Investigation of an Outbreak of *Clostridium difficile* Infection in a General Hospital by Numerical Analysis of Protein Patterns by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

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Received 5 August 1993/Returned for modification 8 November 1993/Accepted 16 December 1993

One hundred forty-five cultures of *Clostridium difficile*, including strains from an apparent nosocomial outbreak of infection, were characterized by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell proteins. Each protein pattern was characterized by the presence of one to three dense bands which were highly reproducible. The first 100 strains (in chronological order) were used as the basis for a numerical analysis which divided the strains into 17 phenons (EP types 1 to 17). The protein patterns of the remaining 45 strains were identified to type by comparing their individual patterns against a data base made up of the protein patterns of the first 100 strains. EP type 1 was the most common, with 70 of 139 (50%) patient isolates having this pattern type, and it accounted for 26 of 35 strains (74%) from patients in a medical teaching ward from which the outbreak was believed to have originated. This type was also found in a high proportion of isolations in a number of other medical and oncology wards, but the majority of these isolates occurred subsequent to the infections on the initial outbreak ward. This technique can therefore provide a method for tracing the possible spread of epidemic strains in hospitals and other institutions and may contribute to a better understanding of the epidemiology of *C. difficile*.

*Clostridium difficile* has been widely implicated as the major cause of pseudomembranous colitis and diarrhea associated with antimicrobial (AAD) and/or antineoplastic chemotherapy in adult hospitalized patients (2, 18, 23). In the most serious form of the disease (pseudomembranous colitis), the pathogenic role of *C. difficile* is usually readily apparent (1). Often, however, the classic features of this illness are absent and the role of the organism in infection may be difficult to determine, especially in situations where there is asymptomatic colonization (21).

The spectrum and severity of clinical presentations associated with *C. difficile* may result from different strains varying in their intrinsic ability to cause disease. At least two toxins are produced, enterotoxin (toxin A) and cytotoxin (toxin B), and may provide the organism with much of its pathogenic potential. The possibility of strain-specific differences in virulence has also been suggested in a number of clinical studies (6, 28, 35, 37).

It is apparent that treatment with certain antibiotics, which displace or alter the balance of the normal bowel flora, increases susceptibility to infection by *C. difficile* (34). There is some evidence for person-to-person transmission of *C. difficile* in hospitals (5, 14), and there are documented cases of both environmental contamination and carriage on the hands of health-care workers (17, 20). Taken together, such studies indicate that intestinal disease induced by infection with *C. difficile* is frequently nosocomial in origin. As a consequence, the mode of transmission of *C. difficile* within the hospital has been generally evaluated in epidemic settings (14, 24, 28, 32).

Various methods have been applied to the typing of *C. difficile* for epidemiological evaluations. The aim of this study was to compare the high-resolution one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) whole-cell protein patterns of a number of *C. difficile* isolates from a hospital, both prior to and during a suspected outbreak of infection (26). Although the study was not prospective, it was fortunate that all suspected cases of *C. difficile* diarrhea in the hospital were investigated and strains were isolated and preserved for nearly 2 years prior to the outbreak. A computerized analysis of protein patterns (4) provided an objective evaluation of the technique as a method for differentiating the strains involved and deducing the epidemiology of the outbreak.

(This study was presented in part at the 93rd General Meeting of the American Society for Microbiology, Atlanta, Ga., 16 to 20 May 1993, abstr. L15, p. 547.)

MATERIALS AND METHODS

Sources and strains. A total of 145 strains of *C. difficile* were examined. Of these, 139 were recovered from patients with AAD while the remaining 6 were environmental samples. Strains were isolated over a 3-year period between 2 January 1990 and 2 December 1992 from 14 different wards in a 465-bed Canadian hospital. The 139 strains comprised all the cultures of *C. difficile* isolated from patients in the hospital over this 3-year period. This investigation was prompted when the number of *C. difficile* isolations on a medical teaching ward (ward 385) showed a significant and sustained increase (2 isolates in 1990, 10 in 1991, and 23 in 1992), thereby suggesting an outbreak in progress. The outbreak was considered to have started in late 1991, and since then the epidemic strain (EP type 1) has been transmitted to other wards and has become endemic.

The case definition for *C. difficile* diarrhea and the patient sampling protocol depended on a number of criteria. Isolation of *C. difficile* was generally only attempted when (i) patients...
presented with loose stools (≥3/day) or, in 13 cases, were admitted with diarrhea; (ii) hospital inpatients were, prior to their diarrhea, already on antibiotics known to predispose to this condition; and (iii) cytotoxin and enterotoxin were detected in feces. *C. difficile* was considered the causative agent of the diarrhea if, in addition to the above criteria, it was the only enteric pathogen found. However, since simultaneous culture of liquid stools and toxin assay were performed in all suspected cases of drug-associated diarrhea, some specimens proved culture positive but toxin negative. In such cases, the diarrhea may or may not have been associated with *C. difficile*.

