INFECTION AND IMMUNITY, July 1991, p. 2456–2462
0019-9567/91/072456-07$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 59, No. 7

Correlation of Immunoblot Type, Enterotoxin Production, and Cytotoxin Production with Clinical Manifestations of *Clostridium difficile* Infection in a Cohort of Hospitalized Patients

LYNNE V. McFARLAND,[1,2]* GARY W. ELMER,[2] WALTER E. STAMM,[1,3] AND MAURY E. MULLIGAN[4]

Department of Epidemiology,[1] Department of Medicinal Chemistry,[2] and Department of Medicine,[3] University of Washington, Seattle, Washington 98195, and Infectious Disease Department, Long Beach VA Medical Center, Long Beach, California 90822, and University of California, Irvine, School of Medicine, Orange, California 92668[4]

Received 14 February 1991/Accepted 23 April 1991

To determine whether strain-specific differences in immunoblot type, enterotoxin production, or cytotoxin production correlated with clinical presentation of *Clostridium difficile* infection, we evaluated isolates obtained from 428 prospectively studied hospitalized patients. Of 99 isolates available for immunoblot typing, 61 were recovered from asymptomatic carriers and 38 were from patients with *C. difficile*-associated diarrhea. Of 17 immunoblot types, the seven types comprising the majority of isolates (82 of 99; 83%) were variably associated with disease. Neither the presence of cytotoxin in the stool nor the production of cytotoxin or enterotoxin by isolates in vitro was significantly different for symptomatic versus asymptomatic patients. Selected host factors were more predictive of symptomatic disease than was the specific infecting *C. difficile* strain. These results suggest that variations in the clinical severity of *C. difficile* infection in different patients are not solely strain-specific phenonema related to immunoblot type or to the production of cytotoxin or enterotoxin.

In studies of adult hospitalized patients, acquisition of *Clostridium difficile* results in a spectrum of illness ranging from asymptomatic carriage to diarrhea or colitis (10, 16, 21). Following the 1974 report of Tedesco et al. (32), who described an outbreak of *C. difficile*-associated pseudomembranous colitis, the importance of nosocomial outbreaks of *C. difficile*-associated diarrhea or colitis has been recognized (1, 5, 14, 17). Extended-care facilities have also reported outbreaks of diarrhea caused by *C. difficile* (2, 34). Continued investigation has also documented the high frequency of asymptomatic colonization in some settings (14, 21). It is not clear why some patients develop disease while others are simply colonized with no apparent clinical manifestations. The pathogenic role of *C. difficile* is usually readily apparent for the patients who develop the most severe form of disease, pseudomembranous colitis. However, in many cases, the classic features of this serious illness are absent and it is difficult to determine the role of *C. difficile*, especially in an endemic setting in which asymptomatic colonization is common.

One possible explanation for the variety of clinical presentations associated with *C. difficile* is that specific strains vary in their intrinsic ability to cause disease. Although *C. difficile* isolates can produce up to four virulence factors, only two toxins have been well characterized (19, 27). Enterotoxin (toxin A), which causes fluid accumulation in rabbit ileal loop assays and increases vascular permeability, is thought to play a major role in disease production, but commercially available assays are not generally available at present (6, 12, 19). Cytotoxin (toxin B), which is highly cytotoxic in tissue culture assays, also has a role in pathogenesis. The cytotoxin tissue culture assay is the standard test for diagnosing *C. difficile*-associated colitis or pseudomembranous colitis. However, results may be less predictive of *C. difficile*-associated diarrhea (13, 18, 19). Studies in animals have demonstrated that colonization with a nontoxigenic *C. difficile* strain may be protective prior to challenge with a toxigenic strain (38). Several clinical studies have also suggested the possibility of strain-specific differences in virulence (7, 24, 26, 33, 40).

In this study, we used immunoblotting to type *C. difficile* isolates collected in a prospective study from a large number of hospitalized adults and compared the typing results with clinical manifestations associated with each isolate. Our first objective was to determine whether diarrhea was more frequently observed after acquisition of specific *C. difficile* immunoblot types. Another objective was to determine whether production of toxins was associated with specific immunoblot types. In addition, we examined cytotoxin production (the current "gold standard" for the diagnosis of *C. difficile* disease) and enterotoxin production to assess the predictive values of each toxin for symptomatic disease. Our last objective was to evaluate the relative importance of these factors as they relate to the organism and compare them with host factors that have been shown to be associated with *C. difficile* disease. This study was conducted in an institution in which *C. difficile* is highly endemic and did not encompass a discrete outbreak of pseudomembranous colitis during the time of the study.

MATERIALS AND METHODS

Patient source. All consecutive consenting adult patients admitted to one general medicine ward at Harborview Medical Center, Seattle, Wash., during an 11-month period were enrolled. These patients were studied as part of a prospective evaluation of the nosocomial acquisition and transmission of *C. difficile* (21). All patients had a stool specimen or rectal swab obtained for culture within 48 h of admission to the ward. Patients were interviewed daily until discharge, and clinical signs and symptoms were noted. Every 3 to 5 days, a rectal swab or stool sample was obtained for *C. difficile* culture. If *C. difficile* acquisition or diarrhea oc-

* Corresponding author.

curred, specimens were collected more frequently. Patients were monitored and cultured until discharged from the study ward. Throughout hospitalization, all medications and medical procedures were reviewed.

