Relative Frequency of *Clostridium difficile* in Patients with Diarrheal Disease

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We have studied 161 patients with diarrheal disease to determine the frequency with which *Clostridium difficile* occurs in such patients. *C. difficile* or its toxin or both were detected in stools from 19 patients (11.9%), 17 of whom had previously received antimicrobial agents. Enteric pathogens other than *C. difficile* were recovered less frequently, with *Salmonella* sp., *Giardia lamblia*, and *Campylobacter fetus* being recovered from 4.1, 2.5, and 1.3%, respectively, of the patients studied. These data suggest that *C. difficile* may be frequently encountered in specimens obtained from patients with diarrhea who receive antibiotics and may play a role in diarrheal disease in such patients.

Clostridium difficile has been implicated as an important etiological agent in antimicrobial-associated diarrheal disease and pseudomembranous colitis (1, 3, 11). The pathogenicity of this organism is due to its ability to produce an extracellular protein that has cytotoxic effects on cells in tissue culture (6, 7) and can produce disease in experimental animals similar to that seen in humans (2, 10). Although the ability of *C. difficile* to produce pseudomembranous colitis has been well established (1, 3, 11), the frequency of this organism in a general population with diarrheal disease has not been well established. In addition, the relative frequency of *C. difficile* in comparison to other enteric pathogens, such as *Salmonella* sp., *Shigella* sp., *Yersinia* sp., *Campylobacter fetus*, and intestinal parasites, has not been reported in the United States. In this paper, we present data that relate the frequency of isolation of *C. difficile* to that of other established enteric pathogens from patients with diarrheal disease.

MATERIALS AND METHODS

Patient population. The patients examined in this study were either inpatients or outpatients seen between March 1979 and April 1980 at the North Carolina Memorial Hospital, a tertiary care facility. All patients studied had watery diarrhea, which prompted a request for either bacterial stool culture or stool examination for ova and parasites. Watery diarrhea was defined as stools which took the form of the specimen container and could be easily pipetted with a pasteur pipette. Multiple specimens were often examined, with as many as seven specimens examined

per patient. Cultures for *C. fetus*, *Clostridium difficile*, *Salmonella* sp., *Shigella* sp., and *Yersinia* sp. were performed within 2 h of collection. Specimens for ova and parasites were also routinely examined for parasites within 2 h of collection. Those specimens submitted for parasitological examination that could not be examined within 2 h were mixed with 10% Formalin-0.85% saline solution until examination. Stools that were to be assayed for *C. difficile* toxin were held at -70°C until assay. All of the patients' charts were reviewed for diarrheal disease history and associated antimicrobial therapy.

Culture techniques. Of 77 patients whose specimens were cultured for *C. fetus*, 54 had their stools cultured on Skirrow medium (19). The plate medium was incubated in a candle jar at 37°C and examined daily for 3 days for colonies characteristic of *C. fetus*. Specimens from the remaining 23 patients were cultured on Campy-blood agar plates and in tubes of thioglycolate broth containing the same antibiotics found in Campy-BAP (4). The plate medium was incubated in a large GasPak jar (BBL Microbiology Systems, Cockeysville, Md.) by using two of the three catalyst baskets and two carbon dioxide-hydrogen gas generators. The plated medium was then processed, in the same manner previously described, for specimens obtained from the first 54 patients. The thioglycolate broth was incubated for 18 to 24 h in a water bath at 42°C and then subcultured to Campy-BAP and incubated as previously described. Colonies with morphology similar to that observed with *Campylobacter* colonies were examined by wet mount and, when necessary, further examined by Gram stain. Isolates which showed darting motility and a Gram stain similar to *C. fetus* were identified by using established techniques for *C. fetus* (20).

Stool specimens were examined for *C. difficile* by inoculating portions of each specimen onto a cycloserine-cefoxitin-fructose agar plate (12) which was then incubated in a GasPak jar for 48 h. Colonies ferment-

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ing fructose, not degrading lecithin, and exhibiting a spreading margin were Gram stained and, when appropriate, identified by the anaerobe identification scheme of Holdeman et al. (14).

Stool examinations for ova and parasites were done by direct mounts in saline and Lugol iodine and, on occasion, by use of the trichrome stain (17). Stools were also concentrated by using Formalin-ether concentration techniques (18) or a modified concentration technique in which ethyl acetate was substituted for ether (21). Stool concentrates were suspended in saline and Lugol iodine and examined microscopically.

