JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1994, p. 1963–1969
0095-1137/94/$04.00+0

Vol. 32, No. 8

Comparison of Restriction Endonuclease Analysis, Ribotyping, and Pulsed-Field Gel Electrophoresis for Molecular Differentiation of *Clostridium difficile* Strains

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Received 9 February 1994/Returned for modification 22 March 1994/Accepted 5 May 1994

A combined clinical and molecular epidemiologic analysis of 46 strains of *Clostridium difficile*, including 16 nosocomial isolates from one ward (outbreak ward) plus 17 other nosocomial isolates and 13 community-acquired isolates, was performed. *Hin*dIII digests of total cellular DNA were analyzed by restriction enzyme analysis (REA) and ribotyping; *Sma*I digests were analyzed by pulsed-field gel electrophoresis (PFGE). Isolates were assigned to typing groups on the basis of the profiles detected; isolates with closely related profiles were assigned to subgroups. The 16 isolates from the outbreak ward were resolved by both REA and PFGE into five distinct groups; 13 isolates represented two REA groups and three PFGE groups and two isolates were resolved as distinct groups by both techniques. DNA obtained from one isolate was persistently partially degraded, precluding analysis by PFGE. Seventeen sporadic nosocomial isolates were resolved by REA and PFGE into comparable numbers of groups (i.e., nine groups) and subgroups (i.e., 15 and 14 subgroups, respectively), with two isolates not evaluable by PFGE. The 13 epidemiologically unrelated community-acquired isolates were assigned to 11 groups by REA and to 12 groups by PFGE. Overall, ribotyping identified only nine groups among the 46 isolates. We conclude that REA and PFGE have comparable discriminatory powers for epidemiologic typing of *C. difficile* isolates and that ribotyping is appreciably less discriminatory. For a few isolates, partial DNA degradation prevented analysis by PFGE but not by REA or ribotyping; the cause of the degradation is unknown.

Clostridium difficile causes a spectrum of colonic diseases ranging from antibiotic-associated diarrhea to pseudomembranous colitis and produces significant morbidity among hospitalized patients (4). Sporadic cases of *C. difficile* disease may represent overgrowth of endogenous organisms (7), but nosocomial clusters of cases have been ascribed to interpatient transfer of organisms through contaminated environmental sources as well as by hospital personnel (9, 26). Multiple techniques have been used to identify distinct strains of *C. difficile* and to resolve epidemiologically related cases. The earlier typing systems based on phenotypic traits or on plasmid and phage profiles were insufficient for comprehensive analyses, since phenotypes lack discriminatory power and not all strains are typeable by phage and plasmid studies (10, 14, 16, 21, 33, 37, 38). Subsequently, *C. difficile* typing based on restriction endonuclease analysis (REA) was shown to be reproducible, highly discriminatory, and universally applicable (8, 15, 17, 20). In this technique, total cellular DNA is digested with a frequently cutting restriction enzyme, and the resulting fragments are resolved by agarose gel electrophoresis. However, the visual assessment of hundreds of restriction fragments in a single gel can be difficult and may be confounded by the presence of extrachromosomal DNA (6, 15). Southern blot analysis using a ribosomal probe (ribotyping) detects fewer fragments and can therefore be easier to read, but it may have less discriminatory power (8, 34). Chromosomal DNA digests prepared with an infrequently cutting restriction endonuclease can be resolved by pulsed-field gel electrophoresis (PFGE) into 10 to 20 discernible bands representing the entire chromosome (1, 25). PFGE has been shown to be more discriminatory than ribotyping in the study of several bacterial pathogens, including *Escherichia coli* (1), *Staphylococcus aureus* (29), and *Klebsiella* spp. (23), but has not previously been systematically evaluated for *C. difficile*. The purpose of this study was to compare REA, ribotyping, and PFGE as systems for analyzing epidemiologically related and unrelated isolates of *C. difficile*.

