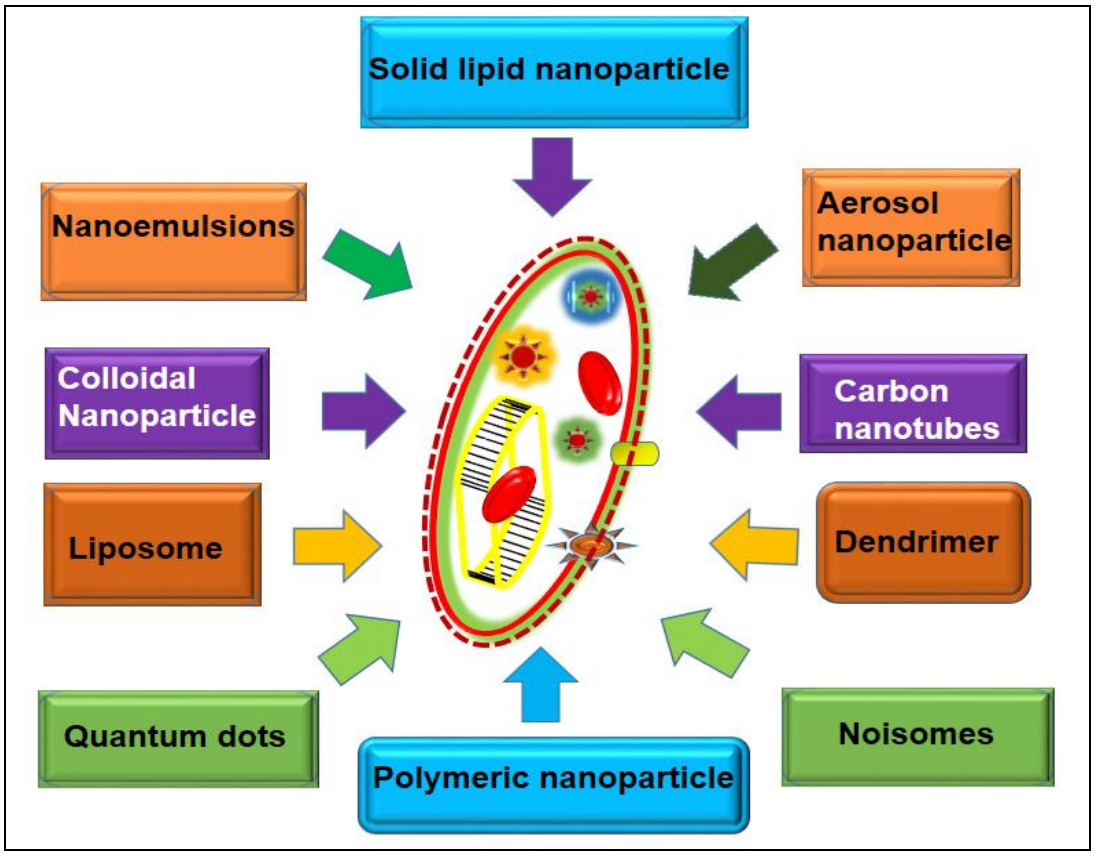

Fig 2.6. Different nanotechnology-based approaches to target the MTBC.

2.5.3 Nanotechnology in MTB vaccination

The vaccination is one of the most credible activities of prophylactic use. According global vaccine action plan (WHO), newborn BCG vaccination is partially effective at protecting children for TB disease. It is poorly defending against TB lung disease. No other single activity, has such an overwhelming effect on reducing mortality rates resulting from juvenile diseases [79]. Despite the discovery of improved vaccines and drugs for TB, about a third of the world and a half of world's immigratory population are still infected with TB. Although in recent years, the incidence has dropped significantly. A nanoparticle has the possible route to fill this clinical gap. They possess several fundamentally unique properties that simplify the induction of the robust immune responses that are needed in an ideal TB vaccine. For example, their nanosize allows these nanoparticles to pass through biological barriers to reach lymph nodes and they can be loaded with proteins and oligonucleotides. The various nanocarriers that are currently explored for use in TB vaccines include lipid,

polymeric, carbohydrate and amino acid nanoparticles as well as viral nanoparticulate vectors. Owing to aerodynamic limitations, the size of nanoparticles employed in inhaled TB vaccines is constrained within the 1–5 μm range [80]. The status of the pipeline for new TB vaccines as reported by WHO in August 2018, including the names of vaccine developers, is shown in Fig. 2.7. There are 12 vaccines in Phase I, II and III trials. It is seen that there are 12 vaccines including MTBVAC, AFC/BCO2, MVA85A, Ad5 Ag85A, RUTI, and H56:IC31, ID93+, RIBSP, Dar-901 Booster, M72/ASo1, VPM1002 and Vaccae.

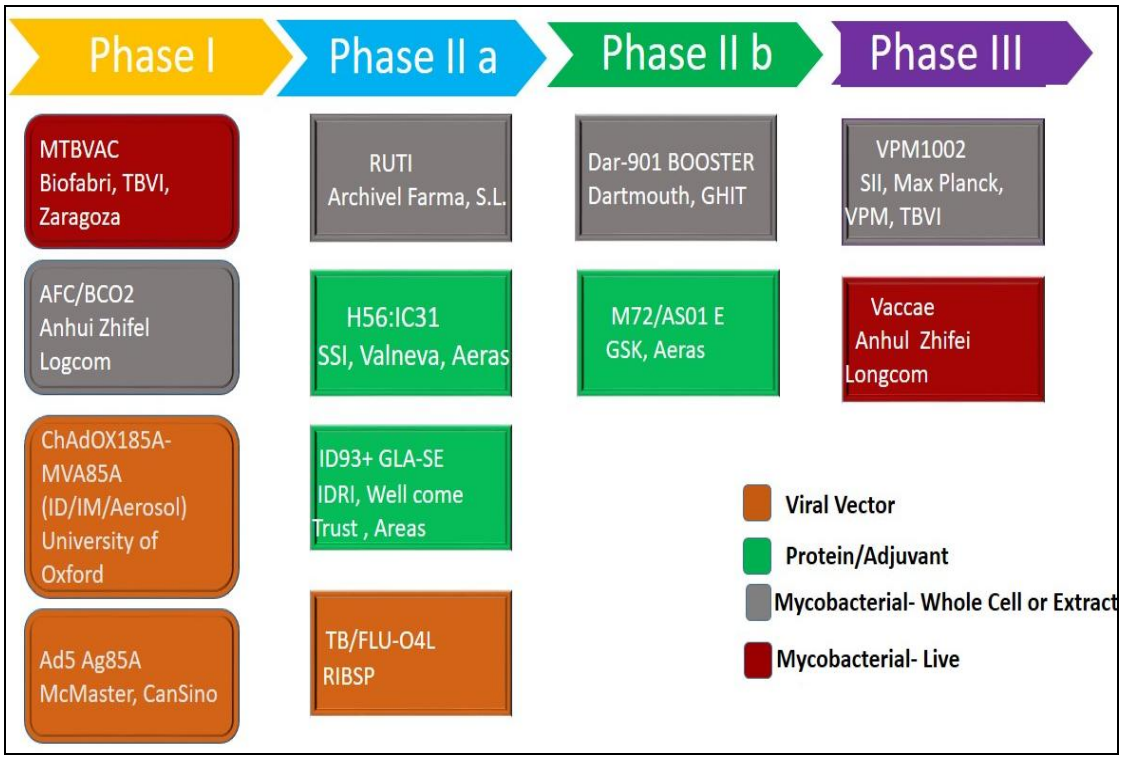

Fig. 2.7 The global development status of pipeline for new TB vaccines, WHO, 2018.

The chitosan nanoparticles increase the immune response afforded by TB vaccines. The synthesized PLGA-polyethyleneimine nanoparticles are conjugated to the TB antigen Rv1733c. When administered in mice, this novel vaccine stimulated T-cell proliferation and IFN- γ induction by (Bivaz-Benita *et al.*) The formulated synthetic TLR-4 agonist glucopyranosyl lipid adjuvant (GLA) into a stable oil-in-water nanoemulsion and subsequently cross-linked with this TB antigen ID 93 (Orr and Coler *et al.*). The polymeric nanoparticles as anti-TB drug carriers are able to enhance therapeutic efficacy with prolonged drug half-life [81]. A successful result of aerosol delivery using nanoparticle technology offers a potentially new platform for

immunization [82]. Guinea pigs, vaccinated with the aerosol treatment and subsequently exposed to TB, have found less than one percent of lung and spleen infection [83]. The new technique enables TB vaccines, and possibly other bacterial and viral-based vaccines to sidestep the traditional problems associated with keeping vaccines in cold storage [84]. Furthermore, a nanotechnology-based immunizing agent adjuvant for TB was developed by the U.S firm, Biosante, in 2002.

2.6 Tuberculosis Nanotechnology: Prevention of MTB

The challenges in the prevention of TB are prompt recognition, separation and provision of services, investigations for TB and referral patients with suspected TB patients [85-87]. Prevention of TB mainly based on screen, educate, separate, investigate, monitor and appraise. Early diagnosis with confirmed TB patients is the initiation within the hindrance of TB.

Tuberculosis control program of WHO has declared that TB is an international emergency, because TB is out of management in several components of the globe. The goals of the TB control program are to reduce mortality, morbidity, and disease transmission until TB no longer poses a threat to public health. The targets of the program are to cure 85% of the sputum smear-positive cases detected and to detect 70 % of existing cases of sputum smear-positive pulmonary MTB [88-91]. The successful TB control depends on health care workers treating TB patient with national TB program framework and full implementation of the DOTS strategies. This means assuring the correct diagnosing and effective treatment of all TB patients. The DOTS strategies may be a fruitful treatment for TB patient. This suggests that health worker follow-up the patient throughout the course of treatment and watches the patient swallow the tablets [92- 95]. The WHO has developed a new strategy, which builds on the success of DOTS and addresses unmet needs, for the global plan to stop TB (2006-2015). The goal to stop TB strategy was to dramatically reduce the global burden of TB by 2015 in line with the millennium development goals and the stop TB partnership targets. The researchers all over the world are working on application of nanotechnology for preventing the tuberculosis. TB is a communicable disease and is spread through air by droplet nuclei. The size of the MTB are in the range of micrometers (uM). In view of this the researchers are targeting the development of nano filters and making their use as mask. However there is a long way to go for finding the solution in this regard.

2.7. Summary and future prospects

Numerous methodologies and techniques have been developed for the diagnosis of MTB. All Nanotechnology based TB diagnostic tests have been tested using synthetic or PCR prepared molecules as targets. Few methods have been applied to the diagnosis of DNA directly in clinical samples and most of them involve real-time PCR. The Nano-biosensor platform used for the diagnosis of MTB DNA targets opens a possibility to target specific fragments of the *IS6110 rpoB* gene. The gold nanoparticle with synthetic probe GP-I and GP-II species-specific oligonucleotide are used for MTB target. This technique have potential for several specific probes identification. It can be labeled using additional metal nanoparticles for signal enhancement (cadmium, lead, zinc, etc.) besides gold nanoparticles. TB infection is leading health problems in the developing countries. The main aim is to find better early detection, therapeutic, and prevention of TB by manufacturing better and more effective short treatment with low toxicity drugs. Nanoparticle-based TB chemo-therapy has a better choice drug regimen and recover TB patients at an early stage. Nanoparticles are more feasible for oral and inhalation ways of drug management and TB Vaccination.

2.8 Conclusions

Most of the conventional methods available for TB detection are just an improvisation of older methods, which are half a century old. Bleaching methodology and optimization of culture techniques are increasing the detection rate but they failed to prove during clinical trials in large scale. New generation molecular nanotechnology-based diagnosis of MTB have been successfully applied in the diagnosis of infectious diseases. The perspectives of nanoparticle-based nanodevice and POC platform, magnetic barcode assay system, cell phone based microscopy platform, paper device, and different types of nano-biosensors for TB detection. The nanomaterials based TB drug delivery, nanotechnology in TB vaccination and finally prevention of MTB. Nanotechnology based TB diagnosis methods have significant advantages over traditional TB diagnosis. Therefore multidisciplinary effort is required from biological, engineered and clinical perspectives for the development of diagnostic, therapeutic and prevention of MTB.

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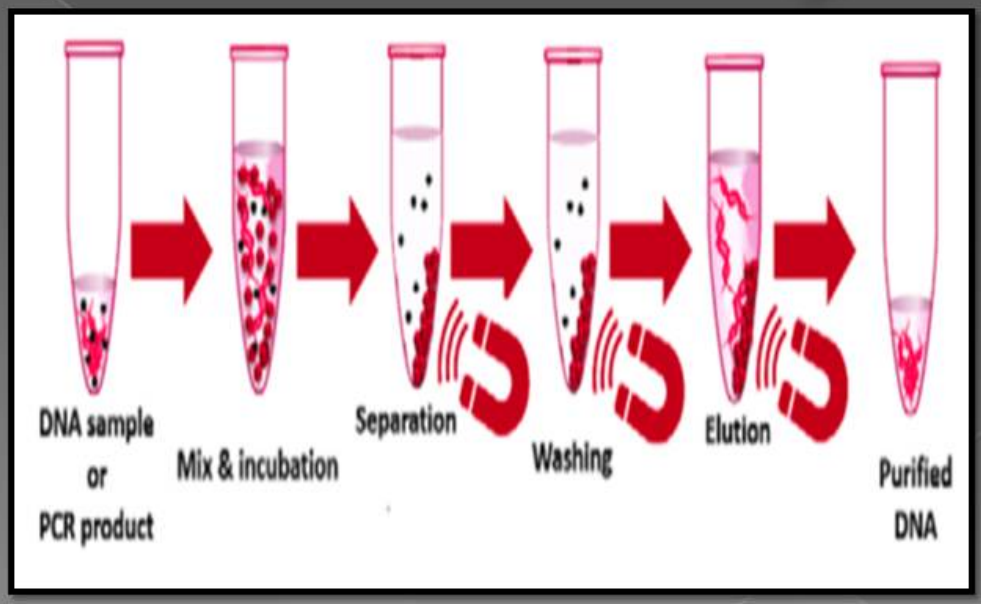
Chapter 3: Isolation of MTB from Clinical Samples



3.1 Introduction

Mycobacterium tuberculosis (TB) is a pathogenic bacteria. The isolation of TB bacteria is a critical process as compared to other bacteria. With all kinds of clinical samples, pulmonary as well as extra-pulmonary TB can be inoculated by primary isolation of *Mycobacterium*. Pulmonary sputum specimens are probable to infect micro-organism as normal flora. The sample pre-treatment is necessary for digesting (liquefaction) and decontamination for earlier inoculation for which a pellet is used for the preparation of AF smear and culture inoculation.

The sample pre-treatment (liquefaction and decontamination) is necessary for earlier inoculation for which a pellet is used for the preparation of AF smear and culture. A major step in the detection of TB PCR (Real Time Polymerase chain reaction) is the isolation of pure DNA (deoxyribonucleic acid) from clinical samples. Tissue and cell ingredients present in biological materials are often inhibitory to PCR, which may affect outcome [1].

Most of the isolation procedures have been established for sputum since it creates most collective and infectious biological specimens. The collection of sputum sample itself is a challenge, as its physical appearances may vary from containing abundant amounts of mucus to being purulent and hemoptysis [2]. The normally employed nucleic acid separation methods include the use of enzymes such as lysozyme and proteinase-K and cetyltrimethylammonium bromide (CTAB). The enzymatic action with organic solvents, detergent-induced lysis, or lysis using guanidinium isothiocyanate (GITC) are used in the extraction process. Another method by Chelex-100 resin is used for nucleic acid isolation [3].

There are several scientific methods available for analysis of biological samples for detection of MTB. The commercial kits have also been develop for analysis of MTB. These are based on isolation of pure DNA for detection with RT-PCR. However, they are overpriced than the conventional methods. The nation like India facing overprice of commercial molecular diagnostic kits. Therefore, there is a need for a simple, reproducible, and inexpensive MTB isolation method for mycobacterial isolation from all types of biological samples [4, 5].

All the existing methods differ in their quality and quantity of the extracted nucleic acid for downstream applications of PCR. Therefore the selection of a simple, reproducible, low-cost technique is truly needed in the field of molecular biology [6]. The source of material and the technique selected for isolation of DNA from MTB

will define the purity and yield of DNA [7]. The tough and complex cell wall structure that is impermeable and difficult to lyse which complicates purification process in comparison to that of bacteria with relatively fragile walls. The mycobacterial cell walls with full amount of polysaccharides, can adversely get influenced by subsequent manipulation of nucleic acid [8, 9].

Therefore the commonly employed method of isolating DNA yield are either low quantity (due to incomplete lysis of bacterial cell wall) or poor quality of mycobacterial DNA, resulting in low sensitivity of the test [10]. Various studies with different clinical specimens are reported a wide range of sensitivity and specificity of the PCR technique for diagnostic purposes. Pure MTB DNA is required for the downstream application in TB PCR diagnosis [11]. Thus in the present investigation we have made an attempt for isolation of MTB from clinical sample.

3.2 Experimental

Total four hundred sputum samples were collected from Dr. D. Y. Patil Hospital, and Research Centre Kolhapur, Miraj Wanless Hospital Miraj and C.P.R. Hospital Kolhapur. This experimental study was carried out in the department of Microbiology Dr. D. Y. Patil, Hospital and Research Centre, Kolhapur. Only AF-positive pulmonary sputum samples (one hundred twenty) processed and tested for TB, real time PCR, AF-stain, and culture method. This experimental study involved all clinical samples tested for tuberculosis from August 2016 to January 2017.

3.2.1 Materials and Methods

Materials:

Sodium chloride (NaCl), Tris- EDTA, sodium dodecyl sulfate (SDS), sodium hydroxide (NaOH), NALC (N-acetyl-L-cysteine) and polyethylene glycol (PEG, MW 8000), were purchased from Thomas Baker (India). Sodium chloride (NaCl), Tris-EDTA, sodium dodecyl sulfate (SDS), sodium hydroxide (NaOH) purchased from Molychem (India).

Ethics approval to conduct this study was granted by the Institutional Ethics Committee Permission was obtained after oral presentation at D. Y. Patil University, Kolhapur, 416006, Maharashtra, India (2016/44/PA-Ph. D).

Inclusion Criteria:

Criteria included clinically diagnosed and radiological documented new cases of pulmonary *Mycobacterium tuberculosis* of any gender and age group or untreated MTB cases, patients with anti-tuberculosis drug therapy in past 6 months.

Exclusion Criteria:

Criteria included patients with anti TB therapy or its constituent's drugs in the past 3 months. The control group was also studied from the patient who had a diagnosis other than pulmonary tuberculosis.

3.2.2 Experimental protocol

Methods: Cross-sectional studies were conducted on clinical and radiological suspected TB patients in the department of Microbiology at Dr. D. Y. Patil Hospital and Research Centre, Kolhapur, Miraj Wanless Hospital Miraj and C.P.R. Hospital Kolhapur. All samples received in the month of August 2016 to January 2017. The samples are scrutinized after AF microscopic examination.

3.2.3 Important safety precautions

All the techniques have been performed in the biosafety cabinet for sample processing, inoculum preparation, AF-smear preparation, making serial dilutions, sub culturing, inoculation of media. As per the recommendation of CDC, we have endorsed a Biosafety cabinet Level (BSL-2) laboratory with negative air pressure and with a suitable aeration system shown in Fig 3.1.

Fig 3.1 Biosafety cabinet: level 2 laboratory

Further as per recommendation of CDC the procedure, including handling of TB cultures, DST, has been carried out in a BSL- 3 laboratory. All the isolation procedure involved in isolation and handling of TB samples are carried out in a BSL-2 laboratory with an exhaust air flow where the bacteria are cleared by an outlet, the aeration is well- adjusted to run right directional air flow inside the room, entry to the chamber is restricted when lab procedure is in progress, and then applies all equipment's endorsed for BSL-3 were compulsory followed as listed in table no. 3.1.

We have compulsory used proper protected gloves, gowns, goggle and appropriate respiratory masks while handling MTB cultures. Proper care was taken of all international safety precautions. The selection of proper mycobacterial disinfectants was followed while cleaning the work area. All collected clinical samples are processed inside the Biosafety cabinet at level 2 laboratory as shown in Fig.3.1

Table 3.1. Clinical sample: partial bio-safety assessment

Method	Biosafety level required	Personal Protective care	Engineer control required	Special equipment required	Aerosol potential
Reading of Smears (Acid fast)	BSL 2	Gloves & Gown	Done on Bench top	-	Slight
Manipulation Of MTB Cultures for Identification	BSL 3	Gown, shoe Covers, lab goggle for eye Protection, Gloves and Respirator	Work process in Micro biology Safety cabinet	Use loops; Use racks to prevent Tipping, Work over Disinfectant-soaked Towel	Significant
Susceptibility testing of MTB	BSL 3 for setup BSL 2 for incubation and reading of Closed, Bottles Plates, Tubes	Setup - Gown, shoe covers, goggle for the eye protection, gloves Respirator paper cover	Work in biological safety cabinet to inoculate plates, bottles, tubes tubes with organism	Avoid aerosol generation when inoculating Tubes & plates	Significant

3.2.4 Study design:

Study design	Cross-sectional
Setting	Department of Microbiology, at Dr. D. Y. Patil Hospital and Research Centre, Kolhapur and tertiary care hospitals.
Specimen	Sputum
Sample size	400
Study Period	August 2016 to January 2017
Duration of study	6 months

3.3 Sputum sample collection and processing

3.3.1 Sputum sample collection

The specimens were collected in a clean and sterile containers with air tight-fitted cap. Instructions were given to the patient for proper collection of sputum and avoiding saliva and need of induced and deep expectorant sputum samples. Early morning minimum two samples were collected on two separate days with a volume of approximately 2-10 ml. In case of new registered patients three sputum samples were collected on sequential days and treated each sample separately [12]. The two disposable plastic airtight sterile containers were supplied to patients. A 50 ml screw cap plastic centrifuge tubes were used. Now a days these containers are available in the commercial market. The criteria of good quality sputum are shown in Fig 3.2.

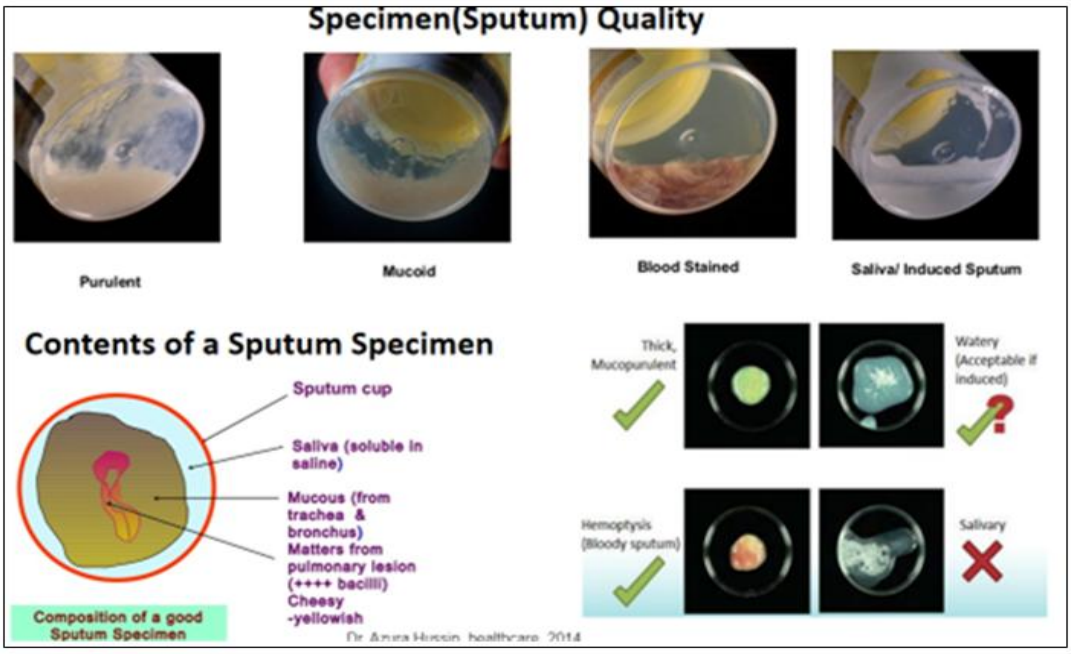


Fig 3.2 Criteria of acceptability of sputum sample

The quality of clinical specimens are important for TB lab diagnosis. The physical appearance of sputum were thick, purulent or blood stained with sufficient quantity of at least 3-5 ml. The upper respiratory tract secretion is originated from the lungs. The pediatric or older patients who are unable to produce sputum may require hypertonic saline. It is suggested that clinical specimens were to be transported to the microscopy laboratory as early as possible, but within 24 hours of collection it is required to keep in proper temperature and to avoid the growth of respiratory organisms.

Total four hundred pulmonary sputum samples were considered in the improvement and authentication parts of this research. The graphical representation of samples collected during study is shown in Fig 3.3. Which involved 120 sputum samples and 280 extra pulmonary clinical samples of human source namely, it consists of Blood (n = 02) Cerebral spinal fluid (CSF: n = 94), Urine (n = 23) pus (n = 32), Broncho alveolar lavage (BAL: n = 34), Fine needle aspirate (FNAC: n = 20), tissue biopsy (n = 37), pleural fluids (n = 38).

These clinical samples were collected from multi-specialty hospital. In our area there are many multi-specialty Hospitals located but we have mainly selected TB centers, including Wanles Hospital Miraj, C.P.R hospital Kolhapur, and Dr. D. Y. Patil Hospital and Research Centre, Kolhapur. The master chart of TB positive patients with sputum samples is given in table 3.4. From the table 3.4 it is seen that there are 60.83 % are male, 39.17 female AFB (acid fast bacilli) positive patients.

All pulmonary sputum samples (n-120) which are involved for acid fast staining, culture and Real Time PCR analysis. Out of two samples collected from each patient. The 1st sample is processed by conventional acid fast staining method, culture and Real Time PCR in the microbiology lab and the second container of sputum sample was used for MTB DNA isolation. Only pulmonary sputum samples are used in our study. Total four hundred samples collected and the highest number of sputum samples (n=120) are involved in this study. The graphical representation of all total samples during the study is shown in Fig. 3.3

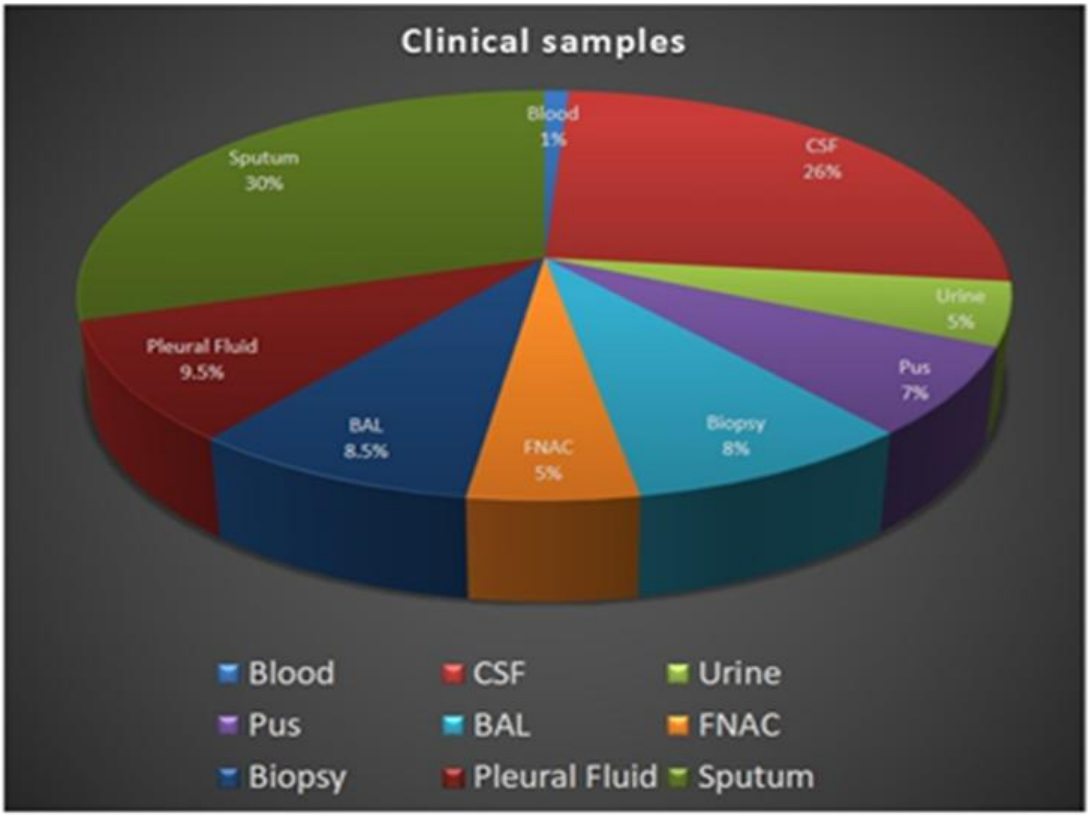


Fig 3.3 Graphical representation of the total samples during the study

3.3.2 Sample transportation and storage

The samples were transported to the microbiology laboratory as quickly as possible because the delay in transport, specifically in warm weather can cause the growth of respiratory bacteria which can result in increased contamination rate [13, 14]. The samples were kept and transported in a container with an ice box to maintain low temperature. The short-term storage of sputum samples and isolates (MTB) recovered from an AF-positive samples were performed by sub culturing to Lowenstein-Jensen (LJ) slants for stored at 4°C until the end of the study. The long-term storage of MTB isolates recovered from positive cultures were stored in duplicate 7H9 broth with glycerol at –70 to –80°C for six months after the conclusion of the study.

3.3.3 Methods for digestion, decontamination and concentration

Many techniques are used for digestion and decontamination throughout the world. The N-acetyl-L-cysteine-sodium hydroxide (NALC) method is probably the most common [15]. There are several other conventional methods which require more time and have some drawbacks. Oxalic acid is used for decontamination of *Pseudomonas* for specimens from cystic fibrosis patients. Sodium Chloride, NaOH method (Petroff's method), Zephiran- trisodium phosphate (Z-TSP) are most commonly used for sample processing and decontamination. A conventional Hcl treatment is used for digestion and decontamination of sputum samples. Other methods like Guanidine thiocynide treatment are also useful for quick liquefaction of sputum sample and AF bacilli which is easy to liberate from thick mucus sputum sample [16]. But the use of Guanidinium thiocyanate is dangerous.

Table: 3.2 Digestion and decontamination methods for sputum processing with their strength and weakness.

Material	Procedure
Sodium Hydroxide (NaOH)	Concentration technique by centrifugation
N-acetyl L cysteine Sodium chloride (NALC)	Mostly used in developed countries - Used in combination with centrifugation
Oxalic acid	Recommended to eliminate *P. aeruginosa* contamination (e.g. in urine, specimens from cystic fibrosis patients)
Cetylpyridinium - Sodium Chloride	For preservation and digestion / decontamination while in transport to the laboratory
Guanidinium isothiocyanate (GITC)	Simple method most used in developed countries, centrifugation required

There are number of method reported in the literature for digestion and decontamination of sputum samples, there are summarized and listed in table 3.2 with their strengths and weakness. In light of the above facts we have used NALC method in the present investigations. The samples were pre-treated with NALC (0.5 % N-acetyl-L-cysteine) and 2% sodium hydroxide for digestion and decontamination. This method also helps to free the bacilli from the mucus cells in which they may be embedded in sputum [17]. Microscopic observation by acid-fast bacilli staining was done by both direct and concentrated smears. The clinical sample processing may differ according to their nature.

NaOH-NALC treatment

This is the standard WHO approved procedure, which is also endorsed by CDC. In this process, 4% NaOH solution is mixed with equal amount of sodium citrate solution (2.9%) to make a working solution (2% NaOH concentration in this solution). Once an equivalent amount of NaOH-NALC-citrate and sputa are mixed, the total concentration of NaOH in the sample was 1%.

Reagent Preparation

• 4% NaOH solution was prepared by dissolving 4g NaOH pellets into 100 ml sterile deionized water.

• 2.9% sodium citrate solution was prepared by dissolving and melting 2.9 g sodium citrate in 100 ml of sterile deionized water and mixed well.

Note: Daily requirement fresh solution was prepared

NALC powder was added to make a final concentration of 0.5% (for 100 ml NaOH-Na citrate solution, add 0.5 g NALC powder).

Procedure:

In brief, sputum samples were mixed with NALC-NaOH solution vortexed and centrifuged at 3000 rpm for 20 min at 4°C. The supernatant was decanted, and sediment was suspended in 2 ml of phosphate buffer at pH 6.8. The sediment was used for AFB staining and nucleic acid extraction. The sample pre-treatment digestion and decontamination by N-acetyl-L-cysteine sodium hydroxide treatment is shown in Fig. 3.4

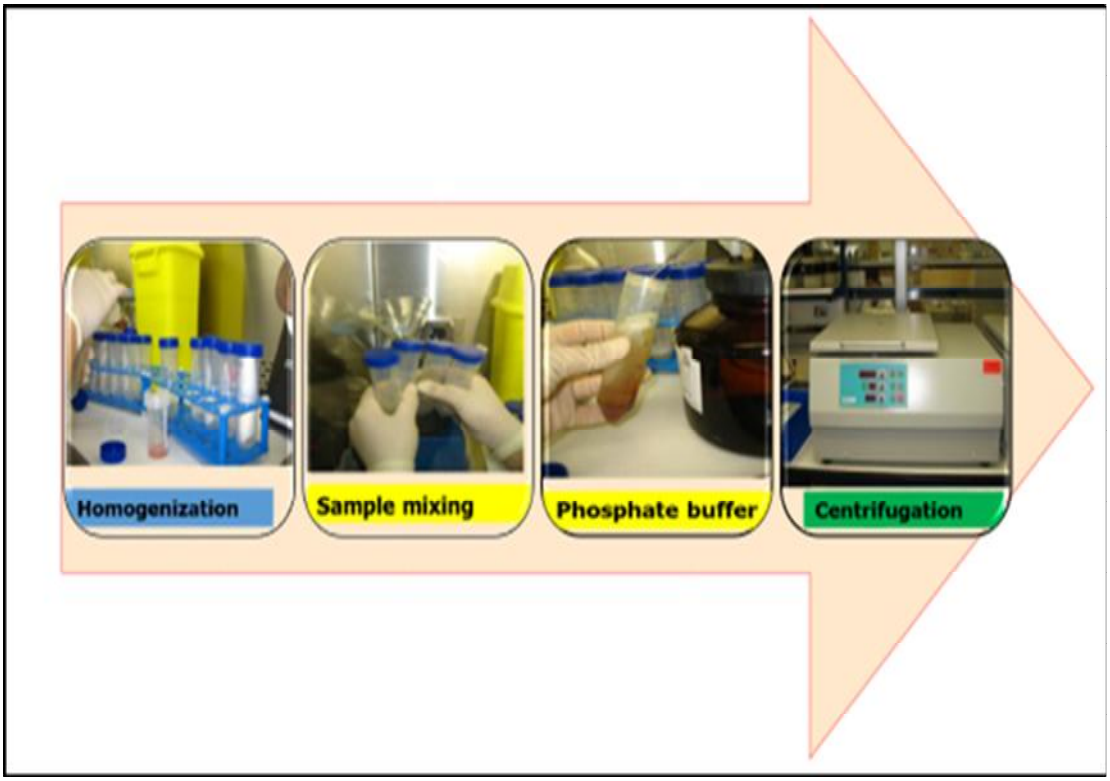


Fig. 3.4 The sequence of sputum pre-treatment by NALC (N-acetyl-L-cysteine) method

3.3.4 Preparation of smear for Microbiological diagnosis

Microscopic examination of an acid fast bacilli (AFB) detection by sputum is the mainstay of pulmonary *Mycobacterium tuberculosis*. Acid fast bacilli for TB diagnosis relies on the stained microscopic smear examination. This conventional smear staining method includes mainly two types [18].

Acid fast stain : basic fuchsin is mixed with phenol (carbolic acid) to form the primary stain i.e. Carbol fuchsin ZN stain and Flourochrome stain – Auramine O / Auramine - Rhodamine.

A) Acid fast stain:-

Acid fast stain method is used bacteria which are not stain by Gram staining or simple method, mostly the MTBC member of genus Mycobacterium, are resistant and can only be observed by acid-fast staining. *Mycobacteria* have an exclusive property of binding to the dye, basic fuchsin so firmly that it resists decolourization with strong alcohol or acids. This property is owing to its high lipid content, i.e. mycolic acid existing in the cell wall of MTB. This is a typical staining property along with their

distinguishing slender, thin beaded shape is valuable in the early stage detection of disease and in the monitor of treatment for TB.

