A comparative study on the application of 3 molecular methods in epidemiological typing of bacterial isolates using MRSA as a prototype

Sahar I. Al-Thawadi, MD, ABMM, George Kessie, MS, Damian Dela Cruz, DVM, Mohammed N. Al-Ahdal, PhD, MASM.

ABSTRACT

Objectives: To evaluate and compare 3 widely used molecular techniques, namely, restriction endonuclease analysis of plasmid deoxyribonucleic acid (REAP), randomly amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) for their suitability and usefulness in the typing and fingerprinting of bacterial isolates.

Methods: Twenty-four epidemiologically unrelated methicillin-resistant *Staphylococcus aureus* (MRSA) isolates were used to evaluate the molecular typing methods (REAP, RAPD and PFGE). The study was conducted at the Research and Diagnostic Laboratories of King Faisal Specialist Hospital and Research Center from January 2002 through January 2003.

Results: Only 20.8% of all isolates studied were of the same genotypes by all 3 methods. Two major clusters of strains each representing 33% of the total number of isolates were identified by REAP analysis. Each of RAPD and PFGE however, identified one major cluster represented by 54% and 83% of the total number of isolates, All 3 typing methods, therefore, showed the clonal genetic relatedness among distant MRSA isolates. However inter-strain comparison of fingerprint data generated from each method revealed differences in clonal representation of the MRSA isolates.

Conclusion: Although a variety of molecular assays are available for typing of bacterial species, there is no single standardized protocol for routine analysis. Reproducibility and interpretation of genotypic data are therefore, highly dependent on methodologies employed by the individual laboratory. Our findings illustrate the importance of using a combination of methods in typing schemes of bacterial isolates. In terms of reproducibility and typeability we found that PFGE is superior to REAP and RAPD and, therefore, more suitable for routine, standardized tracing of nosocomial bacterial isolates.

Saudi Med J 2003; Vol. 24 (12): 1317-1324

The ability to characterize and determine genetic relatedness among bacterial isolates involved in nosocomial infections and infectious disease outbreaks is a prerequisite for epidemiological investigations. Effective control measures are dependent on a thorough assessment of the relatedness of clinical isolates, which requires a typing technique that can be universally applied. Molecular genotypic methods

From the Department of Biological and Medical Research (Al-Thawadi, Kessie, Dela Cruz, Al-Ahdal), Department of Pathology and Laboratory Medicine (Al-Thawadi, Al-Ahdal), King Faisal Specialist Hospital and Research Centre, Riyadh, *Kingdom of Saudi Arabia*.

Received 24th May 2003. Accepted for publication in final form 20th September 2003.

Address correspondence and reprint request to: Dr. Mohammed N. Al-Ahdal, Principal Scientist and Adjunct Professor, Department of Biological and Medical Research, King Faisal Specialist Hospital and Research Centre, PO Box 3354 (MBC-03), Riyadh 11211, *Kingdom of Saudi Arabia*. Tel. +966 (1) 4427867. Fax. +966 (1) 4424519. E-mail: ahdal@kfshrc.edu.sa

were found to be superior to phenotypic methods, since they can detect minute genetic variation and, therefore, are more useful for microbial source tracking. Among various molecular typing methods, 3 have been used extensively, namely, restriction endonuclease analysis of plasmid (REAP), randomly amplified polymorphic deoxyribonucleic acid (DNA) (RAPD), and pulsed-field gel electrophoresis (PFGE). These molecular methods have several advantages over traditional phenotypic typing methods, including higher discriminatory power, broader application to a variety of bacterial species, and at times, speed.[1] Restriction endonuclease analysis of plasmid was among the first few molecular methods to be used as a bacterial typing tool that analyzes the extrachromosomal DNA elements found in many clinical isolates.[2] Randomly amplified polymorphic deoxyribonucleic acid is a polymerase chain reaction (PCR)-based molecular method that has been widely used for typing of a variety of microbes.[3] Short random arbitrary primers (10-20 bases) are used to hybridize with sufficient affinity to various sites on chromosomal DNA sequences at low annealing temperatures, resulting in the amplification of random regions of the bacterial genome.[4] Several DNA fragments of different lengths are generated which can be analyzed by conventional agarose gel electrophoresis. Pulsed-field gel electrophoresis of genomic DNA has been widely used for typing a variety of microorganisms and has been found to have high discriminatory power and reproducibility.[5,6] This technique utilizes restriction enzymes to generate a discrete number of high molecular weight DNA fragments over 50 kb in size. These fragments are then separated by agarose gel electrophoresis that employs electric fields applied alternatively in 2 directions. Such switching allows a more rapid orientation of smaller fragments and, therefore, faster mobility compared to larger ones, thereby providing efficient resolution of the DNA fragments.[7-9]

These molecular techniques have been used on many bacterial species, but most notably on methicillin–resistant *Staphylococcus aureus* (MRSA) since it has remained a major cause of nosocomial morbidity and mortality.[10-17] In the present study, we randomly selected 24 MRSA isolates from our laboratory as a prototype to test and compare these 3 molecular methods (REAP, RAPD, and PFGE). Our aim is to utilize one or more of these molecular methods for future implementation of a typing procedure for genotyping and tracing the sources of significant nosocomial pathogens.

