Evaluation of the performance of two liquid-phase culture media for the diagnosis of pulmonary tuberculosis in a national hospital in Lima, Peru

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KEYWORDS
Tuberculosis; Diagnosis; Sensitivity and specificity; MGIT; Middlebrook; Ogawa

Summary
Objective: To evaluate the diagnostic performance of two liquid-phase culture media for the diagnosis of pulmonary tuberculosis.

Patients and methods: From May to July 2003, sputum samples for culture were obtained from patients with respiratory symptoms attending the Hospital Nacional Cayetano Heredia. These were cultured in Ogawa medium, mycobacteria growth indicator tube (MGIT), and modified Middlebrook 7H9. Results were compared against a composite reference standard.

Results: One hundred sputum specimens from 100 patients were included. Of these, 33 had culture-proven tuberculosis. The sensitivity of MGIT was found to be 100%. The modified Middlebrook 7H9 medium was found to have a sensitivity of 72.73%, while the sensitivity of Ogawa medium was found to be 69.70%. The mean growing time for MGIT was 12.18 days (95% confidence interval 10.24 to 14.12; \( p < 0.01 \) vs. Ogawa and modified Middlebrook 7H9); for modified Middlebrook 7H9 was 16.65 days (95% confidence interval 14.85 to 18.80; \( p < 0.01 \) vs. Ogawa), and for the Ogawa medium 25.74 days (95% confidence interval 22.22 to 29.6).

Conclusions: The liquid culture medium MGIT was superior to the modified Middlebrook 7H9 and the Ogawa media, both in terms of sensitivity and shorter growing time of colonies of \textit{Mycobacterium tuberculosis}. The modified Middlebrook 7H9 medium is significantly faster but comparable in diagnostic performance to Ogawa. Costs remain an issue for MGIT.

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Introduction

Tuberculosis has a high incidence in Peru, estimated at 141.4/100,000 inhabitants. Of the pulmonary cases, which make up the vast majority, approximately 30% have negative acid-fast bacilli (AFB) smears. Currently, solid media cultures such as Lowenstein–Jensen and Ogawa are considered the reference standards for the diagnosis of tuberculosis. Their great advantage is their low cost, however they require a long time for growth and species-typification of mycobacteria, leading to a longer period of infectivity, worsening of the patient’s clinical condition, and loss of productivity. In addition, their sensitivity is relatively low, calculated at between 80% and 85%. For these reasons, numerous attempts have been made to develop faster and more sensitive culture media for Mycobacterium tuberculosis. Many of these have been described in the literature, however, only a few are now routinely used in the diagnosis of tuberculosis because of their requirement for special equipment, as in the radiometric methods, which makes them unaffordable in most developing countries.

The mycobacteria growth indicator tube (MGIT; Becton Dickinson Microbiology Systems), developed relatively recently, is a liquid culture medium that develops an orange fluorescence in the presence of growing mycobacteria. It appears to have a sensitivity equal to or better than that of solid media (especially in smear-negative cases of tuberculosis), requires less time for growth, and has lower costs than radiometric methods. Middlebrook 7H9 is cheaper than MGIT, and has previously been successfully tested.

In the mycobacteria laboratory of the Hospital Nacional Cayetano Heredia, a modification of Middlebrook 7H9, based on the addition of tetrazolium bromide, has been developed and tested with good preliminary results. This is referred to in this paper as modified Middlebrook 7H9.

Based on the previous considerations, we decided to evaluate the diagnostic yield of the liquid media MGIT and modified Middlebrook 7H9 for the diagnosis of pulmonary tuberculosis.

Patients and methods

Setting

This study was undertaken at the Hospital Nacional Cayetano Heredia (HNC), a tertiary-level hospital and national reference center for infectious diseases in Lima, Peru.

Population

From May to July 2003, patients attending the hospital emergency room due to respiratory complaints were considered for the study. We included patients over the age of 14 years with at least two of the following: cough (≥7 days duration), fever (≥7 days duration), weight loss (>3 kg in a month), or hemoptysis. Only patients able to collect sputum samples were included. Patients with a defined diagnosis of active tuberculosis during the last 6 months or already in treatment were excluded.

