ORIGINAL ARTICLE

Rapid culture of Mycobacterium tuberculosis on blood agar in resource limited setting

Murli L. Mathur, Scientist F,[1]
Jyoti Gaur, Field Investigator,[1]
Ruchika Sharma, Field Investigator,[1] *and*
Aruna Solanki, Professor[2]

1) Desert Medicine Research Centre, Pali Road, Jodhpur-342005, India, and

2) Department of Microbiology, Dr. S. N. Medical College, Jodhpur-342003, India.

Correspondence: Murli L. Mathur, Desert Medicine Research Centre, Pali Road, Jodhpur-342005, India.

E-mail: murlimathur@dmrcjodhpur.org

Dan Med Bull 2009;56:208-10

ABSTRACT

Introduction: Sputum samples collected at a teaching hospital of Jodhpur, were processed at Desert Medicine Research Centre, Jodhpur.

The aim was to assess the time required for primary isolation of *Mycobacterium tuberculosis* (Mtb) on locally prepared sheep blood agar slants as compared to Löwenstein-Jensen (LJ) medium.

Methods: Equal volume of homogeneous inoculums prepared from smear positive sputum samples processed with the N-acetyl L-cysteine sodium hydroxide method was inoculated on slants of locally prepared 7% sheep blood agar and LJ medium. These were incubated at 37 °C and were observed daily for growth visible with naked eyes. Time taken in growth of Mtb was compared.

Results: LJ medium recovered 68 of 70 isolates (97.1%) as compared to 66 by blood agar (94.2%). The difference was not significant. Mean time to detect macroscopic colonies of Mtb on blood agar was 13.6±5.2 days as compared to 20.4±5.1 days on LJ medium (p=0.0001). More colonies were observed on blood agar than on LJ medium.

Conclusion: Blood agar slants may be a good substitute of LJ medium for rapid detection of Mtb from sputum in resource limited settings. It may save about one third of the time.

Primary isolation of *Mycobacterium tuberculosis* (Mtb) normally precedes its drug sensitivity determination. In developing countries, Löwenstein Jensen (LJ) medium is the most commonly used medium for culture of Mtb, which is also recommended by WHO [1]. Growth of Mtb on LJ medium is slow and when clinical material like sputum is inoculated on it, macroscopic colonies of Mtb appear in 2-6 weeks and negative culture report cannot be given before eight weeks. Methods for obtaining rapid growth of Mtb from clinical material are being investigated worldwide for rapid determination of drug resistance.

Blood agar is commonly used in most clinical microbiology laboratories because it is inexpensive, simple to prepare and the majority of bacteria can be grown on it. The use of blood agar media for the growth of Mtb was reported early in the last century but has not been mentioned in contemporary microbiology manuals [2-4]. The Clinical Laboratory Standard Institute recommends three methods for susceptibility testing of Mtb, the proportion method on Middlebrook 7H10 agar, BACTEC 460 TB System (Becton Dickinson, Sparks, MD, USA) and BACTEC MGIT 960 (Becton Dickinson) [5]. All these methods require colonies of Mtb grown from clinical material.

Sporadic papers have reported the isolation of Mtb on standard blood-agar [6-8]. Drancourt et al found that Mtb isolates could be easily grown on blood agar [9]. In their study when respiratory material was inoculated, differences in the time of detection and in the number of colonies between egg based Coletsos medium and blood agar were not significant. In a recent study Drancourt et al found median time to detect positive culture of Mtb from clinical specimens on blood agar was only 19±5 days as compared to 26±6 days on BACTEC 9000 MB broth (Becton Dickinson Diagnostic Systems) [10].

Coban et al reported that results of drug susceptibility of Mtb could be obtained on blood agar in two weeks as compared to three weeks on 7H10 Middlebrook agar as the results were the same on blood agar at the end of 14 days and at 21 days [11]. They used isolates of Mtb for drug sensitivity testing in their studies and did not attempt for primary isolation of Mtb on blood agar [11, 12]. In developing countries, whether use of blood agar in place of LJ medium for primary isolation of Mtb would save time is not very clear. The aim of the present study was to find out if primary isolation of Mtb on locally prepared sheep blood agar in place of LJ medium required significantly less time in resource limited settings.