Clinical status. Patients were placed into one of three categories according to their clinical status: (i) asymptomatic carriage of *C. difficile*, (ii) *C. difficile*-associated diarrhea, or (iii) *C. difficile* colitis (either nonspecific or pseudomembranous colitis). *C. difficile*-associated diarrhea was ascertained by daily patient interview and was defined by three criteria: (i) a change of normal bowel habits with three or more loose stools a day for ≥2 consecutive days, (ii) diarrhea not attributed to another apparent etiology (infectious, medicinal, or mechanical) that occurred at the same time as positive cultures, and (iii) symptoms occurring shortly after or during the time that *C. difficile*-positive cultures were obtained. Colitis was defined by using standard endoscopic criteria (9).

Microbiologic methods. Stool specimens or rectal swabs were inoculated onto selective difficile agar plates containing cycloserine (0.25 mg/ml) and cefoxitin (16 mg/ml) (Prepared Media Laboratories, Tualatin, Ore.) and then incubated anaerobically in a GasPak jar (BBL Microbiology Systems, Cockeysville, Md.) for 48 h at 35°C before being examined for characteristic growth (22). In addition to plate cultures, all stool samples (1:10) and rectal swabs (in 0.5 ml of broth) were inoculated into a broth to facilitate the detection of low concentrations of *C. difficile* and to stimulate spore germination. The prereduced peptone broth (Becton Dickinson, Rutherford, N.J.) was supplemented with cefoxitin (39 μg/ml) and 0.1% sodium taurocholate (98% pure; Sigma Chemical Co., St. Louis, Mo.) and then incubated at 35°C for 3 days (4). The identities of the strains were confirmed by using a gas-liquid chromatography method previously reported (13).

Cytotoxin assay. Presence of cytotoxin was measured in vivo with fresh stool specimens (not rectal swab samples) and in vitro with the supernatants of pure broth cultures, using the procedure described by Willey and Bartlett (37) but substituting human tonsil fibroblastic cells. Fresh stool (assayed within 24 h of collection) was diluted 1:3 in Hanks balanced salt and antibiotic solution, centrifuged (3,000 rpm for 10 min), and filtered (0.8-μm pore size). Serially diluted filtered stool (1:1 to 1:1,000) was then added to cell cultures and observed for cytopathic effect (CPE) at 24 and 48 h. The specificity of the CPE was checked by neutralization using *Clostridium sordellii* antitoxin, and a known positive *C. difficile* control was used with every assay. Samples showing CPE in only undiluted (1:3) stool were not considered positive for cytotoxin.

Cytotoxin production by isolates was tested in vitro by inoculating three typical colonies from a difficile plate into a prereduced brain heart infusion broth (Difco, Detroit, Mich.) with an anaerobic venting unit and incubated for 3 days at 35°C. Isolates were transferred at least twice in brain heart infusion broth. Each isolate was allowed to grow for 3 days (for maximum cytotoxin production) and spun (20,000 rpm for 20 min), and the supernatant was assayed for cytotoxin as described above. Isolates showing CPE at dilutions of $>10^2$ (of 1 g of stool or 1 ml of culture supernatant) were classified as high-cytotoxin-producing strains, and strains producing CPE only at dilutions of 10^0 to 10^2/g of stool or ml of culture supernatant were classified as low-cytotoxin-producing strains. The limit of this standard cytotoxin assay is 3 to 5 ng/ml (15). Isolates with low or negative titers were retested at least three times to determine the validity of the result.

Enterotoxin assay. Prior to assay, isolates were transferred twice in brain heart infusion broth and incubated for 3 days at 35°C. The culture supernatant was spun (20,000 rpm, 20 min) and tested for enterotoxin (toxin A) by using the double-sandwich enzyme-linked immunosorbent assay technique described by Mahe et al. (20) and summarized as follows. Microtiter plates (Flow Laboratories, McLean, Va.) were coated with pork antitoxin A, washed, and incubated with the samples to be tested. After washing, rabbit antitoxin (purified according to Sullivan et al. [29]) was added, and the plates were incubated and washed again. Anti-rabbit alkaline phosphatase conjugate and substrate were then added to reveal the enzymatic activity. The limit of detection was approximately 0.2 ng of enterotoxin per ml. Isolates with low titer had <200 ng of enterotoxin per ml, and high-titer isolates produced ≥200 ng/ml. Isolates with low or negative titers were retested at least three times to determine the validity of the result.

Immunoblot typing. *C. difficile* isolates from 99 patients were coded and immunoblot typed by personnel blinded to the clinical status of the patients. Immunoblot typing was performed by using an enzyme-linked immunoelectrotransfer blotting method as previously described (25). Hyperimmune sera prepared by immunizing individual rabbits with a variety of *C. difficile* strains were used as the antibody source. Initial typing was performed by using individual rabbit sera and then, for representative isolates, compared with results obtained with pooled sera.

Statistical methods. Differences in group proportions were assessed using χ^2 (or Fisher's exact test if the sample size was small). Significant differences in mean values were assessed by the Student *t* test. Two-tailed tests of significance at the $P < 0.05$ level were used to determine statistical significance. Predictive value of a positive test was calculated as the proportion of those patients with a positive test or assay with *C. difficile*-associated diarrhea or colitis (36).