Methods. ***Bacterial strains.*** Twenty-four MRSA isolates were randomly selected from a collection of isolates identified in our laboratory. They were subcultured on Muller-Hinton agar plates, supplemented with 4% Nacl and 6ug/ml oxacillin. Resistance to oxacillin was confirmed by the detection of mecA gene using PCR followed by southern blot hybridization as described previously.[18]

Preparation of plasmid deoxyribonucleic acid. Bacteria were grown in 500 ml of Muller-Hinton medium containing oxacillin (6 ug/ml) and incubated at 35°C overnight. Crude preparations of the plasmids were obtained using the alkaline lysis method of Birnboim and Doly,[19] except that lysostaphin (one mg/ml) was substituted for lysozyme in the lysis buffer. The plasmids were purified through a cesium chloride-ethidium bromide equilibrium density gradient ultra centrifugation as described by Sambrook et al[20]

Preparation of chromosomal deoxyribonucleic acid. Chromosomal DNA was prepared from the bacterial isolates according to standard methods. Briefly, harvested cells from one ml overnight cultures were resuspended in 0.5 ml of lysis solution (10 mM Tris-HCl, pH 8.0, 0.1 mg/ml lysostaphin, 0.5 % sarkosyl, 0.1 M ethylenediaminetetraacetic acid (EDTA) and 250 ug/ ml proteinase K). The mixture was incubated overnight at 37°C and extracted with phenol: chloroform: isoamyl alcohol (25:24:1). The DNA was precipitated with cold ethanol and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to a final concentration of one mg/ml.

Restriction endonuclease analysis of plasmid. Five micrograms of purified plasmid DNA was digested with 5 units of the restriction endonuclease enzymes Hind III, Xba I and Msp I (Life Technologies, Gaithersburg, Maryland, United States of America, USA)) at 37°C for a minimum of 4 hours. Appropriate reaction buffer recommended by the manufacturer was used for each enzyme. The reaction was stopped by the addition of 5 ul of 0.1 M EDTA followed by the addition of 2.5 ul of 0.1% bromophenol blue. The digests were loaded onto 1% agarose slab gels (0.5 x 20 x 20 cm) and electrophoresed overnight in running buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.3) at 60 volts. The gels were stained with ethidium bromide (one ug/ml) and photographed under ultra-violet (UV) illumination.

Randomly amplified polymorphic deoxyribonucleic acid. Randomly amplified polymorphic deoxyribonucleic acid analysis was performed using random primers RAPD1, RAPD7, and ERIC2 under standard conditions using a model 480 programmable thermal cycler (Perkin-Elmer Cetus, Norwalk, Connecticut, USA). The reaction mixture contained 20 mM Tris-HC1 (pH 8.8), 10 mM KC1, 2 mM $MgSO_4$, 10 mM (NH^4) 2 SO_4, 0.1% Triton X-100, 0.2 mM of each of dNTPs (dATP, dGTP, dCTP, and dTTP), 100 pmoles of each primer in separate reactions and 100 ng of DNA preparation in a total volume of 50 ul. Reaction mixtures were overlaid with 50 ul of light mineral oil to prevent evaporation. The sequences of primers were RAPD1: 5'-GGTTGGGTGAGAATTGCACG-3', RAPD7: 5'-

Table 1 - Plasmid content of methicillin-resistant *Staphylococcus aureus* (MRSA).

MRSA isolate identification number	n of plasmids found
7,18,23,25,31,32	8
2,3,12,24,29,34	7
9,15,19,20,36	6
6,30	5
14,28,33,37,38	2

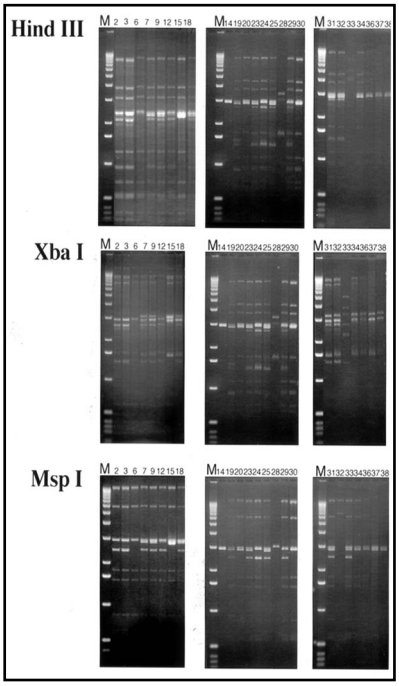

Figure 1 - *Hind* III, *Xba* I, and *Msp* I digestion profiles of plasmid DNA from MRSA clinical isolates. M represents 1 kb DNA ladder. Number above the lanes indicate identities of the 24 MRSA isolates used in the study.

