

## SHEA Position Paper

# How to Select and Interpret Molecular Strain Typing Methods for Epidemiological Studies of Bacterial Infections: A Review for Healthcare Epidemiologists

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### ABSTRACT

Strain typing is an integral part of epidemiological investigations of nosocomial infections. Methods for distinguishing among bacterial strains have improved dramatically over the last 5 years, due mainly to the introduction of molecular technology. Although not all molecular techniques are equally effective for typing all organisms, pulsed-field gel electrophoresis is the technique currently favored for most nosocomial pathogens. Criteria to aid epi-

demologists in interpreting results have been published. Nucleic acid amplification-based typing methods also are applicable to many organisms and can be completed within a single day, but interpretive criteria still are under debate. Strain typing cannot be used to replace a sound epidemiological investigation, but serves as a useful adjunct to such investigations (*Infect Control Hosp Epidemiol* 1997;18:426-439).

Outbreaks of nosocomial infections continue to occur among patients in a variety of healthcare settings.<sup>1,2</sup> Although fungi, viruses, and parasites can cause nosocomial infections, bacterial agents remain the most commonly recognized cause of outbreaks of disease.<sup>1</sup> In hospitals, the use of indwelling catheters, ventilators, and a variety of other medical devices often serve as sources and conduits for bacteria and make efforts to control outbreaks more difficult.<sup>3</sup> In addition, the decreased effectiveness of some antimicrobial agents due to the emergence of resistant bacteria<sup>4</sup> complicates infection control efforts.<sup>5</sup>

Investigations of presumed outbreaks of bacterial infections in hospitals often require strain typing

data to identify outbreak-related strains and to distinguish epidemic from endemic or sporadic isolates.<sup>6</sup> Strain typing data, particularly those that are generated by using newer molecular methods, have been helpful for investigating outbreaks caused by a wide range of bacterial pathogens, including methicillin-resistant strains of *Staphylococcus aureus* (MRSA),<sup>7</sup> vancomycin-resistant enterococci (VRE),<sup>8</sup> *Pseudomonas aeruginosa*,<sup>9</sup> and *Klebsiella pneumoniae*.<sup>10</sup> The molecular approaches, which include pulsed-field gel electrophoresis (PFGE), arbitrarily primed polymerase chain reaction (AP-PCR) assays, and plasmid fingerprinting (PF), have several advantages over traditional typing methods, including higher discriminatory power, broader application to

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a variety of bacterial species, and, at times, speed.<sup>6</sup> While many of these newer methods now are utilized in clinical and reference laboratories around the world, there often is disagreement on how to interpret the results. In most cases, the focus of discussion relates to the degree of variability in the test results that can occur before two isolates are determined to belong to different strains.<sup>11</sup>

The goal of this position paper is to provide healthcare epidemiologists with an overview of the methods available for analyzing bacterial isolates suspected of causing nosocomial outbreaks and to propose criteria for interpreting the results of those studies. The emphasis will be on those molecular methods whose utility has been validated by studies performed in multiple laboratories on large sets of isolates. More traditional techniques, such as staphylococcal phage typing, biotyping, and pyocin typing,<sup>12</sup> are not considered here. It is critical that epidemiologists understand and appreciate that the results of strain typing studies do not substitute for a sound epidemiological investigation. Rather, the laboratory studies and the epidemiological studies of suspected outbreaks should be analyzed in parallel and should be viewed as complementary components of the overall investigation.

## BACKGROUND CONSIDERATIONS

The ability of molecular typing systems to distinguish among epidemiologically unrelated isolates is a reflection of the genetic variation seen in the chromosomal DNA of a bacterial species.<sup>13</sup> Usually, this variability is high, and differentiation of unrelated strains can be accomplished using any of a variety of techniques.<sup>14</sup> However, the factors that enable bacteria to cause infection often are not uniformly distributed within a species. Thus, the organisms most commonly associated with infections often are a smaller subset of the many strains that constitute a species.<sup>15</sup> As a consequence, this subset may exhibit relatively little genetic diversity, and it can be difficult to differentiate among strains even with newer molecular techniques. Methicillin-resistant *Staphylococcus aureus* are an example of this phenomenon. Because most MRSA recovered from patients are derived from a relatively small number of clones,<sup>16</sup> there are a limited number of strain types that can be differentiated regardless of the typing method used. While phenotypic methods often cluster isolates of MRSA into a few broad groups, most molecular methods are capable of differentiating endemic from epidemic strains.<sup>17</sup>

## CHARACTERISTICS OF TYPING METHODS

Typing methods fall into two broad categories: *phenotypic* methods and *genotypic* methods. Phenotypic

methods are those that characterize the products of gene expression in order to differentiate strains. Properties such as biochemical profiles, bacteriophage types, antigens present on the cell's surface, and antimicrobial susceptibility profiles all are examples of phenotypic properties that can be determined in the laboratory. Because they involve gene expression, these properties all have a tendency to vary, based on changes in growth conditions, growth phase, and spontaneous mutation.

Genotypic methods are those that are based on an analysis of the genetic structure of an organism and include polymorphisms in DNA restriction patterns based on cleavage of the chromosome by enzymes that cleave the DNA into hundreds of fragments (frequent cutters), or into 10 to 30 fragments (infrequent cutters), and the presence or absence of extrachromosomal DNA. Genotypic methods are less subject to natural variation, although they can be affected by insertions or deletions of DNA into the chromosome, the gain or loss of extrachromosomal DNA, or random mutations that may create or eliminate restriction endonuclease sites.

All typing systems can be characterized in terms of typeability, reproducibility, discriminatory power, ease of performance, and ease of interpretation.<sup>6</sup> The characteristics of a number of typing methods are presented in Table 1. Typeability refers to the ability of a technique to assign an unambiguous result (type) to each isolate. Although nontypeable isolates are more common with phenotypic methods, they have been recognized with most methods. For example, with PFGE, a technique that is almost uniformly applicable to bacteria,<sup>11</sup> some strains of *Clostridium difficile* remain nontypeable because the chromosomal DNA is degraded, presumably by endogenous nucleases, before it can be cleaved properly by the restriction endonucleases used in the PFGE protocol.<sup>18</sup>

A reproducible method is one that yields the same results upon repeat testing of a bacterial strain. In the context of an epidemiological study, this means that the same strain recovered from epidemiologically linked patients will give the identical (or nearly identical) typing result. Poor reproducibility may reflect technical variation in the method or biologic variation occurring during in vivo or in vitro passage of the organisms to be examined. Over time (a few weeks to years, depending on the species), the typing patterns produced by DNA-based methods, such as PFGE and AP-PCR, will show some minor, natural variation.<sup>19,20</sup> Thus, when analyzing results, it is important to consider the length of time over which the bacterial isolates were collected.

