ADVANCES IN THE DIAGNOSIS OF TUBERCULOSIS AND DRUG RESISTANCE

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Disclosure: No potential conflict of interest.
Received: 26.04.2013 **Accepted:** 12.08.2013
Citation: EMJ Respir. 2013;1:129-134.

ABSTRACT

Countries in which tuberculosis (TB) is common are countries with lower economical conditions and less laboratory opportunities, so diagnostic tests for tuberculosis should not only be rapid and sensitive, but they should also be cheap, reliable and easily applicable. Current initiatives targeting the development of new diagnostic tests have increased the pace of identification and testing of a number of potentially useful innovations. Novel diagnostic methods for use in TB epidemiological studies are highly desirable. Detection of mycobacterial species (excluding *Mycobacterium tuberculosis*) using molecular methods is cheaper and simpler than conventional cultural detection, however, culture is still the gold standard in the diagnosis of *Mycobacterium tuberculosis* (MTB); even molecular and non-molecular tests cannot replace it. Results of molecular and non-molecular methods should be evaluated together with culture, microscopy, and clinical findings. When using these tests (conventional methods for example microscopy and culture; phagotypic and chromatographic methods; molecular methods for example nucleic acid amplification techniques (NAAT) and solid-phase hybridisation assays; serology) in a country with limited economical resources, cost/effectiveness analysis should be made carefully. This review examines the recent advances in the diagnosis of MTB in humans.

Keywords: Tuberculosis, diagnosis, treatment, drug resistance.

INTRODUCTION

Tuberculosis (TB) continues to be one of the most important causes of disease worldwide. Studies on diagnosis, treatment and prevention of disease have been increasing because of this. In 2011, there were an estimated 8.7 million incident cases of TB (range, 8.3 million–9.0 million) globally, equivalent to 125 cases per 100,000 in the population. Most of the estimated number of cases in 2011 occurred in Asia (59%) and Africa (26%); smaller proportions of cases occurred in the Eastern Mediterranean Region (7.7%), the European Region (4.3%) and the Region of the Americas (3%). Of the estimated 8.7 million incident TB cases in 2011, only 66% were diagnosed and notified to national TB control programmes, due, in part, to inadequate laboratory capacity in many low and middle income countries.[1]

TB is caused by a bacterium called *Mycobacterium tuberculosis* (MTB). Once inhaled, the bacteria reach the lungs and grow slowly over several weeks. In over 80% of people, the immune system kills the bacteria and they are removed from the body. In a small number of cases, a defensive barrier is built around the infection but the TB bacteria are not killed and lie dormant. This is called latent tuberculosis; the person is not ill and is not infectious. If the immune system fails to build the defensive barrier, or the barrier fails later, latent tuberculosis can spread within the lung (pulmonary tuberculosis), into the lymph glands within the chest (intrathoracic respiratory tuberculosis), or develop in any part(s) of the body to which it has spread (extrapulmonary tuberculosis). Because TB can affect many sites in the body, there can be a wide range of symptoms, some of which are not specific and may delay diagnosis.[2] Typical symptoms of pulmonary TB include chronic persistent cough

(of 3 weeks or more), sputum production (sometimes with haemoptysis), chest pain (TB pleurisy), and shortness of breath.[2,3]

TB, in parts other than the lungs, has symptoms which depend on the site, and may be accompanied by intermittent fever or weight loss. TB is a possible diagnosis to be considered in anyone with intermittent fever, weight loss, night sweats, fatigue or weakness, and other unexplained symptoms. Latent tuberculosis without disease, however, has no symptoms. The diagnosis of TB is suspected from a combination of context, symptoms, clinical signs and investigations. TB is diagnosed in a number of ways. Tissue samples from biopsies may show changes which suggest TB, as do certain X-ray changes, particularly on chest X-rays. Definite diagnosis is achieved by culturing the TB bacterium from sputum or other samples. This not only confirms the diagnosis, but also shows to which of the TB drugs the bacterium is sensitive.[2] However, low sensitivity of microscopic examination, and 2 to 6 weeks duration of culturing has resulted in a search for more rapid and sensitive diagnostic methods.

