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# Diagnosis of Tuberculosis in Children (Part 1): Demonstration of the Causative Organism

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*Atypical mycobacterium*
*Auramine- Rhodamine*
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*Cartridge-based nucleic acid amplification test*
*Fluorescent stains*
*Gene Xpert™*
Laboratory Diagnosis
*Lipoarabinomannan*
*Loop-mediated isothermal amplification (LAMP)*
*MGIT culture media*
Mycobacterium
*Polymerase chain reaction*
*Sample collection*
*Sample transport*
Tuberculosis
Ziehl-Neelsen staining

**Abbreviations**
(See - Appendix 1)

## Abstract

*Tuberculosis (TB), despite being a preventable and treatable infection, still remains a major cause of mortality worldwide. Delay in establishing the diagnosis significantly contributes to the mortality. Early diagnosis, especially in children, is faced with several challenges. This article reviews the current knowledge and challenges of TB diagnosis.*

*The philosophy of TB diagnosis is based on two principles: (1) demonstration of the causative organism in the host, and (2) demonstration of the host-reaction to the invading pathogen. Staining and culture demonstrate intact bacilli, while molecular methods demonstrate cellular fragments of mycobacteria. Staining techniques use a unique property of mycobacterial cell wall that resists decolourization with acid-alcohol. Fluorescent stains (e.g. auramine-rhodamine) are better than the conventional Ziehl-Neelsen stain. When the bacterial load is too low to be detected in stained smears, culturing is used to increase the bacterial density. The WHO recommends Bactec MGIT-960™ medium because of its better yield than the conventional Lowenstein-Jensen medium.*

*Dead or multiplying bacilli shed their genetic materials (DNA and RNA) as well as cell wall antigens into the host secretions or circulation. Techniques like polymerase chain reaction amplify the miniscule amount of bacterial nucleic acids and facilitate their detection. The specificity and sensitivity of these techniques are often greater than 90%. Hence, the WHO recommends cartridge-based nucleic acid amplification test (CB-NAAT) as the preferred investigation of childhood TB. Lipoarabinomannan, a cell wall glycolipid specific to mycobacteria, is a useful urinary biomarker of TB, especially in HIV patients.*

"*More human lives have been lost to tuberculosis than to any other disease.*" - *Stewart Cole* [1]

## INTRODUCTION

**T**uberculosis (TB), despite being a preventable and treatable infection, continues to be a major cause of mortality worldwide. Its incidence is 127 per 100,000 of the population. In 2022, over 1.25 million children developed TB and 214,000 died of the infection.[2] Notably, 80% of these deaths occurred in children under the age of 5 years, and 96% of them had never received any anti-tubercular treatment (ATT).[2] Obtaining accurate statistics of childhood TB is challenging for several reasons, including under-recognition of the disease, difficulties in confirming the diagnosis, and under-reporting to national TB programs. Early diagnosis is crucial not only for timely treatment but also to halt disease transmission by identifying and isolating the source of infection.[3] This article (the first of a two-part series) reviews the available methods and the challenges of diagnosing TB in children.

## DIAGNOSTIC CHALLENGES

Several factors contribute to the diagnostic difficulties of pediatric TB.[4,5] The chief among them is the non-specific nature of clinical and radiological features of TB, which are often confused with that of other microbial infections and tumors. Demonstration of the causative organism in children is challenging as they typically have paucibacillary disease. The mycobacterial load is directly proportional to the age of patients. For example, sputum smears were positive for acid-fast bacilli (AFB) in only 7% of children, as compared to 52% in adults.[6] Smear positivity is 14% in children aged 5-14 yr while it drops to 0.5% in those aged 0-4 yr.[6] The diagnostic yield of mycobacterial cultures and molecular tests is also less than 25-40% in children as compared to 60-85% in adults.[7]

Clinical manifestation of TB is a spectrum, ranging from latent tuberculosis infection (LTBI) to active miliary disease. TB can occur in any organ and it is sometimes classified as pulmonary TB (PTB) and extrapulmonary TB (EPTB). Innumerable permutation of these clinical presentations poses unique diagnostic challenges. For example, LTBI is a dormant infection without any overt symptoms. Hence, a clinical suspicion is rarely raised.[8] Sensitivity of TB screening tests seldom exceeds 85% and screening an entire population is impractical due to huge economic implications.

Co-morbidities such as acquired immune deficiency syndrome (AIDS) not only alter the clinical manifestations of TB, but also interfere with the diagnostic tests, especially those based on immunological reactions.[9] Social stigma of TB, lack of awareness, poverty, non-availability of diagnostic tools and lack of expertise are challenges peculiar to resource-limited countries, where TB is endemic.[10] Last, but not least, is the shared morphological and immunological properties of typical and atypical mycobacteria that make differential diagnosis difficult, if not impossible.

## TYPICAL AND ATYPICAL MYCOBACTERIA

Mycobacteria are categorized into three groups: the *Mycobacterium tuberculosis (MTB)* complex, *Mycobacterium leprae* (not relevant to this review article), and non-tubercular mycobacteria (NTM). Typical mycobacteria refer to the species included under the MTB complex that are morphologically similar, but vary in their host specificity, pathogenicity, genetic make-up and biochemical reactions. For example, *Mycobacterium bovis* infects cattle; but can be a zoonosis in human beings. The two subtypes of *Mycobacterium africanum* (East and West African types) have special affinity to black races and are responsible for 50% of TB in sub-Saharan countries. Perplexingly, it is seldom seen outside Africa.[11] *Mycobacterium canettii* specifically affects lymph nodes in children.[12]

Atypical mycobacteria, also known as NTM,[13-17] are mostly non-pathogenic environmental species.

They rarely cause opportunistic infections, particularly in susceptible individuals such as children, patients with human immunodeficiency virus (HIV) infection and those undergoing immunosuppressive treatment.[18-20](Table 1) They may also contaminate laboratory samples and cause diagnostic confusion.[21,22] NTM mimic typical MTB in many aspects including clinical manifestations and staining properties; yet they differ in their genetic make-up, culture characters and drug susceptibility. NTM are classified based on their growth rates (slow vs. rapid growers) and their ability to produce pigments (chromogens vs. non-chromogens).[22] Diagnosis of NTM can be challenging due to their complex morphology and growth characteristics.[18,20]

Table 1. Organs affected by various mycobacteria [#]

