An Update on the Laboratory Diagnosis of Tuberculosis

Khurshid Ahmad¹, Farhat Khurshid²
¹ Consultant Microbiologist, Islamabad Diagnostic Centre; Scientist Emeritus, National Institute of Health, Islamabad
² Department of Microbiology, Islamabad Diagnostic Centre, Islamabad

Introduction

Tuberculosis is one of the most serious diseases afflicting mankind since times immemorial; it has been present in all ages and climates.¹ Using the most modern DNA techniques, presence of Mycobacterium tuberculosis has been confirmed in the lesions from the lungs of a 1000-year old mummy in Peru.² Recent decades have witnessed resurgence in tuberculosis, both in the developing as well as in developed countries – more people are currently dying of TB than at any other time in human history. According to WHO estimates, one third of the entire human population is presently infected with TB, most of it in developing countries; a person is newly infected every second. Up to 8 million individuals develop active TB infection, with 2.9 million deaths, annually.

Among the 22 countries that have the highest burden of TB cases in the world Pakistan is listed in the top eight.³ Prevalence of pulmonary tuberculosis in Pakistan among adults over the age of 15 years stands confirmed at 295 per 100,000 populations.⁴ At least 400,000 new TB cases occur every year including an alarming 15000 cases of multi-drug resistant tuberculosis. These disturbing statistics call for a redoubling of efforts to properly diagnose and treat all those who have been infected with tuberculosis.

Mycobacteria

Microorganisms belonging to the genus Mycobacterium are thin, non-motile, non-sporing and non-capsulated rods measuring 0.2-0.4 x 2-10um. Three characteristic features differentiate these organisms from other pathogenic bacteria: a) they cannot be stained by the classical Gram stain, b) they are acid-fast – once stained, they resist decolourisation by acid - hence the name Acid Fast Bacilli or AFB; both these characteristics owe their occurrence to the high lipid content of the cell wall, and c) they have extremely slow growth in culture – 2-8 weeks as opposed to 18-24 hours for other common pathogens. Currently the genus Mycobacterium comprises more than 70 recognized species.⁵ Based on their fundamental epidemiological attributes, mycobacterial species are divided into two major groups:

- **Mycobacterium tuberculosis** complex (MTB Complex). Comprises 3 major species:
  - Mycobacterium tuberculosis
  - Mycobacterium bovis (including M. bovis BCG)
  - Mycobacterium africanum

- **The Non-tuberculous Mycobacteria** (NTM) – also called Atypical Mycobacteria: All mycobacterial species other than MTB complex have been included in this group.⁶

Species belonging to Mycobacterium tuberculosis complex occur in humans (and animals); although all three are capable of causing disease, Mycobacterium tuberculosis is the predominant species responsible for tuberculosis in humans. Differentiation of these three species for routine microbiological diagnosis is not only difficult and complicated but also of little or no medical importance in the diagnosis and clinical management of the disease except when required for epidemiological and public health purposes.³

Tuberculosis primarily affects the lungs. Infection occurs by inhalation of infectious airborne droplet nuclei containing Mycobacterium tuberculosis exhaled during coughing and sneezing (even speaking and singing) by “open” cases of TB lungs. Therefore pulmonary tuberculosis is by far the commonest form of disease produced by these organisms; also it has probably the smallest infective dose of any infectious disease – inhalation of a single viable organism of MTB complex has been shown to lead to infection.⁷ In a small percentage of infected patients, extra-pulmonary TB occurs which may affect the lymph nodes, gastrointestinal tract, central nervous system (meningitis), bones and joints, peritoneum, genitor-urinary tract, pericardium etc.

Outcome of primary infection with MTB complex is variable; a) in the majority the cellular immune system of the host is able to contain the organisms; bulk of the organisms is killed; those that survive are surrounded by T cells and macrophages to form granulomas which limit their multiplication and spread. After a variable period of time 15-20% of these people develop clinical tuberculosis. b) In others the mycobacteria do not produce disease but lie within the granulomas in a dormant state for years and decades. Such people have a latent TB infection (LTBI).
but not clinical tuberculosis. c) A variable percentage of primary infections (up to 5%) develop directly into progressive primary tuberculosis. Thus tuberculosis can be **active** which is symptomatic and highly infectious or it can be **latent and inactive** without any symptoms. Individuals with latent TB infection are at the risk of developing active disease whenever their immune status changes (e.g. due to immunosuppressive therapy or HIV infection) or clinical circumstances are altered. Each person having a LTBI has about 10% chance of progressing to active disease.

