Antimicrobial Agents and Chemotherapy

Rapid susceptibility testing of Mycobacterium tuberculosis by bioluminescence assay of mycobacterial ATP.

L E Nilsson, S E Hoffner, and S Anséhn

1988. Rapid susceptibility testing of Mycobacterium tuberculosis by bioluminescence assay of mycobacterial ATP.. Antimicrob. Agents Chemother. 32(8):1208-1212. doi:10.1128/AAC..

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1988, p. 1208–1212
0066-4804/88/081208-05$02.00/0

Vol. 32, No. 8

Rapid Susceptibility Testing of *Mycobacterium tuberculosis* by Bioluminescence Assay of Mycobacterial ATP

LENNART E. NILSSON,[1]* SVEN E. HOFFNER,[2] AND STEFFAN ÅNSÉHN[1]

Department of Clinical Bacteriology, University Hospital, S-581 85 Linköping,[1] and Department of Bacteriology, National Bacteriological Laboratory, S-105 21 Stockholm,[2] Sweden

Received 23 February 1988/Accepted 23 May 1988

Mycobacterial growth was monitored by bioluminescence assay of mycobacterial ATP. Cultures of *Mycobacterium tuberculosis* H37Rv and of 25 clinical isolates of the same species were exposed to serial dilutions of ethambutol, isoniazid, rifampin, and streptomycin. A suppression of ATP, indicating growth inhibition, occurred for susceptible but not resistant strains within 5 to 7 days of incubation. Breakpoint concentrations between susceptibility and resistance were determined by comparing these results with those obtained by reference techniques. Full agreement was found in 99% of the assays with the resistance ratio method on Lowenstein-Jensen medium, and 98% of the assays were in full agreement with the radiometric system (BACTEC). A main advantage of the bioluminescence method is its rapidity, with results available as fast as with the radiometric system but at a lower cost and without the need for radioactive culture medium. The method provides kinetic data concerning drug effects within available in vivo drug concentrations and has great potential for both rapid routine susceptibility testing and research applications in studies of drug effects on mycobacteria.

Testing of *Mycobacterium tuberculosis* susceptibility to antimycobacterial agents on the basis of growth on solid media requires 3 to 4 weeks of incubation (2, 3). Among these methods, the resistance ratio method on Lowenstein-Jensen medium (2) is most used in Europe, while the plate proportional method on 7H10 agar (3) is the main method used in the United States. To achieve more rapid susceptibility testing, alternative techniques based on bacterial growth in broth cultures have been adapted. The rapid radiometric system (BACTEC) quantifies $^{14}CO_2$ produced by mycobacteria growing in broth containing ^{14}C-labeled palmitic acid (4). Results are provided after 1 week of incubation, and susceptibility is defined as a 99% reduction of the metabolic activity of a drug-exposed culture compared with that of an unexposed control culture (9). This technique is widely used, and the results are in good agreement with those of reference methods on solid media (4, 8–11).

The firefly bioluminescence assay of bacterial ATP has been used for studies of effects of antimicrobial agents on various bacteria (6, 7) and for rapid susceptibility testing of *M. tuberculosis* (1).

The aim of this study was to evaluate this technique for rapid susceptibility testing of *M. tuberculosis* by comparing it with the resistance ratio method on Lowenstein-Jensen medium and with the rapid radiometric system (BACTEC).

MATERIALS AND METHODS

Bacterial strains. *M. tuberculosis* H37Rv and 25 clinical isolates of the same species with different patterns of susceptibility to antimycobacterial agents were obtained from the culture collection of the National Bacteriological Laboratory.

Antimycobacterial agents. Stock solutions of 10,000 μg of active drug per ml were prepared from ethambutol (Cyanamid of Great Britain Ltd., Gosport, England), isoniazid (Ferrosan, Malmö, Sweden), rifampin (Ferrosan), and streptomycin (Glaxo Laboratories Ltd., Greenford, England).

Radiometric method. Antimycobacterial drug solution (0.1 ml) was added to a BACTEC vial containing 2 ml of Middlebrook 7H12 tb medium (Johnston Laboratories, Inc., Towson, Md.) to give final concentrations of 5 μg of ethambutol, 0.2 μg of isoniazid, 2 μg of rifampin, and 4 μg of streptomycin per ml. Each vial was inoculated with 0.1 ml of a bacterial suspension comparable to the McFarland no. 1 standard. The same inoculum was added to one drug-free vial (control A), and for determining the 1% proportion of resistance, another drug-free vial (control B) was inoculated with a 100-fold dilution of that inoculum. The amount of $^{14}CO_2$ produced as a result of the bacterial metabolic activity was measured in a BACTEC 460 instrument (Johnston Laboratories). The result was expressed as a growth index. A culture was considered resistant to a tested drug when the daily increase of the growth index of that culture in a drug-containing vial was greater than that of the culture in the diluted control vial (control B), when the growth index of the culture in this vial was at least 30. Results of tests with cultures in which the recorded growth index of control B was below 30 after a week of incubation were disregarded, and the test was repeated with a higher inoculum.

