

Blood agar for susceptibility testing of *Mycobacterium tuberculosis* against first-line drugs

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SUMMARY

OBJECTIVE: To evaluate the performance of blood agar for the susceptibility testing of 50 *Mycobacterium tuberculosis* clinical isolates against isoniazid (INH), rifampicin (RMP), streptomycin (SM) and ethambutol (EMB).

DESIGN: The activity of the drugs was determined by the proportion method on blood agar instead of Middlebrook 7H10 agar according to Clinical Laboratory Standard Institute recommendations. The final concentrations of INH, RMP, SM and EMB were 0.2 µg/ml, 1 µg/ml, 2 µg/ml and 5 µg/ml, respectively.

RESULTS: The results were compared with the radiometric proportion method as the reference, and the agreements were determined as 100% for INH and RMP, 92% for SM and 96% for EMB. The specificity,

sensitivity, positive predictive value and negative predictive value were 90.4% and 97.5%, 100% and 90%, 66.6% and 90% and 100% and 97.5% for SM and EMB, respectively, while these values were 100% for INH and RMP. The results of susceptibility testing were obtained on the 14th day of incubation.

CONCLUSION: According to this preliminary study, our results suggest that blood agar can be used as an alternative medium for the susceptibility testing of *M. tuberculosis* strains against INH, RMP, SM and EMB in resource-limited countries. However, further studies are needed before implementing the method in diagnostic laboratories.

KEY WORDS: *Mycobacterium tuberculosis*; proportion method; blood agar

THE SPREAD of multidrug-resistant tuberculosis (MDR-TB, defined as resistance to at least isoniazid [INH] and rifampicin [RMP]) in the world, especially in developing countries, remains a major public health problem. Early diagnosis of TB and rapid detection of drug resistance is urgently needed for the prevention and control of MDR-TB. There is therefore a need for rapid determination of the resistance profile of *Mycobacterium tuberculosis* clinical isolates, and many different methods have been described for this purpose.¹⁻⁴

The Clinical Laboratory Standard Institute (CLSI) recommends three different methods for susceptibility testing of *M. tuberculosis*, including the proportion method on Middlebrook 7H10 agar, BACTEC 460 TB System (Becton Dickinson, Sparks, MD, USA) and BACTEC MGIT 960 (Becton Dickinson).⁵ Although the susceptibility testing results can be obtained in 4-7 days by automated systems, the proportion method requires an average of 3 weeks to detect the results. Because automated systems are labour-

intensive, expensive, generate radioactive waste and are not always available, many investigators have been trying to improve reliable, rapid and inexpensive methods.¹⁻⁴

In recent years, it has been reported that *M. tuberculosis* isolates could be easily grown on blood agar in 1-2 weeks.⁴ The results of our first report showed that blood agar can be used as an alternative medium for the susceptibility testing of *M. tuberculosis* against INH and RMP.⁶ In the present study, the performance of blood agar instead of Middlebrook 7H10 agar was evaluated for susceptibility testing of *M. tuberculosis* clinical isolates against INH, RMP, streptomycin (SM) and ethambutol (EMB).

MATERIALS AND METHODS

Bacterial strains

A panel of 50 clinical isolates of *M. tuberculosis* with known drug susceptibility profiles was tested. H37Rv (ATCC 27294) was used as a control strain. The re-

Table 1 Resistance profiles of *M. tuberculosis* clinical isolates

Resistance types	Isolates (n)
INH	3
SM	1
INH+RMP	9
INH+SM	1
INH+EMB	1
INH+RMP+SM	3
INH+RMP+EMB	6
INH+SM+EMB	1
INH+RMP+SM+EMB	2
Total	27

INH = isoniazid; SM = streptomycin; RMP = rifampicin; EMB = ethambutol.

sistance profiles of all isolates are summarised in Table 1. We tested 27 resistant strains and 23 fully susceptible strains. All strains were freshly subcultured onto Löwenstein-Jensen (LJ) medium before use and were tested blindly by the proportion method on blood agar.

Preparation of inoculum

Inoculum was prepared from freshly grown colonies from LJ medium. Three or four colonies were transferred to a tube containing 3–4 ml 7H9 broth and 6–9 sterile glass beads. Tubes were vigorously agitated on a vortex mixer and clumps were allowed to settle for 30–45 min. The supernatants were transferred to sterile tubes. These supernatants were adjusted with phosphate buffer saline to equal the density of 1 McFarland standard for use as the standard inoculum.⁵

Anti-tuberculosis drugs

INH, RMP, SM and EMB were purchased from Sigma (Steinheim, Germany). The stock solutions were prepared, filter sterilised and kept at -80°C .

