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Application of Molecular Techniques to the Study of Hospital Infection

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INTRODUCTION

Nosocomial infections are an important source of morbidity and mortality in hospital settings, afflicting an estimated 2 million patients in United States each year. This number represents approximately 5% of hospitalized patients and results in an estimated 88,000 deaths and 4.5 billion dollars in excess health care costs (45, 67, 139, 179). Although viruses, fungi, and parasites are recognized as sources of nosocomial infections, bacterial agents remain the most commonly recognized cause of hospital-acquired infections (67, 121).

Increasingly, hospital-acquired infections with multidrug-resistant pathogens represent a major problem in patients. Several risk factors for acquiring an infection have been commonly cited, including the presence of underlying conditions (such as diabetes, renal failure, or malignancies), long hospitalizations, surgical procedures, receipt of prior antimicrobial therapy, and the presence of indwelling catheters. Major antimicrobial resistance problems are typically associated with gram-positive nosocomial pathogens, which include glycopeptide (vancomycin)-resistant enterococci (125, 172, 173, 187, 280, 304), methicillin-resistant *Staphylococcus aureus* (MRSA) (15, 229, 253), and, more recently, glycopeptide-intermediate and -resistant *S. aureus* (255). Among the gram-negative bacilli, extended-spectrum-beta-lactamase-producing strains of *Escherichia coli* and *Klebsiella pneumoniae* and fluoroquinolone-resistant strains of *Pseudomonas aeruginosa* and *E. coli* have been the primary concerns (180, 182, 183, 231, 242, 258, 278, 279, 286).

Understanding pathogen distribution and relatedness is essential for determining the epidemiology of nosocomial infections and aiding in the design of rational pathogen control methods. The role of pathogen typing is to determine if epidemiologically related isolates are also genetically related. Historically, this analysis of nosocomial pathogens has relied on a comparison of phenotypic characteristics such as biotypes, serotypes, bacteriophage or bacteriocin types, and antimicrobial susceptibility profiles. This approach has begun to change over the past 2 decades, with the development and implementation of new technologies based on DNA, or molecular, analysis. These DNA-based molecular methodologies, which will be examined extensively in this review, include pulsed-field gel electrophoresis (PFGE) and other restriction-based methods, plasmid analysis, and PCR-based typing methods. The incorporation of molecular methods for typing of nosocomial patho-

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gens has assisted in efforts to obtain a more fundamental assessment of strain interrelationship (1, 7, 8, 55, 82, 103, 105–111, 138). Establishing clonality of pathogens can aid in the identification of the source (environmental or personnel) of organisms, distinguish infectious from noninfectious strains, and distinguish relapse from reinfection. Many of the species that are key hospital-acquired causes of infection are also common commensal organisms, and therefore it is important to be able to determine whether the isolate recovered from the patient is a pathogenic strain that caused the infection or a commensal contaminant that likely is not the source of the infection. Likewise, it is important to know whether a second infection in a patient is due to reinfection by a strain distinct from that causing the initial infection or whether the infection is likely a relapse of the original infection. If the infection is due to relapse, this may be an indication that the initial treatment regimen was not effective, and alternative therapy may be required.

INVESTIGATING NOSOCOMIAL INFECTIONS

A number of the nosocomial infections are endemic, or sporadic, infections, which constitute the background rate of infection in the institution. Most nosocomial infections are endemic and are the focus of most infection control efforts. On the other hand, epidemic infections are defined by the occurrence of infections at a rate statistically significantly higher than the background rate of infections. Epidemiologic investigations of nosocomial infection are typically triggered by an increase in the prevalence of infection associated with a particular pathogen species, a cluster of infected patients, or the identification of an isolate that has a distinctive antimicrobial susceptibility pattern.

There are a number of key factors that are essential in an epidemic investigation, including the recognition that a problem exists, establishment of a case-control definition, confirmation of cases, and completion of the case findings. The investigation is typically multifaceted, involving infection control specialists, infectious disease physicians, laboratory personnel, and often pharmacists (208). The laboratory can serve as an early warning system for epidemiologic surveillance. Once an aberration is detected, an investigation can be initiated. The basic investigation includes the collection of critical data and specimens, the initiation of empirical control measures, the identification of affected persons, and then a review of characteristics of the representative case patients in relation to time, person, and place. In the case-control portion of the investigation, characteristics of affected persons are compared with those of a similar but unaffected population. The pathogens associated with the outbreak are characterized using molecular typing methods to assist in the clinical epidemiologic assessment. A hypothesis about causation is then generated about the epidemiology of the infection, including mode of spread, reservoirs and vectors, and specific control measures initiated. Ongoing surveillance is necessary to evaluate the effectiveness of infection control measures and the efficacy of the treatment regimen.

COST-EFFECTIVE APPLICATION OF TYPING METHODS

The integration of molecular typing with conventional hospital epidemiologic surveillance has been proven to be cost-effective due to an associated reduction in the number of nosocomial infections (28, 71, 119, 122, 207–209). Cost-effectiveness is maximized by the collaboration of the laboratory, through epidemiologic typing, and the infection control department during outbreak investigations (207–209, 241, 256, 305, 316, 318). Molecular techniques can be very effective in tracking the spread of nososcomial infections due to genetically related pathogens, which would allow infection control personnel to more rationally identify potential sources of pathogens and aid infectious disease physicians in the development of treatment regimens to manage patients affected by related organisms. Therefore, the use of molecular tests is essential in many circumstances for establishing disease epidemiology, which leads to improved patient health and economic benefits through the reduction of nosocomial infections.

The importance of molecular testing in epidemic investigations of hospital pathogens is well established. Recent information also suggests that the use of an integrated laboratory assessment of drug-resistant pathogens can have an impact on rates of endemic infection and can be cost saving. Northwestern Memorial Hospital in Chicago, Illinois, instituted an in-house molecular typing program to rapidly assess microbial clonality and integrated the typing into its infection control program (207–209). The effectiveness of their integrated infection control program was assessed by examining data on nosocomial infections during a 24-month period before and a 60-month period following implementation of the program. Following implementation, infections per 1,000 patient days fell 13 percent, and the number of hospitalized patients with nosocomial infections decreased 23 percent. The rate of infection fell to 43 percent below the national average, and approximately 50 deaths were avoided during the 5-year period. The cost of implementing the typing program was $400,000 per year; however, this expenditure led to a savings of $5.00 for each dollar spent on the program because of the large reduction of nosocomial infections.

The cost savings realized by Northwestern Memorial Hospital and others (183, 187) through the incorporation of molecular testing in the infection control program for endemic nosocomial infections is associated with the ability to enact early interventions following the identification of pathogen clonality, which could be an early indication of an outbreak. Conversely, the determination of the unrelatedness of isolates (sporadic infections), avoids triggering unneeded and costly epidemic investigations. Cost reduction was also accomplished by earlier recognition of person-to-person spread of isolates compared to that with traditional surveillance, thus potentially preventing the spread to additional patients. To further illustrate the value of molecular typing programs for limiting pathogen spread and reducing health care costs, a study by the Centers for Disease Control and Prevention (6, 81) evaluated the costs and benefits of the PulseNet molecular subtyping based surveillance system. The study examined the investigation by the Colorado state public health laboratories of a 1997 *E. coli* O157:H7 outbreak in which contaminated frozen ham-

burger patties implicated in the outbreak were recalled. If only 15 cases were averted by the recall, the PulseNet system in Colorado would have recovered all of the start-up costs for the system plus the costs for 5 years of operation. The molecular subtyping system becomes even more cost-effective if the resources that would have been wasted on epidemiologic investigations of sporadic cases of *E. coli* O157:H7 infections are taken into account. Likewise in the hospital setting, the early detection of a potential outbreak could trigger enhanced investigation and infection control strategies to limit future infections, while for sporadic infections, costly outbreak investigations could be minimized.