Resistance ratio method. Series of tubes with Lowenstein-Jensen medium containing twofold dilutions of ethambutol (0.5 to 16 μg/ml), isoniazid (0.06 to 8 μg/ml), rifampin (5 to 16 μg/ml), and streptomycin (2 to 64 μg/ml) were inoculated with the strains to be tested and the susceptible standard strain H37Rv. The ratio between the MICs for the strain to be tested and the standard strain H37Rv was calculated. A ratio of 4 or higher indicated resistance (2).

Bioluminescence method. Dilutions of the antimycobacterial agents were prepared in 7H9 broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with OADC (oleic acid bovine albumin [factor V], dextrose, catalase) enrichment (BBL) and 0.05% Tween 80. Samples (0.5 ml) of these dilutions were added to series of test tubes. Colonies of *M. tuberculosis* from Lowenstein-Jensen medium were suspended in 2 ml of supplemented 7H9 broth in tubes containing glass beads. After vortexing, the tubes were left for 5 min

* Corresponding author.

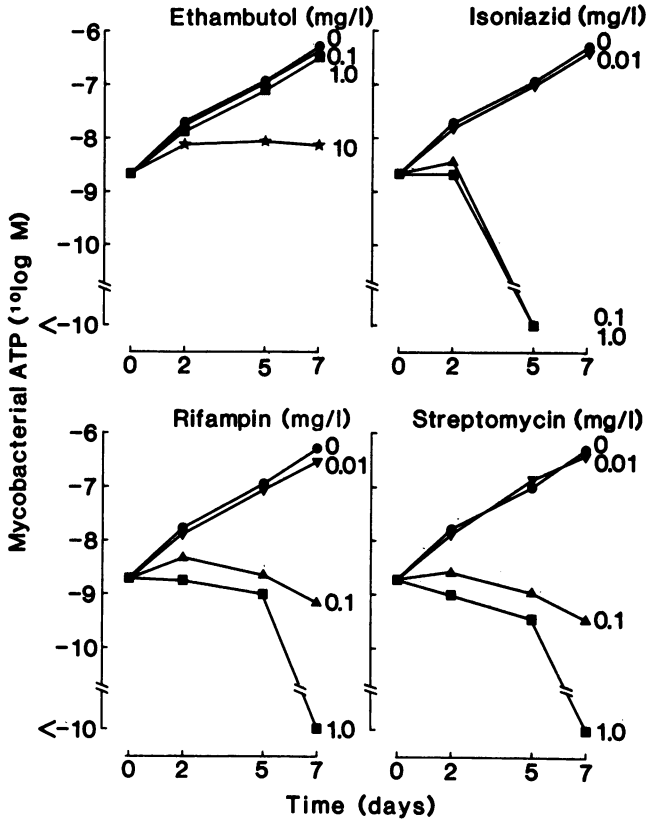

FIG. 1. Mycobacterial ATP in cultures of *M. tuberculosis* H37Rv exposed to various concentrations of ethambutol, isoniazid, rifampin, and streptomycin.

