Comparison of Conventional Lowenstein Jensen Medium and Middlebrook Biphasic Medium for isolation of Mycobacterium Tuberculosis

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ABSTRACT

Introduction: For many centuries tuberculosis (TB) has been the most important of human infections in its global prevalence. It remains one of the world's deadliest communicable diseases. The present study was attempted to assess the feasibility of using Middlebrook biphasic medium (MB) as primary isolation medium for mycobacteria. It is compared with the Lowenstein Jensen (LJ) medium, which is gold standard, for their recovery and growth rate.

Material and methods: Total 250 sputum samples from clinically diagnosed cases of pulmonary tuberculosis were studied. These were collected from Revised National Tuberculosis Control Programme (RNTCP) Centre of Bharati Hospital, Sangli. All the samples were decontaminated by Petroff's method. Each sample was subjected to ZN staining and it was simultaneously inoculated on both LJ and MB medium (Middlebrook 7H11 agar slant + Middlebrook 7H9 broth) for their recovery from sputum and growth rate i.e. time required for the visible growth of mycobacterium after subculture on both LJ and MB medium. The growth from cultures was confirmed by ZN staining and they were further identified by conventional biochemical tests.

Results: We have evaluated and compared MB biphasic system and LJ medium. Biphasic system showed the recovery of mycobacteria in 41 samples as against 35 samples on LJ medium after incubation for 28 and 33 days respectively and for growth rate it took 17 and 21 days on MB and LJ medium respectively.

Conclusion: Biphasic media requires less days for recovery and growth of *M. tuberculosis*. Hence it is superior to LJ medium for use in clinical Mycobacteriology laboratory.

Keywords: *M. tuberculosis*, LJ medium, Middlebrook Biphasic medium (MB), Recovery rate, Growth rate.

INTRODUCTION

For many centuries tuberculosis (TB) has been the most important of the human infections in its global prevalence. It remains one of the world's deadliest communicable diseases.[1] In India the statistics of tuberculosis is calculated as per the Revised National Tuberculosis control programme (RNTCP).[2] The WHO statistics for 2014 gives an incidence of 2.2 million cases of tuberculosis for India out of a global incidence of 9 million.[3] It is estimated that about 40% of the Indian population is infected with *M.tuberculosis*, the vast majority of whom have latent rather than active tuberculosis.[1] Deaths from TB are preventable, if diagnosed and treated early.

Laboratory confirmation and proper follow up is extremely important. Although the introduction of amplification techniques in mycobacteriology laboratory provides faster and more accurate detection of Mycobacterium tuberculosis complex (MTB), culture still represents a decisive step for diagnosis, treatment and control of tuberculosis. A combination of solid and liquid media is currently regarded as the "gold standard" for primary isolation of mycobacteria. Turnaround time not exceeding 21 to 31 days after specimen collection is recommended for MTB identification and drug susceptibility testing.[4]

Smear and culture is the corner stone of diagnosis of tuberculosis. In India the availability of amplification techniques is still out of reach for the poor in whom tuberculosis is common. We undertook one-year study as an attempt to compare Biphasic Middlebrook medium with LJ to find out a medium which has shorter turnaround time and is feasible for use in smaller laboratories.

MATERIAL AND METHODS

Two fifty sputum samples from patients attending RNTCP center in Bharati Vidyapeeth Medical College were studied during a period of one year. These patients were clinically diagnosed as pulmonary tuberculosis. Few had radiological evidence suggestive of tuberculosis. A prior approval of Institutional ethical committee was taken for the study and informed consent was taken from all the patients participating in the study.

LJ medium (HI media M168) was prepared as per manufacturer's instructions. MB system was prepared in two stages. For the solid phase Middlebrook 7H11 agar (Hi media-M511) was used. The sterile OADC (Hi media) was added into this medium and slants were prepared in 30 ml screw capped bottles as per manufactures instructions.[5] For the fluid phase Middlebrook 7H9 broth base (Hi media-M198) was used. Glycerol and OADC supplement (Hi media FD019) was added as per manufacturer's instruction.[6] Sterility test of media were done by incubating them for 72 hours to rule out contamination.[7] Quality check of the media was done by inoculating H37RV strain of M. tuberculosis.[6]

The patients were asked to cough into a sterile wide mouthed container. The specimens were immediately transported to the microbiology laboratory. The sample selection was done according to Barlett's grading system for assessing the quality

