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TECHNICAL SERIES

Mycobacterium Tuberculosis

AFB Staining, Culture and Sensitivity

...Setting trends

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Introduction

Humans are very susceptible to the *Tuberculosis* infection but are remarkably resistant to the *Tuberculosis* disease; which is dependent largely on the state of the hosts immune system. Of all the *Mycobacterial* species *Mycobacterium tuberculosis* remains the most common cause of pulmonary tuberculosis and remains the most virulent of all the *Mycobacterial* species.

The disease, as now well known, is highly contagious. Although the disease involves all susceptible individuals, the incidence is higher amongst disadvantaged minorities. Industrialization, increased crowded housing and nutritional deprivation have influenced the spread. With the emergence of HIV and resultant immunocompromise, TB has emerged as a major killer not only in the third world countries but is also resurging in the western world. According to World Health Organisation (W.H.O.) reports, each year an estimated eight million new cases of *Tuberculosis* occur, leading to three million deaths; and almost a third of the world's population is infected by the causative organism, *Mycobacterium tuberculosis*.

According to a study, in India, the number of *Tuberculosis* patients is increasing at the rate of 1.5 million per year, and a quarter of these are sputum positive. Thus, about 40 per cent of all Indians are infected with *Mycobacterium tuberculosis*.

With the emergence of the multiple drug resistant strains due to poorly administered therapeutic measures and patient non-compliance, *Mycobacterium tuberculosis* is challenging its containment, on the basis of empirical treatment alone.

Brief Microbiology

The genus *Mycobacterium* is composed of slow growing organisms, which are "acid fast". Currently about 55 species of *Mycobacteria* are recognized. They are non-motile, slightly curved or straight rods (0.2 – 0.6 X 1-10 mm) and may occasionally demonstrate branching. The organisms are aerobic and have a gram positive cell wall, although they do not gram stain well.

The *Mycobacteria* contain a lipid rich cell surface which includes true waxes and glycolipids. 60-90 carbon, long chain Mycolic acids, unique to the *Mycobacterial* cell wall are responsible for their:

- Acid fastness;
- Failure to react with gram stains ;
- Resistance to the action of antibodies and complement.

The four species in the *Mycobacterium tuberculosis* complex are *M. tuberculosis*, *M. microtic*, *M. africanum* and *M. bovis*. Laboratories can use biochemical tests for

differentiation between isolated strains.

Diagnosis of *Mycobacterium tuberculosis* infection

The diagnosis of *Tuberculosis* is often made on the basis of clinical symptoms, chest X-ray and sputum AFB, since available tests based on immunological principles for *Mycobacterium tuberculosis* diagnosis have yet to over come the problem of poor sensitivity and specificity associated with them. For the time being, speedy and appropriate laboratory diagnosis of *Tuberculosis* infection through AFB staining, culture and sensitivity have more and more important role to play in sensitive detection and appropriate treatment of patients with *Tuberculosis*. However, sample collection, preparation, processing techniques and detection methods employed have a profound effect on the sensitivity and specificity of the results for the detection of *Mycobacterium tuberculosis* infection by AFB and culture methods.

Specimen Selection

A critical factor in the ability of laboratories to isolate *Mycobacterium tuberculosis* is obtaining appropriate specimen for AFB smear and culture. Approximately 85% of the TB cases are pulmonary. However many patients cannot produce sputum spontaneously and alternative respiratory tract specimens such as induced sputum, gastric lavage or fiberoptic bronchoscopy may be needed. As the proportion of patients with extra-pulmonary form of *Tuberculosis* is increasing, adequate specimen from extra pulmonary sites need to be provided.

Recommendations for Sample Collection for *Mycobacterial* isolation and Acid Fast Staining