Risk index. A risk index was calculated from a multivariate model quantifying relative risks of host factors and in-hospital exposures described previously (23). The model is given by the equation: $\log P = \beta_1$ (age) $+ \beta_2$ (severity of underlying disease) $+ \beta_3$ (cephalosporins) $+ \beta_4$ (penicillins) $+ \beta_5$ (gastrointestinal stimulants) $+ \beta_6$ (enemas). The index was based on an additive model, and values for host factors were as follows: β_1 equaled 5.8 (for ages 41 to 60), 9.6 (for ages 61 to 75), and 7.9 (for ages over 76); β_2 equaled 5.2 (for extreme illness); β_3 equaled 2.1 (if exposure to cephalosporins was for 1 week or less); β_4 equaled 3.4 (for 8 to 14 days of penicillin use); β_5 equaled 3.1 if the patient was exposed to stimulants; and β_6 equaled 3.3 if the patient was exposed to enemas. All other nonsignificant factors were coded zero if not otherwise defined above. High-risk hosts were those patients having a risk index of >15, and low-risk hosts had a risk index of ≤15.

RESULTS

Study population. Of the 428 study patients, 112 had cultures positive for *C. difficile* at some time during their stay on the study ward. Eighty-three (74%) acquired *C. difficile* while on the study ward, and the remainder were colonized upon admission. The 99 strains available for typing were isolated from 61 patients who were asymptomatic carriers (62%), from 35 patients with diarrhea (35%), and from 3 (3%) with colitis (2 with nonspecific colitis and 1 with

TABLE 1. Diagnostic factors characterizing patients with in *C. difficile*

Patient group	Diagnostic factor: Microbiologic: Mean CFU/g of stool	Stool cytotoxin No.	Stool cytotoxin %	Isolate cytotoxin No.	Isolate cytotoxin %	Isolate enterotoxin No.	Isolate enterotoxin %	Clinical: Fever >37.5°C No.	Fever >37.5°C %	Fecal leukocytes No.	Fecal leukocytes %	Abdominal pain No.	Abdominal pain %	Nausea or vomiting No.	Nausea or vomiting %	Leukocytosis[a] No.	Leukocytosis[a] %	Duration of diarrhea (days)
Asymptomatic carrier (n = 61)	9.0×10^4	35	57	54	88	53	86	25	41	2	4	7	12	12	20	9	15	0
Symptomatic																		
C. difficile diarrhea (n = 35)	7.0×10^6	26	74	32	91	32	91	21	60	8	40[b]	5	14	12	34	7	20	6.7 ± 5.9
C. difficile colitis or pseudomembranous colitis (n = 3)	8.3×10^6	2	67	3	100	3	100	1	33	2	67[b]	2	67[b]	2	67[b]	2	67	10 ± 13[b]

[a] Above 15,000 leukocytes per mm^3; some patients were missing clinical measurements, and percentages reflect this.
[b] $P < 0.05$ compared with asymptomatic patient data.

pseudomembranous colitis). The majority of symptomatic patients did not have colitis (Table 1), but the diarrhea associated with *C. difficile* was severe in that it was prolonged (mean = 7 days), and fecal leukocytes were found significantly more often in patients with diarrhea (8 of 20, or 40%) than in asymptomatic carriers (2 of 50, or 4%; Fisher's exact test, $P < 0.01$). Patients with *C. difficile* diarrhea and colitis were grouped together because the primary physician felt that endoscopic examinations were warranted only for 13 patients, and microbiologic and clinical parameters given in Table 1 were similar for each group. Of the 38 patients with symptomatic disease, 7 were treated with vancomycin, 2 were treated with metronidazole, 7 had the inciting antibiotic discontinued, and 22 had no therapeutic intervention.

Association of immunoblot type with presence of disease. Overall, the 99 clinical isolates examined were classified into 17 distinct immunoblot groups (Table 2). Reliability of the immunoblot typing method was tested by comparing different sources of rabbit sera and blinded duplicate isolate submissions. The specific immunoblot patterns that were obtained with pooled sera used as the antibody source were different from those obtained with rabbit sera prepared against a single immunizing isolate, but clinical isolates were classified into the same immunoblot type regardless of the source of the sera. Duplicate samples of clinical isolates prepared by using blinded random codes gave identical immunoblot results in all cases.

The association between clinical status of the patient and immunoblot types is presented in Table 2. The most frequently isolated immunoblot types (types 1, 2, 4, and 5) were variably associated with disease. The most common immunoblot type (type 1) was associated with disease in 11 symptomatic patients but was also recovered from 16 asymptomatic carriers. Several immunoblot types were recovered only from symptomatic patients (types 6, 10, 17, and 18) or only from asymptomatic carriers (types 3, 11, 12, 13, 15, and 16), but the numbers of isolates in these types were small.

TABLE 2. Immunoblot type frequencies by clinical status of *C. difficile*-colonized patients

Immunoblot type	Strains from: Asymptomatic carriers No.	Asymptomatic carriers %	Symptomatic patients No.	Symptomatic patients %	Total
1	16	59	11	41	27
2	11	73	4	27	15
3	5	100	0		5
4	10	62	6	38	16
5	6	60	4	40	10
6	0		1	100	1
7	3	60	2	40	5
9	1	25	3	75	4
10	0		1	100	1
11	1	100	0		1
12	1	100	0		1
13	1	100	0		1
14	2	40	3	60	5
15	3	100	0		3
16	1	100	0		1
17	0		2	100	2
18	0		1	100	1
Total	61		38		99

Production of toxins by immunoblot type. Of the 17 different immunoblot types, 11 (65%) of the groups were composed of isolates that always produced detectable cytotoxin in vitro, 5 types (29%) had variable cytotoxin production (types 1, 2, 4, 5, and 14), and the single isolate of type 12 (6%) did not produce detectable cytotoxin (Fig. 1). Within specific immunoblot types, there were no statistical differences in the amount of cytotoxin produced for isolates from symptomatic versus asymptomatic patients ($\chi^2 = 1.6$, $P = 0.44$, df = 2).

