NEW DIAGNOSTIC METHODS OF TUBERCULOSIS: FEASIBILITY AND APPLICABILITY IN THE FIELD

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Summary

Tuberculosis (TB) remains one of the major causes of death from a single infectious agent worldwide of great concern for TB control is the emergence of drug resistance. Since there is no cure for some multidrug-resistant strains of *Mycobacterium tuberculosis*, there is concern that they may spread around the world, stressing the need for additional control measures, such as new diagnostics, better drugs for treatment, and a more effective vaccine. Pulmonary TB can be diagnosed by its symptoms, chest radiography, and sputum smear microscopy and by cultivation of *M. tuberculosis*, which is considered as the gold standard. Recent advances in molecular biology and molecular epidemiology, and a better understanding of the molecular basis of drug resistance in TB, have provided new tools for rapid diagnosis; however, the high cost of most of these techniques, and their requirement for sophisticated equipment and skilled personnel have precluded their implementation on a routine basis, especially in low-income countries. Other nonconventional diagnostic approaches proposed include the search for biochemical markers, detection of immunological response and early detection of *M. tuberculosis* by methods other than colony counting. In the present article, some of these approaches will be reviewed and the feasibility for their implementation in diagnostic laboratories will be discussed.

Keywords: Bactec, resazurin, nitrate, proportion method etc.

Introduction

The latest estimates of the global burden of TB show that there were 9.27 million new cases of TB in 2007 (including 1.37 million cases among HIV-positive people), 1.32 million deaths from TB in HIV-negative people with an additional 0.46 million TB deaths in HIV-positive people, and 13.7 million prevalent cases. There were 0.5 million cases of MDR – TB.[1]

The diagnosis of mycobacterial infections remained practically unchanged for many decades and probably would have not progressed at all without the unexpected resurgence of tuberculosis (TB) which characterized the last twenty years of the 20th century. With microscopy lacking wide margins for improvement, the areas which most benefited from the renewed interest in TB were culture and identification, while a completely new approach emerged, aimed towards the direct detection of mycobacterial nucleic acids in clinical specimens. In the present article, some of these approaches will be reviewed and the feasibility for their implementation in diagnostic laboratories will be discussed.[2]

1] Automated culture methods: [2]

Although known for decades, liquid media for cultivation of mycobacteria had never attracted the attention of mycobacteriologists. In fact, the ability of a liquid medium to support a faster growth was heavily hampered by its susceptibility to contamination. The use of antimicrobial combinations suitable of inhibiting the growth of the whole spectrum of potential contaminants (Gram-positive and Gram negative bacteria as well as fungi) represented a turning point. During the same period, automation was taking its first steps in microbiology, with blood cultures leading the field.

1.1] BACTEC TB-460 [2,3,4,5.7,8,9,10,11,12,15,22]

The BACTEC TB-460 system (Becton Dickinson, Sparks, MD) was the first, and for many years the only, automated approach in mycobacteriology. It makes use of a

radiometric instrumentation developed for blood cultures with the broth bottles replaced by vials containing a medium specific for mycobacteria.

The principle:

The medium: A modified Middlebrook 7H9 medium is used, in which one of the
components, palmitic acid, is radiolabeled with 14C. Contamination is controlled by the addition, prior to use, of a mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA) reconstituted with a poly-oxyethylene solution. The use of such a combination of antibiotics does not eliminate the decontamination step, which needs to be performed before inoculation of the samples. The vials containing the medium remain sealed through the whole culture process and the specimen is inoculated by puncturing the rubber septum with a needle.

Instrumentation: Once the paired needles have perforated the rubber septum of the vial, the gaseous phase is aspirated and replaced with air containing 5 % CO2. The aspirated gas is analyzed by a β-counter to quantify the eventual presence of radiolabeled CO2.

The rationale: When viable mycobacteria are present in the culture vial, the radiolabeled palmitic acid is metabolized and radioactive CO2 is liberated into the gaseous phase.

The performance:
The BACTEC TB-460 was first commercialized in 1980 and soon became popular worldwide. It is not a fully automated system, as the vials, which are held in an external incubator, must be loaded into the instrument for reading. The reading is usually performed twice a week during the first 15 days of incubation, and weekly thereafter, until the 42nd day. BACTEC TB-460 is still used in many laboratories worldwide.

Disadvantages: 1] The increasing cost of radioactive waste disposal

2] The interest of the manufacturer to promote newly developed alternative systems.