GTGGATGCGA-3', and ERIC2: 5'-AGGTAAGTGACTGGGGTGAGCG-3' as previously described.[21] Polymerase chain reaction was initiated by the addition of 2 units of Vent (exo-) DNA Polymerase (New England BioLabs, Beverly, Massachusetts, USA). After an initial denaturation for 5 min at 94°C, 35 cycles of amplification was carried out. Each cycle consisted of denaturation at 94°C for one min, annealing at 25°C for 1 min and extension at 74°C for 2 min. Amplified DNA was stored at 4°C. Amplified products (10 µl) were analyzed on 1.5% agarose gel electrophoresis in TBE buffer (45 mM Tris base, 45 mM boric acid, one mM EDTA, pH 8.3), stained with ethidium bromide (1 µg/ml), and photographed under UV light.

Pulsed-field gel electrophoresis. A modification of the method of Matushek et al[22] for standard chromosomal DNA preparation and extraction was used. A single bacterial colony was grown overnight in 5 ml of trypticase soy broth. Cells were harvested by centrifugation at 3,000 rpm for 10 minutes and then resuspended in 2.5 ml of PIV buffer (1 M NaCl, 10 mM Tris HCl, pH 7.4). A 0.5 ml aliquot of the cell suspension was combined with 0.5 ml of melted 1.6% low melting point agarose (LMP). This mixture was then pipetted into 300 µl plug mold (Bio-Rad Laboratories, Richmond, California, USA) and allowed to solidify at 4°C for 30 min. Each plug was placed into one ml of lysis solution (6 mM Tris HCl, pH 7.4, 1 M NaCl, 10 mM EDTA, pH 7.5, 0.5% Brij 58, 0.2% deoxycholate, 0.5% sodium lauroyl sarcosine), to which lysozyme (Sigma, St. Louis, Missouri, USA) at 0.5 mg/ml, RNase A (DNase free; Sigma) at 10 mg/ml and 100 µg/ml of lysostaphin (Sigma) were freshly added on the day of the experiment. Plugs were incubated overnight at 37°C in a water bath. After incubation, the lysis solution was replaced with one ml of ESP solution (10 mM Tris-HCl, pH 7.4, 1 mM EDTA), to which proteinase K (Sigma) at a final concentration of 100 mg/ml and 1% of sodium dodecyl sulfate were freshly added on the day of the experiment. The plugs were incubated overnight at 50°C in a water bath. Plugs were washed twice with 5 ml of diluted TE buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA) for 30 min and placed in 2 ml of fresh TE buffer for storage at 4°C until digestion by restriction endonuclease. A plug slice (2-4 mm wide) was suspended in a total volume of 250 µl of the appropriate restriction enzyme buffer and incubated for 15 min on a shaker at 4°C. The buffer was discarded and replaced with a freshly prepared buffer containing 30 units of Sma I (Life Technologies). Digestion mixtures were incubated overnight at 25°C. At the end of incubation; plug slices were washed with diluted TE buffer for one hour at 37°C in a water bath before loading into the gel. The digested DNA was electrophoresed in 1% a garose gel (Sigma) using TBE buffer in a CHEF-DR "II cell (Bio-Rad Laboratories) for 21 hours at 200 V with switching times ramped from 1-20 s. The gel was stained with ethidium bromide (one ug/ml) and photographed under UV light. Lambda DNA concatemers (New England BioLabs, 50-1000 kb) were used as the molecular weight markers.

Results. ***Restriction endonuclease analysis of plasmid.*** The plasmid content of the isolates is shown in **Table 1**. All of the 24 MRSA isolates were typeable with REAP **(Figure 1)**. Each strain was given a unique capital letter code by each of the 3 enzymes,

Table 2 - Methicillin resistant *staphylococcus aureus* (MRSA) strain types as defined by restriction endonuclease analysis of plasmid deoxyribonucleic acid.

