Comparison of a novel bilayered medium with the conventional media for cultivation of *Mycobacterium tuberculosis*

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**Background & objectives:** There is resurgence of tuberculosis in recent years in spite of availability of comprehensive multidrug therapy. Conventional culture media require a long time for the appearance of growth of *Mycobacterium tuberculosis*, while the other methods are expensive. Hence, a rapid low cost and safe bilayered medium was developed for early growth and sensitivity testing of *M. tuberculosis* and the results were compared with those on Lowenstein Jensen medium, Middlebrook 7H10 and Kirchner’s liquid media.

**Methods:** A specially designed bilayered medium, consisting of a lower layer of Lowenstein Jensen medium without malachite green and a top layer of Middlebrook 7H 10 medium with added antibiotics and antifungal agents was prepared. Sputum from clinically suspected cases of tuberculosis, pleural fluid and pus samples were inoculated on the bilayered medium along with the inoculation on other conventional media after proper decontamination and concentration of the samples. Antibiotic sensitivity pattern was determined against a few rapidly growing control and test strains by disc diffusion technique and the results could be recorded by 3 to 7 days.

**Results:** Statistically significant (P< 0.001) isolation rate was obtained on this bilayered medium when compared with the other three media, being 81.7 per cent growth by 7 days. Antibiotic sensitivity test could be recorded by 3 days in case of the rapidly growing strains on this medium, and by 7 days in case of *M. tuberculosis* strains.

**Interpretation & conclusions:** Bilayered medium produced rapid growth earliest by 48 h, higher isolation rates were achieved as compared to the other conventional media and drug sensitivity testing could also be carried out successfully. Thus, the bilayered medium can be used for obtaining early culture report.

**Key words** Acic fast bacilli - antimycobacterial agents - bilayered medium - *Mycobacterium tuberculosis*

In recent years there has been a resurgence of tuberculosis in spite of the availability of multidrug therapy1. In developed countries it is due to immigration from places with high prevalence of tuberculosis, drug abuse and infection with HIV2. However, in the developing countries it is due to poverty, malnutrition, irregular and insufficient treatment3. Conventional media like Lowenstein-Jensen (L-J) medium, Kirchner’s liquid medium and Middlebrook
7H10 and 7H9 pose problems of contamination, low recovery rate from clinical specimens and biohazards during sample processing for preparation of smears and cultivation. Automated detection processes like BACTEC AFB system, Septichek AFB system for Mycobacterium along with Mycobacteriast Growth Indicator tube assay are complex to handle, highly expensive for routine laboratories, and pose problems of disposal of radioactive substances. Also, identification of growth by nucleic acid probes may show false positive results if there is any contamination during processing.

Hence it was realized that an inexpensive medium is required for rapid and safe cultivation of M. tuberculosisthat might also permit evaluation of drug sensitivity pattern. The clinicians would then obtain early report of growth, which is not usually obtained before 3-6 wk, by conventional culture media also select appropriate drugs from sensitivity testing report and would help the patients considerably to undergo rational treatment much sooner. Work has been initiated in this regard both in India and abroad for rapid and non expensive cultivation of mycobacteria.

The present study was taken up to develop a new cultivation medium for safe and rapid cultivation of M. tuberculosist along with evaluation of drug sensitivity testing at a shorter period of time and compare the results with conventional media.

**Material & Methods**

The study was conducted at Department of Microbiology, Nil Ratan Sircar Medical College, Department of Microbiology, Calcutta Medical College, Division of Microbiology; Department of Pharmaceutical Technology, Jadavpur University and Department of Microbiology, Herbicare Healthcare Bio-Herbal Research Foundation, Kolkata. Procedures done in BSL as per recommendations.

**Strains:** Reference standard strains M. smegmatis 1546, M. fortuitum 1529, M. tuberculosis H37Rv, H37R, were obtained from Dr V.M. Katoh, then Director, National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra, India. Although M. smegmatis is non pathogenic and M. fortuitum can occasionally become pathogenic to cause a pulmonary disease, these were used as reference strains as these are known rapid growers and were used for comparison in drug sensitivity testing by disc diffusion method as the drugs would diffuse out completely by 3 days.

**Media:** The various chemical and biological components of the media were obtained from Oxoid, UK. Antibiotic Rifampicin was obtained from Medispan Limited, Chennai, INH was obtained from Vitapure Limited, Mumbai.