MATERIALS AND METHODS

Study population. A prospective epidemiologic study of *C. difficile* colonization and diarrhea was initiated at New England Deaconess Hospital (NEDH) to investigate the persistently high incidence of *C. difficile* diarrhea (~15 cases per 1,000 admissions). Patients admitted or transferred to two wards (one predominantly medical and one predominantly vascular-surgical) and three intensive care units (two surgical and one medical) constituted the study population. From January through May 1991, for patients enrolled in the study, specimens for culture were collected by rectal swab within 72 h of admission to the study sites and then weekly until discharge.

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During the study period, all NEDH patients who had diarrhea and whose stools were positive for cytotoxin also had samples taken for culture for *C. difficile*. Details of the epidemiologic analyses for the entire study population have been presented elsewhere (30, 31).

An isolate was considered community acquired if *C. difficile* was recovered from stool cultures obtained within 72 h of admission and the patient had not been hospitalized within the previous 60 days. An isolate was designated NEDH-acquired if (i) the initial culture specimen was negative and a subsequent culture specimen obtained >72 h after admission was positive; (ii) the initial culture specimen was positive but the patient had been hospitalized at NEDH within the previous 60 days; (iii) the initial culture specimen was positive and a culture specimen obtained >72 h after admission yielded a different strain, in which case the subsequent isolate was considered NEDH acquired; or (iv) for nonstudy patients for whom no initial culture specimen was obtained, diarrhea began >72 h after admission and a positive culture specimen was obtained. An isolate was designated as acquired at another hospital if a culture specimen obtained within 72 h of admission to NEDH was positive and the patient had been hospitalized elsewhere within the previous 60 days.

The present study examined 46 isolates selected to include patients with different epidemiologic exposures. The incidence of *C. difficile* diarrhea was highest on the ward housing predominantly patients on the vascular-surgical service (outbreak ward); 16 of the isolates analyzed in this study were cultured from patients located on this ward, including five symptomatic patients hospitalized in January 1991, three symptomatic patients hospitalized over a 6-week period between 21 March and 3 May, 1991, and eight asymptomatic patients admitted over the course of the epidemiologic study. The remaining 30 isolates included 17 additional nosocomially acquired isolates (5 cultured from patients on other NEDH wards and 12 cultured from patients at the time of transfer to NEDH from other hospitals) and 13 community-acquired strains.

Microbiologic characterization. All primary cultures were performed at NEDH with rectal swabs or stool specimens. Agar plates containing cycloserine (500 μg/ml), cefoxitin (16 μg/ml), and fructose supplemented with sodium taurocholate were incubated at 37°C for 48 h in an anaerobic jar (31). Presumptive isolates of *C. difficile* were identified by characteristic odor and colony morphology. Toxigenicity was determined with an in-house cytotoxin B tissue culture assay (13) and an enzyme immunoassay for toxin A (Premier Meridian Diagnostics, Cincinnati, Ohio); nontoxigenic strains were confirmed to be *C. difficile* with a latex agglutination test (Culturette CDT; Becton Dickinson, Cockeysville, Md.) and by biochemical testing (RapIDS AnaII System; Innovative Diagnostic Systems, Inc., Atlanta, Ga.). After isolation of *C. difficile*, three colonies were sampled and stored at room temperature in chopped meat media. REA typing was performed at the Minneapolis VA Medical Center; samples from original isolates were transported overnight in chopped meat media. PFGE and ribotyping were done at the Boston VA Medical Center. Single colonies of strains to be typed were isolated at NEDH on anaerobic blood agar plates and inoculated into 5 ml of brain heart infusion broth under CO_2 with a cannula and grown overnight. The following morning the turbid broth was subcultured (1:33 dilution) into a fresh brain heart infusion broth under anaerobic conditions and immediately transported to the Boston VA Medical Center. PFGE and ribotyping were performed without knowledge of source, prior REA results, or epidemiologic relationship of the isolates.