**Isolation, culture media, and conventional biochemical tests.** *C. difficile* strains were isolated from feces by inoculation onto selective CCFA medium (12) and incubation anaerobically for 48 h at 35°C. *C. difficile* strains were presumptively identified by their characteristic ground-glass appearance and yellow-green fluorescence under long-wave UV light on CCFA medium (12). They were further recognized by their odor, Gram stain, and biochemical test results (15). A rapid slide agglutination test (Scrobact; Disposable Products Pty. Ltd., South Australia, Australia) was used to confirm the identification.

For production of protein samples, cultures were grown on fastidious anaerobe agar (LAB 90 FAA; Lab M, Bury, England) supplemented with 5% (vol/vol) defibrinated horse blood for 24 h at 37°C in an anaerobic cabinet (Don Whitley Scientific Ltd., Shipley, Yorkshire, England).

**Toxin assay.** Analysis of feces and culture supernatant for the presence of *C. difficile* toxin B was performed by tissue culture cell assay (9, 10). The cell line used was human foreskin fibroblasts in microwells (Barlows cytotoxicity assay; Baxter Healthcare Co., West Sacramento, Calif.). Testing for toxin A in feces and culture supernatants was by the enzyme immunoassay (Premier; Meridian Diagnostic Inc., Cincinnati, Ohio) method.

**Preparation of protein samples, electrophoresis, staining, and scanning of gels.** Bacterial samples (10 mg [wt weight]) were harvested directly from plates into 150 μl of lysozyme (3 mg/ml) and incubated for 2 h at 37°C. An equal volume of double-strength lysis buffer (4) was then added, and the sample was heated at 100°C for 10 min in a heating block. Protein sample extraction, gel preparation, and electrophoresis were as described previously (4).

The stained protein patterns in the dried gels were scanned with the equipment and methods described by Costas (4). The absorbance range was set from 0.1 to 1.2 absorbance units.

**Analysis and computation of similarity.** Analysis and computation of similarity were as described by Costas (4), except that the protein patterns were corrected for gel-to-gel variation by segmented linear correction using 17 discernible marker positions on the reference pattern and linear correction was carried out within each of the 16 defined segments for each track on the calibrated gel. The length-corrected traces on the reference gel were composed of 445 absorbance values (after removal of the initial and final bands), and the background cutoff was set at 0.4. The best fit between each pair of traces was obtained by laterally shifting one corrected trace with respect to the other in single-point steps of 160 μm up to three points on either side of the initial alignment. The analysis was based on the whole of the protein pattern and included proteins to 31.5 kDa.

A data base was created with the patterns of the first 100 strains of *C. difficile* received in chronological order. These represented the full diversity of protein pattern types observed in the strains of the present study. The strains (CD36/92 to CD136/92) were those used in the cluster analysis. The remaining 39 strains (CD262/92 to CD305/92) were allocated to a particular EP type by using an identification program. Utilizing the Pearson product moment correlation coefficient, the program calculated and ordered those five strains in the data base having the highest similarity to the unknown. Final placement of an unknown strain to a particular EP type was determined by the highest similarity achieved and subsequently confirmed by introduction into the data base and unweighted pair group with mathematical averages (UPGMA) clustering. Identity was accepted only if the top strains selected were of a single type and they gave similarities well above the cutoff threshold for phonon formation.

**RESULTS**

**Study population.** All of the study population were symptomatic in that they fulfilled the selection criteria detailed earlier. However, 11 patients with diarrhea harbored strains that were nontoxicogenic. The majority of the population were elderly, with 77% of cases among patients ≥60 years of age (range, 13 to 93). The male-to-female ratio was 1:1.3. Conditions prior to the onset of diarrhea included pneumonia, solid tumors, and hematological malignancies. The antibiotics being administered to the patients comprised primarily cephalosporins, clindamycin, penicillins (ampicillin and amoxicillin), and metronidazole (administered parenterally); some patients were also receiving antineoplastic drugs.

