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Comparison of PCR-Based Approaches to Molecular Epidemiologic Analysis of *Clostridium difficile*

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Representative isolates of the 10 serogroups of *Clostridium difficile* and 39 clinical isolates (30 toxigenic and 9 nontoxigenic), including 5 isolates from a confirmed nosocomial outbreak, were analyzed by using two previously described arbitrary-primer PCR (AP-PCR) molecular typing methodologies (AP-PG05 and AP-ARB11) and PCR ribotyping. The two AP-PCR methods investigated gave comparable results; AP-PG05 and AP-ARB11 identified 8 and 7 groups among the serogroup isolates and classified the clinical isolates into 21 and 20 distinct groups, respectively. PCR ribotyping also identified 8 unique groups among the serogroup isolates but classified the clinical isolates into 23 groups. In addition, when results obtained by the PCR methods were compared with typing data generated by pulsed-field gel electrophoresis (PFGE), PCR ribotyping and PFGE were found to be in agreement for 83% (29 of 35) of isolates typeable by both techniques while AP-PG05 was in agreement with PFGE for 60% (20 of 33) and AP-ARB11 was in agreement with PFGE for only 44% (17 of 36). These results indicate that PCR ribotyping is a more discriminatory approach than AP-PCR for typing *C. difficile* and, furthermore, that this technique generates results that are in higher concordance with those obtained by using an established method for differentiating isolates of this organism on a molecular level than are results generated by using AP-PCR.

Clostridium difficile is the etiologic agent of pseudomembranous colitis and the principal cause of antibiotic-associated diarrhea (AAD) (2). This latter manifestation of *C. difficile* disease is one of the major nosocomially acquired infections in the United States (10), and several large outbreaks associated with interpatient transfer of this organism have been documented (4, 12).

Many different techniques have been employed to help determine genetic relatedness among *C. difficile* isolates; a number of recent publications have focused on the use of PCR-based approaches for identifying intraspecies genetic variation in this organism, including so-called arbitrary-primer PCR (AP-PCR) (1, 7, 13, 16) and PCR ribotyping (3, 5). In AP-PCR, genomic DNA is amplified under relatively nonstringent annealing conditions and, most commonly, with only a single, short (10 to 15 nucleotides) primer of arbitrary sequence to initiate amplification (19). Following electrophoretic separation of fragments, banding pattern comparison is used to classify isolates into related and unrelated groups. In contrast to AP-PCR, PCR ribotyping amplifies, via the use of highly specific primers and stringent amplification conditions, the 16S-23S rRNA intergenic spacer region of isolates (8). Isolates of *C. difficile* may possess up to 16 alleles of the rRNA gene cluster, with the principal allelic feature being the length of the intergenic spacer region (5). Banding patterns revealed by gel electrophoresis of the products of PCR ribotyping represent the rRNA allele composition of that isolate and can be used to differentiate epidemiologically related and unrelated isolates (3, 5).

The attraction of all PCR-based techniques in comparison with conventional molecular epidemiologic methods like restriction endonuclease analysis and pulsed-field gel electrophoresis (PFGE) is that because of the speed and ease with which results can be generated, analyses of large numbers of isolates from potential outbreaks can rapidly be performed. Decreasing the turnaround time for generation of typing results could enhance significantly the inherent epidemiologic value of the data obtained (11). If one of the PCR-based typing systems thus far described can be shown to be comparable to conventional molecular epidemiologic approaches in terms of discriminatory power and typability, its application could provide valuable and timely assistance in efforts to control the spread of *C. difficile* in hospitals.

In the current study, two previously described AP-PCR methodologies, referred to here as AP-PG05 (16) and AP-ARB11 (7), were compared with PCR ribotyping for their ability to differentiate isolates of *C. difficile*. Isolates were also typed by a conventional molecular epidemiologic method, namely, PFGE, principally to ascertain to what extent PCR-based detection of genomic variation in *C. difficile* correlates with variation detected by restriction endonuclease-mediated probing of this organism's genome.