Specimen type.	Specimen requirements.	Special instructions.	Unacceptable specimen.
Abscess contents, aspirated fluid.	As much as possible in syringe with Luer tip cap.	Cleanse skin with alcohol before aspirating sample. Laboratory may provide 7H9 broth / Kirchner medium for transport of small volumes of aspirates.	Dry swab.
Blood.	10 ml SPS (yellow top) blood collection tube or 10 ml Isolator tube.	Disinfect site as for routine blood culture. Mix tube contents immediately after collection. SPS is preferred anticoagulant. Heparinized blood is also acceptable.	Blood collected in EDTA, which greatly inhibits *Mycobacterial* growth even in trace amounts. Coagulated blood.
Body fluids (pleural, pericardial, peritoneal).	As much as possible (10-15 ml min.) in sterile container or syringe with Luer tip cap. Collect bloody specimens into SPS blood collection tubes.	Disinfect site with alcohol if collecting by needle and syringe.	----
Bone.	Bone in sterile container without fixative or preservative.	----	Specimen submitted in formalin.
Bone marrow.	As much as possible in SPS blood collection tube or 1.5 ml in pediatric Isolator tube.	Collect aseptically. Mix SPS tube contents immediately following collection.	----

Bronchoalveolar lavage or bronchial washings.	≥5 ml in sterile container.	Avoid contaminating bronchoscope with tap water. Saprophytic mycobacteria may produce false-positive culture or smear results.	---
Bronchial brushings.	Sterile container or Middlebrook 7H9 broth, or Kirchner medium.	---	---
CSF	≥2 ml in sterile container.	Use maximum volume attainable.	---
Gastric lavage fluid.	≥5-10 ml in sterile container. Collect in the morning soon after the patient awakens in order to obtain sputum swallowed during sleep.	Collect fasting early morning specimen on three consecutive days. Use sterile saline. Adjust to neutral pH with 100 mg of sodium carbonate immediately following collection. Laboratory should provide collection tube containing sodium carbonate.	Specimen that has not been neutralized.
Lymph node.	Node or portion on sterile container without fixative or preservative.	Collect aseptically, and avoid indigenous microbiota. Select caseous portion if available. Do not immerse in saline or other fluid or wrap in gauze.	Specimen submitted in formalin.
Skin lesion.	Submit biopsy specimen in sterile container without fixative or preservative. Submit aspirate in syringe with Luer tip cap.	Swabs in transport medium (Amies or Stuarts) are acceptable only if biopsy sample or aspirate is not obtainable. For cutaneous ulcer, collect biopsy sample from periphery of lesion, or aspirate material from under margin or lesion.	Dry swab.
Smear on slides.	Smear specimen over 1.5 by 1.5 cm area of clear slide.	Heat fix smears. Transport in slide container taped closed and labeled BIOHAZARD.	---
Sputum.	5-10 ml in sterile, wax-free disposable container. Collect an early morning specimen from deep, productive cough on at least three consecutive days. Do not pool specimens. For follow-up of patients on therapy, collect at weekly intervals beginning three weeks after initiation of therapy.	For expectorated sputum, instruct patient on how to produce sputum specimen as distinct from saliva or nasopharyngeal discharge. Have patient rinse mouth with water before collecting sputum to avoid contaminating specimen with food particles, mouthwash, or oral drugs, which may inhibit the growth of mycobacteria. For induced sputum, use sterile hypertonic saline. Indicate on request if specimen is induced sputum.	24 hour pooled specimens; saliva.
Stool.	≥1 g in sterile, wax-free, disposable container.	Collect specimen directly into container, or transfer from bedpan or plastic wrap stretched over toilet bowl. Wax from container may produce false positive smear.	Frozen specimen. Utility of culturing stool for acid-fast bacilli remains controversial.

* Adapted from Tuberculosis; A Clinical Handbook, 1st Edition 1995, Edited by Larry I. Lutwick.

Sample Concentration and Decontamination

Specimens obtained from sterile sites such as CSF, peritoneal or pleural fluids do not require decontamination. However most specimens for AFB smear and culture are from the respiratory tract and do contain mixed microbial flora.

Successful recovery of *Mycobacteria* depends upon properly collected specimen and suppression of contaminating bacteria.

Since mucous traps AFB and protects other organisms from effective decontamination a combination of 2% NaOH (decontaminant) and 0.5% N-acetyl-L-cysteine (mucolytic agent) is preferably employed. Neutralization of strong decontaminating solutions before using the sample for AFB stain and culture is usually accompanied with sequential buffered wash of the concentrated sample because if the pH of the concentrate remains alkaline or acidic it can destroy the culture medium and prevent the growth of *Mycobacteria* and staining efficiency of the AFB smears. The buffered wash also helps in reducing the specific gravity of specimen and sediments the *Mycobacterium* more effectively.