Restriction endonuclease digestion. Whole-cell DNA was prepared as previously described (8, 9, 28). Briefly, brain heart infusion broth was inoculated with a single colony from an anaerobic blood agar plate and then incubated overnight. Cells were washed in TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), resuspended in 0.1 ml of TE with lysozyme (50 mg/ml; Sigma), incubated for 30 min at 35°C, mixed with 0.5 ml of GES solution (guanidine thiocyanate, 0.6 g/ml; EDTA, 100 mM; sarcosyl, 0.5%, vol/vol), incubated for 10 min at room temperature, mixed with 0.75 ml of ammonium acetate (7.5 M), and held on ice for 10 min. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with cold 2-propanol. For restriction digestion, DNA (10 to 20 μl) was incubated with *Hin*dIII (Bethesda Research Laboratories, Gaithersburg, Md.) according to the manufacturer's recommendations, except that 20 U of enzyme was used and 3 μl of spermidine (100 μg/ml; Sigma) was added. The resulting restriction fragments were resolved in a 0.7% agarose gel as previously described; the gel was stained with ethidium bromide and photographed under UV light.

PFGE analysis. The application of PFGE to the study of the molecular epidemiology of bacterial isolates has recently been described in detail (25). The procedure was applied with the following specific modifications. Isolates were inoculated into prereduced brain heart infusion broths and incubated at 37°C in a stationary position, and the optical density was monitored hourly in a Spectronic 21 spectrophotometer (Milton Roy Company, Rochester, N.Y.). When growth reached mid-exponential phase (optical density at 540 nm ≥ 0.500), typically 7 h after inoculation, the organisms were pelleted at 4°C and then processed as previously described (25). *C. difficile* DNA in agarose was digested with *Sma*I (New England Biolabs, Cambridge, Mass.), and the resulting macrorestriction fragments were resolved by PFGE. In our experience, overnight broths, although suitable for other bacterial species (25), yield insufficient DNA in agarose for PFGE analysis of *C. difficile* isolates.

The gels were electrophoresed for 22 h in a contour-clamped homogeneous electric field apparatus (CHEF DRII; Bio-Rad, Richmond, Calif.) at 6.0 V/cm, with initial and final switch times of 20 and 70 s, respectively, and linear ramping. The gels were stained with ethidium bromide and photographed under UV light. *Sma*I-digested *S. aureus* (ATCC 8325) was used as a molecular weight size standard (25).

Ribotyping. One agarose plug prepared as described above was washed in TE buffer and then melted at 65°C. The molten agar was allowed to cool to 42°C, digested with β-agarase (New England Biolabs), and subsequently restriction digested with *Hin*dIII (New England Biolabs) according to the manufacturer's recommendations. The digests were electrophoresed at 1.2 V/cm for 16 h under constant field conditions in a 0.8% agarose gel (SeaKem GTG; FMC, Rockport, Maine) with Tris-acetate-EDTA running buffer. Subsequently, the DNA in the gel was transferred to a Duralon-UV membrane (Stratagene, La Jolla, Calif.) in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) using a vacuum blotter (Bio-Rad). The membranes were then baked at 80°C for 30 min and UV cross-linked by using 20 mJ/cm^2 (UV Stratalinker 2400; Stratagene). Hybridization and washes were done at 65°C in a hybridization chamber (Robbins Scientific, Sunnydale, Calif.) by using the manufacturer's standard protocol. Probing was performed with the 7.5-kb *Bam*HI fragment isolated from pC6, which includes the entire *rrnB E. coli* operon of *E. coli* K-12 (5).

Definitions of groups and subgroups. For each method, isolates were independently assigned a group and a subgroup

designation; "subgroups" in this article correspond to "types" in a previous paper describing REA (8). In the initial analysis of REA patterns, isolates were assigned to subgroups by direct visual comparison of *Hin*dIII restriction digests; isolates with substantially similar banding patterns among fragments of <11.5 kb were assigned to the same group, with subgroups determined by the specific pattern of fragments of ≥11.5 kb (18).

