

# Immunoblots and Plasmid Fingerprints Compared with Serotyping and Polyacrylamide Gel Electrophoresis for Typing *Clostridium difficile*

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Two new methods for typing *Clostridium difficile*, immunoblotting and plasmid fingerprinting, were compared with serotyping and polyacrylamide gel electrophoresis (PAGE). Of these methods, immunoblotting was found to be the most valuable for use in a comprehensive typing system. More groups could be distinguished by immunoblotting than by serotyping or PAGE. Immunoblotting results were also more reproducible and distinctive than results by PAGE. Plasmid fingerprinting was an excellent marker for plasmid-bearing strains, but it had limited use because many isolates lacked plasmids. A unique plasmid profile observed for one group of isolates correlated with differences in phenotypic characteristics resolved by immunoblot analysis but not by serotyping or PAGE. Preliminary attempts to correlate typing results with pathogenicity of isolates were not successful but underscored the need for future studies to include careful assessment of the clinical significance of isolates.

Typing of *Clostridium difficile* is important for epidemiologic studies and may, ultimately, be an important tool to define fully the pathogenesis of *C. difficile*-associated disease. Various methods have been developed for typing the organism. These methods include susceptibility testing, serogrouping, polyacrylamide gel electrophoresis (PAGE), immunochemical fingerprinting, bacteriocin and bacteriophage susceptibilities, and PAGE of radiolabeled proteins; some of these have been used to confirm the role of *C. difficile* as a nosocomial pathogen (3, 5, 6, 11, 15, 17, 19, 21, 26). However, important questions remain to be answered. Asymptomatic acquisition of *C. difficile* has been documented (24), and nontoxicogenic isolates have been identified (9, 13, 25). The epidemiologic and clinical importance of strain variation is not yet fully defined. There is a continuing need to evaluate typing methods that can be correlated with epidemiologic and clinical data.

In this report we describe two methods, immunoblotting and plasmid fingerprinting, developed in two different laboratories. To make an initial assessment of the utility of these methods compared with other typing methods, we selected isolates to be studied by the two methods and compared the results with those obtained with a serotyping system and with PAGE typing.

## MATERIALS AND METHODS

**Selection of *C. difficile* strains.** A total of 53 cultures of *C. difficile* were assigned code numbers in Minnesota before they were typed as unknowns in Los Angeles. Of these, 36 represented only 12 different organisms that were randomly numbered and submitted in triplicate. The remaining 17 isolates represented single organisms recovered from different patients. In all, there were 29 different isolates recov-

ered from 24 adult patients who were evaluated clinically at the Minneapolis Veterans Administration (VA) Medical Center from 1982 to 1985. Nine isolates (isolates 834, 835, 840, 844, 849, 857, 884, 887, 898) were considered to be epidemiologically related. Basic clinical and epidemiologic data about the isolates are presented in Table 1. In all cases, *C. difficile* was considered a possible pathogen. Patients had diarrhea (a minimum of six unformed bowel movements over a period of 36 h) and had received antimicrobial therapy (8).

Isolates were initially recovered on selective media (7) and identified by standard methods, including Gram stain, colonial morphology, and gas-liquid chromatography (12, 20).

**Cytotoxin assays, latex testing, and plasmid fingerprinting.** Cytotoxin assays, latex testing, and plasmid profiles were performed at the Minneapolis VA Medical Center. The cytotoxin that was present in 7-day broth cultures was assayed by methods based on those described by Chang et al. (4); however, HEP-2 tissue culture was used, and the broth culture filtrate was diluted to give quantitative results. In addition, a commercial latex test (Marion Scientific, Div. Marion Laboratories, Inc., Kansas City, Mo.) which detects the bacterial protein that is present in *C. difficile* and several other bacterial species was also used to assay the dilutions of the broth cultures.

