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PCR Amplification of rRNA Intergenic Spacer Regions as a Method for Epidemiologic Typing of *Clostridium difficile*

CHARLES P. CARTWRIGHT,[1]* FRIDA STOCK,[1] SUSAN E. BEEKMANN,[2]
ESTHER C. WILLIAMS[1] AND VEE J. GILL[1]

Microbiology Service, Clinical Pathology Department,[1] and Hospital Epidemiology Service,[2]
Warren G. Magnuson Clinical Center, National Institutes of Health,
Bethesda, Maryland 20892

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From January to March 1993, a suspected outbreak of antibiotic-associated diarrhea occurred on a pediatric oncology ward of the Clinical Center Hospital at the National Institutes of Health. Isolates of *Clostridium difficile* obtained from six patients implicated in this outbreak were typed by both PCR amplification of rRNA intergenic spacer regions (PCR ribotyping) and restriction endonuclease analysis of genomic DNA. Comparable results were obtained with both methods; five of the six patients were infected with the same strain of *C. difficile*. Subsequent analysis of 102 *C. difficile* isolates obtained from symptomatic patients throughout the Clinical Center revealed the existence of 41 distinct and reproducible PCR ribotypes. These data suggest that PCR ribotyping provides a discriminatory, reproducible, and simple alternative to conventional molecular approaches for typing strains of *C. difficile*.

Clostridium difficile, in addition to being the etiologic agent of pseudomembranous colitis, is the major cause of nosocomially acquired diarrhea in the United States (15), and a number of large outbreaks of *C. difficile*-associated disease have been reported (6, 17). Various phenotypic and genotypic methods have been used to type *C. difficile* strains, including antibiogram pattern comparison, serotyping, plasmid analysis, immunoblotting, restriction endonuclease analysis (REA) of genomic DNA, and arbitrary primer PCR (AP-PCR) (1, 5–7, 9, 19). Unfortunately, those methods with well-established discriminatory power and reproducibility (REA and immunoblotting) lack the ease and robustness necessary for them to be employed in the routine clinical microbiology laboratory.

Molecular analysis of the rRNA gene cluster has proved extremely valuable for both taxonomic, via sequence comparison (2), and epidemiologic, via conventional ribotyping (13), investigation of microorganisms. Species-specific sequence polymorphisms can be detected in PCR-amplified fragments of the 16S-23S rRNA intergenic spacer region, and analysis of these sequences has been of considerable use in differentiating closely related members of a number of bacterial genera (16). In addition to sequence polymorphisms in the spacer region, certain organisms possess multiple alleles of the rRNA gene cluster (*Escherichia coli*, for example, has seven such alleles [11]), with considerable interallelic variation occurring in the length and sequence of the 16S-23S rRNA spacer (3). Kostman et al. (12) recently showed that PCR-mediated amplification of the intergenic spacer region of isolates of *Pseudomonas cepacia* yielded a variable number of amplified fragments and further demonstrated that epidemiologically unrelated and related strains could be differentiated on the basis of the pattern of fragments generated by this technique, which they termed PCR ribotyping. Kostman and coworkers hypothesized that the different PCR ribotype patterns reflected strain-dependent variation in the possession of different rRNA alleles.

In a series of elegant experiments, Gürtler (8) recently demonstrated that *C. difficile* possesses 14 different rRNA alleles, each differing in 16S-23S intergenic spacer length, and showed that, at least with a limited number of isolates, considerable variation existed in the number of different alleles present in epidemiologically unrelated strains. The purpose of the present study was to evaluate the potential of PCR ribotyping as a tool for typing *C. difficile* and tracking nosocomial outbreaks of disease caused by this organism.

