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Resazurin Microtiter Assay Plate: Simple and Inexpensive Method for Detection of Drug Resistance in *Mycobacterium tuberculosis*

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A method for detecting multidrug-resistant *Mycobacterium tuberculosis* by using a reduction of resazurin is described. Eighty clinical isolates were evaluated against isoniazid and rifampin; results at 7 days were compared with those of the proportion method. Specificity and sensitivity were excellent. The method is simple, inexpensive, and rapid and might be used with other antituberculosis drugs.

Multidrug-resistant (MDR) tuberculosis (TB), defined as resistance to isoniazid (INH) and rifampin (RIF), is a severe problem for TB control. Recent reports document the global emergence of highly resistant *Mycobacterium tuberculosis* strains, particularly in countries of Eastern Europe (7, 36). The emergence of MDR TB highlights the need for drug susceptibility testing (DST), patient management, and drug resistance surveillance. Early diagnosis is essential for starting an effective treatment regimen and reducing its transmission in the population.

In countries with low resources, DST involves conventional culture methods with Löwenstein-Jensen (L-J) and Middlebrook agars and requires 3 to 6 weeks to yield results (1, 2, 16). Faster methods which use liquid media, such as the BACTEC radiometric method and the mycobacteria growth indicator tube method, require radioisotopes (27, 28), expensive equipment and media, or commercial products not always available in most developing countries (34). Recently, molecular methods for rapid detection of drug resistance have appeared (6, 30, 31, 35). However, their costs and the requirement for equipment and skilled personnel have precluded their routine implementation in countries with low resources.

Colorimetric assays employing oxidation-reduction indicators for DST have been previously used with mycobacteria (11, 21, 38). A simple method employing Alamar blue for DST of *M. tuberculosis* was recently described (23). Resazurin, an oxidation-reduction indicator, has been used to assess viability and bacterial contamination and to test for antimicrobial activity (3, 17, 29). Since Alamar blue has been recently identified as resazurin in cell cytotoxicity studies (22), we have standardized and evaluated a microplate method which uses the reduction of resazurin for DST to INH and RIF in clinical isolates of *M. tuberculosis* from low-income countries. The results were compared to those of the proportion method (PM) on L-J medium.

Eighty clinical isolates were studied: 65 from TB patients from a region of Peru with a high prevalence of MDR TB and 15 from the Tuberculosis Reference Laboratory at the Instituto Nacional de Laboratorios de Salud, La Paz, Bolivia. American Type Culture Collection (Manassas, Va.) reference strains were used as controls. INH and RIF (Sigma-Aldrich NV/SA, Bornem, Belgium) solutions were prepared at concentrations of 1 mg/ml in distilled water and 10 mg/ml in methanol, respectively, filter sterilized, and frozen until used. Resazurin sodium salt powder (Acros Organic N.V., Geel, Belgium) was prepared at 0.01% (wt/vol) in distilled water and filter sterilized; it can be stored at 4°C for 1 week. The resazurin microtiter assay (REMA) plate method was performed in 7H9-S medium containing Middlebrook broth, 0.1% Casitone, and 0.5% glycerol and supplemented with oleic acid, albumin, dextrose, and catalase (Becton-Dickinson). INH and RIF solutions were thawed and diluted in 7H9-S medium. Serial twofold dilutions of each drug in 100 μl of 7H9-S medium were prepared directly in 96-well plates at concentrations of 1.0 to 0.03 μg/ml for INH and 2.0 to 0.06 μg/ml for RIF. Growth controls containing no antibiotic and sterility controls without inoculation were also included. The inoculum was prepared from fresh L-J medium in 7H9-S medium adjusted to a McFarland tube no. 1 and then diluted 1:20, and 100 μl was used as an inoculum. The plates were covered, sealed in plastic bags, and incubated at 37°C in the normal atmosphere. After 7 days of incubation, 30 μl of resazurin solution was added to each well, incubated overnight at 37°C, and assessed for color development. A change from blue to pink indicates reduction of resazurin and therefore bacterial growth. The MIC was defined as the lowest drug concentration that prevented this color change. Breakpoint drug concentrations defining drug resistance were determined for each drug. The PM was performed on L-J medium according to standard procedures (1, 2, 16).

The INH MICs for all 54 *M. tuberculosis* isolates determined to be resistant to INH by PM were at least 0.25 μg/ml with the REMA plate method, and the MICs were 1.0 μg/ml or higher for 45 (83%) of the isolates. MICs of 0.06 μg/ml or lower were found by using the REMA plate method for 25 out of 26 isolates determined to be susceptible to INH by PM (Table 1).