Screening for plasmids was performed by a modification of the method described by Portnoy et al. (16). *C. difficile* was grown anaerobically for 24 h in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.), followed by centrifugation. The cell pellets were suspended in 20% (wt/vol) sucrose solution and centrifuged again. Cell pellets were then suspended in 1 ml of the same sucrose solution and incubated with 0.5 ml of 30 mg of lysozyme per ml for 30 min at 37°C. Lysis buffer (6 ml; 1% sodium dodecyl sulfate in TE buffer [1 μM EDTA and 10 μM Tris; pH 7.2]; pH 12.3) were added with 20 inversions. Lysates were subsequently

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neutralized by the addition of 3 ml of 2 mM Tris (pH 7.0), and 1.6 ml of 5 M NaCl was added to precipitate chromosome-membrane complexes that were then pelleted ( $20,000 \times g$  for 30 min) after 1 to 2 h on ice. Supernatants were mixed with isopropanol at  $-20^{\circ}\text{C}$  for 0.5 h to precipitate total nucleic acids. Nucleic acids were pelleted ( $3,000 \times g$  for 20 min) and dried at room temperature for 30 min. Plasmid DNA was purified by isopycnic banding in cesium chloride-ethidium bromide gradients (18) and was separated by electrophoresis in 0.7% agarose gels with Tris acetate buffer at 25 V (1.9 V/cm) for 16 h. A modified rapid method was also used to screen for the presence of plasmids before detailed plasmid fingerprints were determined (1).

**Serotyping, PAGE, and immunoblotting.** Serotyping, PAGE, and immunoblotting were performed at the West Los Angeles VA Medical Center. Serotyping and PAGE of EDTA extracts were performed by methods described previously (14). Four antisera that were prepared by intravenous immunization of New Zealand White rabbits with four different isolates of *C. difficile* were stored at  $-70^{\circ}\text{C}$  until use. Isolates for serotyping were grown in reduced brain heart infusion broth (BBL) for 24 h; washed three times with saline; and in suspensions of  $10^9$  organisms per ml, mixed with equal volumes of twofold dilutions of antisera in microtiter plates. The highest dilution of each antiserum sample that displayed agglutination with the isolate was determined after incubation at  $37^{\circ}\text{C}$  for 24 h. Serotyping was performed by determining the pattern of agglutination titers observed when the test organism was agglutinated with the panel of four antisera. For PAGE, 15 ml of brain heart infusion broth was inoculated with two to three colonies of *C. difficile* and incubated at  $37^{\circ}\text{C}$  for 24 h. Cells were washed in 10 ml of 0.15 M phosphate-buffered saline (pH 7.4) twice. EDTA extracts were then prepared by adding 100  $\mu\text{l}$  of 70 mM EDTA to each tube of washed cells and then by incubating them for 30 min at  $45^{\circ}\text{C}$  and centrifuging at  $1,500 \times g$  for 15 min. The supernatant EDTA extract was stored at  $-20^{\circ}\text{C}$  for later use. The protein concentration was standardized to 35  $\mu\text{g}/50 \mu\text{l}$ . Prior to PAGE, 50  $\mu\text{l}$  of EDTA extract was mixed with 12  $\mu\text{l}$  of sample buffer (0.5 ml of glycerol, 0.25 ml of 20% sodium dodecyl sulfate, 0.05 ml of beta-mercaptoethanol, and 0.05 ml of 0.1% bromophenol blue per ml) and then heated for 15 min at  $65^{\circ}\text{C}$ . Electrophoresis was performed with a 12.5% running and a 5% stacking gel at 20 A for the stacking gel and 40 A for the running gel for a total of 6 h. Molecular weight standards were included.

