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ORIGINAL ARTICLE

Localization and Characterization of *MupA* Gene in High and Low-Level Mupirocin Resistant Methicillin Resistant *Staphylococcus Aureus*

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Staphylococcus aureus, Methicillin resistance, Mupirocin resistance, MupA gene

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ABSTRACT

Background: *Two types of mupirocin resistance among methicillin resistant Staphylococcus aureus (MRSA) have been reported; low-level mupirocin resistance (LL-MR), and high-level mupirocin resistance (HL-MR). The mupA gene is typically located on mobile genetic elements which facilitate the resistance dissemination.* ***Objective****: The aim of this work was to identify the mupA gene location, as well as the restriction fragment length polymorphism (RFLP) patterns in high and low-level mupirocin resistant MRSA.* ***Methodology:*** *This study was conducted on 100 MRSA isolates; seven of them were mupirocin resistant. The E test was used to identify high and low level mupirocin resistance. Amplification of mupA gene in total and plasmid DNA was performed. We also detected the spacer region (trsLM–IS257-like–mupA) in the 7 isolates by PCR then we investigated its RFLP patterns.* ***Results:*** *Four MR MRSA isolates had low level resistance, their MupA gene was located on chromosomal DNA, whereas, three isolates showed high level MR, their MupA gene was located on plasmid DNA. Four types of different RFLP patterns of the spacer region were identified; type-1 included two LL-MR isolates, each of type-2 and 3 included both HL-MR and LL-MR isolates, and type-4 included one HL-MR isolate.* ***Conclusions:*** *Staphylococcus aureus mupA gene responsible for LL-MR is located on the chromosome while that responsible for HL-MR is plasmid-mediated. The spacer region variations appear to occur in both chromosomal and plasmid-located mupA gene regardless the type of mupirocin resistance.*

INTRODUCTION

*M*ethicillin resistant *Staphylococcus aureus* (MRSA) is among the most common human pathogens. It can cause mild to fatal disease, spread locally and globally, colonize numerous human body parts and persist in various environments outside hosts. Over the last decade, MRSA strains have become endemic worldwide[1,2]. MRSA carriage, with the anterior nares as the primary predilection site, is an important risk factor for subsequent endogenous staphylococcal infection that rates between 76% and 86%[2,3].

Resistance to methicillin implies resistance to all *β*-lactam antibiotics and is correlated with the development of increased resistance to other non-*β*-lactam antibiotic agents[4]**.** One of the few drugs that are still effective against MRSA is mupirocin. Mupirocin (pseudomonic acid A), produced by *Pseudomonas fluorescens,* is an analogue of isoleucine which competitively binds to isoleucyl-transfer RNA synthetase, thereby inhibiting protein synthesis[5]. It was first introduced in the United Kingdom in 1985 and because of its success, it has been widely used to treat various staphylococcal skin infections and to eradicate nasal carriage of methicillin-susceptible *S. aureus* (MSSA) and MRSA[6]. Although this topical agent was considered safe, effective, less costly and lacked the undesirable effects on normal body flora associated with oral antimicrobial agents, two years after its introduction, resistance to mupirocin was reported[7]. Mupirocin resistance in adults ranges from 2 to 28% [8]. Two types of mupirocin resistance have been elucidated according to the minimum inhibitory concentration (MIC); low-level mupirocin resistance (LL-MR) and high-level mupirocin resistance (HL-MR). LL-MR with an MIC of 8–256 µg/ml is more common and caused by spontaneous point mutational events in the native chromosomal isoleucyl-transfer RNA synthetase-encoding gene which is located on the bacterial chromosome. These mutations are typically stable and non-transferable, and occur as a result of exposure to increasing concentrations of mupirocin. HL-MR with an MIC of ≥512 µg/ml is mediated by a plasmid-encoded *mupA* gene which is carried on the mupirocin resistance (Mupr) plasmid. A second novel staphylococcal isoleucyl-tRNA synthetase enzyme (*ileS2*) is encoded by *mupA* gene[9,10]. The *mupA* gene is typically located on mobile genetic elements, which likely facilitates the dissemination of this resistance mechanism. Nasal carriage of mupirocin-resistant MRSA is a health threat because of the increased failure rates for nasal decolonization of LL-MR MRSA, yet some strains (at

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least with an MIC of 32µg/ml) are eradicated[11]. On the other hand, HL-MR MRSA has been associated with decolonization failure[12]; it cannot be eradicated with mupirocin and constitutes a serious problem especially when it is resistant to methicillin[13].

