# Journal of Medical Sciences

ISSN 1682-4474

science alert

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Review Article

J. Med. Sci., 10 (5): 124-129
1st July, 2010

# Evolutionary Development of Molecular Tools in the Diagnosis of *Mycobacterium tuberculosis*: A Review

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Nucleic acid amplification technologies such as PCR are revolutionizing the detection of infectious pathogens such as tuberculosis (TB). Amplification technology offers the potential for the diagnosis of TB in a few hours with a high degree of sensitivity and specificity. However, molecular assays neither replace nor reduce the need for conventional smear and culture, speciation and antibiotic sensitivity assays. It is likely that future detection systems may be sufficiently sensitive to detect small numbers of pathogens without any amplification. Whatever the format and instrumentation for the assays, the impact of molecular testing on patient care will be substantially greater in the future.

**Key words:** *Mycobacterium tuberculosis*, molecular tools, diagnosis

***JMS (ISSN 1682-4474) is an International, peer-reviewed scientific journal that publishes original article in experimental & clinical medicine and related disciplines such as molecular biology, biochemistry, genetics, biophysics, bio-and medical technology. JMS is issued eight times per year on paper and in electronic format.***

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## INTRODUCTION

Tuberculosis is an airborne infectious disease that is preventable and curable which is caused by mycobacteria. In humans *Mycobacterium tuberculosis* mainly attack the lungs but can also affect the gastrointestinal tract, central nervous system, lymphatic system, circulatory system, genitourinary system, bones, joints and even the skin (Davies, 2002). Other mycobacterium such as *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium canetti*, *Mycobacterium microti* also cause tuberculosis but these species are less common in humans (Reed, 1957; Kraus *et al*., 2001). Symptoms of tuberculosis depend on where in the body the *Mycobacterium tuberculosis* is growing. TB in the lungs may cause symptoms such as a chronic cough, pain in the chest, coughing up blood or sputum (phlegm from deep inside the lungs). Other symptoms of active TB disease are weakness or fatigue, weight loss, no appetite, chills, fever and sweating at night. *Mycobacterium tuberculosis* (MTB) was discovered by Robert Koch in 1882 and his studies on tuberculosis won him Noble prize for Physiology and Medicine in 1905 (Sakula, 1882; VanGuilder *et al*., 2008).

## TYPES OF MTB

MTB is the type of strain of the genus *Mycobacterium* and at that time there was only one other member of the genus, the non-culturable *M. leprae*, the causative agent of leprosy (Hansen, 1974). The genus was named after the fungus-like growth of MTB in liquid culture (Snewin *et al*., 2002).

**Epidemiology:** Over the past years considerable amount of work has been done on tuberculosis. A major development in the diagnosis of TB was the introduction of several Nucleic-Acid Amplification (NAA) techniques, such as the Polymerase Chain Reaction (PCR) that has been widely evaluated (Abe *et al*., 1993). There exist other commercially available amplification methods for direct detection of *M. tuberculosis* in clinical samples, like Real Time Polymerase Chain Reaction (RT-PCR) and COBAS Amplicor (www.medscape.com).

## METHODS FOR DIAGNOSIS OF MTB

**Traditional culture and sensitivity test:** The confirmation of presence of drug resistance is obtained only by culture and sensitivity test. The culture and sensitivity test of the bacteria can be done with the help of either 7H10, 7H11 Middle- brook or on LJ Media. If patient is known smear positive, the drug is incorporated into the medium and results are usually available within 6 to 8 weeks. In sputum negative patients, first the culture of the bacteria is essential and in case the culture comes positive, the susceptibility tests are carried out on the cultured bacilli. This usually takes 12 to 16 weeks. Since these traditional methods are time consuming and may delay the treatment by at least 2 to 4 months, newer and rapid methods of culture and sensitivity of the mycobacteria have been developed.

**Slide culture:** Mycobacteria are fixed on slide and then transferred to the liquid medium containing the drug. This is incubated at 37° for one week and the growth is examined microscopically. It gives sensitivity results within 8 to 10 day.

**Egg Enriched Sheep Blood Media (EESBM):** In this, egg enriched sheep blood medium is used instead of LJ medium for rapid isolation of organisms by using slide culture, when sputum positive samples are used, the results of drug susceptibility are available within 8-10 days. The results of culture and sensitivity tests of mycobacteria by using EESBM are almost as par traditional culture as LJ medium.