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of sputum samples on microscopy. Samples suggestive of lack of evidence of active inflammation or salivary contamination were rejected and a repeat fresh sample was collected.[8] All the laboratory work was done in inoculation hood and biosafety cabinet. The sputum was subjected to decontamination and concentration by Petroff's technique.[2]

The ZN smears were prepared from concentrated sputum. 0.5 ml of concentrated decontaminated sputum was inoculated on to LJ medium and both slant and broth of MB medium. The media were incubated aerobically at 37^{0}C. They were inspected daily for contamination for period of 10 days. After a week of incubation, the MB medium was tilted on alternate days for one week for first two weeks and thereafter once a week for inoculating the slant.[5]

Recovery of *M. tuberculosis* was the time of visible growth after inoculation.

LJ medium showed the growth of typical buff colored, raised colonies of *M. tuberculosis* with rough surface. On Biphasic medium translucent tiny colonies appeared on Middlebrook 7H11 agar slant and serpentine cords were seen in Middlebrook 7H9 broth.

ZN smears were prepared from colonies and broth showing growth. The identification of mycobacteria was done by conventional biochemical tests.[9]

Smears were also prepared from broth and media showing no growth to avoid false negative results.

STATISTICAL ANALYSIS

Mean and S.D. of recovery and growth rate in days was calculated. Z test (Standard error of difference between two means) was applied to find out the significant difference in days for recovery and growth rate for biphasic and LJ medium.

RESULTS

Out of total 250 samples studied, 70(28%) samples showed presence of acid fast bacilli in ZN staining and 180(72%) samples were negative for acid fast bacilli (Table-1). Out of 250 sputum samples, the growth of mycobacteria was obtained on total 35 LJ media (Figure-1) and 41 Biphasic media (Figure-2). In 41 samples growth was obtained by the 5th week of incubation on middlebrook Biphasic medium, whereas only 13 cultures were positive on LJ by 5th week. For the rest 21, it took 6 weeks for the bacteria to grow on LJ medium (Table-2). All these isolates were further confirmed as *M. tuberculosis* by standard biochemical tests. In our study we did not find any nontuberculous mycobacteria.

Table-3 shows the number of days required for recovery of *M. tuberculosis*. The recovery days were calculated by taking mean of recovery days of all the samples showing growth on LJ and Biphasic media. Statistical analysis using unpaired t test was done. P value is calculated. There is a statistically significant difference in mean days for recovery on BP and LJ, i.e. Mean recovery days for biphasic medium were significantly less than LJ medium for *M. tuberculosis* (P=0.002). This shows that biphasic media requires less days for recovery.

Mean days required for growth of *M. tuberculosis*. (I.e. time required for growth after subculture) on biphasic media were 16.59 while that on LJ medium were 21.17. There is a statistically significant difference in mean days for growth on BP and LJ medium (P=0.000) (Table-4). i.e. growth was earlier in biphasic medium than in LJ medium.

DISCUSSION

Tuberculosis still remains a major health problem in India. It accounts for 30% of global TB Burdon.[10] It is the most common cause of death due to single infectious agent. Rapid diagnosis is important for treatment and containment of the disease. Now a days rapid tests like BACTEC, septic check AFB system and MGIT have become available, but not so commonly in rural settings. The high cost of these methods is a major hurdle.

Microscopy and culture still form the corner stone of diagnosis of tuberculosis. Hence we worked on a system which has a shorter turn over time for recovery and growth of *M. tuberculosis*. The conventional LJ medium was compared to composite MB system for recovery. The present study was attempted to assess the feasibility of using biphasic medium as primary isolation media for mycobacteria i.e. recovery and its growth.

In our study a total of 250 cases of pulmonary TB from RNTCP were studied. The most common presentation was fever and cough seen in 144(69.4%) of the cases followed by weight loss 40(16%). Out of these 31 (12.4%) had radiological evidence of

Total no. of sputum samples	Positive	Negative
250 (100%)	70 (28%)	180 (72%)

Table-1: ZN Staining- Smear findings

Figure-1: Growth of M.tuberculosis on LJ

Figure-2: Growth of M.tuberculosis on Middlebrook Biphasic medium (MB)

