



Molecular Diagnostic Tests Used in the Diagnosis of Tuberculosis



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Abstract

The definitive diagnosis of tuberculosis (TB) is dependent on the isolation, identification and drug susceptibility testing of the causal agent *Mycobacterium tuberculosis* by cultivation. Smear microscopy has poor sensitivity and culture is slow to yield results. The resurgence of tuberculosis worldwide has been accompanied by an increase in the incidence of multidrug-resistant (MDR) tuberculosis on all continents. At the same time, a number of other nontuberculous mycobacterial (NTM) species are emerging as causes of disease. A quick and correct diagnosis of symptomatic tuberculosis is critical for the control of this serious disease. The nucleic acid amplification techniques (NAATs) and other molecular biology methods (i.e. DNA hybridization, DNA sequencing, etc.) are essential in today's laboratory practices for detection and characterization of mycobacteria. The use of NAATs in the routine detection of mycobacteria allowed a fast and accurate detection of the *Mycobacterium* species within 24 hours. The methods are widely used for the identification of mycobacteria, detection of the mutations in the resistance genes as well as the molecular epidemiological studies. The availability of new kits, and accumulated experience with nucleic acid amplification techniques for *M. tuberculosis* detection in most laboratories, have yielded improved sensitivity and specificity of these tests.

Keywords: Tuberculosis; Diagnosis; Serological tests

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is an old and serious infectious disease in humans, and it is still a major public health problem worldwide. It is estimated that nearly 1 billion people will be newly infected with TB between 2000 and 2020 and, furthermore, two hundred million people will develop disease and 35 million will die from TB within this period [1-4]. The definitive diagnosis of tuberculosis is dependent on the isolation, identification and drug susceptibility testing of the causal agent *Mycobacterium tuberculosis* by cultivation. Smear microscopy has poor sensitivity and culture is slow to yield results. A quick and correct diagnosis of symptomatic tuberculosis is critical for the control of this serious disease [5-8]. The resurgence of tuberculosis worldwide has been accompanied by an increase in the incidence of multidrug-resistant (MDR) tuberculosis on all continents. At the same time, a number of other nontuberculous mycobacterial (NTM) species are emerging as causes of disease [9-17]. The late 1990's have brought significant changes to clinical mycobacteriology with the introduction of broth-based cultivation and molecular biological methods. Molecular diagnostic tools for TB have evolved quickly with new innovations which can provide unprecedented opportunities for the rapid, sensitive and specific diagnosis of *M. tuberculosis* in clinical specimens and the status of its drug sensitivity. However, the value of any new method can

be estimated only on the basis of comparisons with conventional techniques currently available in clinical laboratories [18-24].

The nucleic acid amplification techniques (NAATs) and other molecular biology methods (i.e., DNA hybridization, DNA sequencing, etc.) are essential in today's laboratory practices for detection and characterization of mycobacteria. NAATs are a molecular system that detects small amounts of genetic material from the micro-organism (DNA or RNA). The nucleic acid must be extracted from the clinical specimen and afterwards amplified by PCR [21,23]. Using the nucleic acid amplification techniques, in theory, a single mycobacterial DNA can be amplified with an enzymatic reaction to the detectable amount in a very short period of time [22-24]. The use of NAATs in the routine detection of mycobacteria allowed a fast and accurate detection of the *Mycobacterium* species within 24 hours. The methods are widely used for the identification of mycobacteria, detection of the mutations in the resistance genes as well as the molecular epidemiological studies. The insertion element IS 6110 and the 16S rDNA are the most common targets used. Other regions used for amplification include the prob gene encoding the b-subunit of the RNA polymerase, the gene coding for the 32kD protein, the rec A gene, the hsp65 gene, the DNAJ gene, the sod A gene and the 16S-23S rRNA internal transcriber spacer

[22-30]. There are many nucleic acid amplification techniques as commercial or in-house prepared kits for *M. tuberculosis* from clinical samples. The availability of new kits, and accumulated experience with nucleic acid amplification techniques for *M. tuberculosis* detection in most laboratories, have yielded improved sensitivity and specificity of these tests.