For the final analysis of REA patterns in this study, isolates were organized into groups and subgroups on the basis of a quantitative fragment pattern similarity index (SI) (8). Isolates were run in a gel with up to eight strains which had been previously assigned to REA subgroups and whose patterns were judged to be similar to the pattern of the new isolate. The REA patterns were analyzed by visually comparing each 1-mm segment of the top 60 mm of DNA fragments on the same gel (typically including fragments ranging from 30 to 2 kb). An SI was calculated as the percentage of identical segments among the total of 60 segments analyzed. Isolates whose patterns were indistinguishable (SI = 100) from that of an existing subgroup were assigned to that subgroup; isolates with an SI between 90 and 99 relative to existing subgroups were assigned to a new subgroup within that group. Isolates with an SI of <90 relative to existing subgroups were assigned to a new group and became reference strains for future comparisons. REA groups were designated by one or two capital letters and REA subgroups were designated by arabic numerals.

Ribotypes and PFGE patterns were used to assign isolates to groups and subgroups on the basis of differences in the numbers and sizes of restriction fragments detected. Isolates that differed by one or two band shifts consistent with a single genetic event (restriction site mutation, insertion, deletion, or inversion) were designated as subgroups within the same group; isolates that differed by multiple changes not consistent with a single genetic event were assigned to different groups (25). All gels included molecular weight size standards to facilitate comparisons; all subgroup assignments were confirmed by running the isolates in the same gel. Ribotype groups were designated by uppercase letters and subgroups were designated by arabic numerals; PFGE groups were designated by roman numerals and subgroups were designated by lowercase letters.

RESULTS

Reproducibility. In preliminary studies comparing REA and PFGE, we noted that two isolates (698A and 730A) assigned to different REA groups were assigned to the same group by PFGE, while other isolates (e.g., 288A and 159) representing those REA groups were assigned to different, unrelated PFGE groups (Fig. 1A). Taken together, these results implied that isolates representing different genetic lineages (as resolved by one technique) could converge to the same genetic lineage (as resolved by another method). Such a conclusion is inconsistent with the fundamental premise that DNA-based analyses differentiate among isolates on the basis of divergent genetic variation (1, 24). That is, although different methods may vary in their ability to detect evolutionary divergence and, consequently, in their discriminatory power, isolates assigned to a particular genetic lineage by one technique should not be assigned to substantially different, unrelated lineages by an alternative approach.

We considered that these incongruent results could represent either variations in reproducibility or technical errors, such as the mislabeling of strains, occurring during the course of processing in the two separate laboratories. Consequently,

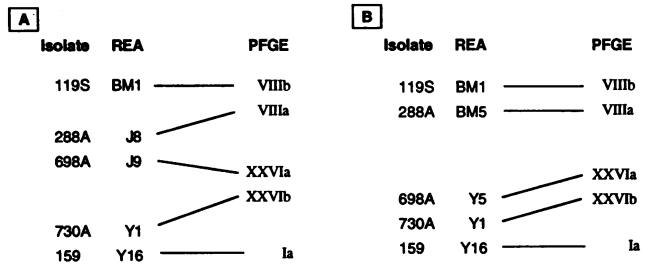

FIG. 1. Increased correspondence between PFGE and REA methods for typing *C. difficile* isolates associated with application of SI procedure for interpreting REA profiles. In panel A, REA groups and subgroups were assigned on the basis of visual inspection; in panel B, REA groups and subgroups were assigned on the basis of SI (as described in the text). Each panel represents a separate analysis in which isolates were examined independently by both methods. Isolate 159 is representative of the strain detected among symptomatic patients on the outbreak ward in January 1991 (see the text for details).

we reexamined these isolates plus additional strains representing multiple different groups and subgroups. Duplicate aliquots of each isolate were prepared simultaneously, coded, and sent blinded to the two typing laboratories. On the repeat PFGE analysis, each of the isolates was assigned to the same group and subgroup as previously. Reexamination by REA, now analyzed by using the SI, assigned four isolates (including two shown in Fig. 1) to groups different from those in the original study; the results for the remaining eight isolates were unchanged. For each of the isolates, the original and repeat restriction digests showed the same pattern of restriction fragments, indicating that the change in assignments was related to different methods of interpreting the digest profiles. The nonparallel classifications were resolved by using the revised REA assignments (Fig. 1B). We conclude that the SI provides a more reliable and consistent method for analyzing the REA digests than direct visual comparison.