Procedures

A clinical questionnaire, physical examination, and chest X-ray were done. All patients volunteering for HIV testing were tested with ELISA-HIV. Each patient, was asked to collect a simple sputum sample (without any kind of induction). An acid-fast smear using Ziehl–Neelsen staining was performed.

Decontamination was done (detailed below), and the treated samples were cultured in Ogawa, a solid-phase medium, and in two liquid-phase media: MGIT and modified Middlebrook 7H9. All the samples were processed in the mycobacteria laboratory of HNC.

Specifications of culture media

First, decontamination of sputum samples was performed. The volume of the sample was measured and an equivalent volume of 4% sodium hydroxide and N-acetyl-L-cysteine (NALC) sodium citrate solution was added. The solution was vortex-mixed for 10 minutes. Next, the tubes were inverted and the personnel in charge assured that the sample was totally liquefied. Incubation was done at 37 ºC for 10—15 minutes. Sterile distilled water was added to complete 50 ml and it was vortex-mixed again. This was centrifuged at 3000 g for 15—20 minutes. The supernatant was decanted and 1—2 ml distilled sterile water was added to the sediment and mixed again. Of this solution, 0.5 ml was inoculated in each of the liquid media.

MGIT medium (Becton Dickinson Microbiology Systems) was constituted of 4 ml of Middlebrook 7H9 plus casein peptone, 110 µl of oxygen-sensitive fluorescent indicator, 0.5 ml of OADC (oleic acid, albumin, dextrose, and catalase), and 110 µl of antimicrobial supplement PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) according to the manufacturer’s instructions. The vials were evaluated daily with ultraviolet light of 365 nm wavelength (using a Wood’s lamp). Those vials that developed fluorescence comparable to a positive chemical control (0.4% sodium sulfate in an empty MGIT tube) were considered positive.

The modified Middlebrook 7H9 consisted of the combination of liquid Middlebrook 7H9 medium associated with tetrazolium bromide, a qualitative colorimetric indicator in a proportion of 50 µl of solution (1 mg/ml) per 200 µl of Middlebrook 7H9. After inoculation of the treated sample, the culture vials were incubated at 37 ºC. On the seventh day, 0.5 ml of the reagent tetrazolium bromide was added to the culture, after which changes in the color were assessed daily. The development of a blue or purple staining was considered a positive result. If no color change developed, an additional 0.5 ml tetrazolium bromide was added on the 14th day and daily evaluation continued.

Ogawa cultures were done following the conventional methodology. The appearance of visible colonies was considered a positive result. All three cultures were read daily until signs of growth (definition of positivity) or until 8 weeks in the case of negative results.

After a positive result, the samples were re-evaluated using Ziehl–Neelsen to verify the presence of AFB. Typification of M. tuberculosis was based on the standard methodology (niacin production, nitrate reduction, and catalase enzyme production).
In the case of growth in MGIT or modified Middlebrook 7H9 and not in Ogawa, we carried out a ‘reculture’ in Ogawa medium to confirm that the mycobacteria recovered were M. tuberculosis. By ‘reculturing’ we mean taking an aliquot of 0.5 ml of a MGIT or modified Middlebrook 7H9 culture showing positivity and seeding it into a new Ogawa tube to assess growth in the latter, with subsequent species identification if a positive result was found. In these cases, PCR were also performed to evaluate the presence of M. tuberculosis genetic material.

Readings of the three types of culture media were done independently. In order to achieve a blinded analysis, one of our researchers, in charge of the seeding of the cultures, assigned different codes for each of the three culture vials coming from the same sample. Afterwards, an independent biologist performed all the readings.

Polymerase chain reaction

We used the commercially available Amplicor M. tuberculosis PCR test (Amplicor MTB; Roche Diagnostic Systems). The procedures were done following the same methodology as reported by Osores et al. In brief, 1 ml aliquots of culture medium in which mycobacteria grew were taken. This was followed by concentration and treatment with proteinase K for 12 hours and then purification of the sample for obtaining the DNA employing silica filters (Qiagen method). Finally, 4 μl aliquots of the purified sample were extracted for the PCR using extension primers that amplify the insertion segment IS6110.

