ORIGINAL ARTICLE

# DNA Fingerprinting of Methicillin-Resistant *Staphylococcus aureus* (MRSA) by Pulsed-Field Gel Electrophoresis (PFGE) in a Teaching Hospital in Malaysia

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## Summary

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been prevalent in our hospital over the last three years. Differentiation among MRSA strains by DNA typing in addition to antibiotic resistance pattern surveillance is crucial in order to implement infection control measures. The aim of this study was to characterize MRSA isolates from patients admitted to Hospital Universiti Kebangsaan Malaysia (HUKM) by phenotypic (analyses of antibiotic susceptibility pattern) and genotypic (PFGE) techniques to determine the genetic relatedness of the MRSA involved and to identify endemic clonal profiles of MRSA circulating in HUKM. Seventy one MRSA strains collected between January to March 2000 from patients from various wards in HUKM were tested for antimicrobial resistance and typed by pulsed-field gel electrophoresis (PFGE). Four major types of PFGE patterns were identified (A, B, C and D) among MRSA strains. Two predominant PFGE types were recognised, Type A (59.2%) and Type B (33.8%). Most of these strains were isolated from ICU, Surgical wards and Medical wards. MRSA strains with different PFGE patterns appeared to be widespread among wards. Strains with the same antibiotype could be of different PFGE types. Most of isolates were resistant to ciprofloxacin, erythromycin, gentamicin and penicillin. One isolate with a unique PFGE pattern Type D and susceptible to gentamicin was identified as a different clone. Some isolates obtained from the same patient showed different PFGE subtypes suggesting that these patients were infected/colonized with multiple MRSA strains. PFGE analysis suggests that MRSA strains with different PFGE types was propagated within our hospital. The relationship between antibiotic susceptibility and PFGE patterns was independent. The ability of PFGE technique in differentiating our MRSA strains make it a method of choice for investigating the source, transmission and spread of nosocomial MRSA infection, and thus an appropriate control programme can be implemented to prevent the spread of MRSA infection.

*Key Words:* MRSA, pulsed-field gel electrophoresis (PFGE), Malaysia

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## Introduction

Nosocomial infection caused by methicillin-resistant *Staphylococcus aureus* (MRSA) has been a major problem in large medical centers. It has become one of the most significant nosocomial pathogens throughout the world and is capable of causing a wide range of hospital infections[1]. These infections are sometimes life-threatening for patients with severe underlying conditions, despite intensive care. MRSA have a propensity to spread rapidly among patients and staff. There have been a number of reports of MRSA carried by hospital staff causing invasive infection in patients[2,3].

In Hospital Universiti Kebangsaan Malaysia (HUKM), MRSA remains a major clinical problem. Patients infected with MRSA not only have to stay longer in hospital but also require more expensive treatment. MRSA has therefore been designated an alert organism and all patients with MRSA are identified and followed up by the Infection Control nurses. The MRSA rate in HUKM, expressed as the number of infected or colonised patients per 100 discharges, in three years were 1.1 (1998), 0.62 (1999) and 0.6 (2000). Although it indicates some improvement in controlling the MRSA spread, the number is still more than the acceptable threshold that has been set as 0.5/100 admissions for large tertiary hospitals like HUKM[4].

Epidemics of nosocomial infections attributable to MRSA are difficult to control and require strict preventive measures with continous epidemiologic surveillance[5]. Although bacterial identification to species level and determination of the antibiotic susceptibility patterns may be useful, it is frequently not sufficient to determine the epidemiologic relationship among isolates of MRSA. In order to prevent nosocomial transmission, type-investigation of the prevailing MRSA is necessary. DNA typing can be used to confirm or refute the relatedness of isolates and to plan MRSA control programmes[6]. The analysis of chromosomal DNA restriction patterns by pulsed-field gel electrophoresis (PFGE) have come to be regarded as a useful method for investigation the source, transmission, and spread of nosocomial MRSA infection[7,8,9].

This study was designed to characterize MRSA isolates from patients admitted to HUKM by phenotypic (analyses of antibiotic susceptibility pattern) and genotypic (PFGE) techniques to determine the genetic relatedness of the organisms involved and to identify endemic clonal profiles of MRSA circulating in HUKM.

