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In Vitro Susceptibility of *Clostridium difficile* Isolates from Patients with Antibiotic-Associated Diarrhea or Colitis

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In vitro susceptibility tests were performed on 84 strains of *Clostridium difficile* to 11 antimicrobial agents. All isolates were from the stools of patients with antibiotic-associated diarrhea or colitis in which there was a cytopathic toxin that was neutralized by *Clostridium sordellii* antitoxin. Over 95% of the strains were susceptible to vancomycin, penicillin G, ampicillin, and metronidazole at concentrations of 4 μg/ml. Susceptibility to clindamycin was variable; 60% of the strains were susceptible at 1 μg/ml, and 9% were resistant at 128 μg/ml. Studies of individual isolates showed that a major portion of the strains were relatively susceptible to the antimicrobial agent implicated in causing the disease.

Recent studies have implicated *Clostridium difficile* as the causative agent of antimicrobial agent-associated pseudomembranous colitis. Evidence to support this conclusion is based on the high isolation rate of *C. difficile* in stools (2, 3, 8, 10, 13, 14), the demonstration of a clostridial cytotoxin in these specimens (2, 3, 13, 14), and studies showing that *C. difficile* produces a similar or identical cytotoxin in vitro (4). Additionally, experimental animal work indicates that *C. difficile* causes an anatomically similar disease in hamsters after antimicrobial agent administration (5). The lesion of this disease may be reproduced with an intracecal injection of either broth cultures or partially purified *C. difficile* cytotoxin (15).

Previous studies have shown that the role of *C. difficile* and its cytotoxin in diarrheal disease is almost exclusively restricted to patients with antimicrobial agent exposure (2, 3, 14). One interpretation of this observation is that the organism is resistant to the agent administered and thus flourishes while competing components of the colonic floral are suppressed (6, 11, 18). This concept is supported by previous studies in animals which showed that clindamycin administration to hamsters resulted in typhlitis involving clindamycin-resistant strains of *C. difficile* (5). A similar observation was noted with tetracycline administration in these animals (18). Recent work has shown that ampicillin also produces a comparable lesion in hamsters, but the strains of *C. difficile* recovered in stools proved susceptible to ampicillin at levels of 0.5 μg/ml or less. This observation suggested that superinfection with resistant strains was an overly simplistic explanation for the pathophysiology of antibiotic-induced colitis in the animal model.

The purpose of the present report was to test antimicrobial susceptibilities of *C. difficile* isolates recovered from the stools of patients with antibiotic-associated diarrhea or colitis and to correlate these findings with the pathogenesis of the disease.

MATERIALS AND METHODS

Source of *C. difficile* isolates. All strains of *C. difficile* were recovered from stools of patients with antibiotic-associated diarrhea or colitis. Each of these specimens contained a cytotoxin which was neutralized by *Clostridium sordellii* antitoxin by the techniques described below. Stool cultures to recover *C. difficile* were made by a modification of the method described by George et al. (9). Samples of 0.1 ml of four, serial 100-fold dilutions (10^{-2} to 10^{-8}) were plated onto brucella base blood agar and brain heart infusion agar containing 5% sheep blood, 250 μg of cycloserine per ml, and 10 μg of cefoxitin per ml. Organisms with the typical colonial morphology of *C. difficile* were isolated and identified by established procedures (12). These isolates were tested for in vitro production of a cytotoxin which is neutralized by *C. sordellii* antitoxin, using chopped meat-glucose broth cultures (Scott Laboratories, Inc., Fiskeville, R.I.) after incubation in an aerobic chamber for 5 days.

Tissue cultures. Toxin assays were performed on stools and broth cultures of *C. difficile* isolates. Tissue cultures were made with human embryonic lung fibroblasts (HEM Research, Rockville, Md.) in flat-bottomed microtiter wells containing 50 μl of medium 199 (Microbiological Associates, Walkersville, Md.) supplemented with 5% fetal calf serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 U/ml). The samples were centrifuged at 3,000 × *g* for 15 min, and the supernatant was filtered with a 0.45 μm membrane filter. Samples of 25 μl of the cell-free supernatant were combined with 25 μl of phosphate-buffered saline and added to the wells. Duplicate wells were inoculated with 25-μl samples combined with 25 μl of

C. sordellii antitoxin (Bureau of Biologics, Rockville, Md., lot S1 or 40067-3647). Results were read at 24 h. The criteria for a positive assay were typical actinomorphic changes affecting at least 50% of the cells and the complete neutralization of cytopathic changes by the antitoxin.

Antibiotic susceptibility testing. Organisms identified as *C. difficile* were subcultured with chopped meat-glucose broth and streaked for purity onto brucella base blood agar. Five to six representative colonies were then inoculated into 5 ml of prereduced, anaerobically sterilized, supplemented brain heart infusion broth (Scott Laboratories, Inc.), and incubated anaerobically for 6 to 8 h. Serial dilutions were then performed with supplemented brain heart infusion broth to achieve a turbidity approximating a no. 0.5 McFarland standard. Quantitative cultures indicated an inoculum of 10^5 to 10^6 viable colony-forming units per ml.