Reference standard

We developed a composite reference standard for the diagnosis of pulmonary tuberculosis in a similar fashion to that reported by Somoskovi and Magyar and Chew et al., considering positive cultures the ones that reported positivity and seeding it into a new Ogawa tube to assess growth in the latter, with subsequent species identification if a positive result was found. In these cases, PCR were also performed to evaluate the presence of M. tuberculosis genetic material.

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Ethical aspects

All the patients gave an informed consent. The study had the approval of the ethics committee of HNCH.

Results

One hundred and four patients were included in the study. Complete information and adequate sputum samples were obtained for 100. Pulmonary tuberculosis was diagnosed in 33 (33%). Among the baseline characteristics, age had a skewed distribution (skewness = 0.8) with a median of 32 years and a range of 64 years. Sixty-four percent of patients were male, and 8% of patients had a positive serology for HIV. Thirty-four percent had had contact with somebody having pulmonary tuberculosis and 29% had a history of a previous episode of tuberculosis. Forty-eight percent of the patients had a history of hemoptysis. The most frequent radiological findings were the presence of apical infiltrates, interstitial infiltrates, and cavities. Clinical variables associated with culture positivity were weight loss, fever, miliary infiltrate, and pneumothorax (p < 0.01 for all of them).

Out of the 33 culture-positive patients, 20 (60.6%) were smear-positive. Ten presented growth in MGIT or modified Middlebrook 7H9 but not in Ogawa. The ‘reculturing’ in Ogawa was positive in all of them.

Evaluation of MGIT medium

We obtained mycobacterial growth in all 33 cases. The average growing time was significantly shorter in MGIT than in modified Middlebrook 7H9 and Ogawa media (Table 1). The average growing time for smear-positive specimens was significantly lower than for smear-negative ones (10.55 days vs. 13.6 days, p = 0.02). The sensitivity was 100% for this culture medium according to our definition.

Evaluation of modified Middlebrook 7H9 medium

We obtained mycobacterial growth in 24 cases. The average growing time was significantly shorter than the average for Ogawa (Table 1). The growing time for smear-positive specimens was also significantly shorter than for smear-negative ones (15 vs. 20.67 days; p = 0.047). The overall sensitivity was 72.73%, being 80% for smear-positive cases and 58.33% for smear-negative cases.

Evaluation of the Ogawa medium

We obtained mycobacterial growth in 23 cases. The growing time for smear-positive specimens was not significantly different than for smear-negative ones (24.25 vs. 25.24 days, p = 0.97). The overall sensitivity was 69.70%; sensitivity for smear-positive patients was 84.21%, while for smear-negative patients was 46.15%.

Table 1 Comparison of growing times among the three culture media

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Average growing time (days)</th>
<th>95% Confidence interval</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGIT</td>
<td>12.18</td>
<td>10.24—14.12</td>
<td>&lt;0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Modified Middlebrook 7H9</td>
<td>16.65</td>
<td>14.85—18.80</td>
<td>&lt;0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ogawa</td>
<td>25.74</td>
<td>22.22—29.60</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Against the other two culture media.<br><sup>b</sup> Against Ogawa.
Concordance

The value of kappa statistic for concordance was 0.76 between MGIT and Ogawa, 0.78 between MGIT and modified Middlebrook 7H9, and 0.75 between modified Middlebrook 7H9 and Ogawa. All the values were highly significant ($p < 0.01$). Tables 2 and 3 show the distribution of discordant results.

Polymerase chain reaction (PCR)

We used the PCR technique as already mentioned in samples that were positive in MGIT or modified Middlebrook 7H9 and negative in Ogawa, together with reculturing in Ogawa media. Out of the 10 that met these criteria, only two were negative for PCR. As these were positive in Ogawa reculturing, we classified them also as pulmonary tuberculosis.