Immunoblotting was based on the method for enzyme-linked immunoelectrotransfer blotting described by Tsang et al. (23). After heating at  $65^{\circ}\text{C}$  for 15 min with sample buffer, EDTA extracts were subjected to PAGE as described above and then transferred to nitrocellulose paper in the reservoir of a Trans-Blot cell (Bio-Rad Laboratories, Richmond, Calif.) containing Tris, glycine, and methanol. After 16 h of blotting at 10 V, the paper was washed four times with phosphate-buffered saline-Tween at  $40^{\circ}\text{C}$  with shaking and then reacted with one of the four rabbit antisera (diluted 1:500) at room temperature for 1 h with shaking. Each bacterial sample was tested with each of the four antisera. The four strains used for immunization were obtained from two adults in California with colitis (antisera 1178 and 4597), one adult in Texas with antimicrobial agent-associated diarrhea (antiserum 1181), and one adult in California with asymptomatic colonization (antiserum 3998). After three washes with phosphate-buffered saline-Tween, a commercial, specific, second antiserum immunoglobulin G (IgG) fraction of goat anti-rabbit IgG whole molecule conjugated

with peroxidase (Sigma Chemical Co., St. Louis, Mo.) was added for 1 h with shaking. The paper was then washed three times and soaked in a mixture of 3,3'-diaminobenzidine and hydrogen peroxide.

## RESULTS

In all cases, serotyping, PAGE, and immunoblotting results were related (Table 1). For example, PAGE group c isolates were invariably serotype I; PAGE group d isolates were always serotype III; PAGE groups a, b, and e were all serotype II; and PAGE groups h, f, and g were distinctive serotypes.

Serotype I isolates had agglutination titers of less than 1:32 (or  $2^5$ ) with antisera 1178 and 4597, but titers of approximately 1:1,028 (or  $2^{10}$ ) with antisera 1181 and 3998 (Fig. 1). Other distinctive patterns defined the remaining serotypes.

Four different blot patterns were obtained for each isolate when they were tested with the four antisera, but the patterns were invariably correlated. It was determined that the use of two antisera, 1178 and 3998, was sufficient to distinguish among the 10 immunoblot groups.

A comparison of PAGE and immunoblot results demonstrated that groups were much more readily distinguished by immunoblots and that it was possible to identify subgroups of isolates that were indistinguishable by PAGE and serotyping. Differences that are subtle and sometimes difficult to reproduce by PAGE were pronounced and consistently reproducible by immunoblotting. For example, isolate 287 (PAGE group a) and isolate 855 (PAGE group h) were distinguished only by a band at approximately 38,000 daltons for PAGE group a that was absent in PAGE group h (Fig. 2), a difference that was not always reproducible. In contrast, the immunoblots with antiserum 1178 (Fig. 3) displayed a striking complex of three dark bands in the 50,000-dalton region for isolate 287 and a single band in this region for isolate 855. A second antiserum, 3998 (Fig. 4), was less useful for confirming differences suggested by PAGE but allowed subgrouping of isolates that typically appeared identical by PAGE. Isolates 265, 834, 838, and 898 were all serotype III and PAGE group d. Although they appeared slightly different (Fig. 2), they usually had identical PAGE patterns. They were, however, always differentiated by immunoblotting with the high-molecular-weight (above 90,000) double band of isolates 834 and 898 (Fig. 4).

In one case the subgrouping that was identified by immunoblotting was reflected by minor differences in PAGE results. The PAGE pattern of isolate 472 (indicated as c+ in Fig. 2) was slightly more complex than those of other PAGE group c isolates.

The six plasmid fingerprints observed among the study isolates are illustrated in Fig. 5. All serotype I and PAGE group c isolates lacked plasmids, with the exception of isolate 472 (Fig. 5, lane B). This was the isolate that was slightly different by PAGE but clearly distinctive by immunoblotting. Serotype II isolates fell into three PAGE groups, only one of which (group b) contained plasmids (Fig. 5, lane C). Serotype III, PAGE group d isolates could be separated into two immunoblot groups, d' and d''. All immunoblot d' isolates lacked plasmids, whereas all immunoblot d'' isolates had identical plasmid fingerprints (Fig. 5, lane D). Three isolates that were unique in all typing systems, isolates 831, 839, and 835, each had unique plasmid fingerprints (Fig. 5, lanes E to G).

