Use of Multiple Primers in RAPD Analysis of Clonal Organisms Provides Limited Improvement in Discrimination

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ABSTRACT

Randomly amplified polymorphic DNA (RAPD) analysis using two or more primers has been reported to provide additional discriminatory ability over one primer used individually. This may be of particular application in epidemiological typing of clonal organisms, such as Shiga toxin-producing E. coli *O157, where strain differentiation can be difficult. Using four arbitrary primers individually, and in all possible permutations,* E. coli *O157 isolates and other arbitrarily chosen* E. coli *strains were typed using RAPD analysis. For most nonclonal strains, the use of two primers resulted in increased differentiation between isolates; however, more than two primers did not increase further the discriminatory capacity.* E. coli *O157 isolates that produced virtually identical profiles using one primer did not show increased differentiation when using two or more primers, demonstrating that in some cases, where strains of an organism are highly related, there is limited advantage to using more than one primer in RAPD analysis.*

INTRODUCTION

Randomly amplified polymorphic DNA (RAPD) analysis is a modification of a typical PCR in that a single, short, arbitrary sequence primer is used to produce amplification products characteristic of the template DNA (14). Primer length of 10–12 nucleotides is typically used. The method requires no previous knowledge of the target genome sequence or biochemistry and can therefore, in theory, support DNA amplification from any organism. Genomic differences between strains alter the size of a DNA segment between two priming sites that manifest as polymorphic bands in the profiles obtained. Following electrophoresis, fingerprints are typically visualized by ethidium bromide staining of agarose gels.

Current epidemiological typing methods are variable in their speed, technical complexity, cost, and ability to reliably discriminate between bacterial strains. Some methods may also require relatively large amounts of high-quality nucleic acid template. However, RAPD analysis is capable of generating discriminatory and reproducible fingerprints from low concentrations of crudely extracted DNA. Initial concerns about the reproducibility of RAPD typing have been addressed by the development of robust protocols for a wide range of bacterial species; thus, the method is finding increasing acceptance as a typing method. Genetic fingerprinting is important in epidemiology for reliably tracing the source and mode of transmission of pathogens. An epidemiological typing method must be capable of reproducible discrimination between different strains and identification of identical isolates. The ability to discriminate between strains of certain medically important pathogens such as Shiga toxin-producing *E. coli* (STEC) O157 and *Salmonella* spp. is often complicated by the clonal nature of the group; therefore, a discriminatory method must be capable of detecting the often minimal amount of variation available. Modification of the RAPD protocol has included the use of two or more primers in the reaction in an effort to increase its discriminatory capacity (9). While using two primers may increase differentiation, it is not currently known whether adding additional primers will increase further the discriminatory capacity of RAPD analysis.

In this study, *E. coli* isolates (includ-

Table 1. Strains of Shiga Toxin-Producing *E. coli* Typed by RAPD Analysis Using One or More Primers

Strain Identification No.	Serotype	Additional Information
Very similar		
908418	STEC O157	PT 8, Stx 1 and 2
908507	STEC O157	PT 2, Stx 2
909112	STEC O157	PT 4, Stx negative
910833	STEC O157	PT 34, Stx negative
910887	STEC O157	PT 21, Stx 2
Dissimilar		
909802	STEC O157	PT 34, Stx negative
912151	STEC O157	PT 2, Stx 2
908906	STEC O157	PT 2, Stx 2
910634	STEC O157	PT 32, Stx 2
904907	STEC O157	PT 8, Stx ?
Arbitrarily chosen		
7.242	Not determined, uropathogenic isolate	-
7.303	Not determined, uropathogenic isolate	-
739944	Not determined, uropathogenic isolate	-
912549	Not determined, uropathogenic isolate	-
913334	Not determined, uropathogenic isolate	-

STEC, Shiga toxin-producing *E. coli*; EPEC, enteropathogenic *E. coli*; PT, phage type; and Stx, Shiga toxin.

ing strains representing enteropathogenic and enterohaemorrhagic isolates) were analyzed using a RAPD protocol employing up to four 10-mer primers in all possible combinations. A quantitative assessment of the differential ability of each primer combination was determined from a similarity matrix generated using the band-matching coefficient of Dice and unweighted pair group method with arithmetic mean (UPGMA) clustering (11).

