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Restriction Endonuclease Analysis of Nosocomial Isolates of *Clostridium difficile*

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A total of 110 clinical isolates of *Clostridium difficile* were analyzed by agarose gel electrophoresis by using both bacterial restriction endonuclease analysis (REA) and plasmid profiles. A total of 72 isolates were divided into 12 groups according to their REA patterns. Some 38 isolates exhibited unique patterns. Pattern A occurred in 20% of isolates. Isolates with patterns B, E, and G were cytotoxin negative. The remaining groups were cytotoxin positive. Multiple isolates obtained from two stool specimens were studied to examine the variation in REA profiles found in single specimens. In these specimens no variation in REA profiles was found. The stability of *C. difficile* was studied by examining sequential in vitro subcultures of a single isolate and strains isolated over a 4-month period from two long-term carriers. REA patterns were stable over time, both in vitro and in vivo. Because plasmid DNA was observed in 53% of isolates, plasmid profiles alone could not be used to study the spread of *C. difficile*; however, they were necessary for the interpretation of REA patterns in some instances.

Clostridium difficile is the major etiologic agent in antibiotic-associated diarrhea (2). Multiple clusters of nosocomially acquired infections have been described before (10, 11, 15, 18, 24, 25, 30), some of which have been associated with isolates from the environment (12, 15) or isolates from the stools of health care workers (15). Several investigators have attempted to distinguish isolates of *C. difficile* by using phage typing (26), protein profiles (12, 21, 27, 31), serotyping (8, 21), and plasmid analysis (20, 33).

Restriction endonuclease analysis (REA) of bacterial DNA has been used to study the epidemiology of several bacteria (29), including *Corynebacterium diphtheriae* (22), *Corynebacterium* spp. group JK (14), *Vibrio cholerae* (13), *Salmonella typhi* (17), *Legionella pneumophila* (28), *Neisseria meningitidis* (16), *Citrobacter diversus* (19), and *Campylobacter jejuni* (6, 23). We adapted the techniques described by Bradbury et al. (5, 6) to gain insight into the molecular epidemiology of this organism by using both REA and plasmid analysis.

MATERIALS AND METHODS

Analysis of stored isolates. A total of 110 clinical isolates from our stored collection were analyzed by using both plasmid profiles and bacterial REA. Results were correlated with cytotoxin production.

Analysis of multiple isolates from the same stool specimen. Two stool specimens from different patients were chosen for study. When numerous colonies of *Clostridium difficile* were present, 10 colonies per specimen were frozen in skim milk. When less than 10 colonies were apparent, all colonies were frozen in skim milk for REA.

Stability studies. Two stability studies were undertaken. The first was an in vitro study. Isolate 426, a plasmid-free isolate obtained from a patient admitted to the Toronto General Hospital, was subcultured 9 times onto blood agar. After each subculture, one colony was frozen in skim milk and one colony was subcultured onto blood agar. After passage 9, all isolates were subjected to REA.

The second stability study was a longitudinal study of the in vivo carriage of isolates. Stool samples from two patients (Cd-1 and Cd-3) were cultured weekly for *C. difficile* and remained positive for prolonged periods. Stored isolates from these two patients were analyzed by REA and plasmid profiles.

Isolation of *C. difficile* and toxin studies. Specimens were inoculated onto cycloserine-cefoxitin-fructose agar with sodium taurocholate (TCCFA) and into Buchanan enrichment broth (7, 32). When primary plates were negative at 48 h, the enrichment broth was subcultured onto TCCFA. Both primary plates and subcultures were reexamined after a second 48-h anaerobic incubation period. Stool samples and isolates were assessed for cytotoxicity by using WI-38 cells and standard techniques (2).

Growth of isolates for REA and plasmid profiles. Cultures were incubated anaerobically for 24 h at 35°C on blood agar plates. They were then inoculated into anaerobic brain heart infusion (BHI) broth containing 1.6% glycine and incubated anaerobically at 35°C for 15 h.

