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Testing Susceptibility of Multidrug-Resistant *Mycobacterium tuberculosis* to Second-Line Drugs by Use of Blood Agar▽

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In this study, the susceptibilities of 35 multidrug-resistant (MDR) *Mycobacterium tuberculosis* clinical isolates to second-line drugs, including kanamycin (KM), rifabutin (RBU), ofloxacin (OFX), *p*-aminosalicylic acid (PAS), capreomycin (CAP), clofazimine (CFM), and ethionamide (ETH), were investigated on blood agar according to CLSI recommendations. Compared with the results of the Bactec 460 TB system, agreement was 100, 100, 97, 100, 100, 100, and 86% for KM, RBU, OFX, PAS, CAP, CFM, and ETH, respectively. Compared with the results of the proportion method, agreement was 100, 100, 97, 100, 97, 100, and 77% for KM, RBU, OFX, PAS, CAP, CFM, and ETH, respectively.

Multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) are major public health problems, especially in developing countries (13, 18, 21). Rapid susceptibility testing is critical for early diagnosis of MDR- and XDR-TB and the initiation of effective regimens (14). The agar proportion method performed on Middlebrook 7H10 and 7H11 agars is the reference method for susceptibility of *Mycobacterium tuberculosis* according to CLSI recommendations (16). The Bactec 460 TB system (Becton Dickinson Diagnostic Systems, Sparks, MD), Bactec MGIT 960 (Becton Dickinson Diagnostic Systems, Sparks, MD), and Versa TREK system (formerly known as ESP II; Trek Diagnostic Systems, West Lake, OH) are cleared for used by the U.S. FDA for testing *M. tuberculosis* susceptibility to first-line drugs (16). It has recently been demonstrated that blood agar can be used for routine culture and testing of *M. tuberculosis* susceptibility to first-line drugs (1–4, 6, 7, 15).

In this study, the susceptibilities of 35 MDR *M. tuberculosis* clinical isolates against the second-line drugs kanamycin (KM), rifabutin (RBU), ofloxacin (OFX), *p*-aminosalicylic acid (PAS), capreomycin (CAP), clofazimine (CFM), and ethionamide (ETH) were investigated on blood agar, and results were compared to those obtained using the Bactec 460 TB system and the proportion method on Middlebrook 7H10 agar, performed according to CLSI recommendations.

Clinical MDR-TB strains were obtained from the Istanbul Faculty of Medicine, Department of Microbiology and Clinical Microbiology, Istanbul, Turkey. Isolates were identified by the Bactec NAP test as *M. tuberculosis* complex. Susceptibility to first-line drugs was determined by the radiometric method (Bactec 460 TB system) (19, 20). *M. tuberculosis* H37Rv was used as a control strain. All drugs were obtained from the manufacturers in a chemically pure form. Stock antibiotic solutions and subsequent dilutions were prepared. All stock solutions except CFM, which was stored in the dark at room temperature, were stored at −70°C in small aliquots.

The agar proportion method using Middlebrook 7H10 and blood agar was performed with concentrations of 10, 5, 5, 1, 2, 1, and 2 μg/ml for CAP, ETH, KM, CFM, OFX, RBU, and PAS, respectively (9, 10, 12, 16, 17).

Middlebrook 7H12 broth (Bactec 12B; Becton Dickinson Microbiology Systems) was used for radiometric testing (5, 11, 16, 19, 20). Second-line drugs (KM, RBU, PAS, CAP, CFM, OFX, and ETH) were tested by using single critical concentrations. Concentrations of second-line drugs tested in the Bactec 460 TB system were 1.25, 1.25, 5, 0.5, 2, 0.5, and 4 μg/ml for CAP, ETH, KM, CFM, OFX, RBU, and PAS, respectively (9, 10, 12, 16, 17).

The inoculum was prepared from freshly grown colonies on Löwenstein-Jensen medium. The supernatant of each isolate was adjusted to a 1 McFarland standard. The agar proportion method was performed on both Middlebrook 7H10 agar and blood agar (infusion agar, Becton Dickinson) separately according to CLSI recommendations (16). The plates were monitored twice a week by the naked eye. Resistance was defined as growth of a colony count of >1% on drug-containing quadrants in comparison to drug-free quadrants (16).