| Mycobacterial species | Affected organs |
|---|---|
| ***Typical mycobacteria*** | |
| *M. africanum* | All organs |
| *M. bovis* | All organs (Zoonosis) |
| *M. canettii* | LN |
| *M. caprae,* | All organs (Zoonosis) |
| *M. pinnipedii* | All organs |
| *M. suricatae* | All organs (Zoonosis) |
| *M. tuberculosis* | All organs |
| ***Atypical mycobacteria*** | |
| *M. abscessus †* | Lung, SS, Bone, LN*, DD* |
| *M. arupense* | Bone |
| *M. asiaticum* | Lung* |
| *M. aubagnense* | Lung |
| *M. avium §* | Lung, LN, DD, SS, Bone* |
| *M. bacteremicum* | Bone |
| *M. boenickei* | SS, Bone |
| *M. bolleti* | Lung |
| *M. brisbanense* | SS, Bone |
| *M. canariasense* | Bone |
| *M. celatum* | Lung, DD* |
| *M. chelonae †* | LN, DD, SS, Bone, Lung* |
| *M. chimaera* | Heart |
| *M. conspicuum* | DD* |
| *M. cosmeticum* | Bone |
| *M. flavescens* | DD |
| *M. fortuitum †* | Lung, LN, DD, SS, Bone |
| *M. genavense* | Colon, Lung*, DD* |
| *M. goodii* | Heart valves |
| *M. gordonae* | SS |
| *M. haemophilum* | DD, SS*,Bone*,Lung*, LN* |
| *M. heraklionense* | Bone |
| *M. hiberniae ‡* | SS, Bone |
| *M. houstonense* | SS, Bone |
| *M. immunogenum †* | DD*, SS*, Bone* |
| *M. intracelluare §* | Lung, LN, DD, SS, Bone* |
| *M. iranicum* | Bone |
| *M. kansasii* | Lung, Bone, DD, LN*, SS* |
| *M. kumamotonense* | Bone |
| *M. lentiflavum* | DD |
| *M. longobardum* | Bone |
| *M. mageritense* | SS |
| *M. malmoense* | Lung, LN, DD, SS, Bone* |
| *M. marinum* | SS, Bone, DD* |
| *M. massiliense* | Lung |
| *M. monacense* | Bone |
| *M. mucogenicum †* | DD |
| *M. neoaurum* | Heart |
| *M. neworleansense* | SS, Bone |
| *M. non-chromogenicum‡* | SS, Bone* |
| *M. peregrinum* | SS, Bone |
| *M. phocaicum* | Lung |
| *M. porcinum* | SS, Bone |
| *M. salmoniphilum* | Bone |
| *M. scrofulaceum* | LN, Lung*, DD* |
| *M. senegalense* | SS, Bone |
| *M. shimoidei* | Lung* |
| *M. simiae* | LN, SS, Bone, DD*, Lung* |
| *M. smegmatis †* | SS, LN, Lung*, Bone* |
| *M. szulgai* | SS, Bone, Lung*,LN*,DD* |
| *M. terrae ‡* | SS, Bone |
| *M. thermo-resistibile* | Bone |
| *M. triviale ‡* | SS, Bone |
| *M. ulcerans* | SS, Bone |
| *M. vaccae* | SS |
| *M. wolinskyi* | Heart |
| *M. xenopi* | Lung, Bone, DD* |

*# Source: Griffith[13], Sharma[14], Tran[17], Bittner[15], Brown[16], Pennington[21]*
*DD-Disseminated disease, LN-Lymph node, M-Mycobacterium, SS-Skin and soft tissue.*
** Uncommon organs to be affected*
*† Rapid growers (<7 days on subculture), others are slow growers*
*§ Combinedly known as M. avium intracelluare complex*
*‡ Combinedly known as M. terrae complex*

## PRINCIPLES, CLASSIFICATION AND OVERVIEWS

Basically, TB is diagnosed either by demonstrating the causative organism in clinical samples or by demonstrating the host-reaction to the invading pathogen. (Tables 2 & 3) Microscopic examination of stained smears is the simplest way of detecting mycobacteria. It is suitable for luminal and celomic TB from where infected body secretions can easily be sampled. Although it can be done with fine needle aspiration (FNA) specimens of soft tissues, the diagnostic yield will be relatively poor (18-36%) as compared to secretions (48-75%).

The threshold limit of bacterial load in the clinical sample that can be detected by stained smears is 5000-10000 AFB/ml. If the bacterial density is less than this limit (paucibacillary disease), it has to be increased by culture. Alternatively, fluorescent stains or immunochemical stains may be used to increase the diagnostic yield. Demonstration of the causative organism in histology is extremely difficult even when these special stains are used. This is due to extremely low bacterial density in tissue specimens, thick microscopic sections, and indistinguishable staining pattern of the host cytoplasm from that of the mycobacteria. The common pitfall of diagnosing TB by demonstrating MTB is its inability to distinguish environmental NTM contaminants and real pathogens.

Dead or multiplying AFB shed their cellular debris (genetic materials and cell wall components) into the body fluids and blood circulation. Detection of these cellular fragments, now popularly known as liquid biopsy, provides indirect evidence for the presence of MTB in the host.[23] Modern molecular techniques, such as the polymerase chain reaction (PCR), amplify the miniscule amount of genetic material and facilitate easy detection of species-specific DNA or RNA sequences. As the sensitivity of this approach is usually very high (80-95%), the World Health Organization (WHO) recommends rapid, automated cartridge-based nucleic acid amplification test (CB-NAAT) as the preferred

Table 2: Diagnosis of tuberculosis: Tests based on causative organism

| Goal of test | Examples |
|---|---|
| Detection of intact organism | Smear study<br>Hot stains<br>Ziehl-Neelsen stain<br>Wade-Fite Stain<br>GMS stain<br>Cold stains<br>Kinyoun stain<br>Hallberg stain<br>Gabbett stain<br>Tison stain<br>Zhao stain<br>Fluorescent stains<br>Auramine-O<br>Auramine-Acridine orange<br>Auramine-Rhodamine<br>Immunochemical tagged stains<br>pAbBCG<br>Anti-MPT64 antibody<br>Electron microscopy<br>Culture*<br>Solid medium<br>Liquid medium<br>Indicator medium<br>Fine needle aspiration cytology<br>Immunohistochemistry |
| Detection of bacterial DNA or RNA | Polymerase chain reaction (PCR)<br>LAMP<br>Reverse transcription-PCR<br>Truenat™<br>CB-NAAT<br>GeneXpert MTB/RIF™<br>GeneXpert MTB/RIF Ultra™<br>GeneXpert MTB/XDR™<br>GeneXpert Omni/Edge™ |
| Detection of bacterial cell wall antigen | Lipoarabinomannan (LAM) |

** See Table 4 for more details*
*CB-NAAT-Cartridge-based nucleic acid amplification test; GMS-Grocott methenamine silver; LAMP- Loop-mediated isothermal amplification; Truenat-Taqman RTPCR based nucleic acid test*

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Table 3: Diagnosis of tuberculosis: Tests based on host reaction

| Goal of test | Examples |
|---|---|
| Direct proof of tissue changes | Conventional histopathology<br>Immunohistochemistry<br>Fine-needle aspiration cytology<br>Imprint cytology |
| Indirect proof of tissue changes (Imaging) | Radiography<br>Ultrasonography<br>Computed Tomography Scan<br>Magnetic Resonance Imaging<br>Positron Emission Tomography<br>Isotope scans |
| Demonstration of in-vivo cell mediated immunity | Mantoux test<br>Tine test†<br>Heaf test† |
| Demonstration of in-vitro cell mediated immunity | CD4+ T-cell response to Mycobacterium† |
| Demonstration of in-vivo humoral immunity | Anti TB IgG (against Mce1A)<br>Anti TB IgM |
| Demonstration of in-vitro humoral immunity | Interferon-gamma release assays (IGRA)<br>QFT Gold (QFT-G)™ ELISA<br>QFT Gold-Plus (QFT-Plus)™<br>QFT Gold-in-tube (QFT-GIT)™<br>T-SPOT.TB assay<br>TB-Feron ELISA™<br>A-60 ELISA<br>MagPlas ELISA |
| Detection of host-based biomarkers† | Diacetylspermine<br>Hydroxykynurenine<br>Mass-to-charge ratio 241.0903<br>MPT64<br>N-acetylhexosamine<br>Neopterin<br>PPE17 (Rv1168c) protein<br>Sialic acid<br>Ureidopropionic acid<br>*(Continued)* |
| Detection of non-specific inflammatory markers | Erythrocyte sedimentation rate<br>Leukocyte Count<br>C-Reactive Protein<br>Adenosine deaminase<br>Interleukins (IL-6)<br>Lactate dehydrogenase (LDH)<br>VEGF†<br>Tumor necrosis factor (TNF) |
| Non-biological inference interface | Artificial intelligence (AI) |

