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# Antibiograms and Molecular Subtypes of Methicillin-Resistant *Staphylococcus aureus* in Local Teaching Hospital, Malaysia

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**The objectives of this study were to determine the antibiotypes, SCC*mec* subtypes, PVL carriage, and genetic diversity of MRSA strains from a tertiary hospital. Sixty-six MRSA strains were selected randomly (2003, 2004, and 2007) and tested for the Panton–Valentine leukocidin gene, *mec*A gene, and SCC*mec* type *via* a PCR. The antibiograms were determined using a standard disc diffusion method, and the genetic diversity of the isolates was determined by PFGE. Thirty-four antibiograms were obtained, with 55% of the 66 strains exhibiting resistance to more than 4 antimicrobials. All the isolates remained susceptible to vancomycin, and low resistance rates were noted for fusidic acid (11%), rifampicin (11%), and clindamycin acid (19%). The MRSA isolates that were multisensitive (n=12) were SCC*mec* type IV, whereas the rest (multiresistant) were SCC*mec* type III. Only two isolates (SCC*mec* type IV) tested positive for PVL, whereas all the isolates were *mec*A-positive. The PFGE was very discriminative and subtyped the 66 isolates into 55 pulsotypes (F=0.31–1.0). The multisensitive isolates were distinctly different from the multidrug-resistant MRSA. In conclusion, no vancomycin-resistant isolate was observed. The Malaysian MDR MRSA isolates were mostly SCC*mec* type III and negative for PVL. These strains were genetically distinct from the SCC*mec* type IV strains, which were sensitive to SXT, tetracycline, and erythromycin. Only two strains were SCC*mec* IV and PVL-positive. The infections in the hospital concerned were probably caused by multiple subtypes of MRSA.**

**Keywords:** MRSA, PFGE, SCC*mec* types, *mec*A, PVL gene

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major nosocomial pathogen in Malaysia and worldwide. The prevalence of MRSA in Malaysia ranged from 17% in 1986 to 40% in 2000 [16]. MRSA is often resistant to all penicillins, penems, carbapenems, and cephalosporins. Antimicrobial agents that are effective against MRSA vary between hospitals, as each hospital has its own unique resistance pattern [18].

Thus, knowledge of the clonal relationship of MRSA and its antimicrobial susceptibility patterns can contribute to hospital infection control efforts in monitoring and limiting the spread of MRSA in and between hospitals. Various methods have already been used to type and study the clonality of MRSA, such as PFGE, multilocus sequence typing (MLST) [3], SCC*mec* typing [8], and multiple-locus variable number of tandem repeats analysis (MLVA) [19]. Among these methods, PFGE using SmaI is considered the gold standard for genotyping MRSA and has been carried out by numerous researchers to study the epidemiology of MRSA. In Malaysia, PFGE has already been applied to type MRSA [12–14]. An MRSA status is based on the presence of a staphylococcal cassette chromosome *mec* (SCC*mec*) element carrying the *mec*A gene, which is responsible for methicillin resistance [5]. SCC*mec* elements have two essential components: the *ccr* gene complex (*crr*) and *mec* gene complex (*mec*). Five main subtypes have also been defined based on the combination of these two genes: SCC*mec* type I [class B *mec* and type 1 *ccr*], type II [class A *mec* and type 2 *ccr*], type III [class A *mec* and type 3 *ccr*], type IV [class B *mec* and type 2 *ccr*], and type V [class C *mec* and type 5 *ccr*] [8].

The Panton–Valentine leukocidin (PVL) gene is one of the toxin genes produced by *S. aureus*, and MRSA infection associated with PVL includes skin sepsis and necrotizing pneumonia [21].

Accordingly, the objectives of this study were to determine the SCC*mec* types, PVL, and genetic diversity as determined by PFGE of MRSA isolates from outbreaks and sporadic cases of infection from a local teaching

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hospital to establish a clonal relationship among isolates, as there is limited information on this important nosocomial pathogen in a hospital setting.

