Cycloserine-cefoxitin fructose agar (CCFA), CCFA with horse blood and taurocholate, and cycloserine-cefoxitin mannitol broth with taurocholate and lysozyme (CCMB-TAL) were compared for recovery of *Clostridium difficile* from 120 stool specimens. Compared to CCFA, CCFA-HT enhanced *C. difficile* growth and improved recovery by 4%. In a separate study, 9% (8/91) of stool samples previously *C. difficile* negative on plate medium were *C. difficile* positive when cultured in CCMB-TAL.

C. difficile colony appearance on CCFA-HT agar is nonhemolytic, 2 to 6 mm in diameter from 24 to 72 h, creamy yellow to gray-white in color, and irregular. Colonies have a coarsely mottled to mosaic internal structure, a matte or dull surface, green fluorescence under long-wave UV light, and a para-cresol odor. From experience, breakthrough colonies on CCFA and CCFA-HT look much different and are generally much smaller than *C. difficile* colonies. Final identification was performed by current methods (8, 9), including the use of special potency disks and proline, determination of the absence of aerobic growth, and Gram stain. If needed, further confirmation utilized API 20A kits (bioMérieux, St. Louis, MO) or 16S rRNA gene sequencing (10).

**CCFA/CCFA-HT/CCMB-TAL comparison study.** A total of 120 sequential fecal samples were studied. On the day of culture, samples were transferred from the freezer into an anaerobic chamber. After samples were thawed, one drop of sample was added directly to CCMB-TAL. Another 0.25 ml of the sample was ethanol shocked by mixing it with an equal volume of ethanol and allowing it to stand at room temperature for 10 to 20 min. Two drops of the ethanol-shocked sample was plated onto CCFA and CCFA-HT. Plates were streaked for isolation into 4 equal quadrants. All media were incubated at 37°C. Relative colony size on the two plate media and growth (0 to 4+ according to quadrant growth) were recorded at 20 to 24, 48, and 72 h. CCMB-TAL was examined at 24 to 72 h for yellow color predicting the presence of *C. difficile*. Yellow tubes were

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TABLE 3 Summary of C. difficile growth versus contamination breakthrough for 93 sample-paired CCFA-HT and CCFA plates at 24 and 48 h

<table>
<thead>
<tr>
<th>Growth of mediuma</th>
<th>C. difficile</th>
<th>Breakthrough</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of specimens positive (%)b</td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>HT &gt; CCFA</td>
<td>61 (66)</td>
<td>58 (62)</td>
</tr>
<tr>
<td>HT = CCFA</td>
<td>27 (29)</td>
<td>28 (30)</td>
</tr>
<tr>
<td>HT &lt; CCFA</td>
<td>5 (5)</td>
<td>7 (8)</td>
</tr>
</tbody>
</table>

a CCFA-HT > CCFA, CCFA-HT growth greater than CCFA; CCFA-HT = CCFA, CCFA-HT growth equal to CCFA; CCFA-HT > CCFA, CCFA-HT growth less than CCFA.
b Boldfaced values highlight the lack of change between 24 and 48 h that might be expected.

subcultured onto CCFA, which was the standard comparator medium for the study.

In all, 78% (93/120) of the fecal samples were C. difficile positive in at least one of the three media (Table 2). Of the 93 C. difficile-positive cultures, 99% (92/93) were positive at 24 h on CCFA-HT and 100% at 48 h. However, on CCFA, only 90% (84/93) were positive at 24 h, and 96% (89/93) were positive at 48 to 72 h. At 24 h, of the nine specimens that were C. difficile negative on CCFA, five were C. difficile positive in CCMB-TAL. At 48 to 72 h, four specimens were C. difficile negative on CCFA; of these, two were C. difficile positive in CCMB-TAL at 48 h and one more at 72 h, demonstrating enhanced recovery of C. difficile in CCMB-TAL for these samples. To determine if taurocholate may have increased growth results from CCFA-HT, 24- and 48-h cultures were examined when the CCFA-HT growth was greater than or equal to 2+ more growth than CCFA and vice versa. In each set, the REA types and their proportions were consistent with those of the clinical trial as a whole.

CCMB-TAL cultures were C. difficile positive in 86% (80/93) of the samples at 24 h and 90% (84/93) and 92% (86/93) at 48 h and 72 h, respectively. Of the seven specimens that remained C. difficile negative in CCMB-TAL at 72 h, two were also negative on CCFA at 72 h; however, all were C. difficile positive on CCFA-HT at 24 h. Isolates that grew on plate media from the seven samples that were CCMB-TAL negative included three isolates from the REA BI group and four with nonspecific REA types. Although these findings represent too small a sample for statistical analysis, the BI group was found in a proportion similar to that in the clinical trial as a whole.