## Materials and Methods

*Bacterial strains*

A total of 71 MRSA isolates were collected from clinical samples from January to March 2000. These strains were isolated from 45 patients from various wards in HUKM, which is a large teaching hospital with about 650 beds. Isolates were obtained from blood (15 isolates), pus swab (32 isolates), tracheal aspirates (13 isolates), throat swab (9 isolates) and cerebrospinal fluid (2 isolates). These isolates were isolated from patients from various wards; ICU (14 isolates), Medical ward (18 isolates), Surgical ward (14 isolates), Burn Unit (4 isolates), Paediatric ward (5 isolates), Orthopaedic ward (10 isolates), High Dependency ward (2 isolates), Trauma ward (1 isolate), Orthopaedic clinic (2 isolates) and Opthalmology clinic (1 isolate). The hospital microbiology laboratory performed the collection and initial identification of bacterial isolates.

*Determination of methicillin resistance and antimicrobial susceptibility testing*

Methicillin resistance was determined by the presence of a zone of $\leq$ 10 mm around a 1 μg oxacillin disc after 24 hours of incubation at 35°C on a Muller Hinton agar plate as recommended by NCCLS[10]. Susceptibility to different antimicrobial agents was performed by the disc diffusion method according to National

Committee for Clinical Laboratory Standard (NCCLS) guidelines[10]. The following antimicrobial agents were tested: ciprofloxacin, erythromycin, fucidic acid, gentamicin, penicillin, chloramphenicol, rifampicin, clindamycin, mupirocin and vancomycin.

*Genomic DNA analysis by PFGE*

A well isolated colony of each isolate was inoculated into 5 ml of trypticase soy broth and incubated overnight at 37°C with shaking. The broth culture was adjusted to a concentration of 1 x $10^9$ cfu/ml. One mililiter of each culture was harvested by centrifugation in an eppendorf tube. The pelleted cells were washed in 1 ml TE buffer (10 mM Tris-HCl, 50 mM EDTA; pH 7.5), and then resuspended in 0.5 ml of the same buffer. Two hundred microliter of the suspension were mixed with 200 µl of 2% pre-warmed low-melting point agarose (Sigma) and 0.0015% lysostaphin (Sigma) added and mixed well before being allowed to solidify in a plug mold (Bio-Rad Laboratories). The solidified plugs were removed from the mold and placed into 2 ml of ES buffer (1% N-laurylsarcosine in 0.5 M EDTA, pH 8.0) containing 0.05% proteinase K (Sigma) and incubated at 55°C overnight with gentle shaking. The plugs were then washed with TE buffer (10 mM Tric-HCl, 1 mM EDTA; pH 8.0) at 4°C. A slice of the plug was cut and digested with 40 units of *SmaI* enzyme (New England Biolabs) according to the manufacturer's instruction. The plugs containing the restricted DNA were inserted into 1.2% agarose gel in 0.5x TBE buffer, and restriction fragment were separated using a contour-clamped homogenous electric field system (CHEF-DR III) from Bio-Rad Laboratories. Electrophoresis was performed for 18 hours with pulse time of 5s to 10s followed by 15s to 20s. The gels were stained with ethidium bromide and photographed under UV light using gel documentation equipment (Gel Doc 1000).

Differences between isolates were determined by visual comparison of DNA fragments.

## Results

PFGE after restriction with *SmaI* resolved genomic DNA of 71 MRSA isolates into 4 main distinct PFGE patterns (A, B, C and D) as shown in Table I. Assuming that a single base mutation in the chromosomal DNA could introduce maximally a three-fragment difference in the restriction pattern[8], strains showing more than three-fragment variations were assumed to represent major patterns (assignment of capital letters), while one- to three- fragment differences were considered to represent subtypes (capital letter with numerical subcode) (Fig I. A and B). PFGE pattern type A was seen in 42 strains (59.2%), which could be further classified into seven subtypes (A1 to A7), of which subtype A1 represented the majority (27 isolates, 64.3%). PFGE type B had 5 subtypes (B1 to B5) and appeared in 24 strains (33.8%). Four strains (5.6%) showed PFGE type C, which could be subdivided into subtypes C1 and C2. PFGE type D was found only in a single isolate which showed susceptibility to gentamicin (Table II). The PFGE type A and B appeared to be widespread among wards of HUKM. Most of these strains were isolated from ICU (5 isolates of type A and 9 isolates of type B), Surgical wards (8 isolates of type A and 6 isolates of type B) and Medical wards (15 isolates of type A and 2 isolates of type B). The PFGE type C were isolated from Paediatric (2 isolates) and Orthopaedic (2 isolates) ward, while PFGE type D was isolated from a patient in a Medical ward. Single or few isolates of the subtypes A and B were obtained from the other wards.