## Materials and Methods

### Bacterial Isolates

Sixty-six isolates identified as MRSA in coagulase tests, biochemical tests, and methicillin disc sensitivity tests by the microbiology laboratory in the study hospital were used in this study. A retrospective analysis of the clinical records for 2003 showed an increase in the isolation rate between July and August 2003, for Orthopedic Ward 8U. Six isolates (0307-0308) from these two months, designated as "outbreak" isolates, were available for analysis. Another 19 isolates from January to September 2003 were also randomly picked for further analysis, including 5 strains recovered from hand swabs of staff nurses working in Orthopedic Ward 8U during the same period. Sporadic isolates (n=41) were randomly obtained from various hospital wards for years 2004 (n=29) and 2007 (n=12). In 2007, an increasing number of strains sensitive to a combination of sulfamethoxazole–trimethoprim, tetracycline, and erythromycin (SXT–TE–E) were noted and 12 were randomly selected for comparison.

### Antimicrobial Susceptibility Tests

The antimicrobial susceptibility tests were performed using a disc diffusion method as recommended by the Clinical and Laboratory Standards Institute [2]. Thirteen antimicrobial agents were tested: oxacillin (1 μg), penicillin (1 μg), erythromycin (15 μg), clindamycin (2 μg), vancomycin (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), rifampicin (5 μg), fusidic acid (10 μg), tetracycline (30 μg), netilmicin (30 μg), amikacin (30 μg), and sulfamethoxazole–trimethoprim (25 μg). *S. aureus* ATCC 25923 was used as the quality control. The antibiograms were clustered into dendrogram using BioNumerics Version 5.0 (Applied Maths, Inc.).

### Polymerase Chain Reaction Detection of *mec*A, PVL Gene, and SCC*mec* Types

Crude DNA was prepared by the direct boiling of a 50-μl cell suspension at 99°C for 5 min. The cell lysate was then briefly centrifuged and 5 μl of the supernatant (approx. 10 ng DNA) was used for a PCR. A monoplex PCR was carried out with *mec*A gene primers (5' AAAATCGATGGTAAAGGTTGGC 3' and 5' AGTTCT GCAGTACCGGATTTGC 3') using the PCR cycling previously described by Murakami *et al.* [10]. A separate monoplex PCR was carried out with PVL gene primers (luk-PV-1 5' GTAAAATGTCTG GACATGATCCA 3' and luk-PV-2 5' CAAC/GTGTATTGGATAGC AAAAGC 3') using the PCR cycling conditions described by Lina *et al.* [7]. The PCR products were resolved on a 1.5% agarose gel at 100 V. Confirmation of the amplicons was determined by DNA sequencing and the results were checked against the NCBI blastn (http://www.ncbi. nlm.nih.gov).

The SCC*mec* element typing was determined by multiplex PCR identification of the *ccr* (cassette chromosome recombinase) allele and *mec* complex class, as described by Milheirico *et al.* [8]. The PCR was performed in a total volume of 50 μl containing a 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTPs, and 1 U of *Taq* DNA polymerase (Promega). Based on optimization experiments, the primer concentrations were modified as follows: primers CIF2 and kdp, 0.2 μM of each; *mec*I, RIF5, *ccr*C, SCC*mec*V JI, 0.4 μM of each; SCC*mec* III JI, 0.48 μM; and *dcs*, *ccr*B2, 0.8 μM of each. The PCR products (5 μl) were analyzed by horizontal electrophoresis on 3.0% (w/v) agarose gels, followed by staining with ethidium bromide (0.5 μg/ml). Five positive-control MRSA strains, NCTC10442, N315, 2112, JCSC4469, JCSC2172, JCSC4788, and WIS, were used for SCC*mec* types I, II, III, IV, and V, respectively (Table 1 and Fig. 1).

