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Identification Techniques for Tuberculosis

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HUMAN

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ABSTRACT

World's One-third population have been infected with *M. tuberculosis*, with new infections occurs at a rate of about one per second. In 2007, there were found 13.7 million chronic active cases, while in 2010, there were found 8.8 million new patients and 1.5 million deaths related with T. B., mostly occurring in developing countries. So the Antitubercular Testing Techniques are the helpful in determining the potent drug for treatment of TB. By the Antitubercular testing Techniques the detection of the sensitivity of the antitubercular entities enables the management of tuberculosis.

INTRODUCTION

Tuberculosis, MTB are alternatives names for TB (short for tubercle bacillus) is a common infectious disease caused by various strains of mycobacteria, such as *Mycobacterium tuberculosis*.[1] Tuberculosis generally attacks the lungs, but can also affect other parts of the body.

It is spread through the air when people who have TB infection through cough, sneeze, or otherwise transmit through their saliva and air.[2] Many times such type of infections are asymptomatic and unknown, but about one in ten unknown infections progresses to active disease, if which is not treated at the specific time , which kills more than 50% of those so infected.

Many of the *mycobacteria* are resistance to the tuberculosis treatment so need of the Antitubercular Testing Techniques is required.

Aim of work:-

- The main objective of this topic is to study the different Antitubercular Testing Techniques.
- Understand the importance Antitubercular Testing Techniques.
- The various techniques to evaluate the Antituberculosis agent.

Drug Resistance:-

Resistance occurs when a person becomes infected with repeated strains of TB. A person who is fully susceptible to TB may develop secondary (acquired) resistance during therapy. Secondary resistance occurs due to incomplete or proper treatment, and non-compliance of patients or using low-quality medication [3] Drug-resistant of TB is a serious public health issue in many developing countries. When drug resistance occurs treatment is longer and requires more expensive drugs. MDR-TB means a resistance to the two most effective first-line TB drugs i.e. rifampicin and isoniazid. drug-resistant TB is also occurrs due to three or more of the six classes of second-line drugs.[4] Drug-resistant TB, which was firstly observed in 2003 in Italy.[5] so that the Antitubercular testing techniques are required.

Advances in diagnosis of drug Resistance [6]

1) Genotypic method	2) Phenotypic method
- DNA sequencing	- Phage-based assay
- Solid-phase hybridization Techniques	- Calorimetric method
- Microassays	- The nitrate reductase assay
- Real-time PCR technique	

Genotypic method:-

Searching for genetic determinants of resistance rather than resistance phenotypes.

Two basic steps:

Molecular Nucleic Acid Amplification Assay (NAA) eg.PCR to amplify sections of the M.Tb. Genome known to be altered in resistant strains.

a) Assessing the amplified products for specific mutations correlating with resistance.

Advantages:-

1. Shorter turnaround time.
2. No need for growth of the organism.
3. Less Biohazard risk.
4. Feasibility for automation.
5. Possibility for direct application to clinical specimens.

Common loci for Resistance [6]:-

Sr.No.	Drug	Mutant gene	Frequency
1.	Rifampicin	Rpob	96 %
2.	INH	KatG /inhA	75-80 %
3.	Streptomycin	Rpsi	65 -75 %
4.	Pyrazinamide	pnCA	~70 %
5.	Ethambutol	embB	~70 %

Conventional method of antitubercular testing techniques:-

The conventional method of the antitubercular testing method is done by the using conventional media used for antimycobacterial susceptibility testing. The first method, known as the Method of Proportion, uses Middle brook and Cohn 7H10 Agar.

1] Historically, the Method of Proportion (MOP) procedure has included susceptibility testing of *M. tuberculosis* using two concentrations of antimicrobials. The National Committee for Clinical Laboratory Standards (NCCLS) [7] continues to recommend that the MOP test procedure it includes two concentrations of the primary drugs for testing except rifampicin.