1.2] The BACTEC MGIT960 system [2, 6,7, 8 ,13,14]

The BACTEC MGIT960 system (Becton Dickinson, Sparks MD) uses the technology of the previously developed blood culture instrument. The original system (BACTEC 9000) was first adjusted to support mycobacterial cultures but was sequently completely redesigned to process tubes.

The principle:

The medium: Mycobacteria Growth Indicator Tube (MGIT) is a modified Middlebrook 7H9 medium in which a supplement is added at the moment of use. The supplement is a mixture of oleic acid, albumin, dextrose, and catalase (OADC) enrichment and the same PANTA antibiotic mixture used in the radiometric system. The presence of PANTA does not do away with the decontamination step, which needs to be done before inoculation. As the tubes containing the medium are screw-capped, no needle is needed for inoculation. A silicon film embedded with a ruthenium salt is present at the bottom of the tube as a fluorescence indicator

The instrumentation: Incubator and reader are combined in a single cabinet. The bottom of each tube, stimulated by ultraviolet light, is monitored by a fluorescence reader. Fluorescence-emitting tubes are reported as positive. It is also possible to use the MGIT tubes without instrumentation, by holding the tubes in a normal incubator and observing the fluorescence under a Wood's lamp.

The rationale: The oxygen normally present in the medium quenches the natural fluorescence of the ruthenium salt. If viable mycobacteria are present in the tube, oxygen is consumed due to their metabolism, the quenching effect lowers accordingly, and the bottom of the tube fluoresces when exposed to ultraviolet light.

The performance:
The BACTEC MGIT960 is a typical walk-away instrumentation which monitors the tubes at one-hour intervals, alerts when they become positive and signals the end of the incubation period.

Advantages: clearly faster and more sensitive than solid media.

1.3] VersaTREK/ESP system: [2, 4,15,16]
The VersaTREK (previously known as the ESP system II) uses the technology of a previously developed blood culture system and is commercialized by Trek Diagnostic Systems.

The principle:

The medium: It uses a modified Middlebrook 7H9 medium to which the OADC enrichment must be added. Two different antimicrobial mixtures are available. The first one, also known as AS, includes polymyxin B, azlocillin, fosfomycin, nalidixic acid, and amphotericin B. The second contains polymyxin B, vancomycin, nalidixic acid, and amphotericin B (PVNA). Usually, AS is used for specimens originating from sterile samples or with a low risk of contamination, while PVNA is used for heavily-contaminated samples. The presence of such antimicrobial mixtures for contamination control does not eliminate the decontamination step, which needs to be performed before inoculating the sample. The bottles of medium hold a cellulose sponge whose large surface area allegedly improves growth. Bottles are inoculated through a rubber septum by means of a syringe.

The method: In the ESP susceptibility test system, lyophilized PZA powder (Trek Diagnostics) was rehydrated with 25 ml of PZA reconstitution fluid according to the manufacturer's instructions, and aliquots were frozen at -70°C. One milliliter of the reconstituted drug was inoculated into an ESP Myco bottle. The final concentration of PZA in the test bottle was 300µg/ml. One milliliter of the

rehydration fluid was inoculated into the drug-free control bottle. The primary ESP Myco bottle served as the inoculum source. Positive bottles may be used as the inoculum source up to 72 h after producing a positive signal. An aliquot was removed from the ESP Myco bottle and diluted 1:10 with normal sterile saline. A 0.5-ml aliquot of this suspension was used to inoculate the drug-containing and control bottles. The control and drug-containing bottles were placed into the ESP instrument, where they are monitored for changes in gas pressure.

The instrumentation: Incubator and reader are combined in a single cabinet which also shakes the bottles during the incubation. The pressure within each bottle is monitored by a manometer through a proper connector. Cultures presenting a decreased headspace pressure are reported as positive.

The rationale: If viable mycobacteria are present in the bottle, the oxygen consumption due to their metabolism reduces the internal pressure. An isolate is considered resistant to PZA if the drug-containing bottle signals positive within 3 days of the control bottle becoming positive. Conversely, an isolate is reported to be susceptible to PZA if the drug-containing bottle fails to signal within 3 days of the control becoming positive.

The performance:
VersaTREK/ESP system is a typical walk-away instrumentation which continuously monitors the bottles, alerts when they become positive and signals the end of the incubation period.