**Tuberculosis and HIV/AIDS**

HIV/AIDS infection has played a crucial role in the resurgence of tuberculosis particularly in Africa and South Asia. TB and HIV/AIDS are fatally synergistic. Immunity to MTB complex is primarily cell-mediated. Because of suppressive effect of HIV on cell mediated immune function, co-infection with HIV greatly increases the risk of developing active tuberculosis with rapidly developing primary disease, instead of latent infection, and markedly accelerates its progress. WHO estimates that up to 40% of AIDS patients in Africa and South Asia die of tuberculosis.

**Safety Precautions**

Organisms of MTB complex carry a very high risk of laboratory acquired infection (Hazard Group 3). Even the simplest procedures like making AFB smears should be undertaken using disposable gloves and a face mask. Extreme care should be taken when sterilizing the inoculating loop after making smear. Any amount of sputum remaining in the loop spurts in the flame and may create aerosols. This maneuver should be performed using a hooded flaming device. It is safer to use a tooth pick instead of wire loop which can be discarded in disinfectant without flaming. Beyond this, all procedures on TB specimens should be carried out in a bio-safety level 2 facility (Class II safety cabinet). WHO further advises that all laboratories handling TB cultures, must have Biosafety level 3 (BSL-3) facilities.

**Collection and Transport of Clinical Specimens**

Successful detection and isolation of MTB complex depends on the type and quality of the specimen obtained from the patient which in turn is determined by the clinical presentation of the case.

**Pulmonary tuberculosis**

Spontaneously produced early morning sputum from a deep and vigorous cough is the specimen of choice. It should be collected in a clean (not necessarily sterile), dry, wide-mouthed, leak-proof container and should be free from saliva. Patients should be asked to cover their mouths carefully during sputum collection or they should be asked to collect it in a bath room. Up to three specimens on three successive days are usually recommended for detection of AFB by smear microscopy; at least one, if not all three, must be early morning sample. Other pulmonary specimens may include broncho-alveolar lavage, broncho-alveolar brushings and trans-tracheal aspirate.

**Extra-pulmonary Tuberculosis**

Most common specimens from cases of extra-pulmonary tuberculosis consist of body fluids (including pleural, peritoneal, pericardial, synovial and CSF), urine and pus. Others include lymph nodes, bone marrow, gastric aspirate and tissues. From cases of renal tuberculosis, mid-stream urine is collected on three successive days. Specimens are transported to the laboratory immediately, at most within 2 hours of collection. In case of delay, they should be refrigerated.

**Diagnostic Modalities Currently Available**

The clinical microbiology laboratory is faced with the challenge of providing a **rapid, reliable and cost effective diagnosis** of both active and latent tuberculosis. Techniques presently available include the following:

**Conventional techniques**

1. AFB Smear Microscopy
2. Tuberculin Skin Test (TST) – (Mantoux test)
3. AFB Culture – Lowenstein-Jensen (LJ) solid medium

**New technologies**

1. BACTEC MGIT 960 Culture System (Becton Dickinson, USA)
2. Interferon Gamma Release Assays – IGRAs
   a. Quantiferon TB Gold
   b. T-SPOT.TB
3. Nucleic acid Amplification Tests (NATs) - PCR

**Serological tests**

1. Rapid Immunochromatographic assays (ICTs)
2. Enzyme-linked Immunosorbent Assays (ELISA)

Except for the basic procedures of smear microscopy and tuberculin skin test, most of the advanced technologies, including culture, conventional as well as MGIT, described herein belong to the domain of specialized tuberculosis laboratories.

**AFB Smear Microscopy**

In Pakistan and other resource poor countries with a high TB prevalence, smear microscopy continues to be the mainstay of TB diagnosis. Because of its simplicity and minimum requirements in terms of equipment and supplies,
it is by far the most widely used procedure in TB diagnostics not only in detecting TB cases but also in establishing their infectiousness.

