

Diagnosis and Management of Tuberculosis

Chapter 5

Tuberculosis and its Diagnosis: A Review

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1. Introduction

Mycobacterium genus covers more than 140 species, these species are divided into three major sets, that is, *M.tuberculosis complex* (MTBC), *M.leprae*, and mycobacteria other than tuberculosis (MOTT) [1]. The MTBC comprises of five species including *M.tuberculosis*, *M.bovis*, *M.africanum*, *M.canetti*, and *M.microti* that causing tuberculosis (TB) [2]. *M.tuberculosis*, is the primary cause of TB and it is the most prominent members of the MTBC. It is an obligate human pathogen and are the leading causes of death all over the globe [3]. The size of the *Mycobacterium* is less than 5 μ and the generation time of this bacterium is 18-24 hours [4]. They are obligate aerobes. It resists decolonization dyes like Sulphuric acid and acid alcohol because its cell wall contains mycolic acid. Therefore, this bacillus is also termed as “Acid fast bacilli” [5].

More than one third population of the world's has been infected with TB caused by MTBC, arising new infections at the speed of one per second. Annually, *M.tuberculosis* infected around 2 billion people all over the world. More than 9-10 million develop active TB disease and almost 2-3 million died because of this miserable disease [6]. Pakistan ranks fifth among high burden countries (HBC) and ranks 4th regarding the estimated number of multidrug resistance tuberculosis (MDR-TB) cases worldwide. The estimated prevalence is 341 per 100,000 populations and incidence rate is 270 per 100,000 populations respectively. The mortality rate of TB is 26 per 100,000 populations [7].

M.tuberculosis transmitted from one person to another through the air. Therefore, it is very highly transmissible disease [8]. TB cases occur predominantly in the economically most productive age group between 15 to 49 years [9]. As TB is very infectious especially in terms of pulmonary TB, therefore quick and exact diagnosis is very significant component of TB treatment and control.

2. Ziehl nelson (ZN) Staining

Conventional methods available for diagnosis of TB and the most important and easily available test is Ziehl nelson (ZN) staining for sputum [10].

2.1. Principle

This test is based on the property of acid-fastness. Mycolic acid causing acid fastness is the basic component present in the MTBC cell wall. The basic dye carbol fuchsin binds with the mycolic acid and this dye did not washed off with decolourizer i.e. strong acid or acid alcohol. Counter stain used are methylene blue which produce contrast in background.

2.2. Reagents

1% Carbol fuchsin solution, 25% H_2SO_4 solution, 0.1% methylene blue solution

2.3. Methodology

Smear was prepared by means of wire loop from the samples and leave in the open air to be air dried. Smear was heat fixed and then placed for staining on staining rack. Filtered Carbol fuchsin was put on the prepared slide for atleast 5 minutes. Slide was heated underneath with flame until steam appeared. Remove the stain from the slide by tilting by means of forceps. Clean tap water was used for washing the slide. Acid solution was added to the slide for 3 minutes. Rinsed with clean tap water again. In the last step, methylene blue solution was added for 1 minute. Clean tap water was used for washing and wait for drying which is then ready for microscopic examination and appears as pinkish colour rod in a blue background (Fig.1).

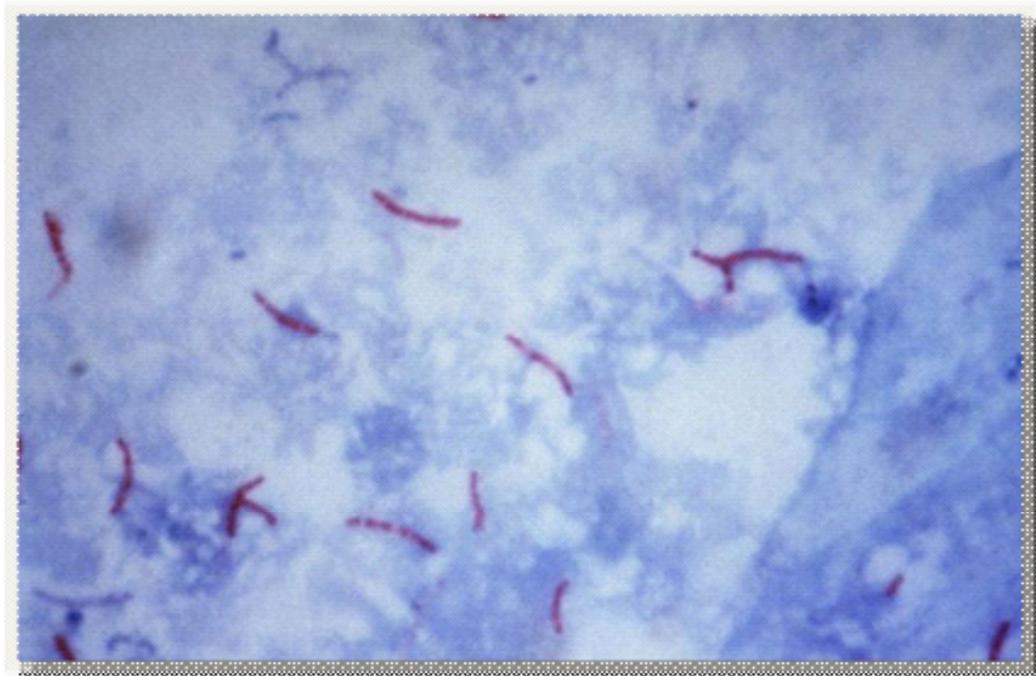


Figure 1: AFB showing in ZN staining

2.4. Reporting

Recording	Results
No AFB seen per 100 fields	Negative
1-9 AFB seen per 100 fields	exact figure reported
10–99 AFB seen per 100 fields	1+
1–10 AFB seen per field (count only 50 fields)	2+
7 10 AFB seen per field (count only 20 field)	3+

- Positive results was indicated by red ink and result must provide to the concerned on priority basis.
- “AFB not seen” used for a negative report while the result were not reported as “No TB” in case of negative result.

