Rapid Enumeration and Identification of Stressed Fecal Coliforms

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ABSTRACT

A rapid (72 h) method for enumeration and subsequent identification of fecal coliforms is described. The procedure consists of plating a food suspension in an agar-repair medium (trypticase soy agar pour plates preincubated at 35°C for 2 h, then overlaid with violet red bile agar) incubated at 45.5°C. Fecal coliforms can be enumerated within 24 h and then confirmed as *Escherichia coli* within 48 h by subculturing typical colonies to an IMViC agar plate. Feasibility and applicability of the procedure were demonstrated by comparing the efficiency of recovery of both stressed and non-stressed cells on the agar repair medium with conventional agar and liquid most-probable-number media.

The inability of bacteria exposed to sublethal stresses to grow in selective media used for their enumeration has been reported (1.4-9,11). Various methods have been proposed, in recent years, to recover these injured (stressed) bacteria in both liquid and solid media (1.4-9,11). While these methods may have improved the sensitivity of detection, they generally did not reduce the time of analysis.

We conceived a rapid (72 h) test scheme for isolation and identification of *Escherichia coli* which was inspired by two recent developments in methods for recovery and identification of coliforms. The first development was an agar-repair method for enumeration of "total" coliforms at 35°C (6). By using this same method at 45.5°C, we proposed to selectively isolate and enumerate injured "fecal" coliforms within 24 h. The resulting colonies could then be confirmed as *E. coli* within 48 h on a newly devised IMViC agar plate (7). Completion of the test in only 72 h will reduce by as much as 7 days, the time required to identify *E. coli* by the conventional most-probable-number method (7). Methods previously proposed for enumeration of fecal coliforms at "elevated temperatures" (44.5°C) made no provision for recovery of injured cells (2,10) and they underestimated cell densities by as much as 20% (10).

Development of rapid analytical methods is a major need in research supported by the military because the delay of food shipments and storage of food pending analytical results is costly and causes numerous administrative and operational problems. The purpose of this study was to demonstrate the feasibility of the proposed rapid (72 h) test scheme and to compare the agar-repair medium (6) with conventional media commonly used for coliforms and *E. coli* in particular.

MATERIALS AND METHODS

**Media**

All media were purchased from Difco Laboratories, Detroit, MI. Media were autoclaved at 121°C for 15 min except violet red bile agar (VRBA) which was steamed at 100°C. The agar repair medium (VRBA/TSA) consisted of 10-12 ml of trypticase soy agar (TSA) overlaid, after an appropriate preincubation period, with an equal volume of VRBA as described by Speck and Ray (6). The IMViC agar plate was prepared as cited by Powers and Latt (3). IMViC agar media prepared in advance were distributed into test tubes in the proper volume and refrigerated no longer than 30 days. All dilutions were made in Butterfield's phosphate buffer (7).

**Unstressed and stressed cells**

Unstressed cells were grown in 5 ml of tryptic soy broth (TSB) and enumerated after incubation at 35°C for 24 h. Stressed cells were from the remainder (4 ml) of the same cultures which were immediately frozen at −40°C for 24 h, thawed by placing the tubes in water at 3°C and subsequently enumerated in the same manner as the unstressed cells.

**Differentiation of coliforms on VRBA at 45.5°C**

Fifty-six coliform cultures were streaked on the surface of VRBA with a 3-mm bacteriological loop in a manner to obtain isolated colonies. Plates were then overlaid with 4-5 ml of tempered VRBA and incubated in an air incubator at 45.5°C for 48 h. Duplicate plates were also incubated at 35°C to serve as growth controls. Plates were examined for presence or absence of growth, color and size of colonies, and presence or absence of a halo around colonies. The coliform cultures tested were the following: 26 *E. coli* strains including three enteropathogenic *E. coli*; 12 *Enterobacter* strains including four *Escherichia aerogenes*, five *E. cloacae* and three *E. agglomerans*; four *Citrobacter* strains including three *C. freundii* and one *C. diversus*; 14 *Klebsiella* strains, including 11 *K. pneumoniae*, two *K. ozaenae*, one *K. rhinoscleromatis*. All cultures streaked on VRBA were grown in trypticase soy broth at 35°C for 24 h.