MATERIALS AND METHODS

Isolates tested. Representative isolates of each of the 10 *C. difficile* serogroups (A, B, C, D, F, G, H, I, K, and X) originally described by Toma and colleagues (18) were generously provided by Haru Kato, Institute of Anaerobic Bacteriology, Gifu University School of Medicine, Gifu, Japan. Clinical isolates used in this study were cultured from stool or rectal swabs obtained from patients at either the Warren G. Magnuson Clinical Center, National Institutes of Health, Bethesda, Md. (NIH) (16 toxigenic and 4 nontoxigenic isolates), or the New England Deaconess Hospital, Boston, Mass. (15 toxigenic and 4 nontoxigenic

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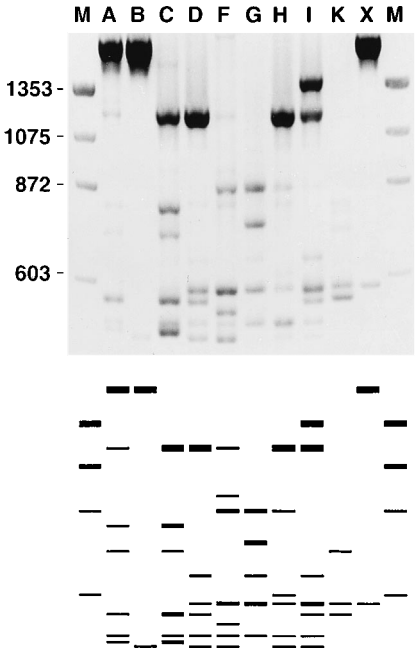

FIG. 1. AP-PG05 banding patterns (top) and Dendron software-generated representations of patterns (bottom) obtained with serogroup isolates. Lanes A to X contain the serogroup isolates; letters above these lanes correspond to the Delmee serogroup designations. Lanes M contain molecular size markers (*Hae*III digest of ϕx174 [Stratagene]). Numbers are in base pairs.

isolates). Five of the isolates from the NIH have been described previously and were obtained from patients implicated in a nosocomial outbreak of *C. difficile* AAD (3).

Organism isolation and characterization. Clinical isolates were identified as *C. difficile* and their toxigenicity was determined as described previously (3).

DNA extraction for PCR. Isolates were inoculated into 10 ml of chopped-meat–glucose (CMG) broth and cultured anaerobically for 18 h at 35°C. Aliquots of broths (200 μl) were then inoculated into prereduced brain heart infusion (BHI) (10 ml) broth and cultured anaerobically at 35°C for 4 h. Organisms were harvested, and DNA was extracted and purified as described previously (3).

AP-PCR. Two independent AP-PCR protocols were performed on all isolates. Primers (synthesized by Research Genetics, Huntsville, Ala.) used were those described by Killgore and Kato (AP-ARB11; 5′-CTAGGACCGC-3′) (7) and Silva et al. (AP-PG05; 5′-AGCCCAGCTATGAAC-3′) (16). AP-PCR was performed as described in the original publications, with the following modifications. All PCRs were performed in an MJ Research PTC200 thermal cycler (MJ Research, Watertown, Mass.). Amplification parameters for AP-ARB11 consisted of an initial denaturation of template at 94°C for 30 s followed by 50 cycles of amplification, with each cycle consisting of 30 s at 94°C, 30 s at 36°C, and 30 s at 72°C. For AP-PG05, 2 low-stringency cycles consisting of 30 s at 94°C, 30 s at 26°C, and 60 s at 72°C were followed by 55 cycles with 50°C as the annealing temperature. Resolution of bands by agarose gel electrophoresis and subsequent generation of negative photographic images were performed as described previously (3).

PCR ribotyping. PCR ribotypes of isolates were obtained as described previously (3).

PFGE analysis. PFGE analysis of isolates was performed as described by Kristjánsson et al. (9).

