Update on the laboratory diagnosis of tuberculosis

This article focuses on the microbiological diagnosis of tuberculosis.

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Tuberculosis (TB) is a major health problem in South Africa, with incidence rates in some areas exceeding 900/100 000 population.[1] The TB epidemic is fuelled by the co-existing HIV epidemic and complicated by a considerable number of drug-resistant cases, both multidrug resistant (MDR) and extremely drug resistant (XDR). The early diagnosis of TB is therefore crucial. This article discusses some of the current laboratory tests available for the diagnosis of TB. It focuses on microbiological tests and omits potentially valuable tests from other laboratory disciplines, such as chemistry or histology.

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Specimen collection

Diagnostic tests for TB can be performed on a variety of specimen types even though sputum is the most common specimen submitted. The procedure of sputum collection is potentially hazardous as it generates infectious aerosols. It should therefore take place in a well-ventilated room or even outdoors if possible. An induced sputum specimen, produced by nebulisation with hypertonic saline, is valuable in patients unable to produce sputum. In children, gastric washings are the traditional sample, but induced sputum may be superior, with a single induced sputum sample being equivalent to three gastric washings.[2]

Fine-needle aspiration biopsy of lymph nodes is an excellent practical method of obtaining material for microscopy in cases of TB and malignancy. The material can also be inoculated directly by the operator into liquid TB culture media, provided it is done under aseptic conditions. Use of commercial media for direct inoculation is expensive and sometimes wasteful as these media can easily be contaminated with skin flora, but locally produced 'TB transport bottles'[3] currently being developed are cheaper.

Blood and bone marrow samples, taken in cases of suspected disseminated mycobacterial disease, can be inoculated directly into specific mycobacterial blood culture bottles. Other suitable specimen types include biopsies, cerebrospinal fluid, urine, pus swabs and stool. All specimens from non-sterile sites require decontamination before inoculation to prevent the overgrowth of other more rapidly growing micro-organisms. Despite this process some specimens, particularly from stool, may become contaminated.

Diagnostic methods

Microscopy

Microscopy for the detection of acid-fast bacilli (AFB) remains a vital tool in the laboratory diagnosis of TB as it is cheap, rapid and detects the most infectious cases. Slides are stained with carbolfuchsin (e.g. Ziehl-Neelsen (ZN) stain) or fluorochrome dyes (auramine stain) and examined with light and fluorescent microscopy, respectively, as shown in Fig. 1.

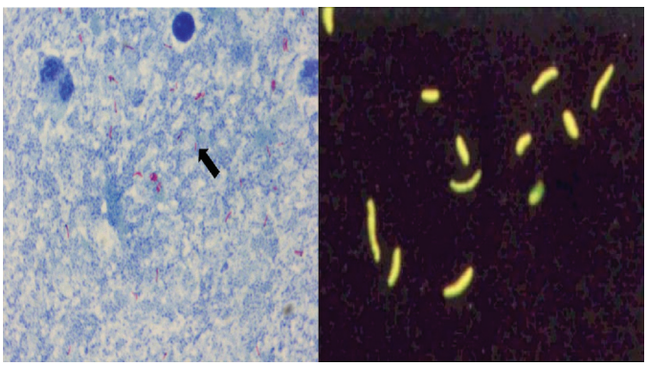

Fig. 1. Microscopy for acid-fast bacilli (AFB) using Ziehl-Neelsen (ZN) stain and light microscopy (left), and auramine stain and fluorescent microscopy (right).

In areas of high TB prevalence microscopy is considered relatively specific for *Mycobacterium tuberculosis* (90 - 95%), but its disadvantage is limited sensitivity (40 - 60%), particularly for the pauci-bacillary disease that occurs in extrapulmonary TB or in children or immunocompromised individuals.

Fluorescent microscopy is preferred to light microscopy for sputum smears, as it is more sensitive but equally specific and requires less laboratory time per slide.[4] The development of light-emitting diodes (LEDs), which are far cheaper and easier to maintain than the mercury vapour lamp traditionally used as a light source, is likely to enable increased use of fluorescent microscopy in resource-limited settings.[5,6]

Culture remains the gold standard for the diagnosis of TB, particularly in immunocompromised smear-negative patients.