**Rapid (24 h) enumeration of stressed fecal coliforms**

One ml of an appropriate cell suspension was pipetted into 100 x 15 mm petri plates, in triplicate, and poured with 10-12 ml of TSA. Following a preincubation period of 2 h at 35°C, the TSA was overlaid with 10-12 ml of VRBA as recommended for total coliforms (6). Selective enumeration of fecal coliforms was accomplished by reincubating the agar-repair plates (VRBA/TSA) directly at 45.5°C (air incubator) for 24 h. Typical colonies (red, greater than 0.5 mm and surrounded by a halo) were counted as fecal coliforms and were transferred to IMViC agar plates (3) to be identified as *E. coli*.

**Forty-eight-hour IMViC agar plate test**

Typical colonies from the agar-repair medium incubated at 45.5°C were transferred to IMViC agar plates as previously described (3). IMViC agar plates were incubated at 35°C for 48 h. Following addition of appropriate reagents *E. coli* was identified by the typical IMViC
Comparative recovery of E. coli on VRBA and the agar-repair medium (VRBA/TSA)

One ml of six replicate 24 h, TSB cultures of E. coli ATCC 11775, diluted to obtain between 100 and 300 colonies, was plated into six sets of triplicate petri plates. For each culture, two sets of plates were poured with 10-12 ml of VRBA and four sets were poured with 10-12 ml of TSA. As soon as the agar solidified, all VRBA plates were overlaid with an equal volume of VRBA. Then one set of VRBA plates and one set of TSA plates (not overlaid) were incubated directly at 35 C and 45.5 C. Two remaining sets of TSA plates (repair plates) were preincubated at 35 C for 2 h, then were overlaid with 10-12 ml of VRBA (6) and reincubated at 35 C and 45.5 C. At both temperatures TSA (not overlaid) served as growth control upon which colony counts were taken as 100% recovery. Incubation at both 35 C and 45.5 C was accomplished in carefully controlled air incubators. The procedure was repeated with freeze stressed cells from the same cultures.

Comparison of the agar-repair medium at 45.5 C with the most-probable-number procedure

Recovery of cells from 24 E. coli strains on the agar-repair medium at 45.5 C (air incubator) as described above, was compared with recovery by the conventional 3-tube most-probable-number (MPN) estimate (7). Both media were inoculated in parallel with unstressed and freeze-stressed cells grown in TSB at 35 C for 24 h. In the MPN procedure, 1 ml of appropriately diluted cultures was first inoculated into three lauryl sulfate tryptose (LST) broth tubes per dilution and incubated at 35 C for 48 h. Dilutions were calculated to yield positive and negative end points. Gassing LST tubes were subcultured to EC medium with a bacteriological loop (3 mm) and incubated at 45.5 C for 48 h in a water bath. Percent recovery on the agar-repair medium was relative to the MPN estimate in EC medium taken as 100% recovery.

RESULTS

Table 1 shows the growth of 56 coliform cultures on VRBA at 45.5 C (air incubator). Of the 26 E. coli cultures tested, 22 grew and produced typical colonies (red, greater than 0.5 mm in diameter and surrounded by a "halo" of precipitated bile). None of the Enterobacter or Citrobacter cultures grew and only five of the 14 Klebsiella cultures grew. All five were strains of K. pneumoniae which produced red colonies, but only three strains produced colonies surrounded by the characteristic "halo" and only one strain produced colonies larger than 0.5 mm. The other four strains produced pin-point colonies which would not be counted as coliforms on VRBA. These results show that E. coli will grow on VRBA at 45.5 C while the other coliforms either do not grow or produce small atypical colonies, easily differentiated from E. coli.

