Improved 18-Hour Methyl Red Test


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Standard methods for the methyl red (MR) test are not practical for routine use in clinical laboratories because of the necessarily prolonged incubation period. When read after overnight incubation, the usual MR test is often equivocal or falsely positive. The present study demonstrates the importance of standardizing the total volume of broth, the size of the vessel in which the cultures are incubated, and the density of the inoculum. In very small volumes of broth, cultures are better exposed to atmospheric oxygen, and thus MR-negative organisms tend to revert the initial acidic pH much more quickly than in deeper, large-volume broth cultures. In the proposed technique, a single colony was inoculated into a 0.5-ml amount of MR-Voges Proskauer (VP) broth (13- by 100-mm tube), and, after 18 to 24 hr at 37 C, one drop of MR was added. With this technique, the broth cultures produced either a definite red (positive) or yellow (negative) color, whereas various shades of orange were frequently observed when larger volumes of broth were tested after only 1 to 2 days of incubation. With 6.0-ml broth cultures, 18-hr MR tests were totally unreliable, but 18-hr tests in 0.5 ml of broth were comparable to the standard MR test performed after 5 days of incubation and superior to those performed after 48 hr in 6.0-ml broth cultures. With the proposed technique, the MR test can be incorporated readily into the routine scheme for identification of Enterobacteriaceae.

In a buffered peptone-glucose broth, the Enterobacteriaceae will initially produce acid by-products of glucose metabolism. Most genera will maintain a low pH as incubation continues, but some coliform bacilli can further metabolize these initial acidic products and the pH then tends to return toward neutrality. Clark and Lubs (3) first suggested that methyl red (MR) could be used as a pH indicator for distinguishing between those organisms which produce a low terminal pH (MR-positive) and those with higher terminal pH values (MR-negative). The MR test has become a standard biochemical procedure used in the characterization of the Enterobacteriaceae.

If the MR test is performed too early, the results are often equivocal or falsely positive since MR-negative organisms may not have had the opportunity to completely utilize the initial acidic products that first accumulate. Therefore, Clark and Lubs (3) recommended that MR tests should be carried out with broth cultures incubated for 5 days at 30 C. Since that time, several studies have been undertaken to standardize the methodology (4, 6, 7). Most investigators recommend that broth cultures should be held at least 3 to 5 days before MR tests are performed. Edwards and Ewing (5) suggest that with most clinical isolates satisfactory results can be obtained after 48 hr at 37 C, but when the results at 48 hr are questionable the tests should be repeated with 4 to 5 days of incubation. Because of the requirement for prolonged incubation, the MR test is not suitable for routine use in clinical laboratories, where time and accuracy are both of critical importance. Since the standard MR test is not dependable with 18- to 24-hr broth cultures, a reliable method for performing the MR test after overnight incubation is desirable.

Previous studies confirmed the observation that reliable Voges-Proskauer (VP) tests can be obtained after overnight incubation, if the total volume of MR-VP broth is reduced to 0.5 ml (1, 2). The present study explored the possibility that the closely related MR test could also be completed accurately, but more rapidly, with small-volume broth cultures. This report describes a reliable MR test which can be completed after 18 hr of incubation. Results with this method are comparable to those observed with standard 5-day
broth cultures and are clearly more accurate than results obtained with MR tests performed on 48-hr broth cultures.

MATERIALS AND METHODS

The organisms used in this study were recent clinical isolates, identified according to Edwards and Ewing (5). The MR solution was prepared by dissolving 0.5 g of MR in 300 ml of 95% ethanol and then adding 200 ml of distilled water. Six-milliliter volumes of MR-VP broth (Difco) were prepared in screw-cap tubes (16 by 125 mm), 0.5-ml volumes were transferred aseptically to tubes (13 by 100 mm), and 3.0-ml volumes were transferred to tubes (16 by 125 mm) just before use. Each tube of broth was inoculated with one colony picked from 18- to 24-hr growth on MacConkey agar (MAC) or blood-agar (Tryptase Soy Agar, Bio-Quest, with 5% defibrinated sheep blood). After the indicated period of incubation at 37 C, one drop of MR was added to the 0.5-ml culture tubes, three drops were added to the 3.0-ml culture, and six drops were added to the 6.0-ml broth cultures. Tests were recorded as positive if a red color developed immediately, negative if yellow, and equivocal if an orange to light brown color appeared.