AFB smear microscopy may be performed directly or after concentration.

**Direct smears**
A loopful from a thick purulent portion of the sputum is spread evenly on a slide to make a smear taking care that it should neither be too thin nor too thick. A slide which has already been used for sputum smear and washed should never be reused for making a fresh smear. The smear is air-dried and fixed by gently heating the slide on the flame.

**Concentration method:** AFB can be concentrated by centrifugation after treating the sputum with an equal volume of 5% sodium hypochlorite (commercial bleach) for 10-15 minutes which also renders the specimen non-infectious. Concentration technique significantly increases the sensitivity of smear microscopy (by up to 50%) for the detection of AFB in sputum.

**Staining**
Smears are stained by the standard Ziehl-Neelsen technique or by Auramine-O stain (if facilities for fluorescent microscopy are available). ZN staining is a hot stain procedure as it requires heating the slide for better penetration of stain into the mycobacterial cell wall.

**Examination, interpretation and reporting of AFB smears**
ZN stained smears are examined under oil immersion. While applying oil on the smear the slide should never be touched with oil applicator or the nozzle of oil-containing bottle. A small oil droplet should be dropped on the smear without touching it. Examination of smears should be carried out by scanning at least 300 oil immersion fields (roughly equivalent to 3 horizontal sweeps over an area 2 cm x 1 cm) before giving a negative result. Oil should be wiped off from the objective before examining the next smear, especially in case of a positive smear; AFBs from a positive smear may come off the slide and float in the oil remaining on the objective and may give a false positive result while examining the next smear. In a well stained smear, typical acid fast bacilli, when present, appear as red or purple, slightly curved rods, 2-8 um long, against a crisp blue background of mucous threads and debris. Bacilli of MTB complex often appear beaded or banded (Fig.1).

**Reporting**
The following information should be included in the final report: Specimen quality (Thick, mucoid, muco-purulent, muco-salivary, blood stained etc), staining method used (ZN stain), quantification or grading of result.

If positive, result should be reported only as “Acid Fast Bacilli seen” with quantification/grading as follows (WHO / CDC):

<table>
<thead>
<tr>
<th>Number of AFB / Oil Field</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 9 AFB / oil field</td>
<td>(++++)</td>
</tr>
<tr>
<td>1 – 9 AFB / oil field</td>
<td>(+++)</td>
</tr>
<tr>
<td>1 – 9 AFB / 10 oil fields</td>
<td>(++)</td>
</tr>
<tr>
<td>1 – 9 AFB / 100 oil fields</td>
<td>(+)</td>
</tr>
<tr>
<td>1 – 2 AFB in the entire smear</td>
<td>Doubtful. Repeat on fresh specimen</td>
</tr>
</tbody>
</table>

**Sensitivity and specificity of AFB smear microscopy**
An AFB stained smear from clinical specimens requires at least 5000-10000 bacilli per ml for detection. In skilled and experienced hands, overall sensitivity and specificity of smear microscopy is excellent – up to 80% and 100% respectively. There have been suggestions that sputum smear microscopy may be more reliable for evidence-based diagnosis of pulmonary tuberculosis than X-ray chest which may often result in over-diagnosis.

Use of direct ZN microscopy for spinal, pleural and other body fluids for detection of AFB is an unrewarding exercise; some experts consider it a wasteful use of time and energy as these samples are rarely positive. Many laboratories have stopped reporting direct AFB smears on CSF and other effusions. However a highly specialized test, adenosine deaminase level, is now available for the reliable diagnosis of tuberculous effusions particularly the pleural and ascetic effusions.

**AFB Fluorescent microscopy**
Fluorescent staining using auramine O stain is more sensitive than conventional ZN staining but this advantage is more than offset by the high cost of fluorescent microscope and reagents as well as the requirement of highly trained and experienced staff for interpretation. It has been strongly recommended that all positive results of auramine staining must be confirmed either by a second trained observer or by staining the same smear with ZN stain.

