

Full Length Research Paper

Pulsed-field gel electrophoresis (PFGE) as an epidemiological marker for typing of methicillin-resistant *Staphylococcus aureus* recovered from KSA

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In this study, pulsed field gel electrophoresis (PFGE) was used for genomic DNA finger printings of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates. Thirty strains of *S. aureus* collected from major hospital laboratories and public health centers, Riyadh, King Saudi Arabia were tested phenotypically by conventional methods and genotypically by multiplex-PCR for direct detection of *S. aureus* 16S rRNA and *mecA* genes. The chromosomal DNA of the isolates was examined by using pulsed-field gel electrophoresis (PFGE) using Smal. Smal cut the chromosomal DNA of the examined MRSA into 9 to 13 fragments, moreover, 16 chromosomal digestion patterns were observed out of the 30 examined isolates. The first pulsed field gel electrophoresis (PFGE1) contains 9 strains recovered from soft tissue infections and surgical wound infections. The second one (PFGE 2) contains 4 MRSA isolates, 3 of which were recovered from skin and soft tissue infections, while one was recovered from wound infection. Moreover, there are 3 chromosomal digestion patterns (PFGE 3, 4 and 5), each pattern involved two strains of MRSA which were recovered from surgical wound infections. A dendrogram of percent similarity, revealed three major clusters, the first cluster containing four groups (17 strains). The second cluster contains one group (12 strains), while the third cluster contains only one strain recovered from deep abscess.

Key words: Fingerprinting, *Staphylococcus aureus*, pulsed field gel electrophoresis, nosocomial infection, multiplex-PCR.

INTRODUCTION

Staphylococcus aureus is one of the most important pathogens causing hospital acquired nosocomial infections (Gardam, 2000; Begier et al., 2004). The prevalence of hospital acquired methicillin-resistant *S.*

aureus (HA-MRSA) differs among different countries and different hospitals, but once MRSA strains are introduced into a hospital they may become endemic. Moreover, MRSA are also emerging in the community and the prevalence of these strains become increase rapidly (Chambers, 2001; Shukla, 2004). The percent of resistant *S. aureus* to methicillin reached 40%, and up till now, it increases year by year. Most of the HA-MRSA treated with vancomycin may in turn increase the risk for selection of vancomycin resistant isolates (Herwaldt,

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1999). Therefore, it is very essential to make complete identification of clonal spread within hospitals (Herwaldt, 1999; Tenover et al., 2001). A molecular typing technique have been used with increasing frequency in studies of the epidemiology of MRSA and also for a better understanding of the evolutionary relationships among MRSA clones (Crisostomo et al., 2001; Enright et al., 2000; Oliveir et al., 2001).

The ideal molecular typing technique must be rapid, easy to perform, reproducible, and capable of demonstrating differences between strains of MRSA with great discriminatory power (Arbeit, 1999). Therefore, several molecular methods have been used with varying success (Schmitz et al., 1998; Tenover et al., 1994). Recently, a simple fast technique called the random amplified polymorphic DNA (RAPD) assay was proposed for genetic analysis (Williams et al., 1990). In this technique, single short primers with arbitrary nucleotide sequences are used in a polymerase chain reaction to amplify genomic DNA. The profiles obtained after electrophoretic separation of the amplification products can be used to fingerprint strains of various prokaryotic and eukaryotic species (Mazurier et al., 1992; Williams, 1990). Among the techniques put forward for typing MRSA, pulsed-field gel electrophoresis (PFGE) of *Sma*I restriction fragments has been the most applied one in epidemiological studies (Aires de Sousa et al., 1998; Lemaitre et al., 1998). PFGE is a valuable tool for epidemiological investigation of nosocomial infections caused by MRSA (Aucken et al., 2002; Bannerman et al., 1995; Deplano et al., 2000). This technique is based on the digestion of bacterial DNA with restriction endonucleases that recognize few sites along the chromosome, generating large fragments of DNA (10 to 800 kb) that cannot be separated effectively by conventional electrophoresis. In PFGE, the orientation of the electric field across the gel is periodically changed (pulsed), allowing DNA fragments on the order of megabase pairs to be effectively separated according to size (Feil et al., 2003; Haley et al., 1995; Murchan et al., 2003). Consequently, PFGE allows for the comparison of chromosomal DNA with much simpler profiles than those generated by high-frequency restriction endonucleases (Haley et al., 1995). Therefore, PFGE has proven itself to be robust enough to type strains with great resolution, and is considered the "gold standard" technique for typing MRSA (Schmitz et al., 1998).