MATERIAL AND METHODS

Blood was collected from jugular vein of sheep and 25 ml of it was mixed with 5 ml of sodium citrate (3.8%) solution. It was mixed well and stored at 2-8 °C, 100 ml of deionized water with 4.4 g of Columbia blood agar base (Central Drug House Ltd. New Delhi, India) and 1 ml of crystal violet solution (0.01%) was boiled to dissolve the medium completely. It was sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 minutes and cooled to 45-50 °C, 7 ml of citrated sheep blood was mixed in it. To avoid growth of contaminants two solutions were added to the sample: 1) 90 microlitres of nystatin solution (prepared by dissolving 18 mg nystatin dissolved in 9 ml methanol) and 2) 50 microlitres of antibiotic solution (prepared by dissolving 88.8 mg Polymixin-B, 5 mg trimethoprim and 20 mg nalidixic acid). Contents were mixed well by vortexing for a minute and approximately 12-13 ml was quickly dispensed to each of 30 ml, flat bottom culture tubes (McCartney bottles).

These bottles were kept obliquely and allowed to cool till slants of blood agar were ready. Instead of using petri plates, we used McCartney bottles for blood agar to avoid desiccation. These bottles were incubated at 37 °C for 24 hours for sterility check and those showing any growth were discarded. Remaining blood agar slants were then stored at 2-8 °C. LJ medium was prepared as per WHO guidelines [1] using locally available ingredients.

Smear positive sputum samples of cases of pulmonary tuberculosis were obtained from laboratory of K. N. Chest Hospital Jodhpur on the day of collection from patient. These were processed with the

Growth of *Mycobacterium tuberculosis* on blood agar slants.

N-acetyl L-cysteine sodium hydroxide (NALC-NaOH) method for homogenisation and decontamination. In brief equal volumes of sodium hydroxide (4%) and sodium citrate (2.9%) solutions were mixed. To this mixture N-acteyl-L-cysteine was added to make a final concentration of 0.5%; 5 ml of this mixture was added to 5 ml sputum; contents were mixed well and kept at room temperature for 15 minutes. Then these were centrifuged for 15 minutes at 3000 rpm. Supernatant was discarded in 5% Lysol and sediment was washed with deionized water. The sediment pellet was resuspended in 7H9 Middlebrook broth or deionized water by vortexing to make a homogeneous inoculum; 50 microlitres of this inoculum was inoculated on LJ slants as well as blood agar slants in triplicate. These McCartney bottles were sealed with parafilm.

The inoculated slants were incubated at 37 °C for eight weeks. These were observed daily for appearance of macroscopic growth. The growths observed on LJ medium as well as blood agar slants were observed after keeping the slants in diffuse sunlight for about one hour and no pigmentation was ever observed. Time to grow macroscopic colonies on LJ slants and blood agar slants as monitored by naked eye examination of McCartney bottles was recorded. Paired t test was used to compare number of days taken in appearance of visible growth on blood agar and LJ medium. Smears prepared from colonies grown were stained by the Ziehl-Neelsen method and were examined microscopically.

RESULTS

After treating with the NALC-NaOH method, 70 sputum samples were inoculated on three blood agar (7% sheep blood) slants as well as three slants of control LJ medium. Growth patterns were not different within three slants of any medium. However Mtb colonies were light grayish, glistening and easily recognized against red blood agar as compared to cream-coloured rough colonies on the green LJ medium. Colonies did not appear on blood agar from four sputum specimens which were smear grade +1 and showed colonies on LJ slants. Two sputum samples did not grow colonies of MTB on LJ medium, and showed growth on blood agar only. LJ medium recovered 68/70 isolates (97.1%) as compared to 66/70 by blood agar (94.2%). The difference was not significant. Rate of contamination was 1.6% on blood agar and 7.8% on LJ medium, the difference may be because of antibiotics added to blood agar; 64 sputum samples grew visible colonies of Mtb on LJ medium as well as blood agar. Time to grow macroscopic colonies on LJ medium and blood agar was compared in these samples.

