Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from major hospitals in Riyadh, Saudi Arabia

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**Abstract:** The few studies that have reported the incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) in Saudi Arabia have indicated that a diverse number of circulating MRSA strains have been detected in several major hospitals. Thus, this study was designed to track the presence of MRSA strains in major hospitals in Riyadh, Saudi Arabia, and perform comparative chromosomal DNA analysis of MRSA strains for epidemiological investigation using pulsed-field gel electrophoresis (PFGE). Correlation of the PFGE types generated with microbiological and clinical data of the isolates was attempted. Screening for decreased susceptibility to vancomycin among the isolates was also done. A dendogram was generated using PFGE macrorestriction fragments and 6 types were identified (M1–M6) with M1 being predominant and widespread. A clear link between PFGE types and some clinical and microbiological data available for the strains was found. For example, M1 was statistically associated with male patients, whereas the unique types were associated with female patients, M2 was associated with isolates from wounds and age group <5 years, and M4 was associated with isolates from patients admitted to intensive care units. M5 was highly correlated with low sensitivity to linezolid. No vancomycin-resistant isolates were detected.

**Key words:** MRSA, PFGE, molecular typing, Saudi Arabia.

**Introduction**

After the initial discovery of methicillin-resistant *Staphylococcus aureus* (MRSA) in the UK in 1961 (Jevons 1961), it has subsequently been identified from many countries worldwide (Hiramatsu et al. 2001). The first MRSA outbreak was reported in 1963 in a European hospital (Stewart and Holt 1963). Now MRSA has become an established nosocomial pathogen, with hospital-based outbreaks occurring worldwide (CDC 1999; Enright et al. 2002).

In Saudi Arabia, it is not known when MRSA was first recognized, as an ongoing national surveillance program for MRSA does not exist. The National Library of Medicine search identified only 35 reports of MRSA from 1990 through April 2007 from Saudi Arabia. This relatively low number of reports is in contrast to both the United Kingdom (480 reports) and the United States (826 reports) over com-


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parable periods. A recent study from 7 hospitals in Riyadh indicates that the prevalence of MRSA among Staphylococcus aureus isolates ranges from 12% to 49% with most of the tested isolates giving a prevalence of 27%–33% (Baddour et al. 2006).

Bacterial strain typing distinguishes epidemiologically related or clonal isolates from unrelated isolates. Typing plays an important role in understanding the epidemiology of MRSA and evaluating the effectiveness of infection control and antimicrobial prescribing measures. Pulsed-field gel electrophoresis (PFGE), by far the most widespread molecular typing tool in developed countries, is considered to be the method of choice for DNA fingerprinting of MRSA and other bacterial pathogens (Gordillo et al. 1993; Bannerman et al. 1995).

It appears that the documented data regarding MRSA in Saudi Arabia are relatively scarce despite its prevalence and endemicity in many facilities across the Kingdom. Therefore, the aim of this study was to track the presence of MRSA strains in major hospitals in Riyadh, Saudi Arabia. The performance of comparative chromosomal DNA analysis of MRSA strains for epidemiological was investigated using PFGE, which will help further studies for the ideal ways to combat and control the spread of MRSA. An attempt was made to detect the possible correlation between PFGE types and clinical and microbiological data. Finally, screening for decreased susceptibility to vancomycin among the isolates was done.

Materials and methods

We conducted an active, prospective laboratory surveillance during the period from January 2004 through December 2005 to identify MRSA isolates from patients at 7 tertiary care hospitals in Riyadh, namely, Riyadh Medical Compound (hospital 1), Security Forces (hospital 2), Military (hospital 3), King Faisal Specialist (hospital 4), King AbdulAziz University (hospital 5), King Fahad Medical City (hospital 6), and King Khalid University Hospitals (hospital 7) and their affiliated outpatient clinics. Approximately 512 MRSA isolates have been procured. MRSA strain ATCC 33591 was used as a control strain. Isolates were identified as Staphylococcus aureus by standard microbiological procedures (Kloos and Bannerman 1999). Medical records were reviewed to document patient demographic data, underlying conditions, risk factors, antibiotics given, and previous history of MRSA infection or colonization.