Another important aspect post decontamination is the specimen concentration and relative centrifugal force applied to the specimen.

Improvement in correlation between specimen showing a positive smear for AFB and a positive culture has been demonstrated by increasing the centrifugal force applied to pellet the specimen.

Effect of Centrifugal Force on positive smears / cultures for *Mycobacteria*

Specimen	Relative Centrifugal Force (*g*)		
	1260	3000	3800
Positive smears	1.8%	4.5%	9.6%
Positive cultures	7.1%	11.2%	11.6%
Correlation of positive smears/ cultures	25%	40%	82%

* Adapted from Clinical Diagnosis & Management by Laboratory Methods, Todd, Sanford & Davidsohn, 17th Edition 1998, Edited by John Bernard Henry.

Thus proper decontamination and preparation of specimen is crucial to AFB detection by culture and AFB staining.

The AFB Smear

The sensitivity of AFB smear for specimen from extra pulmonary sites is lower than from sputa. The lipid–containing cell walls of *Mycobacteria* have a unique characteristic in binding carbolfuchsin stain so tightly that it resists de-staining with strong decolorizing agents such as strong alcohols and strong acids. This "acid-fast" staining reaction of *Mycobacteria*, along with their unique beaded and slightly curved shape, is a valuable aid in the early detection of infection and monitoring of therapy.

It has been estimated that there must be 10,000 acid-fast bacilli per milliliter of sputum to be detected by microscopy. Patients with extensive disease will shed large numbers of *Mycobacteria* and show a good correlation between a positive smear and a positive culture. In patients with minimal or less advanced disease, the correlation of positive smears to positive cultures may range from 30 to 80 per cent.

Acid-fact stains performed on a weekly basis are also useful in following the response of patients to drug therapy. After drugs are started, cultures will became negative before smears, indicating that the bacilli are injured sufficiently to prevent replication but not to the point of preventing binding of the stain. With continued drug treatment, more organisms are killed and fewer shed, hence monitoring the number of stainable organisms in the sputum during treatment can provide an early and objective measure of response.

It should be noted that in patients receiving antimycobacterial therapy not all stainable organisms are viable. Should the number of organism fail to decrease after therapy is started, the possibility of drug resistance must be considered. Additional cultures should be taken and drug susceptibility studies obtained.

Two types of acid-fast stains are frequently used:

1. Carbolfuchsin based stains;
2. Fluorochrome based stains.

1. **The carbolfuchsin stains,** so called because of the reagent formed by mixing of the stain Basic Fuchsin with the disinfectant Phenol (carbolic acid). Carbolfuchsin stained *Mycobacteria* appear bright red / pinkish against a bluish background.

Two procedures using carbolfuchsin based stains are in common use:

(a) Three component Ziehl-Neelsen, or "hot stain", and
(b) Three component Kinyoun or "cold stain".

The Kinyoun stain is a modification of the classical Ziehl-Neelsen “hot stain”. The classical Ziehl-Neelsen “hot stain” requires application of heat to the fixed smears flushed with the stains during staining process, where as the Kinyoun stain does not require the application of heat and is less tedious to perform and standardize.

Recent advances in staining techniques have been reported where the cold Kinyoun stain has been further modified to accommodate the decolorizer within the counter stain. The novel two component two step stain is time, labor and cost saving, more user friendly and easy to standardize. It also has good correlation with the classical Ziehl-Neelsen “hot stain” and AFB cultures.

2. **The fluorochrome based stains** for AFB comprise of Auramine O, sometimes used in combination with a second fluorochrome stain, Rhodamine.

Smears stained with Auramine O can be scanned using a 25 **x** objective. Fluorochrome-stained *Mycobacteria* appear bright yellow against a dark background obtained by counterstaining with potassium permanganate, thereby permitting the slide to be scanned under the lower magnification without loosing sensitivity. The sharp visual contrast between the bright colored *Mycobacteria* and the dark background offers a distinct advantage in scanning a much larger area of the slide during the same time necessary for looking at the carbolfuchsin stain.

When using the Auramine stain, a significantly larger area of the smear can be scanned in the same period of time used to scan a carbolfuschin-stained smear.