Mean time to detect macroscopic colonies of Mtb on blood agar was 13.6±5.2 days as compared to 20.4±5.1 days on LJ medium (p=0.0001) (Table 1). The median time to culture positivity of Mtb was 13 days (range: 5-25) on blood-agar slants and 19 days (range: 12-31) on LJ slants (p=0.0001). More colonies were observed on blood agar than on LJ medium.

DISCUSSION

The present study has highlighted that time to detect Mtb from smear positive sputum specimens was found to be significantly shorter with blood agar slants (13.6±5.2 days) as compared to LJ medium (20.4±5.1 days). Many evaluation studies have been carried out comparing performance of automated and semi-automated complex culture systems in terms of detection time for Mtb. Some of them have compared detection time for Mtb from smear positive sputum samples on a particular culture system and on LJ medium [13-21]. Primary isolation of Mtb from smear positive sputum samples by the VersaTREK system took 19.8±11.2 days [13], by the Liquid Bio FM (BIO-RAD) medium it took 10.4 (3-33) days [14], by the Biphasic system (Middlebrook 7H11 agar slant+Middlebrook 9H broth) it took 21±4.4 days [15], by MGIT 960 it took 9 (7-11) days [21], 11.9 days [16] and 12.6 days [18], by the MB-Check culture system (liquid phase) it took 14.8±8.0 days [19], by BACTEC 9000 MB it took 9 (4-12) days [20], and by BACTEC 460 TB it took 7 (3-27) days [21], 7 (4-12) days [20] and 13.8 days [18] in various studies. Looking at these observations made by using sophisticated equipments, expensive kits and skilled manpower, use of blood agar slants appears to be fairly simple and has shown a fairly comparable performance as far as time to detect Mtb is concerned.

Mean time to detect Mtb from smear positive pulmonary sample on LJ slants has been found to be in the range of 19-24 days by most workers [14, 16, 19-21]. Use of blood agar slants in place of LJ medium is likely to reduce this time to approximately two thirds in developing countries. Blood agar slants have shown sensitivity of 94.2% in the present study, which was not significantly different from that of LJ medium and is acceptable in resource limited settings.

The present study has a limitation as it did not assess performance of blood agar slants with smear negative sputa or extrapulmonary specimens. Furthermore performance of blood agar was not compared with BACTEC or other sophisticated systems, which detect Mtb from a significantly higher proportion of clinical samples as compared to LJ medium. Since sensitivity of blood agar slants was comparable to LJ slants in the present study, these may only be a good substitute of LJ medium and not of more sensitive systems like BACTEC, which are not readily available in developing countries. The present work is therefore a small step indicating utility of blood agar in the place of LJ medium for rapid detection of Mtb from sputum in resource limited settings.

Table 1. Time to detect macroscopic colonies of *Mycobacterium tuberculosis* (Mtb) on blood agar and Löwenstein-Jensen (LJ) medium.

	Blood agar	LJ medium	p-value
Mean time to detect macroscopic colonies of Mtb	13.6±5.2 days	20.4±5.1 days	0.0001
Median time to detect macroscopic colonies of Mtb	13 days (range 5-25)	19 days (range 12-31)	0.0001

CONCLUSIONS

Blood agar slants may be a good substitute of LJ medium for rapid detection of Mtb from sputum in resource limited settings, where sophisticated systems like BACTEC are not available. It may save about one third of the time.