MATERIALS AND METHODS

Epidemiologic identification of *C. difficile* cluster. Hospital infection control data revealed a significant increase in the frequency of hospital-acquired cases of *C. difficile* diarrhea, defined as symptoms plus a positive test for *C. difficile* cytotoxin in stool, occurring in pediatric oncology patients during the months of January and February 1993 (Fig. 1). To aid in the identification of nosocomial outbreaks, cases of *C. difficile* diarrhea occurring in patients admitted to the hospital more than 72 h prior to diagnosis were arbitrarily regarded as hospital acquired. Cases occurring in outpatients or inpatients admitted sometime within the 72-h period preceding diagnosis were designated as community acquired. By these criteria, seven hospital-acquired cases were tightly clustered over a 10-day period in February 1993, and *C. difficile* isolates from six of these patients were available for analysis. Six toxigenic isolates obtained from community-acquired cases of *C. difficile* diarrhea occurring during the same time period were used as controls.

Isolates used for prospective study of PCR ribotypes. PCR ribotype analysis was performed on 102 isolates of *C. difficile*, obtained from stool cultures of 73 symptomatic patients at the National Institutes of Health Clinical Center, collected during the period from May 1993 to February 1994. Of these isolates, 91 were toxigenic and 11 were nontoxigenic.

Stool culture and cytotoxin testing. All *C. difficile* isolates included in the outbreak investigation and prospective study of PCR ribotypes were obtained from stool specimens submitted to the laboratory for *C. difficile* cytotoxin testing. The presence of *C. difficile* cytotoxin in specimens was detected by demonstration of the cytopathic effect of stool filtrates on a human foreskin fibroblast cell line and confirmed by neutralization with high-titer *Clostridium sordelli* antitoxin (Baxter Diagnostics, Inc., Deerfield, Ill.). To isolate *C. difficile*, specimens were inoculated onto cycloserine-cefoxitin-fructose agar, and cultures were incubated for 48 h at 37°C in an anaerobic chamber. Isolates were identified on the basis of their colonial morphology and biochemical profiles obtained with commercial test systems (AN-Ident and API 20A; Analytab Products Inc., Plainview, N.Y.). In vitro toxigenicity of isolates was determined by assaying for the presence of cytotoxin in chopped meat glucose broth culture supernatants. All *C. difficile* isolates were stored in chopped meat glucose broth.

DNA extraction. Isolates were inoculated into 10 ml of prereduced fastidious broth (4) and cultured anaerobically for 18 h at 35°C. Organisms were harvested by centrifugation, washed in lysis buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA), and resuspended in 0.5 ml of lysis buffer containing

* Corresponding author. Phone: (301) 496-4433. Fax: (301) 402-1886.

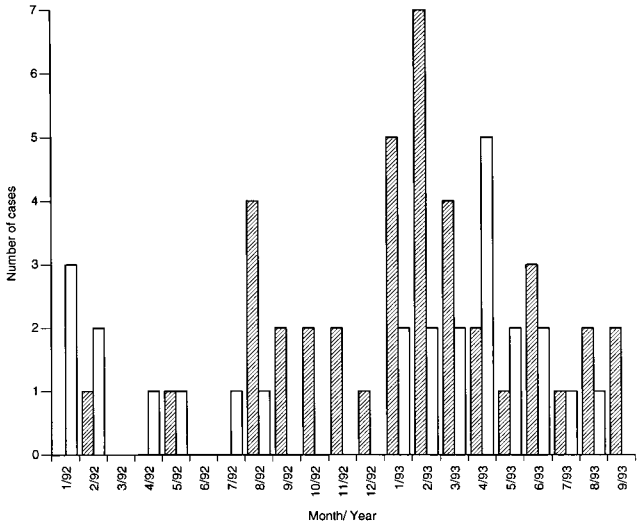

FIG. 1. Occurrence of hospital-acquired (hatched bars) and community-acquired (open bars) *C. difficile*-associated diarrhea on a pediatric oncology ward at the Clinical Center Hospital, National Institutes of Health.

lysozyme (10 mg ml^{-1}). After 30 min of incubation at 37°C, sodium dodecyl sulfate (3% [wt/vol], final concentration) was added, and incubation continued for a further 15 min at 37°C. DNA was then purified by extraction with phenol:chloroform:isoamyl alcohol (25:24:1), ethanol precipitated, dried under vacuum, and resuspended in 50 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). DNA extracts were stored at −70°C.