In this study, we applied the pulsed field gel electrophoresis (PFGE) technique, for epidemiological study of nosocomial MRSA infections in the hospitals of Riyadh, King Saudi Arabia.

MATERIALS AND METHODS

Bacterial isolates

Thirty strains of *S. aureus* collected during the period of 2009 from major hospital laboratories and public health centers, Riyadh, King

Saudi Arabia were tested phenotypically by conventional methods and genotypically by multiplex-PCR for direct detection of *S. aureus* 16S rRNA and *mecA* genes of *S. aureus* (Jo-Ann McClure et al., 2006), including 10 strains recovered from skin and soft tissue infection, 8 strains recovered from abscess and 12 strains recovered from surgical wound infection. All isolates were identified according to colonial and microscopical morphology, catalase and coagulase production and novobiocin sensitivity.

Antimicrobial susceptibility test

Antimicrobial susceptibility test to a range of antimicrobial agents, conducted with the following disks (oxacillin, methicillin, chloramphenicol, tetracycline, fusidic acid, gentamicin, erythromycin, ciprofloxacin, clindamycin, Rifampicin and vancomycin), was done adopting the Kirby-Bauer disk diffusion method using Muller-Hinton broth and agar and antibiotics disks (Oxoid Limited, Hampshire, England) according to the recommendations of Clinical Laboratory Standards Institute (CLSI), formally National Committee for Clinical Laboratory Standards, (NCCLS), in 2006.

Extraction of DNA from bacterial isolates

The bacterial isolates were re-suspended in 400 µl Tris-EDTA buffer (pH 8.0) and heated in heat block at 105°C for 25 min, and the DNA was extracted according to the method of Moussa and Shible, (2009).

Multiplex PCR for detection of *S. aureus* species specific 16S rRNA, *mecA* gene

Two sets of primer pairs were used, the first one was Staph756F (5' ACTCTGTTATTAGG GAAGAACA-3') and Staph750R (5'-CCACCTTCTCCGG TTTGTCACC-3'), which could amplify 756 base pair fragments specific for 16S rRNA of *S. aureus* (which serves as an internal control), the second one was *MecA*1(5'-GTAGAAATGACTGAACGTCCGATAA-3') and *MecA*2 (5'-CCAATTCACATTGT TTCGGTCTAA-3'), which could amplify 310 base pair fragments specific for *mecA* gene. The primer design, reaction mixtures and the thermal cyler was adjusted according to (Jo-Ann McClure, et al., 2006)

Plasmid DNA analysis

Plasmid DNA from 30 strains mentioned before was extracted by the modified alkaline lysis method. An overnight culture was harvested, washed with saline-EDTA solution (0.15 M NaCl, 10 mM EDTA [pH 8.0]), resuspended in 0.1 ml of glucose-TE buffer (50 mM glucose, 25 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0]) containing chropeptidase (1 mg/ml; Wako Pure Chemical Co.), and incubated for 30 min at 37°C. To this, 0.2 ml of 0.2 M NaOH-1% (w/v) sodium dodecyl sulfate was added, and the mixture was allowed to stand on ice for 10 min; then 0.15 ml of 3 M potassium-5 M acetate was added, and the mixture was cooled for another 10 min on ice. The mixture was centrifuged at 12,000 × g for 5 min, and the supernatant containing plasmid DNA was extracted once with buffer-saturated phenol mixture. The DNA was electrophoresed through a 0.9% agarose gel, stained with ethidium bromide, and photographed. Lambda/HindIII DNA was used as the size standard.

