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# Use of Arbitrary Primer PCR To Type *Clostridium difficile* and Comparison of Results with Those by Immunoblot Typing

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**An arbitrarily primed PCR (AP-PCR) assay was used to type *Clostridium difficile* isolates from a hospital outbreak of antibiotic-associated diarrhea. Forty-one isolates were separated into nine groups, with 66% falling into one group; no other group contained more than 10%. Comparison of AP-PCR grouping with that when the immunoblot technique was used showed agreement for 33 of 34 isolates typed by both techniques, and AP-PCR grouped seven isolates that were not typeable by immunoblotting.**

*Clostridium difficile* is frequently implicated in outbreaks of nosocomial antibiotic-associated diarrhea and colitis (1, 5, 7). Efforts to understand the epidemiology of this important nosocomial pathogen have resulted in a variety of approaches for developing useful typing techniques. These include serotyping, plasmid profiles, polyacrylamide gel electrophoresis, immunoblotting, and restriction endonuclease analysis (4, 6, 8, 9, 11–13, 15). Most of these techniques are based on comparison of electrophoresis gel banding patterns of proteins or nucleic acids and require labor-intensive reagent preparation and/or extraction procedures. We present a simplified typing technique based on the PCR with a single short arbitrary primer. The technique has been variously referred to as DNA amplification fingerprinting (2), arbitrarily primed PCR (AP-PCR) (14), and random amplified polymorphic DNA analysis (16).

AP-PCR makes use of a single primer approximately 10 nucleotides in length, whose sequence is chosen arbitrarily. Because of its short length and the low-stringency PCR conditions, the primer binds at various sites on either strand of the target DNA, resulting in a series of PCR products with different sizes that may be separated on an agarose gel. McMillin and Muldrow (10) applied this technique to a half-dozen *C. difficile* strains and found a variety of banding patterns by using several different arbitrary primers.

***C. difficile* strains and sample preparation.** The forty-one strains examined in this study were from a group of 50 strains isolated from patients during a hospital outbreak of colitis and diarrhea and were previously described and typed by Western immunoblotting (8).

Samples were prepared from overnight growth of *C. difficile* on anaerobic blood agar. Early in the study, DNA was extracted with mutanolysin and proteinase K. Titration with early primers indicated optimal template amounts to be from 25 ng to 50 ng of DNA for a total PCR volume of 25 μl. With primer ARB11, it was found that boiling one to two colonies for 5 min in TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and adding 5 μl to the PCR mixture gave comparable banding patterns. A disposable 1-μl loop was used to scoop one to two colonies of bacterial growth from the agar and suspend it in 500 μl of 10 mM TE (pH 8.0). Each sample was mixed to homogeneity on a vortex mixer and heated in a boiling water bath for 5 min. No effort was made to quantitate the amount of DNA in the boiled colony samples. Samples were cooled on ice and used immediately or stored at −20°C.

**Arbitrary primer.** Several 10-mer arbitrary primers were prepared by the Core Facility of the Centers for Disease Control and Prevention and tested by our laboratory. We chose primer ARB11 because of its distinctive patterns and reproducibility. ARB11 has the sequence 5′ CTAGGACCGC 3′.

**Amplification conditions.** The PCR conditions used were those of Williams et al. (16), with slight modification of some reactant concentrations. Stronger and more consistent banding patterns were observed when primer and polymerase concentrations were increased. Amplification was conducted in a volume of 25 μl containing 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 100 μM (each) dATP, dGTP, dCTP, and dTTP, 2 μM primer, 2 U of *Taq* polymerase (Perkin-Elmer Cetus), and 5 μl of sample. Samples were amplified on a Perkin-Elmer Cetus Model 480 Thermocycler for 35 cycles at 95°C for 1 min, 36°C for 1 min, and 72°C for 2 min.

**Analysis.** Amplification products were analyzed by agarose gel electrophoresis. Fifteen microliters of product mixed with 3 μl of loading buffer (6× loading buffer is 0.25% bromophenol blue and 15% Ficoll in water) was applied to a 1.5% agarose gel in 0.5× Tris-borate-EDTA buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA [pH 8.4]) cooled to 18°C. The gel was subjected to 200 V until the bromophenol blue dye marker migrated 20 cm. φX174 replicative form DNA-*Hae*III fragments were used as DNA molecular weight markers. The banding patterns of all lanes were visually compared, and isolates showing identical patterns or variation only in very faint bands were considered the same type.