The results obtained by serotyping, PAGE, immunoblotting, and plasmid fingerprinting were reproducible, despite

TABLE 1. Clinical and epidemiologic data plus analysis of strains by serotyping, PAGE, immunoblotting, and plasmid fingerprinting

Group and patient no. <sup>a</sup>	Isolate no.	Clinical data <sup>b</sup>	Yr	Serotype	PAGE pattern	Blot <sup>c</sup>	Plasmid pattern <sup>d</sup>
<b>Group 1</b>							
2	265	PMC	1982	III	d	d'	None
3	247, 256	PMC	1982	III	d	d'	None
14	838	AAD	1985	III	d	d'	None
12	834	AAD	1985	III	d	d''	2.0, 3.5, 4.1, 5.6, 6.5, 7.1
13	835	AAD	1985	III	d	d''	2.0, 3.5, 4.1, 5.6, 6.5, 7.1
16	840	AAD	1985	III	d	d''	2.0, 3.5, 4.1, 5.6, 6.5, 7.1
17	844	AAD	1985	III	d	d''	2.0, 3.5, 4.1, 5.6, 6.5, 7.1
18	849	AAD	1985	III	d	d''	2.0, 3.5, 4.1, 5.6, 6.5, 7.1
20	857	AAD	1985	III	d	d''	2.0, 3.5, 4.1, 5.6, 6.5, 7.1
21	884	AAD	1985	III	d	d''	2.0, 3.5, 4.1, 5.6, 6.5, 7.1
22	887	AAD	1985	III	d	d''	2.0, 3.5, 4.1, 5.6, 6.5, 7.1
23	898	AAD	1985	III	d	d''	2.0, 3.5, 4.1, 5.6, 6.5, 7.1
15	839	AAD	1985	VI	g	g'	7.4, 8.2
<b>Group 2</b>							
6	287, 288	AAD(?)	1983	II	a	a'	None
5	275, 290	AAD	1983	II	b	b'	7.1, 7.8, 8.3, 9.3, 9.9, 11.9
7	291, 294	AAD(?)	1983	II	e	e'	None
<b>Group 3</b>							
1	242, 252	PMC	1982	I	c	c'	None
4	269	AAD(?)	1983	I	c	c'	None
8	445	AAD(?)	1982	I	c	c'	None
9	472	AAD(?)	1982	I	c+	c''	6.7
10	775	AAD(?)	1985	I	c	c'	None
24	905	AAD	1985	I	c	c'	None
11	831	AAD(?)	1985	V	f	f'	8.5, 14.3, 19.5
19	855	AAD	1985	IV	h	h'	22.3

<sup>a</sup> Group 1 isolates were fecal cytotoxin positive and isolate cytotoxin positive, group 2 isolates were fecal cytotoxin negative and isolate cytotoxin positive, and group 3 isolates were fecal cytotoxin negative and isolate cytotoxin negative. Two isolates in group 3 were recovered from swabs so that there was inadequate specimen for fecal cytotoxin assay; fecal cytotoxin assays were presumed to be negative for these specimens.

<sup>b</sup> Clinical diagnoses are abbreviated as follows: PMC, pseudomembranous colitis confirmed histologically; AAD, antibiotic-associated diarrhea; AAD(?), other possible cause of diarrhea present (such as prior surgery or quinidine therapy).

<sup>c</sup> Immunoblot patterns are described as follows. If two immunoblots were differentiated within one PAGE group, one was designated with a prime and the other was designated with a double prime.

<sup>d</sup> Plasmid patterns were characterized by bands that migrated to the regions (in kilobases) indicated.

the prolonged storage of isolates. The 12 isolates studied in triplicate gave identical results, with one exception. It was considered that the one inconsistency in the 36 evaluations was due to a labeling error.

