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GUEST COMMENTARY

Factors Affecting Reliability and Reproducibility of Amplification-Based DNA Fingerprinting of Representative Bacterial Pathogens

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Arbitrary amplification of polymorphic DNA sequences has increasingly been reported as a method for the genetic characterization of microorganisms, and there are many variations of this technique. Arbitrarily primed PCR (AP-PCR), random amplified polymorphic DNA (RAPD) analysis, and DNA amplification fingerprinting are the main contenders (8, 9, 79, 81).

These methods all use quite different approaches, but the underling theory is the same in that arbitrary DNA sequences are used as single primers that target an unspecified genomic sequence in order to generate a genetic profile. Amplification is conducted at low annealing temperatures, which allows for mismatches and thus permits arbitrary primer sequences to bind nonspecifically as well as specifically to the DNA template. Amplimers are generated whenever two correctly oriented copies of the primer are close enough for the PCR to proceed efficiently. Venugopal et al. (76) provide some insight into the molecular nature of RAPD analysis and the mismatch annealing of primers. Unlike regular PCR, where an increase in DNA and/or primer concentration is expected to increase the concentration of existing products, these methods may amplify new targets or reduce amplification of previous ones (61). The resulting profile is thus a combination of artifactual variation mixed with true polymorphism, and recent focus has been placed on recognizing and correcting for these artifacts (41, 42, 47).

A recent search of Medline to identify the evolution in the use of these techniques has revealed an exponential increase over the past 5 years. The majority of researchers use the RAPD technique, and one of the most common applications in microbiology has been for the inter- and intraspecies discrimination of microbial isolates. A review by Caetano-Anollés (7) highlights these methods and encompasses them under the global term multiple arbitrary amplicon profiling (MAAP).

Theoretically, arbitrary primers will generate a consistent amplification pattern for related strains of a species, and it has been commonly accepted that any polymorphisms observed between related individuals or strains are due to loss of priming sites by mutation, deletion, or insertion of genetic elements (61, 81). However, a recent publication has indicated that amplification of unrelated, comigrating RAPD products can occur due to preferred synthesis of unrelated loci (69), and completely unbiased estimators do not appear possible (47). These errors in estimating similarities would have a major impact on any phylogenetic analysis, and several researchers have endeavored to reduce artifacts through experimental manipulation. Consequently, although these methods are shown to have some value in rapidly discriminating between individual isolates or identifying outbreak strains, the interlaboratory reproducibility of arbitrary amplification protocols leaves much to be desired. They can be affected by many of the same factors influencing regular PCR such as Mg^{2+} concentration and PCR conditions, as well as DNA extraction methods, batch-to-batch variation in primer synthesis, ratio of DNA template concentration to primer concentration, the model of thermocycler used, and the supplier and concentration of *Taq* DNA polymerase. For these reasons caution must be exercised when comparing and interpreting like data between laboratories. Table 1 summarizes the scope of the problems with these techniques and provides references that provide further information.

As an alternative to this arbitrary approach, known conserved regions can be amplified with single DNA primers in a way which gives rise to polymorphic DNA fingerprints. Repetitive DNA motifs are particularly amenable to this approach, and several highly conserved intergenic repetitive consensus nucleotide sequences that exploit this principle have been reported in the literature. Initially identified in enteric bacteria, similar sequences have now been revealed in many diverse eubacterial species (78). The 33- to 40-bp repetitive extragenic palindromic (REP) elements have been discovered in the genomes of *Escherichia coli* and *Salmonella typhimurium* and are present at approximately 500 to 1,000 copies, occupying up to 1% of the bacterial genome (70). These sequences have also been referred to as palindromic units (27, 28, 31). The 124- to 127-bp enterobacterial repetitive intergenic consensus (ERIC) elements have also been discovered in the genomes of the aforementioned organisms as well as other gram-negative species and are present at approximately 30 to 150 copies (35). These sequences have also been called intergenic repetitive units (68). The 154-bp BOX element was discovered in the genome of *Streptococcus pneumoniae* and is present at approximately 25 copies. From 5′ to 3′, BOX elements are composed of three subunits: the 59 nucleotides of box a, the 45 nucleotides of box b, and the 50 nucleotides of box c (49). They appear to be the gram-positive equivalent of the REP and ERIC sequences and are the first such example of repetitive elements to be described in gram-positive organisms. All these motifs are genetically stable and differ only in their copy num-

