Sensitive and Selective Culture Medium for Detection of Environmental Clostridium difficile Isolates without Requirement for Anaerobic Culture Conditions

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Effective and easy-to-use methods for detecting Clostridium difficile spore contamination would be useful for identifying environmental reservoirs and monitoring the effectiveness of disinfection. Culture-based detection methods are sensitive for detecting C. difficile, but their utility is limited due to the requirement of anaerobic culture conditions and microbiological expertise. We developed a low-cost selective broth medium containing thioglycolic acid and 1-cystine, termed C. difficile brucella broth with thioglycolic acid and 1-cystine (CDBB-TC), for the detection of C. difficile from environmental specimens under aerobic culture conditions. The sensitivity and specificity of CDBB-TC (under aerobic culture conditions) were compared to those of CDBB (under anaerobic culture conditions) for the recovery of C. difficile from swabs collected from hospital room surfaces. CDBB-TC was significantly more sensitive than CDBB for recovering environmental C. difficile (36/41 [88%] versus 21/41 [51%], respectively; P = 0.006). C. difficile latex agglutination, an enzyme immunoassay for toxins A and B or glutamate dehydrogenase, and a PCR for toxin B genes were all effective as confirmatory tests. For 477 total environmental cultures, the specificity of CDBB-TC versus that of CDBB based upon false-positive yellow-color development of the medium without recovery of C. difficile was 100% (0 false-positive results) versus 96% (18 false-positive results), respectively. False-positive cultures for CDBB were attributable to the growth of anaerobic non-C. difficile organisms that did not grow in CDBB-TC. Our results suggest that CDBB-TC provides a sensitive and selective medium for the recovery of C. difficile organisms from environmental samples, without the need for anaerobic culture conditions.

Contaminated environmental surfaces are an important source of Clostridium difficile transmission (1). However, many studies have demonstrated that environmental disinfection is often suboptimal in health care facilities (1–3). Effective and easy-to-use methods for the detection of spore contamination would be useful for improving environmental disinfection interventions by identifying environmental reservoirs and providing a means to monitor the effectiveness of disinfection (2). Commercial real-time PCR assays are easy to use and widely available. However, commercial PCR assays may not be sufficiently sensitive for detecting low levels of environmental contamination (4). Culture-based detection methods are sensitive for detecting C. difficile, but their utility is hindered by the requirement of anaerobic culture conditions and microbiological expertise. Recently, Curry et al. (5) demonstrated that combining selective anaerobic broth culture preamplification with a confirmatory real-time PCR assay for toxin genes provided a sensitive method for detecting asymptomatic carriers of C. difficile. This approach can be used for the detection of environmental contamination, but its limitations include the need for anaerobic culture conditions and the expense of the PCR assay.

Thioglycolic acid and 1-cystine are reducing agents that consume oxygen in medium and facilitate the growth of obligate anaerobes (6). Here, we tested the hypothesis that a modified selective broth culture medium containing thioglycolic acid and 1-cystine as reducing agents would provide a sensitive and selective method for the recovery of C. difficile from environmental specimens under aerobic culture conditions. We evaluated the utility of a commercial PCR assay for toxin B genes, an enzyme immunoassay for toxins A and B, an enzyme immunoassay for glutamate dehydrogenase, or a C. difficile latex agglutination assay as confirmatory tests for positive broth specimens. Our goal was to develop an effective assay that would be inexpensive and easy to use.

MATERIALS AND METHODS

C. difficile strains. Two C. difficile strains were studied. Strain VA 17 is an epidemic restriction endonuclease analysis (REA) type BI strain, and strain VA 11 is an REA J-type strain.

Preparation of spores. The spores were prepared by growth on Duncan-Strong agar medium, as previously described (7). The spores were stored at 4°C in sterile distilled water until use. The spores were confirmed by phase-contrast microscopy and malachite green staining to be 98% free of vegetative cells or cell debris.

