TEST EVALUATION

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TEST

Hybridization Assay for Mycobacteria

BACKGROUND

Pulmonary tuberculosis caused by Mycobacterium tuberculosis (Mtb) continues to be a major source of morbidity and mortality in the United States. (1) Furthermore, with the onset of the AIDS epidemic, infections by the Mycobacterium avium-intracellulare complex (MAI) has been recognized to result in pulmonic and systemic disease in this immunocompromised population. (2,3,4,5) Diagnosis of tuberculosis is essential in order to institute appropriate therapy to reduce morbidity and mortality, and to minimize the risk of disease transmission. Diagnosis of M. avium-intracellulare infection among AIDS patients is important in order to make a differential diagnosis from among several pulmonary and systemic illnesses to which AIDS patients are susceptible, including lymphoma, Pneumocystis carinii pneumonia, and disseminated Kaposi's sarcoma. A specific distinction between infection by M. tuberculosis versus avium-intracellulare is also critical because M. tuberculosis infection is amenable to antibiotic therapy, while a reproducibly effective antibiotic regimen for M. avium-intracellulare infection has not yet been achieved. (6)

The gold-standard of diagnosis of mycobacterium infection has been, and remains, culturing the organisms from secretions or biopsies. However, detection of the presence of mycobacteria in culture may take from two to eight weeks, and identification of the specific mycobacterium species may take three to twelve additional weeks. Clearly, this procedure is not adequate to meet the goals of diagnosis mentioned above. Without a more rapid diagnosis, the clinician is faced with the options of instituting presumptive anti-mycobacterial therapy with drugs that have significant morbidity, and/or proceeding to invasive procedures to try to make a diagnosis.
Classically, rapid diagnosis has been achieved by chest x-ray and/or acid-fast staining of sputum. Chest x-ray is a useful adjunct but is not conclusive due to low specificity and sensitivity. Acid-fast stains of sputum do not distinguish between MTb and MAI infections, and have a low specificity (reported to be from 22% to 75%). (7) Furthermore, although a specificity of almost 100% can be attained, specificity remains highly dependent on the experience and skill of involved personnel. Clearly, it would be desirable to have a sensitive and specific method for rapid diagnosis of MTb and MAI infections.

Gen-Probe has developed a sensitive and specific Rapid Diagnostic System whereby mycobacteria species in general, and MTb complex in particular, can be detected in sputum in two to three hours. Furthermore, colonies of MTb complex can be distinguished from those of MAI complex after growth either in broth or on agar, also in two to three hours. Individual species within these two groups cannot be distinguished, but these distinctions are not clinically important.

**BASIS FOR THE GEN-PROBE "MYCOBACTERIUM RAPID DIAGNOSTIC SYSTEM"**

The Gen-probe Rapid Diagnostic System for Mycobacterium is based on detection of portions of ribosomal RNA, specific to the organism in question, by liquid hybridization. The keys to the success of this procedure are (i) the presence of ribosomal RNA in thousands of copies per cell, and (ii) the utilization of liquid hybridization rather than hybridization onto a fixed matrix such as nitrocellulose filter paper. (8)

Although ribosomal RNA genes are known to be highly conserved among evolutionarily related organisms, Gen-Probe has succeeded in isolating and synthesizing DNA from specific portions of the ribosomal genes of the MTb complex and MAI complex species groups. These single-stranded DNA probes can recognize and hybridize specifically to ribosomal RNA extracted from mycobacterial species within these two groups. The detection of ribosomal RNA rather than ribosomal (or other specific) DNA genes is a great advantage because of the vast abundance of copies of ribosomal RNA per cell as compared to the corresponding DNA genes. This relative abundance increases proportionally the sensitivity of the procedure and the rate of hybridization. Ribosomal RNA is already single-stranded and hence the nucleic acids released from the mycobacteria do not have to be denatured. The use of liquid rather than filter hybridization increases the rate of hybridization and decreases the amount of DNA probe required.

An additional key to the success of this procedure is a rapid and efficient method to separate hybridized probe from non-hybridized probe. Hybridization of RNA with complementary single-stranded DNA results in a stable DNA-RNA double-stranded "hybrid" polynucleotide molecule. Hydroxyapatite crystals bind
double-stranded, but not single-stranded, polynucleotide molecules under the appropriate conditions, and hence can be used to separate these two components of the hybridization reaction mixture. Gen-Probe has bound hydroxyapatite crystals to a solid matrix which can be added to liquid hybridization reaction mixture as a suspension. Double-stranded DNA:RNA hybrid molecules bind to the hydroxyapatite and can be pulled out of solution and physically separated from unhybridized DNA and RNA left in solution. One Gen-Probe procedure uses centrifugation to spin down the hydroxyapatite, and another procedure uses a magnet to attract and separate hydroxyapatite attached to magnetized matrix.