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TABLE 1. Experimental parameters found to influence amplicon profiles in MAAP analysis, plus additional references[a]

Parameters observed to influence MAAP profiles and reproducibility	References citing confirmatory observations
Mg concn	1, 13, 14, 18, 60, 63
Taq polymerase concn	1, 5, 14, 64
Source	1, 10, 57, 67
DNA concn	1, 14, 79
Preparation	11, 17, 53, 59, 60, 79
Secondary structure	7, 10
Strain-to-strain variation	No studies found
Genome size	No studies found
Primer concn	1, 8, 48, 60
Primer length	7, 8, 10, 48, 81
G+C content of primer	7, 8, 10, 38, 81
Purity and source	No studies found
Batch-to-batch variation	71
Importance of primer selection	3, 14, 30, 79
Primer/template ratio and interaction	7, 10, 13, 18, 48, 61
Thermocycler	
Annealing temp	7, 10, 18, 38, 60, 79
Cycle no. and time	1, 13, 38, 48, 60
Extension time	38, 60
Denaturing time	14, 38
Model	13, 30, 48, 57, 64
Temp across block	14, 44
RNA contamination	18, 59, 83
Type of gel	3, 53
Interlaboratory	
Due to *Taq* polymerase	57, 67
Due to thermocycler	14, 48, 57, 64
Due to all variables	60, 66

[a] MAAP has been described previously (7). Items in boldface type represent those factors cited most often as contributing to variability.

ber and chromosomal locations between species, thus making them a desirable target for strain differentiation (35). Collectively, primers targeting these repetitive elements have been referred to as rep-PCR (46).

The present study investigates an application of the ERIC II primer designed from the central inverted repeat of the ERIC consensus sequence located in extragenic regions of various enterobacteria (35). Although it was originally found to yield desirable amplification patterns in gram-negative enteric species, most gram-positive species showed minimal amplification (78). One group of researchers, however, used these primers to discriminate gram-positive methicillin-resistant *Staphylococcus aureus* by using a low-stringency annealing temperature of 25°C for 40 cycles (71–73). This low-stringency annealing approach was maintained by some of the same researchers with gram-negative organisms as well (19, 25, 74, 75). The published protocol used an annealing temperature below even that used with most arbitrary primers. It is postulated that the ERIC primer used under these conditions, although in theory less arbitrary, may still bind nonspecifically to the template DNA and is therefore subject to all the problems with regard to reproducibility that are known to occur with methods that use arbitrary primers (Table 1). In our evaluation of this method, we tested the effects of variable DNA template concentration, primer concentration, *Taq* DNA polymerase, and annealing temperature on the ERIC-based DNA profile in order to assess the reliability and reproducibility of this low-stringency approach. Although we used several different organisms as template DNA in assessing the stability of low-annealing-temperature ERIC-PCR, only the results for the clinical isolates of *S. aureus* and *Helicobacter pylori* are presented because they provide good examples of stability among gram-positive and gram-negative organisms.

METHODS AND RESULTS

Bacterial DNA was extracted by the standard methods described previously (65). *S. aureus* cells were preincubated in a lysis buffer containing lysostaphin (2 mg/ml; Sigma Chemical Co., St. Louis, Mo.) instead of lysozyme and were incubated at 37°C for 30 min before continuing with the standard protocol. Several aspects of the PCR were manipulated to assess the impact on reproducibility with the exception of the constant use of 3 mM $MgCl_2$ and 250 μM (each) dATP, dGTP, dCTP, and TTP (Sigma Chemical Co.). The resulting amplicons were separated by agarose gel electrophoresis and were analyzed by using the BioImage Workstation and the Whole Band Analysis Software (Bio Image, Ann Arbor, Mich.).

S. aureus and *H. pylori* DNA template concentrations were varied from 5 μg to 0.5 pg per 50-μl reaction volume to assess the impact on the ERIC-primed DNA profile at a low annealing temperature. Results indicated that the range of DNA concentrations which produced a stable pattern for *S. aureus* was between 0.5 μg and 50 ng, and that for *H. pylori* was 0.5 μg and 5 ng. When the concentration was reduced to 5 ng or less for *S. aureus* and 0.5 ng or less for *H. pylori*, extra bands that directly corresponded to the background bands in the control tubes were visible. Most notable are the bands around 290 and 630 bp (Fig. 1A, lanes B to D, and Fig. 1B, lanes B to D). Below 0.5 ng for *S. aureus* and 50 pg for *H. pylori*, little template amplification was detected.

