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Variability in presence–absence scoring of AP PCR fingerprints affects computer matching of bacterial isolates

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Abstract

Sources of variation in scoring bands in arbitrarily primed PCR (AP PCR) fingerprints of bacterial isolates were identified, and their effect on computer matching of fingerprints was determined. *E. coli* and five *Salmonella* serotypes were fingerprinted. PCR reactions and gel electrophoresis analysis of PCR products were replicated, including comparisons in the same gel and in different gels. Bands in the images were assigned by two different people on a presence–absence basis. Variations in scoring the images occurred at all levels, and prevented correct identification of isolates. *E. coli* was distinguished from *Salmonella*, but discrimination among different *Salmonella* serotypes and between two isolates of the same serotype was poor. Our results suggest that computer analysis of AP PCR fingerprints scored on a presence–absence basis may not correctly match isolates. Side-by-side visual comparison of isolates is recommended. © 1997 Elsevier Science B.V.

Keywords: Bacteria; PCR; DNA fingerprinting; *Salmonella*

1. Introduction

DNA fingerprinting of bacteria using the polymerase chain reaction (PCR) is becoming increasingly popular because it is simple, rapid and sensitive to DNA sequence differences among isolates [1]. Two primary PCR fingerprinting methods are arbitrarily-primed PCR (AP PCR) [2,3] and PCR based on repetitive DNA elements (rep-PCR) [4–6]. Both AP PCR and rep-PCR produce fingerprints in agarose gels consisting of bands that vary in intensity. Faint bands that may not be reproducible from day to day or in different laboratories create doubts that PCR fingerprints can correctly identify isolates.

Two approaches to matching PCR fingerprints have been reported. The most common and most conservative approach for comparing two isolates has been to perform PCR on both isolates together in a thermal cycler using the same master mix of reagents, and to analyze the PCR products visually side-by-side in the same gel. By this method, two fingerprints have been determined to be the same or different based on an overall visual impression. Using this approach, AP PCR and rep-PCR fingerprints have been considered reproducible [1,7,8]. Berg et al. [1] believed that occasional lack of reproducibility of AP PCR was caused by "suboptimal or non-constant amplification conditions."

The second approach to matching has been to enumerate the bands in each fingerprint and to estimate their length in base pairs. Usually bands

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have been scored on a presence–absence basis, disregarding band intensity. This approach has reduced fingerprints to data that can be analyzed and compared by computer. Typically, pairwise similarities have been estimated between isolates, and dendrograms have been created to show relatedness [5]. Interpretation of fingerprints by computer, if successful, creates the potential for identification of isolates in different laboratories from fingerprint patterns stored in databases [5]. It has not been determined, however, whether PCR fingerprinting is robust enough to permit laboratory-to-laboratory comparisons.

The objective of the research reported here was to identify sources of variability influencing correct identification of known isolates from their AP PCR fingerprints. For that purpose, fingerprints of increasing expected similarity were produced in order to establish the level of discrimination of a protocol that includes AP PCR amplification, gel electrophoresis separation of PCR products, and computer analysis of images. Therefore, *E. coli* and different *Salmonella* serotypes were fingerprinted, including two different isolates of one serotype and two template preparations of one isolate. In addition, PCR reactions were replicated, products were separated by gel electrophoresis in triplicate, and gels were scored by two different scientists. Results were displayed as dendrograms.