The conjugative plasmid responsible for mupirocin resistance possesses transmission-related genes in a cassette-like pattern, including *mupA* gene, insertion sequences (IS) and the transfer gene complex (*trs*). IS*257* is present in staphylococci associated with plasmid and/or chromosomal genes encoding resistance to several antibacterial drugs such as aminoglycosides, tetracycline, methicillin and mupirocin[14].

The aim of this work was to investigate the phenotypic and genotypic patterns of mupirocin resistance in MRSA. It also aimed to identify the *mupA* gene location, either plasmid or chromosomal in high and low-level mupirocin resistant MRSA. We also aimed at investigating the restriction fragment length polymorphism (RFLP) patterns of the spacer regions between the transfer gene complex (*trs*) and *mupA*.

METHODOLOGY

This study was conducted on 100 MRSA isolates; 50 obtained from nasal swabs from health care workers and 50 from different clinical specimens from hospitalized patients at Kasr Al-Aini Hospital after taking the consent of all participants. Ethical approval was obtained from the ethical committee of Faculty of Medicine, Cairo University.

Isolation and identification of *S. aureus:*

Samples were cultured on blood agar and mannitol salt agar aerobically for 24 hours at 35°C. Isolates that exhibited delayed fermentation of mannitol were re-incubated overnight before excluding being *S. aureus*[15]. Yellow pigmented β-hemolytic colonies grown on blood agar and yellow colonies on mannitol salt agar were subjected to Gram stain, catalase test, slide coagulase and tube coagulase tests to be identified as *S. aureus*.

Identification of MRSA by disc diffusion susceptibility test:

MRSA was identified by testing *S. aureus* isolates for their *in-vitro* susceptibility to cefoxitin discs (30µg) (Oxoid, England) by disc diffusion method as recommended by CLSI guidelines[16]. Zone of inhibition ≥ 22 mm indicates sensitivity to cefoxitin while ≤ 21 mm indicates resistance.

Antimicrobial susceptibility testing for mupirocin resistance in MRSA:

Disc diffusion method using 5 and 200µg mupirocin discs:

All MRSA isolates were tested by disc diffusion method using 5 µg mupirocin discs as a first step to detect mupirocin resistance, zone of inhibition ≤ 14 mm indicated mupirocin resistance. Isolates that showed resistance to 5 µg mupirocin discs were subjected to 200 µg mupirocin discs to test for the presence of high level resistance. Absence of zone of inhibition around the edge of the 200 µg mupirocin disc indicated high-level mupirocin resistance[16,17].

Mupirocin E-test:

The MICs of MRSA isolates that showed either low-level or high-level mupirocin resistance by disc diffusion method were detected using the E-test (AB, BioMérieux., France) to confirm the type of mupirocin resistance. The E-test was performed according to the manufacturer's instructions. Low-level resistance by the E-test was considered when MIC was 8-256 µg/ml, whereas high-level resistance was considered when MIC was ≥ 512 µg/ml)[16,17]

Genotypic Detection of *mupA* gene:

