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SHORT REVIEW

ADVANCES IN THE LABORATORY DIAGNOSIS OF *MYCOBACTERIUM TUBERCULOSIS*

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Abstract
Mycobacterium tuberculosis (MTB), the agent of human tuberculosis remains a leading cause of mortality globally. Its resurgence during the last two decades is a reflection of its opportunistic relationship with HIV. The challenges associated with the disease are enormous and often debilitating. The role of clinical and research laboratories is central and significant in this regard as prompt and adequate diagnosis are key factors in the management and control of the disease.

Key words: *Mycobacterium tuberculosis, challenges, multidrug resistance, laboratory investigation*

Résumé
Mycobactérie tuberculose, un agent de la tuberculose humaine est toujours une cause majeure des morts dans le monde. Cette maladie qui est resurgie dans les deux derniers siècles, a un grand rapport opportunistic avec le (HIV). La maladie est pleine de défis qui sont à la fois énorme et ennuyant. Dans cet égard, les laboratoires et les hôpitaux jouent un rôle central et significatif, car les maîtres mots dans le control et la maîtrise de cette maladie c'est le diagnostic suffisant et prompt.

Mots clés: *Mycobactérie tuberculose, les défis, des enquêtes laboratoires, la résistance aux produits pharmaceutiques*

Introduction

The rising trend of (MTB) infection including cases of multi drug resistant (MDR) strains reported in the developing nations especially the Sub Saharan Africa has been attributed to failure to implement effective TB control program.[1] The DOTS[2,3] (Directly observed therapy short course) which advocates case detection and treatment is central, in the global TB control initiative while the diagnostic/research laboratories provide useful information in; the diagnosis of primary TB, treatment monitor, species identification, drug susceptibility testing and strain typing by DNA fingerprinting necessary for contact tracing and epidemiological studies.

Routine identification of tubercle bacilli by microscopic examination of clinical specimens stained by Ziehl Neelsen's method is recommended as confirmatory for TB diagnosis.[4] This is quite useful particularly for laboratories unable to perform further diagnostic and identification procedures. However, the present trend and pattern of MTB infection which include its opportunistic and lethal synergy with HIV, therapeutic complications due to emergence of drug resistant MTB strains and infection by non tuberculous mycobacteria species (NTM species are inherently resistant to normal doses of anti tuberculosis drugs)[5] necessitate the scaling up of routine diagnostic procedures at least to include culture, identification of causative Mycobacterial species and drug susceptibility test.

This article is an overview of laboratory diagnosis of MTB infection and a call for nations in sub Saharan Africa with high TB burden to scale up diagnostic procedures beyond the routine Ziehl Neelsen's staining method. Nigeria for example has limited laboratories proficient for standard mycobacteriological examination.

Laboratory Identification Methods

Methods for the identification of MTB are categorized as conventional (smear microscopy and culture) and non conventional (automated and molecular) methods.

Conventional methods

Smear microscopy

Smear microscopy for AFB is an important preliminary step in the identification of MTB. It is simple to perform, specific for acid fast bacilli and serves as standard laboratory identification method for detection of MTB in routine laboratories lacking the facilities for Mycobacterial culture and further identification procedures. The cheap and simple Ziehl Neelsen's staining for acid fast bacilli is widely used. Other methods include; the Kinyoun's acid fast staining and Auramine Rhodamine fluorescene staining. Their application may be as direct smear or by concentration method.[6] The latter is a centrifugation-sedimentation process after sputum digestion. It is considered to yield higher positive results than the direct sputum smear which involves the direct use of clinical sample to prepare smear for AFB staining. It has been estimated that about 50%-80% of pulmonary tuberculosis will have positive sputum smears.[4]

The direct microscopy method is generally used in routine clinical laboratories lacking the appropriate biosafety facilities for protection and infection containment.

The 5% sodium hypochlorite (bleach) concentration method[6,7] has been recommended for use in preference to the direct AFB method. Bleach performs a dual function of sputum digestion and mycobactericidal activity thus making the specimen relatively safe to handle in the absence of a biosafety cabinet.

Routine laboratories lacking the standard biosafety facilities may need to comparatively review the efficacy of the direct smear microscopy and the bleach concentration methods with a view to improve the detection of AFB in sputum.