2. Materials and methods

E. coli and five different *Salmonella* serotypes were fingerprinted (Table 1). Two different isolates of one serotype (*S. newport*) and two template preparations of one isolate (*S. typhimurium*) were included. Template DNA was prepared by pelleting cells from a nutrient broth culture (optical density ~0.6 at 590 nm), washing the pellet in 1 M NaCl, resuspending in sterile distilled water, and lysing in a thermal cycler for about 10 min at 98°C. A 1:10 dilution of this lysate was used as template for AP PCR, performed with minor modifications of conditions optimized in our laboratory [9]. A 50 μl AP PCR reaction consisted of 5 μl bacterial cell lysate (approximately 5×10^6 CFU), 5 μl of GeneAmp® 10X Buffer II (Roche Molecular Systems, Branchburg NJ) (500 mM KCl, 100 mM Tris–HCl, pH 8.3), 6 μl of 25 mM $MgCl_2$, 5 μl of dNTP mix (2.5 mM), 1 μl of 40 μM primer stock (5′ CGT–GGG–GCC–T 3′), 0.5 μl AmpliTaq® polymerase (Roche Molecular Systems) (5 U/μl), and 27.5 μl sterile deionized water. The *Taq* polymerase was added during an initial eight minute denaturation cycle at 94°C. There were 40 cycles of amplification in a DNA Thermal Cycler (Perkin Elmer, Foster City CA) consisting of denaturation at 94°C for one minute, primer annealing at 40°C for 1 minute, and product extension at 72°C for two minutes.

Table 1
E. coli and *Salmonella* isolates fingerprinted by AP PCR

	Sample	Source	
1	*E. coli*	ATCC 15524	
2	*S. typhimurium*	ATCC 23565	DNA preparation 1
3	*S. typhimurium*	ATCC 23565	DNA preparation 2
4	*S. newport*	Environmental	Isolate 1
5	*S. newport*	Environmental	Isolate 2
6	*S. senftenberg*	Environmental	
7	*S. anatum*	Environmental	
8	*S. agona*	Environmental	

Replicate template preparations were made for one *S. typhimurium* strain. Two different *S. newport* isolates were fingerprinted. PCR was performed in duplicate for each sample. Aliquots of each duplicate PCR reaction were run in triplicate in agarose gels and scored by two different technicians.

All PCR reactions were run together using the same master mix, thus minimizing amplification conditions as a variable. Replicate PCR tubes were run for each template preparation. Three aliquots from each PCR replicate tube were run in agarose gels, two aliquots in the same gel and a third aliquot in a different gel. Positions in gels were randomized. Three gels were required to analyze all fingerprints. The gels were stained together in the same ethidium bromide solution on a rotating shaker and digitally scanned by The Imager™ (Oncor/Appligene, Gaithersburg MD), using the same settings for focal length, aperture, exposure, and contrast. The scanned images were analyzed using the Bio Image™ Whole Band Analyzer (Bio Image, Inc., Ann Arbor, MI). Although there is the option to permit the computer to score the bands in an image, we have found manual scoring more reliable. Thus, bands were scored visually on a presence–absence basis using the cursor to mark the location of each band ob-

served. Two scientists scored each image. Scored images were examined to determine the sources of variability in scoring and matching isolates and the effect of this variability on correctly matching known isolates.

3. Results

The first variable examined was how two different scientists scored the same images. There is little hope that a computer will correctly match fingerprints of identical isolates if their fingerprints are not scored 'correctly.' In this test, both scientists scored computer images of the same three gels containing 48 fingerprints. They were instructed to use the cursor to mark the location of every band they could distinguish from the background. Both complained of difficulties in deciding about faint bands and were undecided about several bands. From two to eleven bands were detected per fingerprint. Half the fingerprints (24) were scored the same by each person. In the other 24 fingerprints, one person always detected from one to three more bands per fingerprint than the other person. Scoring biases were also apparent. Because aliquots of PCR products were randomized in gels, two complex fingerprints from the same *S. agona* template happened to be run in adjacent lanes in the same gel. Believing that the images were identical, each person admitted being influenced to score the same bands in both. However, one person detected eleven bands in each fingerprint; the other detected nine bands in each.

Next, each person was asked to score a fourth image without knowing that it was a computer-generated duplicate image of one they had already scored. One person scored eight of sixteen fingerprints differently the second time, the other person scored four of sixteen differently. Interestingly, the person who scored fewer bands overall scored the duplicate image more consistently than the other person. Fig. 1 illustrates the variability in scoring a replicate fingerprint image by the two scientists. In a few cases, the scientists positioned the cursor in noticeably different locations when scoring the same bands in replicate images (Fig. 1). In our experience, a 30 to 50 bp difference in the size estimate of a band can easily result from small differences in