ZN staining (Smear grading)	Scanty		+		++		+++		Total no. of Smear positive sputum samples		Total no. of Smear negative sputum samples	
Total no. of sputum samples n==250	8(11%)		31(44.2%)		17(24.28%)		14(20%)		70		180	
Positive in days(week)	L J	MB	L J	MB	L J	MB	L J	MB	L J	MB	LJ	MB
7 days (1st week)	-	-	-	-	-	-	-	-	-	-	-	-
14 days (2nd week)	-	-	-	-	-	-	-	-	-	-	-	-
21 days (3rd week)	-	-	-	01	-	01		03	-	05	-	-
28 days (4th week)	-	02	01	03	02	02	02	01	05	09	-	01
35 days (5th week)	02	02	03	09	01	08	01	06	08	27	1	2
42 days (6th week)	01	-	07	-	06	-	05	-	21	-	2	-
49 days (7th week)	01	-	-	-	-	-	-	-	01	-	-	-
Total	04	04	11	13	09	11	08	10	35	41	03	03
L J - Lowenstein Jensen Medium, MB- Middlebrook biphasic medium												

Table-2: Recovery of M. tuberculosis in L J and Middlebrook Biphasic medium from sputum

Recovery in days		
	Biphasic medium	L J medium
Mean	27.76	33.31
S.d.	8.10	7.57
Z value	3.014	
P value	0.002	

Table-3: The table shows the number of days required for recovery of *M. tuberculosis*. The recovery days were calculated by taking mean of recovery days of all the samples showing growth on LJ and Biphasic media.

Growth in days		
	Biphasic medium	L J medium
Mean	16.59	21.17
S.d.	1.79	1.53
Calculated Z value	12.210	
P value	0.000	

Table-4: Table shows the mean days required for growth of *M. tuberculosis*. (I.e. time required for growth after subculture) on LJ and Biphasic media.

tuberculosis.

Out of 250 clinical cases, on ZN staining sputum smear positivity was 28% (70samples) and 72 %(180 samples) were found to be negative on microscopy (Table-1). Percentage of smear positivity is differently reported by different workers accounting for 23% to 62.9% respectively.[5,11] Bacilli are shed out in sputum when a necrotic caseating cavitatory lesion communicates with the airway. Moreover, it requires 10,000 bacilli per ml of sputum for the ZN smear to be positive.[12] The grading of the smears gives us an idea of the bacterial load. More the number of bacilli more is the infectivity of the patient. It depends upon variety of factors such as the time of collection, the number of samples which are taken, the nature of the samples, the treatment with antituberculous drugs, its duration and the method of grading which was used.[13] In our study, a large number of patients were on the antitubercular treatment for variable time periods. This might have had impact on the bacterial load and the culture positivity. Majority of patients were 1(+) i.e. 31(44.2%) which ranked highest (Table-2). Only 12% had radiological evidence of tuberculosis.

On comparing the recovery of the bacilli from sputum, *M. tuberculosis* could be recovered from 41 sputum samples in MB medium and only in 35 samples on LJ medium. In 6 sputa the growth was negative on LJ medium. In 3 ZN smear negative samples the culture came positive in both LJ and MB, but in LJ they were recovered one week later than MB. (Table-2)

The LJ medium did not support any growth as early as 3 weeks whereas in 5 specimens growth of *M. tuberculosis* was obtained on MB medium in 3 weeks from specimens. All 41 cultures were positive by 5 weeks in MB whereas only 13 cultures (5 in 4th week and 8 in 5th week) were positive on LJ. For rest 21 it took 6 weeks for the bacteria to grow on LJ medium. So there is a considerable time lag between growth in MB and on LJ. This difference in recovery time was statistically significant. (Table-3) This may be due to the use of liquid phase, which permits an increase in concentration of an initially small number of bacteria in the broth to serve repeatedly as inoculum for the agar surface in MB biphasic medium.

The rate of growth of all 41 strains when sub cultured on both media also showed significantly earlier growth in MB Biphasic medium (Table-4). i.e. *M. tuberculosis* was grown almost 6-7 days earlier in biphasic medium as compared to LJ medium after subculture. This difference in growth rate in both media is also statistically significant.

CONCLUSION

We feel that MB Biphasic medium could be well adapted for early recovery of *M. tuberculosis* with ease of performance and reliability. It does not require gas supplies or radioactive tracers and enables recovery of the mycobacteria without special equipment in small and peripheral laboratories. With further additional studies, its use can be upgraded for susceptibility testing also.

It is not only comparable with the conventional LJ medium, but significantly better for recovery and growth of *M. tuberculosis.* It is safer and self-contained and can be used easily in rural laboratories.

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