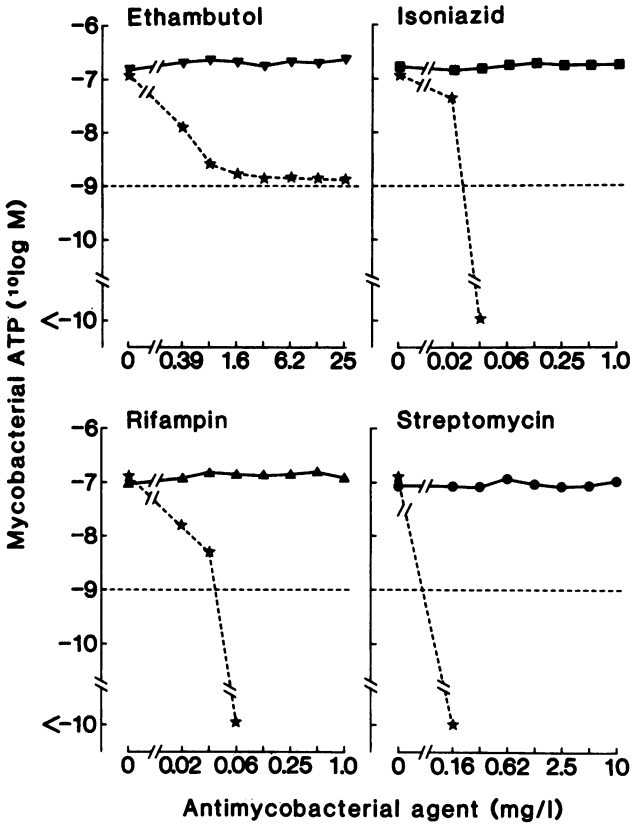

FIG. 2. Dose-dependent effects of ethambutol, isoniazid, rifampin, and streptomycin on mycobacterial ATP in cultures of *M. tuberculosis* H37Rv (★), 1018/83 (▼), 1500/82 (■), 9612/85 (▲), and 2916/85 (●) after 5 days of incubation. The horizontal dotted lines indicate the mycobacterial ATP of the inocula.

in order for larger particles to settle. The supernatant was diluted to a McFarland no. 4 standard, corresponding to approximately 10^9 CFU/ml.

After further dilution to the indicated inocula, 0.5 ml was added to tubes containing antimycobacterial agents, and the tubes were incubated at 37°C.

Extraction of mycobacterial ATP. A 50-μl sample from the mycobacterial culture was pipetted into 500 μl of boiling 0.1 M Tris buffer (pH 7.75) containing 2 mM EDTA. After heating for 90 s in a LKB Biocal 2073 incubator (LKB Products, Bromma, Sweden), the extracts were cooled to room temperature before assay of mycobacterial ATP.

Bioluminescence assay of mycobacterial ATP. ATP monitoring reagent (100 μl) (LKB-Wallac, Turku, Finland) was added to each extract (550 μl), and the light intensity was measured in a 1250 Luminometer (LKB-Wallac) and recorded on a 1250 Display (LKB-Wallac). Sample ATP levels were calculated by using assay of standard amounts of ATP as reference, and correction for background luminescence was made.

RESULTS

The mycobacterial ATP levels in cultures of *M. tuberculosis* H37Rv, exposed to 10-fold dilutions of the antimycobacterial agents, are shown in Fig. 1. Growth inhibition was recorded after 2 days of exposure to effective drug concentrations. A decrease below the ATP level of the inoculum, indicating a bactericidal effect, occurred after 5 to 7 days of incubation with isoniazid, rifampin, and streptomycin (Fig. 1).

M. tuberculosis H37Rv and four clinical isolates, classified as resistant by the resistance ratio method, were exposed to various concentrations of antimycobacterial agents for 5 days (Fig. 2). A dose-dependent decrease in mycobacterial ATP levels was found in cultures of the susceptible reference strain (H37Rv) after exposure to all the agents (Fig. 2). At high concentrations of isoniazid, rifampin, and streptomycin, the ATP levels decreased below the ATP level of the inoculum, indicating bactericidal effects. The ATP levels in the cultures of the resistant isolates were not affected by the agents, even at the highest concentrations tested (Fig. 2).

Inocula of approximately 10^4 and 10^6 CFU/ml of 25 clinical isolates of *M. tuberculosis* were exposed to serial twofold dilutions of the antimycobacterial agents for 7 days. The mycobacterial ATP level was plotted as the percentage of the ATP level in an unexposed control culture (ATP index). The results obtained with 10^4 CFU/ml are shown in Fig. 3. These results were used to find concentrations that discriminated susceptible and resistant strains by comparing the results with those obtained by the resistance ratio method and the radiometric system (BACTEC) with 5 μg of ethambutol, 0.2 μg of isoniazid, 2 μg of rifampin, and 4 μg of streptomycin per ml. At the discriminating concentration (breakpoint), the ATP index had to be less than 0.1 to classify the strain as susceptible by the bioluminescence assay. By using this criterion, the following breakpoints were defined (micrograms per milliliter): ethambutol, 2.5; isoniazid, 0.25; rifampin, 0.25; and streptomycin, 0.31 (Fig. 3).