The antibiotic resistance patterns of the 71 MRSA isolates showed 10 different types (Type 1 to 10) as shown in Table II. All MRSA isolates were resistant to erythromycin and penicillin G. The majority were also resistant to ciprofloxacin (95.7%) and gentamicin (98.6%). The percentages of isolates resistant to chloramphenicol, clindamycin, rifampicin and fusidic acid were 23.4%, 16.9%, 15.5% and 12.7%, respectively.

Resistance to mupirocin and vancomycin was not observed. Strains with PFGE type A exhibited all the antibiotypes except antibiotypes 8, 9 and 10, with the majority exhibiting antibiotype 1 (17 isolates) and type 2 (14 isolates). Fourteen strains of PFGE type B showed antibiotype 1.

We also examined the PFGE patterns of multiple MRSA strains isolated from different sources from the same patient. As shown in Table III, the majority of the patients had isolates with the same PFGE pattern. In 3 patients more than one subtype was obtained. One patient (patient 2) with subtypes of PFGE type A (from cerebrospinal fluid) had an additional isolate of subtype B1 (from tracheal aspirate). Some of the patients (patient 2, 3, 5, 6 and 9) were transferred to other wards and were still found to be MRSA carriers after transfer. Patient 16 was transferred in from another hospital to HUKM and was colonized by MRSA strain with PFGE type C.

**Table I: MRSA PFGE major patterns and subtypes**

| PFGE major pattern (no. of isolates) | PFGE subtypes (no. of isolates) | No. of fragment difference compared to subtype A1 |
|---|---|---|
| A (42) | A1 (27) | - |
| | A2 (1) | 1 |
| | A3 (2) | 2 |
| | A4 (3) | 2 |
| | A5 (2) | 2 |
| | A6 (5) | 2 |
| | A7 (2) | 2 |
| B (24) | B1 (13) | 5 |
| | B2 (3) | 4 (1*) |
| | B3 (2) | 6 (2*) |
| | B4 (4) | 4 (3*) |
| | B5 (2) | 6 (3*) |
| C (4) | C1 (2) | 6 |
| | C2 (2) | 7 (2**) |
| D (1) | | 14 |

*, No. of fragment difference compared to subtype B1
**, No. of fragment difference compared to subtype C1