Detection of methicillin resistance

This was carried out according to Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) guidelines using oxacillin agar screen test. If any growth (more than one colony) was detected, the isolate was considered oxacillin or methicillin resistant (NCCLS 2004).

Surveillance of MRSA with decreased vancomycin susceptibility

Vancomycin resistance was tested for by using vancomycin agar screening test as recommended by the CLSI. Any isolate growing 2 or more colonies on this agar was considered positive (NCCLS 2004). Minimal inhibitory concentrations (MICs) for the isolates were determined by the E-test (AB-Biodisk, Solna, Sweden). The criteria used were those of the NCCLS in which sensitive isolates have an MIC £ 4, intermediate isolates have an MIC = 8–16, and resistant isolates have an MIC ≥ 32 μg/mL (NCCLS 2004).

PFGE

A preliminary comparative study has been carried out between PFGE according to the Matushek technique (Matushek et al. 1996), the standardized European technique (Murchan et al. 2003), and the Canadian standardized protocol (Mulvey et al. 2001); the Matushek has been validated and used for the present study, as it gave more consistent separation of the bands and better migration down the agarose gel. The protocol followed is described briefly as follows.

Extraction of DNA

A single colony of MRSA was grown overnight in tryptic soy broth at 37 °C in a shaking incubator. Bacteria were harvested by centrifugation at 3000 rpm/min for 10 min. Cells were resuspended in 2.5 mL PIV buffer (1 mol/L NaCl, 10 mmol/L Tris–HCl, pH 7.4). A volume of 0.5 mL of the cell suspension was mixed with 0.5 mL of 1.6% low-melting-point agarose (Sigma) and pipetted into a 300 μL plug mold. The gel was solidified at 4 °C. Plugs were released into 1 mL lysis solution (0.5 mg lysozyme, 10 mg RNase, 100 μg lysisostaphin, 6 mmol/L Tris–HCl, 1 mol/L NaCl, 10 mmol/L EDTA, 0.5% w/v Brij 58, 0.2% w/v deoxycholate, 0.5% w/v sodium lauryl sarcosine, pH 7.5) and incubated overnight at 37 °C. Lysis solution was replaced with 1 mL ESP (100 μg proteinase K, 10 mmol/L Tris–HCl, 1 mol/L NaCl, 10 mmol/L EDTA, 1% SDS, pH 7.4) and plugs were incubated overnight at 50 °C. Plugs were washed twice in TE buffer (10 mmol/L Tris–HCl, 0.1 mmol/L EDTA, pH 7.4) for 30 min each. They were then stored in fresh TE at 4 °C. A slice of the plug 2–4 mm wide was placed in 30 units of Smal enzyme at 25 °C overnight. Plug slices were washed for 1 h in TE at 37 °C. Smal digests the genome of Staphylococcus aureus into 15–20 restriction fragments with sizes ranging from 10 to 700 kb. The enzyme recognition sequence is CCC GGG.

Electrophoresis

Plugs were inserted into the wells of a 1% agarose prepared in 0.5× TBE buffer (0.9 mol/L Trizma base, 0.9 mol/L boric acid, 20 mmol/L EDTA, 1 L dH2O). Lambda PFGE markers (50 μg/mL) (50–1000 kb) (New England BioLabs, USA) were inserted into appropriate wells in each gel. Smal digested DNA from Staphylococcus aureus ATCC 33591 was used as an internal standard next to concatemeric bacteriophage lambda DNA. Intergel comparison was done via the BioNumerics 2.0 software (Applied Maths, Sint-Martens-Latem, Belgium). Wells were over layered with low-melting-point agarose and allowed to solidify at 4 °C. Gel was placed in the PFGE machine (CHEF DR III) (Bio-Rad Laboratories, Hercules, California, USA) adjusted with the following conditions: temperature, 14 °C; run time, 21 h; volts, 200 V (6 V/cm); and pulse time, initial switch time 1 s and final switch time 20 s. Gels were stained with 0.5 μg/mL ethidium bromide and gel images
were digitized through a UV gel image acquisition camera (Gel Doc XR; Bio-Rad Laboratories).