**Tuberculin Skin Test (TST) – Mantoux test**
Next to smear microscopy, tuberculin skin test, in spite of its limitations, is probably still the most commonly performed test for detection of tuberculosis. Introduced by Robert Koch nearly 100 years ago, it is still used as a standard screening test in the United State for latent TB infection. Reaction is based on the premise that infection with MTB complex elicits delayed type hypersensitivity based on cell mediated immune response to antigenic components of MTB complex. In the standard Mantoux skin test, 0.1 ml of purified protein derivative (PPD) from tubercle bacilli containing 5 tuberculin units is injected intradermally on the forearm. After 72 hours, the site is examined for induration (area of firmness as a result of influx of immune cells). The diameter of induration (not erythema which is usually present with or without induration) is measured. Result is
reported according to the interpretative criteria given in table 1. A positive Mantoux skin test establishes exposure to tuberculosis but does not distinguish between active and latent infection. Infection with non-tuberculous mycobacteria and BCG vaccination may yield a false positive tuberculin skin test; BCG effect may last for several years. On the other hand a negative skin test does not rule out the presence of tuberculosis.

Cultural methods

Specimen processing: Sputum and other specimens from sites containing resident flora require decontamination and digestion before culture. 4% NaOH or a mixture of NaOH and N-acetyl l-cystein – NALC (a mucolytic agent) not only decontaminates but also digests and liquefies the mucoid material and organic debris which may wall off AFBs from nutrients in the medium. Specimens from sterile sites do not require decontamination. They should only be concentrated by centrifugation.

Conventional AFB Culture

A definitive diagnosis of tuberculosis requires culture which is still considered to be the gold standard for laboratory diagnosis of tuberculous disease. It is much more sensitive than microscopy, being able to detect as low as 10 viable tubercle bacilli per ml of specimen. However, the conventional culture on egg-based solid LJ medium has a long detection time, taking anywhere from 4 to 8 weeks for the growth to appear.

BACTEC MGIT (Mycobacterial Growth Indicator Tube) consists of modified Middlebrook 7H9 broth medium which is known to yield better recovery and faster growth of MTB complex.

| Table 1: Interpretation of Mantoux skin test (5 tuberculin units) |
|--------------------------|-----------------|------------------|
| Area of induration       | Person tested   | Result           |
| 0 – 4 mm                 | All individuals | Negative         |
| 5 – 9 mm                 | - Healthy       | Borderline       |
|                          | individuals     | positive         |
|                          | - Close contacts of active TB cases | Positive |
|                          | - HIV positive | Positive         |
|                          | individuals     | Positive         |
|                          | - Injecting drug users | Positive |
|                          | All individuals |                  |
| = / > 10 mm              |                  |                  |

A fluorescent compound embedded in silicon sensor at the base of the tube monitors oxygen consumption by growing AFB if present in the specimen. Growth of AFB and resulting O₂ depletion is detected as an increase in fluorescence. Fluorescence can be detected visually in UV light, manually by Mini-MGIT or automatically by the MGIT 960 system (Beckton Dickensen, USA) in which the instrument monitors fluorescence every 60 minutes and gives a signal when detected.

<table>
<thead>
<tr>
<th>Table 2: Summary of diagnostic procedures for tuberculosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test</td>
</tr>
<tr>
<td>AFB smear</td>
</tr>
<tr>
<td>microscopy</td>
</tr>
<tr>
<td>Culture</td>
</tr>
<tr>
<td>Medium</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>MGIT</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>IFN-γ release assays - (IGRAs) – T-SPOT.TB, Quantiferon TB</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Bacteriophage based assay -FASTplaque TB</td>
</tr>
<tr>
<td>NA amplification tests – PCR TB</td>
</tr>
<tr>
<td>Rapid Serological tests- ICTs – 20 - 30 minutes</td>
</tr>
<tr>
<td>Conventional ELISA</td>
</tr>
</tbody>
</table>

A schematic representation of the processing of sputum by smear microscopy and AFB culture is given in Figure 2. Footnote: ICT: Immuno-chromatographic Test, IGRA: Interferon Gamma Release Assay, LJ: Lowenstein-Jensen Medium, LTBI: Latent Tuberculosis Infection, MGIT: Mycobacterial Growth Indicator Tube, NTM:: Non-tuberculous Mycobacteria, PCR: Polymerase Chain Reaction, TST: Tuberculin Skin Test. Mean detection time in the MGIT system is 11 – 13 days. Growth is confirmed by ZN staining of a smear from the
culture tube which will show AFBs in groups or cords (cording).