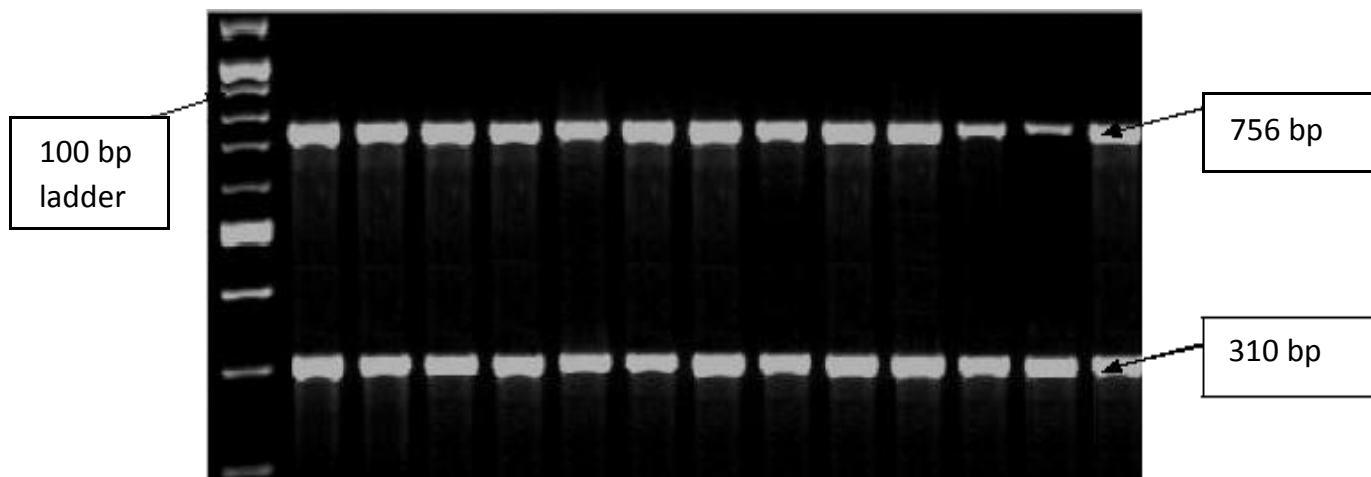


Figure 1. Agarose gel electrophoresis showing multiplex 756 bp fragments specific for *Staphylococcus aureus* 16s rRNA and 310 bp fragments specific for *mecA* gene.

Pulsed field gel electrophoresis

Preparation of DNA

The isolates were grown on tryptic soy agar plates (Becton, Dickinson and Company; Sparks, MD USA) at 37°C overnight. Bacterial cells were suspended in 2.5 ml PIV buffer (1 M Tris NaCl; 10 mM Tris-HCl, pH 7.4) (Sigma; St. Louis, MO USA) and vortexed gently. Aliquots of 500 µl of these suspensions were mixed with 500 µl of 1.6% low melting point agarose (LMP) (Invitrogen; Carlsbad, CA USA) dissolved in sterile water. This agar-cell suspension mixture was immediately distributed into the wells of the plug molds and allowed to solidify at 4°C for 30 min. The plugs were then transferred into tubes containing 1 ml of 1X Lysis buffer (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; 0.5 mg/ml lysozyme; 10 mg/ml RNase A) (Sigma; St. Louis, MO USA) and incubated overnight at 37 °C water bath. The lysis buffer was replaced with 1 ml ESP buffer (10 mM Tris-HCl (pH 7.4); 1 mM EDTA; 100 µg/ml Proteinase K; 1 % SDS) (Sigma; St. Louis, MO USA) and the mixture was incubated overnight at 50 ° C water bath. Bacterial plugs were washed 4 times with 5 ml TE (10 mM Tris-HCl, pH 7.4; 0.1 mM EDTA) (Sigma; St. Louis, MO USA) for 30 minutes each wash at room temperature (Sambrook et al., 1989).