PCR ribotyping. PCR ribotyping was performed with primers complementary to conserved regions of the 3′ end of the 16S and 5′ end of the 23S rRNA genes (12). PCR mixtures consisted of buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% [wt/vol] gelatin; pH 8.3), 200 μM each deoxynucleoside triphosphate, 10 pmol of each primer, 2.5 U of *Taq* polymerase, and 2 μl of diluted DNA extract (1:50 in sterile H_2O), in a total volume of 20 μl. After the template was denatured for 30 s at 94°C, DNA was amplified for 30 cycles. Each cycle consisted of 15 s at 94°C, 15 s at 55°C, and 15 s at 72°C. Amplified products were separated by agarose gel electrophoresis (1.5% [wt/vol]; 50 V for 2 h) and visualized by UV transillumination following ethidium bromide staining. Prints made from photographs of negative images of gels were used to compare ribotypes; this technique enabled easier discrimination of banding patterns (see Fig. 2, 4, and 5). Minor, presumably artifactual, bands revealed by this photographic technique (see Fig. 5) were disregarded during analysis of banding patterns.

REA. Genomic DNA for REA was extracted as described for PCR ribotyping. Aliquots of purified DNA (20 μl; approximately 5 to 10 μg) were digested for 3 h at 37°C with 20 U of *Hin*dIII restriction endonuclease (New England Biolabs, Beverly, Mass.) in a total volume of 60 μl. Digested fragments were separated by agarose gel electrophoresis (0.8% [wt/vol] agarose; 50 V for 18 h) and visualized and photographed as described for PCR ribotyping.

RESULTS

PCR ribotyping and REA of outbreak strains. The results of PCR ribotype analysis of the *C. difficile* isolates obtained from patients implicated in the outbreak of *C. difficile* diarrhea are shown in Fig. 2. Five of the six isolates (O-1, O-2, O-3, O-4, and O-6) possessed an identical PCR ribotype (later classified as type III); the PCR ribotype of isolate O-5 was clearly different (type XII). Results comparable to those obtained with PCR ribotyping were generated by REA. Although minor interisolate differences in REA patterns were discernible (Fig. 3), isolates O-1 to O-5 all clearly belong to the same REA group (defined as >90% similarity by Clabots et al. [5]), while O-6 belongs to a different group. The six control isolates, obtained from presumed community-acquired cases of *C. difficile* diarrhea occurring during the months of January to March 1993, all gave different PCR ribotypes (Fig. 4) and REA patterns (data not shown); of these control isolates, one, namely, C-1, gave the same ribotype pattern (III) as the majority of outbreak strains.

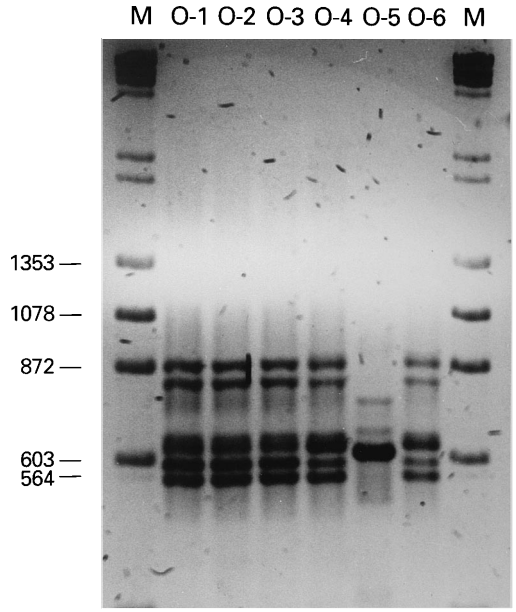

FIG. 2. PCR ribotypes of *C. difficile* isolates (lanes O-1 through O-6) obtained from six patients implicated in an outbreak of *C. difficile*-associated diarrhea. Lanes M, molecular weight markers (*Hae*III digest of φX174 [Bethesda Research Laboratories]). Numbers are in base pairs.