The discriminatory power of a technique refers

TABLE 1  
CHARACTERISTICS OF TYPING SYSTEMS\*

Typing System	Proportion of		Discriminatory Power	Ease of Interpretation	Ease of Performance
	Strains Typeable	Reproducibility			
Biotyping	All	Poor	Poor	Moderate	Easy
Antimicrobial susceptibility patterns	All	Good	Poor	Easy	Easy
Serotyping	Most	Good	Fair	Moderate	Moderate
Plasmid fingerprinting <sup>†</sup>	Most	Good	Good	Moderate	Moderate
REA of chromosomal DNA with conventional electrophoresis	All	Good	Good	Difficult	Moderate
RFLP analysis with DNA probes <sup>‡</sup>	All	Excellent	Moderate to Excellent	Moderate	Difficult
PFGE	All	Excellent	Excellent	Moderate	Moderate
AP-PCR <sup>§</sup>	All	Good	Good	Moderate	Moderate

Abbreviations: REA, restriction endonuclease analysis; RFLP, restriction fragment-length polymorphism; PFGE, pulsed-field gel electrophoresis; AP-PCR, arbitrarily primed polymerase chain reaction.

\* Adapted from reference 6.

<sup>†</sup> For isolates of *Staphylococcus aureus* and coagulase-negative staphylococci, plasmid analysis with restriction digestion often is required. For the gram-negative organisms, such as *Klebsiella*, *Enterobacter*, or *Serratia*, whole-plasmid analysis without restriction digestion often provides sufficient discriminatory power.

<sup>‡</sup> As applied to IS6110 analysis of *Mycobacterium tuberculosis* and ribotyping.

<sup>§</sup> As applied to *S aureus*, *Clostridium difficile*, and selected *Enterobacteriaceae*. Note that interpretive criteria for AP-PCR have yet to be standardized.

to its ability to differentiate among epidemiologically unrelated isolates, ideally assigning each to a different type. Traditional phenotypic methods, such as antibiogram typing, serotyping, and biotyping, frequently show lower discriminatory power than newer molecular methods.<sup>6,13</sup>

Ease of performance reflects the cost of specialized reagents and equipment, the technical complexity of a method, and the effort required to learn and to implement the technique in the laboratory. Most molecular methods require purchase of new equipment, some of which is costly (\$4,000-\$20,000). However, these methods are learned easily and are widely applicable to a variety of species. Many traditional methods also involve considerable costs in labor and materials, but are restricted to a single or relatively few species. For example, bacteriophage typing, which is used primarily for *S aureus* and a few other bacterial species, requires the maintenance of bacteriophage stocks that constantly must be replenished and titered, a process that is both time-consuming and labor-intensive.

Finally, ease of interpretation refers to the effort and experience required to obtain useful, reliable typing information using a particular method. At present, the interpretation of the results of molecular methods remains an area of active discussion. However, this is in contrast to methods such as bacteriophage typing and pyocin typing, which require significant expertise to perform and interpret and

often still yield ambiguous results.

## INDICATOR TECHNIQUES (PHENOTYPIC METHODS)

### Biotyping

In the 1960s and early 1970s, identification of bacterial species frequently was undertaken using racks of tubes representing a variety of biochemical tests, and the variability of certain tests, such as indole, H<sub>2</sub>S, or pigment production, served as markers for particular strains. Thus, biotyping emerged as a useful tool for epidemiological investigations. Today, identification of bacterial species normally is accomplished by using a combination of biochemical and immunologic tests, many of which now are performed using commercial kits or automated devices. However, biotyping using automated methods relies on a variety of novel substrates, and some of these tests, such as carbohydrate fermentations, are highly variable even within isolates of the same strain. Thus, biotyping, like most phenotypic methods, has only modest reproducibility, because microorganisms can alter unpredictably the expression of many cellular products. Moreover, contemporary biotyping typically has poor discriminatory power and cannot differentiate among some of the current nosocomial problem pathogens, such as enterococci, where biochemical diversity is uncommon. Occasionally, outbreaks are observed that are caused by bacterial strains that represent unusual species or unusual biotypes of com-

mon species, for example, H<sub>2</sub>S-producing isolates of *Escherichia coli*. In such situations, additional typing techniques may not be needed. However, even clusters of unusual isolates may not always indicate a common-source outbreak, as indicated by a recent report in which four isolates of *Leptotrichia buccalis*, an unusual anaerobic gram-negative bacillus, recovered from blood cultures of four different bone marrow transplant patients, were found to be unrelated by PFGE and fatty-acid profile analysis.<sup>21</sup> The investigation of the suspected outbreak revealed that each of the patients had undergone dental manipulations prior to developing bacteremia. In addition, all of the patients had been placed on prophylactic antimicrobial agents to which the *L. buccalis* isolates were resistant. Thus, each neutropenic patient developed bacteremia with his own endogenous strain of *L. buccalis*, which served as an opportunistic pathogen. Nonetheless, it should be noted that outbreaks can, in some cases, be caused by multiple pathogens.

#### **Antimicrobial Susceptibility Patterns**

Antimicrobial susceptibility patterns also have relatively poor discriminatory power, because antimicrobial resistance is under tremendous selective pressure in healthcare institutions<sup>22</sup> and often is associated with mobile genetic elements (eg, transposons and plasmids).<sup>23</sup> Changes in antibiograms also may reflect spontaneous point mutations, such as seen with fluoroquinolones.<sup>24</sup> Thus, isolates that are epidemiologically related and otherwise genetically indistinguishable may manifest different antimicrobial susceptibilities due to acquisition of new genetic material over time<sup>25</sup> or the loss of plasmids.<sup>26</sup> Conversely, unrelated isolates may have indistinguishable resistance profiles, which may represent acquisition of the same plasmid by multiple species (a "plasmid outbreak").