References

1. Laboratory services in tuberculosis control part III culture. Global Tuberculosis Programme, World Health Organization. Geneva, Switzerland: World Health Organization, 1998:47-52.
2. Lambi EA. Medium selection and incubation for the isolation of Mycobacteria, In: Isenberg HD, ed. Clinical microbiology procedures handbook. Vol. 1. Washington, D.C.: American Society for Microbiology, 1993:3.6.1-3.6.8.
3. Metchock BG, Nolte FS, Wallace Jr. RJ. Mycobacterium. In: Murray PR, Baron EJ, Pfaller MA et al, eds. Manual of clinical microbiology. 7th ed. Washington, D.C.: American Society for Microbiology, 1999:399-437.
4. Manual on isolation, identificaton and sensitivity testing of Mycobacterium tuberculosis. 2nd ed. Bangalore: National Tuberculosis Institute, Govt. of India, 1998:45-66.
5. Susceptibility testing of Mycobacteria, Nocardia, and other aerobic actinomycetes: approved standard. Document M24-A. Wayne, PA: National Committee for Clinical Laboratory Standards, 2003.
6. Arvand M, Mielke ME, Weinke T et al. Primary isolation of Mycobacterium tuberculosis on blood agar during the diagnostic process for cat scratch disease. Infection 1998;56:254.
7. Gil-Setas A, Mazon A, Alfaro J et al. Blood agar, chocolate agar, and Mycobacterium tuberculosis. J Clin Microbiol 2003;41:4008.
8. Tarshis MS, Weed WA, Kinsella PC et al. Further experience with a new blood medium for the cultivation of Mycobacterium tuberculosis. Am J Public Health 1955;45:1157-61.
9. Drancourt M, Carrieri P, Gévaudan MJ et al. Blood agar and Mycobacterium tuberculosis: the end of a dogma. J Clin Microbiol. 2003;41: 1710-1.
10. Drancourt M, Raoult D. Cost-effectiveness of blood agar for isolation of mycobacteria. PLoS Negl Trop Dis 2007;1:e83.

11. Coban AY, Bilgin K, Uzun M et al. Susceptibilities of Mycobacterium tuberculosis to isoniazid and rifampin on blood agar. J Clin Microbiol 2005;43:1930-1.
12. Coban AY, Bilgin K, Uzun M et al. 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 2008;46:4095-8.
13. Falconi FQ, Suárez LI, López Mde J et al. Comparison of the VersaTREK system and Löwenstein-Jensen medium for the recovery of mycobacteria from clinical specimens. Scand J Infect Dis 2008;40:49-53.
14. Ramarokoto H, Rasolonavalona T, Ratsimba L et al. Evaluation of a rapid culture method on liquid Bio FM (BIO-RAD) medium for the isolation of mycobacteria. Int J Tuberc Lung Dis 2007;11:898-903.
15. Ghatole M, Sable C, Kamale P, Kandle S, Jahagirdar V, Yemul V. Evaluation of biphasic culture system for mycobacterial isolation from the sputum of patients with pulmonary tuberculosis. Indian J Med Microbiol 2005;23:111-3.
16. Diraa O, Fdany K, Boudouma M et al. Assessment of the Mycobacteria growth indicator tube for the bacteriological diagnosis of tuberculosis. Int J Tuberc Lung Dis 2003;7:1010-2.
17. Kocazeybek BS. Comparison of the BBL-mycobacteria growth indicator tube method with culture in the diagnosis of tuberculosis and evaluation of the resistance patterns of isolated strains to four major drugs. Chemotherapy 2002;48:64-70.
18. Somoskövi A, Ködmön C, Lantos A et al. Comparison of recoveries of mycobacterium tuberculosis using the automated BACTEC MGIT 960 system, the BACTEC 460 TB system, and Löwenstein-Jensen medium. J Clin Microbiol 2000;38:2395-7.
19. Palacios JJ, García JM, Ferro J et al. Comparison of MB-check and Löwenstein-Jensen media for recovery of mycobacteria. Microsc Res Tech 1997;38:512-8.
20. Zanetti S, Ardito F, Sechi L et al. Evaluation of a nonradiometric system (BACTEC 9000 MB) for detection of mycobacteria in human clinical samples. J Clin Microbiol 1997;35:2072-5.
21. Casal M, Gutierrez J, Vaquero M. Comparative evaluation of the mycobacteria growth indicator tube with the BACTEC 460 TB system and Löwenstein-Jensen medium for isolation of mycobacteria from clinical specimens. Int J Tuberc Lung Dis 1997;1:81-4.