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ORIGINAL ARTICLE

# Rapid Molecular Identification of Hospital-acquired Methicillin Resistant *Staphylococcus aureus* (HA-MRSA) Lineages

*[1]Enas Mamdouh Hefzy* and [2]Gamal Mohamedin Hassan*

[1]Lecturer of Medical Microbiology and Immunology, Faculty of Medicine, Fayoum University, Fayoum, Egypt
[2]Associate Professor at Genetics Department, Faculty of Agriculture, Fayoum University, Fayoum, Egypt

***Key words:***

***MRSA, RM test, mecA, pvl***

## ABSTRACT

***Background**: MRSA infection accounts for high percentage of all S. aureus infections in most healthcare facilities. Epidemiological typing is usually done to identify outbreaks or track local spread of strains, and this helps in infection control programmes. A restriction-modification test (RM test) is a rapid, simple and inexpensive method to classify HA-MRSA strains into their appropriate clonal lineage.* **Objective(s):** *Phenotypic and genotypic identification of MRSA isolates from clinical specimens at Fayoum University Hospital (FUH). Detection of mecA and pvl genes by PCR and using RM test to classify HA-MRSA strains into their appropriate clonal lineage were also performed.* **Methodology:** *Seventy non duplicated S. aureus isolates collected from various clinical specimens at FUH were screened for methicillin-resistance and antibiotic resistance profile. Duplex PCR reaction was done for mecA and pvl genes detection and three RM tests for detection of MRSA isolates clonal lineage were also performed.* **Results:** *MRSA prevalence among clinical S. aureus isolates was 57.1%. 17.5% were pvl$^+$. Five different lineages were detected (CC1, CC5, CC8, CC22 and CC45). Clonal lineage CC22 was the predominant one. CC30/ST36 was not detected at all. Eight isolates (20%) were negative for this test. pvl gene was detected in three different clonal lineages; CC5, CC22 and CC1 in variable proportions.* **Conclusion:** *RM tests are simple, easy and inexpensive tests that are suitable for detection of clonal lineages of MRSA isolates at healthcare settings with limited resources. Effective infection control programmes are needed to control the rapidly spreading MRSA at Egyptian hospitals.*

## INTRODUCTION

One year after the introduction of methicillin, the first methicillin-resistant *Staphylococcus aureus* (MRSA) was reported in England in 1961 [1]. This created treatment challenges as MRSA infection accounts for high percentage of all *S. aureus* infections in most healthcare facilities [2]. Methicillin resistance occurs due to the expression of modified penicillin-binding proteins termed PBP2a and PBP2'which are encoded by *mecA* geneamong MRSA isolates. The *mecA* gene is found on a mobile staphylococcal cassette chromosome *mec* element (*SCCmec*) which exists in MRSA as well as methicillin resistant coagulase negative *Staphylococci* [1].

Healthcare-associated MRSA (HA-MRSA) strains affect hospitalized patients and those chronically ill. The emerging community associated MRSA (CA-MRSA) strains was firstly seen in the 1990s. It causes mainly skin and soft tissue infections in healthy adults and children [3]. The differentiation between HA-MRSA and CA-MRSA is indistinct from the clinical and epidemiological points of view. CA-MRSA has type IV or V *SCCmec*, which are smaller than *SCCmec* types I–III typically found in HA-MRSA [4, 5]. Second, virulence determinants such as PVL toxin and phenol-soluble modulins (PSMs) are commonly found in CA-MRSA than in HA-MRSA [6]. Also, antibiotic susceptibility patterns and molecular typing tests can differentiate between HA-MRSA and CA-MRSA strains [4].

The bi-component, pore-forming exotoxin Panton Valentine leucociden (PVL) is encoded by two genes, *lukF-PV* and *lukSPV* and transmitted by bacteriophages. Almost all CA-MRSA strains carry *pvl* genes also a small percentage of methicillin-sensitive *S. aureus* (MSSA) clinical isolates does. PVL has an essential role in bacterial fitness, virulence and transmissibility [3].