It was first labelled by two German pathologist Friedrich Neelsen (1854–1898) and bacteriologist Franz Ziehl (1859–1926). The direct inspection of pulmonary sputum for AFB (acid fast bacilli) by ZN stained smears using traditional microscopy is a regular procedure in the diagnosis of pulmonary tubercular bacilli [19, 20]. At least one hundred microscopic fields have to be inspected per slide (at 1000×magnification). The acid fast stain can reveal AFB only if the sample contains >10,000 bacilli / ml. This test is highly precise in high burden countries [21, 22]. This method is used for staining smears made from specimen if fluorochrome staining is not available. It is also used to stain fluorochrome positive smears for confirmation, and for staining smears made from positive cultures as shown in Fig. 3.5 (a).

Advantages: Acid fast stain has been preferred due to its relative simplicity, high specificity, and low cost of reagents, simple microscope requirements, easy to perform and does not require expertise.

Disadvantages: An experienced technician may spend over 10 min per slide to rule out TB. Overall, over 30 min may be needed for preparation and examination of each slide, thus limiting the number of slides that technicians can be expected to reliably examine daily (~20–30). Various factors affect AF-microscopy sensitivity, low quality smear preparation and improper staining may be of poor quality; and it may not be possible to examine the recommended number of fields per slide due to fatigue and excessive workload. Both issues are related to the overall time required for the examination of each slide. These factors diminish the sensitivity of ZN microscopy, and TB cases may be missed. Its usefulness is questionable in patients with reduced pulmonary cavity formation or reduced sputum bacillary load, such as in children and HIV co-infected patients [23].

Protocol of acid fast staining:

• Flooded the slide with Carbol fuchsin stain.

↓

• Slide was heated gently until steam rises

↓

• Stained for 5-10 minutes; allowed to cool.

↓

• Slide was washed gently with water.

↓

• Decolorized with acid alcohol for 2 minutes

↓

• Washed gently with water. Drained excess water.

↓

• Poured Methylene Blue (counter stain) on the smear and left for 2 minutes.

↓

• Washed gently with water. Drained excess water.

↓

• Air-dried and observed microscope under oil immersion objective.

B) Auramine O / Auramine – Rhodamine: (Flourochrome stain)

Certain dyes called fluorochromes, have the property of becoming excited (raised to a higher energy level) after absorbing UV light (shorter wavelength). These excited molecules return to their normal state, releasing excess energy in the form of visible light (emission) of longer wavelength. This property of becoming self-luminous is called **fluorescence.** The emission of light occurs within nano- seconds after absorption of light (shorter wavelength)**.** The fluorochrome dyes for mycobacteria are auramine O/Auramine-Rhodamine. The identification of mycobacteria with auramine O/Auramine-Rhodamine stain is due to the affinity of the mycolic acid in the cell walls for the fluorochromes [24, 25]. The dye binds to the mycobacteria and appear as bright yellow, luminous rods against a dark background [26].

All acid-fast organisms are stained with Auramine O/ Auramine-Rhodamine stain. Both dead and alive bacilli are stained. Doubtful slides stained with Auramine O/ Auramine-Rhodamine were re-stained with ZN method and examine to confirm morphology. This re-staining is done only when the smears are rechecked after 48-72

hours of primary reading of smears. However, once smears have been stained by ZN staining, they were not used for fluorescent microscopy (FM). Fluorescent stained smear was read within 24 hours of staining because of disappearing. This is attributed to the fact that stained smears have a tendency to fade on exposure to light. The slides were stored in the slide box to avoid exposure to light. Alternatively, were stored wrapped in brown or black paper and kept away from light [27]. The fluorochromes stains have been used because of their increased sensitivity and speed.

One of the most common quantitative reporting procedures recommended by the CDC and used by us. Number of AFB seen report 0 Negative 1-2 AFB / Whole Smear Doubtful positive. Confirm by observing another smear from the same specimen or from another specimen from the same patient. If positive smear is doubtful with only 1-2 AFB seen on the whole smear, stain and examine another smear made from the same patient. Doubtful fluorochrome stained positive smears were confirmed by Ziehl-Neelsen or any other Carbol fuchsin method. It is important to run a positive and a negative quality control slide with each batch of stains [28, 29]**.** The comparative grading of AF reporting is shown in table No.3.3

Table 3.3: Comparative AFB grading

RNTCP ZN staining Grading (using 100x oil Immersion objective and 10x eyepiece)	**Auramine O Fluorescent Staining grading (using 20 or 25x objective and 10x eyepiece)**	**Reporting /Grading**
>10 AFB/field after examination of 20 fields	>100 AFB/field after examination of 20 fields	Positive, 3+
1-10 AFB/field after examination of 50 fields	11-100 AFB/field after examination of 50 fields	Positive, 2+
10-99 AFB/100 field	1-10 AFB/ field after examination of 100 fields	Positive, 1+
1-9 AFB/100 fields	1-3 AFB/100 fields	Doubtful positive /repeat
No AFB per 100 fields	No AFB per 100 fields	Negative

Microscopic observation of acid fast bacilli were seen under a 40X objective lens. The yellow colour rod shaped bacilli were seen for all (120) sputum samples. The typical micrograph of yellow colour rod shape acid fast bacilli is shown in Fig. 3.5 (b).

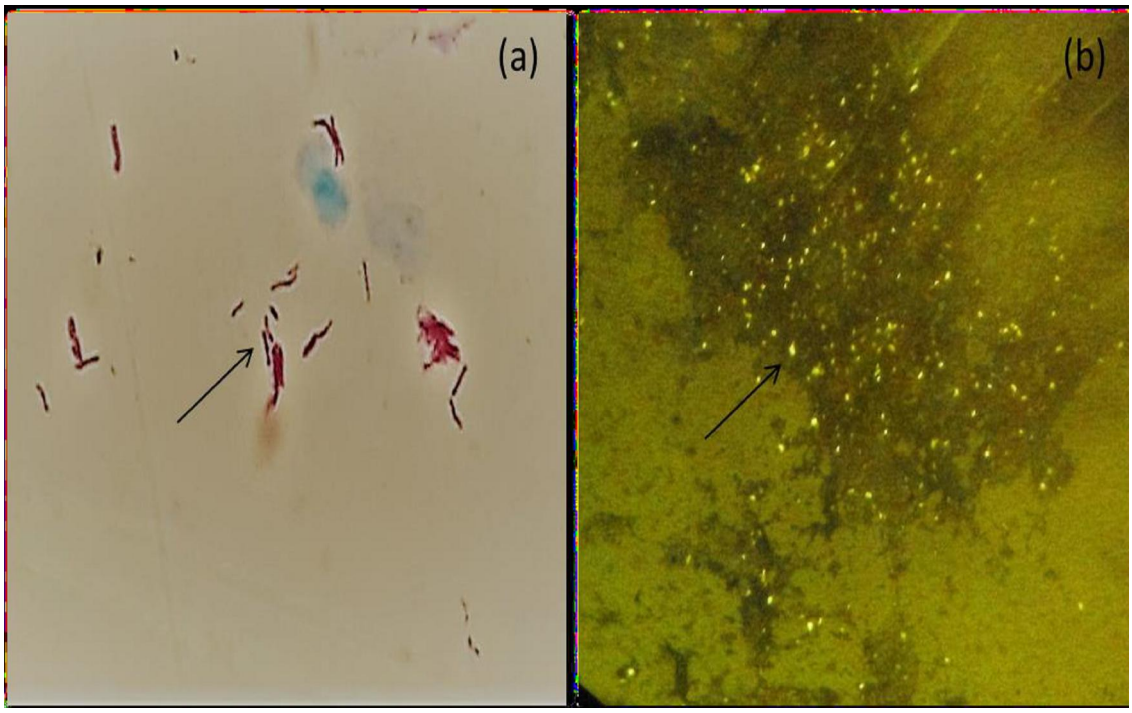


Fig. 3.5 (a) ZN stain, red color AFB (b) Auramine O stain yellow color AFB

Auramine-O staining protocol:

Primary stain- Auramine-O stain (15 min)

↓

Rinsed with water

↓

Decolorized- O.5 % acid alcohol (2 min)

↓

Rinsed with water

↓

Secondary stain- O.5 % potassium permanganate (2 min)

↓

Rinsed with water

↓

Air dried

↓

Observed under a 40X objective lens

3.4 Culture examination and identification

Of a total four hundred specimens examined, 280 were extra pulmonary clinical samples of human source. 94 CSF, 2 blood, 23 urine, 32 pus, 34 BAL, 20 FNAC, 37 biopsy, 38 pleural fluids and 120 pulmonary sputum samples. After preparing smears all sputum specimens were digested and decontaminated by using Petroff's method as per standard protocol. The digested and decontaminated samples were centrifuged at 3000 rpm for 15 minutes [29, 30]. Each pulmonary sputum samples were used for culture examination and identification. The supernatant was carefully discarded in a disinfectant container, leaving approximately 0.5 ml of sediment. The two loops of suspension were inoculated on LJ medium. The inoculated medium was incubated aerobically at 37ºC and examined at weekly intervals until growth occurred.

Primary culture techniques:

Mycobacterial culture provides the definitive diagnosis of TB and is considered as the gold standard. Culture techniques can detect only viable bacilli and sensitivity is 10-100 bacilli/ ml. [31] Culture increases the number of TB cases found, often by 30-50% and detects cases earlier, often before they become infectious. It also helps in strain identification and subsequent performance of drug susceptibility. The generation time of MTB is 18-22 hours hence it grows slowly on solid media [32, 33]. Furthermore the growth requirements are specific like high protein content and defined salts, glycerol and asparagines. Three main groups of culture media are available for tubercle bacilli:-1) Egg based media-Lowenstein Jensen Medium, (M. wheeler *et.al.*). 2) Agar based media – Middlebrook 7H10 and 7H11 with supplements, 3) Liquid media- Middlebrook 7H 9 or 7H12.

1) Egg based media: Egg malachite green medium (Lowenstein's medium for *MTB*)

Monopotassium phosphate, KH2PO4..... 1.2 g
Magnesium sulfate, MgSO4..... 0.12 g
Magnesium citrate..... 0.3 g
L-asparagine..... 1.8 g
Glycerol, A.C.S..... 0-40 ml
Distilled water to make..... 300 ml
Potato flour .... 15 g
Fresh eggs (weighed in shell) ..... 10 eggs or 500 g
Malachite green, 2 per cent solution..... 10 ml

1) Lowenstein-Jensen (LJ) Medium is most widely used for the culture**.** Colonies appeared rough, tough and buff after an incubation of 4-8 weeks at 37 ^{0}C.

2) Agar based media:

When Middlebrook 7H10 or 7H11 medium is used for isolation, cultures were incubated in an atmosphere of 10 % CO_2. Exposure of Mliddlebrook 7H10 or 7H11 agar to either daylight or heat results in release of formaldehyde in sufficient concentrations to inhibit the growth of mycobacteria.

3) Liquid media:

Middlebrook 7H 9 or 7H12 are available as a liquid medium to which were added growth supplements like glycerol, Tween 80 and albumin. The liquid medium has advantages of faster growth. Many automated systems have been designed which use liquid media not only for the detection of the MTB but also for anti-TB drug susceptibility testing.

All the pulmonary sputum samples was reported positive for *Mycobacterium tuberculosis* or NTM. Some sputum samples shows delay in growth. After 6 to 8 weeks the yellow colour rough single isolated muciod colonies are seen indicates all specimens are reported positive of MTB.

3.5 Results and Discussion

Out of four hundred total sputum samples, of these, one hundred twenty acid fast (AF) positive (grade 1^+, 2^+, 3^+ and 4^+) TB patient having clinical and radiological strong evidence. In our present study, 60.83 % (73) patient were male and 39.17 % (47) were female patients. Showed more prevalence in male patient in our study. The 18 % young patients with age group (21-30 y) are mostly affected for pulmonary tuberculosis. In this age group, according to AF grade 16 TB patients found strong 3 $^+$AF smear positive, 2 $^+$ total two TB patient, strong positive 4^+ two TB patient and 1 $^+$ TB total 6 patient detected. As compared to other age group this young age group TB patient were more in number may be due to family history of TB and alcohol and tobacco.

Master chart and graphs are prepared by using analysis of data in the present study. MS Excel 2007 data analysis is done in graph pad, instat software. The Means of all quantitative variables which follows a normal distribution are compared by unpaired t-test and which do not follow normal distribution are compared by "Wilcoxion signed rank test". Probability ($P<0.05$) is considered statistically

significant. Arithmetic mean: It gives the average value of total of data given. It obtained by adding together all items and dividing this total by the total number of TB patients. According to statistical analysis of clinical samples in the present study, it was found that out of 120, 73 male patients and 47 female patients were TB positive, which showed that, vast difference between them. In this study, 15 patients were in the age group 11-20 y, highest 35 TB patients are seen in 21-30 age group. Ziehl Neelsen staining were performed on smears of all sputum samples and distributed in grade wise, which shows grade I-8 patients, grade II- 2 patients, and grade III-2 patients and grade IV–O patients in the age group 11-20, are shown in Fig. 3.6

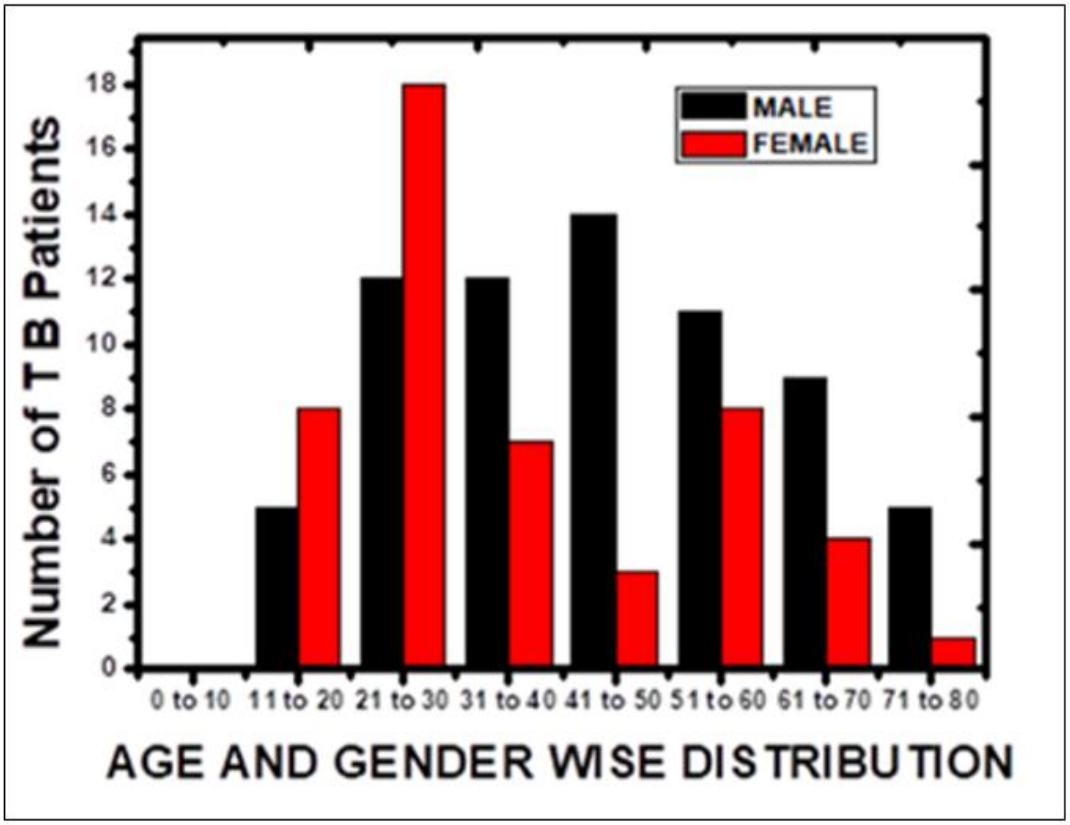


Fig. 3.6 Distribution of tuberculosis cases

All the pulmonary sputum samples was reported positive for *Mycobacterium tuberculosis* or NTM. The cultures were examined after 1 week to detect rapidly growing mycobacteria. After 3 to 4 weeks, positive cultures of the MTB as well as other slow growing mycobacteria were detected. Cultures were examined up to 8 weeks to detect very slow growing mycobacteria including MTB

before judging the cultures to be negative. The results are recorded and shown in Fig.3.7

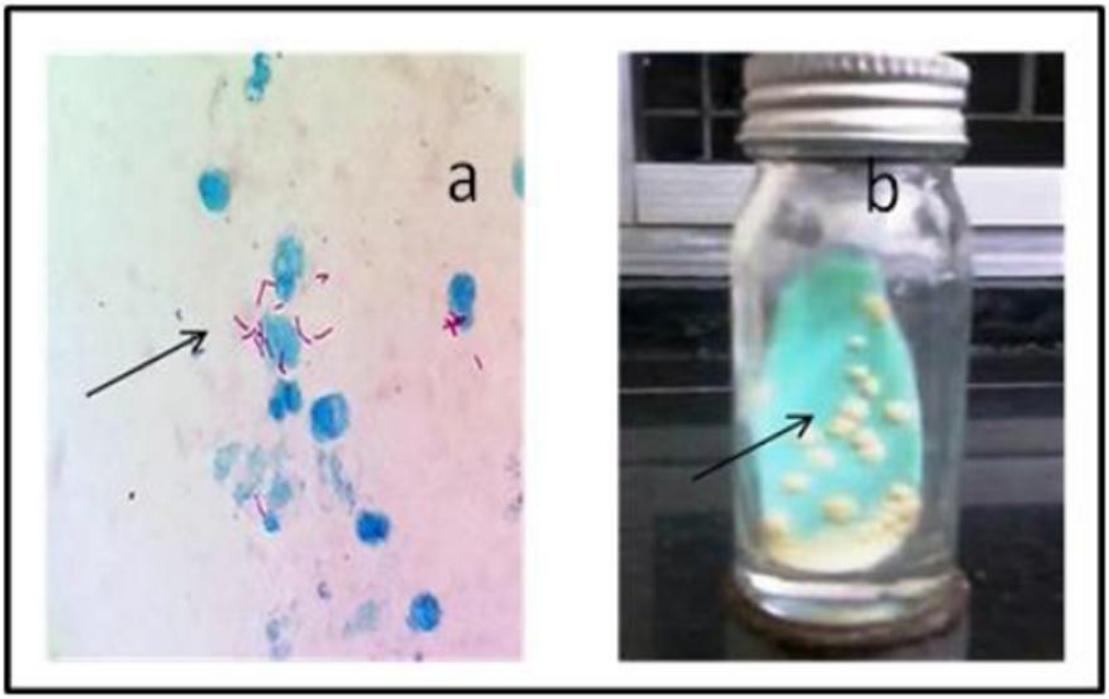


Fig. 3.7 (a) Acid fast bacilli, (b) yellow color colonies of MTB on L.J. Media

3.6 Conclusions:

This study reveals that sputum processing with NALC treatment will result in quick recovery of MTB. AF-microscopy is a simple, rapid, and reliable method. It is a promising approach for the routine identification of mycobacteria in the specialized laboratory. Culture method is still gold standard method. For rapid diagnosis of TB automation is required to reduce the time by using alternative to radiometric system. In conclusion non-stop improvements in medical Microbiology and molecular diagnostics require novel methods for isolation of MTB.

Table 3.4: Master chart of TB positive patients

SR. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POSITIVE GRADE	REAL TIME PCR	HIV
1	45	M	SPUTA	N	N	N	N	Y	+	Y	N
2	38	M	SPUTA	N	N	N	N	Y	+	Y	N
3	66	F	SPUTA	N	N	N	N	Y	+	Y	N
4	26	M	SPUTA	N	N	N	N	Y	3+	Y	N
5	30	M	SPUTA	N	N	N	N	Y	3+	Y	N
6	37	F	SPUTA	N	N	N	N	Y	2+	Y	N
7	60	M	SPUTA	N	N	N	N	Y	3+	Y	N
8	17	F	SPUTA	N	N	N	Y	Y	3+	Y	N

SR. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POSITIVE GRADE	REAL TIME PCR	HIV
9	35	F	SPUTA	N	N	N	N	Y	3+	Y	N
10	51	M	SPUTA	N	Y	Y	Y	Y	2+	Y	N
11	71	M	SPUTA	N	N	Y	Y	Y	3+	Y	N
12	20	M	SPUTA	N	N	N	N	Y	1+	Y	N
13	22	M	SPUTA	N	N	Y	Y	Y	3+	Y	N
14	40	M	SPUTA	N	N	N	N	Y	1+	Y	N
15	15	F	SPUTA	N	N	N	N	Y	2+	Y	N

SR. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POSITIVE GRADE	REAL TIME PCR	HIV
16	47	F	SPUTA	N	N	N	N	Y	3+	Y	N
17	62	F	SPUTA	N	N	N	N	Y	1+	Y	N
18	23	F	SPUTA	N	N	N	N	Y	1+	Y	N
19	40	M	SPUTA	N	N	Y	Y	Y	3+	Y	N
20	50	M	SPUTA	N	N	N	Y	Y	3+	Y	N
21	45	M	SPUTA	N	N	N	Y	Y	1+	Y	N
22	38	M	SPUTA	N	N	N	Y	Y	3+	Y	N

SR. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POSITIVE GRADE	REAL TIME PCR	HIV
23	65	F	SPUTA		N	N	N	Y	1+	Y	N
24	65	M	SPUTA	N	N	N	N	Y	1+	Y	N
25	22	M	SPUTA		N	N	N	Y	2+	Y	N
26	18	F	SPUTA	N	N	N	N	Y	2+	Y	N
27	66	F	SPUTA	N	N	N	N	Y	3+	Y	N
28	20	M	SPUTA	N	N	N	N	Y	1+	Y	N
29	26	M	SPUTA	N	N	Y	Y	Y	3+	Y	N

SR. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POSITIVE GRADE	REAL TIME PCR	HIV
30	28	M	SPUTA	N	N	N	N	Y	1+	Y	N
31	14	M	SPUTA	N	N	N	N	Y	1+	Y	N
32	50	M	SPUTA	N	N	N	N	Y	1+	Y	N
33	60	M	SPUTA	N	N	N	N	Y	3+	Y	N
34	21	F	SPUTA	N	N	N	N	Y	1+	Y	N
35	25	M	SPUTA	N	N	N	N	Y	3+	Y	N
36	25	F	SPUTA	N	N	N	N	Y	3+	Y	N

SR. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POSITIVE GRADE	REAL TIME PCR	HIV
37	40	F	SPUTA	N	N	N	N	Y	1+	Y	N
38	18	F	SPUTA	N	N	N	N	Y	1+	Y	N
39	40	F	SPUTA	N	N	Y	Y	Y	3+	Y	N
40	30	M	SPUTA	N	N	N	N	Y	3+	Y	N
41	40	F	SPUTA	N	N	N	N	Y	1+	Y	N
42	39	M	SPUTA	N	N	N	N	Y	3+	Y	N
43	17	F	SPUTA	Y	Y	N	N	Y	3+	Y	N
44	26	M	SPUTA	N	N	N	N	Y	3+	Y	N

SR. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POSITIVE GRADE	REAL TIME PCR	HIV
45	43	M	SPUTA	N	N	N	N	Y	3+	Y	N
46	58	M	SPUTA	N	N	N	N	Y	2+	Y	N
47	25	F	SPUTA	N	N	N	N	Y	1+	Y	N
48	26	F	SPUTA	N	N	N	N	Y	3+	Y	N
49	62	M	SPUTA	N	N	N	N	Y	3+	Y	N
50	61	F	SPUTA	N	N	N	N	Y	3+	Y	N
51	35	M	SPUTA	N	N	N	N	Y	1+	Y	N
52	42	M	SPUTA	N	N	N	N	Y	3+	Y	N
53	17	F	SPUTA	Y	N	N	N	Y	3+	Y	N

SR. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POSITIVE GRADE	REAL TIME PCR	HIV
54	19	F	SPUTA	N	N	N	N	Y	3+	Y	N
55	42	M	SPUTA	N	N	N	N	Y	1+	Y	N
56	75	M	SPUTA	N	N	N	N	Y	1+	Y	N
57	23	M	SPUTA	N	N	N	N	Y	2+	Y	N
58	32	M	SPUTA	N	N	N	N	Y	1+	Y	N
59	20	F	SPUTA	N	N	N	N	Y	1+	Y	N
60	74	M	SPUTA	N	N	N	N	Y	2+	Y	N
61	51	M	SPUTA	N	N	N	N	Y	2+	Y	N
62	23	F	SPUTA	N	N	N	N	Y	1+	Y	N

SR. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POSITIVE GRADE	REAL TIME PCR	HIV
63	75	M	SPUTA	N	N	N	N	Y	1+	Y	N
64	58	F	SPUTA	N	N	N	N	Y	3+	Y	N
65	27	F	SPUTA	N	N	N	N	Y	1+	Y	N
66	65	M	SPUTA	N	N	N	N	Y	1+	Y	N
67	45	M	SPUTA	N	N	N	N	Y	1+	Y	N
68	36	M	SPUTA	N	N	N	N	Y	1+	Y	N
69	17	F	SPUTA	N	N	N	N	Y	1+	Y	N
70	58	F	SPUTA	N	N	N	N	Y	1+	Y	N
71	28	M	SPUTA	N	N	N	N	Y	3+	Y	N

SR. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POSITIVE GRADE	REAL TIME PCR	HIV
72	49	M	SPUTA	N	N	N	N	Y	3+	Y	N
73	27	F	SPUTA	N	N	N	N	Y	3+	Y	N
74	21	F	SPUTA	N	N	N	N	Y	3+	Y	N
75	46	M	SPUTA	N	N	N	N	Y	1+	Y	N
76	37	M	SPUTA	N	N	N	N	Y	1+	Y	N
77	55	F	SPUTA	N	N	N	N	Y	1+	Y	N
78	24	M	SPUTA	N	N	N	N	Y	3+	Y	N
79	27	F	SPUTA	N	N	N	N	Y	3+	Y	N
80	35	F	SPUTA	N	N	N	N	Y	3+	Y	N

SR. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POSITIVE GRADE	REAL TIME PCR	HIV
81	60	M	SPUTA	N	N	N	N	Y	1+	Y	N
82	27	F	SPUTA	N	N	N	N	Y	3+	Y	N
83	34	F	SPUTA	N	N	N	N	Y	3+	Y	N
84	34	M	SPUTA	N	N	N	N	Y	3+	Y	N
85	60	M	SPUTA	N	N	N	N	Y	1+	Y	N
86	41	F	SPUTA	N	N	N	N	Y	3+	Y	N
87	41	M	SPUTA	N	N	N	N	Y	2+	Y	N
89	40	M	SPUTA	N	N	N	N	Y	3+	Y	N
90	55	M	SPUTA	N	N	N	N	Y	1+	Y	N

SR. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POSITIVE GRADE	REAL TIME PCR	HIV
91	21	F	SPUTA	Y	N	N	N	Y	2+	Y	N
92	27	M	SPUTA	N	N	N	N	Y	3+	Y	N
93	69	M	SPUTA	N	N	N	N	Y	1+	Y	N
94	27	F	SPUTA	N	N	N	N	Y	1+	Y	N
95	60	M	SPUTA	N	N	N	N	Y	1+	Y	N
96	27	F	SPUTA	Y	N	N	N	Y	3+	Y	N
97	20	M	SPUTA	Y	N	N	N	Y	3+	Y	N
98	55	F	SPUTA	Y	N	N	N	Y	3+	Y	N
99	22	M	SPUTA	Y	N	N	N	Y	2+	Y	N

SR. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POSITIVE GRADE	REAL TIME PCR	HIV
100	55	M	SPUTA	Y	N	N	N	Y	3+	Y	N
101	79	F	SPUTA	Y	N	N	N	Y	3+	Y	N
102	40	M	SPUTA	Y	N	N	N	Y	2+	Y	N
103	45	M	SPUTA	Y	N	N	N	Y	3+	Y	N
104	65	M	SPUTA	Y	N	N	N	Y	3+	Y	N
105	65	M	SPUTA	Y	N	N	N	Y	4+	Y	N
106	20	M	SPUTA	Y	N	N	N	Y	3+	Y	N
107	42	M	SPUTA	Y	N	N	N	Y	4+	Y	N
108	22	M	SPUTA	Y	N	N	N	Y	4+	Y	N

SR. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POSITIVE GRADE	REAL TIME PCR	HIV
109	19	M	SPUTA	Y	N	N	N	Y	3+	Y	N
110	68	M	SPUTA	Y	N	N	N	Y	4+	Y	N
111	22	M	SPUTA	Y	N	N	N	Y	4+	Y	N
112	40	F	SPUTA	Y	N	N	N	Y	3+	Y	N
113	29	F	SPUTA	Y	N	N	N	Y	2+	Y	N
114	29	F	SPUTA	Y	N	N	N	Y	3+	Y	N
115	40	M	SPUTA	Y	N	N	N	Y	3+	Y	N
116	43	F	SPUTA	Y	N	N	N	Y	3+	Y	N
117	60	M	SPUTA	Y	N	N	N	Y	3+	Y	N

SR. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POSITIVE GRADE	REAL TIME PCR	HIV
118	70	M	SPUTA	Y	N	N	N	Y	2+	Y	N
119	43	F	SPUTA	Y	N	N	N	Y	2+	Y	N
120	79	M	SPUTA	Y	N	N	N	Y	3+	Y	N

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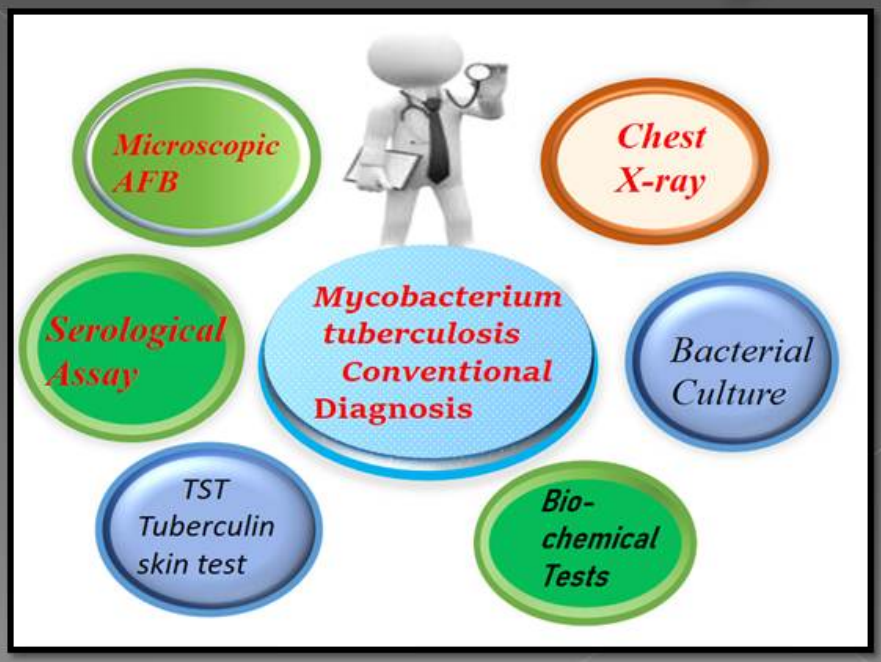
Chapter 4: Detection of MTB with conventional methods



4.1 Introduction

This chapter highlights existing conventional TB diagnostic methods for the detection and identification of TB in the clinical diagnostic laboratory [1]. The methods contains pulmonary chest X-ray diagnosis, tuberculin test (TT), serology based TB diagnosis, microscopy and culture which traditionally were followed by phenotypic-based detection methods. However, molecular based TB diagnosis methods are considered the gold standard for both the fast TB detection directly from clinical samples as well as for the identification of TB following culture. In addition, this chapter provides an overview of conventional methods used in the clinical laboratory for TB diagnosis [2].

Tuberculosis (TB) is an infectious disease caused by MTB that can affect any organs in the body, although the vast majority of cases involve only the lungs. The burden of tuberculosis is high in developing countries, where diagnosis of latent TB, drug resistant TB and HIV co-infected MTB among for children remains still a challenge [3]. Different conventional techniques are available but have their own limitations and may cause delay in the diagnosis of pulmonary and extra-pulmonary TB. MTB cultivation and determination of the phenotype are still the "gold standard'' but it requires time consuming and also have low detection limit. Accurate TB diagnosis test is still a challenge. In medical practice, fast and early pulmonary TB detection remains to be a challenge for clinicians [4].

4.2 Experimental

4.2.1 Materials and methods

From August 2016 to January 2017 we received 400 pulmonary sputum samples from the department of Microbiology laboratory, Dr. D. Y. Patil Hospital and Research Centre, Kolhapur, Wanless Hospital, Miraj and C.P.R. Hospital Kolhapur. Of these one hundred twenty patients had confirmed pulmonary tuberculosis. We reviewed chest X-ray from the patients who were acid fast smear positive. The study was approved by the Institutional ethics committee, D. Y. Patil University, Kolhapur.

4.2.2 Medical History of TB

After showing a medical history, the physician were inquired if any signs of TB infection are present consequently, for how long and if nearby known exposure to a person already infected with TB. The important information on whether the individual has been identified in the earlier with latent tuberculosis infection (LTBI)

[5]. The physicians may also interact with local health division for evidence on whether a patient has an earlier history of TB. If the patient were treated earlier for TB regimen or have undergone incomplete anti-TB therapy, or TB infection may reappear and may be drug-resistant. It is very important to study demographic factors (e.g., occupation, racial group, country of origin, age group and civilization) that may raise the patient's risk of being vulnerable to TB infection [6]. The physician should regulate if the patient has causal medical disorders, diabetes or specific HIV (Human Immunodeficiency Virus) infection that raises the risk for developing to TB infection in those latently infected with TB. The physician may collect the valuable information during physical examination, also take information during body examination. The habits of the patient like chewing of tobacco or whether the patient is alcoholic, including family history like whether any known positive case or old patient is treated with TB in his family members [7].

4.2.3 Physical examination

A physical examination is an important part of the assessment of any patient. It can't be used to rule out TB infection, nevertheless it can offer valued information about the patient's general complaints. After physical examination the physician may decide to select various techniques used for TB diagnosis. The physician may be rule out if the patient is early diagnosed or under anti-TB treatment [8]. The tuberculosis signs and symptoms is listed in table No.4.1

Table No.4.1 Signs and symptoms of pulmonary and extra pulmonary TB

Signs of pulmonary TB	**Symptoms of extra pulmonary TB**
More than 3 weeks cough	Tuberculosis Meningitis
Cough with blood	Renal TB
Fever	Fever
Loss of appetite	Loss of appetite
Weight loss	Weight loss
Chest pain	Larynx TB
Night sweats	Night sweats

4.2.4 Conventional TB diagnostic tests

The choice of the best appropriate tests for detection of TB infection should be established on the reasons and the background for testing, test accessibility, and total cost value of testing [9]. Presently, there are two main existing methods for the detection of TB. Most traditional methods used for diagnosis of TB are pulmonary sputum smear and culture based techniques [10]. The conventional TB diagnosis tests largely depend on acid fast microscopy and Chest X-ray. Both these tests have limitations which is a tiresome process and need skilled persons to handle the investigation. The first choice of TB diagnosis by ZN stain has been preferred due to its relative simplicity, high specificity, low cost of reagents, simple microscope requirements, easy to perform and does not require expertise. An experienced technician may spend over 10 min per slide to rule out TB. Overall, over 30 min may be needed for preparation and examination of each slide, thus limiting the number of slides that technicians can be expected to reliably examine daily (~20–30). These factors diminish the sensitivity of ZN microscopy and the TB cases may be missed. Its usefulness is questionable in patients with reduced pulmonary cavity formation or reduced sputum bacillary load, such as in children and HIV co-infected patients [11].

The patient with a productive cough that lasts for 2 weeks or more or with unsolved chronic fever with sudden weight loss should be assessed for tuberculosis infection. The chest X-ray is the primary radiological evaluation to confirm pulmonary TB patients. Almost every TB patients with long-lasting cough have an X-ray chest radiograph during their diagnostic assessment for pulmonary chest infection [12]. It is important to rule out several forms of extra-pulmonary TB diagnosis, e.g. pleural, vertebral and joint tuberculosis. X-ray allows detection of TB in resource limited settings as recommended by the WHO. Chest X-ray (CXR) is the commonly used method to find TB, but it is done in conjunction with tuberculin skin test (TST). Acute pulmonary TB can be easily diagnosed with CXR image. The extra-pulmonary TB cannot be detected by CXR. This test shows more sensitivity but less specificity. Latest study results have proven that chest radiographs are the ideal methodology for finding pediatric Tuberculosis [13].