In the near future, it is likely that additional resources will be developed for use of molecular testing in the early detection of nosocomial drug-resistant pathogens (162). Stosor et al. have demonstrated the capacity for rapid, sensitive detection of vancomycin-resistant enterococci on rectal swabs from colonized patients by using PCR-based methods (261). The cost of the PCR method was equal to the cost of 1 day of isolation precautions. Similar rapid tests for detection of resistant staphylococci by molecularly based assays are now commercially available and are used clinically in a number of hospitals for the detection of MRSA isolates. A recent study by Harbarth et al. found that the use of PCR detection versus standard culture methods cut down the time for identification of MRSA by a factor of 4, i.e., in medical intensive care patients from 106 to 23 h and in surgical intensive care patients from 87 to 21 h (124). Therefore, the incorporation of molecular methods and microbial genotyping is among the recommended guidelines for infection control in hospitals and has been found to be medically useful and economically justified.

CHARACTERISTICS OF TYPING METHODS

There are a number important attributes for successful typing schemes: the methodologies should be standardized, sensitive, specific, objective, and subject to critical appraisal. All typing systems can be characterized in terms of typeability, reproducibility, discriminatory power, ease of performance and interpretation, and cost (in terms of time and money) (198). Typeability refers to the ability of a technique to assign an unambiguous result (type) to each isolate. Nontypeable isolates are more common with phenotypic methods but can also occur with genotypic methods. The reproducibility of a method refers to the ability to yield the same result upon repeat testing of a bacterial strain. 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. The discriminatory power of a technique refers to its ability to differentiate among epidemiologically unrelated isolates, ideally assigning each to a different type. In general, phenotypic methods have lower discriminatory power than genotypic methods. Most molecular methods require costly material and equipment but are relative easy to learn and are applicable to variety of species. On the other hand, phenotypic methods also involve costs in labor and material and are restricted to a few species; for example, antisera for *Salmonella* serotyping will not work to type gram-positive organisms.

PHENOTYPIC METHODS

The earliest methods that were used to identify and type organisms were based upon their phenotypic characteristics. One of the most widely utilized techniques is biotyping, or the differentiation of strains based on properties such as differences in biochemical reactions, morphology, and environmental tolerances. Biotyping is often used to help determine the species of microorganisms based upon their abilities to utilize components in different growth media and carry out certain chemical reactions, but it can also be used to separate members of a particular species due to biochemical differences among the organisms. Biotyping is now routinely performed in laboratories using automated systems designed for species identification.

Antimicrobial susceptibility testing is a common practice in the clinical microbiology laboratory. The resultant antibiogram indicates the pattern of in vitro resistance or susceptibility of an organism to a panel of antimicrobial agents (17, 230). Antimicrobial susceptibility testing is typically performed using either automated broth microdilution or disk diffusion methods. Disk diffusion methods are not used as commonly as they once were because of the lack of automation for testing. Microdilution testing provides a quantitative measure of the MIC, which is defined as the lowest concentration of the antimicrobial agent that inhibits the growth of the organism. Both disk diffusion and broth dilution have been carefully standardized and are therefore quite reproducible within and between laboratories. In most epidemiologic studies the antibiogram has limited value because isolates that are not genetically and epidemiologically related may have the same susceptibility pattern. In fact, in many situations different genotyping methods are used to study the distribution of these antimicrobial resistance phenotypes throughout hospital environments.

Serotyping uses a series of antibodies to detect antigens on the surface of bacteria that have been shown demonstrate antigenic variability (12, 149, 210). Serotyping methods have been used for decades for the taxonomic grouping of a number of bacterial pathogen species and remain important for typing *Salmonella*, *Legionella*, *Shigella*, and *Streptococcus pneumoniae* isolates. Serotyping also has been shown to have epidemiologic value in differentiating strains within species of nosocomial pathogens such as *Klebsiella* and *Pseudomonas*. There are a number of different ways in which serotyping can be performed; each varies the way in which the antibody-antigen reactions are detected. Often direct antibody-antigen agglutination is used, in which a bacterial cell suspension is mixed with panels of antibodies. Based upon agglutination profiles, the serotype is determined. Additionally, for organisms such as *S. pneumoniae* the quellung test is used, in which test antibodies bind to the corresponding capsular antigens and induce swelling of the capsule, which can be observed with microscopy (12).

Bacteriophage and bacteriocin typing as epidemiologic tools are limited to bacteria. Bacteriophage (phage) typing classifies bacteria based on the pattern of resistance or susceptibility to a certain set of phages (130, 131, 161, 243). Bacteriophages are viruses that are able to attach to the cell walls of certain bacteria, enter, multiply, and lyse the cells. The differential ability of phages to infect certain cells is based upon the avail-

ability of corresponding receptors on the cell surface for the phage to bind. Often different strains of pathogens have a different cohort of receptors, leading to variable lysis profiles. Bacteriophage typing has some drawbacks due to a lack of widespread availability of biologically active phages and the technical difficulty of performing the technique, but the method has been applied to a number of bacteria associated with nosocomial infections, such as *S. aureus*, *P. aeruginosa* and *Salmonella* species. Additionally strains can be typed based on their susceptibility to a set of heterogeneous substances (generally proteins) that are produced by other bacteria. These inhibitory compounds, or bacteriocins, often limit the growth of closely related species. Bacteriocin typing has had limited utility because of drawbacks similar to those of phage typing, but it has been used for typing *P. aeruginosa* (7). Additionally, an analogous approach has been developed for *Candida* species (particularly *C. albicans*).

The use of phenotypic methods for the characterization of nosocomial pathogens has been useful for our understanding of pathogens; however, these methods have drawbacks that limit their utility for highly discriminatory typing of microorganisms. Limitations of serotyping include a lack of availability of certain antisera and problems with standardization of different methods. Biotyping often lacks discriminatory power because of variations in gene expression and random mutations that may alter biologic properties of microorganisms. Biotyping cannot differentiate among strains where biochemical diversity is uncommon, such as the enterococci, and therefore the utility of biotyping in epidemiologic studies is quite limited. Bacteriophage typing is labor-intensive, and the method often demonstrates poor reproducibility and standardization. When other phenotyping methods fail, bacteriocin typing may have some utility for organisms not easily typed, such as *P. aeruginosa* and *Candida* species. Despite these limitations, phenotypic characterization continues to play a vital role in the overall management of infectious diseases. For example, routine antimicrobial susceptibility testing by the clinical microbiology laboratory may uncover a unique pattern of antimicrobial resistance, which frequently serves as early warning of potential disease problems among patients.