*† Not routinely practiced now*

*CD - Cluster differentiation; ELISA-Enzyme linked immuno sorbant assay; QFT-QuantiFERON-TB; T.SPOT-TB assay-Tuberculosis-specific enzyme-linked immuno-spot assay; MagPlas-Magnetoplas-monic; VEGF- Vascular endothelial growth factor*

diagnostic method in pediatric TB where the AFB load is typically low.[5,24] Nonetheless, the high sensitivity of molecular methods is also a disadvantage that they leading to over diagnosis. They simply detect cell debris rather than live bacilli. Thus, false positivity is resulted from the residual debris that persist even after several months of successful treatment.[25] Further, many of the children with incipient and subclinical TB detected by CB-NAAT, may not proceed to develop clinical infection thanks to the immune system.[26]

Among the diagnostic methods based on the host response to bacterial invasion, histopathology is the most reliable. Unique antigens and chemicals expressed by MTB cause a distinct inflammatory reaction with granuloma formation. Histological demonstration of this characteristic tubercle is diagnostic of TB, particularly in endemic areas. Histology is suitable for TB affecting any organ, especially the extra-luminal lesions. However, it requires a surgical operation to secure the specimen. FNA is a surrogate of histopathology, but it is inferior to tissue biopsy in terms of detecting granuloma (49% vs. 98%), demonstration of AFB (9% vs. 17%) and culture positivity (40% vs. 70%).[27] Very rarely other diseases (e.g. sarcoid-

osis) may mimic TB in histology, especially in the evolving phases of granuloma.[28]

Ability of the host immune system to mount an attack on mycobacteria could be an indirect evidence of microbial invasion. The humoral and cell-mediated immune responses can be measured either in-vitro or in-vivo. (Table 3) However, these antibody-based tests tend to over diagnose clinical infection, as they are also positive in healthy individuals with adequate immunity against TB. Further, in immuno-compromised children these tests will be negative despite active infection. For these reasons, the WHO discourages these tests in routine practice. Intradermal tuberculin test is an exception to this notion and it is widely used as screening tool because of its simplicity and cost-effectiveness. Antibody-based tests are also useful in diagnosing LTBI, assessing the effectiveness of anti-TB vaccination and predicting the risk of developing TB in high-risk individuals such as the contacts.[29]

Apart from the foregoing specific evidences of mycobacteria, several indirect, non-specific investigations may raise a suspicion of TB. The chief among them are radiological shadows caused by tubercular granuloma and its sequelae. They are studied using a variety of modalities such as plain or contrast radiography, ultrasonography, computed tomographic (CT) scan, magnetic resonance imaging (MRI), positron emission tomography (PET) and scintigraphy. Although they can identify lesions of internal organs, the images are neither pathognomonic of TB, nor are detectable in the early stage of the disease. Nevertheless, they are important tools for screening for TB and monitoring the therapeutic progress.

TB, being an infectious disease, causes an increase in the serum levels of certain inflammatory markers (e.g. erythrocyte sedimentation rate, adenosine deaminase). Despite being non-specific, they are often used as corroborative indirect evidences of chronic inflammation. They are also useful in monitoring the response to ATT. As they are cheap and easy to do, they are widely used as surrogates for complex molecular tests.

From the foregoing description, it is obvious that no single test can confidently establish or exclude the diagnosis of TB. Complementation of more than one test and correlation of clinical features are the essential principles of TB diagnosis.

## SPECIMEN SAMPLING

### Sample Collection and Its Types

Accurate diagnosis of TB depends on appropriate collection, transportation and handling of the specimen.[30] It is essential to avoid contamination of NTM from environmental sources (e.g. tap water), as this will result in false-positives. TB is clinically classified into pulmonary and extrapulmonary infections. Extrapulmonary TB may be luminal (affecting the gastrointestinal and genito-urinary tracts), celomic (involving pleura, peritoneum, pericardium and meninges), soft tissual and bony infections. Specimens appropriate for diagnosing TB depend upon the affected site.

Sample collection is relatively non-invasive in luminal TB as sputum, urine and stool can be obtained easily. But they are at a high risk of contamination with commensal flora and hence require at most care in collection and handling.[31] Samples of extra-luminal lesions are generally not contaminated; but they require varying degrees of invasive sampling, which ranges from needle aspiration to open biopsy by craniotomy or thoracotomy. In case of celomic TB, aspiration of pleural, pericardial, ascitic or cerebro-spinal fluid provides an uncontaminated specimen. Soft tissue and bone TB requires FNA, core-needle biopsy or tissue sampling by endoscopic or open biopsy. The biopsy specimen should be dispatched to the lab in normal saline for mycobacterial detection, and formalin for histology. Cut surface of the biopsied tissue may be pressed against a glass slide for

imprint cytology. Homogenate of biopsied tissue may be used for culture and other microbiological studies.

## Microbial Sampling of Pulmonary TB

*Sputum sampling*

Sputum is the best material to detect the causative organism in aero-digestive TB. Originally examination 3 sputum smears obtained on two days was recommended.[32] In the 'Spot–Morning–Spot' strategy, a spot sample is collected at the first hospital visit, followed by an early morning sample collected at home, and a third spot sample collected during the second visit. However, for operational convenience WHO now recommends only 2 spot samples (Spot-Spot) collected on the same day. Although early morning sputum was held to have higher concentrations of AFB, recent studies confirmed that spot samples are also reliable.[125]

*Induced sputum sampling*

When young children cannot produce enough sputum by expectoration, several induction techniques are employed to increase the sputum yield. They include pre-sampling inhalation of bronchodilators, nebulization with 3-5% hypertonic saline and nasopharyngeal aspiration.[33-35] Lung Flute™ is a new device that works on the principles of oscillatory positive expiratory pressure (OPEP) facilitating sputum collection in young children.[36] (Fig. 1) The reeds in it generate 18–22 Hz sound waves at 110–115 dB with 2.5 $cmH_2O$ pressure.[37] These vibrations travel through the tracheobronchial tree, loosen mucus and enhance mucociliary clearance of the sputum.

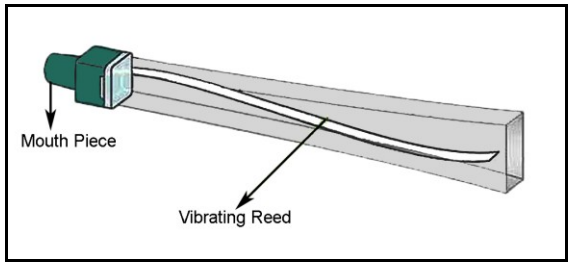


**Fig 1.** *Lung Flute™ for induced sputum collection*

*Gastric Aspirate sampling*

Young infants who have not learnt to spit sputum tend to swallow it. Hence, the standard practice in them is to collect two to three fasting gastric aspirates on consecutive mornings.[38,39] About 5-10 ml of gastric content should be aspirated using a nasogastric tube. If the secretions are inadequate for sampling, saline lavage can be used to augment the yield.[40] It is essential to neutralize the acidity of gastric samples by adding sodium bicarbonate before dispatching them to the laboratory.[41,42]

*Stool sampling*

Drancourt recommended against routine use of gastric aspirate because, stool samples are equally effective in detecting the swallowed AFB.[43] Stool samples, though diagnostic of gastrointestinal and pulmonary TB, are notorious to be contaminated with NTM and colonic commensals. They should be collected from diapers or cling-wrap placed on the toilet seats, and a nylon water-proof material should be used for infants with liquid stools.[44,45]

*Endoscopic sampling*

Broncho-alveolar lavage done using bronchoscopy is specifically useful in sputum-negative PTB or paucibacillary disease.[46] However, it is invasive, costly, requires endoscopic expertise and is not universally available.[47].