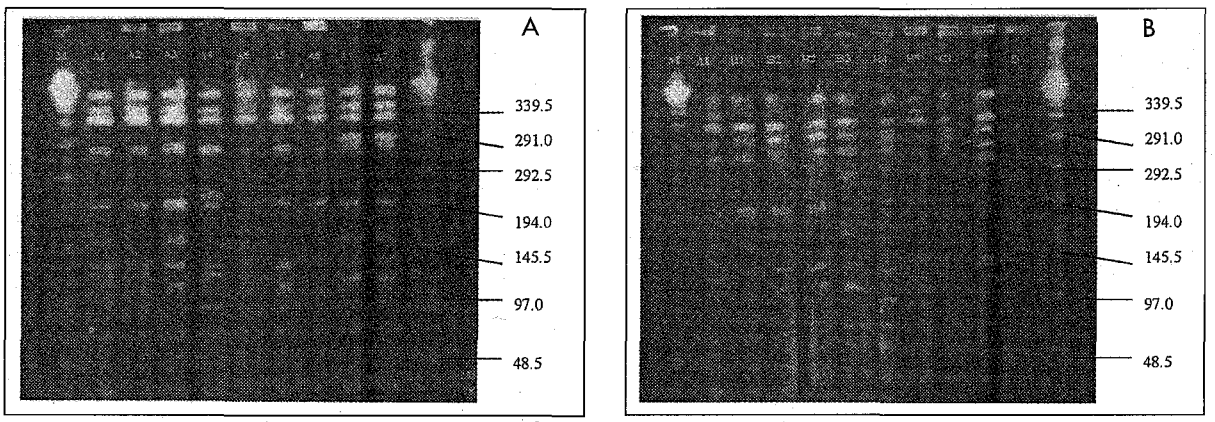


**Fig 1. PFGE patterns observed in MRSA strains from HUKM. Lane M, marker (kilo base pair)**

**Table II: Antimicrobial susceptibility pattern of MRSA isolates**

| Antimicrobial susceptibility pattern* | | | | | | | | | | Antibiotype | PFGE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Cip | Ery | FA | GN | PG | Chl | Rif | Cc | Mup | Va | (no. of isolates) | (no. of isolates) |
| R | R | S | R | R | S | S | S | S | S | 1(31) | A1 (10) |
| | | | | | | | | | | | A3 (2) |
| | | | | | | | | | | | A4 (1) |
| | | | | | | | | | | | A6 (4) |
| | | | | | | | | | | | B1 (10) |
| | | | | | | | | | | | B2 (1) |
| | | | | | | | | | | | B3 (1) |
| | | | | | | | | | | | B4 (2) |
| R | R | S | R | R | R | S | S | S | S | 2 (17) | A1 (10) |
| | | | | | | | | | | | A2 (1) |
| | | | | | | | | | | | A4 (2) |
| | | | | | | | | | | | A6 (1) |
| | | | | | | | | | | | B1 (2) |
| | | | | | | | | | | | B2 (1) |
| S | R | S | R | R | S | R | S | S | S | 3 (2) | A1 (2) |
| R | R | S | R | R | S | S | R | S | S | 4 (9) | A1 (4) |
| | | | | | | | | | | | A5 (1) |
| | | | | | | | | | | | B5 (2) |
| | | | | | | | | | | | C2 (2) |
| R | R | S | R | R | R | S | R | S | S | 5 (1) | A1 (1) |
| R | R | S | R | R | S | R | R | S | S | 6 (1) | A5 (1) |
| R | R | R | R | R | S | R | S | S | S | 7 (7) | A7 (2) |
| | | | | | | | | | | | B5 (3) |
| | | | | | | | | | | | C1 (2) |
| S | R | S | R | R | S | S | S | S | S | 8 (1) | B1 (1) |
| R | R | R | R | R | S | S | S | S | S | 9 (1) | B5 (1) |
| R | R | R | S | R | S | R | R | S | S | 10 (1) | D (1) |

* Cip, Ciprofloxacin; Ery, Erythromycin; FA, Fusidic acid; GN, Gentamicin; PG, Penicillin G; Chl, Chloramphenicol; Rif, Rifampicin; Cc, Clindamycin; Mup, Mupirocin; Va, Vancomycin.

**Table III: PFGE patterns of 43 MRSA strains isolated from 17 patients**

| Patient | Isolate | Site of isolation* | Date of isolation (day/mo/year) | Ward** | PFGE type |
|---|---|---|---|---|---|
| 1 | 1 | Blood | 17/1/2000 | Surg3 | A1 |
| | 2 | Blood | 21/1/2000 | Surg3 | A1 |
| 2 | 3 | CSF | 9/2/2000 | Surg4 | A4 |
| | 4 | CSF | 14/2/2000 | Surg4 | A1 |
| | 5 | T/Asp | 14/2/2000 | ICU | B1 |
| 3 | 6 | T/Asp | 21/2/2000 | ICU | A1 |
| | 7 | T/Asp | 26/2/2000 | Surg4 | A1 |
| 4 | 8 | T/Asp | 21/2/200 | Med5 | A1 |
| | 9 | Throat swab | 24/2/200 | Med5 | A1 |
| | 10 | | 6/3/2000 | Med5 | A1 |
| 5 | 11 | Pus swab | 7/3/2000 | Burn | A1 |
| | 12 | Pus swab | 14/3/2000 | Oftal Clinic | A1 |
| | 13 | Pus swab | 28/3/2000 | Surg3 | A1 |
| 6 | 14 | T/Asp | 9/3/2000 | ICU | A1 |
| | 15 | Throat swab | 27/3/2000 | Paed3 | A1 |
| 7 | 16 | Pus swab | 17/3/2000 | Med3 | A1 |
| | 17 | Throat swab | 29/3/2000 | Med3 | A1 |
| 8 | 18 | Pus swab | 22/3/2000 | Burn | A1 |
| | 19 | Pus swab | 27/3/2000 | Burn | A1 |
| 9 | 20 | Blood | 20/1/2000 | Med2 | A6 |
| | 21 | Blood | 7/3/2000 | Med2 | A6 |
| | 22 | Blood | 10/3/2000 | Med3 | A4 |
| | 23 | Blood | 30/3/2000 | Med3 | A6 |
| 10 | 24 | Pus swab | 9/3/2000 | Orthopaedic | A5 |
| | 25 | Pus swab | 9/3/2000 | Orthopaedic | A5 |
| 11 | 26 | T/Asp | 27/1/2000 | ICU | B1 |
| | 27 | T/Asp | 2/2/2000 | ICU | B1 |
| | 28 | Throat swab | 4/2/2000 | ICU | B2 |
| 12 | 29 | Blood | 29/1/2000 | Surg2 | B1 |
| | 30 | Blood | 12/2/2000 | Surg2 | B1 |
| 13 | 31 | Blood | 1/2/2000 | Surg3 | B1 |
| | 32 | Throat swab | 3/2/2000 | Surg3 | B1 |
| | 33 | Blood | 11/2/2000 | Surg3 | B1 |
| 14 | 34 | T/Asp | 2/2/2000 | ICU | B4 |
| | 35 | Blood | 31/3/2000 | ICU | B4 |
| 15 | 36 | Pus swab | 2/3/2000 | Orthopaedic | B5 |
| | 37 | Pus swab | 13/3/2000 | Orthopaedic | B5 |
| | 38 | Pus swab | 20/3/2000 | Orthopaedic | B5 |
| | 39 | Pus swab | 23/3/2000 | Orthopaedic | B5 |
| 16 | 40 | Pus swab | 1/3/2000 | Paed3 | C1 |
| | 41 | Pus swab | 3/3/2000 | Paed3 | C1 |
| 17 | 42 | Pus swab | 24/2/2000 | Orthopaedic | C2 |
| | 43 | Pus swab | 26/2/2000 | Orthopaedic | C2 |