Total DNA (chromosomal and plasmid DNA) extraction from the MR MRSA isolates was performed using Simply P Total Nucleic Acid Extraction Kit (Bioflux, CA, USA) according to the manufacturer's instructions. Plasmid extraction was also done using GF-1 Plasmid DNA Extraction Kit (Vivantis, Selangor, Malaysia) according to the manufacturer's instructions. Both extracted total DNA and plasmid were subjected to PCR amplification of the *mupA* gene using the following primers; mupA (5' TAT ATT ATG CGA TGG AAG GTT GG 3') specific for 1645 bp to 1667 bp and mupB (5' AAT AAA ATC AGC TGG AAA GTG TTG 3') specific for 2079 bp to 2103 bp[18]. Total reaction volume was 25 µl; 12.5 µl of PCR master mix (Qiagen, USA) was added to 1 µl of each primer (equivalent to 20 picomol/µl), 5 µl of the template DNA and finally 5.5 µl RNase free distilled water to complete the reaction volume. The PCR amplification was performed under the following conditions: an initial denaturation step at 95 °C for 5 minutes followed by 35 cycles run at 95 °C for 30 sec, at 57 °C for 60 sec, and at 72 °C for 30 sec and finally one cycle of 72 °C for 10 minutes. A clinical MRSA isolate proved to have *mup A* gene was used as a positive control, and sterile distilled water was used as a negative control, both controls were included in each PCR run. The amplification was performed in Biometra T Gradient personal thermal cycler (Analytik Jena, Germany). PCR products were analyzed on 2% agarose gel stained with ethidium bromide and visualized under UV illumination using Biometra TI1 Gel Documentation System (Analytik Jena, Germany). Visual detection of DNA bands of the expected molecular mass (458 bp) was indicative of the presence of *mupA* gene either carried on chromosomal or plasmid DNA.

Amplification and Restriction fragment length polymorphism of Spacer regions between the transfer gene complex (*trs*) and mupA:

The spacer region starting from the transfer gene complex (trs) to mupA gene (*trsLM*–IS*257*-like–*mupA*) was amplified by PCR using the *mupA*-2 (R-AAC ATG GCC AGC ATG AGG AAG GC) and *trsLM* (F-CTG

ACC GAA GGT ATT GCA GAC GG) primers[14]. The amplified PCR products were digested with *Eco*RI according to *Yoo et al.*[14] and visualized on a 2% agarose gel after ethidium bromide staining.

Statistical Analysis:

Data were analyzed using statistical package SPSS (Statistical Package for the Social Sciences) version 25. Data were summarized in terms of frequencies (number) and relative frequencies (percentages) for categorical data. A probability value (p value) ≤ 0.05 was considered statistically significant.

RESULTS

Detection of mupirocin resistance among MRSA isolates:

Among the 100 MRSA isolates included in this study, there were seven (7%) mupirocin resistant isolates. Five of the mupirocin resistant isolates were retrieved from patients; one of them was HL-MR, and the remaining four were LL-MR. While among the 50 MRSA isolates obtained from health care workers, there were 2 isolates (4%), both of them were identified as HL-MR. Both disc diffusion and mupirocin E-test showed similar results (R-value = +1.0 and P-value < 0.001). Characteristics of the studied mupirocin resistant MRSA isolates are shown in Table 1.

Table 1: Characteristics of the studied mupirocin resistant MRSA isolates

Isolate number	Source	Mupirocin resistance level	Mupirocin MIC
87	Carriers	HL-MR	1024
17	Carriers	HL-MR	1024
13	Cases	LL-MR	8
15	Cases	LL-MR	12
32	Cases	LL-MR	12
35	Cases	LL-MR	12
54	Cases	HL-MR	1024

Genotypic detection of *mupA* gene:

The four LL-MR MRSA isolates were positive for *MupA* gene in total DNA extracts but not in plasmid extracts, indicating its location on chromosomal DNA. Whereas, the three HL-MR MRSA isolates were positive for *MupA* gene in both total DNA and plasmid extracts, indicating its location on plasmid DNA (Figures 1 and 2).

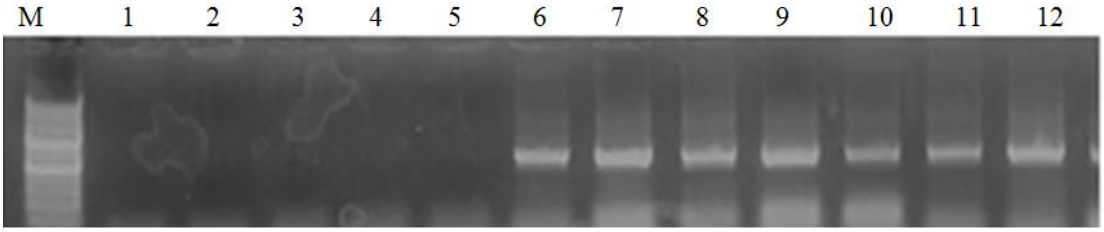

Figure 1: Agarose gel electrophoresis of PCR products of *mupA* gene from extracted total DNA showing bands at 457 bp

