STUDIES ON EARLY DETECTION OF MYCOBACTERIUM TUBERCULOSIS USING NANOTECHNOLGY

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ABSTRACT

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 pair, clumps or chains .The use cell wall of MTB consist lipid layer composed with mycolic acid, polysaccharides, peptidoglycans plasma-membrane, lipoarabinomannan,phosphatidylinositol and mannoside . The MTB is comprised by a remarkably protein rich cell wall . The peptiogycan 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 . TB is caused by bacteria which is spread from person to person through air . However , HIV reduces the sensitivity of TB microscopy because ofimmediate killing of TB bacilli by alveolar macrophages. This is achieved using different bactericidal mechanism such as reactive nitrogen species and oxygen interemediate . However , the bacilli may survive in 25-505 of infected individual and continue to divide with macrophages cytoplasma . The macrophages then present the mycobacterial antigen to CD4+ lymphocytes.

INTRODUCTION.

Tuberculosis (TB) is the disease caused by mycobacterium bacilli (MTB) . TB has existed for millennia and remains global health problem _According to the latest global report an estimated 1.6 million people died from TB IN 2021 . In 2021 , an estimated 10.6 million people fell ill with TB worldwide .TB is the 13th leading cause of death and second leading infectious killer after COVID-19 (above HIV/AIDS)_Which means that 39% of latest cases have been 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 _About 64% of drug sensitive TB patient were newly diagnosed and notified to national TB control programs. An estimated 22 million lives saved through the use of directly observed treatment (DOTS) and the ‘ STOP TB Strategy ‘ recommended by WHO_Most of the conventional methods used for detecting Mycobacterium tuberculosis bacilli depend on microscopic examination and

culture techniques , which involves tedious processes, require 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

The present MTB detection method involves smear microscopy ,culture and molecular techniques .Ziel-Neelsen (ZN) sputum staining relies upon on the ability of MTB to resist acid de-colorization for its excessive mycolic acid content .It depend 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 take 6 to 8 weeks to confirm TB on (L J)Lowenstein Jensen media .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 require 5-10x10-3 bacilli/ml for detection of MTB .In addition ,phenotypic identification such as culture andbiochemical studies include colony morphology ,pigment production ,urease test ,niacin test, nitrate test ,reductiontest ,catalase activity ,pyraziamide test and growth in the presence of p-Nitrobenzoic acid Although molecular detection method show high sensitivity and specificity they are expensive ,time consuming,require sophisticated laboratory infrastructure and highly trained personnel. A new approach for rapid,safe and reproducidle identification of MTB infection is real-time polymerase chain reaction (RT-PCR) .The success of final amplication and Detection of Nucleic Acid Ampplication Test (NAAT) which depends on successful DNA extraction from pulmonary sputum sample. Which become activated and initate a cell mediated response. Such activated maphages become enlarged and differentiated into what are known as epitheliod cells populated with many other cell types such as neutrophils, dendritic cells,natural killer cells and fibroblasts. It is generally believed that main purpose of granuloma is to wall off the bacteria in the host resulting in containmaent or cure 90% of individual. However this view has recently been revised and the granuloma is now thought to have a role in the dissemination of infection .Considering that the granuloma is mainly a protective structure in host , and that the CD4+ lymphocytes plays an important role in the foration of granuloma.

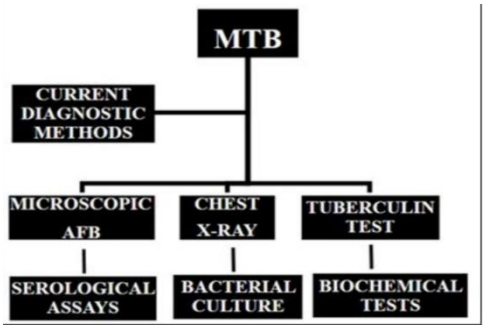
MATERIAL AND METHODS.

The study was conducted from 31 March 2022 to 1 January 2023 in the Swastik diagnostic laboratory and Depatment of Molecular Biology .

Methods :

All the traditional method are less sensitive and require more sample volume. According to WHO, serological blood test are not recommended due to false positive and false negative percentages of MTB detection tests. Culture method are still considered gold standard but they take 3 to 4 weeks to identify TB. The various diagnostic methods for MTB detection are ;

1. MTB
2. MICROSCOPIC AFB
3. CHEST X-RAYS
4. TUBERCULIN TEST
5. SEROLOGICAL ASSAYS
6. BACTERIAL CULTURE
7. BIOCHEMICAL TEST



Schematic representation of MTB detection by Conventional method

Sputum sample collection :-

The specimens were collected in a clean and sterile containers with air tightfitted 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 . The two disposable plastic airtight sterile containers were supplied to patients.