**Fig. 1: Sputum smear strongly positive for AFB – ZN stain**

![Sputum smear strongly positive for AFB – ZN stain](image)

**Fig. 2: Suggested flow diagram for processing of sputum samples for AFB**

![Suggested flow diagram for processing of sputum samples for AFB](image)

**Fig. 3: T-SPOT.TB test showing “spot forming effector T cells”. Each spot represents an IFN-γ-producing T cell in response to TB antigens**

![T-SPOT.TB test showing “spot forming effector T cells”. Each spot represents an IFN-γ-producing T cell in response to TB antigens](image)

**Interferon Gamma Release Assays – IGRAs**

Interferon-gamma release assays (IGRAs) are based on the principle that the T-cells of individuals who have acquired TB infection respond to re-stimulation with *M. tuberculosis* by secreting interferon gamma (IFN-γ). These assays employ specific antigens of *M. tuberculosis* (ESAT-6 & CFP-10) which are shared neither by BCG strains nor (with rare exceptions) by non tuberculous mycobacteria, making these assays virtually specific for MTB complex. The QuantiFERON-TB Gold (Cellestis U.K. - uk@cellestis.com) measures the IFN-γ produced in response to these *M. tuberculosis* antigens in whole blood. In contrast, the T-SPOT.TB assay (Oxford Immunotec UK) detects the number of “spot forming effector T cells” in peripheral blood which produce IFN-γ in response to the above antigens (Fig 3). Like Mantoux skin test, IGRAs do not distinguish between active and latent TB infection. However, unlike skin test, these assays are not affected by BCG and by most non-tuberculous mycobacteria.

**Nucleic acid Amplification Tests (NATs) - PCR**

NATs have been successfully used for the direct detection of MTB in sputum and blood. In spite of being rapid, highly sensitive and specific, these tests suffer from a number of limitations: they are too expensive to be used in countries where they are most needed, require special expertise and may not be specific for active infection as DNA from a dead organism can be amplified and detected by PCR with equal efficiency. Assays from Roche and Gen-Probe have been approved by FDA for use on AFB-smear-positive specimens only because of less than optimum sensitivity on smear-negative specimens.

Recently WHO has endorsed a NA assay for TB control programmes in developing countries by the name of GeneXpert MTB/RIF. It is an automated, cartridge-based NA amplification assay specifically for the simultaneous detection of MTB complex as well as rifampicin resistance directly from sputum samples in less than two hours.

**Bacteriophage-based assay – FASTplaque TB**

FASTPlaque TB (Biotec UK) utilizes the ability of a specific mycobacteriophage (Actiphage) to lyse and destroy viable MTB complex in decontaminated sputum. The test takes 48 hours to complete but is technically demanding and can only be used for sputum specimens.

**Serological tests**

Antibody-based serological tests for the detection of antibodies to MTB complex, including immunochromatographic assays and ELISA, are simple to use, comparatively inexpensive and easily available. Despite widely being used in routine laboratories, these tests have important limitations as clearly stated in the following WHO recommendations:

- “It is strongly recommended that these commercial tests not be used for the diagnosis of pulmonary and extra-pulmonary diagnosis.”
Currently available commercial sero-diagnostic tests (also referred to as serological tests) provide inconsistent and imprecise results.

There is no evidence that existing commercial serological assays improve patient outcomes, and high proportions of false positive and false negative results may have an adverse impact on the health of the patients.

A summary of diagnostic procedures for tuberculosis and their clinical correlations is given in table 2.

Acknowledgments

We are grateful to Dr. Aftab Ahmad for the use of his excellent camera microscope for taking picture of AFB smear (Fig. 1) and to Mr. Najam Farooq for providing picture of a positive T-Spot.TB test performed at Islamabad Diagnostic Centre (Fig. 3)

References