Epidemiological typing is usually done to track strains local spread and to identify outbreaks, and this helps in infection control programmes. It is also done to identify local, national or universal trends in strain evolution and dissemination. Consequently, lineage detection is extremely important because it is comparable and reproducible [7] as most localities have a

****Corresponding Author:***
Enas Mamdouh Hefzy
Department of Medical Microbiology and Immunology, Faculty of Medicine, Fayoum University, Fayoum, Egypt, 63514,
E-mail:emh01@fayoum.edu.eg, Tel:+201006441809,

limited number of predominant MRSA lineages in its hospitals [8].

There are about ten major human lineages dominating among *S. aureus* populations [9]. CC5 is the major HA-MRSA lineage [10] that is present all over the world and sometimes it is pandemic [11]. The CA-MRSA lineages include CC8 and CC1 which contains the first observed *pvl*-positive strain [11]. CC22, CC30 and CC45 are other pandemics [10].

The genetic tests; *spa* typing, multi locus sequence typing (MLST), the widely used pulsed field gel electrophoresis (PFGE), and whole genome microarray are time consuming, expensive and technically demanding, so, they are not used in large scale by epidemiological studies or diagnostic laboratories [10]. A restriction-modification (RM) test has now been developed that is simple, inexpensive and rapid as it can be performed by standard laboratory equipment in a few hours with accurate determination of the HA-MRSA lineages. This test is based on three PCRs reactions for *hsdS* gene variants. This specific region within the *S. aureus* genome is very stable and discriminatory [12] but it is particularly variable according to lineage [7], so it is an early indicator of new emerging lineages [10].

The available data about the MRSA strains clonal lineage that could be running in Egypt is scarce. Accordingly, the objective of this study is phenotypic and genotypic identification of MRSA isolates from clinical specimens at Fayoum University Hospital (FUH), the largest tertiary referral hospital at Fayoum Governorate, and using RM test as a rapid, simple and inexpensive method to classify HA-MRSA strains into their appropriate clonal lineage.

## METHODS

For the present study, seventy non duplicated *S. aureus* isolates collected from various clinical specimens between November 2015 and March 2016 at FUH were screened for methicillin-resistance and antibiotic resistance profile. Duplex PCR reaction was done for *mecA* and *pvl* genes detection and three RM tests for detection of MRSA isolates clonal lineage were also performed.

**_S. aureus_ isolation and identification**

*Staphylococci* were isolated from clinical specimens (urine, blood, pus, catheter, surgical wound and soft tissues infections, bone, exudates and respiratory samples). These specimens were first cultured on blood agar and mannitol salt agar (MSA) at 37°C for 48h. Identification of *S. aureus* was confirmed by routine microbiological methods such as Gram staining, growth on MSA, catalase and coagulase production and ß-haemolysis.

**Methicillin susceptibility test**

Phenotypically, MRSA isolates were identified by its resistance to cefoxitin disc on Mueller-Hinton agar plates (Oxoid, England) as recommended by the Clinical and Laboratory Standards Institute (CLSI) [13]. For this, a cefoxitin disk (30 µg) was used. Zone diameter was measured after 24 h. A zone diameter of $\leq$ 21 mm was assigned as MRSA and that with a zone diameter of $\geq$ 22 mm was assigned as MSSA [13].

**Antimicrobial susceptibility testing of MRSA isolates**

Susceptibility testing of MRSA isolates to a panel of antibiotics was performed according to the recommendations of the CLSI [13]. Antibiotics and their susceptibility breakpoints are displayed in Table 2.

**Genomic DNA extraction**

Genomic DNA was extracted from *S. aureus* according to the protocol described by Ida *et al.*, 2001 [14]. Briefly, centrifugation of 1mL of overnight cultures was done at 6,000×g for 3 min. The pellets then were resuspended in 100µL of lysis solution [20 mM Tris–HCl, 140 mM NaCl, 5 mM EDTA (pH 8.0)]. Addition of three units of lysostaphin (Sigma Chemicals) and incubation of the suspension at 37 °C for 3h was done. Then 200µL of distilled water was added. An equal volume of phenol–chloroform (1:1, pH 8) was added after incubation at 95°C for 5 min, and mixed vigorously, and the mix was centrifuged at 10,000×g for 4 min. The aqueous phase was transferred into a fresh tube, and DNA was precipitated with 3M sodium acetate and ice cold isopropanol for 30 min. After the final centrifugation, the pellet was washed with 80 % ethanol. The pellet was then dried and resuspended in 50µL of TE buffer. The extracted DNA stored frozen at -20°C until used.