Susceptibility tests were performed with microtiter plates (Cooke Engineering Co., Alexandria, Va.) (1). Serial twofold dilutions in 50 μl of supplemented brain heart infusion broth were prepared in the wells with the following agents: penicillin G (Eli Lilly & Co., Indianapolis, Ind.), cephalothin (Eli Lilly & Co.), cefoxitin (Merck Sharp & Dohme, West Point, Pa.), vancomycin (Eli Lilly & Co.), gentamicin (Schering Corp., Bloomfield, N.J.), chloramphenicol base (Sigma Chemical Co., St. Louis, Mo.), clindamycin hydrochloride (The Upjohn Co., Kalamazoo, Mich.), tetracycline (Bristol Laboratories, Syracuse, N.Y.), ampicillin (Sigma Chemical Co.), metronidazole (Amersham Corp., Arlington Heights, Il.), and erythromycin (USPC, Inc., Rockville Md.). The wells were inoculated with 50 μl of the test isolate, as was an additional growth control well which contained no antimicrobial agents. A stock strain of *Clostridium perfringens* (no. 249) was tested with the antimicrobial agents in each experimental run as a control. The trays were sealed with a sterile lid, incubated in an anaerobic chamber, and read at 16 to 18 h. The minimum inhibitory concentration was the lowest concentration of the antimicrobial agent which showed no visible growth.

Clinical correlations. Clinical records were reviewed to determine endoscopic observations and the antibiotic treatment history before the onset of symptoms.

RESULTS

Clinical observations. Isolates of *C. difficile* were tested from 84 patients with diarrhea ascribed to antimicrobial agent usage. In each instance, the stool source of the isolate was shown to contain a cytotoxin which was neutralized by *C. sordellii* antitoxin. Broth cultures of all strains also contained a cytotoxin which was neutralized by this antitoxin, suggesting that these organisms accounted for cytopathic changes with the original specimen. Endoscopic studies showed that 60 patients had pseudomembranous colitis, 2 had colitis without pseudomembrane formation, and 14 had erythema and edema of the intestinal mucosa; 8 individuals failed to undergo endoscopic examination. Antimicrobial histories in these 84 patients implicated the following agents: clindamycin (26 patients), ampicillin (23 patients), cephalosporins (12 patients), sulfamethoxazole-trimethoprim (3 patients), penicillin G (2 patients), erythromycin (2 patients), chloramphenicol (1 patient), and tetracycline (1 patient); 14 patients received various combinations of these agents.

In vitro susceptibility. Results of in vitro susceptibility testing are summarized in Table 1. These show that 99% of the organisms were susceptible to ampicillin, penicillin G, metronidazole, and vancomycin at concentrations of 4 μg/ml or less. Chloramphenicol inhibited 72% of the strains at 1 μg/ml, and all but two of the isolates were susceptible to 32 μg/ml. Tetracycline was active against most strains, with 89% being susceptible to 1 μg/ml. Susceptibility to clindamycin was variable and is discussed in more detail below. Erythromycin showed a bimodal distribution: 91% of strains were susceptible to 2 μg/ml, whereas 8% were resistant at 64 μg/ml. Cefoxitin, cephalothin, and gentamicin were relatively inactive against *C. difficile*.

Correlation of in vitro susceptibility and agent implicated. Susceptibility of *C. difficile*

TABLE 1. *Susceptibility of 84 strains of C. difficile to 11 antimicrobial agents*

Drug	Cumulative % inhibited at concn (μg/ml) of:							
	1	2	4	8	16	32	64	128
Ampicillin	82	93	99	100				
Cefoxitin	0	0	0	0	15	44	59	95
Cephalothin	0	0	0	11	49	90	97	100
Chloramphenicol	72	79	87	92	93	98	100	
Clindamycin	60	70	77	86	86	87	87	91
Erythromycin	89	91	92	92	92	92	92	93
Gentamicin	0	0	0	1	6	10	36	74
Metronidazole	98	99	99	99	99	99	99	99
Penicillin G	92	99	99	99	100			
Tetracycline	89	89	90	91	93	97	98	100
Vancomycin	86	96	99	100				

isolates from 26 patients with clindamycin-associated diarrhea showed that 11 were susceptible to 1 μg of clindamycin per ml, whereas 6 were resistant at 64 μg/ml. Resistance to clindamycin was increased in this group compared with isolates from patients in whom other antimicrobial agents were implicated (Fig. 1). All strains of *C. difficile* were relatively susceptible to ampicillin regardless of the antimicrobial agent implicated as the cause of diarrhea or colitis. Of the 23 strains recovered from patients with ampicillin-associated diarrheal complications, 20 were susceptible to 1 μg of ampicillin per ml, and all were susceptible to 2 μg/ml. The median minimum inhibitory concentration of cephalothin was 16 μg/ml for *C. difficile* isolates recovered from the 12 patients with diarrhea ascribed to cephalosporins; one isolate in this group was resistant at 64 μg/ml. The number of patients in whom other antimicrobial agents were implicated is too small for a meaningful analysis. Nevertheless, it is noteworthy that the strains recovered from the five patients who had received tetracycline, erythromycin, or penicillin were all susceptible to 2 μg/ml or less for each of these agents. The strain of *C. difficile* recovered from the patient with chloramphenicol-associated pseudomembranous colitis was susceptible at the 32 μg/ml level. Susceptibility to sulfamethoxazole-trimethoprim was not tested.