GENOTYPIC METHODS

In recent years, molecular or genotypic techniques have received increased attention as means of analyzing epidemiologic interrelationships. Figure 1 provides a comparison of the methods for major genotypic techniques used in the study of hospital infections. In the examination of the genotypic methods for their application to the study and control of hospital-acquired infections, the goal of genotyping studies is that epidemiologically related isolates collected during an outbreak of nosocomial disease are able to be linked to one another. In other words, isolates involved in a nosocomial outbreak are genetically related and thus originate from the same strain. Therefore, the use of strain typing in infection control decisions is based on several assumptions: (i) isolates associated with the outbreak are recent progeny of a single (common) precursor or clone, (ii) such isolates will have the same genotype, and (iii) epidemiologically unrelated isolates will have different genotypes. There are a few exceptions to these assumptions. For example, epidemiologically unrelated isolates may have similar or indistinguishable genotypes if there is limited genetic diversity within a species or subtype or if the genotyping method is not adequate to distinguish among the nonclonal isolates. In other cases, genetic events (mutation, plasmid acquisition, etc.) may occur during the outbreak, so it may not be enough to know if strains are identical or not but rather may be necessary to know how related (or not) are the isolates. The assumptions and exceptions for use of the different typing methods are discussed below.

PFGE

The chromosome is the most fundamental component of identity of the cell and therefore represents a preferred measure for assessing strain interrelatedness. One approach has been to digest chromosomal DNA with restriction enzymes, resulting in a series of fragments of different sizes that form different patterns when analyzed by agarose gel electrophoresis. Differences in these patterns are referred to as restriction fragment length polymorphisms (RFLPs). Enzymes used to cleave DNA often recognize numerous sites within the bacterial chromosome, resulting in too many band fragments to efficiently and accurately compare following conventional agarose gel electrophoresis. More recently, restriction enzymes that cleave chromosomal DNA less frequently have been utilized for analysis. The resulting DNA fragments are too large to be separated by conventional agarose gel electrophoresis. A number of alternative methods, generally classified as PFGE, are capable of separating these large DNA fragments (11, 44, 47, 94, 233, 245, 246). In conventional agarose gel electrophoresis, DNA molecules that are more than 40 to 50 kb in size fail to migrate efficiently. By periodically changing the direction of the electrical field in which the DNA is separated, PFGE allows the separation of DNA molecules of over 1,000 kbp in length (often referred to as megabase-sized DNA). PFGE methods differ in the way the pulsed electric field is delivered to the agarose gel. Two of the most commonly utilized approaches are contour-clamped homogenous electric field (CHEF) and field inversion gel electrophoresis (44, 94). Field inversion gel electrophoresis utilizes a conventional electrophoresis chamber in which the orientation of the electric field is periodically inverted by 180°. CHEF uses a more complex electrophoresis chamber with multiple electrodes to achieve highly efficient electric field conditions for separation; typically the electrophoresis apparatus reorients the DNA molecules by switching the electric fields at 120° angles. CHEF has been used to evaluate the spread of various antimicrobial-resistant bacteria. The finding of isolates that have identical or related restriction endonuclease patterns suggests spread from single strains.

To interpret DNA fragment patterns generated by PFGE and transform them into epidemiologically useful information for typing nosocomial pathogens, the clinical microbiologist must understand how to compare PFGE patterns and how random genetic events can alter these patterns. Ideally, the PFGE isolates representing an outbreak strain will be indistinguishable from each other and distinctly different from those of epidemiologically unrelated strains. If this occurs, the outbreak is relatively easy to identify. Alternatively, random genetic

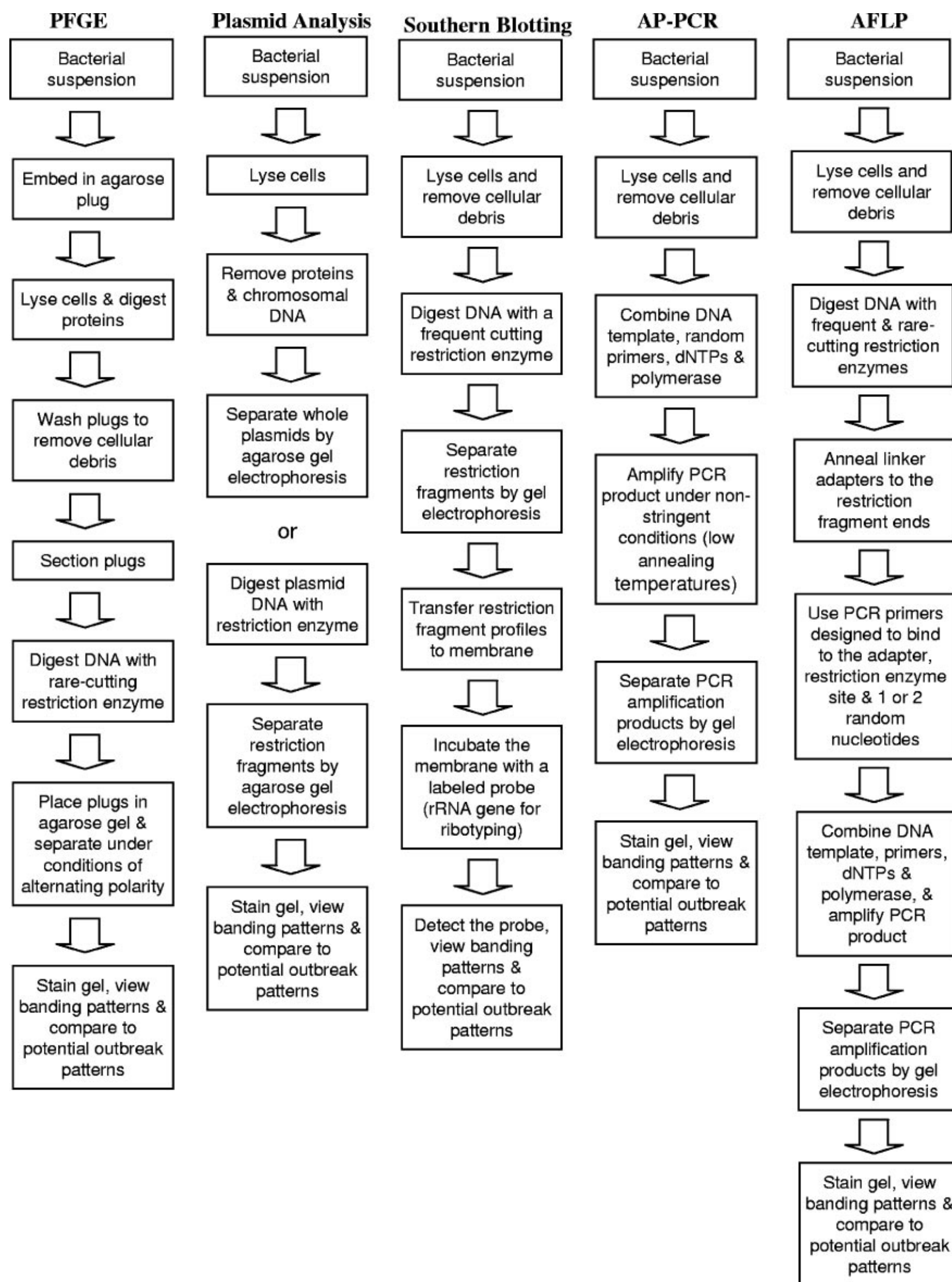

FIG. 1. Flow chart comparison of the different procedural steps used for various molecular typing techniques. dNTPs, deoxynucleoside triphosphates.

events, such as point mutations or insertions and deletions of DNA, that can alter the restriction profile obtained during the course of an outbreak can occur (123, 218, 281). The purpose of interpretative criteria is to establish a guide for distinguishing true differences in strains from random genetic polymorphisms that may occur over the time of a given nosocomial outbreak. Appropriate interpretative criteria provide consistent, objective guidelines for correlating restriction pattern

variations observed between individual isolates and the putative outbreak strain and provide an estimate of the likelihood that the isolate is part of the outbreak (9, 106, 106, 263, 275–277). This correlation focuses on the number of genetic events required to generate the observed pattern variation. Because only a small portion of the organism's genetic component is undergoing analysis, isolates that give identical results are classified as "indistinguishable," not "identical."

