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Amplification of rRNA for Assessment of Treatment Response of Pulmonary Tuberculosis Patients during Antimicrobial Therapy

DOUGLAS F. MOORE,[1]* JANIS I. CURRY,[1] CAROLINE A. KNOTT,[2] AND VIVIAN JONAS[2]

Public Health Laboratory, Orange County Health Care Agency, Santa Ana, California 92706,[1] *and Gen-Probe Incorporated, San Diego, California 92121*[2]

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The time course of persistence of *Mycobacterium tuberculosis* as measured by detection of rRNA, acid-fast bacillus (AFB) smear, and culture was determined for pulmonary tuberculosis patients during antimicrobial therapy. Twenty-three patients who were initially AFB smear positive and who subsequently completed a course of antimicrobial therapy were selected for the study. Sequential specimens were tested by AFB smear, culture, and rRNA amplification (Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test [MTD]). The initial diagnostic specimens of all patients were positive by culture; those of 22 patients (96%) also were positive by MTD. Overall, MTD results remained positive longer than both smear and culture results. The median times to the last positive test result were 9 days for AFB smear, 26 days for culture, and 30 days for MTD. The last positive test result was the AFB smear result in 4% of cases, the culture result in 22%, and the MTD result in 52%. Fifty-six percent of patients had a period of shedding of noncultivable *M. tuberculosis* which was detected by MTD after culture results had converted to negative. This noncultivable period lasted 7 to 245 days. All three tests became reproducibly negative before the end of therapy and remained negative during follow-up for up to 1 year. These results indicate that during successful antimicrobial therapy, *M. tuberculosis* is eliminated in sputum samples as measured by amplification of rRNA, as well as by AFB smear and culture. No long-term rRNA carrier state was detected. While the time course of clearance of *M. tuberculosis* measured by rRNA overall was longer than with the two traditional tests, the rRNA test results allow sensitive and precise measurement of the clearance of noncultivable *M. tuberculosis* from respiratory specimens. This attribute may allow rRNA testing to be useful in clarifying patient response to antimicrobial therapy.

Many investigators have demonstrated the ability of nucleic acid amplification assays to supplant the acid-fast bacillus (AFB) smear and culture for laboratory diagnosis of *Mycobacterium tuberculosis*. Studies have focused on the rapid detection of *M. tuberculosis* for initial diagnosis of pulmonary tuberculosis. Assays which amplify ether DNA or RNA have been utilized for this purpose. The performance characteristics of transcription-mediated amplification (amplification of rRNA) (1, 4, 13, 17, 19, 21, 22), as well as laboratory-based PCR (7, 10, 26) and commercially based PCR (2, 6, 8, 18, 22), have been demonstrated. Sensitivities ranging from 82 to 100% and specificities ranging from 98 to 100% have been documented (1, 2, 4, 6–8, 10, 13, 17–19, 21, 22, 26). The performance characteristics of these assays, combined with a short turnaround time and commercial availability, ensure that use of these assays will become more widespread in the near future.

AFB smear and culture results are utilized to determine response to antimicrobial therapy, as well as initial diagnosis. Because the turnaround time for culture results is long, most initial clinical and public health management decisions are made on the basis of the AFB smear result, despite its low sensitivity and lack of specificity (3). Nucleic acid amplification tests have been shown to be more sensitive than the AFB smear and can be completed in 8 h or less (1, 6, 13, 18). Thus, it should be possible to utilize nucleic acid amplification test results, as well as AFB smear results, to guide patient therapy and public health decisions. A good understanding of the time frame of *M. tuberculosis* persistence as measured by nucleic acid amplification compared with culture and AFB smear is necessary to enable use of these tests for these purposes. Little has been published concerning this usage. Several researchers have demonstrated that in certain patients *M. tuberculosis* DNA, as detected by PCR, can be found after culture results have turned negative (10, 11, 26). Since these studies were not designed to address *M. tuberculosis* nucleic acid persistence, the data available are very limited in scope. In a study assessing use of PCR to monitor patients on therapy, Kennedy et al. demonstrated that PCR test results of successfully treated patients converted to negative but those of patients with a relapse became positive again (14). An in vitro study utilizing *M. smegmatis* treated with antibicrobial agents concluded that detection of rRNA was correlated with cell viability (20). The present study was performed to determine the time frame of *M. tuberculosis* persistence, as measured by detection of rRNA compared with culture and AFB smear in patients undergoing antimicrobial therapy. This can provide a basis for determination of the usefulness of rRNA detection to monitor patient response to therapy, as well as provide data to determine the meaning of a positive Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test (MTD; Gen-Probe, San Diego, Calif.) result when AFB smear and culture results are negative.