Lane M: 100 bp DNA ladder
Lane 1-5: Negative control
Lanes 6, 7, 8, 9: LL-MR MRSA (isolates # 13, 15, 32, 35 respectively)
Lanes 10, 11, 12: HL-MR MRSA (isolates # 17, 54 and 87 respectively)

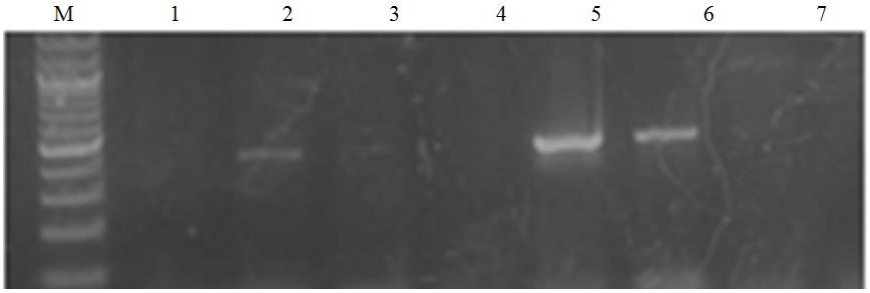

Fig. 2: Agarose gel electrophoresis of PCR products of *mup A* gene from extracted plasmid DNA showing bands at 457 bp

Lane M: 100 bp DNA marker (100-1000)
Lanes 1, 3, 4, 7: LL-MR MRSA showing no bands (isolates # 13, 15, 32, 35 respectively)
Lane 2, 5, 6: HL-MR MRSA showing bands at 457 bp (isolates # 17, 54 and 87 respectively)

Amplification and RFLP of the spacer region:

The spacer region (*trsLM*–IS*257*-like–*mupA*) was found in the seven MR MRSA isolates (Figure 3). We identified four *Eco*RI RFLP patterns among the seven studied isolates. RFLP pattern type-1 included two LL-MR MRSA isolates, each of RFLP patterns type-2 and 3 included one LL-MR and one HL-MR MRSA isolate, and RFLP pattern type-4 included one HL-MR MRSA isolate (Figure 4).

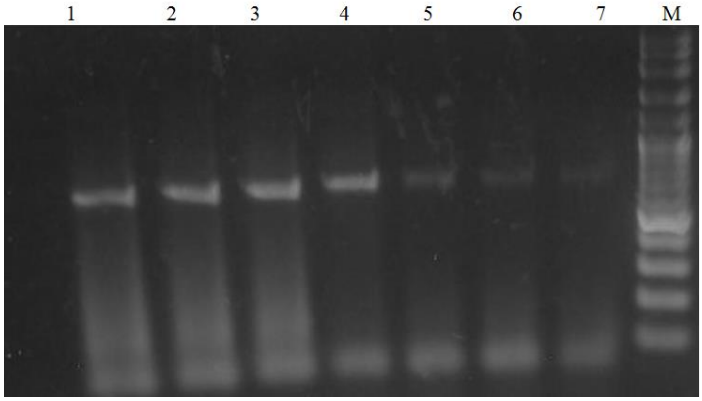

Fig. 3: Agarose gel electrophoresis of the seven MR MRSA isolates after amplification of the DNA sequence of the spacer region (*trsLM*–IS*257*-like–*mupA*)
Lane M: 1000 bp DNA ladder (1000-10000)
Lanes 1-7: isolates # 13,15,17,32,35,54,87 respectively