Guidelines proposed by Tenover et al. are often used to for the interpretation of PFGE (276). With these guidelines, a banding pattern difference of three fragments could have occurred due to a single genetic event and thus these isolates are classified as highly related, differences of four to six restriction fragments are likely due to two genetic events, and differences of greater than seven restriction fragments are due to three or more genetic events. Isolates that differ by three fragments in PFGE analysis may represent epidemiologically related subtypes of the same strain. Conversely, isolates differing in the positions of more than three restriction fragments may represent a more tenuous epidemiologic relation. A number of studies using PFGE and other typing methods indicate that single genetic events, such as those that may alter or create a new restriction endonuclease site or DNA insertions/deletions associated with plasmids, bacteriophages, or insertion sequences, can occur unpredictably even within the time span of a well-defined outbreak (1 to 3 months) (9, 231, 276). With the detection of two genetic variation events by differences in fragment patterns compared to the outbreak strain, the determination of relatedness to an outbreak falls into a gray zone. The results may indicate that these isolates are related (especially if isolates were collected over a long period of time, such as 3 to 6 months), but there is also a possibility that strains are unrelated and not part of the outbreak.

Often in a nosocomial disease outbreak situation, analysis of PFGE patterns is done using a software program such as BioNumerics (Applied Maths, Kortrijk, Belgium) or one a number of other programs that are available for the analysis of DNA fingerprint data. Rementeria et al. compared the results from three such software packages (GelCompar version 4.0 [Applied Maths], Molecular Analyst Fingerprinting version 1.0 [Bio-Rad, Hercules, CA], and BioImage version 3.2 [BioImage Corp., Ann Arbor, MI]) and manual visualization and found that each method produced acceptable results for gel analysis; however, there were some differences among the detected genotypes determined by each of the methods (220). While unaided visual inspection of a small number of isolate profiles can be done, the software programs have the capability to normalize banding patterns over multiple gels and store the data in databases, so it is feasible to compare a large numbers of strain profiles over time. Most analysis programs also contain algorithms that allow for phylogenic analysis of strains, which allows for the detection of strain evolution and ancestral relationships among isolates (13, 70, 95, 141). In general, strains are considered identical if they show 100% similarity and are considered clonally related if they show greater than 80% similarity (generally comparable to a three-fragment difference as noted above). The typical phylogenic output is the dendrogram, which provides a visual representation of strain lineages and of genetic similarities and differences between groups. A sample dendrogram for community-associated MRSA isolates from southeastern Michigan is shown in Fig. 2.

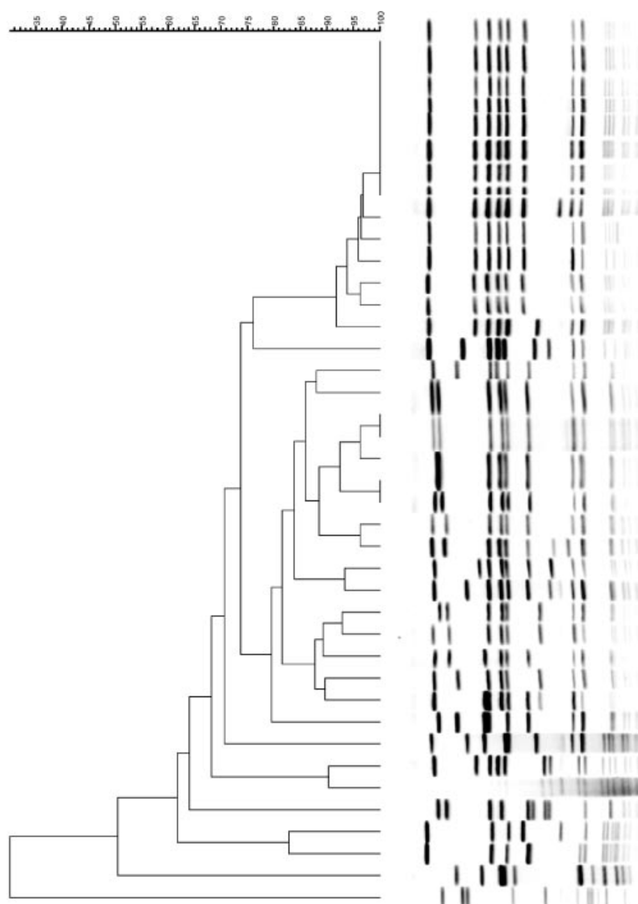

FIG. 2. Dendrogram of pulsed-field gel electrophoresis analysis of community-associated methicillin-resistant *Staphylococcus aureus* isolates (from this study).

PFGE has been applied to at least 40 pathogens or pathogen groups (2–5, 18–20, 22–41, 43, 46–53, 57–68, 75–78, 87, 89–91, 101–103, 105–115, 120, 126, 128, 129, 135, 136, 140, 143–145, 148, 151–153, 156–159, 166–170, 175, 176, 178, 184, 185, 187, 189–193, 199–205, 211, 213, 215, 216, 222–227, 244, 247–250, 260, 262, 264, 271, 272, 283, 289, 310, 311, 313, 314, 320–323). CHEF systems have also been used for typing of *Candida* species (64, 69, 74, 236–238, 301–303, 326). In general PFGE is one of most reproducible and highly discriminatory typing methods available, and it generally is the method of choice for many epidemiologic evaluations. In fact, because of its ability to type organisms, standardized PFGE protocols have been developed by the PulseNet national food-borne disease surveillance network for *E. coli* 0157:H7, nontyphoidal *Salmonella* and *Shigella* isolates, and *S. aureus* as part of the public health laboratory surveillance of nosocomial pathogens (6, 133, 268).

Southern Blot Analysis-Ribotyping

In addition to the use of rare-cutting restriction enzymes and PFGE to utilize RFLP for isolate typing, Southern

blotting can be used to make the number of bands resolved manageable for interpretation. As described above, typical restriction enzyme digestion of chromosomal DNA results in the generation of too many fragments to efficiently analyze and make genetic comparisons among strains. To get around this limitation, Southern blotting can be used. Briefly, the bacterial DNA is digested using a frequent-cutting restriction enzyme, the DNA fragments are separated by agarose gel electrophoresis, and then the fragments are transferred (blotted) onto a nitrocellulose or nylon membrane (257). Next, a labeled (colorimetric or radioactive) piece of homologous DNA is used to probe the membrane. Under the appropriate conditions, the probe hybridizes to a complementary base pair, and the banding patterns are resolved through the detection of the probe label. The discriminatory power of this method is related to the copy numbers of the targeted genetic elements in the bacterial genome and their distribution among the restriction fragments following electrophoresis. Variations in the number and sizes of fragments detected are used to type the microorganisms. One of the most common targets for Southern blotting is the gene for the rRNA, and the targeting of the rRNA gene is referred to as ribotyping. Typically, the discriminatory power of ribotyping has been shown to be less that of PFGE or some PCR-based methods (described below); however, a variety of organisms have been studied using this method. A potential benefit of ribotyping is that it can be very highly automated, reducing the amount of human capital needed to perform the technique and limiting user variability (14). One such automated system is the RiboPrinter microbial characterization system (Qualicon, Inc., Wilmington, DE). Southern blotting has also been used to detect specific antimicrobial resistance genes or virulence factors.