While microscopy is a relatively simple technique, there is increasing awareness of the need for quality assurance procedures to monitor all its aspects. Allowing sufficient time to examine slides adequately (5 - 10 minutes recommended before declaring a slide negative) has been shown to increase the sensitivity of sputum smear microscopy.[7] A recent systematic review determined that the increase in sensitivity of microscopy on a third sputum smear was less than 5%.[8] Accordingly, the World Health Organization (WHO) no longer recommends a third smear, thereby reducing laboratory workload and allowing for more careful examination of the initial two smears.

Culture

Culture remains the gold standard for the diagnosis of TB, particularly in immuno-compromised smear-negative patients. Liquid-based commercial culture systems, e.g. the Mycobacteria Growth Indicator Tube (MGIT, Becton Dickinson) system, are now standard practice in many TB laboratories in South Africa in both the public and private sectors, the chief advantages being the more rapid turnaround time (10 - 14 days for growth, compared with ≥3 weeks on solid media) and improved sensitivity (Fig. 2). The disadvantage is that contamination rates may be higher with some of the liquid-based systems.[9] Alternative culture methods have been developed, e.g. microscopic observed direct susceptibility (MODS) assay[10] and colorimetric media, but none is currently in routine diagnostic use.

Speciation of cultured mycobacteria can be done using traditional phenotypic methods, but is currently frequently achieved more rapidly using genotypic methods. Depending on the methods used, isolates may be identified to group level, e.g. *M. tuberculosis* complex, or to species level. Where bacille Calmette-Guérin (BCG) disease is suspected, the laboratory should be informed so that specific tests which differentiate *M. bovis* BCG from the *M. tuberculosis* complex can be used.

Drug-susceptibility testing (DST)

Phenotypic DST is based on a comparison of the rate of growth of mycobacteria in the presence or absence of a standardised concentration of antibiotic. Phenotypic DST can be performed on solid or liquid media, the latter giving more rapid results within approximately 14 days.[9] Since DST is usually performed indirectly, i.e. from organisms already cultured from the primary specimen, this time period is additional to time needed for initial isolation. The reliability of DST for first- and second-line drugs has increased over recent years as methods have become more standardised. However, some drugs still remain problematic, most notably pyrazinamide. Phenotypic testing remains the gold standard for detecting resistance due to known and unknown mechanisms.

Genotypic testing relies on the detection of gene mutations responsible for drug resistance; it is therefore not useful if the mutation is unknown. For example, the sensitivity for detection of rifampicin resistance is >98% as almost all resistance is due to mutations in the *rpoB* gene, whereas sensitivity for detection of isoniazid resistance is lower due to the existence of multiple mechanisms of resistance, some of which are still unknown. Genotypic testing is more rapid than phenotypic testing and can also frequently provide a result even if the culture is contaminated. The drawbacks are cost and the requirement for more sophisticated laboratory infrastructure and expertise. Genotypic testing can also be applied directly to clinical specimens. This is discussed further in the following section.