MATERIALS AND METHODS

Patient selection criteria and study design. Sequential, new, smear-positive patients presenting at the Orange County Health Care Agency Pulmonary Disease Clinic were selected for the study. Patients who completed a course of antimicrobial therapy were retained. Patients were treated with a standard four-

* Corresponding author. Mailing address: Orange County Public Health Laboratory, 1729 W 17th Street, Santa Ana, CA 92706. Phone: (714) 834-8385. Fax: (714) 834-7968.

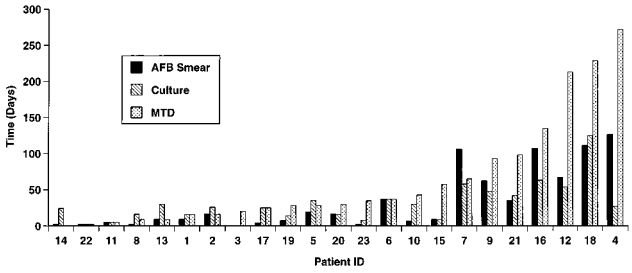

FIG. 1. Time to the last positive test result by patient. Patient order is based on increasing time to the last positive MTD result. ID, identification.

drug regimen (isoniazid, rifampin, pyrazinamide, and ethambutol). Pyrazinamide was discontinued after 3 months or when improvement was noted. Treatment was altered on the basis of patient response and laboratory results (culture, AFB smear, and drug susceptibility) as necessary. Standard laboratory testing was completed, and an aliquot of each specimen was frozen at −70°C. The MTD was performed retrospectively after the patient identifications had been removed. Review of patient charts was carried out to determine history, clinical diagnosis, therapy, and outcome.

Specimen collection and laboratory techniques. All specimens were induced sputum samples taken with an Ultra-Neb 99 nebulizer (DeVilbiss, Somerset, Pa.) with a 0.45% NaCl solution and a 50-ml sterile conical tube collection kit (Sage Products, Crystal Lake, Ill.). Specimens were kept at 4°C and received by the laboratory within 2 h. The majority of specimens were processed within 24 h by a standard *N*-acetyl-L-cysteine sodium hydroxide method (15) with addition of 1.0 ml of 0.2% bovine albumin (Becton Dickinson Microbiology Systems, Cockeysville, Md.), 45.5 U of penicillin G per ml, and 9% LaMotte Wide Range Indicator (LaMotte Chemical Company, Chestertown, Md.) to the final pellet, followed by titration to pH 6.8 to 7.2 with 0.5 N HCl. For each specimen, two Lowenstein-Jensen tubes (Becton Dickinson Microbiology Systems) were inoculated with a 0.1-ml specimen, a BACTEC 12B bottle was inoculated with 0.5 ml, and a smear was made for fluorochrome staining. The remainder of the sample was frozen at −70°C. Fluorochrome staining was performed by standard procedures (15). Tube cultures were examined weekly for 6 weeks, BACTEC vials were read daily for 7 days and biweekly for an additional 5 weeks. Positive cultures were quantitated, and acid-fast isolates were identified by standard biochemical techniques (15), DNA-RNA hybridization (Accu-Probe; Gen-Probe), or high-performance liquid chromatography (5). Antimicrobial susceptibility testing was done by standard techniques (15). The transcription-mediated amplification test (MTD) was performed in accordance with the package insert. Culture and smear results were not known by the investigators performing the MTD. Statistical analysis was done with Student's *t* test.

RESULTS

Patient demographics. Twenty-three patients, including 16 males (ages, 20 to 72 years) and seven females (ages, 25 to 65 years), made up the patient population. The majority of the patients were Hispanic ($n = 10$) and Southeast Asian ($n = 10$); two were Indian, and one was Caucasian. Twenty-one of the 23 patients were foreign born (10 were from Mexico and Central America, 10 were from Southeast Asia, and 2 were from India). Fifteen of the 23 patients were infected with *M. tuberculosis* strains susceptible to all primary drugs. Seven patients had strains resistant to a single drug (isoniazid, three; streptomycin, three; ethambutol, one), and one strain was resistant to all primary drugs. Six patients had previously been treated for tuberculosis. Patients remained on antimicrobial therapy for a median of 373 (range, 220 to 856) days. A mean of 15 (range, 6 to 26) specimens from each patient were analyzed. Twenty-two (96%) of 23 patients had initial diagnostic specimens which were positive by MTD, as well as by culture and AFB smear.