As a standard reference for evaluating results with the rapid tests, the method described by Edwards and Ewing (5) was performed with each culture. That is, 6-ml broth cultures were tested after 48 hr at 37 C, and if the results were equivocal or if *Klebsiella* or Enterobacter was MR-positive, the tests were repeated with 6-ml broth cultures incubated for 5 days at 37 C. A few strains were tested after incubation at room temperature as well as 37 C.

To investigate some of the variables which could influence this test, the pH of growing broth cultures was measured with a model 10 pH meter (Corning Glass Works, Corning, N.Y.) attached to a continuous recording apparatus (VOM-5, Bausch & Lomb Inc., Rochester, N.Y.) with a semimicro combination electrode (Corning Glass Works).

RESULTS

To investigate the effect of reducing the total volume of MR-VP broth, three strains were selected: an MR-positive *Escherichia coli* and two strains of *Klebsiella* (one MR-negative after 72 hr and one which consistently gave equivocal results after 72 hr in 6.0 ml of broth). All three strains were grown in 0.5 ml and in 6.0 ml of MR-VP broth, and the pH was continually monitored during incubation at 37 C (Fig. 1). As expected, both strains of *Klebsiella* first produced an acidic environment, but as incubation continued the pH began to rise. Methyl red develops a bright red color at a pH below 5.0, yellow at a pH above 5.8, and various shades of orange between pH 5.0 and 5.8. In 6.0-ml broth cultures, the MR-negative *Klebsiella* strains would have been MR-positive or equivocal if tested after 18 to 24 hr, but after 48 to 72 hr one would have been MR-negative and the other equivocal (Fig. 1). When the total volume of broth was reduced to 0.5 ml, the two *Klebsiella* strains initially deminished the pH very rapidly and reverted toward alkalinity much more quickly so that both strains would have been MR-negative after only 12 hr of incubation. When similar studies were carried out with 3.0-ml MR-VP broth cultures, the pH tended to revert somewhat more rapidly than with 6.0-ml broth cultures, but the differences were not remarkable.

To evaluate further the effect of altering the volume of broth, period of incubation, and presence of fermentable carbohydrates in the medium from which the inoculum was obtained, tests were performed with 50 *E. coli*, 50 *Klebsiella* sp., 34 Enterobacter cloacae, and 16 *E. aerogenes*, by using the eight methods outlined in Table 1. All 50 *E. coli* and three of the *Klebsiella* sp. were clearly MR positive with all eight methods, and the three *Klebsiella* sp. were also positive in 5-day broth cultures. False-positive and equivocal results were observed with the remaining 97 MR-negative isolates. The source of inoculum did not appear to influence the results greatly, but in larger volumes of broth there were fewer equivocal results when lactose-fermenting colonies were selected from MAC than when the inoculum was obtained from an agar medium free from carbohydrates (BAP). As anticipated, MR tests with 6.0-ml broth cultures were occasionally unsatisfactory after 48 hr of incubation, and after 18 hr the test was quite unreliable. The number of equivocal results with 18-hr cultures was markedly reduced when smaller volumes of broth were used, and false-positive tests were eliminated by using 0.5-ml broth cultures.