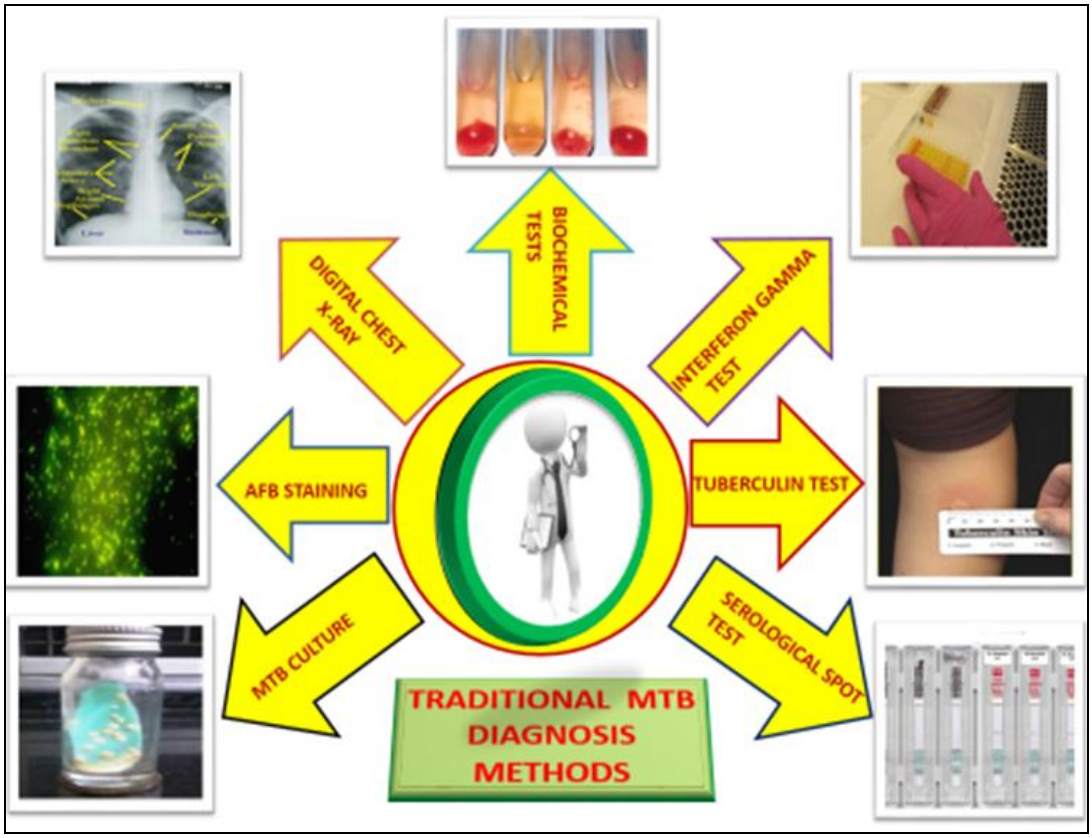


Fig 4.2. Schematic representation of conventional MTB diagnostic methods

4.2.5 Study using Chest X-ray

Total four hundred chest X-rays reports were initially obtained from each hospital within 3 months of the acid fast smear test. The films were studied individually by expert radiologists and pulmonary chest consultants for the patient's bacteriology reports. After screening four hundred patients, one hundred twenty TB patients with previous lung infection such as chronic TB were confirmed. None of the patients registered in this experiment were associated with HIV (Human Immunodeficiency Virus) infection. The schematic representation of conventional TB diagnosis methods are shown in Fig.4.2.

4.2.6 Tuberculin test

The tuberculin skin test or Monteux test is based on the detection of a cutaneous delayed type of hypersensitivity response. The PPD (purified protein derivative) test is a tool for screening TB infection. TST (tuberculin skin test) is one of the main test which is used worldwide [14]. A standard dose of PPD is 5 tuberculin unit (TU) is injected intradermal on forearm position and read after 48 hours. The injected site become red or it is called erythema due to an immune reaction to the

antigen, if the diameter of skin induration is >10 mm then TST positive is shown in Fig 4.3

The PPD reactivity to TST designates TB infection and can support TB diagnosis amongst the persons belonging to a group with low rate of infection, e.g. individuals in developing nations. In this groups with high incidence of infection, the analytical value of a positive test is lowest, to be of abundant value in approving TB diagnosis [15, 16].

The sensitivity of the TST is high, though specific conditions (e.g. Malnutrition and HIV infection) can suppress the reactivity of TT, ensuing in reduced sensitivity of the test amongst the actual people who are at risk of tuberculosis infection. The assessment of a positive TT test in the TB diagnosis is frequently decreased in emerging countries, where immunization of BCG exposure to environmental NTM (Nontuberculosis mycobacteria) result in decreased test specificity [17].

4.3 Results and discussion

However, this technique required minimum $10x10^3$ bacteria/ml present in the samples, to allow bacilli detection by smear microscopy. The microscopic observation revel AF-positive smear grade 1^+ to grade 3^{+++} according to RNTCP (revised national tuberculosis control programme) guideline and the results are confirmed by microscopic observation. The present study has shown that patients with an AF positive grade 3^+ sputum not only need a delay of TB management in the intensive phase more often than those with AF positive grade 1^+ or 2^+ and scanty. The AF positive smear grade proportion is found to be higher in 3^+ patients.

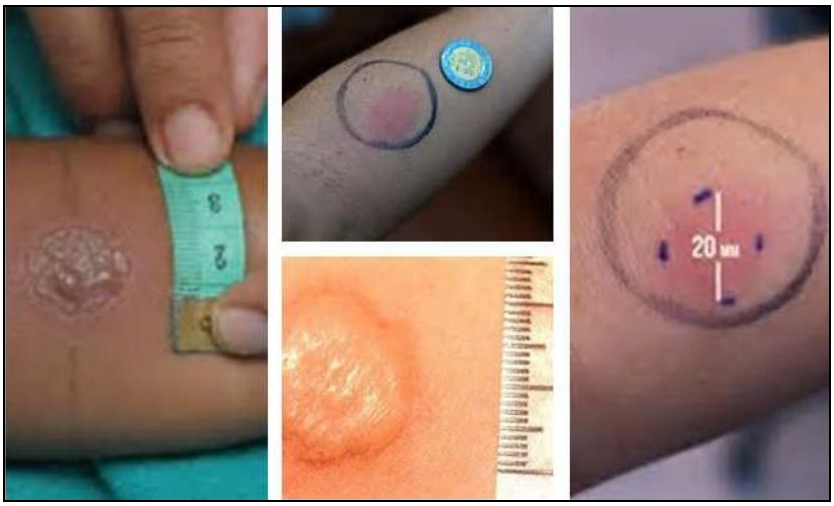


Fig. 4.3 Tuberculin skin test

4.3.1 Radiology Chest X-ray

Radiological chest X-ray performance is negotiated in various cases. Radiology branch also delivers vital information for managing and follow-up of these TB patients and is very useful for nursing complications. The chest X-ray is suitable, but is not precise for diagnosis of pulmonary TB and can be regular even when the infection is present [18].

The master chart of TB Chest X-ray positive patients and sputum AFB positive patient is given in table 4.3. From the table 4.3 it is seen that out of four hundred, one hundred twenty patients was confirmed TB positive by Chest X-ray confirmed by radiologist. The age and gender wise distribution Chest X-ray study is shown in Table 4.2. On the basis of statistical analysis age group of 21-30 show maximum number of X-ray TB positive, in which 15 patients are female and 14 patients are male. The lowest number of X-ray TB patient were detected in 71-80 age group (1 female and 4 male patients).

Thus, it cannot offer a sure independent analysis and desires to be followed by pulmonary sputum analysis. The lymphadenopathy is very rare and the cavitation is the seal of the post-primary TB appearances, if radiology is partial of all patients [19, 20]. In the posterior and apical segments of the superior lobes poorly defined and patchy, consolidation and in the larger segment of the inferior lobe is also commonly seen. Though, post-primary infection activity cannot be precisely measured by x-ray chest radiography.

The radiographic constancy for six months and in case of negative sputum cultures is the greatest indicator of inactive infection. The expressive terms ‘inactive’ TB were rejected in service of radio graphically constant TB, as viable bacilli may continue to despite satisfactory therapy. A significant task for X-ray radiology is to control whether these residual results are indicative of active disease. For this, purpose chest X-ray has partial value, in such cases a lesion is constant. A stable lesions can contain active tubercle bacilli [21].

The chest X-ray is the foremost diagnostic tool for assessing pulmonary TB, chest computed tomography (CT) is usually required to identify well lesions to outline equivocal lesions, or to assess problems. The chest X-ray and CT chest is a real diagnostic technique when basic films are regular, and it offers valued information for handling the disease. CT Chest can add valuable evidence for the diagnosis of bacterial activity. The branching cavitation, opaqueness, are clear marks of active TB,

infection is complete by examining AF - sputum for the occurrence of bacilli. The typical TB patient infected Chest X-ray shown in Fig. 4.4 and table No.4.2.

Table No.4.2 Chest X-ray TB positive patients

Age group	**Total TB positive at chest X-ray patient**	**Gender**	
11-20	14	F	04
		M	10
21-30	29	F	15
		M	14
31-40	18	F	09
		M	09
41-50	15	F	05
		M	10
51-60	14	F	03
		M	11
51-60	14	F	03
		M	11
61-70	13	F	05
		M	08
71-80	05	F	01
		M	04

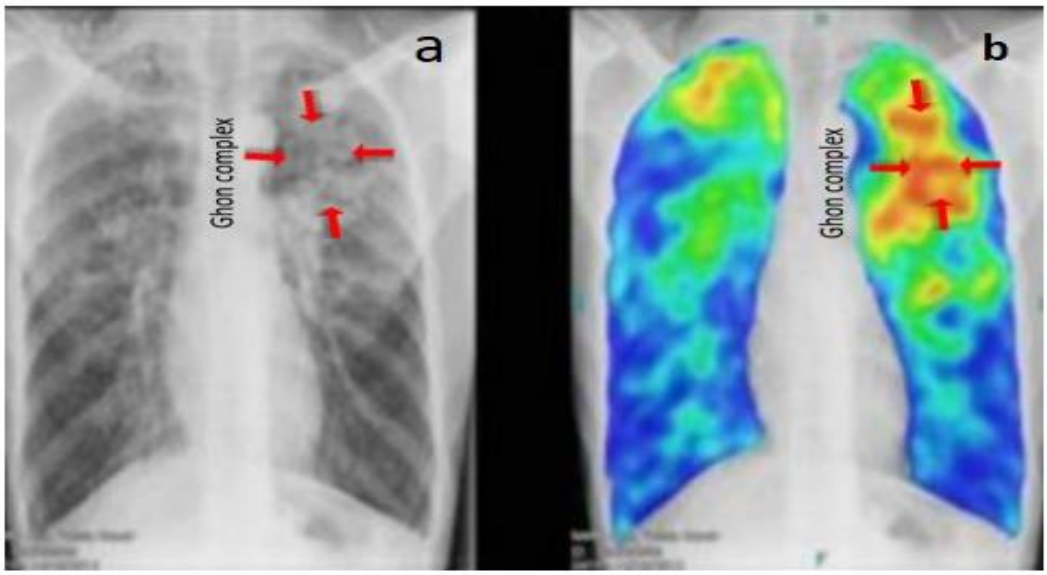


Fig 4.4 [a]Chest X-ray, [b] Dual -energy Chest radiography

4.3.2 Interpretation of tuberculin skin test (TST) results

Total One hundred sputum positive patient were tested CXR and TST. For TST results all positive sputum individuals showed induration of above 10 mm. An individual who has been defenceless to the microbes is expected to stand an increase reply in the skin inclosing the bacterial protein. The delayed type of hyper sensitivity reaction is observed by assessing the diameter of induration. The interpretation of TST test remains problematic and controversial. Numerous issues like age, immunological status co-existing infection are the impact of its results. The highest care was taken while making the judgement of TST according to centre for disease control (CDC) guideline, TST interpretation depends on two factors, dimension in millimeters of the induration. Individual's risk of existence infected with TB and development to disease. TST is used only for screening for TB patients with correlation of CXR and sputum positive. This is used only for screening for TB patients with companion of CXR and sputum positive smears.

Positive TST reactions:

[a] An induration of 5 mm or more millimeters is considered positive in

1. HIV-infected persons
2. A recent contact of a person with TB disease
3. Persons with fibrotic changes on Chest X-ray consistent with prior TB

4. Patients with organ transplants
5. Persons who are immunosuppressed for other reasons (e.g., taking the equivalent of >15 mg/day of prednisone for 1 month or longer, taking TNF-a antagonists)

[b] An induration >10 millimeters is considered positive in

1. Recent immigrants (< 5 years) from high-prevalence countries
2. Injection drug users
3. Residents and employees of high-risk
4. Laboratory technicians
5. Persons with clinical conditions that place them at high risk
6. Children < 4 years of age
7. Infants, children, and adolescents exposed to adults in high-risk categories

[c] An induration of 15 or more millimeters: is considered positive in any individual, including persons with no known risk factors for TB. However, targeted skin testing programs were conducted among high-risk groups.

Limitations of the test: Although widely used the test has several limitations. A positive reaction may be observed in both latent and active TB infection, therefore, it is unreliable in differentiating whether the person is currently having TB or had been infected in the past or at carrier stage. Confirmatory tests such as chest X-ray, sputum culture, or both are usually done to rule out an active TB infection.

False-Positive- TST reactions: Some persons may react to the tuberculin skin test even though they are not infected with *M. tuberculosis*. The causes of these false-positive reactions may include, but are not limited to, the following:

1. Infection with Nontuberculosis mycobacteria (NTM)
2. Previous BCG vaccination
3. Incorrect method of TST administration
4. Incorrect interpretation of reaction
5. Incorrect bottle of antigen used

False-Negative TST Reactions: A negative TST result usually signifies that the individual has never been exposed to *M. Tuberculosis*. However, there are factors that may cause a false-negative result or diminished ability to respond to tuberculin even if the persons are infected with *M. tuberculosis.* The reasons for these false-negative reactions may include, but are not limited to, the following:

1. Cutaneous anergy (anergy is the inability to react to skin tests because of a Weak immune system)
2. Recent TB infection (within 8-10 weeks of exposure)
3. Very old TB infection (many years)
4. Very young age (less than 6 months old)
5. Recent live-virus vaccination (e.g., measles and smallpox)
6. Overwhelming TB disease
7. Some viral illnesses (e.g., measles and chicken pox)
8. Incorrect method of TST administration
9. Incorrect interpretation of reaction

4.3.3 Bacterial culture

Culture and drug sensitive test method is still "gold standard "method for diagnosis of MTB. Samples are pre-treated with NaOH-N-acetyl Cysteine based method for proper digestion and decontamination of sputum samples. Several solid culture media are used for isolation, identification, species differentiation and DST (drug sensitive test) as Lowenstein-Jensen (LJ) middle brooks 7H10 and blood agar. Because MTB is a slow growing bacteria, it requires minimum 2 and 6 weeks for liquid and solid culture medium. The details of bacterial culture detection is described in chapter 3 section 3.4

Development TB diagnosis test:

1. To simplify and improve detection of TB cases, containing AF smear-negative and pediatric tuberculosis, extra-pulmonary TB, over improved sensitivity and specificity and better-quality available.

2. To develop accurate, safe, simple, fast and low cost tests that can be achieved at the point-of-care level of the tertiary health care system and that yield very quick results within two hours.

3. To enable more real monitoring of TB therapy.

4. To rapidly identify drug resistance multidrug resistant TB together with first- and second-line anti-TB drugs.

5. To reliably identify latent (LTBI) infection and regulate the danger of development of active TB disease, allowing the sensible use of protective therapy.

4.4 Conclusions

Conventional TB diagnosis methods of AF-smear microscopy and TB culture remain the gold standard for identifying pulmonary TB; nevertheless, reduced performance of these old traditional methods demands for more sensitive and specific techniques. In this study the young age group (21-30) showed maximum number of TB positive patients. The TST showed best results in correlation with chest x-ray especially in pediatric patients. Most of the conventional methods available for TB detection are just an improvisation of older techniques, which is a half a century old technique. The conventional TB diagnosis methods are found less sensitive and requires more time. Bleaching methodology and optimization of culture techniques were increasing the detection rate, but they failed to prove during clinical trials in large scale.

Table 4.2: TB Chest X-ray, Acid fast staining and HIV

Sr. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POS. GRADE	HIV
1	45	M	SPUTA	N	N	N	N	Y	+	N
2	38	M	SPUTA	N	N	N	N	Y	+	N
3	66	F	SPUTA	N	N	N	N	Y	+	N
4	26	M	SPUTA	N	N	N	N	Y	3+	N
5	30	M	SPUTA	N	N	N	N	Y	3+	N
6	37	F	SPUTA	N	N	N	N	Y	2+	N
7	60	M	SPUTA	N	N	N	N	Y	3+	N
8	17	F	SPUTA	N	N	N	Y	Y	3+	N
9	35	F	SPUTA	N	N	N	N	Y	3+	N

Sr. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POS. GRADE	HIV
10	51	M	SPUTA	N	Y	Y	Y	Y	2+	N
11	71	M	SPUTA	N	N	Y	Y	Y	3+	N
12	20	M	SPUTA	N	N	N	N	Y	1+	N
13	22	M	SPUTA	N	N	Y	Y	Y	3+	N
14	40	M	SPUTA	N	N	N	N	Y	1+	N
15	15	F	SPUTA	N	N	N	N	Y	2+	N
16	47	F	SPUTA	N	N	N	N	Y	3+	N
17	62	F	SPUTA	N	N	N	N	Y	1+	N
18	23	F	SPUTA	N	N	N	N	Y	1+	N
19	40	M	SPUTA	N	N	Y	Y	Y	3+	N

Sr. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POS. GRADE	HIV
20	50	M	SPUTA	N	N	N	Y	Y	3+	N
21	45	M	SPUTA	N	N	N	Y	Y	1+	N
22	38	M	SPUTA	N	N	N	Y	Y	3+	N
23	65	F	SPUTA	N	N	N	N	Y	1+	N
24	65	M	SPUTA	N	N	N	N	Y	1+	N
25	22	M	SPUTA	N	N	N	N	Y	2+	N
26	18	F	SPUTA	N	N	N	N	Y	2+	N
27	66	F	SPUTA	N	N	N	N	Y	3+	N
28	20	M	SPUTA	N	N	N	N	Y	1+	N
29	26	M	SPUTA	N	N	Y	Y	Y	3+	N

Sr. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POS. GRADE	HIV
30	28	M	SPUTA	N	N	N	N	Y	1+	N
31	14	M	SPUTA	N	N	N	N	Y	1+	N
32	50	M	SPUTA	N	N	N	N	Y	1+	N
33	60	M	SPUTA	N	N	N	N	Y	3+	N
34	21	F	SPUTA	N	N	N	N	Y	1+	N
35	25	M	SPUTA	N	N	N	N	Y	3+	N
36	25	F	SPUTA	N	N	N	N	Y	3+	N
37	40	F	SPUTA	N	N	N	N	Y	1+	N
38	18	F	SPUTA	N	N	N	N	Y	1+	N
39	40	F	SPUTA	N	N	Y	Y	Y	3+	N

Sr. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POS. GRADE	HIV
40	30	M	SPUTA	N	N	N	N	Y	3+	N
41	40	F	SPUTA	N	N	N	N	Y	1+	N
42	39	M	SPUTA	N	N	N	N	Y	3+	N
43	17	F	SPUTA	Y	Y	N	N	Y	3+	N
44	26	M	SPUTA	N	N	N	N	Y	3+	N
45	43	M	SPUTA	N	N	N	N	Y	3+	N
46	58	M	SPUTA	N	N	N	N	Y	2+	N
47	25	F	SPUTA	N	N	N	N	Y	1+	N
48	26	F	SPUTA	N	N	N	N	Y	3+	N
49	62	M	SPUTA	N	N	N	N	Y	3+	N

Sr. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POS. GRADE	HIV
50	61	F	SPUTA	N	N	N	N	Y	3+	N
51	35	M	SPUTA	N	N	N	N	Y	1+	N
52	42	M	SPUTA	N	N	N	N	Y	3+	N
53	17	F	SPUTA	Y	N	N	N	Y	3+	N
54	19	F	SPUTA	N	N	N	N	Y	3+	N
55	42	M	SPUTA	N	N	N	N	Y	1+	N
56	75	M	SPUTA	N	N	N	N	Y	1+	N
57	23	M	SPUTA	N	N	N	N	Y	2+	N
58	32	M	SPUTA	N	N	N	N	Y	1+	N
59	20	F	SPUTA	N	N	N	N	Y	1+	N

Sr. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POS. GRADE	HIV
60	74	M	SPUTA	N	N	N	N	Y	2+	N
61	51	M	SPUTA	N	N	N	N	Y	2+	N
62	23	F	SPUTA	N	N	N	N	Y	1+	N
63	75	M	SPUTA	N	N	N	N	Y	1+	N
64	58	F	SPUTA	N	N	N	N	Y	3+	N
65	27	F	SPUTA	N	N	N	N	Y	1+	N
66	65	M	SPUTA	N	N	N	N	Y	1+	N
67	45	M	SPUTA	N	N	N	N	Y	1+	N
68	36	M	SPUTA	N	N	N	N	Y	1+	N
69	17	F	SPUTA	N	N	N	N	Y	1+	N

Sr. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POS. GRADE	HIV
70	58	F	SPUTA	N	N	N	N	Y	1+	N
71	28	M	SPUTA	N	N	N	N	Y	3+	N
72	49	M	SPUTA	N	N	N	N	Y	3+	N
73	27	F	SPUTA	N	N	N	N	Y	3+	N
74	21	F	SPUTA	N	N	N	N	Y	3+	N
75	46	M	SPUTA	N	N	N	N	Y	1+	N
76	37	M	SPUTA	N	N	N	N	Y	1+	N
77	55	F	SPUTA	N	N	N	N	Y	1+	N
78	24	M	SPUTA	N	N	N	N	Y	3+	N
79	27	F	SPUTA	N	N	N	N	Y	3+	N

Sr. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POS. GRADE	HIV
80	35	F	SPUTA	N	N	N	N	Y	3+	N
81	60	M	SPUTA	N	N	N	N	Y	1+	N
82	27	F	SPUTA	N	N	N	N	Y	3+	N
83	34	F	SPUTA	N	N	N	N	Y	3+	N
84	34	M	SPUTA	N	N	N	N	Y	3+	N
85	60	M	SPUTA	N	N	N	N	Y	1+	N
86	41	F	SPUTA	N	N	N	N	Y	3+	N
87	41	M	SPUTA	N	N	N	N	Y	2+	N
89	40	M	SPUTA	N	N	N	N	Y	3+	N
90	55	M	SPUTA	N	N	N	N	Y	1+	N

Sr. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POS. GRADE	HIV
91	21	F	SPUTA	Y	N	N	N	Y	2+	N
92	27	M	SPUTA	N	N	N	N	Y	3+	N
93	69	M	SPUTA	N	N	N	N	Y	1+	N
94	27	F	SPUTA	N	N	N	N	Y	1+	N
95	60	M	SPUTA	N	N	N	N	Y	1+	N
96	27	F	SPUTA	Y	N	N	N	Y	3+	N
97	20	M	SPUTA	Y	N	N	N	Y	3+	N
98	55	F	SPUTA	Y	N	N	N	Y	3+	N
99	22	M	SPUTA	Y	N	N	N	Y	2+	N
100	55	M	SPUTA	Y	N	N	N	Y	3+	N

Sr. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POS. GRADE	HIV
101	79	F	SPUTA	Y	N	N	N	Y	3+	N
102	40	M	SPUTA	Y	N	N	N	Y	2+	N
103	45	M	SPUTA	Y	N	N	N	Y	3+	N
104	65	M	SPUTA	Y	N	N	N	Y	3+	N
105	65	M	SPUTA	Y	N	N	N	Y	4+	N
106	20	M	SPUTA	Y	N	N	N	Y	3+	N
107	42	M	SPUTA	Y	N	N	N	Y	4+	N
108	22	M	SPUTA	Y	N	N	N	Y	4+	N
109	19	M	SPUTA	Y	N	N	N	Y	3+	N
110	68	M	SPUTA	Y	N	N	N	Y	4+	N

Sr. NO	AGE	GENDER	SAMPLE	PAST TB	FAMILY H/O	TOBACCO	ALCOHOL	X-RAY	AFB-POS. GRADE	HIV
111	22	M	SPUTA	Y	N	N	N	Y	4+	N
112	40	F	SPUTA	Y	N	N	N	Y	3+	N
113	29	F	SPUTA	Y	N	N	N	Y	2+	N
114	29	F	SPUTA	Y	N	N	N	Y	3+	N
115	40	M	SPUTA	Y	N	N	N	Y	3+	N
116	43	F	SPUTA	Y	N	N	N	Y	3+	N
117	60	M	SPUTA	Y	N	N	N	Y	3+	N
118	70	M	SPUTA	Y	N	N	N	Y	2+	N
119	43	F	SPUTA	Y	N	N	N	Y	2+	N
120	79	M	SPUTA	Y	N	N	N	Y	3+	N

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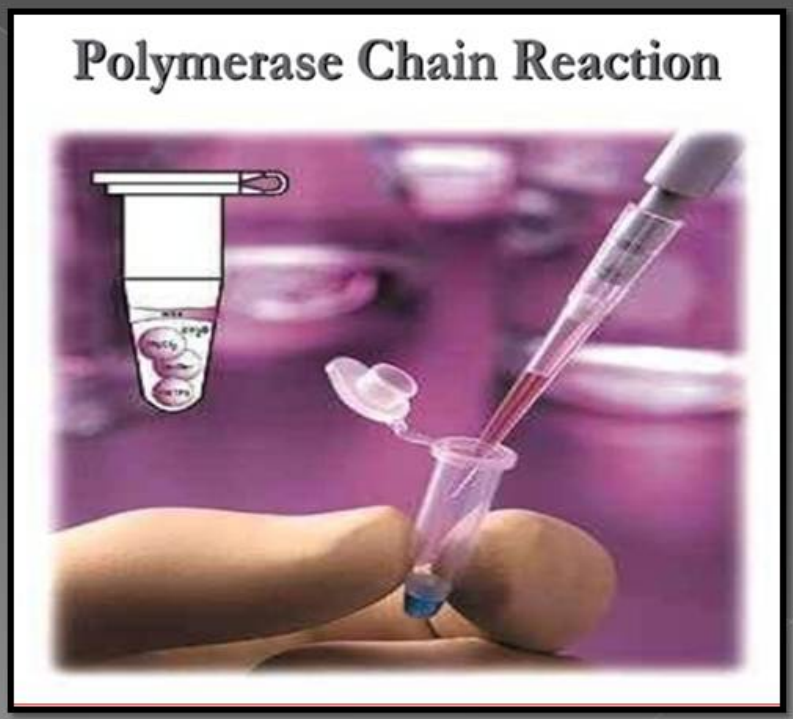
Chapter 5: Detection of MTB with Real time PCR



5.1 Introduction

Polymerase chain reaction (PCR) is one of the safe and reproducible new approach for the rapid and early stage identification of TB infection. The PCR method is a direct and continuous identifying process for MTB in clinical specimens [1]. In this study, PCR method was used for the quantification of MTB DNA in pulmonary sputum samples. It is a simple laboratory technique to obtain multiple copies of specific DNA fragments even from samples containing only minute quantities of DNA or RNA. The name, polymerase chain reaction is derived from the DNA polymerase used to amplify a piece of DNA by *in vitro* enzymatic replication. This process is known as a "chain reaction". The PCR based techniques have revolutionized the field of molecular biology and have seen a modified and are widely used in microbiology, biotechnology, genetics, molecular diagnostics, and clinical, forensic and environmental laboratories [2].

Real time PCR is a recent modification of the standard PCR technique, which combines the objectivity of fluorescence detection with the simplicity of a basic PCR assay. This method is used for the detection and quantitation of amplified PCR product as the reaction progresses in 'real time'. This new approach is based on the incorporation of a fluorescent dye, DNA-intercalating dyes SYBR Green or sequence-specific oligonucleotide chemistry such as TaqMan probes are increase in fluorescence signal generated during the PCR is directly proportional to the separation of amplicons detection [3].

Real time PCR has earned wider acceptance due to several advantages that it offers. Use of a closed system, leading to the reduced risk of carryover contamination, improved sensitivity, and reproducibility, reduced turnaround time, wide dynamic range of target detection, and feasibility for quantitation are a few of the advantages of this method. It is accepted as the "Gold standard" for diagnosis of several pathogens, and quantification of viral load in clinical samples- an indicator of active infection, disease progression and therapeutic response to antiviral drugs [4].

The substantial monetary investment required for real time PCR instrumentation and reagents is a major stumbling block for its routine use in most diagnostic laboratories. However, it is cost-effective in high throughput laboratories and can become a feasible option for many other laboratories as more indigenous and less expensive commercial kits are made available in the near future. In a conventional PCR, the standard protocol involves the exponential amplification of a

specific DNA sequence which is achieved through a series of cycles, each comprising of three steps- denaturation, annealing and extension [5]. Some of the generally used variants of PCR are discussed below.

5.2 Real Time PCR

5.2.1. Polymer chain reaction:

Real time PCR is a very quick and accurate analysis tool for early detection of TB. In a country like India with such high burden of TB and limited resources for diagnosing TB, PCR is found to be very rapid and sensitive method to aid in early diagnosis, treatment, and cure of TB. The PCR is a molecular technology, the main aim to amplify a single copies of the DNA to thousands or millions of copies. This technique developed in 1983 by kary Mullis. PCR employs two main reagents, Primers (single stranded DNA fragments: oligonucleotides and a DNA polymerase.

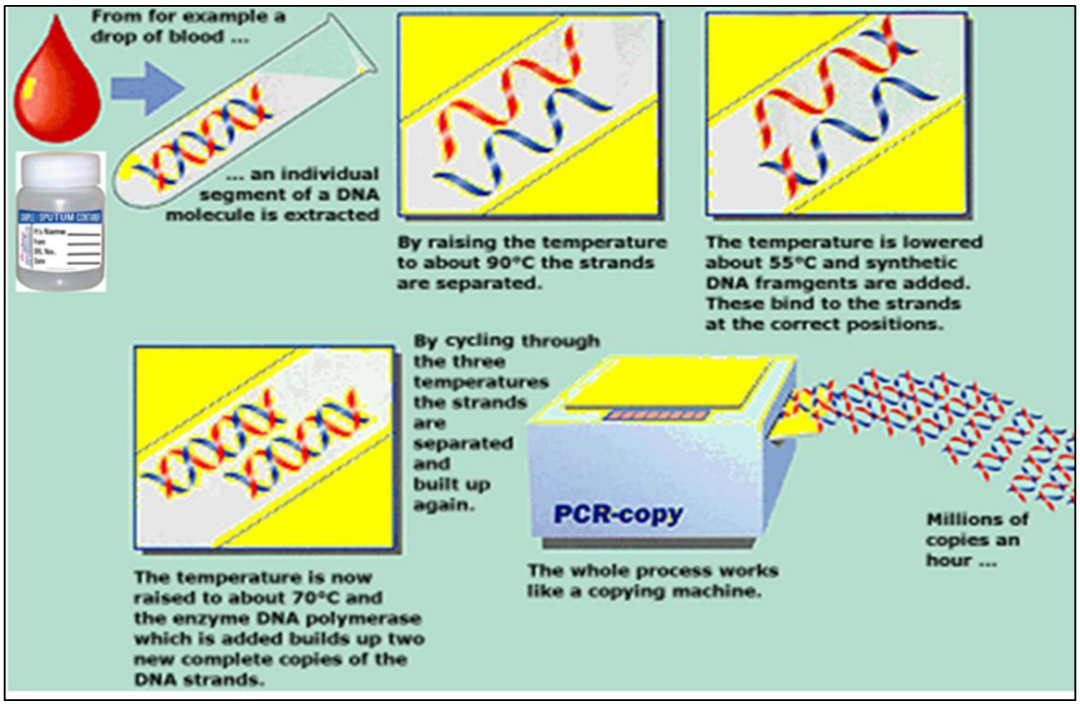


Fig.5.1 The schematic representation of Polymer Chain Reaction: DNA amplification

The DNA molecule is extracted and processed by using thermal cycler. In this reaction the three steps involved. The strands are separated and built up again by raising the temperature to about 90 ^{0} C for 10 minutes, the strands are separated again the temperature is lowered about 55^{0} C for 20 seconds and synthetic DNA fragments

are added. These bind to the strands at the correct positions. By cycling through the three temperature the strands are separated and built up again. The temperature is then raised to about 70 ^{0}C for 15 seconds and the enzyme DNA polymerase which is added and builds up two new copies of the DNA strand. The whole process works like a copying machine and millions of copies generated within hours. The schematic representation of PCR: DNA amplification layout is shown in Fig 5.1. It consists of mainly 4 steps: initial activation, denaturation, annealing and extension.

5.2.2 Principle of Real –Time PCR

The target detection of MTB DNA, the PCR amplifies a specific area of a DNA strand. Most real time PCR methods amplify DNA fragments of between up to 40 kbp. The quantity of amplified product is determined by the obtainable substrates in the reaction, which become restrictive as the reaction progresses. The robust assay exploits for newer RT-PCR technique called Taqman principle. During PCR, forward and reverse primers hybridize to a specific sequence product [6]. A TaqMan probe, which is contained in the same reaction mixture and which consists of an oligonucleotide labeled with a 5'-reporter dye and a downstream, 3'-quencher dye, hybridizes to a target sequence within PCR product. A Taq polymerase which possesses 5'-3'exonuclease activity cleaves the probe. The reporter dye and quencher dye are separated upon cleavage, resulting in a fluorescence for the reporter [7]. Thus, the increase in fluorescence is directly proportional to the target amplification during PCR.

The use of PCR in molecular diagnostics has increased to the point where it is now accepted as the gold standard for detecting nucleic acids from a number of origins and it has become as essential tool in the research laboratory. Real time PCR has created a wider acceptance of the PCR due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carryover contamination [8]. The method utilizes a pair of synthetic oligonucleotides or primers, each hybridizing to the one spanning a region that will be exponentially reproduced. The hybridized primer acts as a substrate for a DNA polymerase most commonly derived from the thermophilic bacterium called *Thermus aquaticus* (Taq), which creates a complementary strand via the sequential addition of De-oxy nucleotides. The schematic representation of PCR optical detection layout shown in Fig 5.2 It consists of 1.halogen tungsten lamp, 2 excitation filters, 3intensifier, 4 sample plate, 5 ccd detector.

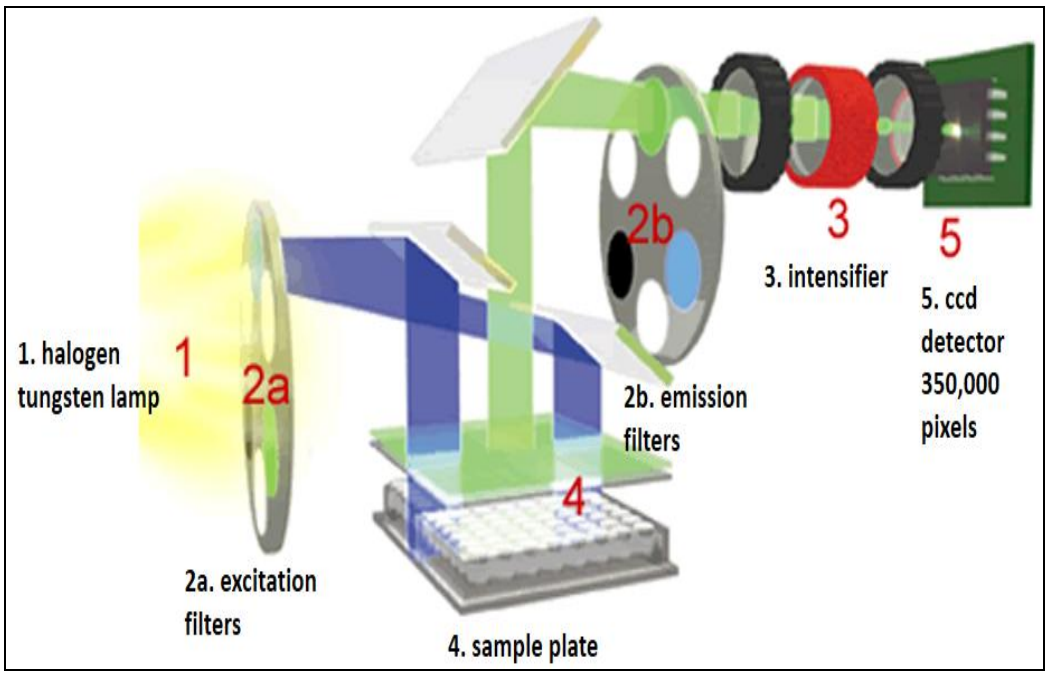


Fig. 5.2 Schematic representation of PCR optical detection layout

5.2.3 Applications of PCR

The applications of PCR in infectious disease diagnostics include specific or broad-spectrum pathogen detection, evaluation of emerging infections, surveillance, detection of agents of bioterrorism and antimicrobial resistance profiling. In the field of infectious diseases, PCR can be an attractive strategy as it yields rapid results, has high throughput along with high sensitivity and specificity. The PCR based techniques have revolutionized the field of molecular biology and have witnessed an exponential growth in the recent years. These techniques have been extensively modified and are widely used in clinical microbiology, virology, bacteriology, mycology, Parasitology forensic medicine, biotechnology and allied fields, genetics, and environmental laboratories, besides several other applications [9].