MRSA isolate identification number	Strain type	n of isolates
2,3,7,9,12,18,31,32	AAA	8
15,20,23,24,25,29,34,36	BAA	8
6,30	BBA	2
37,38	EEE	2
14	CDD	1
19	DAC	1
28	GDD	1
33	FCB	1
Total	**8 patterns**	**24 isolates**

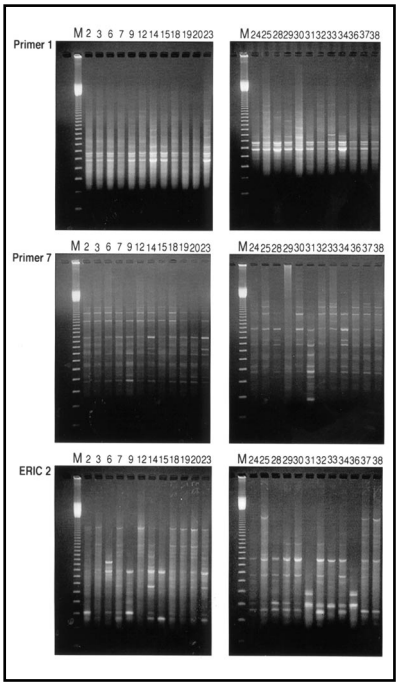

Figure 2 - Randomly amplified polymorphic deoxyribonucleic acid fingerprints generated from genomic DNA of the MRSA isolates with primer 1, primer 7, and ERIC 2. M represents 100 bp DNA ladder. Numbers above the lanes indicate identities of the 24 MRSA isolates used in the study.

Hind III, Xba I and Msp I, which were able to classify the 24 MRSA isolates into 7 (A-G), 5 (A-E), and 5 (A-E) distinct strains, for each enzyme. By visual inspection of the banding patterns, any strain showing greater than 2 band difference was given different letters.[23] Combined analysis of the banding patterns of the 3 enzymes gave a total of 8 distinct strains. Each strain had a 3-letter code starting with the pattern assigned by Hind III, Xba I and Msp I. Three similar letter codes in a single strain signify that the 3 restriction enzymes gave the same banding pattern for that particular strain as shown in **Table 2**. Two major clusters of strains were found by REAP analysis. Strain type AAA and strain type BAA each represented 33.3% of the isolates. Strain types BBA and EEE, each represented 8.3%. The other types CDD, DAC, GDD and FCD each represented 4.2% of the total number of the isolates.

Randomly amplified polymorphic deoxyribonucleic acid. In RAPD analysis, the DNA fingerprints of the strains were compared for similarity by visual inspection of the banding patterns (**Figure 2**). Two fingerprints were considered different when at least one band difference was present in one of the patterns. Variations in band intensity were not considered to be differences. Bands that were too faint to be reproduced were not considered.[24] The fingerprint types were transformed to a 3-letter code (one letter per type per primer). The three-letter code was applied, and new types were defined only when more than one primer gave a different result. Subtypes were defined in case of a single change; such as from AAA to AAC.21 Amplification with RAPD 1 primer generated 12 patterns (A-L), RAPD 7 primer 9 patterns (A-I), and ERIC 2 generated 8 patterns (A-H). Combined analysis allowed the discrimination of 15 distinct strains including 3 subtypes identifiable by a 3-letter code for the generated patterns of RAPD1, RAPD7 and ERIC2 primers (**Table 3**). One major cluster was identified by RAPD analysis, which are strain type AAA and 3 subtypes (AAC, AAD and AAG). This strain represented 54.2% of the total isolates. All of the other strain types represented 4.2% of the total number of the isolates.

Pulsed-field gel electrophoresis. Pulsed-field gel electrophoresis analysis. Electrophoresis of SmaI digests yielded well-resolved patterns consisting of 12-14 fragments per isolate (**Figure 3**). Only 3 major PFGE genotypes were obtained. The interpretation of PFGE banding patterns was carried out visually. A genotype was defined as any fragment pattern, which varied from another pattern with regard to the number of the DNA fragments. Two or more isolates were defined as the same if they showed identical pattern. Isolates that showed patterns different in 1-3 bands were considered as subtypes or genetically related to the major type and were given the same letter code as the major type with a number. Strains showing more

Table 3 - Methicillin-resistant *staphylococcus aureus* syndrome (MRSA) strain types as defined by randomly amplified polymorphic deoxyribonucleic analysis.