Restriction enzyme digestion

The plug slices of 3 × 5 mm-wide were placed in a 1.5 ml microcentrifuge tube containing 200 µl of 1X restriction buffer (NE Buffer 4) (New England Biolabs; Ipswich, MA USA) with 50 U of SmaI (New England Biolabs; Ipswich, MA USA), and incubated overnight at room temperature (Sambrook et al., 1989).

Electrophoresis

Plug slices were washed for 30 min with 0.5X TBE (Sigma; St. Louis, MO USA) and subsequently inserted on the well of 1% agarose gel and the wells were overlaid with 1% LMP agarose dissolved in 0.5X TBE. After solidification, the gel was run with CHEF-DRIII apparatus (Bio-Rad; Hercules, CA USA) in 3 L of 0.5X

TBE buffer at the following conditions: Initial switch time 1 s, final switch time 20 s; running time 22 h; gradient 6 V/cm; angle 120; temperature 14°C. Gel was stained for 30 min in 300 ml of sterile distilled water containing 1 µg/ml of ethidium bromide (Sambrook et al., 1989).

RESULTS

Antimicrobial susceptibility patterns

Susceptibility to methicillin and oxacillin

All the tested strains were resistant phenotypically to methicillin and oxacillin which was confirmed by multiplex-PCR using the specific primers of *mecA* resistance gene as shown in Figure 1.

Fifteen strains out of 30 tested strains (50%) were resistant to chloramphenicol, 13 strains (43.3%) were resistant to tetracycline, 11 strains (36.6%) were resistant to fusidic acid, 13 strains (43.3%) were resistant to gentamicin, 14 strains (46.67%) were resistant to Clindamycin, 18 strains (60%) were resistant to erythromycin. While 10 strains (33.3%) were resistant to ciprofloxacin while all isolates were susceptible to vancomycin.

Multiplex PCR for detection of *S. aureus* species specific 16S rRNA and *mecA* genes

Multiplex PCR for detection of *S. aureus* species specific 16S rRNA and *mecA* genes were performed. All the 30 strains (100%) previously identified phenotypically as *S. aureus* with bacteriological examination were positive for amplification of 756 base fragments specific for 16S rRNA of *S. aureus* using Staph756 F and Staph750 R primers, also all the tested strains were positive for *mecA*