Enterotoxin production also varied within immunoblot types (Fig. 2). Of the 27 isolates capable of high-titer enterotoxin production, 13 (48%) were found in types 1 and 4. Immunoblot type 2 was composed of a significantly higher frequency of enterotoxin-negative isolates than were other immunoblot types. Of the 11 isolates with no detectable enterotoxin, 8 (73%) were of type 2 (Fisher's exact test, $P = 5.0 \times 10^{-6}$), but enterotoxin-negative type 2 isolates were equally frequent in asymptomatic carriers and symptomatic patients (Fisher's exact test, $P = 0.66$). Each isolate with negative enterotoxin results was retested three times, and the results were identical.

Association of cytotoxin with presence of disease. We compared both the presence of cytotoxin in fresh stool and the ability of a patient's isolate to produce cytotoxin in vitro with the presence or absence of disease. The frequency of detectable stool cytotoxin was higher in patients with diarrhea or colitis (28 of 38, or 74%) than in asymptomatic carriers (35 of 61, or 57%; $\chi^2 = 2.03$, $P = 0.15$), but this difference was not significant. The frequency of cytotoxin

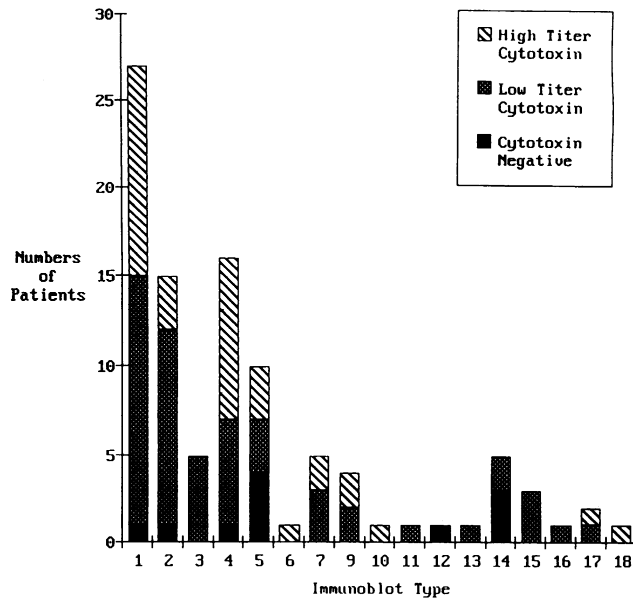

FIG. 1. Frequency of cytotoxin production by isolates from patients with *C. difficile* carriage, diarrhea, or colitis by immunoblot type. High-titer cytotoxin is defined as CPE present at dilutions of >1:100 per ml of culture filtrate; low-titer cytotoxin had CPE at dilutions less than or equal to 1:100.

production in vitro was higher in both groups than was frequency of cytotoxin production in the stool but was similar among isolates from patients with diarrhea (35 of 38, or 92%) and asymptomatic carriers (54 of 61, or 88%; Fisher's exact test, $P = 0.42$). Further analysis comparing the amount of cytotoxin produced by an isolate in vitro

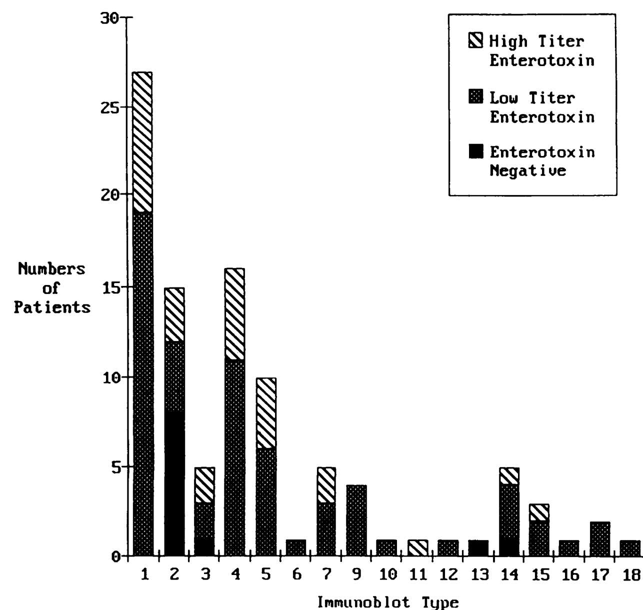

FIG. 2. Frequency of enterotoxin production by isolates from patients with *C. difficile* carriage, diarrhea, or colitis by immunoblot type. Low-titer enterotoxin production is defined as <200 ng of enterotoxin per ml; high titer is defined as ≥200 ng/ml.