**Table 1.** Antimicrobial resistance of MRSA isolates.

| Antimicrobial agent | 2003 (n=25) | 2004 (n=29) | *p*-Value[a] | SXT–TE–E–sensitive isolates (2007) (n=12) | All isolates (n=66) |
|---|---|---|---|---|---|
| | No. of resistant isolates (%) | No. of resistant isolates (%) | | n (%) | No. of resistant isolates (%) |
| Oxacillin | 25 (100) | 29 (100) | 0.463[b] | 10 (67) | 64 (100) |
| Penicillin | 25 (100) | 29 (100) | -[c] | 12 (100) | 66 (100) |
| SXT[d] | 25 (100) | 23 (79) | 0.025[b] | 0 (0) | 48 (73) |
| Tetracycline | 8 (32) | 23 (79) | 0 | 0 (0) | 31 (47) |
| Erythromycin | 24 (96) | 29 (100) | 0.463[b] | 8 (67) | 61 (92) |
| Gentamicin | 24 (96) | 25 (86) | 0.358[b] | 1 (8) | 50 (76) |
| Amikacin | 21 (84) | 22 (76) | 0.459 | 1 (8) | 44 (67) |
| Netilmicin | 4 (16) | 12 (41) | 0.042 | 0 (0) | 16 (24) |
| Ciprofloxacin | 24 (96) | 29 (100) | 0.463[b] | 9 (75) | 62 (94) |
| Rifampicin | 1 (4) | 7 (24) | 0.108[b] | 0 (0) | 8 (12) |
| Fusidic acid | 1(4) | 6 (21) | 0.108[b] | 0 (0) | 7 (11) |
| Vancomycin | 0 (0) | 0 (0) | - | 0 (0) | 0 (0) |
| Clindamycin | 2(8) | 9 (31) | 0.036 | 1 (8) | 12 (18) |

[a]By Chi-square test.
[b]By Fisher's exact test.
[c]*p*-Value could not be computed because variables were constant.
[d]sulfamethoxazole–trimethoprim.

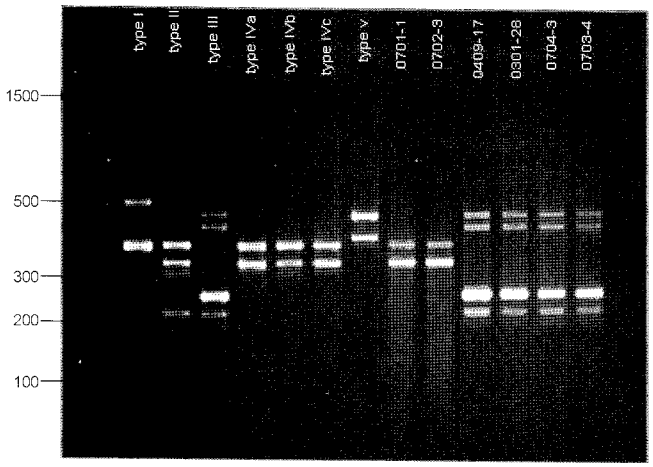


**Fig. 1.** Amplification patterns obtained from SCC*mec* typing using multiplex PCR.

### Macrorestriction of Chromosomal DNA by Pulsed-Field Gel Electrophoresis (PFGE)

The macrorestriction of the chromosomal DNA was carried out as described by Mulvey *et al.* [9] with some modifications. An aliquot of a standardized cell suspension ($1\times10^8$ CFU/ml) was mixed with an equal volume of 1% Seakem Gold agarose containing lysozyme (0.1 mg/ml) and lysostaphin (0.1 mg/ml) to form gel plugs. The gel plugs were incubated in a lysis buffer (50 mM Tris, 50 mM EDTA, 1% Sarcosyl) for 3 h at 37°C, and then further lysed with a fresh lysis buffer containing proteinase K (1 mg/ml) for another 4 h at 55°C. After washing the plugs with a TE buffer (pH 8, 0.1 M Tris, 0.5 M EDTA), a slice of the DNA plug (2 mm) was digested with 10U SmaI at 25°C for 4 h. PFGE of the SmaI-digested DNA was performed with a CHEF DRII (BioRad) using pulsed times of 2.2 to 63.8 sec for 26 h. The PFGE patterns were analyzed using GelComparII (Applied Maths, Inc.) to generate dendrograms based on the unweighted pair group arithmetic means method. The quantitative differences among the profiles were determined by the Dice coefficient, F.

### Statistical analysis

The statistical package for social sciences (SPSS) version 11.5 was used to analyze the data. A comparison of the categorical variables and percentages between groups was performed using a Pearson Chi-square test or Fisher's exact test, as appropriate. The threshold for a significant difference was designated as a $p$ value of <0.05. All the tests were two-tailed.