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 200 hundred pulmonary sputum samples were taken from patients who registered in Swastik Diagnostic Laboratory Jammu for MTB test.

All pulmonary sputum samples 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 200 hundred samples collected and the highest number of sputum samples are involved in this study.

EXPERIMENTAL

Materials and methods :

From 31 MARCH 2022 TO APRIL2023 we received 200 pulmonary sputum samples from the department of Microbiology Department of Swastik Diagnostic Laboratory . Of these 100 hundred twenty patients had confirmed pulmonary tuberculosis. We reviewed chest X-ray for the patients who were acid fast smear positive. 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)

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 .

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 . The tuberculosis signs and symptoms is listed in table No.1

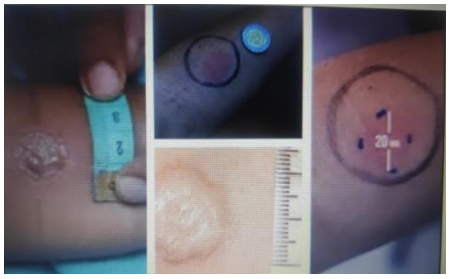


Table No.1 Sign and Symptoms of Pulmonary and extra pulmonary TB

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
Chest Pain	Weight loss
Night sweats	Larynx TB
Weight loss	Night sweats

Direct sputum microscopy

Acid fast sputum microscopy was developrd more than 100 year ago. This technique require the examination of fresh 2-4 ml pulmonary sputum samples. The thick smear is prepared and stained by Ziehl and nelson method (ZN) . This method require trained medical technician for observation of acid fast bacilli under microscopic examination. The presence of red color rod shape bacteria results acid fast positive sputum.

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 .

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.

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 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 . 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 . 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.

RESULTS & OBSERVATION

AFB STAINING RESULT

AFB – microscopy is indicated for suspected cases of tuberculsosis
Positive microscopy confirms the presence of acid- fast bacilli.
Acid fast bacteria will be red after staining while non acid fast bacteria will stain blue/green with counterstain .
smear positive: at least one positive sputum smear
smear negative: three or more negative smear
smear intermediate: less than three ssputum smear collected and of those available , all were negstive
Microscopic observation of acid fast baciili were seen under a 40x objective .The rod shaped

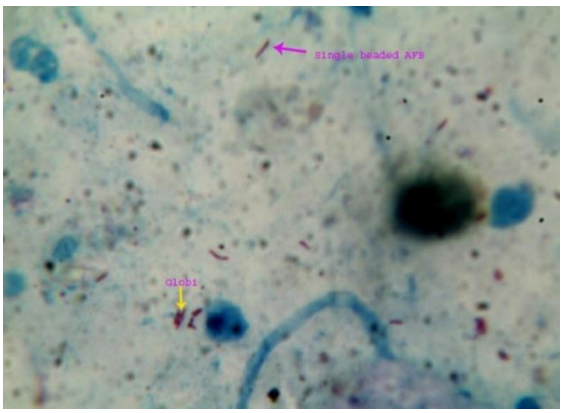


Figure 14 : showing AFB globi pattern or single beaded

However , this technique required minimum 10x103 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.

. **TUBERCULIN SKIN TEST RESULT** :-

The test is positive if there is a bump of a certain size where the fluid were injected.

Total 19 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.

Table :- T.B Chest X-Ray , Acid Fast Staining

S.NO	AGE	GENDER	SAMPLE	PASTTB	FAMILY H/O	XRAY	AFB-POS-GRADE	TABACCO
1	45	M	SPUTAM	N	N	Y	+	N
2	38	M	SPUTAM	N	N	Y	+	N
3	66	F	SPUTAM	N	N	Y	+	N
4	26	M	SPUTAM	N	N	Y	3+	N
5	30	F	SPUTAM	N	N	Y	3+	N
6	37	M	SPUTAM	N	N	Y	2+	N
7	60	M	SPUTAM	N	N	Y	3+	Y
8	17	F	SPUTAM	N	N	Y	2+	N
9	35	F	SPUTAM	N	N	Y	3+	N
10	51	M	SPUTAM	N	N	Y	3+	Y
11	71	M	SPUTAM	N	N	Y	3+	Y
12	20	M	SPUTAM	N	N	Y	3+	N

13	22	M	SPUTAM	N	N	Y	1+	N
14	40	M	SPUTAM	N	N	Y	3+	N
15	15	F	SPUTAM	N	N	Y	2+	N
16	47	F	SPUTAM	N	N	Y	1+	N
17	62	F	SPUTAM	N	N	Y	1+	N
18	23	F	SPUTAM	N	N	Y	1+	N
19	40	M	SPUTAM	N	N	Y	3+	Y

3 Results of RTPCR FOR MTB

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.