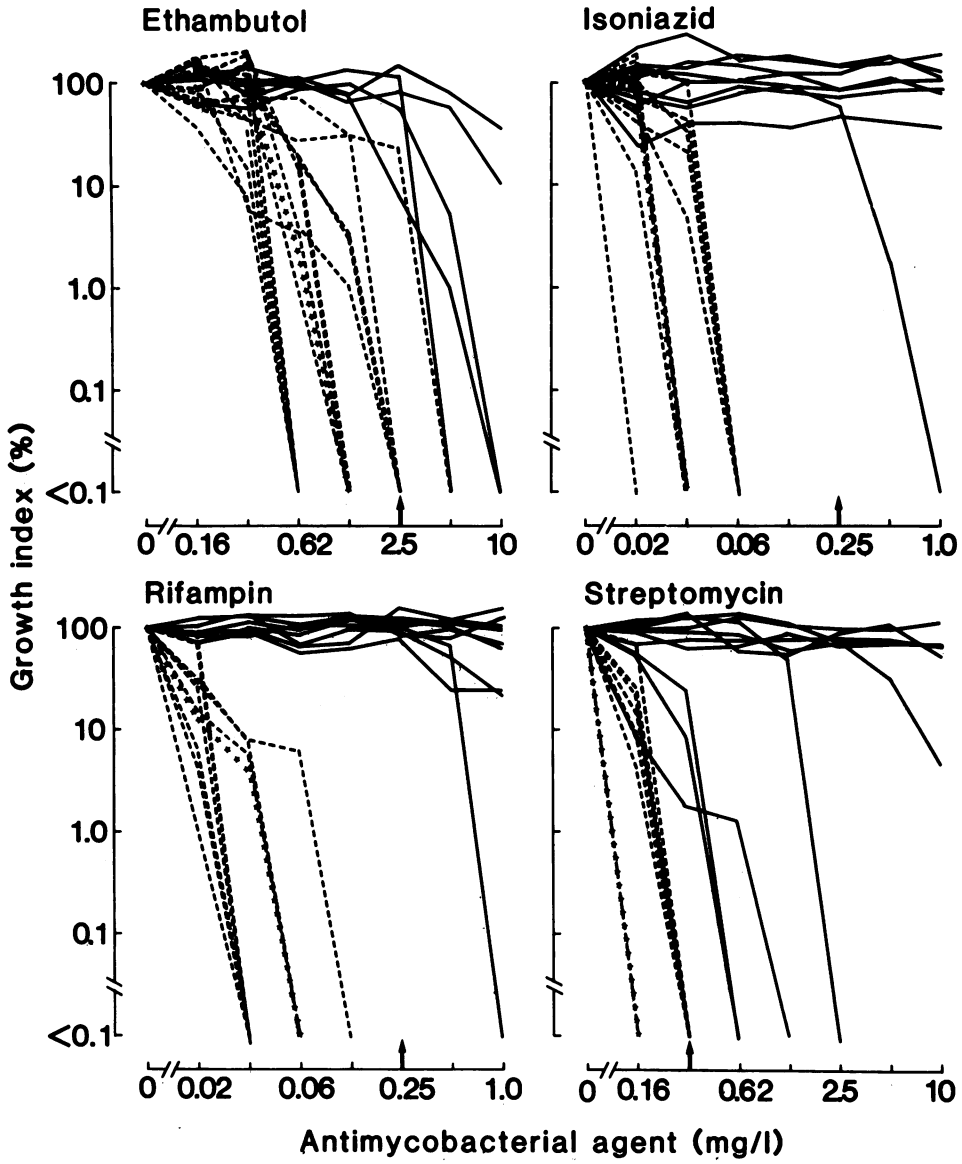

FIG. 3. Mycobacterial ATP in cultures of *M. tuberculosis* H37Rv (★) and in cultures of 25 clinical isolates of the same species classified as susceptible (---) or resistant (——) to ethambutol, isoniazid, rifampin, and streptomycin by the resistance ratio method. The mycobacteria (10^4 CFU/ml) were exposed to the antimycobacterial agents for 7 days, and the mycobacterial ATP levels in the cultures were plotted as percentages of each unexposed control culture. The defined breakpoint for each agent is indicated with an arrow.

Full agreement between the bioluminescence assay and the resistance ratio method was found in 103 of 104 susceptibility tests (99%), and with the concentrations indicated above, 102 tests (98%) were in full agreement with the radiometric system (Table 1). One strain (15867/81) was classified as susceptible to streptomycin (4 μg/ml) by the radiometric system but resistant by the other two methods (Table 1). Another strain (9630/83) was classified as resistant to ethambutol by the bioluminescence assay but susceptible by the resistance ratio method and the BACTEC system (ethambutol, 5.0 μg/ml) (Table 1).

