Comparison of Methods for Recovery of *Clostridium difficile* from an Environmental Surface

BRIAN P. BUGGY,[1,2]* KENNETH H. WILSON,[1] AND ROBERT FEKETY[2]

Department of Internal Medicine, Ann Arbor Veterans Administration Medical Center,[1] and Department of Internal Medicine, University of Michigan,[2] Ann Arbor, Michigan 48109

Survival of *Clostridium difficile* in an aerobic environment is possible because of spore formation. When sodium taurocholate is substituted for the egg yolk of a selective medium, cycloserine-cefoxitin-fructose-agar (CCFA), enhanced recovery of *C. difficile* spores is shown. This selective medium (TCCFA) does not improve recovery of vegetative forms. In this study, dry and saline-moistened swabs, adhesive paddles, and Rodac plates containing CCFA and TCCFA were compared in their ability to recover *C. difficile* spores from an inoculated surface. Rodac plates grew 20 to 25 times as many spores on TCCFA as on CCFA. Saline-moistened swabs recovered fewer organisms than Rodac plates. Dry swabs and adhesive paddles rarely recovered spores. Prereduction of agar in an anaerobic chamber was not necessary for optimal spore recovery. Optimal growth of vegetative *C. difficile* required prereduced media. Agar prereduced for 2 h supported the growth of 12 *C. difficile* isolates as well as agar prereduced for 18 h. Vegetative cells of *C. difficile* survived for only 15 min in room air. Use of Rodac plates containing TCCFA is preferred for detection of *C. difficile* spores in the hospital environment.

Clostridium difficile is an important cause of antibiotic-associated colitis (1), which occurs most frequently in seriously ill, hospitalized persons. Antimicrobial (1, 8) or antineoplastic (2, 3) therapy may promote the overgrowth of *C. difficile*, which then may elaborate its toxin. The organism in many cases may derive from healthy intestinal carriers. It can be isolated from the stools of 3 to 7% of healthy adults (4, 5, 13) and from as many as 20% of patients receiving antibiotics who do not have diarrhea (16). However, the occurrence of hospital outbreaks of *C. difficile*-associated colitis (8, 14, 17) has suggested that nosocomial acquisition of the organism may play an important role in the epidemiology of this disease. Experimental observations of the hamster model of antibiotic-associated colitis support the concept of cross-infection. When hamsters were housed in facilities designed to prevent cross-infection, the frequency of enterocolitis with clindamycin usually was lowered (10). However, when the animals were sheltered in facilities where *C. difficile* can be cultured easily from room equipment and caretakers, enterocolitis was easily induced with clindamycin (15).

C. difficile has been isolated from the hospital environment in several studies, using cycloserine-cefoxitin-fructose-agar (CCFA), a medium selective for *C. difficile* (6). Survival of *C. diffi-*

cile in an aerobic environment is possible because of the ability of this obligate anaerobe to form aerotolerant spores. Use of the above selective agar containing sodium taurocholate (TCCFA) instead of egg yolk, has been shown to recover a much higher number of *C. difficile* spores than did CCFA (18). To standardize methods for epidemiological study of *C. difficile*-associated colitis, we compared several methods for the recovery of *C. difficile* spores from an inoculated surface, using CCFA, TCCFA, and a selective broth. The need for overnight prereduction of media in an anaerobic chamber before use and the survival on an environmental surface of both vegetative and spore forms of the organism were also evaluated.

MATERIALS AND METHODS

Bacterial isolates. Two isolates of *C. difficile* were identified by standard methods (7). Strain K-302 was isolated from a surface in a pediatric ward at the University of Michigan as part of an earlier environmental study from this institution (9). It is toxigenic and was utilized in a spore preparation (vide infra). Strain VPI-2018 (obtained from Tracy Wilkins) was originally isolated at the Virginia Polytechnic Institute, is nontoxigenic, and has never been successfully induced to sporulate in this laboratory. Eleven other *C. difficile* isolates (from patient specimens and the hospital environment) were used to evaluate the effect of prereduced agar on bacterial growth.