ERIC II primer concentrations ranging from 0.007 to 1.0 μM were tested by using 250 ng of *S. aureus* or *H. pylori* template DNA. The minimum primer concentration required for amplification was found to be 0.145 μM for *S. aureus* and 0.03 μM for *H. pylori*, and no amplification was detected below these levels. The optimal primer concentration was 0.18 to 0.7 μM for *S. aureus* and 0.25 to 1.0 μM for *H. pylori*. These levels generated DNA profiles suitable for analysis, and only slight

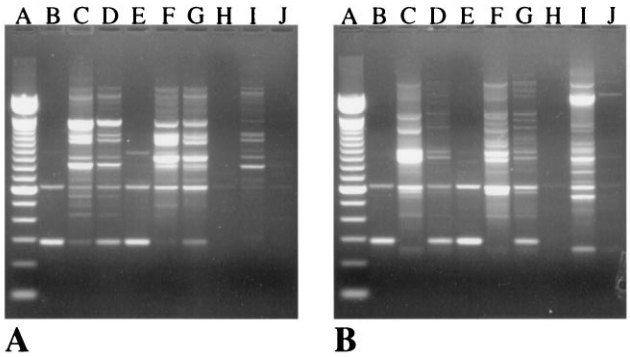

FIG. 1. Effect of temperature and DNA template concentration on amplification patterns using ERIC II primer (35) with *Taq* DNA polymerase (Gibco-BRL). Lane A, 100-bp ladder (Gibco-BRL); lanes B to D, 25°C annealing temperature; lanes E to G, 40°C annealing temperature; lanes H to J, 55°C annealing temperature; (A) *S. aureus* DNA as template DNA at 0 (B, E, H), 50 (C, F, I), and 5 (D, G, J) ng. (B) *H. pylori* DNA as template DNA at 0 ng (B, E, H), 50 ng (C, F, I), and 50 pg (D, G, J).

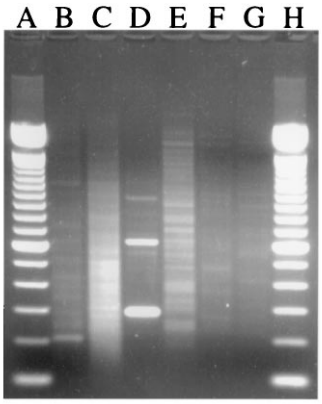

FIG. 2. Amplification in water blanks using ERIC II primer (35) and *Taq* DNA polymerase from various suppliers at an annealing temperature of 25°C. Lanes A and H, 100-bp ladder (Gibco-BRL). *Taq* DNA polymerases were from Promega (lane B), Appligene (lane C), Gibco-BRL (lane D), Boehringer Mannheim (lane E), Stratagene (lane F), and Perkin-Elmer (lane G).

variations were observed in the patterns. The banding profiles for both species showed significant changes when the primer concentration was increased from 0.5 to 1.0 μM, and for both species there was a decline in the intensity and number of bands as the primer concentration was reduced below 0.5 μM, with the effect being more pronounced in the *S. aureus* samples.

Six brands of *Taq* DNA polymerase were studied for their effects on the reproducibility of the ERIC-PCR. They were from Appligene (BP 72; F-67402 Illkirch-France), Boehringer Mannheim (Montréal, Québec, Canada), Gibco-BRL (Burlington, Ontario, Canada), AmpliTaq (Perkin-Elmer), Promega, and Stratagene (La Jolla, Calif.). It was observed that in control reaction tubes lacking experimental DNA template, there was still distinct DNA banding or smears. This phenomenon was observed with all enzymes tested, and the background patterns differed for each source of *Taq* polymerase (Fig. 2). Further study showed that even when different tubes of *Taq* polymerase with the same lot number were used as controls, different amplification patterns were produced (Fig. 3). These background patterns are highly related to the concentration of *Taq* DNA polymerase used. As the concentration decreases, the background pattern changes from a smear to distinct bands. In general, this background pattern did not seem to interfere with the experimental results, because the bands or smears could not normally be detected in the DNA profile when template DNA was added to the reaction mixture. However, it is unclear whether this is true in all cases, because some strains appeared to contain bands of variable intensity directly corresponding to those in the controls when certain brands of *Taq* polymerase were used. The explicit example of this occurs in the case of the Gibco-BRL *Taq* DNA polymerase, where two fragments at 290 and 630 bp in the DNA profiles of *S. aureus* and *H. pylori* were also noted in the corresponding control when lower levels of template DNA were used (Fig. 1). It was difficult to interpret the effect of the background of other brands of *Taq* polymerase due to the quantity of bands produced or the fact that the background came up as a smear. The Gibco-BRL brand had three very obvious and distinct bands.

