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Evaluation of Agar-Based Medium with Sheep Sera for Testing of Drug Susceptibility of *Mycobacterium tuberculosis* to Isoniazid, Rifampin, Ethambutol, and Streptomycin

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The performance of sheep sera instead of sheep blood in agar-based media was investigated for susceptibility testing of *Mycobacterium tuberculosis* against primary drugs. The levels of agreement between agar-based medium supplemented with sheep sera and the proportion method on Middlebrook 7H11 agar as the reference method for determining susceptibility to isoniazid (INH), rifampin (RIF), ethambutol (EMB), and streptomycin (STR) were 98.4, 98.4, 95.3, and 100%, respectively.

Tuberculosis is a major public health problem, particularly in developing countries. Multidrug-resistant tuberculosis (MDR-TB) is one of the factors that reduce the efficacy of tuberculosis control programs (1). Early identification of drug-resistant *Mycobacterium tuberculosis* is the first and most crucial step in the fight against tuberculosis. The existence of an available and cheap method for susceptibility testing may help to prevent and reduce dissemination of MDR tuberculosis and may increase as the efficacy of tuberculosis control programs as well (2).

The agar proportion method performed with Middlebrook 7H10-11 agar is used as a reference method for susceptibility testing of *Mycobacterium tuberculosis*. The results are calculated by comparing the colony number in a drug-containing tube to the colony number in a drug-free tube. If this ratio is >1%, the bacteria are considered resistant to the drug (3). It was reported that *Mycobacterium tuberculosis* colonies may grow on blood agar (4). This report led to further research on the use of blood agar for cultivation of *M. tuberculosis* (5, 6). In addition, it was noted that blood agar can be used instead of Middlebrook 7H10-11 agar or Löwenstein-Jensen (LJ) media for susceptibility testing of *M. tuberculosis* (7–11). However, there is a disadvantage to the use of blood agar for susceptibility testing of *M. tuberculosis* due to the fact that the color of the medium is red and the medium is not as transparent as 7H10-11 medium. As a consequence, monitoring of the bacterial growth (e.g., microcolonies) is difficult. In this study, sheep sera were used instead of sheep blood for resolution of this problem and the performance of the blood agar-based medium with sheep sera for determining the susceptibility of *M. tuberculosis* to isoniazid (INH), rifampin (RIF), ethambutol (EMB), and streptomycin (STR) was investigated.

A total of 64 *M. tuberculosis* isolates from our collection were tested for their antimicrobial susceptibilities. In addition, H37Rv-ATCC 25618 (susceptible to all drugs), ATCC 35838 (resistant to RIF), and ATCC 35837 (resistant to EMB) were used as control strains. The susceptibility testing was performed according to CLSI recommendations. All bacteria were tested on Middlebrook7H11 agar as a reference method and on blood agar base medium with sheep sera as a new medium.

Middlebrook 7H11 agar (Becton, Dickinson and Company, Sparks, MD) was prepared according to the manufacturer's instructions; after it was sterilized, the supplement (oleic acid-albumin-dextrose-catalase [OADC]) was added at 10%. Each antibiotic was added from stock solutions. The final concentrations of INH, RIF, EMB, and STR were 0.2 μg/ml, 1 μg/ml, 7.5 μg/ml, and 2 μg/ml, respectively (3). Once the drug was added, 1 ml of medium with drug was dispensed into 24-well plates (TPP; Tissue Culture Testplate, Switzerland) and this was repeated for each drug. Growth control medium without drug was also prepared in 24-well plates for each bacterium. All plates were stored at +4°C until use.

Blood agar-based medium (bioMérieux, Marcy l'Etoile, France) was prepared according to the manufacturer's instructions; after it was sterilized, sheep sera (Pendik Veteriner Kontrol Enstitusu; Pendik, Istanbul, Turkey) were added to the medium at 5%. Each antibiotic was added from stock solutions. The final concentrations of INH, RIF, EMB, and STR were 0.2 μg/ml, 1 μg/ml, 4 μg/ml, and 2 μg/ml, respectively (11). After drug was added, 1 ml of medium with drug was dispensed into 24-well plates for each drug. Growth control media without drug for each strain were also prepared in 24-well plates. All plates were stored at +4°C until use.

Inoculums were prepared from fresh colonies on Löwenstein-Jensen culture medium. Each inoculum was adjusted to McFarland no. 1 turbidity. All inoculums were then diluted to 1:100 in the test wells before use.

The susceptibility testing was performed according to CLSI recommendations. Briefly, 20 μl of bacterial inoculums was added to each well and all inoculated plates were sealed. All inoculated plates of 7H11 agar were incubated at 37°C under conditions of 5% CO_2. Incubation of plates with blood agar-based medium with sheep sera was done at 37°C under conditions of normal atmospheric air. The results for each isolate and drug were recorded according to CLSI recommendations (3).

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TABLE 1 Results determined using blood agar-based media with sheep sera compared with results by the reference method, agar proportion on 7H11 agar[a]

		Reference method (agar proportion on 7H11 agar)						
Drug	BBMSS	S	R	Sensitivity (%)	Specificity (%)	Major discrepancy (%)	Very major discrepancy (%)	Agreement (%)
INH	S	40	1	100	95.8	0	2.5	98.4
	R	0	23					
RIF	S	44	1	100	95	0	2.2	98.4
	R	0	19					
EMB	S	55	3	100	66.7	0	5.4	95.3
	R	0	6					
STR	S	51	0	100	100	0	0	100
	R	0	13					

[a] BBMSS, blood agar-based medium with sheep sera; INH, isoniazid; RIF, rifampin; EMB, ethambutol; STR, streptomycin; S, susceptible; R, resistant.