MATERIALS AND METHODS

Strains

Human *E. coli* clinical strains were received from Good Hope Hospital and the Public Health Laboratory Service at Birmingham Heartlands Hospital, West Midlands, UK. Genomic DNA was extracted using a method based on phenol chloroform extraction and quantified by measuring the absorbance at 260 nm as described by Hilton et al. (6) and diluted to 10 ng/µL with sterile distilled water.

During preliminary work, primer 1254 was used to screen human *E. coli* strains (Figure 1). Based on the profiles produced, *E. coli* O157 strains were classified into two groups: "very similar" (strains producing profiles that differed by one or two bands when subtyped using RAPD analysis but contained known genomic polymorphisms on the basis of toxin and phage typing) and "dissimilar" (strains producing profiles that had three or more band differences). "Arbitrarily chosen" strains were picked from the available culture collection and generated unrelated profiles (Table 1). These three groups of *E. coli* were then subtyped using all possible primer combinations.

RAPD Amplification

The PCR was carried out in a 25-µL volume containing 10× PCR buffer (10 mM Tris-HCl, pH 8.3, 3.5 mM $MgCl_2$, 25 mM KCl), 200 µM dNTPs (Bioline, London, UK), 2.4 mM primer [1247: 5′-AAGAGCCCGT-3′, 1254: 5′-CCGCAGCCAA-3′, 1283: 5′-GCGATCCCCA-3′, or 1290: 5′-GTGGATGCGA-3′ (1)], 1.25 U *Taq* DNA polymerase (Life Technologies, Paisley, UK), and 20 ng DNA template. In reactions involving more than one primer, the total concentration of primer added remained constant but was divided equally between the primers. Reactions were amplified in a Progene thermal cycler (Techne, Cambridge, UK) as follows: one cycle of 4.5 min at 94°C, followed by five cycles of 30 s at 94°C, 1 min at 22°C, and 2 min at 72°C, and 35 cycles of 30 s at 94°C, 30 s at 32°C, and 2 min at 72°C. Reactions using primers 1290 and/or 1247 had an annealing temperature of 28°C. The amplification was concluded with 5 min at 72°C, and the reactions were stored at 4°C until analysis.

Profile Analysis

PCR products were size separated by electrophoresis in 1.2% agarose and recorded using the IS500 digital imaging system (Flowgen, Kent, UK). Digitally captured RAPD fingerprints were analyzed using GelCompar software (Version 4.0; Applied Maths, Belgium) using the band-matching coefficient of Dice and UPGMA clustering to determine profile relatedness (11). Upon comparison, the relatedness of each profile was described as a percentage similarity and expressed in a similarity matrix. The mean percentage similarity of all compared profiles for a given primer combination was then calculated from the matrix to provide a measure of discriminatory capacity.

RESULTS AND DISCUSSION

Molecular typing of STEC O157 is important in allowing the comparison of strains for epidemiological purposes. Not only can outbreaks be confirmed and their potential source identified but also subtyping may be useful for clinical follow-up of a patient. Many currently used subtyping methods are not ideal because of constraints of time, equipment, and reagents used; thus, analysis may be restricted to only a small number of isolates or specialized laboratories. In contrast to some of the current methods, RAPD analysis compares favorably as a molecular subtyping method.

In this study, RAPD analysis using four primers in all possible permutations was used to subtype human *E. coli* strains, including enterohaemorrhagic and enteropathogenic isolates. The RAPD analysis protocol used was the product of systematic optimization of the reaction and thermal cycling con-