Kirchner's liquid medium (KLM) was prepared as described by Laidlaw, distributed in 2 ml amounts in Bijou bottles, autoclaved and sterile chloramphenicol solution was added at 50 µg/ml concentration in cold condition. Lowenstein-Jensen Medium (LJM) was prepared according to the method described by Laidlaw. For Middlebrook 7H10 agar (7H10) medium 100 ml of distilled water was added to 2.16 g of the agar base and 1 ml of Analar glycerol, pH was set at 6.5; it was distributed in 5 ml amounts in large Bijou bottles, autoclaved and kept in water bath at 50 °C. To each bottle, chloramphenicol was added at 50 µg/ml concentration and allowed to solidify in a sloping position. The slopes were then kept at 4 °C and utilized within 1 month.

Bilayered medium (bilayered) consisted of a lower layer of LJM (without malachite green); 5 ml of the medium was poured in each 30 ml McCartney bottle and inpsissated at 75 – 80 °C for one hour for three consecutive days.

The upper layer consisted of Middlebrook 7H10 agar medium (Oxoid) along with added antibiotics, antifungal agents to avoid contamination; triphenyltetrazolium chloride was used to indicate the growth of bacteria by its change of colour on reduction. To 100 ml of Middlebrook 7H10 agar base (without malachite green) 1 ml of glycerol was added and the pH was adjusted to 6.5. This was distributed in 2 ml amounts in test tubes, autoclaved and kept in water bath at 50 °C to avoid solidification, so that the antibiotics when added would not be inactivated. With the help of micropipette having sterile tips, the following antibiotics were added – chloramphenicol (50 µg/ml), polymyxin B sulphate (15 µg/ml), carbenicillin (15 µg/ml) and tetrazolium salt (200 µg/ml) as indicator. The medium was stored at 4 °C and used up to 1 month.

**Reagents**

(i) Phosphate buffer saline (PBS) – was prepared according to standard protocol. To this, the following antibiotics were added – carbenicillin (30 µg/ml), polymyxin B sulphate (30 µg/ml), chloramphenicol (150 µg/ml), and itraconazole (1 µg/ml). PBS was kept stored in the refrigerator at 4 °C after proper sterility check up.

(ii) 2 per cent NaOH solution

(iii) Normal saline (0.9%)
(iv) Pipettes were specially designed to draw inoculum by capillary action as no teats were used to reduce the chance of aerosol formation. The narrow end was slightly bent over the flame in such a way that there was proper apposition of this end with the inner wall of the bottle containing the medium. Cotton plug was applied over the broad end. (v) Masks – Specially designed masks made by authors were used with a layer of lint in between two layers of towel for effective blockade against inhalation of mycobacterium. Full sleeved, full length gowns were routinely worn. (vi) Cotton swabs and formalin were kept ready in dropping bottles in case of accidental splashing of inoculum on the working table. A formalin jar was also kept on the working table for disposal of pipettes and loops after use.

Procurement of clinical materials: Samples (sputum, pleural fluid, pus in suspected cases of tuberculosis) were collected from the DOT clinic (Directly Observed Treatment short course), in Chest Medicine Department, S.S.K.M. Hospital, Kolkata, and different wards and OPD of S.S.K.M. Hospital, Kolkata. Grading was made as + to ++++ according to Joklik et al.4

The samples were collected from May 2005 to April 2006.

A total of 71 clinical cases sputum positive for AFB and chest X-ray suggestive of tuberculosis were included; sputum samples were collected from 66. Of these, 60 were sputum positive for AFB and 6 were sputum negative but having clinical history suggestive of tuberculosis; 4 samples were obtained as pleural fluid in case of pleural effusion and 1 was a pus sample. Samples were collected after obtaining written consent of individual patients. Clinical details of the patients regarding age, sex, history of persistent cough for more than 3 wk, weight loss, pyrexia, rise of temperature particularly in the evening, night sweat, chest pain, loss of appetite, shortness of breath, malaise, haemoptysis, family history of tuberculosis and history of intake of antitubercular medicines were recorded. Besides, pleural fluid samples and pus samples were also collected.

Smear examination: Each smear (direct from untreated specimen and from concentrated deposit) was prepared on a glass slide in a drop of formalin taking proper precautions, dried and heat fixed and then stained with Ziehl-Neelsen (ZN) method and graded4. The growths were confirmed as that of Mycobacterium spp. after performing different biochemical tests like niacin test, nitrate reduction test and catalase test15. Photographs were taken in a binocular Olympus microscope (Japan).

Liquefaction and decontamination: Each sample was decontaminated by modified Petroff’s method (2% NaOH)13,14. Sterile glass beads were added to each sample and the pots were gently rotated for homogenization and adequate digestion of sputum. Neutralization of the sample by acid was not carried out to avoid even minimum injury to the mycobacteria. This served as the inoculum. Inoculation was done in pairs in each of the four media. For LJ, 7H10 and bilayered media the bottles were kept in a slanting position for about 2 h for flow of the inoculum over the surface of the slant. Incubation was at 37°C in a vertical position.