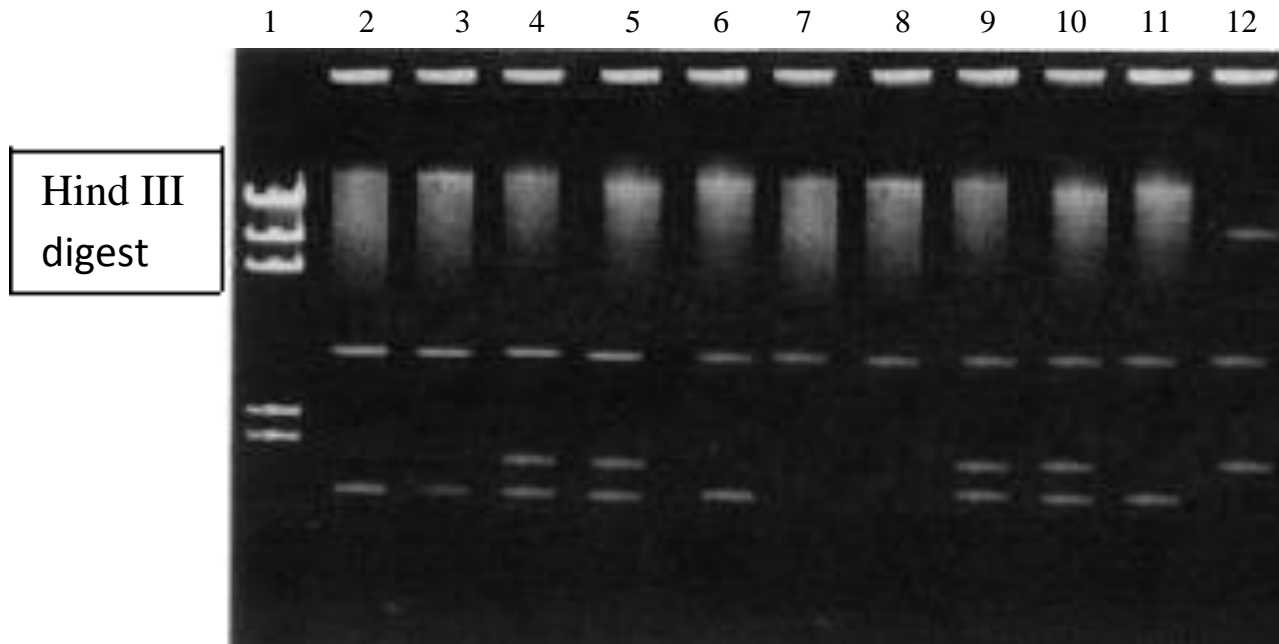


Figure 2. Agarose gel electrophoresis of plasmid DNA from MRSA strains. Lane 1 contains marker DNA fragments (lambda/HindIII).

gene using MecA1 and MecA2 primers and showed amplification of 310 bp fragments as shown in Figure 1.

Plasmid DNA analysis

The 30 strains of MRSA when tested with plasmid DNA analysis showed four patterns of plasmid profile. The first profile (P1) contains 3 plasmids of molecular weight; 25, 3.5 and 1 kbp respectively, the second profile (P2), contains 4 plasmids of molecular weight; 25, 3.5, 1.5 and 1 kbp, the third plasmid profile (P3) contains 2 plasmids of molecular weight; 25 and 3.5 kbp while the fourth plasmid profile (P4), contains 3 plasmids of molecular weight; 10, 3.5 and 1.5 kbp as shown in Figure 2.

Chromosomal DNA analysis by PFGE

Small cut the chromosomal DNA of the examined MRSA into 9 to 13 fragments, moreover 16 chromosomal digestion patterns were observed from the 30 examined isolates as shown in Figure 3. One of them (PFGE 1) containing 9 strains recovered from soft tissue infections and surgical wound infections. The second one (PFGE 2) contains 4 MRSA isolates, 3 of them recovered from skin and soft tissue infections while the other one recovered from wound infection. Moreover, there are 3 chromosomal digestion patterns (PFGE 3, 4 and 5) each one of them containing two strains of MRSA all of them recovered from surgical wound infections. A dendrogram

of percent similarity, calculated with Dice coefficients from the PFGE data using a cutoff of 80%, revealed three major clusters, the first cluster containing four groups (17 strains). The second cluster contains one group (12 strains), while the third cluster containing only one strain recovered from deep abscess as shown in Figure 4.

DISCUSSION

The real time PCR had been used for amplification of a portion of the 16S rRNA for rapid and specific detection of the most common *Staphylococcal* species (including *S. aureus* and *S. epidermidis*). Due to the high costs of equipment and reagents, use of the real time PCR technology has generally been limited to the larger microbiology laboratories. Jo-Ann McClure et al. (2006) developed a new multiplex assay to aid with the early identification of MRSA strains. This assay targets the *staphylococcus* genus-specific 16S rRNA gene, the *luk S/F-PV* and the *mecA* gene. This assay was shown to be 100% accurate and reliable. Moreover this assay is easily amenable to routine clinical use in any molecular biology laboratory with PCR capabilities. One of the main objectives of this study was the use of PCR for rapid and specific detection of 16S rRNA specific for *S. aureus* and the *mecA* gene specific for methicillin / oxacillin resistant, therefore, multiplex –PCR using two primer pairs, one targeting 16s rRNA and the other targeting *mecA* gene were used according to the methods explained by Jo-Ann McClure et al. (2006). Results observed in Figure 1,