Some of the resistant strains had lower ATP indices than other resistant strains at high concentrations of the antimycobacterial agents (Fig. 3). Most of these strains had comparable low delta growth indices, determined by the radiometric system and low ratios, determined by the resistance ratio method (Table 1).

DISCUSSION

This study shows that growth of *M. tuberculosis* can be monitored by bioluminescence assay of mycobacterial ATP and that growth monitored by this method in antimicrobial agent-exposed cultures allows for rapid susceptibility testing.

In susceptibility testing of bacteria, it is important to use an inoculum of the proper size. If it is too small, the proportion of resistant bacteria necessary for the arbitrary criteria for resistance will not be detected. On the other hand, if the inoculum is too large, the growth of resistant variants, present in low frequencies, will simulate resistance. Therefore, we used both small (10^4 CFU/ml) and large (10^6 CFU/ml) inocula. The bioluminescence assay detected effects on mycobacterial growth within 5 to 7 days with both inocula, but when the larger inoculum (10^6 CFU/ml) was

TABLE 1. Susceptibility of *M. tuberculosis* to ethambutol, isoniazid, rifampin, and streptomycin by the resistance ratio method (RR), the radiometric method (BACTEC), and the bioluminescence assay (ATP)

Strain	Result for[a]:											
	Ethambutol			Isoniazid			Rifampin			Streptomycin		
	RR	BACTEC	ATP	RR	BACTEC	ATP	RR	BACTEC	ATP	RR	BACTEC	ATP
H37Rv		S	S		S	S		S	S		S	S
11 Clinical isolates	S (1)	S	S	S (1)	S	S	S (1)	S	S	S (1)	S	S
9612/85	S (1)	S	S	S (1)	S	S	R (32)	R	R	S (1)	S	S
7426/84	S (1)	S	S	S (1)	S	S	S (1)	S	S	R (4)	R[b]	R[c]
3769/84	S (1)	S	S	S (1)	S	S	S (1)	S	S	R (4)	R[b]	R[c]
2656/84	S (1)	S	S	S (1)	S	S	S (1)	S	S	R (8)	R	R
4895/84	S (2)	S	S	S (1)	S	S	S (1)	S	S	R (8)	R	R
2916/85	S (1)	S	S	S (1)	S	S	S (1)	S	S	R (16)	R	R
5638/85	S (2)	S	S	R (160)	R	R	R (16)	R	R	S (1)	S	S
5412/84	S (2)	S	S	R (160)	R	R	R (8)	R	R	S (2)	S	S
9630/83	S (1)	S	R[c]	R (64)	R	R	R (16)	R	R[c]	R (16)	R	R
6005/85	R (4)	R[b]	R[c]	R (4)	R[b]	R[c]	R (32)	R	R	S (1)	S	S
1500/82	R (8)	R	R	R (32)	R	R	R (8)	R	R	R (8)	R	R
4302/83	R (8)	R	R	R (32)	R	R	R (8)	R	R	R (8)	R	R
S262/86	R (4)	R[b]	R[c]	R (64)	R	R	R (8)	R	R	R (8)	R	R
15867/81	R (8)	R[b]	R[c]	R (80)	R	R	R (8)	R	R	R (8)	S	R[c]

[a] S, Susceptible; R, resistant. Numbers in parentheses are resistance ratios.
[b] Lower delta growth index than most other resistant strains.
[c] Lower ATP indices at high drug concentrations than most other resistant strains.

used (data not presented), the breakpoints, which discriminated resistant from susceptible bacterial strains, had to be shifted toward higher concentrations of the drugs, and because of relatively high ATP levels of the inoculum, the ATP index at the breakpoint concentrations had to be higher than when the smaller inoculum (10^4 CFU/ml) was used. The optimal assay was obtained when the smaller inoculum was used, which is in agreement with the observations presented by Beckers et al. (1).

There was a pronounced discrimination between resistant and susceptible strains for isoniazid and rifampin by the bioluminescence assay (Fig. 3). This made it easy to choose breakpoints that gave full agreement with the reference methods. However, the effects at low concentrations of rifampin on mycobacterial ATP were more pronounced in the bioluminescence assay than in the other two methods. This is in agreement with results presented by Beckers et al. (1) using Dubos-Tween-albumin broth. The pronounced effect of rifampin at low concentrations is probably due to the presence of Tween 80 in the growth media (1; see above).

