Use of Bile-Esculin Agar for Rapid Differentiation of Enterobacteriaceae

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Bile-esculin agar has been used for several years for the presumptive identification of group D streptococci. All members of the Enterobacteriaceae family will also grow on this medium, but only certain ones can hydrolyze esculin to 6,7-dihydroxy-7-methylcoumarin, which reacts with iron to produce a characteristic blackening of the medium. One thousand and six cultures from clinical specimens representing 20 genera were isolated and identified. Heavy inocula from fresh pure culture isolates on heart infusion agar were placed on bile-esculin agar slants and incubated at 35 C. The slants were examined at 4 h and again at 18 h for esculin hydrolysis. Shigella, Salmonella, Arizona, Proteus mirabilis, Proteus morganii, Providencia alcalifaciens, and Providencia stuartii all produced negative results. Klebsiella pneumoniae, Enterobacter aerogenes, Serratia marcescens, and Serratia rubidaea produced a positive reaction in 4 h. The other remaining eight genera exhibited varying results. The use of this medium in conjunction with triple sugar iron-lysine iron agar has been of great value in differentiating the Klebsiella-Enterobacter-Serratia group from other Enterobacteriaceae.

Facklam et al. (2-4) have published numerous articles in recent years proclaiming the usefulness of bile-esculin medium in differentiating the group D streptococci.

Weatherall and Dible (10) stated that esculin medium per se was first introduced into bacteriological work in 1909 by Harrison and vander Lack, who used the method for recognizing fecal types of Bacillus coli. In 1924, Rochaix (7) applied the test to the differentiation of enterococci from other streptococci. Utilizing the esculin base, Meyer and Schönfeld, 1926 (6), then added bile. In their study they found that 61 of 62 enterococci were able to grow and split esculin, whereas other streptococci could not.

Swan (8) in his study used the esculin medium containing 40% bile salt. He reported that a positive reaction on bile-esculin medium correlated with a serological group D precipitin reaction.

Organisms hydrolyze esculin to 6,7-dihydroxy-7-methylcoumarin. Coumarin then unites with iron present in the medium to form a black compound, which is indicative of a positive test.

A preliminary study published by Wasi lauskas (9) showed that some of the gram-negative bacilli would also grow on bile-esculin medium, but only certain of these would produce the characteristic blackening observed with enterococci. Since members of the Klebsiella-Enterobacter-Serratia group were known to hydrolyze esculin (5), bile-esculin medium was evaluated for its usefulness in distinguishing members of the group from the other Enterobacteriaceae.

The purpose of this study was to expand upon and further investigate the findings of Wasi lauskas (9) and to determine what value this test has in clinical and hospital laboratories in differentiating members of the Enterobacteriaceae.

MATERIALS AND METHODS

Cultures. The majority of the isolates used in this study were obtained directly from clinical specimens or were submitted to our laboratory as subcultures from hospital laboratories for further identification. Seventy-five cultures were kindly supplied from the stock culture collections of the Center for Disease Control, Atlanta, Ga., and the Maine State Department of Health Laboratory.

Medium. The bile-esculin agar (Difco) was prepared according to the manufacturer's instructions, except that horse serum was not added to the sterilized medium (2). Slants were prepared by dispensing 7-ml volumes in screw-cap tubes (16 by 125 mm). A heavy inoculum (4-mm loop) from pure culture isolates grown for 18 h at 35 C on heart infusion agar (Difco) slants was inoculated on bile-esculin agar slants and incubated at 35 C for 18 h. The slants were examined for characteristic blackening at 4 h and again at 18 h.
A positive reaction on bile-esculin was recorded when blackening could be seen at the site of heavy inoculum in 4 h or 18 h. Negative reactions were recorded when no blackening occurred within 18 h.

**Biochemical identification.** Isolates were identified by their reactions on triple sugar iron agar, lysine iron agar, urea agar. Andrades base sugars (dextrose, sucrose, lactose, salicin, adonitol, dulcitol, rhamnose, raffinose, and inositol), phenylalanine deaminase, lysine and ornithine decarboxylase, arginine dehydrodase, malonate, Simmon’s citrate, indol, methyl red, Voges-Proskauer, and oxidase according to the scheme of Edwards and Ewing (1). The preceding differential media (Difco) were prepared according to the manufacturer's instructions.