2.5. Advantages

The advantages of sputum smear microscopy are Quick, Robust, low-cost, at the door step of the majority of patients and require low level infrastructure.

2.6. Disadvantages

The disadvantages of sputum smear microscopy are need large number of bacilli is required i.e. low sensitivity up to 10^4 – 10^5 bacilli/ml, detects both dead and viable bacilli, does not distinguish tubercle bacilli from other mycobacteria, cannot distinguish between species of mycobacterium and no indication of drug susceptibility testing.

3. Auramine Staining

Auramine staining is another staining technique used examined by fluorescence microscopy [11].

3.1. Principle

Auramine staining technique is used for detection of AFB by microscopy. Fluorescence microscopy is used when the slide was stained with the auramine staining technique. In this staining, auramine which is the primary stain binds with mycolic acids of the cell wall. Strong acids, alcohol were used as decolourization as it does not release primary stain from the mycobacterial cell wall and bacilli seen bright yellow colour. Potassium permanganate was used as a contrast but blue ink was preferred for counter staining.

3.2. Reagents and methodology

The slide was flooded with auramine (0.1%) for 15 minutes and then washes with clean

tape water. Decolourizing solution i.e. acid-alcohol (0.5%) was flooded on the slide for 3 minutes and again washed with tape water. Slide was flooded with 0.5% potassium permanganate or 10% blue ink (Counter staining) for 1 minutes and rinse with tape water.

The fluorescence stained slide was kept in a box or folder in the dark because fluorescence fades quickly when these slides exposed to light. Therefore read as soon as possible.

The fluorescent lamp of the microscope was switched on for 5 minutes. The other ordinary lamp was off. Then rotate the nose piece so that the 20X objective of the microscope is in the light path. Checking for the strong blue light; if not, open the fluorescent light beam diaphragm. First check the positive control slide and then the test slide under the objective of the microscope. Always 40 X objectives were used for confirmation of bacilli (Fig.2).

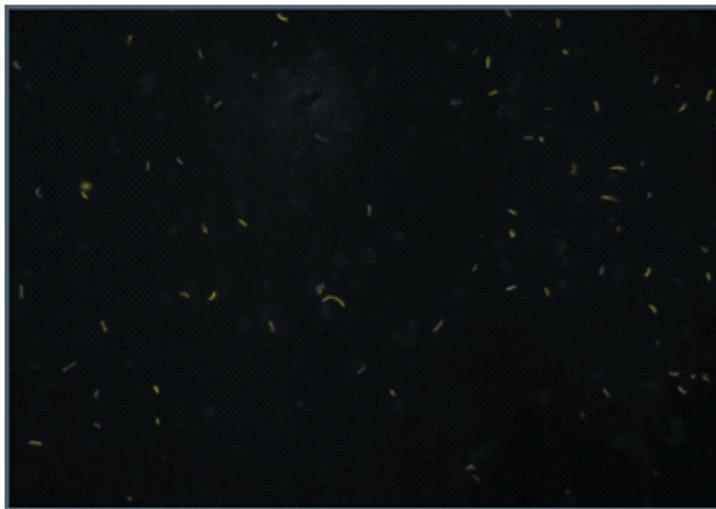


Figure 2: AFB showing in Auramine O fluorescent staining

3.3. Reporting comparison between ZN staining and Auramine staining

ZN staining	Auramine O fluorescent staining	Reporting /Grading
>10 AFB per field seen in 20 fields	>100 AFB per field seen in 20 fields	Positive, 3+
1-10 AFB per field seen in 50 fields	11-100 AFB per field seen in 50 fields	Positive, 2+
10-99 AFB seen per 100 field	1-10 AFB seen in 100 fields	Positive, 1+
1-9 AFB seen per 100 field	1-3 AFB seen in 100 fields	doubtful positive /repeat
No AFB seen in 100 fields	No AFB seen in 100 fields	Negative

3.4. Advantages

Fluorescence microscopy is better and more rapid than that of the ZN staining microscopy and it is very useful for huge work load setting. It is also more delicate for paucibacillary specimens, as it requires less effort for examination of maximum fields.

3.5. Disadvantages

However, a stable power supply is required, greater expertise of the lab technician in reading and even microscope adjustment. Moreover, a regular supply of the bulbs is also a factor in maintaining this microscopy which is so costly and short lived. Cheaper microscope using halogen lamps have less inflexible requirements.

4. Lowenstein Jenson (LJ) Media

Lowenstein Jenson (LJ) media is use for the culturing of *M.tuberculosis*. There are three main ingredients in the preparation of LJ media i.e. Salt solutions, malachite green and egg homogenate. For the production if the solid surface of the media salt solutions and egg homogenate were mixed. Malachite green was added which would help to prevent the growth of contaminants. **Stone brink media** used for the growth of *M.bovis* have the same composition as LJ medium, only pyruvate is added instead of glycerol. Pyruvate enhanced the growth of *M.bovis*.