Advantages: 1] VersaTREK in comparison with solid media and similar automated and semi-automated competitor systems performs better than solid media but shows no substantial advantage over other systems.
2] Mycobacterial blood cultures can also be performed with the VersaTREK system. However, whole blood cannot be used and a previous treatment is required to obtain sediment for inoculation.

1.4] BacT/Alert 3D [2, 12,17,18]

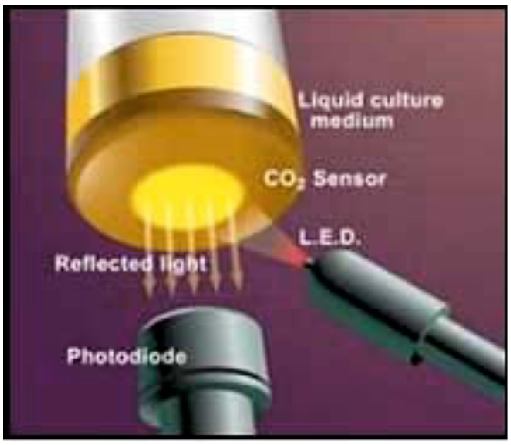
BacT/Alert 3D (previously known as MB/BacT) is commercialized by bioMérieux and uses the technology of a previously developed blood culture system.

The principle:

The medium: A modified Middlebrook 7H9 medium is used in which a supplement, a mixture of OADC enrichment and polymyxin B, amphotericin B, nalidixic acid, trimethoprim, vancomycin and azlocillin, is added at the moment of use. The presence of such contamination-controlling antibiotics does not eliminate the decontamination step needed before inoculation. The bottles of medium have a CO2 sensor at the bottom and are inoculated through a rubber septum by means of a syringe.

The instrumentation: Incubator and reader are combined in a single machine which does not shake the bottles during incubation. The CO2 sensor is impacted by a light whose reflected ray is monitored by a photodiode. Bottles producing specific changes in the intensity of the reflected light are reported as positive.

The rationale: If viable mycobacteria are present in the bottle, the CO2 produced by their metabolism causes a change in the color of the sensor, from green to yellow, which alters the intensity of the reflected light ray.



BacT/Alert technology

The performance: BacT/ALERT 3D is a typical walk-away instrumentation which monitors the bottles at 10-min intervals, alerts when they become positive, and signals the end of the incubation period.

Advantages: 1] It is faster and more sensitive than conventional media.
2] The system is also suitable for mycobacterial blood cultures, provided proper bottles are used; no previous treatment of the blood is required.

2] Nucleic acid amplification methods[2,19,22]
When the polymerase chain reaction (PCR) methodology took its first steps into diagnostic microbiology, M. tuberculosis have the potential to benefit from this novel technique. The rapid diagnosis of TB is possible by this method.

2.1] In house methods for diagnosis of tuberculosis: [2,20]
One of the first findings on the way to developing a PCR technique aimed at *M. tuberculosis* detection was that, although different targets were investigated, none of them were suitable for differentiating *M. tuberculosis* from other species belonging to the *M. tuberculosis* complex. In fact, the differentiation of such species is of very limited relevance from the clinical and therapeutic point of view. Alternative amplification methods were developed. Most successful were the reverse transcriptase PCR, the ligase chain reaction, and the strand displacement amplification.

2.2] Commercial methods [2,,22]
In the last few years, several amplification methods have been commercialized; only four methods have gained worldwide diffusion and been widely validated by international studies, although one of them (LCx, Abbott) is no longer on the market.

1. Amplified MTD[2,12,22]
Amplified Mycobacterium tuberculosis Direct Test (AMTD), developed by Gen-Probe (San Diego, CA, USA), is an isothermal (42°C) transcriptase-mediated amplification system.

2. Amplicor MTB Test [2,,21]
The Amplicor MTB Test (Roche Molecular Systems, Basel, Switzerland) relies on standard PCR. The amplification and detection steps are carried out automatically by the Cobas Amplicor instrument.

3. BD ProbeTec ET[2,23]
The BD ProbeTec ET (Becton Dickinson, Sparks, MD) uses DNA polymerase and isothermal strand displacement amplification to produce multiple copies of IS6110, an insertion element unique to M. tuberculosis complex.

Advtantages: Specificity is good.

Disadvantages: 1] The unsatisfactory sensitivity is major limitation.
2] Paucibacillary specimens also detected by molecular amplification.
3] Time consuming and also raises the risk of contamination due to which false positive results.
4] The sediment of samples contains substances inhibiting the amplification process and no method available for its neutralization.