## RESULTS

### Bacterial Isolates

The 2003 isolates recovered from the patients in the ICU wards were from skin/wound swabs (n=12, 18%), fluid/secretion (n=2, 3%), upper respiratory sites (n=6, 9%), sputum (n=1, 2%), and others (tissue, bone) (n=4, 6%). The sporadic isolates (2004/2007) were recovered from skin/wound swabs (n=14, 21%), fluid/secretion (n=11, 17%), sputum (n=4, 6%), blood (n=8, 12%), and others (tissue, bone) (n=4, 6%) and were from the following wards: orthopedic (n=9, 22%), geriatric (n=7, 17%), medical (n=11, 27%), ICU (n=3, 7%), pediatric (n= 2, 5%), dialysis (n=2, 5%), oncology (n=2, 5%), and others (n=5, 2%).

### Antibiograms

The resistance rates of the isolates towards antimicrobial agents are summarized in Table 1. The rates of antimicrobial resistance between the outbreak (2003) and sporadic isolates (2004, 2007) were significantly different ($p<0.05$) for sulfamethoxazole–trimethoprim, tetracycline, netilmicin, and clindamycin. Thirty-four antibiograms were obtained with 55% of the 66 strains exhibiting resistance to more than 4 antimicrobials. All the isolates remained susceptible to vancomycin, and low resistance rates were noted for fusidic acid (11%), rifampicin (11%), and clindamycin acid (19%). Two isolates were resistant to all antibiotics, except for vancomycin, where one of the isolates was recovered from a wound swab of a patient diagnosed with osteomyelitis, and the other recovered from the tissue secretion of a patient. Based on a 70% similarity, two clusters were obtained. All SXT–TE–E-sensitive isolates were clustered together. It was also noted that isolates that were resistant to fusidic acid were also resistant to rifampicin. No correlation was observed between the antibiograms and the source or wards from which the isolates were obtained.

### PCR Detection of *mec*A, PVL Genes, and SCC*mec* Types of Malaysian Strains

All 66 isolates were *mec*A positive, yet only 2 (3%) of the 66 MRSA harbored the PVL gene (433-bp amplicon). A DNA sequencing analysis of the *mec*A and PVL amplicons confirmed their identities. The two PVL-positive results were SXT–TE–E-sensitive isolates from the year 2007. Only two SCC*mec* types were observed among the Malaysian isolates tested, Type III (n=52) and Type IV (n=12) (Fig 1). Two strains were untypeable, as they did not show any characteristic bands when compared with the positive-control strains. The 12 isolates that were SCC*mec* type IV were all 100% sensitive to SXT, tetracycline, amikacin, fusidic acid, vancomycin, rifampin, netilmicin, and gentamycin, yet exhibited a varying resistance to erythromycin, ciprofloxacin, and oxacillin.

### Genotyping by PFGE

The SmaI-digested chromosomal DNA of MRSA gave 13 to 17 bands (Fig. 2) and subtyped the 66 isolates into 55 PFGE profiles (PFPs). The 6 "outbreak" isolates were closely related, with a difference of fewer than 4 bands (F=0.90). In contrast, the PFPs of the sporadic isolates (F=0.31–1.0) and isolates with phenotype SXT–TE–E (F=0.15–1.0) were more diverse with a difference of more than 4 bands. Thus, evidence of possible cross-contamination in the orthopedic ward was indicated by indistinguishable profiles of isolates