4.1. Preparation

Reagents used in the preparation of LJ and stone brink media are salt solution, 2% malachite green solution, Hen's egg.

4.2. Methodology

Cleaned the work area with disinfectant and washed the eggs. The eggs were dipped in 70% ethanol for 15 minutes and leave them to dry. Every egg was broken separately into a sterile flask to check its quality. The eggs were transferred into another sterile beaker and mixed well by means of a stirrer. To obtain the desired volume sufficient eggs were added. Then salt solution and malachite green solution were added. Mixed gently all three solutions to avoid bubbles and attain homogeneity. Filtered through sterile cotton gauze and transferred 6 to 8 ml volumes into sterile McCartney bottles. Kept all the tubes in the inspissator at a position to form the proper slopes and coagulated at 80°C for 45 minutes. After inspissation, remove the tubes and then allowed to cool which was ready for use.

4.3. Culture

M.tuberculosis identification through culture techniques is presently the benchmark for diagnosis [12]. Culture examination is better as compared with microscopy because upto 20-50% chances of bacilli increases. Culture can detect up to 10 bacilli per ml of the sample. The time required in culture diagnosis and with the increase rate of negative results in paucibacillary specimens are the main restrictions. There are several methods for attaining early growth of *M.tuberculosis* have been developed during the last two decades [13]. The most important

method of these is decontamination of the specimens using N-acetyl L-cysteine-Sodium hydroxide (NALC–NaOH) method [14-15].

4.4. Reagents

The reagents used in the NALC–NaOH method for processing the sputum samples are the following.

4% NaOH solution, 2.9% trisodium citrate solution, NALC powder. NALC was prepared on daily basis. Took 0.25 g of NALC powder and add it in 50 ml Tri-sodium–NaOH solution.

While for the preparation of Phosphate buffer having pH 6.8, took Na_2HPO_4 and KH_2PO_4 in a combination that the final pH of the buffer was 6.8.

4.5. Methodology

NALC–NaOH concentration method is the best culture process method for sputum samples decontaminated [14-15]. Sodium hydroxide acts as a decontaminating agent while NALC function as a mucolytic agent. Heavy metal ions attached by sodium citrate which may be present in the samples because they attached to NALC and NALC was non-functional by the presence of these ions. About 5 ml samples but not less than 2ml along with equal volume of the NALC–NaOH solution was transferred to the falcon tube and tightened the cap of the tube. Vortexed for 20 seconds and incubate for 15 minutes for the sample to decontaminate at room temperature. After incubation, filled the tube with phosphate buffer upto 50ml and then vortexed. Centrifuged the tubes at 3000 rpm for 15 minutes. Remove the supernatant carefully from the tube by means of a funnel into a discordant which containing 5% phenol. The sediment was resuspended by adding 0.3ml of phosphate buffer.

The sediment was inoculated on two slopes of Lowenstein Jenson (LJ) media and also with liquid media 7H9 broth using MGIT (Mycobacteria growth indicator tube) system.

The LJ media was transferred to incubator and wait for up to eight weeks at 37°C for the appearance of any visible colonies i.e. typically rough, tough and buff in colour by continuously reading for atleast one time every week (Fig.3). While in the MGIT system if the tube is contaminated, negative, positive then it detect automatically (Fig.4). The contaminated cultures in either media were discarded in the first week of incubation. In case of solid culture, if there was no growth observed after 8 weeks incubation declared as negative and after 6 weeks incubation in case of MGIT system.

4.6. Advantages

Culture detects up to 10 bacilli per ml of the sample depending on the technique used, upto 30-50% case detection rate improves over microscopy, definite and excellent diagnosis of extra pulmonary tuberculosis, MTBC species detection, allows drug susceptibility testing, allows drug resistance surveys and epidemiological studies and confirms TB in HIV+ patients.



Figure 3: Colonies of *Mycobacterium tuberculosis* on LJ media are typically rough, tough and buff in colour.

4.7. Disadvantages

Slow growth of *M. tuberculosis*, high cost, delays results, more sensitive to technical deficiencies, greater need for infrastructure i.e. qualified staff, equipment.

5. Drug Susceptibility Testing

5.1. Principle

Drug susceptibility testing using the proportion method [16] for *M. tuberculosis* along with the members of MTBC i.e. *M.africanum* and *M.bovis* against anti-TB drugs on LJ medium is also very important method. This technique is a standard method against which other methods judged. In this technique, high concentrations of viable infectious bacilli are under test, the minimum quantity of which is 10⁹ bacilli/ml so great expertise and personal protection is very important and mandatory. Therefore, this method must be carried out only in bio safety level 3 labs which is strongly recommended by WHO and it is only access to authorized personal with maximum restriction.

As the name indicates, this method measures the proportion of resistant bacilli present in a sample which is totally different from the concept of resistance in routine clinical microbiology. The strain should be declared as susceptible when it is below a certain proportion and resistant when above that specific proportion. This proportion of the drug is known as critical proportion. The lowermost concentration of the anti TB drug indicates resistance of clinical

importance in the medium at which the growth of acid fast bacilli appears is termed as critical proportion. Growth control is also used as a control to inhibit unwanted growth on the culture medium but it is without test drug to check it with medium containing the critical concentration of the drug.