When the low-stringency annealing ERIC-PCR was tested for the effect of changes in concentration of *Taq* polymerase, slight variations in the number of amplified bands and their intensities were observed for all bacterial strains, and these patterns differed depending on the source of the *Taq* polymerase used. *S. aureus* and *H. pylori* were most stable between 10 and 2.5 U/50-μl reaction volume. These variations could be caused from pipetting volume variations and/or the specific activity of the enzyme. When pipetting error was reduced by virtue of serial dilutions of *Taq* polymerase, some variations in intensity of the profiles were still observed, but these were not as pronounced as when different volumes of enzyme were removed directly from the tube.

When Boehringer Mannheim *Taq* DNA polymerase was compared with Gibco-BRL *Taq* DNA polymerase by using *S. aureus* DNA, the Boehringer Mannheim brand gave consistently more intense bands in the DNA profile. When the Gibco-BRL *Taq* polymerase was increased to 5 U/50-μl reaction volume, the same two bands which were only observed previously at 2.5 U and low template levels showed up as high as 250 ng of template DNA/50-μl reaction volume, revealing that the concentration of the *Taq* enzyme is a critical factor for reproducible banding and the effect of the background. Appligene *Taq* DNA polymerase did not work as well as the others in all cases, supporting the view that different *Taq* polymerases have significantly different enzyme activities.

In order to assess the effect of different annealing temperatures on the specificity of the ERIC II primer, all tests were repeated at 40 and 55°C (the theoretical melting temperature of the ERIC II primer). For most of the *Taq* DNA polymerase used, when the annealing temperature was increased from 25 to 40°C, the background banding was reduced, and at 55°C the background banding was almost entirely eliminated. In some cases (i.e., Gibco-BRL; see Fig. 1), however, the background banding was more apparent at 40°C but dropped off dramatically at 55°C. Although the range over which the patterns remained stable by using the aforementioned variables was not affected, the DNA profiles did vary significantly in number and intensity of bands, and at 55°C some strains of *S. aureus* did not amplify satisfactorily (Fig. 1). No variations were found to occur between three identical models of Perkin-Elmer 480 thermocyclers used in this study; however, variations between models have been documented previously (30, 48).

Scaling up the reaction volume to 100 μl affected the pattern that had been standardized at 50 μl (data not shown), and it is assumed that similar problems would occur if the reaction volume were scaled down to 25 μl.

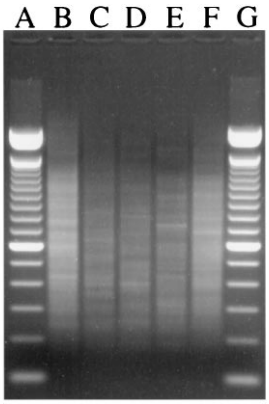

FIG. 3. Variation in amplification of water blanks using ERIC II primer (35) and different aliquots of Boehringer Mannheim *Taq* polymerase from the same lot number. Lanes A and G, 100-bp ladder.

DISCUSSION

MAAP techniques have been developed to allow for the detection of DNA sequence polymorphism with a single short primer of an arbitrary nucleotide sequence (usually a 5- to 20mer) in a DNA amplification system. These primers provide an opportunity to produce information with regard to genetic profiling in the absence of any knowledge of the genomic sequence. They have been used in and recommended for epidemiological investigations in microbiology, the generation of genetic markers for assessing genomic diversity, linkage mapping, development of species-specific probes, inheritance studies, and population genetics and, foremost, as an alternative typing method and intraspecies subtyping system. However, in determining the usefulness of a scheme for typing microorganisms, five criteria must be considered: typeability, reproducibility, discriminatory power, ease of performance and interpretation, and expense. With arbitrary DNA amplification, identification of a suitable primer that provides consistent and reproducible results is difficult and discriminatory power is uncertain (50). It is the nonspecific aspect of the technique that hinders reproducibility, and many further factors must be considered, including primer selection, interaction between primer and target DNAs, ion concentration, thermocycler machine, and source of *Taq* DNA polymerase (Table 1).