#### **Serotyping**

Serotyping, a nonmolecular method, uses a series of antibodies to detect different antigenic determinants on the surface of the bacterial cell. Serotyping is one of the classic strain typing techniques that has been used over the years for epidemiological studies of many species of bacteria. It remains a key method for typing isolates of *Salmonella*, *Shigella*, and pneumococci. However, maintaining stocks of typing sera (including the >2,200 antisera required for definitive *Salmonella* typing) is a major limitation of this method. Because of the association of certain *Salmonella* serotypes with foodborne disease, and the association between specific pneumococcal serotypes and invasive disease,

particularly in children, serotyping continues to be a valuable typing technique. Nonetheless, PFGE has been shown to resolve distinct clonal strains within individual serotypes of both *Salmonella* and pneumococci, thus indicating that it is a more discriminatory typing tool.<sup>27,28</sup>

#### **GENOTYPIC METHODS**

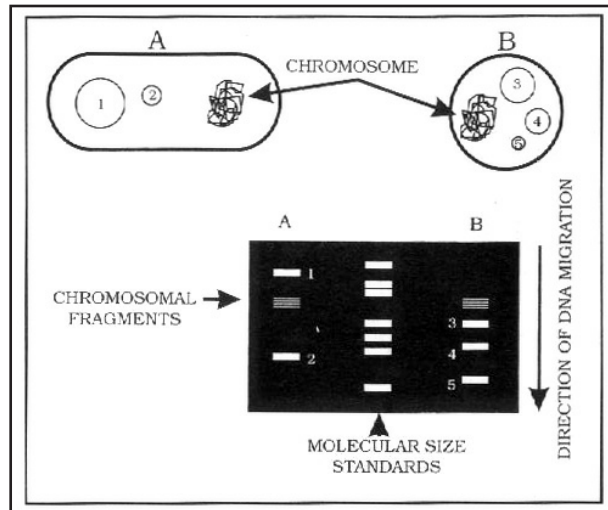
Over the last several years, six molecular techniques have emerged as the methods of choice for typing bacterial isolates. They are PF; restriction endonuclease analysis (REA) of plasmid DNA; REA of chromosomal DNA using frequent cutting enzymes and conventional electrophoresis; restriction fragment-length polymorphism (RFLP typing) analysis using DNA probes; PFGE; and AP-PCR and other related nucleic acid amplification-based typing methods. In addition, PCR-DNA sequencing methods are just beginning to be utilized.

#### **Plasmid Fingerprinting**

Plasmid fingerprinting was the first molecular method to be used as a bacterial typing tool.<sup>29,30</sup> Plasmids are extrachromosomal DNA elements that are present in most clinical isolates and can be identified readily by simple cell lysis procedures followed by agarose gel electrophoresis of the lysates (Figure 1).<sup>31</sup> The number and size of the plasmids present is used as the basis for strain identification. This strain typing technique has been used successfully for analysis of outbreaks of nosocomial infections<sup>29</sup> and community-acquired infections<sup>32</sup> caused by a variety of species of gram-negative rods.

#### **REA of Plasmid DNA**

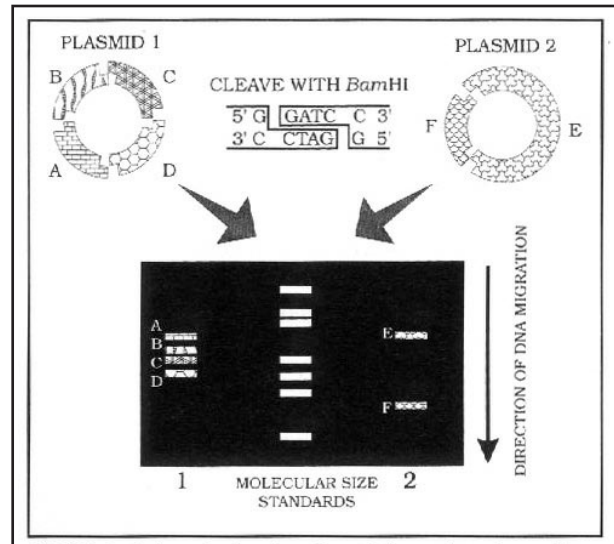
Some strains of bacteria contain only a single large plasmid, often in the size range of 100 to 150 kilobases (kb). Because it is difficult to differentiate plasmids in this size range, especially those that vary by only 10 kb to 15 kb, some investigators have added a restriction endonuclease digestion step to try to increase the discriminatory power of agarose gel electrophoresis (Figure 2).<sup>33</sup> While this can be helpful, large plasmids produce many restriction fragments, which can make interpretation more difficult, especially when multiple large plasmids are present. Thus, for gram-negative rods, the REA step no longer is performed in most laboratories. However, for analysis of staphylococci, where the plasmids typically are <50 kb, REA appears to increase the discriminatory power of the analysis, because the number of restriction fragments generated usually is <20.<sup>33</sup> Digestion also makes the patterns of the restriction fragments produced from



**FIGURE 1.** Schematic drawing of the plasmid fingerprinting technique using agarose gel electrophoresis. The oval on the left signifies a typical gram-negative rod, and the circle on the right signifies a typical gram-positive coccus. Cells are lysed using detergents at high pH, the chromosomal DNA is removed, and the plasmid DNA is applied to an agarose gel that then is stained and photographed. Some chromosomal fragments usually are visible on the gel and serve as an internal molecular size standard (approximately 12-15 kb). Plasmid DNA within the bacteria is shown in circular form. Each plasmid is numbered within the cell, and its corresponding position in the agarose gel is indicated by the same number.

staphylococcal plasmids easier to analyze than the undigested profiles, which often show multiple forms for plasmids of less than 15 kb, because circular and linear forms of the plasmid migrate at different rates than the covalently closed circular form. Plasmid fingerprinting is technically simple to perform and requires relatively inexpensive equipment (\$1,500-\$3,000). At this time, the method is used primarily as an alternative technique for staphylococcal isolates, which frequently carry multiple plasmids, and for selected species of *Enterobacteriaceae*, which often have large distinctive plasmids.

When applying the plasmid fingerprinting technique, investigators must be aware of two confounding factors. First, it is possible that plasmids can spread to multiple species of bacteria, causing a plasmid outbreak in which unusual antibiograms are recognized in multiple species. This has been recognized both in gram-negative rods and in staphylococci.<sup>25,34</sup> Second, it is important to appreciate that the structure of individual plasmids and the plasmid content of a particular strain may vary over time. This variability reflects two factors: over time, plasmids can be lost spontaneously or acquired from other organisms, and plasmids frequently carry smaller mobile genetic elements (transposons and insertion sequences) that promote duplications and

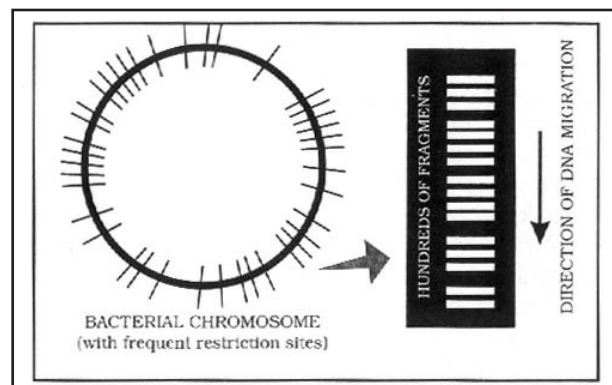


**FIGURE 2.** Schematic drawing of restriction endonuclease digestion of two unique plasmids, followed by agarose gel electrophoresis. Different-sized restriction fragments are denoted by the different patterns.