Growth was observed in KLM as turbidity from 10th day onwards; while it could be seen in LJ from 3rd wk onwards, incubation was up to 8 wk before cancellation of failure of growth13. In 7H10 medium growth appeared earliest by the end of 2nd wk15 incubation was up to 8 wk. In the bilayered medium growth could be detected after 48 h through transmitted light. In 4-5 days, visible growth appeared, bottles were discarded with ‘no growth’ after 30 days.

Antibiotic sensitivity test by disc diffusion technique: Growth from rapidly growing M. smegmatis 1546 and M. fortuitum 1529 along with M. tuberculosis H_{37}Rv, H_{37}Ra and isolates obtained from clinical specimens was observed. With the help of a metal loop the growth from each bottle was transferred to a flat bottomed round flask containing 1 ml of sterile distilled water and glass beads. The flask was shaken manually for 2 min, 4 ml sterile distilled water was then added and allowed to stand for 1 min. The homogenous portion was removed with a Pasteur pipette, transferred to a sterile tube, visually compared with McFarland Standard 1, with addition of distilled water, if necessary, for adjustment of opacity (104 standard was equivalent to 0.1 mg of bacterial mass/ml)16. This was further diluted to 103, from which 0.2 ml was allowed to flow over the bilayered slant and dried for 30 min. Two drug impregnated discs were placed over the surface with adequate spacing and pressed gently to ensure full contact with media. Testing was made in bottles since such a medium cannot be prepared in a petridish.

The amounts (µg) of rifampicin/discs were 0.25, 0.5, 1 and 5 and that of INH were 0.01 and 0.02. The bottles were incubated at 37°C and observed everyday upto 7 days.

Statistical analysis was carried out according to χ2 test.
Results

Each sputum specimen was first examined microscopically in a direct smear prepared from the untreated specimen and then in a smear made from a centrifuged concentrated deposit obtained after the specimen was treated and homogenized (Fig. 1). Of the 71 cases of tuberculosis, 60 showed presence of AFB through microscopic examination by Z-N staining. All the specimens including those from 11 sputum negative cases were inoculated in KLM, LJM, 7H10 and bilayered media.

The growth of mycobacteria appeared within 10-16 days in KLM (Table I). The growth was recorded only as turbidity in this medium and it was followed by Z-N staining for confirmation. In LJM, growth appeared frequently after 3 wk; however, some specimens took a longer period for exhibiting growth (6-8 wk). The medium 7H10 proved more supportive for tubercle bacillus, since growth could be observed in many cases within 2 wk (Table I).

Growth in the bilayered medium was detected earliest by 48 h with the help of a hand lens against transmitted light. There was either earlier appearance of colour change followed by appearance of translucent colonies or vice versa. The formation of red colour in the medium was due to reduction of the tetrazolium indicator demonstrating growth. The colonies were translucent, small, partially above the surface of the media and partially seen submerged in the top layer. With further incubation confluent growth was produced (Fig. 2).

Typical mycobacterial morphology was obtained by Z-N staining. Small, straight or slightly curved non-

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**Table 1. Comparison of isolation rate of Mycobacterium spp. from clinical specimens in different media**

<table>
<thead>
<tr>
<th>Media</th>
<th>Sputum</th>
<th>No. of isolates showing</th>
<th>Time taken for growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Contamination</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Growth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KLM</td>
<td>+ve</td>
<td>36</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(60)</td>
<td>(13.3)</td>
</tr>
<tr>
<td></td>
<td>-ve</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(45.4)</td>
<td>(36.2)</td>
</tr>
<tr>
<td>LJM</td>
<td>+ve</td>
<td>40</td>
<td>3-8 weeks</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(66.7)</td>
<td>(11.7)</td>
</tr>
<tr>
<td></td>
<td>-ve</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(54.5)</td>
<td>(18.1)</td>
</tr>
<tr>
<td>7H10</td>
<td>+ve</td>
<td>37</td>
<td>2-6 weeks</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(61.66)</td>
<td>(13.8)</td>
</tr>
<tr>
<td></td>
<td>-ve</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(36.2)</td>
<td>(36.2)</td>
</tr>
<tr>
<td>Bilayered</td>
<td>+ve</td>
<td>49</td>
<td>3-12 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(81.7)</td>
<td>(10)</td>
</tr>
<tr>
<td></td>
<td>-ve</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(63.6)</td>
<td>(9.1)</td>
</tr>
</tbody>
</table>

Data in parenthesis show per cent; *P<0.001 according to χ² test
No. of sputum +ve cases=60; No. of sputum -ve cases=11
*P<0.001 compared to other media

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Fig. 1. Ziehl-Neelsen staining of sputum showing acid fast bacilli. Smear was made from direct sputum. 1000x.