Time to conversion of test results. The time to the last positive AFB smear, culture, or MTD result for all patients is presented in Fig. 1. The MTD result was slower to convert to negative than the AFB smear and culture result for the majority of patients. As summarized in Table 1, the MTD had the highest median and mean times to the last positive result and the longest maximum time to the last positive result and was the last positive test in 52% of cases. For the 16 patients whose positive MTD results persisted longer than the AFB smear results, the median length of time of positivity past the last positive AFB smear result was 30 days. For the 13 patients whose positive MTD results persisted longer than their positive culture results, the median length of time of positivity past the last positive culture result was 45 days (Table 1).

Figure 2 presents the kinetics of conversion from positive to negative test results for all patients. The initial rate of conversion to negative test results was fastest for the AFB smear, slower for culture, and slowest for MTD. Fifty percent of all patients' AFB smear results were negative after day 9, compared with day 26 for culture and day 30 for MTD. Ninety percent of patients had their last positive test result on or before day 107 for the AFB smear, day 58 for culture, and day 213 for MTD. While the initial rate of AFB smear result conversion for the population was very rapid, the rate slowed at 60% of patients and was intermediate between those of culture and MTD results for the final 40%.

Detection of noncultivable *M. tuberculosis*. Many of the patients had periods of shedding of noncultivable *M. tuberculosis*. Specimens from these patients were positive by AFB smear or MTD or by both tests after culture results had converted to negative. A period of noncultivable *M. tuberculosis* shedding was detected by AFB smear in 5 patients (22%) and by MTD in 13 patients (56%), including all 5 patients in whom shedding was detected by AFB smear. The median durations of these noncultivable-mycobacterium-shedding periods after the culture result had converted to negative were 44 (range, 13 to 99) days for the 5 patients in whom shedding was detected by AFB smear and 45 (range, 7 to 245) days for the 13 patients in whom shedding was detected by MTD. The pattern of test results for these patients is represented by Fig. 3D and E.

Effects of previous treatment and drug-resistant strains on clearance times. The 6 patients who had been previously treated (patients 4, 7, 12, 15, 18, and 23 in Fig. 1) had mean times to the last positive test result of 70, 47, and 145 days for the AFB smear, culture, and MTD, respectively, compared with mean times of 20, 25, and 35 days for the AFB smear, culture, and MTD, respectively, for the 17 patients with no previous treatment. However, these differences were not sta-

TABLE 1. Comparison of time to last positive test result and difference in time to last positive MTD result

Test	% of last positive result[a]	Median no. of days to last positive result	Mean no. of days to last positive result	Maximum no. of days to last positive result	Median no. of days (range) from last positive result to last positive MTD result
AFB smear	4	9	33	126	30 (7–146)[b]
Culture	22	26	31	125	45 (7–245)[c]
MTD	52	30	64	272	

[a] The last positive test was often a combination of tests: AFB smear, culture, and MTD, 13%; culture and MTD, 9%.
[b] For the 16 patients whose MTD results were positive longer than their AFB smear results.
[c] For the 13 patients whose MTD results were positive longer than their culture results.

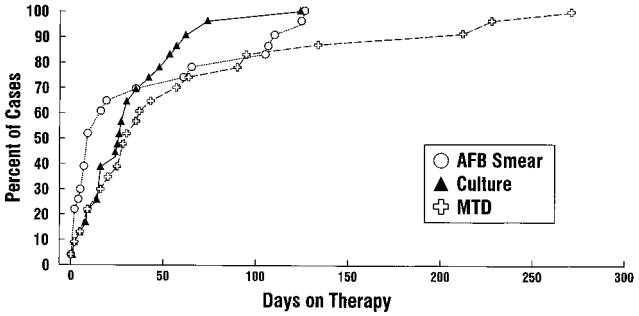

FIG. 2. Time of last positive test result for all patients. Symbols: ○, AFB smear; ▲, culture; ✣, MTD.

tistically significant ($P > 0.05$). There was no statistically significant difference ($P < 0.05$) in clearance times due to resistance to a single drug, ethnicity, and gender. The one patient (no. 18) with a multiply resistant strain had the second longest conversion time for AFB smear and MTD results. She also had a history of previous treatment and was treated with primary drugs for 90 days before therapy was switched to secondary drugs. Patients 4 and 12, who had the longest and third longest conversion times for MTD results, both had been previously treated, in Vietnam and Korea, respectively. The details of their earlier therapy were not available.