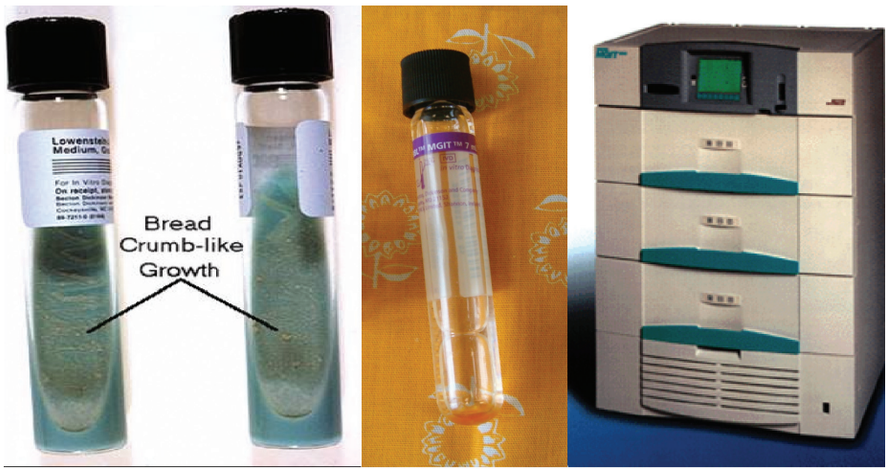

Fig. 2. Mycobacterial culture on solid Lowenstein-Jensen media (left), commercial liquid culture Mycobacteria Growth Indicator Tube (MGIT, Becton Dickinson) (centre), and automated MGIT machine holding 960 tubes (right).

Rapid tests

Rapid diagnostic tests,[11] which can be performed directly on clinical specimens, can be divided into two types, namely nucleic acid amplification tests (NAAs) and the phage-based tests. Both types of tests can be used for the diagnosis of TB and for the rapid detection of drug resistance.

Nucleic acid amplification tests

NAAs for the diagnosis of TB amplify nucleic acid regions specific to *M. tuberculosis* (complex). A number of commercial systems are available, their disadvantage being cost, particularly in resource-limited settings. Cheaper in-house PCR assays have been used in many laboratories in the past, although the trend is to move towards commercial assays because of their greater standardisation and consistency. NAAs show high specificity but only moderate sensitivity, particularly in smear-negative samples. A positive result may therefore not add much value to the diagnosis in a smear-positive individual in a high-prevalence area, while a negative result is not sufficient for the exclusion of TB in the same setting.

NAAs for the detection of drug resistance target mutations responsible for drug resistance. To date they have focused on detection of rifampicin and isoniazid resistance in order to facilitate the rapid detection of MDR TB. Line-probe assays use reverse hybridisation to detect the amplified DNA which is bound to complementary sequences or probes embedded on plastic strips. Multiple gene sequences are targeted and amplified and the absence of wild-type sequences and presence of mutation sequences confirm the presence of resistance, as shown in Fig. 3. Commercially available line-probe assays include the INNO-LiPA Rif.TB kit (Innogenetics NV, Ghent, Belgium) and the GenoType MTBDR plus assay (Hain Life-Science GmbH, Nehren, Germany). In a recent study,[12] carried out on more than 500 smear-positive sputum samples in a busy diagnostic laboratory in South Africa, the GenoType MTBDR plus assay demonstrated