Polymerase chain reaction (PCR) is a method for reproducing specific DNA chains in a tube, which was an advancement for the diagnosis of agents, and which can hardly be cultured or which cannot be cultured at all.[4] Countries in which TB is most common are countries with lower economical conditions and less laboratory opportunities, so diagnostic tests for tuberculosis should not only be rapid and sensitive, but they should also be cheap, reliable, and easily applicable.[5] Novel diagnostic methods for use in TB epidemiological studies are highly desirable.[6,7] Current initiatives targeting the development of new diagnostic tests, new drugs, and new vaccines have increased the pace of identification and testing of a number of potentially useful innovations.[8,9] Detection of mycobacterial species (excluding *Mycobacterium tuberculosis*) using molecular methods is cheaper and simpler than conventional cultural detection, however, culture is still the gold standard in the diagnosis of TB; even molecular tests cannot replace it.[1,4,5] This review examines the recent advances in the diagnosis of MTB in humans.

LATENT TB INFECTION

Accurate identification of latent TB infection (LTBI) is the key to prevention of the disease among persons at risk. The tuberculin skin test (TST) was, until recently, the only tool available for detecting LTBI. Although the TST has proven to be useful in clinical practice, it has several major limitations.[10] Another advanced method to detect cellular immune response is the measurement of IFN-γ (interferon-gamma), excreted by T cells stimulated with MTB antigens (Interferon-gamma release assays [IGRAs]). New version antigens (early secreted antigen target-6 [ESAT-6] and culture filtrate protein-10 [CFP-10]) were coded by genes located on the RD1 (the region of difference 1) segment of MTB genome and are more specific for MTB than PPD.[11,12] Two IFN-γ assays, based on RD1 antigens, are available as commercial kits: the QuantiFERON®-TB Gold assay and the T-SPOT®.TB. As there is still no gold standard for the diagnosis of LTBI, these assays potentially may serve as a routine diagnosis test other than TST to identify people with LTBI.[13]

QuantiFERON®-TB Gold assay (for diagnosing both latent infection and active disease) is a whole-blood, ELISA-based test, whereas the T-SPOT®. TB test uses peripheral blood mononuclear cells and ELISPOT technology. The QuantiFERON®-TB Gold assay comes in two formats: a 24-well culture plate format and a newer, simplified, in-tube format. QuantiFERON®-TB Gold In-Tube (which also includes, in addition to ESAT-6 and CFP-10, the antigen TB7.7) test (Cellestis Limited, Carnegie, Victoria, Australia, 2007) and the T-SPOT®.TB test (Oxford Immunotec Limited, Abingdon, United Kingdom, 2008) are both newer tests.[14] A recent study has compared QuantiFERON®-TB Gold In-Tube, T-SPOT®.TB and TST in 373 HIV-infected patients, reporting that IGRAs were more sensitive than TST for the diagnosis of MTB infection in this category of patients.[15] The world Health Organization (WHO) advises against the use of IGRAs over TSTs as a diagnostic test in low and middle-income countries with typically high TB and/or HIV burdens.[16] Specificity of both tests for the determination of LTB in BCG-vaccinated are higher than TST.[17] Because of this high specificity, IGRA can be helpful in cases with cross reactivity due to BCG as the interpretation of TST could be difficult. IGRA may help to decrease false-positive results and can thereby increase the efficacy of LTB screening.[18,19]

MICROBIOLOGICAL DIAGNOSIS IN TUBERCULOSIS

Conventional Methods

Though studies to discover new diagnostic tests for TB have gained speed worldwide, detection of TB cases are still based on sputum acid-fast bacilli (AFB) culture, radiological findings and clinical symptoms. At present, 57% of all TB cases are

diagnosed bacteriologically, therefore the quality of presently applied methods needs to be increased. Some success has been achieved in this subject.[20]