Continuity of negative test results. For every patient, all test results, including rRNA amplification, became negative during the course of therapy and were negative before therapy was discontinued. Patients had a median of 9 (range, 1 to 14) specimens negative by MTD after conversion to a negative result. The specimens producing these negative MTD results were collected over a median period of 385 (range, 26 to 715) days. Specimens were collected from 10 patients after cessation of therapy. These specimens were also uniformly negative when tested by AFB smear, culture, and MTD. The median period of negative MTD results after cessation of therapy was 197 (range, 10 to 385) days for these 10 patients. Therapy was continued past the last positive test result for at least 145 days for AFB smear, 190 days for culture, and 118 days for MTD. None of the patients later relapsed to active tuberculosis.

Patterns of test results. The complete test results for five representative patients shown in Fig. 3 demonstrate the most common patterns of test results over time. For patients 2, 6, and 22, all test results converted to negative in under 10 days. While individual test results correlated for patients 6 and 22, they did not for patient 2 while following the same pattern. Patient 4 represents patients with an early conversion of culture followed by an extensive period of shedding of noncultivable organisms, detected first by smear and rRNA and then by rRNA alone. Patient 12 also had a noncultivable-organism-shedding period with a gap of negative results ended by a single positive rRNA result.

All results were examined to determine if three sequential negative test results would accurately predict final conversion from a positive to a negative test result. Four patients had a period in which three or more sequential negative test results were followed by a positive test result. This occurred once for culture, twice for the AFB smear, and three times for MTD. The calculated predictive values of three negative specimens signaling conversion are 91% for the AFB smear, 96% for culture, and 87% for MTD. Four negative results were 96% predictive for the AFB smear, 96% predictive for culture, and 91% predictive for MTD. Five negative test results were 100% predictive of final conversion to negative results for all of the tests.

DISCUSSION

Conversion of AFB smear and culture results from positive to negative for patients undergoing antimicrobial therapy is well established (9, 25). In this study, the median and mean times to the last positive AFB smear were 9 and 33 days, respectively. This conversion gives timely feedback to the clinician on the efficacy of antimicrobial treatment. The data presented here demonstrate that conversion from positive to negative results also takes place for a test which detects *M. tuberculosis* rRNA. However, the time course of the conversion of MTD results to negative was longer than that for AFB smear or culture in the majority of patients. For every patient, the rRNA test results converted to negative before antimicrobial treatment was completed. Once the rRNA test results converted to a negative state, they remained negative, as did AFB smear and culture results. However, there was a variability of individual test results for some patients, as shown in Fig. 3. This could be the result of variations in the shedding of organisms or in the efficiency of specimen collection. Even with this variability, the data presented here indicate that three negative results are ≥96% predictive of conversion for all tests while five negative results are 100% predictive.

There was no evidence of an extended rRNA carrier state. Of interest was the detection by MTD of a noncultivable-organism-shedding period in 56% of the patients. In these patients, rRNA was detected after culture results had converted to negative. This noncultivable-organism-shedding pe-