3] Genetic identification methods [2]
Following the extraordinary development of molecular methods, the identification of mycobacteria, previously based on phenotypic investigations, suddenly started to rely on genotypic methods. Different genetic approaches developed in research laboratories became rapidly popular in diagnostic laboratories and some of them were transformed into commercial diagnostic kits.
3.1] PCR restriction-enzyme analysis[2,,24]
3.2] DNA probes: 3.2.1]AccuProbe [2,,25]
3.2.2] Line probe assays: Three commercial methods are available,
3.2.2-A] INNO-LiPA MYCOBACTERIA (Innogenetics, Ghent, Belgium) [2, 11,26,27]
3.2.2-B] GenoType Mycobacterium (Hain, Germany) [28]
3.2.2-C] GenoType MTBC (Hain, Germany).

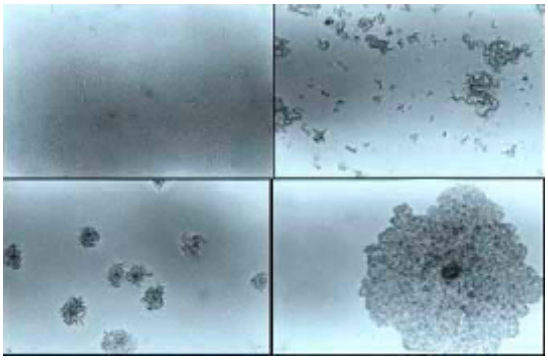
4] Non-conventional phenotypic diagnostic methods[2]

4.1] Phage-based assays:
The phage-based assay relies on the ability of *M. tuberculosis* to support the growth of an infecting mycobacteriophage.

4.2] The micro-colony method[2,30,31]
The micro-colony method or thin-layer agar technique is an old method for culturing and identifying mycobacteria; it allows both rapid detection and presumptive identification of isolates based on the characteristic morphology of mycobacteria in culture, and has been proposed as an inexpensive alternative method for the rapid detection and culture of mycobacteria.

Advantages: 1] Sensitivity was 92.6% for thin layer 7H11 agar.
2] Time for detection of 11.5 days.

Disadvantages: The contamination rate was 5.1%.



Microcolonies of M. tuberculosis after, 4, 6, 8, and 15 days of culture (Courtesy J. Robledo)

4.3] Microscopic observation broth-drug susceptibility assay (MODS) [2,32,33,34]

The principle: The method is based on the observation of the characteristic cord formation of *M. tuberculosis* visualized microscopically in liquid medium with the use of an inverted microscope.

Method: Briefly, broth cultures were prepared in 24-well tissue-culture plates (Becton Dickinson), each containing 720 µl of decontaminant, Middlebrook 7H9 broth (Becton Dickinson), oxalic acid, albumin, dextrose, and catalase (OADC) (Becton Dickinson), and polymyxin, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA) (Becton Dickinson). For each sample, 12 wells were used: in 4 control wells, no drug was added, and each of the remaining 8 wells contained one of four drugs at one of two concentrations tested. The cultures were examined under an inverted light microscope at a magnification of 40x every day (except Saturday and Sunday) from day 4 to day 15, on alternate days from day 16 to day 25, and twice weekly from day 26 to day 40.

The Rationale: Positive cultures were identified by cord formation, characteristic of *M. tuberculosis* growth, in liquid medium in drug-free control wells.

Advantages: 1] The sensitivity for the detection of M. tuberculosis was 97.8 % compared to 89.0 % for the automated mycobacterial culture, and 84.0 % for Löwenstein-Jensen medium ($P < 0.001$);
2] The median turnaround time was 7, 13, and 26 days for MODS, the automated culture system, and Löwenstein-Jensen medium, respectively ($P < 0.001$).
3] The method is rapid, inexpensive, sensitive, and specific method for *M. tuberculosis* detection and susceptibility testing.

Limitations: The requirement for an inverted microscope, which is necessary to observe the cord formation in liquid medium.