The Bactec 460 TB system revealed that all isolates were susceptible to KM and CFM. RBU, OFX, PAS, CAP, and ETH resistance was detected in 27, 1, 3, 2, and 17 isolates, respectively. All isolates were susceptible to KM and CFM on Middlebrook 7H10 agar; resistance was detected in 27, 1, 3, 1, and 14 isolates for RBU, OFX, PAS, CAP, and ETH, respectively. On blood agar, all isolates were susceptible to KM and CFM; resistance was detected in 27, 2, 3, 2, and 22 isolates for RBU, OFX, PAS, CAP, and ETH, respectively.

Blood agar results were compared with the results obtained using the Bactec 460 TB system and the proportion method on Middlebrook 7H10 agar. Agreement, sensitivity, specificity, positive predictive value, and negative predictive value are shown in Tables 1 and 2. The results of susceptibility testing were obtained on the 21st day of incubation by the proportion method on blood and Middlebrook 7H10 agar as recommended by the CLSI. The results of susceptibility testing by the

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TABLE 1. Comparison of blood agar results and Bactec 460 TB system results[a]

Drug	Result on blood agar	Bactec 460 TB system			% specificity	PPV (%)	NPV (%)	% agreement
		No. of resistant isolates	No. of susceptible isolates	% sensitivity				
KM	Resistant	0	0	100		100		100
	Susceptible	0	35					
RBU	Resistant	27	0	100	100	100	100	100
	Susceptible	0	8					
OFX	Resistant	1	1	97	100	100	50	97
	Susceptible	0	33					
PAS	Resistant	3	0	100	100	100	100	100
	Susceptible	0	32					
CAP	Resistant	2	0	100	100	100	100	100
	Susceptible	0	33					
CFM	Resistant	0	0	100		100		100
	Susceptible	0	35					
ETH	Resistant	17	5	72	100	100	77	86
	Susceptible	0	13					

[a] KM, kanamycin; RBU, rifabutin; OFX, ofloxacin; PAS, *p*-aminosalicylic acid; CAP, capreomycin; CFM, clofazimine; ETM, ethionamide; PPV, positive predictive value; NPV, negative predictive value.

Bactec 460 TB system were obtained on the 5th day of incubation for 31 isolates and on the 8th day for 4 isolates.

Recently, it has been reported that blood agar could be used for the isolation of *M. tuberculosis* from clinical isolates (6, 7, 15). In 2003, Drancourt et al. (6) reported that *M. tuberculosis* grows on blood agar within 1 to 2 weeks. Moreover, they emphasized that it is a time-saving, cost-effective, more sensitive method and at least as rapid as the automated method. Mathur et al. (15) assessed the time required for primary isolation of *M. tuberculosis* on sheep blood agar compared with the time required for isolation on Löwenstein-Jensen medium. They reported that the median time to detect *M. tuberculosis* on blood agar was 13 days, while it was 19 days on Löwenstein-Jensen medium. In their study, more bacterial colonies were observed on blood agar than on Löwenstein-Jensen medium. Coban et al. (1) demonstrated that blood agar may be used as an alternative medium for testing the susceptibility of *M. tuberculosis* to INH and RIF. They compared the results obtained from blood agar and the Bactec 460 TB system. Susceptibility results were recorded on day 14, and agreements were 94.1 and 100% for INH and RIF, respectively. In another study, Coban et al. (2) evaluated the performance of blood agar for testing the susceptibility of *M. tuberculosis* to INH, RIF, STM, and ETM. The results were obtained on the 14th day of incubation, and the agreements with the radiometric proportion method were determined as 100, 100, 92, and 96% for INH, RIF, STM, and ETM, respectively. In a collaborative study, Coban et al. (3) evaluated the performance of blood agar for testing the susceptibility of *M. tuberculosis* to first-line drugs. Susceptibility test results were reported on the 14th day for 100 isolates and on the 21st day of incubation for 47 isolates. The agreements with radiometric or the agar proportion method were 94.5, 96.5, 93.1, and 87.7% for INH, RIF, STM, and ETM, respectively. Yildiz et al. (23) evaluated the