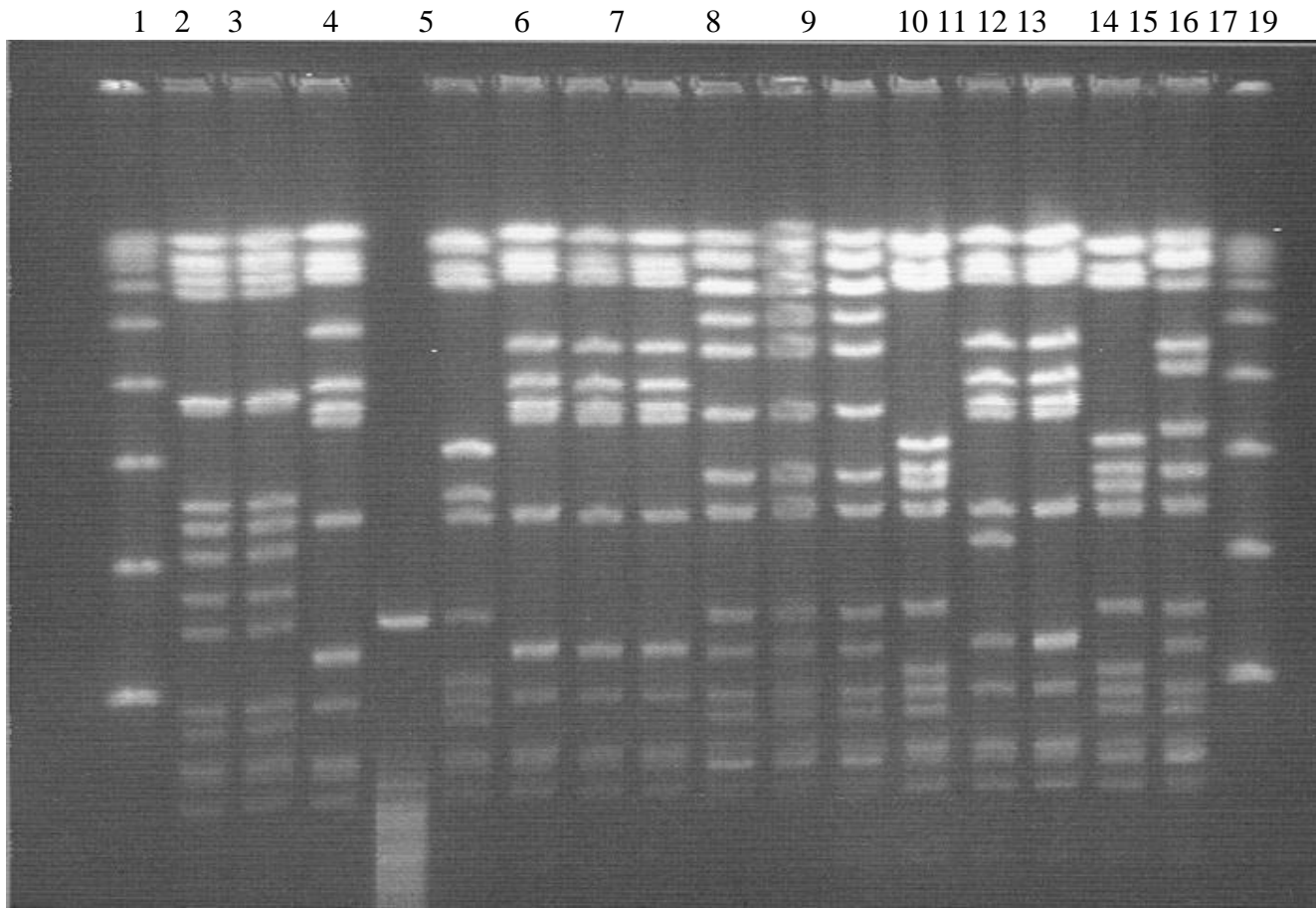


Figure 3. PFGE separation of restriction fragments of the MRSA genome digested with SmaI. A total of 16 MRSA strains from three different hospitals were tested for chromosomal digestion.

revealed positive amplification of 756 base pair fragments specific for 16S rRNA of *S. aureus* and 310 base pair fragments specific for *mecA* gene with all the examined isolates with 100% sensitivity and specificity, confirming the standard microbiological techniques used for evaluation of such strains.