4.4] Analysis of cell wall mycolic acids[2,35]

Mycobacteria have unusually high lipid content in their cell wall. Such lipids include mycolic acids and other saturated and unsaturated fatty acids. Mycolic acids are branched, long-chain fatty acids present in the cell wall of a limited number of genera; they exhibit the maximum length in the genus Mycobacterium. The analysis of the lipid content of the mycobacterial cell wall has been widely used for identification purposes. The various techniques used are

based on the physical partitioning between two phases (stationary and mobile) of single lipids present in the mycobacterial cell wall. The extraction of the lipids from the bacterial colonies is the preliminary step in all the techniques described below.
1] Thin-layer chromatography (TLC) 2] Gas-Liquid Chromatography (GLC)
3] High-Performance Liquid Chromatography (HPLC)

Advantages: 1] This can be performed by thin-layer chromatography and by high-performance liquid chromatography, that it can be completed in a few hours, 2] It is relatively inexpensive, and can identify a wide range of mycobacterial species.

Disadvantage: The initial investment in the cost of the equipment.

 # Sensitivity Test Methods[36]
During the 1960s, there was much discussion about the methods used in drug sensitivity tests (DSTs). The World Health Organization meetings reported three different methods for performing DSTs; the absolute concentration method, the resistance ratio method and the proportion method.

5] Proportion method[37,38,39]
Drug susceptibility of isolates to isoniazid, rifampicin, streptomycin and ethambutol performed by this method. Briefly, LJ media with drug incorporated in various concentrations and plain LJ medium for control were prepared. The growth from a 3-4 wk old culture was scraped with a loop and bacterial suspension was made in sterile distilled water, vortexed and matched with McFarland opacity tube No. l. Dilutions of 10~2and 10"3 were made and inoculated on both the control and drug containing media and incubated at 37^0C. The first reading was taken after 28 days of incubation and the second on 42 day. The percentage resistance (R) was calculated as the ratio of the number of colonies on the drug containing media to those on the control medium.

The Rationale:
R (%) = No. of colonies on drug media/ No. of colonies on control medium ×100
If R = >1 per cent, the isolate was taken as resistant

5.1] Agar proportion method [37,38]
The agar proportion method, as recommended by the National Committee for Clinical Laboratory Standards was performed to determine the percentage of *M. tuberculosis* organisms resistant to each of the concentrations of antimycobacterial agents tested. Briefly, 7H10 agar medium (Difco) was prepared, autoclaved at 121°C at 15 lb/in^2 for 15 min, and cooled to 50 to 56°C. INH, RIF, and EMB were added to the medium to yield final concentrations of 0.2, 1.0, and 5.0 µg of INH/ml, 1.0 µg of RIF/ml, and 5.0 µg of EMB/ml. Subsequently, 5.0 ml of each concentration of antimycobacterial containing medium was dispensed into labeled quadrants of sterile petri dishes. One quadrant per plate was reserved for 7H10 medium free of any antimycobacterial agent to serve as a growth control. Upon solidification of the medium, the plates were inoculated with 0.1 ml of 10^2 and 10^4 dilutions of a suspension of each *M. tuberculosis* isolate equivalent to a McFarland 1.0 standard. The inoculated plates were then incubated at 37°C in the presence of 5% CO_2 for 3 weeks.

The Rationale: Each strain was classified as susceptible to a drug if the number of colonies that grew on the drug-containing medium was <1% of the number of colonies grown on the control plate and resistant if the number of colonies was >10%. In cases where two drug concentrations were tested in the agar proportion method, a strain was classified as intermediate if it showed resistance to the lower drug concentration but was susceptible to the higher drug concentration.

6] The resistance ratio method [39]
The resistance ratio (RR) method utilizes the ratio of the minimum inhibitory concentration (MIC) for the patients' strain to the MIC of the drug-susceptible reference strain, H37Rv, both tested in the same experiment. Inclusion of

the reference strain in each experiment is not only for quality control but also to standardize the results by taking into account the test variations within certain permissible limits. This feature makes the RR method the most expensive of the three conventional methods that use solid media. Reading after 4 weeks of incubation defines "growth" on any slope as the presence of 20 or more colonies, and MIC is defined as the lowest drug concentration in the presence of which the number of colonies is less than 20. The range required for the test strain is determined by the variation in the MIC of H37Rv, and by the need to determine a resistance ratio of 2 or less for sensitive strains and a resistance ratio of 8 or more for resistant strains.

7] The absolute concentration method [39]

The absolute concentration method was used originally to determine the MIC of isoniazid and of the drug that will inhibit growth, i.e. fewer than 20 colonies by the end of 4 weeks.