Enthusiasm for the carbolfuchsin and fluorochrome staining methods varies between laboratories, with different professionals strongly partial to one method or the other. Specificity for *Mycobacteria* seems to be the same for both.

The crucial factors in maximizing smear sensitivity and specificity are:

- Centrifugation of digested fluid specimen at a minimum of 3000 g;
- The smear should be prepared on a new clean undamaged glass slide;
- Scanning of at least 300 fields per slide;
- The reporting of the AFB smear should be preferably done according to the C.D.C., USA method, or as per the National Reference Institution norms.

Quantitation Scale for Acid-Fast Bacillus Smears according to stain used

Carbolfuchsin (X 1,000)	Fluorochrome (X 250)	Quantity Reported
No AFB/300 fields	No AFB/30 fields	No AFB seen
1-2 AFB/300 fields	1-2 AFB/30 fields	Doubtful; repeat test
1-9 AFB/100 fields	1-9 AFB/10 fields	Rare (1+)
1-9 AFB/10 fields	1-9 AFB/ field	Few (2+)
1-9 AFB/ field	10-90 AFB/ field	Moderate (3+)
>9 AFB/ field	>90 AFB/ field	Numerous (4+)

* Adapted from American Thoracic Society; Am. J. Respir. Cirt. Care Med.; Vol 161, pp 1376-1395, 2000.

However, Indian Reference Institutions recommend reporting after 5 minutes of examination covering about 100 fields. Grading is done as follows:

Number of Acid Fast Bacilli observed	Report
No Acid Fast Bacilli	Negative
1-10 Acid Fast Bacilli	Actual Number
>10 Acid Fast Bacili	+
Masses of Acid Fast Bacilli in several fields	++

* Adapted from National Tuberculosis Institute Monograph Series:1, Manual for Establishment & Functioning of a Tuberculosis Culture Laboratory, Govt. Of India National Tuberculosis Institute, No. 8, Bellary Road, Bangalore 560 003, August 1993.

Smears with fewer than 3 AFB per slide account for about 85% of false positive smear reporting and are considered doubtful. A repeat specimen should be registered. However *Mycobacterium tuberculosis* infection must be considered for any patient with repeat smear AFB positive regardless of the number of AFB observed.

Factors Influencing Sensitivity & Specificity of AFB Smears

False Positive Results

- **Acid fast particles other than tubercle bacilli**

Occasionally, a sputum specimen or smear may contain particles that are acid-fast i.e., when treated with the Ziehl-Neelsen method, they retain the red stain (carbolfuchsin) and resist decolorization with acid-alcohol. These red particles may sometimes resemble tubercle bacilli. They include certain food particles (e.g., waxes, oils), precipitates, other micro-organisms, inorganic materials and artifacts.

Food particles: To eliminate these, the patient should rinse their mouth with pure water and clean their teeth (without using tooth-paste or disinfectant) before producing the sputum specimen. It is even better if the patient produced the specimen before breakfast or on an empty stomach.

Precipitated stains: Though these are quite easy to differentiate from acid-fast bacilli, they may hamper reading or occasionally mislead an inexperienced microscopist. Precipitates can be removed by filtration of staining solutions. However, it is safer to use freshly prepared solutions, filled into carefully cleaned bottles, rather than stale staining solutions.

Saprophytic acid-fast bacilli: These occur in soil and water, and may occasionally get into the specimen or smear during processing. This can be avoided by using distilled or boiled water from scrupulously clean containers.

Mycobacterium kansasii or Nocardia species: These occasionally occur in specimens. When they cause pulmonary disease, they are usually present in large numbers.

Spores of Bacillus subtilis: These are very rare, mostly of ovoid shape, and larger than tubercle bacilli.

Fibers & pollens: Fibers, including those of wood, cotton, filter paper and bamboo, usually occur singly, most often in only one microscopic field. The pollen of certain pine trees is seen as short, coccoid rods occurring very rarely in specimens.

Scratches on the slide: Scratches may sometimes retain the red stain and confuse beginners. They are usually seen in parallel rows, are generally longer than acid-fast bacilli, and are undulated. They can be identified easily, because they are found in a deeper layer on the slide, below the smear disappearing when the cells

(e.g., leucocytes) in the smear get focused on.