TABLE 2. Comparison of blood agar results and Middlebrook 7H10 agar results[a]

Drug	Result on blood agar	7H10 agar			% specificity	PPV (%)	NPV (%)	% agreement
		No. of resistant isolates	No. of susceptible isolates	% sensitivity				
KM	Resistant	0	0	100		100		100
	Susceptible	0	35					
RBU	Resistant	27	0	100	100	100	100	100
	Susceptible	0	8					
OFX	Resistant	1	1	97	100	100	50	97
	Susceptible	0	33					
PAS	Resistant	3	0	100	100	100	100	100
	Susceptible	0	32					
CAP	Resistant	1	1	97	100	100	50	97
	Susceptible	0	33					
CFM	Resistant	0	0	100		100		100
	Susceptible	0	35					
ETH	Resistant	14	8	61.9	100	100	63.6	77
	Susceptible	0	13					

[a] KM, kanamycin; RBU, rifabutin; OFX, ofloxacin; PAS, *p*-aminosalicylic acid; CAP, capreomycin; CFM, clofazimine; ETM, ethionamide; PPV, positive predictive value; NPV, negative predictive value.

performance of sheep blood and human blood agar for testing the susceptibility of *M. tuberculosis* to INH using the proportion method. Their results were obtained on incubation days 6 to 8 in both media; they showed that both media can be used as an alternative medium for the susceptibility testing of *M. tuberculosis*.

To the best of our knowledge, there is no report which tests the performance of blood agar in determining the susceptibility of *M. tuberculosis* against second-line drugs. The results of the present study demonstrated that blood agar may be useful as an alternative medium for determining the susceptibility of *M. tuberculosis* clinical isolates to second-line drugs (except ETH). Coban et al. (4) determined the susceptibilities of *M. tuberculosis* to first- and second-line antituberculosis drugs by the Etest by using different media, including 7H11, blood, and chocolate agars. They noted that chocolate agar showed insufficient growth, while results on blood agar were comparable to those on Middlebrook 7H11 agar.

In conclusion, susceptibility testing results of *M. tuberculosis* strains against second-line drugs, except ETH, on blood agar showed excellent agreement compared with two gold standard methods. A limitation of this study is that the majority of tested *M. tuberculosis* isolates were not resistant to second-line drugs. Further studies with more resistant strains are needed before the implementation of blood agar for susceptibility testing of *M. tuberculosis* strains against second-line drugs in diagnostic laboratories.