By adding a carefully controlled inoculum of M. tuberculosis to the control and drug-containing media. Media containing several sequential dilutions of each drug are used, and resistance is indicated by the lowest concentration

8] Colorimetric methods[40,55]

8.1] Microdilution Resazurin Assay (MRA) [41,42,43,45]

Method: The REMA plate method was carried out as described by Palomino *et al*. Briefly, the inoculum was prepared from a fresh colony on LJ in 7H9 medium supplemented with 0.1% casitone/0.5% glycerol/10% OADC, A growth control containing no antibiotic and a sterile control without inoculation were also prepared on each plate. Two hundred microliters of sterile water was added to all perimeter wells to avoid evaporation during incubation.Turbidity of inoculum adjusted to a turbidity equivalent to that of a McFarland no. 1 standard and diluted 1:10. The microdilution test was performed in 96-well

plates. Two-fold dilutions of each drug were prepared in the test wells in complete 7H9 broth, the final drug concentrations being: isoniazid 128–0.00625 mg/L, rifampicin 128–0.0625 mg/L, streptomycin 128–0.125 mg/L and ethambutol 128–0.25 mg/L. Twenty microlitres of each bacterial suspension was added to 180 µL of drug-containing culture medium. Control wells were prepared with culture medium only and bacterial suspension only. The plates were sealed and incubated for 7–14 days at 37°C. The range of concentrations tested for nicotinamide was 8–2000 mg/L. The plate was covered, sealed in a plastic bag and incubated at 37°C in normal atmosphere. After 7 days of incubation, 30 µL of resazurin solution was added to each well, and the plate was reincubated overnight. A growth control containing no antibiotic and a sterile control without inoculation were also prepared on each plate. Two hundred microliters of sterile water was added to all perimeter wells to avoid evaporation during incubation. Then, at day 8, a change in color from blue (oxidized state) to pink (reduced state) indicated the growth of bacteria.

The Rationale: MIC was defined as the lowest drug concentration that prevented resazurin colour change from blue to pink. Each MIC was determined three times in duplicate experiments. Viable counting from control wells and from test wells was performed onto 7H11 agar plates.

Advantages:1] MRA is a rapid, inexpensive, low technology procedure, suitable for susceptibility testing of first- and second-line antitubercular drugs and for screening new antitubercular compounds against *M. tuberculosis* clinical strains.
2]The MRA method a safe, rapid and reliable assay; the resazurin solution we employed in the microplate test MRA did not need stabilizing agents, its stability being always ensured by diluting the frozen stock solution when needed for the test.
3] By means of MRA the drug susceptibility of different *Mycobacterium* species, including *Mycobacterium avium*. clinical strains can be evaluated.

8.2] MTT assay[43,44,45,55]

This method was carried out as described by Abate *et al.* A stock solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] at a concentration of 5 g/L was prepared in PBS, pH 6.8, and kept at 4 °C in the dark. Formazan solubilization buffer was prepared by mixing 1:1 (v/v) 20% sodium dodecyl sulphate (SDS) and a solution of 50% *N*,*N*-dimethylformamide (DMF). The inoculum was prepared as described above for the REMA plate method and the drug concentration ranges used were the same. Preparation of the 96-well plates was identical as described for the REMA plate. After 7 days of incubation at 37 °C, 10 µL of the MTT solution (5 g/L) was added to each well and the plate was re-incubated overnight. If a violet precipitate (formazan) appeared, 50 µL of the SDS/DMF solution was added to the wells and the plate re-incubated for 3 h. A change in colour from yellow to violet indicated growth of bacteria and the MIC was interpreted as in the REMA plate.

8.3] Microplate Alamar Blue assay[46,47,48,54]

Two hundred microliters of sterile deionized water was added to all outer-perimeter wells of sterile 96-well plates to minimize evaporation of the medium in the test wells during incubation. The wells received 100 µl of 7H9GC broth, one hundred microliters of 2× drug solutions should add. By using a multichannel pipette, 100 µl was transferred and the contents of the wells were mixed well. Identical serial 1:2 dilutions were continued 100 µl of excess medium was discarded from the wells. Final drug concentration ranges were as follows: for INH, 0.031 to 8.0 µg/ml; for RMP, 0.0156 to 4 µg/ml in initial tests and 0.062 to 16 µg/ml on repeat testing; for SM, 0.125 to 32 µg/ml; and for EMB, 0.5 to 128 µg/ml. One hundred microliters of *M. tuberculosis* inoculum was added to the wells by using an Eppendorf repeating pipette (yielding a final volume of 200 µl per well). Thus, the wells served as drug-free (inoculum-only) controls. The plates were sealed with Parafilm and were incubated at 37°C for 5 days. Fifty microliters of a freshly prepared 1:1 mixture of 10× Alamar Blue (Accumed International, Westlake, Ohio) reagent and