## Microbial Sampling of Extrapulmonary TB

Demonstration of mycobacterium in extrapulmonary TB (EPTB) is often difficult due to low mycobacterial load and anatomical inaccessibility.[48] The choice of diagnostic sample depends on the anatomical site of the lesion.[49] Frequently EPTB is diagnosed by molecular diagnostics or by invasive (endoscopic or open) biopsy.

*Fine-Needle Aspiration (FNA) Sample*

FNA provides satisfactory sample for a wide range of investigations such as smear staining, culture, molecular diagnostics, immunochemistry and cyto logy.[50] Of particular interest, cytology is a surro-

gate of histopathology. Unlike histology, FNA cytology does not show the arrangement pattern of inflammatory cells. Yet, demonstration of typical cells such as AFB, epithelioid cells, Langhans giant cells and caseation necrosis are sufficient to diagnose TB in endemic areas.[51] FNA can be done at bedside and is highly suitable for lymph nodal, intrathoracic, intracranial and abdominal lesions wherein a major surgery for securing specimen can be avoided. FNA can be done blindly or under imaging guidance. It is not ideal for luminal TB and lesions less than 1 cm in diameter. FNA will also miss the diagnosis in HIV-positive children infected with *Mycobacterium avium* as they evoke poor granulomatous reaction.[50]

*Imprint Smears*

Imprint cytology was first described by Dudgeon and Patrick in 1927. It is suitable for solid organs with TB granuloma. Cut section of the surgically excised specimen is pressed against a glass slide, which is then processed by routine staining.[52] It is cost-effective, quick, universally available and efficient. It accurately diagnosed 93% of tubercular lymphadenitis.[52] It avoids the need for frozen sections, thus preventing cryostat contamination.

### Specimen Transport

Specimen with live AFB should be sent to laboratory as soon as possible. If delay is inevitable, they should be refrigerated. For delays exceeding 3 days, an equal volume of 1% cetyl pyridinium chloride in 2% sodium chloride should be added to the clinical sample, which can keep AFB viable for 8 days at the room temperature.[53,54] Recently, special transport media such as Wright medium for FNA samples[55] and PrimeStore™ for molecular studies [56] have been introduced.

Infectious specimens intended to be transported must be packaged in accordance with national biosafety guidelines and relevant international regulations.[57] Mycobacterial cultures should be transported to reference laboratory using solid media in screw-cap tubes. Cultures on Petri dishes or in liquid media should not be shipped. Mycobacterial cultures are generally classified by WHO as Category A (high risk). However, for surface transport, they can be considered as Category B (moderate risk).[58] For transporting Category B samples, the WHO recommends them to be kept in a leak-proof primary container that is surrounded by successive layers of absorbent materials, secondary leak-proof container, cushioning materials and a sturdy outer packing. The outer pack should be clearly labeled with a biohazard symbol and appropriate handling instructions.[58] (Fig. 2)

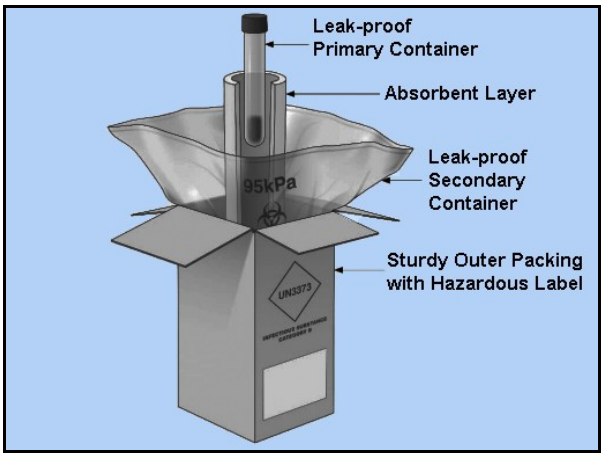


**Fig 2.** *Triple packaging of Category B bio-hazardous specimens for transport (WHO 2022)*

## DEMONSTRATION OF MYCOBACTERIUM - STAINING

### Principle of Acid-Fastness

Gram's stain that is routinely used in bacteriology cannot stain mycobacteria due to the waxy coating on their cell wall.[59] Hence, they are called "Gram-ghost bacilli" (neither positive, nor negative). A phenol-based stain or a fluorescent stain is needed to visualize mycobacteria.

In staining the cell wall, two different techniques are used to penetrate the waxy barrier. In the hot-staining process, the specimen mixed with appropriate stain is slightly warmed (not boiled). In the

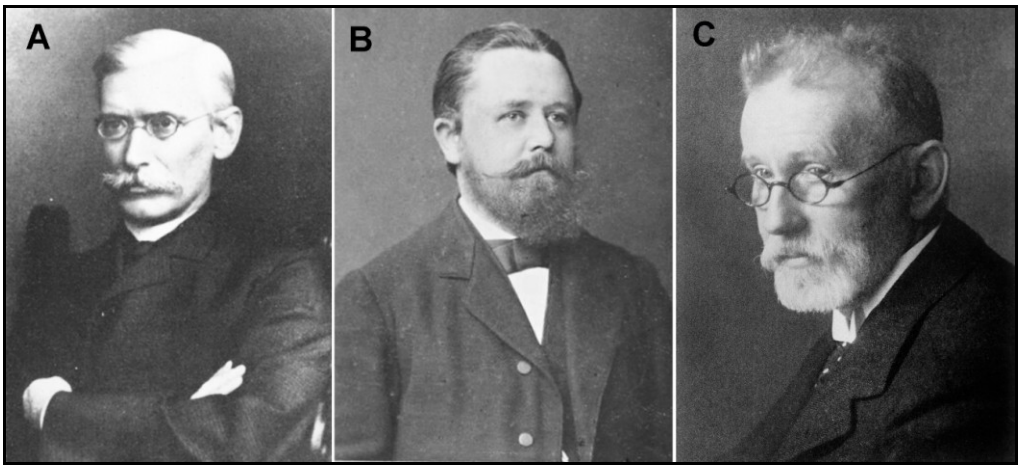


**Fig 3.** *Men who made Mycobacterium visible by staining techniques: (A) Franz Ziehl - 1882 (B) Friedrich Neelsen - 1883 and (C) Paul Ehrlich - 1882. (Sources: Fig. A is reproduced under Fair Use doctrine from Bishop and Neumann, Tubercle 1970; Fig. B is a public domain image from Sachsen Digital Museum - Object ID 8041; Fig. C is a public domain image from the Prints and Photographs division of the United States Library of Congress, digital ID hec.04709)*

cold-staining method, concentration of the staining chemical is increased in lieu of heating. Once fixed to the cell wall of mycobacteria, these stains cannot be removed by exposing them to acids - hence the name 'acid-fast bacilli'. This unique property is due to the presence of mycolic acid, peptidoglycan and arabinogalactan in the cell wall of mycobacteria.[59,60] Only a few other microbes like *Nocardia* and *Brucella* share this unusual character and hence acid-fastness is considered to be conclusive of mycobacteria.[60] Contaminating bacteria that are not acid-fast, are made visible by a counter stain.