5.2.4 Detection of infectious diseases

The real time PCR permits for fast and extremely specific detection of infectious diseases, as well as those caused by *Mycobacterium tuberculosis* infection. The innovative RT-PCR permits identification of slow-growing *Mycobacterium tuberculosis* bacilli, anaerobic bacterium and viruses. The main idea for RT-PCR diagnostic applications in medical science are to detect infectious agents and also the sensitivity of non-pathogenic from infective strains by virtue of specific genes. It

also allowed the detection of low quantity of disease organisms (both live and dead), in clinical samples [10, 11].

5.2.5 Types of PCR, Quantitative and Qualitative analysis

This is one of the most common variants of polymer chain reaction used today

[a] Hot start PCR: One of the major drawbacks of conventional PCR is specificity that could arise through activities if Taq polymerase enzyme amplifying non-specific targets .This problem is overcome by using several strategies wherein the enzyme gets activated only at higher temperatures [12].

[b] Universal PCR/ Broad range: Very frequently, one comes across a problem where PCR is to design to target all species belonging to a genus or even to detect the presence of all different types' bacteria in a clinical sample, desired results could be achieved by using conserved sequences of certain genes to design the primers [13].

[c] Multiplex PCR: in microbiology, as a part of a diagnostic protocol of polymicrobial infections, detection of multiple organisms has to be done simultaneously. In such situations, performing PCR separately for individual microbe would be expensive and time consuming. To overcome this problem, primers can be designed in such a way that they can be simultaneously used in a single reaction to detect anywhere between 2 and 10 different microbes at one go [14].

[d] Nested PCR: Increased sensitivity can be offered by modifying the PCR protocol, which could be very useful in clinical applications, especially where the same samples are limited in quality. Nested PCR is a modification that increases the specificity and sensitivity of PCR. Here two pairs of primers are used to amplify a single target in two separate runs. The second pairs of primers, designed to bind slightly inside the binding sites of the first pair, will amplify the product of the first PCR in a second round of amplification [15].

[e] Quantitative PCR: Even though real time PCR is most ideal for quantitating a template, conventional PCR can also be used for this purpose. It may not be very accurate, but still can provide useful information under certain circumstances. This could be achieved by using either external standards or internal standards such as various housekeeping genes, since the data is not obtained in terms of absolute copy numbers, it is called as relative quantification [16].

[f] Reverse transcriptase PCR: In clinical microbiology, one has to ideal deal with situations where there is a need to look at the expression levels of cellular transcripts such as cytokines or detection of RNA viruses in body fluids [17]. In these

experiments, the nucleic acid is available in the form RNA. The RNA will have to be converted into complementary DNA by using enzymes derived from retroviruses and the process is called reverse transcription [18]. The cDNA thus obtained will be then subjected slandered PCR using the regular protocol. This protocol can be adapted to real time PCR also for quantification of the data. There are several other variations such as inverse PCR, booster PCR, asymmetric PCR, touchdown and touch-up PCR that have been used for different purposes [19, 20].

5.3 Experimental

5.3.1 Materials and methods

In this PCR experiment we have used DNA purification kit, primers, oligonucleotides were purchased from integrated DNA technology (IDT), USA and NALC (N-acetyl-L-cysteine) were purchased from Thomas Baker India.

5.3.2 Sample pre- treatment

Sputum samples collected from the patient as listed in table 5.2 are studied for their PCR analysis. This study were confirmed by conventional microscopic observation by using Acid-fast staining using ZN (Ziehl-Neelsen) stain. The pulmonary sputum samples are obtained from clinical and radiological evidence of tuberculosis and these samples are selected for DNA extraction. All specimens were treated with 0.5 % N-acetyl-L-Cysteine (NALC) / 2% NaOH method for digestion and decontaminated and concentrated by centrifuging at 3000 rpm for 15 minutes. Supernatant is decanted, and transferred the sediment in 2 ml of phosphate buffer pH 6.8. The sediment is used for ZN stain and DNA extraction [21].

5.3.3 MTB DNA extraction

The pretreated sample were kept at 80^0C for 10 minutes for inactivation of *Mycobacteria*. DNA was extracted from all positive sputum samples (n=90) as listed in table 5.2. Conventional chloroform-phenol method, commercially available DNA kit (QIAGEN) and MNP based method were used for DNA extraction. A small part of pulmonary sputum was used for DNA extraction. For magnetic DNA extraction method after cell lysis, 100 µL magnetic nanoparticle (Fe_3O_4) is added into the micro centrifuge tube, MNPs are immobilized by an external magnet, by using elution buffer and DNA was separated and kept in -20 ºC [22].

5.3.4 Detection of MTB DNA with Real time PCR

(a) Real time PCR conditions

The isolated DNA from clinical sputum samples are processed for amplification by real-time PCR for *in- vitro* diagnostic use. Total 25 µl reaction volume of master mix it contains 12 µl (R1) super mix, magnesium solution, MTB complex 2.5µl (R 2) and internal control IC-1 (R 3) RG 0.5 µl and 10 µl extracted DNA samples. The PCR grade water used as a negative control. Cycling conditions were 1 cycle initial activation at 95°C for 10 min, a number of cycles 45 cycles, denaturation at 95°C for 15 seconds, annealing at 60°C for 20 seconds and an extension step at 72°C for 15 seconds [23]. Real time PCR conditions are shown in table no.5.1

Table No. 5.1: # Real time PCR conditions

PCR-steps	Cycle	Temperature	Time
Initial activation	1 cycle	95°C	10 minutes
Denaturation	1 cycle	95°C	15 minutes
Annealing	45 Cycle	60°C	20 seconds
Extension	45 Cycle	72 °C	15 seconds

(b) Amplification of MTB species genes with IS6110 primers

According to Genome Diagnostics Pvt. Ltd. PCR, primers were used. The probes and primers were purchased from Genome Diagnostics Pvt. Ltd. India, MTBC- specific primers were used to amplify a targeted *IS6110*, the Accession number LC005454.1. An internal control (IC) plasmid genomic DNA used to detect PCR inhibition in the extracted MTB DNA (n=90). The schematic representation of amplification of MTB species gene by is *IS6110* primers shown in Fig 5.3. The process is summarized in three steps

1. ds DNA separation, 2. Primer annealing, 3. Optimal extension

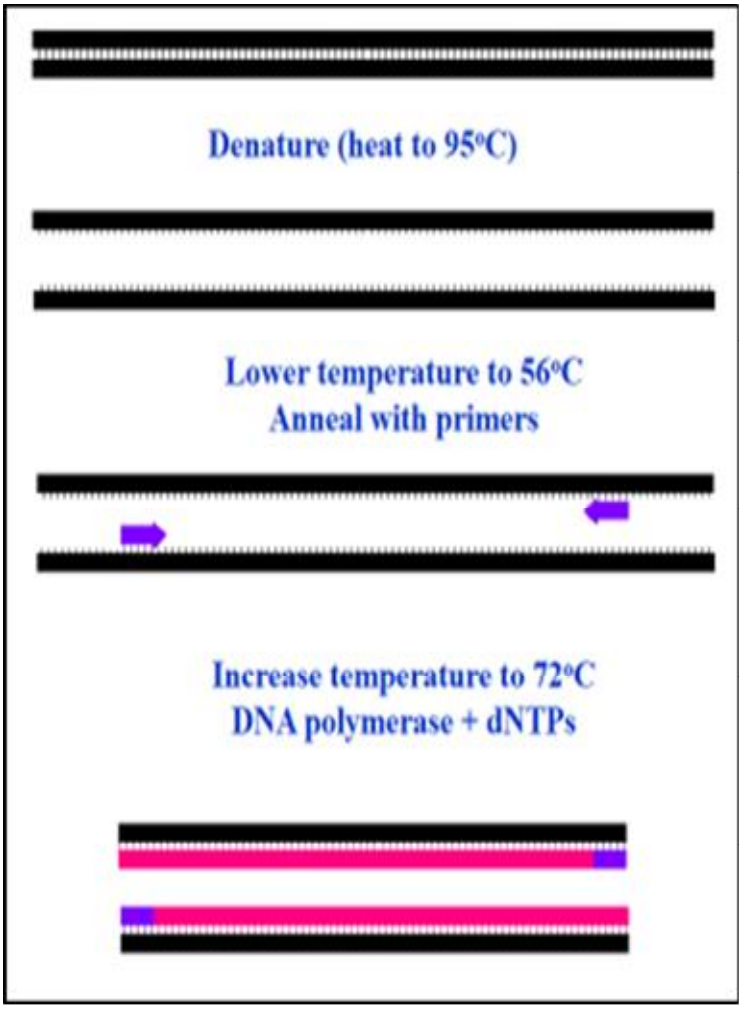


Fig. 5.3 Schematic representation of Amplification of MTB species genes by *IS6110* primers

5.4 Results and discussion:

5.4.1 Detection of MTB patient with Real time PCR (Quantification of PCR amplicons)

The patients are diagnosed for MTB disease by using collection of sputum samples and extracting their DNA. The analysis of DNA of the samples is studied with RT-PCR. As per the principle of RT-PCR the outcomes of the measurement is recorded in terms of fluorescent intensity is dependent on the number DNA present in the sample. These DNA are increased and measured in terms of number of cycles for non TB patient. The variations of fluorescent intensity with number of cycles is shown in Fig. 5.4.2. It is seen that there is sharp increase in fluorescent intensity at 28.33 cycles crossing the plot of control sample from the nature of the graph and data collected from the RT PCR is given in the table adjacent to the figure. It is seen that

the sample is positive with MTB detected of 1.3 x 10^{03} cfu/ml. The onset of the sudden rising is denoted as Ct value. The Ct from the theory of PCR Ct is inversely proportional to initial concentration of MTB DNA in the sample. The variation of Ct values for different patient depend on the severity of TB infection. The typical plots for two different patients are shown in Fig 5.4.3 and 5.4.4. The Ct values for 90 patients obtained from results of RT-PCR are listed in table 5.2.

$$C_t \alpha \frac{1}{N_i} \quad \text{...1}$$

Where, N*i* is the initial concentration of MTBDNA in the sputum sample. This indicates the sensitivity of the TB disease. Higher the initial concentration lower is the Ct value. From table 5.2 it is seen that the lowest Ct value of the order of 22 is found in 17 TB patients (Sr. No. 35, 54, 56, 57, 58, 60, 71, 73, 75, 78, 80, 82, 83, 84, 86, 87,& 90)out of the these 17 patient 11 are male and 6 are female. The most TB affected age group is 21-30 years. This indicates that 19 % population of the TB patient is in the serious stage. The results of real time PCR is shown in Fig. 5.4.1 Further the highest magnitude of Ct value was observed the order of 35 in patient number 4 & 6. This indicates the lowest value of MTB DNA present in TB patient can also be detected at the early stage. The negative results RT-PCR melting curve is shown in Fig. 5.4.1 of Samples contain MTB DNA and Fig 5.4.2. (JOE/Yellow channels: the samples contain internal control and FAM (Green) channel: the sample contain MTB DNA). Our graph result shows positive in Ct value is (26.33) the melting curve of positive results shown in Fig 5.4.3.

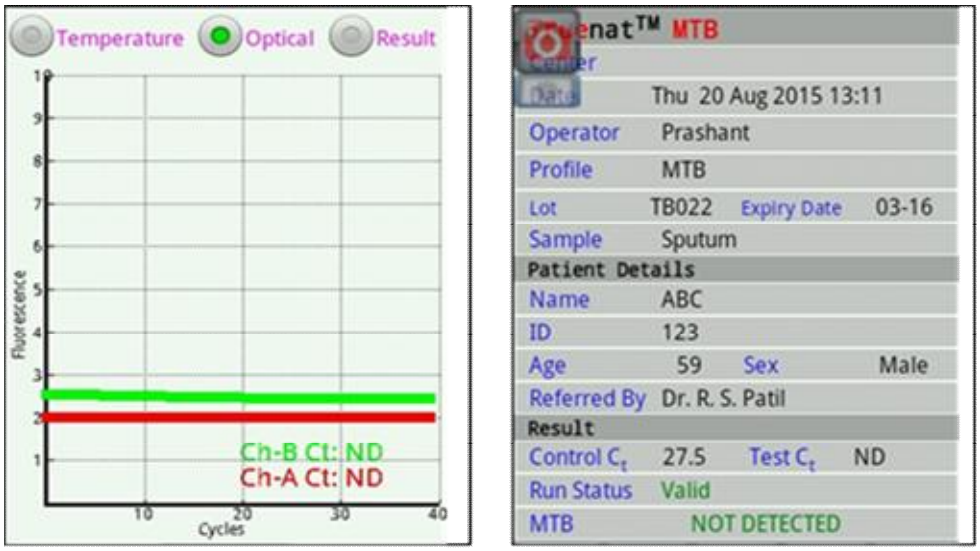


Fig. 5.4.1 Melting curve of negative MTB result

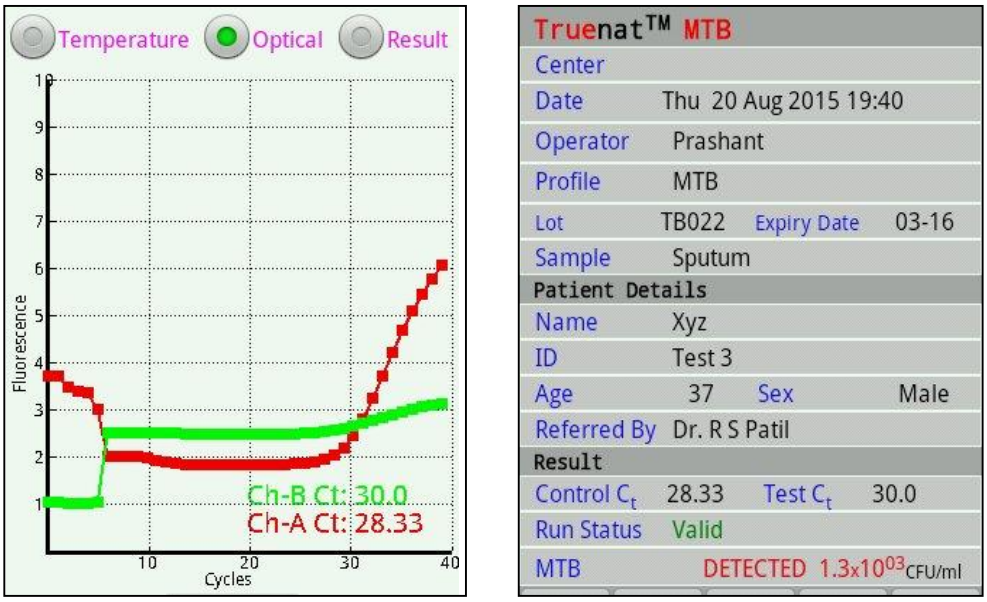


Fig. 5.4.2 CT value and melting curve of positive MTB result

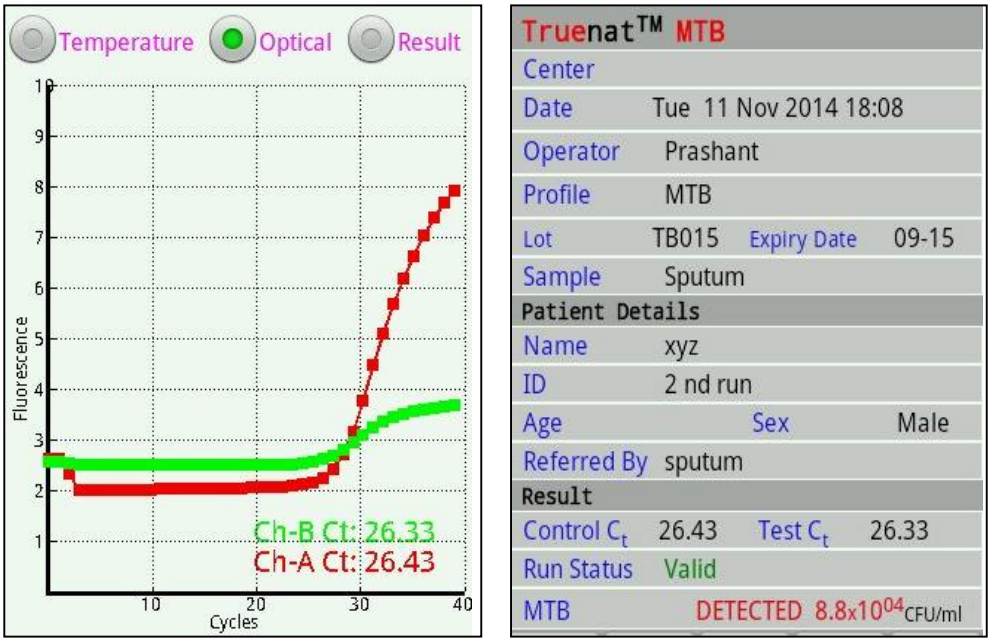


Fig. 5.4.3 Melting curve of positive MTB result

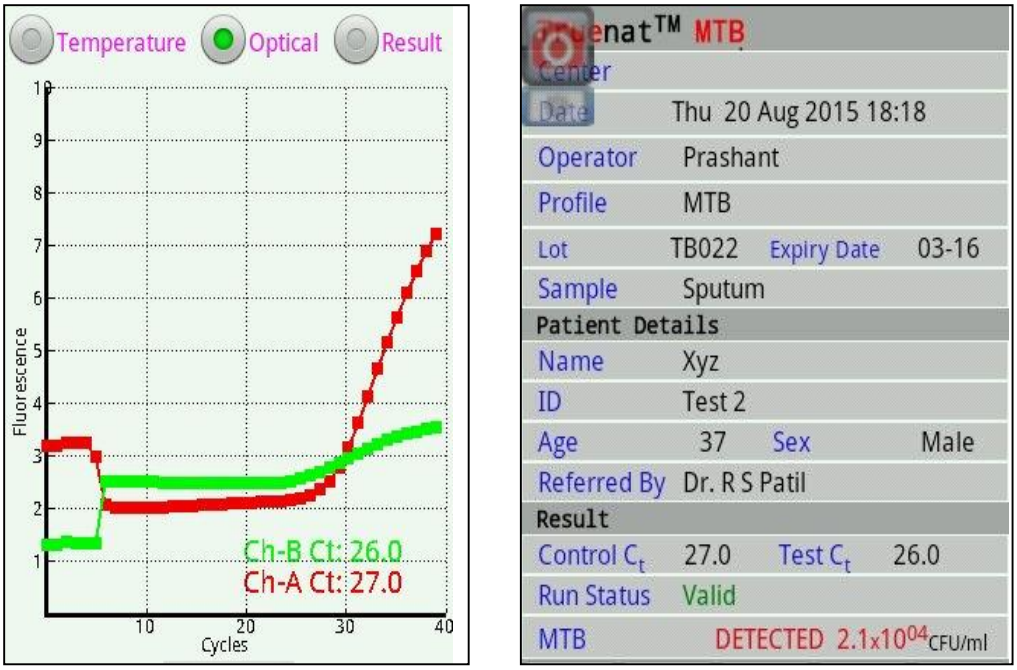


Fig. 5.4.4 CT value and melting curve of positive MTB result

5.4.2 Comparison of TB patient based on CT value

The real-time PCR amplification was performed in a total volume of 20 µl that contained 10 µl of 2× thunderbird probe qPCR mix (Toyobo), 3.0 µl of primer and the TaqMan® probe mixture, 5 µl of template DNA, and PCR grade H_2O added to give a final volume of 20 µl for each sample. Positive and negative controls were included throughout the procedure. No-template controls with PCR grade H_2O instead of template DNA were incorporated in each run under the following conditions: 95 °C for 3 min and 40 cycles of 95 °C for 20 second and 60 °C for 40 second in single real-time PCR. The bacterial load was quantified by determining the cycle threshold (CT), the number of PCR cycles required for the fluorescence to exceed a value significantly higher than the background fluorescence. A positive result was indicated when the CT value was less than 35 after observing the signal formation of wavelength from each channel [24-26].

The figure 5.4.1 shows melting curve of negative MTB results, it suggests that after 20 PCR cycles the MTB results show negative. The control melting curve also shows melting negative results (Ct value 27.5). It also proves the test is valid and negative MTB results confirm against the control value.

The figure 5.4.2 shows positive MTB results. The control Ct values 28.33 and test Ct value 30. The melting curve of positive MTB results after 30 cycles the

fluorescence of positive melting curve slightly increases and detect the MTB target ($1.3x10^{03}$ CFU/ml.)

The figure 5.4.3 shows positive MTB results. The control Ct values 26.43 and test Ct value 26.33. The melting curve of positive MTB results after 26.33 cycles the fluorescence of positive melting curve slightly increases and detects the mycobacterial target ($8.8x10^{04}$ CFU/ml.)

The figure 5.4.4 shows positive MTB results. The control Ct values 27 and test Ct value 26. The melting curve of positive MTB results after 26 cycles the fluorescence of positive melting curve slightly increases and detects the MTB target ($2.1x10^{04}$ CFU/ml.). The sensitivity and specificity and rapidity of real time PCR test in diagnosis of *Mycobacterium tuberculosis* disease shown in this study should inspire the use of this technique in routine MTB diagnosis. We compared the performance of different age group, gender, and AF positive pulmonary sputum samples for early diagnosis of TB [26]. The master chart of all positive sputum smear AFB grade (n=90) and RT-PCR Ct values were listed in table 5.2**.**

5.5 Conclusions

Real time PCR prove rapid, sensitive and specificity for pulmonary tuberculosis with turn-around time 2 h compared with conventional culture based methods that takes between 2 to 6 weeks for TB detection. Real time PCR showed rapid and accurate early detection of TB. The Ct values of positive TB (26.43) cycles and detected the MTB target ($1.3x10^{03}$ CFU/ml). We compared the performance of different age group, gender and acid fast positive pulmonary sputum smear AFB grade (n=90) and real time PCR Ct values are listed in table 5.2. It is seen that lowest Ct values of the order of 22 is found in 17 TB patients (11 male and 6 female). The most TB affected age group is 21-30 years. Above these findings our results shows real time PCR assay donate considerably for an early TB detection and use an control on the anti-TB drug management and TB control. The efficacy of this assay for MTB diagnosis was equal with bacterial culture, thus it can be implemented in a tertiary care setting.

Table 5.2 AFB grade and Real time PCR Ct values

SR NO	AGE	GENDER	AFB-POSITIVE GRADE	REAL TIME PCR	CT VALUES
1	45	M	+	y	30
2	38	M	+	Y	29
3	66	F	+	y	30
4	26	M	3+	y	35
5	30	M	3+	y	33
6	37	F	2+	Y	35
7	60	M	3+	y	32
8	17	F	3+	Y	33
9	35	F	3+	Y	30
10	51	M	2+	Y	28
11	71	M	3+	Y	27
12	20	M	1+	Y	25
13	22	M	3+	Y	30
14	40	M	1+	Y	27
15	15	F	2+	Y	30
16	47	F	3+	Y	30
17	62	F	1+	Y	29
18	23	F	1+	Y	28
19	40	M	3+	Y	30
20	50	M	3+	Y	32
21	45	M	1+	Y	30
22	38	M	3+	Y	28
23	65	F	1+	Y	30
24	65	M	1+	Y	27
25	22	M	2+	Y	28
26	18	F	2+	Y	27
27	66	F	3+	Y	32
28	20	M	1+	Y	30

SR NO	AGE	GENDER	AFB-POSITIVE GRADE	REAL TIME PCR	CT VALUES
29	26	M	3+	Y	28
30	28	M	1+	Y	28
31	14	M	1+	Y	25
32	50	M	1+	Y	25
33	60	M	3+	Y	26
34	21	F	1+	y	21
35	25	M	3+	Y	22
36	25	F	3+	Y	27
37	40	F	1+	Y	28
38	18	F	1+	Y	30
39	40	F	3+	Y	28
40	30	M	3+	Y	27
41	40	F	1+	Y	25
42	39	M	3+	Y	24
43	17	F	3+	Y	27
44	26	M	3+	Y	26
45	43	M	3+	Y	24
46	58	M	2+	Y	25
47	25	F	1+	Y	23
48	26	F	3+	Y	25
49	62	M	3+	Y	24
50	61	F	3+	Y	23
51	35	M	1+	Y	22
52	42	M	3+	Y	26
53	17	F	3+	Y	25
54	19	F	3+	Y	22
55	42	M	1+	Y	25
56	75	M	1+	Y	22
57	23	M	2+	Y	24
58	32	M	1+	Y	22
59	20	F	1+	Y	23

SR NO	AGE	GENDER	AFB-POSITIVE GRADE	REAL TIME PCR	CT VALUES
60	74	M	2+	Y	22
61	51	M	2+	Y	24
62	23	F	1+	Y	24
63	75	M	1+	Y	23
64	58	F	3+	Y	24
65	27	F	1+	Y	23
66	65	M	1+	Y	24
67	45	M	1+	Y	23
68	36	M	1+	Y	23
69	17	F	1+	Y	24
70	58	F	1+	Y	23
71	28	M	3+	Y	22
72	49	M	3+	Y	23
73	27	F	3+	Y	22
74	21	F	3+	Y	23
75	46	M	1+	Y	22
76	37	M	1+	Y	23
77	55	F	1+	Y	24
78	24	M	3+	Y	22
79	27	F	3+	Y	23
80	35	F	3+	Y	22
81	60	M	1+	Y	24
82	27	F	3+	Y	22
83	34	F	3+	Y	23
84	34	M	3+	Y	22
85	60	M	1+	Y	24
86	41	F	3+	Y	22
87	41	M	2+	Y	22
88	40	M	3+	Y	24
89	61	M	2+	Y	24
90	55	M	1+	Y	22

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Chapter 6: Synthesis, Characterization and application of SPIONs in DNA extraction and detection of MTB with PCR

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DETECTION OF MYCOBACTERIUM TUBERCULOSIS FROM PULMONARY SPUTUM SAMPLE USING SPION MEDIATED DNA EXTRACTION METHOD

D. V. Sawant, R. A. Bohara, R. S. Patil, S.H. Pawar*

6.1 Introduction

Tuberculosis (TB) is a respiratory disease caused by *Mycobacterium tuberculosis*. [1]. There may be an urgent requirement for fast and price effective diagnostic approach. The distinguishing characteristic of all *Mycobacterium* species is that the cell wall is thicker than in lots of other different bacteria, being hydrophobic, waxy, and rich in mycolic acids [2]. Pulmonary tuberculosis represents around 85% of TB cases, at the same time as much extra pulmonary TB cases are very low (15%) [3, 4]. TB detection method remains a challenging task for both clinicians and Microbiologists because of the low sensitivity of conventional acid-fast staining methods. In this background, in recent year's one new approach for rapid, safe and reproducible identification of MTB infection is real-time polymer chain reaction (RT-PCR). The success of final amplification and detection of DNA depends upon extraction from clinical samples is important. Presently nanotechnology based DNA extraction and detection methods are highlighted [5].

Nanotechnology is the influence of individual atoms and molecules as the manipulation of matter with at least one dimension sized from 1 to 100 nanometers. The branch of nanotechnology that deals with dimensions and tolerances of less than 100 nanometers. The goal of nanotechnology precisely manipulating atoms and molecules for fabrication of macro scale products, similarly now mentioned to as molecular nanotechnology. The interdisciplinary background of nanotechnology has important effect in many applications specifically medical finding. The unique, physical, chemical, electrical and optical features of nanomaterials for many diagnostic purpose such as early detection of TB and targeted management of disease [6].

Magnetic nanoparticles (MNPs) have shown unlimited potential in various fields. When the size of the MNPs becomes lesser from few nanometers to a couple of tenth of nanometers, depending on the material, it becomes superparamagnetic appearances. SPIONs (superparamagnetic iron oxide nanoparticles) are single domain particles with all their magnetic moments united in the same way, and with a short relaxation time. Due to their distinctive features, SPIONs are intensively industrialized and have found many uses in in bio-sensing, targeted drug delivery, magnetic resonance imaging, cell separation, DNA (deoxyribonucleic acid) separation, biological entities (cell, protein, nucleic acids, enzyme, bacterial, virus, etc.) SPION primarily based MTBDNA extraction technique is straightforward to

synthesize, it provides a vast surface area, and do not affect the structure and properties of DNA. Magnetic properties and biocompatibility, is helpful in magnetic extraction of DNA. Magnetic adsorption techniques are used for the isolation of DNA from pulmonary sputum samples [7].

From positive sputum samples we demonstrated SPION mediated MTB DNA extraction method. It proves to be a rapid, cheap, it is done by a single test tube, without the use of toxic chemicals enzymes. The extracted MTB DNA samples from this technique are worthy for Real-Time PCR detection [8]. The potential uses of SPIONs use as MRI (magnetic resonance imaging) contrast agent, target drug delivery and imaging, cellular tracking, magnetic cell separation technologies, biological entities (protein, nucleic acids, enzyme, bacteria, virus, etc.) imaging probe, microfluidic device [9,10]. In the present investigation we have attempted to develop an advanced SPION-mediated DNA extraction approach; which may be rapid less expensive and be achieved in a single test tube. The SPIONs used are helpful for generating magnetic field gradients and hence the extracted DNA from this method are pure and useful for real time PCR detection [11,12].The success of final amplification and detection of Nucleic Acid Amplification test (NAAT) depends on successful DNA extraction from pulmonary sputum samples [13-15].

6.2 Experimental

6.2.1 Material

Analytical grade chemicals were used for the preparation of SPIONs mainly Ferrous chloride ($FeCl_2$) and Ferric chloride ($FeCl_3$) were purchased from Molychem India, Sodium chloride (NaCl), Tris- EDTA, sodium dodecyl Sulfate (SDS), Sodium hydroxide (NaOH), NALC (N-acetyl-L-cysteine) and Polyethylene glycol (PEG, MW 8000), were purchased from Thomas Baker, India.

6.2.2 Synthesis of Fe_3O_4 MNPs by Co-precipitation method

Most of the communal methods include the use of co-precipitation, thermal decomposition, hydrothermal synthesis, micro emulsion, sonochemical synthesis, and sonochemical synthetic for the synthesis of high quality of iron oxide NPs and its schematic representation of synthesis of Fe_3O_4 MNPs by co-precipitation method is shown in Fig 6.1.

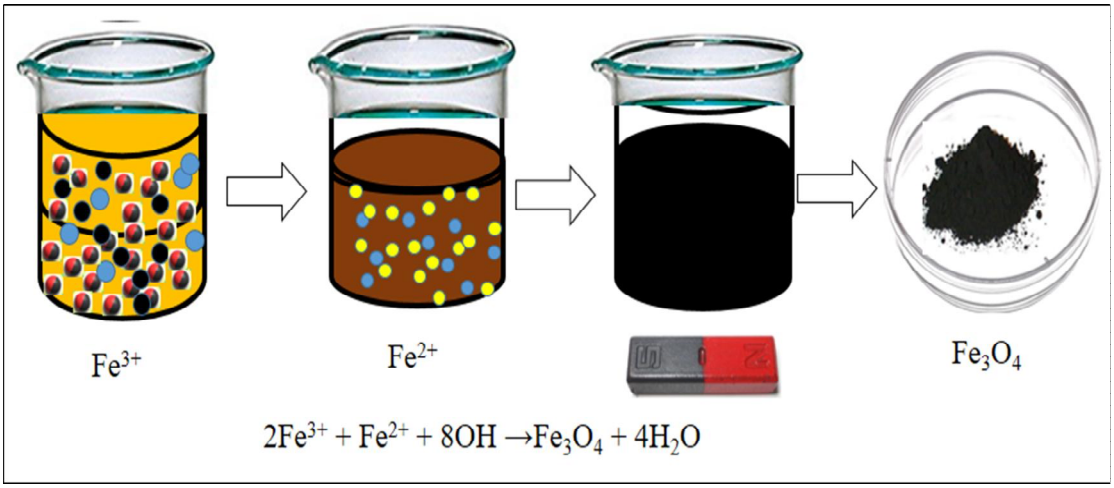


Fig. 6.1 Schematic representation of synthesis of Fe_3O_4 MNPs by co-precipitation method

Chemical co precipitation has the potential to meet the increasing demand for the direct preparation of well dispersed (water-base) Fe_3O_4 nanoparticles. The synthesis of uniform-sized nanocrystals with controllable sizes is very important because the properties of these nanocrystals depends strongly on their dimensions. Furthermore all biomedical applications require that the nanoparticles should have high magnetization values, with sizes smaller than 100 nm, and a narrow particle size distribution [16].

Synthesis of Fe_3O_4 by Co-precipitation method

The raw material SPIONs were prepared by conventional co-precipitation method as reported earlier by kans k *et al* [17]. The Co-precipitation technique was used for the synthesis of SPIONs. Ferrous chloride ($FeCl_2$) and Ferric chloride ($FeCl_3$) were used as a precursors for the reaction in 2:1 (v/v) proportions. In this process, the salt solution of the required metallic elements is reduced by 0.1 NaOH solutions. The reactants were mixed at a temperature of 90˚C. The appearance of black precipitate was indicated the formation of iron oxide nanoparticles. The precipitate was separated by using external magnets and washed thoroughly with double distilled water. The possible reaction taking place is:

$$2Fe^{3+} + Fe^{2+} + 8OH \rightarrow Fe_3O_4 + 4H_2O \quad \text{... (1)}$$

Functionalization of SPION

Cetyltrimethyl ammonium bromide (CTAB) coated SPIONs were taken into a round bottom flask and aqueous ammonia, ethanol, and double distilled water was added into the flask. The mixture was allowed to stir and was added to it.

Nanoparticles of Fe_3O_4 were collected through magnetic separation. Finally, they were isolated by centrifugation, washing several times with double distilled water and drying [18].

6.2.3 Structural analysis of Fe_3O_4 MNPs

The XRD properties of SPION was studied by X-ray diffractometer (Rigaku D/Maxila Diffractometry, USA) and it is shown in Fig.6.2. It shows diffraction peaks at 2θ = 30.23˚, 35.73˚, 43.15˚, 53.69˚, 57.46˚, 63.0˚ which are assigned to (220), (311), (400), (422), (511) and (531). All the diffraction peaks are assigned with standard database in (JCPDS file No. 19-629) and it reveals phase pure SPIONs nanoparticles are successfully obtained. The crystallite size of material is estimated by using Debays Sherrer formula and it is 9.7 for a most intense peak (311).

$$crystalline\ size\ d = \frac{0.9 * \lambda}{\beta . cos\theta} \quad \text{.... (2)}$$

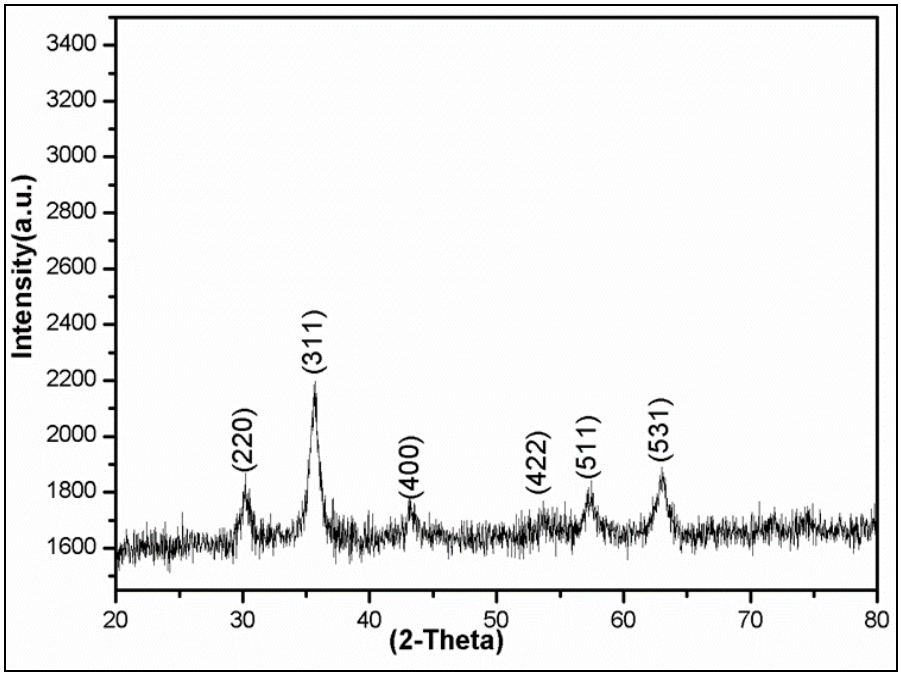


Fig. 6.2 XRD pattern obtained from Fe_3O_4 MNPs

6.2.4 Fourier Transform Infrared Spectroscopy (FT-IR) analysis of Fe_3O_4 MNPs

FT-IR spectrum of SPION is recorded in the range of 400 to 4000 $_{CM}^{-1}$ and it is shown in Fig.6.3. The band observed at 567 $_{CM}^{-1}$ is assigned to the vibration of the Fe- O bonds which is related to the magnetite phase. In additional, the band observed at 1626 $_{CM}^{-1}$ and 3407 $_{CM}^{-1}$ are assigned to the stretching vibration of the hydroxyl group which is present on the surface of SPION. The bands observed at 1461 $_{CM}^{-1}$ and 1626 $_{CM}^{-1}$ are assigned to stretching vibrations of carboxyl group (Coo-). The band observed at 2852 $_{CM}^{-1}$ and 2921 $_{CM}^{-1}$ are assigned to asymmetric and symmetric CH_2 stretching vibration of O-H. [19].

