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Minireview

Molecular Diagnosis of Mycobacteria

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Tuberculosis is one of the leading infectious diseases in the world and is responsible for more than 2 million deaths and 8 million new cases annually. Because of the slow growth rate of the causative agent *Mycobacterium tuberculosis*, isolation, identification, and drug susceptibility testing of this organism and other clinically important mycobacteria can take several weeks or longer. During the past several years, many molecular methods have been developed for direct detection, species identification, and drug susceptibility testing of mycobacteria. These methods can potentially reduce the diagnostic time from weeks to days. Currently, two nucleic acid amplification methods, the Enhanced *Mycobacterium tuberculosis* Direct Test (Gen-Probe) and the Amplicor *Mycobacterium tuberculosis* Test (Roche Diagnostic Systems), have been approved by the Food and Drug Administration for direct detection of *M. tuberculosis* from clinical specimens. PCR-based sequencing has become commonly used to identify many mycobacterial species. DNA probes have been widely used for species determination of the most commonly encountered mycobacteria. High-density oligonucleotide arrays (DNA microarrays) also have been applied to simultaneous species identification and detection of mutations that confer rifampin resistance in mycobacteria.

The genus *Mycobacterium* consists of the members of the *Mycobacterium tuberculosis* complex and >80 species of nontuberculous mycobacteria, including pathogenic, opportunistic, and nonpathogenic species. The most important species of this genus is *M. tuberculosis*, the causative agent of tuberculosis (TB).[4] TB is one of the leading infectious diseases in the world and is responsible for more than 2 million deaths and 8 million new cases annually *(1)*. *M. avium* is the most common nontuberculous mycobacterium found in clinical specimens. Infections caused by this species can be clinically significant, especially when associated with AIDS *(2)*. The major difference between TB and other mycobacterial infections is that *M. tuberculosis* is transmitted from person to person. Thus it is particularly important to diagnose TB as rapidly as possible. In addition, antibiotic treatment varies according to the species of mycobacterium.

Although the initial diagnosis of mycobacterial disease often is based on clinical data, definitive diagnosis usually involves the isolation and identification of the infecting organism in the laboratory. The usual laboratory procedure for clinical specimens involves decontamination and digestion of the specimen, microscopic examination for the presence of acid-fast bacilli (AFB), isolation of the organism by culture, and identification and drug susceptibility testing of the recovered organism. Because of the slow growth rate of mycobacteria, isolation, identification, and drug susceptibility testing can take several weeks or longer.

During the past 10 years, several molecular methods have been developed for direct detection, identification, and susceptibility testing of mycobacteria. These methods can potentially reduce the diagnostic time from weeks to days. The aim of this review is to briefly describe the currently available methods and their impact on diagnosis and management of TB.

Direct Detection of Mycobacteria from Specimens

Many mycobacterial species, including *M. tuberculosis*, grow extremely slowly in the laboratory and require 3–8 weeks of incubation on solid medium or at least 2 weeks in a radiometric liquid culture system (BACTEC). This

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[4] Nonstandard abbreviations: TB, tuberculosis; AFB, acid-fast bacilli; NAA, nucleic acid amplification; FDA, Food and Drug Administration; E-MTD, Enhanced *Mycobacterium tuberculosis* Direct Test; Amplicor, Amplicor *Mycobacterium tuberculosis* Test; RIF, rifampin; INH, isoniazid; and LiPA, Line Probe assay.

slow growth often leads to a delay in TB diagnosis. Nucleic acid amplification (NAA) methods allow for detection of mycobacterial DNA or RNA directly from the specimens before the culture results are available.

The Food and Drug Administration (FDA) has approved two NAA tests for direct detection of *M. tuberculosis* from clinical specimens. These are the Enhanced *Mycobacterium tuberculosis* Direct Test (E-MTD; Gen-Probe, San Diego, CA) and the Amplicor *Mycobacterium tuberculosis* Test (Amplicor; Roche Diagnostic Systems, Inc., Branchburg, NJ).

AMPLICOR

The Amplicor test is based on the PCR. In this assay, mycobacterial DNA is amplified with genus-specific primers formulated on the basis of the 16S rRNA gene. After amplification, the amplicons are denatured to form single strands and added to a microtiter plate containing a bound, *M. tuberculosis* complex-specific oligonucleotide probe. An avidin-horseradish peroxidase conjugate then binds to the bound, biotin-labeled amplicons. The conjugate then reacts with peroxide and 3,3′,5,5′-tetramethylbenzidine in dimethylformamide to form a color complex. The results are measured with a photometer. False-positive results produced by carryover contamination are prevented by the incorporation of dUTP coupled with uracil-*N*-glycosylase restriction. After the clinical specimen has been processed with the standard *N*-acetyl-L-cysteine–NaOH decontamination method, the Amplicor results are available in ~6.5 h. An automated version of this test is available in Europe (Cobas Amplicor).