Four different Gen-Probe kits are available, each utilizing different specific ribosomal DNA probes. They are designed to detect (i) mycobacterial species in sputum, (ii) MTb complex organisms in sputum, (iii) MTb complex organisms in culture, and (iv) MAI complex organisms in culture.

STEPWISE PROCEDURE (9)

The Rapid Diagnostic System involves (i) growth of bacteria on appropriate agar or broth, or alternatively solubilization of sputum samples; (ii) lysis of bacteria to release ribosomal RNA into solution; (iii) hybridization with added $^{125}$I-labeled DNA probe; (iv) separation of hybridized from non-hybridized DNA probe; (v) quantitation of hybridized probe; (vi) calculation of percent of added probe that has hybridized; and (vii) interpretation of results.

(i) Solubilization: A minimum of one cc of sputum can be processed with "Solubilizer" and "Digest Reagent" provided by the Gen-Probe kit.

(ii) Lysis of mycobacteria: Bacteria are lysed using "Lysing Reagent" provided by the kit, and sonication. Solubilized sputum, or bacterial colonies from agar or broth, diluted in water to a McFarland #1 density, are used. Colonies as small as 1 mm in diameter can be suspended in 0.3 cc water for lysis.

(iii) Hybridization: $^{125}$I-labeled single-stranded DNA probe is added to lysed bacteria and the reaction mixture is incubated at 72°C in a water bath for one or two hours (depending on the source of the sample).

(iv) Separation: Hydroxyapatite linked to a solid matrix (a trade secret) is added to the reaction mixture, which is then incubated at 72°C for another five minutes. Hybridized nucleic acid is pulled out of solution by centrifugation, or by a magnet (depending on the particular kit in use), and is washed in buffer supplied by the kit.
Detection: The number of counts per minute (cpm) of 125-I-labeled DNA probe in double-stranded hybridized form (pulled out of solution attached to hydroxyapatite) is determined on a gamma counter. This numerical value equals "Sample cpm". A volume of 125-I-labeled DNA probe equal to that initially added to the reaction mixture is counted in parallel, and this equals "Total cpm". Background cpm is also measured and subtracted from both values.

Calculation: The "percent hybridization" equals: Sample cpm/Total cpm, multiplied by 100.

Interpretation of results: If no mycobacterial ribosomal RNA is present in the reaction mixture, the Calculated Percent Hybridization should be 0. If mycobacterial ribosomal RNA is indeed present in the reaction mixture, a percent hybridization greater than 0 and up to 100 would be obtained. A value of 100% hybridization would be obtained if an amount of mycobacterial RNA equal or greater to the amount of 125-I-labeled ribosomal DNA is present in the reaction mixture, and the reaction mixture is allowed to go to completion. Since the amount of radioactively-labeled DNA probe and the time of incubation are fixed for rapid detection, the reaction is never allowed to go to completion, and intermediate values of hybridization are obtained. These intermediate values are proportional to the concentration of mycobacterial ribosomal RNA present in the specimen.

Each of the four kits have different guidelines for interpretation. For instance, the kit for Mycobacterium species detection in sputum instructs that any value of hybridization greater than 3.6% is to be interpreted as indicating the presence of Mycobacterium species in the sputum. This cut-off level can detect as little as 5x10^-5 ug of MTb ribosomal RNA and 1x10^-4 ug MAI ribosomal RNA per assay. Values of less than 3.6% but greater that 1.2% hybridization are designated equivocal (i.e. if mycobacterial ribosomal RNA is present in the specimen, it is there in too low concentration to be distinguished from background cpm.) When some of these equivocal sputums are cultured, some grow out mycobacterium species, while others are culture negative. A value of less than 1.2% is indicative of the absence of mycobacterium in the sputum specimen, but of course does not exclude the possibility of mycobacterial infection in the patient since the mycobacterial organisms are irregularly shed in the sputum. It is recommended that at least three early morning sputum samples should be analyzed if negative or equivocal results are obtained.