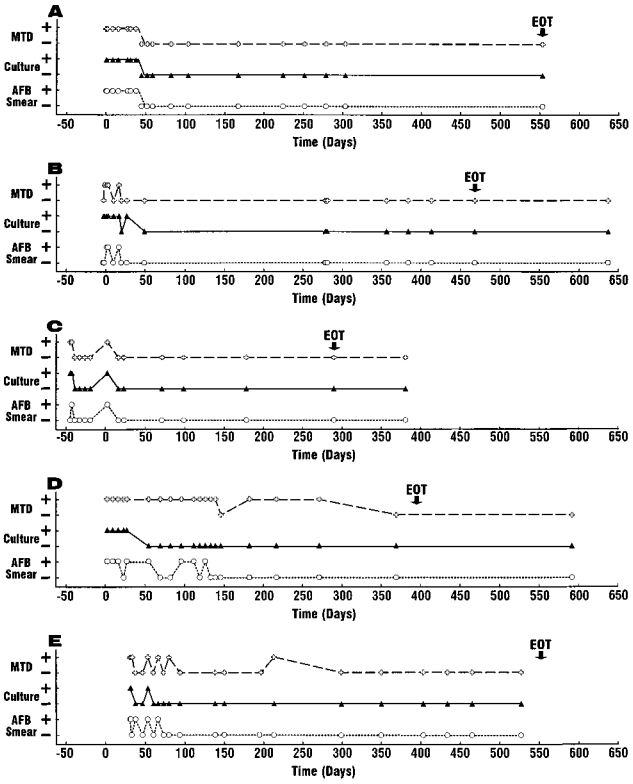

FIG. 3. Serial MTD, culture, and AFB smear results for samples from patients 6 (A), 2 (B), 22 (C), 4 (D), and 12 (E). Zero time is the day of initiation of antimicrobial therapy. EOT, end of therapy.

riod can be extensive; the maximum time seen in this study was 245 days but is usually much shorter (the median duration was 45 days). A noncultivable-organism-shedding period was also detected by AFB smear but in only 22% of the patients. Overall, these results are similar to the PCR results reported by Kennedy et al. for successfully treated patients (14).

The composition of the patient population studied here may have affected the results. Only initially smear-positive patients were enrolled in the study, and only patients who completed therapy were retained for this study. In our clinic, approximately 50% of the patients are smear negative on initial presentation, so our patient sample was biased to more heavily infected patients. Because of the aggressive treatment and the selection of patients who completed therapy, all of the patients in this group were clinically determined to have completed therapy successfully and suffered no relapses. Thus, this study represents a longitudinal view of traditional and rRNA testing results for a more heavily infected group of patients who successfully completed therapy. For other patient populations, the results may be slightly different. For smear-negative tuberculosis cases, the time for conversion to negative culture and MTD results will most likely be less than that reported here. Also, because there is a lighter *M. tuberculosis* load in smear-negative patients, the percentage of patients shedding noncultivable organisms will most likely be lower and the shedding periods may be shorter. Preliminary (unpublished) data from our laboratory indicate that noncompliant patients who do not respond to therapy remain MTD positive, as well as AFB smear and culture positive, longer than did the patients in this study.

The noncultivable-organism-shedding period detected by AFB smear in this study is very similar in incidence and duration to those described in previous studies (9, 12, 16, 23). However, this is the first report describing the quantitation of such a shedding period with an rRNA amplification test. Researchers have previously presented data which support the existence of such a period on the basis of both PCR (2, 10, 11, 14, 18) and MTD (13). In some instances, the effect of the noncultivable-organism-shedding period is seen as initial "false-positive" specimens when nucleic acid amplification test and culture results are compared. On discrepant analysis, it is often seen that these specimens were collected from a patient with documented tuberculosis on antimicrobial therapy (2, 7, 13, 18). It appears that these patients were in a noncultivable-organism-shedding period when the specimens were collected. A recent in vitro study utilizing *M. smegmatis* concluded that rRNA decays rapidly after cell death while DNA is stable for longer periods (20). Whether a noncultivable DNA period is longer or shorter than the noncultivable rRNA period in vivo and if it reproducibly converts to negative test results for patients on antimicrobial therapy, as seen here for rRNA, must be determined by future investigations.

The data presented here reinforce results from several clinical trials which indicate that detection of *M. tuberculosis* rRNA, as well as positive AFB smear and culture results, is indicative of an active infection and a diseased state (1, 4, 13, 17, 19, 21, 22). In this study, the last positive MTD result occurred a minimum of 118 days before patients were considered cured and therapy was discontinued on the basis of standard clinic protocols. There is no evidence of an extended latency state after successful treatment that can be detected by MTD. These results are similar to a longitudinal PCR study of patients treated for *Chlamydia trachomatis* in which no long-term carrier state was seen (24).

No statistically significant effect on clearance time was seen when patients were grouped by gender, ethnicity, resistance to a single drug, or previous treatment. However, the three patients with the longest clearance times by MTD had had previous therapy and one of these was infected with a multiply resistant strain. These three patients were all foreign born, and details of their earlier treatment were not available. Patient 7 had several positive AFB smear results with negative MTD results. This was most likely due to an inhibitor of the MTD, since the sensitivity of the MTD for smear-positive samples in our laboratory is 99% (13). To detect inhibitors, our current protocol calls for retesting of all AFB smear-positive, MTD-negative samples by using the package insert protocol for inhibitor detection.