Analysis of banding patterns. To aid comparison of patterns produced by PCR methods, computer-generated images of gels were obtained by using the Dendron software package (Solltech Inc., Oakdale, Iowa). This enabled both elimination of gel artifacts and direct, side-by-side comparison of all patterns generated. Since the number of bands produced by both AP-PCR and PCR ribotyping varied considerably among isolates, patterns were compared visually rather than by using the similarity index algorithm built into the Dendron software. PCR groups contained isolates producing patterns that were identical or differed by only one band, presumably consistent with variation occurring as a result of no more than a single genetic event. Each individual pattern within a designated PCR group was identified as a subgroup. Isolates from which only a single band was amplified by a particular PCR method were considered untypeable by that method and excluded from further comparison. To improve the objectivity of this comparison, independently coded isolate sets were used for each of the PCR typing procedures. Only after all typing results were complete were the identities of isolates revealed. PFGE patterns were compared by a technique described in detail in a previous publication (9). Isolates showing band differences consistent with a single genetic event were assigned to the same group, and indistinguishable isolates were assigned to the same subgroup; the use of such interpretive criteria for PFGE was recommended in a recent publication by Tenover and colleagues (17).

RESULTS

Typing of serogroup strains. Both original agarose gel electrophoresis patterns and the Dendron-generated images derived from them, obtained by using the different PCR methods for typing the Delmee serogroup strains, are shown in Fig. 1 (AP-PG05), 2 (AP-ARB11), and 3 (PCR ribotyping). AP-PG05 (Fig. 1) identified eight distinct groups. Two groups each contained two subgroups, with isolates A and C and isolates D and I being grouped together. AP-ARB11 identified seven unique groups (Fig. 2). Isolate G was considered nontyped by this method, giving only a single band. None of the groups identified with AP-ARB11 contained subgroups; however, isolates A and C and isolates I and X gave identical patterns. PCR ribotyping (Fig. 3) identified eight groups; as with AP-PG05, two of the groups contained two subgroups. PCR ribotyping clustered isolates A and C together; in addition, isolates G and H appeared to belong to the same group.

Typing of outbreak isolates. The isolates chosen for testing in this study included five that had previously been implicated in a nosocomial outbreak of *C. difficile* AAD at the NIH (3). Both AP-PG05 and PCR ribotyping placed all five isolates in the same group, while only four of these isolates appeared related by AP-ARB11. Of the 34 nonoutbreak isolates tested, AP-ARB11 placed 1, PCR ribotyping placed 4, and AP-PG05 placed 8 in the same group as the outbreak isolates. The reference method, PFGE, also included all five outbreak isolates in the same group, and an additional three nonoutbreak isolates appeared to be related to the outbreak cluster by this method.

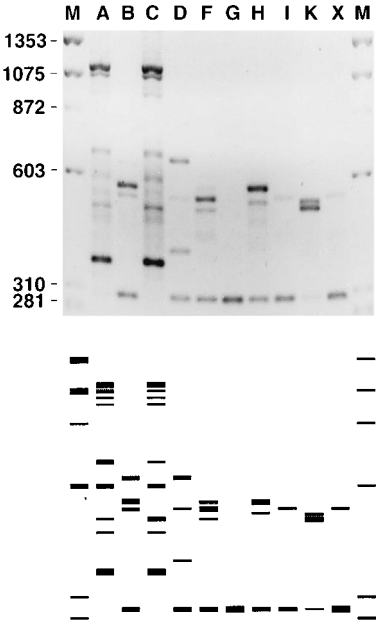

FIG. 2. AP-ARB11 banding patterns (top) and Dendron software-generated representations of patterns (bottom) obtained with serogroup isolates. Lanes A to X contain the serogroup isolates; letters above these lanes correspond to the Delmee serogroup designations. Lanes M contain molecular size markers (*Hae*III digest of ϕx174 [Stratagene]). Numbers are in base pairs.

