Molecular Biology Today 2001. 2(1): 5-10.

Computer-Based Analysis of RAPD (Random Amplified Polymorphic DNA) Fingerprints for Typing of Intestinal *Escherichia coli*

Forough Nowrouzian*, Agnes E. Wold and Ingegerd Adlerberth

Department of Clinical Bacteriology, Göteborg University, Guldhedsgatan 10, S-413 46 Göteborg, Sweden

Abstract

A random amplified polymorphic DNA (RAPD) PCR method was developed for the identification of *Escherichia coli* strains in the normal human intestinal microflora. Bacteria amounting to approximately one tenth of a colony were added directly to the PCR mixture and disrupted by heating. Taq polymerase was added and PCR was run using a single 10nt primer. The PCR products were separated by polyacrylamide gel electrophoresis, which gave complex band patterns suitable for computer-aided cluster analysis using the Pearson products moment correlation coefficient (i) and the unweighted pair group method with arithmetic averages (UPGMA). The intestinal *E. coli* flora of five infants followed from birth to 6 months' age was analyzed by RAPD. Two isolates were considered to be members of different strains if showing less than 80% similarity in this analysis, as strain differentiation based on this cut-off point coincided largely with strain grouping based on multilocus enzyme electrophoreis. This RAPD method should, thus, be suitable for epidemiological studies of the intestinal *E. coli* flora.

Introduction

Microbial ecology denotes the study of complex microbial communities, such as the microflora inhabiting the human large intestine. The normal intestinal microflora does not only contain an immense number of bacterial species. At a given point in time, several clones, or strains, of the same species may also coexist. For example, an individual typically harbours one to ten different *E. coli* strains simultaneously (Vosti *et al*., 1964; Ochman *et al*., 1983). Some of these strains have the capacity to persist in the colonic microflora for extended periods of time (resident strains), while others are not capable of long term colonization (transient strains) (Sears *et al*., 1949; Sears and Brownlee, 1951; Sears *et al*., 1956). Thus, the study of the normal human microflora does not only require techniques for the reliable identification of different bacterial species, but also methods to distinguish between different strains of the same species. *E. coli* strains in the normal intestinal microflora have been identified by extended serotyping (Sears and Brownlee, 1951; Sears *et al*., 1956), by multilocus enzyme electrophoresis (MLEE) (Ochman *et al*., 1983; Whittam *et al*., 1983; Wold *et al*., 1992; Adlerberth *et al*., 1998) or by biotyping (Kühn and Möllby, 1986; Tullus *et al*., 1991).

During the last years, a number of typing methods based on the heterogeneity of bacterial DNA have been developed. Restriction fragment length polymorphism (RFLP) is regarded as the most sensitive method for strain identification (Dorn and Angrick, 1991; Samadpour *et al*., 1993). However, this method is complicated, time-consuming and expensive (Williams *et al*., 1990; Pfaller, 1991; Birch *et al*., 1996), and has not been applied to analyses of the intestinal microflora.

Random Amplified Polymorphic DNA (RAPD) is a PCR based method which can be used to distinguish between strains within a species. One or a few short primers of arbitrary sequence are allowed to bind under low stringency conditions to various sites on both strands of the template DNA. The PCR reaction yields a series of products of varying size, which may be separated by gel electrophoresis. The band pattern represents a "genetic fingerprint" characterizing a particular bacterial strain (Welsh

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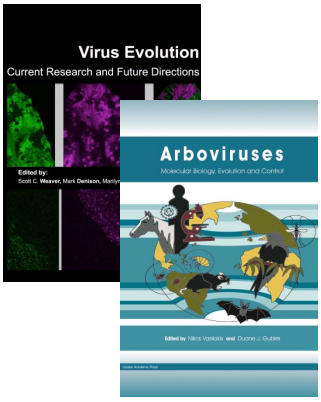