MRSA isolate identification number	Strain type	n of isolates
2,3,7,12,15,18,19,20,37,38	AAA	10
6	AAC	1 (subtype of AAA)
9	AAD	1 (subtype of AAA)
36	AAG	1 (subtype of AAA)
14	CBB	1
23	DBB	1
24	ECF	1
25	HDE	1
28	BEF	1
29	GEH	1
30	FDH	1
31	IFG	1
32	JGH	1
33	KHD	1
34	LIH	1
Total	**15 patterns**	**24 isolates**

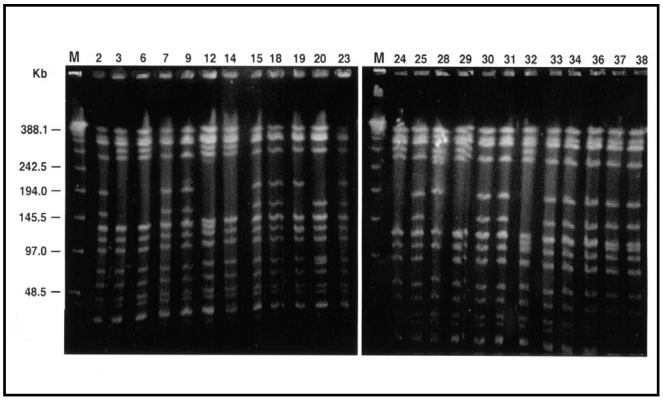

Figure 3 - Agarose gel showing pulsed-field gel electrophoresis patterns of S*ma* I digested genomic deoxyribonucleic of MRSA syndrome clinical isolates. M represents lambda DNA concatemers used as molecular weight markers. Numbers above the lanes indicate identities of the 24 MRSA isolates used in the study.

Table 4 - Methicillin-resistant *staphylococcus aureus* strain types as defined by pulsed-field gel electrophoresis (PFGE) analysis.

MRSA isolate identification number	Strain type	n of isolates
2,7,9,15,18,19,20,23,25 30,31,33,34,36,37,38	A	16
3,6,24,32	A1 (subtype of A)	4
12,14	B	2
22,29	C	2
Total	**4 patterns**	**24 isolates**

than 3 band difference were considered genetically different.[25-27] Among the 24 MRSA isolates analyzed, only 3 PFGE patterns (3 strains and one subtype) were found (**Table 4**). Strain A and its subtype A1 were the dominant strains, representing 83.3 % of the total number of the isolates.

Combined analysis. Five isolates (isolate number: 2,3,7,9, and 18), representing 20.8% of all isolates studied, were of the same genotype by all 3 typing methods. Both PFGE and RAPD identified 7 isolates (isolate number: 6, 15, 19, 20, 36, 37, and 38), representing 29.2% of all isolates studied, as belonging to the same genotype. Two isolates (isolate number: 31 and 32), representing 8.3% of all isolates studied, were found to have the same genotype by PFGE and REAP. RAPD and REAP methods showed that one isolate (isolate number: 12), representing 4.2% of all isolates studied, was found to have the same genotype. Nine isolates (isolates number: 14, 23, 24, 25, 28, 29, 30, 33, and 34), representing 37.5% of all isolates studied, showed different genotypes by each of the 3 methods.

Discussion. Several techniques for tracing and typing microbial pathogens have been used over the years, including traditional and, the more sophisticated molecular methods. Phage typing, biotyping, antibiogram, and serotyping are among the traditional methods that have been useful in epidemiological studies of infectious agents. Nevertheless, these methods were found to be labor intensive, slow for practical values, and variable. In view of this, and utilizing the recent knowledge in molecular biology, newer molecular techniques have been adopted and constantly evaluated, modified and revised. In this study, we evaluated 3 most common molecular methods for typing and fingerprinting DNA of microbial origin using some MRSA isolates. Our main aim was to adopt one or more of these methods for our routine bacterial DNA fingerprinting.

Although many investigators have reported the usefulness of plasmid analysis[17,23,25,28] concerns remain