Microscopy

Staining of AFB is widely used all over the world as it is fast, simple, inexpensive and an easy laboratory method. Globally, the rate of treatment success for the 2.7 million new cases of sputum smear-positive pulmonary TB who were treated in the 2010 cohort was 87%. It is also impressive that as the size of the global treatment cohort grew from 1.0 million in 1995 to 2.7 million in 2010, the treatment success rate progressively improved. Globally, the rate of treatment success was 85% in 2010.[1] Although carbol fuchsine staining is preferred routinely, more sensitive fluorochrom stains are also used, which enable a more rapid screening of the preparations. Using these methods, called 'flourenscent AFB staining' or 'fluorochrome AFB staining', a wider field can be screened with a lower magnification power, and time needed to screen all preparations can be decreased up to approximately ten times. They are therefore advised for laboratories examining a large number of samples daily for diagnosis and follow-up of patients during treatment.[21] Recent literature reviews have confirmed that it may also be beneficial in ordinary microscopy. This technique could be further improved by attaching a stronger light source called an ultra-bright light-emitting diode.[20]

Culture examinations

Despite the development of various new techniques for rapid diagnosis, the gold standard for diagnosis nowadays is still culturing[22] as, for example, 2 live Bacilli ml-[1] was reported to be enough for culture positivity.[23] A systematic review demonstrates that these liquid cultures (BACTEC™ 460TB, Septi-Chek™ AFB) are more rapid and sensitive than solid medium cultures (Lowenstein-Jensen, Middlebrook7H-10,7H11). The mean time to detection was 12.9 days by BACTEC™ MGIT™ 960, and 15 days with BACTEC™ 460TB, compared with 27 days with Lowenstein-Jensen solid medium.[20] An Indian study showed that the BACTEC™ 460TB radiometric method obtained 87% of the positive results within 7 days and 96% within 14 days. Therefore, the BACTEC™ 460TB method is considered cost-effective in countries endemic for TB.[24] BACTEC™ 460TB (Becton Dickinson, Sparks, MD, USA) has been considered the best method for rapid testing of susceptibility of MTB to major anti-tuberculous drugs such as rifampicin, isoniazid, ethambutol, pyrazinamide, and streptomycin.[25]

Phagotypic Methods

Novel diagnostic tests using mycobacteriophages to identify MTB from biological specimens, require only 2 days of turnaround time in the laboratory. There are two methods: luciferase reporter phage assay (LRP) and the phage amplified assay (PhaB or MAB). Both are simple, rapid and rather cheap techniques requiring few pieces of equipment.[19,20] They have a high specificity, but lack sufficient sensitivity to conventional culture techniques.[19,20]

Phage-based assays are available as commercial kits. For diagnosis, the FASTPlaque-TB® (Biotec Laboratories Ltd., Ipswich, UK) assay can be directly used on sputum specimens. A variant, the FASTPlaque-TB-MDRi® kit, is designed to detect rifampicin resistance in culture isolates. An advanced version of this kit, FASTPlaque-TB-Response®, has been developed for the detection of drug resistance (e.g. rifampicin) directly from sputum specimens.[26]

Chromatographic Methods

Direct MTB identification from clinical samples has been attempted by using different chromatography methods to detect tuberculostearic acid (TBSA) alone or in combination with other structural components of the mycobacterial cell wall. Fast gas chromatography mass spectrometry (GC-MS) and new immunochromatographic assays (based on the MPT-64 antigen) are used,[27] but they do not yet represent a significant alternative for the rapid diagnosis of TB from clinical specimens.

Molecular Methods

Many fast and easy molecular methods with high sensitivity and specificity have been developed in the last 25 years to detect and identify mycobacteria directly in the specimens or by cultivation. These methods are nucleic acid amplification (NAA), nucleic acid hybridisation and nucleic acid chain analysis.[28]

Nucleic acid amplification techniques (NAAT)

These are molecular systems aimed to determine MTB complex from the clinical specimens.[20] Nucleic acid amplification tests are used in smear-positive cases to differentiate TB from atypical mycobacteria in developed countries with a low incidence of TB and in regions with high HIV infection rates.