**Typing of C. difficile.** One-dimensional SDS-PAGE of whole-cell protein extracts of the 145 isolates studied produced patterns containing about 40 discrete bands with molecular masses of 18 to 100 kDa. Most of the bands were relatively weakly stained; a number of these were common to all strains and were therefore species specific. However, there were several prominent bands in the region between 33 and 55 kDa, and the mobility of between one and three of these varied between strains. The patterns served both to group strains when common bands were evident and to differentiate these groups on the basis of pattern heterogeneity. Representative PAGE protein patterns for each EP type are illustrated in Fig. 1. The protein patterns of the isolates examined were highly reproducible both within and between gels. Replicate protein samples of the reference strain and molecular weight protein standards, run on each gel, gave similarity values of 94.3% ± 2.1% and 95.0% ± 2.4%, respectively.

Numerical analysis of PAGE total protein profiles based on the determination of the Pearson product moment correlation coefficient and UPGMA clustering revealed that at the 77% similarity (S) level, the first 100 *C. difficile* isolates formed a total of 17 distinct phenons, as shown in the dendrogram (Fig. 2). Each phenon represented a different protein profile type, i.e., EP types 1 to 17. Two subphenons were evident for type 9, subtypes 9a and 9b, and some heterogeneity was also evident within EP type 14 from a visual inspection of the banding patterns. The remaining strains were assigned to a particular type after comparison of their individual patterns against the data base. The EP types of all strains are given in Fig. 3.

Three types, 1, 4, and 5, accounted for 70% (97 of 139) of all isolates. Type 1 was the most common (70 of 139), although 55 of these were isolated in a single year, 1992. The remaining 14 types were each represented by up to seven isolates, and of these, five types were represented by single strains. The distribution of the EP types over the period of the study is shown in Fig. 3.

The six environmental isolates were recovered from two wards (385 and 497) on which the outbreak type, EP type 1,
Distribution of EP type 1 by ward. Five wards (385, 395, 396, 497, and 498) accounted for 73% (102 of 139) of all isolates of C. difficile, with the largest proportion occurring on ward 385. In wards 385 and 396, EP type 1 accounted for more than 74% of the 35 and 13 isolations, respectively (Fig. 3). However, although 26 and 18 isolates were made on wards 497 and 498, respectively, of these only 46 and 22% were EP type 1. Ten strains were isolated on ward 395 (medical oncology), but none were EP type 1.

Production of toxin by EP type. Of the 17 different EP types, 15 were composed of strains that were always toxigenic. In the remaining two groups (EP types 14 and 15; 11 strains) all strains were nontoxigenic.

DISCUSSION

This study confirmed that C. difficile diarrhea is, in the main, an infection of the elderly and is associated with prior antimicrobial therapy. It remains to be determined, however, whether C. difficile-associated diarrhea is an endogenous disease (which implies overgrowth of the organism in a susceptible host) or an exogenous disease or both. Thirteen patients in this study had AAD and harbored the organism on admission. The remaining 112 patients developed diarrhea while in the hospital.

Although this study was not prospective and was confined only to hospitalized symptomatic patients, it did survey the normal background incidence of C. difficile in such patients for some 2 years prior to the commencement of an apparent outbreak. Before August 1991, 44 isolations were made, representing a multiplicity of types of C. difficile (i.e., 16 of the 17 EP types defined in this study [all except EP type 2]). None were particularly predominant, but EP type 4 occurred in the largest numbers (nine isolates; compared with eight isolates of EP type 1). While 112 patients clearly developed diarrhea in the hospital, we do not know whether they carried these strains on admission and overgrowth occurred after treatment with inciting antimicrobial agents. Alternatively, they may have acquired the organism in the hospital either from the environment or by person-to-person contact (staff or other patients). The prospective study of McFarland et al. (21) showed that 13 of 17 types were isolated from asymptomatic patients on admission to the hospital but that many of these same types were subsequently recovered from symptomatic patients, the implication being that many cases of AAD associated with C. difficile are imported to the hospital endogenously by the patients. It is likely that, prior to the outbreak, this was also the case in our study. However, there is some evidence of cross-infection, as one of the more predominant types, EP type 4, was concentrated on ward 395.

Sporadic isolations of EP type 1 were made on five wards in the 2 years prior to the onset of the outbreak, and there was no association with any particular ward. It is apparent that EP type 1 was responsible for an initial outbreak of infection on ward 385. The outbreak, defined here as eight cases with the same C. difficile type on a single ward within a period of 8 weeks, was considered to have started in November 1991. From this time, 15 of 19 subsequent isolations from ward 385 were also of EP type 1. Analysis of the sequence of types seen over time shows that once established on ward 385, EP type 1 then spread to cause further secondary outbreaks successively through wards 376, 396, 497, and 486. EP type 1 became widely distributed in the hospital, accounting for 62 of 95 (65%) of all isolations and being found in 12 of the 14 wards. This type has now become endemic in a number of the wards in the hospital.
and despite the larger number of isolations of C. difficile, the number of other types has declined from 16 to 10. Clearly, in this outbreak situation, isolates must have been acquired nosocomially from an exogenous source. This is supported by the typing results which show that of the 13 patients admitted with C. difficile diarrhea, none had EP type 1 strains. Further evidence for this is provided by a patient who was admitted to ward 497, where she had diarrhea which yielded EP type 5 and maintained this strain even after subsequent transfer to ward 385. One month later, she had acquired the outbreak strain EP type 1. Isolates of EP type 1 were subsequently obtained from environmental samples from wards 385 and 497. There is no simple explanation for EP type 1 to have suddenly become more prevalent and to cause the outbreak of infection. Increased dissemination or enhanced virulence of the strain or increased spore survival may be the explanation.