deletions of DNA segments. Both plasmids and transposons often include antimicrobial resistance determinants and thus are subject to considerable selective pressure within hospitals due to antimicrobial agent use.<sup>22</sup> In general, plasmid fingerprinting is most useful for epidemiological studies that are limited both temporally and geographically. In selected instances, plasmid fingerprinting may complement other techniques, such as PFGE analysis, by providing a basis for differentiating isolates that are related genotypically but are separated epidemiologically by moderate time periods, such as several months.<sup>35</sup>

### **Gel Electrophoresis Techniques for Analysis of Chromosomal DNA**

There are two methods of typing organisms based on fragment patterns produced by cleaving chromosomal DNA with restriction endonucleases. The first method, often referred to as conventional electrophoresis, uses a restriction enzyme that cuts the chromosome into hundreds of pieces (frequent cutter), followed by standard agarose gel electrophoresis. Fragments that are 25 kb to 0.5 kb are resolved into a discernible banding pattern, although a single band may contain fragments of similar size from several different areas of the chromosome. Larger fragments coalesce at the top of the gel or do not migrate into the gel. The second method, PFGE, uses enzymes that cut chromosomal DNA infrequently, generating from 10 to 30 bands, followed by a novel form of electrophoresis that can



**FIGURE 3.** Schematic drawing of restriction endonuclease analysis of chromosomal DNA using conventional electrophoresis. The box on the right represents the banding pattern of hundreds of fragments after conventional agarose gel electrophoresis. Each band may contain a number of unique chromosomal fragments of similar size.

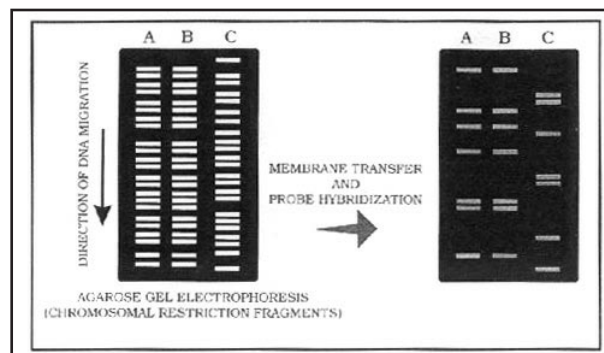
separate fragments from 1 kb up to 1,000 kb (1 megabase).<sup>36</sup> Each method, and a variation of the conventional electrophoresis method, is described in greater detail below.

#### **REA of Chromosomal DNA With Frequent Cutting Enzymes and Conventional Electrophoresis**

Each restriction endonuclease cleaves DNA at a particular sequence of nucleotides that may be repeated numerous times around the chromosome. The number and size of the restriction fragments generated by digesting a given piece of DNA reflects the frequency and distribution of the restriction sites. In conventional REA, endonucleases with frequently occurring sites in the bacterial genome are used to digest total DNA (plasmid and chromosome), thereby generating hundreds of fragments ranging from approximately 0.5 to 50 kb in length (Figure 3). Such fragments can be separated by size using agarose gel electrophoresis, and the pattern can be detected by staining the gel with ethidium bromide (or other dyes) and photographing under ultraviolet light. Different strains of the same bacterial species have different REA profiles (depicted as a series of bands on agarose gels) because of variations in their DNA sequences. All isolates are typeable by REA; however, it can be very difficult to interpret the complex profiles, which consist of hundreds of bands that may be indistinct or overlapping. Although the approach has been applied to many species, at this time, its primary use is as an alternative technique for analyzing *C difficile*.<sup>37</sup>

#### **RFLP Analysis Using DNA Probes**

In this technique, chromosomal restriction digests produced by frequent cutting enzymes are separated by conventional agarose gel electrophoresis, as

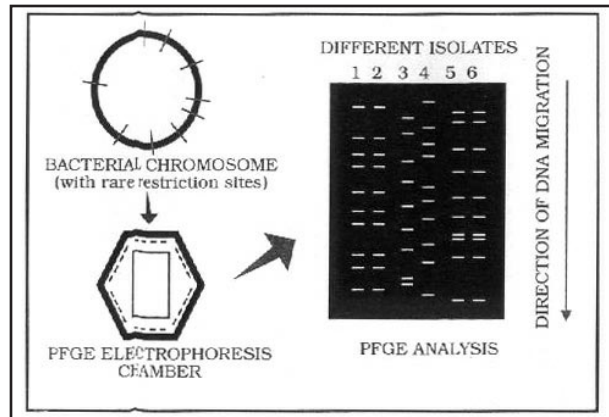


**FIGURE 4.** Schematic drawing of restriction fragment-length polymorphism analysis using a DNA or RNA probe, such as IS6110 or ribosomal RNA. The box on the left represents agarose gel electrophoresis of chromosomal DNA cleaved with a restriction endonuclease, and the box on the right represents the nylon filter to which the DNA has been attached and hybridized with a specific probe. Only the DNA fragments on the nylon filter that bind the probe can be visualized. The organisms represented in lanes A and B are indistinguishable, whereas the isolate represented in lane C is a different strain.

described above, and then the DNA fragments are transferred onto a nitrocellulose or nylon membrane (Figure 4).<sup>38</sup> The DNA on the membrane then is hybridized with a specific chemically or radioactively labeled piece of DNA or RNA (a probe), which binds to the relatively few fragments on the membrane that have complementary nucleic acid sequences. Variations in the number and size of the fragments detected by hybridization are referred to as RFLPs.

One common typing method that uses chromosomal DNA preparations and a ribosomal RNA probe is ribotyping.<sup>39</sup> Because all bacterial isolates have one or more chromosomal rRNA operons distributed around the chromosome, and because those sequences are highly conserved, essentially all bacterial isolates can be typed using probes directed to the DNA sequences that encode the rRNA loci using a single rRNA probe.<sup>39</sup> However, enthusiasm for this system has diminished, because the approach has proven to be only moderately discriminatory.<sup>40-42</sup>

Restriction fragment-length polymorphism analysis using the DNA insertion element IS6110 currently is the method of choice for typing isolates of *Mycobacterium tuberculosis*.<sup>43</sup> An insertion element is a piece of DNA with a defined structure that is able to move independently and to insert in multiple locations in plasmids or chromosomal locations, but does not contain antimicrobial resistance genes or genes involved in pathogenesis. IS6110 is present in essentially all isolates of *M tuberculosis*, and, because insertion sequences are mobile, the number and chromosomal locations of the insertions vary



**FIGURE 5.** Schematic drawing of pulsed-field gel electrophoresis, in which chromosomal DNA is cleaved with a rare cutting enzyme followed by electrophoresis, using a unique chamber and current switching protocol. The box on the right is the agarose gel showing the very large DNA fragments derived from the unique electrophoresis chamber.

greatly from strain to strain. The approach has proven reliable and discriminatory and, in most cases, can distinguish successfully sporadic and clustered cases of tuberculosis.<sup>44</sup> However, for isolates with fewer than five copies of *IS6110*, the method has relatively poor discriminatory power and must be supplemented by studies using other probes.