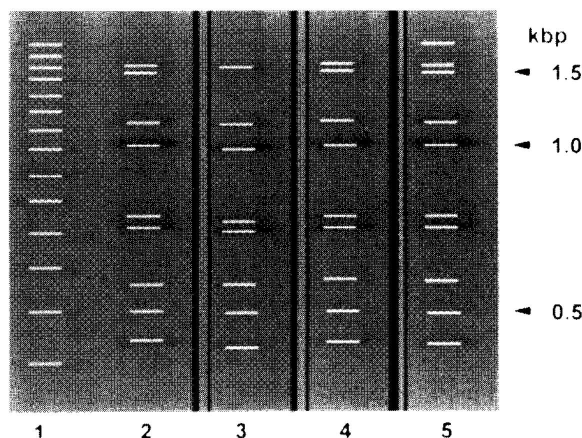

Fig. 1. Composite showing variability in scoring the same fingerprint image (*S. agona*) by two scientists. White marks indicate assigned band locations. Lane 1, 123 bp ladder. Lanes 2 through 5 are computer generated replicate images of a single lane. Lanes 2 and 3, scoring by the first scientist. Lanes 4 and 5, scoring by the second scientist. Overall, three different patterns were detected from the same image. In addition, there were slight differences in marked band locations.

cursor placement during scoring, and can affect subsequent computer matches of images.

Differences in scoring replicate aliquots of the same PCR reaction run in the same gel versus different gels were examined next. The contents of each PCR reaction tube were partitioned into three aliquots, two run in the same gel and a third in a different gel. The gels were stained together and scanned identically. Thus, each person scored three randomized lanes from each of sixteen PCR reactions. One person scored all three lanes the same in only six of sixteen cases. The other scored them the same in only four cases. The two aliquots run in the same gel were no more likely to be scored the same than aliquots in different gels. An interesting result was that not all serotypes were equally easy to score. Each person detected the same four-band pattern in all six *S. senftenberg* fingerprints examined. Bands in fingerprints of this serotype were distinct and well separated in the gel. This result suggests that certain primers may produce better fingerprints from certain serotypes.

Finally, dendrograms were created to determine how well the different isolates were discriminated in spite of variability in scoring. First, band sizes in

each scored fingerprint were estimated using the Bio Image™ Whole Band Analyzer. Pairwise similarities between fingerprints were calculated using a simple index, essentially the ratio of matching bands to mismatches. The 'stringency' of matching was varied by changing the percent deviation, i.e., the allowed relative difference in the estimated sizes of two bands considered a match (range from 2 to 4%). Fingerprints were clustered using average linkage (unweighted pair group method with arithmetic averages, or UPGMA) [10]. Fig. 2 is a dendrogram (2% deviation, UPGMA) showing the similarities among the fingerprints scored by one of the scientists. Fingerprint profiles are shown on the right. The dendrogram consists of three gel electrophoresis replicates of the contents of eight different PCR reactions.

E. coli (EC) fingerprints were easily distinguished from *Salmonella*, even though the number of bands scored ranged from two to four per *E. coli* fingerprint. The *Salmonella* serotypes were not well discriminated from each other, however. Only two *S. senftenberg* (SF) fingerprints (from the same gel) were recognized as 100% similar even though all

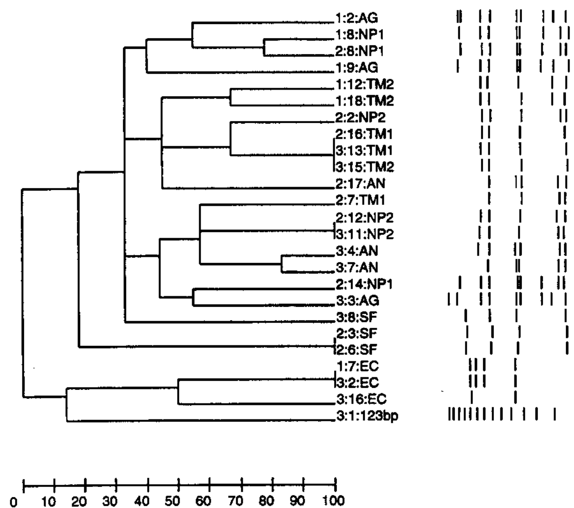

Fig. 2. Dendrogram showing groupings of AP PCR fingerprints scored visually by one scientist. The bottom profile is 123 bp ladder. A scale of percent similarities is along the horizontal axis. Lane designations are gel:lane:serotype. AG=*S. agona*. NP=*S. newport* (two isolates, NP1 and NP1). TM=*S. typhimurium* (two template preparations, TM1 and TM2). AN=*S. anatum*. SF=*S. senftenberg*. EC=*E. coli*.