Modified medium for culture of C. difficile in room air. The base medium that was modified for the purposes of this study was C. difficile brucella broth (CDBB). Under anaerobic conditions, CDBB was previously shown to stimulate the germination and outgrowth of C. difficile spores at a rate comparable to that with cycloserine-cefoxitin-fructose broth, and an agar formulation was as sensitive and selective as cycloserine-cefoxitin-fructose agar for the recovery of C. difficile from stool spec-

Received 19 March 2014 Returned for modification 14 April 2014 Accepted 18 June 2014 Published ahead of print 25 June 2014
TABLE 1 Formulation of *Clostridium difficile* brucella broth with thioglycolic acid and l-cysteine

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brucella broth</td>
<td>28.0 g</td>
</tr>
<tr>
<td>Vitamin K&lt;sub&gt;1&lt;/sub&gt; solution</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Hemin solution</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Sodium bicarbonate solution</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>d-Mannitol</td>
<td>6.0 g</td>
</tr>
<tr>
<td>Neutral red solution (1%)</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Sodium taurocholate</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>d-Cycloserine</td>
<td>500.0 mg</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>16.0 mg</td>
</tr>
<tr>
<td>Agar</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Thioglycolic acid (mercaptoacetic)</td>
<td>1.0 g</td>
</tr>
<tr>
<td>l-Cysteine</td>
<td>1.0 g</td>
</tr>
</tbody>
</table>

Tubes (Fisher Scientific). Agitation was kept to a minimum. After 72 h of incubation at 37°C, specimens with a color change from red to yellow were plated onto prereduced CDBA plates inside the anaerobic chamber to determine if *C. difficile* was present. The experiments were repeated 9 times.

To assess selectivity, *Clostridium sporogenes* (strain ATCC 11437), *Clostridium perfringens* (strain ATCC 131124), and *Bacillus subtilis* (ATCC 6051) spores and facultative organisms, including *Enterococcus faecium* (strain C68) (8), methicillin-resistant *Staphylococcus aureus* (clinical isolate), *Staphylococcus warneri* (ATCC 14990), and *Candida glabrata* (ATCC 90030), were incubated in CDBB, CDBB-TC (incubated in room air incubator), and CDBB-TC (incubated inside the anaerobic chamber) at 37°C for 72 h. The samples that underwent a color change from red to yellow were plated onto blood plates to assess the growth of the organisms.

Comparison of sensitivity and selectivity of CDBB-TC and CDBB for detecting environmental contamination on hospital wards. We evaluated the sensitivity and selectivity of CDBB-TC versus CDBB for detecting environmental contamination. BD BBL CultureSwabs (Becton, Dickinson) with 2 swab prongs were applied to surfaces in *C. difficile* infection (CDI) patient rooms and on portable equipment. The swabs were premoistened in Dey-Engley neutralizing broth (Remel Products, Lenexa, KS) and applied to a 5 by 10 cm area of the surfaces. One prong was transferred to the anaerobic chamber and inoculated into prereduced CDBB inside the anaerobic chamber, and the other was inoculated into CDBB-TC in room air and placed in an incubator in room air. The cultures were incubated at 37°C for 72 h. All specimens that underwent a color change from red to yellow were plated onto prereduced CDBA inside the anaerobic chamber and incubated for 72 h. Yellow colonies with the typical appearance were streaked for isolation onto blood plates and were confirmed to be *C. difficile* on the basis of the typical odor and appearance of the colonies and by a positive reaction using a *C. difficile* latex agglutination assay. For a subset of 18 positive CDBB-TC specimens, confirmatory testing was performed on an aliquot from the bottom of the tube using a commercial PCR assay (Xpert *C. difficile*, Cepheid, Sunnyvale, CA), enzyme immunoassays (ELA) for glutamate dehydrogenase and toxins A and B, and a *C. difficile* latex agglutination assay (Microgen Bioproducts, Camberley, United Kingdom) for the detection of *C. difficile* somatic antigens.

To evaluate false-positive cultures (i.e., cultures that turned yellow but did not grow *C. difficile* when plated on CDBA), colonies from CDBA that were not consistent with *C. difficile* based on color and morphology were transferred to blood plates and identified by using the RapID ANA II system (Remel Products, Lenexa, KS) for obligate anaerobes or the Vitrek 2 system (bioMérieux, Durham, NC) for facultative organisms; if cultures that turned yellow did not yield growth on CDBA, the medium was plated on blood plates to assess growth. Organisms that were identified were then inoculated into CDBB and CDBB-TC to reassess growth and color change.