10% Tween 80 was added to well. The plates were reincubated at 37°C for 24 h. If control well turned pink, the reagent mixture was added to all wells in the microplate (if the well remained blue, the reagent mixture would be added to another control well and the result would be read on the following day). The microplates were resealed with Parafilm and were incubated for an additional 24 h at 37°C, and the colors of all wells were recorded. A blue color in the well was interpreted as no growth, and a pink color was scored as growth. The MIC was defined as the lowest drug concentration which prevented a color change from blue to pink.

Advantages: 1] Rapid test and their use of low technology, and their low cost.
2] Method of choice for drug susceptibility testing of *M. tuberculosis.*

9] Nitrate reductase assay [43,47,49,50,51,52,53,55]
NRA was performed as described by Golyshevskaia et al11 and Angeby et aln. The following critical concentrations were used: 0.2µg/ml for INH, 40 µg/ ml for RIF, 4 µg/ml for STR and 2.0 µg/ml for EMB.

Method: Fresh subculture (1 µl loops of bacteria) from isolates of M. tuberculosis grown on LJ medium was taken and vortexed in 3ml of phosphate buffer saline (PBS, pH 7.4) and turbidity was adjusted according to McFarland standard no.1. Part of the suspension was diluted 1:10 in PBS. For each isolate, 0.2 ml of suspension was inoculated into the tubes containing LJ medium with potassium nitrate (KNO3 and the antitubercular drugs; 0.2 ml of the 1:10 dilution was inoculated into drug free media (LJ media) containing KNO3 which served as growth controls. Tubes in triplicate were incubated at 37°C for 14 days and 0.5 ml of a mixture of three reagents (25 µl of concentrated HCl, 50 µl of 2% sulphanilamide and 50 µl of 1% n-1-napthyl-ethylenediamine dihydrochloride) was added to one drug-free control tube after 7 days of incubation. If its colour changes to pink then tubes with drugs were tested.

The Rationale: An isolate was considered resistant if there was colour change (pink or deep red to violet) in the drug tube in question greater than in the 1:10 diluted growth control on the same day. If the tubes did not show any colour change and remains the same, these were further incubated for 10 days and for 14 days.

Advantages: 1] It is rapid, inexpensive and easy to perform as it does not require much instrumentation; it could be used routinely in laboratories in developing countries for drug susceptibility testing of M. tuberculosis.
2] Apply to the test directly to microscopy positive sputa, thus drastically reducing the time needed for detection.

10] Flow cytometric susceptibility testing[54,56,57,58]
Aliquots of 0.9 ml of each actively growing *M. tuberculosis* isolate were transferred to 2.0-ml polypropylene screw-cap microtubes (Sarstedt). The tubes were inoculated with different concentrations of standard drugs. Drug-free controls of *M. tuberculosis* were also prepared by inoculating them with 0.1 ml sterile phosphate-buffered saline (pH 7.4). Subsequently, the suspensions were incubated for 24, 48, or 72 h at 37°C in an environment of 5% CO_2. After incubation, 0.2 ml of each assay suspension was removed and placed in a sterile 2.0-ml screw-cap microtube containing 30 µl of 8% paraformaldehyde (pH 7.4). Samples were then mixed and held at room temperature for 40 min before being analyzed with a Bryte HS flow cytometer with WinBryte software (Bio-Rad Laboratories, Hercules, Calif.).After paraformaldehyde treatment, *M. tuberculosis* cells were detected and differentiated from non-*M. tuberculosis* particles in 7H9 medium by using forward and side angle light scatter signals. Electronic noise and background particles in the 7H9 medium were excluded from analysis by adjusting the threshold monitor listed on the WinBryte software program. Forward- and side-angle light scattering was then used to analyze *M. tuberculosis* cells that were incubated with or without antimycobacterial agents. For each sample acquired, the flow cytometer provided a histogram profile relating to the number of *M. tuberculosis* organisms in each

of 2,048 logarithmic channels of increasing light scattering, a measurement of the number of *M. tuberculosis* events/microliter and a contour plot of forward- versus side-angle light scattering. Five thousand events were acquired for each sample.

The rationale: An isolate was considered susceptible by the flow cytometric method if the number of *M. tuberculosis* organisms (events) in the drug-containing medium was reduced 25, 45, and 50% or more after exposure to EMB, INH, and RIF, respectively, compared to the number of tubercle bacilli in the drug-free control.

