Microculture in Biphasic Medium with Silicone-coated Slides for Isolation of Mycobacteria

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The study reported here, seeking to develop a simple, practical, sensitive, and inexpensive technique for microbial diagnosis of tuberculosis, used a combination of biphasic media and microculture techniques to augment the sensitivity of traditional culture methods.

A total of 540 sputum samples (5 mL each) were obtained from 180 patients with suspected tuberculosis in Mexico City. These samples were treated with Hanks reagent, neutralized with 25% HCl, and centrifuged. In each case the resulting residue was combined with liquid media (Sula medium or a phosphate-buffered control solution) and was inoculated into a bottle containing a solid medium (Löwenstein-Jensen-Holm or Middlebrook). A silicone-coated slide appropriate for culture of hydrophobic mycobacteria was inserted in each bottle, and the cultures (examined weekly) were incubated at 37 °C until the first macroscopic bacterial growth was detected or for up to eight weeks if none was detected. When such growth was detected, or at the end of eight weeks, each slide was withdrawn from the bottle, sterilized, stained by Kinyoun's method, and examined microscopically.

Following 2–4 weeks of incubation, macroscopic bacterial growth was detected in 71 bottles and was confirmed by microscopic examination of the corresponding slides. No macroscopic bacterial growth was found in any of the remaining 469 bottles, but microscopic growth was observed on 77 of the slides examined after eight weeks. The authors conclude that this method represents a noteworthy improvement over standard culture methods in terms of bacterial isolation and suggest that its ease, economy, and practicality make it suitable for application in developing countries.

The serious public health problem posed by tuberculosis has worsened notably in recent years, as attested by morbidity and mortality records in both industrialized and developing countries (1, 2). Detection of the tubercle bacillus through microscopic examination or culture of sputum samples continues to be of fundamental importance for diagnosis and control of the disease. Two procedures, the molecular polymerase chain reaction (PCR) technique (3, 4) and automated radiometry (BACTEC) (5, 6),
have also proven highly useful in this regard. However, these techniques' high complexity, cost, and/or use of radioactive material has kept them from being practical options in the rural areas of developing countries.

Studies have recently been conducted on the growth of mycobacterial microcolonies before they are macroscopically visible in solid media (7). Although various slide culture techniques had previously been described (8–10), none gained general acceptance because of the high levels of contamination observed and the tendency for the bacilli to become detached from the slide, thus increasing the risk of transmission. In addition, a microculture technique was developed in which *Mycobacterium tuberculosis* adhered strongly to a test tube's hydrophobic silicone surface (11). Using a modification of this latter liquid media technique employing silicone-treated glass slides, it was possible to obtain microscopic bacillary growth in suspensions of *M. tuberculosis* cultures within 2–7 days, depending on the concentration of the inoculum. It was also possible to observe small colonies macroscopically in 5–15 days, as compared to 10–40 days when solid media were employed (12).

Attracted by the practical advantages of the biphasic medium for blood cultures developed by Ruiz-Castañeda (13) and the sensitivity shown by a microculture technique (12) for *M. tuberculosis*, we tested a modification of both methods that sought to enhance detection of mycobacteria isolated from clinical samples by introducing a silicone-coated slide into the culture flask. The biphasic media employed were solid Löwenstein-Jensen-Holm culture medium or Middlebrook 7H10 agar medium (14) combined with Sula liquid medium (15) or phosphate buffer solution (see Table 1). The aim of the study was to compare microscopic growth on the silicone-coated slides with macroscopic growth in the biphasic medium in an effort to develop a simple, practical, sensitive, and inexpensive technique for the microbiologic diagnosis of tuberculosis.

**MATERIALS AND METHODS**

To prepare the slides using the technique proposed by Higashi et al. (12), 26 mm ×

<table>
<thead>
<tr>
<th>Biphasic culture media</th>
<th>No. of samples</th>
<th>Cultures positive at 2–4 weeks (samples macroscopically and microscopically positive)</th>
<th>Cultures macroscopically negative after 8 weeks</th>
<th>Samples microscopically positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>(%)</td>
<td>No. of macroscopically negative samples</td>
</tr>
<tr>
<td>Löwenstein-Sula</td>
<td>194</td>
<td>27</td>
<td>(13.9)*</td>
<td>167</td>
</tr>
<tr>
<td>Middlebrook-Sula</td>
<td>132</td>
<td>19</td>
<td>(14.4)*</td>
<td>113</td>
</tr>
<tr>
<td>Löwenstein-buffer</td>
<td>214</td>
<td>25</td>
<td>(11.7)*</td>
<td>189</td>
</tr>
<tr>
<td>Total</td>
<td>540</td>
<td>71</td>
<td>(13.1)</td>
<td>469</td>
</tr>
</tbody>
</table>

*Observed differences between the results obtained with the three different biphasic media were not statistically significant (*P* = 0.71).