**Effect of thioglycolic acid on stimulation of germination of *C. difficile* spores.** To investigate potential explanations for the increased recovery of environmental *C. difficile* by CDBB-TC compared with that by CDBB, we tested the hypothesis that thioglycolic acid stimulates the germination of *C. difficile* spores, with the degree of germination based on the pH. Spore germination was compared in sterile water (control) versus sterile water supplemented with 1 mg/ml thioglycolic acid alone or in combination with 1 mg/ml lysozyme and at pH 5 versus 7.6. To assess germination, spores (10<sup>6</sup> CFU) were added to 1 ml of each solution and incubated in room air at 37°C for 1 h, and then 100-μl aliquots were subjected to heat shock at 80°C for 5 min in a water bath (activated spores are killed at 80°C, whereas nongerminated spores are not). After heat shock, the samples were serially diluted and plated onto prereduced CDBA in the anaerobic chamber at 37°C for 72 h, and the counts were calculated. All experiments were repeated in triplicate.
RESULTS

Germination and outgrowth of C. difficile in CDBB versus CDBB-TC. As shown in Fig. 1, the rate and extent of C. difficile spore germination and outgrowth were similar in CDBB-TC and CDBB (Fig. 1). Approximately 90% of the spores (1 log) germinated within 30 min, based upon susceptibility to alcohol.

Comparison of sensitivity and selectivity of CDBB-TC and CDBB in the laboratory. CDBB-TC was equivalent to CDBB for the recovery of C. difficile spores from inoculated swabs (Fig. 2). In CDBB-TC and CDBB consistently yielded positive results from swabs inoculated with ≥2 log_{10} CFU of spores. Positive results were obtained from a subset of swabs inoculated with 1 log_{10} CFU of spores; 3 of 9 (33%) runs were positive for CDBB versus 7 of 9 (78%) for CDBB-TC (P = 0.15). Positive cultures for CDBB were yellow throughout the medium tube, whereas positive CDBB-TC cultures were yellow at the bottom of the tube.

None of the organisms used to assess selectivity (C. sporogenes, C. perfringens, B. subtilis, E. faecium, methicillin-resistant S. aureus, S. warneri, or C. glabrata) grew in CDBB-TC incubated in room air. However, the C. sporogenes and C. perfringens strains grew in CDBB and in CDBB-TC that was incubated inside the anaerobic chamber.

Comparison of sensitivity and selectivity of CDBB-TC and CDBB for detecting environmental contamination on hospital wards. Environmental swabs were collected from 477 total surfaces (345 from surfaces in CDI patient rooms and 132 from portable equipment). As shown in Fig. 3, 41 (9%) of the cultures were positive by CDBB-TC and/or CDBB based on a subculture onto CDBA yielding C. difficile: 16 of the cultures were positive from both culture media, 20 were positive from CDBB-TC only, and 5 were positive from CDBB only. For environmental samples that were negative by one culture medium but positive by the other, confirmatory testing of the negative samples by C. difficile latex agglutination assay, ELISA for glutamate dehydrogenase and toxins A and B, and PCR for toxin B genes was consistently negative.

If any positive culture with confirmed C. difficile was considered the gold standard, CDBB-TC was significantly more sensitive than CDBB (36/41 [88%] versus 21/41 [51%]; P = 0.006). For 18 culture-positive specimens from CDBB-TC subjected to other confirmatory tests, 18 (100%) aliquots from the bottom of the CDBB-TC tube tested positive by each of the other confirmatory tests, including C. difficile latex agglutination, PCR for toxin B genes, and ELISA for glutamate dehydrogenase and toxins A and B. For 8 culture-negative CDBB and 8 culture-negative CDBB-TC tubes, the broth from the bottom of the tubes was negative by the same confirmatory tests.

For the 477 total environmental cultures, the specificities of CDBB-TC and CDBB based upon false-positive yellow-color development of the medium without the recovery of C. difficile were 100% (0 false-positive results) and 96% (18 false-positive results), respectively (P = 0.0001). The 18 false-positive cultures for CDBB were attributable to the growth of non-C. difficile anaerobic organisms that did not grow in CDBB-TC in room air. Fourteen colonies from false-positive cultures that were not consistent with C. difficile were identified as Fusobacterium spp. (n = 6), C. perfringens (n = 3), Clostridium septicum (n = 2), Clostridium tertium (n = 1), Streptococcus constellatus (n = 1), and Bacteroides fragilis (n = 1); 4 additional isolates were unable to be subcultured. The inoculation of these organisms into CDBB or CDBB-TC inside the anaerobic chamber resulted in a yellow color and growth, whereas inoculation into CDBB-TC with incubation in room air did not.