Plasmid Analysis

Plasmid typing was the first molecular method to be used as a bacterial typing tool (10, 80, 164, 181, 240, 273, 274, 325). Plasmids are self-replicating, often-transferable extrachromosomal DNA elements in the prokaryote cytoplasm. Typing is performed through the isolation of plasmid DNA and comparison of the numbers and sizes of the plasmids by agarose gel electrophoresis. Some bacteria have large plasmids in the range of 100 to 150 kb, making their separation difficult; for these strains, the addition of a restriction endonuclease digestion step following plasmid isolation will often aid in typing because multiple fragments are generated, which makes interpretation of strain relatedness more feasible. Plasmid restriction is also commonly used for the analysis of staphylococci and enterococci, whose plasmids are typically less than 50 kb in size. The inclusion of restriction enzyme analysis increases the discriminatory power of plasmid analysis.

Evaluation of plasmid content is not generally useful in delineation of strain relatedness. An exception may be when isolates from a suspected nosocomial outbreak have three or more plasmids in common (excluding open circular or linear forms, which may appear as additional light bands for plasmids under 15 kb in size), as is commonly seen with strains of coagulase-negative staphylococci, *K. pneumoniae*, and other gram-negative bacilli; in this case one can say with a degree of confidence that they are epidemiologically related without further testing. With fewer plasmids the discriminatory power of the test is decreased. Plasmid dissemination could be suspected when clonally unrelated isolates, either detected by PFGE or different species, have similar antimicrobial resistance profiles.

Plasmid analysis has been applied in clinical situations to determine the evolution and spread of antibiotic resistance among isolates with different PFGE profiles or among different species of organisms within hospitals (78, 92, 280). Plasmids are not generally helpful in for differentiation between endemic and epidemic strains, because plasmids are often mobile extrachromosomal DNA fragments that can be acquired and deleted. A consequence of this plasmid mobility is that epidemiologically related isolates can exhibit different plasmid profiles. Many plasmids carry antibiotic resistance determinants that are contained within mobile genetic elements (transposons) that can move in or out of plasmids and the chromosome, allowing for the DNA composition of a plasmid potentially to change rapidly. The analysis of plasmid content is limited to investigations in which a plasmid epidemic is responsible for the spread of a resistance trait. The selective pressure for nosocomial organisms to express antibiotic resistance may cause such plasmids to spread rapidly among strains and among different species.

To evaluate the potential for plasmid dissemination among clonally unrelated isolates, isolation of resistance plasmids and restriction analysis are essential. A transposon epidemic is suggested when isolates from different species, or isolates of the same species with differing PFGE and profiles plasmid contents, have similar resistance genes. Further analysis by PCR and DNA sequencing of transposon content by insertion sequence evaluation have been useful to establish the potential of a transposon epidemic. In a study of the epidemiology of vancomycin resistance in *Enterococcus*, the glycopeptide resistance element Tn*1546* in 124 VanA *E. faecium* clinical isolates from 13 Michigan hospitals was evaluated using PCR fragment length polymorphism (77). Prior to the study it was observed that there was considerable PFGE profile heterogeneity among vancomycin-resistant *E. faecium* strains even in epidemiologically related isolates (280). Plasmid analysis among the isolates showed a diversity of plasmids; however, the Tn*1546* elements were identified as related, suggesting the presence of a transposon epidemic (77). A combination of PFGE, plasmid analysis, and PCR analysis of Tn*1546* was needed to elucidate the epidemiology of these nosocomial pathogens. Many enteric gram-negative organisms, such as *Serratia marcescens*, have only a single, often highly conserved plasmid, which demonstrates similar fragment patterns after restriction endonuclease digestion even in epidemiologically unrelated strains; therefore, it would be recommended to use additional typing techniques for these or similar isolates.

Typing Methods Using PCR

PCR is a biochemical in vitro reaction that permits the synthesis of large quantities of a targeted nucleic acid sequence (188). The procedure requires template DNA from the organism being typed, two complementary oligonucleotide primers

that are designed to flank the sequence on the template DNA to be amplified, and a heat-stable DNA polymerase. The PCR primers serve as the starting point for the polymerase to add the bases that make up a strand that is complementary to the template. A growing number of organisms have been studied using this approach (99, 100, 116, 118, 134, 155, 174, 194, 195, 234, 254, 261, 265, 266, 267, 269, 282, 285, 288, 292–299, 300, 306–308, 312). Each amplification cycle consists of a heat denaturation phase in which double-stranded DNA is melted into single strands, an annealing phase where the primers bind to the single-stranded target sequences, and an extension phase. It is during the extension phase, in which the copy number of the DNA is doubled, that the DNA synthesis proceeds from the primers along the template strands, generating copies of the original double-stranded DNA molecule.

Multiplex PCR. In order to increase the efficiency of PCR typing and reduce reagent costs, multiple sets of primers can be included in a single reaction tube in a process termed multiplex PCR (96, 98). A key strategy in the development of a multiplex PCR assay is the design of the primers. Primers must be designed such that all of the primers have very close annealing temperature optimums, and the amplification products that they produce need to be of noticeably different sizes to facilitate interpretation. If the amplification products were too close in size, it would be difficult to determine the identity of the amplification product. An additional concern with multiplex PCR is that the mixing of different primers can potentially cause interference in the amplification process, thus making optimization of the reaction difficult, especially as the number of primer pairs in the reaction mixture increases.

Nested PCR. When there is an extreme need for sensitivity and specificity in PCR, the process of nested PCR can be carried out. Nested PCR involves the sequential use of two PCR primer sets. The first primer set is used to amplify a target sequence (which increases the sensitivity for the second primer set); the amplicon generated then serves as the template for a second amplification using primers internal to those of the first amplicon. This secondary amplification proceeds only if the intended target was initially amplified; if the primary amplification was nonspecific, the secondary amplification would not occur (increased specificity). A major drawback of nested PCR is that the reaction vessel needs to be opened in order to add the second primer set, increasing the potential for contamination of the work environment with amplified DNA.

AP-PCR. Arbitrarily primed PCR (AP-PCR) and the very similar randomly amplified polymorphic DNA assay are variations of the PCR technique in which a random primer, which is not targeted to amplify any specific bacterial DNA sequence, is used for amplification (88, 147, 165, 235). The key to the random priming is that low annealing temperatures are used (at least initially) during amplification, allowing imperfect hybridization at multiple random chromosomal locations to occur and initiate DNA synthesis. Amplification will continue if two of the primers bind in close enough proximity to one another on the complementary strands to allow synthesis of the DNA fragment. Although the method is much faster than many of the other typing methods for nosocomial pathogens, it is much more susceptible to technical variation than most other methods. Slight variations in the reaction conditions or reagents can lead to difficulty in reproducibility of results and to differences in the band patterns generated. Therefore, trying to make comparisons among potential outbreak strains can be very problematic. When tightly controlled, AP-PCR can provide a high level of discrimination, especially when multiple amplifications with different primers are performed.