Typeability. As expected, all isolates could be analyzed by REA and by ribotyping. However, three isolates could not be assessed by PFGE because the DNA prepared in agarose was consistently degraded into random fragments ranging from 100 to 200 kb in size. This mild degradation prevented the resolution of the large restriction fragments generated by digestion with infrequently cutting enzymes, such as *Sma*I, although these preparations produced distinct, reproducible *Hin*dIII restriction digests suitable for REA or ribotyping. Multiple attempts to avoid this degradation, including either immediately washing the organisms in EDTA and/or proteinase K or immediately heating them to 65°C with or without EDTA, were unsuccessful. Neither of these procedures adversely affected the isolation of DNA from the *S. aureus* control strain. This phenomenon has been noted by other investigators preparing DNA from *Clostridium perfringens* (12) and from other clinical isolates of *C. difficile* (36) for PFGE analysis. The problem appears to be particularly prevalent among clostridial isolates. In studies involving >300 isolates of the family *Enterobacteriaceae*, we have observed similar degradation with only two strains of *Klebsiella*; we have never observed it among >400 isolates of staphylococci (2).

Discriminatory power. The discriminatory power of the different techniques is indicated by the number of distinct typing groups defined among the isolates studied and the total number of distinct patterns detected (i.e., subgroups). Overall, REA and PFGE gave generally comparable results, while ribotyping identified approximately half as many distinct pat-

TABLE 1. Discriminatory power of different molecular methods for typing *C. difficile* isolates

Isolates	*n*	No. of groups/no. of subgroups identified by:		
		Ribotyping	REA	PFGE[a]
Nosocomial, outbreak ward	16	2/5	5/7	5/6
Nosocomial, sporadic	17	4/10	9/15	9/14
Community acquired, sporadic	13	7/12	11/13	12/13
Total	46	9/16	18/30	20/28

[a] One isolate from the outbreak ward and two other nosocomial isolates could not be typed by PFGE (see the text for details).

terns (Table 1). Among the total series of 46 strains, PFGE discerned slightly more groups than REA (20 versus 18, respectively). The three isolates that were nontypeable by PFGE all represented a single REA group, with each isolate assigned to a different subgroup. Consequently, overall, REA distinguished a slightly greater number of distinct patterns than PFGE (30 versus 28, respectively).

Among the 16 isolates from the outbreak ward, four of the five isolates from the patients with diarrhea in January 1991 were indistinguishable by each method (isolates 20s, 2s, 10s, and 159) (Fig. 2 through 4); the fifth isolate (12s) was indistinguishable by PFGE and was assigned to a related subgroup by REA. The three isolates obtained from patients with diarrhea on the outbreak ward during March through May were also indistinguishable from each other by all three methods but represented a distinctly different strain. Among the eight isolates from asymptomatic patients on the outbreak ward, REA and PFGE both detected three additional groups and ribotyping detected one.