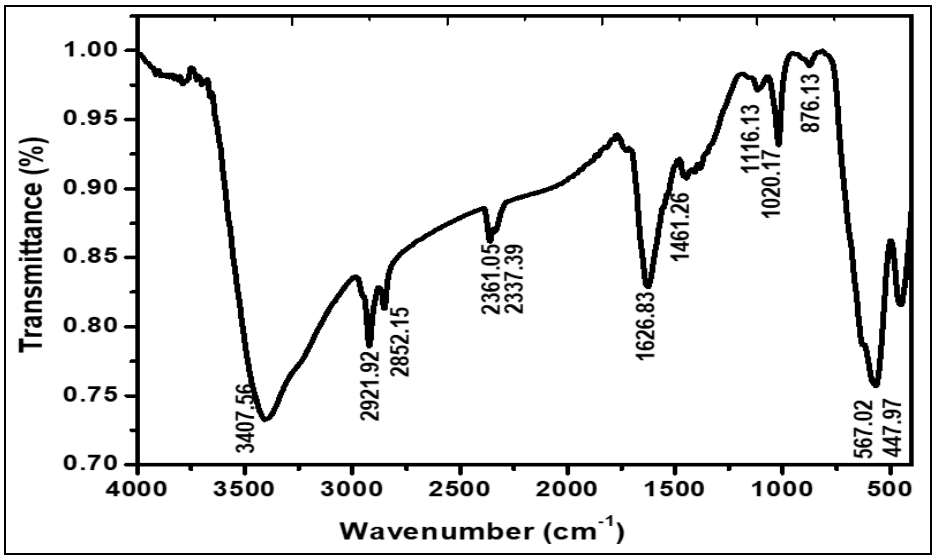


Fig 6.3 FT-IR of Fe_3O_4 MNPs

6.2.5 Field emission scanning electron microscope of Fe_3O_4 MNPs

Surface morphology of SPIONs are studied by using FE-SEM (Field emission scanning electron microscopic) it is shown in Fig 6.4 (a). The bare SPIONs were observed in a spherical and granular shape, however its agglomeration are observed due to dipole dipole interaction. The observed granular size of SPION (43.23nm) is shown in Fig 6.4 (b).

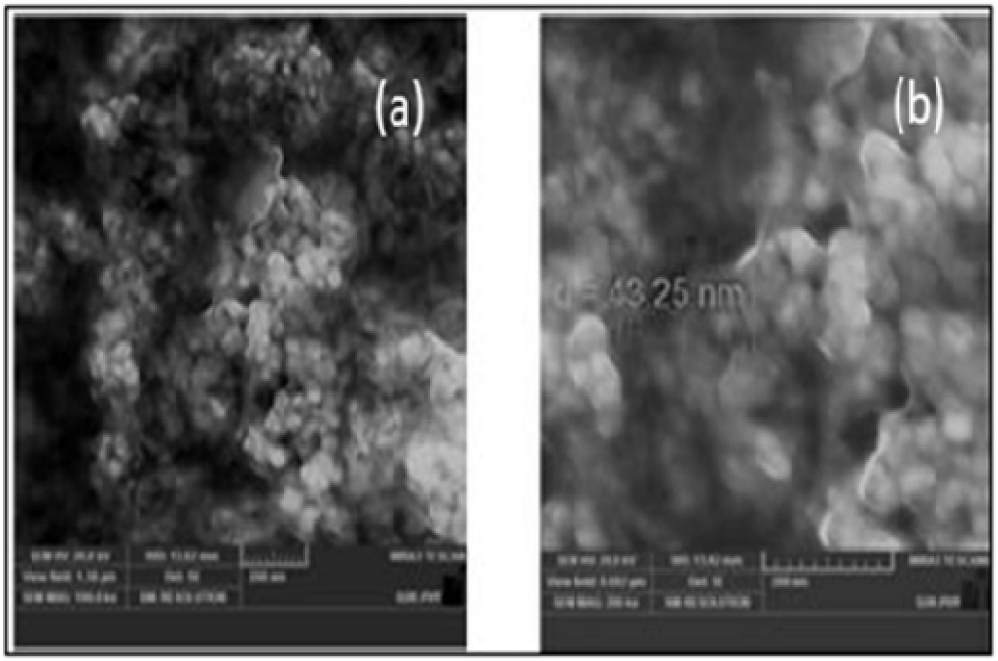
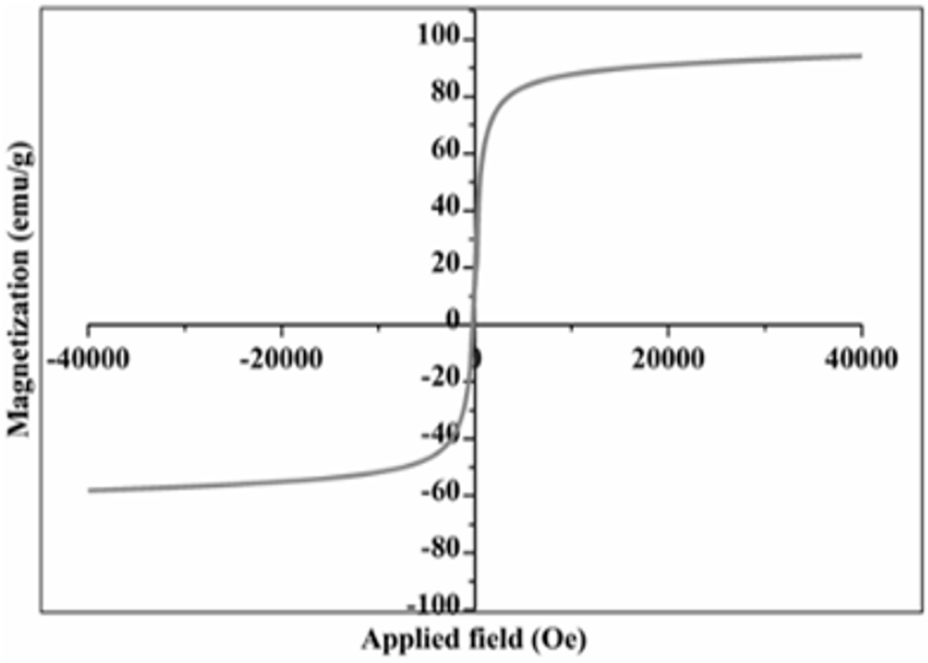


Fig 6.4 Field emission scanning electron microscopy of Fe_3O_4 MNPs

6.2.6 Magnetic measurement of Fe_3O_4 MNPs

The magnetic properties of SPION were studied using a vibrating sample magnetometer. SPIONs usually show superparamagnetic behavior below 20 nm size, which is characterized by zero coercively and remenance. The room temperature M–H curve of samples is shown in Fig 6.5. It can be seen that the Ms Value of the sample is 84 emu g^{-1}. The vibrating sample magnetometer (VSM) curve clearly indicates the super paramagnetic nature of the material which is highly desirable for biomedical operation because larger magnetic particles aggregated after exposure to a magnetic field [20, 21].



6.5 *M-H* loops of Fe_3O_4 MNPs

6.2.7 Processing of pulmonary sputum sample with SPION

The sputum samples were collected from Dr. D. Y. Patil hospital and Research Centre Kolhapur, in a sterile container. Total four hundred samples were collected of these one hundred twenty were sputum smear positive for TB. Samples are pre-treated with NALC (0.5 % N-acetyl-L-cysteine) and 2 % sodium hydroxide for digestion and decontamination. In our protocol, we have used NALC method [22]. Microscopic acid-fast staining direct and concentrated smears were prepared from sputum samples. In brief, samples were mixed with NALC-NaOH solution in a vortex mixture, centrifuged at 3000 rpm for 20 min at 4°C. The supernatant decanted, and transferred the sediment in 2 ml of phosphate buffer pH 6.8. The sediment was used for AFB staining and DNA extraction [23, 24].

6.3 Extraction and purification of MTB

a) Chloroform-phenol method: DNA was isolated from sputum sample using a standard method described previously reported. DNA was finally suspended in 50 µl TE buffer and stored at -20°C [25].

b) Commercial kit method: DNA isolated from sputum sample using the spin column method according to the manufacturer's instructions. DNA was finally suspended in 50 µl TE buffer and stored at -20°C.

c) Extraction by using SPIONs: 200 µl of concentrated liquefied sputum were centrifuged for 15 minutes at 13000 rpm at 4ºC. Pellet was suspended in 200 µl nuclease free water, vortex and boiled at 100 ºC for 20 minutes, then cooled to room temperature. Supernatant was transferred to a new 1.5 ml sterile micro centrifuge tube. 200 µl lysis buffer (10mM Tris-HCl, 0.5 mM EDTA, 10%SDS, pH 7.6) and 10µl proteinase-K (20 mg/ml) were added and incubated in a water bath at 56 ºC for 2 hrs. Samples were centrifuged at 10,000 rpm at 4ºC for 20 minutes. Supernatant was transferred to a new test tube containing 500 µl binding buffer (25% polyethylene glycol 8000 MW) and 100 µl SPIONs. Mixed by gently inverting and allowed to stand at room temperature for 5 minutes.

Using an external magnet supernatant was removed. The magnetic pellet was washed with cold 70 % Ethanol and repeated for 2 to 3 times. 100 µl elution buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 8.0) was added to elute bound DNA by incubation at 56 ºC for 5 minutes with gentle agitation. Centrifugation was done at 13000 rpm for 5 min and 50 µl elute was collected in a new micro-centrifuge tube. A buffer containing the extracted DNA was transferred carefully into a fresh micro centrifuge tube and stored at -20°C.

d) **Commercial magnetic bead extraction method**: DNA was isolated from sputum sample using the magnetic beads according to the manufacturer's instructions; nucleic acids are re-suspend in the elution buffer. DNA was transferred carefully into a fresh micro centrifuge test tube and stored at -20°C [26].

6.3.1 Assessing the purity of DNA by using UV-visible spectroscopy.

There are numerous techniques for the isolation of MTB DNA from sputum sample each method having its own benefit as well as limitations. MTB DNA extraction is usually affected by various factors such as incomplete cell lysis, adsorption, desorption techniques to a particular material, chemical substances, and sample pre-treatment processThe purity and yield of the extracted DNA were analyzed using UV-visible spectroscopic assessment, where A_{260}/A_{280} ratio gives protein contamination. The concentration of DNA can be determined by the equation,

$$1\ OD_{260}\ \text{unit} = 50\ \mu g/ml\ \text{for double stranded DNA} \quad \ldots\ldots (3)$$

6.3.2 Real time PCR

Real time PCR was carried out by using extracted MTB DNA. All extracted DNAs were submitted for SYBR green based real time PCR. The probes and primers were purchased from Genome Diagnostics Pvt. Ltd., India (Accession number LC005454.1.) The reaction system contained 25 µl of total sample volume, in which 12.5 µl of MTB complex super mix, 2.5 µl Mg. Solution. MTB complex, internal control 0.5µl, 10 µl DNA was added to appropriate tube. Finally, tubes were kept in the thermal cycler Rotor Gene 2000/3000/6000, (Corbett Research Australia).The reaction condition was 10 min.at 95°C: 45 cycles of 20 s at 60°C and 15 s at 72°C [27].

6.3.3 Agarose Gel Electrophoresis

Agarose gel (2%) electrophoresis was carried out using a horizontal gel electrophoresis unit. All the extracted DNAs were analyzed and observed using gel image system (Syngene- USA). The running buffer was 1X TAE; electrophoresis was carried out at 60 mA at 100-120V for 2 hours [28].

6.4 Results and Discussion

6.4.1 SPION assisted isolation of DNA

Magnetic adsorption techniques have been used for isolation of DNA from pulmonary sputum samples. Here, SPION were used for magnetic separation as well as solid phase support to adsorb DNA. It provides a high surface area for adsorption of biomolecules and allows a greater response to the applied magnetic field and easy separation and manipulation of SPION [29]. Magnetic particles binding with DNA to the surface of SPIONs are due to the electrostatic interaction between positively charged SPIONs and the negative charged DNA molecule. Surface to volume ratio was very high, and increase rapidly as the relevant dimension decreases for a 3 nm diameter, particles approximately 90% of the atoms reside on the surface. DNA was the molecule with a backbone of phosphodiester linkages hydroxy and amino groups present on the surface of SPIONs. It reacts with molecules bearing hydroxyl, carboxyl, phosphate, and sulfate groups through formation of hydrogen bonds [30-32]. Phosphate groups were present in phosphodiester bond, and these were responsible for imparting a negative charge to DNA. Electrostatic and hydrogen

bonding were responsible for adsorption of DNA, the presence of protonated amino group $–NH3^+$ and hydrogen bonding and carboxyl group with DNA [33].

6.4.2 Evaluation of DNA extraction and its comparison with SPION method

The quality, quantity and timing process of the extraction technique obtained by SPION method were compared with conventional chloroform-phenol and commercial kit based methods. The purity of DNA sample was measured by A_{260} / A_{280} ratio, with absorption spectrum from 220-350 nm wavelength measured by using bio-spectrophotometer (Cary 60 Agilent Technology, USA). The concentration of the DNA in the sample was determined by the equations (One OD $_{260}$ Unit =50 μg/ml for double stranded DNA.

The average A_{260} /A_{280} ratio and yield of SPION recovered DNA was approximately 1.8 to 2.0 indicates, least protein contamination, obtained by SPIONs. By comparing the ratio between conventional and commercial kit methods table 6.1 shows our novel DNA purification method using SPION is better than other methods, eluted MTB DNA produced highest yield 985.3 ng/μl. The absorbance A_{260}/A_{280} ratio is 1.72 and 1.87 which indicates DNA is pure and free from protein [34, 35]. The purity and yield of the extracted DNAs were measured by UV-visible bio-spectrophotometer, which is shown in Fig 6.6.

Magnetic separation has established considerable attention in biomedical research because of its important properties of large surface/volume ratio, biocompatibility and low toxicity [36, 37]. In the present study, we have developed a SPION-mediated MTB extraction method and compared it with conventional and commercial kit based method. The main advantage of our method is rapid and accomplished in a single test tube [38, 39].

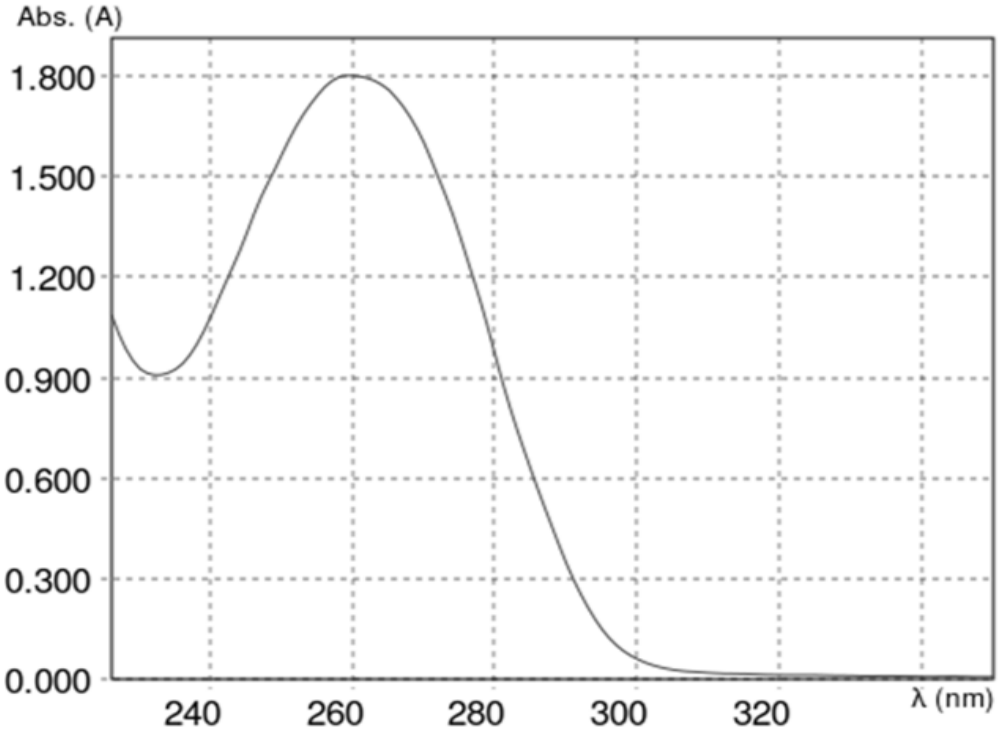


Fig 6.6. UV-visible spectrum of eluted MTB DNA by SPION.

The conventional chemical methods are time consuming and require trained person and the yield of DNA is very low [40-42]. Therefore, based on the quantity and quality of DNA extracted, the SPION mediated DNA extraction method was better than conventional and commercial kit based method. The average A_{260} /A_{280} ratio was approximately 1.8 to 2.0 indicating, least protein contamination, obtained by SPIONs. The details are shown in Table 6.1. We developed MTB DNA extraction method of positive sputum sample using SPION and its superiority has brought to focus for the first time through study. This method is even applicable for small sample volume and even low bacterial load. The rapid and efficient extraction of sputum sample is suitable for molecular identification techniques such as real time PCR [43]. Magnetic isolation methods are simple, rapid, sensitive and environmentally friendly, are suitable for routine laboratory use, but also hold potential for building of automatic sputum DNA extraction systems for several diagnostic purposes [44].

Table 6.1: DNA isolation and quantification of results by Bio-spectrophotometer

Method	Samples	DNA Yield (ng/ µl)	A260/A280
chloroform-phenol	1	65.6	2.21
	2	66.9	2.24
	3	96.9	2.23
Commercial	1	315.5	1.54
	2	375.5	1.14
	3	233.1	1.52
SPION	1	**985.3**	1.72
	2	843.4	1.72
	3	664.3	1.87
Mag.Bead	1	789.0	1.51
	2	622.4	1.89
	3	584.3	1.73

DNA diluted 1:100 in nuclease free water for spectrophotometric analysis

6.4.3 Agarose Gel Electrophoresis

The isolated MTB DNA were analysed using agarose gel electrophoresis experiment and discovered below the gel picture. Each MTB DNA band show with at the same position (Fig 6.7). This result indicated that the eluted MTB DNA is pure. Eluted DNA shows no smears or low molecular weight band were detected, indicating the absence of RNA contamination

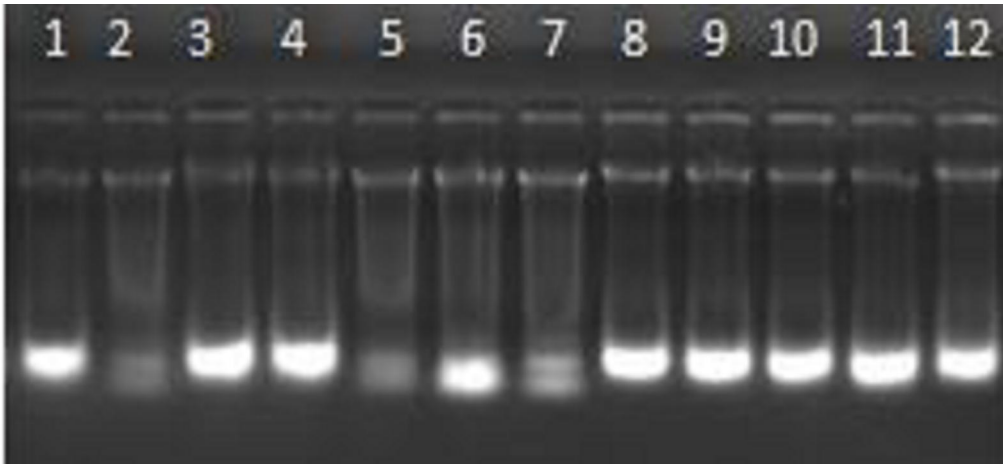


Fig 6.7 Electrophoresis of MTB DNA, Lane [1-3] chloroform-phenol, lane [4-6] Commercial kit, lane [7-9] SPIONs and lane [10-12] magnetic beads

6.4.4 Real time PCR (RT- PCR)

The sensitivity of all extracted DNA samples was studied using Real time Rotor Gene 2000/3000/6000, (Corbett Research Australia). MTBC- specific primers were used to amplify a target IS6110, the Accession number LC005454.1. An internal control (IC) plasmid genomic DNA was used to detect PCR inhibition in the extracted MTB DNA. All results are MTB positive confirmed by Real time PCR, Ct value shows for SPION (21.3) was lower as compared to the commercial kit, indicates more amount of target nucleic acid is found. In real time assays, undergo 40 cycles of amplification.

6.4.5 Analysis of results for clinical sample

The results of real time PCR is shown in Fig. 6.8. (a) Melting curve analysis FAM/Green channel: the samples contain MTB DNA. JOE / Yellow channels: the samples contain internal control.

Fig. 6.8 (b) shows quantitative analysis of MTB positive results (Ct value 21.3). The average Ct value was determined from a curve generated from a plot of cycle number versus fluorescence of the template control. The Ct is inversely proportional to initial concentration of the test DNA in sample.

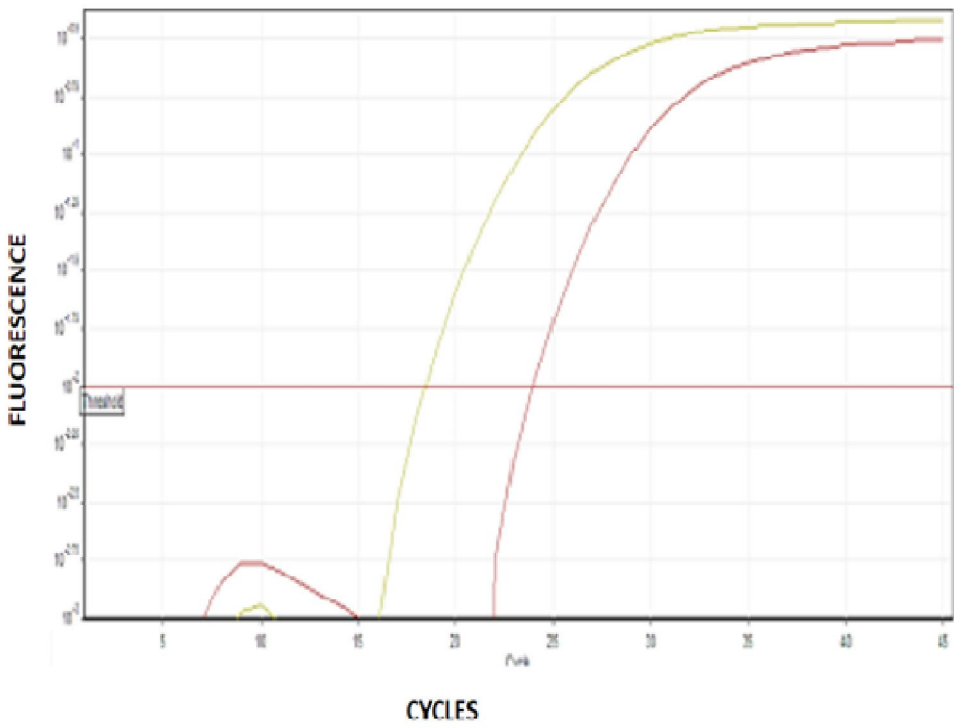


Fig 6.8[a] Quantitative analysis of MTB DNA: RT-PCR melting curve

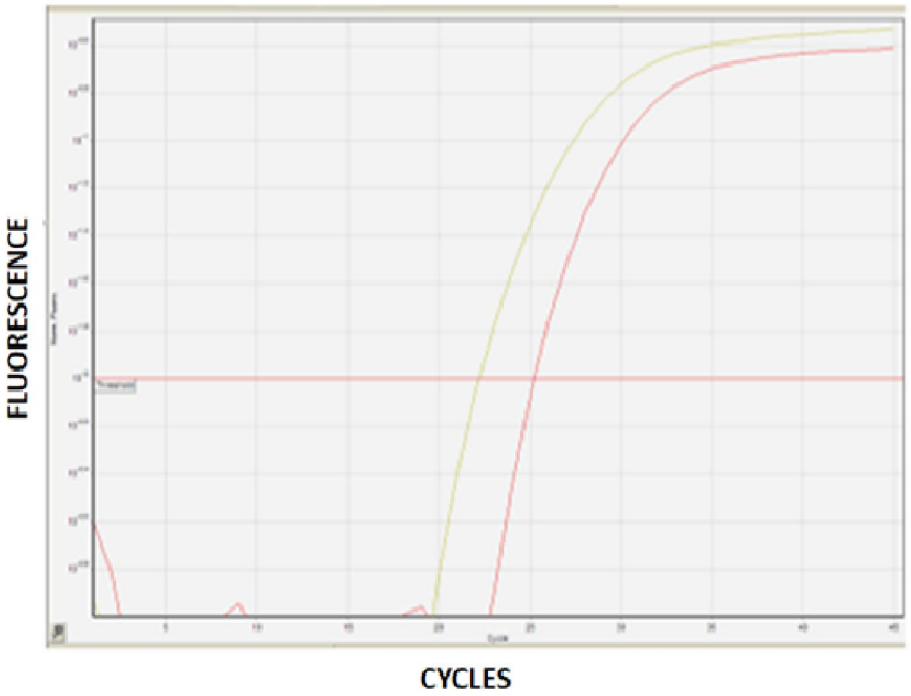


Fig 6.8 [b] Quantitative analysis of MTB DNA: RT-PCR Positive result

6.4.6 Ct values of DNA extraction method

The mean Ct values for four preparations were such as evaluated (27.5) for phenol chloroform, 23.8 for commercial kit, 21.3 for SPION and 24.8 for magnetic bead method. These result indicated that all sample are positive for MTB, however Ct value for SPION (21.3) is lower as compared to commercial kit and magnetic bead. The Phenol chloroform organic method Ct value 27.5 is high as compared to other extracted methods because it may contain PCR inhibitor. SPION mediated DNA extraction techniques is shown in Fig.6.9.

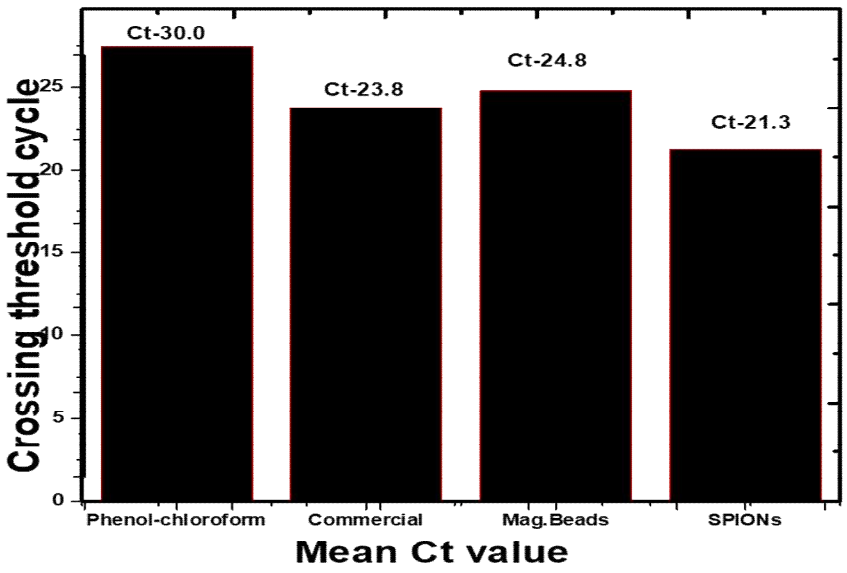


Fig. 6.9: Ct values of DNA extracted using different methods

6.5 Conclusions:

The SPIONs were successfully synthesized by co-precipitation method and they were characterized by X-ray diffractometer study. The crystallite size of material is found to be 9.7nm.The FT-IR spectrum of SPION shows that the band observed at 567 $_{CM}^{-1}$ is assigned to the vibration of the Fe- O bonds which is related to the magnetite phase. The surface morphology of SPION were observed in spherical and granular shape. The magnetic measurement of SPIONs show superparamagnetic behavior below 20 nm size. SPIONs are used further for MTB DNA extraction. The quality of MTB DNA was measuring by using UV-visible bio-spectrophotometer and are found to increase the DNA yield (985.3 ng/ul) as compared to conventional (96.9 ng/ul), commercial (375.5 ng/ul), commercial magnetic bead (789.0 ng/ul). The extracted positive MTB DNA samples were processed by Real time PCR assay. All results are MTB positive and confirmed by Real time PCR and the Ct value for SPION (21.3) was lower as compared to the commercial and conventional kit base methods indicates more amount of target DNA is found.

In this study we have developed SPION medicated MTB DNA extraction from positive sample and detected with Real time PCR and its superiority has brought to focus for the first time through this study. This method definitely stand to be the

one of the new, safe, cost effective and better alternative and holds tremendous potential to replace the current technique.

Table 6.2: Real time PCR with Ct values with SPION

SR NO	AGE	GENDER	AFB-POSITIVE GRADE	REAL TIME PCR	CT VALUES WITH SPION	YIELD OF DNA
1	21	F	2+	Y	20	978
2	27	M	3+	Y	20	890
3	69	M	1+	Y	21	884
4	27	F	1+	Y	20	678
5	60	M	1+	Y	21	847
6	27	F	3+	Y	20	669
7	20	M	3+	Y	20	974
8	55	F	3+	Y	21	678
9	22	M	2+	Y	22	844
10	55	M	3+	Y	21	687
11	79	F	3+	Y	22	981
12	40	M	2+	Y	21	845
13	45	M	3+	Y	21	674
14	65	M	3+	Y	20	672
15	65	M	4+	Y	21	984
16	20	M	3+	Y	21	889
17	42	M	4+	Y	20	889
18	22	M	4+	Y	21	900
19	19	M	3+	Y	21	882
20	68	M	4+	Y	22	788
21	22	M	4+	Y	21	802
22	40	F	3+	Y	20	884
23	29	F	2+	Y	20	889

24	29	F	3+	Y	21	901
25	40	M	3+	Y	20	981
26	43	F	3+	Y	21	884
27	60	M	3+	Y	20	789
28	70	M	2+	Y	22	814
29	43	F	2+	Y	20	902
30	79	M	3+	Y	21	890

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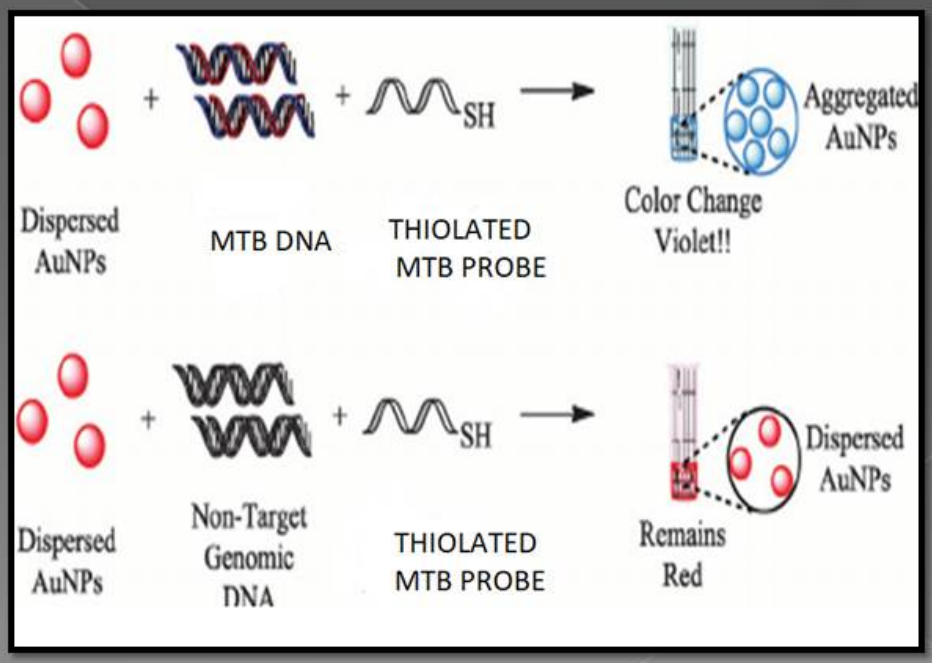
Chapter 7: Detection of MTB with colorimetric Gold Nanobiosensor



7.1 Introduction

Nanobiotechnology is now providing effective devices for microbiological laboratory diagnosis. Several nanoparticles (NP) produced methodologies towards finding MTB infection are with extra sensitivity [1]. Recently, scientists developed a new method which is cost effective, such as NPs constructed methods which are projected to change growth through the years permitting to satisfy the needs faced in the area. Gold nanoparticles (Au-NPs) fabricated with oligonucleotide were considerably applied for identification of *Mycobacterium* infection [2]. The color changes and agglutination ideas of gold nano probes are now ready in the absence or presence of the precise gene order. The incidence of the opposite genetic order stops agglutination which resulted in no change in color (red color band at 525 nm). Whereas the absence of exact nucleic acid gene target order show NPs agglutination after salt addition and the color of the solution turns red to purple (red shift of the LSPR wavelengths 600-650nm) [3].

In the current study, we are trying to show Au-nano probe assay exhibiting in MTB detection. Oligonucleotides capping with gold for assessing specific pathogens. This idea covers the colorimetric finding to appropriate MTB spot test with the advance detection potential of this method [4]. Here, we report advanced thiol bond chemistry attached with single stranded deoxyribonucleic acid on metal probe for color change observation to identify *Mycobacterium tuberculosis* [5].

7.2 Experimental

7.2.1 Materials and Methods

In the current work Gold (III) chloride hydrate ($HAuCl_4H_2O$) used as a gold precursor, were obtained from S. Aldrich. Thermo Fisher Company supply tri-sodium citrate. All materials were used without further purification, primers and oligonucleotides were purchased from integrated DNA technology (IDT) New Delhi, India.

Reagent and Positive control MTB DNA: Au-Nanoprobe sequences were designed using Mylab life solution Pvt. Ltd. (India) 2017-18/D/95 relative gene sequence.

7.2.2 Synthesis of citrate capped gold nanoparticles

The precursor of hydrated -gold nano seed was produced as per earlier reported method (Tabrizi A, *et. al*. 2009). A gold (III) chloride hydrate and tri-sodium citrate dehydrate liquid was set by final applications of 2.5 x 10.4 M and 38.8 mM,

correspondingly, in 20 ml of d/d water. To above mixture 1% trisodium citrate (1ml) was added till the color becomes wine red color. Approximately 13.4 nm diameter GNP (gold nanoparticle) were prepared. By using a clean glassware rinsed with Nanopure H_2O and then dried in the oven prior to use for synthesis [6].

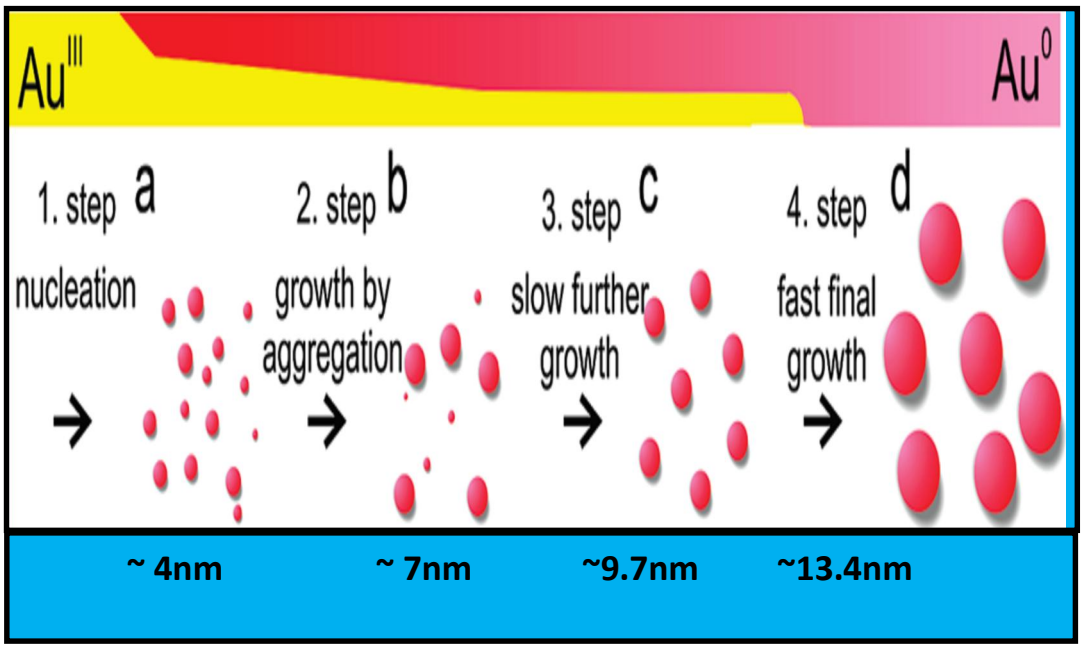


Fig 7.1 Diagram design for the reduced development of AuNps

7.2.3 Mechanism of sodium tri-citrate reduction method

A widespread range of promising procedures in gold nanoparticles; areas like medical devices, biotechnology etc. where has forcefully considered nanoscale resources. A chemically prepared gold nanoparticle precursor is dissolved by $HAuCl_4$, by a reducing agent. While in maximum cases, the additional reducing agent is needed to stop further growth of the gold particles [7, 8]. Where as in most cases a further stabilizing is required to prevent agglomeration or further growth of gold nanoparticles.

