Use of Sodium Taurocholate to Enhance Spore Recovery on a Medium Selective for *Clostridium difficile*

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Isolation of *Clostridium difficile* from fecal specimens has been facilitated by the development of a selective and differential medium, cefoxitin-cycloserine-fructose agar (CCFA). We substituted 0.1% sodium taurocholate for the 2.5% egg yolk in CCFA and compared the growth of 15 isolates of *C. difficile* on the resulting medium with growth on conventional CCFA. The taurocholate-containing medium (TCCFA) quantitatively recovered vegetative forms of *C. difficile* in the same numbers as CCFA medium. Recovery of spores was a mean $1.7 \log_{10}$ higher on TCCFA than on CCFA. Thirty-six of 60 patient stool specimens growing *C. difficile* gave a heavier growth on TCCFA than on CCFA, and 9 failed to yield *C. difficile* on CCFA. TCCFA detected spores at 75 colony-forming units per ml from artificially inoculated fecal specimens when conventional stool culturing techniques were used. Fluorescence of colonies of *C. difficile* was more intense on TCCFA than on CCFA. TCCFA was simpler to prepare and, overall, was more sensitive than CCFA.

Clostridium difficile is the most common cause of antibiotic-associated colitis in humans (3, 4). Isolation of this organism from fecal specimens has been facilitated by the development of cefoxitin-cycloserine-fructose agar (CCFA), a selective and differential medium containing 2.5% egg yolk (5). Use of a medium containing egg yolk is useful for detecting lecithinase and lipase activities of clostridia. *C. difficile*, however, produces neither enzyme, and the need for egg yolk in a selective medium for this organism has not been established. Although CCFA medium has been reported to allow detection of as few as 2×10^3 colony-forming units of *C. difficile* per ml of feces (5), we have found it relatively insensitive at detecting spores. Failure of viable clostridial spores to grow on agar media is not unique to *C. difficile*. Raibaud et al. reported that they can augment the outgrowth of clostridial spores from feces by adding 1% sodium taurocholate to media (9), and they have shown that *C. difficile* spores may grow more reliably on such media (8). In the present study we deleted the 2.5% egg yolk in CCFA medium and added 0.1% sodium taurocholate. We evaluated the effect of these changes on quantitative recovery of *C. difficile* spores and vegetative forms, detection of *C. difficile* in fecal specimens, and fluorescence of colonies of *C. difficile* growing on agar medium.

MATERIALS AND METHODS

Bacterial isolates. Fifteen isolates of *C. difficile* from the University of Michigan, identified by standard

methods (6), were tested. Isolate 49A was recovered from a hamster dying of antibiotic-associated cecitis. Isolate K-302 was recovered from the environment of a pediatric ward. All other isolates were obtained from patients' fecal specimens that were positive for *C. difficile* toxin.

Agar media. CCFA medium containing egg yolk was prepared as previously described (5). The basal medium (B) was prepared in the same way as CCFA but without egg yolk. The taurocholate-containing medium (TCCFA) consisted of B with 0.1% (wt/vol) sodium taurocholate (Mann Research Laboratories, New York, N.Y.) added before autoclaving. All media to be compared contained cefoxitin and cycloserine, were made simultaneously, and were prerduced together in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.). Poured plates were inoculated within 5 days or discarded.

Sporulation medium. Sporulation medium contained 90 g of Trypticase Peptone (BBL Microbiology Systems), 5 g of Proteose Peptone no. 3 (Difco), 1 g of $(\text{NH}_4)_2\text{SO}_4$, and 1.5 g of Tris in 1 liter of distilled water. The pH was adjusted to 7.4 at 37°C with 1 M NaOH; the medium was autoclaved at 121°C for 20 min and the prerduced for at least 24 h. Initial studies had shown this medium to yield a microscopic count of over 10^7 *C. difficile* spores per ml after 48 h of incubation.

Viable counts. Serial 10-fold dilutions of broth cultures were made using prerduced Trypticase soy broth with 0.04% Na_2CO_3 (wt/vol) to compensate for 5% CO_2 in the anaerobic atmosphere. A 0.1-ml sample of each dilution was plated on prerduced media in 10-cm petri dishes and cultured anaerobically for 48 h. Microscopic counts were performed with a Petroff-Hausser chamber under phase contrast.