All isolates of serotypes I, IV, and V were negative by cytotoxin assay with titers of <1:10 (Table 1). The titers of cytotoxin also appeared to correlate with typing results for

other isolates. All serotype III isolates had cytotoxin titers of  $\geq 1:5,120$ . Serotype II isolates fell into three groups: PAGE group a isolate titers were 1:20,480, PAGE group b isolate titers were  $\leq 1:320$  and PAGE group e isolate titers were 1:2,560. Because three PAGE groups consisted of isolates

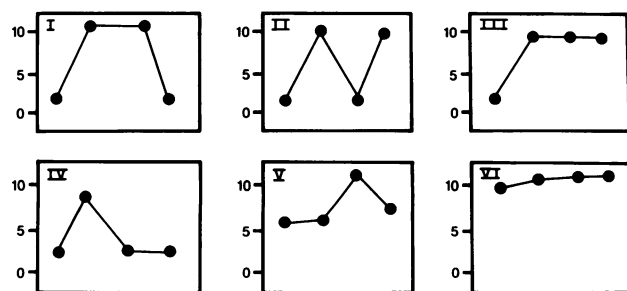


FIG. 1. Patterns of agglutination titers used to determine the serotypes of *C. difficile*. Roman numerals indicate serotypes. Titers of agglutination (0 to 10) are expressed as the highest twofold dilution showing agglutination (thus, a titer of 5 = 2<sup>5</sup>, or a 1:32 dilution, and a titer of 10 = 2<sup>10</sup>, or a 1:1,028 dilution). Antisera 1178, 1181, 3998, and 4597 were used.

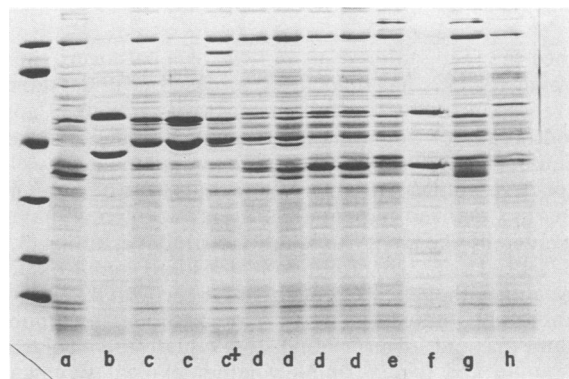


FIG. 2. PAGE patterns of selected isolates of *C. difficile*. Letters indicate PAGE group types. Molecular weight standards in lane 1 are, from the top, 92,500, 66,200, 45,000, 31,000, 21,000, and 14,400. Actual isolate numbers are given in Fig. 3 and 4.

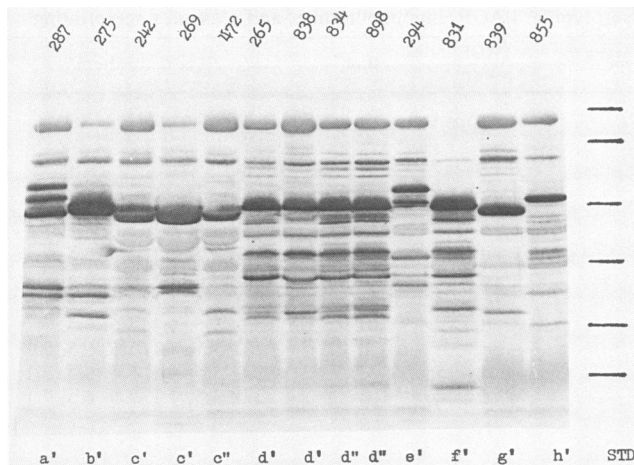


FIG. 3. Immunoblots with antiserum 1178. Primes indicate immunoblot subgroups. The molecular weight standards (STD) indicated by the bars on the right are, from the top, 130,000, 75,000, 50,000, 39,000, 27,000, and 14,400. Isolate numbers are given at the top of the lanes.

from single individuals, however, this may be only an apparent correlation. There was no correlation between the presence of plasmids and cytotoxin titers.

It does not appear that our antisera detected cytotoxin *per se*. We could distinguish no bands common to cytotoxin-positive isolates that were absent in the cytotoxin-negative isolates.