Guidelines for identification of MTb complex in sputum also contain the equivocal category, but those for identification of MTb or MAI from culture only have positive (greater than 10% hybridization) and negative (less than 10%) categories.
SENSITIVITY AND SPECIFICITY (9)

The designation of an equivocal category when analysing sputum samples makes it difficult to compare the Gen-Probe Rapid Diagnostic System with acid-fast staining. Acid fast staining of sputum has up to almost a 100% specificity for detection of mycobacterium species, but specificity will depend on the experience and skill of the personnel screening the slide. Published sensitivities also vary from 22% to 75%. In a study of 294 sputums analysed by Gen-Probe with the mycobacterium species kit for sputums, 50 specimens (17%) gave equivocal results. In the remaining 244 specimens, Gen-probe reports a sensitivity of 85% and a specificity of 100%. Similarly, in a study of 371 sputum samples, 42 samples (11%) yielded equivocal results with the MTb complex kit for sputum. In the remaining 329 samples, sensitivity was 92.5% and specificity was 100%.

To compare this data with that of acid fast staining of sputum, it would be appropriate to include the equivocal sputums in the negative category to maintain specificity at 100%. The sensitivity of the Gen-Probe kits for sputum calculated this way is 67-68%.

When tested with a variety of pure cultures of reference strains of other bacteria, all four Gen-Probe kits were specific for the appropriate organism, with the exception that the kit for detection of mycobacterium species in sputum detected the presence of Rhodococcus bronchialis. Excess E. coli or N. asteroides ribosomal RNA added to the reaction mixtures did not interfere with detection of mycobacterial ribosomal RNA.

CLINICAL UTILITY

The unique clinical utility of the Rapid Diagnostic System is the ability to rapidly distinguish MTb complex-induced tuberculosis from MAI complex-induced tuberculosis-like pulmonary disease in AIDS patients. This can be done by testing sputum with the Gen-Probe kit for mycobacterial species (or by performing an acid fast stain on the sputum). If this is positive, the sputum is tested with the Gen-Probe kit for MTb complex. As mentioned above, distinguishing MTb from MAI infections is important with regard to therapeutic decisions. The Gen-Probe kit cannot distinguish individual member species within the MTb complex, but this distinction is not usually clinically important since M. tuberculosis is the overwhelming pathogen in this group.

From a laboratory standpoint, the Rapid Diagnostic System for detection of mycobacterial species in sputum has a higher sensitivity than acid fast staining and hence can be considered for use as a screen to identify sputums that do not contain mycobacteria, thus eliminating the need to culture these negative sputums. A protocol can be adopted that provides for culturing
only those sputums that yield positive or equivocal results with the Gen-Probe kit. However, it must be borne in mind that some cases of mycobacterial infection will be missed using this protocol, since, after excluding sputums in the equivocal category, the Rapid Diagnostic System for detection of mycobacterial species in sputum only identifies 85% of culture positive sputums as containing mycobacteria.

Even if this protocol is not adopted, the Gen-Probe mycobacteria kit for sputum is a good, and perhaps superior, alternative to acid fast staining because of its equal or greater sensitivity at a high specificity as compared to reported values for the acid fast stain. In non-immunocompromised patients, a positive result with the Gen-Probe mycobacteria kit for sputum may be taken as good evidence for MTb infection, but if desired, the Gen-Probe kit for detection of MTb in sputum can also be used for additional specificity.

If the Gen-Probe Rapid Diagnostic System is introduced into a laboratory to test sputums for the presence of mycobacteria, the same equipment can be used to identify cultured isolates of mycobacteria as being MTb complex or MAI complex. Gen-Probe sensitivities and specificities for detection of cultured mycobacteria are 100%, and the technique is faster (2 hours) as compared to biochemical identification (weeks) or BACTEC NAP identification (days). (8) It also has the theoretical advantage of positive identification of MAI complex, which the BACTEC system cannot do.

A decision to implement the Gen-Probe system to assay sputums or cultured isolates will depend on the prevalence of AIDS and tuberculosis in the population to be tested. (10) If these prevalences are high, the Rapid Diagnostic System offers a distinct advantage to acid fast staining of sputums to quickly identify patients infected with MTb complex. In the absence of a high percentage of AIDS patients, a decision to implement the Gen-Probe system would hinge on a cost-effect analysis as compared to alternative methods. Factors that must be considered include the prevalence of tuberculosis in the population in question, the number of specimens tested, the shelf-life of various reagents, and the sensitivity and specificity of acid fast staining of sputums at a given institution.
REFERENCES


9. Gen-Probe Rapid Diagnostic System Package Inserts