The overall sensitivity of the Amplicor test (compared with culture) for respiratory specimens is 79.4–91.9%, the specificity is 99.6–99.8%, the positive predictive value is 92.6–96.6%, and the negative predictive value is 98.6–98.7% (Table 1). However, the sensitivity for smear-negative specimens is somewhat lower, 40.0–73.1% *(3–5)*. Therefore, the Amplicor test has been approved by the FDA only for direct detection of *M. tuberculosis* in AFB smear-positive respiratory specimens. Chin et al. *(6)* reported that the sensitivity of the Amplicor test was similar to that of culture (58% vs 56%) for detecting *M. tuberculosis* from respiratory specimens when the clinical case definition of TB was used as the reference standard. However, Al Zahrani et al. *(7)* reported that although the Amplicor test had excellent specificity (100%), it was less sensitive than culture (42% vs 73%) for diagnosis of minimal active pulmonary TB (patients suspected of having TB but without spontaneous sputum or with AFB-negative smears).

E-MTD

The E-MTD test is based on the transcription-mediated amplification system developed by Kwoh et al. *(8)*. In this assay, rRNA is released from the target cells by sonication, and a promoter-primer binds to the rRNA target. Reverse transcriptase is then used to copy rRNA to a cDNA-RNA hybrid. The initial RNA strand is degraded, and a second primer binds to the cDNA and is extended, leading to the formation of double-stranded cDNA, which is then transcribed by DNA-directed RNA polymerase to produce more rRNA molecules. The new transcripts serve as templates for reverse transcription and further amplification. The RNA amplicons are detected with an acridinium ester-labeled DNA probe in a solution hybridization assay. Importantly, the amplification procedure is isothermal and the reaction is performed in a single tube, which helps to reduce carryover contamination. After

Table 1. Comparison of commercially available, FDA-approved NAA tests for detection of *M. tuberculosis* complex organisms.

Test	Amplicor	E-MTD
Manufacturer	Roche	Gen-Probe
Amplification technique	PCR	TMA[a]
Target	16S rDNA	rRNA
Analytical sensitivity	≥20 organisms/reaction[b]	NA
Clinical sensitivity	79.4–91.9%[c]	90.9–95.2[d]
Clinical specificity	99.6–99.8%[c]	98.8–100%[d]
Sensitivity for AFB smear-negative specimens	40.0–73.1%[c]	83–100[d]
Positive predictive value	92.6–96.6%[c]	83.3–100%[d]
Negative predictive value	98.6–98.7%[c]	98.4–99.6%[d]
Control for amplification inhibitors	Yes	No
Prevention of carryover contamination	Yes	No
Turnaround time after specimen decontamination	6.5 h	3.5 h
Instrumentation needed	Thermocycler, photometer	Heat block, luminometer
FDA-approved use	AFB smear-positive respiratory specimens	AFB smear-positive and smear-negative respiratory specimens

[a] TMA, transcription-mediated amplification; NA, not available.
[b] Based on manufacturer's claim.
[c] Based on Refs. *(3–5)*.
[d] Based on Refs. *(9–11)*.

standard decontamination of the clinical specimen, the E-MTD test can be completed in 3.5 h.

The E-MTD test has been reported to perform well with both AFB smear-positive and smear-negative specimens. The overall sensitivity (compared with culture) for respiratory specimens is 90.9–95.2%, the specificity is 98.8–100%, the positive predictive value is 83.3–100%, and the negative predictive value is 98.4–99.6% *(9–11)*. In a study where the performance of the E-MTD test was assessed based on the degree of clinical suspicion of TB, the greatest utility was documented for patients with an intermediate clinical suspicion of having TB *(12)*. The E-MTD test is FDA-approved for detection of *M. tuberculosis* in both AFB smear-positive and smear-negative specimens.

Scarparo et al. *(13)* compared the performance of the E-MTD and the Cobas Amplicor tests with 486 respiratory and nonrespiratory specimens obtained from 323 patients. No significant differences were observed between the results of the assays. However, it was noted that although the turnaround time is shorter for the E-MTD test, the Amplicor test can be fully automated and has an internal control for monitoring amplification inhibitors.