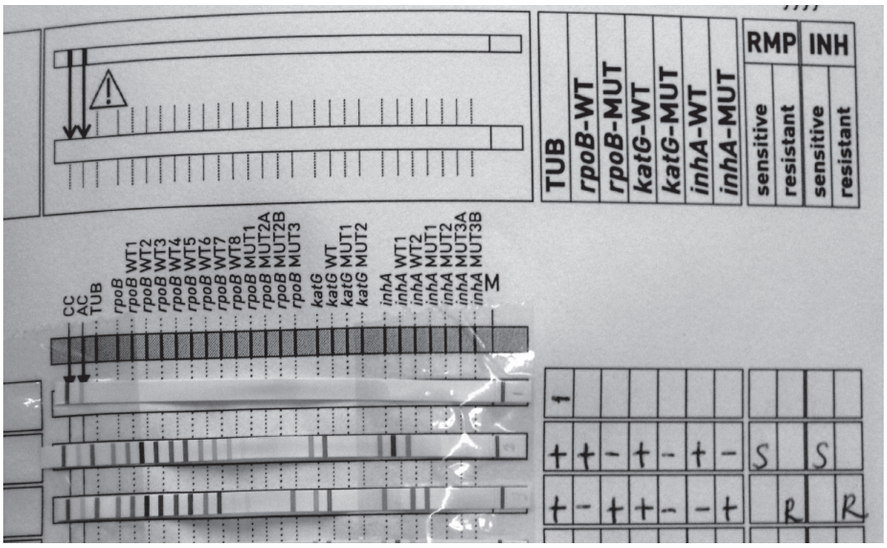

Fig. 3. Examples of line-probe assays for identification and susceptibility testing of M. tuberculosis *(GenoType MTBDR plus assay, Hain Life-Science, Nehren, Germany). The* M. tuberculosis *(TUB) zone hybridises with all members of* M. tuberculosis *complex; hence the absence of a TUB band in sample 1 excludes the presence of* M. tuberculosis *complex. The presence of wild-type sequences (WT) and the absence of mutation sequences (MUT) indicate susceptibility of the isolate to the relevant drug, as seen in sample 2. Sample 3 is resistant to rifampicin, as shown by the absence of* rpoB *WT8 and the presence of* rpoB *MUT3, and resistant to isoniazid, as shown by the absence of* inhA *WT1 and the presence of* inhA *MUT1.*

excellent sensitivity and specificity for the detection of MDR TB. While genotypic susceptibility tests are expensive and require considerable laboratory infrastructure and expertise, they have recently been endorsed by the WHO for use in high-prevalence low-income countries. The South African Department of Health has also approved their use and it is likely that these tests will shortly become part of routine diagnostic practice within the public sector.

Phage-based assays

These assays use mycobacteriophages to infect any viable *M. tuberculosis* present in a sample. The remaining extracellular phages are killed by the addition of a viricide, and the protected intracellular phages are subsequently detected as clear areas or plaques in a lawn of rapidly growing indicator mycobacteria. An alternative, more rapid detection system uses genetically engineered bacteriophages with a luciferase reporter gene that allows detection through the emission of light. The assay can be adapted for rapid DST by incorporating exposure to rifampicin before infection with phages. Rifampicin resistance is detected if the number of plaques from the rifampicin-exposed sample is similar to the number generated from the unexposed sample.

Phage assays can provide results within 2 days. They are technically relatively simple, putting them within the capability of many smaller laboratories. When used on culture isolates the phage-based assays show good sensitivity and specificity. However, their performance on direct clinical specimens has been relatively poor and it seems likely that they will be displaced by NAAs in the South African setting.

Immune-based diagnosis of TB

Tuberculin skin test

The tuberculin skin test (TST), involving the intra-dermal inoculation of a known amount of tuberculin protein, followed by the recording and interpretation of the subsequent induration, measures some aspects of the host's cell-mediated immune response to TB. The interpretation of the test is complicated by cross-reactions in persons either vaccinated with BCG or exposed to environmental mycobacteria, and false-negative results can occur owing to anergy. Most importantly, the TST does not differentiate latent infection from TB disease although it is still often used as an adjunctive diagnostic test in the paediatric setting.

Interferon-gamma release assays (IGRAs)

The newer alternatives to the TST are the interferon-gamma (IFN-γ) release assays, which measure the IFN-γ response of sensitised T-cells to specific *M. tuberculosis* antigens.[13] The use of more specific antigens, such as early secreted antigen target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10), which are encoded by genes located in the unique region of difference 1 (RD1) segment of the *M. tuberculosis* genome, reduces cross-reactions with the BCG vaccine and other non-tuberculous mycobacteria.

Two IFN-γ assays based on RD1 antigens are currently available commercially, namely the QuantiFERON-TB Gold, which is a whole-blood ELISA-based test, and the T SPOT-TB test based on ELISPOT methodology, which counts the number of T-cells secreting IFN-γ.

IGRAs are more specific than TSTs, particularly in persons vaccinated with BCG; they also give a more objective quantitative response, and remove the need for patient follow-up at specific times. Serial testing is possible, without boosting of the immune response which may occur with repeated TST testing. IGRAs have been approved for use in the USA instead of TSTs. However, it should be stressed that IGRAs only indicate infection with *M. tuberculosis* and cannot differentiate between latent infection and active disease.