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TABLE 1. MICs of INH and RIF for 80 *M. tuberculosis* isolates determined by using the REMA plate method

Result with PM (*n*)	No. of isolates for which INH MIC (μg/ml) was:						No. of isolates for which RIF MIC (μg/ml) was:				
	≤0.03	0.06	0.25	0.5	1.0	>1.0	≤0.06	0.125	0.25	0.5	>2.0
INH resistant (54)			3	6	13	32					
INH susceptible (26)	18	7				1					
RIF resistant (49)										1	48
RIF susceptible (31)							14	7	10		

The only discordant isolate was determined to be susceptible by PM, but the MIC for it was higher than 1 μg/ml with the REMA plate method. On repeated testing, it was found to be susceptible by both methods. It was also susceptible by the mycobacteria growth indicator tube method. Based on these results, the tentative breakpoint concentration of INH was defined as 0.25 μg/ml.

RIF MICs for all 49 isolates shown to be resistant to RIF by PM were at least 0.5 μg/ml with the REMA plate method, and the MICs for all but one (48 out of 49) were higher than 2 μg/ml. MICs for all 31 isolates shown to be susceptible by PM were 0.25 μg/ml or lower when tested by the REMA plate method (Table 1). A tentative breakpoint concentration of RIF was defined as 0.5 μg/ml. For INH, 79 out of 80 results were concordant, yielding specificity and sensitivity of 96.2 and 100%, respectively, with predictive values for susceptibility and resistance of 100 and 98.2%. As all 80 results were concordant for RIF, the specificity, sensitivity, and predictive values were all 100%.

Timely detection of initial drug resistance is very important for the outcome of TB treatment (4, 18, 20, 24), and both the Centers for Disease Control and Prevention and the British Thoracic Society recommend that DST be performed on isolates from all new patients (15, 32).

Although DOTS (directly observed treatment short course) can reduce the emergence of drug resistance (37), it has been found that patients with initial resistance to INH and RIF treated with standard DOTS developed resistance to other drugs (9, 26). DST on initial isolates from new patients is also an essential component of the DOTS-plus strategy (8, 14). In low-income countries DST methods use solid media, such as L-J and Middlebrook agars, on which the proportion of resistant bacteria can be quantitatively determined (13), taking 3 to 6 weeks to obtain results (16). Alternative methods employing liquid media have been described previously (27, 34, 39). Here we describe the initial results with a rapid method that uses the reduction of resazurin as an indicator of mycobacterial growth. This REMA plate method is simple to perform and inexpensive, giving results after 1 week of incubation that are comparable to those of other DST methods which use liquid media (27, 34, 39). MICs were higher than 2 μg/ml for 98% of RIF-resistant isolates, allowing easy discrimination of RIF resistance. All of these isolates were also resistant to INH, so testing for RIF resistance alone with the REMA plate method would have identified all of the MDR-TB isolates (19, 33). Receiver operating characteristic curve analysis (12) gave areas under the curve of 0.973 for INH (95% confidence interval, 0.909 to 0.996) and 1.00 for RIF (95% confidence interval, 1.000), demonstrating an excellent capacity to differentiate resistant and susceptible isolates.

Colorimetric methods have been used for DST in yeast (25) and bacteria (40), including *M. tuberculosis* (5, 10, 23, 38). This REMA plate method is very similar to the Alamar blue assay, interpretation of results is very easy, and correlation with the PM was excellent. Since it is also tested in liquid medium, it has not been implemented as a direct DST method due to the contamination problems that may arise. Being a nonproprietary product and cheaper than Alamar blue, it could be easily implemented in low-resource settings; it has the added advantage that it doesn't require uptake by the bacterial cell (17). One main concern with this type of test is biosafety; it has been shown, however, that the test can be easily adapted to a closed-tube format, thereby avoiding this problem (38). In summary, we have found the REMA plate method to be a rapid, simple, and inexpensive technique for the detection of MDR TB isolates that could be adapted for DST with other anti-TB drugs, particularly in low-income countries.