By conventional staining techniques, AFB will be seen as pink or red rods on the contrasting blue background of the counter stain. It is to be noted that acid-fast mycobacteria may also turn acid-labile (negative AFB), if cellular synthesis of mycolic acid is inhibited,[59] as it is in nutrient-deficient cultures, isoniazid treatment and mutant strains. Rapidly multiplying virulent bacilli (e.g. aggressive disease as in HIV patients) as well as non-dividing dormant bacilli (e.g. LTBI) do not produce enough mycolic acid and hence they do not exhibit acid-fastness. This phenomenon is known as the Koch Paradox.[59] Acid-lability of MTB adds yet another challenge to the diagnosis of LTBI. For the same reason, detection of AFB in patients undergoing ATT will also be difficult. Acid-lability could be a proxy indicator of isoniazid susceptibility.

### Hot Stains

The most popular hot-staining is the Ziehl-Neelsen protocol.[61] It was originally developed by Paul Ehrlich (1854–1915) and later modified by Franz Ziehl (1857–1926) and Friedrich Karl Adolph Neelsen (1854-1894).[32] (Fig. 3) In Ziehl-Neelsen technique 0.75% carbol fuchsin is used as primary stain that stains bacilli with pink or red colour and the counterstain methylene blue gives a blue background. (Fig. 4) It is easy to perform and has a sensitivity of 70% and specificity of 97%. False positives are due to contamination from laboratory equipments or dietary fibers. False negatives are due to too thick or thin smears, overheating of stains and poor standardization of the technique.

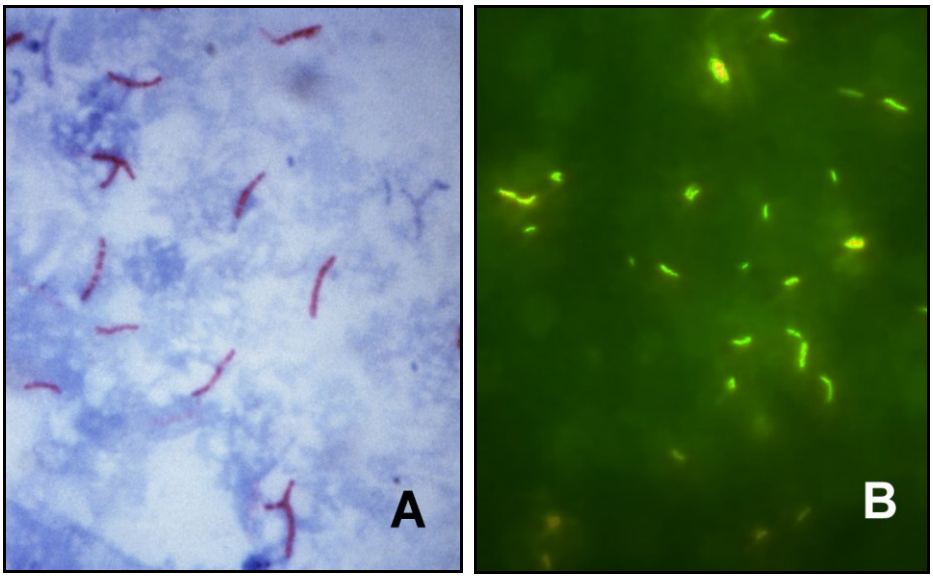


**Fig 4.** *Staining of mycobacteria (A) Ziehl-Neelsen stain (Magnification 1000X) showing pink coloured mycobacteria; (B) Auramine-Rhodamine stain showing bacilli as brilliantly fluorescent yellow or green rods. (Sources of images: Fig. A is public domain image from US Center for Disease Control and Prevention - Public Health Image Library ID #5789 credited to Dr. George P. Kubica. Fig. B courtesy of Dr. Ajay Kumar Chaurasiya @ https://medicallabnotes.com)*

Although Ehrlich originally used 30% nitric acid, and Neelsen used 25% sulphuric acid as decolorizing agents, a weak 3% hydrochloric acid has been found to reduce false negativity as it removes less stain from the bacterial cell wall, thus making it easily visible.[63] Recently, controlled heating with microwave oven [64] has been suggested to reduce false negativity that is attributable to overheating.

### Cold Stains

Generally hot stains more effectively penetrate the waxy layer of mycobacterial cell wall and provide a better colour contrast of the bacilli against the blue background.[65] However, technical errors in heating may result in destruction of the bacterial cells. Hence, Gabbett in 1887 described a technique of cold staining. Kinyoun's staining is similar to Ziehl-Neelsen method, except that the concentration of carbol fuchsin is 3.1% rather than 0.75%.[66] Hallberg method uses carbol-Nachtblau (midnight-blue) solution as the primary stain and carbol fuchsin as the counterstain resulting in blue colour bacilli on red background (inverse of Ziehl-Neelsen appearance). Tison used Nachtblau as the primary stain and orange-G as the counterstain (blue bacilli on orange background). Colour contrasting plays a major role in reducing false negatives. Thus, blue bacilli against orange background are less likely to be missed as compared to pink bacilli against dark blue background.[67] Hok combined Kinyoun's primary stain and Gabbett's decolorizing agent. Zhao used dioxogen to fix the primary stain in lieu of heating.[60,68]

Grocott's methenamine silver (GMS) stain is rarely used to visualize non-viable mycobacteria that are not acid-fast. It is also useful in intracellular bacilli engulfed by the host macrophages, AFB obscured by inflammatory cells and paucibacillary TB.

### Fluorescent Stains

Ziehl-Neelsen stain is a poor technique to demonstrate mycobacteria in tissue sections or when the bacterial load is less than 5000-10000 AFB/ml of the specimen.[59] This limitation can be overcome by using a fluorescent stain, such as auramine-O,

instead of carbol fuchsin in the Ziehl Neelsen protocol. The diagnostic threshold of fluorescent stains is as low as 500-1000 AFB/ml of specimen which is 10 times lower than that of the conventional stains.[69] Combining two fluorescent stains that bind different components of the bacterial cell has been shown to improve the diagnostic yield. For example, auramine-O that binds to the mycolic acid of the bacterial cell wall can be combined with either acridine orange that binds to nucleic acids (RNA and DNA) or rhodamine-B that binds to mitochondrial membrane and DNA. SYBR-Gold™ is a novel stain that has a strong affinity to nucleic acids of AFB.[70]

Several studies have attested to the superiority of fluorescent stains over conventional stains.[71-73] Sensitivity and specificity of auramine-rhodamine staining exceed 95% and it diagnoses 10-25% of cases that are missed by the conventional Ziehl-Neelsen technique.[71,72] Several factors contribute to the improved diagnostic yield: (1) Bright yellow or orange fluorescence of viable bacilli and green glow of non-viable mycobacteria against a dark background is easy to recognize even by colour blinded individuals. (Fig. 4) (2) Fluorescent stains bind to not only the mycolic acid in the bacterial cell wall but also to nucleic acid and mitochondrial membranes inside the cell.[59] Thus, they resist decolourization better than conventional stains. (3) Fluorescent preparations are read typically under low magnification, thus enabling examination of a larger field in a shorter time.

Auramine-rhodamine staining is ideal for culture-negative or paucibacillary TB, as it is in children. Its disadvantages are high cost, need for a fluorescence microscope and false-positivity due to excessive background fluorescence from organic debris in the specimen. (Fig 5) In resource-poor settings, light-emitting diode (LED) microscope is shown to be a cheaper alternative to expensive fluorescence microscope.[74,75] Distracting background fluorescence can be reduced by using suitable counter-

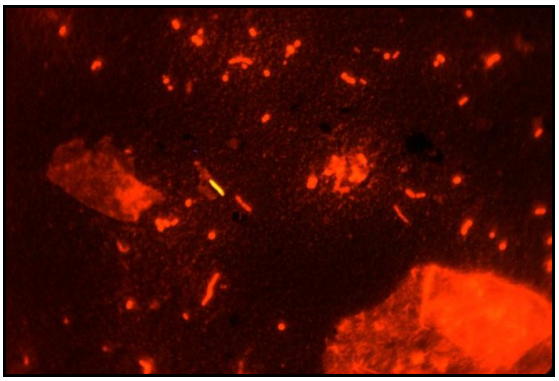

**Fig 5.** *Mycobacteria stained with auramine-acridine orange (Magnification 900X). Note the distraction caused by excessive background fluorescence of organic debris. (Source: Public domain image from Public Health Image Library of the US Center for Disease Control and Prevention, PHIL.ID 6468)*

stains such as $KMnO_4$, methylene blue, malachite green, blue ink or toluidine blue.