**BACTEC system:** It is radiometric detection of mycobacterial growth with the help of sophisticated gamma camera by using radioactive 14C. It gives results of culture and sensitivity within 10-14 days.

**Luciferase Reporter Mycobacteriophage test (LRM test):** This is one of the newer and rapid methods used for accurate culture and susceptibility testing. This test uses mycobacteriophage, a virus that infects *M. tuberculosis* and has a cloned gene for production of luciferase reporter enzyme. The LRM (Luciferase mycobacteriophage) when mixed with culture of bacterial cells results in production of light. The procedure of the test includes growth of mycobacteria in the drug treated medium to which LRM particles are added and then emitted light is measured. If drug kills the mycobacteria no light is produced, revealing sensitivity of the organism to the drug. If mycobacterium is resistant to the drug, the cell remains unaffected and light will be produced. This test is extraordinarily sensitive, specific and rapid. It provides results of sensitivity of *M. tuberculosis* to various drugs within 48 to 72 h.

## MOLECULAR DIAGNOSIS OF MTB

**Restriction Fragment Length Polymorphism (RFLP):** These tests are used to categories and compare isolates of *Mycobacterium tuberculosis*. Patients isolates can be compared and can be categorized into drug sensitive/multiple drug resistant and treatment can be started on that basis.

**DNA finger printing:** These have been observed not to change during development of drug resistance.

**Single Stranded Confrontation Polymorphism in conjunction with PCR (SSCP PCR):** This test is very useful for primary drug sensitivity. INH, RMP, SM resistance can be per- formed directly and gives results in 48 h. Currently this test is being tried.

**Ligase chain reaction:** This test utilizes enzyme DNA Ligase to unite two strands of DNA to combine as double strand. By this method it is possible to detect mismatch of nucleotide.

**Real time-PCR:** Real-Time PCR is also called quantitative real time polymerase chain reaction (Q-PCR/qPCR) or kinetic polymerase chain reaction in molecular biology. It is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule enabling both detection and quantification of one or more specific sequences in a DNA sample.

The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is detected as the reaction progresses in real time and the main advantage of RT-PCR is its speed in giving results (1.5-2 h after DNA extraction) and the decrease in the risk of contamination as both reaction and detection occur in the same tube (Logan *et al*., 2009). Two common methods for detection of products in real-time PCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA and (2) sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target (Udvardi *et al*., 2008). The main advantage of RT-PCR is its speed in giving results and the decrease in the risk of contamination as both the reaction and detection occur in a single tube (Palomino, 2005).

Several authors have developed real time-PCR assays that provide rapid detection of target sequences of *Mycobacterium tuberculosis* in clinical specimens with variable sensitivity and very high specificity (Broccolo *et al*., 2003; Cleary *et al*., 2003; Takahashi and Nakayama, 2006; Pounder *et al*., 2006).

Molecular methods have been reported to have different sensitivities in the diagnosis of pleural tuberculosis and this may in part be caused by the use of different methods of DNA extraction. Our study compares 9 DNA extraction systems (7 manual and 2 automatic) for analysis with RT-PCR in an experimental model of pleural tuberculosis. The automated method is more expensive, but requires less time. Almost all the false negatives were because the difficulty involved in extracting MTB DNA, as in general all the methods studied are capable of eliminating inhibitory substances that block the amplification reactions. The method of MTB DNA extraction used affects the result of diagnosis of pleural tuberculosis by molecular methods. DNA extraction systems that have shown to be effective in pleural liquid should be used (Santos *et al*., 2010).

The AFB primers were as follows: 5'GCGTGCTTAACACATGCAAGTC-3' and 5'TCCTCCTGATATCTGCGCATTC-3'. DNA from each culture was extracted and subjected to PCR and sequencing analysis, the results were obtained in three days. One specimen showed the presence of *Mycobacterium mucogenicum* and the contaminant turned to a mycobacterium species that was identified by a different PCR sequencing analysis with a set of universal bacterial primers. The other specimen matched well for *Mycobacterium avium*. In contrast, the conventional method used by the reference laboratory eventually confirmed the same species, but not until 7 and 13 weeks later.

The timely results from the PCR sequencing method helped our clinicians make management decisions as early as possible. The efficiency and cost effectiveness of using the mycobacterial PCR sequencing method in community hospital setting should be investigated further (Drury *et al*., 2006).