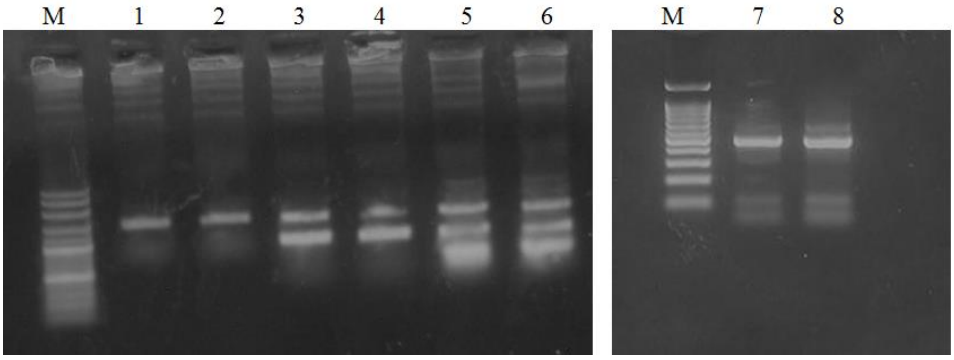

Fig. 4: Agarose gel electrophoresis showing four RFLP patterns of the seven MR MRSA isolates after digestion by *Eco*RI
Lane M: DNA marker (250-10000)
Lanes 1 and 2: isolates 13, 15: one band (RFLP type-1)
Lanes 3 and 4: isolates 17*, 32: two bands (RFLP type-2)
Lanes 5 and 6: isolates 35, 54*, three bands (RFLP type-3)
Lane 7 and 8: isolate 87*: three bands (RFLP type-4)
* HL-MR MRSA

DISCUSSION

In this present study, seven (7%) out of the 100 MRSA isolates were mupirocin resistant; four (4%) were LL-MR and three (3%) were HL-MR. Lower rate of mupirocin resistance among MRSA isolates (2%) was reported by several studies[19-21]. A study by Chen et al. reported that MR resistance among MRSA isolates was 5.1%[22]. On the other hand, higher rates were reported recently. The prevalence of mupirocin resistant *S. aureus* in Africa in two recent studies (14%, 23%) is worrisome and there is a need for data on administration and use of mupirocin[23,24]. Furthermore, in Egypt, the prevalence of mupirocin resistance among MRSA strains was 11.6% in Suez Canal University Hospital, Ismailia. MRSA strains were isolated from surgical wound and urinary tract infections of the patients and from nasal swabs of the healthcare workers[25]. Also

another Egyptian study reported MR among MRSA isolated from surgical site infection to be 16.7%[26]. *McDanel et al.* showed that mupirocin resistance was identified in 12% of MRSA isolates, with 9% of the isolates exhibiting HLMR. HLMR rates per their facility ranged from 0 to 31%[27]. A study by *Hayden et al*. reported that 7.5% of MRSA isolates expressed HLMR and 7.1% expressed LLMR[28]. *Abdulgader et al*. showed that the prevalence of HLMR amongst MRSA was 4%, while that of LLMR was 18%[24]. *Kizerwetter-Świda et al.* reported three HL-MR among 140 methicillin-resistant staphylococci isolates from dogs and cats. One of the HL-MR isolates was identified as MRSA and was isolated from dogs. Thus, monitoring of mupirocin resistance in staphylococci of animal origin, especially in methicillin-resistant isolates, is strongly recommended[29].

Curing and transfer experiments revealed that a 38-kb plasmid is a conjugative plasmid encoding high-level mupirocin resistance. Furthermore, these experiments revealed that one isolate carried both plasmid-borne HL-MR and chromosomal LL-MR. On the other hand, the *mupA* gene was detected on the 38-kb plasmid DNA but not on the chromosomal DNA of the LL-MR isolates, suggesting that different genes encoded low- and high-level mupirocin resistance in these isolates[30].

Typing based on phenotypic and genotypic methods play an important role in understanding the epidemiology of MRSA and evaluating the effectiveness of infection control and antimicrobial prescribing measures[31]. In the present study, *mupA* gene was detected by PCR using *mupA*-specific primers. All of the mupirocin resistant MRSA produced a PCR product of the expected size (458 bp). The *mupA* gene was amplified from total DNA extracts but not from plasmid extracts in the four LL-MR MRSA isolates indicating that it is located on chromosomal DNA. Whereas, the three HL-MR MRSA isolates were positive for *mupA* gene in both total DNA extracts and plasmid extracts indicating that it is located on plasmid DNA.