Scraped and pick up portions from all colonies of the culture medium with a sterilized loop.

5.2. Methodology

Transferred all the colonies to a screw capped glass tube containing glass beads having diameter of 3mm approximately. Gently shake the tube. 2 drops of sterile saline or distilled water was added and vortex. Wait for 15-30 minutes so that larger aggregates of bacteria were settled down. Aseptically transferred the supernatant to another tube and turbidity must match with McFarland turbidity standard No.1. If it is too turbid then sterile distilled water was added. If it is insufficiently turbid then do not add more cells because mycobacterial cells are very hard to homogenize. Let the suspension to settle down and discard some of the upper portion to concentrate cells. The turbidity of the suspension was adjusted by adding distilled water.

A strain is declared as resistant when the drug containing medium contains colonies more than that of growth control with the 1% in oculum and it is sensitive when the colonies are less than that of growth control. A strain of *M.tuberculosis* H₃₇Rv is used as control. H₃₇Rv is the strain which is sensitive to all first line drug of TB.

6. Nitrate Reduction Assay

Nitrate Reduction Assay is a type of biochemical test used in the biochemical identification of MTBC species. The nitrate reduction test is also used for the Drug Susceptibility Testing of MTBC. It is based on the colour change in which nitrate is converted to nitrite result in the production of colour change. The presence of this colour change can be detected by means of specific reagents. Nitrate reduction assay uses this colour change actually produce by nitrite as indication of growth in the drug susceptibility test [17].

6.1. Principle

M.tuberculosis is the strong nitrate reduction producer and reduce nitrate to nitrite measured by colorimetric method. *M.bovis* showed a negative test or a weak producer. Isolated colonies and pure cultures must be used for this test to avoid false positive test [18].

6.2. Requirements

Sodium nitrate substrates in buffer having pH 7.0, hydrochloric acid solution, 0.2%, sulfanilamide solution, 0.1% N-naphthylethylene diamine solution are the reagents used in the

nitrate reduction test.

6.3. Methodology

Took 0.2ml sterile distilled water in a tube and add pure colonies from LJ media and stone brink media to the tube. Then 2ml NaNO_3 solution was added and incubated at 37°C in water bath for 2 hours. After incubation, one drop of hydrochloric acid solution, 2 drops of 0.2% sulfanilamide solution and two drops of (0.1%) N-naphthylethylene diamine solution was added to the tubes. The change in colour from pink to red was noted as nitrite producer.

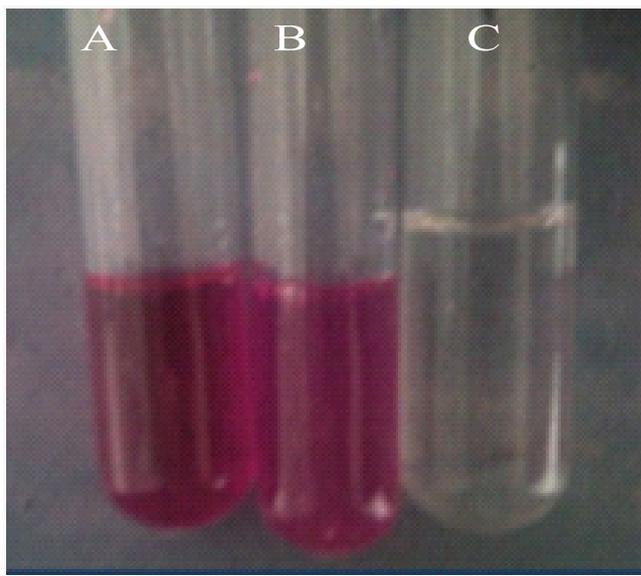


Figure 8: Nitrate reduction by *M.tuberculosis* (A) Positive, (B) control and (C) Negative.

7. Niacin Test

7.1. Principle

Niacin test is the biochemical test that identifies the accumulation of niacin in culture media and is used as for the detection of *M.tuberculosis*.

Nicotinic acid present in the niacin was detected in the niacin test and it is one of the important factors in the identification of *M.tuberculosis* in the culture media because almost all the strains of *M.tuberculosis* excrete a large amount of niacin (nicotinic acid). Niacin test must be only carried out on pure culture of *M.tuberculosis* otherwise it produces false results. The best results obtained from 22 to 28 days old culture on LJ media having more than 50 colonies. This test must be processed in a BSC as dealing with the pure cultures.

7.2. Methodology

Took colonies from the culture and transfer to a sterile tube. 1 ml of sterile distilled water was added to the culture and tightens the caps of the tube. The tubes were placed horizontally so the fluid covers the entire surface of the medium with the fluid. Place the tube on

the room temperature for 30 minutes. Upright the slants and wait for 5 minutes so the fluid was drain to the bottom. 0.5 ml of fluid was transferred to the clean screw cap tube. The strip was inserted to the fluid having the identification mark at the top. The tube was closed immediately. Wait for 15 to 20 minutes at room temperature and agitating it occasionally. Observe for yellow colouration of the liquid in the bottom of the tube indicate a positive test against a white background.