**RESULTS**

Table 1 shows the results of 1,069 cultures on bile-esculin agar representing 20 species of the family Enterobacteriaceae. It should be noted that five species (Klebsiella pneumoniae, Enterobacter aerogenes, Enterobacter cloacae, Serratia liquefaciens, Serratia marcescens, and Serratia rubidaea) produced 100% positive results in 4 h, whereas seven species (Shigella sonnei, Salmonella sp. Arizona hinshawii, Proteus mirabilis, Proteus morganii, Providencia alcalifaciens, and Providencia stuartii) produced 100% negative results. Escherichia coli, Citrobacter freundii, Citrobacter diversus, Enterobacter hafniae, and Proteus vulgaris did not exhibit blackening in 18 h in at least 70% of the cultures tested. Enterobacter cloacae, Enterobacter agglomerans, and Proteus rettgeri were the only organisms which yielded equivocal results.

The reactions of esculin have been well documented by Edwards and Ewing (1). No discrepancies were observed between the bile-esculin medium results in our laboratory and that of esculin hydrolysis obtained in other laboratories.

Wasilauskas (9) suggested that the time required for an isolate to give a positive reaction was directly proportional to the size of inoculum; therefore, it was decided that a 4-mm loopful of 18-h growth from a slant constituted a constant “heavy” inoculum. It should be emphasized that the heavy inoculum need not be taken only from a heart infusion agar slant. The 18-h growth on triple sugar iron agar or lysine iron agar may also be used. An ancillary study was performed on 65 cultures to determine if the age of the culture might alter the reactions on bile-esculin. Bile-esculin slants were inoculated from the same stock culture slant every day for 7 days. No difference in reactions was observed. Practical use of bile-esculin, however, would dictate that fresh slants (18 h) should always be used.

### Table 1. Bile-esculin agar reactions of Enterobacteriaceae

<table>
<thead>
<tr>
<th>Enterobacteriaceae</th>
<th>No. tested</th>
<th>4 h</th>
<th>18 h</th>
<th>4 h</th>
<th>18 h</th>
<th>4 h</th>
<th>18 h</th>
<th>4 h</th>
<th>18 h</th>
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<td><strong>Positive</strong></td>
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<td>23</td>
<td>101</td>
<td>2</td>
<td>18</td>
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<td>1</td>
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<td>3</td>
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<td>80</td>
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<tr>
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<td>6</td>
<td>1</td>
<td>18</td>
<td>24</td>
<td>4</td>
<td>72</td>
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<td>44</td>
<td>20</td>
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<td>15</td>
<td>46</td>
<td>22</td>
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<tr>
<td>Proteus rettgeri</td>
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<td>2</td>
<td>4</td>
<td>33</td>
<td>22</td>
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<td>Proteus morganii</td>
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<td>31</td>
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</tr>
<tr>
<td>Providencia alcalifaciens</td>
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<td>0</td>
<td>14</td>
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<td>0</td>
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<td></td>
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</tr>
<tr>
<td>Providencia stuartii</td>
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<td>0</td>
<td>10</td>
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<td>0</td>
<td>100</td>
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<td><strong>Klebsiella pneumoniae</strong></td>
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<td>149</td>
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<td>Enterobacter aerogenes</td>
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<td>39</td>
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<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td></td>
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<td>Serratia liquefaciens</td>
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<td>0</td>
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<tr>
<td>Serratia marcescens</td>
<td>55</td>
<td>55</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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</table>
DISCUSSION

A comparison of bile-esculin agar made according to manufacturer's instruction (with horse serum) and a modified bile-esculin agar which contained no horse serum was made by Facklam et al. in 1973. They found that both media yielded similar results. The omission of horse serum decreased the cost and simplified the preparation of the medium without sacrificing value throughout the study.

The most accurate method for identifying members of the family Enterobacteriaceae utilizes extensive and time-consuming procedures that have been established for the differentiation of these organisms (1). However, the earlier a physician can be made aware of the bacterial etiology of a disease and of the identity of the organism(s), the earlier he can initiate specific chemotherapy. Therefore, there have been continuing investigations of test procedures to obtain presumptive results rapidly and accurately.