REA of *C. difficile*. Total cellular DNA was extracted and subjected to REA by the methods described by Bradbury et al. (6) with the following modifications. The lysis buffer consisted of 25% sucrose, 0.05 M NaCl, 0.005 M cyclohexanediaminetetraacetate, and 0.5 M Tris hydrochloride (pH 8) (TCS buffer) with 3 mg of lysozyme per ml. After the addition of lysis buffer, the mixture was incubated for 30 min at 35°C. After the addition of sodium dodecyl sulfate, the mixture was incubated for 20 min at 35°C. Precipitation of DNA in cold absolute alcohol was carried out at −20°C overnight. Restriction digests were performed by using *Cfo*I according to the instructions of the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Electrophoresis was carried out with a 0.7% agarose gel.

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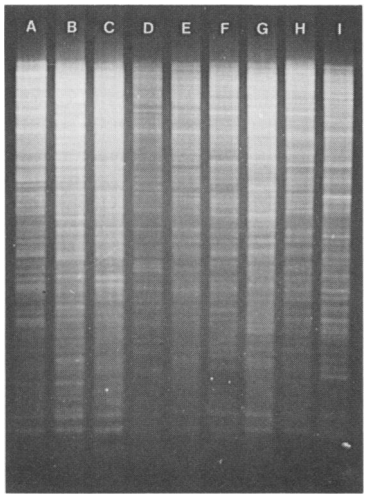

FIG. 1. Agarose (0.7%) gel electrophoresis of total cellular DNA from *C. difficile* isolated from patients at the Toronto General Hospital. This reference gel demonstrates the nine major REA profiles. DNA was digested with the restriction endonuclease *Cfo*I.

For optimal interpretation, the negative of a photograph (Polaroid) of the REA pattern was viewed with backlighting. When REA patterns were compared, the patterns were initially compared with plasmid profiles, and bands that comigrated with plasmid bands were discounted. Isolates that electrophoresed on different gels which appeared to share the same pattern were subjected to REA a second time and were electrophoresed on the same agarose gel to confirm that the patterns were indistinguishable by using *Cfo*I.

Plasmid analysis of *C. difficile*. Plasmids were extracted from a 1.5-ml culture of *C. difficile* grown in BHI broth with 1.6% glycine by the methods described by Bradbury et al. (5) with the following modification. Plasmid extraction buffer I was modified to contain TCS buffer with 10 mg of lysozyme per ml. Electrophoresis was performed on a 0.7% agarose gel.

RESULTS

Stored isolates. It was possible to analyze the DNA restriction patterns of all 110 isolates studied by using the enzyme *Cfo*I. Patterns were reproducible when isolates were retested after storage in cooked meat for several months. A total of 72 isolates were divided into 12 groups: 9 major groups, consisting of three or more isolates sharing the same REA pattern, and 3 minor groups, consisting of two isolates each. Some 38 isolates (34%) exhibited unique patterns. A reference gel containing the REA patterns of the nine major groups is shown in Fig. 1. All REA patterns that occurred in two or more isolates, the number of isolates in each group, and cytotoxicity status are listed in Table 1. Strains exhibiting patterns B, E, and G were cytotoxin negative. Strains belonging to the remaining common patterns were cytotoxin positive. The most common pattern was pattern A, which occurred in 20% of isolates.

Plasmid bands were observed in 53% of the stored isolates. The number of bands per strain ranged from 1 to 7. Plasmids ranged in size from <1.8 to 86 megadaltons. Most plasmid profiles were unique, with the exception of five groups of isolates. Strains 81, 75, 64, 63, and 21 had the same plasmid profile. Strains 24 and 76 also shared the same plasmid profile. They were isolated from patients that were admitted to the same ward 18 months apart. In three situations strains with the same plasmid profile were isolated from patients on different wards (strains 43 and 5, strains 71 and 50, and strains 39 and 31). Although plasmid profiles and REA patterns correlated in three groups, different REA patterns were found among strains in two groups (Table 2).

TABLE 1. Restriction endonuclease patterns of nosocomial isolates of *C. difficile*

Pattern	No. of isolates	Toxicity status
A	22	+
B	5	−
C	4	+
D	4	+
E	9	−
F	7	+
G	5	−
H	7	+
I	3	+
J	2	+
K	2	+
L	2	+

Analysis of multiple isolates from the same stool specimen. The REA patterns of 10 isolates from a single stool specimen from patient Cd-64 (Fig. 2) were indistinguishable, as were the REA patterns of four isolates from a single stool specimen from patient Cd-33.