Recently, laser scanning confocal microscopy has been shown to be useful in detecting bacilli in the tissue sections and smears stained with antibody-tagged fluorescent stains.[76] It is claimed that confocal microscopy can detect even a single bacillus in the specimen. [76]

### Immuno-Fluorescent Stains

The diagnostic yield of fluorescent stains can be further improved by tagging them with specific antibodies against mycobacteria. These antibodies guide the staining chemical to the target cell avoiding organic debris in the background. Commonly used tagging antibodies are polyclonal anti-BCG antibodies (pAbBCG)[77] and anti-Mycobacterium Protein Tuberculosis-64 (MPT64) antibodies.[78]. MPT64 is highly specific to MTB and thus can differentiate it from other NTM. Immunofluorescent stains, instead of staining the entire cell wall surface of bacilli, simply bind a part of it. Thus immunofluorescent stains just locate the bacilli rather than revealing their actual structure. (Fig. 6) Presence of mycobacteria can only be inferred from the reddish brown dots of fluorescence rather

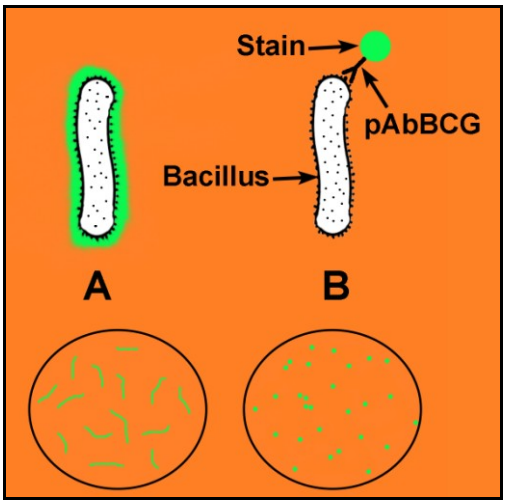


**Fig 6.** *Principles of regular fluorescence staining (A) and immunofluorescence staining (B). The top panel shows the mechanism of staining and the lower panel shows the pattern of microscopic appearance. pAbBCG - polyclonal anti-BCG antibody. (Courtesy of Elanthamizh)*

than by seeing the actual bacilli. Therefore, results are evaluated based on the intensity of staining.[78]

None of the aforesaid stains, except the MPT64 immuno-fluorescent stain, can differentiate MTB and NTM, as they both share the same cell wall structures.[79] The sensitivity of staining techniques varies depending on several factors, including the pathogen load and specimen processing protocols. It is important to note that all the staining methods simply indicate the presence of mycobacteria in the specimen without suggesting anything about pathogenicity or species identity.[21]

## DEMONSTRATION OF BACILLI - CULTURE

The critical density of AFB that can be detected by Ziehl-Neelsen stain and fluorescent stains are 5000-10,000 AFB/ml and 500-1000 AFB/ml respectively. If the bacterial density of the clinical sample is less than these critical limits, it should be increased by culturing techniques before AFB can be recognized in smears. As little as 10-100 AFB/ml can be successfully cultured.[80] Further, culturing also allows species identification and testing of antimicrobial susceptibility. Hence, in 2007, the WHO declared culture as the gold standard of TB diagnosis.[81]

Several solid and liquid media are available for culturing mycobacteria[82] (Table 4) Among them the WHO now recommends Mycobacteria Growth Indicator Tube (MGIT)-960 liquid medium as it supports rapid growth and easy identification.[83] However, solid media are still widely used, as they are cost-effective.[84] AFB grows faster in the agar-based media than in the egg-based media.[85] The grayish or creamy glistening colonies are easily visible in the dark-red background of blood-agar or the green background of malachite containing egg-agar media. (Fig. 7)

Table 4. Mycobacterial culture media

| |
|---|
| **Solid media** |
| Blood-agar medium |
| Lowenstein-Jensen Egg-agar medium |
| MB Redox™ medium |
| Middlebrook medium* |
| Ogawa egg agar medium |
| Petragnani medium |
| Ribonucleic acid medium |
| Thin layer agar (TLA) medium |
| **Liquid media** |
| BacT/ALERT™ |
| Bactec MGIT-960†™ |
| Bactec MGIT-320™ |
| Bactec 9000™ |
| Bactec-460™ |
| Bactec™ Myco/F |
| Dubos medium |
| Kirchner medium |
| Proskauer - Beck medium |
| Sauton medium |
| VersaTREK™ |

**7H10 and 7H11 are two modifications of Middlebrook medium*

*† It is a modification of Middlebrook broth*

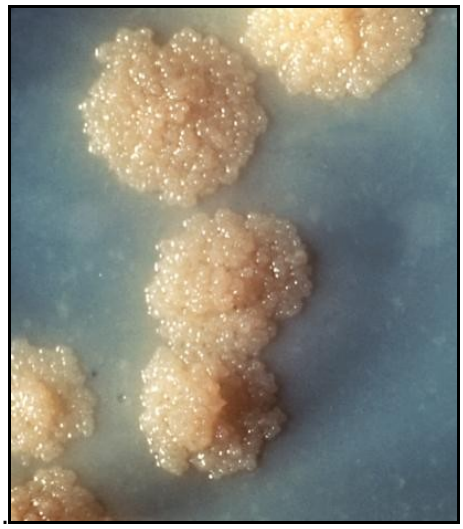

**Fig 7.** *Mycobacterial colonies in egg-based medium. Malachite green in the medium enhances the colour contrast for easy recognition of creamy glistening colonies. (Source: Public domain image from Public Health Image Library of the US Center for Disease Control and Prevention, PHIL.ID 6468)*

Various species of mycobacteria differ in their doubling time and they generally take 15-20 hr to complete one cycle of cell division.[69] Thus, visible growth in culture may take as long as 6-8 weeks, with an average of 14 days for liquid media and 25 days for Lowenstein-Jensen medium. The shortest duration is 9 days for the Bactec-460™ medium.[86] Overgrowth of contaminating commensals may mask the mycobacterial colonies. This is avoided by using selective media with added antibiotics to suppress commensals.

### Indicator Media

Species identification of mycobacteria is done by their characteristic chemical reactions. Indicator media take advantage of this property and facilitate easy identification of MTB.[87] Indicators may be chromogenic (causing colour change) or non-chromogenic. For example, MTB turns the dark-blue colour of BIO-FM media into violet.[88,89] By bacterial oxy-reduction colorless tetrazolium salt in MB-Redox™ medium turns into pink, red or violet formazan.[90] In Bactec-460™ media, mycobacteria generate radioactive carbon dioxide from the radio-labeled carbon of palmitic acid and the resultant radioactivity is measured by a Geiger-Muller counter. Subsequent versions, such as the Bactec-9000MB™, are based on generation of non-radioactive carbon dioxide that is detected by spectrometry.[91] In Bactec MGIT™, the bottom of the tube is coated with oxygen-avid fluorochrome pigments. When oxygen present in the liquid media is completely utilized by mycobacteria, the uninhibited pigments start glowing brilliantly.[92] (Fig. 8) VersaTREK™ is based on detecting the pressure changes of the culture tube caused by fermented gas from bacterial action. A major disadvantage of all patented indicator media is their prohibitive cost.