Analysis of bacterial plasmids was the first molecular technique used for the epidemiological investigation of MRSA. This technique consists in the extraction of plasmid DNA and subsequent separation of this DNA by electrophoresis in agarose gels. It is an easily executed and interpreted technique. Therefore the 30 strains of MRSA were examined for plasmid DNA analysis result observed in Figure 2 revealed four pattern of plasmid profile, the first profile (P1), contain 3 plasmids of molecular weight; 25, 3.5 and 1 kbp respectively (8 strains), the second profile (P2), contains 4 plasmids of molecular weight; 25, 3.5, 1.5 and 1 kbp (12 strains), the third plasmid profile (P3) contains 2 plasmids of molecular weight; 25 and 3.5 kbp (2 strains) while the fourth plasmid profile (P4), contains 3 plasmids of molecular weight; 10, 3.5 and 1.5 kbp (8 strains). It is

necessary to say that most of the strains having at least two plasmids of the same molecular weight indicate the great similarity of such strains; therefore, the plasmid profile analysis could help in epidemiological investigation of nosocomial infections caused by MRSA (Archer and Mayhall, 1983; Zuccarelli et al., 1990; Ichiyama et al., 1991; Trindade et al., 2003). However it has several limitations, especially the fact that plasmids can be spontaneously lost or readily acquired by bacteria. Consequently, epidemiologically related isolated can display different plasmid profile (Hartstein et al., 1995). Moreover, many plasmids carry resistance determinants contained in transposons that can be readily lost or acquired, quickly altering the composition of plasmid DNA. Another limitation is the number of plasmids present in these isolates, usually one or two, which leads to poor discrimination between them (Arbeit, 1999; Weller, 2000).

PFGE is a valuable tool for epidemiological investigation of nosocomial infections caused by MRSA (Aucken et al., 2002; Bannerman et al., 1995; Deplano et al., 2000). This technique is based on the digestion of