*CSF, Cerebrospinal fluid; T/Asp, Tracheal aspirate

**ICU, Intensive Care Unit; Surg, Surgical ward; Med, Medical ward; Paed, Paediatric ward

## Discussion

*Staphylococcus aureus*, in particular MRSA has been one of the more serious and problematic nosocomial pathogens in many hospitals[1]. It was first described in England in 1961[11], but has become an increasingly frequent cause of nosocomial infection worldwide since the late 1970s[12,13]. To prevent the spread of the organism, it is important to know what type of MRSA is epidemiologically prevalent and their spread. For the purpose of differentiating isolates, various techniques and methods, such as antibiograms, ribotyping, phage typing, plasmid fingerprinting, PCR-based methods and analysis of chromosomal DNA restriction patterns by PFGE[14,15,16,17] have been developed. At present, PFGE has emerged as the most accurate method for MRSA genotyping[18]. This technique provides an overall view of the organization of the bacterial genome[19].

Screening with several restriction enzymes to cut the genomic DNA of MRSA had been done by Satoshi et al.1991[20]. It was found that *Sma*I cut the genomic DNA of MRSA into a convenient number of fragments (15 - 20 fragments) ranging from 30 to 1,500 kb. Other enzymes cut it into a large number of fragments or a small number of fragments. In the present study, we choose the *Sma*I enzyme to cut the genomic DNA of MRSA isolates.

Among the 71 MRSA isolated and analysed in this study, 4 major types of PFGE pattern were identified in the hospital over a duration of 3 months. There are obviously two major groups of MRSA strains prevalent in the hospital. PFGE type A was found to be the most common type circulating and was observed in 52.9% of isolates. The second most common was PFGE type B, observed in 33.8% of isolates. PFGE type A and B were generally distributed in all wards. This indicates that MRSA strains with PFGE type A and B were the endemic strains and there is a clonal cluster of MRSA strains in the hospital. No particular strain of MRSA was unique to a specific ward. More MRSA strains were isolated in the ICU, Surgical and Medical wards. A comparison of the PFGE type A and B allowed the identification of genetic similarities. The PFGE type B showed differences of four to six bands when compared with PFGE type A. These differences can be explained by changes consistent with two independent genetic events (simple insertion or deletion of DNA or the gain or loss of restriction sites)[21]. Using the criteria in interpreting chromosomal DNA pattern produced by PFGE proposed by Tenover et. al. 1995, these strains were considered to be possible related to the epidemic clone type A, suggesting that the isolates may be derived from the same genetic lineage. Four MRSA strains with PFGE type C were isolated from 2 patients. Subtype C1 was isolated from patient 16 from the Paediatric ward. The patient colonized with subtype C1 was transferred in from another hospital to HUKM, and the referring hospital may have been the source of the strain. However, an MRSA strain with PFGE subtype C2 was isolated on another ward (Orthopaedic ward) on 24th and 26th February, whereas the subtype C1 was isolated on 1st and 3rd March. It is thus possible that the PFGE subtype C2 strain was introduced via a transfer of an unrecognised MRSA carrier prior to identification of the subtype C1. Subtype C2 showed a two band difference when compared with subtype C1 (Fig IB.) which is consistent with changes due to a single genetic event (i.e. a point mutation or an insertion or deletion of DNA)[21]. This subtype C2 is considered to be closely related to the subtype C1 strain. These two subtypes C1 and C2 had different antibiotic susceptibility pattern with antibiogram type 7 and 4, respectively. In this situation, phenotypic method of these isolates cannot confirm their clonal origin relationship which is shown by their distinct antibiotype. However, DNA typing with PFGE clearly showed the relationship between these two subtypes and suggests the possibility that subtype C2 had originated from subtype C1.