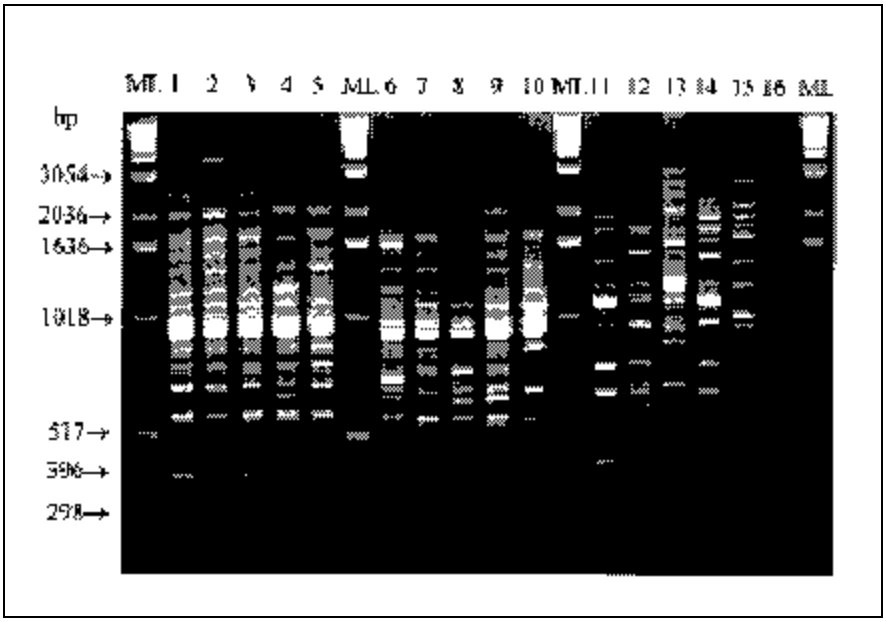

Figure 1. Example of RAPD analysis profiles amplified from genomic DNA of human *E. coli* strains using primer 1254. Lane 1, 908418; lane 2, 908507; lane 3, 909112; lane 4, 910833; lane 5, 910887; lane 6, 909802; lane 7, 912151; lane 8, 908906; lane 9, 910634; lane 10, 904907; lane 11, 7.242; lane 12, 7.303; lane 13, 739944; lane 14, 912549; lane 15, 913334; lane 16, no DNA control; ML, 1-kb DNA ladder (Life Technologies).

Table 2. Results of the Quantitative Assessment of Differential Ability of Each Primer Combination Determined Using the Band-Matching Coefficient of Dice and UPGMA Clustering

Primer(s) Used	Very Similar	Dissimilar	Arbitrarily Chosen
1254	96.7	82.0	74.4
1290	94.9	73.5	69.9
1283	88.6	76.6	67.9
1247	97.1	61.2	60.0
Average	**94.3**	**73.3**	**68.1**
1290 and 1254	92.3	73.9	68.9
1290 and 1247	94.6	75.3	58.0
1290 and 1283	93.9	63.7	70.0
1254 and 1247	99.1	79.8	64.4
1254 and 1283	92.9	74.5	58.4
1283 and 1247	85.2	56.9	62.5
Average	**93.0**	**70.7**	**63.7**
1290, 1254, and 1283	92.4	67.8	64.4
1290, 1254, and 1247	93.9	75.0	64.1
1290, 1247, and 1283	88.6	72.2	65.2
1254, 1247, and 1283	96.6	70.6	61.7
Average	**92.9**	**71.4**	**63.9**
1290, 1247, 1283, and 1254	95.9	70.3	63.5

Figures represent percentage similarity between the profiles (the higher the figure, the more related).

ditions (7). Strains were characterized in duplicate over widely different times, and comparison of the resulting PCR products confirmed the reproducibility of the technique. Analysis by RAPD produced useful arrays of 14–26 PCR products with 100% typability.

From an available culture collection, five STEC O157 strains were chosen based on the similarity of their RAPD fingerprints to other STEC O157 strains, five STEC O157 strains were chosen based on their atypical profiles when compared to other STEC O157 profiles, and five other *E. coli* isolates were arbitrarily chosen (Table 1).

The discriminatory capacity of each primer combination is shown in Table 2. Initial work using a single primer to screen the strains illustrated the clonal nature of *E. coli* O157. "Very similar" strains shared on average 94% genomic similarity, compared to only 68% for the arbitrarily chosen *E. coli* strains. It has been proposed that the *E. coli* O157 serogroup forms a clone complex within which there is limited recombination of chromosomal genes, resulting in limited polymorphism when studying interstrain variation (13).