TABLE 1. Number of *C. difficile* spores recovered with Rodac plates

Spore density[a]	Mean colony count per cm^2 ± SEM (range)[b]	
	CCFA	TCCFA[c]
680	49.6 ± 14.8 (4–120)	>300 (>300)
68	9.5 ± 2.7 (0–32)	245 ± 55.5 (128–295)
6.8	2.4 ± 0.7 (0–9)	58.6 ± 13.7 (17–144)
0.68	0.4 ± 0.2 (0–3)	7.8 ± 0.2 (0–21)
0.068	0	0.4 ± 0.08 (0–1)

[a] Number of viable spores applied per square centimeter.

[b] Mean of 25 Rodac plates of each medium (agar surface area of a Rodac plate = 25 cm^2).

[c] $P < 0.001$ for TCCFA compared with CCFA at all dilutions.

TABLE 2. Number of *C. difficile* spores recovered with saline-moistened swabs

Spore density[a]	Mean colony count per cm^{2b} ± SEM (range)		XTB (no. of tubes turbid/ no. inoculated)[c]
	CCFA	TCCFA	
680	8 ± 3.9 (0–25)	80 ± 24 (30–140)	12/12
68	0.5 ± 0.4 (0–4)	8 ± 3 (0–21)	11/12
6.8	0	0.9 ± 0.4 (0–3)	7/10
0.68	0	0.3 ± 0.6 (0–1)	5/10
0.068	0	0.12 ± 0.04 (0–1)	0/10

[a] Number of viable spores applied per square centimeter.

[b] Mean of 12 plates of each medium (area sampled with each swab = 4 cm^2).

[c] XTB broth = TCCFA without agar. Assayed at 48 h.

Agar media. CCFA agar medium was prepared as previously described (6). TCCFA agar medium consisted of the substitution of 0.1% (wt/vol) sodium taurocholate (Mann Research Laboratories, New York, N.Y.) for the egg yolk of CCFA (18). Other crude preparations of taurocholate should not be assumed to have the same activity (K. Wilson unpublished data). The taurocholate was added before autoclaving. Both media contained cefoxitin (16 μg/ml) and cycloserine (500 μg/ml), were made simultaneously, and were prereduced for 18 h before use in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.). In certain experiments, the media were not reduced until after inoculation. A selective broth (XTB) was also used, consisting of TCCFA prepared without agar (but with cefoxitin and cycloserine). Media were inoculated within 3 days of preparation.

Sporulation medium. A sporulation medium was prepared as previously described (18). Earlier studies have shown this medium to yield a microscopic count of over 10^7 *C. difficile* spores per ml after 48 h of incubation. The number of viable spores present in the suspension used was ascertained by quantitative cultures each time a sample was used. All spores used in these experiments were grown in a single batch and then pipetted into sterile 2-ml glass vials and flame sealed. Vials were immersed in water at 56°C for 10 min and then frozen at −20°C until use. Previous studies have shown that vegetative forms are eliminated by this procedure (18).

Culture methods. (i) Spore recovery. The stock spore preparation of strain K-302 was thawed, and 10-fold dilutions in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) were spread evenly with a sterile glass rod over the sampling surface, a pane of sterile 0.25-in. (ca. 0.64-cm)-thick window glass having an area of 784 cm^2. All sampling procedures were performed after overnight drying had occurred. Sterile cotton swabs were used either dry or moistened with 5 drops of sterile, non-bacteriostatic 0.9% NaCl. They were rubbed vigorously on the glass over an area of 4 cm^2 for 5 s and then taken into the anaerobic chamber. CCFA and TCCFA were then streaked in four quadrants, and XTB broth was inoculated by cutting the swab tip with sterile scissors and allowing it to fall into the broth. A separate swab was used for each agar

plate or broth. Adhesive paddles (Falcon Plastics, Oxnard, Calif.) were pressed against the glass surface, taken into the anaerobic chamber, and inoculated into XTB broth. Ten to 15 replicate samples were taken at each dilution. Rodac contact plates (Falcon Plastics), filled with approximately 17 ml of either CCFA or TCCFA, were pressed firmly onto the glass surface without sliding or twisting and taken promptly into the anaerobic chamber. Twenty-five Rodac plates of each agar were evaluated at each spore dilution.

(ii) Timed survival. A 100-fold dilution of an overnight turbid growth of strain VPI-2018 in Trypticase soy broth was used in the timed survival experiment. This strain has never been induced to sporulate in our laboratory, and growth thus represents vegetative forms. The organism was spread over the glass plate, as above, and impression cultures were made with Rodac plates. TCCFA was compared with CCFA at 1, 5, 15, 30, and 60 min. One-half of the plates had not been prereduced before use. The plates were taken immediately after impression into the anaerobic chamber.