CLINICAL UTILITY

The clinical utility of the commercial NAA tests has been discussed in a recent review *(14)* and in guidelines published by the FDA *(15)*. In brief, it is recommended that sputum specimens be collected on 3 different days for AFB smear and culture. The NAA test should be performed on the first specimen collected, the first smear-positive specimen, and additional specimens if needed. If the first specimen is both AFB smear-positive and NAA-positive, the patient can be presumed to have TB. However, if the specimen is smear-positive but NAA-negative, a test for inhibitors should be performed. This option is available in the Amplicor test, but if the E-MTD test is used, a specimen to which *M. tuberculosis* DNA has been added must be analyzed. If inhibitors are not detected and additional specimens remain NAA-negative, the patient can be presumed to have nontuberculous mycobacteria. However, if inhibitors are detected, the NAA test does not offer any diagnostic help. If a specimen is smear-negative and NAA-positive and the same result is obtained with an additional specimen, the patient can be presumed to have TB. In the case that all sputum specimens remain smear-negative and NAA-negative, the patient can be presumed to be not infectious; however, this does not exclude the possibility of active TB, and clinical judgment must be used in decisions regarding TB therapy *(14, 15)*.

With respect to extrapulmonary TB, clinical diagnosis often is uncertain, and NAA tests could provide important information to the clinician. Although the currently available NAA tests have been approved for respiratory specimens only, they have also been used to test several different types of nonrespiratory specimens. In general, the performance of both tests with nonrespiratory specimens has been similar to their performance with respiratory specimens *(13, 16, 17)*. NAA tests also appear to be useful for early identification of *M. tuberculosis* complex for all specimen types (except for blood) grown in liquid cultures *(18, 19)*. However, the NAA tests must be validated in house if they are used for other than respiratory specimens.

In conclusion, the currently available NAA tests can enhance diagnostic speed, but they do not replace AFB smear or culture. Because the tests can only detect *M. tuberculosis*, cultures are still needed for identification of nontuberculous mycobacteria and for drug susceptibility testing. Because the tests cannot distinguish between live and dead organisms, they cannot be used to monitor TB therapy. Clinicians should interpret the NAA test results based on the clinical situation, and the tests should usually be performed at the request of the clinician.

Identification of Mycobacterial Species from Culture

Mycobacterial isolates have traditionally been identified to the species level based on their reactions in a series of phenotypic and biochemical tests. However, the biochemical reactions of isolates of the same species may vary from each other and from time to time, and in many cases no definitive identification is obtained. Because biochemical testing is slow, cumbersome, and may yield ambiguous results, laboratories are increasingly using molecular methods for species identification.

DNA PROBES

Commercial DNA probes (AccuProbe; Gen-Probe Inc.) have been available for some time for identification of clinically important mycobacterial species, including *M. tuberculosis* complex, *M. avium*, *M. intracellulare*, *M. avium* complex, *M. kansasii*, and *M. gordonae*. The tests are based on species-specific DNA probes that hybridize with rRNA released from bacteria. The probes are labeled with acridinium ester, and results are measured with a luminometer. For culture-positive specimens, the turnaround time for the method is ~2 h. The method is very easy to perform, and no special instrumentation is needed. The probes have been evaluated extensively in clinical practice and are rapid, sensitive, and specific *(20, 21)*. The probes can also be combined with the BACTEC or other liquid-based culture systems to further decrease the time needed for species identification *(22, 23)*. However, probes are not available for all pathogenic mycobacterial species, and those isolates must be identified by other methods. In addition, the *M. tuberculosis* complex probe cannot differentiate between the members of this complex (*M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, and *M. microti*).

PCR-BASED SEQUENCING

PCR-based sequencing has become the gold standard for identification of mycobacterial species. The method consists of PCR amplification of mycobacterial DNA with genus-specific primers and sequencing of the amplicons.

The organism is identified by comparison of the nucleotide sequence with reference sequences. As commonly practiced, only one sequencing reaction is needed for a definitive identification. This method also allows for direct detection of mycobacterial species that cannot be grown on conventional laboratory culture media, and several previously unrecognized species have been identified *(24, 25)*.

The target most commonly used is the gene coding for the 16S rRNA. This gene is present in all bacterial species and contains both conserved and variable regions, making it an ideal target for taxonomic purposes. The 16S rRNA gene has been sequenced from a large number of mycobacterial species, and the identification method based on this gene has been evaluated extensively in diagnostic laboratories. Sequencing of two hypervariable regions of the 16S rRNA gene allows for identification of the majority of mycobacterial species. However, members of the *M. tuberculosis* complex cannot be distinguished. Similarly, an important pathogen, *M. kansasii*, has a sequence identical to that of a nonpathogenic species, *M. gastri*, and additional 16S rRNA gene regions need to be sequenced to differentiate *M. marinum* from *M. ulcerans (26, 27)*.