- **Contamination through the transfer of bacilli from one smear to another**

It may happen that acid-fast bacilli are transferred accidentally from a positive slide to a negative one, when several slides are treated simultaneously in staining or decolorisation tanks. This can be avoided by processing each slide separately, e.g., on a rack. Such racks are usually made of wire and can be decontaminated easily by flaming.

Acid-fast bacilli may also be transferred accidentally when the glass rod or dropper used for placing immersion oil on the slide touches the surface of a positive slide and rubs off some material. The same can happen when blotting paper is used for drying several stained smear consecutively. Therefore the blotting paper should not be used at all, or for no more than one slide. The oil dropper should not touch the smear, and the oil should be allowed to drip freely on to the slide. For the same reason, the surface of the slide should not be rubbed with the oil immersion objective. Before a new slide is examined, the oil should be wiped off the lens with a piece of cotton tissue or, even better, with special lens-cleaning paper.

When microscopy is used for the detection of acid-fast bacilli, slides should never be used more than once.

False Negative Results

False negative results are commonly due to deficiencies in the preparation of the smear, in staining, and in scanning. Adequate collection of the specimen and subsequent selection of sputum particles are essential to the preparation of a smear and should receive special attention.

Deficiencies leading to false negative results include the following:

- **Inadequate sputum collection**

The patient is sometimes not told clearly enough what constitutes a proper sputum specimen and how he should produce one. It must be made clear to him that saliva and nasopharyngeal discharge are unsuitable for examination. Patients should be encouraged and given time to produce bronchial sputum from the “depths of the chest”. If repeated attempts have failed, tickling of the inner surface of the epiglottis or trachea with a swab, or intratracheal instillation of 5-10 ml of cool saline or sterile water may provoke a vigorous cough with sputum. Other techniques to stimulate the production of sputum, such as aerosol induction, gastric aspiration, and

bronchoscopy, require more complex equipment or special skills.

If a patient discharges acid-fast bacilli in his sputum, these are more likely to be found in a specimen produced in the early morning than in one produced later in the day. If early-morning sputum in required, the patient should be given a container and instructed to place in it the very first sputum he produces in the morning, before breakfast and before taking any medicaments.

- **Improper storage of sputum specimens and stained smears**

Acid-fast bacilli may lose their acid-fastness as a result of exposure of the specimen to direct sunlight, radiation (e.g., ultraviolet light), excessive heat, or storage for more than a week in hot and dry conditions.

If Ziehl-Neelsen stained smears have to be stored for re-examination, the immersion oil must be washed from the smears with xylol because the immersion oil removes the stain from the acid-fast bacilli.

Fluorochrome stained smears will lose their fluorescence with storage.

- **Failure to select suitable sputum particles for smear preparation**

Tubercle bacilli are most likely to be found in little blobs ("lentils") of greenish-grey or yellowish matter of a thick, creamy consistency. (Such blobs usually consist of dead caseous tissue eliminated from a cavity in the lung). If the sputum is not treated by a special concentration procedure involving centrifugation, these blobs have to be carefully separated from the rest of the sputum and transferred to a slide. They can be seen more easily in the sputum against a dark background.

- **Inadequate preparation of smear or staining of slides**

False negative results may be obtained also when:
(a) Too little material has been spread on the slide, so that the smear is too thin;
(b) The smear is too thick, so that sufficient light cannot pass through it;
(c) The slide has been over heated when fixing the smear;
(d) The smear has not been sufficiently fixed and parts of the material have been washed off;
(e) The staining with carbolfuchsin was too short or was overdone by boiling;
(f) The counterstaining was too intensive, so that the acid-fast bacilli have been obscured;
(g) Staining and counterstaining times have not been followed precisely.

- **Inadequate examination of the smear**

If the scanning is done erratically or too briefly, too few fields may be examined. (Occasionally the examiner is unable to distinguish the red-stained acid-fast bacilli because of color blindness or other visual disturbances).

- **Other reasons for false results**

Administrative errors: Such errors may include:
(a) Misidentification of patients, misspelling of names, or confusion of names or of codes numbers of specimens and slides;
(b) Mistakes in labeling containers;
(c) False recording of reporting.