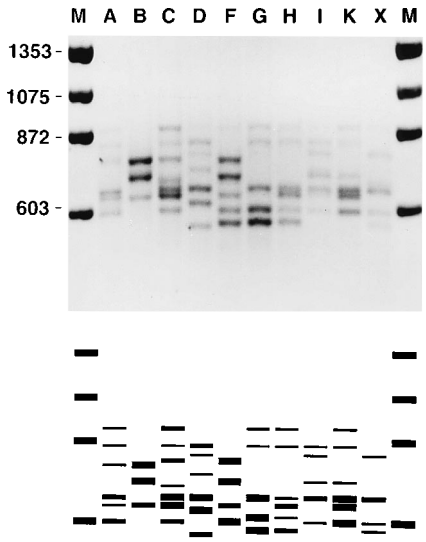

FIG. 3. PCR ribotypes (top) and Dendron software-generated representations of ribotypes (bottom) obtained with serogroup isolates. Lanes A to X contain the serogroup isolates; letters above these lanes correspond to the Delmee serogroup designations. Lanes M contain molecular size markers (*Hae*III digest of ϕx174 [Stratagene]). Numbers are in base pairs.

Discriminatory power of PCR methods. The results obtained with the different PCR techniques for typing both outbreak-related and unrelated isolates are summarized in Table 1. All three techniques produced an approximately comparable number of groups and subgroups (Table 1). The frequency with which epidemiologically unrelated isolates were clustered together was, however, somewhat lower with PCR ribotyping than with AP-PCR. Eighteen of the 23 groups (78%) identified by PCR ribotyping contained only a single isolate, compared with 14 of 21 (66%) for AP-PG05 and 11 of 20 (55%) for AP-ARB11. Computer-generated images of representative examples of the patterns produced by each method are shown in Fig. 4 (AP-PG05), 5 (AP-ARB11), and 6 (PCR ribotyping). Relatively few isolates were deemed nontypeable by any of the PCR methods utilized, with the arbitrary criterion of failure to generate more than a single amplified band. The frequency with which isolates were deemed nontypeable was slightly higher, however, with AP-ARB11 than with either of the other two methods (Table 1). Correlation of typing results with toxigenicity of isolates was significantly greater for PCR ribotyping than for either AP-PCR method. Of the nine nontoxigenic isolates tested, only one was included in a group containing toxin-producing isolates by PCR ribotyping. In contrast, four (AP-PG05) and five (AP-ARB11) nontoxigenic isolates were included in groups containing toxigenic strains when AP-PCR was used to delineate typing groups.

Concordance of results obtained by PCR methods with those obtained by PFGE. Of the 39 isolates included in this study, 36 were typeable by PFGE. The 36 typeable isolates could be differentiated into 21 distinct groups and 31 subgroups (data not shown). By PFGE analysis, no nontoxigenic strains were included in groups containing toxigenic isolates. Allocation of isolates to particular groups by the three PCR-based typing strategies were compared with PFGE allocations, primarily to enable assessment of the degree of concordance between established and experimental approaches to assessing genetic variation in *C. difficile*. The groupings of 29 of the 35 isolates (83%) typeable by both PCR ribotyping and PFGE were in agreement, 60% (20 of 33 isolates) correlation was observed between AP-PG05 and PFGE, and only 17 of 36 isolates (44%) typed by AP-ARB11 and PFGE were assigned concordantly.

TABLE 1. Results of typing 39 clinical isolates by the different PCR methodologies

PCR method	No. of groups/ no. of subgroups	No. of nontypeable isolates	Mean no. of bands/ pattern (range)[a]
AP-PG05	21/25	1	5.1 (2–9)
AP-ARB11	20/24	3	3.8 (2–6)
PCR ribotyping	23/25	1	5.0 (3–7)

[a] Nontypeable isolates are not included.