The exact physical state of *M. tuberculosis* rRNA during the noncultivable-organism-shedding period demonstrated in this study is not known. A noncultivable-organism-shedding period detected by MTD when the AFB smear result is also positive suggests that intact organisms with detectable rRNA sequences are present. They do not reproduce in culture because they are either totally nonviable or so damaged that they cannot grow on laboratory media. *M. tuberculosis* detected in a noncultivable state by MTD only may also be intact but at a concentration too low for detection by the AFB smear. It is also possible that there could be a period during which *M. tuberculosis* rRNA is detectable while there is no intact organism but before all of the rRNA is degraded. Determination of the condition of noncultivable *M. tuberculosis* cells remains for further studies. Whatever the outcome of these studies, it has been demonstrated here that the duration of the noncultivable-organism-shedding period is finite for patients on a successful treatment regimen.

The results of this study indicate that rRNA becomes nondetectable in patients responding to therapy before therapy was discontinued. Therefore, the use of the MTD to monitor therapy and as a final "test of cure" is a theoretical possibility. However, before this can be considered, additional studies need to be carried out to corroborate the existing data, compare conversion times for successfully treated patients versus nonresponders, and establish the minimum time period after rRNA conversion for continuation of therapy. Until these studies are carried out, an understanding of the kinetics of rRNA test conversion in successfully treated patients allows the MTD to be helpful in certain instances. One example is in the initial diagnosis of partially treated patients. These patients are often culture negative and may be AFB smear positive or negative. The results presented here indicate that a patient may have a positive rRNA test result, due to shedding of noncultivable organisms, which would establish the diagnosis.

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REFERENCES

1. **Abe, C., K. Hirano, M. Wada, Y. Kazumi, M. Takahashi, Y. Fukasawa, T. Yoshimura, C. Miyagi, and S. Goto.** 1993. Detection of *Mycobacterium tuberculosis* in clinical specimens by polymerase chain reaction and Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test. J. Clin. Microbiol. **31:** 3270–3274.
2. **Beavis, K. G., M. B. Lichty, D. L. Jungkind, and O. Giger.** 1995. Evaluation of Amplicor PCR for direct detection of *Mycobacterium tuberculosis* from sputum specimens. J. Clin. Microbiol. **33:**2582–2586.
3. **Benneson, A. S.** 1995. Tuberculosis, p. 488–497. *In* A. S. Benenson (ed.), Control of communicable disease in man, 16th edition. American Public Health Association, Washington, D.C.
4. **Bodmer, T., A. Gurtner, K. Schopfer, and L. Matter.** 1994. Screening of respiratory tract specimens for the presence of *Mycobacterium tuberculosis* by