Reading errors: Reader or observer error, which is mainly due to visual or psychological reasons, occurs in practically all diagnostic, clinical and laboratory work. The nature of this phenomenon, sometimes called the "human factor", is to a large extent unknown. Nevertheless, under certain conditions it is measurable. The degree and frequency of error-overreading as well as under-reading varies from one person to another and also within the same individual at different times.

Inter-individual reader variations in smear microscopy has been repeatedly studied and its frequency has been found relatively low compared, for instance, with inter-individual error in say, chest radiography.

It seems likely that many reader errors would be avoided if each microscopist were properly trained and strongly advised to report what he actually saw, and never what he thought he was expected to see. However, discrepancies in the results of smear microscopy are far more often due to deficient sputum collection and smear preparation than due to reader error.

AFB Culture and Isolation

The modern bacteriology has many mycobacteriological media available to it. An ideal medium should be able to produce rapid and abundant growth, enhance phenotype characteristics, inhibit the growth of contaminants and should be usable for antimicrobial techniques. However despite advances, the isolation of *Mycobacterium tuberculosis* is still a slow process ranging from 10 days to 8 weeks.

Solid media: L. J. medium produces a slightly higher rate of TB isolation however it is prone to slant contamination. A good L. J. medium is non-selective, light green in colour, smooth slant without bubble formation so as to view *Mycobacterial* growth

easily. The concentration of Malachite green is critical for achieving a good color contrast for visualisation of *Mycobacterial* colonies. Sub optimal concentration of Malachite green in the medium produces higher contamination rates where as excessive Malachite green can suppress and delay the *Mycobacterium* growth itself.

Agar based medium such as Middlebrook are transparent, allow quicker examination of colony morphology. Middlebrook is more resistant to contamination and produces growth of *Mycobacterium tuberculosis* faster than L.J. medium. Some commercially available 7H11 medium have been modified to increase the amount of Malachite green. Laboratory workers should be careful to determine this, for while the increase content of aniline dye retards growth of contaminating bacteria, it can also inhibit the growth of *Mycobacterium.*

Exposure of 7H10 to strong light or storage of the media at 2-8^{0}C for more than four weeks can be associated with deterioration and release of formaldehyde. The presence of formaldehyde results in a very inhibitory media with little or no growth of *Mycobacteria.*

Both Middlebrook 7H10 and 7H11 can be used for *Mycobacterial* drug susceptibility testing, although 7H11 is preferred.

When laboratories rely primarily on solid medium it will take a minimum of 3 weeks to produce colonies of *Mycobacterium tuberculosis*.

Liquid media: Such as Middlebrook 7H9, Dubos Tween albumin broth and Kirchner medium have been developed for the enrichment of growth of small number of *Mycobacteria.* They are valuable in isolating bacteria from uncontaminated specimen such as CSF, pleura and peritoneal fluids. There is an increased growth rate of *Mycobacterium tuberculosis* in liquid medium. Inclusion of Antibiotic cocktails such as PACT (Polymyxin B, Amphotericin B, Carbenicillin, Trimethoprim) or PANTA (Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin) is required to make the liquid media sufficiently inhibitory to the growth of other bacteria and fungi especially when sputum specimens are used.

It is recommended internationally that specimen for *Mycobacterial* culture should be inoculated in both types of media. According to DIN and DZK guidelines atleast 3 different media should be inoculated, and atleast one of them being a liquid medium.

The different composition of the media and combination of different media have an

impact on the yield and positive cultures, thereby increasing sensitivity of culture & *Mycobacterial* isolation.

Recent Indian studies have also indicated that *'Lowenstein-Jensen'* medium and *'Kirchner's'* liquid medium are the best combination for the isolation of *Mycobacteria* from specimens other than sputum.

Ideally the cultures are incubated at 36±1°C; with an atmosphere of 5-10% of CO_2 being stimulating to the growth of *Mycobacteria*.

Radiometric media: Developed in 1970, represent a significant improvement in the rapid isolation of *Mycobacterium tuberculosis*. Detection time is directly proportional to the number of metabolically active bacteria present and the metabolic rate is influenced by the type of specimen, number of organisms, therapy status of patient, decontamination procedures and the incubation temperature.

The average time for reporting the isolation of *Mycobacterium tuberculosis* using radiometric technique is reportedly 22±9 days as compared to 31±9 days for solid media.