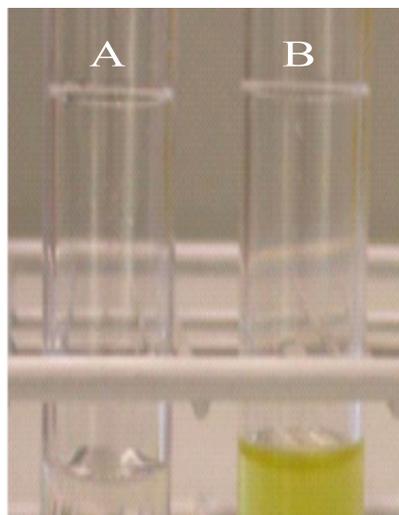


Figure 9: Niacin test by *M.tuberculosis* (A) Negative and (B) Positive.

8. Catalase Test

8.1. Principle

Catalase test is useful in the identification of *M.tuberculosis* because it detects the catalase activity from their colonies. This test was performed only in the Bio safety level 3 lab as recommended by the WHO because live and infectious bacteria are in used. The heat stability of the catalase activity was measured at 68 °C and it is one of the key factors in the identification of *M.tuberculosis*. Pure cultures of the tubercle bacilli having minimum of 14 days old was used, otherwise it produces false results.

Catalase breaks down hydrogen peroxide resulting in the production of water and oxygen. This oxygen was appearing as in the form of oxygen bubbles in the liquid suspension. These bubbles indicate the presence of all mycobacteria except isoniazid resistant bacilli and were reported as positive test.

8.2. Requirements

Phosphate buffer (pH 7.0), containing two reagents i.e. Reagent 1 consists of KH_2PO_4 (9.07 g) dissolve in 1000 ml distilled water and Reagent 2 consists of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (23.68 g) dissolve in 1000 ml distilled water. 38.9 ml of reagent 1 was mixed with 61.1 ml of reagent 2 and check pH with a pH meter. Hydrogen peroxide (H_2O_2), Tween 80 solution, 10 ml Tween 80 was mixed with 90 ml distilled water and autoclave at 121°C for 10 minutes. It is used for

up to 3 months when store in the refrigerator. 30% hydrogen peroxide and 10% Tween 80 was mixed in equal volume. This suspension is called as substrate. It is very important to prepare a freshly prepared substrate to reduce the chances of false result.

8.3. Methodology

0.5 ml of phosphate buffer was dispensed into screw cap test tubes. Scraped several bacterial colonies from LJ media and emulsify in the buffer. Place one tube was placed in the water bath at 68 °C for 20 minutes and the other tube at room temperature. The heated tube was allowed to cool to room temperature. Then 0.5 ml of freshly prepared Tween 80 peroxide substrate was added to each tube and loose the cap of the tube. Note the bubbles formation. Care must be taken that don't shake the tube because tween 80 alone forms bubbles on shaking producing false positive results.

8.4. Results

If bubbles appear in the unheated tube and not in the heated tube, the result was interpreted as test strain belongs to the MTBC.

If bubbles appear in both tubes i.e. heated and unheated then the result was reported as this strain produces heat stable catalase and is unlikely to be a member of the MTBC.

If no bubbles appear in both tubes, then the test is reported as the test strain is catalase negative.

M.terra which is a saprophytic strain used as negative control.



Figure 10: Catalase test

9. Mycobacteria Growth Indicator Tube (MGIT)

MGIT is the fully automatic system for detection of *M.tuberculosis* and drug susceptibility testing of MTBC. MGIT System was compared with LJ media for detection and isolation of mycobacteria by many researchers [19-20]. Similarly, drug susceptibility testing by MGIT has been thoroughly studied [19].

9.1. Principle

It consists of liquid medium embedded with oxygen quenched fluorescent material containing silicone at the bottom of the tube. The free O₂ in the tube is utilized during bacterial growth and is replaced by CO₂. With exhaustion of O₂, the fluorochrome is no longer inhibited resulting in fluorescence at the bottom of the tube that could be detected by MGIT instrument. The intensity of fluorescence is directly proportional to the extent of O₂ reduction and rate of bacterial growth.

9.2. Methodology

This method has been developed by Becton and Dickinson. The growth is detected in the tube by a non-radioactive detection system using fluorochrome [21]. MGIT system produce improved yield and much faster growth of mycobacteria as compared to LJ media by using liquid broth medium also known as modified Middle brook 7H9 broth base. The volume of this medium is sterilized terminally by autoclaving. This media is ready for the growth of mycobacteria after adding MGIT OADC (Oleic acid, Albumin, Dextrose and Catalase) or MGIT 960 Growth Supplement. This growth supplement is vital component for growth of almost all mycobacteria, especially MTBC. Contamination is suppressed by the addition of the MGIT PANTA. PANTA consists of Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim and Azlocillin.