**Fig 8.** *Bactec MGIT™ culture media showing brilliant fluorescence at the bottom of the tubes indicating the presence of mycobacteria. (Courtesy: Prof Thasneem Banu, Chennai)*

### Animal Inoculation

Diagnostic animal inoculation with rabbit, monkey, guinea pig or hamster was done in the past for growing MTB and testing its drug susceptibility.[93] However, this is no longer practiced due to the complexity of the procedure, high cost and ethical concerns.

## DETECTION OF GENETIC MATERIAL

DNA and RNA fragments of mycobacteria have several signature sequences such as rpoB (RNA polymerase-β subunit),[94] 16S-rRNA gene,[95] hsp-65 (heat-shock protein-65) gene,[96] 32-kDa protein gene and ITS (internal transcribed spacer)[97] Detecting these unique nucleotide sequences is diagnostically as accurate as demonstrating the whole organism. These molecular techniques are quick, easy to do and reliable; but are costly.

The DNA and RNA fragments are shed by the dead or dividing bacteria into the infected body fluids from which they can be isolated by centrifuging the sample. As the DNA or RNA fragments thus retrieved will be too miniscule to be identified, they need to be amplified before being detected by agarose gel electrophoresis technique. Polymerase chain reaction (PCR), real-time reverse transcription PCR (RT-PCR), loop-mediated iso-thermal amplification (LAMP) and cartridge-based nucleic acid amplification test (CB-NAAT) are some of the molecular techniques used in amplifying nucleic acid segments.

### Polymerase Chain Reaction (PCR)

PCR is the traditional and reliable technique of detecting the genetic material of mycobacteria.[98] DNA extracted from the clinical sample is added to a PCR mixture which contains probes (a short-chain nucleotide tagged with a fluorescent stain that binds the amplified target-DNA fragment and helps in measuring them), primers (another short-chain nucleotide that binds to the segment of interest in the target-DNA template and initiates elongation of the synthesized complement nucleotide sequence), deoxy-nucleoside tri-phosphate (dNTP - the building block of nucleic acid chain), Taq DNA polymerase (the enzyme needed to add dNTP to the growing chain of nucleic acid from the primer), nuclease-free water, buffer and $MgCl_2$. The process of nucleic acid amplification involves the following steps: (1) Denaturation (unwinding of the bacterial double stranded DNA into single strand templates), (2) Annealing (attachment of primer to the template DNA segment of interest) and (3) Polymerization (synthesis of new DNA segments that is complement to the template DNA strand by the adding dNTP to lengthen the original primer segment). (Fig. 9) These molecular events are externally controlled by heating and cooling the PCR mixture. At optimal temperature, approximately 1000 dNTP units can be polymerized per minute. A set of temperature fluctuations that results in the synthesis of one complete set of new DNA strands is called one thermocycle. By repeating the thermocycles, more and more copies of the DNA strands can be generated. Typically with each thermocycle, the target DNA fragments in the PCR mixture get doubled by geometric proportion. Usually, 30-40 thermocycles are required to produce a sufficient amount of DNA segments that can be easily detected. Thus, 40 thermocycles will generate 1,099,511,627,776 copies ($2^{40}$) of the original double-stranded target-DNA segment. These amplified copies of DNA should be separated from other impurities by gel electrophoresis. Probes with fluorescent stains (e.g. SYBR-Green™, TaqMan™) bind the amplicons (multiplied DNA copies) and make them visible as bright fluorescent dots under ultraviolet transillumination or Southern blotting. [98-100]

Nested PCR is a technique in which two sequential PCR reactions are carried out to amplify the target DNA segment.[101] It has greater sensitivity and specificity than conventional single-step PCR. Line probe assay (LPA) is a variant of the PCR in which the amplicons are recognized by naked eyes when they react with probe chemicals on a membrane strip and cause color precipitates.

Liquid specimens such as sputum, cerebrospinal fluid (CSF), bronchial lavage, pleural fluid, urine and gastric aspirates are ideal for PCR.[98] The commonly used primers are $TB_{41}$ or $TB_{42}$ (targeting IS6110 sequence; probed with $TB_{43}$) and $MT_1$ or $MT_2$ (probed with $MT_3$).[98] Appropriate selection of primers, probes and thermal fluctuations

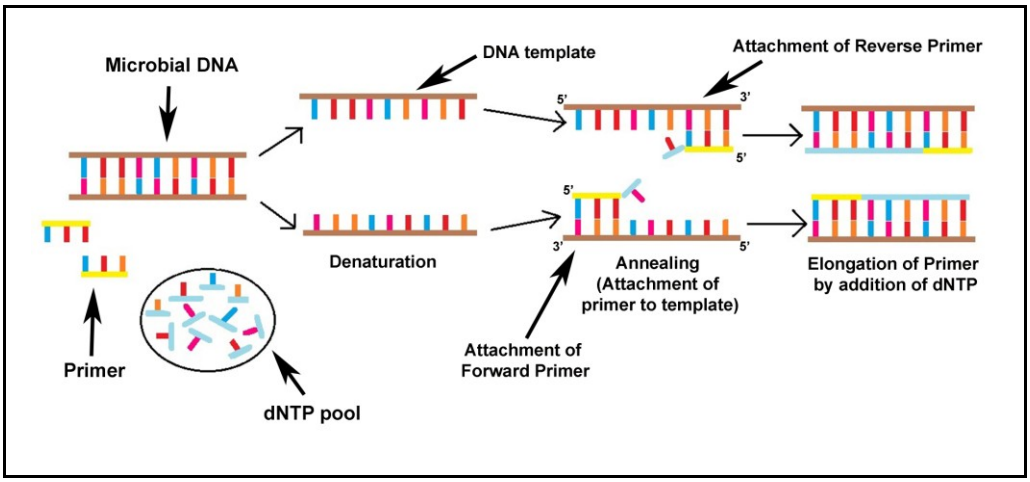


**Fig 9.** *Schematic diagram showing the principle of Polymerase Chain Reaction (Courtesy of Srinidhi)*

are essential for accurate results. With excessive heating or cooling, the primer will bind the target-DNA template at a wrong place, thus causing false negatives. PCR inhibitors (Table 5) such as hemoglobin and urea interfere with the polymerase reaction, resulting in false negativity.[102] Hence, blood and urine samples are generally considered unsuitable for PCR studies. However, newer protocols of specimen preparation annul these inhibitor effects. For example, use of citrate as anticoagulant in blood samples effectively replaces EDTA or heparin which are PCR inhibitors.[102] Similarly, bile salts in stool samples can be neutralized by adding activated charcoal.[102] PCR of blood, urine and stool samples are now considered as reliable diagnostic tools for childhood tuberculosis.[103] As PCR multiplies the template DNA fragment several billion times within few hours, it will also amplify contaminating DNA fragments leading to false positivity. Hence, extreme caution is needed to avoid contamination during sample handling and preparation of the PCR mixture.