Discussion

The development of new rapid, cheap, and accurate culture media for the diagnosis of tuberculosis constitutes a research priority for this disease. Among recently developed media, MGIT has shown promise results in several studies, but the main limitation for its use is still high cost. Searching for cheaper and technologically easier alternatives led us to evaluate, in addition to the MGIT, the association of a known liquid medium (Middlebrook 7H9) plus a colorimetric indicator of mycobacterial growth (tetrazolium bromide) in our setting.

In concordance with many other studies, we found the best sensitivity for the MGIT medium; however, there are also some papers suggesting that MGIT is only equivalent in performance to the conventional solid media. We think the reported differences are partly due to the definition of the reference standard, the prevalence of tuberculosis (higher in the studies finding MGIT equivalent to solid media), and the type of specimen (clinical versus laboratory ones). In our study, many of the cases diagnosed by MGIT but not by Ogawa, were paucibacillary in the smear or smear-negative. This may suggest that the additional benefit offered by MGIT in comparison to solid media can be mainly observed in cases with relatively low bacillary burden.

Modified Middlebrook 7H9 had a slightly better performance than Ogawa, though not significantly superior. But interestingly, when analyzing discordant pairs, we found five cases that presented growth in modified Middlebrook 7H9 but not in Ogawa and four cases growing in Ogawa but not in modified Middlebrook 7H9. This suggests that both methods can be complementary, and an appealing alternative would be the combination of both in a mixed biphasic culture medium. The evaluation of the performance of such a culture merits further research, given the low cost of both methods and the feasibility of its application in low resource and low technique-availability settings.

Middlebrook 7H9 is also the culture medium employed in studies evaluating the performance of microscopic-observation drug-susceptibility (MODS) assays. These have found a higher sensitivity of this medium in comparison to ours. Some methodological differences, such as the reading technique (direct observation versus inverted-light microscopic observation) and the addition of tetrazolium bromide can account for part of the observed difference, but most of the discrepancy is probably due to the fact that those studies did not use MGIT as a component of the reference standard, and so not only the sensitivity for Middlebrook 7H9 but also that of Lowenstein–Jensen was above 80%.

A striking finding was the relatively low sensitivity of the Ogawa method (around 70%), mostly due to our definition of the reference standard, consistent with other studies that also used MGIT as a reference standard or as a part of it. We emphasize that methods were used to avoid the false positive cultures by reculturing all the specimens that did not grow in Ogawa but did in MGIT or Middlebrook, and performing species identification and PCR in these.

As found by other researchers, the mean growing time was significantly shorter in MGIT medium compared with modified Middlebrook 7H9 and also shorter when compared to Ogawa. Of note, growing times depended on the bacillary burden (for MGIT smear-positive cases 10.55 days versus 13.60 for smear-negative specimens). But we must take into account the small sample size.

One of the aspects that merits consideration is the choice of Ogawa as the solid medium, while the most commonly used solid medium is Lowenstein–Jensen. Ogawa is considered equivalent or even slightly better, and cheaper. Another important feature is our definition of the reference standard. We chose a positive result in Ogawa or else a positive result in any of the liquid media but with a positive reculturing in Ogawa. We did this because we considered, according to the literature, that the current reference standard (solid medium) could be less sensitive than the new diagnostic tool (liquid medium), and indeed finally found that it was. Combinations of solid and liquid media cultures as a reference standard have already been used and we believe that in the near future the paradigm of the solid medium as the reference standard for diagnosis of tuberculosis will be modified as a result of scientific progress.

With respect to the possibility of extrapolating our results, it should be taken into account that we studied a population...
with a high prevalence of tuberculosis, hence the results of our study may not be valid in populations with different prevalences of tuberculosis or HIV infection, where local validation of these media is needed.

In conclusion, our findings show that the MGIT medium had the higher diagnostic yield as well as the lowest time required for growth of *M. tuberculosis* among the three evaluated media, while modified Middlebrook 7H9 had similar sensitivity but a significantly lower time required for growth than Ogawa. Given its affordability, with costs comparable to conventional solid media cultures, modified Middlebrook 7H9 could be considered as a sensitive alternative to these, meriting further validation in other settings.

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