**Molecular methods**

***Primers and preparation for the duplex PCR assay***

Primers used in this study were obtained from Invitrogen™ (Life Technologies, USA). Sequences of the primers and predicted PCR product size are listed in (Table1). Master mix was obtained from (One PCR™, GeneDirex, Taiwan). All PCR reactions were run in the thermal cycler 2720 (Applied Biosystems, USA). The molecular marker used was a 100 bp ladder and all amplicons were observed under UV radiation.

***Molecular detection of mec*A *and pvl genes***

MRSA identification was confirmed for the presence of *mec*A gene. Duplex PCR assay was used for amplification of *mec*A and *pvl* genes was performed according to Stegger *et al.*, 2012 [15].

PCR reaction was done in a total volume of 25µl reaction tube containing 8.5µl PCR $H_2O$, 12.5µl 2x PCR Master Mix, one µl primer mix1 Forward (0.5 µl of each primer), one µl primer mix2 Reverse (0.5 µl of each primer) and 2µl of template DNA. A positive PCR control and a negative control, with no target DNA, were included in the reaction. The amplification programme included the following: an initial denaturation step at 94°C for five min; 30 cycles of denaturing at 94°C for 30 min, annealing step at 59°C

for one min and extension at 72°C for one min; and a final extension at 72°C for ten min. Eight µL of the final product for each samples were analysed by electrophoresis on ethidium bromide stained 2% agarose gel.

***Detection of S. aureus clonal lineages by RM tests***

Three PCR reactions were performed; each reaction included one forward primer and two different reverse primers. So each reaction could lead to one of three findings; a small PCR product, a large PCR product or no product according to the reaction described by Cockfield *et al.*[10]. PCR reactions were performed in a total volume of 25µl containing 10µl PCR H2O, 12.5µl 2x PCR Master Mix, 1.5 µl primer mix (0.5 µl of each primer) and 1µl of template DNA. The following cycling conditions were used: denaturation at 94°C for five min, followed by 35 cycles of 94°C for 30 sec, 56°C for 30sec and 72°C for two min. A final elongation step was performed at 72°C for 7 min. Ten µL of each amplicon were analysed by agarose gel electrophoresis (1.5% agarose gel), stained with ethidium bromide (0.2 µg/mL).

**Table 1: Target genes, nucleotide sequences and sizes of amplicons for the *mecA*, *pvl* genes and restriction modification (RM) clonal lineages gene-specific oligonucleotide primers**

| Test | Primer name | | Sequence | Product size(bp) |
|---|---|---|---|---|
| *mecA* | Forward primer | *mecA* P4 | TCCAGATTACAACTTCACCAGG | 162 |
| | Reverse primer | *mecA* P7 | CCACTTCATATCTTCTAACG | |
| *pvl* | Forward primer | *pvl*-F | GCTGCACAAAACTTCTTGGAATAT | 85 |
| | Reverse primer | *pvl*-R | AGGACACCAATAAATTCTGGATTG | |
| RM | Forward primer | AF | AGGGTTTGAAGGCGAATGGG | |
| test1 | Reverse primers | AR30 | CAACAGAATAATTTTTTAGTTC | 203 |
| | | AR22 | TCAGAGCTCAACAATGATGC | 990 |
| RM | Forward primer | AF | AGGGTTTGAAGGCGAATGGG | |
| test2 | Reverse primers | AR45 | GGAGCATTATCTGGTGTTTTCC | 722 |
| | | AR1 | GGGTTGCTCCTTGCATCATA | 1037 |
| RM | Forward primer | BF | CCCAAAGGTGGAAGTGAAAA | |
| test3 | Reverse primers | BR8 | CCAGTTGCACCATAGTAAGGGTA | 680 |
| | | BR5 | TCGTCCGACTTTTGAAGATTG | 1071 |

**Statistical Analysis of Data**

Obtained data were analyzed using Statistical Package for Social Science (SPSS) version 16. Descriptive statistics were used to describe variables. Percent and proportions were used for qualitative variables

**Ethical Considerations**

The study was designed and performed according to the ethical standards mentioned in the Declaration of Helsinki, and its modifications thereafter.