The result of smear microscopy is vital for clinical and epidemiological evaluation since it gives a quantitative estimation of the number of bacilli being excreted and thus an insight into the degree of infectivity as well as the severity of the disease.

Grading of sputum samples by direct smear microscopy:[1,6]

1-9 AFB /100 oil immersion fields: Actual AFB counts should be recorded (scanty)

10-99 AFB per oil immersion fields: +

1-10 AFB per field in at least 50 fields: ++

>10 per field in at least 20 fields: +++

The smear microscopic method is however limited by; its inability to differentiate MTB from NTM species, ability to detect only the actively growing population of the bacilli (about 10^4 bacilli/ml of sputum)[4] while paucy- bacillary cases often turn out negative, ability to detect AFB of other microbial genera such as Norcadia species which may give false positive findings.[6]

Culture

Culture is considered a gold standard in clinical bacteriology. It entails the cultivation of clinical specimens on synthetic media for the purpose of isolating the causative agent of a disease condition. Mycobacterial culture is more sensitive than smear microscopy as it is able to detect as few as 10 bacilli per ml of digested concentrated clinical specimen.[4] Cultivation of MTB complex group (*M. tuberculosis, M. africanum, M. bovis, M. microti and M. canetti*) is difficult and time consuming, requiring 3 – 8 weeks for primary isolation from clinical samples and 4–6 weeks for drug susceptibility tests.[6] Cultures of mycobacterial species are however needful for performance of biochemical identification tests, drug susceptibility test and specific molecular methods requiring the use of genomic DNA (eg, Restriction fragment length polymorphism; RFLP).

Various mycobacteriological culture media are available. These are categorised as; egg-potato-based, egg-based (Lowenstein -Jensen's) and agar based (Middle Brook 7H-10, Middlebrook 7H-11 and Dubois Oleic albumin) media.

Non conventional methods

Automated methods (radiometric and non radiometric)

Automated methods are designed to yield results of primary cultures and drug susceptibility tests within the shortest possible period (4- 21 days). Their ability to combine efficiency and reproducibility overcomes most of the limitations of the conventional methods. Automated machines are commercially available from different manufacturers. These include;

The BACTEC 460 TB System (TB BACTEC), a radiometric procedure which measures $^{14}C_{02}$ released during metabolism of ^{14}C-fatty acid substrate by growing bacteria. The amount of ^{14}C02 released is

expressed as growth index (GI) on a scale of 0 to 999. In the presence of an antimicrobial agent, inhibition of daily GI is considered as susceptibility of test organism to the drug.[8,9] The handling and disposal of radioactive medium in this technique is however considered a disadvantage.

The mycobacteria growth indicator tube (MGIT) 960 (Becton Dickinson USA) uses a Middlebrook 7H9 broth in 7 ml plastic tube and a fluorescence quenching-based oxygen sensor embedded at the bottom of the tube. The procedure involves initial, fluorescence quenching by presence of dissolved oxygen in the broth. Subsequently, growing mycobacterial cells in the inoculated tubes consume the oxygen, producing fluorescence under 365nm UV illumination. The system continuously monitors the tubes to detect increase in fluorescence and automatically determines and interprets results. When used for drug susceptibility test, a set of drug containing and drug free (growth control) media are inoculated with the test strain. Comparison of records in the test and control tubes are automatically done and reported as susceptible or resistant.[10,11]

MB/BacT ALERT (BioMerieux, Durham) is a fully automated and non radiometric system that utilizes a bottle containing a colorimetric sensor embedded in its bottom. Carbon dioxide produced by microbial metabolism causes reduction in pH of medium and changes the sensor color from dark green to yellow. The color change is continuously monitored and promptly reported by the instrument.[12,13]

Reports of various studies have indicated the efficiency of the different machines.[14-17] particularly in the shorter turn out time (4-21 days) compared to that of the conventional culture method (3- 8 weeks). Their application may however be limited to research and reference laboratories due to the high cost of procuring the equipment and regular consumables.