PCR is the first developed method. There are two commercially available molecular systems based on NAA for AFB positive airway specimens by direct observation of fresh specimens. One is Amplicor® MTB (Roche Diagnostic Systems) based on PCR. The other is Amplified MTB® Direct Test (AMTD) based on GenProbe-Transcription-mediated amplification (TMA). Amplicor MTB® is used in smear-positive airway specimens and AMTD is used both in smear-positive and negative airway specimens. In addition to these, there are a lot of commercially available molecular tests based on NAA. Some methods based on strand-displacement amplification (SDA) are BD ProbeTec™ (BD Diagnostic assay), Abbott LCx MTB assay (Ligase chain reaction-LCR; Abbott Laboratorıes, Chicago, IL), commercial kits for Real-Time PCR (LightCycler®-Roche, iCycler®-BioRad, ABI PRISM7000®-Applied Biosystem) instrument, COBAS TaqMan® MTB test, and nucleic acid chain amplification (NASPA) based methods produced by DNA•STRIP® technology, which includes GenoType® Mycobacteria Direct Test (Hain Lifescience, Germany).[23]

A half automatic method based on SDA principle is BD ProbeTec™, which has a primary amplification target of IS6110 insertion chain and 16S rRNA gen. Sensitivity and specificity of this test is good in smear-positive airway samples.[29] Commercial systems such as GenoType® Mycobacteria Direct Test (Hain Lifescience, Nehren-Germany) and Real-Time PCR (LightCycler™ system-Roche Diagnostics, Indianapolis, IN) are also molecular methods which have been used more and more in rapid diagnosis of tuberculosis. Special probes such as fluorescence resonance energy transfer (FRET) are used in some of the methods and are specific for MTB.[23] NAA-based tests are rapid tests with high sensitivity and specificity in positive direct observed fresh airway specimens and high specificity and low sensitivity in negative fresh specimens.[30-32]

In addition to the developments in clinical laboratory tests, DNA fingerprint methods are helpful with showing laboratory contaminations and epidemiological studies.[19,20]

Rapidly developed drug resistance can also be shown with molecular methods. Phenotypic (culture-based) and genotypic (nucleic acid amplification testing–based) methods have been developed to detect drug-resistant TB, however, first-generation tests were rarely available in TB-endemic areas, were poorly standardised, and had slow turnaround times. Genotypic drug-susceptibility testing (DST) for firstline agents is accurate for RIF and isoniazid (INH) but less reliable for streptomycin, ethambutol, and pyrazinamide.[33,34] Automated liquid culture systems and molecular line probe assays are recommended by WHO as the current gold standard for first-line DSTs.[34] The most important drug for the development of rapid drug resistance is probably rifampicin. Strains resistant to rifampicin are detected to be also resistant to many drugs because most rifampicin-resistant isolates are also isoniazid-resistant. Detection of rifampicin resistance in the early stages can be important for the management of treatment.[35] There is an instrument (GeneXpert® Xpert MTB/RIF, Cepheid, Sunnyvale, CA, U.S.A) detecting rifampicin resistance using PCR directly without a prior procedure of the specimen. In this assay, high sensitivity and specificity are obtained for the detection of MTB, and the few studies performed to date have also observed a good response as regards resistance to rifampin.[36,37] The Xpert MTB/RIF assay was rapidly endorsed by WHO in December 2010 for use in TB, multidrug-resistant TB, and TB/HIV-endemic regions using a risk-based approach to testing.[38]