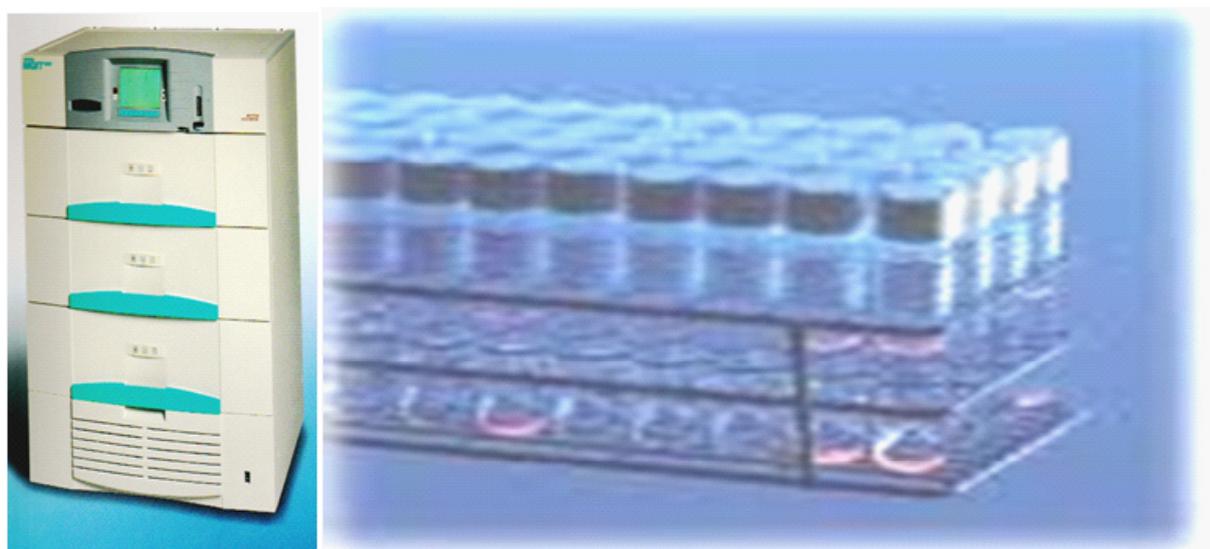


Figure 4: MGIT 960 Automated System with fluorescence seen in the positive tubes

10. Drug Susceptibility Testing on MGIT

10.1. Principle

Same principle is applied for the drug susceptibility testing on MGIT system. The test culture that required drug susceptibility testing is inoculated into two MGIT tubes. To one MGIT tube added a specific amount of test drug and compared it with other tube having no drug. Fluorescence in the MGIT tube is suppressed in case when the test drug is active against the mycobacteria and it inhibits the growth. While the fluorescence increased after the growth control will grow uninhibited. This growth is then monitored by the MGIT instrument as susceptible or resistant.

10.2. Methodology

The contaminated or mixed cultures may be concluded as positive by using liquid media. When the modified Middlebrook 7H9 broth base was declared positive by the instrument then a smear is prepared from the bottom of the tube containing the culture. Blood agar and chocolate agar plate can be used for the purity of a positive liquid culture and for the identification of possible mixed mycobacterial cultures as these are difficult to detect in the liquid media.

10.3. Advantage

MGIT increases the case detection by 10% over solid media are the main advantage of this technique.

10.4. Disadvantage

The limitation is more prone to contamination of this technology.

11. Microscopic Observation Drug Susceptibility Assay (MODS)

11.1. Principle

The decontaminated processed sputum specimens inoculated in Middlebrook 7H9 medium were introduced in 24 well plates for the presence of microcolonies by an inverted light microscope as this is much earlier than visible colonies appearance on the solid media. The isoniazid and rifampicin were also detected for the rapid identification of multidrug resistant (MDR-TB) using the same 24 well plates.

MODS detect and identify *M.tuberculosis* along with isoniazid and rifampicin susceptibility by liquid culture directly from the samples [22].

There are two main advantages of this techniques regarding growth of *M.tuberculosis*:

(1) Liquid media produces faster growth of *M. tuberculosis* as compared to solid LJ media.

(2) *M.tuberculosis* typical cording characteristic appearance in liquid media.

11.2. Reagents

The reagents used in the process are the decontamination reagents for the sputum process are PBS (PH 6.8), NaOH (4%) and Na-Citrate (2.9%), NALC (0.5%) which is NALC-NaOH solution.

Middlebrook 7H9 broth base containing Glycerol and Casitone (4.5 ml aliquotes in glass tubes), OADC; supplement (oleic acid, albumin, dextrose, catalase) commercially prepared, PANTA which is Lyophilized antibiotic supplement i.e. Polymyxin-B, Amphotericin-B, Nalidixic acid, Trimethoprim, Azlocillin) and antibiotic isoniazid & Rifampicin stock solutions (8mg/ml). Aliquotes of 20ul were prepared and store at -20°C for 6 months.

11.3. Methodology

Sputum samples having volume of not less than 2ml were decontaminated using NALC-NaOH concentration method [19-20]. Sputum samples along with equal volume of the NALC-NaOH solution were added to the tube. Tighten the cap of the tube and vortexed. Leave the tube for 15 minutes at room temperature to decontaminate the samples. Phosphate buffer was added upto 50ml of the tube and again vortexed. The tubes were centrifuged at 3000 rpm for 15 minutes. The supernatant was carefully removed through a funnel into a discordant containing 5% phenol. The pellet was resuspended by adding 0.3ml of phosphate buffer.

OADC was added to 7H9 medium i.e. 7H9-OADC in a clean room or Biological Safety Cabinet (BSC). PANTA was reconstituted and add to tubes with 7H9-OADC. The tube now consists of 7H9-OADC-PANTA. The resuspended pellet was added to the 7H9-OADC-PANTA. Antibiotic working solutions was prepared in a 24 well plate. The antibiotic working solutions was added to plated samples.

The plate was closed with lids and sealed each in a ziplock bag. Placed in incubator at 37°C. Read the plates on day 5 of incubation by means of inverted microscope. Plates are examined within the transparent and sealed zip lock bags and not opened.