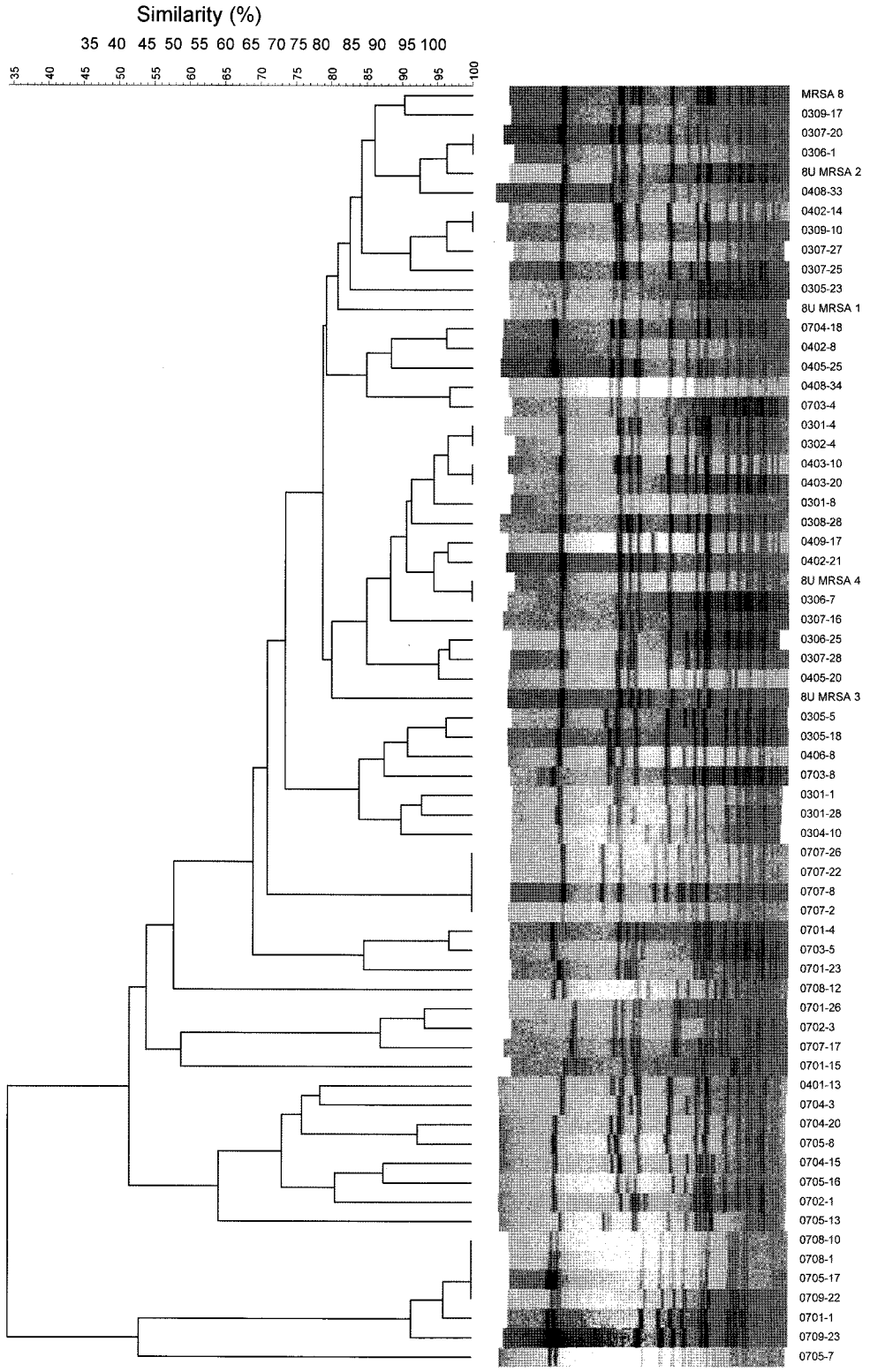


**Fig. 2.** Dendrogram of PFGE SmaI-digested chromosomal DNA profiles of MRSA.

from healthcare workers and wound swabs from patients (isolates MRSA 4 and 0306-7 and MRSA 2 and 0306-1). Overall, there was a diversity of PFGE genotypes of MRSA. One outbreak isolate, 0307-20, exhibited the same PFP as another isolate, 0306-1, isolated in June 2003, a month prior to the outbreak period in the orthopedic ward.

All the SXT–TE–E-sensitive isolates and isolates sensitive to sulfamethoxazole–trimethoprim, tetracycline, netilmicin,

rifampicin, fusidic acid, gentamicin, and amikacin tended to be clustered together and distinct from the multidrug-resistant MRSA.

## DISCUSSION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important nosocomial pathogen in Malaysian hospitals. Thus, the characterization of these strains is important for local epidemiology and surveillance. In this study, all the MRSA strains isolated from various tissues harbored the *mec*A gene, and two were oxacillin-sensitive. However, since not all isolates that carry the *mec*A gene can express it *in vitro* [15], a cefoxitin disc instead of an oxacillin disc has been recommended for a phenotypic resistance test, as it is a better inducer of *mec*A [2].