**Dendograms**

Gel images done for all samples were digitized through a UV gel image acquisition camera (Gel Doc XR; Bio-Rad Laboratories) and saved as TIFF images. Intergel comparison was performed with the BioNumerics version 2.0 analytical software (Applied Maths, Sint-Martens-Latem, Belgium). DNA fragments on each gel were normalized using the molecular weight standards run on each gel to allow comparisons between different gels. Dendograms were generated for combined gel images. A 1.75% band tolerance and an optimization of 4% were selected for use during comparisons of DNA profiles. Cluster analysis was performed by the unweighted pair-group method using arithmetic averages and DNA relatedness was calculated based on Dice coefficient. Similarity coefficient of 80% was applied to the generated dendogram, above which strains were considered to be closely related; this was previously found to correlate with 1–6 band differences on visual examination (Struelens et al. 1992). Control strain MRSA ATCC 33591 was used to assure adequate PFGE band separation.

**Statistical analysis**

Data management was performed using Microsoft Excel 2003 software and statistical analysis was done using SPSS software version 11. Association between categorical variables was examined by \( \chi^2 \) test. Variables were summarized by frequencies and percentages. Multiple logistic regression was used to predict the probability of each PFGE type by the using the following variables as predictors: hospital, patient age group, gender, colonization versus infection, risk factors for infection, site of isolation of MRSA, being inpatient or outpatient, and the hospital service where the patient was admitted.

**Results**

Isolates obtained from selected hospitals with different geographical locations within Riyadh during the period from January 2004 through December 2005 gave the following results.

For all the acquired isolates, screening for oxacillin resistance has been redocumented using the oxacillin agar screening test to rule out any methicillin-sensitive *S. aureus* (MSSA).

Screening for vancomycin resistance showed that until now, no such isolates have been detected. Results of the MIC testing using the E-test (AB-Biodisk, Solna, Sweden) showed that all isolates were susceptible to vancomycin with no evidence of reduced susceptibility to the drug. The MICs to vancomycin fell in the range from 0.25 to 3 \( \mu \)g/mL with most isolates in the 1 and 1.5 \( \mu \)g/mL groups (data not shown). This is reassuring and indicates that vancomycin-resistant *S. aureus* (VRSA) has not yet been established in the Saudi hospitals included in the study.

For all the isolates procured, molecular typing was performed by PFGE by *SmaI* restriction enzyme using the Matushek technique (Fig. 1). An intralinkage homology level of 80% between patterns was assumed as the cutoff for defining a close genetic relationship between strains and was used to define the clusters. Band tolerance was set at 1.75%. A dendogram has been generated using the BioNumerics 2.0 software. According to the results, we categorized our isolates into 6 major groups designated M1 through M6 from the 7 hospitals studied; type M1 was the most prevalent (187 isolates, 36.5%), together with a group of MRSA that apparently did not belong to any clear grouping according to the set parameters and was thus designated unique as shown in Table 1. Figure 2 shows a dendogram of representatives of the 6 PFGE types as well as the unique types. To check the reproducibility of the Matushek technique, *SmaI* digested DNA from *S. aureus* ATCC 33591 was used as an internal standard next to concatemeric bacteriophage lambda DNA. Intergel comparison between markers and *S. aureus* ATCC 33591 from different gels was performed via the BioNumerics 2.0 software using the same parameters used for the test isolates. This showed excellent reproducibility with all the markers and control strains showing over 97% homology except for those from one gel, which were 90% homologous with the rest so that gel was excluded and the isolates were resubjected to PFGE once again whereby the markers fell back into the homologous range of the other markers and the isolates were thus reincluded in the dendogram.