Effect of thioglycolic acid on stimulation of C. difficile spore germination. As shown in Fig. 4, minimal germination (based upon a reduction in spores at 80°C) of C. difficile spores occurred in sterile water or in 1 mg/ml thioglycolic acid alone or in combination with 1 mg/ml lysozyme at pH 5 (P = 0.82); however, at pH...
7.6, a significant reduction in spores occurred at 80°C in 1 mg/ml thioglycolic acid (P < 0.0001), with no further enhancement by lysozyme (P = 0.74).

**DISCUSSION**

Our findings demonstrate that a broth medium containing thioglycolic acid and L-cysteine provides a sensitive and selective method for the culture of *C. difficile* from environmental specimens without the need for anaerobic culture conditions. The medium is easy to prepare. Moreover, the requirement for microbiological expertise is minimal because positive broth samples based on color change can be confirmed as *C. difficile* by latex agglutination assay or EIA for glutamate dehydrogenase, or as toxigenic *C. difficile* using commercial PCR assays for toxin genes or enzyme immunoassays for toxins.

The fact that *C. difficile* was recovered more frequently from environmental swabs inoculated into CDBB-TC than into CDBB was unexpected. In contrast, Wilcox and Dave (9) recently reported that lysozyme enhanced the recovery of *C. difficile* from environmental samples, but preexposure to alkaline thioglycolate did not further improve recovery. Our results suggest that a potential explanation for the increased recovery of environmental spores is the stimulation of germination by thioglycolic acid. At pH 7.6 but not pH 5, thioglycolic acid stimulated the germination of *C. difficile* spores based upon the susceptibility of spores to killing by heating to 80°C. Others have demonstrated that pH may influence the germination of *C. difficile* spores, with an optimum pH range for germination being 6.5 to 7.5 (10). It is possible that thioglycolic acid stimulates the germination of a fraction of spores from the environment that exist in a superdormant state (i.e., spores that do not germinate in response to common germinants) (11). Alternatively, some studies have suggested that thioglycolic acid exposure may sensitize spores to the activity of lysozyme, presumably by rupturing disulfide bonds and increasing the penetration of lysozyme to the site of action (12–14).

The finding that CDBB-TC was more selective than CDBB was also unexpected. The selectivity of the medium is an advantage, because extra work and expenses are required in the evaluation of false-positive cultures that turn yellow due to the growth of organisms other than *C. difficile*. A variety of anaerobic organisms grew in CDBB and caused a color change to yellow; these organisms did not grow in CDBB-TC incubated in room air but did grow in CDBB-TC incubated inside the anaerobic chamber. We postulate that some of these organisms may be more fastidious than *C. difficile* in their requirement for obligate anaerobic conditions (i.e., CDBB-TC incubated in room air may be sufficiently anaerobic to grow *C. difficile* but not the other *Clostridium* spp. that grew only under anaerobic conditions).

In addition to being easy to prepare, CDBB-TC is relatively inexpensive. Based upon the current pricing of ingredients from the listed distributors, the costs for one culture would be $2.99, including the medium ingredients ($0.50/10 ml), BD BBL CultureSwabs ($2.00/swab), and polystyrene tubes ($0.49/tube). The addition of a confirmatory test to be used on tubes showing a
change in color to yellow would increase costs a modest degree. If the *C. difficile* latex agglutination test were used, it would add $5.24 for each confirmatory test. If all of the 8% of CDBB-TC tubes that exhibited a color change in the current study had been confirmed using the latex agglutination test, the total cost per 100 environmental cultures would have been $340.92 (versus $299 if no confirmatory testing were done).

Our study has some limitations. The medium is qualitative and does not provide an assessment of the burden of contamination. The sensitivity of the swabs for recovering spores may be reduced in comparison to that for sponges (15). Environmental samples were collected from one institution only. Additional larger studies are needed in multiple institutions. Finally, we tested only one commercial real-time PCR assay. There may be variability in the sensitivities of the different nucleic amplification tests (16).

ACKNOWLEDGMENTS

This work was supported by a Merit Review grant from the Department of Veterans Affairs to C.J.D. and by a grant from the Agency for Healthcare Research and Quality (R18 HS20004-01A1) to C.J.D.

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