## RESULTS

**MRSA isolates and antibiotic susceptibility pattern**

Out of the seventy non-duplicated *S. aureus* isolates, forty isolates (57.1%) were found to be MRSA by cefoxitin disc screening test and confirmed by detection of *mecA* gene by PCR. Half or even more of the MRSA isolates were resistant to gentamycin (55%), tetracycline (60%) and ciprofloxacin (50%). Lower prevalence of resistance to erythromycin (40%), clindamycin (40%), cotrimoxazole (47.5%) and chloramphenicol (35%) was observed. None of the isolates was resistant to vancomycin, teicoplanin or linezolid. Antibiotic susceptibility pattern of the isolates is demonstrated in table 2.

**Table 2: Results of Antibiotic susceptibility testing of MRSA isolates**

| *Antibiotic* | *Zone diameter for resistance* | *Resistant isolates Total = 40* |
|---|---|---|
| Gentamycin (10µg) | ≤12 | 22(55%) |
| Erythromycin (15µg) | ≤13 | 16(40%) |
| Tetracycline (30µg) | ≤14 | 24(60%) |
| Ciprofloxacin (5µg) | ≤15 | 20(50%) |
| Clindamycin (2µg) | ≤14 | 16(40%) |
| Cotrimethoxazole (1.25/23.75µg) | ≤10 | 19(47.5%) |
| Chloramphenicol (30µg) | ≤12 | 14(35%) |
| Rifampicin (5µg) | ≤12 | 8(20%) |
| Vancomycin (30µg) | ≤14 | 0 |
| Teicoplanin (30µg) | ≤10 | 0 |
| Linezolid (30µg) | ≤20 | 0 |

***mecA* and duplex PCR reaction**

Among the forty tested isolates that were reported as MRSA by cefoxitin disc susceptibility test, *mecA* gene was detected among all of it. *pvl* gene was detected in 7(17.5%) of MRSA isolates (Figure 1).

**RM tests**

According to the three PCR reactions included in the RM tests which were performed to detect variable MRSA clonal lineages, five different lineages were detected (table 3& figure 2). Clonal lineage CC22 was the most prevalent as it was detected in 15 (32.5%) isolates. CC30/ST36 was not detected at all. CC5, CC8/239 CC45 and CC1 were detected in 17.5%, 12.5%, 7.5% and 5% respectively. Eight isolates (20%) were negative for this test. *pvl* gene was detected in three different clonal lineages; CC5, CC22 and CC1 in variable proportions (table 3)

**Table 3: Prevalence *pvl* gene among of the different clonal lineages detected by restriction modification (RM) test**

| | Restriction modification (RM) test | | | | | | |
|---|---|---|---|---|---|---|---|
| | Test 1 | | Test 2 | | Test 3 | | |
| ***pvl*** **(Total=7)** | **CC30/ST36** (203 bp) | **CC22** (990 bp) | **CC45** (722 bp) | **CC1** (1037 bp) | **CC8/239** (680 bp) | **CC5** (1071 bp) | **Negative RM test** |
| **Positive** | 0 | 3(7.5%) | 0 | 2(5%) | 0 | 1(2.5%) | 1(2.5%) |
| **Negative** | 0 | 12(30%) | 3(7.5%) | 0 | 5(12.5%) | 6(15%) | 7(17.5%) |
| **Total** | 0 | 15(37.5%) | 3(7.5%) | 2(5%) | 5(12.5%) | 7(17.5%) | 8(20%) |

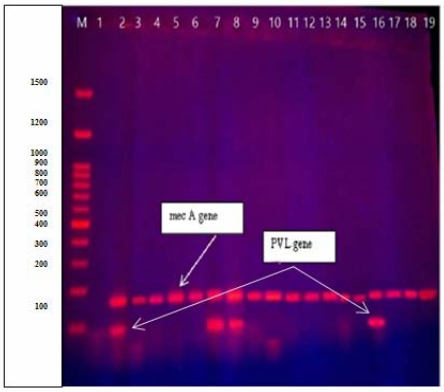


**Figure 1:** Agarose gel electrophoresis pattern of duplex PCR products for MRSA with amplification of *mecA* (162 bp) and *pvl* (85 bp) genes. M: molecular base pair marker (100bp-1500pb); lane 1: negative control; lane 2*: pvl-mecA* positive control; lanes 3-6, 9-15, 17-19 are non-*pvl*-MRSA; lanes 7, 8 and 16 are *pvl*$^{+}$MRSA.