Spore preparation. Forty-eight-hour cultures of *C.*

TABLE 1. Relative recovery of spores and vegetative forms for 15 isolates of *C. difficile*

<i>C. difficile</i> inoculum grown on:	Heat shock	Recovery ^a on medium ^b :		
		TCCFA	CCFA	B
Sporulation medium	+	6.4 ± 0.6 (5.3–7.3)	4.7 ± 0.7 (3.1–5.6)	2.9 ± 0.8 (1.8–4.7)
	–	7.6 ± 0.4 (6.7–8.5)	7.5 ± 0.5 (6.5–8.5)	7.5 ± 0.5 (6.5–8.5)
Brain heart infusion	+	3.5 ± 2.0 (1.0–7.0)	Not tested	Not tested
	–	8.1 ± 0.1 (8.0–8.3)	8.1 ± 0.1 (8.0–8.3)	8.1 ± 0.1 (8.0–8.3)

^a Log₁₀ mean viable count ± SD (range).

^b TCCFA = CCFA with 0.1% sodium taurocholate substituted for egg yolk; CCFA = usual CCFA including egg yolk; B = CCFA medium without egg yolk.

difficile grown at 37°C in 10 ml of sporulation medium were diluted and quantitatively cultured. The remainder of the 48-h culture was then removed from the anaerobic chamber, and 0.5-ml samples were pipetted into sterile, 2-ml glass vials, which were flame-sealed. Vials were totally immersed in water at 56°C for 10 min and then placed on ice and taken into the anaerobic chamber. Initial studies indicated that vegetative forms were rapidly eliminated by this procedure, and the yield of spores was higher at 56°C than at 70°C.

Culture of patient specimens. Fecal specimens were sent to our lab specifically for toxin testing and culture for *C. difficile*. Our tissue culture assay for *C. difficile* toxin has been described previously (10). For culture, specimens were taken into the anaerobic chamber, sampled with sterile cotton swabs, and streaked onto CCFA and TCCFA plates. The inoculated plates were then cross-streaked three times with a sterile loop. Confirmation of isolates resembling *C. difficile* was by the standard methods used to identify anaerobes (6).

Inoculation of stool with *C. difficile*. To compare the sensitivity of CCFA and TCCFA media with watery stool, we mixed equal portions of normal stool with sterile water. The mixture was reduced for 6 h in the anaerobic chamber, and then 75 colony-forming units of spores or 160 colony-forming units of vegetative forms were added per ml. Spores and vegetative forms were each mixed with four stool samples, and each resulting suspension was cultured in duplicate. The culture technique was the same as that used for patient specimens.

Fluorescence of colonies. Ten isolates of *C. difficile* were inoculated simultaneously onto CCFA, TCCFA, and B media. Fluorescence was observed at 20, 26, 48, and 72 h. The room was totally darkened so the observer could not identify the type of medium in a given plate, and a Woods lamp was held approximately 10 in. (ca. 25 cm) from the culture plates. Each of the three culture plates was subjectively ranked from most to least fluorescent for each isolate.

RESULTS

Broth cultures. Table 1 shows viable counts obtained from broth cultures before and after heat shocking. Microscopic spore counts on the suspension before heat shocking showed a mean

log of 7.4 ± 0.15 (standard deviation) spores per ml. Samples from the sporulation medium before heating gave essentially the same viable count whether quantitated on TCCFA, CCFA, or B medium. After heat shocking, however, the suspension yielded more on TCCFA than on CCFA (mean 1.7 log₁₀; range 0.5 to 3.5 logs difference). The difference between the yields on TCCFA and B was even more marked (mean 3.5 log₁₀; range 2.4 to 4.7 logs difference).

Since the microscopic spore counts in sporulation medium were nearly the same as the total viable count, we also tested a more predominantly vegetative preparation. This was done to assure that vegetative forms indeed grew equally well on all three media. Overnight cultures in brain heart infusion broth were used. Table 1 shows growth of vegetative *C. difficile* from this preparation to be equivalent on the three media. After heat shock, the *C. difficile* count dropped a mean of 4.6 logs on TCCFA, indicating that the yield of log 8.1 before heating had been due to vegetative forms. In brain heart infusion preparations, the spore concentration was too low to be counted microscopically.