Stability studies. When the original isolate of strain 426 and all nine subcultures were subjected to REA, the REA patterns were indistinguishable by using *Cfo*I (Fig. 3).

Although strains isolated on 27 May 1985 and 3 June 1985 from patient Cd-1 contained two plasmid bands, and strains isolated on 17 May 1985, 10 June 1985, 25 July 1985, and 3 August 1985 contained no plasmids, the REA patterns remained constant. Strains from patient Cd-3 did not exhibit plasmid DNA. All restriction endonuclease patterns from strains isolated between 6 June 1985 and 27 September 1985 were indistinguishable.

DISCUSSION

A successful subtyping system must be applicable to all isolates of the species under study and should be stable and

TABLE 2. Correlation of *C. difficile* isolates sharing the same plasmid profile with REA pattern

Plasmid group	Isolate	Size (megadaltons) of plasmids	REA pattern
I	81	4, 7.2	A
I	75	4, 7.2	A
I	64	4, 7.2	Unique
I	63	4, 7.2	Unique
I	21	4, 7.2	A
II	24	2.3, 4.1, 6, 19	A
II	76	2.3, 4.1, 6, 19	A
III	43	4.2, 7.8, 20, 28.5, 47	Unique
III	5	4.2, 7.8, 20, 28.5, 47	Unique
IV	71	4.1, 6.4, 7, 12, 19	C
IV	50	4.1, 6.4, 7, 12, 19	C
V	39	20, 26.5, 47	B
V	31	20, 26.5, 47	B

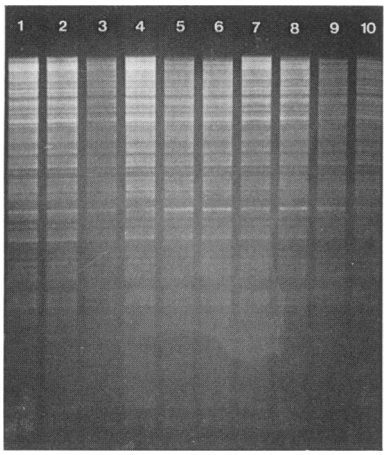

FIG. 2. Agarose (0.7%) gel electrophoresis of total cellular DNA extracted from 10 *C. difficile* isolates taken from a single stool specimen from patient Cd-64. All isolates exhibited the same REA pattern when restricted by *Cfo*I.

discriminative. In addition, groupings should correlate with important phenotypic markers. To facilitate the study of nosocomial acquisition of *C. difficile*, both restriction endonuclease analysis and plasmid profiles were used to develop a system which subdivided isolates of *C. difficile* based on genotypic differences.

Initial technical problems associated with lysis of *C. difficile* isolates and poorly resolved REA profiles were overcome by using two strategies. Allcock et al. (1) found that they could increase the rate of protoplast formation of *Clostridium acetobutylicum* by preconditioning cells by growth in clostridium basal medium with 0.4% glycine. DNA extraction was optimal when 1.6% glycine was added to anaerobic BHI broth, enabling complete lysis of *C. difficile*. Background staining was minimized when the initial incubation period in BHI broth with glycine was decreased from 24 to 15 h. The nonspecific staining that we observed may have been due to the production of DNase by *C. difficile*, because DNase production is known to occur in other *Clostridium* species and has been reported to affect plasmid analysis (4).

A subtyping system which subdivides isolates by toxin production is essential if these systems are to be used to study the nosocomial spread of *C. difficile*. Using *Cfo*I, we were able to divide 72 of 110 strains of *C. difficile* into 12 distinct groups. Strains grouped together by REA with *Cfo*I did not differ in cytotoxicity status.

The stability of REA patterns was demonstrated both in vitro for nine subcultures and in vivo over a 4-month period in two chronic carriers. These results are in agreement with those of Khabbaz et al. (14), who demonstrated that *Corynebacterium* spp. group JK could be isolated from the rectum of three patients at 3, 6, and 9 months after the initial culture without demonstrable changes in REA pattern. Similarly, Bjorvatn et al. (3) showed both that the REA pattern of a single isolate of *N. meningitidis* remained unchanged following 29 successive passages in vitro and that a single patient maintained colonization with a strain of *N. meningitidis* with the same REA pattern for 13 months.