### Real-Time Reverse Transcription-PCR

In PCR, signature DNA copies of the template DNA fragments in clinical samples are multiplied. In real-time reverse transcription polymerase chain reaction (RT-PCR), complement DNA (cDNA) strands are reverse transcribed from the messenger RNA (mRNA). The cDNA is simultaneously amplified by PCR for easy detection.[104] Template RNA extracted from centrifuged clinical samples is mixed with RT-PCR mixture containing primer, probe, manganese diacetate,dNTP, reverse transcriptase enzyme, DNA polymerase, uracil nucleotide glycosidase and EZ buffer. The reaction takes place in a thermocycler and subsequent steps of identification of the cDNA are similar to PCR technique.[105] Its sensitivity and specificity are 82% and 99% for PTB; 70% and 99% for EPTB.[106]

Table 5. List of PCR inhibitors

| | |
|---|---|
| Bile salt | Lactoferin |
| Calcium ions | Lipids |
| Collagen | Melanin |
| Detergents | Myoglobin |
| Drugs like acyclovir | Phenol |
| EDTA anticoagulant | Plasmin |
| Glycogen | Polysaccharides |
| Hemoglobin | Proteinase |
| Heparin | Sodium salt |
| Hormones | Urate |
| Immunoglobulin G | Urea |

*Source: Schrader [102]*

### Loop-Mediated Isothermal Amplification (LAMP)

LAMP is another technique of nucleic acid amplification that differs significantly from conventional PCR. Unlike PCR, where thermal fluctuations are used to control polymerization reaction, in LAMP nucleic acid synthesis occurs at a constant temperature.[107] Instead of one primer as in PCR, LAMP uses 4-6 primers (inner and outer primers). This increases its specificity and reduces reaction time. LAMP can synthesize $10^9$ copies of the target DNA segment within 1 hr, while the same would require 5-6 hr in conventional PCR.[107] LAMP uses special polymerization enzymes that have high strand displacement activities besides replication. LAMP can be used to amplify both DNA and RNA. Concurrent usage of more than one primer and probe causes a peculiar problem of false positivity from primer-primer or primer-probe polymerization.[108]

### Cartridge Based Nucleic Acid Amplification Test

Even nano-level contamination of environmental nucleic acid debris during the preparation of PCR mixture may lead to false positivity. To overcome this disadvantage Cepheid company, in collaboration with Foundation for Innovative New Diagnostics (Geneva), developed automated cartridges containing PCR mixtures. This cartridge-based nucleic acid amplification test (CB-NAAT) is also known by its brand name Gene-Xpert™ MTB/RIF (RIF stands for Rifampicin sensitivity). Like conventional PCR, Xpert is intended to detect rpoB gene that is linked with rifampicin sensitivity. This gene is present in all bacteria; but its length and nucleotide sequence differ between various species. GeneXpert MTB is intended to detect MTB specific rpoB gene, thus implying the presence of mycobacteria. In addition, recognized mutations of the gene will predict the bacterial resistance to rifampicin therapy. Xpert MTB has several technical variations such as Xpert MTB/RIF Ultra (a 6-color method) and Xpert MTB/XDR (a 10-color method).

As the reagents are readily available in cartridges and the process is automated, GeneXpert™ results can be obtained in just 45 min as compared to 4-6 hr with conventional PCR. Pre-prepared cartridges annul environmental contamination and enhance specificity by reducing false positives. Hence, the WHO recommends CB-NAAT as initial diagnostic test of choice in pediatric TB.[109] CB-NAAT is also suitable for stool samples and gastric aspirates in children.[110] They are suitable for both PTB and EPTB.[111,112]

False positive results may occur due to sampling error, detection of NTM DNA (which share similar sequence with MTB) or small amount of contaminating MTB DNA from previous samples.[25] False-negative results may occur due to low bacterial load ($<$0.25 CFU/ml),[113] presence of PCR inhibitors in the sample,[102] polymerization between primers and probes, and certain specific mutations in rpoB region.[114]

## PATHOGEN-BASED BIOMARKERS

Mycobacteria release their surface antigens and secrete certain specific proteins into the host circulation. They may also get excreted in urine depending on their filtration size. By detecting these substances in blood or urine, the presence of mycobacteria can be inferred. Lipoarabinomannan (LAM), 38-kiloDalton (kDa) antigen, 16-kDa antigen and MTB12 are promising biomarkers in TB diagnosis.[115]

### Lipoarabinomannan (LAM)

LAM is a glycolipid that forms the cell wall of MTB. They are shed into the host circulation when the bacilli undergo cell division or death.[116,117] LAM is 17.4kDa in size which is less than the 30kDa cutoff value of glomerular filtrates. Hence, LAM is easily excreted in urine irrespective of the organ affected. Detection of LAM more than $>$0.615 ng/ml in urine[118] or $>$2.29 pg/ml in blood samples[119] is considered diagnostic of TB. In children, urinary LAM assay is appealing in the background of difficult sample collection in that age group. Urinary LAM value is directly proportional to the bacterial

load.[120] LAM is highly elevated in disseminated disease and immuno-compromised patients.[121] In the late stages of HIV infection, impaired function of podocyte in nephrons also increases the urinary excretion of LAM. In paucibacillary disease such as pediatric TB and LTBI, LAM is generally considered unreliable.[120] However, urinary LAM detection using chemi-luminescent immunoassay (e.g. Fuji LAM™, Alere-Determine-LAM™) instead of using the Enzyme-linked immuno-sorbent assay technique has been found to increase the diagnostic yield in children and HIV negative patients.[122-124]

## CONCLUSION

Diagnosis of TB in children is often challenging because of low bacterial load. The critical bacterial density necessary for detection is 5000-10000 AFB/ml for conventional Ziehl-Neelsen staining, 500-1000 AFB/ml for fluorescent staining, 10-100 AFB/ml for cultures while 0.25 CFU/ml is sufficient for nucleic acid amplification tests. Hence, the WHO recommends CB-NAAT (Gene Xpert MTB/RIF Ultra™ or Gene Xpert MTB/XDR™) as the preferred first-line investigation of TB in children. CB-NAAT is also advantageous in predicting drug resistance to rifampicin. Several host and pathogen based biomarkers are currently under investigation which may facilitate point-of-care diagnosis of TB in the future.

### Addendum

Tests based on host reaction to invading mycobacteria will be reviewed in the part-2 of this article. It includes histopathology, fine-needle aspiration cytology, radiological imaging, antibody-based tests, host-based biomarkers and indicators of inflammation.

## APPENDIX-1

### Abbreviations

AFB - Acid-fast bacilli
AIDS - Acquired immuno deficiency disease
ATT - Anti-tubercular treatment
BCG - Bacillus Calmette-Guérin
CB-NAAT - Cartridge based nucleic acid amplification test
CD - Cluster of differentiation
CT - Computed tomography
DNA - Deoxy-nucleic acid
dNTP - deoxynucleoside triphosphate
ELISA - Enzyme-linked immuno-sorbent assay
EPTB - Extrapulmonary tuberculosis
FNA - Fine-needle aspiration
HIV - Human immunodeficiency virus
LAM - Lipoarabinomannan
LAMP - Loop-mediated isothermal amplification
LPA - Line Probe Assay
LTBI - Latent tuberculosis infection
MGIT - Mycobacterial growth indicator tube
MRI - Magnetic resonance imaging
MTB - *Mycobacterium tuberculosis*
MPT64 - anti-Mycobacterium Protein Tuberculosis-64
NTM - Non-tubercular mycobacteria
pAbBCG - Polyclonal anti-BCG antibodies
PCR - Polymerase chain reaction
PTB - Pulmonary tuberculosis
QFT- QuantiFERON TB Gold test™
RNA - Ribonucleic acid
RT-PCR - Reverse transcription polymerase chain reaction
TB - Tuberculosis
Truenat™ -Taqman RTPCR based nucleic acid test
WHO - World Health Organization

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