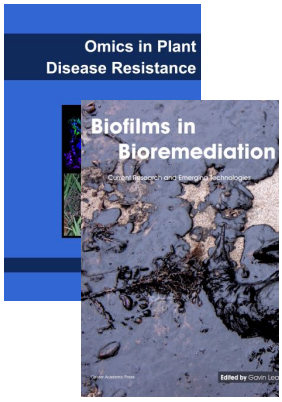

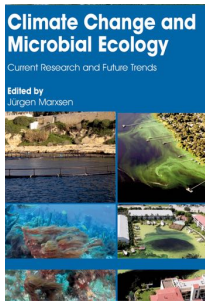

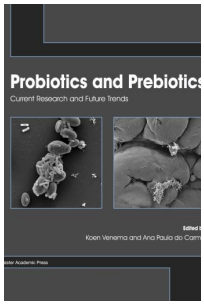

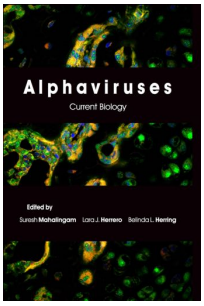

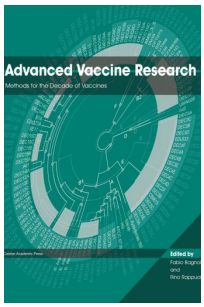

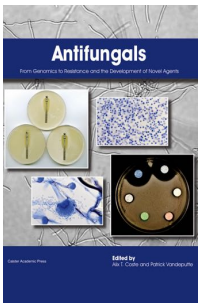

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and McClelland, 1990).

RAPD has lower discriminatory capacity than RFLP (Johansson *et al.*, 1995) and a single primer has mostly not proven sufficient to yield band patterns complex enough to permit the separation of different *E. coli* strains (Birch *et al.*, 1996; Kärkkäinen *et al.*, 1996). A solution has been to run a series of RAPD analyses with different primers and combine the patterns (van Belkum *et al.*, 1994; Desjardins *et al.*, 1995; Kärkkäinen *et al.*, 1996; Pacheco *et al.*, 1997a), but this reduces the speed and cost effectiveness of the method.

The aim of the present study was to design a RAPD typing method suitable for rapid screening of intestinal *E. coli* isolates directly from primary cultures. A single primer should be used, and computer-aided analysis employed to exclude observer bias. Such a method was developed, using a collection of intestinal *E. coli* previously analyzed by multilocus enzyme electrophoresis.

Results

Comparison Between Agarose and Polyacrylamide Gel Electrophoresis

Figure 1 shows the results of RAPD analysis of ten different intestinal *E. coli* strains using one 10-mer primer (primer no. 10, Kit A, Operon Technologies). The PCR products were separated either by agarose or by polyacrylamide gel electrophoresis. The band patterns of the ten strains differed clearly from one another using either polyacrylamide (Figure 1a) or agarose gel electrophoresis (Figure 1 b), but the band patterns were much more complex with the former than the latter method. Specially, fragments in the low molecular weight range (234-453 bp) did not show up on the agarose gel (Figure 1a, b).

DNA Extraction

We compared prior lysis of bacteria for obtaining template DNA (Johansson *et al.*, 1995), with a simplified procedure in which a small amount of bacteria from an agar-grown colony were suspended directly in the PCR mixture and disrupted by heating before adding the Taq polymerase. In our hands, the simplified method gave as clear and reproducible band patterns as prior lysis of bacteria (data not shown). In order to assess the number of bacteria we had added to the PCR mixture, we performed viable counts. In six experiments, the number of bacteria picked was found to vary between $2¥10^7$ and $4¥10^7$. We next prepared bacterial suspensions of varying concentrations and analyzed by PCR. The PCR
pattern was found to be stable over the above range (data not shown).

Computer Aided Analysis of RAPD Patterns for the Identification of Intestinal *E. coli* Strains

We studied the applicability of the RAPD method for typing of intestinal *E. coli* strains by re-analyzing isolates from a longitudinal study of the *E. coli* flora of five newborn infants. The isolates from each child were previously analyzed by multilocus enzyme electrophoresis (MLEE) and grouped into different strains, of which some yielded repeated isolates (resident strains) and others were only found once (transient strains) (Adlerberth *et al.*, 1998).