Unlike random priming, rep-PCR is not arbitrary. It has been designed to target known conserved and repetitive DNA sequences (78). Studies by ERIC-based PCR analysis (with primers with average lengths of about 22 oligonucleotides) indicate that this technique can effectively discriminate between closely related stains which may be indistinguishable by other subtyping methods (25, 36, 46, 78). Most researchers have used the ERIC primer at annealing temperatures and under PCR conditions close to those outlined in the original paper (78); however, a small group of researchers were found to use a low-specificity annealing temperature for various purposes (19, 25, 71–75). It is important to stress that under these conditions, the results obtained with ERIC primers are sensitive to the same factors as those described for arbitrary primers due to the fact that it is possible for primers to nonspecifically combine with the DNA template. In all these instances the annealing temperature was even lower than any used for applications involving arbitrary primers.

Caution is recommended in applying rep-PCR at low annealing temperatures to the study of gram-positive organisms. A preferable target in these organisms may be the recently discovered BOX elements (49), because REP and ERIC sequences have not been demonstrated in gram-positive organisms and have been shown to have little homology with them (78). The rep-PCR appears best suited for gram-negative species at annealing temperatures approaching the theoretical melting temperature of the primer. Since REP sequences only appear to be characteristic of certain enterobacterial chromosomes such as those of *E. coli* and *S. typhimurium* (32) and other closely related enteric bacteria (26, 62), they may not be as well suited as ERIC sequences for the development of general primers targeting bacterial species. ERIC sequences are found in most enterobacteria (35), and therefore, the primers targeting these regions are more flexible. However, REP-like sequences have recently been reported within the genomes of members of the family *Legionellaceae* (22), *Enterobacter aerogenes* (12, 23), *Pseudomonas putida* (33), and *Mycobacterium bovis* (15), and researchers have indeed attempted to extend the use of REP primers to gram-positive organisms in an epidemiological setting under normal REP conditions (77).

In the present study, data on DNA concentrations indicate that the template concentration can affect the ERIC-based amplification pattern at all annealing temperatures. At low annealing temperatures, however, reducing the DNA template concentration below a critical level can produce extra bands that were not previously detectable (Fig. 1). It appears that these bands are related to the background amplification, and if so, this becomes a problem at low template and low temperature levels because competition for priming sites is reduced and specificity is lowered. Therefore, it is essential to standardize template concentrations. This would discourage the use of whole-cell bacterial cultures, which have been claimed to have been used successfully in RAPD analysis (43, 51, 52, 56). We feel this practice should be avoided, since growth variability from strain to strain could influence the DNA profile. Also, whole-cell preparations may contain nucleases that can degenerate the DNA template, thus adding further variability to the PCR product. The PCR product may also degenerate over time unless EDTA is added or the product is stored at −20°C (24). Whole-cell rep-PCR, on the other hand, has been shown to work successfully at the higher annealing temperatures for which the primer was designed (45, 46, 82). We have found the use of a fluorometer combined with the Hoechst 33258 dye (Sigma Chemical Co.) to be superior to spectrophotometry in accurately determining DNA concentrations.

Primer concentration is a very sensitive factor for reproducibility. As the primer concentration is reduced, bands drop off in number and intensity. When the primer concentration is too high, mispriming may occur more frequently and produce greater nonspecific amplification; low primer concentrations may result in no amplification or low product yields (13, 16). These minimum, maximum, and ideal ranges appear to be closely correlated with the species being examined, and some bacterial species are more sensitive to changes. It is therefore recommended that appropriate optical density readings be taken before using any primers to guarantee that the optimal concentrations are accurately stated.

It appears that it is the ratio of the primer concentration to template DNA concentration that is most critical (29). The titer of the template DNA concentration should be carefully determined against a fixed primer concentration to obtain ideal conditions. This should be done for each different organism or when the DNA varies in quality or the DNA extraction procedures vary (13). The optimal and minimum primer concentrations for the organisms used here support the view that the two species have very different homologies with the ERIC primer. The banding patterns generated with staphylococcal DNA are likely based on nonspecific amplification, as opposed to those generated with *H. pylori*, which appears to contain the target sequence. Although it has been noted that the optimal DNA concentration varies for different species (60), this could simply be due to the arbitrary nature of the primer used. Some primers interact differently with various DNA templates, and a primer that works well for one species may be less well suited for use with another. This is most likely due to loss of potential binding sites, and therefore, a higher DNA concentration is required to achieve the same level of amplification, provided that all other variables remain constant. This emphasizes the importance of considering the primer-template interaction and suggests that some bacterial genera may be inherently more amenable to MAAP techniques.