Current nucleic acid amplification methods to detect *Mycobacterium tuberculosis* are complex, labor-intensive and technically challenging. We developed and performed the first analysis of the Cepheid Gene Xpert System's MTB/RIF assay, an integrated hands-free sputum-processing and real-time PCR system with rapid on-demand, near-patient technology, to simultaneously detect *M. tuberculosis* and rifampin resistance. Analytic tests of *M. tuberculosis* DNA demonstrated a Limit of Detection (LOD) of 4.5 genomes per reaction. Specificity rose to 100% after correcting for a conventional susceptibility test error. In conclusion, this highly sensitive and simple-to-use system can detect *M. tuberculosis* directly from sputum in less than 2 h (Helb *et al*., 2010).

A real-time PCR assay using the Light Cycler (LC) instrument for the specific identification of

*Mycobacterium tuberculosis* complex (MTB) was employed to detect organisms in 135 Acid-Fast Bacillus (AFB) smear-positive respiratory specimens and in 232 BacT/ALERT MP (MP) culture bottles of respiratory specimens. This real-time MTB assay is sensitive and specific; a result was available within 1 h of having a DNA sample available for testing (Miller *et al*., 2002).

Several environmental mycobacteria have been shown to be important human pathogens linked to immunomodulation especially in relation to effect on vaccination. Hence identification of mycobacteria to the species level is not only relevant to patient management but also to understand epidemiology of mycobacterial diseases and effect on vaccination. of 119 (85.7%) isolates and the remaining 17 isolates (14.3%) were confirmed by 16S rRNA sequencing also. These 119 environmental mycobacterial isolates, included several potentially pathogenic species such as *M. fortuitum*, *M. chelonae*, *M. avium*, *M. marinum*, *M. manitobense*, *M. kansasii* and others belonged to nonpathogenic species, *M. terrae*, *M. smegmatis* and *M. flavescens*. *M. chelonae* was isolated from water samples only whereas *M. fortuitum* was isolated from both water as well as soil samples. This combination strategy using PCR-RFLP and 16S rRNA sequencing may be useful for characterization of mycobacteria from similar environmental settings from other parts of world (Parashar *et al*., 2009).

Mycobacteria cause a variety of illnesses that differ in severity and public health implications. The differentiation of *Mycobacterium tuberculosis* from nontuberculous mycobacteria (NTM) is of primary importance for infection control and choice of antimicrobial therapy. Despite advances in molecular diagnostics, the ability to rapidly diagnose *M. tuberculosis* infections by PCR is still inadequate, largely because of the possibility of false-negative reactions. All clinically significant mycobacteria were detected; the mean melting temperatures. This real-time PCR assay with melting curve analysis consistently accurately detected and differentiated *M. tuberculosis* from NTM. The specific melting temperature also provides a suggestion of the identity of the NTM present, when the most commonly encountered mycobacterial species are considered. In a parallel comparison, both the LightCycler assay and the COBAS Amplicor *M. tuberculosis* assay correctly categorized 48 of 50 specimens that were proven by culture to contain *M. tuberculosis* and the LightCycler assay correctly characterized 3 of 3 specimens that contained NTM (Shrestha *et al*., 2003).

The Seeplex™ TB Detection-2 assay (Rockville, MD) is a nested endpoint PCR for the *Mycobacterium tuberculosis* complex (MTBC) targets IS6110 and MPB64 that utilizes dual priming oligonucleotide technology. When used to detect the presence of MTBC DNA in formalin-fixed paraffin-embedded tissue specimens, the sensitivity and specificity of this assay is equivalent to a labor-intensive traditional endpoint PCR assay and is more sensitive than a commercial real-time PCR assay. (Drews *et al*., 2008).

The conventional culture technique for diagnosis of extra pulmonary tuberculosis is time consuming. In order to find a sensitive and rapid technique nested Polymerase Chain Reaction (nPCR) targeting the conserved MPB 64 gene of *Mycobacterium tuberculosis* was evaluated for detection of *M. tuberculosis* DNA directly from clinical specimens of extra pulmonary origin. A total of 400 clinical specimens from clinically suspected cases of extra pulmonary tuberculosis and 30 control specimens of nontuberculous aetiology were processed by smear and culture and by nPCR technique for detection of *M. tuberculosis*. All the 30 control specimens were negative by nPCR. The nPCR using MPB64 gene primers might be a rapid and reliable diagnostic technique for detection of *M. tuberculosis* genome in clinically suspected extra pulmonary tuberculosis specimens, as compared to the conventional techniques (Therese *et al*., 2005).