using the Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test. J. Clin. Microbiol. **32:**1483–1487.
5. **Butler, R. W., K. C. Jost, Jr., and J. O. Kilburn.** 1991. Identification of mycobacteria by high-performance liquid chromatography. J. Clin. Microbiol. **29:**2468–2472.
6. **Chin, D. P., D. M. Yajko, W. K. Hadley, C. A. Sanders, P. S. Nassos, J. J. Madej, and P. C. Hopewell.** 1995. Clinical utility of a commercial test based on the polymerase chain reaction for detecting *Mycobacterium tuberculosis* in respiratory specimens. Am. J. Respir. Crit. Care Med. **151:**1872–1877.
7. **Clarridge, J. E., R. M. Shawar, T. M. Shinnick, and B. B. Plikaytis.** 1993. Large-scale use of polymerase chain reaction for detection of *Mycobacterium tuberculosis* in a routine mycobacteriology laboratory. J. Clin. Microbiol. **31:**2049–2056.
8. **D'Amato, R. F., A. A. Wallman, L. H. Hochstein, P. M. Colaninno, M. Scardamaglia, E. Ardila, M. Ghouri, K. Kim, R. C. Patel, and A. Miller.** 1995. Rapid diagnosis of pulmonary tuberculosis by using Roche AMPLICOR *Mycobacterium tuberculosis* PCR test. J. Clin. Microbiol. **33:**1832–1834.
9. **East African/British Medical Research Councils.** 1973. Controlled clinical trial of four short-course (6-month) regimens of chemotherapy for treatment of pulmonary tuberculosis. Lancet **1079:**1331–1339.
10. **Forbes, B. A., and K. E. S. Hicks.** 1993. Direct detection of *Mycobacterium tuberculosis* in respiratory specimens in a clinical laboratory by polymerase chain reaction. J. Clin. Microbiol. **31:**1688–1694.
11. **Hermans, P. W., A. R. J. Schuitema, D. Van Soolingen, C. P. H. J. Verstynen, E. M. Bik, J. E. R. Thole, A. Kolk, and J. Van Embden.** 1990. Specific detection of *Mycobacterium tuberculosis* complex strains by polymerase chain reaction. J. Clin. Microbiol. **28:**1204–1213.
12. **Hobby, G. C., A. P. Holman, M. D. Iseman, and J. M. Jones.** 1973. Enumeration of tubercle bacilli in sputum of patients with pulmonary tuberculosis. Antimicrob. Agents Chemother. **4:**94–104.
13. **Jonas, V., M. J. Alden, J. I. Curry, K. Kamisango, C. A. Knott, R. Lankford, J. M. Wolfe, and D. F. Moore.** 1993. Detection and identification of *Mycobacterium tuberculosis* directly from sputum sediments by amplification of rRNA. J. Clin. Microbiol. **31:**2410–2416.
14. **Kennedy, N., S. H. Gillespie, A. O. S. Saruni, G. Kisyombe, R. McNerney, F. I. Ngowi, and S. Wilson.** 1994. Polymerase chain reaction for assessing treatment response in patients with pulmonary tuberculosis. J. Infect. Dis. **170:**713–716.
15. **Kent, P. T., and G. P. Kubica.** 1985. Public health mycobacteriology. A guide for the level III laboratory. Centers for Disease Control, Atlanta.
16. **Kim, T. C., R. S. Blackman, K. M. Heatwole, T. Kim, and D. F. Rochester.** 1984. Acid-fast bacilli in sputum smears of patients with pulmonary tuberculosis. Am. Rev. Respir. Dis. **129:**264–268.
17. **Miller, N., S. G. Hernandez, and T. J. Cleary.** 1994. Evaluation of Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test and PCR for direct detection of *Mycobacterium tuberculosis* in clinical specimens. J. Clin. Microbiol. **32:**393–397.
18. **Moore, D. F., and J. I. Curry.** 1995. Detection and identification of *Mycobacterium tuberculosis* directly from sputum sediments by Amplicor PCR. J. Clin. Microbiol. **33:**2686–2691.
19. **Pfyffer, G. E., P. Kissling, R. Wirth, and R. Weber.** 1994. Direct detection of *Mycobacterium tuberculosis* complex in respiratory specimens by a target-amplified test system. J. Clin. Microbiol. **32:**918–923.
20. **Van Der Vliet, G. M. E., P. Schepers, R. A. F. Schukkink, B. Van Gemen, and P. R. Klatser.** 1994. Assessment of mycobacterial viability by rRNA amplification. Antimicrob. Agents Chemother. **38:**1959–1965.
21. **Vlaspolder, F., P. Singer, and C. Roggeveen.** 1995. Diagnostic value of an amplification method (Gen-Probe) compared with that of culture for diagnosis of tuberculosis. J. Clin. Microbiol. **33:**2699–2703.
22. **Vuorinen, P., A. Miettinen, R. Vuento, and O. Hällström.** 1995. Direct detection of *Mycobacterium tuberculosis* complex in respiratory specimens by Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test and Roche Amplicor Mycobacterium Tuberculosis Test. J. Clin. Microbiol. **33:**1856–1859.
23. **Warring, F. C., Jr., and U. Sutramongkole.** 1970. Nonculturable acid-fast forms in the sputum of patients with tuberculosis and chronic pulmonary disease. Am. Rev. Respir. Dis. **102:**714–724.
24. **Workowski, K. A., M. F. Lampe, K. G. Wong, M. B. Watts, and W. E. Stamm.** 1993. Long-term eradication of *Chlamydia trachomatis* genital infection after antimicrobial therapy. JAMA **270:**2071–2075.
25. **Yeager, H., Jr., J. Lacy, L. R. Smith, and C. A. LeMaistre.** 1967. Quantitative studies of mycobacterial populations in sputum and saliva. Am. Rev. Respir. Dis. **95:**998–1004.
26. **Yuen, K. Y., K. S. Chan, C. M. Chan, B. S. W. Ho, L. K. Dai, P. Y. Chau, and M. H. Ng.** 1993. Use of PCR in routine diagnosis of treated and untreated pulmonary tuberculosis. J. Clin. Pathol. **46:**318–322.