However the radiometric system is more labor intensive, requires disposal of radioactive material and still cannot detect some *Mycobacterium tuberculosis* isolates that can only be detected on agar slants. Some laboratories prefer to use L.J. slants as a backup to Radiometric media. Considering the cost aspects and the fact that *Mycobacterium tuberculosis* is largely a problem of the third world, use of radiometric media is still restricted and use of solid and liquid media is widely practiced.

Susceptibility Testing of *Mycobacterium tuberculosis*

Resistance to antitubercular agents was recognised soon after their introduction in early 1960s, and standardized methods for antimicrobial susceptibility have been developed. Routine laboratory susceptibility testing of primary TB isolates has not been generally suggested unless drug resistance in a particular community exceeds 5%. However with the resurgence of TB drug resistance, C.D.C. USA has recently recommended that susceptibility tests should be performed on all primary isolates.

In a recent Indian study, a total of 3181 samples were processed for isolation of tubercle bacilli; and 707 samples were culture positive. The pattern of drug resistance is shown in the following table:

Pattern of Drug Resistance for *Mycobacterium tuberculosis*

Drug	Per cent Resistance
Isoniazid	30.41
Rifampin	58.55
Streptomycin	46.95
Ethambutol	3.67
D Cycloserine	24.32
Kanamycin	14.42
Ethionamide	60.67
Amikacin	15.84
Ciprofloxacin	7.49

* Adapted from Bombay Hospital Journal; Drug Resistance in Tuberculosis; by Lina Deodhar et al. April 1999.

In India, it has been observed that private practitioners use different drug regimens to treat tuberculosis and very few regimens match with the standard (recommended by W.H.O.).

The problem of acquired drug resistance (ADR) is truly man made. Poor administered *Tuberculosis* control programme, inadequate dosages, monotheraphy, insufficient durations of treatment, irregularity in drug intake, frequent defaults are some of the common reasons for emergence of ADR. In addition, HIV is quickening the pace at which *Tuberculosis* is spreading. Therefore, *Tuberculosis* is becoming the leading killer disease of HIV-positive people.

Clinicians should ensure that *Mycobacterium tuberculosis* susceptibility tests are carried out for patients:

- Who fail to respond after 3 months of treatment;
- Who do not convert to having negative smears after 3 months of treatment; with regimens that included INH and Rifampin, and 5 months for treatment without INH and Rifampin.
- Whose smears demonstrate increasing number of AFB after an initial decrease;
- Patients whose cultures do not become negative after 4-6 months;
- Patients who relapse.

TB susceptibility testing has three main goals:

- It provides data as to what drug should be used for treatment;
- Screens for drug resistance;
- Measures incidence and prevalence of drug resistance within the community.

Susceptibility Testing Methodology

Susceptibility tests can be performed directly, from a smear positive specimen, or indirectly, from the growth of colonies from the specimens. The former has the advantage of measuring the sensitivity prior to cultivation on laboratory media. The direct method also produces results more rapidly but; because of uncertainty on the species of *Mycobacterium*, and due to less control of the viable inoculum size, the results require confirmation with an indirect test, the direct test is not generally utilized.

Three methods make use of critical concentrations to define drug resistance and can be performed directly or indirectly:

- Absolute concentration method;
- Resistance ratio method;
- Proportion method.

The absolute concentration method determines if 1% or more of an inoculum will grow after being cultured on media containing critical concentrations of a drug on the plate. It requires growth of the patient strain on drug free medium to demonstrate the viability, but does not compare the colony numbers on drug free and drug containing media so that the inoculum must be carefully standardized.

The resistance ratio is similar to the absolute concentration method except that the patient strain is compared with the growth of a standard laboratory strain. Results are reported as the ratio of the MIC of the patient strain to that of the laboratory strain. A patient strain with a ratio of 8:1 is considered resistant, while 4:1 is suggestive of resistance. This method is more tolerant to variation in concentrations of drugs within different batches of media.

The proportion method compares the growth of a patient strain in the presence and absence of a drug. If 1% or more of the inoculum produces colonies on media that contains an agent at the critical concentration compared with controls, the isolate is considered to be resistant. This method is the most popular and is relatively simple to perform and interpret.