Prospective analysis of PCR ribotypes. PCR ribotypes consisted of patterns comprising 1 to 12 bands, with the size of bands varying from approximately 500 to 1,100 bp (Fig. 2, 4, and 5). By using the criterion of two bands' difference as the minimum necessary to assign isolates to different PCR ribotypes, the toxigenic strains could be classified into 33 visually distinct PCR ribotypes and the nontoxigenic strains could be classified into 8 PCR ribotypes. Toxigenic and nontoxigenic isolates possessed mutually exclusive PCR ribotypes; thus, a

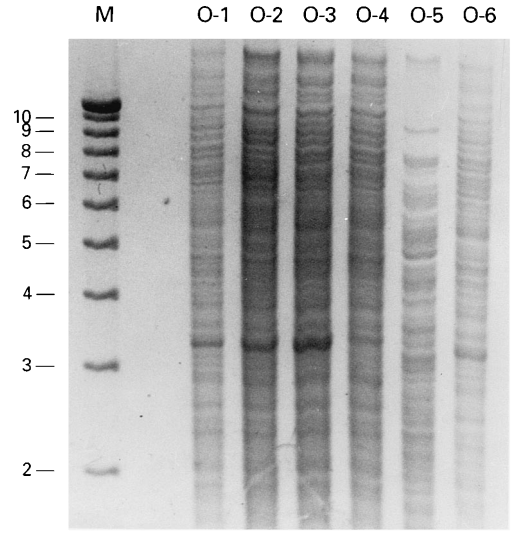

FIG. 3. REA of *C. difficile* isolates (lanes O-1 through O-6) obtained from six patients implicated in an outbreak of *C. difficile*-associated diarrhea. Lane M, a molecular weight marker (1-kb ladder [Bethesda Research Laboratories]). Numbers are in kilobases.

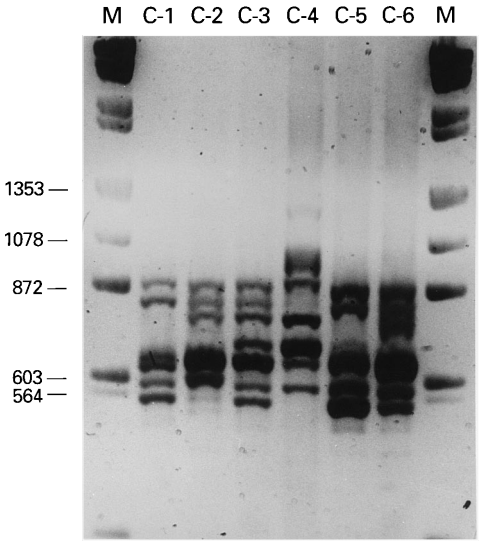

FIG. 4. PCR ribotypes of *C. difficile* isolates (lanes C-1 through C-6) obtained from six patients with community-acquired, *C. difficile*-associated diarrhea diagnosed during the months of January to March 1993. Lanes M, molecular weight markers (*Hae*III digest of ϕX174). Numbers are in base pairs.

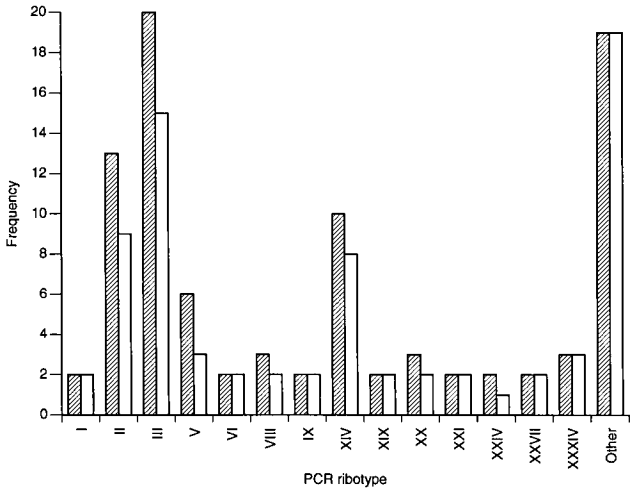

FIG. 6. Frequency distribution of PCR ribotypes among toxigenic strains of *C. difficile* isolated from symptomatic patients at the Clinical Center Hospital, National Institutes of Health. The numbers of isolates with a given ribotype are shown in hatched bars, and the numbers of patients are shown in open bars.