The reaction situations are often understood as a four stage nuclear progress. The first part is often separated into 2 phases. The first stage might be a fast development of nuclei (step a in Figure 7.1) monitored by the union of the nuclei into larger atoms (step b in Figure 7.1). The 3 phase contains relaxed dispersal development of atoms continue by current reduction of gold precursor moreover as an extra union (step c in Figure 7.1) subsequently gold particles produce quickly to their fast and final growth (step d in figure 7.1).

7.2.4 Characterization of gold nanoparticle (AuNPs)

Au-Nps were studied for their structural and phase identification techniques by using X-ray Diffractometry (XRD) and DLS (Dynamic light scattering), Optical characterization done by UV- visible spectroscopy and FT-IR, and Morphological characterization was done by FE-SEM (Field emission transmission electron microscopy). An electrochemical study was done by using cyclic Voltammetry study (CV) and finally molecular biological techniques done by agarose gel electrophoresis study and real time PCR.

7.2.5 Gold nanoparticle-probe-conjugation

Conjugation of Au-NPs by better-quality Oligo-nucleotides was constructed on earlier labeled technique (Mirkin's strategy). Total 600 mL AuNPs was centrifuged at 12000 rpm for 15 min, and the concentrated pellet was suspended into 12 ml phosphate buffer 10 mM (pH 7) and 32.5 µl thiol modified oligonucleotide (GP-1/GP-2) was added in a 900 µl of AuNPs suspension. The mixture was kept for aging for 16 hours at 25°C. After addition of 100 µl of 0.1M NaCl. The mixture was centrifuged 5 min at 13000 rpm. The obtained oily red color precipitate was washed with 0.1M NaCl and 10 mM PBS solution (pH 7). 0.3M NaCl (100µl) was added to 10 mM phosphate buffer solution (pH 7). Finally, the capping of gold with Oligo was confirmed through FT-IR and UV visible spectroscopy studies [9, 10].

7.2.6 *In-situ* pulmonary sputum test, sample preparation and its bacterial counts

The sputum samples were collected from Dr. D. Y. Patil Hospital and Research Centre, Kolhapur, in a sterile container. The samples were pre-treated with NALC (L-cysteine- N-acetyl 0.5 %) and 2% NaOH for digestion and decontamination. In brief, samples were mixed with NALC-NaOH solution using a vortex mixture, centrifuged at 3000 rpm for 20 min at 4°C. The sediment was mixed with 2 ml of phosphate buffer (pH 6.8). The sediment was later used for DNA extraction [11].

7.2.7 MTB DNA extraction from pulmonary sputum sample

The MTB suspensions were first treated with NALC method and mixed with identical volume of buffer (Tris-HCl buffer, pH 8). The mixture was vortexed well, and separated at 13000 rpm for ten minutes, re-suspended and processed with lysis

buffer at 95°C for 20 minutes. Pure DNA was collected and suspended with buffer and stored at -20°C [12].

7.2.8 NGS (Next Generation sequencing) sequencing and Data analysis

Total DNA isolated from laboratory from pulmonary sputum samples was used for 16S rRNA gene amplicon sequencing [13, 14]. Universal bacterial primers specific for the V3 region of the 16S rRNA gene, MTB DNA fragments were used for PCR amplification [15]. The amplified products were detected by running 1% agarose gel and quantified according to the manufacturer's instructions.

Briefly, the sequences were assembled using FLASH and clustered under 97% sequence similarity threshold using UCLUST closed orientation OTU option SILVA 123 file (released on July 2015). Taxonomic assignments were done using Silva 123 reference database [16]. To evaluate alpha diversity, diversity indices such as Shannon-Wiener, Simpson's, Goods coverage and Chao1 were calculated. Unifracmetrics were calculated to estimate the beta diversity [17, 18].

NGS statistics accessibility:

The sequences were obtained from high throughput sequencing determination. This data is submitted to NCBI and which are available under SRA accession: SRP 133027 and Bio project ID: PRJNA 434486.

7.2.9 q-PCR for Mycobacterial population count

The abundance of important bacterial taxa was confirmed by quantification of total and specific bacterial population using quantitative real time PCR in terms of copy numbers of 16S rRNA gene. Target groups of microbes, gene sequences, and size of amplicon are concise in Table 7.1**.** Total qPCR quantitative assays were done as labeled previously wherein, every genus below attention, three biological replicates with duplicate technical replicates of each were setup (10µl each), inclosing suitable couple of primers, metagenomic DNA (50ug) and SYBR green master mix. Reactions were run on RT-PCR system (Applied Bio-systems, US), by successive PCR settings initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 60°C for 1minutes. The average curves were produced from sequential dilutions of a well-known absorption of PCR products. Moreover, melting curve study was done at the completion of qPCR cycles to confirm the intensification specificity. The average values of the test samples were used for accounts of verified gene amounts for every

genus by average curves produced below parallel conditions. The RT- PCR efficacy was sustained above (90 %) with a correlation coefficient (>0.99).

7.3 Results and Discussion

7.3.1 Bacterial diversity assessment in upper respiratory tract samples

After the high-throughput sequencing resulted into total 92964 usable quality sequences (distribution counts for A: 37460, and B: 55504), and segregated into 3808 Operational Taxonomic Units (OTUs) by 97% similarity cut-off. OTUs based analyses were further revealed the diversity measure assessments. Alpha diversity indices and inter-sample variations were recorded in **Table 7.1**. There were 09 total phylum reported; whereas *Firmicutes* phylum was abundant (range: 39.7% to 67.2%) followed by viz. *Bacteroidetes, Fusobacteria, Proteobacteria, Actinobacteria, TM7*, *Cyanobacteria, Synergistetes* and SR1. Furthermore total 50 genus level taxons were recover and mentioned in **Supple. Table 7.1**, and sharing pattern was shown in **supple. Fig. 7.4 a.** Top 10 genus abundant wise present in respiratory tract were demonstrated in **Fig. 7.4 b**. Here are some common genus found has mostly denoted as 'core microbiome' and we got total 29 core genus in our tested cohort samples.

7.3.2 Quantification surveillance of total and MTB bacteria in upper respiratory Tract:

Quantitative surveillance had been demonstrated through the molecular qPCR assays. Some molecular markers used for this surveillance study to depict the total and *Mycobacterium* genus population (**Table 7.2**). The entire microbial counts based on 16S rRNA gene (product size 172 bp), and *Mycobacterium* genus count was constructed on the *IS6110* gene specific to the *Mycobacterium* only (product size 172 bp, Fig.7.5). The IS6110 gene specific to the *Mycobacterium* were utilized for the qPCR positive template standard with INS 1/2 and IS6110 F/R primer derived products, amplification plot and standard curve as depicted in **Supplementary Fig.7.2 and 7.3.**

7.3.3 Structural analysis of gold nanoparticle (AuNPs)

The crystalline arrangement of the gold nanoparticles was studied using XRD. Fig.7.2 presents an XRD spectrum of GNPs. It is clear that there is a diffraction strong peak located at $2\theta = 38^{\circ}$, 44.25°, 64.5°, and 77.5°, correspond to the planes (111), (200), (220), and (222), respectively. These peaks are characteristics of the crystalline structure of AuNPs.

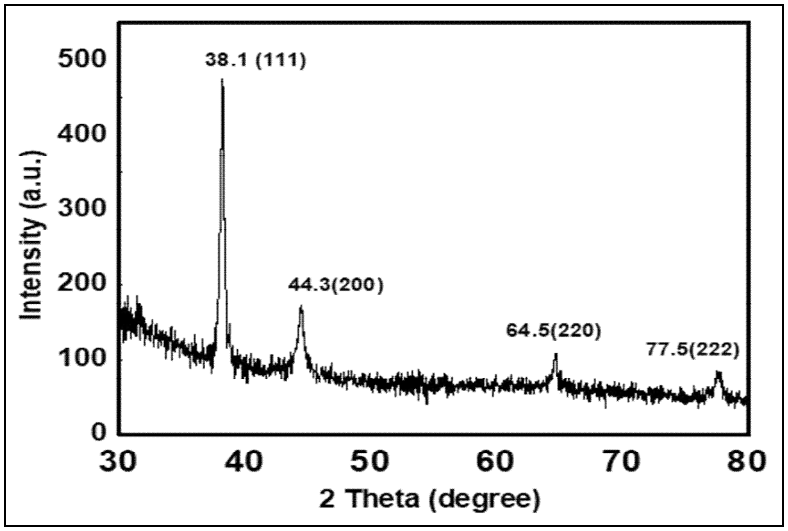


Fig. 7.2 X-ray diffraction pattern of AuNPs

7.3.4 FT-IR of AuNPs

The transmission IR spectrum of the synthesized gold nanoparticle is shown in Fig.7.3. It is observed that peaks at 758 and 875 cm^{-1} are finger print of the synthesized GNPs. This revealed that the successful formation of the GNPs by tri-sodium citrate reduction method. OH stretching band at 3467^{-1} was found on the other hand the peak at 1632 cm^{-1} are due to the presence of citrate and amine [19].

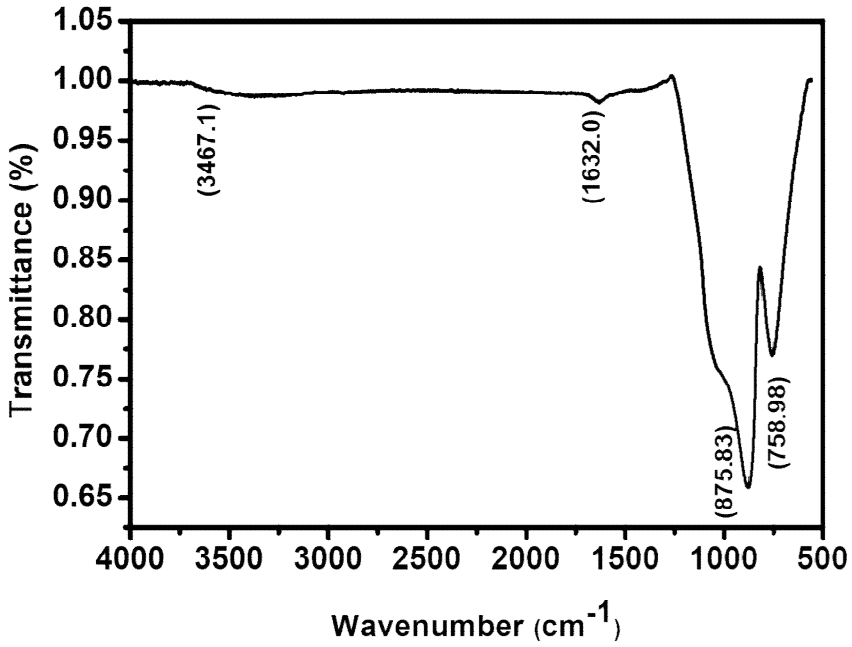


Fig 7.3 FT-IR spectrum of AuNPs

7.3.5 Light scattering technique of AuNPs

The dimension of atom dimension and size scattering of AuNPs was supported by DLS test. Fig 7.4 displays (NICOM DLS) characteristic dimension and size scattering of the trisodium citrate produced AuNPs [20]. The typical hydrodynamic dimension of the isolated trisodium citrate method is 13.4 nm and 100% of the atom size scattering by using NICOM software.

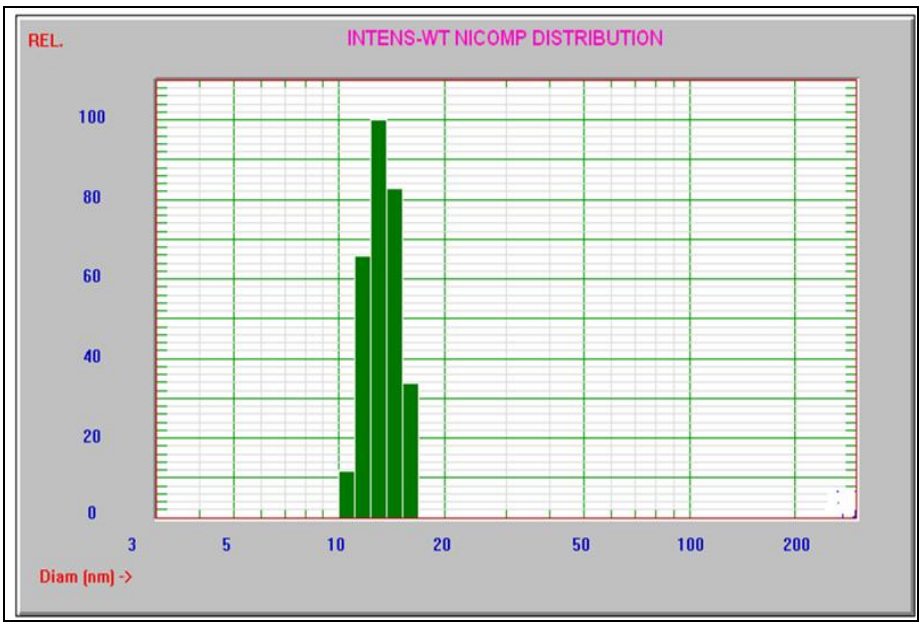


Fig 7.4 Dynamic light scattering of AuNPs

7.3.6 Absorption spectrum of gold nanoparticle

The first step in characterizing the prepared colloidal solution of gold nanoparticles was to test its absorption spectrum via the UV-Visible spectrophotometer [21]. Part of the sample was diluted and tested by the UV-Visible-spectrophotometer to obtain its concentration range. Fig.7.5 displays the typical concentration range of AuNPs obtained in our experiments. It is clear that the absorption spectrum peaks up at the wavelength 522 nm. The absorption peak at this specific wavelength is considered as a fingerprint of colloidal gold- nanoparticle. The red wine color of the prepared colloidal GNPs solution is a result of absorbing specific parts of the visible light spectrum, mainly at the surface Plasmon resonance which corresponds to the green visible light. However, what appears to us is the complementary color which is red wine [22, 23].

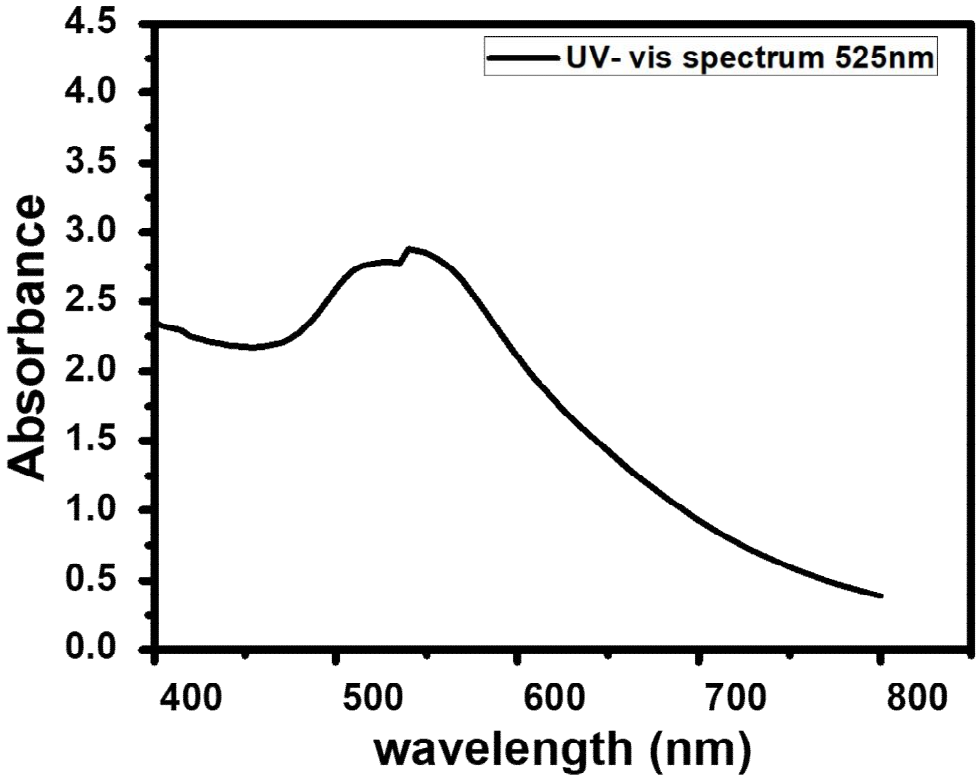


Fig. 7.5 UV-Vis spectrum of AuNPs

7.3.7 Field emission scanning electron microscopy (FE-SEM) of AuNPs

The AuNPs are air-dried in aluminium foil using magnetic heating plate by drop cast method and subjected to field emission scanning electron microscopy using Nova Nano SEM NPEP 303. The spherical shaped gold nanoparticle size at 500 nm with 100 Kx magnification is shown in Fig 7.6.

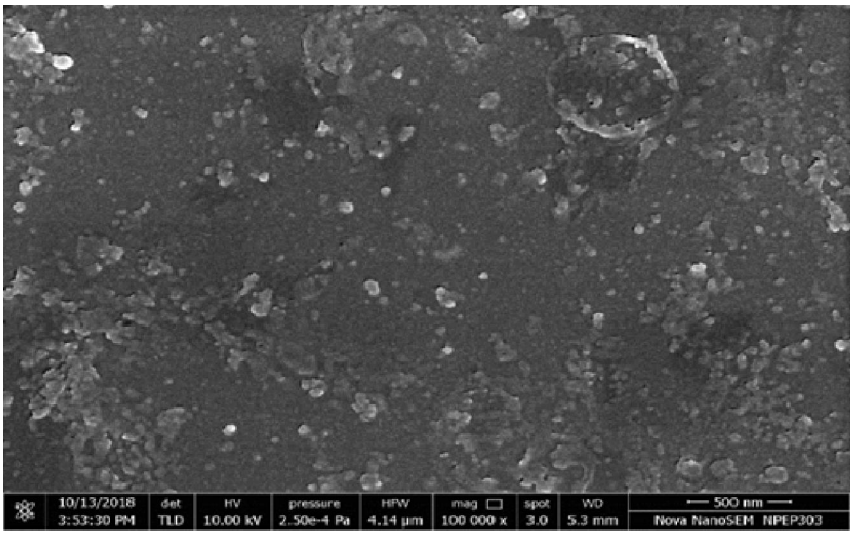


Fig. 7.6 FE-SEM of AuNPs magnification at (100 kx)

7.4 Gold nanoparticle probe bioconjugation

7.4.1 Fabrication of colorimetric gold nano-biosensor

The average 13.4 nm diameter of gold nanoparticles with two sets are tailored with 3'- or 5'alkylthiol. Gp-1 and Gp-2 Oligo product are mixed and incubated. After 16 hours, the pellet was separated in 10 mM PBS (pH 8) along with 0.1 M NaCl [24, 25]. The probe bioconjugation of GNP is shown in Fig 7.7.

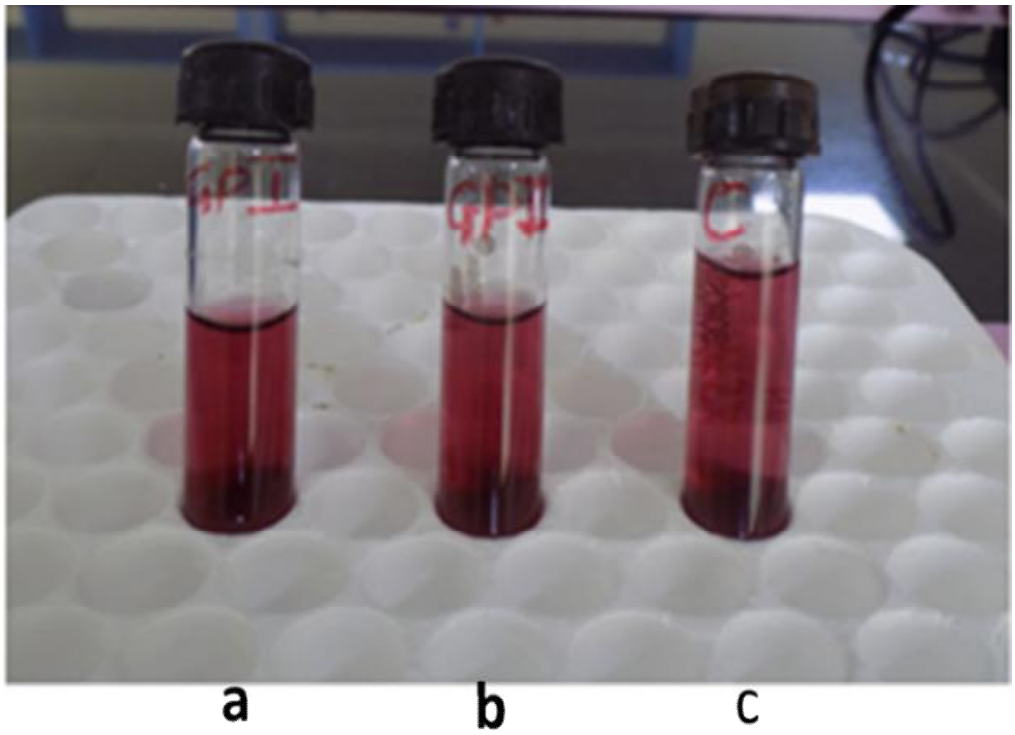


Fig. 7.7 Bio-conjugation of GNPs [a] GP-1 conjugate, [b] GP-2 conjugate and [C] Bare GNPs

7.4.2 Probe-nanoparticles quantitative assay

The colorimetric test were done in an absolute capacity of 30µl comprising gold Nanoprobe at an absolute absorption of 2.5 nM in 10 mM phosphate buffer (pH 8) and complex produced at concluding DNA absorption of 17.7 µg/mL. The combination was excited at 95°C for 5 minutes and then cooled at 25°C for 5 minutes. For both oligoprobes, the test involved on the UV visible spectrophotometer contrast of a "Blank". The $MgCl_2$ was added to each reaction, and after 30 minutes at 25 °C. The colour change was observed (red to purple). The UV-Vis spectral changes with different DNA target detection is shown in Fig.7.8.

7.4.3 Amplification of target DNA with AuNPs probes

Two probe (GP-1 and GP-2 in 0.5 M PBS, 1:1 ratio, final nanoparticle concentration =3.61 nM) to target DNA with 17.7 ng/ul. Target DNA was 110 bp sequence of IS6110 gene amplified by PCR and then denatured at high temperature. Hybridization are done at 95°C for ten minutes, followed by the PCR plate at 25°C for 1 hour. 5 ul of 0.7 M, $MgCl_2$ are added and again re-hybridization by 25°C for 1 h. Finally colour change from red to purple with precipitations was observed. This colour alteration is specifically owing to a red colour modification in the SPR from λ max = 520 to 570 nm.

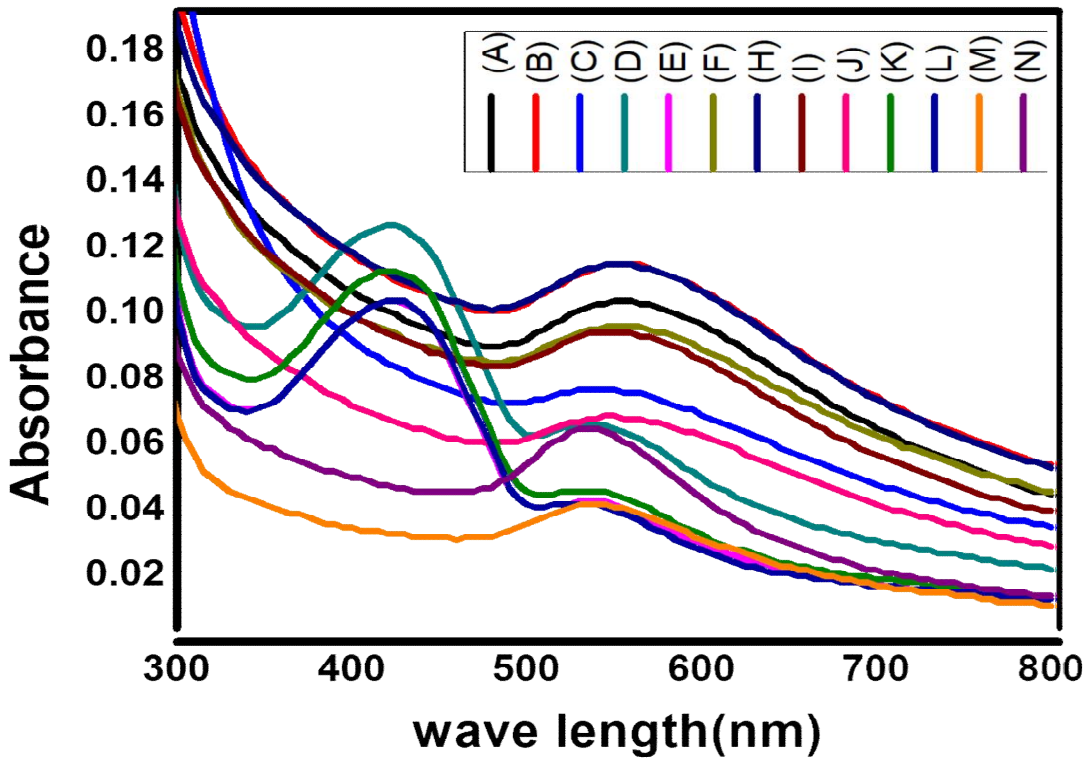


Fig. 7.8 UV-Vis. Spectra of gold nanoparticles probe aggregate solution which show the spectral changes with different DNA target detection.

Sample (A): UV graph for 5ml. bulk AuNPs, (B): AuNps initial used for preparation for 5ml bulk,(C): AuNps initial used for preparation 1 for 5ml bulk, (D): AuNPs $MgCl_2$ for 5ml bulk,(E): GP1 initial used for preparation 1 for 5ml bulk, (F): GP1 nonreactive for 5ml bilk (H): GP1 template negative for 5ml bulk, (I): GP1 without $MgCl_2$ for 5ml bulk, (J): GP1 10^{10}copies for 5ml bulk,(K): GP1 10^2 copies for 5ml bulk, (L):GP1 10 copies for 5ml bulk, (M): sample A for 5ml bulk , (N): **Sample (B)** for 5ml bulk preparation.

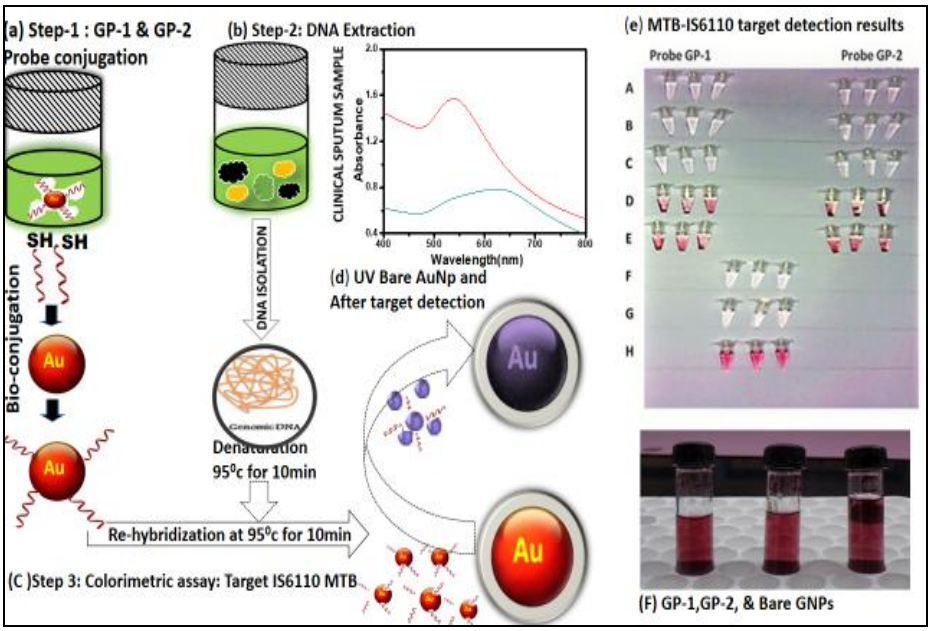


Fig. 7.9 (a): Step1: Schematic representation of the concept for colorimetric detection of target, DNA gold nanoparticle probe (GP-1 and GP-2) conjugation, (b) Step: 2 DNA extraction (c) Step 3: Colorimetric assay: MTB Target IS 6110, (d) UV-Vis spectra bare GNPs and after target detection. (e) Actual target detection MTB – IS6110 results: (A)MTB DNA $3.03X10^{10}$ (B) MTB DNA $3.03X10^{2}$ (C) MTB DNA 30.3 (D) Sample A 10979 (E) Sample B 11983 (F) Nonreactive template (G) Template Negative (H) Bare GNPs

7.4.4 Detection limit of assay

After PCR amplification target MTB gene (*IS6110*) deoxyribonucleic acid intensified from MTB was measured by AuNPs probe test. Amplified DNA were quantified and serially diluted to detect 10 Femtomoles of an oligonucleotide and determined MTB DNA (1.95×10^{-2} ng/mL). At this least count the detection of target *IS6110* MTB DNA by GP-1 and GP-2 Oligo probe spectral color changes and agglutination are shown in Fig. 7.8.

7.4.5 Target detection of MTBC and MTB strains

The DNA fragments of *IS6110* specific to MTBC **(*Mycobacterium tuberculosis* complex)** and MTB were used as the detection target in this experiment. The initial experiments are performed by addition of two sets of primers GP-1 and GP-2. The primer pairs, G-IS6110F/G-IS6110R are used in amplification of specific DNA fragments of MTBC and MTB [26, 27]. Exact 110 bp DNA fragment are amplified from *IS6110*. The minimum quantity of genomic DNA required for the

aggregation of gold nanoparticle probes and colour change from red to purple and the SPR are shifted from λ max = 520 to 570nm. In the presence of target DNA concentration of at least count was 1.95×10^{-2} ng/ml was detected at 10 Femtomoles of an oligonucleotide for MTB determination.

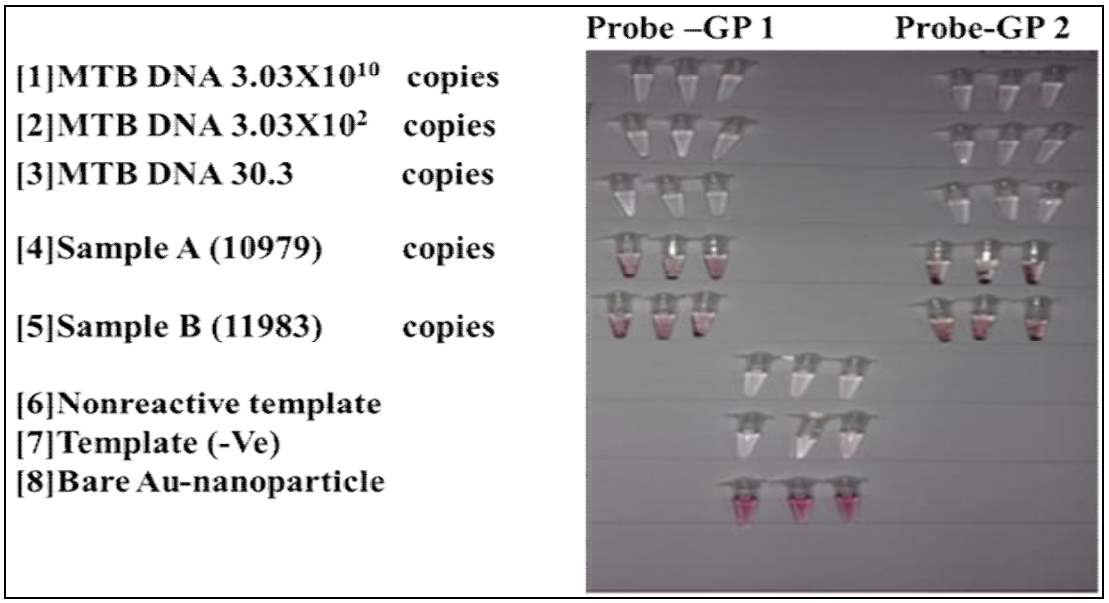


Fig. 7.9.1 Detection of target IS6110 MTB by using GP-1 and GP-2 probe

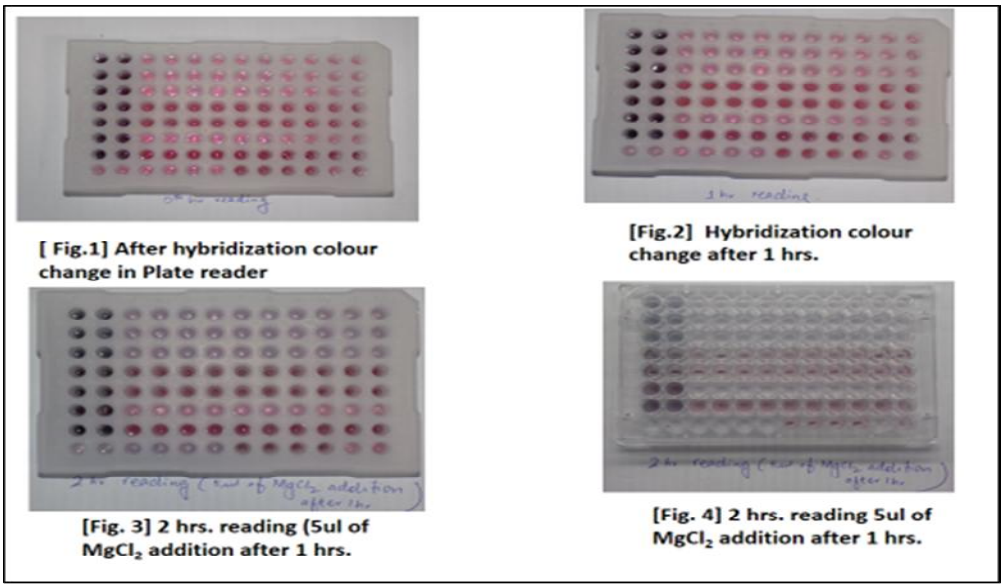
7.4.6 Sensitivity and specificity of GP-1 and GP-2 probes

The least amount of target DNA required for mycobacterial detection were determined by serial dilution of PCR product consisting GP-1 and GP-2 probe and certain concentration of target DNA incubated at 55°C for DNA hybridization for 2 h. The absorbance (OD) of the solution was measured by UV-Visible spectrophotometer. Owing to red modification in the SPR of GNPs the positive reaction means a dynamic change of colour from red to purple and the GNP probe aggregation was determined [28, 29]. On the other hand, the colour and absorption pattern are not changed if specific target DNA are absent. The concentration of 1.95×10^{-2} ng/ml for MTB DNA (10 Femtomoles) that are easily detectable by naked eye and defining the minimum detection limit of the assay shown in Fig.3. The spectral alterations with different DNA target are detected in Fig.7.9.1 to 7.9.4.

7.5 Naked eye colorimetric detection of MTB target (*IS6110*)

The colorimetric assays were performed in a final volume of 30 µL containing Au-Nanoprobe at a final concentration of 2.5 nM in 10 mM phosphate buffer (pH 8) and multiplex PCR product at final DNA concentration of 17.7 µg/mL. The mixture

was heated up at 95°C for 5 min and then cooled down to 25°C for 5 min. For each probe, the assay consisted on the spectrophotometric comparison of a "Blank" (without DNA), 10 mM phosphate buffer (pH 8), 0.1 M NaCl; non-related control containing non-complementary DNA; and the samples [30]. The pre-determined $MgCl_2$ concentration was added to each reaction, and after 30 min at RT for colour development seen red to purple by naked eye, further the mixtures and the blank assayed by UV-visible spectroscopy in a micro plate reader (Fluo- star omega). For calibration, each set of Au Nanoprobe was tested against purified simplex PCR.



[Fig.1] After hybridization colour change in Plate reader

[Fig.2] Hybridization colour change after 1 hrs.

[Fig. 3] 2 hrs. reading (5ul of $MgCl_2$ addition after 1 hrs.

[Fig. 4] 2 hrs. reading 5ul of $MgCl_2$ addition after 1 hrs.