The incubation temperature strongly influences the MR reaction obtained with *E. hafnia* (Hafnia
sp.) and with *E. liquefaciens*. Two strains of each of these two species were also tested with the methods described above. Each method was performed in duplicate, one set incubated at 37 C and the other at 25 C. Results with the 0.5-ml, 18-hr method were identical to those obtained with 6.0-ml, 5-day broth cultures. Tests with the other 18-hr, 3.0- or 6.0-ml broth cultures were often equivocal and therefore less reliable. Two other *Enterobacter* sp. were also tested in this way since they had been tentatively identified as *E. hafnia* because 48-hr, 6.0-ml broth cultures were clearly MR-positive at 37 C and negative at 25 C. These two isolates were VP-positive at 37 and at 25 C. When retested with the 0.5-ml, 18-hr MR test and after 5 days in the larger volumes, both strains were MR-negative at 37 and at 25 C. These two cultures would have been misidentified if 48-hr broth cultures were used routinely and if 4- to 5-day cultures were tested only when equivocal results were obtained at 48 hr. The routine use of 0.5-ml, 18-hr broth cultures or 6.0-ml, 4- to 5-day cultures would have avoided this complication.

MR tests with the 18-hr, 0.5-ml broth cultures were evaluated further in comparison with 48-hr, 6.0-ml broth cultures. A total of 425 coliform bacilli (153 *E. coli*, 190 *Klebsiella* sp., 52 *E. cloacae*, 26 *E. aerogenes*, 2 *E. liquefaciens*, and 2 *E. hafnia*) were tested by both methods with single colonies selected from overnight cultures on MAC as inocula. Table 2 presents the results of these 18-hr tests compared to 48-hr, 6.0-ml cultures which were repeated after 5 days when the results were equivocal or when *Klebsiella* or *Enterobacter* were positive. Only one “false-positive” test was observed with the 18-hr method. An *E. coli*, which was MR-positive in 0.5-ml broth, was equivocal when tested after 48 hr in larger volumes and MR-negative after 5 days of incubation. The 18-hr MR test was equivocal with only two *E. cloacae*. In contrast, 48-hr broth cultures yielded 7 false positive tests (4 *Klebsiella* and 3 *E. aerogenes*) and 12 equivocal tests (7 *Klebsiella*, 2 *E. aerogenes*, 2 *E. cloacae*, and 1 *E. coli*).

### Table 1. Effect of broth volume, incubation time, and source of inoculum on the methyl red test

<table>
<thead>
<tr>
<th>Volume of broth (ml)</th>
<th>Incubation time (hr)</th>
<th>Source inocula*</th>
<th>MR test results$^b$</th>
<th>Positive</th>
<th>Equivocal</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>18</td>
<td>Mac</td>
<td>53 (0)*</td>
<td>2</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BAP</td>
<td>53 (0)</td>
<td>2</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>18</td>
<td>Mac</td>
<td>56 (3)</td>
<td>6</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BAP</td>
<td>57 (4)</td>
<td>5</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>18</td>
<td>Mac</td>
<td>65 (12)</td>
<td>25</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BAP</td>
<td>66 (13)</td>
<td>34</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>48</td>
<td>Mac</td>
<td>56 (3)</td>
<td>4</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BAP</td>
<td>54 (1)</td>
<td>10</td>
<td>86</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Inoculum: one colony from an 18- to 24-hr culture on MacConkey agar (Mac) or Trypticase soy agar with 5% sheep blood (BAP).

$^b$ Number of tests positive, equivocal, or negative with each method, by using 53 MR-positive isolates (50 *E. coli* and 3 *Klebsiella* sp.) and 97 MR-negative isolates (47 *Klebsiella* sp., 34 *E. cloacae*, and 16 *E. aerogenes*).

$^c$ Numbers in parentheses represent the number of false-positive tests with strains which were MR-negative after 2 to 5 days by using the procedure of Edwards and Ewing (5).