Use of non-prereduced media. To ascertain whether prereduction of media in an anaerobic environment was necessary, quantitative cultures of both K-302 and VPI-2018 were performed with CCFA and TCCFA which had been either prereduced for 18 h or brought into the anaerobic chamber for various periods before inoculation. The growth of VPI-2018 was assessed on agar which had been reduced for 5, 15, 30, and 120 min before plating. Spores (K-302) were inoculated onto plates which had been prereduced for 18 h and also onto non-prereduced agar plates outside of the chamber and then incubated anaerobically. Eleven other isolates of *C. difficile* (patient and hospital environmental isolates) were also grown on agar media that had been prereduced overnight or for 2 h. All cultures were incubated at 37°C in the anaerobic chamber for 48 h before quantitation. Broth cultures were scored as turbid or clear. Individual colony counts were performed on the agar media. Confirmation of isolates recovered as *C. difficile* was performed by standard methods (7). The two-tailed Student's *t* test was used to analyze results.
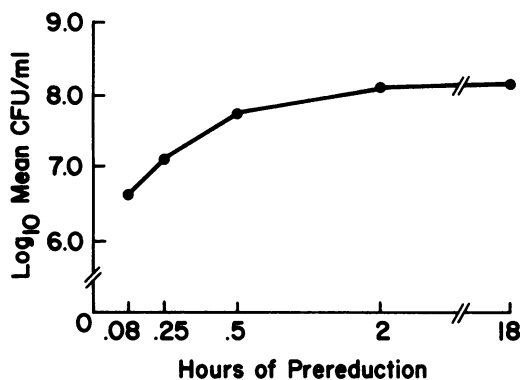
FIG. 1. Effect of prereduction of media on growth of *C. difficile* vegetative cells of strain VPI-2018. Pooled data of CCFA and TCCFA (standard error of the mean at each point was between 0.01 and 0.16). n = 10 at each time interval. P < 0.001 between 0.08 and 0.25, 0.25 and 0.5, and 0.5 and 2.0 h.

RESULTS

Agar and broth recovery. The quantitative recovery of *C. difficile* spores with Rodac plates is shown in Table 1. At all spore concentrations tested, TCCFA yielded growth of 20 to 25 times as many spores as did CCFA (P < 0.001), and thus a much higher percentage of the inoculum was recovered with it than with CCFA. The recovery with CCFA ranged from 0.3 to 2.3%, whereas with TCCFA 14.5 to 45.8% of the inoculum was recovered. Table 2 illustrates the recovery of *C. difficile* spores with saline-moistened swabs streaked onto TCCFA or CCFA and XTB broth. TCCFA grew spores at all concentrations and significance was reached when compared with CCFA at all but the two lowest spore concentrations (P < 0.001). Mean percent recovery with moist swabs (number of tubes or plates positive/number inoculated) and XTB broth (63%) was slightly higher than with TCCFA (56%). Both media, however, recovered significantly more spores than the 22% with CCFA (P < 0.001 by chi square).

Dry swabs infrequently recovered spores, even when concentrations were $10^7/cm^2$. Broth cultures and solid media were equally insensitive when inoculated with dry swabs. Adhesive paddles rarely yielded spores at any concentration. When saline-moistened swabs were used to inoculate XTB broth containing either 0.1 or 1.0% sodium taurocholate, there was no enhancement of spore recovery with 1.0% taurocholate.

At the lowest two spore concentrations, TCCFA-Rodac plates were positive significantly more often than saline-moistened swabs inoculated either into XTB broth or onto TCCFA agar (95 and 33% of TCCFA-Rodac plates sampled

TABLE 3. Effect of prereduction of media on growth of vegetative forms of 11 *C. difficile* isolates

Medium	Log_{10} of mean CFU/ml ± SEM[a] at given prereduction time	
	2 h	18 h
CCFA	8.15 ± 0.03	8.15 ± 0.02
TCCFA	8.11 ± 0.03	8.11 ± 0.04

[a] n = 22 for each medium at each time.

were positive; 50 and 0% of XTB broth tubes inoculated were positive; 31 and 13% of TCCFA agar plates were positive at 0.68 and 0.068 spores per cm², respectively; P < 0.05 by chi square).