Interpretation of AP-PCR results is sometimes difficult, because variation of AP-PCR cannot be tightly coupled with specific genetic events. Therefore, the principles defined previously for PFGE cannot be applied to typical AP-PCR patterns (154, 239). If strains being typed have identical fragment patterns or patterns with three or more fragment differences, the interpretation is more straightforward; they are either clonal or unrelated, respectively. But there are no criteria in for interpreting a change in the size of a single band or the intensity of several bands. For strains with one or two band differences from an outbreak strain, it may be necessary to try alternate primers or to vary the reaction conditions to determine if the differences are true differences or due to problems with reproducibility. To combat this problem, 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 epidemiologic data or independent typing studies. Such analysis is available for relatively few species; these include *S. aureus* and *Clostridium difficile* (16, 59). For strains in these species 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. A multicenter study demonstrated that although participating laboratories obtained different AP-PCR products, the same epidemiologic clusters were identified satisfactorily (290).

AFLP. Amplified fragment length polymorphism (AFLP) is a typing method that utilizes a combination of restriction enzyme digestion and PCR (21, 42, 56, 137, 196, 270, 287, 309, 315, 317). In the AFLP procedure, the DNA is digested with two different restriction endonucleases, usually chosen so that one cuts more frequently than the other. This restriction strategy generates a large number of fragments. In order to make the interpretation of the results more feasible, only a specific subset is used for isolate comparison. The subset is generated by linking adapter sequences to the ends of the restriction fragments extending the length of the known end sequences. PCR primers are designed to hybridize to the adapter sequence, the remaining restriction site sequence, and an additional one or two nucleotides of the unknown template sequence. The addition of each nucleotide, chosen at random, to the end of the primer reduces the number of fragments that will be amplified by a factor of four. Following PCR, the reaction products are separated by gel electrophoresis and their banding patterns are resolved. The method utilizes the benefits of RFLP analysis with the increased sensitivity of PCR to generate profiles that are reproducible and relatively easy to interpret and compare to those for other isolates from a nosocomial outbreak by using a software program such as BioNumerics.

Other PCR-associated typing methods. In many bacterial species there are repetitive DNA sequences spread throughout the genome. PCR primers can be designed for these elements

to amplify the genomic DNA between the repetitive elements when two of the elements are in relatively close proximity (308). The regions located between the repeated elements often vary in size due to difference among separate strains, and thus fragments of different sizes will be amplified, creating unique profiles following gel electrophoresis. These different banding patterns are compared to one another to genotype the organisms (102). Repetitive-element PCR systems have been developed for a variety of hospital-associated pathogens, including *E. coli* (132), *S. aureus* (228, 254), and the enterococci (214).

Additional methods that can be used to type hospital-acquired pathogens and that rely on PCR include variable-number tandem repeat (VNTR) typing and spoligotyping for *Mycobacterium tuberculosis*. *M. tuberculosis* has a region in its genome that is rich in direct repeats interspaced with nonrepeat sequences or "spacer oligonucleotides" (142). Spoligotyping exploits the conserved repeats as PCR primer targets to amplify the spacer sequence. The products are amplified with biotinylated primers and used to hybridize with a panel of synthetic oligonucleotides, representing known spacer sequences, which are bound to a membrane. The PCR products bind to complementary oligonucleotide sequence, providing a hybridization profile used to genotype the different organisms (127, 142). VNTR typing utilizes a feature of many bacterial genomes which contain short, repetitive tandem sequences. The copy number of these VNTR sequences often varies among unrelated strains and can be used for genotyping. Often, fluorescently labeled PCR primers are designed to amplify the whole repeat region. Following amplification, the PCR products are separated (often with an automated sequencer if fluorescently labeled primers were used) and sized to determine the number of repeats present. Typically, multiple repeat regions are analyzed to determine the genotype (163, 197). There are a number of bacterial species of hospital-associated pathogens for which VNTR typing techniques are available, and a review by Lindstedt et al. provides an in depth overview of VNTR typing of many pathogens (163).

COMPARISON AND SELECTION OF TECHNIQUES

When evaluating the use of molecular typing methods in the study of hospital-associated infections, one should first decide whether there is a real need to use a molecular epidemiologic approach to study the situation in question. The molecular characterization of nosocomial isolates generates data regarding the interrelatedness of isolates. In an individual patient, the use of molecular characterization can assist in separating relapse from reinfection or, in the case of bacteremia, whether the organism is from the infection or contamination. In a group of isolates the characterization of isolates by a method such as PFGE can assist in the establishment of clonal relationships that are essential in assessing the question of patient-to-patient spread of an organism. Plasmid or transposon analysis of strains is used when there is suspicion of dissemination of a particular resistance gene or set of genes. Since the performance of molecular testing can result in a significant expenditure of time and money, there should always be a clear objective for use and a desire to limit strain typing to only the isolates needed to define the clinical or epidemiologic entity. In the analysis of nosocomial infections, detective-like problem solving skills are often required. While it may be useful to repetitively type isolates to increase discrimination, most studies have shown that repetitive typing or use of a second method does not significantly alter the epidemiologic assessment, provided that the isolates are typeable and appropriate controls (isolates from epidemiologically related and unrelated patients) are used in the analysis. The best method often depends not only on the specific epidemiologic situation but also on the resources available.

When comparing the different molecular methods, it is important to consider what each method is actually assessing. With PFGE, the restriction fragment patterns reflect the distances between infrequent restriction sites located around the chromosome; therefore, for the method to detect differences between isolates, changes in the chromosome must affect the restriction sites and/or the distances between them. PFGE analysis provides relatively global chromosomal overview, scanning more than 90% of the chromosome (the sum of the restriction fragment sizes), but it has only moderate sensitivity, since minor genetic changes may go undetected. Conversely, PCR-based methods generally survey relatively limited regions representing less than 10% of the chromosome (the sum of the amplicon sizes). Since PCR products are usually relatively small (equal to or less than 5 kb), electrophoretic analysis can detect even small genetic changes affecting their size; however, if the change occurs outside of the region amplified, it will not be detected. It is accepted that some basic level of genetic change may be expected to occur in nosocomial pathogens as they move from patient to patient (a single genetic change event). With PFGE, a single detectable chromosomal change would result in a difference of at least two band positions. PCR methods would minimally detect a difference of one position (such as an addition, loss, or shift in electrophoretic migration of a single band). The greater the genetic difference between isolates, the more likely that they are epidemiologically unrelated.

Additionally, there is a need to have an understanding of the underlying genetic diversity of the species of microorganism being typed to have a more full understanding of the typing results. In some instances minor genetic differences may be of epidemiologic significance in more clonal organisms that, by definition, have low degree of genetic variability. A key way to accomplish this understanding of diversity is through the development of large isolate databases, either in-house or through a central clearinghouse. The expense of generating a large in-house database can be prohibitory; therefore, there has been a push toward the development of centralized databases. This process has been very successful with the PulseNet system for typing food-borne pathogens (268) but has been slower to develop for common hospital-associated patterns. Recently, CDC has begun to develop a national database for the MRSA isolates and has set up a naming scheme for a number of MRSA clones. This system is modeled after PulseNet and will involve state health departments along with CDC (177). Additional databases are available for other typing methods, such as multilocus sequence typing (MLST) (discussed below). The online databases (available at www.mlst.net) provide standardized sequence typing nomenclature for comparison among laboratory personnel across the globe.