TABLE 3. Cytotoxin production by *C. difficile* isolates from asymptomatic carriers and symptomatic patients

Cytotoxin titer[a]	Asymptomatic carriers		Symptomatic patients	
	No.	%	No.	%
None	7	11.5	3	7.9
Low	35	57.4	18	47.4
High	19	31.1	17	44.7
Total	61		38	

[a] Low, CPE at $\leq 10^2$/ml of culture filtrate; high, CPE at $>10^2$/ml of culture filtrate.

versus the presence or absence of disease (Table 3) also failed to show any significant differences between strains from asymptomatic carriers and symptomatic patients ($\chi^2 = 1.92$, $P = 0.4$). For 7 (18%) of the symptomatic patients, there was no detectable stool cytotoxin, but the isolates were found to produce cytotoxin in vitro. Nineteen (31%) of the carriers had no detectable stool cytotoxin but were found to have isolates capable of cytotoxin production in vitro.

The ability of cytotoxin assays to distinguish between patients with *C. difficile* diarrhea or colitis and patients with asymptomatic carriage was thus limited. The positive predictive value for *C. difficile* diarrhea in stool cytotoxin assays was 28 of 63 (44%), and that for cytotoxin production by an isolate was 35 of 89 (39%).

Association of enterotoxin with presence of disease. The frequency of enterotoxin production by isolates in vitro was similar for isolates from patients with asymptomatic carriage and those with symptomatic disease. Of the 38 isolates from symptomatic patients, 35 (92%) produced enterotoxin in vitro, compared with 53 (87%) of the 61 isolates from asymptomatic carriers (Fisher's exact test, $P = 0.32$). In addition, the median enterotoxin production by isolates from symptomatic patients (77 ng/ml) was not significantly different from the median in isolates from carriers (139 ng/ml; $\chi^2 = 1.6$, $P = 0.2$).

Interaction with host factors. With use of a multivariate model, patients were classified as high-risk hosts exposed to several known *C. difficile* risk factors or low-risk hosts not exposed to multiple risk factors. Patients with symptomatic disease were more frequently found to be high-risk hosts. Eighteen (47%) of patients with symptomatic disease had a risk index of >15, and significantly fewer ($n = 12$, or 20%) of the asymptomatic carriers were categorized as high-risk hosts ($\chi^2 = 5.8$, $P = 0.02$). Data stratified by the host risk factor index suggested that high-risk patients made susceptible by exposure to multiple risk factors may not require isolates capable of high toxin production to cause symptomatic disease (Table 4). In high-risk patients, isolates capable of producing high titers of cytotoxin were slightly more frequent in symptomatic patients ($n = 6$, or 33%) than in asymptomatic carriers ($n = 1$, or 8%) ($P = 0.12$). In low-risk patients, isolates capable of high-titer cytotoxin production were significantly more common in symptomatic patients ($n = 11$, or 55%) than in carriers ($n = 12$, or 24%) ($P = 0.02$). Isolates capable of high enterotoxin production were cultured more commonly from carriers than from symptomatic patients regardless of host risk category (Table 4). There was no difference in the infecting strain's immunoblot type between high-risk and low-risk hosts.

TABLE 4. Interactions of host risk factor index, production of toxin by *C. difficile* isolates, and production of disease

Factor	Symptomatic patients (n = 38)	Asymptomatic carriers (n = 61)	P value
High-risk hosts[a]			
Total no. of isolates	18	12	
No. (%) with high-titer production of:			
Cytotoxin	6 (33)	1 (8)	0.12
Enterotoxin	3 (17)	6 (50)	0.06
Low-risk hosts[b]			
Total no. of isolates	20	49	
No. (%) with high-titer production of:			
Cytotoxin	11 (55)	12 (24)	0.02
Enterotoxin	1 (5)	17 (35)	0.01

[a] Multivariate host risk factor index of >15.
[b] Multivariate host risk factor index of ≤15.

DISCUSSION

Because acquisition of *C. difficile* may be associated with a variety of clinical manifestations ranging from asymptomatic carriage to life-threatening colitis, diagnostic tests that indicate the presence of the organism (or its toxins) do not ensure that there is a pathogenic role for *C. difficile*. One possible explanation is that different strains vary in pathogenicity. As early as 1982, a nosocomial outbreak was found to be associated with isolates of *C. difficile* with a distinctive antibiotic susceptibility pattern (2). More recent studies of *C. difficile* outbreaks have suggested that there may be an association between the isolate type, clinical manifestations, and production of toxins by *C. difficile* (7, 26, 28, 35, 40). Wren and colleagues (40), using electrophoresis of radiolabelled isolates as first described by Tabaqchali et al. (31), found that two of the nine types identified always produced detectable cytotoxin and enterotoxin whereas two other types were invariably non-toxin producers. However, the numbers of isolates in some of their groups were small, and five of their types varied in production of toxins (from nondetectable to high-level production). Toma et al. (35), using the serotyping method of Delmée et al. (8), studied 246 isolates and classified 98% of them into 15 serogroups. They reported evidence for strain-specific cytotoxin production and pathogenicity on the basis of their finding that six serogroups were recovered mainly from symptomatic adults and were almost always cytotoxigenic (range 86 to 100%), whereas two serotypes were recovered from asymptomatic neonates and children and were invariably cytotoxin negative. Despite these associations, one serogroup (G) that had previously been recovered by Delmée and colleagues predominantly from neonates and children was, in the study of Toma et al., most often isolated from symptomatic adults. Other evidence for strain-specific pathogenicity was provided by Tabaqchali's report that an epidemic strain was more commonly recovered from symptomatic patients (88%) than from asymptomatic carriers (38%) (30). Confirmation that pathogenic strains can be reliably identified might offer the possibility for improvement of the currently unsatisfactory diagnostic tests. However, before one accepts the premise that pathogenicity of *C. difficile* may be primarily a strain-specific phenomenon, it is important to consider that previous studies may not have evaluated asymptomatic adult carriers (7, 26, 35) or were generally conducted during outbreaks, when a single strain might be expected to be recovered from a disproportionate number of symptomatic patients (24, 40).