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REFERENCES

1. **Canetti, G., S. Froman, J. Grosset, P. Hauduroy, M. Langerova, H. T. Mahler, G. Meissner, D. A. Mitchison, and L. Sula.** 1963. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. Bull. W. H. O. **29:**565–578.
2. **Canetti, G., W. Fox, A. Khomenko, H. T. Mahler, N. K. Menon, D. A. Mitchison, N. Rist, and N. A. Smelev.** 1969. Advances in techniques of testing mycobacterial drug sensitivity and the use of sensitivity tests in tuberculosis control programs. Bull. W. H. O. **41:**21–43.
3. **Carter, R. A., S. A. Ericsson, C. D. Corn, P. R. Weyerts, M. G. Dart, S. G. Escue, and J. Mesta.** 1998. Assessing the fertility potential of equine semen samples using the reducible dyes methylene green and resazurin. Arch. Androl. **40:**59–66.
4. **Coates, A. R., and D. A. Mitchison.** 1983. The role of sensitivity tests in short-term chemotherapy. Bull. Int. Union Tuberc. **58:**111–114.
5. **Collins, L., and S. G. Franzblau.** 1997. Microplate Alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. Antimicrob. Agents Chemother. **41:**1004–1009.
6. **De Beenhouwer, H., Z. Lhiang, G. Jannes, W. Mijs, L. Machtelinckx, R. Rossau, H. Traore, and F. Portaels.** 1995. Rapid detection of rifampicin resistance in sputum and biopsy specimens from tuberculosis patients by PCR and line probe assay. Tuber. Lung Dis. **76:**425–430.
7. **Espinal, M. A., A. Laszlo, L. Simonsen, F. Boulahbal, S. J. Kim, A. Reniero, S. Hoffner, H. L. Rieder, N. Binkin, C. Dye, R. Williams, and M. C. Raviglione.** 2001. Global trends in resistance to antituberculosis drugs. N. Engl. J. Med. **344:**1294–1303.
8. **Farmer, P., and J. Y. Kim.** 1998. Community based approaches to the control of multidrug resistant tuberculosis: introducing "DOTS-plus." BMJ **317:**671–674.
9. **Farmer, P., J. Bayona, M. Becerra, J. Furin, C. Henry, H. Hiatt, J. Y. Kim,**