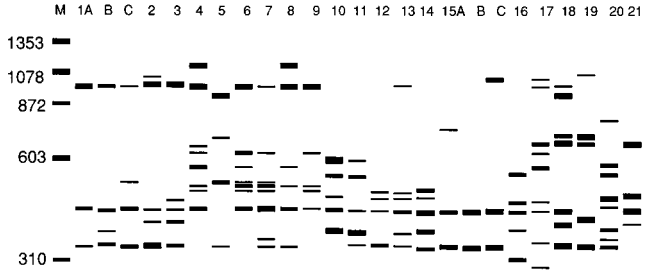

FIG. 4. Dendron software-generated representations of group and subgroup banding patterns obtained with clinical isolates by using AP-PG05. Lane M, molecular size markers (*Hae*III digest of ϕx174 [Stratagene]). Numbers are in base pairs.

DISCUSSION

A considerable number of studies describing applications of PCR for the molecular epidemiologic analysis of *C. difficile* have been published (1, 3, 5, 7, 13, 16). In the current investigation, we sought to compare several of these published techniques using both standardized serogroup isolates and a set of clinical isolates characterized both epidemiologically (into outbreak- and non-outbreak-related isolates) and molecular genetically, by PFGE. The results seem to indicate that of the two principal PCR methods so far advocated, PCR ribotyping is at least the equal of AP-PCR in discriminatory power and is considerably more reliable; typing results obtained by this technique were in much higher concordance with those generated by PFGE than were those generated by either of the two AP-PCR approaches examined.

In general, the results indicated that all PCR methods are somewhat less discriminatory than PFGE, mirroring the find-

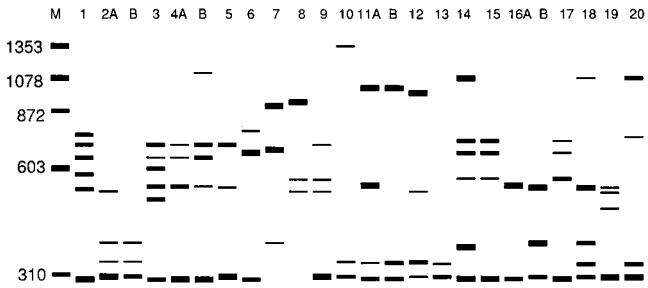

FIG. 5. Dendron software-generated representations of group and subgroup banding patterns obtained with clinical isolates by using AP-ARB11. Lane M, molecular size markers (*Hae*III digest of ϕx174 [Stratagene]). Numbers are in base pairs.

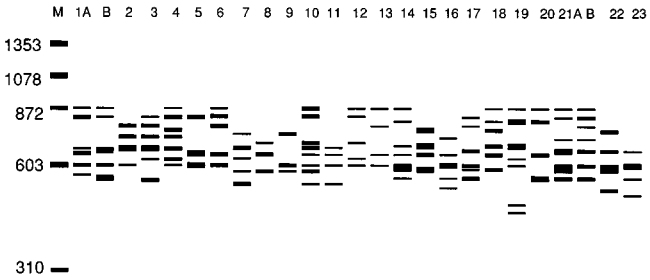

FIG. 6. Dendron software-generated representations of group and subgroup banding patterns obtained with clinical isolates by using PCR ribotyping. Lane M, molecular size markers (*Hae*III digest of ϕx174 [Stratagene]). Numbers are in base pairs.