REFERENCES

1. **Coban, A. Y., K. Bilgin, M. Uzun, N. Tasdelen Fisgin, A. Akgunes, C. Cekic Cihan, A. Birinci, and B. Durupinar.** 2005. Susceptibilities of *Mycobacterium tuberculosis* to isoniazid and rifampin on blood agar. J. Clin. Microbiol. **43:**1930–1931.
2. **Coban, A. Y., C. C. Cihan, K. Bilgin, M. Uzun, A. Akgunes, E. Cetinkaya, and B. Durupinar.** 2006. Blood agar for susceptibility testing of *Mycobacterium tuberculosis* against first-line drugs. Int. J. Tuber. Lung Dis. **10:**450–453.
3. **Coban, A. Y., A. Martin, M. Uzun, K. Bilgin, J. C. Palomino, and B. Durupinar.** 2008. Evaluation of blood agar for susceptibility testing of *Mycobacterium tuberculosis* against first-line antituberculous drugs: results from two centers. J. Chemother. **20:**388–390.
4. **Coban, A. Y., K. Bilgin, M. Uzun, A. Akgunes, A. Yusof, and B. Durupinar.** 2008. Comparative study for determination of *Mycobacterium tuberculosis* susceptibility to first- and second-line antituberculosis drugs by the Etest using 7H11, blood, and chocolate agar. J. Clin. Microbiol. **46:**4095–4098.
5. **Della-Latta, P., and I. Weitzman.** 1998. Mycobacteriology, p. 169–203. *In* H. D. Isenberg (ed.), Essential procedures for clinical microbiology. ASM Press, Washington, DC.
6. **Drancourt, M., P. Carrieri, M. J. Gevaudan, and D. Raoult.** 2003. Blood agar and *Mycobacterium tuberculosis*: the end of a dogma. J. Clin. Microbiol. **41:**1710–1711.
7. **Drancourt, M., and D. Raoult.** 2007. Cost-effectiveness of blood agar for isolation of mycobacteria. PloS Negl. Trop. Dis. **28:**e83.
8. Reference deleted.
9. **Inderlied, C. B., and K. A. Nash.** 1996. Antimycobacterial agents: in vitro susceptibility testing, spectra of activity, mechanisms of action and resistance, and assays for activity in biologic fluids, p. 127–175. *In* W. Lorian (ed.), Antibiotic in laboratory medicine, 4th ed. Williams and Wilkins, Baltimore, MD.
10. **Inderlied, C. B., and M. Salfinger.** 1999. Antimycobacterial agents and susceptibility tests, p. 1601–1623. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, R. H. Yolken (ed.), Manual of clinical microbiology, 7th ed. ASM Press, Washington, DC.
11. **Isenberg, H. D. (ed.).** 1992. Clinical microbiology procedures handbook, 1:5.13.1–5.14.24. ASM Press, Washington, DC.
12. **Kent, P. T., and G. P. Kubica.** 1985. Public health mycobacteriology. A guide for the level III laboratory, division of laboratory training and consultation. CDC Press, Atlanta, GA.
13. **Lemus, D., A. Martin, E. Montoro, and J. C. Palomino.** 2004. Rapid alternative methods for detection of rifampicin resistance in *Mycobacterium tuberculosis*. J. Antimicrob. Chemother. **54:**130–133.
14. **Lin, S. Y. G., E. Desmond, D. Bonato, W. Gross, and S. Siddiqi.** 2009. Multicenter evaluation of Bactec MGIT 960 system for second-line drug susceptibility testing of *Mycobacterium tuberculosis* complex. J. Clin. Microbiol. **47:**3630–3634.
15. **Mathur, M. L., J. Gaur, R. Sharma, and A. Solanki.** 2009. Rapid culture of *Mycobacterium tuberculosis* on blood agar in resource limited setting. Dan. Med. Bull. **56:**208–210.
16. **NCCLS.** 2003. Susceptibility testing of mycobacteria, nocardia, and other aerobic actinomycetes. Approved standard M24-A. NCCLS, Wayne, PA.
17. **Pfyffer, G. E., D. A. Bonato, A. Ebrahimzadeh, W. Gross, J. Hotaling, J. Kornblum, A. Laszlo, G. Robert, M. Salfinger, F. Wittwer, and S. Siddiqi.** 1999. Multicenter laboratory validation of susceptibility testing of *Mycobacterium tuberculosis* against classical second-line and newer antimicrobial drugs by using the radiometric Bactec 460 technique and the proportion method with solid media. J. Clin. Microbiol. **37:**3179–3186.
18. **Shenoi, S., and G. Friedland.** 2009. Extensively drug-resistant tuberculosis: a new face to an old pathogen. Annu. Rev. Med. **60:**307–320.
19. **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:**908–913.
20. **Siddiqi, S. H.** 1995. Bactec 460 TB system. Product and procedure manual, revision D. Becton Dickinson Microbiology Systems, Sparks, MD.
21. **Tortoli, E., D. Dionisio, and C. Fabbri.** 2004. Evaluation of moxifloxacin activity in vitro against *Mycobacterium tuberculosis*, including resistant and multidrug-resistant strains. J. Chemother. **16:**334–336.
22. Reference deleted.
23. **Yildiz, C., M. Ulger, G. Aslan, and G. Emekdas.** 2007. Isoniazid susceptibilities of *Mycobacterium tuberculosis* on blood agar. Saudi Med. J. **28:**975–977.