The PVL gene codes for the leukocidin toxin and is associated with community-acquired MRSA (CA-MRSA). In this study, PVL was only found in 2/66 strains, which was not surprising as the majority of the strains were known to be hospital-acquired MRSA. Meanwhile, 52% of the strains were SCC*mec* type III and mostly multidrug-resistant. A study by Chongtrakool *et al.* [1] showed that SCC*mec* III is common in 8 Asian countries. Among the 12 SCC*mec* type IV MRSA, two isolates were also PVL-positive and multisensitive to erythromycin, sulfamethoxazole–trimethoprim, tetracycline, amikacin, rifampicin, and fusidic acid, where one of these isolates was recovered from tissue from an infected finger, whereas the other was from a shoulder swab from a patient diagnosed with pneumonia. These characteristics are typical of community-acquired MRSA, as a separate study by Sam *et al.* [17] reported similar features (SCC*mec* IV, PVL+, multisensitive) for 9 isolates from the same hospital, implying that the CA-MRSA strains have emerged much earlier than reported [17].

Nosocomial MRSA is often multidrug-resistant towards sulfamethoxazole–trimethoprim, tetracycline, erythromycin, and gentamicin [11]. In this study, 9% of the isolates were simultaneously resistant to fusidic acid and rifampicin, where the isolates were from wound swabs (50%), blood (17%), and fluid/secretion (33%), with one from 2003 and four from 2007, indicating an increase in the occurrence of these isolates. No clinical data were available to ascertain if the patients were receiving fusidic acid or rifampicin therapy. In another study, Norazah *et al.* [13] reported an isolation rate of 5% fusidic-acid- and rifampicin-resistant MRSA from another hospital. In Malaysia, fusidic-acid- and rifampicin-resistant MRSA have increased over time, as the rate of resistance about 20 years ago was less than 1%. In Malaysia, vancomycin is recommended to treat serious MRSA infections, whereas fusidic acid and rifampicin are used in combination for the treatment of bacteremia and cardiovascular infections caused by MRSA. Thus, simultaneous resistance to these two antibiotics is not surprising owing to continued selective pressure.

In this study, 18% and 92% of the isolates were resistant to clindamycin and erythromycin, respectively, although resistance to both agents is mediated by a similar mechanism. The susceptibility of the isolates to clindamycin could be due to the fact that there is a lack of exposure to this drug, since clindamycin is not frequently prescribed in the hospital where the isolates were obtained.

According to Tenover *et al.* [20], an outbreak is the increased incidence of an infectious disease in a specific place during a given length of time that is above the baseline rate for the given time-frame and place. In this study, the outbreak was marked by an increase of MRSA-positive patients in the months of July and August 2003. The 6 outbreak isolates exhibited 6 different profiles with a difference of 1–3 bands, indicating multiple subtypes of a predominant clone. Lessing *et al.* [6] demonstrated that multiple strains of MRSA can circulate in a hospital during an outbreak. A previous study of an outbreak in the Special Care Nursery at the UMMC hospital showed that the isolates were genetically different from the surgical ward isolates [4].

The possibility of cross-contamination between a healthcare worker and a patient could have occurred. One MRSA isolate, MRSA-4, from a staff nurse exhibited the same indistinguishable pattern as an isolate recovered from the wound of a patient. MRSA can also spread from patient to patient through colonized hands of healthcare workers during contact with patients or contaminated materials or medical devices. Thus, the compliance of healthcare workers as regards hand hygiene and infection control regulations is of utmost importance

The indistinguishable clinical isolates (0707-8, 0707-22, and 0707-26) from the same month (July) and same ward (Geriatric 13U) were in one cluster, suggesting the circulation of a particular clone in that ward. However, the transmission of MRSA clones between wards was also indicated, based on indistinguishable isolates. For example, a cerebral spinal fluid isolate, MRSA 0403-10, and sputum isolate, MRSA 0403-20, from Medical Ward 6U and Dialysis Ward 8D, respectively, had similar PFGE profiles. Overall, the MRSA isolates recovered from sporadic cases were very diverse. Forty-one sporadic isolates had 32 PFPs and formed 5 different clusters. Whether this was due to the importation of isolates from other healthcare institutions or the community (non-healthcare-related institutions) requires further investigation. This may also reflect the microevolution of MRSA in a hospital setting. Notwithstanding, further research involving all MRSA isolated in a hospital for an extended period of time needs to be done to address this hypothesis.

In conclusion, although most of the MRSA isolates from the teaching hospital were multidrug-resistant, there were

increasing numbers of multisensitive ones that were genetically distinct from the MDR MRSA. Only two SCC*mec* types (Type III and IV) were noted in this study. Effective hospital control practices need to be reinforced to avoid cross-contamination.

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