Due to extraneous contamination of *Taq* DNA polymerase, all six brands of enzyme used in this study showed background amplification when tested in control blanks containing no experimentally introduced DNA template. Frequent contamination of *Taq* DNA polymerase was first recognized in 1990 with the advent of universal rRNA gene primers (4), and methods

for eliminating this have now been suggested (34, 54). Since this was the same time that arbitrary primers were becoming popular, the potential impact of *Taq* polymerase on the reproducibility of the method was not given much consideration. Many researchers still do not appear to be running the appropriate control blanks in order to account for any affect of background. Exogenous contamination of *Taq* DNA polymerase has been observed by those who choose to run the appropriate controls, but it has been reported that this background does not affect amplification of the DNA sample (57, 81). We find, however, that this is not so clear-cut and suggest that the background may be a function of the primer and brand of *Taq* polymerase being used, especially with arbitrary primers (i.e., some primers may not amplify the contaminating DNA in the enzyme). It is therefore very important to run control blanks that look for the primer-template interaction in the *Taq* polymerase and account for any background bands. The fact that different patterns result from using different tubes of *Taq* DNA polymerase, even tubes with the same lot number (Fig. 3), indicates that this contamination may arise from various sources. Our observations support the findings of Hughes et al. (34), who sequenced the contaminating DNA from the *Taq* polymerase and found that it appeared to be derived from more than one strain or species of eubacterial DNA. However, it could not be identified as coming from the host bacteria from which it was extracted, *Thermus aquaticus*, nor the cloning vector, *E. coli* (34).

Background patterns that vary from a smear to distinct banding as the *Taq* polymerase concentration is reduced can be explained by the fact that as the *Taq* polymerase concentration decreases, there is less enzyme activity as well as less contaminating DNA for the primer to amplify. Because this amplification is nonspecific, the primer must find regions of greater and greater homology in order to hybridize long enough for the polymerase to bind. Therefore, fewer and more pronounced bands are produced. In a recent outline of the RAPD protocol, del Tufo and Tingey (13) found that these hazy smears, which partially or completely obscure the amplified DNA, can be controlled by adjusting the ratio of primer to template DNA in order to ensure that the DNA template is saturated with primer (13). Williams et al. (81) reported resolving the smears by reducing *Taq* polymerase or DNA concentrations. Although we did not attempt to verify this with an experimental DNA template, it would help explain our findings with regard to the *Taq* DNA polymerase. The pattern itself is likely a product of the source and condition of the DNA contamination. Bell and DeMarini (2) suggest that these observed smears are random-length high-molecular-weight fragments created from extension and random termination of annealing events involving the annealing of the 3′ OH ends of the amplified product to genomic template or each other after most of the oligonucleotide primer has been converted to PCR product. This implies that the generation of nonspecific amplification products can be prevented by altering the primer/template ratio or the number of PCR cycles, or both (14).

The extra bands noted in the DNA profile (Fig. 1) suggest that the background pattern may affect the resulting ERIC-based pattern at low annealing temperatures when low template levels are present or high *Taq* polymerase concentrations are used. Since the use of different concentrations or different sources of *Taq* DNA polymerase resulted in variations in the numbers and intensities of bands, proper enzyme selection and standardization are essential for obtaining consistency with the ERIC-PCR at low annealing temperatures or with MAAP techniques in general. Since the compositions of the storage buffers for the various brands of *Taq* polymerase are virtually identical (see manufacturers' literature accompanying each *Taq* DNA polymerase), it can only be higher enzyme activity that accounts for variations. This view is in agreement with that of Schierwater and Ender (67), who made similar statements. Caetano-Anollés et al. (10) claimed to have optimal reproducibility when they used a truncated derivative of *Taq* polymerase called the Stoffel fragment. This is a highly thermostable, recombinant DNA polymerase that lacks the 289 N-terminal amino acids and has no associated exonuclease activity (10). Although we did not test this particular brand, we found no significant increases in stability between the directly purified preparation and the cloned preparation called Ampli*Taq* from Perkin-Elmer. In a study by Meunier and Grimont (57), the variations in the DNA profile with regard to the supplier of *Taq* DNA polymerase also did not appear to be influenced by whether or not the *Taq* polymerase was cloned or directly purified. They also recognized that the composition of the enzyme reaction buffer was of no significance (57). Since it can be shown that variations in enzyme activity from one supplier's *Taq* polymerase to the next or between tubes may affect the DNA profile, it may be worth initially pooling a batch of *Taq* DNA polymerase to achieve optimal reproducibility.