First, we sought to determine a cut-off point, below which two isolates could be considered as representing two different strains, instead of two isolates of the same strains. To this end, we compared strain grouping with RAPD, using different cut-off points, with results using MLEE. Figure 2 demonstrates the RAPD analysis of 22 intestinal *E. coli* isolates from one child. Employing 80% as the cut-off level, the 22 isolates were grouped into nine strains ("A"-"G") by RAPD. Previously, these isolates were grouped by MLEE into nine electrophoretic types, "I" to "IX" (child no 1, Figure 3). Thus, using 80% as cut-off, grouping of 21 out of 22 isolates from this child agreed between the two methods. The exception was one isolate ("D"/"V", child no 1, Figure 3). Using RAPD this isolate was grouped together with another isolate, comprising the "D" strain. Using electromorphic typing, the same isolate was instead grouped with a third isolate, these two isolates composing the strain "V". Other cut-off point (e.g.75% or 85%) gave less agreement between the two methods.

Comparisons between the two methods for analysis of the intestinal flora of all five infants are shown in Figure 3. To a large extent, the results from the two methods agreed, although isolates not distinguished by MLEE were separated using RAPD in eight cases. In three cases, isolates showing different enzyme electrophoretic patterns were not separated by RAPD.

Attempts to use agarose gel band patterns for computer-based analysis were not successful. Thus, repeated analyses of a single isolate of *E. coli* often gave electrophoretic patterns which, despite looking very similar to the naked eye, showed less similarity in computer-based analyses than two separate strains.

Discussion

The aim of the present study was to develop a RAPD-based typing method for *E. coli* suitable for identification of individual *E. coli* strains in the normal intestinal microflora. Similarity analyses of the electrophoretic patterns of the PCR products should be computer-based, in order to facilitate comparisons of large numbers of isolates and to eliminate inter-individual observer variation.

It was impossible for us to obtain band patterns in agarose gels that could be used for computer-aided analysis, but possible if the PCR products were instead separated on polyacrylamide gels and visualised by silver staining, which yielded much more complex bands. A sufficiency large number of bands is a prerequisite for adequate performance of computer-based band pattern analysis (Seward *et al.*, 1997).

In one case a close relationship between two isolates (strain "D", Figure 2) was suggested by the program although the isolates looked different to the naked eye. The sensitivity of the program to background may cause this type of mismatch. This observation emphasises the continued need for manual assessment and interpretation of automated results as well as the need to ensure that findings are based on sufficiently complex fingerprints (Seward *et al.*, 1997).

Despite the fact that polyacrylamide gel electrophoresis is more complicated than agarose gel electrophoresis, speed and cost-effectiveness were nevertheless increased, since the complex band patterns obtained permitted us to use a single primer for typing of *E. coli* strains. Although there are some previous reports on RAPD characterization of *E. coli* strains using a single primer and agarose electrophoresis, none of these studies have used computer-aided analysis of band patterns (Alos *et al.*, 1993; Wang *et al.*, 1993). Thus, we could not successfully apply computer based separation of *E. coli* strains when using one of these reported methods (Wang *et al.*, 1993). Indeed, the use of multiple primers has been a prerequisite for efficient separation of *E. coli* by RAPD in most studies (van Belkum *et al.*, 1994; Desjardins *et al.*, 1995; Birch *et al.*, 1996; Kärkkäinen *et al.*, 1996; Pacheco *et al.*, 1997b), which limits the usefulness of the methods.

The DNA extraction step was eliminated to increase speed and versatility. A small amount of bacteria, just enough to be clearly visible by the naked eye, was picked from a colony and suspended directly in the PCR mixture. The bacteria were disrupted by heating in the PCR mixture before the heat sensitive Taq polymerase was added. The use of *E. coli* whole-cell preparations for PCR has been described previously, e.g. in repetitive element sequence-based PCR (rep-PCR) for the typing of *E. coli* (Woods *et al.*, 1993) and in multiplex PCR assays (Yamamoto *et al.*, 1995). In the present study, the amount of bacteria added to the PCR mixture was found to vary between $2¥10^7$ and $4¥10^7$, in which range the PCR patterns showed satisfying stability. In accordance, Pacheco *et al.* found that similar amplification products were obtained over a rather large template concentration range (Pacheco *et al.*, 1996), and a number of studies report reproducible RAPD results without prior quantification of bacterial DNA (Lawrence *et al.*, 1993; Wong *et al.*, 1994; Birch *et al.*, 1996; Seward *et al.*, 1997). Although standardizing the amount of template DNA may increase reproducibility (Ellsworth *et al.*, 1993; Muralidharan and Wakeland, 1993; Brikun *et al.*, 1994; Cavé *et al.*, 1994), we believe that the possibility of directly screening bacteria from primary cultures gives a strong advantage to our simplified method.