To determine whether clinical manifestations of *C. difficile* are strictly strain-specific phenomena, we examined isolates recovered from a large number of patients at a facility in which *C. difficile* is an endemic pathogen and included asymptomatic carriers in our study population. Unfortunately, we did not have the opportunity to evaluate many patients with biopsy-documented colitis because endoscopy was performed for only 13 of the 38 symptomatic patients. This reflects the fact that many physicians are reluctant to subject patients to the potential hazards of this procedure and depend on other, noninvasive diagnostic tests. Our low frequency of colitis may also be due to the absence of a large outbreak during the time of the study. Thus, our data reflect the behavior of endemic strains which may be of lower virulence potential than strains associated with epidemic outbreaks of diarrhea or colitis. Another factor that may have resulted in the low frequency of detected colitis was the early recognition and prompt treatment of 42% of patients with *C. difficile* diarrhea; this may have prevented the progression to colitis or pseudomembranous colitis.

By comparing symptomatic patients (with and without documented colitis) with asymptomatic carriers, we sought to determine whether these two groups could be differentiated on the basis of strain identification. We did not find a direct correlation between immunoblot typing results and a patient's clinical status. We did find that some types were never associated with disease and some were always associated, but the numbers of strains within those immunoblot types were small. Much more significant was the finding that the most common immunoblot types were variably associated with disease. Thus, we conclude that immunoblot typing results alone cannot generally be used as a predictor of *C. difficile* disease. Our results do not preclude the possibility that specific strains of *C. difficile* are nontoxigenic and nonpathogenic or that specific strains are associated with distinctive clinical features or with epidemics. However, among adults hospitalized in a setting with a high prevalence of *C. difficile* disease, the variable pathogenic role of the organism cannot be ascribed simply to strain differences. In fact, asymptomatic carriers may be the source of isolates of identical immunoblot type that are subsequently associated with symptomatic disease in hospital contacts (21). Unfortunately, there have been few prospective studies or surveys which have sought hospitalized patients with asymptomatic carriage of *C. difficile*. It would be useful to evaluate a population with a low rate of *C. difficile* to determine the role of carriage in nosocomial transmission and to examine the strains recovered in that setting.

It was also of note that the in vitro ability of an isolate to produce toxins was not consistent within a given immunoblot type. Other investigators, using different typing systems, have also reported variable toxin production by isolates of the same type (25, 30, 35). It has also been shown that even individual isolates may not be consistent in their production of toxins. Haslam and colleagues reported that toxin production by individual organisms may be altered by changes in the nutrients and conditions of culture (11). Mahe and colleagues found that not only production of toxins but also pathogenicity in an animal model could be markedly changed by alterations in diet (20). They showed that an isolate that was associated with universal fatality in conventionally fed gnotobiotic mice caused no deaths in mice fed a

specific semisynthetic diet. Toxin production was also affected by the animals' diets. These important experiments suggest possible explanations for the lack of correlation between typing results, pathogenicity, and toxigenicity.

We also found that clinical status was not significantly associated with the detection of cytotoxin in the stool or with in vitro production of cytotoxin by the isolates. In general, assays of stool detected cytotoxin less frequently than did assays of broth cultures of isolates in vitro. We did find that isolates from symptomatic patients produced high levels of cytotoxin more frequently (45%) than did isolates from asymptomatic carriers (31%), but this difference was not significant and stool cytotoxin assays did not reflect this phenomenon.

A positive stool cytotoxin assay is considered to be useful for predicting antibiotic-associated colitis but appears to be much less valuable for predicting disease in patients with *C. difficile*-associated diarrhea (1, 13, 18). Isolates from asymptomatic carriers and symptomatic patients had similar frequencies of in vitro cytotoxin production (88 and 92%, respectively). It has long been accepted that despite the strong association between histologically confirmed colitis and *C. difficile*, many cases of antibiotic-associated diarrhea cannot be attributed to this organism (1). Our study, which documents that *C. difficile* and its toxins are also detected frequently in asymptomatic patients, supports the evidence that currently available clinical diagnostic tests alone are not sufficient for detecting *C. difficile* disease. Diarrhea is common among hospitalized patients; if other causes are not excluded and currently available diagnostic tests indicate the presence of *C. difficile*, a pathogenic role may be erroneously ascribed. Clearly, the accurate diagnosis of *C. difficile*-associated disease requires the integration of clinical assessment with laboratory results. It is also important to consider that the predictive value of a test is related to the prevalence of disease and that the value of a positive test may be very different in a setting in which *C. difficile* is not an endemic pathogen. Indeed, as discussed by Bennett and colleagues (3), these issues should be considered when any diagnostic test is evaluated.

We found that host factors as reflected by the multivariate risk index appeared to be important determinants of clinical manifestations. High-risk hosts were more likely than low-risk hosts to be symptomatic. Isolates capable of high-titer cytotoxin production were more commonly cultured from low-risk hosts. These data may imply that higher titers of cytotoxin are needed to produce disease in noncompromised hosts. This risk index may also reflect other host differences such as intestinal cell turnover, receptor availability, or local immunity.