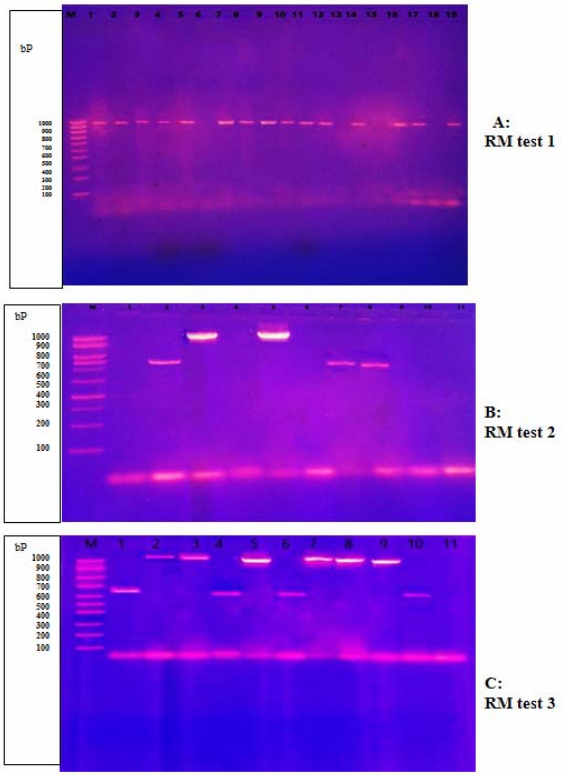


**Figure 2:** Agarose gel electrophoresis pattern of PCR products from the three RM tests, M: molecular base pair marker (100bp-1000pb). **A (RM test 1):** lanes 6, 13, 15 and 18 are negative, and lanes 1- 5, 7-12, 14, 16, 17 and 19 had a band with molecular weight 990 bp (CC22). **B (RM test 2):** Lanes 1, 4, 6 and 9-11 are negative, lanes 3 and 5 had a band with molecular weight 1037 bp (CC1), and lanes 2, 7 and 8 had a band with molecular weight 722 bp (CC45**). C (RM test 3):** Lane 11 is negative in RM test 3, and lanes 2, 3, 5, 7, 8 and 9 had a band with molecular weight 1071 bp (CC5), and lanes 1, 4, 6 and 10 had a band with molecular weight 680 bp (CC8).

## DISCUSSION

Recently, MRSA has been reported as the most frequently detected antibiotic-resistant pathogen around the world, including the Middle East and North Africa [16]. The annual death rate because of MRSA in the United States is estimated as the highest for any infectious pathogen [17]. There is scarce and insufficient molecular epidemiological data about spread of *S. aureus* and MRSA in the Middle East, including Egypt [16, 18-20]. Also, the running strains were found to be fluctuating due to the introduction of new strains with the universal exchange of several clones [2].

In this study, we described the prevalence of MRSA and we used RM tests as simple, rapid tests for epidemiological identification of clinical isolates collected from FUH. The overall MRSA prevalence in this study was 57.1%. This concurs with Al Laham *et al.,* 2015, who found 56.3% prevalence of MRSA in Gaza [21].

In a previous study by the same authors which has investigated the prevalence of MRSA nasal carriage among healthcare workers at FUH, a carriage rate of 13.5% was found. This high prevalence of MRSA carriage may accounts for the high prevalence of nosocomial MRSA described in the current study [22].

Prevalence of nosocomial MRSA in the Eastern Mediterranean countries was the highest among the Mediterranean countries [21]. A meta-analysis study aimed to reduce the increasing spread of invasive MRSA infections from Eastern and Southern Mediterranean countries from 2003 to 2005, found an overall high prevalence in Egypt (52%), with statistically significant increasing course was observed in the Egyptian hospitals [23]. This finding was supported by observations of this study.