Solid-phase hybridisation assays

Species detection of mycobacteria using probe technology can also be done; AccuProbe® (Gen-Probe-San Diego, CA), line probe assays (LiPA, Innogenetics, Ghent, Belgium ve Bayer Diagnostics, Tarrytown), and GenoType® MTBC (Hain Lifescience, Nehren-Germany) are the most commonly used products for this. Line probe assays are developed for the identification of cultured mycobacteria and for the detection of drug-resistant mutants. The first version of reverse hybridisation is LiPA mycobacteria assay (Innogenetics); probes for 16S and 23S sites of mycobacteria are used in this method, and MTB complexes, MAI complexes, *M. avium, M. intracellulare, M. kansasii, M. chelonae, M. gordonae, M. xenopi, and M. scrofulaceum* can be detected. The test takes approximately 3 hours. It was reported that PCR, following reverse hybridisation, gave very successful results in smear-positive direct clinical specimens.[39] In addition to identification of species, genetic determinants of rifampicin resistance can also be detected with reverse hybridisation of direct clinical specimens.[40] Rifampicin resistance can be examined using commercially available INNO-LiPA Rif TB (Innogenetics, Ghent, Belgium) and GenoType MTBDR (Hain Lifescience, Germany) kits.[41] Duration of these tests is approximately 3 to 4 hours and the results are 90% concordant with the classical

drug sensitivity results. WHO subsequently issued a statement supporting the use of MTBDR plus directly on smear-positive samples for rapid detection of drug resistance. Limitations of the assay include reduced sensitivity for detection of INH resistance compared with rifampicin resistance.[42,43]

In situ hybridisation is another method which has been used for molecular diagnosis for a number of years. It is called fluorescence *in situ* hybridisation (FISH), if fluorescence is used as detector molecule, and chromogenic *in situ* hybridisation (CISH), if detection of the probe is performed with a secondary reaction and colour.[23] DNA chain analysis is accepted as the gold standard and the most reliable method in the identification of mycobacteria, which can be used to detect species of mycobacteria and genetic mutations responsible for drug resistance. With the commercially available MicroSeq® (Applied Biosystems, Inc (ABI), Foster City, CA), DNA chain setting of a 500 bp part of the 16S rRNA gene is possible. This test is mostly used for research rather than for routine laboratory examinations because it is cheap but difficult and needs experience to be performed. DNA microarray is used for the identification of *Mycobacterium* species and the detection of mutations related to antibiotic resistance; because it is very expensive, it is a molecular method mostly used in research.

Some genotyping methods used for diagnosis and typing of mycobacteria like spoligotyping, MİRU-VNTR (Variable Number Tandem Repeats of mycobacterial interspersed Repetitive Units) are primarily used in research. Molecular methods should especially be used in smear-negative patients with clinically suspected tuberculosis and especially to examine airway specimens. To come to a conclusion for diagnosis in a short time, specimen AFB positive and NAA test positive patients should be accepted as TB. For extra-lung specimens more examinations are needed.

Serology

These are methods based on detection of antibodies produced against MTB antigens like ES-31, ES-43, EST-6 antigen 5, antigen 60, P32 and lipoarabinomannan by ELISA. No serological first-line methods are currently used for TB, particularly due to variability in results and cross-reactivity with environmental mycobacteria, which leads to false-positive results.[30] The sensitivity of these tests is high in patients with smear-positive disease, but much lower in children, patients with extra-pulmonary disease, HIV infection or smear-negative cases. Moreover, these tests cannot reliably distinguish latent infection from active disease or different species of mycobacteria.[30] LAM-ELISA may be a suitable option for the diagnosis of human immunodeficiency virus (HIV)-associated TB in urine specimens from patients with low CD4 cell counts.[44] MTB antigen detection provides direct evidence of TB; LAM, 65 Kd, 14 Kd antigens were widely used; it is very quick and easy to perform, but the main limitation is low sensitivity.[45]

In addition to these methods, detection of enzymes (adenosine, deaminases, lysosomes) excreted from various cells are also used in the diagnosis of MTB. But these methods could not replace classical culture.[23]

CONCLUSION

Culture is still the gold standard in the diagnosis of TB, even molecular and non-molecular tests cannot replace it. When using molecular tests for diagnosis, it should be kept in mind that there may be many problems arising because of user applications, and quality control standards should be applied exactly. Results of these tests should be evaluated together with culture, microscopy and clinical findings. It should be kept in mind that inappropriate specimens or a single finding may not mean anything. There should be good communication between the physician and the laboratory, and clinical findings should be followed closely. This not only enables a quick diagnosis and treatment, but also a control on infection. Using these tests in a country with limited economical resources, cost/effectiveness analysis should be made carefully.

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