* **Pre-treatment** The figure below depicts MTB positive patient. Blue line shows Positive value of Patient.i.e 23.33.This blue line shows positivity rate in a particular patient.

IC internal Control value i.e., 28.0,

Green lines shows CT value which is ND (Not Detected)

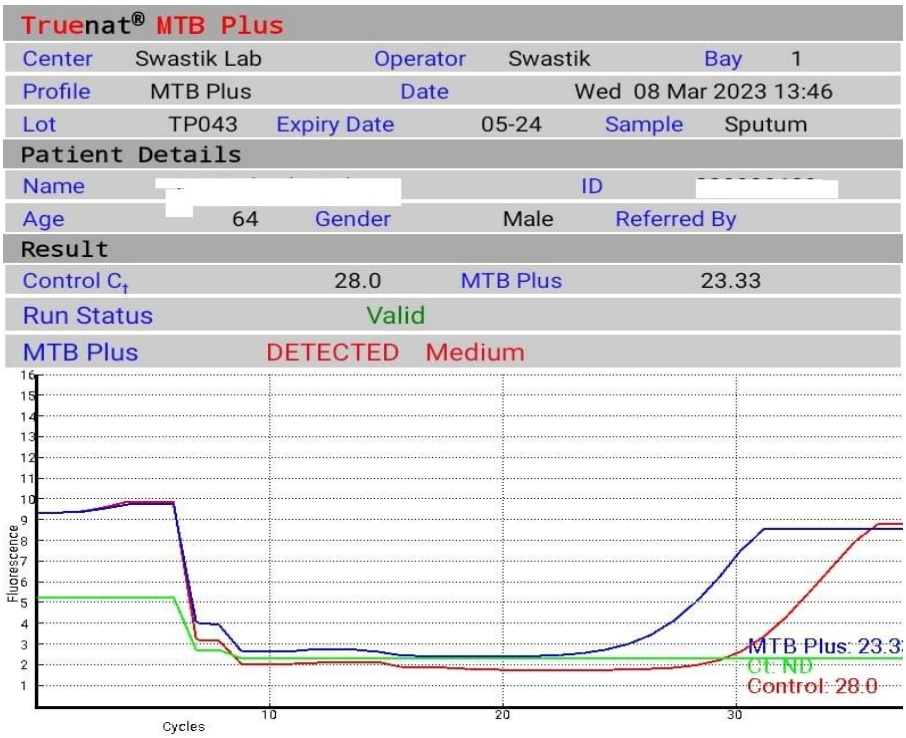


Figure: MTB PCR positive for patient

2. The figure below depicts MTB negative patient .Bue line shows positivity rate in a particular patient which is negative in the figure below. Red lines show IC Internal controlvalue i.e., 29.17 Green line shows CT value which is ND (NOT DETECTED).

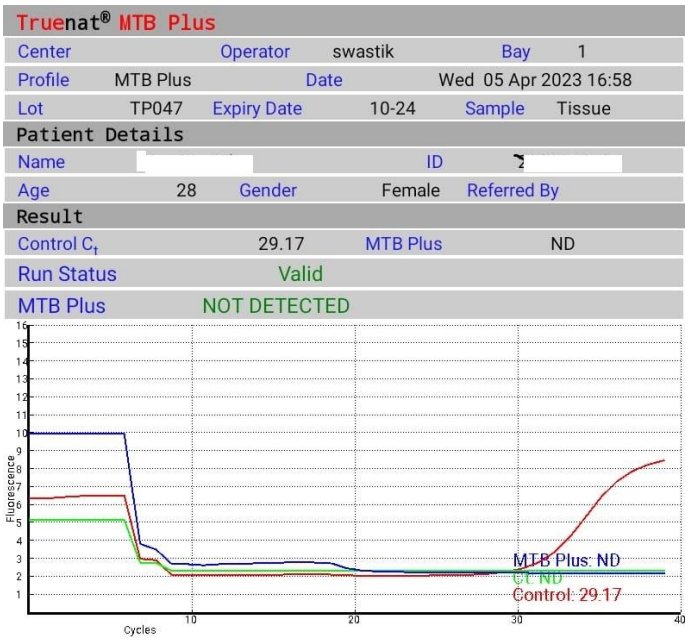


Fig : MTB PCR negative for patient

DISCUSSION. Nanotechnolgy has greatly contributed to majaor advance in computing and electronics, leading to faster , smaller,and more portable systems that can manage and store larger amount of information.

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.

The sample is positive with MTB detected of 1.3 x 1003 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.,one is positive (DETECTED) and other is not detected .

The Ct values for 100 patients obtained from results of RT-PCR , Of these 100 hundred twenty patients had confirmed pulmonary tuberculosis. We reviewed chest X-ray for the patients who were acid fast smear positive. 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)

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

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