C. Mitnick, E. Nardell, and S. Shin. 1998. The dilemma of MDR-TB in the global era. Int. J. Tuberc. Lung Dis. **2:**869–876.
10. **Franzblau, S. G., R. S. Witzig, J. C. McLaughlin, P. Torres, G. Madico, A. Hernandez, M. T. Degnan, M. B. Cook, V. K. Quenzer, R. M. Ferguson, and R. H. Gilman.** 1998. Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue assay. J. Clin. Microbiol. **36:**362–366.
11. **Gomez-Flores, R., S. Gupta, R. Tamez-Guerra, and R. T. Mehta.** 1995. Determination of MICs for *Mycobacterium avium-M. intracellulare* complex in liquid medium by a colorimetric method. J. Clin. Microbiol. **33:**1842–1846.
12. **Hanley, J. A., and B. J. McNeil.** 1982. The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology **143:**29–36.
13. **Heifets, L. B., and G. A. Cangelosi.** 1999. Drug susceptibility testing of *Mycobacterium tuberculosis*: a neglected problem at the turn of the century. Int. J. Tuberc. Lung Dis. **3:**564–581.
14. **Iseman, M. D.** 1998. MDR-TB and the developing world—a problem no longer to be ignored: the WHO announces 'DOTS Plus' strategy. Int. J. Tuberc. Lung Dis. **2:**867.
15. **Joint Tuberculosis Committee of the British Thoracic Society.** 1998. Chemotherapy and management of tuberculosis in the United Kingdom: recommendations 1998. Thorax **53:**536–548.
16. **Kent, P. T., and G. P. Kubica.** 1985. Public health mycobacteriology. A guide for the level III laboratory. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, Atlanta, Ga.
17. **Mann, C. M., and J. L. Markham.** 1998. A new method for determining the minimum inhibitory concentration of essential oils. J. Appl. Microbiol. **84:** 538–544.
18. **McCray, E., C. M. Weinbaum, C. R. Braden, and I. M. Onorato.** 1997. The epidemiology of tuberculosis in the United States. Clin. Chest Med. **18:**99–113.
19. **McNerney, R., P. Kiepiela, K. S. Bishop, P. M. Nye, and N. G. Stoker.** 2000. Rapid screening of *Mycobacterium tuberculosis* for susceptibility to rifampicin and streptomycin. Int. J. Tuberc. Lung Dis. **4:**69–75.
20. **Mitchison, D. A., and A. J. Nunn.** 1986. Influence of initial drug resistance on the response to short-course chemotherapy of pulmonary tuberculosis. Am. Rev. Respir. Dis. **133:**423–430.
21. **Mshana, R. N., G. Tadesse, G. Abate, and H. Miorner.** 1998. Use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide for rapid detection of rifampin-resistant *Mycobacterium tuberculosis*. J. Clin. Microbiol. **36:**1214–1219.
22. **O'Brien, J., I. Wilson, T. Orton, and F. Pognan.** 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur. J. Biochem. **267:**5421–5426.
23. **Palomino, J. C., and F. Portaels.** 1999. Simple procedure for drug susceptibility testing of *Mycobacterium tuberculosis* using a commercial colorimetric assay. Eur. J. Clin. Microbiol. Infect. Dis. **18:**380–383.
24. **Park, S. K., C. T. Kim, and S. D. Song.** 1998. Outcome of chemotherapy in 107 patients with pulmonary tuberculosis resistant to isoniazid and rifampin. Int. J. Tuberc. Lung Dis. **2:**877–884.
25. **Pfaller, M. A., and A. L. Barry.** 1994. Evaluation of a novel colorimetric broth microdilution method for antifungal susceptibility testing of yeast isolates. J. Clin. Microbiol. **32:**1992–1996.
26. **Portaels, F., L. Rigouts, and I. Bastian.** 1999. Addressing multidrug-resistant tuberculosis in penitentiary hospitals and in the general population of the former Soviet Union. Int. J. Tuberc. Lung Dis. **3:**582–588.
27. **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.
28. **Siddiqi, S. H., J. P. Libonati, and G. Middlebrook.** 1981. Evaluation of rapid radiometric method for drug susceptibility testing of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **13:**908–912.
29. **Smith, C. F., and D. E. Townsend.** 1999. A new medium for determining the total plate count in food. J. Food Prot. **62:**1404–1410.
30. **Takiff, H.** 2000. Molecular mechanisms of drug resistance, p. 77–114. *In* I. Bastian and F. Portaels (ed.), Multidrug-resistant tuberculosis. Kluwer Academic Publishers, Dordrecht, The Netherlands.
31. **Telenti, A., P. Imboden, F. Marchesi, T. Schmidheini, and T. Bodmer.** 1993. Direct, automated detection of rifampin-resistant *Mycobacterium tuberculosis* by polymerase chain reaction and single-strand conformation polymorphism analysis. Antimicrob. Agents Chemother. **37:**2054–2058.
32. **Tenover, F. C., J. T. Crawford, R. E. Huebner, L. J. Geiter, C. R. Horsburgh, Jr., and R. C. Good.** 1993. The resurgence of tuberculosis: is your laboratory ready? J. Clin. Microbiol. **31:**767–770.
33. **Traore, H., K. Fissette, I. Bastian, M. Devleeschouwer, and F. Portaels.** 2000. Detection of rifampicin resistance in *Mycobacterium tuberculosis* isolates from diverse countries by a commercial line probe assay as an initial indicator of multidrug resistance. Int. J. Tuberc. Lung Dis. **4:**481–484.
34. **Walters, S. B., and B. A. Hanna.** 1996. Testing of susceptibility of *Mycobacterium tuberculosis* to isoniazid and rifampin by mycobacterium growth indicator tube method. J. Clin. Microbiol. **34:**1565–1567.
35. **Williams, D. L., L. Spring, T. P. Gillis, M. Salfinger, and D. H. Persing.** 1998. Evaluation of a polymerase chain reaction-based universal heteroduplex generator assay for direct detection of rifampin susceptibility of *Mycobacterium tuberculosis* from sputum specimens. Clin. Infect. Dis. **26:**446–450.
36. **World Health Organization.** 2000. Anti-tuberculosis drug resistance in the world: report no. 2 prevalence and trends. The WHO/IUATLD Global Project on Anti-Tuberculosis Drug Resistance Surveillance. WHO/CDS/TB/2000.278. World Health Organization, Geneva, Switzerland.
37. **World Health Organization.** 2001. Global tuberculosis control. WHO report 2001. WHO/CDS/TB/2001.287. World Health Organization, Geneva, Switzerland.
38. **Yajko, D. M., J. J. Madej, M. V. Lancaster, C. A. Sanders, V. L. Cawthon, B. Gee, A. Babst, and W. K. Hadley.** 1995. Colorimetric method for determining MICs of antimicrobial agents for *Mycobacterium tuberculosis*. J. Clin. Microbiol. **33:**2324–2327.
39. **Yew, W. W., S. C. Tong, K. S. Lui, S. K. Leung, C. H. Chau, and E. P. Wang.** 2001. Comparison of MB/BacT system and agar proportion method in drug susceptibility testing of *Mycobacterium tuberculosis*. Diagn. Microbiol. Infect. Dis. **39:**229–232.
40. **Zabransky, R. J., A. R. Dinuzzo, and G. L. Woods.** 1995. Detection of vancomycin resistance in enterococci by the Alamar MIC system. J. Clin. Microbiol. **33:**791–793.