Fig. 2. Growth of *M. tuberculosis* 21 on bilayered medium and LJM.
sporing acid fast rods (deep red in colour) that did not branch or produce hyphae were seen throughout the smear (Fig. 3).

It may be noted that the frequency of occurrence of growth in all the media was greater in the 60 sputum positive specimens that showed presence of AFB. However, among 11 sputum negative cases appearance of growth was observed more in the bilayered in comparison to other media.

Considering growth, no growth and contamination, significant difference existed among all the media ($P<0.001$, $\chi^2$ test). Bilayered medium produced significantly higher growth ($P<0.001$) compared to other media (Table 1). However, contamination in bilayered was not significantly different from that observed in other media.

**Effect of rifampicin and INH on Mycobacterium spp:**

The rapid grower $M. fortuitum$ 1529, which is known to be occasionally pathogenic was sensitive to rifampicin at 0.5 $\mu$g/disc and to INH at 0.02 $\mu$g/disc; both $M. tuberculosi$s $H_3R_6$ and $H_3R_5$ were highly sensitive to rifampicin and less sensitive to INH. The clinical isolates $M. tuberculosi$s 39 was resistant to both antibiotics while $M. tuberculosi$s 21 and 52 exhibited sensitivity to rifampicin at a concentration of 5.0 $\mu$g/disc; INH had no effect on these isolates (Table II).

**Table II. Antibiotic sensitivity pattern of Mycobacterium spp. on bilayered medium**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Action of antitubercular drugs exhibiting inhibition</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Rifampicin (µg/disc)</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>$M. tuberculosi$s H$_3R_6$</td>
<td>-</td>
</tr>
<tr>
<td>$M. tuberculosi$s H$_3R_7$</td>
<td>+</td>
</tr>
<tr>
<td>$M. tuberculosi$s 21</td>
<td>+</td>
</tr>
<tr>
<td>$M. tuberculosi$s 39</td>
<td>+</td>
</tr>
<tr>
<td>$M. tuberculosi$s 52</td>
<td>+</td>
</tr>
<tr>
<td>$M. fortuitum$ 1529</td>
<td>+</td>
</tr>
</tbody>
</table>

INH, Isonicotinyl hydrazine (isoniazid); +, growth; -, no growth

**Discussion**

The genus Mycobacterium comprises a variety of microorganisms that exhibit diverse nutritional types. These can be rapid growers, slow growers or even non-growing like $M. leprae$. Certain heterotrophic mycobacteria can be cultivated even in nutrient agar. Although some non-pathogenic mycobacteria like $M. smegmatis$ and $M. phlei$ can produce distinct visible colonies on LJ medium within 48-72 hours, majority of mycobacteria, particularly the pathogens take a much longer period to demonstrate their growth. Thus a mosaic of nutritional types exists among these organisms, many of which prefer a mixture of nutritional factors. Based on such observations a combination of various nutritive agents was therefore provided in a particular medium for making their growth most optimum as well as rapid.

The new bilayered medium contained a bottom layer of LJ medium without malachite green. The upper layer was 7H10 agar containing antibiotics and antifungal agents to prevent the growth of contaminants. The indicator triphenyltetrazolium chloride present in the top layer induced rapid growth of mycobacteria that was noticed by change of the colour due to reduction. This indicator is also known for its beneficial role in supporting cultivation of mycobacteria. Addition of malachite green in the lower LJ medium was avoided since that might interfere with the red colour that always developed in the top layer with the growth of various mycobacteria. The lower layer of LJ containing egg and starch provided nutritive support to the mycobacteria since the upper layer of 7H10 agar is a totally synthetic medium containing primarily inorganic salts.
The new bilayered medium has thus provided a unique system of cultivating all mycobacterial species except M. leprae from clinical material with in 2-5 days along with the drug sensitivity testing. This finding corroborates the observations made by others with biphasic media. However, tetrazolium salt in the top layer has made our medium a unique and advantageous system. Since every diagnostic laboratory may not be able to possess the BACTEC system of growing mycobacteria from clinical samples, this bilayered medium will provide a simple, cost effective culture medium for such laboratories. Further work are in progress to cultivate many more mycobacteria from clinical specimens and effect of other antimonycobacterial drugs in the present medium.

References

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