After 5 to 9 days of incubation, mycobacterium growths are like small curved commas shaped and spirals. The colony formation of mycobacterium usually turned to cords and then later develops to irregular tangled. The results are declared as positive, when two or more than two colonies are detected in each well i.e. ≥ 2 CFU.

11.4. Reporting

When there is no colony detected after day 5 incubation, then the drug free well are further incubated and continue reading daily or on the alternate day depending upon the workload until two or more colonies are detected (≥ 2 CFU). When the drug free well was positive then observe the growth of the wells containing isoniazid and rifampicin on the same day. While if no growth detected till day 15 then continue the incubation to 18 and atleast to day 21. At day 21, if the result is still negative the final report was reported as negative.

In case of only 1 CFU detected in drug or drug free well then report the result as “indeterminate”. The drug well should only read when the drug free well was positive and not read when the drug free well was negative or indeterminate. In case of bacterial or fungal contamination appearance in the well then the sample should be re-decontaminated, or a fresh sample was requested and the result was reported as un-interpretable.

11.5. Interpretation

The Resistance of this method is defined as when two or more than two colonies are detected in the drug containing wells on the same day that both drug-free wells are positive.

All positive cultures are detected within 21 days and $>98\%$ within two weeks thus a negative MODS culture at three weeks is a confident rule-out.

11.6. Advantage and Disadvantage

This technique is very simple, the liquid media sensitivity has great advantage over solid media for TB detection, characteristic growth of *M. tuberculosis* specificity, short duration for the detection of drug susceptibility testing and low cost of reagents are the major advantages.

MODS are recommended only for smear positive samples and not for smear negative specimens are the only limitation.

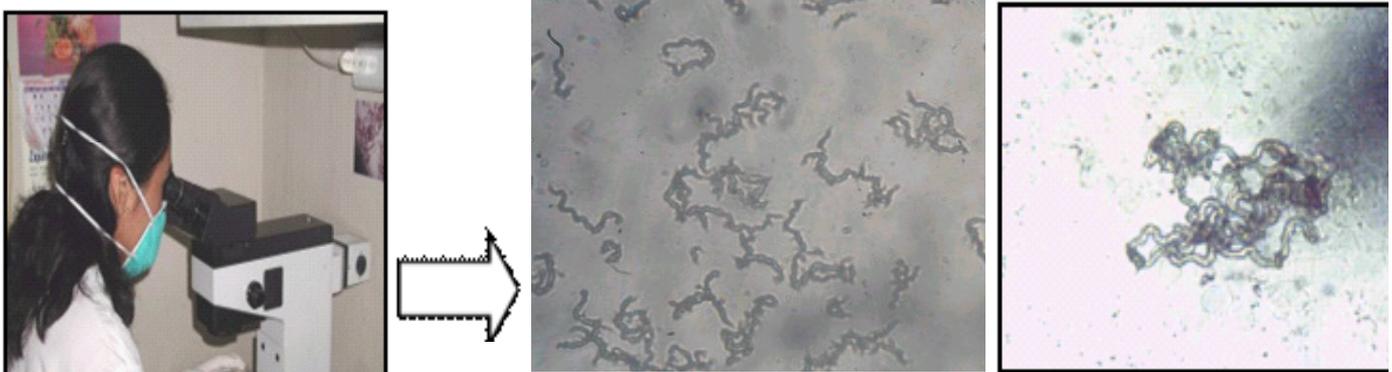


Figure 5: MODS: Inverted Microscope showing colony morphology of AFB

12. Line Probe Assays (LPA)

12.1. Principle

LPA was first of all introduced in 2004, identifies MTBC and simultaneously identifies mutations in the *rpoB* gene as well as mutations in the *katG* gene for high-level isoniazid resistance.

LPA is currently the useful and faster available technology for the rapid identification of Rifampicin and isoniazid resistance, but the only disadvantage of this technology is that it is only used in smear-positive specimens. It is technically complex procedure, extensive infrastructure required because the chances of cross-contamination. It is only suitable and available at reference laboratory [23].

12.2. Methodology

The main steps involved in the LPA are *M.tuberculosis* DNA was extracted from culture isolates or directly from smear positive specimens. Next, DNA was amplified by polymerase chain reaction (PCR) cycles of the resistance determining region of the gene using biotinylated primers was performed. After amplification was performed, a strip containing specific oligonucleotide probes was used for hybridization of the labeled the PCR products. Colorimetric method was used for the identification of these captured labeled hybrids. These labeled hybrids shows the identification of *M.tuberculosis* along with the detection of wild-type and mutation probes for isoniazid and rifampicin resistance [24].

If one of the target regions shows mutations, the amplicons will not hybridize with the relevant probe. Mutations are detected by the presence of mutation probe and lack of wild-type probes. A coloured band was detected on the strip after post hybridization reaction at the site of probe binding which is clearly observed by human eye [25]. The different types of *Mycobacterium* species identified by line probe assay (Fig. 6).

12.3. Advantage

Smear negative specimens are not recommended on the LPA. This technology is only applied to and validated in direct smear positive samples and culture isolates of *M.tuberculosis* complex which is isolated from smear negative and smear positive samples.

12.4. Disadvantage

Three rooms are required for the performance of LPA in Laboratory i.e. DNA extraction room, pre-amplification room and post-amplification room. In these rooms unidirectional flow of work, only authorized personal allowed and cleaning procedure must be established to reduce amplicons contamination which leads to false positive results.