### PFGE

Pulsed-field gel electrophoresis was first described in 1984 as a tool for examining the chromosomal DNA of eukaryotic organisms (Figure 5).<sup>36</sup> Subsequently, PFGE has proven to be a highly effective molecular typing technique for many different bacterial species.<sup>6,13,14,19</sup> In this method, the bacterial genome, which typically is 2,000 to 5,000 kb pairs in size, is digested with a restriction enzyme that has relatively few recognition sites and thus generates approximately 10 to 30 restriction fragments ranging from 10 to 800 kb. Essentially all of these fragments can be resolved as a pattern of distinct bands by PFGE, using a specially designed chamber that positions the agarose gel between three sets of electrodes that form a hexagon around the gel. Instead of applying an electric current to the gel in a single direction, as is done in conventional electrophoresis, in PFGE, the current is applied first in one direction from one set of electrodes, then shifts to the second set of electrodes for a short period of time (a pulse), and then shifts to the third set of electrodes. Thus, the electric field that causes the DNA to migrate in the gel is provided in pulses that alternate from three sets of electrodes. This causes the DNA to wiggle through the gel, and the back-and-forth movement results in the higher level of fragment resolution seen

with the technique. All species are typeable by PFGE, although the isolation of intact chromosomal DNA is technically difficult for some species. As noted above, the chromosomal DNA of some strains of *C difficile* spontaneously degrades during the cell lysis procedure, making this typing approach impractical.<sup>18</sup> PFGE has been applied successfully to a wide range of bacterial species, both gram-positive (eg, staphylococci, enterococci, and mycobacteria) and gram-negative (eg, *E coli*, other *Enterobacteriaceae*, and pseudomonads).<sup>11</sup> In general, PFGE is one of the most reproducible and highly discriminatory typing techniques available and is currently the typing method of choice for many species. The major difficulties associated with PFGE relate to the technical demands of the procedure and initial cost of the equipment. Preparation of suitable genomic DNA requires 1 to 3 days, depending on the organisms tested, and the equipment required (including the electrophoresis apparatus and transilluminator) costs between \$10,000 and \$20,000. However, once the method is operational in a laboratory, it can be applied readily to a wide range of species with only minimal modifications. The interpretation of PFGE gels is relatively straightforward, and consensus guidelines for correlating variations in restriction profiles with epidemiological relatedness were published recently.<sup>11</sup>

### Typing Methods Using PCR

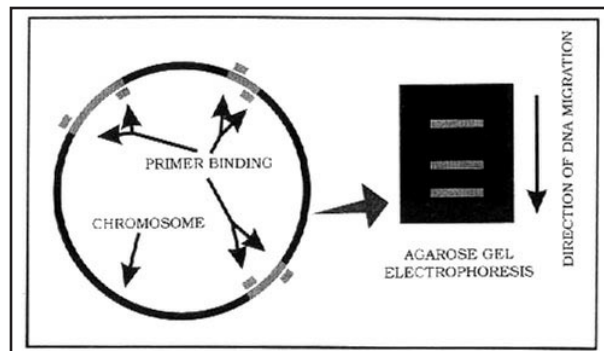
Polymerase chain reaction, which has been used for several years for the direct detection of many types of infectious agents in clinical samples,<sup>45</sup> has been adapted for use as a typing tool.<sup>46-48</sup> The hallmark of PCR is the ability to produce literally millions of copies of a particular DNA segment with high fidelity within 3 to 4 hours' time. The procedure requires template DNA (or RNA if a reverse transcriptase step is used initially), which may be present in the sample in minute quantities; two oligonucleotide primers, which flank the sequences on the template DNA to be amplified (thus defining the starting points for DNA polymerase activity); and a heat-stable DNA polymerase. Efficient amplification is accomplished readily for templates of less than 2,000 base pairs, although templates as large as 35 kb now can be amplified by using newer polymerases. A typical PCR assay requires approximately 3 hours to complete 30 cycles, where each cycle consists of a heat denaturation phase, in which double-stranded DNA is melted into single strands; an annealing phase, in which the primers bind to the target sequences on the single strands; and an extension phase, in which DNA synthesis proceeds from the primers along each strand of the template DNA,

thereby generating two new double-stranded copies of the original template. After 30 such cycles, a single initial copy of template DNA theoretically can be amplified to 1 billion copies.

### Arbitrarily Primed PCR

Arbitrarily primed PCR, also referred to as the randomly amplified polymorphic DNA assay, is a variation of the PCR technique employing a single short (typically 10 base pairs) primer that is not targeted to amplify any specific bacterial DNA sequence.<sup>46</sup> Rather, at low annealing temperatures, the primer will hybridize at multiple random chromosomal locations and initiate DNA synthesis. If one copy of the primer binds to one strand of DNA, and another copy of the primer binds on the opposite strand of DNA but in proximity of the first primer, a DNA fragment will be synthesized and amplification of that fragment will occur (Figure 6).<sup>46</sup> The resulting PCR products will represent a variety of different-sized DNA fragments that are visualized by agarose gel electrophoresis. This approach has remarkable general applicability and has been applied to typing eukaryotic species, as well as many bacterial species.<sup>48</sup>

The reproducibility and discriminatory power of AP-PCR is a subject of active discussion and investigation. Recent reports have described specific primers and conditions for analyzing isolates of *C difficile*<sup>49</sup> and *S aureus*,<sup>50</sup> and have provided formal assessments of the procedure. These studies have indicated that, although AP-PCR is appreciably faster than other typing systems (often 20 to 30 organisms can be completed in a single day), the method is much more susceptible to technical variation than is routine PCR employing primers directed at known sequences. In AP-PCR there can be substantial variations in the efficiency with which the primers initiate DNA synthesis at a particular site, depending on even slight variations in the pH or ionic strength of the buffers used, the temperature of the reaction, or the source of the DNA polymerase.<sup>48,50,51</sup> Consequently, on independent amplifications of the same strain, the number of copies generated from a particular locus can be appreciably different, yielding wide variations in the intensities of individual fragments. These factors can make it difficult to obtain reproducible patterns and interpretations with AP-PCR, particularly when attempting to compare isolates tested on different days. At this time, the most reliable results are obtained when a set of isolates is tested in a single amplification reaction and analyzed on a single electrophoretic gel. The comparison of AP-PCR fragment patterns obtained by testing the same isolates in different laboratories often is problematic, as was shown recently in a multicenter



**FIGURE 6.** Schematic drawing of arbitrarily primed polymerase chain reaction (AP-PCR) technique. The box on the right represents an agarose gel showing the products of amplification using PCR with a random primer. When the primer binds in two places on opposite strands of DNA in proximity to one another, a fragment is generated that can be recognized by agarose gel electrophoresis.

study.<sup>50</sup> Furthermore, standard guidelines for interpretation of AP-PCR are not yet available, and the general principles that are emerging for the interpretation of other molecular typing data, such as for PFGE, cannot be applied readily.