Salmonella and *Shigella* spp. were cultured by inoculating 5% sheep blood, MacConkey, and Hektoen agar plates (BBL) and a GN enrichment broth with each specimen. The GN enrichment broth was subcultured after 6 to 18 h incubation at 37°C onto a Hektoen and MacConkey agar plate. The blood agar plate was used to detect overgrowth of the bowel flora with gram-positive organisms or yeasts. The MacConkey and Hektoen plates were examined for non-lactose-fermenting or hydrogen sulfide-producing organisms. Three colonies of each suspicious colony type were screened by using a triple sugar iron agar, a lysine iron agar, and a phenylalanine agar slant (8). Organisms with biochemical reactions consistent with those of either *Salmonella* or *Shigella* species were identified to serogroup by using standard biochemical and serological tests (8).

Yersinia enterocolitica was cultured by a cold enrichment method. After primary culture onto the media described above for *Salmonella* and *Shigella* spp., a stool swab was placed in 1.0 ml of phosphate-buffered saline and incubated for 3 weeks at 4°C. Subcultures were made weekly onto MacConkey and salmonella-shigella agar, and these plates were incubated at room temperature for 72 h. These culture plates were examined daily for non-lactose-fermenting colonies similar to *Y. enterocolitica*. These isolates were characterized biochemically by using triple sugar iron agar or phenylalanine agar and two tubes of motility-indole-ornithine medium; one tube was incubated at room temperature and the other was incubated at 37°C. Organisms with biochemical reactions consistent with those of *Y. enterocolitica* were identified by using appropriate biochemical tests (13). All dehydrated media used during the course of this study were supplied by either Difco Laboratories, Detroit, Mich., or BBL, unless otherwise noted.

C. difficile toxin assay. Frozen stool specimens were thawed in a water bath at 37°C. A stool sample (ca. 0.5 ml) was placed in a plastic snap-cap tube (12 by 75 mm) and diluted with an equal volume of phosphate-buffered saline containing 500 mg of cycloserine. This mixture was mixed thoroughly and then centrifuged at $2,500 \times g$ for 10 min. The stool supernatant was removed and used for the toxin assay. Those supernatants that either remained cloudy or contained particulate matter after centrifugation were filtered through a 0.45- μ m membrane filter (Acrodisc; Gelman Sciences, Inc., Ann Arbor, Mich.) before assay. Either MRC-5 or WI-38 cells lines were grown in minimal essential medium supplemented with 10% fetal calf serum and 30 μ g of gentamicin per ml. The cell mono-

layers were prepared by placement of 0.2 ml of cell suspension (at ca. 10^5 /ml) into each well of a 96-well flat-bottomed microtiter tissue culture plate (Costar, Cambridge, Mass.) and incubation at 37°C in 5% CO₂ until a monolayer formed. The assay for *C. difficile* toxin was performed by placing 50 μ l of 1:2 and 1:10 dilutions of each stool supernatant onto duplicate monolayers of one of the two fibroblast cell monolayers.

The diluted stool supernatant fluids were incubated with the cell monolayers at 37°C in 5% CO₂ for 48 h. The monolayers were examined at 12-h intervals during the incubation period. Those monolayers showing no morphological change after 48 h were considered negative. Those monolayers showing actinomorphous changes (2) in >50% of the cell monolayer treated with the diluted stool supernatant were considered positive, and a neutralization assay was performed on those supernatants. Monolayers showing toxic changes such as cell lysis or cell fusion were also tested in the neutralization assay. Stool supernatants which caused contamination of the monolayers by microorganisms were filter sterilized as described above and again assayed.

Culture supernatant from a known toxigenic strain of *C. difficile* grown anaerobically in prereduced chopped meat medium (Scott Laboratories, Fiskeville, R.I.) at 37°C for 48 h was used as the positive standard for this assay. This toxigenic strain was kindly provided by V. R. Dowell of the Centers for Disease Control, Atlanta, Ga.

Neutralization assay. Confirmation of the presence of *C. difficile* toxin in patient specimens required the neutralization of cytotoxic activity with specific antibody. Supernatants showing actinomorphous or toxic changes at a 1:10 dilution were diluted in phosphate-buffered saline-cycloserine to give a final dilution of 1:10. The diluted supernatants were then mixed with a 1:10 dilution of antiserum to *C. difficile* toxin or a 1:10 dilution of fetal calf serum. The *C. difficile* antitoxin (9) used in this study was kindly supplied by Tracy Wilkins of the Virginia Polytechnic Institute and State University. This mixture was incubated at room temperature for 30 min, and 100 μ l of the mixture was assayed as described above. Specimens causing cultured cells to demonstrate rounding in the presence of fetal calf serum and no rounding in the presence of *C. difficile* antitoxin were considered positive for the toxin.