The occurrence of strains with different levels of resistance to ethambutol and streptomycin made it more difficult to define breakpoints of these agents. This has also been discussed in comparisons between the radiometric method and methods based on growth on solid media (4, 12).

Overall, the results obtained by the bioluminescence method were shown to be in good agreement with the results of both the resistance ratio method (99%) and the radiometric system (98%). The main advantages of the bioluminescence assay compared with the resistance ratio method are the rapidity of the ATP assay and the available kinetic data concerning drug effects within achievable in vivo drug concentrations. Several similarities were found between the bioluminescence assay and the radiometric system. The results are available in the same amount of time; both techniques are based on broth cultures, which allows for the use of drug concentrations obtainable in vivo; and both methods are suitable for routine use in a mycobacteriological laboratory. The advantages of the bioluminescence assay are the lower initial cost of the analytical equipment, the lower reagent cost per analysis, and the use of nonradioactive culture medium.

We conclude that the bioluminescent assay has great potential both for routine susceptibility testing of *M. tuberculosis* and, because of the kinetic data obtained, for research applications in the study of mycobacterial resistance, screening of new drugs, and drug synergy.

ACKNOWLEDGMENTS

This work was supported by a grant from the Swedish National Association Against Heart and Chest Diseases.

We are grateful to B. Persson and M. Nilsson for their skilled technical assistance.

LITERATURE CITED

1. **Beckers, B., H. R. M. Lang, D. Schimke, and A. Lammers.** 1985. Evaluation of a bioluminescence assay for rapid antimicrobial susceptibility testing of mycobacteria. Eur. J. Clin. Microbiol. **4**:556–561.
2. **Canetti, G., S. Froman, J. Grosset, P. Hauduroy, M. Langerova, H. T. Mahler, G. Meissner, D. A. Mitchinson, and L. Sula.** 1963. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. Bull. W.H.O. **29**:565–578.
3. **Kent, P. T., and G. P. Kubica.** 1986. Public health mycobacteriology: a guide for the level III laboratory. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta.
4. **Laszlo, A., P. Gill, V. Handzel, M. M. Hodgkin, and D. M. Helbecque.** 1983. Conventional and radiometric drug susceptibility testing of *Mycobacterium tuberculosis* complex. J. Clin. Microbiol. **18**:1335–1339.
5. **Middlebrook, G., Z. Reggiardo, and W. D. Tigertt.** 1977. Automatable radiometric detection of growth of *Mycobacterium tuberculosis* in selective media. Am. Rev. Respir. Dis. **115**: 1066–1069.
6. **Nilsson, L.** 1978. New rapid bioassay of gentamicin based on luciferase assay of extracellular ATP in bacterial cultures. Antimicrob. Agents Chemother. **14**:812–816.
7. **Nilsson, L.** 1985. Bioluminescent assay of bacterial ATP as a tool in clinical microbiology, p. 448–454. In K.-O. Habermehl

(ed.), Rapid methods and automation in microbiology and immunology. Springer-Verlag KG, Berlin.
8. **Roberts, G. D., N. L. Goodman, L. Heifets, H. W. Larsh, T. H. Lindner, J. K. McClatchy, M. R. McGinnis, S. H. Siddiqi, and P. Wright.** 1983. Evaluation of the BACTEC radiometric method for recovery of mycobacteria and drug susceptibility testing of *Mycobacterium tuberculosis* from acid-fast smear-positive specimens. J. Clin. Microbiol. **18**:689–696.
9. **Siddiqi, S. H., J. P. Libonati, and G. Middlebrook.** 1981. Evaluation of a rapid radiometric method for drug susceptibility testing of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **13**: 908–912.
10. **Snider, D. E., R. C. Good, J. O. Kilburn, L. F. Laskowski, R. H. Lusk, J. J. Marr, Z. Reggiardo, and G. Middlebrook.** 1981. Rapid drug susceptibility testing of *Mycobacterium tuberculosis*. Am. Rev. Respir. Dis. **123**:402–406.
11. **Vinckè, G., O. Yegers, H. Vanachter, P. A. Jenkins, and J. P. Butzler.** 1982. Rapid susceptibility testing of *Mycobacterium tuberculosis* by a radiometric technique. J. Antimicrob. Chemother. **10**:352–354.
12. **Woodley, C. L.** 1986. Evaluation of streptomycin and ethambutol concentrations for susceptibility testing of *Mycobacterium tuberculosis* by radiometric and conventional procedures. J. Clin. Microbiol. **23**:385–386.