### PCR-DNA Sequencing

Although infrequently used at this time, DNA sequencing of hypervariable gene sequences has shown promise as a typing tool.<sup>52,53</sup> While this technique currently is not feasible for most laboratories, the availability of less expensive DNA sequencing machines may make this technique more accessible in the future.

### WHEN TO USE STRAIN TYPING

Bacterial strain typing data are most effective when they are collected, analyzed, and integrated into the results of an epidemiological investigation. The preferred typing methods for various organisms are presented in Table 2. The hospital epidemiologist should initiate strain typing studies in consultation with the hospital infection control laboratory or the hospital microbiology staff when investigating a potential outbreak of an infectious disease.<sup>73</sup> This may be triggered by a noticeable increase in the rate of isolation of a particular pathogen, a cluster of infections on a particular ward, or the recognition in the clinical microbiology laboratory of multiple isolates with an unusual biotype or antibiogram. Strain typing data should supplement, and not replace, a carefully conducted epidemiological investigation. In some cases, typing data can effectively rule out an outbreak and thus avoid the need for an extensive epidemiological investigation. In other cases, strain typing data may reveal the presence of outbreaks caused by more than one strain. However, undue reliance on

**TABLE 2**  
PREFERRED STRAIN TYPING TECHNIQUES FOR NOSOCOMIAL AND COMMUNITY-ACQUIRED PATHOGENS

Species	Reference Method*	Alternative Methods	References
<i>Staphylococcus aureus</i>	PFGE	AP-PCR, PF	7, 11, 17, 33, 35, 50, 54
Coagulase-negative staphylococci	PFGE	PF	11, 26, 55
<i>Streptococcus pneumoniae</i>	PFGE	Serotyping	28, 56, 57
Enterococci	PFGE	—	8, 42, 55, 58
<i>Escherichia coli</i> , † <i>Citrobacter</i> , <i>Proteus</i> , <i>Providencia</i>	PFGE	AP-PCR	11, 19, 20, 32, 59
<i>Klebsiella</i> , <i>Enterobacter</i> , <i>Serratia</i>	PFGE	PF‡	10, 11, 29
<i>Salmonella</i> , <i>Shigella</i>	Serotyping	PFGE	11, 27, 30, 60, 61
<i>Pseudomonas aeruginosa</i>	PFGE	—	9, 11, 62-66
<i>Burkholderia</i> , <i>Stenotrophomonas</i> , <i>Acinetobacter</i>	PFGE	—	11, 67, 68
<i>Clostridium difficile</i>	AP-PCR	REA, PFGE§	18, 37, 41, 49
<i>Mycobacterium tuberculosis</i>	IS6110 RFLP	REP-PCR	43, 44, 69, 70
Mycobacteria other than tuberculosis	PFGE	—	11, 71, 72

Abbreviations: PFGE, pulsed-field gel electrophoresis; AP-PCR, arbitrarily primed polymerase chain reaction; PF, plasmid fingerprinting (with or without restriction analysis); REA, restriction endonuclease analysis of chromosomal DNA using conventional electrophoresis; RFLP, restriction fragment-length polymorphism typing using IS6110; REP-PCR, repetitive-element polymerase chain reaction.

\* Recommendations are based on published studies and consensus opinions of experts.

† *E coli* O157:H7 must be identified by serotyping.

‡ For the gram-negative organisms, whole-plasmid analysis without restriction digests often is sufficient.

§ Many strains of *C difficile* are nontypeable by PFGE due to DNA degradation.

strain typing in the absence of epidemiological data is an inefficient use of laboratory resources.<sup>11,73</sup>

#### SETS OF ISOLATES MOST APPROPRIATE FOR TYPING STUDIES

Sets of epidemiologically related isolates representing a putative outbreak spanning a period of 1 to 3 months are appropriate organisms for strain typing studies. This implies that the infection control staff and hospital epidemiologist already have made a preliminary assessment and have determined the need for strain typing data. One should not test sporadic isolates, except as epidemiologically unrelated controls (see below). The results of testing sporadic isolates often are misleading and frequently lead to wasted resources in both the microbiology laboratory and the infection control service. Most of the techniques described above can be applied effectively to 10 to 30 isolates per day, especially in dedicated infection control laboratories.

#### Control Strains

In attempting to detect single-strain outbreaks involving species that may be endemic, eg, MRSA or multiply resistant gram-negative bacilli, it is important to include epidemiologically unrelated isolates or historical controls to help differentiate endemic problems from new outbreaks. Outbreaks caused by more than one organism (which occur with considerably lower frequency than those caused by single

strains) are much more complex to analyze and require large numbers of additional control strains in order to recognize the outbreak strains. Although endemic strains may cause clusters of infections, the inclusion of historical controls from previous outbreaks can help determine whether the recent isolates are likely to represent a new outbreak. Additional controls include the testing of well-characterized strains, such as *S aureus* NCTC 8325, *E coli* MG1655, and *Enterococcus faecalis* OG1RF,<sup>11</sup> to ensure that each step of the typing method is working properly.

#### INTERPRETATION

Interpretation of strain typing results is facilitated greatly by an appreciation of the molecular basis of genetic variability of bacteria and the technical factors that can affect results. Three assumptions usually are made: isolates representing the outbreak strain are the recent progeny of a single (or common) precursor; such isolates will have the same genotype; and, epidemiologically unrelated strains will have different genotypes. Ideally, strain typing will provide a clear, objective basis for identifying the outbreak strain and distinguishing it from epidemiologically unrelated isolates.