The practical applicability of the RAPD method was tested by re-analyzing *E. coli* isolates previously

grouped into different strains using multilocus enzyme electrophoresis (MLEE). These isolates were obtained from a longitudinal study of the intestinal *E. coli* flora of newborn infants (Adlerberth *et al*., 1998). MLEE is based on differences in electrophoretic mobility between isoforms of cytoplasmic "housekeeping" enzymes. It is a well established and reliable, but expensive and time consuming method (Ochman *et al*., 1983; Selander *et al*., 1986), which has been used in several epidemiological studies of intestinal *E. coli* (Whittam *et al*., 1983; Wold *et al*., 1992; Adlerberth *et al*., 1998). In our hands, RAPD using polyacrylamide electrophoresis and computer-aided analysis of band patterns showed good agreement with electromorphic typing for the identification of intestinal *E. coli* strains, but was many times less labour intensive and
expensive. We did not compare with a third method, such as RFLP, regarded as the most sensitive typing system for a number of different bacterial species (Wachsmuth, 1986). We believe that although RFLP may be superior to distinguish between strains which are very closely related, such a sensitive method is not required to characterize the normal intestinal *E. coli* flora, which in a certain individual represents a rather heterogeneous collection of strains (Caugant *et al*., 1983).

Preliminary data indicate that the RAPD patterns of *E. coli* strains are stable upon intestinal colonization. Thus, ten different *E. coli* strains which were allowed to colonize germ-free rats for two weeks could be identified in cultures of the intestinal contents using their RAPD patterns (V. Herías, unpublished). *In vivo* stability of RAPD patterns has also been reported by others (Cavé *et al*., 1994; Kärkkäinen *et al*., 1996). This supports that RAPD is a method well suited for epidemiological studies of intestinal *E. coli*.

Experimental Procedures

Bacteria

A collection of *E. coli* isolated from the intestinal microflora of five newborn infants was used to develop and test the RAPD method (Adlerberth *et al*., 1998). Sampling of the rectal flora was performed on 12 occasions during the first 6 months of life. The isolates of each child had previously been grouped into different *E. coli* strains using multilocus enzyme electrophoresis (MLEE) based on the isoenzyme pattern of the following eight enzymes: malate dehydrogenase, 6-phospogluconate dehydrogenase, adenylate kinase, phosphoglucose isomerase, glucose 6-phosphate dehydrogenase, mannose phosphate isomerase, phenylalanyl-leucine peptidase and leucylglycyl-glycine peptidase. Using this method a number of different *E. coli* strains could be identified in each infant. Strains persisting in the intestinal flora for a period of time contributed several consecutive isolates, while transient strains were represented by a single isolate (Adlerberth *et al*., 1998).

Selection of Primer

Twenty different 10-mer primers (kit A, Operon Technologies, Alameda, CA) and the 20-mer primer D14307 (GGTTGGGTGAGAATTGCACG) described by Wang *et al*. (Wang *et al*., 1993) were tested for their capacity to discriminate between eight different *E. coli* strains from the collection of infant intestinal *E. coli.*

Primer no. 10 (GTGATCGCAG) was chosen because it generated the most discriminatory RAPD profiles for the eight *E. coli* strains. The 20-mer primer D14307 yielded less informative band patterns than primer no. 10, both under the PCR conditions described in the original paper (Wang *et al*., 1993) and under the conditions described below.

Optimization of PCR Reactants

Different concentrations of primer (4-9 mM), dNTP (0.1-0.4 mM) (Perkin Elmer, Branchburg, NJ), $MgCl_2$ (1.5-6 mM) (Perkin Elmer) and Taq polymerase (1-3 U/ml) (Perkin Elmer) were tested, and the following concentrations were found to be optimal: 6 mM of primer, 0.1 mM dNTP, 2 U/ml Taq polymerase and 1.5 mM $MgCl_2$. Higher concentration of $MgCl_2$ decreased the numbers of informative bands (data not shown).