Fig.7.9.2 (a) Colour change after target IS6110 detection

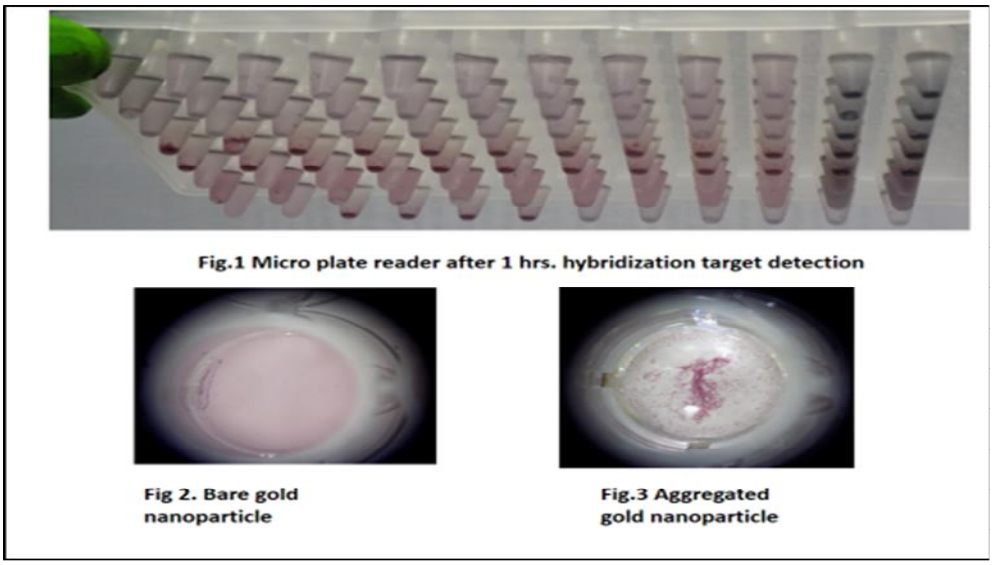


Fig.1 Micro plate reader after 1 hrs. hybridization target detection

Fig 2. Bare gold nanoparticle

Fig.3 Aggregated gold nanoparticle

Fig.7.9.2 (b) Colour change after target IS6110 detection

Fig 7.9.3 (a) Electrochemical Label free assay: Cyclic voltammetry (CV) SpE electrodes

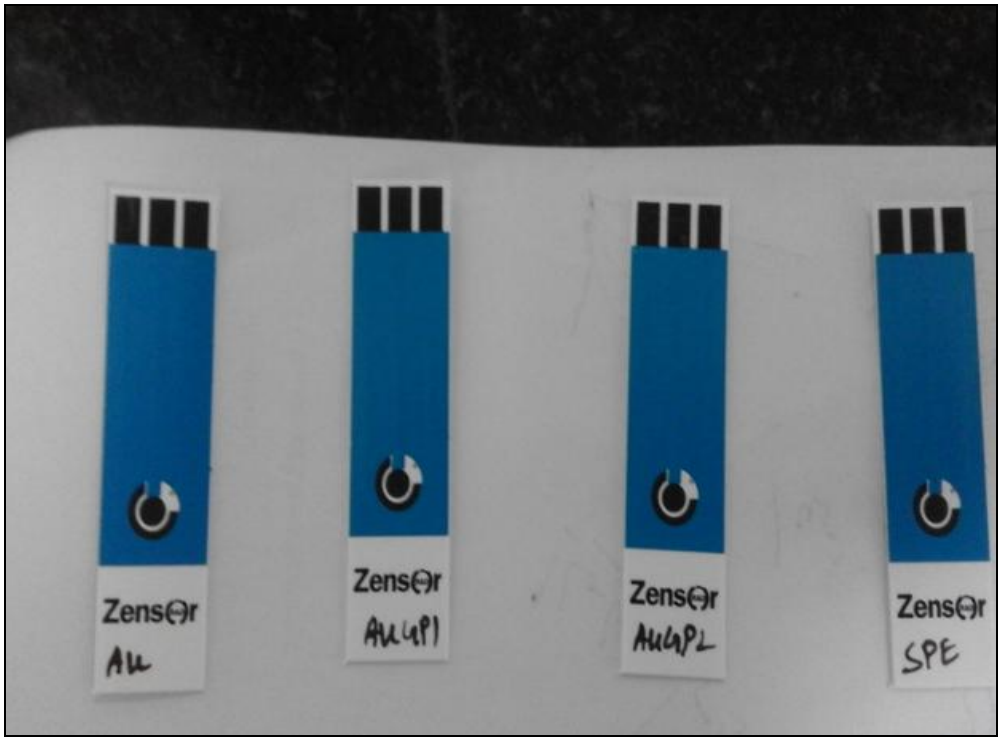


Fig 7.9.3 (b) Electrochemical Label free assay: Cyclic voltammetry (CV) SpE electrodes

7.5.1 Electrochemical Label free assay: cyclic voltammetry

We have further analyzed MTB target detection study by conducting electrochemical assay using portable electrochemical work station (Zensor Simulator, ECAS 100) and Screen Printed Carbon 3 in 1 electrodes, SPE (Zensor TE-100A), with carbon as both working and counter electrodes and silver as pseudo reference electrode . At first, the electrodes were drop coated with 2 µl of colloidal AuNPs and dried at 60 ºC for 30 minutes to make Au-SPE as a control for electrochemical studies. Further, 4 µl GP1 and GP2 AuNP probes were drop coated on other electrodes and incubated for 30 minutes to produce Au-SPE-GP1 and Au-SPE-GP2 respectively. After completion of reaction, 2 µl each target DNA on to Au-SPE-GP1 and Au-SPE-GP2 and kept at 90 ºC for hybridization for 10 minutes, and subsequently allowed to cool at room temperature for 30 minutes.

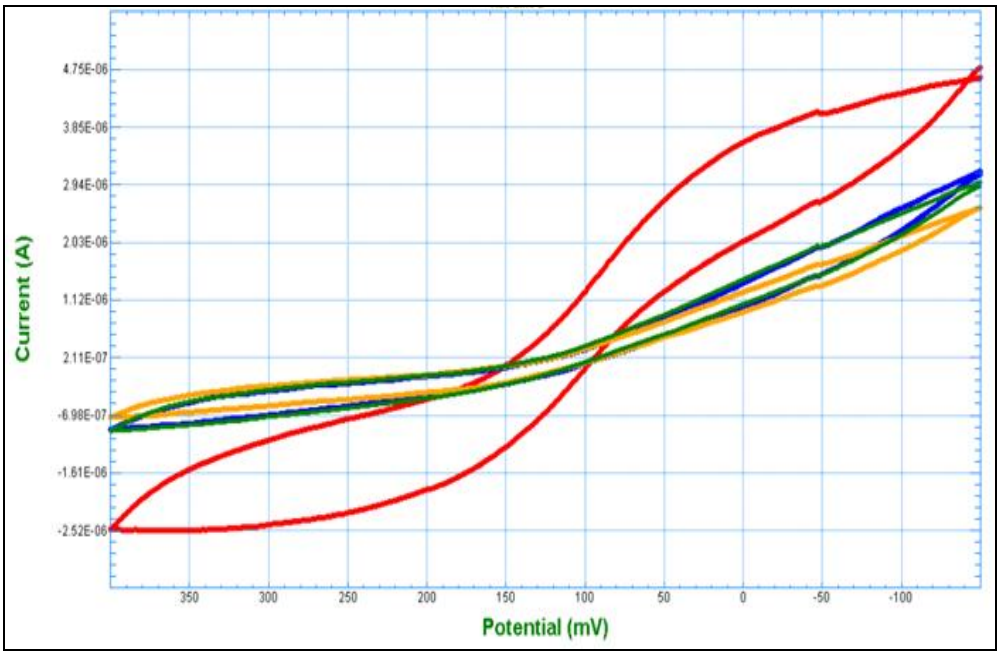


Fig.7.9.4 Cyclic voltammograms for Au-SPE (red), Au-SPE-GP1 (blue), Au-SPE-GP1-T (golden yellow), and Au-SPE-GP2-T (green) in the occurrence of 5 mM $K_3Fe(CN)_6$ in 0.1M KCL solution with a scan rate of 50 mV/s.

All the probes were electrochemically characterized by using 5 mM $K_3Fe(CN)_6$ in 0.1M KCL solution. The CV studies (Fig.4) showed the probes interaction towards common bench mark redox couple, $K_3Fe(CN)_6$. The control electrode (Au-SPE) depicted redox behavior while comparing with respect to GP1 modified electrode (Au-SPE-GP1). It is well known that, the introduction of negatively charged

biological moiety reduces the electrochemical interaction towards an anionic probe (herein $K_3Fe(CN)_6$). Interestingly, DNA target (Au-SPE-GP1-T) showed a sluggish electron transfer towards $K_3Fe(CN)_6$, than the probe GP2 (Au-SPE-GP2-T, green trace). It should be noted that, the GP1 probe easily binds with the MTB DNA target, obviously resulting an increased amount of resistance towards the electron transfer. On the other hand GP2 doesn't interact; hence there is no change in the voltammogram while comparing with Au-SPE-GP1-T. In other words, the voltammograms is more or less similar to Au-SPE-G1 (blue). We use a portable negative system of electrochemical workstation as shown in the Fig 7.9.3 and 7.9.4.

7.5.2 Limitation of present detection technique

All molecular techniques requires pure DNA which is free from protein lipid. Although extensively used these days, direct sputum samples are not use for detection of MTB. Deoxyribonucleic acid are first separated from the sample which is time consuming and tedious which require skilled work and well set BSL-II laboratory facilities [31, 32]. Most wide used technique for DNA amplification is PCR assay. This assay includes a particular primer combined to amplify a singular genomic target specific sequence for analysis. Following PCR, a range of post-amplification strategies area unit accustomed evaluate the merchandise like direct sequence analysis, use of genus or species specific probes, and utilization of restriction accelerator analysis of the product, e.g., fragment length polymorphism analysis (RFLP). Even though these post-amplification strategies are shown to be useful for the analysis of microbes, sequence analysis is taken into account a particularly helpful technique for the identification of microbe species because of its wide range application to a range of species.

7.6 Conclusions:

There is a non-stop and strenuous demand for powerful diagnostic device to identifying pathogens at point-of-care. Gold nanobiosensor design to detect with a specific detection of MTB target by using a single Au-Nanoprobe. In a single reaction detects multiple targets without loss of sensitivity was demonstrated successfully in present investigation. The gold nanoparticle were successfully synthesized by citrate reduction method and they were characterized by X-ray diffraction study, FTIR, DLS, UV-visible spectrum, FE-SEM of gold nanoparticle.

The concept applied here may easily extended to different pathogens and targets. It is a cost reduction process and enlarge the procedure to vast range of molecular testing for pathogens at point of need. In conclusion, spherical GNPs with multifunctional properties were offer unique opportunities for their use in the development of nanodevice used for early finding of TB, the AuNps probe established with robust medical applications. The outcomes of this assay can be realized by both straight remark or by bio spectrophotometry. The device is potential for use in regions of high MTB occurrence.

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Supp. File:

Table No.7.1 16s amplicon sequencing of MTB sample

Target gene	Product size	Sample 1	Sample 2	Comments
16S rRNA gene	180 bp	5.321E+06	2.114E+05	Total Bacterial population at clinical sample
IS6110 gene (INS1/INS2 primers)	245 bp	2.272E+03	4.014E+02	*Mycobacterium* complex population at clinical sample
IS6110 gene (IS6110F/ IS6110R primers)	110 bp	3.468E+03	4.656E+02	*Mycobacterium* complex population at clinical sample
IS6110/ 16S rRNA gene	NA	0.065 %	2.202%	Share of *Mycobacterium* complex population

Table 7.2 Primer sequence

Target / GenBank Accession	*M. tuberculosis* RRDR
	CP003248.2
5'-Forward-3' AACCGACGACATCGACCACTTCGGCAACCG	
5'-Reverse-3' CCAGCGCCGACAGTCGGCGCTTGTGGGTCAA	

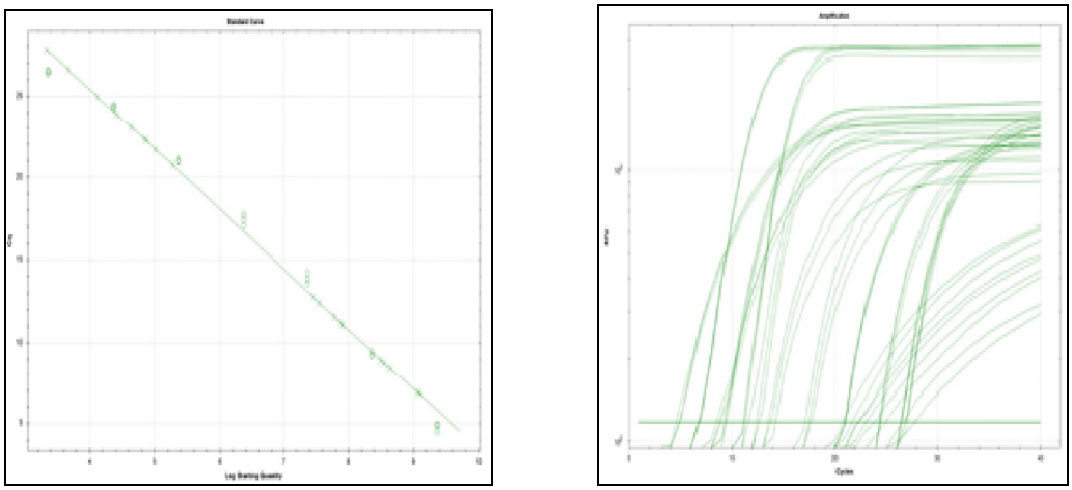

Fig.7.3 TB PCR Melting curve analysis

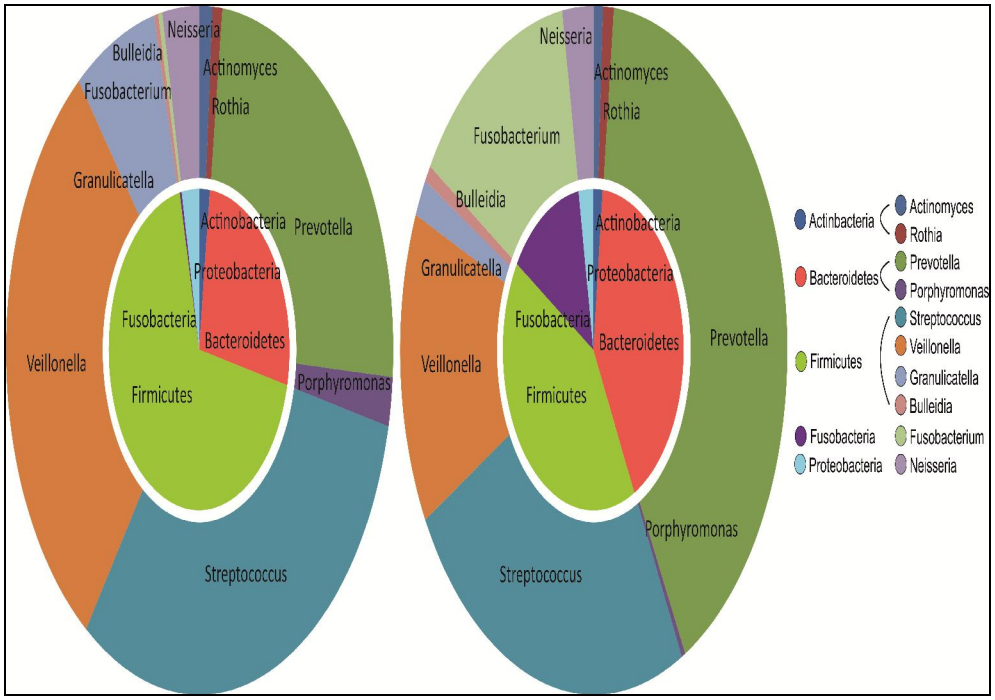

Fig.7.4 a: Distribution of 10 most abundant bacterial genus and their respective phylum found in tested samples.

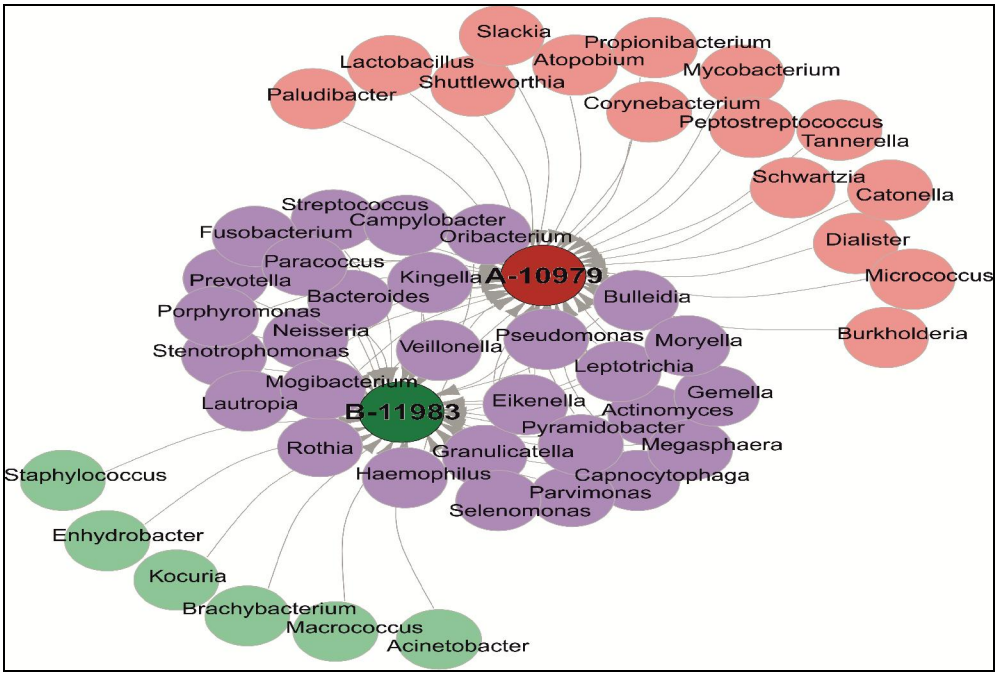

Fig 7.4 b Distribution bacterial genus and their respective phylum found in tested samples

Fig 7.5 Gel electrophoresis of MTBDNA

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Chapter 8 : Comparative Study of conventional, Real time PCR and Nanotech assisted MTB Detection methods

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Original Article

EVOLUTION OF NANOTECH ASSISTED PCR DIAGNOSIS OF *MYCOBACTERIUM TUBERCULOSIS* AND ITS ASSESSMENT WITH CONVENTIONAL METHODS

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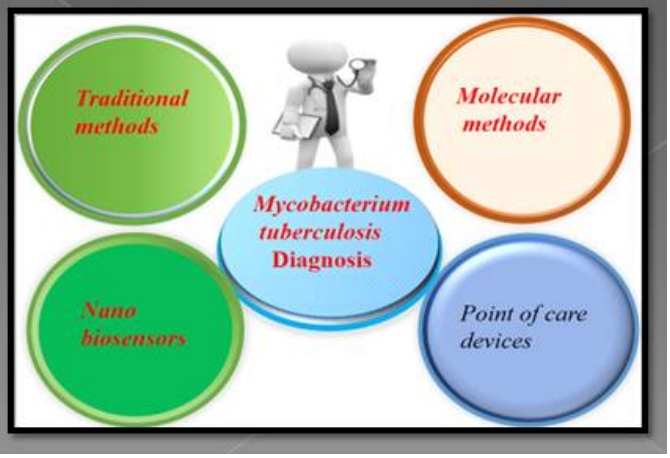

8. 1 Introduction

This chapter includes all conventional MTB diagnostic and molecular diagnostic methods. In this section comparative study includes analysis of MTB DNA extraction methods, including conventional chloroform phenol DNA extraction, the commercial kit based method and magnetic bead method Vs. magnetic nanoparticle based method. Results are compared with quantification of MTB DNA and newly developed colorimetric gold nano-biosensor with qPCR. Finally, we concluded that colorimetric nano-biosensor results are equivalent with q-PCR method. Nowadays molecular procedures are extensively used in the quick analysis of transmissible diseases. PCR is the ideal method for this purpose. Obtaining adequate and lipid free pure DNA is vital for the PCR. Different DNA extraction procedures such as phenol-chloroform, commercial kit base, magnetic method, have been used successfully for DNA isolation from clinical samples [1]. However, meanwhile gram-positive bacteria have a thicker layer of peptidoglycan and mycobacteria have complex glycolipids in their cell walls, for the isolation of DNA. By using MNp MTB DNA extraction method high and pure lipid free DNA can successfully extracted for downstream application an on PCR analysis. The MNP assisted method provides important advantages such as decreasing the cost and the decrease extraction time [2].

8.2 Experimental

MTBDNA was extracted using various methods, mainly conventional phenol-chloroform method, commercial method, commercial magnetic bead method and our newly developed form the MNP base method.

8.2.1 Conventional Chloro-phenol methods

Sample pellet was dispersed in 10 mM Tris-1mM EDTA buffer containing 0.1 % Tween 80 and 2 mg/ml lysozyme. The tube was incubated for 2 h at 37°C with intermittent shaking and centrifuged. The pellet was lysed by suspending it in TE buffer containing 100 µg of Proteinase K/ ml and 1% (w/v) sodium dodecyl sulphate and incubating for 1 h at 37°C. DNA was extracted by adding an equal volume of TE saturated phenol: chloroform: iso-amyl alcohol (25:24:1 v/v/v). The aqueous phase was transferred to another tube and 0.1 volume of cold 3 M sodium acetate (pH 5.2) was added. The sample was mixed by inversion and placed on ice for 10 min before centrifugation for 10 min. The supernatant fluid was transferred to another tube and DNA was precipitated by the addition of 20 µg of acrylamide/ml, 0.05 volumes of 3M

sodium acetate and 2.5 volume of ethanol, washed, dried, dissolved in 25 µl sterile triple distilled water [3].

8.2.2 Commercial kit based methods

The commercial kit based method contain readymade TE buffer, and proteinase K, the DNA was eluted in 200ul of TE buffer. According to manufactures guidelines. The bacterial pallet was re-suspended in 200 ul TE buffer, vortex and then kept at 56°C for 20 minutes and the again vortexed and centrifuged at 13000 rpm for 15 minutes, supernatant was transferred to micro tube in another sterile micro test tubes. DNA was collected in TE buffer and stored at -20°C.

8.2.3 Nanotech assisted MTB DNA extraction method

The pellet was re-suspended in 200µl nuclease free water, vortexed and boiled at 100ºC for 20 minutes, then cooled to room temperature. Supernatant transferred into new 1.5 ml sterile micro centrifuge tube, 200µl lysis buffer (10mM Tris-HCl, 0.5 mM EDTA, 10 % SDS, pH 7.6) and 10µl proteinase-K (20 mg/ml) were added and incubated in a water bath at 56 ºC for 2 hrs. Sample was centrifuged at 10,000 rpm at 4ºC for 20 minutes. Supernatant was transferred to a new test tube containing 500 µl binding buffer (25% polyethylene glycol 8000 MW) and 100 µl SPIONs. Mixed by gently inverting and allowed to stand at room temperature for 5 minutes. Using external magnet supernatant was removed. The magnetic pellet was washed with cold 70% Ethanol was added for 2 to 3 times. 100 µl of elution buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 8.0) was added to elute bound DNA by incubation at 56 ºC for 5 minutes with gentle agitation. Centrifuge at 13000 rpm for 5 min and collect 50µl elute in the new micro-centrifuge tube. A buffer containing the extracted DNA was transferred carefully into a fresh micro centrifuge tube and stored at -20°C [4].

8.2.4 Commercial magnetic bead extraction method:

DNA was isolated from sputum sample using the magnetic beads according to the manufacturer's instructions; nucleic acids were re-suspended in the elution buffer. DNA was transferred carefully into a fresh micro centrifuge test tube and stored at -20°C.

8.3 Results and Discussion

8.3.1 Comparative analysis of MTB DNA extraction

There are numerous techniques for the isolation of MTB DNA from sputum sample, each method having its own benefits as well as limitations. MTB DNA extraction is usually affected by various factors such as incomplete cell lysis,

adsorption, desorption techniques to a particular material, chemical substances, and sample pre-treatment process [5]. The quality, quantity and timing process of the extraction technique obtained by SPION method were compared with the conventional chloroform-phenol and commercial kit base methods, The purity of DNA sample was measured by A_{260} / A_{280} ratio, with absorption spectrum from 220-350 nm wavelength measured by using bio-spectrophotometer (Cary 60 Agilent Technology, USA). The concentration of the DNA in the sample was determined by the equations (One OD 260 Unit =50 µg/ml for double stranded DNA. The average A_{260} /A_{280} ratio and yield of SPION recovered DNA was approximately 1.8 to 2.0 indicates, least protein contamination, obtained by SPIONs [6-9]. By comparing the ratio between conventional and commercial kit methods, our novel DNA extraction method using SPION are better than other methods. The amount of eluted MTB DNA produced highest MTB DNA yield of 985.3 ng/µl. The Absorbance A_{260}/A_{280} ratio was 1.72 and 1.87 which indicates that DNA is pure and free from protein. The purity and yield of the extracted DNAs were measured by UV-visible bio-spectrophotometer [10].

8.3.2 Statistical analysis of DNA extraction

Statistical significance of four different methods for DNA extracting methods was analyzed. The efficiency of each DNA extracting protocol was compared using the data analysis package included within graph pad instat software. The mean of quantitative variables which follow a normal distribution were compared by unpaired t-test (table 8.1 to 8.3). Mean of quantitative variables which do not follow normal distribution are compared by using the Wilcoxon signed rank test ($P<0.0001$**) and they are listed in tables No. 8.4 to 8.6. The probability is considered statistically significant*. The mean crossing threshold (Ct) values for the four extracted methods were positive for real-time PCR. The highest Ct values obtained were 27.5 for phenol-chloroform, 23.8 for commercial kit, 21.3 for MNP and 24.8 for magnetic bead. These results indicated, all samples are positive for MTB but the Ct value shows 21.3 for MNPs is lower as compared to commercial kit and magnetic beads. For phenol-chloroform, organic method Ct value 27.5 is higher as compared to other extracted methods as it has contain PCR inhibitor. MNP mediated DNA extraction technique proved to be rapid, inexpensive and robust as compared to the commercial kit [11, 12].

8.3.3 Evaluation of extraction methods with real time PCR

The yield of DNA was compared with the different extraction methods. These results are listed in tables 8.1 to 8.6 and figure 8.1. It was found that MNP based cell separation was more efficient than the conventional phenol-chloroform method and commercial methods in (P<0.0001**) results were statistically significant. To summarize the results with different extraction methods in combination with DNA purification from MNp cell separation, the conventional chloroform-phenol method consistently produced the highest Ct value in contrast to manufactures protocol. MNP method gives lowest Ct value and gives highest pure DNA yield.

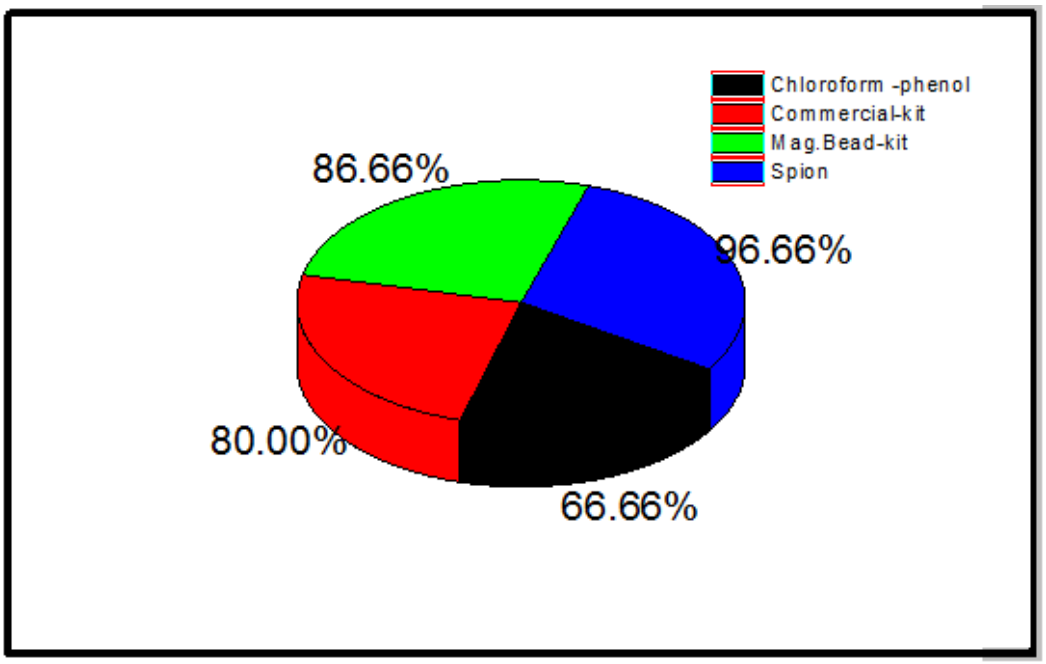

Fig. 8.1 Distribution of samples in percentage

Comparison of DNA extraction methods and the efficiencies of DNA extraction protocols were compared with the yield of DNA of the different extraction methods. These results are listed in table 8.1 to 8.6. It was found that MNP based cell separation was more efficient than the conventional phenol-chloroform method and commercial methods (P<0.0001**) results were considered statistically significant. To summarize the results with different extraction methods in combination with DNA purification from MNp cell Separation, the conventional chloroform-phenol method consistently produced the highest, Ct value in contrast to manufactures protocol. MNP method gives lowest Ct value and gives highest pure DNA yield [13- 15].

8.3.4 Purity and yield of DNA (A_{260}/A_{280})

Data analysis is performed by graph pad instat software. Mean of quantitative variables which follow a normal distribution were compared by unpaired t-test. Mean of quantitative variables which do not follow normal distribution were compared by using the Wilcoxon signed rank test P<0.0001** data was considered statistically significant.*

Table: 8.1 The Yield of MNPs-DNA method Vs Chloroform-phenol by unpaired t-test

	MNPs- METHOD ($\bar{X}$±SD)	Chl.-phenol ($\bar{X}$±SD)	P-value test	Statistical Test used
Yield of DNA	1.742 ± 0.043	2.224 ± 0.097	P<0.0001**	Unpaired t-test
N	30	30		
SEM	0.0078	0.0178		
C.I.	1.726 - 1.758	2.188 - 2.261		

$\bar{X}$± SD denotes the values in mean ± STD deviation values of yield DNA are expressed in: $\bar{X}$± SD, (n=30), p<0.05 is considered statistically significant, *shows statistically significance.

The mean ratio of MNPs and chloroform-phenol method were compared by unpaired t-test which is statistically significant. Ratio by MNPs method is significantly more than chloroform-phenol method (P < 0.0001**)

Table: 8.2 The Yield of MNPs method Vs. Commercial kit method Unpaired t-test

	MNPs-METHOD ($\bar{X}$±SD)	Commercial Kit ($\bar{X}$±SD)	P-value	Statistical test used
Yield of DNA	1.742 ± 0.043	1.43 ± 0.1348	P<0.0001**	Unpaired t-test
N	30	30		
SEM	0.0078	0.0246		
C.I.	1.726 -1.758	1.382 - 1.483		

$\bar{X}$± SD denotes the values in mean ± STD deviation values of yield DNA are expressed in: $\bar{X}$± SD, (n=30), p<0.05 is considered statistically significant, *shows statistically significance.

The mean ratio of MNPs and commercial kit method were compared with unpaired t-test which is statistically significant. Ratio by MNPs method is significantly more than commercial kit method ($P < 0.0001$**).

Table: 8.3 The Yield of MNPs-DNA method Vs. Magnetic Bead Unpaired t-test

	MNPs-METHOD ($\bar{X}$±SD)	Mag.Bead ($\bar{X}$±SD)	P-value	Statistical test used
Yield of DNA	1.742 ± 0.043	1.713 ± 0.14	P<0.0001**	Unpaired t-test
N	30	30		
SEM	0.0078	0.02668		
C.I.	1.726 - 1.758	1.65 - 1.76		

$\bar{X}$± SD denotes the values in mean ± STD deviation values of yield DNA are expressed in: $\bar{X}$± SD, (n=30), $p<0.05$ is considered statistically significant,

*shows statistically significance.

The Mean ratio of MNPs and magnetic bead methods were compared by unpaired t-test which is statistically significant. Ratio by MNPs method is significantly more than magnetic bead method ($P < 0.0001$**).

Table 8.4 The Yield of MNPs method Vs. Chl.-phenol method by Wilcoxon signed rank test

	MNPs-METHOD ($\bar{X}$±SD)	**Chl. phenol ($\bar{X}$±SD)**	**P-value**	**Statistical Test used**
Yield of DNA	845.633 ± 101.02	75.34 ± 8.895	P<0.0001**	Wilcoxon test
N	30	30		
SEM	18.443	1.624		
C.I.	807.92 – 883.35	72.026 - 78.668		

$\bar{X}$± SD denotes the values in mean ± STD deviation values of yield DNA are expressed in**:** $\bar{X}$± SD, (n=30), $p<0.05$ is considered statistically significant,

*shows statistically significance.

The mean ratio of MNPs and chloroform-phenol method are compared by Wilcoxon signed rank test which is statistically significant. Ratio by MNPs method is significantly more than chloroform-phenol method (P < 0.0001**).

Table: 8.5 The Yield of MNPs method Vs. Comm. kit method by Wilcoxon signed rank test

	MNPs-METHOD ($\bar{X}$±SD)	Com.Kit ($\bar{X}$±SD)	P-value	Statistical test used
Yield of DNA	845.633 ± 101.02	331.4 ± 44.487	P<0.0001**	Wilcoxon S. Rank
N	30	30		
SEM	18.443	8.122		
C.I.	807.92 – 883.35	314.79 - 348.01		

$\bar{X}$± SD± SD denotes the values in mean ± STD deviation values of yield DNA are expressed in**:** $\bar{X}$± SD, (n=30), p<0.05 is considered statistically significant,

*shows statistically significance.

The mean ratio of MNPs and commercial kit method were compared by Wilcoxon signed rank test which is statistically significant. Ratio by MNPs method is significantly more than commercial kit method (P < 0.0001**)

Table: 8.6 The Yield of MNPs method Vs. Mag. bead method by Wilcoxon signed rank test

	MNPs-METHOD ($\bar{X}$±SD)	Mag.Bead ($\bar{X}$±SD)	P-value	Statistical Test used
Yield of DNA	845.633 ± 101.02	680.86 ± 84.92	P<0.0001**	Wilcoxon test
N	30	30		
SEM	18.443	8.122		
C.I.	807.92 – 883.35	649.16 – 712.57		

$\bar{X}$± SD denotes the values in mean ± STD deviation values of yield DNA are expressed

In: $\bar{X}$± SD, (n=30), $p<0.05$ is considered statistically significant, *shows statistically significance.

The mean ratio of MNPs and magnetic bead kit methods were compared by Wilcoxon signed rank test which was statistically significant. Ratio by MNPs method is significantly more than magnetic bead kit method ($P < 0.0001$**).

PCR diagnosis of *M. tuberculosis* infection is the best molecular technique, it proves specificity, and sensitivity and rapid PCR test. In this study, we focus on the evolution of nanotech assisted PCR diagnosis of *M. tuberculosis* and its assessment with the conventional methods for the detection of *M. tuberculosis* [16]. Conventional methods for bacteriological detection of TB are still based on microscopic visualization of AFB in sputum samples, for growing this micro-organism in the culture media for later identification. The sputum smear microscopy method is most commonly used in the detection of the pulmonary tuberculosis because it is simple, rapid, and inexpensive despite its low sensitivity [17]. In India, where culture methods, are still used as the gold standard, this method is time-consuming and delays diagnosis. It may take one to two months because the organism is slow growing. Mostly culture is performed in the positive smear in the second month of treatment, MDR (multi-drug resistant) TB patient with previous history, immunosuppressed patients [18].

Nanotechnology based methods have the advantage of being earlier than culture-based methods and reduce the delay for TB detection. Nanotech methods are now more reliable compared to conventional methods. In this study RT-PCR detected a higher number of confirmed TB cases. Real-time PCR results depend on quality, quantity and of DNA. In our study, the combination with magnetic nanoparticle with real time PCR is an effective tool for detection of *M. tuberculosis* directly from pulmonary sputum sample [19, 20].

The sample distribution according to age and gender-wise, varied greatly as a maximum number of sputum sample when compared to extra pulmonary sample. The more number of AF positive samples and culture positive could be recognized in significantly more number of sputum samples. Occasionally, problems with false positive real-time PCR results, are reported may be due to variations between laboratories due to technical differences and cross contamination. PCR method is highly sensitive and specific, for low bacilli containing samples. The disadvantage of

PCR is its high cost, molecular lab infrastructure and skilled technologist [21-23]. At present-day, in India real-time PCR is suitable as a public tool for rapid and early detection of TB. Nanotech assisted PCR with higher efficiencies has been evolved. In the present investigations, it is attributed to more significant and innovative properties of nanoparticles than that of magnetic beads [24].

8.4 Conclusions

The success of final amplification and detection of DNA depends on the extraction of good quality DNA free from protein, lipids and RNA. The extraction protocol using the magnetic nanoparticle showed the best results in terms of quantification and sensitivity of real-time PCR amplification. The MNP-DNA extraction with NALC followed by *IS6110* target amplification has proved to be an effective tool for early detection of *M. tuberculosis* from a pulmonary sputum sample.

Future trends:

The extraction protocol by using MNP are better than other conventional and commercial kit method. We were able to extract highest yield of DNA (845.6ng/ul). The extraction yield of DNA can give more results by using other super paramagnetic properties. Recently, however many more MNPs with core structure have shown higher magnitudes of magnetisms and needs to be tried for such kind of studies.