Antibiotic sensitivity patterns for these isolates was determined previously (Baddour et al. 2006) and a correlation between these patterns and PFGE types was sought. The antibiotics tested were vancomycin (V), quinupristin/dalfopristin (Q), linezolid (L), gatifloxacin (G), chloramphenicol (C), mupirocin (M), gentamicin (N), and sulphamethoxazole/trimethoprim (S). PFGE M1 type was commonly associated with antibiotic pattern VQLGCMNS (46/187, 24.6%) followed by antibiotic pattern VQLGM (39/187, 20.9%) then pattern VQLGCMN (20/187, 10.7%) (\( P \leq 0.001 \)). On the other hand, most of PFGE M2 were associated with antibiotic pattern VQLCMNS (70/105, 66.7%) (\( P < 0.001 \)). M3 correlated with pattern VQLGC...
and M4 was almost equally associated with patterns VQLGCM and VQLCM (27/73, 37.0% and 28/73, 38.4%, respectively) ($P < 0.05$). M5 was associated with pattern VQLGCM (9/29, 31.0%) and VQLGM (6/29, 20.7%). M6 were associated with pattern VQLGCMNS (2/7, 28.6%). Almost 1/4 of the unique isolates associated with pattern VQLGMNS (20/84, 23.8%) ($P < 0.001$). No significant association was found between individual antibiotic resistance and the PFGE types except for a high correlation between M5 and low sensitivity to linezolid (3.9%, 19/29) as opposed to the high sensitivity of the other PFGE types to linezolid (96.1%, 468/483) ($P < 0.005$).

M1 was associated with male patients, whereas the unique PFGE types were associated with female patients ($P < 0.005$). M2 was significantly associated with age group <5 years, $P \leq 0.005$, as 25% of M2 belonged to this age group. No significant correlation was found between the PFGE type and the source of the isolates being from inpatients or outpatients. M2 was associated with isolates from wounds (47.9%, $P < 0.005$). M4 was associated with isolates from patients admitted to ICUs (40.4%, $P \leq 0.005$). Other than that, no particular risk factors correlated with any of the PFGE types and no difference was noted in isolates coming from patients clinically infected or merely colonized ($P > 0.05$).

Within the tested hospitals, 53.6% (96/179) of hospital 1 isolates belonged to M1, whereas 31.9% (23/72) of hospital 2 isolates belonged to M2. In hospital 3, 53.6% (37/69) of the isolates belonged to M2. Hospital 4 gave 34.4% isolates that belonged to M4 (22/64), whereas 32.8% (21/64) had unique PFGE patterns. Eighty percent (4/5) of hospital 5 isolates belonged to M2 (the small number of isolates acquired from this hospital, however, confounds statistical relevance). From hospital 6, 45.9% (34/74) belonged to M1. Finally, 40.8% (20/49) of hospital 7 isolates belonged to M1 followed by 34.7% (17/49) from M2. It seems that types M1 and M2 are quite prevalent in the tested hospitals and these associations were statistically significant when compared with the prevalence of the other PFGE types ($P < 0.005$).

Multivariate analysis was performed taking into account hospital of MRSA isolation, patient sex, age group, site of isolation, colonization versus infection, risk factors, inpatient–outpatient, and hospital service in which the patient was admitted. These variables were used jointly to predict the probability of each PFGE pattern. Correcting for the covariables showed that regarding M1, it was found to be more commonly associated with hospital 1 than with other hospitals ($P < 0.05$). Hospital 2 was next in significant association with M1 ($P < 0.05$). No other significant association was delineated by multivariate analysis except for a significant association between hospital 4 and M5 ($P < 0.05$).