Presumptive identification of certain members of the family Enterobacteriaceae can be made from reactions observed on primary differential media, namely, triple sugar iron agar, lysine iron agar, and Christiansen's urea. With the addition of bile-esculin agar, inoculated at the same time as the urea, many organisms can be ruled in or out by the result obtained in 4 h (the same time urease activity is determined).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Bile-esculin agar reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSI LIA</td>
<td>Negative</td>
</tr>
<tr>
<td>K/A K/A</td>
<td>Shigella sonnei</td>
</tr>
<tr>
<td>Urease</td>
<td>Escherichia coli (80%)*</td>
</tr>
<tr>
<td>H,S</td>
<td></td>
</tr>
<tr>
<td>K/A K/A</td>
<td>Citrobacter freundii (94%)</td>
</tr>
<tr>
<td>Urease</td>
<td>Citrobacter diversus (80%)</td>
</tr>
<tr>
<td>H,S a</td>
<td></td>
</tr>
<tr>
<td>K/A R/A</td>
<td>Proteus vulgaris (83%)</td>
</tr>
<tr>
<td>Urease</td>
<td>Proteus mirabilis</td>
</tr>
<tr>
<td>H,S</td>
<td></td>
</tr>
<tr>
<td>K/A (K)</td>
<td>Proteus morganii</td>
</tr>
<tr>
<td>Urease</td>
<td>Proteus rettgeri (44%)</td>
</tr>
<tr>
<td>H,S a</td>
<td></td>
</tr>
<tr>
<td>K/A R/A</td>
<td>Providencia stuartii</td>
</tr>
<tr>
<td>Urease</td>
<td>Providencia alcalificiens</td>
</tr>
<tr>
<td>H,S</td>
<td></td>
</tr>
<tr>
<td>A/A K/A</td>
<td>Enterobacter cloacae (20%)</td>
</tr>
<tr>
<td>Urease</td>
<td>Enterobacter agglomerans</td>
</tr>
<tr>
<td>H,S</td>
<td>(32%)</td>
</tr>
<tr>
<td>(A) K/A</td>
<td>Enterobacter hafniae (72%)</td>
</tr>
<tr>
<td>Urease</td>
<td>E. hafniae (24%)</td>
</tr>
<tr>
<td>H,S</td>
<td>Enterobacter aerogenes</td>
</tr>
<tr>
<td>K/N(K)</td>
<td>Serratia marcescens</td>
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<tr>
<td>H,S</td>
<td>Serratia rubidaea</td>
</tr>
<tr>
<td>K/A K/N(K)</td>
<td>Salmonella Arizona</td>
</tr>
</tbody>
</table>

* TSI, Triple sugar iron agar; LIA, lysine iron agar; K, alkaline; A, acid; d, + or -; R, red (deamination); N, neutral.

* See discussion.
Table 2 illustrates a schematic system for the differentiation of members of the family Enterobacteriaceae.

*E. coli* was placed in the first group of reactions only because it is closely associated with *Shigella*. It was not meant to limit the reactions of this organism to only those *E. coli* which do not ferment lactose or decarboxylate lysine. This study was made on all completely identified organisms and not on the individual differences of those organisms.

To what extent a system of differentiation may be implemented at the level of clinical bacteriological practice depends on the methodology required for identification of the various members of the microbial family. It should be emphasized that the proposed schema is not meant to be a means for complete identification but merely a directional signal. Application of this four-tube system permits the laboratorian to be very selective in determining additional biochemicals necessary to complete an identification. Our system permits a great number of possible organisms to be eliminated. Occasionally aberrant forms are observed which do not fit into the schema, but they are easily identifiable by additional tests. This system has been used in our laboratory for identifying a large majority of cultures of *Enterobacteriaceae* isolated from clinical specimens such as urine, wounds, abscesses, etc. for the past 2 years.

The methodology described provides a realistic approach to the handling of *Enterobacteriaceae* cultures with a savings in time and laboratory resources, without sacrificing accuracy.

**LITERATURE CITED**