†Observed differences between the results obtained with the control (Löwenstein-buffer) medium and the other two media were statistically significant (*P* < 0.001).
76 mm slides were cut lengthwise into three parts using a diamond-tipped tool. The original technique was modified by substituting a 500 mL precipitate beaker for the aluminum test tube rack throughout the entire procedure. The slides were degreased with a mixture of sulfuric acid and potassium dichromate for 12 hours. They were then washed discretionally with tap water, rinsed in distilled water, dried at room temperature, washed with petroleum ether, and again dried at room temperature. Next they were immersed in a 2% (V/V) solution of dimethyl silicone (Watson-Philips) in carbon tetrachloride. After 15 minutes the solvent was allowed to drain off and the slides were dried by evaporation at room temperature before being incubated at 300 °C for one hour to stabilize the silicone film.

To prepare the solid media, 5 mL portions of the Löwenstein-Jensen-Holm and Middlebrook 7H10 media (24) were placed in 60 mL McCartney bottles and subsequently tested for sterility.

To procure specimens for testing, a total of 540 sputum samples were taken from 180 patients at the National Institute of Social Security and Services for State Workers (Instituto de Seguridad y Servicios Sociales de los Trabajadores del Estado—ISSSTE) in Mexico City who were suspected of having pulmonary tuberculosis. Expectoration was provoked with a saline aerosol administered by an ultrasonic nebulizer (De Vilbiss) (16). Five mL of sputum from each sample were then treated with an equal volume of Hanks reagent (17) containing 0.5% N-acetyl L-cysteine (18). After 20 minutes the sample was neutralized with 25% hydrochloric acid and centrifuged at 2,800 rpm for 20 minutes. The supernatant was removed and the resulting bacterial pellet was suspended in 5 mL of Sula liquid medium or a control solution (phosphate buffer with a pH of 6.8). All of the suspension was transferred to a bottle containing solid medium, and a sterilized silicone-coated slide was inserted into each bottle.

Using this procedure, the 540 clinical samples were introduced into three types of biphasic media as follows: 194 into Löwenstein-Sula, 132 into Middlebrook-Sula, and 214 into Löwenstein-buffer. The cultures were incubated at 37 °C and examined weekly for eight weeks. As soon as any macroscopic bacterial growth was detected, the silicone-coated slide was withdrawn from the biphasic medium, placed in a test tube, and sterilized with 5% phenol or by autoclaving at 121 °C for 10 minutes. It was then stained using Kinyoun’s technique to prepare it for microscopic examination. Samples that failed to exhibit any macroscopic bacterial growth (either in the solid medium or on the slide) continued being incubated for eight weeks, at the end of which all the silicone-coated slides were removed, sterilized, stained, and subjected to microscopic examination.

The overall statistical analysis for the study was carried out using Cochrane’s nonparametric Q test for k related samples. In conducting the test, it was assumed that all samples classified as positive by macroscopic examination of the solid medium would be found positive by microscopic examination of the slide, a hypothesis that was subsequently confirmed. The statistical test was applied to each sample separately with an alpha risk (P = 0.05). To evaluate the results obtained using the biphasic medium, a second statistical analysis was conducted using the chi-square test for independent samples—evaluating first the results of culturing on solid medium and then the results of microculturing on silicone-coated slides (19).

RESULTS

As indicated in Table 1, 71 of the 540 sputum samples yielded macroscopic bacterial growth in both the solid and liquid phases (see Photo 1) during the second, third, or fourth weeks of incubation. The same number of silicone-coated slides showed bacte-
rial growth after a similar period (10–28 days). Microscopic examination of these slides after staining with Kinyoun’s method showed typical growth in the form of loose or tight serpentine chains (Photo 2), with bacilli aligned in a parallel fashion along the longitudinal axis and with more abundant growth occurring on the portion of the slide next to the surface of the liquid medium, the bacilli adhering firmly to each slide despite exposure to the phenol or autoclave and Kinyoun’s stain.
The remaining 469 samples revealed no macroscopic growth in the biphasic media after eight weeks of incubation, at which time a culture is normally deemed negative. However, staining and microscopic examination of the slides corresponding to these 469 samples showed varying amounts and formations of acid-fast bacilli, from isolated bacilli to actual microcolonies (see Photo 3) on 77 slides, but with no formation of serpentine growth patterns. Of the 77 slides showing bacterial growth, 36
had been immersed in the Löwenstein-Sula medium, 27 in the Middlebrook-Sula medium, and 14 in the control (Löwenstein-buffer) medium.