Several other target genes have been characterized for this purpose. The genes coding for the 32-kDa protein *(28)*, the 65-kDa heat shock protein *(29)*, and the 16S-23S rRNA internal transcribed spacer *(30)* contain enough sequence diversity to distinguish all clinically important mycobacteria except for the members of the *M. tuberculosis* complex. These target genes also allow for differentiation of *M. kansasii* and *M. gastri*. In addition, because of the intraspecies variation observed in the 65-kDa protein gene, this target can also be used for distinguishing clones of certain mycobacterial species *(31)*.

DNA MICROARRAYS

High-density oligonucleotide arrays (DNA microarrays) offer the possibility of rapid examination of large amounts of DNA sequences with a single hybridization step. This approach has recently been applied to simultaneous species identification and detection of mutations that confer rifampin resistance in mycobacteria. This technique is based on hybridization of fluorescently labeled PCR amplicons generated from bacterial colonies to a DNA array containing nucleotide probes. The bound amplicons emit a fluorescent signal that is detected with a scanner. The probes used in this array are based on 82 unique 16S rRNA sequences that allow for discrimination of 54 mycobacterial species and 51 sequences that contain unique *rpoB* gene mutations. The method correctly identified 67 of the 70 isolates representing 27 mycobacterial species. All three *M. szulgai* isolates were identified as *M. malmoense* because of an error in the probe sequence. The turnaround time for this method when performed on culture positive specimens was only 4 h *(32, 33)*.

Molecular methods offer many advantages over conventional methods in the identification of mycobacterial species. The results are obtained rapidly, are reliable and reproducible, and even mixed or contaminated cultures can be analyzed. The probes are already widely used in clinical laboratories for the identification of the most common mycobacterial species. Because automatic DNA sequencers and the programs used for analyzing sequence data have become technically simpler, the PCR-based sequencing method is now being used in many mycobacterial reference laboratories as the routine method for species assignment. The DNA microarray method holds great promise for the future because it is easy to perform, it can be readily automated, and it allows for identification of a large number of mycobacterial species in one reaction.

Identification of Antibiotic Resistance-associated Mutations

Drug-resistant *M. tuberculosis* isolates are a serious threat to TB control because only a few effective drugs are available for treatment of this disease. *M. tuberculosis* acquires drug resistance by antibiotic selection of mutations that occur randomly at chromosomal loci. No plasmids or transposable elements (horizontal gene transfer) are involved in this process. Individual nucleotide changes (point mutations) confer resistance to single drugs, and the stepwise accumulation of these mutations leads to multidrug-resistant TB. Drug-resistant strains emerge when chemotherapy is intermittent or otherwise inadequate.

After the primary culture results are available, conventional drug susceptibility testing performed on solid medium takes ~2–4 weeks. When the radiometric BACTEC drug susceptibility testing system is used, several weeks are needed to obtain results. Advances in molecular biology have made it possible to investigate the genetic mechanisms of drug resistance in *M. tuberculosis* and to develop methods for rapid detection of mutations associated with resistance. The currently available methods have mostly been developed for detection of rifampin (RIF) resistance because the genetic basis of RIF resistance in *M. tuberculosis* is fairly simple and well characterized, whereas the molecular basis of resistance to other first-line TB drugs is much more complex. Moreover, resistance to RIF can often be used as a marker of multidrug-resistant TB.

GENETIC BASIS OF RESISTANCE

The genetic basis of resistance to the five first-line TB drugs is described here in brief. A detailed review of this topic has recently been published *(34)*. RIF is a semisynthetic derivative of rifamycin that is used as a first-line TB drug. RIF binds to the β subunit of RNA polymerase, encoded by the *rpoB* gene, and inhibits transcription initiation. Virtually all (96%) RIF-resistant isolates have point mutations in an 81-bp region of this gene, and these mutations are absent in susceptible isolates, making it an ideal target for development of molecular drug susceptibility testing methods *(34)*.