AP-PCR separated the 41 examined isolates into nine groups, numbered 1 to 9 in Table 1. Seven of these groups (groups 1, 2, 3, 4, 5, 8, and 9) are shown in Fig. 1. As can be seen in Table 1, group 1 contained 66% (27 of 41) of the isolates; also note that all of these isolates were the same immunoblot type. Of the remaining 14 isolates distributed among eight groups, seven had been untypeable by the immunoblot technique with existing antisera. With AP-PCR, these seven isolates were assigned to four different groups.

Of the 34 isolates typed by both AP-PCR and immunoblot-

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TABLE 1. Grouping *C. difficile* isolates by AP-PCR compared with immunoblotting

| Immunoblot group | No. of isolates typed as AP-PCR group: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| G1 | 27 | | | | | | | | |
| G2 | | | 1 | | | | | | |
| H | | | | | 4 | | | | 1 |
| I | | | | | | | | 1 | |
| U[a] | | 2 | | 1 | | 3 | 1 | | |

[a] U, untypeable by immunoblotting.

ting, there was agreement in the grouping of 33. The one disagreement was the AP-PCR group 9 isolate, which immunoblotting included in group H along with the four AP-PCR group 5 isolates. All four AP-PCR group 5 isolates were nontoxigenic by cell culture assay, and two were from the same patient. However, the group 9 isolate was toxigenic. Of those isolates that were untypeable by immunoblotting, the two AP-PCR group 2 isolates and the three group 6 isolates were from single patients.

Outbreaks of nosocomial disease caused by *C. difficile* are relatively common in many hospitals (1, 3, 4, 7). Although there are several effective typing systems that have been described for this organism, most have proven to be too cumbersome for routine clinical laboratories. Therefore, we chose to pursue the use of AP-PCR as a typing tool.

Williams et al. (16) showed AP-PCR to have potential for broad application in producing genetic markers in plants and bacteria, and McMillin and Muldrow (10) showed that AP-PCR could produce banding patterns when applied to the genome of *C. difficile*. However, not every arbitrary primer produces a useful banding pattern. We tried several 10-mer primers against the *C. difficile* genome, including two that McMillin and Muldrow (10) reported as giving unique banding patterns against six *C. difficile* isolates. One of these, ARP 6, did not separate the four isolates we used for screening purposes. The other, ARP 2, gave unique patterns, but most of the differences were in the weaker bands. We chose the ARB11 primer for routine use because it gave clear differences among isolates of different immunoblot groups, and grouping of isolates could be reproduced when grouping was done several months apart with stored extracts.

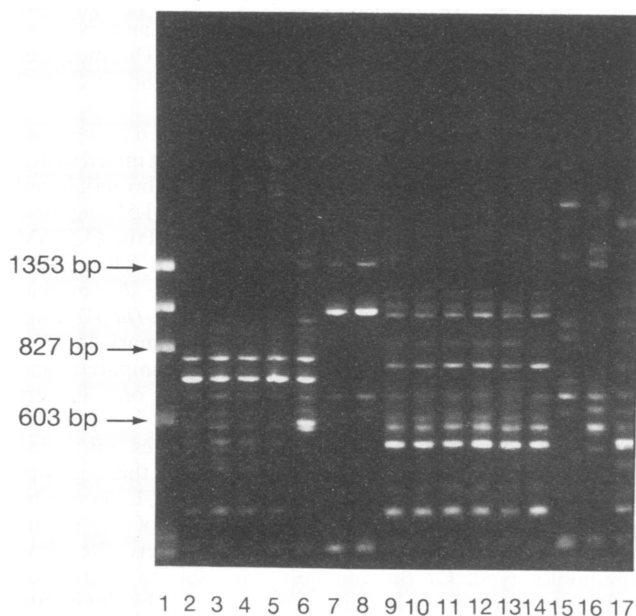


FIG. 1. AP-PCR banding patterns of *C. difficile*. Lanes: 1, molecular weight marker; 2 to 5, group 5; 6, group 4; 7 and 8, group 2; 9 to 14, group 1; 15, group 3; 16, group 9; 17, group 8.

This comparison shows AP-PCR to be at least the equal of the immunoblot technique in discrimination, and AP-PCR was able to group seven isolates that were untypeable by immunoblotting. The one discrepancy between immunoblotting and AP-PCR was a toxigenic strain grouped with four nontoxigenic strains by immunoblotting and grouped separately by AP-PCR. Other highly discriminatory typing techniques such as restriction endonuclease analysis have grouped toxigenic and nontoxigenic isolates together (3).

With AP-PCR, 20 samples were easily processed from extraction to gel photography in <1.5 working days, the limiting factor being the use of a single 20-well gel. PCR and electrophoresis required about 4 h each, and the gels were stained for 10 min and destained from 1 to 2 h before being photographed. In contrast, the immunoblotting technique required from 3 to 4 working days.

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