Determination of toxin titers. Toxin titers were determined for each stool and culture supernatant fluid which was proven to be positive by the neutralization assay. Serial 10-fold dilutions of the toxin sample from 10^{-1} to 10^{-6} were prepared in phosphate-buffered saline-cycloserine, and 50 μ l of each dilution was inoculated onto duplicate monolayers of either MRC-5 or WI-38 fibroblasts. The cells were examined for 48 h at 12-h intervals. The last dilution to give greater than 50% rounding at 48 h was the toxin titer. When the culture supernatant from the control organism was used, the two cell lines proved to be equally susceptible to the toxin.

RESULTS

Patient population. Of the 161 patients studied, 91 (57%) developed diarrheal disease outside the hospital. Eighty-six patients (54%) were male, and 124 patients (76%) were over 25 years of age. A total of 129 patients (80%) had diarrheal disease of less than 4 weeks in duration; 61 of these patients (47%) had diarrhea associated with antimicrobial therapy, whereas 68 (53%) had diarrhea that was not associated with antimicrobial therapy. There were 32 patients studied who had diarrheal disease of greater than 4 weeks in duration; 16 of these patients had chronic inflammatory bowel disease, and 16 had chronic diarrhea of unknown etiology.

Culture results. Specimens from the 161 patients were examined for the presence of *C. difficile* or its toxin. Specimens from 77, 113, 145, and 85 patients were examined for *C. fetus*, intestinal parasites, *Salmonella* and *Shigella* spp., and *Yersinia* sp., respectively. Specimens from 139 patients were examined for three or more of the five groups of organisms. As can be seen in Table 1, *C. difficile* or its toxin was encountered in 12% of the patients. *Salmonella* sp. and *Giardia lamblia* were the only other organisms which were observed to occur in more than one patient. *Y. enterocolitica* and *C. fetus* subsp. *jejuni* were isolated from one patient each. *Shigella* sp. was not detected in any patient specimen. No patient studied had more than one enteric pathogen detected in his or her stools. Five additional patients who received prior antibiotic therapy had stool cultures that revealed an absence of *Enterobacteriaceae* and a predominance of *Pseudomonas aeruginosa* or yeasts.

Detection of *C. difficile* or its toxin. Of the 161 patients studied, specimens of 110 were examined for both *C. difficile* and its toxin; specimens of 51 were tested for the presence of toxin only. The results of culture and toxin assays performed on the 19 patients whose specimens

yielded *C. difficile* or its toxin were as follows. In 13 patients, both the toxin and the organism were present, in 4, only the toxin was detected, and in 2, only the organism was detected. Of the four patients in whom only *C. difficile* toxin was detected, two were not cultured for the organism and two were culture negative. One of the two patients who was toxin positive and culture negative had been treated with oral vancomycin for 24 h before culture. *C. difficile*-associated diarrhea in these four patients was diagnosed by detecting *C. difficile* toxin in their stool specimen(s). The relative concentration of *C. difficile* toxin was determined for stool specimens obtained from these 4 patients and for the 13 patients whose specimens were positive for both toxin and culture. These 17 specimens were positive for toxin at dilutions ranging from 10^{-1} to 10^{-5} . Specimens from 11 of 17 patients yielded positive toxin assays with dilutions $\geq 2 \times 10^{-2}$. No difference was observed in the levels of toxin present in stool specimens that were culture positive when compared with those that were culture negative.

Two patients had *C. difficile* detected in their specimens by culture only. Assay for toxin on these two stools was negative on three separate occasions. Both strains were tested to determine if they produced toxin in vitro. One of the two strains produced a cytotoxin when it was grown anaerobically in prereduced 1% glucose-chopped meat broth. The toxin titer produced by this organism in culture was 5×10^{-2} . The patient from whom this strain was recovered had mild diarrheal disease which resolved without therapy. The other strain that was recovered from a specimen that did not contain free toxin failed to produce the toxin when grown anaerobically. This latter strain did not produce toxin when grown in chopped meat-1% glucose, Schädler broth, thioglycolate broth, and Trypticase soy broth (BBL Microbiology Systems). The patient suffered from several daily episodes of severe diarrhea which resolved after empiric treatment with oral vancomycin. *C. difficile* could not be recovered from cultures of stool specimens obtained after completion of 5 days of vancomycin therapy.