### Discussion

Because the procedures are slow and laborious, molecular typing (e.g., PFGE) is usually used a posteriori to track the course of nosocomial infections in an already established outbreak. However, their value in epidemiological database generation is profound. To improve the speed of typing, DNA-sequence-based approaches such as the multilocus sequence typing (MLST) are becoming more frequently used. However, MLST is not suitable for routine surveillance of MRSA because of the high costs involved and the low discriminatory power compared with PFGE.

PFGE is a powerful discriminatory tool for epidemiological investigations and various protocols have been published for MRSA categorization. In the present study, the standardized European protocol was not adopted because of poor discrimination.
band migration requiring great efforts to harmonize protocols and is therefore only partially successful in generating reproducible results (Murchan et al. 2003). The standardized Canadian protocol was not without problems as well, and these problems with band resolution have been previously reported by Mulvey et al. (2001) when implementing their technique and was also reported from another study group in Riyadh (S. Althawadi, M. Alahdal, M. Bohol, personal communication, 2005).

M1 followed by M2 have been isolated from all included hospitals, indicating their widespread existence. This finding does not fully support the results of a previous study that reported the dominance of a single PFGE type in the Kingdom of Saudi Arabia (van Belkum et al. 1997). That study, however, involved testing a small number of isolates by PFGE (7 only) and inferred that conclusion mainly from random amplified polymorphic DNA (RAPD) analysis, although they do state that results for RAPD did not correlate with PFGE for the same strains. Çırlan et al. (2005) also state the predominance of a multilocus type, ST 239, seeming to be the prevailing MRSA clone. They also state that PFGE analysis for that sequence type clone was diversified. However, those studies dealt with only a limited number of isolates and no previous study from Saudi Arabia has involved a comparable number of isolates tested by PFGE. Interestingly, Udo et al. (2006) report similar findings from Kuwait in which they also found 6 different PFGE patterns among their selected isolates. They state that one PFGE type and its subtypes were shared by isolates from the 6 tested hospitals. This is alarming in the sense that these particular clones seem to be able to very effectively fill niches that were essentially uninhabited by MRSA in the recent past. Understanding genetic relatedness becomes more challenging when the strain study population is larger, separated further in time, and recovered from a larger geographic area. From the results of the current study, it appears that M2 is gaining footing in the Saudi hospitals studied and is competing with the previously predominant strain for widespread existence.

Furthermore, there seems to be a clear link between PFGE types versus some clinical and microbiological data available for the strains. For example, M1 was statistically associated with male patients, whereas the unique types were associated with female patients; M2 was associated with isolates from wounds and age group <5 years, and M4 was associated with isolates from patients admitted to ICUs. M5 was highly correlated with low susceptibility to linezolid.

Given the results shown by this study, it might be prudent to assume that M1, being the most prevalent of the PFGE types, would be suspected as the culprit, especially in hospitals 1 and 2 and if the isolate comes from a male patient. In such a case, starting empirical treatment with one of the antibiotics VQLGM, which are common to all the antibiotic patterns are associated with this type. On the other hand, female patients from hospital 4 would be suspected to bear M5, which was shown to be statistically associated with these conditions by multivariate and univariate analysis. In such cases, physicians should refrain from using linezolid because of the low susceptibility. Patients under 5 years of age suffering from wound infections caused by MRSA from hospitals 2, 3, 5, and 7 could be infected by M2. Most of those isolates (66.7%) would be susceptible to the antibiotics VQLGCMNS, thus giving several treatment options. ICU patients from hospital 4 suffering from infections by S. aureus could be victims of M4 and almost 75% of those would be susceptible to VQLCM. Thus, combining the results of the statistical analyses would help in some cases to predict the PFGE type and thus empirical treatment could be started based on the most probable antibiotic susceptibility pattern.

The main focus of the current report was determination of the MRSA genotypes circulating in the country. Further studies are required to position the MRSA genotypes among the international clones.

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