Advantages: 1] Increases biosafety and the assay does not depend on use of FDA for obtaining results.
2] Instead, the numbers of mycobacteria in the assay suspensions with or without exposure to antimycobacterial agents are determined 72 h after initiation of testing.
3] This biologically safer procedure is still more rapid than the BACTEC-460 system.
4] Simplicity and low cost.

11[Agar Diffusion assay[59]
The common disc or well diffusion assays in antimicrobial assays of natural products are used to evaluate extracts of new compounds, but are merely an indication that there is growth inhibition at some unknown concentration along the concentration gradient.

Disadvantage: Mycobacteria having a very lipid –rich, hydrophobic cell wall, are often more susceptible to less polar compounds.

12] Macro and micro agar dilution [59]
Testing of known concentrations of compounds in an agar medium allows for the quantitation of activity and the determination of a MIC. Most mycobacteria including mycobacterium tuberculosis will grow well Middlebrook 7H11 agar supplemented with oleic acid, albumin, dextrose and catalase.

Disadvantage:-Required at least 18 days to visibly detect growth of the colonies.

13] Septi-check AFB method[60]

The Septi-check system consists of a capped bottle containing 30ml of middle-brook 7H9 broth under enhanced (5-8%) co2. a paddle with agar media enclosed in a plastic tube and enrichment broth containing glucose, glycerin,oleic acid, pyridoxal, catalase, albumin, polyoxyethylene40 stearate, azlocillin,nalidixic acid,trimethoprim,polymyxin B, amphotericin B. One side of the paddle is covered with nonselective middlebrook 7H11agar, the reverse side is divided into two sections: one contains 7H11 agarwith para-nitro-a-acetylamino-b-hydroxypropiophenone(NAP) fordifferentation of *M.tuberculosis* from other mycobacteria, the other section contains chocolate agar for detection of contaminants.

Advantages: simultaneous detection of *M.tuberculosis* ,non-tuberculous mycobacteria (NTM), other respiratory pathogens and even contaminants and better result as compared to other methods.

Discussion

With traditional methods, such as the indirect or direct proportion method, it could take 3 to 4 weeks to obtain susceptibility results The time needed to obtain these results represents a potential danger to patients, health workers, and the community. The development of rapid and inexpensive new methods for resistance detection is an urgent priority for the management of MDR TB in the world, especially in low-resource countries where most cases of MDR TB occur. The turn around time (TAT) is important in order for the patient to receive an appropriate treatment.

The BACTEC 460 system requires isotopes and heavy equipment, consequently is not feasible in most resource-poor settings. The commercial MGIT system, the non-radiometric alternative method is reliable, but still expensive to implement in low-resource countries. The three low cost methods: Nitrate reductase assay, MTT and

Resazurin have been successfully tested in previous studies and might become inexpensive alternative methods for rapid and accurate detection of drug resistance of MDR TB strains. Owing to their higher level of agreement with the gold standard methods, they seem to have the potential to provide rapid detection. These methods do not need any sophisticated equipment, are simple to perform, reduce the time to achieve results compared to the Proportion method and could be implemented in laboratories with limited resources. One disadvantage of the MMT and resazurin assays is their biosafety, since the plates use liquid medium and could generate aerosols. It has been shown recently that this format can be adapted to screw-cap tubes to avoid this situation.

Conclusion

Using liquid medium-based culture systems such as the BACTEC 460 TB system Mycobacteria Growth Indicator Tube MGIT 960, BacT/ALERT 3D or ESP Culture System II to perform indirect susceptibility tests (which require a pure culture of *Mycobacterium tuberculosis*), the results are available anywhere after 9 to 30 days. If susceptibility tests are performed directly from clinical specimens with these systems, the time needed for results is between 4 and 23 days. Although rapid, these methods require expensive substrates and equipment and are therefore not feasible in most developing countries. The results obtained with the MGIT system, nitrate reductase assay and the colourimetric methods (MTT and resazurin) were available on average in 10 days as with the BACTEC 460 TB system. The time of detection for each method was almost the same. The INNO LIPA Rif TB kit was used to confirm the resistance pattern of these strains but cannot be used in routine practice in low resource countries due to its high cost. A rapid and inexpensive nitrate reductase assay (NRA) for the drug susceptibility testing (DST) of *M. tuberculosis*.

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