that the use of an extra chromosomal marker may be misleading. Owing to gain or loss of plasmids over time in different bacterial populations, including MRSA,[29-31] the method is limited to strains which bear several plasmids.[32] However, many isolates carry only one or 2 plasmids, which provides poor discriminatory power for REAP technique.[30] In addition to the disadvantages mentioned above, we feel that this technique remains cumbersome and requires skilled manpower. Randomly amplified polymorphic deoxyribonucleic acid analysis is inexpensive, efficient and no prior nucleotide sequence information of DNA is necessary.[21] It requires very small quantities of DNA, which is especially important when dealing with Gram-positive species where chromosomal preparations are difficult and low concentrations of nucleic acids are usually achieved during extraction.[4] It is a relatively simple method for the rapid identification of strains from endemic and epidemic situations, especially when its use is combined with the use of computerized database that enables one to make correlations between strains analyzed at different times.[33] It can be utilized to determine variations in DNA sequences among closely related species.[4,34] Although the RAPD analysis is easy to perform with a single primer, it becomes more cumbersome when the number of primers increases. Sometimes the intensities of the discriminating bands obtained with the RAPD assay are weak, making the analysis of the different profiles difficult.[35] Our experience with RAPD revealed that it is difficult to standardize in spite of the many advantages cited above. Pulsed-field gel electrophoresis has a discriminatory power that is almost equivalent to or higher than RAPD.[17] Pulsed-field gel electrophoresis can be used to type all bacterial and yeast species, making it the most suitable for molecular typing in clinical laboratories.[23] The wide spread use of PFGE has been hampered by the fact that is a time-consuming procedure, requiring specifically trained people and sophisticated equipments. The need to follow precise standard protocols and to agree on ways to compare restriction patterns obtained in different laboratories has also been seen as a limitation in PFGE typing.[36] In contrast to other bacterial species, the polymorphism provided by PFGE is relatively low as of a high degree of genetic relatedness between MRSA strains. Prevost et al[37] have found only 26 different fingerprints among 239 MRSA isolates from 142 patients identified by PFGE. In a previous study we have shown that 87 out of 94 (93%) of MRSA isolates from Saudi patients analyzed by PFGE belonged to a single clonally related lineage of MRSA.[27] Nevertheless, our extensive work with this technique enables us to classify it as the most reliable among the 3 methods we studied.

The importance of typing *S.aureus* or other bacterial species for epidemiological reasons is acknowledged. However, the choice of the right typing method is often difficult as of inherent technical and theoretical limitations.[32] Our findings are in agreement with other reports where a parallel evaluation of currently available typing systems with staphylococcal isolates demonstrated that no single typing method, including PFGE, clearly prevailed over the others and ultimately, a combination of 2 methods may be the most efficacious.[23,38] It is suggested that one method that would be sensitive and rapid (such as RAPD) to identify all potential patients or sources may be used to screen the isolates early in an epidemiological evaluation, followed by another method (such as PFGE) for detailed strain differentiation. Criteria that are set to determine the suitability of typing schemes include typeability, reproducibility, discriminatory power, and ease of use and interpretation. The choice depends on the resources available in the laboratory and the level of expertise of the personnel involved in the testing. The strength and the weakness of the technique should be understood before results are generated.

Due to the importance of MRSA, it is used in this study for the purpose of illustrating our experience with these methods, even though the number of isolates used is rather small. All 3 typing methods have proven the clonal genetic relatedness observed among distant MRSA isolates. Each method grouped the majority of the isolates into one major genotype. However inter-strain comparison of fingerprint data generated from each method revealed differences in clonal representation of the MRSA isolates. When the PFGE data are compared with RAPD data, it is demonstrated that RAPD differences are not necessarily reflected in the macrorestriction profile by PFGE. This can be explained by the genetic variations as screened with either of these 2 procedures. We have demonstrated previously that PFGE and RAPD provide adequate methods for the reliable discrimination of MRSA strains from diverse sources.[27] Randomly amplified polymorphic deoxyribonucleic acid can be used as a first screen for the rapid detection of genetic polymorphism among strains of geographically distinct MRSA. Finally, our results reaffirm the usefulness of molecular typing as an aid in elucidating the epidemiology of MRSA and are consistent with our previous study that demonstrated the existence of a single, widely spread clone of MRSA in the Kingdom of Saudi Arabia.[27] Multiple typing methods may also be needed to definitively support the contention that epidemiologically related isolates are clonal and possibly cross-transmitted.[28] Our findings demonstrate the importance of using a combination of methods in typing scheme of bacterial isolates. Pulsed-field gele lectrophoresis and RAPD analysis are considered to be the most reliable and reproducible whole-genome typing procedures, with even increased resolution when combined analyses are performed.[39,40]