CCFA-HT colony size was 1.7 times larger on average than CCFA at 24 and 48 h. C. difficile growth and contamination breakthrough for sample-paired CCFA-HT and CCFA plates were similar at 24 and 48 h (Table 3). Quantity of growth on CCFA-HT was ≥1 quadrant more than that on CCFA for 66 and 62% of the cultures at 24 and 48 h, respectively. The proportion of REA types from cultures where CCFA-HT growth was ≥1 quadrant more than CCFA did not vary significantly from that of this study as a whole.

The amount of breakthrough for the majority of cultures on CCFA-HT and CCFA was consistent at 24 and 48 h; 74% and 62%, respectively. All subcultures from the selective plates resulted in good growth, unlike other reports of selective formulations for C. difficile that demonstrated some inhibition (11).

CCMB-TAL enrichment study. To examine the enrichment value of CCMB-TAL, 91 previously plate culture-negative samples were inoculated into CCMB-TAL directly without ethanol shock, incubated at 37°C, and examined at 24, 48, and 72 h for yellow color and turbidity. All yellow or turbid cultures were plated onto CCFA-HT and incubated for up to 72 h before being discarded as negative for C. difficile.

Of the 91 CCMB-TAL cultures from previously negative plate culture specimens, 41 (45%) of the tubes appeared pink and clear and were discarded as negative. Two (2%) were pink and turbid but grew non-C. difficile breakthrough organisms upon subculture to CCFA-HT. Forty-eight (53%) of the CCMB-TAL tubes appeared yellow and cloudy, and of these, eight grew C. difficile and the remainder grew breakthrough organisms upon subculture to CCFA-HT. Six of the eight C. difficile-positive cultures were turbid and changed the indicator to yellow after 24 h, with the remainder turning yellow at 48 h. The eight C. difficile-positive cultures reflect 9% of the total specimens cultured and 17% of the CCMB-TAL tubes that changed the indicator to yellow. Four isolates were of the REA-type BI group, which was similar to the BI group percentage (45%) of the clinical trial as a whole. The remaining isolates included one Y group, and three isolates were nonspecific REA groups.

Of these eight CCMB-TAL-positive specimens, five patients

TABLE 1 Ingredients of CCFA, CCFA-HT, and CCMB-TAL

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>CCFA</th>
<th>CCFA-HT</th>
<th>CCMB-TAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar (g)</td>
<td>15.0</td>
<td>15.0</td>
<td></td>
</tr>
<tr>
<td>Cycloserine (mg)</td>
<td>500.0</td>
<td>250.0</td>
<td>500.0</td>
</tr>
<tr>
<td>Cysteine (g)</td>
<td></td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Fructose (g)</td>
<td>6.0</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>Horse blood (ml)</td>
<td>70.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysozyme (mg)</td>
<td></td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>Mannitol (g)</td>
<td></td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>Neutral red (g)</td>
<td>0.03</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Sodium taurocholate (g)</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

a All media contain cefoxitin (15.5 mg), magnesium sulfate (0.1 g), potassium phosphate (1.0 g), proteose peptone no. 2 (40.0 g), sodium chloride (2.0 g), sodium phosphate (5.0 g), and distilled water (1,000 ml).

TABLE 2 CCFA/CCFA-HT/CCMB-TAL comparison study and results of 93 C. difficile culture-positive specimens

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>CCFA-HT</th>
<th>CCFA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of positive specimens (%)</td>
<td>No. of negative specimens (no. TAL+)</td>
</tr>
<tr>
<td>24</td>
<td>92 (99)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>48</td>
<td>93 (100)</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>93 (100)</td>
<td></td>
</tr>
</tbody>
</table>

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had had no CDI therapy 24 h prior to the start of the study, while
the other three patients were treated with metronidazole on the
day of specimen collection, suggesting possible enhanced enrich-
ment of C. difficile in the latter three samples.

**Toxin studies.** The suitability of C. difficile direct toxin testing
using 24- and 48-h colonies taken from CCFT-HT-grown colo-
nies was tested similarly to the method of Delmee et al. (6). Seven
known toxin-producing strains of C. difficile representing REA
groups BI, K, G, BK, J, Y, and CF were cultured on CCFA-HT.
Three colonies of each were transferred into 200 µl diluent and
tested using the C. difficile TOX A/B II kit (TechLab, Princeton,
NJ) per the manufacturer’s directions for liquid fecal samples.
Two of seven toxin-positive strains, REA types BI and J, gave trace
positive results at 24 h when tested directly from CCFA-HT colo-
nies. Colonies from all seven REA type strains tested strongly toxin
positive at 48 h.

Improved recovery of C. difficile at 24 h can be accomplished by
use of CCFA-HT. For those cultures with significantly more
growth on CCFA-HT than on CCFA, there was no correlation
with REA type, even with those C. difficile strains that are known to
produce greater amounts of spores. CCMB-TAL offers an effective
culture enrichment medium for specimens that contain fewer or-
ganisms and potentially for environmental sampling. Direct toxin
testing of colonies from CCFA-HT cultures provides a rapid tox-
in-testing tool for patients whose fecal samples have been toxin
negative previously.

**ACKNOWLEDGMENTS**

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