Patient specimens. A total of 161 consecutive patient specimens were cultured on CCFA and TCCFA (Table 2). The clinical status of these patients varied considerably and included patients with antibiotic or chemotherapy-induced diarrhea, those with exacerbations of inflammatory bowel disease, and patients on various antibiotic drug studies. *C. difficile* was isolated from 61 specimens. Of positive specimens, 85% yielded *C. difficile* on CCFA, whereas 98% were positive on TCCFA. Of the 50 specimens yielding *C. difficile* on both media, 27 gave a heavier growth on TCCFA. Most specimens yielding an equivalent growth on the two media came from the University of Michigan or another institution involved in a collaborative study and were processed or frozen the day of specimen collection. On the other hand, most specimens giving heavi-

TABLE 2. Comparison of growth on TCCFA and CCFA of *C. difficile* in fecal specimens from patients

Growth ^a		No. of specimens
TCCFA	CCFA	
+	+	50
—	—	101
+	—	9
—	+	1

^a +, *C. difficile* detected; —, *C. difficile* not detected.

er growth on TCCFA came from other outside institutions ($\chi^2 = 7.25$, $P < 0.01$) where prompt handling was less certain. Isolates of *C. difficile* growing more abundantly on TCCFA did not yield more spores than other isolates when grown in sporulation medium. Spores from such isolates also did not grow preferentially on TCCFA any more than other isolates did. These results suggest that where growth on the two media was equivalent, prompt handling of specimens had led to persistence of vegetative forms. The one specimen yielding *C. difficile* only on CCFA grew three colonies and was from an asymptomatic patient receiving topical clindamycin. The toxin test was negative.

When *C. difficile* was mixed with stool as described in Materials and Methods, the mean number of vegetative forms recovered was 16.3 on CCFA (range 10 to 24) and 16.4 on TCCFA (range 7 to 32). The mean number of spores recovered on CCFA was 1.1 (range 0 to 3), and that on TCCFA was 6.9 (range 3 to 10; $P < 0.01$, Student's *t* test). Thus, as expected, the two media were equally sensitive at recovering vegetative forms from feces, but TCCFA was superior at recovering spores.

Fluorescence. *C. difficile* consistently displayed more intense fluorescence when growing on TCCFA than when growing on either CCFA or B. This finding held throughout the 72 h of observation.

DISCUSSION

We have described a selective medium for *C. difficile* in which 0.1% sodium taurocholate was added to conventional CCFA medium without egg yolk. The resulting medium was simpler to prepare than CCFA. Spores of *C. difficile* grew more abundantly on TCCFA agar, and vegetative forms grew equally well on the two media. Although the effect was not as marked as that of taurocholate, egg yolk also increased the recovery of spores over the rate of recovery on CCFA basal medium.

The finding that sodium taurocholate facilitates outgrowth of spores suggests a possible

mechanism by which the normal flora may antagonize *C. difficile*. The absence of primary conjugated bile acids in the normal colon is well documented (1) and is due to the deconjugating activity of many components of the normal gut flora. It is possible that degradation of taurocholic acid by the normal flora is a factor leading to resistance of the normal gut to colonization with *C. difficile*. This possibility should be investigated further.

For some applications, the superiority of TCCFA over CCFA is clear. There are no published data on the use of selective media to determine the carrier rate of *C. difficile* in normal humans. TCCFA would be an appropriate medium to use in studying the normal carrier rate because it would provide for the best known recovery of both vegetative cells and spores. Environmental contamination with *C. difficile* has been suggested to be a significant factor in transmission of this pathogen (2, 7). Since forms persisting in contaminated environments are probably mostly spores, published data on the use of CCFA to isolate environmental contaminants probably underestimate the magnitude of environmental contamination with *C. difficile* (2, 7).

In our study, TCCFA medium was more sensitive for detecting *C. difficile* in patient specimens, and usually yielded a heavier growth than CCFA medium. Three of nine specimens growing *C. difficile* only on TCCFA medium also contained *C. difficile* toxin. This finding suggests that the presence of *C. difficile* was a significant finding in at least some patients yielding the organism only on TCCFA agar. Our experience indicates that TCCFA medium may be particularly useful when fecal specimens are not handled optimally and when vegetative forms lose viability from prolonged exposure to air. However, the definitive diagnostic test for antibiotic-associated colitis remains colonic biopsy, and the finding of *C. difficile* or even its toxin in the stool of a patient with antibiotic-associated diarrhea has not been shown to establish the diagnosis of antibiotic-associated colitis. The relative roles of CCFA and TCCFA in the diagnosis of antibiotic-associated colitis should be studied.

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