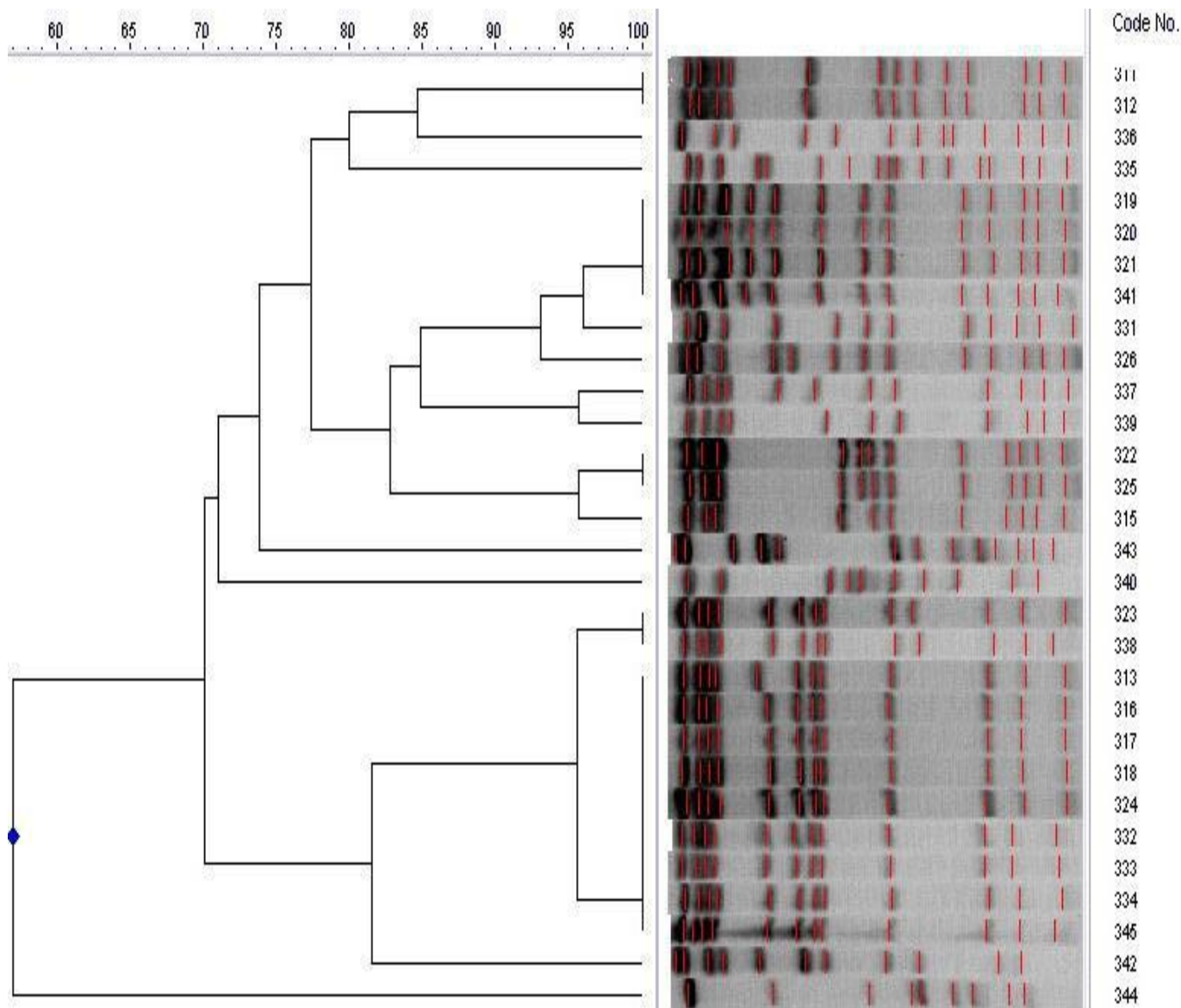


Figure 4. A dendrogram of percent similarity, revealed three major clusters, the first cluster containing four groups (17 strains). The second cluster contains one group (12 strains), while the third cluster containing only one strain recovered from deep abscess.

bacterial DNA with restriction endonucleases that recognize few sites along the chromosome, generating large fragments of DNA (10 to 800 kb) Consequently, allows for the comparison of chromosomal DNA with much simpler profiles than those generated by high-frequency restriction endonucleases (Haley et al., 1995). SmaI cut the chromosomal DNA of the examined MRSA into 9 to 13 fragments, moreover 16 chromosomal digestion patterns were observed from the 30 examined isolates. A dendrogram based on the degree of similarity between isolates, revealed three major clusters, the first cluster containing (17 strains). The second cluster contains (12 strains), while the third cluster containing only one strain recovered from deep abscess, which indicate the ability of PFGE to be used for

epidemiological investigation of nosocomial infections caused by MRSA (Aucken et al., 2002; Bannerman et al., 1995; Deplano et al., 2000). However, there are limitations for the use of PFGE, such as the long time interval until the final results are obtained and the high cost of reagents and specialized equipment used for this technique (Haley et al., 1995). Even though the total number of bands generated is relatively small, there are problems in the interpretation of results, especially in inter-laboratory studies, as small differences in electrophoresis conditions can alter the distance travelled by each band, complicating the comparison between isolates submitted to electrophoresis in different gels (Cookson et al., 1996; Van Belkum, 1998). However, these limitations do not prevent PFGE from being

considered an extremely useful technique used in the characterization of outbreaks (Haley et al., 1995).

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