Timed survival. Recovery of vegetative strain VPI-2018 from the glass surface was not significantly different with CCFA and TCCFA at 1, 5, and 15 min. Drying of the suspension was complete within 5 to 10 min. The counts dropped rapidly at successive time intervals, and no organisms were cultured with either agar at 30 or 60 min. Plates that were prereduced recovered a greater number of colonies per plate at each time interval than did the non-prereduced plates (P < 0.001 by Mann-Whitney test). Prereduced plates recovered organisms at the 15-min interval, whereas non-prereduced plates did not.

Use of non-prereduced media. In these comparisons the vegetative strain (VPI-2018) was inoculated onto CCFA and TCCFA inside the anaerobic chamber. Some plates had been prereduced in the chamber for 18 h and others were brought into the chamber for 5, 15, 30 and 120 min before inoculation. There was no significant difference between the two media in quantitative growth of this strain. Increased growth was observed as media were reduced for 5, 15, and 30 min, but there was no further increase with plates that had been prereduced for at least 2 or 18 h (Fig. 1). Eleven other isolates of *C. difficile* also showed no difference in growth after 2 or 18 h of prereduction (Table 3). Spores inoculated in an aerobic environment on non-prereduced agar and then grown in an anaerobic chamber grew significantly better on TCCFA than on CCFA (Table 4). TCCFA grew approximately 100 times as many colony-forming spores as did CCFA on both prereduced and non-prereduced agars (P < 0.001). Although colonies appeared to grow faster on prereduced TCCFA, the actual mean colony counts at 48 h were equal.

DISCUSSION

We compared several techniques for the recovery of *C. difficile* spores from an inoculated surface to determine the optimal method for detection of *C. difficile* in the environment. Previous reports demonstrating the presence of

TABLE 4. Effect of prereduction of media on growth of spores of *C. difficile* spore strain K-302

Medium prereduced	Log$_{10}$ of mean CFU/ml ± SEM (n = 11)	
	CCFA	TCCFA
No	5.42 ± 0.08	7.20 ± 0.02[a]
Yes	5.82 ± 0.06[b]	7.21 ± 0.05[a]

[a] $P < 0.001$ of quantitative spore growth on TCCFA compared with CCFA.

[b] $P < 0.001$ for effect of prereduction of CCFA.

C. difficile in the hospital environment (4, 9, 11, 12) used CCFA. TCCFA grew 20 to 25 times as many spores as CCFA after aerobic inoculation and sampling. Thus, previous studies with CCFA may have underestimated the true density of *C. difficile* in a hospital environment. The use of saline-moistened swabs detected spores present at low concentrations but prevented quantitation of the number of spores present and required additional steps in handling (saline moistening, cutting swab tips into broth) and interpretation (additional subculturing to obtain pure colonies). TCCFA-Rodac plates, however, easily sampled a larger area than swabs, recovered spores efficiently at very low concentrations, allowed their precise quantitation, and were easy to prepare and transport to a sampling site. Dry swabs and adhesive paddles were not useful.

Although prereduction of agar for 2 h was required for optimal recovery of vegetative cells of *C. difficile* (Fig. 1), prereduction of media was not necessary for maximal recovery of spores on TCCFA (Table 4). Optimal spore growth on CCFA seemed to require prereduction of agar, although the difference was <1 log$_{10}$ (Table 4). That the spore population of *C. difficile* may be the most important in nosocomial acquisition of *C. difficile*-associated diarrhea is supported by the brief (<15 min) survival of a large inoculum of purely vegetative forms. This short survival is probably due to a combination of exposure to oxygen and desiccation. Viable organisms (presumably spores) can be recovered for at least 5 months from an unused hospital floor (9).

Small numbers of *C. difficile* from an exogenous source (human or environmental contacts) may be important in nosocomial transmission of *C. difficile*-associated diarrhea and colitis. Several clusters of infection in hospitalized patients (8, 9, 14, 17) and experience with disease transmission in animals (10, 15) support this possibility. With saline-moistened swabs, *C. difficile* spores were detected more often on TCCFA or in a selective broth than on CCFA and therefore may be useful for environmental detection. However, TCCFA-Rodac plates were more convenient than swabs to use, recovered more spores, and allowed better quantitation of spores recovered from a given area. Preliminary studies in patient rooms have shown that TCCFA-Rodac plates are frequently positive when CCFA-Rodac plates are not (B. P. Buggy, and R. Fekety, unpublished data). Therefore, we suggest that TCCFA-Rodac plates show the most promise for detection of *C. difficile* spores in a hospital environment.