Preparation of blood agar

A blood agar based medium (Oxoid, Unipath Ltd, Basingstoke, Hampshire, UK) was prepared according to the manufacturer's instructions. After sterilisation, it was cooled to $45\text{--}50^{\circ}\text{C}$ and supplemented with defibrinated sheep blood (5%, v/v). The appropriate volume of diluted stock solutions was incorporated into sterile blood agar medium to achieve the desired final concentrations of INH ($0.2\ \mu\text{g/ml}$), RMP ($1\ \mu\text{g/ml}$), SM ($2\ \mu\text{g/ml}$) and EMB ($5\ \mu\text{g/ml}$). Five millilitres of medium with drugs were dispensed quickly into sterile glass plates, allowed to solidify and stored at $+4^{\circ}\text{C}$ until used. Blood agar medium without drug was prepared for the growth control.^{5,6}

Susceptibility testing on blood agar

Susceptibility testing was carried out according to CLSI recommendations.⁵ The inoculum was adjusted to McFarland standard No. 1 and diluted 1:100. One hundred microlitres of diluted inoculum were inoculated on blood agar media with and without drugs. All plates were incubated at 37°C overnight, and then the plates were sealed, placed in plastic bags and incubated at 37°C in 5–10% CO_2 . The plates were examined for contamination during the first week, and re-examined after 10, 14 and 21 days of incubation. Each growth was also examined for tubercle bacilli by Ziehl-Neelsen staining. Resistance was defined as growth on drug-containing media $>1\%$ of the growth of drug-free control media for INH, RMP and EMB, and $>10\%$ for SM.

RESULTS

The results of the study are summarised in Table 2. The agreements were 100% for INH and RMP, 92% for SM and 96% for EMB. Specificity, sensitivity, positive predictive value (PPV) and negative predictive

Table 2 Comparison of the radiometric proportion method results with the results of blood agar

	Results on blood agar agreement %	Radiometric proportion method		Sensitivity %	Specificity %	PPV %	NPV %
		Resistant	Susceptible				
INH							
Resistant	100	26	0	100	100	100	100
Susceptible		0	24				
RMP							
Resistant	100	20	0	100	100	100	100
Susceptible		0	30				
SM							
Resistant	92	8	4	100	90.4	66.6	100
Susceptible		0	38				
EMB							
Resistant	96	9	1	90	97.5	90	97.5
Susceptible		1	39				

PPV = positive predictive value; NPV = negative predictive value; INH = isoniazid; RMP = rifampicin; SM = streptomycin; EMB = ethambutol.

value (NPV) were 90.4% and 97.5%, 100% and 90%, 66.6% and 90% and 100% and 97.5% for SM and EMB, respectively, and 100% for INH and RMP. There were six discrepant results: four isolates determined as susceptible to SM by the BACTEC 460 TB system were resistant to SM on blood agar; one isolate determined as susceptible to EMB by the BACTEC 460 TB system was resistant to EMB on blood agar, and the other isolate resistant to EMB by the BACTEC 460 TB system was found to be susceptible on blood agar. In this study, the plates were examined in the 7th, 10th, 14th and 21st days of incubation. In the 10th day of incubation, colonies of 37 out of 50 isolates were visible macroscopically on blood agar, but the susceptibility testing results could not be evaluated. However, on the 14th day of incubation, all results were noted but incubation was prolonged to the 21st day. By the 21st day of incubation, the results had not changed.

DISCUSSION

Blood agar is commonly used in all clinical microbiology laboratories because it is cheap, simple to prepare and many species can be grown on it. It was recently reported that blood agar could be used for isolation of *M. tuberculosis*.⁷⁻⁹ Drancourt et al. reported that *M. tuberculosis* isolates were easily grown on blood agar in an average of 1–2 weeks and that they routinely inoculated 10 000 samples onto blood agar instead of egg-based medium for the diagnosis of TB every year.⁴ In our first study,⁶ we evaluated the performance of blood agar instead of 7H10 agar for susceptibility testing of *M. tuberculosis* clinical isolates against INH and RMP by the proportion method. The agreements were 100% for RMP and 94.1% for INH. Specificity, sensitivity, PPV and NPV were 100% for RMP and respectively 71.4%, 100%, 93.1% and 100% for INH. Susceptibility test results were also obtained on the 14th day of incubation. In our study, we evaluated blood agar for its suitability for susceptibility testing; we believe that further studies are needed for further information. Limitations of this study are that the critical concentration was not found halfway between the highest MICs for suscep-

tible strains and the lowest MICs for resistant strains, and comparison was only made with Bactec 460 TB susceptibility results and not solid media, such as 7H10 agar.