Table 2 shows the percent recovery, after incubation for only 24 h, of unstressed and freeze stressed cells of E. coli ATCC 11775 on VRBA, TSA overlaid with VRBA (agar-repair medium) and TSA not overlaid. The percent recoveries shown are the average of six replicate cultures and are relative to the counts on TSA taken as 100% recovery. While recovery of unstressed cells on VRBA was 78 and 84% at 35 and 45.5 C, respectively, recovery of the same cells stressed by freezing at 40 C was only 4.3 and 10.4% at these respective temperatures. However, recovery of both unstressed and stressed cells was greater than 100% on the agar-repair medium at 35 and 45.5 C. Growth on the agar-repair medium was typical of E. coli except that colonies were not as large as colonies on VRBA and halos surrounding colonies were not as strong, although they were clearly discernable.

Incubation for 48 h did not alter the count or the appearance of colonies on the agar-repair medium (data not shown) and is not recommended because it dries the agar to the point of splitting. There was no discernible evidence of the agar drying after incubation for only 24 h at 45.5 C.

These data indicate that the agar-repair medium (VRBA/TSA) incubated as described, will effectively recover injured fecal coliforms at 45.5 C within 24 h. While E. coli did grow on VRBA alone, at 45.5 C, recovery was poor, especially when cells were stressed.

Tables 3 and 4 compare the growth and average recovery at 45.5 C of 24 strains of E. coli on the agar-repair medium and in conventional MPN media (LST broth incubated at 35 C followed by subculturing to EC broth incubated at 45.5 C in a water bath) tested in parallel. Table 3 shows that the average count (×107) of

TABLE 1. Differentiation of coliforms on violet red bile agar (VRBA) at 45.5 C:

<table>
<thead>
<tr>
<th>Coliform organism</th>
<th>No. of cultures</th>
<th>Positive growth</th>
<th>Halo ( bile reaction)</th>
<th>Red or pink colony</th>
<th>Colony size &gt; 0.5 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>26</td>
<td>22/26 (85%)</td>
<td>21/22 (95%)</td>
<td>22/22 (100%)</td>
<td>22/22 (100%)</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>12</td>
<td>0/12 (0%)</td>
<td>0/12 (0%)</td>
<td>0/12 (0%)</td>
<td>0/12 (0%)</td>
</tr>
<tr>
<td>Citrobacter</td>
<td>4</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
<td>0/4 (0%)</td>
</tr>
<tr>
<td>Klebsiella</td>
<td>14</td>
<td>5/14 (36%)</td>
<td>3/5 (60%)</td>
<td>5/5 (100%)</td>
<td>1/5 (20%)</td>
</tr>
</tbody>
</table>

TABLE 2. Recovery of E. coli cells on agar media:

<table>
<thead>
<tr>
<th>Medium</th>
<th>Unstressed</th>
<th>Stresseda</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35 C</td>
<td>45.5 C</td>
</tr>
<tr>
<td>VRBA</td>
<td>78</td>
<td>84</td>
</tr>
<tr>
<td>VRBA/TSA</td>
<td>103</td>
<td>109</td>
</tr>
<tr>
<td>TSA</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

aAverage of 6 replicates cultures relative to counts on TSA taken as 100% Incubation time was 24 h.

EC, E. coli ATCC 11775.

bAgar-repair medium: cultures were poured in TSA, preincubated at 35 C for 2 h, overlaid with VRBA and reincubated for 24 h at the indicated temperatures.
TABLE 3. Comparison of an elevated temperature agar repair method with the 3-tube MPN procedure for recovery of unstressed E. coli.

<table>
<thead>
<tr>
<th>Results</th>
<th>Agar repair medium&lt;sup&gt;a&lt;/sup&gt; EC Test at 45.5 C, at 45.5 C</th>
<th>3-tube MPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average count per ml (x 10&lt;sup&gt;7&lt;/sup&gt;)</td>
<td>114</td>
<td>88</td>
</tr>
<tr>
<td>Percent recovery&lt;sup&gt;b&lt;/sup&gt;</td>
<td>129</td>
<td>100</td>
</tr>
<tr>
<td>No. positive cultures/total</td>
<td>23/24</td>
<td>14/24</td>
</tr>
<tr>
<td>No. giving higher counts/total</td>
<td>16/24</td>
<td>6/24</td>
</tr>
</tbody>
</table>

<sup>a</sup>TSA was preincubated at 35 C for 2 h, overlaid with VRBA and reincubated at 45.5 C for 24 h.
<sup>b</sup>Average of 24 E. coli cultures.