Molecular methods

Molecular methods are genetic procedures that make use of genetic materials (DNA or RNA) to detect specific proteins or genes of the test organism using specific probes or short stranded oligonucleotides (primers) complementary to the test DNA strand.

The high degree of DNA polymorphism, repetitive DNA sequences and presence of insertion sequences (IS) characteristic of MTB strains have been used as basis for the study of strain-strain relatedness/diversity of MTB.[18-21] Primer sequences of specific regions in the MTB genome have been done and cloned for use in detection identification and typing from clinical samples and cultures.[22]

Insertion sequence 6110 polymerase chain reaction based diagnostic method

The IS6110 PCR technique is useful in the rapid detection of MTB complex strains in clinical specimens from naturally sterile anatomical sites with minimal bacillary load, often undetectable by the conventional methods. E.g. CSF, pleural effusion, joint and marrow taps, gastric washings. The method is cost-effective and simple to perform. It is specific, sensitive, reproducible and able to generate results within hours.[23,24]

The target DNA is PCR amplified using IS6110 primers sequences. The amplified product is electrophoresed using 2% agarose and observed by UV illumination for DNA band of 123 base pair.

Molecular epidemiology/genotyping

Molecular epidemiology has been defined as the integration of molecular techniques to track specific strains of pathogen with conventional epidemiological approaches to understanding the distribution of disease in populations.[22]

Molecular genotyping of MTB strains is useful in outbreak investigations, comparison of isolates from different laboratories and geographical regions and contact tracing.[25,26] The short repetitive DNA sequences and the insertion sequences characteristic of MTB strains have been used as basis for designing specific probes used in different techniques.[22] Studies on the use of different methods such as the Restriction fragment length polymorphism,[27] spoligotyping,[21] MIRU VNTR[28] (mycobacterial interspersed repetitive units- Variable number of tandem repeats) and other PCR based techniques have been reported.[29]

The goal, ultimately is to have a general understanding of regional and global transmission of MTB isolates and for strain tracking especially those showing peculiar or unusual characteristics,

Drug susceptibility test (DST)

Performance of drug susceptibility test is useful for the diagnosis of drug resistant and multi drug resistant MTB isolates.

Drug resistant MTB: Resistance to one or more than one of any anti tuberculosis drug

Multi drug resistance MTB (MDR): Resistance to isoniazid and rifampicin with or without resistance to other anti tuberculosis drugs.

Several techniques based on phenotypic and genotypic principles have been developed for determination of DST of MTB. Three (phenotypic) methods are generally used in routine practice: The absolute concentration method, resistance ratio method and proportion method. The tests can be performed by the direct inoculation of concentrated specimen onto a set of drug containing media or indirectly by using bacterial suspension of pure cultures obtained from growth on synthetic media (Lowenstein-Jensen, 7H10 or 7H11 agar).

The automated methods already described are

comparatively more advantageous in producing results within days to a few weeks.

Genotypic methods are useful in the understanding of the molecular basis of drug resistance. The methods which include; DNA sequencing,[29-31] Real-Time PCR,[32] DNA Microarrays,[33,34] are used for rapid detection of mutations associated with specific and multi drug resistant tuberculosis.

Conclusion

The challenges associated with the present day mycobacterial infection are issues of global concern. The reliance on smear microscopy as the sole laboratory diagnostic test common in low income nations needs re-evaluation in the light of the rising trend of tuberculosis associated with HIV/AIDS and possible rise in prevalence of MDR MTB in a community. Nationals that have high burdens of TB and HIV infections (Notably, The Sub Saharan and some Asian countries) should scale up laboratory investigatory procedures to include the essentials such as culture and drug susceptibility test.

There is need to differentiate MTB complex strains (slow growers) from NTM (fast growers) to enable prompt eradication of infecting strain from the host.

The IS6110 diagnostic PCR for detection of MTB in clinical specimens is cost-effective, easy to perform and affordable for some routine laboratories in the developing world.

Although the DNA fingerprinting methods are research procedures, their importance and possible application should be considered as epidemiological records are vital for successful national infectious disease prevention and control program.

Collaboration should be established between laboratories with minimal facilities and the research or reference laboratories to enable further identification and typing of key isolates particularly those that seem to defy antituberculosis chemotherapy.

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