CONCLUSION

In this study the performance of blood agar was evaluated for susceptibility testing of *M. tuberculosis* strains against INH, RMP, SM and EMB. The results for SM were poor. More experiments are needed to confirm these results, as the study was done with only 50 strains. According to these results, it was concluded that blood agar can be used as an alternative medium in resource-limited countries. However, further multicentre studies are needed before implementation in diagnostic laboratories.

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RÉSUMÉ

OBJECTIF : Evaluer dans 50 isolats cliniques de *Mycobacterium tuberculosis* les performances de l'agar au sang pour les tests de sensibilité à l'égard de l'isoniazide (INH), de la rifampicine (RMP), de la streptomycine (SM) et de l'éthambutol (EMB).

SCHÉMA : On a déterminé l'activité de ces médicaments par la méthode des proportions sur agar au sang à la place d'agar Middlebrook 7H10 selon les recommandations du CLSI (*Clinical Laboratory Standards Insti-*

tute). Les concentrations finales d'INH, RMP, SM et EMB ont été respectivement de 0,2 µg/ml, 1 µg/ml, 2 µg/ml et 5 µg/ml.

RÉSULTATS : Les résultats ont été comparés comme référence avec la méthode radiométrique des proportions, et l'on a déterminé les taux de concordance comme étant à 100% pour INH et RMP, 92% pour SM et 96% pour EMB. La spécificité, la sensibilité et les valeurs prédictives positive et négative ont été respectivement de

90,4% et 97,5%, de 100% et 90%, de 66,6% et 90% et de 100% et 97,5% pour SM et EMB, alors que ces valeurs ont été de 100% pour INH et RMP. Les résultats du test de sensibilité ont été obtenus au 14^{ème} jour d'incubation. **CONCLUSION** : Selon cette étude préliminaire, nos résultats suggèrent que l'agar au sang peut être utilisé comme

technique alternative pour les tests de sensibilité des souches de *M. tuberculosis* à l'égard d'INH, RMP, SM et EMB dans les pays à ressources limitées. Toutefois, des études complémentaires s'imposent avant la mise en œuvre de cette méthode dans les laboratoires de diagnostic.

RESUMEN

OBJETIVO : Evaluar el rendimiento de las pruebas de sensibilidad a isoniacida (INH), rifampicina (RMP), estreptomycin (SM) y etambutol (EMB) en placas de agar sangre, en 50 aislados clínicos de *Mycobacterium tuberculosis*.

MÉTODO : Se determinó la actividad de estos medicamentos mediante el método proporcional en placas de agar sangre, en lugar de utilizar el agar con Middlebrook 7H10, según lo recomienda el CLSI (*Clinical Laboratory Standards Institute*). Las concentraciones finales utilizadas fueron 0,2 µg/ml de INH, 1 µg/ml de RMP, 2 µg/ml de SM y 5 µg/ml de EMB.

RESULTADOS : Se utilizó como método de referencia el sistema proporcional radiométrico y se encontró una concordancia de los resultados del 100% para INH y

RMP, del 92% para SM y del 96% para EMB. Para SM y EMB, la especificidad fue del 90,4% y del 97,5%, la sensibilidad del 100% y del 90%, el valor pronóstico positivo del 66,6% y del 90% y el valor pronóstico negativo del 100% y del 97,5%, respectivamente. Estos valores fueron del 100% para INH y RMP. Los resultados de las pruebas de sensibilidad se obtuvieron al 14^a día de incubación.

CONCLUSIÓN : Los resultados de este estudio preliminar indican que puede utilizarse el agar sangre como un medio alternativo para las pruebas de sensibilidad de las cepas de *M. tuberculosis* a INH, RMP, SM y EMB, en países con escasos recursos. Se precisan, no obstante, estudios complementarios antes de poner en práctica este método en los laboratorios de diagnóstico.