The recommended low concentrations for the MOP procedure are the critical concentrations for these drugs. The critical concentration is defined as the drug concentration that allows the interpretation of a result as either resistant or susceptible. An isolate is determined resistant if 1% or more of the test population grows in the presence of the critical concentration of the drug. The high drug concentration is used to profile the degree of resistance within the population. This result provides information to the physician to assist in determining whether a modification to the therapy regimen is necessary. [7]

2] Drug susceptibility testing in *mycobacterial* isolates is performed in agar and Lowenstein-Jensen (LJ) medium which is considered as a **'gold standard'**. The bacterial suspension was prepared by picking 2-3 loopful (3mm internal diameter) of colonies into 0.2ml sterile distilled water in a screw capped (approximately 4mg moist weight of the growth on LJ medium). The colonies on the drug-free medium expressed as a percentage. One percent or more of growth is considered as resistant to all drugs. [8]

The list of new method of Antitubercular testing techniques is given below:-

1] *In-vitro* antimycobacterial drug susceptibility testing of non-tubercular *mycobacteria* by tetrazolium microplate assay.

2] Evaluation of rapid MTT tube method for detection of drug susceptibility of *Mycobacterium tuberculosis.*

3]

A] BacT Alert 3D system.

B] BACTEC 460.

4] Nitrate reductase assay (NAA).

5] Resazurin microtiter plate assay (REMA).

6] Mycobacteriophage assay.

1] *In-vitro* antimycobacterial drug susceptibility testing of non-tubercular *mycobacteria* by tetrazolium microplate assay. [9, 10]

Principle:-

Tetrazolium microplate assay [TEMA], which is a colorimetric method. The formation of the colour due to the tetrazolium dye (TTZ).-living cell shows the colour formation and these are less susceptible to the drug.

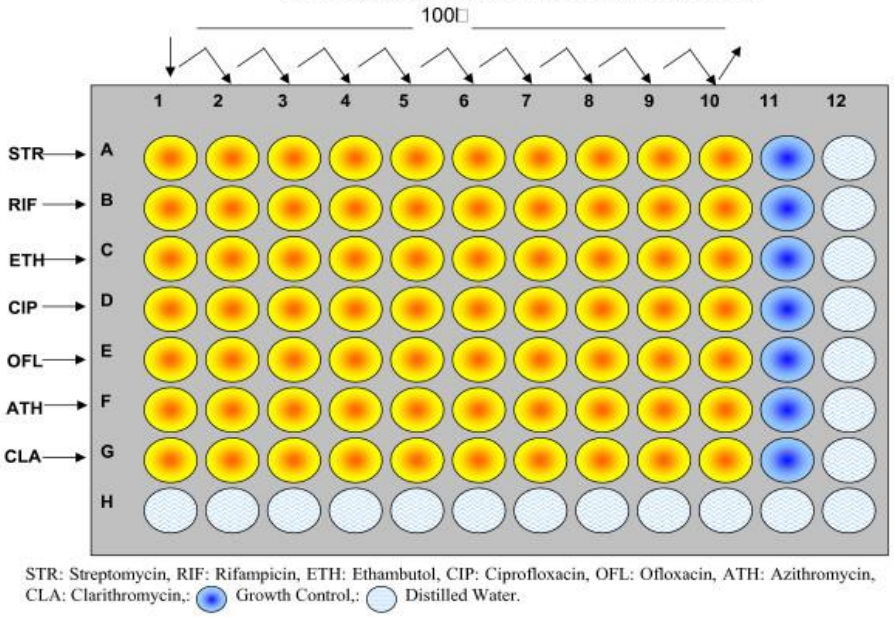
Procedure:-

- The *mycobacterial* inoculums are prepared from a log-phase culture of the Non-tubercular mycobacteria (NTM), NTMs on L-J slants and their turbidity is adjusted to McFarland Standard No.1.
- Middle brook 7H9 broth is added to columns 2 to 11 in rows A to G(labelled on microtitre plates)
- The 2x concentration of drug were added to columns 1 and 2.
- The Antibiotics were serially diluted two-fold in consecutive columns by transferring except for column 10, where excess medium discarded.
- The final drug concentrations in the well were added to wells in rows A to G in columns 1

to 11.

- The wells in column 11 served as an inoculums-growth control with no drugs.
- The plates were incubated at 37°C for 5 days.
- On day 5, 50 µl of the tetrazolium dye is added to well A11 and the plate then incubated at 37°C for 24 hrs.
- A change in colour from yellow to purple indicated growth of bacteria and the MICs was interpreted visually.