Genotypic detection of *mupA* gene using conventional PCR is a useful, rapid method for identifying mupirocin resistance in staphylococci[18]. The chromosomal location for *mupA* gene conferring low-level mupirocin resistance was first reported by *Ramsey et al. (1996)* in three mupirocin resistant *S. aureus* isolates, they also detected the gene in one high-level mupirocin-resistant isolate contained a copy of the gene on the plasmid [32]. *Aqel et al*. detected *mupA* gene only in HL-MR MRSA isolates but not in LL-MR isolates[33]. *Chen et al*. also reported that *mupA* is the mechanism of high-level MR resistance among MRSA[22].

High-level mupirocin resistance has been found to be located on a 41.1 kb conjugative plasmid in South Africa. Furthermore, the transfer of high-level mupirocin resistance has been demonstrated by the conjugative transfer of the plasmid alone or with the co-transfer of a plasmid encoding resistance to cadmium. It is recommended that methicillin-susceptible *S. aureus* (MSSA) and MRSA should be routinely tested for mupirocin resistance[13].

In the present study, we detected the spacer region (*trsLM*–IS*257*-like–*mupA*) in the seven MR MRSA isolates. Previous reports demonstrated the *trs* gene responsible for horizontal plasmid transfer on a conjugative staphylococcal plasmid. *Yoo et al. (2010)* confirmed the presence of the spacer region (*trsLM*–IS*257*-like–*mupA*) in all of the methicillin resistant staphylococci. In spite of the variations of the Mupr plasmid, *mupA* is located on a specific DNA fragment on this plasmid. This finding confirms the fact of dissemination of a conserved sequence of the resistance transposon containing *mupA* gene[14].

We also identified four *Eco*RI RFLP patterns of the spacer region cassette among the seven studied isolates. The three HL-MR MRSA isolates were found in patterns type-2, 3 and 4. Whereas, the four LL-MR MRSA isolates were distributed among RFLP patterns type-1, 2 and 3. These results indicate high rate of mutations in *trsLM*–IS*257*-like–*mupA* nucleotide sequences that occur in both low level and high level MR MRSA. Noteworthy, four different RFLP banding patterns of the spacer region (*trsLM*–IS*257*-like–*mupA*) among staphylococci have been previously reported. The reason behind the variations between RFLP patterns has been conferred to sequence deletion adjacent to the *mupA* locus of the *trs* gene complex. Interestingly, one or two *Eco*RI restriction enzyme sites have been observed close to the *mupA* locus (upstream of the *mupA* gene and just downstream of the *trsLM* gene)[14].

Spontaneous loss of *mupA*-mediated high-level mupirocin resistance was observed in *S. aureus*, although being PCR-positive for *mupA* gene. This finding has been attributed to a single base-pair deletion that resulted in a frame-shift mutation and loss of the functional protein. Reversion to the wild-type allele and restoration of high-level resistance has been observed with high frequency, indicating the transient nature of *mupA* polymorphisms. Such strains, given the high reversion frequency, would readily become resistant at high levels upon initial exposure to the drug and would likely result in treatment failures [34]. Furthermore, exposure to sub-inhibitory concentrations of mupirocin has led to rapid induction of LL-MR in 46% of MRSA isolates as well as mutations of *mupA* gene in 75%. Reversion to mupirocin sensitive state has been reported after incubation in the absence of mupirocin but with no reversion to the susceptible wild-type primary sequence. These findings highlight the need for careful use of this drug and the need to closely monitor for the emergence of resistance [35].

CONCLUSION

We confirmed the location of *mupA* gene on the chromosomal and plasmid DNA in LL-MR and HL-MR MRSA, respectively. The various distributions of the four identified RFLP patterns of *trsLM*–IS*257*-like–*mupA* cassette among LL-MR and HL-MR MRSA isolates indicate a high rate of mutations of this region. These variations appear to occur in both chromosomal and plasmid-located *mupA* gene and regardless the type of mupirocin resistance. Testing of MSSA and MRSA for mupirocin resistance should be performed routinely to facilitate the early detection and control of mupirocin resistance. The rising emergence of mupirocin resistance and subsequent reduction in its effectiveness necessitate the need for other antibiotics to decolonize the nose in case of MRSA carriage.

Conflicts of interest:
The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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