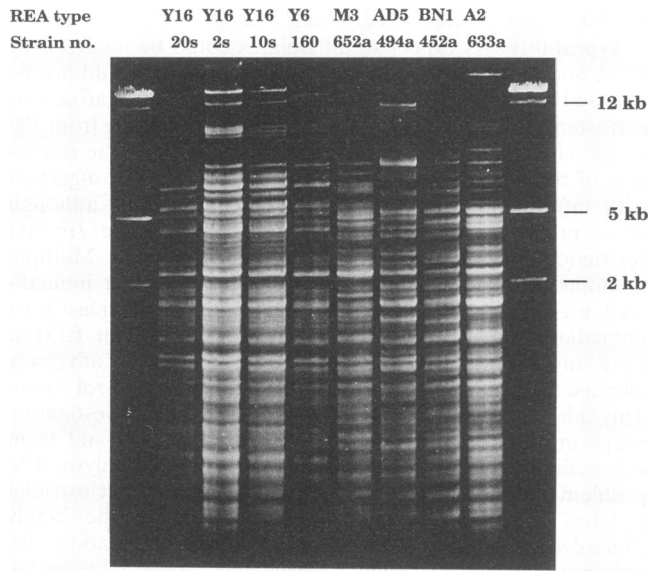

FIG. 2. REA (*Hin*dIII digests) of eight *C. difficile* isolates. Isolates 20s, 2s, and 10s were cultured from symptomatic patients on the outbreak ward in January 1991; isolate 160 was cultured from an asymptomatic patient on the outbreak ward; isolate 652a represents an isolate acquired at another hospital; and isolates 494a, 452a, and 633a represent community-acquired isolates. The extreme left- and right-hand lanes represent *Hin*dIII digests of bacteriophage lambda included as molecular weight standards.

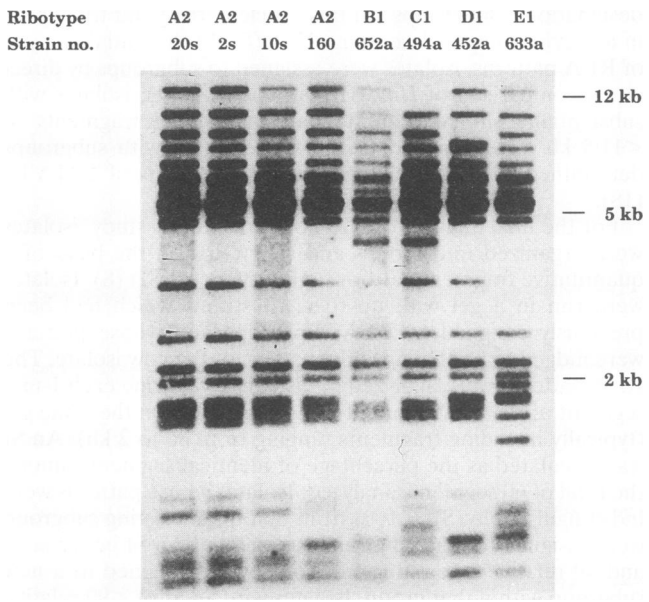

FIG. 3. Ribotype analysis (Southern blot of *Hin*dIII digests probed with an *E. coli* ribosomal operon) of the eight *C. difficile* isolates used for Fig. 2.

Among the 17 sporadic nosocomial isolates there was somewhat greater diversity; REA and PFGE again distinguished comparable numbers of distinct patterns and both were appreciably more discriminatory than ribotyping. As assessed by all three techniques, an isolate obtained from a symptomatic patient transferred from another facility in early January was indistinguishable from the three isolates subsequently cultured from symptomatic patients on the outbreak ward during March through May. Thus, the patient transferred in January appears to have introduced that strain into the hospital. Three other isolates obtained from transferred patients were also assigned to one of the typing groups detected among the isolates on the outbreak ward; however, all of these patients were admitted after isolates of those types had been detected at NEDH, and none had been hospitalized at NEDH in the preceding year.

The greatest diversity was detected among the community-acquired isolates. By PFGE analysis, 12 of these 13 isolates were assigned to different groups; REA detected 11 groups. By both techniques, three isolates were assigned to groups or subgroups detected among the hospital-acquired strains. In each instance, the nosocomial isolate was cultured prior to the community-acquired strain of the same type.

Correlation between toxin assays and molecular typing. Overall, 34 isolates were positive for both toxin A and cytotoxin (toxin B), 11 were nontoxigenic by both assays, and 1 (isolate 555) was negative for toxin A and positive for cytotoxin. Different isolates within the same REA and PFGE group were concordant with respect to toxigenicity, with one exception: REA group A included two nontoxigenic isolates (REA subgroup A2; PFGE group VII) and one toxigenic isolate (REA subgroup A1; PFGE group XII). Isolate 555, a community-acquired isolate cultured from an asymptomatic patient, represented a unique typing group according to all three methods.