In practice, the interpretation of typing data is complicated by the fact that isolates from an ongoing outbreak may demonstrate some, albeit typically limited, genetic variability. The purpose of interpretive criteria is to establish a guide for distinguishing true dif-

**TABLE 3**  
GENERAL PRINCIPLES FOR THE INTERPRETATION OF MOLECULAR TYPING ANALYSES\*

<b>Microbiologic Interpretation Based on Typing Results</b>	<b>No. of Genetic Differences Compared With Outbreak Strain</b>	<b>Typical No. of Fragment Differences Compared to Outbreak Pattern</b>	<b>Epidemiological Correlation</b>
Indistinguishable	0	0	Isolate is part of the outbreak
Closely related	1	2-3	Isolate probably is part of the outbreak
Possibly related	2	4-6	Isolate possibly is part of the outbreak
Different	3	≥7	Isolate is not part of the outbreak

\*Adapted from reference 11.

ferences in strains from the natural genetic variation that occurs over time within a given strain. For illustration, assume that a set of up to 20 putative outbreak isolates has been typed and that the analysis has detected a subset of isolates with a common (modal) type, which is presumed to represent the outbreak strain. Typically, among the other isolates in the set, some have similar types (as represented, for example, by a few band changes in a PFGE pattern), and some are distinctly different types (distinctive PFGE patterns). The interpretative criteria should provide consistent, objective guidelines for correlating the level of variation observed between an individual isolate and the putative outbreak strain with an estimate of the likelihood that the isolate is, in fact, part of the outbreak (Table 3). To provide a generally applicable approach, this correlation focuses on the number of genetic events required to generate the observed typing variation, rather than on the types of specific changes observed in a particular typing system. In the example cited, there is a group of isolates that produce identical typing patterns, ie, the presumed outbreak strain. Because only a small portion of the organisms' genetic complement is undergoing analysis, isolates that give identical results are classified as "indistinguishable," not "identical." A more detailed analysis theoretically could uncover differences in the isolates that appeared to give identical patterns but that were epidemiologically unrelated. However, when a set of epidemiologically linked isolates are analyzed, this is unlikely to occur.

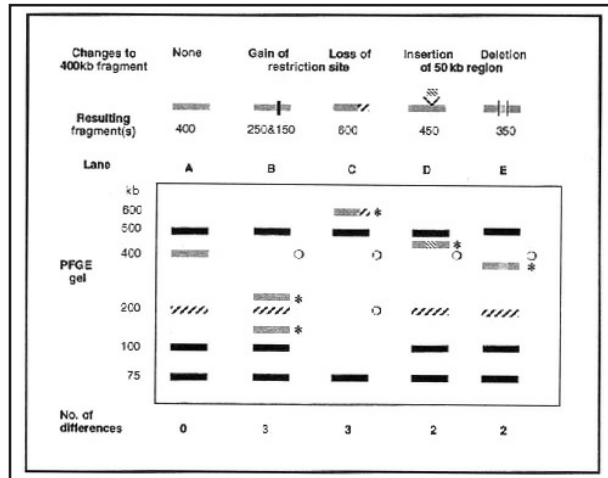
#### ***Classifying Minor Typing Pattern Variations***

The next consideration is how to classify those isolates with minor typing pattern variations. A number of studies using PFGE and other typing methods indicate that single genetic events (ie, random mutations that may destroy or create a new restriction endonuclease site, or deletions or insertions of new DNA such as plasmids, bacteriophages, or insertion sequences)

occur unpredictably even within the time span of a well-defined outbreak (1-3 months). Therefore, interpretive criteria need to accommodate such natural variation. The criteria for interpreting strain typing results are given in Table 3, with their epidemiological corollaries. The changes are depicted in cartoon form in Figure 7. When differences are encountered in the strain typing results, it is important to attempt to ascertain what type of genetic event may have led to the differences. For example, if a single band increases in size, could an insertion of new DNA, such as a bacteriophage or transposon, account for the change in size? Would a random mutation in a restriction endonuclease site account for the appearance of two smaller bands where a single larger band had been seen with other epidemiologically related isolates? Such questions often can be answered by careful analysis of the patterns. If two genetic events have occurred and are recognized through the differences in fragment patterns, the epidemiological interpretation falls into a gray zone. The results may indicate that the isolates are related, especially if they were collected over a long period of time (3-6 months), but there also is a possibility that the strains are unrelated, and the similarity was a result of chance. In such cases, additional information, such as the use of a second strain typing method or supplementary epidemiological analysis, should be sought. This often will help to clarify the relationship among the isolates examined. Finally, isolates that differ by more than three genetic events represent genetically different strains and should be considered unrelated. These general principles have been applied directly to the development of criteria for interpreting PFGE (Table 3; also see reference 11).

#### ***Interpretation of AP-PCR, PF, and RFLP Techniques***

At this time, the interpretation of results generated by AP-PCR, plasmid fingerprinting, and the various nucleic acid probe-based RFLP methods, includ-



**FIGURE 7.** Schematic diagram showing the theoretical changes in pulsed-field gel electrophoresis fragment patterns of an isolate as a result of various genetic events. Lane A, outbreak pattern; Lane B, gain of a restriction site; Lane C, loss of a restriction site; Lane D, insertion of DNA in an existing fragment; Lane E, deletion of DNA from an existing fragment. Open circle (o) indicates fragments present in outbreak pattern and missing in the test isolate after genetic event; asterisk (\*) indicates fragment present after genetic event but absent from outbreak pattern.

ing IS6110, is empiric. For these techniques, the gray zone between “indistinguishable” (identical typing results) and “unrelated” (clearly different results) often is large.<sup>51</sup> In part, this reflects the difficulty of defining the effects of single genetic events in some of these typing systems.

In DNA hybridization-based RFLP studies, such as those using IS6110, two basic mechanisms may alter the patterns observed. The simplest is the generation or loss of a restriction site flanking the insertion sequence (IS) element and a subsequent change in the size of the restriction fragment carrying that element. Such changes often can be defined by performing DNA hybridization using a second DNA probe or by testing DNA cleaved with a different restriction endonuclease. A more complex basis for changes in the RFLP pattern is the loss, gain, or movement of an entire IS element. The initial studies applying IS6110 typing assumed that patterns differing by even a single band represented genetically and epidemiologically different strains. However, more recent data indicates that the IS6110 patterns of isolates representing a definite cluster of *M tuberculosis* cases can differ by single genetic events.

#### Interpretation of PF Results

When isolates from a suspected outbreak have three or more plasmids in common (excluding open circular and linear forms, which sometimes appear as additional light bands for plasmids under 15 kb in

size<sup>31</sup>), as often is seen with strains of coagulase-negative staphylococci, *K pneumoniae*, and other gram-negative bacilli, one usually can say with confidence that they are indistinguishable (genetic interpretation) and therefore likely to be epidemiologically related (epidemiological interpretation) without further testing. With fewer plasmids, the discriminatory power of the test is decreased. Yet, experience with this technique in the 1970s and 1980s, particularly when used to analyze suspected outbreaks of gram-negative organisms causing nosocomial infections, was that, even when only a single plasmid was present, the technique was a very good indicator of strain identity. The problem that was recognized in the 1980s was that of the plasmid outbreak, where the same plasmid could be identified in multiple strains of a species that could be differentiated with other typing techniques. To a degree, with plasmids smaller than 50 kb, the discriminatory power of plasmid fingerprinting can be increased with restriction analysis of the plasmid DNA,<sup>36</sup> which often yields fragment patterns that approach the numbers of bands seen with PFGE, but this does not identify potential plasmid outbreaks. Many enteric organisms, such as *Serratia marcescens*, may have only a single plasmid, and these plasmids often are relatively conserved, showing similar fragment patterns after restriction endonuclease digestion even in epidemiologically unrelated strains. If the plasmid fingerprint and restriction endonuclease data do not fit the epidemiological picture, it may be appropriate to seek data generated by using a different typing technique for analysis of such isolates.