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Chapter 9: Summary and Conclusions

9.1 Introduction

Mycobacterium tuberculosis (MTB) is a deadly communicable infectious disease where 10.4 million people have fallen ill in 2017 (World Health Organization). The development of MTB is sluggish which can hinder the early detection by conventional methods. The great impact of tuberculosis (TB) disease is attributed to the pathogens inherent virulence factors, airborne transmission, prolonged persistence in macrophages and latent infection. These features are the origin of multiple challenges facing both TB diagnosis and treatment, and enhancing the overall complexity of disease management. Other challenges include long-term treatment and lack of an effective vaccine. Reliable early diagnosis of TB is significant in disease control and patient controlling and compliance.

Present TB detection techniques involve smear microscopy, culture, and molecular techniques. TB detection starts with smear staining by acid fast staining. AF staining detects TB within minutes for each specimen, but the sensitivity of this method is low (30–40% positive cases). Irrespective of the smear stain results, all sputum samples are inoculated for L J culture media. The culture method is considered gold standard for TB detection. However, it requires 4 to 8 weeks to confirm its growth.

Automated liquid culture media such as BACTEC MGIT 960 (Becton Dickinson) reduced the detection time of TB (9–16 days). All culture methods still fall underdeveloped from realizing the need for rapid tests and precisely difficult, so that they do not appropriate in low resource setting, where the majority of TB cases arise. The improvement of new diagnostic tools, short turnaround time, cost-effectiveness and ability to differentiate between MTB and other pathogen is therefore highly necessary. Pulmonary sputum sample of 120 TB positive patients as base for field work/ experimental studies.

The aim of the thesis is to develop new methods and novel technology for early diagnosis of *Mycobacterium tuberculosis* by using nanomaterials. The apparent benefits of nano diagnostics involve their skill to produce quick results with the high sensitivity and specificity. The main advantage by using nanomaterials is low cost and to reduce the time for delay in TB diagnosis. Also, this method is used for point of care device in the tertiary healthcare centres. Thus, in the present study, Fe_3O_4 NPs were selected for the effective collection and enriching DNA of MTB from

pulmonary sputum samples. While gold NPs were used for the development colorimetric detection of MTB.

9.2 Competent Components of the Thesis

Tuberculosis (TB) has existed for millennia and remains to be a major global health problem. It causes ill health in millions of people each year. India has a high burden of TB cases, In India, 15 million suffers from TB, of which over 3 million are highly infectious. Half of a million people die from the disease every year, i.e. every two minutes one TB patient dies. Tuberculosis is an ancient disease, and it is estimated to affect one third of the world's population. An estimated 22 million lives are saved through the use of DOTS and the stop TB strategy recommended by the WHO.

The WHO's "End TB" strategy aims to reduce TB deaths by 95%, reduce new cases by 90 % between 2015 and 2035, to ensure that no family is burdened with catastrophic expenses due to TB. Most of the conventional TB detection depends on the microscopic smear examination and culture technique, which involve tedious process and necessary for experienced persons to handle the test. Moreover MTB culture technique takes more time to produce the result varying from several days to months.

The conventional methods are less sensitive that they can detect only half of the active MTB. The conventional TB detection methods, though simple, still depends on AFB microscopy and requires 5,000 – 10,000 bacilli/ml for judgment of MTB. In addition, phenotypic identification such as culture and biochemical study, include velocity of growth; colony morphology; pigment production, urease test, niacin test; nitrate reduction test; catalase activity; Pyrazinamide test: growth in the presence of p-Nitro benzoic acid. With this background, in recent year one new approach for rapid, safe and reproducible identification of MTB infection is real time PCR. It is one of the major competent component of our thesis.

The success of final amplification and detection of nucleic Acid Amplification test (NAAT) depends on successful extraction from pulmonary sputum sample. The RT-PCR (Polymerase chain reaction) is an identical sensitive technique for the detection of communicable disease, but the sensitivity of the assessment is mostly dependent on the productivity of the DNA extraction method. The demand for PCR diagnosis in medical microbiology has highlighted the need for an efficient method of

Nucleic acid (DNA) extraction. Presently magnetic beads are used for MTBDNA extraction, which are polycrystalline in nature and macroscopic.

In recent years the super-paramagnetic nanoparticles such as Fe_3O_4, CoO.5Zn0.5 Fe_2O_4 (CZF), synthesized cobalt ferrite ($CoFe_2O_4$), Ni0.5Zn0.5Fe_2O_4, La0.7Sr0.3MnO_3 (LSMO), MnxMg1−xFe_2O_4, Fe_3O_4 and CS- Fe_3O_4, La0.7Sr0.3MnO_3 (LASMO) can be used for extraction of MTB DNA.

In the present study Fe_3O_4 MNPs has been synthesized and used for MTB DNA detection with higher efficiencies than that of the magnetic beads and tested for clinical samples. Our results are superior than conventional methods. The MNP assisted DNA extraction protocol showed better results in terms of quantification and sensitivity of PCR diagnosis of MTB.

Another new approach that we have attempted in our study is the use of gold nanoparticle. The gold nanomaterial based biomedical application in MTB diagnosis device is developed in our study. Colloidal gold, a classic kind of nanoparticle, have been used to stain glass ever since ancient times. The gold colloid was first explored around 1857 by the distinguished English physicist and chemist Michael Faraday.

Nanoscale properties of AuNPs are used in different fields like optics, electronics, and optoelectronics and biosensor techniques. Gold nanoparticles (NPs), nanodots (NDs), nanorods (NRs) and nanoclusters (NCs) have enormous importance in sensing hazardous anions, cations and drugs due to their stability and size-dependent optical properties. AuNPs based colorimetric approach has emerged as a new strategy for sensing and imaging, bio-molecular interactions due to the visual signal feedback and facile quantification.

The AuNPs are used in most cases, chemically altered for detection of disease specific DNA or RNA with target specific oligonucleotide sequences. Based on a target DNA/RNA interaction with the AuNPs a color change is obtained resulting in the difference between positive and negative samples. In this case, the specific oligonucleotides interaction is of major importance since it is the main factor responsible for differences between positive and negative samples. The surface plasmon resonance (SPR) of gold nanoparticles is responsible for their intense colors. In the solution, monodisperse gold nanoparticles appear red and exhibit a relatively narrow SPR.

There are various types of diagnostic modalities used for MTB detection. We have tried to develop a protocol with a focus on ultrasensitive colorimetric optical

nanobiosensor which is specifically detected MTBC species and MTB. The sputum samples collected from our area Kolhapur, Sangli, Satara and Ratnagiri and Pune zone. The abundance of important bacterial taxa was confirmed by quantification of total and specific bacterial population using quantitative real time PCR in terms of copy numbers of 16S rRNA gene.

We tried to cover all the parameters of colorimetric sensing assay, which include fluorescence spectrometry, DNA analysis, qPCR, amplicon sequencing, a cyclic Voltammetry study using portable base electrochemical analytical as well as CV and impedance base electrochemical work station in department of cyclic voltammetry work station CIR D.Y.Patil university Kolhapur, India. Also in our study as per our knowledge we have first time report addition of $MgCl_2$ into the assay protocol.

In our study, single tube AuNPs based MTB detection assay was developed for the identification of MTB through targeting its 16s rDNA gene. Briefly, extracted DNA is added to the hybridization buffer. The use of thiol-linked ssDNA-modified gold nanoparticles (Au-Nanoprobe) for the colorimetric detection of DNA targets represents an inexpensive and easy-to-perform alternative to common molecular assays.

In this thesis, we report a novel and straightforward optical and colorimetric gold nano particle capped with GP-1 and GP-2 Oligo target for the detection of MTB and MTBC. Thus, the developed colorimetric MTB Oligo probe offered various advantages such as simplicity, reproducibility, stability and selectivity, serving as an effective alternative for optical sensors developed to date

9.3 Summary of thesis

Highlighted in this present study, the total research work of the thesis is distributed into nine chapters. The First two chapters cover the introductory and theoretical review parts and others contain experimental, results and discussion.

Chapter 1. Introduction

The motivation of the research study begins in the first chapter with in-depth understanding of the tuberculosis global health problem. Despite the arrival of scientific improvement in recent years, TB diagnostic method is still based on the old-style method. The standard TB diagnostic method in developing countries involve sputum smear microscopy, which was developed in 1880 and has continued almost

the matching, precise that it relies on the presence of AF-bacilli in the stained smear. This has been resulted in world health organization supporting its efforts to eradicate the global TB burden by the year 2035.

This chapter mainly consists of a history of *Mycobacterium tuberculosis,* TB bacteriology, life cycle and pathophysiology of tuberculosis, tuberculosis vaccines and treatment and all conventional TB diagnosis methods with drawbacks are discussed in this chapter. The last part of this chapter describes about the emerging field of nanotechnology based TB diagnosis methods with various types of biosensor.

Chapter 2. Tuberculosis nanotechnology: Diagnostics, therapeutics and Prevention of *Mycobacterium tuberculosis*

This chapter reviews various nanomaterrials that has been used in TB diagnosis. All types of nanoparticle based Tb diagnosis methods are covered in detail. The nanobiosensor platform used for the diagnosis of MTB DNA target with various types of nanobiosensor, nanomaterial in TB drug delivery, nanotechnology in TB vaccination and finally prevention of TB are mentioned in this chapter.

Chapter 3. Isolation of MTB from clinical samples

Chapter 3 reports various types of isolation methods of MTB from clinical samples with standard operating systems (SOPs). All types of MTB DNA isolation methods, sample collection and processing, storage protocols with importance of biosafety precautions are mentioned in this chapter. Total four hundred sputum samples were collected. Out of four hundred only strong AF positive one hundred twenty sputum were processed and tested for TB, Real time PCR, AF-stain, and conventional culture method. Finally, this study revealed that NALC pre- treatment has resulted in quick recovery of tuberculosis bacilli from sputum sample.

Chapter 4. Detection of MTB with conventional methods

The present study focused on traditional TB diagnostic methods like medical, physical examination, Chest X-ray, tuberculin test (TT), AFB staining method, culture etc. Total four hundred sputum samples selected, out of four hundred one hundred twenty samples are confirmed by X-ray and AF-positive. Finally, new development of TB diagnostic test is mentioned in this chapter

Chapter 5. Detection of MTB with Real time PCR

Chapter 5 reports the quick diagnosis of MTB DNA target with significant improvement and high accuracy. It proves both sensitivity and specificity and generate reports within two hours with the help of real time PCR diagnosis assay. In this chapter the role of TB diagnosis by using real time PCR detection with significance is discussed with various types of PCR their principle and medical applications and finally whole chapter is focused on TB PCR diagnosis. In this chapter methodology of real time PCR, amplification of MTB species gene IS6110 primer quantification of PCR amplicons and melting curve with CT value is discussed for identification of tuberculosis. Total ninety samples were submitted to real time PCR assay. All samples were detected MTB positive by RT-PCR.

Chapter 6. Synthesis, characterization and application of SPIONs in DNA extraction and detection of MTB with PCR

Chapter 6 presents a biological application of MTB DNA detection by MNP method. The present study is focused on MTB DNA isolation methods with Real time PCR diagnosis. The emerging SPION- mediated extraction of MTB DNA technique is developed. Total thirty sputum samples were consider for this method. This technique is rapid, inexpensive, and robust and accomplished in a single test tube. SPION-mediated MTB DNA extraction method produced pure and good quality DNA and are worthy for real-time PCR detection.

In this study, we tried to develop MTB DNA extraction from positive sputum sample using SPION method and its superiority has brought to focus for the first time through this study. The amount of eluted MTB DNA produced highest yield (985.3ng/ul) and the absorbance A_{260}/A_{280} ratio was 1.72 and 1.87 which indicated MTB DNA is pure and free from protein. It is predicted that this method is also applicable for small sample volume and even low bacterial load with this method we can achieve our target at an early stage. The real time PCR Ct value of SPION (21.3) which is lower as compared to other commercial kits. We have concluded that SPION mediated MTBDNA with RT-PCR is more suitable for early detection of MTB.

Chapter 7. Studies on development of Colorimetric gold nanobiosensor for early detection of MTB

This chapter is based on early detection of MTB by naked eye colorimetric observation. The research presented in this chapter mainly consists of two parts, namely (a) Synthesis and characterization of gold nanoparticle and (b) fabrication of colorimetric gold nanobiosensor for early diagnosis of TB. In the first part of the chapter, synthesis of gold by trisodium citrate reduction method is described and in second section the various characterization techniques used are described.

The second part covers the fabrication of colorimetric gold nanobiosensor by using synthetic Oligo nucleotide probes GP-1 and GP-2. The sequence mainly in the IS *6110* gene targeting MTBC. The achieved results are on the proof of concept level.

Finally, we increased the sensitivity and specificity of colorimetric gold probe assay (10 Femtomoles) by using cyclic Voltammetry study (CV). The MTB DNA quantification study was also done. In this study we have selected two DNA samples and detected successfully by colorimetric nanobiosensor. The colorimetric nano-biosensor with qPCR results shows that our method is equivalent to qPCR for early diagnosis of tuberculosis. The entire charts of the present work is represented in Fig.9.1 (a) and Fig 9.1 (b). It shows the step by step flowchart of the experimental part of the thesis.

Chapter 8. Comparative study Conventional, Real time PCR and Nanotech assisted MTB Detections methods:

This chapter covers the comparative study of MTB detections with conventional, Real time PCR and nanotech assisted methods. In this chapter, we have compared the MTBDNA extraction methods. A significant difference is seen in the percentage of different DNA extraction methods, the conventional chloroform-phenol 66.66%, the commercial kit 80%, magnetic bead 86.66% and MNP method 99.66 % found to be significant ($P<0.0001^{**}$).

9.4 Major Conclusions

Out of four hundred clinical suspected patients, only strongly AF positive one hundred twenty-sputum samples were collected and processed for diagnosis of TB by

acid fast staining, culture and real-time PCR tests. The major conclusions are grouped into two parts

Section 1: Synthesis, characterization and application of SPION in DNA extraction and detection of MTB with PCR

1. The SPIONs were successfully synthesized in our laboratory and used for MTB DNA extraction from pulmonary sputum sample and finally detected by real time PCR.

2. The novel DNA purification method using SPION is better than other methods. The amount of eluted MTB DNA produced highest MTB DNA yield 985.3 of ng/µl.

3. SPION mediated MTB DNA extraction shows its superiority over the real time PCR diagnosis of MTB DNA.

4. The MTB DNA target detected Ct value for SPION (21.3) which is lower as compared to the commercial kit, indicating more amount of target nucleic acid is found compared to real time assays of 40 cycles of amplification.

Section: 2 Studies on development of Colorimetric gold nanobiosensor for early detection of MTB

1. The gold nano seed precursor was synthesized successfully in our lab by trisodium citrate reduction method.

2. The gold nanoparticle based biosensor was designed and fabricated specifically to detect MTB DNA target.

3. The sensitivity of this assay was found to detect 10 femtomoles of an oligonucleotide and the detection limit of the assay is 1.95×10^{-2} ng/ml for TB DNA.

4. In conclusion, we successfully developed colorimetric high sensitive and rapid detection of MTB.

5. The outcome of the research may be translated for lab scale to field level trials recommended by **Start-Up India,** is an initiative of the**,** Ministry of Commerce and Industry, (Department for Promotion of Industry and Internal Trade), MsN-Start-Up India Yatra, **Government of India.**

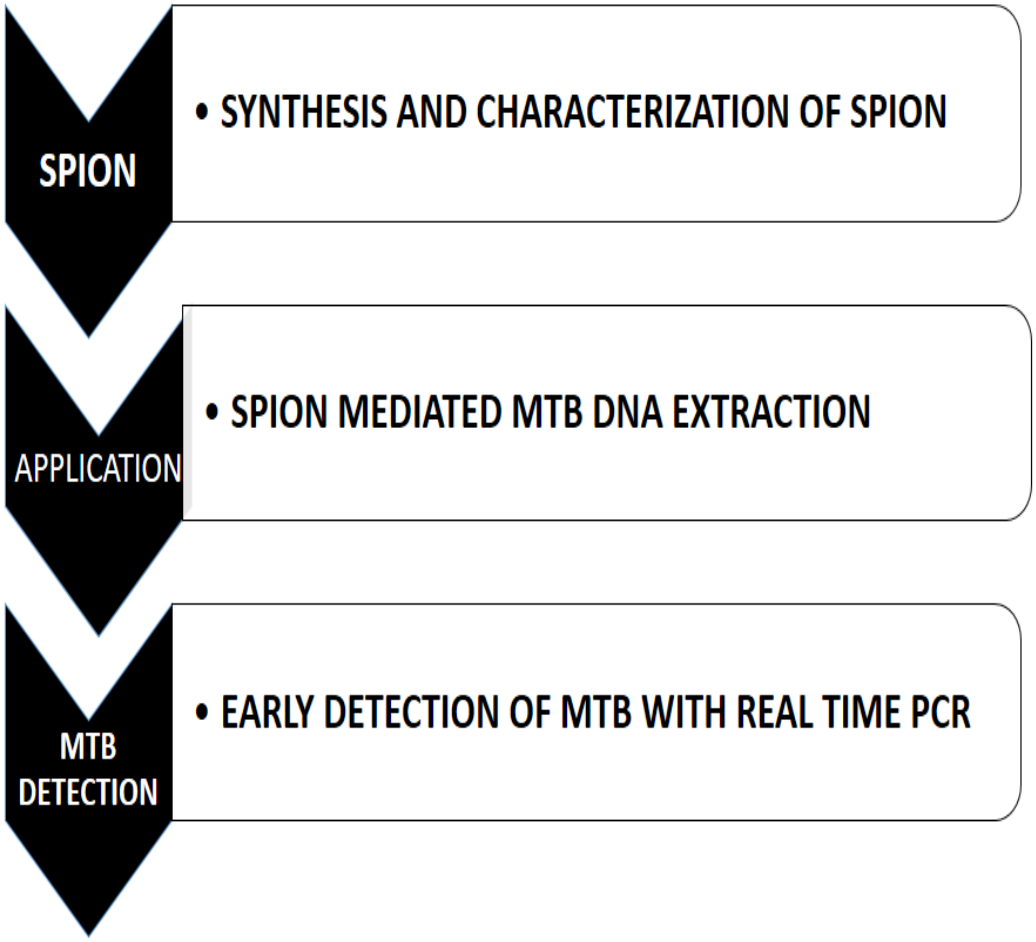

Fig. 9.1 (a) flow chart of SPION synthesis and MTB detection of the present work

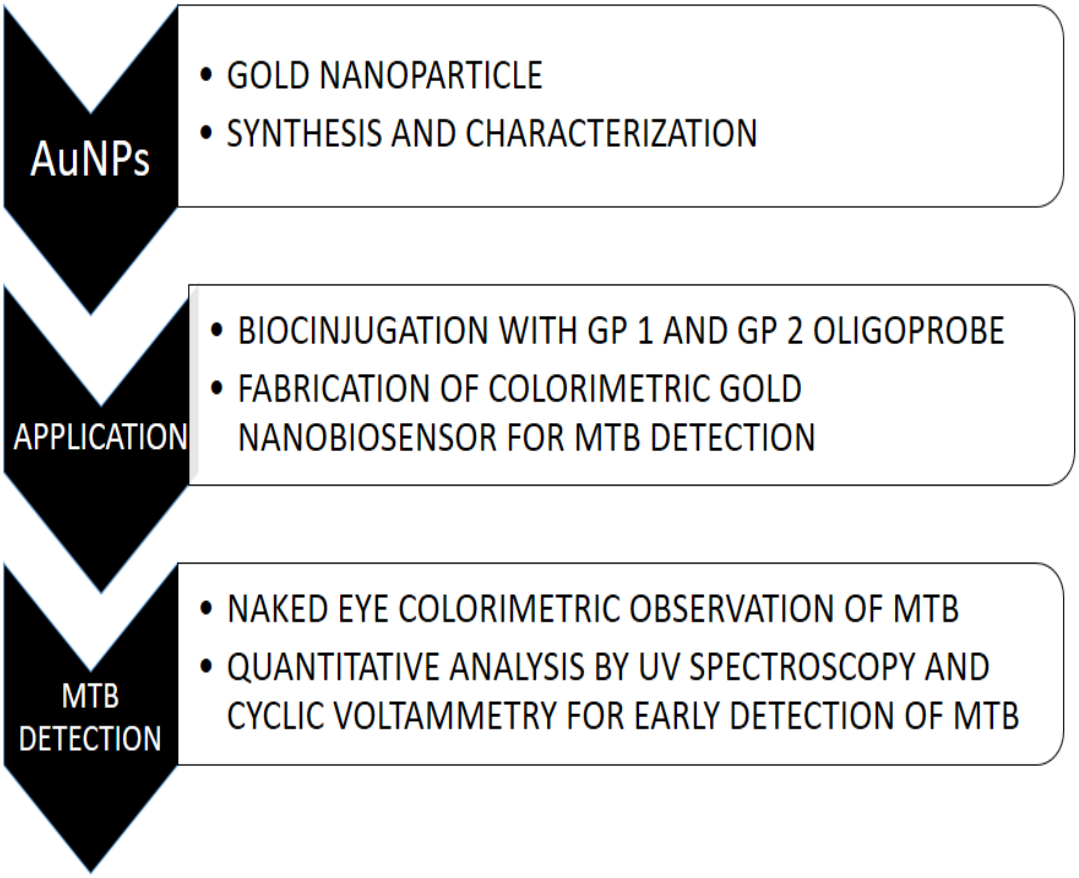

Fig. 9.1 (b) flow chart of AuNPs synthesis and colorimetric MTB detection of the present work

9.5 Future scope of the thesis:

This device, developed in the present investigation definitely stand to be the one of the new better alternatives and hold tremendous potential to replace current technique. In continuation to the present work this device can be scaled up by using simple paper based material for early spot diagnosis of TB in tertiary care centres.

INFORMED CONSENT FORM

I, Mr/Mrs/Ms ……..... Gender …… Age: .....

residing at .....

do hereby confirm that:

I. I have been asked by the student/researcher of D Y Patil Medical College, Hospital and Research Centre, Kolhapur ("the Medical College") whether I wish to participate in a study (research) under the aegis of the Medical College.

II. Purpose and methods of the research in simple language. (It should be written by investigator).

III. Expected duration of participation & frequency of contact, number of participants type of data collection & methods.

IV. Any alternate procedure or treatment should be informed

V. The nature of the study being undertaken by the student/ researcher, as well as the extent of my participation in it, have been duly explained to me in a language that I understand;

VI. The potential risks and consequences associated with this study have also been duly explained to me in a language that I understand;

VII. I also understand that my participation in this study is only for the benefit of advancement in the field of medical research and that at no point in time is my participation being solicited for any pecuniary gain by the researcher or the Medical College;

VIII. I have also been explained that I am in no way obliged to participate in the study and that, once I have agreed to participate in the study, I am still free to withdraw from participation in the study at any point in time upon notifying the Medical College in writing in the prescribed form without assigning any reason;

IX. There will be no financial transaction between myself, the researcher and/or the D Y Patil Medical College for my participation in that study;

X. I have been explained that any data collected out of my participation in the study will only be used for academic purposes and/or for further medical research;

XI. I have also been reassured that any publication of the data collected during the course of the study or any publication of its conclusions, shall be done on a 'no names' basis and shall under no circumstances reveal my personal identity. Any personal details likely to reveal my personal identity shall at all times remain confidential;

XII. I understand that if any accident or undesirable medical complication arises out of a

procedure or treatment done solely for the purpose of research, I will be offered treatment, free of cost, by the D Y Patil Hospital & Research Center, Kolhapur. Any additional compensation considered necessary by the Institutional Ethics Committee may also be given to me.

By affixing my signature/thumb print hereto, I am therefore freely and voluntarily signifying my consent, intent and willingness to participate in the study of the student researcher for the purposes of the postgraduate dissertation under the egis of the Medical College. I also certify that my right to privacy has not been infringed in any manner.

[SIGNATURE/THUMB PRINT OF PARTICIPANT]
DATE:

WITNESSED BY:

(1) NAME: TITLE/CAPACITY: SIGNATURE:	(2) NAME: TITLE/CAPACITY: SIGNATURE:

Name of Project investigator: _____

Address: _____ Contact Number: _____

Signature of investigator:

Name of Project Co- investigator: _____

Address: _____ Contact Number: _____

Signature of investigator:

Helpline Numbers:

Contact Details of Member Secretory: Institutional Ethics Committee

D. Y. PATIL MEDICAL COLLEGE, KOLHAPUR

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Date :

INSTITUTIONAL ETHICS COMMITTEE, D. Y. PATIL MEDICAL COLLEGE, KOLHAPUR.

2016/ 44/PA- Ph.D

Date: 19 SEP 2016

This is to certify that the research project titled

"Studies on early detection of mycobacterium tuberculosis (MTB) using nanotechnology."

Submitted by : **Mr. Deepak V. Sawant**

Under the supervision of appointed Guide (if any): **Prof. Dr. S. H. Pawar**

Has been studied by the Institutional Ethics Committee (IEC) at its meeting held on 09/09/2016 and the student has been granted approval for the study with due effect with the following caveats:

1. If you desire any change in the protocol or standard recording document at any time, please submit the same to the IEC for information and approval before the change is implemented.
2. All serious and/or unexpected adverse events due to the drug/procedures tested in the study must be informed to the IEC within 24 hours and steps for appropriate treatment must be immediately instituted.
3. In case of injury/disability/death of any participant attributable to the drug/procedure under study, all compensation is to be made by the sponsor of the study.
4. The Chief investigator/Researcher must inform the IEC immediately if the study is terminated earlier than planned with the reasons for the same.
5. The final results of the study must be communicated to the IEC within 3 months of the completion of data collection.
6. The researcher must take all precautions to safeguard the rights, safety, dignity and wellbeing of the participants in the study.
7. The researcher must be up to date about all information regarding the risk/benefit ratio of any drug/procedure being used and any new information must be conveyed to the IEC immediately. The IEC reserves the right to change a decision on the project in the light of any new knowledge.
8. Before publishing the results of the study, the researcher must take permission from the Dean of the Institution.
9. The approval is for the Period of 24 months from date of meeting of Institutional Ethics Committee.

Dr. Mrs. Shimpa R. Sharma
(Member Secretary, IEC)

Dr. Mrs. Vasanti Rasam
(Act. Chairperson)

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CURRICULUM VITAE

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1. EDUCATION

Ph.D. Clinical Microbiology

Thesis submitted to D. Y. Patil Education Society (Deemed to be University)

(August 2014 -April 2019)

Title of thesis: "Studies on early detection *Mycobacterium tuberculosis* (MTB) Using nanotechnology".

(a) M.Sc. in Clinical Microbiology

Institution: Punjab Technical University,

Grades: I class (80.80 %) Subjects: (Anatomy, Physiology and Biochemistry,)

Major subject: Clinical Microbiology

Elective subjects: a) Bacteriology (general and systemic) b) Immunology c) Mycology,

d) Virology e) Parasitology f) Clinical Microbiology g) Molecular Microbiology

(b) Advance PG DMLT (MSBTE)

Institution: Maharashtra State Board of Technical Education, Pune.

2. AWARDS & HONOURS

[1] FIRST PRIZE WINNER 2018, Start-Up India,

Start-up India is an initiative of the**,** Ministry of Commerce and Industry, **Government of India.** (Department for Promotion of Industry and Internal Trade)

Healthcare category: Innovative in Healthcare, Government of Maharashtra

[2] Poster presentation Award – "6 th International Oncopathology Seminar'' 2014 At D. Y. Patil University, Kolhapur.

3. PROFESSIONAL SKILLS AND EXPERIENCE

Technical Skills: Medical Laboratory techniques

Molecular Biological Techniques:

DNA, RNA and protein electrophoresis techniques, Genomic DNA isolation, Real time PCR.

Software Skills:

Well versed with software application like Microsoft Office Word, Excel and Power Point.

Computer skills:

Computer fundamentals, MS-DOS, MS-WORD, MS-EXCEL, MSCIT, and BMT (Bachelor of Medical transcription, Pune)

Characterization Techniques:

(a) Expertise in synthesis of nanoparticles.

(b) Instruments handled like X-ray diffractometer and DLS particle size analyser, Scanning electron microscope (SEM), Vibrating sample magnetometer, Fourier transform infrared spectroscopy, UV-Vis spectrophotometer, Electrochemical work station.

Work experience:

(a) Clinical samples in following sections:

1. Culture, identification and antibiotic susceptibility of bacteria in the blood, urine, pus, sputum, throat swab and stool. 2. Virology 3. Mycology 4. Serology 5. PCR and real time-PCR in diagnosis of pulmonary tuberculosis, immunofluorescence. 6. Media 7. Anaerobic bacteriology 8. Mycobacteriology

[b] Experience of handling in various departments:

Working with Instrument like: VITEK / BACTEC, RT-PCR, Haematology, Biochemistry, Serology, Microbiology, day and emergency night lab in Ambika immunofluorescence, Molecular and Pathology laboratory, Prof. Dr. R. S. Patil Kolhapur, Maharashtra.

4. PUBLICATIONS

INTERNATIONAL

1. D. V. Sawant, M.M. Desai, R. S. Patil, and S. H. Pawar, Evolution of nanotech assisted PCR diagnosis of *Mycobacterium tuberculosis* and its assessment with conventional methods, International Journal of Pharmacy and Pharmaceutical Sciences, Feb, 2018, Vol, 10, issue, 133-137.
2. D. V. Sawant, R. A. Bohara, R. S. Patil, S.H. Pawar, Detection of *Mycobacterium tuberculosis* from pulmonary sputum sample using SPION mediated DNA extraction method, Research Journal of life sciences, Bioinformatics, Pharmaceutical and Chemical Sciences, 2018
   DOI - 10.26479/2018.0401.08, 2018 Jan-Dec rjlbpcs 4(1) Page No.91.
3. Deepak Sawant and Shivaji Pawar, Studies on gold nanobiosensor for early diagnosis of *Mycobacterium tuberculosis***,** International Journal of Pharmacy and Biological Sciences, 2019, **(**IJPBS),Vol 9, 77-82.
4. *Mycobacteria tuberculosis*: an overview of traditional, molecular and innovative immerging nanodiagnosis applications, book chapter, CRC press, a Taylor & Francis Group, AAP book, Apple Academic Press., 14 Jan 2019. (Submitted)
5. Quantitative colorimetric recognition of *Mycobacterium tuberculosis* complex bacilli: DNA based gold nanoparticle-probe-conjugates for upper respiratory clinical samples, Deepak Sawant, Mangesh Suryavanshi, Jayachandran Venkatesan K. Sudhakar Prasad and Shivaji Pawar, (Submitted)
6. Tuberculosis Nanotechnology: Diagnostics, Therapeutics, and Prevention of *Mycobacterium tuberculosis,* Deepak V. Sawant and Shivaji H. Pawar, (Submitted)

NATIONAL

7. D.V. Sawant and S. H. Pawar, Conventional and nanotechnology based early stage diagnosis of *Mycobacterium tuberculosis*, Medical Journal D. Y. Patil University, Kolhapur, Vol. IX, Issue 2I, March 2016.
8. D.V. Sawant, S. H. Pawar, SPION based early stage diagnosis of *Mycobacterium tuberculosis*, workshop nuclear technologies for rural development, and there commercialization, ITRDC, Solapur 2017.

9. Deepak Sawant and S. H. Pawar, Colorimetric detection of *Mycobacterium tuberculosis* with gold nanobiosensor, third international conference on nanomaterial synthesis characterization and application ICN 2018 organized by Mahatma Gandhi University, kottayam, Kerala, India

5. **PAPERS PRESENTED AT NATIONAL** / INTERNATIONAL CONFERENCES

[1] UGC sponsored National conference at Rajaram college Kolhapur – recent trend and future prospects in multidisciplinary approaches in microbiology –RTFPM, 4, Oct, 2014, Organised By: Department of Microbiology and Microbiologist Society, India.

[2] 6^{th}, international seminar on recent advances in Oncopathology – 3-4 Jan 2015 Department of Pathology Kolhapur and international society of Oncopathology MMC/Accre. Cert/MED-027, CME Code MM/MAC/2014 Abstract: Application of nanotechnology in cancer detection, **6th International Oncopathology seminars, won third prize in poster presentation**

[3] "Current Trends in Tuberculosis", Department of Microbiology Wanless Hospital, Miraj - 18th Jan 2015, MMC/MAC/2015/B-002236-CME-paraclinical

[4] "Workshop on Application of Statistics in Medical Research"- 20 Jan 2015, D.Y.Patil university Kolhapur, Research cell

[5] "Annual meet on advanced research'' – Amar, 17 march, 2015, D.Y.Patil university Kolhapur, **Poster presentation** - Diagnostic Method – *Mycobacterium tuberculosis*

[6] National conference on "Convergence of Stem cells and Medical Nanotechnology'' 2September 2015, Organised by Centre for Interdisciplinary Research, D.Y.Patil university, Kolhapur, Poster presentation – Isolation MTB DNA from pulmonary sputum sample by SPION.

[7] DYPU Anveshan 2015 – 29 December 2015: **Poster presentation.** Organised by Centre for Interdisciplinary Research, D.Y.Patil University, Kolhapur

[8] "International conference on nanomaterial's and nanotechnology" NANO 2015– 7-10 December 2015, at Tiruchengode, Namakkal (DT), Tamil Nadu, India.7-10 December 2015, Organised by –K. S. Rangasamy college of

Technology. **Oral presentation No.722,** Abstract: Clinical Application of MNPs in Isolation of DNA from sputum sample

[9] National workshop on "Stem Cell molecular biology and bioinformatics" (SMB-2016), Organized by department of stem cell and regenerative medicine, CIR.

[10] "Techniques in Proteomics" Organised by department of Microbiology, Jai Hind college, Mumbai and sponsored by Lady Tata Memorial trusts Mumbai.10 Feb.2017.

[11] Workshop Nuclear Technologies for Rural Development and their commercialization (ITRDC-2017), organised by Centre for research and technology development (CRTDC) and Bhabha atomic research centre , Mumbai and Indian Medical association ,Solapur. **Oral paper presentation.**

[12] Emerging trends in Nanomaterial and their applications (ETNA-2017) **Oral paper presentation** 2 and 3 June 2017.

[13] NCHBT-2017, MCI Sponsored National conference on Haematology and blood transfusion, 18 march 2017 organised by D.Y.Patil University Kolhapur , Department of pathology and Deccan association of pathology – P**oster presentation.**

[14] IC-NACMBM-2017, November 9-11-2017, international conference on nanotechnology addressing the convergence of material science, biotechnology and medical science organised by CIR, D.Y.Patil, University, Kolhapur, **Oral presentation**

[15] Anveshan-2017, 17 November 2017, organised by CIR. D.Y.Patil University Kolhapur, **Poster presentation.**

[16] "One Day workshop on Prime Ministers fellowship scheme for Doctoral Research''. 25 Nov.2016, organized by DST-SERB & CII New Delhi and D.Y.Patil education society deemed university, Kolhapur.

[17] RTMR-2018, 31 Jan 2018, National conference on recent trends in materials research Organised by Department of Physics, Y.C. College warnanagar, Dist. Kolhapur- **Oral paper presentation.**

[18] Third International Conference on nanomaterial synthesis characterization and application ICN 11-13 May, 2018 organized by Mahatma Gandhi University, Kottayam, Kerala, India, **C**olorimetric detection of *Mycobacterium tuberculosis* with gold nanobiosensor, **Oral paper presentation**

[19] NETWAR-2018, National conference on water resources, 27-28 July 2018, CRTDS, Sinhgad institutes, Solapur, Ultrasensitive highly specific colorimetric detection of Nontuberculosis mycobacteria (NTM) in environmental water. **Oral paper presentation**

[20] International Conference on "Empowering Society with Microbial Technology (ICESMT-2019), 7 to 9 February, 2019, TC college campus Baramati, Microbiologist Society, India and Association of Microbiologist of India (AMI) Pune. **Oral paper presentation,** studies on gold nanobiosensor for early diagnosis of *Mycobacterium tuberculosis.*

[21] Anveshan, 5 Dec. 2018, presented research work in the D. Y. Patil (Deemed to be university), Kolhapur-416 006

6. WORKSHOP ATTENDED AT NATIONAL LEVEL

[1] Workshop on- "PCR and Real-time PCR: "Techniques and applications" 2015, Venture Center, Pune.

[2] Workshop – "Hands-on DNA PCR workshop" organised by SDM college of Medical sciences and Hospital, Dharwad-iamm- Karnataka chapter 25-27th February 2016.

[3] Wadhwani Research Centre for Bioengineering (WRCB) Indian Institute of Technology Bombay, third WRCB industrial Day Nov 20, 2018

Reference:

Prof. (Dr.) S. H. Pawar, Emeritus Scientist (CSIR), Distinguished Professor, D. Y. Patil Education Society, Deemed to be University Kolhapur, and Director, Center for Innovative and Applied Research, Anekant Education Society, Baramati- 413 102 (MS) India

E-mail: shpawar1946@gmail.com.

DECLARATION:

I hereby solemnly affirm that all the information furnished by me is true to the best of my Knowledge.

Place- Kolhapur

Date- **(Deepak Vitthal Sawant)**