Fig 1: Diagrammatic illustration of Tetrazolium Microplate Assay



STR: Streptomycin, RIF: Rifampicin, ETH: Ethambutol, CIP: Ciprofloxacin, OFL: Ofloxacin, ATH: Azithromycin, CLA: Clarithromycin,: Growth Control,: Distilled Water.

Diagrammatic illustration of tetrazolium microplate assay

Observation:-

A change in colour from yellow to purple indicated growth of bacteria and the MICs was interpreted visually.

Advantages:-

1] TEMA is effectively used to detect drug resistance in NTM isolates and compare with the APM to which it had a very good concordance.

2] The TEMA results were accurate.

3] Highly reproducible.

4] Rapid to determine the MICs of clinically significant *mycobacteria.*

Disadvantages:-

1] Technically skilled person is required.

2] Special lab is required.

2] Evaluation of rapid MTT tube method for detection of drug susceptibility of Mycobacterium tuberculosis. [11]

Principle:-

This is based on the colorimetric method. A method for detection of inhibition utilises the ability of viable *mycobacterial* cells to reduce MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide) and display living cell in colour i.e live cells convert the yellow tetrazolium salt into a blue formazan. While susceptible cells are unable to do so.

Procedure: [12]

- Inoculums of 10^7cfu/mL are prepared in Middle brook 7H9 medium supplemented with oleic acid, albumin, and dextrose and catalyse.
- For each drug three tubes were used, and one tube for blank. These were incubated at 37^0C for 4 and 7days, after which MTT assay is performed.

Observation:-

Results were read visually and by colorimeter at 570 nm.

Advantages:-

1] The MTT assay was also interpretable by the naked eye.

2] Simple method.

3] Inexpensive assay.

Disadvantages:-

1] Contamination may occur.

3] A] BacT Alert 3D system. [13-20]

Principle:-

The BacT Alert system is an automated antimycobacterial testing method based on the liquid medium so the result accuracy is more in these techniques.

Procedure: - [21]

- The BacT/Alert MP bottle containing the growth of *Mycobacterium tuberculosis* (<36 hours, subculture, then the growth was diluted 1:1 in sterile distilled water).
- This formed the direct growth control of approximately Mc Farland no.2 (DGC).
- A 100-fold dilution of the DGC (0.1ml of DGC + 9.9ml of SDW) was prepared and 0.5ml was added to another BacT/Alert MP bottle and this was the 1% growth control (1% GC).
- These bottles were exposed to various dilution of the drug/NCE to be tested into all drug containing and control bottles.
- At least 3 critical concentration of each of the drugs were tested in the BacT Alert 3D system to determine the ideal concentration for the strains.
- These bottles were incubated in the system at 35ºC for 12 days and monitored every 10 min to detect growth.
- An isolate was considered as resistant if the bottles containing the drug flagged positive at the same time or before the 1% GC.
- If the DGC did not flag positive in 12 days the test was invalidated and had to be repeated.

Observation:-

Results were directly detected by BacT/Alert apparatus.

Advantages:-

1] This is an economical test.
2] The liquid medium higher isolation yields than egg-based media.
3] It is a rapid method and Effective method.
4] Duration required is lesser

Disadvantages

1] Low specificity.

2] Variable sensitivity.

3] Radioisotopes and semi-automated technique are a definite disadvantage. [22]

4] The BACTEC method though requires specialized instrumentation and is not feasible in most resource-poor settings.

5] Liquid media are considerably more expensive.

6] Requires special lab.

B] BACTEC 460[23]

Principle:-

The BACTEC 460 an automated antimycobacterial testing method based on a radiometric method using radioisotope ^{14}C labelled palmitic acid.

Procedure:-[24]

- ^{14}C labelled palmitic acid added to a liquid medium 7H12 Medium.
- Detect *Mycobacteria* by metabolism rather than growth.
- $^{14}CO_2$ detected by the BACTEC 460.[25]
- Growth index is measured.

Observation:-

The result was available in 7-8 days.

Advantages:-

1] Result is obtained within 8 days.

Disadvantages:-

1] Radiation hazard may occur.