All the results obtained in this study are summarized in Table 1. When the results from the blood agar-based medium with sheep sera were compared with the reference method results, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and agreement were 100, 95.8, 97.6, 100, and 98.4% for INH, 100, 95, 97.8, 100, and 98.4% for RIF, 100, 66.7, 94.8, 100, and 95.3% for EMB, and 100, 100, 100, 100, and 100% for STR, respectively.

In all plates, sufficient bacterial growth was obtained in the growth control wells at 14 days of incubation with both methods and all results were noted (Fig. 1). However, incubation was prolonged to the 21st day. It was observed that the results at the 14th and 21st days were the same.

The most important step in the battle against tuberculosis is early determination of drug resistance. Therefore, development of a fast, safe, and inexpensive susceptibility test method is of increasing interest.

In the present study, sheep sera were used instead of sheep blood for preparing of blood agar-based medium and 7H11 agar. The potential limitation of our study is the low number of resistant isolates tested. When the results of blood agar-based medium with sheep sera were compared to the reference method results, sensitivity, specificity, PPV, NPV, and agreement were greater than 90% for INH, RIF, and STR, but specificity was low for EMB (66.7%). Three isolates were found to be susceptible by the blood agar-based medium with sheep sera, while they were resistant by the reference method (Table 1). The five isolates with discordant results were also tested using a Bactec MGIT 960 system. And the same results were observed. EMB susceptibility testing has been difficult to reproduce given MICs near the critical concentration. It was noted that most strains that had microcolonies with EMB in the agar proportion method had EMB-susceptible results with the Bactec 460TB system. However, the significance of microcolonies is unknown (3).

Recently, there has been an increasing interest in molecular methods due to the speed for susceptibility testing of *M. tuberculosis* isolates. However, Folkvardsen et al. (12) noted that the line probe and sequencing were not able to detect the clinically relevant 1% proportion of INH-resistant bacteria. In addition, they identified conventional drug susceptibility testing as the most sensitive method for resistance detection.

The use of serum-containing medium truly makes it easier to detect microcolonies than the use of blood-containing medium. Our previous experiences suggested that microcolonies are diffi-



FIG 1 Bacterial growth on blood agar base medium with sheep sera (A) and on 7H11 agar (B).

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cult to detect on blood-containing medium. This study was designed to solve that problem, and it was observed that colonies are easily detected on serum-containing medium.

In the present study, sheep sera were used instead of sheep blood for easy monitoring of bacterial growth on blood agar-based medium. Our results showed that when the results from the medium with sheep sera were compared with those of the reference agar proportion method, promising agreements were observed for all of the prime antituberculosis drugs.

In conclusion, the results of our study suggest that sheep sera may be used as an enrichment supplement for mycobacterial growth on blood agar-based media.

REFERENCES

1. **Martin A, Portaels F, Palomino JC.** 2007. Colorimetric redox-indicator methods for the rapid detection of multidrug resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. J. Antimicrob. Chemother. **59:**175–183.
2. **Minion J, Leung E, Menzies D, Pai M.** 2010. Microscopic-observation drug susceptibility and thin layer agar assays for the detection of drug resistant tuberculosis: a systematic review and meta-analysis. Lancet Infect. Dis. **10:**688–698.
3. **Clinical and Laboratory Standards Institute (CLSI).** 2011. Susceptibility testing of Mycobacteria, Nocardia, and other aerobic Actinomycetes; approved standard—second edition M24-A2, vol 31, no 5. CLSI, Wayne, PA.
4. **Drancourt M, Carrieri P, Gévaudan MJ, Raoult D.** 2003. Blood agar and *Mycobacterium tuberculosis:* the end of a dogma. J. Clin. Microbiol. **41:** 1710–1711.
5. **Coban AY, Akgunes A, Durupinar B.** 2011. Evaluation of blood agar medium for the growth of mycobacteria. Mikrobiyol. Bul. **45:**617–622. (In Turkish.)
6. **Drancourt M, Raoult D.** 2007. Cost-effectiveness of blood agar for isolation of mycobacteria. PLoS Negl. Trop. Dis. **1:**e83. doi:10.1371/journal.pntd.0000083.
7. **Coban AY, Tanriverdi Cayci Y, Deveci A, Akgunes A, Uzun M, Durupinar B.** 2011. A rapid detection of multidrug-resistant *Mycobacterium tuberculosis* by a nitrate reductase assay on blood agar. Mem. Inst. Oswaldo Cruz **106:**378–380.
8. **Coban AY, Bilgin K, Uzun M, Tasdelen Fisgin N, Akgunes A, Cihan CC, Birinci A, Durupinar B.** 2005. Susceptibilities of *Mycobacterium tuberculosis* to isoniazid and rifampin on blood agar. J. Clin. Microbiol. **43:**1930–1931.
9. **Coban AY, Cihan CC, Bilgin K, Uzun M, Akgunes A, Cetinkaya E, Durupinar B.** 2006. Blood agar for susceptibility testing of *Mycobacterium tuberculosis* against first-line drugs. Int. J. Tuberc. Lung Dis. **10:**450–453.
10. **Coban AY, Bilgin K, Uzun M, Akgunes A, Yusof A, Durupinar B.** 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.
11. **Coban AY.** 2013. Blood agar validation for susceptibility testing of isoniazid, rifampicin, ethambutol, and streptomycin to *Mycobacterium tuberculosis* isolates. PLoS One **8:**e55370. doi:10.1371/journal.pone.0055370.
12. **Folkvardsen DB, Svensson E, Thomsen VØ, Rasmussen EM, Bang D, Werngren J, Hoffner S, Hillemann D, Rigouts L.** 2013. Can molecular methods detect 1% isoniazid resistance in Mycobacterium tuberculosis? J. Clin. Microbiol. **51:**1596–1599.

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