A number of additional difficulties limit the current use of these assays in the South African situation. Firstly, there are still inconsistencies between TSTs and IGRAs, and between the different IGRAs, probably due to their different methodologies. Few longitudinal studies have been performed and fluctuations in readings over time are not fully understood. Their role in certain groups of patients, such as children or immunocompromised individuals, is not known. The significance of a positive IFN-γ test in assessing risk of progression to TB disease in a person with latent TB infection in a high-prevalence setting is uncertain. Consequently there are real concerns that this costly test may be misinterpreted and abused in the South African context and it is therefore not currently recommended as a routine test for the diagnosis of TB.

Antigen detection tests

A variety of antigen detection tests have been developed to facilitate the rapid diagnosis of TB. However, most have not shown proven reliability and cannot be recommended at present.

Serological tests

A number of studies have examined the utility of an antibody-based serological test for TB. However, it is felt that the antibody response to infection with *M. tuberculosis* is relatively heterogeneous, and these tests have shown very poor sensitivity and specificity when single antibodies are used. It is theoretically possible that serological tests designed to detect a panel of different antibodies may be useful; however, no commercial assay yet exists for this purpose.

Quality control issues

Laboratory cross-contamination is always possible and clinicians should bear this in mind if they receive an unexpected positive result. Cross-contamination of TB cultures is facilitated by the complex multi-step

processing required for each TB culture specimen before incubation and by the ability of AFB to survive in the environment. The incidence of culture cross-contamination is usually low (<3%), although variable, being more common with liquid media systems due to their increased sensitivity. Since most cross-contamination is unexpected and probably undetected, laboratories need to have systems in place to detect and minimise it, and improved communication between clinicians and the laboratory is a vital component. Cross-contamination may be confirmed by the use of molecular finger-printing techniques that type suspected isolates.

Contamination of the laboratory environment with amplified DNA is a major risk with nucleic amplification tests, particularly when high volumes of tests are performed. Stringent procedures need to be incorporated into routine practices to prevent this.

Conclusion

Diagnostic laboratories remain the foundation of a national TB control programme; they strive to provide accurate, reliable results as quickly as possible, while balancing this against the costs involved. Health care workers need to respond promptly to laboratory results, in terms of initiation of appropriate treatment and institution of infection control measures and contact tracing. Indeed, the considerable effort currently being made to improve and expand laboratory capacity in the public sector will be useless unless the results are timeously communicated to and acted upon by health care workers in the field. Good communication between clinicians, TB programme officials and the laboratory should facilitate improved outcomes all round.

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In a nutshell

- The early diagnosis of TB is crucial.
- Sputum collection is the most common procedure used for the microbiological diagnosis of TB, but is potentially hazardous and needs to be performed in a well-ventilated environment, preferably outside.
- Fine-needle aspiration biopsy of lymph nodes is an excellent practical method of obtaining material for microscopy for both TB and malignancy.
- Blood and bone marrow samples, biopsies, cerebrospinal fluid, urine, pus swabs and stool may be submitted for microbiological diagnosis of TB.
- Microscopy for the detection of acid-fast bacilli (AFB) remains a vital tool in the laboratory diagnosis of TB as it is cheap and rapid and detects the most infectious cases.
- Fluorescent microscopy is preferred to light microscopy for sputum smears as it is more sensitive, but equally specific, and also requires less laboratory time per slide.
- Culture remains the gold standard for the diagnosis of TB, particularly in immunocompromised smear-negative patients.
- Phenotypic drug-susceptibility testing is based on a comparison of the rate of growth of mycobacteria in the presence or absence of a standardised concentration of antibiotic.
- Nucleic acid amplification tests for the diagnosis of TB amplify nucleic acid regions specific to *M. tuberculosis* (complex).
- Phage-based assays use mycobacteriophages to infect any viable *M. tuberculosis* present in the sample.
- The tuberculin skin test (TST) does not differentiate between latent and active TB.
- The newer alternatives to the TST are the interferon-gamma (IFN-γ) release assays, which measure the IFN-γ response of sensitised T-cells to specific *M. tuberculosis* antigens.
- A variety of antigen-detection tests have been developed to facilitate the rapid diagnosis of TB. However, most have not shown proven reliability and cannot be recommended at present.