In contrast to RIF, the genetic basis of resistance to other TB drugs is more complex. Isoniazid (INH) is a synthetic, bactericidal agent that is used only for treatment of TB because basically all other bacteria are inherently resistant to INH. Alterations in a relatively large number of genes have been associated with INH resistance, but mutations in two genes, *katG* and *inhA*, are found in 75–85% of INH-resistant *M. tuberculosis* isolates. Streptomycin is an aminoglycoside antibiotic that inhibits protein synthesis. Approximately 65–75% of streptomycin-resistant *M. tuberculosis* isolates have mutations in the 16S rRNA gene or the *rpsL* gene, which codes for the ribosomal protein S12. More than 70% of the pyrazinamide-resistant *M. tuberculosis* isolates have mutations in the *pncA* gene, which encodes for pyrazinamidase, an enzyme that converts pyrazinamide to its active form. Ethambutol inhibits the incorporation of essential mycolic acids into the mycobacterial cell wall. Mutations in the *embB* gene are associated with ethambutol resistance in ~70% of resistant isolates *(34)*.

PCR-based sequencing

PCR-based sequencing is the main technique used to elucidate the genetic mechanisms of drug resistance in *M. tuberculosis*. It is the most direct and reliable method for studying mutations and allows for detection of both previously recognized and unrecognized mutations. Unfortunately, the method is not as readily applicable for routine identification of drug resistance mutations as it is for identification of mycobacterial species because many different genes may be involved, as is the case in INH resistance, or the mutations may be scattered in a large segment of the gene. This means that several sequencing reactions need to be performed for each isolate. However, for targets such as *rpoB*, where mutations associated with RIF resistance are concentrated in a very short segment of the gene, PCR-based sequencing is a useful technique *(31)*.

Line-probe assay

The Line Probe assay (LiPA; Inno-Genetics N.V., Zwijndrecht, Belgium) has been developed for rapid detection of RIF resistance. The test is based on the reverse hybridization method, and it consists of PCR amplification of a segment of the *rpoB* gene and denaturation and hybridization of the biotinylated PCR amplicons to capture probes bound to a nitrocellulose strip. The bound amplicons are then detected with alkaline phosphatase-conjugated streptavidin and BCIP/NBT chromogen, producing a color reaction. The LiPA test strip contains five probes for wild-type *rpoB* sequences and four probes for specific *rpoB* mutations, in addition to a conjugate control and *M. tuberculosis* control probes. The interpretation of the banding pattern on the strip allows for identification of *M. tuberculosis* complex and detection of *rpoB* mutations. The test can be performed on *M. tuberculosis* cultures or directly from clinical specimens. The turnaround time for the test is <48 h. Overall concordance of the LiPA test with phenotypic susceptibility testing and direct sequencing, when performed from cultures, has been reported to be good, varying from 92.2% to 99.0% *(35–37)*. Although the LiPA test only detects 4 of the 35 distinct *rpoB* mutations, ~75% of the RIF-resistant clinical isolates carry 1 of the 4 mutations *(34)*, making the LiPA test a useful method for rapid detection of RIF resistance. However, the test cannot be used for detection of rare mutations.

DNA microarrays

DNA microarray technology described for mycobacterial species identification can also be used for rapid detection of mutations that are associated with resistance to TB drugs. Gingeras et al. *(32)* studied 44 RIF-resistant *M. tuberculosis* isolates with a DNA array containing sequences from the *rpoB* gene. A total of 40 isolates had a previously recognized mutation, one new mutation was detected, and no *rpoB* mutations were found in the remaining 3 isolates by either the DNA array or sequencing. These results are in accordance with the data showing that ~4% of the RIF-resistant isolates have an unknown resistance mechanism *(34)*. In a study performed by Troesch et al. *(33)*, 15 RIF-resistant and 1 sensitive *M. tuberculosis* isolate were analyzed with a similar DNA array. All 15 resistant isolates had mutations in the *rpoB* gene, and the mutations were correctly detected with the DNA array. Mutation types included single- and double-base substitutions and three- and six-base deletions; in addition, one previously unrecognized mutation was found. The results were completely concordant with sequencing results *(33)*. A DNA microarray for simultaneous detection of various drug resistance determinants is currently being developed. It contains sequences from the *katG*, *inhA*, *rpoB*, *rpsL*, and *gyrA* (associated with fluoroquinolone resistance) genes *(32)*. This approach has the potential of becoming the most effective and rapid method for detection of drug resistance mutations in *M. tuberculosis*.

Despite the clear advantages that molecular methods offer for drug susceptibility testing, they all suffer from the problem that the genetic basis of resistance is not fully understood for any TB drug for all *M. tuberculosis* isolates. This means that detection of a mutation associated with resistance is clinically relevant, but a lack of mutations in the target gene does not necessarily mean that the organism is susceptible to the drug in question. The currently available molecular methods may aid in rapid detection of mutations associated with drug resistance, but the test results must always be confirmed by phenotypic methods.

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