DISCUSSION

Previous investigators have studied the susceptibility profiles of *C. difficile* to various antimicrobial agents. George et al. reported these data for 39 strains (9), and Fekety studied 15 strains (6). The overall results in these two studies were similar to the findings recorded in the present study. However, both prior reports employed strains in which the vast majority of isolates was from a source other than stools of patients with diarrheal complications of antimicrobial usage.

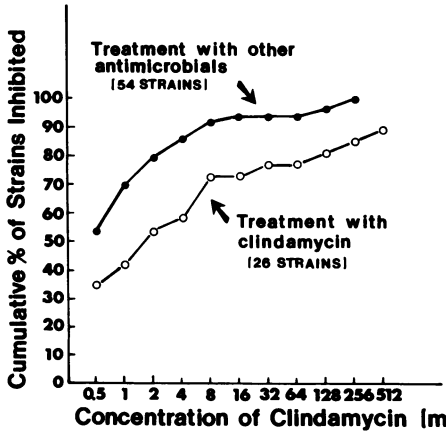

FIG. 1. *Activity of clindamycin against C. difficile isolates from 26 patients who had received clindamycin and 54 patients who had received other antimicrobial agents. (Four patients had received clindamycin in combination with other drugs listed in Table 1 and are not included.)*

The present study was designed to focus attention on susceptibilities of *C. difficile* isolates from patients with antimicrobial agent-associated diarrhea or colitis and to correlate these results with the agent implicated. All isolates were from stools which contained a cytopathic toxin that could be neutralized with *C. sordellii* antitoxin. These specimens were selected to provide some assurance that the *C. difficile* stains tested were responsible for the gastrointestinal complications. This contention was further supported by the demonstration that all isolates produced a similar or identical cytotoxin in vitro.

The results of in vitro susceptibility testing showed that a major portion of *C. difficile* isolates were relatively susceptible to the agent which was causally implicated. Of the 84 strains tested, 68 were from patients who had previously received a single antimicrobial agent. Of these 68 strains, 34 were susceptible to this agent at a concentration of 2 μg/ml. The paradox is well illustrated with ampicillin, which is one of the drugs that is most frequently implicated in causing pseudomembranous colitis due to *C. difficile*. The 84 strains of *C. difficile* in the present study were relatively susceptible to ampicillin, and this includes all 23 strains recovered from stools of patients with ampicillin-associated diarrhea. Curiously, the in vitro activity of ampicillin was remarkably similar to that of vancomycin. To our knowledge, there are no reported cases of pseudomembranous colitis due to vancomycin, and this agent has been found to be remarkably effective in the treatment of pseudomembranous colitis (13, 17).

The interpretation of minimum inhibitory concentrations is obviously complicated by the fact that fecal levels of antimicrobial agents are substantially different from the serum levels which are traditionally used to judge antimicrobial susceptibilities. Antimicrobial levels of the implicated agent were not examined in the specimens which served as the source of the *C. difficile* isolates because the majority were collected several days or weeks after diarrhea was established and the implicated drug had been discontinued. Another consideration is the temporal relationship between the onset of diarrhea and the antimicrobial agent administration. It has been noted that a number of patients with pseudomembranous colitis have the onset of

diarrhea after the antimicrobial agents have been discontinued (16). This poses the possibility that strains of *C. difficile* which are relatively susceptible to the implicated agent could propagate at the time of decreased colonic levels (11). Our findings do not necessarily support this concept since 15 of the 23 patients with ampicillin-associated disease had the antibiotic discontinued due to the onset of diarrhea. Experimental animal studies have shown that oral penicillin administration to hamsters results in high fecal concentrations of β-lactamase and negligible levels of biologically active penicillin and that the animals develop typhlitis during antimicrobial agent administration despite in vitro susceptibility of *C. difficile* to the inducing agent (7). It is possible that a similar mechanism applies to patients receiving ampicillin. Among the patients with clindamycin-associated diarrhea or colitis, 11 had isolates which were susceptible to clindamycin at 1 μg/ml or less, and six of these strains were recovered from patients who noted the onset of symptoms during therapy with clindamycin. Review of these six patients showed that the drug was given parenterally to four and orally to two.

The observations in this study suggest that antibiotic-associated pseudomembranous colitis is more complicated than a simple superinfection with *C. difficile*. However, other factors which may be critical in the interactions among this organism, the fecal flora, and antimicrobial agents remain to be elucidated.

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