Since the different annealing temperatures used resulted in different DNA profiles, this indicates that ERIC-PCR is similar to arbitrary methods when a low annealing temperature is used. Increasing the annealing temperature closer to the theoretical melting temperature increases specificity. With the higher temperatures, more stable and consistent patterns were obtained for *H. pylori*, indicating that the primer has more homology with *H. pylori* than with *S. aureus*. The use of ERIC-PCR at higher annealing temperatures avoids much of the variability that comes with the aforementioned factors.

It has been cited that fluctuations in the number of cycles or cycle time and the use of different brands of thermocycler will often result in different amplification products, and variations between models is well documented (13, 30, 48, 57). In the present case it is possible that an increase in the number of cycles to 45 may have resulted in an increased number of background bands because the intensities of all bands would have increased. It has been further shown that the temperature across the block of some machines may vary as much as 5°C, with the majority of variations occurring in outside wells (30, 44). It is therefore recommended that only the inside core of older models be used and that the use of outside rows be avoided. Given the sensitive nature of low-stringency annealing methods with regard to temperature, this may be a worthwhile precaution.

The RAPD analysis in particular does not appear to be suited for defining the evolution of genetic relationships between organisms, tracking epidemiological relatedness between species, or surveying genetic variation in natural populations due to its inability to discriminate between artifactual variation and true polymorphism. It appears to have limited reliability under strictly defined conditions, and when it is used to solve limited problems, such as typing a collection of unknown isolates which do not necessitate interlaboratory comparisons (57) or use in pedigree analysis where data are obtained on the same day under the same set of conditions (29). However, it does appear to have some merit when it comes to generating genetic markers for linkage mapping (29) and creating species-specific probes (20, 21, 55) where no sequence data are available for the genome in question.

It is important to realize that varying the conditions of the PCR will affect the profile for DNA from all species of mammals, plants, and bacteria; however, it appears that the majority of the reproducibility problems lie with the prokaryotic

DNA. Reports claim that RAPD analysis as applied to eukaryotic DNA is more consistently reproducible possibly due to the larger genome size, which offers more potential binding sites and perhaps greater stability (8, 60, 81). The majority of reproducibility studies have concentrated on plant DNA. Hence, RAPD analysis may be more stable and better suited for larger genomes if a standard set of primers and a standard protocol could be established (64, 81). However, even with wheat DNA, the sensitivity of experimental variables was acknowledged, and it was revealed that consistently reproducible results were only possible with rigorously optimized reaction conditions (14).

For such a seemingly simple assay these arbitrary methods are very complex, and banding patterns can be affected by many factors. The extent to which the experimental parameters are sensitive when using these methods makes them troublesome. Even small changes in these factors can lead to exponential problems due to their interrelatedness. The experimental parameters that affect pattern complexity and reproducibility of the MAAP analysis, and RAPD analysis in particular, are well documented in the literature. Primer-template interaction, secondary structure of the template DNA, efficiency of the heating blocks of various PCR machines, and the supplier of *Taq* DNA polymerase have all been cited as important factors for the efficient amplification and reproducibility of RAPD analysis (Table 1). Thus, a wide range of parameters have been applied to these methods with a variety of organisms, both eukaryotic and prokaryotic, with the intention of establishing ideal conditions. The problem is that researchers claim reproducible results only after exhaustive screening and optimization, but in the hands of other researchers or different DNA extracts those same conditions no longer generate the same pattern. The choice of primers for use in RAPD analysis is one of the most critical factors, and several primers must first be screened. It appears that some arbitrary primers may work better than others and may provide results that are more reproducible (64). The fact that several arbitrary primers give little or no amplification may be due to the presence of extremely rare oligonucleotide sequences, as demonstrated with the DNA of mammals and other organisms (6, 39). It has also been indicated that the use of a combination of oligonucleotide primers in a single RAPD reaction can give more detailed and reproducible patterns (37, 40, 53, 58, 80). For internal use the method would have to be optimized for each arbitrary primer and DNA template used in order to increase the efficiency of the RAPD analysis (38). Even then these optimal conditions, however, may not be reproducible by other laboratories or, as we found, the same laboratory at a future time.