4] Nitrate reductase assay (NAA).

Principle:-

This is test for the presence of enzyme nitrate reductase which causes the reduction of nitrate, in the presence of a suitable electron donor, to nitrite which can be tested for by an appropriate colorimetric reagent**.** [26]

Procedure: - [27]

- *Mycobacterium sp*. were sub-cultured on L-J tube by dispensing two 1-µL loop full of bacteria in 0.5 mL of phosphate-buffered saline (PBS) (pH 7.4) in 7.5-mL screw-cap bottles containing a few 3-mm diameter glass beads and vortexes to obtain a uniform solution to obtain.
- McFarland standard no. 1, Part of the suspension was diluted at 1:10 in PBS. For each strain, 0.2 mL of the undiluted suspension was inoculated into tubes containing the L-J medium with KNO_3 and the antibiotics in duplicates to reduce the risk of contamination and to assess the reproducibility of the test while 0.2 mL of the 1:10 dilution inoculated into three drug-free tubes containing L-J with KNO_3.
- The latter tubes served as growth controls. The tubes were incubated at 37 °C.
- After seven days, 0.5 mL of Griess reagent (Fifty percent (vol/vol) concentrated hydrochloric acid (HCl), 0.2% (wt/vol) sulphanilamide, and 0.1% (wt/vol) n-1-naphthylethylenediamine dihydrochloride were prepared in small volumes and were mixed shortly before use in the reduction of nitrate to nitrite was detected first in control tube no-1 by addition of Griess reagent.
- If any colour change could be observed, the corresponding antibiotic-containing tubes were also tested, and susceptibility results were read.
- If no colour change observed in the growth control tube, this tube was discarded, and the other two control tubes and the antibiotic-containing tubes were re-incubated.
- The procedure was then repeated at day 10, using the second growth control, and if needed, also at day 14, using the last growth control tube.
- The results were classified as negative if no colour change of the medium was observed and positive if pink to violet colour appeared in the medium.
- It is considered to be resistant to a certain drug if there was a colour change in the antibiotic containing tube in question greater than that in the 1:10 diluted growth control on the same day.

Observation:-

The NRA method utilizes the standard detection of nitrate reduction as an indication of growth result was calculated in percentages.

Advantages:-

1] Result were obtained in 7-8 days.

2] Nitrate reductase assay, as observed in the present study was found to be rapid.

3] Inexpensive.

4] Easy to perform. [28]

5] As it does not require much instrumentation it could be used routinely in laboratories in developing countries for drug susceptibility testing of *M. tuberculosis*. [29]

6] It might be possible to apply the nitrate reductase test directly to microscopy positive sputa, thus drastically reducing the time needed for detection of drug resistant M. *tuberculosis*.

Disadvantages:-

1] *Mycobacterium bovis* does not reduce nitrate, therefore the NRA technique is not applicable. [30]

5] Resazurin microtiter plate assay (REMA)

Principle:-[31]

Resazurin microtiter plate assay (REMA) it is an indicator-based method which allows for detection of cell viability scored as a colour change in a microtiter plate format and has been used with different indicators.

Procedure:-

- In a screw cap tube containing a L-J slant with glass ,few drop of saline(to adjust the pH) and Mycobacterial suspension is added.[32]
- Drug/New Chemical Entities of appropriate concentration were added.

- The overall setup of the 96-well microtiter plate for REMA is used where in positive (no drug) and negative (no *mycobacteri*a) growth wells were included in all the assays.
- Growth was evidenced by addition of 20 microliter of resazurin to one of the growth control wells; if a change of colour of dye-indicative of growth seen, 20 microliter resazurin is added to the rest of the wells; otherwise, the plate was incubated for another 24 hr and the process is repeated.
- The final point of the reduction of the dye ,Susceptibility to the tested drugs judged by change of colour Blue to pink indicative of growth, With the MIC recorded as the lowest compound concentration in well which remain blue.

Observation:-

Result where observed by change of colour indicative of growth.
Result may be obtained within 7 to 12 days when vital dyes are used [33, 34].

Advantages:-

1) Less time consuming method.
2) Convienient to use.
3) They are the useful tools for rapid identification of resistance & multidrug resistance *M. tuberculosis* strain.
4) Low cost.
5) Accurate result.