LITERATURE CITED

1. **Bartlett, J. G.** 1979. Antibiotic-associated pseudomembranous colitis. Rev. Infect. Dis. 1:530–539.
2. **Cudmore, M. A., J. Silva, Jr., R. Fekety, M. K. Liepman, and K.-H. Kim.** 1982. *Clostridium difficile* colitis associated with cancer chemotherapy. Arch. Intern. Med. 142:333–335.
3. **Fainstein, V., G. P. Bodey, and R. Fekety.** 1981. Relapsing pseudomembranous colitis associated with cancer chemotherapy. J. Infect. Dis. 143:865.
4. **Fekety, R. F., K.-H. Kim, D. Brown, D. H. Batts, M. Cudmore, and J. Silva, Jr.** 1981. Epidemiology of antibiotic-associated colitis: isolation of *Clostridium difficile* from the hospital environment. Am. J. Med. 70:906–908.
5. **George, W. L., R. D. Rolfe, M. E. Mulligan, and S. M. Finegold.** 1979. Infectious diseases 1979—antimicrobial agent-induced colitis: an update. J. Infect. Dis. 140:266–268.
6. **George, W. L., V. L. Sutter, D. Citron, and S. M. Finegold.** 1979. Selective and differential medium for isolation of *Clostridium difficile*. J. Clin. Microbiol. 9:214–219.
7. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore** (ed.). 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
8. **Kabins, S. A., and T. J. Spira.** 1975. Outbreak of clindamycin-associated colitis. Ann. Intern. Med. 83:830–831.
9. **Kim, K.-H., R. Fekety, D. H. Batts, D. Brown, M. Cudmore, J. Silva, Jr., and D. Waters.** 1981. Isolation of *Clostridium difficile* from the environment and contacts of patients with antibiotic-associated colitis. J. Infect. Dis. 143:42–50.
10. **Larson, H. E., A. B. Price, and S. P. Borriello.** 1980. Epidemiology of experimental enterocecitis due to *Clostridium difficile*. J. Infect. Dis. 142:408–413.
11. **Mulligan, M. E., W. L. George, R. D. Rolfe, and S. M. Finegold.** 1980. Epidemiological aspects of *Clostridium difficile*-induced diarrhea and colitis. Am. J. Clin. Nutr. 33:2533–2538.
12. **Mulligan, M. E., R. D. Rolfe, S. M. Finegold, and W. L. George.** 1979. Contamination of a hospital environment by *Clostridium difficile*. Curr. Microbiol. 3:173–175.
13. **Nakamura, S., S. Nakashio, T. Inamatsu, N. Nishida, N. Taniguchi, and S. Nishida.** 1980. Toxigenicity of *Clostridium difficile* isolates from patients and healthy adults. Microbiol. Immunol. 24:995–997.
14. **Pierce, P. F., Jr., R. Wilson, J. Silva, Jr., V. F. Garagusi, G. D. Rifkin, R. Fekety, O. Nunez-Montiel, V. R. Dowell, Jr., and J. M. Hughes.** 1982. Antibiotic-associated pseudomembranous colitis: an epidemiologic investigation of a cluster of cases. J. Infect. Dis. 145:269–274.
15. **Toshniwal, R., J. Silva, Jr., R. Fekety, and K.-H. Kim.** 1981. Studies on the epidemiology of colitis due to *Clostridium difficile* in hamsters. J. Infect. Dis. 143:51–54.
16. **Viscidi, R., S. Willey, and J. G. Bartlett.** 1981. Isolation rates and toxigenic potential of *Clostridium difficile* iso-

lates from various patient populations. Gastroenterology **81:**5–9.

17. **Walters, B. A. J., R. Stafford, R. K. Roberts, and E. Seneviratne.** 1982. Contamination and cross-infection with *Clostridium difficile* in an intensive care unit. Aust. N.Z.

J. Med. **12:**255–258.

18. **Wilson, K. H., M. J. Kennedy, and R. Fekety.** 1982. Use of sodium taurocholate to enhance spore recovery on a medium selective for *Clostridium difficile*. J. Clin. Microbiol. **15:**443–446.