References

1. Tenover FC, Arbeit RD, Goering RV. How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. ***Infect Control Hosp Epidemiol*** 1997; 18: 426-439.
2. Tenover FC. Plasmid fingerprinting: a tool for bacterial strain identification and surveillance of nosocomial and community-acquired infections. ***Clin Lab Med*** 1985; 5: 413-436.
3. Power EG. RAPD typing in microbiology – a technical review. ***J Hosp Infect*** 1996; 34: 247-265.
4. Dautle MP, Ulrich RL, Hughes TA. Typing and subtyping of 83 clinical isolates purified from surgically implanted silicone feeding tubes by random amplified polymorphic DNA amplification. ***J Clin Microbiol*** 2002; 40: 414-421.
5. Cameron RJ, Ferguson JK, O´Brien MW. Pulsed-field gel electrophoresis is a useful tool in the monitoring of methicillin–resistant Staphylococcus aureus epidemic outbreaks in the intensive care unit. ***Anaesth Intensive Care*** 1999; 27: 447-451.
6. Scott TM, Rose JB, Jenkins TM, Farrah SR, Lukasik J. Microbial source tracking: current methodology and future directions. ***Appl Environ Microbiol*** 2002; 68: 5796-5803.
7. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH et al. Interpreting chromosomal DNA restriction patterns produced by pulsed- field gel electrophoresis: criteria for bacterial strain typing. ***J Clin Microbiol*** 1995; 33: 2233-2239.
8. Gautom RK. Rapid pulsed-field gel electrophoresis protocol for typing of Escherichia coli O157:H7 and other gram-negative organisms in 1 day. ***J Clin Microbiol*** 1997; 35: 2977-2980.
9. Simpson PJ, Stanton C, Fitzgerald GF, Ross RP. Genomic diversity within the genus Pediococcus as revealed by randomly amplified polymorphic DNA PCR and pulsed-field gel electrophoresis. ***Appl Environ Microbiol*** 2002; 68: 765-771.
10. Haddadin AS, Fappiano SA, Lipsett PA. Methicillin resistant Staphylococcus aureus (MRSA) in the intensive care unit. ***Postgrad Med J*** 2002; 78: 385-392.
11. Montesinos I, Salido E, Delgado T, Cuervo M, Sierra A. Epidemiologic genotyping of methicillin-resistant Staphylococcus aureus by pulsed-field gel electrophoresis at a university hospital and comparison with antibiotyping and protein A and coagulase gene polymorphisms. ***J Clin Microbiol*** 2002; 40: 2119-2125.
12. Walker J, Borrow R, Goering RV, Egerton S, Fox AJ, Oppenheim BA. Subtyping of methicillin-resistant Staphylococcus aureus isolates from the North-West of England: a comparison of standardized pulsed-field gel electrophoresis with bacteriophage typing including an inter-laboratory reproducibility study. ***J Med Microbiol*** 1999; 48: 297-301.
13. Santos KRN, Teixeira LM, Leal GS, Fonseca LS, Gontijo Filho PP. DNA typing of methicillin-resistant Staphylococcus aureus: isolates and factors associated with nosocomial acquisition in two Brazilian University Hospitals. ***J Med Microbiol*** 1999; 48: 17-23.
14. Fox AJ, Borrow R, Oppenheim BA. Molecular characterization of methicillin-resistant Staphylococcus aureus. ***Commun Dis Public Health*** 1998; 1: 126-127.
15. van Belkum A, van Leeuwen W, Verkooyen R, Sacilik SC, Cokmus C, Verbrugh H. Dissemination of a single clone of methicillin-resistant Staphylococcus aureus among Turkish hospitals. ***J Clin Microbiol*** 1997; 35: 978-981.
16. Villari P, Farullo C, Torre I, Nani E. Molecular characterization of methicillin-resistant Staphylococcus aureus (MRSA) in a university hospital in Italy. ***Eur J Epidemiol*** 1998; 14: 807-816.
17. Liu PY, Shi ZY, Lau YJ, Hu BS, Shyr JM, Tsai WS et al. Use of restriction endonuclease analysis of plasmids and pulsed-field gel electrophoresis to investigate outbreak of methicillin-resistant Staphylococcus aureus infection. ***Clin Infect Dis*** 1996; 22: 86-90.
18. Murakami K, Minamide W, Wada K, Nakamura E, Teraoka H, Watanabe S. Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction. ***J Clin Microbiol*** 1991; 29: 2240-2244.
19. Birnboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. ***Nucleic Acids Res*** 1979; 7: 1513-1523.
20. Sambrook J, Fritsch EF, Maniatis T. Purification of Plasmid DNA. In: Molecular Cloning: A Laboratory Manual. 2nd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press 1989. p. 1.40-1.52.
21. van Belkum A, Kluytmans J, van Leeuwen W, Bax R, Quint W, Peters E et al. Multicenter evaluation of arbitrarily primed PCR for typing Of Staphylococcus aureus Strains. ***J Clin Microbiol*** 1995; 33: 1537-1547.
22. Matushek MG, Bonten MJ, Hayden MK. Rapid preparation of bacterial DNA for pulsed-field gel electrophoresis. ***J Clin Microbiol*** 1996; 34: 2598-2600.
23. Tenover FC, Arbeit R, Archer G, Biddle J, Byrne S, Goering R et al. Comparison of traditional and molecular methods of typing isolates of Staphylococcus aureus. ***J Clin Microbiol*** 1994; 32: 407-415.
24. de la Puente-Redondo VA, García del Blanco N, Gutiérrez-Martín CB, García-Peña FJ, Rodríguez Ferri EF. Comparison of different PCR approaches for typing of Francisella tularensis strains. ***J Clin Microbiol*** 2000; 38: 1016-1022.
25. Bannerman TL, Hancock GA, Tenover FC, Miller JM. Pulsed-field gel electrophoresis as a replacement for bacteriophage typing of Staphylococcus aureus. ***J Clin Microbiol*** 1995; 33: 551-555.
26. Na'was T, Hawwari A, Hendrix E, Hebden J, Edelman R, Martin M et al. Phenotypic and genotypic characterization of nosocomial Staphylococcus aureus isolates from truma patients. ***J Clin Microbiol*** 1998; 36: 414-420.
27. van Belkum A, Vandenbergh M, Kessie G, Qadri SM, Lee G, van den Braak N et al. Genetic homogeneity among methicillin-resistant Staphylococcus aureus strains from Saudi Arabia. ***Microb Drug Resist*** 1997; 3: 365-369.
28. Hartstein AI, Phelps CL, Kwok RY, Mulligan ME. In vivo stability and discriminatory power of methicillin-resistant Staphylococcus aureus typing by restriction endonuclease analysis of plasmid DNA compared with those of other molecular methods. ***J Clin Microbiol*** 1995; 33: 2022-2026.
29. Branchini MLM, Morthland VH, Tresoldi AT, Nowakonsky AV, Souza Dias MB, Pfaller MA. Application of genomic DNA subtyping by pulsed-field gel electrophoresis and restriction enzyme analysis of plasmid DNA to characterize methicillin-resistant Staphylococcus aureus from two nosocomial outbreaks. ***Diagn Microbiol Infect Dis*** 1993; 17: 275-281.
30. Mulligan ME, Arbeit RD. Epidemiologic and clinical utility of typing systems for differentiating among strains of methicillin-resistant Staphylococcus aureus. I***nfect Control Hosp Epidemiol*** 1991; 12: 20-28.
31. Maslow JN, Mulligan ME, Arbeit RD. Molecular epidemiology: application of contemporary techniques to the typing of microorganisms. ***Clin Infect Dis*** 1993; 17: 153-164.
32. Schlichting C, Branger C, Fournier JM, Witte W, Boutonnier A, Wolz C et al. Typing of Staphylococcus aureus by pulsed-field gel electrophoresis, zymotyping, capsular typing, and phage typing: resolution of clonal relationships. ***J Clin Microbiol*** 1993; 31: 227-232.