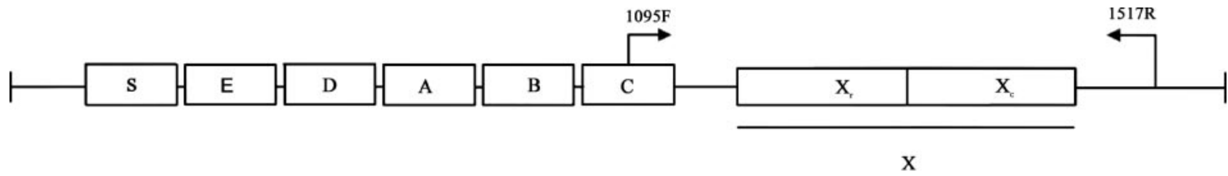

FIG. 3. Protein A gene map. Boxes indicate the genes coding for the initial sequence (S), the immunoglobulin G-binding regions (A to D), a region homologous to regions A to D (E), and the COOH terminus (X), which includes the short sequence repeats (Xr) and the cell wall attachment sequence (Xc). Primers are numbered from the 5′ end of the primer on the forward strand of *Staphylococcus aureus*. (Reprinted from reference 251 with permission.)

There are currently databases for a number of key nosocomial pathogens, including *S. aureus*, *E. faecalis*, *E. faecium*, *E. coli*, and *C. albicans* (www.mlst.net, accessed 6 March 2006). Access to the databases provides an excellent resource to gain an understanding of the background diversity of a species. One of the realizations from examining a large number of MRSA strains is that there is a large degree of clonal homogeneity, and thus laboratory and infection control personnel must take into account the endemic nature of the organism. Thus, even a small change in chromosomal fingerprints may be epidemiologically significant (177). As PFGE provides the broadest genomic overview, it likely remains the method of choice as the initial typing method for most epidemiologic investigation. However, on rare occasions, PFGE is unable to resolve discriminatory profiles for certain organisms, and other typing methods need to be employed to determine the relatedness of the strains (97).

RECENT ADVANCES IN MOLECULAR EPIDEMIOLOGY: NUCLEOTIDE SEQUENCE-BASED ANALYSIS

The rapidly expanding number of sequenced microbial genomes has served as a catalyst for the development of a variety of molecular typing approaches that focus on either single or multiple chromosomal loci. Sequence-based molecular epidemiology is attractive in offering the promise of reproducible typing profiles that are highly amenable to standardization, uniform interpretation, and database cataloging, since they are based on simple quaternary data (A, T, G, and C) (146).

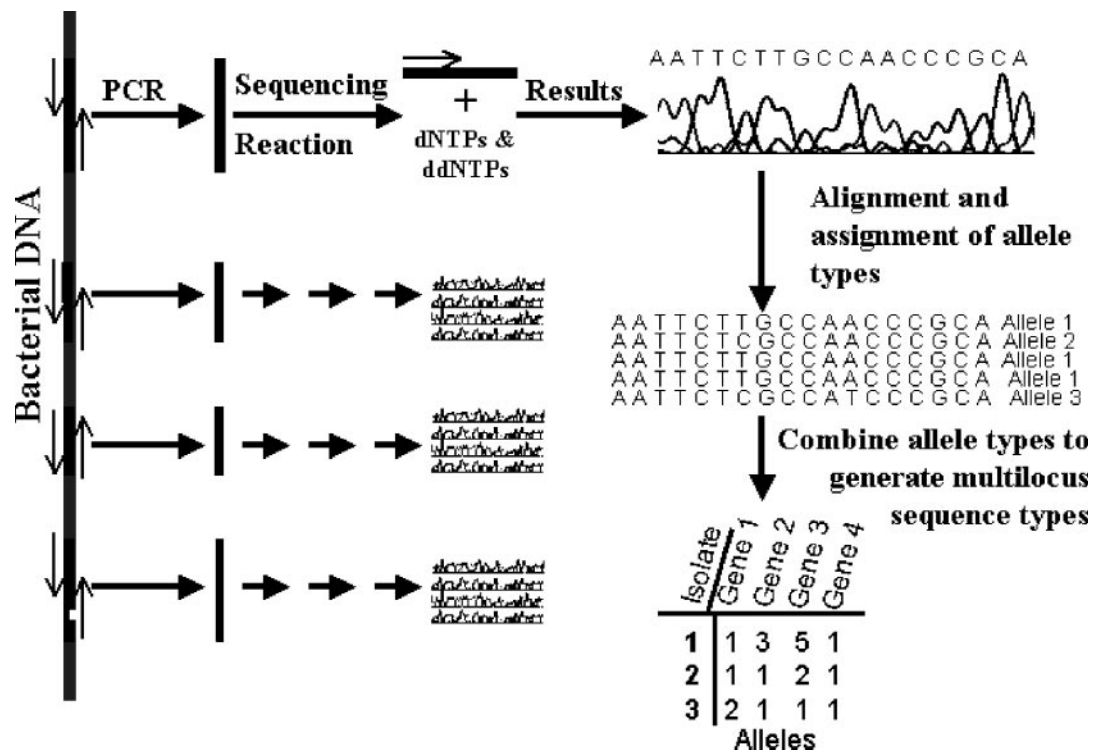

FIG. 4. Diagrammatic representation of the procedure used for MLST. The bacterial DNA sequence at the left represents different gene targets amplified using locus-specific primers (small arrows whose color matches the gene locus). The genes are amplified and sequenced from the locus-specific primers. The nucleotide sequences from the loci are compared to other sequences in an MLST database, and the allele name is assigned. The assigned alleles for each locus are combined to form the multilocus sequence type. dNTPs, deoxynucleoside triphosphates; ddNTPs, dideoxynucleoside triphosphates.