#### Interpretation of AP-PCR Results

For AP-PCR, interpretation of identical fragment patterns and of patterns with three or more fragment differences (there are no restriction enzyme sites to consider in AP-PCR) is straightforward. However, there are no hard-and-fast criteria for interpreting a change in the size of a single band or the intensity of several bands. For strains yielding results such as these, it may be necessary to try alternate primers or to vary the reaction conditions. There is no “universal primer,” although primers such as ERIC1, ERIC2, and repetitive element primers have been used to type several different bacteria and fungi.<sup>51,59,74</sup> The reproducibility and discriminatory power of each primer and amplification protocol need to be validated by analyzing sets of isolates that previously have been well defined by epidemiological data or independent typing studies. Such analyses are available for relatively few species (eg, *S aureus* and *C difficile*). Because variation in AP-PCR cannot be tightly coupled with specific

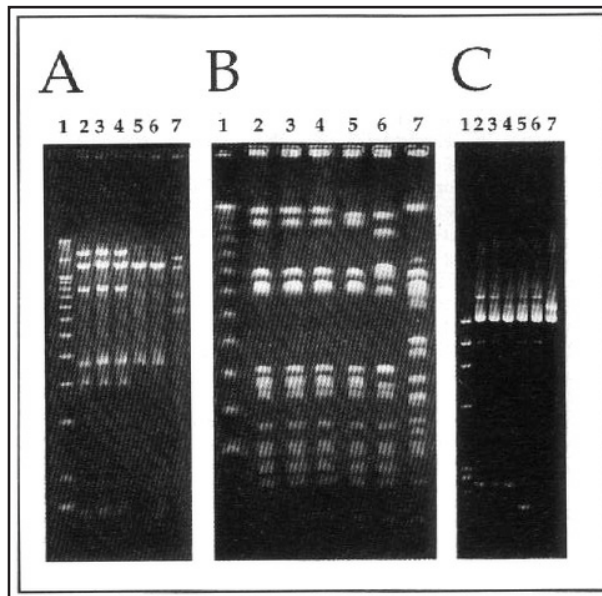
genetic events, the principles defined previously for PFGE cannot be readily applied to typical AP-PCR patterns. The development of generally applicable criteria for interpretation of AP-PCR data remains an area of active investigation.<sup>51</sup> Nevertheless, a recent multicenter study<sup>50</sup> demonstrated that, although participating laboratories obtained different AP-PCR products, the same epidemiological clusters were identified satisfactorily. Thus, where variability of fragment sizes can be demonstrated among epidemiologically unrelated isolates, those showing either no differences or changes only in band intensity can be considered epidemiologically related. Changes in two bands remain difficult to interpret.

### A COMPARISON OF TECHNIQUES

Figure 8 shows the typing results obtained by using three common methods (PF, PFGE, and AP-PCR) to analyze six well-characterized isolates of *S aureus*, for which epidemiological information was available.<sup>17</sup> As previously reported by the CDC,<sup>17</sup> four of the isolates (SB02, SB04, SB06, and SB11) were associated with administration of a contaminated anesthetic. The first three isolates were recovered from the same patient, and the fourth from a different patient. Isolates SB13 and SB17 were unrelated to this cluster, but manifested the same bacteriophage type. As shown in Figure 8, all three methods correctly identified SB02, SB04, and SB06 (lanes, 2, 3, and 4, respectively) as multiple isolates of a single strain. However, only PFGE demonstrated that SB11 (lane B5) was a strain subtype related to the outbreak, while SB13 and SB17 (lanes B6 and B7, respectively) appeared sufficiently different to represent unrelated strains. Conversely, plasmid analysis did not associate isolate SB11 (lane A5) with the outbreak and did not differentiate between SB11 and SB13 (lanes A5 and A6, respectively). Arbitrarily primed-PCR also was unable to distinguish SB11 from SB13 (lanes C5 and C6, respectively). This emphasizes the importance of interpreting the typing data in conjunction with epidemiological data.

At this point in time, we recommend that PFGE be considered the reference method for typing most nosocomial bacterial pathogens (Table 2). Important exceptions to this general recommendation are serotyping for *Salmonella* and *Shigella*, AP-PCR for *C difficile*, and IS6110 RFLP typing for *M tuberculosis*. Alternate techniques can be used when the reference technique is not available or when further discrimination of a set of isolates is desirable.

DNA-based strain typing techniques are a remarkably useful set of tools for complementing the epidemiological analysis of nosocomial outbreaks.



**FIGURE 8.** Interrelationship of *Staphylococcus aureus* strains SB02 (lane 2), SB04 (lane 3), SB06 (lane 4), SB11 (lane 5), SB13 (lane 6), and SB17 (lane 7). The isolates were compared by: (A) *EcoRI* restriction-fragment patterns of plasmid DNA with the 1-kb ladder (Gibco-BRL Life Technologies, Grand Island, NY) plus *EcoRI*-digested lambda DNA as a molecular size standard (lane 1); (B) *SmaI*-digested chromosomal DNA analyzed by PFGE with lambda (48.5-kb) oligomers as a size standard (lane 1); and (C) AP-PCR typing utilizing the ARB11 primer 5' CTAGGACCGC 3' with a *HindIII* digest of lambda DNA as a molecular size standard (lane 1).

This article and the consensus guidelines recently published by the European Study Group on Epidemiological Markers<sup>75</sup> highlight the strengths of molecular typing methods. Pulsed-field gel electrophoresis, in particular, has emerged as a technique that is broadly applicable to most nosocomial pathogens. Interpretive criteria for this technique have been published. Arbitrarily primed PCR has the potential to provide a rapid, alternative typing method, but has not been validated for many species, and standardized interpretative criteria are not yet available. Independent of the typing method used, the application of strain typing to make decisions affecting patient care must be made with an appreciation of the strengths and limitations of molecular methods and only in conjunction with the results of an appropriate epidemiological investigation. The results of strain typing studies produced in a laboratory without the corresponding epidemiological data may be misleading.

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