Prevalence of MRSA infections in other Mediterranean countries as Jordan and Cyprus was 56% and 55% respectively, which parallels our findings [23]. Recent studies in Lebanon [24], Saudi Arabia [25] and Jordan [26] have observed nosocomial MRSA rates of 30%, 50%, and 62% respectively. A nosocomial report from Palestine West Bank found a MRSA prevalence of 8.7% only [27]. Therefore, the MRSA prevalence mentioned in this study can be considered among the highest recognized in our region. In Western Europe, the prevalence of MRSA among *S. aureus* nosocomial isolates was found to be ranged from 5% to 54 % [28].

The present study identified five MRSA-associated clonal backgrounds (CC1, 5, 8, 22, and 45) with CC22 as the predominant clone. CC22 was also found to be the leading clone in a community based nasal carriage surveillance in Palestine that addressed the epidemiology of *S. aureus* in Gaza [29]. The same findings were also described in two studies from Italy[30,31].

Unfortunately, molecular epidemiological data from Egypt are limited [32]. In one study about nasal carriage in outpatients in Saudi Arabia and Egypt [33], MRSA was found in 25% of the Saudi Arabian outpatients and in 32% of the Egyptian outpatients. Prevalence of *pvl*$^+$ MRSA isolates was 12% in Saudi Arabia and 15% of Egyptian isolates which was similar to our results. In this study, four clonal complexes, CC5, CC8, CC22, and CC80, were reported in both Saudi Arabia and Egypt [33]. All these isolates were identified in the current study except for CC80.

However our findings differ from those observed in the Palestine West Bank which have identified CC1, CC5 [18] and CC239 [34] as the dominant clones. Within the Middle Eastern region, CC22 was found as the predominant clones in Kuwait [35], Abu Dhabi [36], and Riyadh [25] with frequencies that ranged from 12.5% to 20%. In Jordan, CC22 isolates were 100% in a community-based carriage study [37]. While in Romania, the most common MRSA strain was CC1-MRSA [38] with high prevalence of *pvl*$^+$ MRSA [38]. The same clone (CC1) was also previously reported from Australia [39] and the Middle East [36, 40].

To assign MRSA clinical isolates to its clonal lineages, the rapid and simple RM tests were used. It provides the most important point of information: the lineage or CC that corresponds to difference in hundreds of genes. In a previous study, the RM tests were accurate and correctly allocated all 102 MRSA isolates to their exact lineage. In the current study, only eight (20%) isolates have tested negative in the three RM tests. This means that the majority of the clones running in our hospital are detectable by this test. Isolates that test negative by this test can be further typed with *spa* typing, MLST, or microarray to identify unusual lineages.

The RM test would be appropriate for infection control units, diagnostic laboratories, and research laboratories dealing with epidemiological analysis of large strain collections. Nonetheless, the RM test could identify the movement of CA-MRSA into hospitals or new lineages emergence within a geographical area or particular healthcare settings.

When subtyping MRSA isolates testing for virulence factors and toxins such as *pvl* could be beneficial. PVL is a virulence factor of CA-MRSA but it is not the only factor responsible for CA-MRSA outbreaks [41]. *pvl* is highly common among CA-MRSA all over the world [42]. For example, in Australia almost all of CA-MRSA isolates were *pvl*$^+$ [43]. In two previous Egyptian studies, 16-19% of MRSA isolates were *pvl*$^+$ which was similar to the present study which found *pvl* gene in 17.1% of MRSA isolates [22, 32]. Generally, search for virulence genes is recommended to predict diseases that can be associated with these virulence factors so appropriate measures could be applied, if possible, for disease management.

## CONCLUSION

RM tests are simple, easy, accurate and inexpensive tests that are suitable for detection of clonal lineages of MRSA isolates at healthcare settings with limited resources. MRSA prevalence among clinical *S. aureus* isolates was 57.1% with 17.5% of it were *pvl*$^+$. CC22 was the predominant lineage. Effective infection control programmes are needed to control the rapidly spreading MRSA at Egyptian hospitals.

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