33. Bart A, Schuurman IGA, Achtman M, Caugant DA, Dankert J, van der Ende A. Random amplified polymorphic DNA genotyping of serogroup A meningococci yields results similar to those obtained by multilocus enzyme electrophoresis and reveals new genotypes. ***J Clin Microbiol*** 1998; 36: 1746-1749.
34. Soto SM, Guerra B, Gonzalez-Hevia MA, Mendoza MC. Potential of three-way random amplified polymorphic DNA analysis as a typing method for twelve Salmonella serotypes. ***Appl Environ Microbiol*** 1999; 65: 4830-4836.
35. Saulnier P, Bourneix C, Prevost G, Andermont A. Random amplified polymorphic DNA is less discriminant than pulsed-field gel electrophoresis for typing strains of methicillin-resistant Staphylococcus aureus. ***J Clin Microbiol*** 1993; 31: 982-985.
36. van Belkum A, van Leeuwen W, Kaufmann ME, Cookson B, Forey F, Etienne J et al. Assessment of resolution and intercenter reproducibility of results of genotyping Staphylococcus aureus by pulsed-field gel electrophoresis of SmaI macrorestriction fragments: a multicenter study. ***J Clin Microbiol*** 1998; 36: 1653-1659.
37. Prevost G, Jaulhac B, Piemont Y. DNA fingerprinting by pulsed-field gel electrophoresis is more effective than ribotyping in distinguishing among methicillin-resistant Staphylococcus aureus isolates. ***J Clin Microbiol*** 1992; 30: 967-973.
38. Tang Y-W, Waddington MG, Smith DH, Manahan JM, Kohner PC, Highsmith LM et al. Comparison of protein A gene sequencing with pulsed-field gel electrophoresis and epidemiologic data for molecular typing of methicillin-resistant Staphylococcus aureus. ***J Clin Microbiol*** 2000; 38: 1347-1351.
39. VandenBergh MFQ, Yzerman EPF, van Belkum A, Boelens HAM, Sijmons M, Verbrugh HA. Follow-up of Staphylococcus aureus nasal carriage after 8 years: redefining the persistent carrier state. ***J Clin Microbiol*** 1999; 37: 3133-3140.
40. van Belkum A, Struelens M, de Visser A, Verbrugh H, Tibayrenc M. Role of genomic typing in taxonomy, evolutionary genetics, and microbial epidemiology. ***Clin***