DISCUSSION

In evaluating typing systems for studying the epidemiology of infectious agents, several basic criteria are relevant—type-

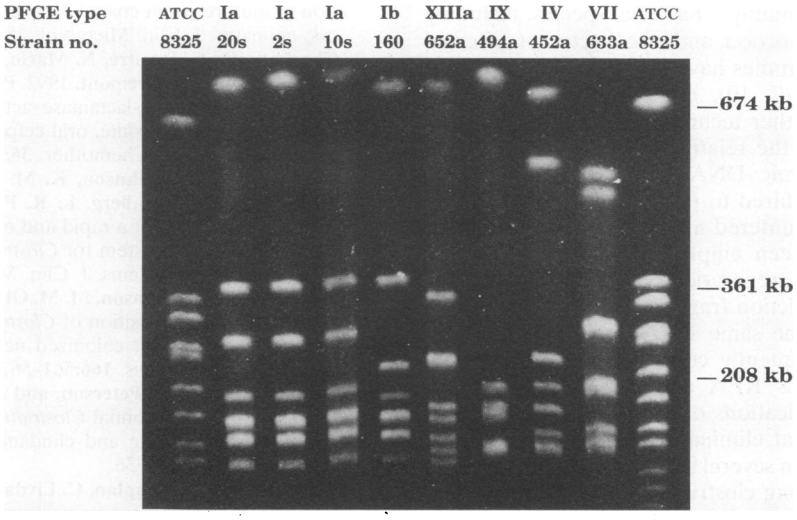

FIG. 4. PFGE analysis (*Sma*I digests) of the eight *C. difficile* isolates used for Fig. 2 and 3. The extreme left- and right-hand lanes represent *Sma*I digests of *S. aureus* ATCC 8325 included as molecular weight standards.

ability, reproducibility, and discriminatory power (24). The organisms causing the infection have to be typeable by the method; the characteristic being assessed by the typing system should be stable, and the method should provide consistent results. The ability to interpret the results easily facilitates applying the technique. Typing systems that are more discriminatory for differentiating among epidemiologically unrelated isolates are more reliable for establishing that isolates of the same type are epidemiologically related. Ideally, the typing method should be inexpensive and readily performed with widely available equipment.

The goal of this study was to analyze a set of epidemiologically well-characterized isolates of *C. difficile* by three different DNA-based techniques—REA, ribotyping, and PFGE. Although all three methods are based on restriction endonuclease digestion of DNA, they differ appreciably in the numbers and sizes of the restriction fragments detected. Both REA and PFGE directly analyze restriction digests of *C. difficile* DNA (~3,600 kb), but REA digests represent hundreds of fragments typically ranging from 0.5 to 30 kb, while PFGE digests comprise 6 to 11 fragments ranging from 10 to 700 kb. In ribotyping, the restriction fragment length polymorphisms associated with the ribosomal operons are detected by probing Southern blots prepared from the REA digests; ribotypes comprise 20 to 24 fragments ranging from 0.7 to 12.2 kb.

Overall, ribotyping was appreciably less discriminatory for analyzing *C. difficile* isolates than REA and PFGE. The latter two methods were comparable for detecting hospital outbreak strains and for differentiating among sporadic nosocomial isolates or epidemiologically unrelated community-acquired isolates. The most appreciable difference between REA and PFGE in this study was the ease of interpreting the different restriction profiles. The discrete number of well-resolved bands detected by PFGE could be readily analyzed by direct visual comparison, whereas the more complex REA patterns required more intensive segmental analysis of the gels to achieve reliable typing results.

The various phenotypic typing systems that have been applied to *C. difficile* isolates have recently been reviewed (35). Antibiotic susceptibility, bacteriocin production and susceptibility patterns, and cytotoxicity all have limited discriminatory power (21, 33, 38). The use of polyacrylamide gel electrophoresis (PAGE) to analyze bacterial proteins is somewhat more effective, particularly when coupled with [^{35}S]methionine labelling or immunoblotting (27, 35). In comparative studies, the discriminatory power of immunoblotting was superior to routine PAGE and serotyping and was inferior to that of REA (8, 27). Typing systems based on the analysis of plasmids and bacteriophage susceptibilities have also been applied to *C. difficile*, but they have limited utility as many strains are nontypeable (10, 27, 33, 38).