The quality of the profile obtained was dependent on the primer(s) used: primer 1247 when used alone did not support amplification as well as the other primers, producing less complex profiles. This may reflect a lack of paired complementary sites to primer 1247 in the *E. coli* genome, potentially leading to many fragments being too large to amplify, as the primer pairs are too widely spaced apart. In addition, primer 1247 has a %GC content of only 60%, compared to 70% for primers 1254 and 1283, which may result in weaker primer-template interactions. Profiles also deteriorated as the number of primers increased. As more primers were added, the total concentration of primer in each reaction remained constant; therefore, excess primer-binding dNTPs cannot explain this observation. Such an approach assumes that each primer is incorporated at an equal rate. However, because of possible differences in the distribution of priming sites for each primer, the PCR may proceed "asymmetrically", in that one primer may be incorporated more often than others and be used up more quickly. Eventually, this would limit the amplification of fragments involving this primer, enabling only fragments using other primer pairs to continue and dominate the resulting PCR product profile. Whether PCR products containing the limiting primer contribute to the profile depends on how quickly the primer is used up—if this happens too early in the reaction, then they may be too faint to be detected.

Profiles obtained when two or more primers were used were different than those obtained using only one primer. They were not a simple summation of profiles generated using each primer individually but contained unique bands. It could be postulated that 50% of fragments might have the same primer at both ends, appearing in either profile generated using the two primers singly, and 50% would be unique, having different primers at the ends (12). However, Welsh and McClelland (12) reported that 68% of fragments contained both primers. It was suggested that fragments primed by two different primers might amplify more efficiently than products primed by a single primer. The latter may form hairpin structures during renaturation as a result of complementary ends and thus be prevented from participating further in amplification.

PCR product size varied with the number of primers used in the reaction: when only one primer was used, PCR product size varied from between 0.2 and 4 kb. However, when two or more primers were used, PCR product size decreased to 0.1–3 kb. The average distance between priming sites on opposite DNA strands would be expected to be shorter when two different primers are used than when only one primer is used (12). Shorter PCR products may also amplify more efficiently than longer ones because of the shorter extension times required and the increased likelihood that the DNA polymerase will complete strand synthesis.

Arbitrarily chosen strains were consistently found to be less related to each other than the *E. coli* O157 strains. The *E. coli* population as a whole shows ex-

tensive genetic diversity in enzyme-encoding genes when studied using multilocus enzyme electrophoresis (10). Strains have also been shown to differ by as much as 23% in genome size, 25% in nucleotide sequence, and 4% in %GC content (2–5). This diversity would provide ample available polymorphism and alter the distribution of primer-binding sites. Shiga toxin-producing *E. coli* O157 strains that produced very similar profiles using only one primer were not distinguished further when two or more primers were used together. This again may reflect the limited amount of genetic diversity within the *E. coli* O157 serogroup. Welsh and McClelland (12) recommended choosing primers that, when used individually, detect the most polymorphisms to maximize discriminatory potential; however, this was not observed in the current study. Primers 1283 and 1290 were the most discriminatory when used individually but were only the fourth most discriminatory when combined in a single reaction. Nevertheless, using two primers increased the discriminatory capacity of RAPD analysis to subtype *E. coli* O157 strains that previously produced "dissimilar" profiles when a single primer was used. As anticipated, using two primers detected polymorphic regions previously missed, yet the primers that performed best when used individually again did not have the highest discriminatory capacity when used together. This observation was also noted but more pronounced in the arbitrarily chosen strains.

Using three or four primers failed to discriminate further between the arbitrarily chosen *E. coli* strains. As more primers were added, the size range of PCR products produced decreased, which did not resolve well in agarose gels and thus required high-percentage polyacrylamide gels to gain useable fingerprints. Polyacrylamide gels are not always practical to use in a busy laboratory, in addition to the safety hazards associated with acrylamide solution and silver nitrate for staining or radioactivity. Combinations of three primers have previously been found to produce reproducible profiles, but the profiles were very similar to those produced by respective pairs of the three primers (9).

Analysis of clonal organisms by RAPD is restricted by the limited amount of genetic heterogeneity existing between strains that may not be detected when using a single primer if the distribution of priming sites is not altered. However, Micheli et al. (9) have suggested that combining more than one primer in RAPD reactions may increase the interstrain discriminatory capacity by targeting previously undetected polymorphisms in the DNA sequences. The data presented here demonstrate that using two primers combined in an optimized RAPD reaction is capable of increasing the discriminatory capacity of RAPD analysis; however, there is limited value in using more than two. When studying clonal organisms, the use of multiple primers offers poor improvement in discriminatory capacity of RAPD protocols. Instead, it may be more advantageous to try other primers used singly. Different primers may vary in their ability to discriminate between strains; therefore, it has been recommended that more than one independent primer is used to try to increase the discriminatory capacity of RAPD analysis (8).

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