Berg et al. (3) have recently published a detailed protocol for RAPD analysis and AP-PCR which highlights many of the concerns with respect to reproducibility. However, they were still not able to successfully reproduce profiles run several months apart or even on a day-to-day basis (3). Confusion prevails when they go on to recommend the use of RAPD analysis to trace genome evolution or track the epidemiology of outbreaks. Other recent protocols suggest that inadequate DNA preparation is the major cause of irreproducibility (53, 59), and along with primer screening for moderately complex patterns and the use of primers in pairwise combination, the reproducibility problem can be solved (53). RNA contamination is also believed to contribute to variability because the arbitrary primer can just as easily combine with the single-stranded RNA molecule, and therefore, the regular treatment of DNA preparations with RNase has been recommended (18, 83). Berg et al. (3) also suggest that newly developed, strongly binding oligonucleotides may reduce variability.

It can be seen from Table 1 that high degrees of standardization and internal control are required to reproducibly carry out MAAP procedures and that the chances of controlling all variables across laboratories are virtually impossible. It is our opinion that interlaboratory data comparison should be avoided, and any attempt to develop formal subtyping schemes based on arbitrary or ERIC-based amplification patterns at low annealing temperatures should not be attempted in view of the inherent variability. This opinion has also been shared and supported by Meunier and Grimont (57) with regard to methods using arbitrary primers in general. Any attempt to standardize conditions should be for the sole purpose of satisfying some immediate internal goal or curiosity, and standard conditions should not be applied to large-scale projects where past results are compared or collated with present and future ones. At this time, we only use RAPD analysis to assess if clinical isolates have any likelihood of being related, as a prelude to the more reliable but laborious methods such as ribotyping, multilocus enzyme electrophoresis, or pulsed-field gel electrophoresis, and only then if the isolates are compared simultaneously in the same amplification protocol to avoid day-to-day variance.

The most obvious and valuable applications of MAAP and rep-PCR lie in the very rapid and relatively discriminatory genomic characterization that is possible in nosocomial settings and outbreak investigations. Application of these techniques will facilitate infection control programs and thereby decrease and limit the spread of pathogens under these circumstances. Of particular concern is the emergence of strains with antibiotic resistance in both community- and hospital-acquired infections. The repetitive elements method appears to have sufficient reproducibility and discriminatory potential to track the spread of these pathogens and to aid in epidemiological studies. The major provision is that factors known to contribute to variability in the observed polymorphism be rigidly controlled and the results subject to careful and cautious interpretation.

The following is a summary of our recommendations for using arbitrary PCR.

(i) Quantify DNA for each organism studied and each extraction method used by ensuring that reliable concentration readings are taken. Fluorometry appears to be superior to spectrophotometry.

(ii) Use of whole-cell extracts should be avoided.

(iii) Oligonucleotide primers should be rigorously screened for priming ability and reproducibility before they are used as genetic markers. Moderately complex patterns appear to be less variable than simpler ones (53).

(iv) Quantify primers for every synthesis reaction. The use of "strongly binding" oligonucleotides may help reduce variability (3).

(v) Titrate DNA against primer concentrations to arrive at an ideal primer/template ratio.

(vi) Standardize the use of *Taq* DNA polymerase by maintaining a constant supplier and pooling the contents of many vials for use as a working stock. The Stoffel fragment is cited as being more stable than other *Taq* DNA polymerases (10).

(vii) Titrate the *Taq* DNA polymerase against the primer/template ratio.

(viii) Standardize the $MgCl_2$ concentration.

(ix) Maintain the use of one thermocycler with a standard set of thermocycling conditions, paying close attention not to vary the annealing temperature or to use outside rows of heating block in first-generation machines.

(x) Always run the appropriate control blanks to account for background. The use of preamplification isopsoralen cross-

linking (54) or "low-DNA" *Taq* polymerase as advertised by the manufacturer may help eliminate or reduce this effect.

We leave the reader with an appropriate quotation: "O! many a shaft at random sent/Finds mark the archer little meant!" (from *The Lord of the Isles* [1815], canto V, st. 18 by Sir Walter Scott [1771 to 1832]).

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