Association of *C. difficile* with antibiotic therapy. Of the 161 patients studied, 61 received antimicrobial therapy before culture. Of these patients, 17 (28%) had *C. difficile* or its toxin present in their feces. No established enteric pathogen was found in specimens obtained from these 61 patients.

Prior antibiotic therapy was not associated with the remaining 100 patients studied, and *C. difficile* was recovered from only two of these

TABLE 1. Recovery of enteric pathogens from patients with diarrheal disease

Organism	No. of patients studied	No. (%) of patients with positive examinations
<i>C. fetus</i>	77	1 (1.3)
<i>C. difficile</i>	161	19 (10.4)
Intestinal parasites ^a	113	4 (3.4)
<i>Salmonella</i> sp.	145	6 (4.1)
<i>Shigella</i> sp.	145	0 (0)
<i>Yersinia</i> sp.	82	1 (1.2)

^a One *Ascaris lumbricoides*, three *Giardia lamblia*.

patients. One of these patients was a 2-year-old female (positive *C. difficile* culture; stool toxin concentration of 2×10^{-1}) diagnosed as having viral gastroenteritis and whose symptoms resolved without antimicrobial therapy. The other patient was a 69-year-old male (stool toxin concentration of 1×10^{-2}) who had diarrhea that resolved with supportive therapy.

Antimicrobial agents most frequently administered to patients with diarrhea that was associated with positive culture or toxin assays for *C. difficile* were penicillins, cephalosporins, and aminoglycosides. Penicillins were administered in 12 of 17 patients who had toxin assays or cultures positive for *C. difficile*. Cephalosporins and aminoglycosides were each given to 11 patients. Prior therapy with clindamycin, chloramphenicol, tetracycline, or trimethoprim-sulfamethoxazole was also associated with two or more patients that developed diarrhea and whose specimens were toxin- or culture-positive for *C. difficile*. Of these 17 patients, 11 received simultaneous therapy with two or more antimicrobial agents, one always being an aminoglycoside. Six additional patients were treated with only one antibiotic which was either a penicillin or cephalosporin. The time of onset of disease after initiation of antimicrobial therapy ranged from 2 to 23 days with an average of 13 days.

The patients who had antimicrobial agent-associated diarrhea with a positive *C. difficile* culture or toxin assay were older than 25 years. Only 5 of 12 patients who had disease caused by other enteric pathogens were older than 25. Three of these latter five patients had *G. lamblia* infections. Neither *C. difficile* nor any other enteric pathogen was found to be associated with diarrheal disease of greater than four weeks duration. All cases of diarrhea caused by established enteric pathogens were outpatients. The two patients who had *C. difficile*-associated disease but did not have a history of prior antimicrobial therapy also developed disease outside the hospital. Of the 17 patients with antimicrobial associated diarrhea with evidence of *C. difficile*, 12 developed symptoms during hospitalization, and five developed symptoms as outpatients. Three of the latter five developed symptoms before hospitalization, whereas the other two developed symptoms less than a month after release from the hospital.

Sigmoidoscopic examinations were performed on 70 of 161 patients studied (Table 2). The examination results were reported as either normal, nonspecific colitis (including inflammatory bowel disease), or pseudomembranous colitis. If the patients with inflammatory bowel disease were eliminated from the *C. difficile*-

TABLE 2. Sigmoidoscopic examination of patients with diarrheal disease

Patient type	No. of patients diagnosed as:		
	Normal	Nonspecific colitis	Pseudomembranous colitis
<i>C. difficile</i> positive ^a			
Antibiotic treatment	3	7	2
No antibiotic treatment	0	1	0
<i>C. difficile</i> negative			
Antibiotic treatment	10	8	0
No antibiotic treatment	13	11	0
Inflammatory bowel disease	2	13	0

^a Individuals had toxin or organism or both present in their stool specimens.

negative patients, *C. difficile*-positive patients had a higher percentage of abnormal sigmoidoscopies (77%) as compared with the *C. difficile*-negative patients (48%). In addition, pseudomembranes were seen on sigmoidoscopy in two of the *C. difficile*-positive patients, but in none of the *C. difficile*-negative patients.