All the macroscopically positive cultures (found positive during the initial 28 days of incubation) were also positive by microscopic examination. Likewise, for these macroscopically positive cultures no statistically significant differences were observed in the results obtained using the three biphasic media (see Table 1). However, at
the end of the conventional eight-week incubation period significantly greater sensitivity \( (P < 0.001) \) was obtained through microscopic slide examination. When these later positive results were included, no significant difference was found between the Löwenstein-Sula and Middlebrook-Sula media \( (P > 0.68) \), but significant differences were found between each of the latter and the control (Löwenstein-phosphate buffer) medium \( (P < 0.001) \). It should be noted that tubercle bacilli tend to proliferate freely in the Sula medium, while in the phosphate buffer solution they do not, unless prolonged contact with the solid medium infuses it with nutrients capable of promoting a modicum of bacterial growth.

**DISCUSSION AND CONCLUSIONS**

The increase in tuberculosis in recent years and the scarce financial resources available for control measures in many developing countries have led us to examine simple ways of increasing the sensitivity of mycobacterial detection. The results of the study reported here show that microscopic examination of silicone-coated slides can increase the sensitivity of microbial culture methods without exceeding the conventional eight-week incubation period. The study did not seek to determine whether the method could improve early identification of *M. tuberculosis*, although this is an important question deserving of further study. Within this context it should be noted that recent studies have concentrated on detecting microcolonies directly in the solid medium, before they are observable macroscopically \( (7, 20) \).

The medium most used in culturing mycobacteria is Löwenstein's, which is egg rather than agar based and typically employed in a tube. The efficacy of Sula's liquid medium for establishing the isolation of mycobacteria has been described by Sula and is similar to that attributed to Löwenstein's medium \( (15, 21) \). We feel that the technique described here permits an increase in sensitivity that is commensurate with the amount of inoculum used. With the customary technique, the inoculum is 0.1 mL; however, in our study all of the pellet obtained from the digested and decontaminated 5 mL sputum sample was used as inoculum—greatly increasing the chances that a larger number of viable bacilli would be obtained. In addition, it is possible that our technique tends to reduce the concentration of certain toxic substances (including therapeutic drugs) in the sputum sample, thereby promoting better growth.

The biphasic medium that served as the control, consisting of Löwenstein's solid medium and phosphate buffer, permitted some bacterial growth but not as much as the Löwenstein-Sula or Middlebrook-Sula media. It appears that the latter can be used interchangeably, because unlike the control medium both satisfy the bacteria's basic nutritional needs in a complementary fashion.

Because of their lipid-rich walls, mycobacteria tend to move into hydrophobic environments \( (11, 12, 22) \), and the silicone-coated surface of the slides used in our study proved no exception. By exploring biphasic culture of *M. tuberculosis* in clinical samples with good results, this work has supported findings of the elegant and pioneering studies by Fisher \( (11) \) and Higashi et al. \( (12) \) that used silicone-coated test tubes and silicone-coated slides, respectively, with liquid media and pure cultures of *M. tuberculosis*. The silicone-coated slide technique has also provided us with good results in the microculture of dermatophytes \( (23) \).

Although the PCR and BACTEC techniques, which have high levels of sensitivity and specificity, have been applied to mycobacterial isolation and detection in recent years, their high cost, sophistication, and (in the case of the BACTEC) use of radioisotopes makes them difficult or impos-
sible to apply from a practical standpoint in developing countries. In contrast, the microculture methods described here have a cost of approximately US$2.00 per sample. Since the materials necessary for applying this technique are available in virtually all laboratories, its use in isolating mycobacteria from clinical samples is recommended. In general this technique seems easy, economical, and practical, and appears to reduce the risk of contagion during the handling of samples. The method, which provides increased sensitivity, can screen a relatively large volume of inoculum, and promotes mycobacterial growth, may prove particularly useful for small laboratories in developing countries.

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REFERENCES

More people died from tuberculosis (TB) in 1995 than in any other year in history, according to a report from the World Health Organization released in March of this year. Nearly 3 million people died from TB in 1995, surpassing the number of deaths in the worst years of the epidemic around 1900, when an estimated 2.1 million people died annually. In Latin America and the Caribbean, PAHO estimates that 75,000 people died from TB in 1995 and 400,000 contracted the disease. Worldwide, TB is now the leading infectious killer of youth and adults. It has become the principal killer of HIV-positive people and kills more women that all causes of maternal mortality combined. Nearly half of the world’s refugees may be infected with TB. At current rates of infection, up to 500 million people could become sick with TB in the next 50 years, many of them with multidrug-resistant strains.

Increased air travel and migration have helped transport the disease throughout the world, including to wealthy countries, which have only recently begun to recognize the threat to their citizens’ health of poor TB treatment practices elsewhere. In 1993, WHO declared a global TB emergency, prompting some governments to increase their response to TB. However, the epidemic continues to outpace these modest efforts.

WHO, PAHO, and the World Bank endorse the strategy known as directly observed treatment, short-course, or DOTS. This strategy — which makes the health workers, not the patients, responsible for ensuring that medicines are taken — can cure nearly 95% of patients at low cost. The challenge now is to encourage countries to adopt, promote, and enforce the DOTS strategy.