ings of previous investigations examining the ability of AP-PCR and PFGE to type isolates of *Staphylococcus aureus* (15). In a previous study, a PFGE technique essentially identical to the one employed here successfully classified 9 of the 10 *C. difficile* serogroup strains into unique groups, the remaining isolate being nontypeable (6). Of the PCR methods tested here, both PCR ribotyping and AP-PG05 resolved the serogroup isolates into 8 groups and 10 subgroups, while AP-ARB11 identified only 7 groups and subgroups. Comparison of the results of typing the clinical isolates shows that PFGE again generated the most subgroups, 31, compared with 25 for PCR ribotyping and AP-PG05 and 24 for AP-ARB11. In the original publication describing AP-PG05 (16), five of the same serogroup strains as used in the current study were typed (A, B, C, D, and F), two groups and four subgroups were identified, and the serogroup A and B isolates generated only a single band. In the present study, AP-PG05 discerned four groups and five subgroups among the same five isolates (Fig. 1). This lack of correlation between the original authors' results and the current findings is due in part to differences in the stringencies of group and subgroup designation criteria. Identical isolates did, however, produce significantly different numbers and sizes of bands in the two investigations, and these differences greatly affected the final typing results. These discrepancies between the results of two studies using the same AP-PCR primer to type identical isolates presumably are indicative of the pronounced effect that interlaboratory variation in reaction conditions can have on this technique.

The most fundamental feature of any typing system is the ability to differentiate outbreak-related and unrelated isolates. In the present study, a cluster of 5 *C. difficile* isolates from a previously described nosocomial outbreak of AAD at the NIH were included among the 39 clinical isolates typed. All five were identified as belonging to the same group by AP-PG05 and PCR ribotyping, but only four of these isolates were clustered by AP-ARB11. Interestingly, two isolates from New England Deaconess Hospital were assigned to the same group as were the NIH outbreak isolates by PCR ribotyping and PFGE, and this strain had been isolated previously from patients involved in outbreaks of AAD at New England Deaconess Hospital. This result demonstrates that common, outbreak-implicated strains of *C. difficile* can be found in geographically disparate hospitals with very different patient populations and presumably indicates that these strains possess certain physiologic characteristics that favor their spread and/or ability to produce disease.

The degree of concordance between experimental and proven typing strategies is an important parameter in determining the reliability of the former. If two equally reliable techniques with comparable discriminatory powers are used to type the same group of isolates, similar patterns of isolate clustering should be observed (9). This similarity in isolate grouping will result in a high degree of concordance between the results obtained by the two methods. In this respect, PCR ribotyping was clearly superior to the AP-PCR methods examined, with 83% agreement between this technique and PFGE compared with 60% for AP-PG05 and 45% for AP-ARB11. One potential criticism of AP-PCR is that since the bands generated are the result of arbitrary and potentially mismatched priming events, differences in banding patterns may occur not as a result of genetic difference but merely as a consequence of variations in experimental conditions beyond the control of the operator (14). These artifactual variations would obviously compromise the value of inferences made regarding the extent of genetic variance among isolates based on the similarity, or lack thereof, of AP-PCR patterns. Our failure to demonstrate a close correlation between typing results generated by the AP-PCR primers used in this study and PFGE appears to support this contention. Both AP-PCR techniques successfully clustered the majority of the outbreak-related isolates together, indicating that these methods could identify a spatially related cluster of epidemiologically linked isolates. However, their failure to include in this group isolates related to the outbreak strain by both PCR ribotyping and PFGE but not spatially or temporally related to the outbreak cluster and their repeated clustering of nontoxigenic and toxigenic isolates lead one to question the overall utility of these methods for molecular epidemiologic analysis of *C. difficile.*

Results obtained by PCR ribotyping appeared to correlate well with those obtained by PFGE, suggesting that variation in rRNA gene cluster allele composition is a parameter by which overall genetic variation in *C. difficile* can be monitored. Although this technique appears to be slightly less discriminatory than PFGE, it has considerable advantages in terms of speed and technical ease. In addition, several investigators have reported significant problems with strain-specific degradation of *C. difficile* DNA occurring during the isolation of nucleic acid for PFGE (6, 9). The inability to type such isolates by PFGE compromises the overall value of this technique for epidemiologic analysis of *C. difficile* and suggests that an alternative, comparably discriminatory genotypic technique for typing this organism would be of considerable value. The apparent reliability and discriminatory power of PCR ribotyping which this study and previous ones have demonstrated (3, 5) suggest that this technique is more likely than AP-PCR to provide such an alternative.

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