Commercially available NAATs

Cobas amplicor MTB test: The Cobas Amplicor MTB Test (Roche Molecular Systems, Basel, Switzerland) is a single unit combining five instruments (automated pipettor, incubator, thermal cycler, wash station and photometer), which enable automated amplification and detection of the *M. tuberculosis* complex. A 584 bp fragment of the 16S ribosomal RNA gene, comprising a species-specific region flanked by genus-specific sequences, is amplified using biotinylated primers [31-35]. The test includes four steps: specimen preparation, PCR amplification, hybridization and detection. In brief, specimens are liquefied and decontaminated with NALC-NaOH. A portion of 50µl of the processed specimen is added to the amplification mixture in amplification tubes containing Taq polymerase, biotinylated primers and abundant dNTPs including deoxyadenosine, deoxyguanosine, deoxycytidine and deoxyuridine (dUTP) in place of deoxythymidine. The amplification process includes denaturation of the double stranded DNA, annealing of the primers and extension of the amplicon sequence, which occur at different temperatures. The procedure is repeated for the required number of cycles, and consequently the copies of the original DNA sequence increase exponentially. Further, after hybridization of *M. tuberculosis*-specific DNA probe, the detection is accomplished by a colorimetric reaction measured with a photometer at wavelength of 660 nm. Absorbance values ≥ 0.35 are scored positive. The detection of the specific amplification product is performed by adding an avidin-enzyme conjugate and a chromogenic substrate [31,32]. The

amplification and detection steps are carried out automatically by the Cobas Amplicor instrument. The method is approved by FDA, USA for testing smear-positive respiratory samples. It includes an internal control, composed of synthetic DNA characterized by identical annealing sequences as the mycobacterial target; when this is not amplified, it signals the presence of inhibitors. The detection of *M. tuberculosis* complex DNA can also be carried out without the Cobas instrument, using a manual kit that, however, does not include an internal control. Other Amplicor kits are available for detection of *Mycobacterium avium* and *Mycobacterium* intra cellular DNA in clinical samples. From the literature review, specificity is close to 100 % while sensitivity ranges from 90% to 100% in smear-positive samples and from 50 % to 95.9 % in smear negative ones [31-43].

Amplified Mycobacterium Tuberculosis Direct test (AMTD)

AMTD is an isothermal (42 °C) transcriptase-mediated amplification system by developed Gen Probe (San Diego, CA, USA). This system is amplifying rRNA (16S rRNA) by DNA intermediates. Briefly, the promoter-primer binds to the target rRNA and the reverse transcriptase enzyme creates DNA copy of the target. rRNA is degraded from the RNA-DNA duplex and the primer 2 anneals to the DNA and new DNA is made. Subsequently DNA-directed RNA polymerase transcribes RNA amplicons from the DNA template. New synthesised amplicons re-enter the TMA process, and repeated replication cycles produce a billion-fold amount of RNA amplicons. The amplicon products are detected with an acridinium ester-labelled DNA probe in a hybridization assay and the results are read by the luminometer [44,45]. The commercial TMA assay (AMTD2, Gen Probe) differs from the Cobas Amplicor test in some respects (Table 1).

Table 1: Differences of the Cobas Amplicor and Amplified Mycobacterium Tuberculosis Direct(AMTD2) assays.

Feature	Cobas Amplicor	AMTD2
Amplification method	PCR ¹	TMA ²
Target	16S r ³ DNA	rRNA
Sample volume(microliters)	50	450
Prevention of carryover contamination	Yes	Yes
Internal control for inhibitors	Yes	No
Assay time after specimen decontamination (hours)	6.5	3.5
Number of samples per run	96	50

¹PCR, polymerase chain reaction; ²TMA, transcription-mediated amplification; ³Ribosomal.

Firstly, thousands of copies of the target rRNA are present in mycobacterial cells compared to 10 to 20 copies of target DNA used in the PCR assay. Thermal-cyclers are not needed and the whole amplification step is carried out on a heating block at 42 °C. The turnaround time is 2.5 hours. No internal control is provided in the kit to monitor the presence of inhibitors. The method is approved by

FDA, USA for testing smear-positive and smear-negative respiratory samples. The overall sensitivity for respiratory specimens was found in the range between 90.9% and 95.2% and the specificity between 97.6% and 100% [44-49].