The average percent injury ranged from 91 to 96 depending on the incubation temperature. The slightly greater percent injury at 35 C was probably due to the unstressed cells was 114 per ml on the agar-repair medium and only 88 per ml in MPN media. The average recovery on the agar-repair medium (VRBA/TSA) was 129% relative to the MPN count in EC medium at 45.5 C, taken as 100% recovery. Twenty-three of the 24 cultures grew on the agar-repair medium (VRBA/TSA), whereas only 14 grew in the MPN media. Eight cultures were actually inhibited by the LST medium at 35 C (data not shown). Comparison of the two media showed that 16 cultures gave higher counts on the agar-repair medium (VRBA/TSA) and only six gave a slightly higher count in MPN media.

Table 4 presents similar results with freeze-stressed cells of the same 24 E. coli cultures. The average count on the agar-repair medium (VRBA/TSA) at 45.5 C was more than twice as high as the MPN estimate at 45.5 C. This resulted in a 238% recovery on the agar-repair medium, relative to the MPN estimate taken as 100%.

DISCUSSION

Previous studies (6,8) have indicated that the agar-repair method could be effectively used at 35 C for enumeration of “total” coliforms from food samples in which the coliforms may be present in an injured state. This study showed that the same method can be used to selectively enumerate stressed “fecal coliforms” by incubating the agar-repair medium directly at 45.5 C, following an initial repair period of 2 h at 35 C. The fecal coliform count can be completed in only 24 h by this procedure. This reduces the time required to estimate the fecal coliform count by the MPN method. The superiority of this method over the MPN method for recovery of fecal coliforms was clearly demonstrated with both stressed (frozen) and unstressed E. coli strains.

Further and more rapid identification of the fecal coliform colonies enumerated on the agar-repair medium was made possible by the recent development of the IMViC agar plate (3). Figure 1, for example, shows how the agar-repair medium and the IMViC agar plate can be combined to form a 72-h test scheme for isolation and identification of E. coli. This scheme reduces, by as much as 7 days, the time required to identify E. coli in foods by the conventional MPN method. The considerable saving in time eliminates many of the administrative and operational problems presented by holding of foods pending the outcome of the lengthy, tedious and inaccurate MPN procedure. The scheme presented in Fig. 1 has the additional advantage of being simple to do and with media and equipment commonly found in the smallest of laboratories.

This study also showed that fecal coliforms could not be accurately enumerated on VRBA alone at elevated temperatures as suggested by other investigations (2), because of the poor recovery achieved, particularly when cells were stressed (Table 2). It was clearly evident from the data presented that stressed coliforms must be allowed a period to recover before being exposed to
FOOD SUSPENSION

\[ \text{ELAPSED TIME} \]

\[ \begin{array}{c|c}
\text{TSA} & 2 \text{ h} \\
35 \degree C & \\
\hline
\text{VRBA OVERLAY} & 24 \pm 2 \text{ h} \\
45.5 \degree C & \\
\hline
\text{FECAL COLIFORM TEST COMPLETED} & \\
\text{IMVIC AGAR PLATE} & 24 \pm 2 \text{ h} \\
35 \degree C & \\
+ + - - \text{ or } - + - - (E. coli) & 72 \pm 2 \text{ h} \\
\end{array} \]

Figure 1. Rapid (72 h) test scheme for isolation of E. coli from foods.

VRBA. In so doing, however, care must be taken to prevent multiplication of cells before they are enumerated, which is a possibility when liquid media are used for repair (6,8). This disadvantage is avoided with solid repair media because colony forming units are immobilized.

The effect on microbiological standards, in the military and other regulatory agencies, of detecting injured organisms not previously detected with conventional methods has yet to be assessed and dealt with. Currently, our work involves evaluation of agar-repair media for recovery of “fecal coliforms” from naturally contaminated foods and improvement of media for differentiation of “fecal coliforms.”

ACKNOWLEDGMENT


REFERENCES