DNA Preparation and Amplification

Two methods were used to obtain bacterial DNA. First, bacteria were cultivated in broth, spun down, washed and lysed before addition to the PCR mixture (Johansson *et al.*, 1995). Later, a simplified method was developed. A small amount of bacteria was picked from an agar-grown colony with the tip of a sterile syringe. The bacteria were suspended in a mixture of primer, nucleotides and $MgCl_2$, in an autoclaved thin-wall reaction tube (Perkin Elmer). The tube was sealed with a drop of mineral oil and the mixture was heated for 10 min at 94° in a thermocycler (Perkin-Elmer Cetus Model 480) in order to disrupt the bacteria.

PCR Analysis

Taq polymerase (Ampli Taq, Perkin Elmer) was added and the PCR reaction was run using the following temperature profile: 94°C for 45 s; 30°C for 120 s; 72°C for 60 s for four cycles followed by 94°C for 5 s; 36°C for 30 s; 72°C for 30 s for 26 cycles (the extension step was increased by 1 s for every new cycle). The PCR reaction was terminated at 72°C for 10 min and thereafter cooled to 4°C (Johansson *et al.*, 1995).

Gel Electrophoresis

For separation of the PCR products, agarose and polyacrylamide gel electrophoresis were compared. Agarose gel electrophoresis was performed using 1.5% agarose gel as previously described (Johansson *et al.*, 1995).

For polyacrylamide gel electrophoresis, 8% ready-made Tris-Glycine gels (Novex, Frankfurt, Germany) were applied in a vertical electrophoresis apparatus and loaded with 6.3 ml of sample and 0.7 ml of loading buffer (Gel loading solution, Sigma). DNA marker VI (Boehringer Mannheim) was used as a molecular weight standard. The electrophoresis was run for 10 min at a constant voltage of 20 V, followed by 90V for 2 h 15 min in a Tris-glycine running buffer (Tris base 0.24 M, glycine 1.9 M, SDS 0.035 M, pH 8.3). DNA was visualized by silver staining (plusOne DNA silver staining kit, Pharmacia Biotech, Uppsala, Sweden) whereafter the polyacrylamide gel was dried (Dry Ease Gel Drying System, Novex).

Computer-Aided Gel Analysis

Agarose and polyacrylamide gels were scanned in a laser densitometer (Studio Scan II Si Agfa, MacForum, Göteborg, Sweden) and analysed by the Gel Compare 4.0 programme (Applied Maths, Kortrijk, Belgium) (Johansson *et al.*, 1995). Similarity matrices were calculated using the Pearson Product-moment correlation coefficient and the isolates were clustered by the unweighted average pair group method (UPGMA) using arithmetic averages (Seward *et al.*, 1997).

Acknowledgements

We thank Siv Ahrné and Mikael Quednau at the Department of Food Technology, Lund University, for expert advice on RAPD and computer-based analysis and we are grateful to Hans-Jürg Monstein at the Department of Clinical Microbiology, Linköping University, for critically reading the manuscript. The study was supported by grants from the Swedish Medical Research Council (no. K98-06X-12612-01A), the Swedish Association for Research on Agriculture and Forestry, the Swedish Foundation for Health Care Science and Allergy Research and the Ellen, Walter and Lennart Hesselman foundation.

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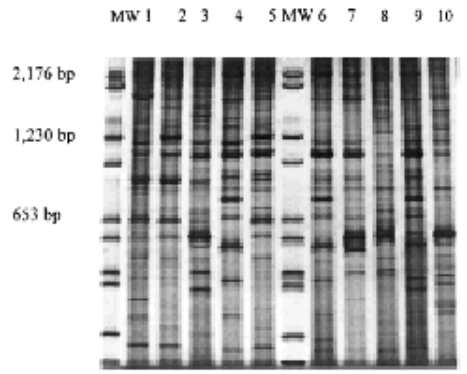
a)



b)