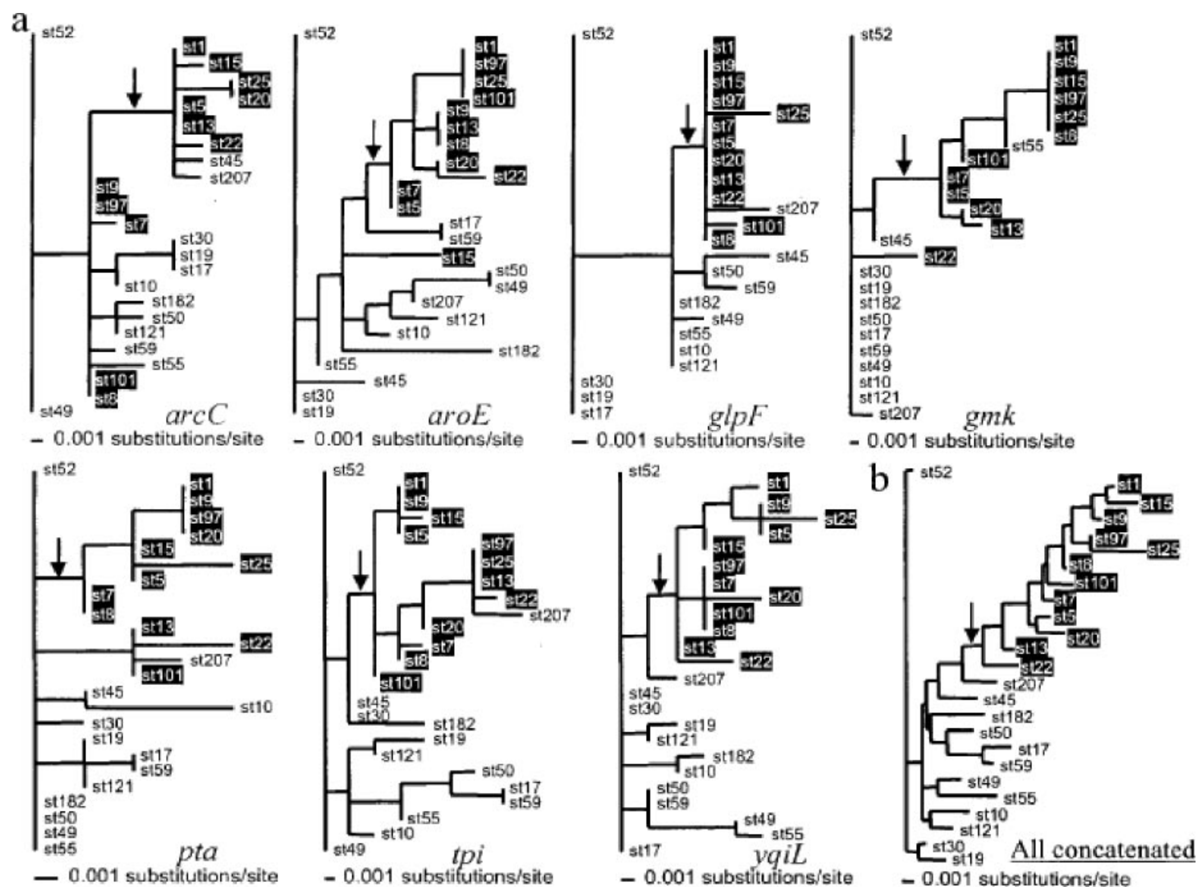

FIG. 5. Distribution of multilocus genotypes, showing multilocus trees for *Staphylococcus aureus*. Trees are shown for each MLST gene and for the concatenated sequences of all seven genes (a) with a sample of 25 diverse strain types (b). Conserved division is marked with an arrow in each tree. (Reprinted from reference 92 with permission.)

SLST

Sequence data for specific loci (genes for virulence, pathogenicity, drug resistance, etc.) from different strains of the same species have revealed variability in a specific gene, such as single-nucleotide polymorphisms and areas with repetitive sequence that demonstrate potential for epidemiologic application. However, the methods are still under development or do not correspond to comparative evaluation detected with other molecular typing techniques such as PFGE (48, 54, 146, 186, 219, 252, 319), and they require further validation with isolates of known epidemiologic interrelationship to gain wider acceptance for typing nosocomial pathogens. At present, the single-locus sequence typing (SLST) approach with most promise involves analysis of a particular region of the staphylococcal protein A gene (*spa*) which is polymorphic (Fig. 3) due to 24-bp repeat sequences that may vary in both the number of repeats and the overall sequence in the polymorphic X or short sequence repeat region (150, 251, 291). Although it is applicable only to *S. aureus*, *spa* typing appears to be very robust, with benefits in throughput, ease of use, and interpretation that tend to balance a lower level of epidemiologic discrimination than that of established genotypic methods such as PFGE (324). At present, no SLST protocol has emerged as a clear stand-alone method for epidemiologic typing. Nevertheless, SLST shows potential for a typing and clearly represents an important and promising sequenced-based approach to epidemiologic analysis. The method at the very least can serve as an adjunct to more definitive genotyping methods, especially for strains in which genetic diversity is not efficiently detected by restriction-based or standard PCR methods.

MLST

Derived, in principle, from multilocus enzyme electrophoresis, MLST utilizes a larger, and potentially more representative, portion of the genome than SLST. MLST compares the nucleotide sequences of internal 400- to 500-bp regions of a series of housekeeping genes (typically seven or more) which are present in all isolates of a particular species. For each gene fragment, genetic polymorphisms in sequences are considered distinct alleles (Fig. 4). Each isolate is defined by the alleles at each of the sequenced housekeeping loci, which together comprise the allelic profile or sequence type. Because there are many potential alleles at each of the loci, it is unlikely that identical allelic profiles will occur by chance. Thus, isolates with the same allelic profile are assigned as members of the same clone. MLST was originally employed to identify hypervirulent lineages of *Neisseria meningitides* (171). However, the

approach has now been applied to a variety of other pathogens, including *S. aureus* (72, 79, 83, 92, 117, 206) and enterococci (259, 284), for the assignment of *S. pneumoniae* strains to major hypervirulent, penicillin-resistant, and multiple-antibiotic-resistant clones (84, 232), and to a large number of other organisms (73, 85, 160, 212, 217, 221). In evaluating MLST it is important to remember that, like multilocus enzyme electrophoresis, the method was originally designed to assess genetic interrelationships in bacterial populations with sufficient opportunity for the sequence of housekeeping genes to diversify, such as in evolutionary studies. However, the epidemiologic window of investigation for hospital outbreaks often represents a relatively short time period. Thus, while MLST excels in identifying broad population-based interrelationships, in a clinical setting it is somewhat less discriminating than typing methods where surveillance is based on a more rapid genomic clock. For example, in the analysis of *S. aureus* (83) and *S. pyogenes* (86), MLST has proved to be less sensitive than PFGE and *emm* typing, respectively. One potential way to overcome this lack of diversity over the short term could be through the sequencing of virulence genes, which will likely be under greater selective pressure to mutate than housekeeping genes (97). As with SLST, MLST demonstrates the potential of sequence-based typing to generate consistent, reproducible isolate profiles that are highly amenable to standardization and database cataloging (93). In comparison to SLST, it is difficult to currently envision MLST in a real-time clinical setting due to the expense, labor, and time involved in surveying multiple (often seven or eight) genes and their corresponding approximately 2,500 bp of sequence that needs to be analyzed to differentiate among multiple isolates (Fig. 5).

CONCLUSIONS

In the future, issues of concern about the emergence of nosocomial infections, increasing antimicrobial resistance, and the increase in morbidity, mortality, and costs associated with these infections will drive the need for refinement of molecular approaches to aid in the diagnosis and epidemiologic analysis of nosocomial infections. The evaluation of hospital-associated infections will continue to rely on clinical infection surveillance as the first step to understanding disease epidemiology and management of infections. It is clear that molecular typing will continue to facilitate this process. The utilization of nucleotide sequence-based typing is still in its infancy, and further information will need to be established about the most useful indicators of strain and resistance gene relatedness. Additionally, the cost-effectiveness of the tests must be evaluated in terms of the overall contribution to nosocomial infection reduction. The most accurate assessment of epidemiologic relationships in a nosocomial setting is always accomplished by careful assessment of all available information. Molecular testing will continue to be an essential tool, for the testing has proven to be cost-effective and medically needed. Molecular typing is a powerful tool in the armamentarium for combating the spread of problem microorganisms in the hospital environment.

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