Accurate diagnosis of intestinal disease due to *C. difficile* continues to be an important and difficult problem. A major recent advance is the development of a probe that appears to consistently differentiate enterotoxin-positive isolates from toxin-negative strains (39). If this tool can be used to detect only toxigenic strains in clinical specimens, it may provide a means to differentiate potential pathogens from nonpathogenic, colonizing strains. Our study suggests, however, that strain differentiation alone may not provide a diagnostic criterion that is sufficient to assign a pathogenic role to *C. difficile* and that we must continue to elucidate host factors that are also clearly implicated as important determinants in the pathogenesis of disease. We reiterate that our results are not in conflict with evidence indicating that strains of *C. difficile* may vary in toxigenicity and pathogenicity; rather, we conclude that in this endemic setting, the variety of clinical manifestations that resulted from *C. difficile* acquisition cannot be explained simply on the basis of strain differences. In epidemic settings, outbreaks have been shown to be due to a specific *C. difficile* strain, but we did not encounter this situation at our hospital. We would agree with colleagues who have referred to this bacterial species as "the difficult *Clostridium*" (35) and suggest that it has not changed its ways.

ACKNOWLEDGMENTS

We thank G. Corthier for generous gifts of pork and rabbit antitoxin A and for assistance in the enterotoxin enzyme-linked immunosorbent assay and Richard Kwok for his technical assistance.

REFERENCES

1. **Bartlett, J. G.** 1990. *Clostridium difficile*: clinical considerations. Rev. Infect. Dis. **12**(Suppl. 2):S243–S251.
2. **Bender, B. S., R. Bennett, B. E. Laughon, W. B. Greenough III, C. Gaydos, S. D. Sears, M. S. Forman, and J. G. Bartlett.** 1986. Is *Clostridium difficile* endemic in chronic-care facilities? Lancet **ii**:11–13.
3. **Bennett, R. G., B. E. Laughon, L. M. Mundy, L. D. Bobo, C. A. Gaydoes, W. B. Greenough, and J. G. Bartlett.** 1989. Evaluation of a latex agglutination test for *Clostridium difficile* in two nursing home outbreaks. J. Clin. Microbiol. **27**:889–893.
4. **Buggy, B. P., C. C. Hawkins, and R. Fekety.** 1985. Effect of adding sodium taurocholate to selective media on the recovery of *Clostridium difficile* from environmental surfaces. J. Clin. Microbiol. **21**:636–637.
5. **Burdon, D. W.** 1982. *Clostridium difficile*: the epidemiology and prevention of hospital acquired infection. Infection **10**:203–204.
6. **Corthier, G., M. C. Muller, G. W. Elmer, F. Lucas, and F. Dubos-Ramare.** 1989. Interrelationships between digestive proteolytic activities and production and quantitation of toxins in pseudomembranous colitis induced by *Clostridium difficile* in gnotobiotic mice. Infect. Immun. **57**:3922–3927.
7. **Delmée, M., and V. Avesani.** 1988. Correlation between serogroup and susceptibility to chloramphenicol, clindamycin, erythromycin, rifampicin and tetracycline among 308 isolates of *Clostridium difficile*. J. Antimicrob. Chemother. **22**:325–331.
8. **Delmée, M., G. Verellen, V. Avesani, and G. Francois.** 1988. *Clostridium difficile* in neonates: serogrouping and epidemiology. Eur. J. Pediatr. **147**:36–40.
9. **Gebhard, R. L., D. N. Gerding, M. M. Olson, L. R. Peterson, C. J. McClain, H. J. Ansel, M. J. Shaw, and M. L. Schwartz.** 1985. Clinical and endoscopic findings in patients early in the course of *C. difficile* associated PMC. Am. J. Med. **78**:45–48.
10. **Gerding, D. N., M. M. Olson, L. R. Peterson, D. G. Teasley, R. L. Gebhard, M. L. Schwartz, and J. T. Lee, Jr.** 1986. *Clostridium difficile*-associated diarrhea and colitis in adults: a prospective case-controlled epidemiologic study. Arch. Intern. Med. **146**:95–100.
11. **Haslam, S. C., J. M. Ketley, T. J. Mitchell, J. Stephen, D. W. Burdon, and D. C. A. Candy.** 1986. Growth of *Clostridium difficile* and production of toxins A and B in complex and defined media. J. Med. Microbiol. **21**:293–297.
12. **Hecht, G., C. Pothoulakis, J. T. LaMont, and J. L. Madera.** 1988. *Clostridium difficile* toxin A perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. J. Clin. Invest. **82**:1516–1524.
13. **Johnson, L. L., F. L. McFarland, P. Dearing, V. Raisys, and F. D. Schoenknect.** 1989. Identification of *Clostridium difficile* in stool specimens by culture-enhanced gas-liquid chromatography. J. Clin. Microbiol. **27**:2218–2221.
14. **Johnson, S., C. R. Clabots, F. V. Linn, M. M. Olson, L. R. Peterson, and D. N. Gerding.** 1990. Nosocomial *Clostridium difficile* colonisation and disease. Lancet **336**:97–100.
15. **Katoh, T., T. Honda, and T. Miwatani.** 1988. Purification and some properties of cytotoxin produced by *C. difficile*. Microbiol. Immunol. **32**:551–564.