BD Probe Tec ET

The BD Probe Tec ET (Becton Dickinson, Sparks, MD) is the Probe Tec ET DTB (Becton Dickinson) test based on the strand

displacement amplification (SDA). In the initial phase (target amplification), amplification is started by two pairs of primers complementary to contiguous sequences delimiting the target. The elongation of the upstream primer, also named bumper, determines the displacement of the simultaneously elongating downstream primer and finally releases the produced amplicon. A restriction site, present in the downstream primer, will also be present in the released amplicon. In the exponential amplification phase, a new primer anneals to the amplicon and, following digestion by the restriction enzyme, the upstream fragment acts as bumper and displaces the downstream fragment [50,51]. It is an automated isothermal method characterized by simultaneous DNA amplification and real-time fluorometric detection of the amplicons. An internal control to detect the presence of inhibiting substances is included in each run. The test performance time is approximately 4 hours after specimen preparation. The system is not yet approved by the US FDA. Kits are also available for the amplification of nucleic acids of *M. avium*, *M. intracellulare* and *Mycobacterium kansasii*. The literature reports a rate of sensitivity ranging from 98.5 % to 100 % for smear positive samples and very variable (0.33%-100%) for smear-negative ones [50-54].

Genotype mycobacteria direct assay

This method includes RNA isolation, isothermal amplification and detection by reverse hybridization. It is based on a DNA strip technology, and in addition to *M. tuberculosis* complex it permits simultaneous detection of *M. avium*, *M. intracellulare*, *M. kansasii* and *M. malmoense* (Hain Life science, Nehren, Germany). This novel assay is based on the nucleic acid sequence-based amplification (NASBA) applied to DNA strip technology. According to the manufacturer, the assay has three steps. The first step consists of isolation of 23S rRNA, the second step includes amplification of RNA by NASBA method, and the third step involves the reverse hybridization of the amplified products on membrane strips using an automated system. The assay has the ability for simultaneous detection of *M. avium*, *M. intracellulare*, *M. kansasii*, *M. malmoense* and MTBC. Isolation of highly specific RNA is achieved by the use of the "magnetic bead capturing" method. This assay is useful, reliable and rapid, with sensitivity and specificity of 92% and 100%, respectively.

LCx MTBC assay

Ligase chain reaction (LCx) (Abbott Laboratories Diagnostic Division, Chicago, USA) is a method based on DNA amplification. The assay uses the ligase chain reaction for amplification of a target sequence within the chromosomal gene that codes for protein antigen b, which is specific for members of the MTBC. Two primers attach to each strand leaving a gap in between, which is filled by the action of DNA polymerase and the primers are linked together by ligase. The first pair of oligonucleotides acts as a template for new complementary oligonucleotides. Detection of the amplicons is carried out by microparticle enzyme assay with the LCx fluorometric analyzer. The LCx MTB assay (ABBOTT LCx probe system) has gone through various modifications [42,57]. This test has no internal control and the main shortcomings have been with

inhibitory substances and lack of sensitivity. The overall sensitivity and specificity of the assay was 74% and 98%, respectively. For smear-positive samples the sensitivity reached 100%, but for smear-negative it was only 57%. In a multicenter evaluation of Amplicor and LCx, the sensitivity of both methods was significantly better when only respiratory specimens were considered (78% and 88%, respectively). When non-respiratory samples were used, the sensitivity was reduced to 59% for Amplicor and 65% for LCx [57-60].

Real time PCR

Real-time PCR systems are the most common used in routine mycobacteriology laboratories. During the laboratory study, these systems allow real-time monitoring of a DNA amplification reaction by measuring an accumulating fluorescence signal. Realtime PCR provided improved sensitivity and specificity, reducing turnaround time and avoiding the use of ethidium bromide-stained gels. Different real-time instruments are now available in the market. In routine laboratory, Real-time PCR detection technology has been widely evaluated pulmonary and extrapulmonary samples for TB. The majority of real-time PCR methods reported to date for mycobacteria focus on detection of the *Mycobacterium tuberculosis* complex. Several publications address the detection of Mycobacteria at the genus level. The risk of contamination is considerably less with real-time PCR compared to conventional PCR, but it still can occur. Specimen to specimen contamination has become a greater challenge than amplified product contamination. The most obvious situation where specimen-to-specimen contamination can occur is with the transfer of specimen to the PCR vessel or to the DNA extraction tube.

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