Gastro-intestinal (GI) TB is often underrated and yet, any kind of delay in prompt initiation, may lead to treatment failure or to developing antibiotic resistance. The significant morbidity and mortality observed in GITB is due to its imprecise features which do not readily suggest a specific diagnosis (3). This abdominal form of TB has an insidious course like any other chronic infectious disease without any specific laboratory, radiological or clinical findings. Due to this non-specificity and great difficulties in its diagnosis a number of rapid investigative methods have been surfacing out to aid in the diagnosis of GITB employing a diverse MTB genomic targets including the IS 6110 insertion sequences (16-19). Undeniably, the PCR systems developed so far have shown good levels of sensitivity (90 to 100%) only on AFB smear -positive samples (20). Our investigation exhibit that real time detection technology using FRET probes present superior sensitivity over conventional detection methodologies for rapid diagnosis of GITB. The results obtained for the group A and B depicted the accuracy of the real time assay as demonstrated by the congruency in the both qualitative and quantitative results. One negative result obtained in group B may be due to the uneven distribution of the MTBC in the tissue. The overall positive index of the samples of group C tested was found to be 36% (Mishra *et al*., 2010).

Rimek *et al.* (2002) compared the performance of two PCR assays, an IS6110-based in-house protocol and the COBAS AMPLICOR MTB PCR (COBAS MTB) system, for the detection of *Mycobacterium tuberculosis* complex in 43 human lymph node samples from 40 patients. For the in-house PCR and the COBAS MTB assays, respectively, sensitivities were 87.5% versus 45.5% ($p<0.05$), specificities were 100.0% versus 91.3% ($p>0.05$) and inhibition rates were 4.8% versus 19.5% ($p<0.05$). For the COBAS MTB system, additional N-acetyl-L-cysteine-NaOH pretreatment of the samples changed neither the inhibition rate nor the sensitivity significantly.

Reischl *et al.* (1998) evaluated the COBAS AMPLICOR PCR system (Roche Diagnostics) for the routine detection of *Mycobacterium tuberculosis* complex (MTBC) in clinical specimens. Diagnostic culture, considered as the reference method, was performed with BACTEC, Lowenstein-Jensen, Stonebrink and Kirchner media. Occasionally MB-Redox, ESP, or MGIT medium was also used. The sensitivity of the COBAS AMPLICOR MTB assay was 96%, versus 48% for smear-negative specimens. By simplifying the work flow through a completely automated amplification and amplicon detection procedure, the COBAS AMPLICOR PCR system proved itself as a very useful component for routine diagnostic procedures.

The current global concern in the treatment of tuberculosis (TB) is the emergence of resistance to the two most potent drugs viz., isoniazid and rifampicin. The level of initial drug resistance is an epidemiological indicator to assess the success of the TB control programme. Though drug resistance in TB has frequently been reported from India, most of the available information is localized, sketchy or incomplete. A review of the few authentic reports indicates that there is no clear evidence of an increase in the prevalence of initial resistance over the years. However, a much higher prevalence of acquired resistance has been reported from several regions, though based on smaller numbers of patients. A strong TB control programme and continuous surveillance studies employing standardized methodology and rigorous quality control measures will serve as useful parameters in the evaluation of current treatment policies as well as the Management of Multidrug Resistant (MDR) TB cases.

**The future of PCR technology:** Thus, the rapid availability of results obtained by molecular assays can be a considerable advantage to patient management in certain situations. More rapid and appropriate treatment can lead to fewer ultimate complications and hospitalization in patients who have the disease and avoidance of unnecessary treatment and isolation of those who do not have TB. Similar pressures for more rapid diagnostic capabilities have driven the development of other molecular assays, particularly those for slow-growing viruses and particularly for the diagnosis of infections of the central nervous system.

New real-time PCR instrumentation is expected to have a very positive impact on the laboratory performance of PCR, but additional changes in the practice of molecular pathology will still be needed. Rather than detection of single microbes, in the future molecular assays will need to be aimed at multiple organisms. Multiplex amplification reactions may be useful for the simultaneous detection of multiple pathogens in a single sample. Alternatively, consensus primers may be used for amplification of related microbes, such as mycobacteria, with species-specific detection performed using probes or DNA arrays.

It is likely that future detection systems may be sufficiently sensitive to detect small numbers of pathogens without any amplification. Whatever the format and instrumentation for the assays, the impact of molecular testing on patient care will be substantially greater in the future.

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