### Table 2. Evaluation of two methyl red (MR) test methods with 425 coliform bacilli

<table>
<thead>
<tr>
<th>Method*</th>
<th>Result</th>
<th>Standard MR test results$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total tests (425)</td>
<td><em>Klebsiella</em> (190)</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>0.5 ml, 18-hr, broth</td>
<td>Positive</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td>Equivocal</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>6.0 ml, 48-hr, broth</td>
<td>Positive</td>
<td>161</td>
</tr>
<tr>
<td></td>
<td>Equivocal</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ All tests inoculated with one colony from an 18- to 24-hr MacConkey agar culture and incubated at 37 C.

$^b$ Procedure of Edwards and Ewing (5), number of strains positive or negative after 48 hr or after 5 days if 2-day tests are equivocal or if positive with *Klebsiella* or *Enterobacter*.

$^c$ Include 52 *E. cloacae*, 26 *E. aerogenes*, 2 *E. liquefaciens*, and 2 *E. hafnia*.
These tests were all inoculated with one colony from an 18- to 24-hr agar plate culture. The following experiment was undertaken to determine the effect of altering the inoculum size. Viable cell counts indicated that one colony suspended in 0.5 ml of broth would yield about $6 \times 10^4$ viable cells per ml, but in 12 times that volume (6.0 ml) the inoculum would be about $5 \times 10^4$ viable cells per ml. Six-milliliter MR-VP broth tubes were seeded with four different inocula, and the pH was measured at hourly intervals for 13 hr and then daily for 4 days. For this study, the *Klebsiella* sp. which gave equivocal results in Fig. 1 was grown in Trypticase Soy Broth without dextrose (BioQuest) and then 10-fold dilutions were prepared in MR-VP broth. Identical results were obtained when this organism was grown in Trypticase Soy Broth (BioQuest) with dextrose and then diluted to give an inoculum of approximately $10^5$ viable cells per ml. Each logarithmic decrease in inoculum size resulted in a significant increase in the lag time before the pH began to drop. Tubes inoculated with approximately $10^5$ to $10^6$ viable cells per ml were much slower in this respect than those receiving the heavier inoculum that would be expected in 0.5-ml broth cultures ($10^7$ to $10^8$ viable cells per ml). Even after 4 days of incubation, those tubes receiving light inocula still demonstrated pH values that would give equivocal MR tests, but those with the heavier inoculum were clearly MR-negative.

Another explanation for the superior results observed in small volumes of broth is the more ready access of atmospheric oxygen to the depths of the medium. Six-milliliter volumes of inoculated broth were distributed into different-sized vessels so that the depth of the medium could be varied without altering the total volume or density of inoculum. Figure 2 presents the results of hourly pH measurements obtained with the *Klebsiella* studied in the previous experiment but this time diluted to give about $10^4$ viable cells per ml. As the depth of the medium was reduced, the pH tended to revert much more quickly, and 6.0 ml of broth incubated in a 50-ml Erlenmeyer flask (ca. 5 mm deep) gave results nearly identical to those observed with 0.5 ml of broth in a tube (15 by 100 mm, ca. 7 mm in depth). After 24 hr of incubation, the culture in a tube (16 by 125 mm) would have given an equivocal MR test (pH 5.3), but the other shallower broth cultures would have been MR negative (pH 6.0). The size of the container in which the broth culture is incubated is certainly another important variable that needs to be considered in standardizing the MR test.

**DISCUSSION**

The MR test is well-established as a useful procedure for characterizing the coliform bacilli. Procedural details have been carefully studied by others (4, 6, 7), and standardized methods have been proposed. In the past, little attention has been paid to the density of inoculum, the total volume of broth, and the size of vessel in which the broth culture is incubated. The present study indicates that all three of these factors are important variables which must be standardized to obtain optimal, reproducible results. Although all of the techniques now recommended call for 2 to 5 days of incubation, many laboratory workers tend to test the broth cultures after 24 to 48 hr. The present study confirms the fact that premature performance of the MR test will result in a few false-positive tests and a number of equivocal results which will be interpreted differently by different people.