Disadvantages:-

1) Assay required high specificity.
2) Require excessive technical skills.

6] Mycobacteriophage assay.[35]

Principle:-

Mycobacteriophages are viruses that efficiently and specifically infect *mycobacteria*. Techniques using phage's for primary detection and DST have been evaluated.[36]

The assay includes the *M. tuberculosis*-specific phage D29, whose replication can be determined by counting the viral particles on fast-growing *Mycobacterium* smegmatis.[37]

Procedure:-

- The assay includes the *M. tuberculosis*-specific phage D29.
- After overnight incubation, the clear plaques are visible on indicator plates harboring the turbid lawn of M. smegmatis.
- Assay needs 1 day for obtaining the results, owing to the development of plaques on the indicator plates.
- The assays are based on the fact that replication of the phage is dependent on viable *mycobacterial* cells.
- In the presence of a drug, the phages are only able to replicate if a drug-resistant strain is present.
- Visualization of the amplified phage's can be performed counting the plaques.

Observation:-

Visualization of the amplified phage's can be performed counting the plaques.

Advantages:-

1] Methods are proven to be rapid.

2] Accurate.

3] Simple, showing high sensitivity and slightly lower specificity.

Disadvantage:-

1] Showing high sensitivity and slightly lower specificity.

2] By contrast, the accuracy of these assays could not be shown when directly applied to sputum specimens, owing to a high rate of non-interpretable results and a high contamination rate.[38,39]

Summary:-

All the techniques of the Antitubercular testing techniques are having the good method and testing accuracy, but according to my view the Tetrazolium microplate assay [TEMA], which is a colorimetric method is easy to understanding and gives the highly reproducible result.
The need of the hour is to have more rapid testing methods for faster analysis of NCE'S.

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Table no:-2 In the table no.2 summary of all techniques are given.

Test	Readout	Time	Advantages	Disadvantages
TEMA	colorimetric	7-8 day	1] Accurate result. 2]Highily reproducible. 3]Rapid to determine the MICs of clinically significant mycobacteria.	1]Technically skilled person is required. 2]Special lab is required.
MTT	colorimetric	7 days	1] The MTT assay was also interpretable by the naked eye. 2] simple method. 3] Inexpensive assay.	1] contamination may occurred
3] A] BacT Alert 3D system.	Automamated	7-8 day	1] This is a Economical test. 2] The liquid medium higher isolation yields than egg based media. 3] It is a rapid methodand Effective method. 4] Less time required.	1] low specificity. 2] Variable sensitivity. 3] Radioisotopes and semi automated technique are a definite disadvantage [1]. 4] The BACTEC method though requires specialized instrumentation and is not feasible in most resource poor settings. 5] Liquid media are considerably more expensive. 6] Requires special lab.
B] BACTEC 460	Automamated	7-8 day	1] Result are obtained within 8 days.	1] The radiation hazard may occur.
NAA	Colorimetric	7-8 day	1] Result where obtained in 7-8 days. 2] Nitrate reductase assay, as observed in the present study was found to be rapid. 3] inexpensive. 4] Easy to perform.[8] 5] As it does not require much instrumentation it	1] Mycobacterium bovis does not reduce nitrate, therefore the NRA technique is not applicable.

			could be used routinely in laboratories in developing countries for drug susceptibility testing of M. tuberculosis.[5] 6] It might be possible to apply the nitrate reductase test directly to microscopy positive sputa, thus drastically reducing the time needed for detection of drug resistant M. Tuberculosis	
REMA	Colorimetric	7-12 day	1) Less time consuming method. 3) They are the useful tools for rapid identification of resistance & multidrug resistance M.tuberculosis strain. 4) Low cost. 5) Acurate result.	1) Assay requird high sspecificity. 2) Require excessive technical skills.
Mycobacterio phage assay.	Visualising		1] Methods are proven to be rapid. 2] Accurate. 3] Simple, showing high sensitivity and slightly lower specificity.	1] Showing high sensitivity and slightly lower specificity. 2] By contrast, the accuracy of these assays could not be shown when directly applied to sputum specimens, owing to a high rate of non interpretable results and a high contamination rate.