Patients can relapse following apparently successful treatment. In accordance with the results of others (3, 16, 24, 27), our data on multiple isolations from single patients show that relapse may be due to either strains of a different type or the same strain. Although the reasons for cases of relapse remain unclear, the acquisition of strains of a different type probably represents exogenous reinfection. However, there remains the possibility that patients, as shown by Sharp and Poxton (31), harbor strains of more than one type. In contrast, other authors (8, 27, 30) found that their patients harbored strains of only a single type, thereby suggesting endogenous recrudescence with the same type after relapse. Of the eight patients involved in probable instances of recrudescence, EP type 1 was the most common. However, since this was the outbreak type and six of the eight cases occurred after the outbreak had become established, exogenous reinfection was a distinct possibility. It should be noted, however, that cases of relapse characterized by different types were sampled at longer time intervals and most occurred prior to the onset of the outbreak, compared with those giving the same type.

Our results show a clear correlation between toxin production and protein type. Recent studies of C. difficile outbreaks have suggested that there may be an association between toxigenicity, type, and clinical presentation (21, 36, 37). This
FIG. 3. Time sequence plot of the distribution of *C. difficile* EP types by ward over the period of study. Each of the 17 types is indicated numerically within each representative symbol. The predominant EP types are as follows: ■, EP 1; ○, EP 4 or 5; ◦, all other EP types. Months are indicated, from January through December, by their initial letters.
correlation does not appear to be clear-cut, since many investigators, using different typing systems, have reported both toxin production and nonproduction by isolates of the same type (7, 21). Delmée et al. (7) reported that in 5 of their 10 serogroups (including serogroup A), some strains were toxin producers and some were not. However, the use of a more sensitive technique—protein typing of serogroup A strains—showed a clear correlation between protein type and toxin production (7), indicating that the use of more-discriminatory typing methods could produce a more complete correlation. Some of our EP types were composed of only small numbers, as was the case with other typing studies (7, 21, 36, 37), and it is possible that the correlation noted here would not be maintained if larger numbers were used. A further bias is introduced by the likelihood that isolates from a single outbreak represent a related set of strains and would all be toxigenic. The relationship between toxin production and diarrhea remains, in any case, controversial, as at least two groups have reported patients with diarrhea but harboring only nontoxigenic strains (22, 28). This reflects our findings with EP types 14 and 15.

Although this study was confined to a single hospital, total protein patterns were used to define 17 EP types. Thus, SDS-PAGE is useful for typing strains of C. difficile and, as in previous studies (7, 13, 25), appears to offer a degree of discrimination better than or similar to that of most other typing schemes (33). Different electrophoretic methodologies have been employed in the typing of C. difficile by protein patterns, and it appears to be the most popular general approach. Typeability is extremely high, and in theory all strains can be typed; in practice, well over 95% of isolates can be typed to previously defined types and any new patterns can be added as new types. Discrimination varies according to technique and the method used to define types. The radiolabeling technique (14, 32) has low discrimination, with only 9 types currently defined compared with more than 17 types by using whole-cell or EDTA-extracted protein patterns (11, 28). The radiolabeled-protein method is more expensive and requires special facilities, while EDTA extraction and immunoblotting (19, 21, 22, 29) involve additional procedures but may not produce any obvious improvement in discrimination.

In conclusion, whole-cell protein typing by SDS-PAGE can be successfully applied to the epidemiologic investigation of C. difficile outbreaks. It is relatively cheap and is not technically complex, and all strains are typeable. The use of a high-resolution scanner coupled with sophisticated computer software for the analysis of patterns, although not obligatory, allows for the objective comparison of large sets of patterns produced on different gels.

ACKNOWLEDGMENTS

We thank the Department of Medical Illustration at the Central Public Health Laboratory for photographic assistance and Bernadette Kucera, infection control coordinator, Henderson Hospital, for assistance in collating patient and clinical data.

REFERENCES