REA is a relatively uncomplicated method for analyzing total cellular DNA and has been successfully applied to several bacterial species, including *Streptococcus pyogenes* (11), coagulase-negative staphylococci (6), and *C. difficile* (15, 17). The method is thus broadly applicable and has proven reasonably discriminatory. However, the numerous restriction fragments that are generated are often poorly resolved, especially fragments of <11 kb (Fig. 2). Consequently, direct interpretation of the profiles may be unreliable for assigning isolates to typing groups and subgroups, as illustrated by the initial analysis in this study. To avoid this pitfall, we now analyze the restriction profile for each isolate by comparing 1-mm segments directly with the restriction profile of a reference strain(s) and thereby generating an SI. This procedure, although somewhat tedious, provides a more objective assessment than does an overall direct visual interpretation, but as the library of known subgroups and groups gets larger, the ability to correctly classify new isolates becomes more difficult. Digital scanning of gels and subsequent analysis using computer software might provide a less labor-intensive means of evaluating the profiles.

Ribotyping is based on a Southern blot analysis of the subset of restriction fragments associated with the ribosomal operons (34). *C. difficile*, like many other bacteria, has multiple ribosomal operons, and the ribotype profiles typically include up to 24 well-resolved bands (Fig. 3). However, as has previously been observed, ribotyping provides only limited discriminatory power for identifying different strains within a species, presumably because the restriction sites both within and flanking the ribosomal operons are relatively well conserved (3, 29).

*Sma*I digests of *C. difficile* DNA comprised 6 to 11 fragments that were well resolved by PFGE; these restriction profiles proved to be both relatively uncomplicated to interpret and highly discriminatory. PFGE has been successfully applied in

epidemiologic analyses of multiple bacterial species, including *E. coli*, staphylococci, enterococci, and mycobacteria (reviewed in reference 25). Recent studies have suggested that it is also applicable to *C. difficile* (7, 19), but the results were not compared with those of other techniques. The limitations of PFGE relate primarily to the relatively long processing time required to isolate genomic DNA in agarose and to the specialized equipment required to perform the electrophoresis. In this study, we encountered an additional complication that has not previously been emphasized. For 3 of the 46 strains, the genomic DNA preparations were mildly degraded, so that distinct *Sma*I restriction fragments for PFGE analysis could not be obtained. The same DNA could be effectively digested with a more frequently cutting restriction enzyme, such as *Hin*dIII, and thus REA and ribotyping were not affected. Numerous modifications of the DNA isolation procedure were unsuccessful at eliminating this problem, which, on the basis of experience in several laboratories, appears to be particularly prevalent among clostridial isolates. The mechanism of this degradation remains unknown but presumably involves a particularly active endonuclease. The observation that all three isolates, which were epidemiologically unrelated, represented the same, distinctive REA group suggests that there is a specific genetic basis for this phenotype.

Previous studies of nosocomial outbreaks of *C. difficile* have identified outbreaks involving a single strain (10, 16, 38). In this study, we detected several distinct strains among the isolates on the outbreak ward, indicating that multiple endemic strains can exist in the hospital simultaneously (30). This observation is compatible with evidence that nosocomial acquisition of *C. difficile* is facilitated by its persistence on environmental surfaces and that it is spread by personnel and by antibiotic pressure (9, 16, 18). The presence of several different strains in the same hospital has also been demonstrated for other endemic nosocomial pathogens, such as methicillin-resistant *S. aureus* (22) and *Legionella pneumoniae* (32). Reproducible, highly discriminatory typing systems such as those described in this study are, therefore, essential for reliably identifying specific nosocomial outbreaks and for aiding investigations of the clinical epidemiology of *C. difficile*-associated diseases.

ACKNOWLEDGMENTS

This work was supported by the Fogarty International Fellowship, National Institutes of Health (M.K.), and the Medical Research Program, Department of Veterans Affairs (R.D.A. and D.N.G.).

We thank Connie Clabots and Lata Venkataraman for technical assistance.

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