Since the disease of most patients who were *C. difficile* culture- or toxin-positive or both was resolved by using either supportive therapy (discontinuing antibiotics, using fluid therapy, and administration of Lomotil) or vancomycin therapy, few follow-up data on this group of patients were available. Almost all of the patients included in this study who had multiple specimens tested had neither *C. difficile* nor an established pathogen recovered. One patient who was positive at admission, by both culture and toxin assay for *C. difficile*, was closely studied for a period of 2 months. She was treated for 2 weeks with oral tetracycline, 3 weeks before admission. At admission, she had severe diarrheal disease. A sigmoidoscopic examination at that time revealed diffuse edema and local inflammation, but no pseudomembranes were seen. She was also culture- and toxin-positive for *C. difficile*. Oral vancomycin (500 mg twice a day) was administered for 5 days, and her diarrhea quickly resolved. Toxin and culture studies 2 weeks after cessation of therapy were both negative. She was readmitted 10 days later with severe diarrhea and, again, was culture- and toxin-positive with a 10-fold increase in her stool toxin titer. She was again treated with the same course of vancomycin previously given and her disease once again resolved. A week after the cessation of therapy she was again culture and toxin negative and did not return with any symptoms for the next 6 months.

DISCUSSION

C. difficile has been implicated as an important etiological agent of antimicrobial-associated diarrhea and pseudomembranous colitis (1-3, 11). However, the relative frequency of this organism in patients with diarrheal disease in comparison to that of other enteric pathogens is not known. Our results indicate that *C. difficile* is an organism that may be commonly recovered from patients with diarrheal disease in our institution. A total of 90% of the *C. difficile*-positive patients were receiving or recently received antibiotic therapy; this supports the view that antibiotic therapy was an important predisposing factor for the establishment of *C. difficile* in the intestine (1-3, 11). Our finding that 100% of patients with pseudomembranous colitis and 27% of patients with antibiotic-associated diarrheal disease harbored *C. difficile* agreed with the finding of Bartlett et al. (3). In addition, we found that the concentrations of *C. difficile* toxin in the stools we examined were similar to those reported by Bartlett et al. (2). These investigators observed that the concentration of toxin varied widely in patients with *C. difficile*-associated pseudomembranous colitis, and they ascribed this finding to the differing number of organisms present in the stool specimens they tested. In our patient population, there appeared to be no correlation between toxin titer and severity of disease. However, patients whose specimens were positive for *C. difficile* had a higher percentage of abnormal sigmoidoscopic examinations when compared with patients whose stools were negative for *C. difficile*, suggesting a role for *C. difficile* in the pathogenesis of disease in our patient population. Further support for such a role in antibiotic-associated diarrheal disease was that therapy effective in pseudomembranous colitis caused by *C. difficile* was also effective in at least one patient with antibiotic-associated diarrhea with positive cultures and toxin assays. Many questions concerning the pathogenesis of antibiotic-associated diarrhea and the possible role of *C. difficile* in this disease remain unanswered.

Two of our patients had stool specimens which did not contain toxin but had cultures that yielded *C. difficile*. One of the patients had a toxigenic organism and mild diarrheal disease which resolved without treatment. The role that *C. difficile* may have played in this patient's diarrhea is difficult to assess. The other patient had severe diarrhea that resolved with oral vancomycin therapy and elimination of a nontoxigenic strain of *C. difficile* from stool specimens. The observation of disease associated with the

presence of a nontoxigenic strain of *C. difficile* may be coincidental or may suggest that the *C. difficile* produces another toxin(s) which may play a role in diarrheal disease. Clostridia, such as *C. perfringens*, produce multiple toxins (16). Therefore, the production of multiple toxins by *C. difficile* would not be unusual.

It has recently been reported that *C. difficile* may play a role in relapse in chronic inflammatory bowel disease (5, 15). None of the 16 patients included in our study who had inflammatory bowel disease and were suffering relapse had stool specimens positive for *C. difficile* or its toxin.

The role for *C. difficile* as a cause of pseudomembranous colitis is well established (1-3, 11). Data presented here and by Bartlett et al. (3) suggest that *C. difficile* may play an important role in the pathogenesis of antibiotic-associated diarrhea. Because of the proven and possible importance of this organism in severe diarrheal disease, clinical laboratories in tertiary care facilities should consider establishing techniques for the detection of *C. difficile* and its toxin.

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