16. **Katz, G. W., S. D. Gitlin, D. R. Schaberg, K. H. Wilson, C. A. Kauffman, S. M. Seo, and R. Fekety.** 1988. Acquisition of *Clostridium difficile* from the hospital environment. Am. J. Epidemiol. **127**:1289–1294.
17. **Keighley, M. R. B.** 1980. Antibiotic-associated pseudomembranous colitis: pathogenesis and management. Drugs **20**:49–56.
18. **Lashner, B. A., J. Todorczuk, D. F. Sahm, and S. B. Hanauer.** 1986. *Clostridium difficile* culture-positive toxin-negative diarrhea. Am. J. Gastrol. **81**:940–943.
19. **Lyerly, D. M., H. C. Krivan, and T. D. Wilkins.** 1988. *Clostridium difficile*: its disease and toxins. Clin. Microbiol. Rev. **1**:1–18.
20. **Mahe, S., G. Corthier, and F. Dubos.** 1987. Effect of various diets on toxin production by two strains of *Clostridium difficile* in gnotobiotic mice. Infect. Immun. **55**:1801–1805.
21. **McFarland, L. V., M. E. Mulligan, R. Y. Kwok, and W. E. Stamm.** 1989. Nosocomial acquisition of *Clostridium difficile* infection. N. Engl. J. Med. **320**:204–210.
22. **McFarland, L. V., and W. E. Stamm.** 1986. Review of *Clostridium difficile*-associated diseases. Am. J. Infect. C **14**:99–109.
23. **McFarland, L. V., C. M. Surawicz, and W. E. Stamm.** 1990. Risk factors for *Clostridium difficile* carriage and *C. difficile*-associated diarrhea in a cohort of hospitalized patients. J. Infect. Dis. **162**:678–684.
24. **McKay, I., J. E. Coia, and I. R. Poxton.** 1989. Typing of *Clostridium difficile* causing diarrhoea in an orthopaedic ward. J. Clin. Pathol. **42**:511–515.
25. **Mulligan, M. E., L. R. Peterson, R. Y. Kwok, C. R. Clabots, and D. N. Gerding.** 1988. Immunoblots and plasmid fingerprints compared with serotyping and polyacrylamide gel electrophoresis for typing *Clostridium difficile*. J. Clin. Microbiol. **26**:41–46.
26. **Pantosti, A., M. Cerquetti, and P. M. Gianfrilli.** 1988. Electrophoretic characterization of *Clostridium difficile* strains isolated from antibiotic-associated colitis and other conditions. J. Clin. Microbiol. **26**:540–543.
27. **Popoff, M. R., E. J. Rubin, D. M. Gill, and P. Boquet.** 1988. Actin-specific ADP-ribosyltransferase produced by a *Clostridium difficile* strain. Infect. Immun. **6**:2299–2306.
28. **Poxton, I. R., B. Aronsson, R. Mollby, C. E. Nord, and J. G. Collee.** 1984. Immunochemical fingerprinting of *Clostridium difficile* strains isolated from an outbreak of antibiotic-associated colitis and diarrhoea. J. Med. Microbiol. **17**:317–324.
29. **Sullivan, N. M., S. Pellett, and T. D. Wilkins.** 1982. Purification and characterization of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**:1032–1040.
30. **Tabaqchali, S.** 1990. Epidemiologic markers for *Clostridium difficile*. Rev. Infect. Dis. **12**(Suppl. 2):S192–S199.
31. **Tabaqchali, S., S. O'Farrell, D. Holland, and R. Silman.** 1984. Typing scheme for *C. difficile*: its application in clinical and epidemiologic studies. Lancet **i**:935–938.
32. **Tedesco, F. J., R. W. Barton, and D. H. Alpers.** 1974. Clindamycin-associated colitis. Ann. Intern. Med. **81**:429–433.
33. **Testore, G. P., A. Pantosti, M. Cerquetti, S. Babudieri, G. Panichi, and P. Mastrantonio Gianfrilli.** 1988. Evidence for cross-infection in an outbreak of *Clostridium difficile*-associated diarrhoea in a surgical unit. J. Med. Microbiol. **26**:125–128.
34. **Thomas, D. R., R. G. Bennett, B. E. Laughon, W. B. Greenough III, and J. G. Bartlett.** 1990. Postantibiotic colonization with *Clostridium difficile* in nursing home patients. J. Am. Geriatr. Soc. **38**:415–420.
35. **Toma, S., G. Lesiak, M. Magus, H. L. Lo, and M. Delmee.** 1988. Serotyping of *Clostridium difficile*. J. Clin. Microbiol. **26**:426–428.
36. **Weiss, N. S.** 1986. Clinical epidemiology: the study of the outcome of illness, p. 16. Oxford University Press, New York.
37. **Willey, S. H., and J. G. Bartlett.** 1979. Cultures for *Clostridium difficile* in stools containing a cytotoxin neutralized by *Clostridium sordellii* antitoxin. J. Clin. Microbiol. **10**:880–884.
38. **Wilson, K. H., and J. N. Sheagren.** 1983. Antagonism of toxigenic *Cl. difficile* by nontoxigenic *C. difficile*. J. Infect. Dis. **147**:733–736.
39. **Wren, B., C. L. Clayton, N. B. Castledine, and S. Tabaqchali.** 1990. Identification of toxigenic *Clostridium difficile* strains by using a toxin A gene-specific probe. J. Clin. Microbiol. **28**:1808–1812.
40. **Wren, B., S. R. Heard, and S. Tabaqchali.** 1987. Association between production of toxins A and B and types of *Clostridium difficile*. J. Clin. Pathol. **40**:1397–1401.