In the present study, the only strain with susceptibility to gentamicin had a different PFGE pattern (type D). This indicates the existence of a different clone of MRSA in the hospital. PFGE technique was necessary to identify individual strain and clearly distinguished and confirmed the unrelated strain of type D with other PFGE type, as showed by its distinct antibiotype. This is our preliminary typing data on the strain, and more MRSA strains with susceptibility to gentamicin will further characterised.

The distribution of MRSA strains with different PFGE pattern in the hospital presumably occurred by cross-infection from patient to patient because of increased frequency of patients transfer from ward to ward. The extensive movement of surgeons, physicians, and other hospital personnel among wards especially between the ICU and the other wards such as Surgical, Medical and Orthopaedic wards in the course of their duties, also contribute to the spread of these multiresistant MRSA strains.

Previous epidemiological study of MRSA in our hospital was performed with the use of phenotypic typing method (antibiotic resistant profile typing) and the identification of new or unusual patterns of antibiotic resistance among bacteria isolated from various patients may raise the suspicion of an outbreak or the presence of a new strain[28]. However, antibiotic susceptibility testing has relatively limited use in epidemiological studies because of phenotypic variation. Antibiotic resistance is also affected by selective pressure in hospitals[22] and the resistance characteristic is often plasmid borne which is unstable over time[23]. In our study, strains with the same PFGE-pattern, have different in antibiotic resistant patterns and strains with different PFGE patterns had similar antibiotic susceptibility pattern (Table II). The PFGE types and antibiotic susceptibility patterns observed among our isolates were not linked, indicating that these two markers were independent. This observation was in agreement with another study[24].

Most of the genomic DNA patterns of repeated MRSA strains from the same patient as shown in Table III have the same PFGE type. This demonstrated a reproducibility of the PFGE technique, in which the genomic DNA patterns of the MRSA strains isolated repeatedly from the same patients at different time remained unchanged. Some of the isolates revealed different PFGE subtypes suggesting that these patients were infected with multiple MRSA strain. Two different subtypes of PFGE patterns (subtypes A and subtype B1) were isolated from different sites (cerebrospinal fluid and tracheal aspirate) from the same patient (patient 2), showing that the patient is colonized by more than one strain. This showed the discriminatory power of the PFGE technique in differentiating MRSA strains. Several types of MRSA could be detected in the same patients, particularly when hospitalized for a long period. The chance of acquiring multiple strain colonization was relatively high, especially in immunocompromised hosts with MRSA bacteraemia[23]. This observation would have a profound impact on surveillance of MRSA because it is generally assumed that when MRSA is isolated from one site, it will be the same as a strain from another site and will not require further investigations[25].

Despite the expense and relative labour intensiveness of PFGE, its high discrimination with absolute typeability and acceptable reproducibility, make it the method of choice for the accurate epidemiologic identification of MRSA infection[26]. The genomic DNA digestion patterns permitted a clear differentiation of the MRSA isolates, even those showing the same antibiogram profiles and may lead to the identification of new clones. The fact that epidemiological investigation enables the identification of clusters and may point to a source of infection and route of transmission makes it important to an infection control programme. The present investigation reveals that presence of 2 prevalent MRSA clones (type A

and B) in the hospital together with a small number of sporadic strains of different types. Infected patients who are readmitted or transferred among wards or from other institutions seem to be the major source of different MRSA strains[27]. The presence of a different clone type D with susceptibility to gentamicin warrants further investigations. More strains of MRSA will be further characterised to determine and confirm the epidemiologically related strains which persist in our hospital in order to further enhance the appropriate control measures for preventing the spread of MRSA.

This study is an initial step in establishing our Infection Control Research programme for typing of the multiresistant bacteria isolates involved in nosocomial infection. The PFGE patterns database of these strains will be compared with any outbreak occur to facilitate the control programmes. Monitoring of the strains is important for understanding why certain clones are widely spread in the hospital.

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