total of 41 different ribotypes were detected among the 102 isolates tested. Representative examples of the 14 most common PCR ribotypes seen in toxigenic isolates are shown in Fig. 5, and the frequency of isolation of the different PCR ribotypes among toxigenic isolates is shown in Fig. 6. Of the 92 toxigenic strains tested, 49 of them (53%) belonged to just four PCR ribotypes (II, III, V, and XIV), with type III being the most common ribotype, accounting for 22% (20 of 92) of toxigenic isolates tested. No spatial or temporal clustering of isolates with the same PCR ribotype was seen, however, and there was no epidemiologic evidence of a potential outbreak of nosocomially acquired *C. difficile* diarrhea in the hospital during the 10-month period in which this study was conducted.

Stability and reproducibility of PCR ribotypes. Repeated subculturing of two toxigenic isolates (types III and XIV) and one nontoxigenic isolate (type IV) (15 to 20 passages in chopped meat glucose broth) did not alter the PCR ribotype (data not shown), suggesting that this is a relatively stable genotypic marker. Furthermore, PCR ribotypes were not affected by either the amount of starting DNA (from 1 ng to 1 μg), the number of cycles (30 to 60), or the thermal cycler (Perkin-Elmer 9600 or MJ Research MiniCycler) used for DNA amplification.

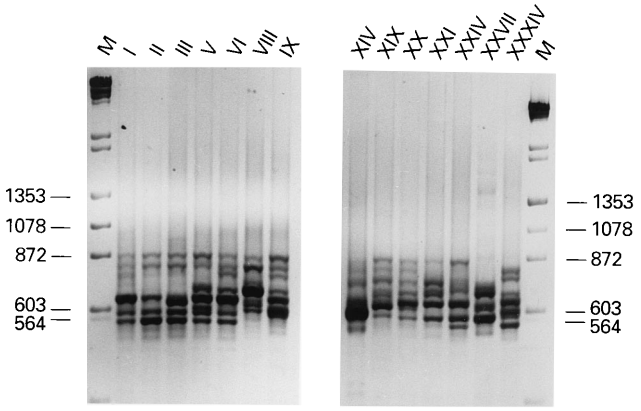

FIG. 5. Representative examples of the 14 most commonly detected PCR ribotypes among toxigenic isolates of *C. difficile* (lanes I through XXXIV). Lanes designated M contain molecular weight markers (*Hae*III digest of ϕX174). Numbers are in base pairs.

DISCUSSION

Tracking of potential nosocomial outbreaks of *C. difficile* diarrhea by conventional infection control methodologies can be an extremely time-consuming exercise, and attempts to differentiate true outbreaks from mere fluctuations in disease incidence are fraught with difficulty. Given these problems, there is an obvious need for a simple, rapid, and reproducible method for typing isolates of *C. difficile*. The present study demonstrates the potential value of PCR ribotyping as a tool for aiding hospital infection control surveillance personnel in evaluating potential nosocomial outbreaks of *C. difficile* disease.

Analysis of multiple isolates obtained from symptomatic patients at the National Institutes of Health revealed the existence of 44 PCR ribotypes within this population, suggesting that this method has sufficient discriminatory power to differentiate related strains from unrelated strains. The most subjective, and therefore controversial, issue with any typing method is the arbitrary decision of how many differences need to be detected in order to identify two isolates as belonging to different strains. Although a single band difference in a PCR ribotype presumably represents the gain or loss of an rRNA allele (8), which is probably a relatively significant genetic event given the stability of PCR ribotypes during in vitro passage, we found that requiring a difference of at least two bands to assign isolates to different ribotypes aided interpretation of patterns without affecting discriminatory power.