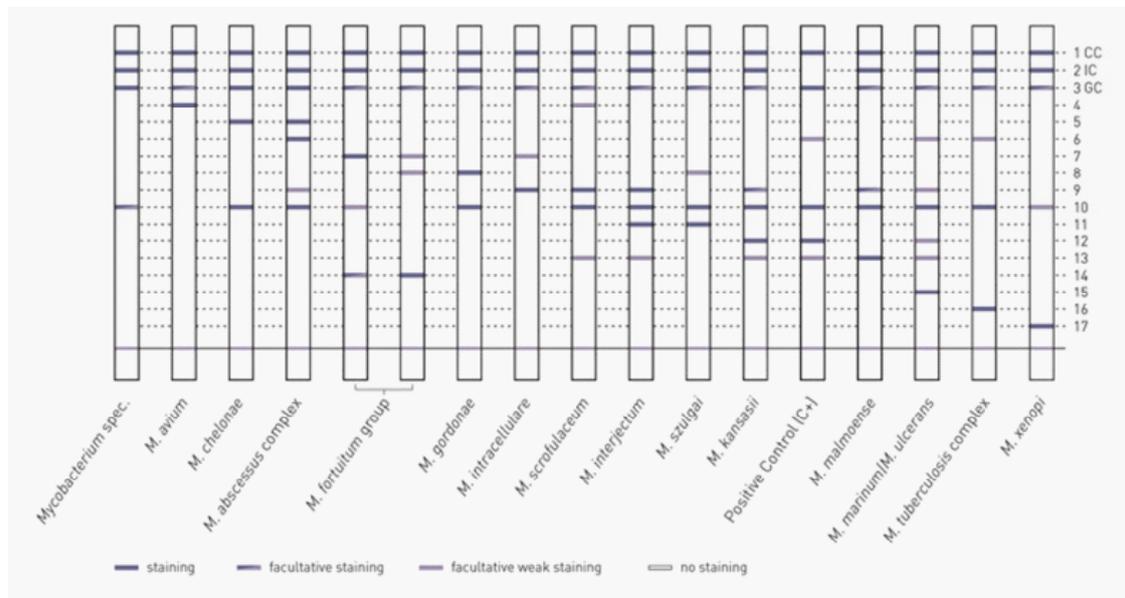


Figure 6: Line Probe Assay showing identification of different organisms

13. GeneXpert

13.1. Principle

GeneXpert was developed in 2009 and it is considered a vital revolution in the competition against TB. A simple molecular test was introduced which is robust and performed outside the laboratory settings for the first time. GeneXpert MTB/RIF assay identified not only *M.tuberculosis* but also rifampicin resistance. The resistance conferring mutations were detected by using three specific primers and five unique molecular probes to confirm a high degree of specificity.

13.2. Methodology

The MTB/RIF assay required less than 2 hours for the results performed directly from specimens. The GeneXpert System automates and integrates DNA extraction/ sample preparation, DNA amplification and detection of the target DNA by using real time polymerase Chain Reaction (PCR). The patient sample was diluted with buffer which was provided with the kits and then incubated for 15 minutes at room temperature. The sample was completely liquefied by mixing to and fro motion after every 5 minutes during incubation. The liquefied sample was transferred to cartridge and gives it to the GeneXpert system. The GeneXpert system gives both summarized and detailed test results in tabular and graphic formats in less than 2 hours.

GeneXpert MTB/RIF system consists of GeneXpert instrument, scanner with barcode reading, computer and GeneXpert software for tests running on different samples and then viewing the results (Fig.7). The test was performed on disposable single use GeneXpert cartridges which hold the PCR reagents and PCR reactions were performed. These cartridges are closed systems so the chances of cross contamination were eliminated between samples. This cartridge include two controls a sample processing control (SPC) which control the processing

of the target bacteria and also monitor the detection of inhibitor in the PCR reaction. The Probe Check Control (PCC) used for the verification of reagent rehydration, proper filling of the PCR tube in the cartridge, probe integrity and dye stability.



Figure 7: GeneXpert- MTB RIF Test showing cartridge and instruments

13.3 Advantage

GeneXpert gives very fast results because an individual can know the result within the same day. It not only diagnoses TB but also detect if the TB of the person is sensitive or resistant to rifampicin. By knowing the status about rifampicin then it is very easily define about the treatment regimen combination and TB can be treated effectively. Up to about 98% Gene Xpert is also more sensitive as compared to other TB tests so it is a very good instrument. It is also detect TB especially in AIDS patient and in people with compromised immune systems because it is often seen that the test is negative for TB even though they are sick with this disease.

13.4. Disadvantages

Gene Xpert is much more costly in comparison with sputum-smear microscopy tests. GeneXpert also needs calibration at least once a year which is also very expensive procedure. Test takes upto two hours without the interruption of electricity for a single second. A computer is also needed for this important instrument. Therefore, it is very essentials that all laboratory setups and hospitals need proper and reliable arrangement and security measures for this important instrument.

14. Future Research Needs

Additional studies to assess the performance of the different tests under program conditions should be conducted. Further research is needed regarding use of the tests for the identification of *Mycobacterium tuberculosis* in multiple clinical circumstances. Studies of test performance should assess specificity, sensitivity, reproducibility, and association of test results

with risk for infection and risk for progressing to TB disease. Comparisons among different basic, culture and biochemical tests are encouraged.

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