three fingerprints appeared to have been scored the same. Most of the *S. typhimurium* fingerprints were grouped together, but only three were judged to be identical, and some were no more than ~40% similar. There were no apparent differences between the two PCR template preparations (TM1 and TM2). Fingerprints of *S. newport*, *S. anatum*, and *S. agona* were not discriminated. Fingerprints of the two different *S. newport* isolates (NP1 and NP2) were generally judged as similar as replicates of either isolate by itself. In addition, gel electrophoresis replicates from the same PCR reaction were generally no more similar than PCR replicates (dendrogram not shown).

4. Discussion

We have shown that variations in presence–absence band scoring are inherent in the analysis of AP PCR fingerprints and that these variations affect the ability of computers to correctly identify isolates by matching their fingerprints. We would expect similar results using rep-PCR. All isolates in this study were known and all fingerprints were gel electrophoresis replicates of PCR reaction replicates. AP PCR reactions were optimized and all reactions were run together in a thermal cycler using the same master mix of reagents. Gels were stained together and scanned using the same settings. The quality of the images was comparable to those usually produced in our laboratory. Nonetheless, only *E. coli* and two *Salmonella* serotypes (*S. senftenberg* and *S. typhimurium*) were generally discriminated by the technique. Four other isolates belonging to three serotypes (*S. newport*, *S. agona*, and *S. anatum*) apparently were similar enough that they could not be discriminated.

Our results suggest that AP PCR fingerprints detected by ethidium bromide staining of agarose gels may not be sufficiently reproducible to permit computer matching using presence–absence scoring. If replicate PCR reactions and replicate gel electrophoresis of PCR products from the same reaction generate fingerprints that are only 40% similar in some cases (Fig. 2), one questions whether a confident identification of isolates can ever be made. It has been shown that AP PCR can indicate that two

strains are different [11]. It is not clear that it can also indicate with confidence that two are identical. Furthermore, it is not known how much similarity should be expected between fingerprints of isolates of the same genus, species, or strain.

Reproducible AP PCR results have been reported by a number of researchers, but in the limited context of comparisons of a few isolates in the same laboratory and often the same gel [7,11–14]. For example, Akopyanz et al. [11] used four different primers to differentiate two strains of *Heliobacter pylori*. The strains were dissimilar enough to be easily discriminated visually in the same gel without any need to score bands. Killgore and Kato [13] considered that two *Clostridium difficile* isolates belonged to the same type if their AP PCR fingerprints were identical by visual inspection or differed only in faint bands. Woods et al. [14] made visual comparisons of fingerprints of *Neisseria meningitidis* isolates in different gels, but confirmed doubtful cases by repeating amplification and by running the products in the same gel.

We are not aware of research indicating that AP PCR or rep-PCR fingerprints from different laboratories have been successfully matched by computers using presence–absence scoring. Yet such long-distance matches are clearly desirable [1]. Reliable matches do not appear attainable as long as non-reproducible faint bands are scored in agarose gels stained with ethidium bromide. Versalovic et al. [5] discussed alternatives to detection in agarose gels stained with ethidium bromide. These include primers with fluorescent labels, or acrylamide gels stained with silver. Versalovic et al. [5] described an alternative to presence–absence scoring using an internal PCR standard to set a threshold for band recognition. Apparently only reproducible bands of a minimum intensity were scored. Improved gel electrophoresis and staining methods combined with a rationale for scoring bands having a minimum intensity is a logical step in making computer matching of PCR fingerprints feasible.

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