One unexpected finding was that cytotoxin-negative isolates 242 and 252 were recovered from a patient with documented pseudomembranous colitis (Table 1). Another cytotoxin-negative isolate, 905, was recovered from a different patient with antibiotic-associated diarrhea who had no other apparent cause for the symptoms. An endoscopy was not performed on the patient, but the standard case definition for antibiotic-associated diarrhea due to *C. difficile* was met.

All isolates gave positive results when tested with the commercial latex test; this test was designed to detect enterotoxin, but subsequently, it has been found to detect a bacterial protein other than enterotoxin (13). Specific assays for enterotoxin were not performed.

## DISCUSSION

Since the recognition of *C. difficile* as a pathogen, the use of typing systems has helped to increase our understanding of disease caused by this organism. Investigations of apparent outbreaks of human disease, animal studies indicating the importance of environmental contamination, as well as prospective studies in which the acquisition of *C. difficile* among hospitalized patients has been documented have all been aided by the ability to identify distinctive strains (3, 10, 15, 17, 19, 21, 22, 26). Important questions about epidemiology and pathogenesis remain to be answered, however. We therefore studied two new typing methods, immunoblotting and plasmid fingerprinting, to evaluate their epidemiologic value and to determine whether clinical significance could be related to typing results.

We found that the results obtained by our typing methods were related, but that immunoblotting distinguished more groups than did serotyping or PAGE and gave better con-

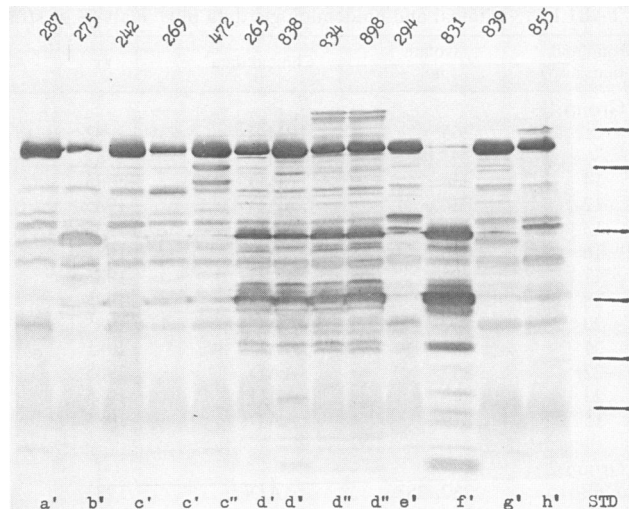


FIG. 4. Immunoblots with antiserum 3998. Data are presented as described in the legend to Fig. 3.

sistency and ease of interpretation. Delmee and colleagues (6) also found a larger number of distinctive strains by PAGE than by their serogrouping methods.

Plasmid fingerprinting, although an excellent marker for plasmid-bearing strains, could not be used as a comprehensive system because many isolates lacked plasmids but could be differentiated by serotyping, PAGE, and immunoblotting. There were strains of serotypes I, II, and III that lacked plasmids. In our study, only 15 of 29 isolates (52%) contained plasmids.