The simple modification of the MR test procedure described in this report not only permits a shortened incubation time but also improves the qualitative distinction between positive and negative reactions. When MR is added to 18-hr, 0.5-ml broth cultures, MR-negative organisms almost always give a definite yellow color and MR-positive strains give a bright red color. In larger volumes of broth, the same organisms often display an orange color reaction somewhere in the spectrum between red and yellow. The interpretation of tests producing such subtle differences in color is rather subjective and less reproducible.
The differences between MR tests inoculated with growth from the two types of agar media used in this study primarily reflected this subjectivity. The source of inoculum had less effect on the MR reaction when the incubation time was increased or when the volume of broth was reduced.

In shallow broth cultures, there is greater access to atmospheric oxygen and thus MR-negative organisms are able to continue catabolism of the acidic intermediate products derived from glucose. As a consequence, the pH reverts much more rapidly in shallow cultures than in deep cultures in which anaerobic metabolism leads to the accumulation of more acidic products. A sufficiently shallow broth culture can be accomplished either by using a large flask or by simply reducing the total volume of broth in a convenient sized tube.

A fairly dense inoculum is necessary for optimal results. One or two colonies suspended in 0.5 ml of broth is optimal, but, with 6.0-ml volumes of broth, 12 to 24 colonies would be needed to obtain the same density of inoculum. Of course, extremely turbid inocula should be avoided since bacterial growth cannot proceed if the inoculum exceeds the maximum cell concentration of about 10^9 viable cells per ml. With each logarithmic decrease in the inoculum size, there is a significant increase in the time required to accumulate enough acidic products to overcome the buffering system in the MR-VP broth. Even after 4 days of incubation, lightly inoculated 6.0-ml broth cultures may fail to detect all MR-negative organisms.

In this study, a small inoculating loop was used for picking isolated colonies from the agar media. Satisfactory results can be obtained if only the center portion of a good-sized colony is transferred to 0.5 ml of MR-VP broth. When dealing with plates inoculated with clinical specimens, there is always the possibility of obtaining mixed cultures by this method, especially when colonies are selected from an inhibitory medium such as MAC. To avoid this problem, colonies are often first subcultured into a small volume of broth (free from fermentable carbohydrates) by touching the top of the colony with an inoculating needle. Then after a few hours of incubation, various test media are inoculated with a drop or loopful of the actively growing broth culture. In our experience, the latter method does not provide a sufficiently dense inoculum for the MR test, and equivocal results are often obtained even with 0.5-ml volumes. The results are much more satisfactory if the MR-VP broth is inoculated with several drops of a turbid suspension or broth subculture or with at least one-half of a colony selected from the original plates or from growth on an agar slant. Our data (Table 1) support the conclusion that the inoculum can be obtained from a medium which contains fermentable carbohydrates since acidic metabolites, which may be present in the inoculum, do not appear to significantly influence the pH of the MR-VP broth.

For the sake of convenience, the MR test is commonly performed at 37 C although 27 to 30 C is often recommended (6, 7). Our results with large-volume broth cultures may have been improved if lower temperatures were used. However, the simple maneuver of reducing the total volume of broth apparently obviates the need for manipulating incubation temperatures. With the clinical isolates we examined, overnight incubation at 37 C appears to be the most practical method of obtaining reliable MR tests. To help identify E. hafnia or E. liquefaciens, MR and VP tests should be performed at 37 and at 25 C, with 0.5-ml volumes of MR-VP broth.

The nature of the work in clinical laboratories does not permit the routine use of tests which require prolonged incubation; thus rapid short cuts are often attempted. For this reason, the MR test is often performed after 18 to 24 hr or, at best, after 48 hr of incubation at 35 to 37 C. The present study demonstrates the difficulties encountered by using this approach (false-positive and equivocal results). However, when 0.5-ml MR-VP broth cultures are used, 18- to 24-hr MR tests are much more reliable. Therefore, this simple modification is ideally suited for routine use in clinical laboratories.

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LITERATURE CITED