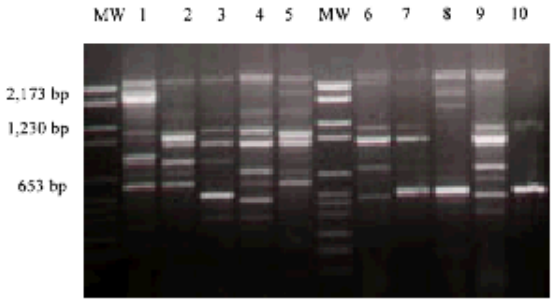


Figure 1. RAPD patterns of ten different *E. coli* strains. The amplified DNA fragments were separated by (a) polyacrylamide gel or (b) agarose gel electrophoresis. The bands were detected by silver staining for polyacrylamide gels and by ethidium bromide staining for agarose gels. The lanes are marked with strain number, or with "MW" for molecular weight standard.

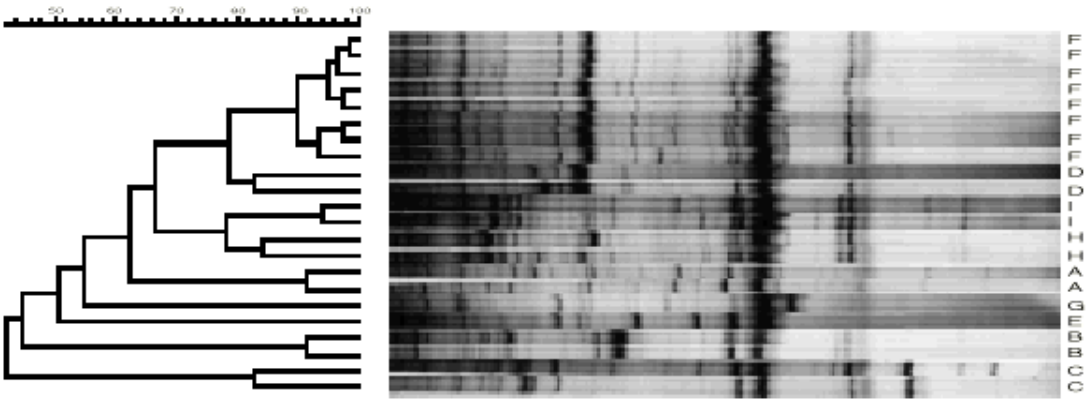


Figure 2. RAPD patterns of 22 *E. coli* isolates obtained from the intestinal flora of child no.1 and a dendrogram showing their relative similarity. The isolates were sampled on different occasions over a six months period and represent different strains, as well as multiple isolates of strains persisting in the microflora. The PCR products were separated by polyacrylamide gel electrophoresis and visualized by silver staining. Band patterns were analyzed using the Gel compare program. Isolates clustering together at a similarity level exceeding 80% were regarded as belonging to the same strain. The nine strains identified by this means are represented vertically by the letters A-I. The strains are identical to those from child no.1 depicted in Figure 3, and are designated by the same letters. The percent similarity is indicated on top of the dendrogram.

Child no.1

RAPD pattern

ET pattern	A	B	C	D	E	F	G	H	I
I	2								
II		2							
III			2						
IV				1					
V				1	1				
VI						8			
VII							1		
VIII								2	
IX									2

Child no.2

RAPD pattern

ET pattern	A	B	C	D	E	F	G	H	I	J	K
I	4	1	2								
II			1	2							
III					2						
IV						2	1				
V								2	1		
VI										4	
VII											2

Child no.3

RAPD pattern

ET pattern	A	B	C	D	E	F	G
I	2						
II		2					
III			1	2			
IV					4		
V						1	
VI							1

Child no.4

RAPD pattern

ET pattern	A	B	C	D	E	F	G
I	2						
II		2					
III			3				
IV				2			
V					2		
VI						1	1

Child no.5

RAPD pattern

ET pattern	A	B	C	D	E	F	G
I	2						
II	2	1	2				
III				1			
IV					2		
V						1	
VI							1

Figure 3. Comparison of the results obtained using RAPD and electromorphic typing (ET) for the grouping of *E. coli* isolates obtained from the intestinal flora of five different infants. Child no. 1 is the one exemplified in Figure 2. The different RAPD profiles identified among the *E. coli* isolates of a child are indicated horizontally by letters and the different electromorphic patterns are indicated vertically by roman numbers. The figures show the number of isolates representing each RAPD/electromorphic pattern.