Susceptibility Testing of *Mycobacteria*

Eleven drugs are used in the treatment of *Tuberculosis*. Five are considered “primary” and include Streptomycin, Isoniazid, Rifampin, Pyrazinamide and Ethambutol, while the remaining six, Ethionamide, Ciprofloxacin, Kanamycin, D cycloserine, para-Aminosalicylic acid and Amikacin are considered “secondary” and used only when resistance develops to the primary drugs.

Although drugs have been incorporated in inspissated egg-based media for conducting susceptibility tests, many laboratories internationally now prefer using Middlebrook 7H11 or 7H10 as a base medium, adding the drugs after cooling the agar to 45^0C. Adding the drugs to the agar medium after autoclaving decreases the loss of activity that can occur in egg-based medium such L.J. during inspissation. An additional loss of drug activity may occur in egg-based media with binding of some agents to egg albumin and other proteins.

Drug Concentrations for Proportion Method Susceptibility Testing using various culture media*

Drug	Drug Concentrations (μg/ml)		
	7H10	7H11	Lowenstein-Jensen
Isoniazid	0.2, 1.0	0.2, 1.0	0.2, 1.0
p-Aminosalicylic Acid	2.0	8.0	0.5
Streptomycin	2.0	2.0	4.0
Rifampin	1.0	1.0	40.0
Ethambutol	2.0	7.5	2.0
Ethionamide	5.0	10.0	20.0
Kanamycin	5.0	6.0	20.0
Capreomycin #	10.0	10.0	20.0
D Cycloserine	20.0	30.0	30.0
Pyrazinamide	50.0	-	100.0

Amikacin & Ciprofloxacin are used in L.J. medium at 20μg/ml & 20μg/ml concentrations respectively, adapted from Bombay Hospital Journal; Drug Resistance in Tuberculosis; by Lina Deodhar et al. April 1999.

* Adapted from Clinical Diagnosis & Management by Laboratory Methods, Todd, Sanford & Davidsohn, 17th Edition 1998, Edited by J. B. Henry and Gradwohl's Clinical Laboratory Methods & Diagnosis; Edited by A. C. Sonnenwirth & L. Jarett. Vol.2, 8th Edition, 1982.

A simplified method for preparing drug susceptibility plates has also been developed. This method uses filter paper disks containing the primary antitubercular drugs, and the test for susceptibility is run in a similar fashion as the Kirby Bauer method for routine drug susceptibility tests.

As discussed, the direct *Mycobacterial* susceptibility test is inoculated from digested and concentrated sputum found to be positive for acid-fast bacilli. The indirect susceptibility test is inoculated from colonies isolated from a primary culture. The direct test will usually give good results only if large numbers of *Mycobacteria* are present in the specimen. The advantage of the direct susceptibility test is an earlier report (three to four weeks) in contrast to the indirect test, which may take up to six to eight weeks. The disadvantage of the direct susceptibility test is that it usually

requires a large number of *Mycobacteria* for successful growth and is often overgrown by large numbers of contaminating bacteria.

Other novel methods of susceptibility testing have been developed based on the mycobacteriophage technique, using the luminiscent luciferase activity. Other researchers have localized specific *Mycobacterium Tuberculosis* mutations responsible for drug resistance. These sites have been used as amplification targets and promise to provide a rapid method for testing the suspetibility of patient isolates to these drugs.

Other Markers

Adenosine deaminase, a surrogate marker, for the diagnosis of *Tuberculosis* has also shown promise. It is based on the measurement of activity of Adenosine deaminase, an enzyme produced by lymphocytes. The test has excellent sensitivity for TB meningitis and for examining pleural infections.The sensitivity and specificity is reported well above 90%, the test is easy to perform and relatively inexpensive.

To conclude, the objective of adapting different types of technology and instruments is to shorten the times for isolation, identification and susceptibility testing of bacteria and other microorganisms has been particularly relevant for *Mycobacteria*. Hopefully alternative methods to the standard procedures now used, could be developed soon enough for routine use, to provide cultures and susceptibility information in a shorter time interval. Till such time the AFB staining, culture and sensitivity remain the Gold Standard for accurate and early diagnosis of *Tuberculosis*, improvements and standardisation of techniques for these classical methods is important for better laboratory diagnosis and clinician information support. Significant cost savings might be effected by a reduction in hospitalization and return of the patient to a productive career.

References and Suggested Reading

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