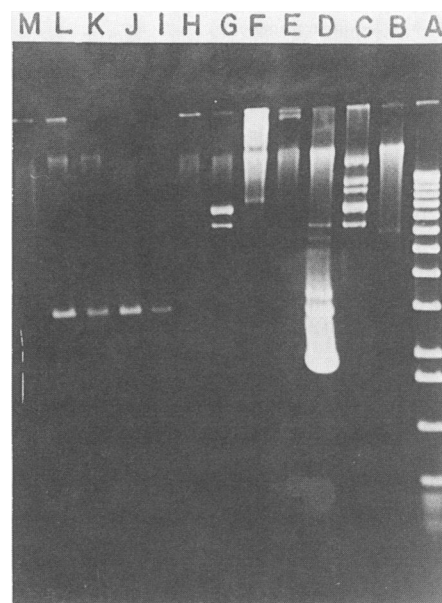


FIG. 5. Plasmid fingerprints of six isolates. Lane A, 1-kilobase (kb) ladder; lane B, isolate 472 with one band at the 6.7-kb region; lane C, isolate 275 with six bands at the 7.1-, 7.8-, 8.3-, 9.3-, 9.9-, and 11.9-kb regions; lane D, isolate 844 with six bands at the 2.0-, 3.5-, 4.1-, 5.6-, 6.5-, 7.1-kb regions; lane E, isolate 855 with one band at the 22.3-kb region; lane F, isolate 831 with three bands at the 8.5-, 14.3-, 19.5-kb regions; lane G, isolate 839 with two bands at the 7.4- and 8.2-kb regions; lanes H to M, the same isolates as in lanes B to G, respectively, examined by a modified screening method (1).

In contrast, plasmid fingerprints clearly differentiated two subgroups of isolates that could not be distinguished by serotyping or PAGE. These were the serotype III and PAGE group d isolates. One subgroup lacked plasmids, whereas the other had a distinctive plasmid fingerprint characterized by bands at the 2.0-, 3.5-, 4.1-, 5.6-, 6.5-, and 7.1-kb regions (Fig. 5). Immunoblotting also differentiated these same subgroups; the isolates with the immunoblot d' pattern lacked plasmids, and those with the immunoblot d'' pattern had the distinctive plasmid fingerprint. All isolates in this study were recovered from patients hospitalized at a single institution in which *C. difficile* has been recognized as a common pathogen since 1980. The immunoblot d'' isolates were recovered during a single year (1985), whereas immunoblot d' strains were recovered before and during 1985. The immunoblot d'' isolates were judged to be epidemiologically related but could not be defined as representing a discrete outbreak in the Minneapolis VA Medical Center with a multiple-year, endemic occurrence of *C. difficile*. Our data are consistent with the possibility that the immunoblot d'' strain that was recovered in 1985 was derived from the endemic immunoblot d' strain. Typing of many more isolates, however, would be required to evaluate this possibility. The use of serotyping and PAGE to identify major groups, with the addition of plasmid fingerprints and immunoblots to identify subgroups that cannot be differentiated by serotyping or PAGE, would be useful for such a study.

Immunoblotting was found to be a new method for the typing of *C. difficile* that should be an excellent epidemiologic tool; it was superior to our serotyping and PAGE systems. The serogrouping method described by Delmee and colleagues (5) is another excellent method but is different from our serotyping method because it employs a panel of antisera that have been absorbed to remove cross-reacting antibodies. That system, however, requires the production of a new antiserum when a new strain is detected, a step that is not necessary with immunoblot typing. The method described by Tabaqchali et al. (21), which uses PAGE of radiolabeled proteins, is also an excellent comprehensive system that identifies strain-specific proteins that are immunogenic (11).

Our initial intent was to evaluate our typing systems not only as epidemiologic tools but also to determine if different strains of *C. difficile* had different clinical significance. However, when we examined the isolates reported in this study (which were all recovered from patients for whom *C. difficile*-associated disease was a consideration), there were unexpected results. We did find correlations between typing results and cytotoxin production: for example, that all serogroup I and PAGE group c isolates were cytotoxin negative. We could not explain, however, why these cytotoxin-negative isolates were recovered from symptomatic patients. Possible explanations, which include the presence of other enteric diseases (24) or intestinal colonization by more than one strain of *C. difficile* (2), could not be investigated for our patients. Studies to determine if pathogenicity is strain related will require not only evaluation of asymptomatic carriers but also careful determination of the pathogenic role of *C. difficile* for symptomatic patients.

We evaluated two new typing methods for *C. difficile*, immunoblotting and plasmid fingerprinting. Immunoblotting appears to be an excellent epidemiologic tool. Plasmid fingerprinting has more limited value because it is useful only for plasmid-bearing isolates. Preliminary attempts to correlate typing traits with clinical significance of isolates were not successful but, rather, indicated that such correlations

must be done with careful attention to confirm the clinical significance of isolates that are studied.

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