Although *Cfo*I was highly discriminative, isolates grouped together cannot be considered the same. As many as three enzymes may be necessary to demonstrate differences in REA patterns. We are presently examining a wide range of enzymes to determine which enzymes will provide additional discriminative value.

It was necessary to examine all strains for plasmid DNA because the presence of plasmids could confound the interpretation of REA patterns. As a typing scheme, however, plasmid profiles could not be used alone to study the spread of *C. difficile* because they were present in only 53% of isolates and because of the great variability of plasmid profiles found in isolates from individual patients. Factors responsible for this variability may include loss of plasmids on storage and the presence of multiple forms of the same plasmid in various preparations. Although other investigators have been able to use plasmid profiles to study isolated outbreaks (33), no investigators have found plasmids in more than 60% of *C. difficile* isolates (20; C. Garrett, S. Lee, D. Gerding, L. Peterson, Program Abstr. 25th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 156, 1985). Moreover, Morris et al. (19) found that plasmid profiles do not reflect phenotypic differences between isolates of *Citrobacter diversus*, while serotyping and REA demonstrate that there is a correlation.

Several other systems have been developed to study the epidemiology of *C. difficile*. The first widely accepted system was phage typing. While it has been used to study epidemics (H. Hächler and J. Wüst, Letter, J. Clin. Microbiol. **20**:604, 1984), it cannot be applied to all isolates at present. Tabaqchali et al. (27) have developed a reference set of nine protein profiles of [^{35}S]methionine-labeled *C. difficile*. This system has been used to study and control an outbreak of *C. difficile*-associated diarrhea in an oncology ward by dividing patients infected with *C. difficile* (12) into cohorts.

Wexler et al. (31) studied the polyacrylamide gel electrophoresis (PAGE) patterns of both cell extracts and EDTA extracts of *C. difficile*. They felt that the former system was too complicated to interpret. Mulligan et al. (21) have expanded the latter system and have described five distinct PAGE profiles of EDTA extracts. Preliminary evidence indicates that these profiles correlate with both toxin A and toxin B production (M. Mulligan, R. Kwok, C. Clabots, and L. Peterson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, I11, p. 166). A major problem associated with PAGE profiles

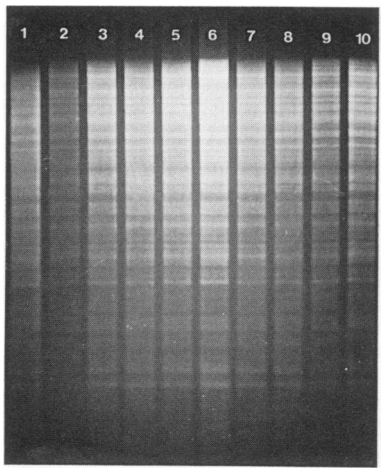

FIG. 3. Agarose (0.7%) gel electrophoresis of total cellular DNA extracted from the same *C. difficile* isolate (426; REA profile E) following nine successive weekly passages. Lane 1, Original isolate 426; lanes 2 to 10, REA profiles of the nine weekly subcultures. All isolates had indistinguishable REA profiles.

has been the comparison of results between laboratories and between gels electrophoresed in the same laboratory.

Recently, Delmee et al. (8) have developed a method of serotyping *C. difficile* by using slide agglutination with adsorbed antisera prepared in rabbits. Of the 10 serogroups described here, 9 correlated with PAGE patterns of cell extracts of proteins and 1 group (group A) was divisible into 12 different PAGE patterns (9). Results of initial work in our laboratory and those of S. Toma (Provincial Health Laboratory, Toronto, Ontario, Canada) suggest that there exists a substantial correlation between the serotyping system developed by Delmee et al. (8) and REA. At this time, however, serotyping is not capable of subdividing organisms based on cytotoxin production (9).

We are continuing to compare our REA system with established phenotypic systems to determine the relationships between these systems and REA. Experience in our laboratory and that of others has demonstrated that REA is highly stable, discriminative, and applicable to all strains of all bacteria examined to date. The use of a genotypic system that is applicable to all species of bacteria will greatly facilitate the study of the spread of infection, both in the community and in institutions.

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