Our finding that a relatively small number of strains are responsible for the majority of cases of *C. difficile* disease in our hospital is in agreement with data presented in previous studies using other strain typing techniques (5, 19); the significance of this finding from an epidemiologic and pathogenesis standpoint remains unclear (14). In view of the strain distribution pattern, it is not surprising that the strain responsible for

the apparent nosocomial outbreak was ribotype III, the most commonly seen ribotype of tested isolates. Interestingly, one of the patients implicated in the nosocomial outbreak by both conventional and molecular epidemiologic analysis had recurrent, severe episodes of *C. difficile* diarrhea in the first 6 months of 1993. Since all four isolates of *C. difficile* obtained from this patient were ribotype III, this patient potentially functioned as the source of the outbreak strain on the pediatric oncology unit. Since the only readily discernible link between this patient and the majority of the other children implicated in the outbreak was use of a communal recreation area, it seems plausible that contamination of equipment in this area may have been the mechanism by which the outbreak strain was spread. Although one of the isolates obtained from a patient with community-acquired *C. difficile* diarrhea also possessed this ribotype, the prevalence of isolates with ribotype III suggests that this patient was not involved in the outbreak and had been correctly categorized by infection control criteria. Clearly, the relatively limited number of strains apparently responsible for the majority of *C. difficile* disease in a given institution makes outbreak detection somewhat problematic, highlighting the fact that any typing system, including PCR ribotyping, can only be used to substantiate evidence obtained in traditional epidemiologic investigations. Effective collaboration between the typing laboratory and infection control staff is essential if the information generated by strain typing of *C. difficile* is to be of any benefit.

PCR ribotyping proved comparable to REA, the most thoroughly investigated of the other molecular typing methods available, in identifying a cluster of related strains isolated from patients suspected to be involved in a nosocomial outbreak. In our prospective study, all isolates tested were typeable by the PCR ribotype technique, and the generation of PCR ribotypes was unaffected by changing multiple experimental parameters. This feature of PCR ribotyping, as well as the ease with which the simple patterns generated could be analyzed, suggests that this technique may provide a robust, yet discriminatory, alternative to REA for typing *C. difficile*. Although Gürtler used denaturing polyacrylamide gel electrophoresis of radiolabelled DNA fragments to separate amplified spacer regions (8), we found that agarose gel electrophoresis followed by ethidium bromide staining, a separation technique that is more compatible with clinical laboratory use, gave sufficient resolution for differentiation of ribotypes (Fig. 2, 4, and 5).

The advantages of PCR over conventional molecular approaches, particularly in terms of speed and simplicity, have led a number of investigators to investigate the PCR-based method known variously as AP-PCR or random amplified polymorphic DNA analysis as a technique for typing organisms, including *C. difficile* (1, 10). Unfortunately, between-isolate differences in AP-PCR patterns do not necessarily reflect characterizable genotypic variation, and the precise patterns obtained are highly dependent on the exact experimental conditions employed (18), making this technique somewhat limited as a standardized typing method. Given the genetic stability and experimental robustness of PCR ribotyping, this provides a more attractive PCR-based option than AP-PCR for strain typing in an organism, like *C. difficile*, that possesses sufficient rRNA alleles with different intergenic spacer lengths to make PCR ribotyping discriminatory.

The relative ease and rapidity with which PCR ribotyping analysis can be accomplished, even on a large number of isolates, have made it possible for us to use this technique on a prospective basis to track trends in strain prevalence in the hospital, generating valuable information for the hospital epidemiology service. The acquisition of diagnostic PCR-based technology by clinical microbiology laboratories seems certain to afford an increasing number of institutions the opportunity to utilize simple, yet reliable, molecular epidemiologic techniques like PCR ribotyping to help discover and track nosocomial outbreaks.

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