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Brief Original Article

Prevalence of inducible clindamycin resistance in methicillin-resistant *Staphylococcus aureus*: the first study in Jordan

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Abstract
Introduction: A high rate of infections with methicillin-resistant *Staphylococcus aureus* (MRSA) has been documented, in both hospital- (HA-MRSA) and community-acquired (CA-MRSA) diseases in Jordan. Erythromycin and clindamycin are considered treatments of choice. However, resistance to erythromycin with false susceptibility to clindamycin *in vitro* may lead to therapeutic failure. Hence, it is mandatory to study the prevalence of inducible resistance to macrolide-lincosamide-streptogramin B ($iMLS_B$) antibiotics conferred by *erm* genes in those bacteria.
Methodology: *S. aureus* isolates were identified morphologically and biochemically, and MRSA were appraised using standard procedures. Induction in resistance to MLS_B antibiotics among MRSA isolates was detected phenotypically using the D-test, and the presence of *erm* genes was revealed by polymerase chain reaction (PCR).
Results: Of 126 collected *Staphylococcus* isolates, 71 (56.3%) isolates were *S. aureus*, of which 55 (77.5%) were MRSA. A total of 43 (78.2%) MRSA-discordant isolates were resistant to erythromycin, of which 33 (76.7%) exhibited the $iMLS_B$ (D-test positive), 2 (4.7%) the MS_B (D-test negative), and 8 (18.6%) the constitutive resistant ($cMLS_B$) phenotypes. Induction of clindamycin resistance was 1.6 times greater in CA-MRSA than in HA-MRSA. Furthermore, *ermA* and *ermC* were significantly prevalent in HA-MRSA and CA-MRSA, respectively.
Conclusions: Continuous surveillance of the MLS_B resistance is important and required before the prescription of clindamycin to treat MRSA infections.

Key words: Clindamycin resistance; $iMLS_B$; MRSA.

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Introduction
In Jordan, methicillin-resistant *Staphylococcus aureus* (MRSA) represents 57%–62% and 19% of clinical and nasal carriage isolates, respectively [1]. MRSA infections are treated with macrolide-lincosamide-streptogramin B (MLS_B) antibiotics, with clindamycin as the drug of choice due to its pharmacokinetic properties [2]. However, resistance to erythromycin (macrolide) in staphylococci is usually associated with resistance to clindamycin (lincosamides) and to type B streptogramin [3]. This cross-resistance to MLS_B antibiotics is mediated by erythromycin ribosomal methylase (*erm*) encoding genes [4]. Three MLS_B phenotypes are known in *S. aureus*, a constitutive resistant phenotype ($cMLS_B$), a clindamycin-susceptible phenotype *in vitro* with inducible resistance *in vivo* ($iMLS_B$), and a clindamycin-susceptible and macrolide-steptogramin B-resistant phenotype (MS_B).

A false susceptibility to clindamycin in $iMLS_B$ MRSA phenotypes may lead to therapeutic failure [5]. Therefore, accurate detection of $iMLS_B$-resistant isolates of *S. aureus in vivo* is a priority concern in therapeutic strategies. Herein, as the first study in Jordan to our best knowledge, this study reports the prevalence of $iMLS_B$, $cMLS_B$, and MS phenotypes with detection of *erm* genes in clinical and nasal carriage MRSA isolates.

Methodology
A total of 126 non-duplicated *Staphylococcus* isolates were obtained from different sources of hospitalized adult Jordanian patients (Al-Karak Hospital and Prince Ali Hospital) in Al-Karak Governorate, Jordan. In addition, nasal swabs of carrier individuals were collected from the same area. Each participant signed a written informed consent document, and the study was approved by the ethics and

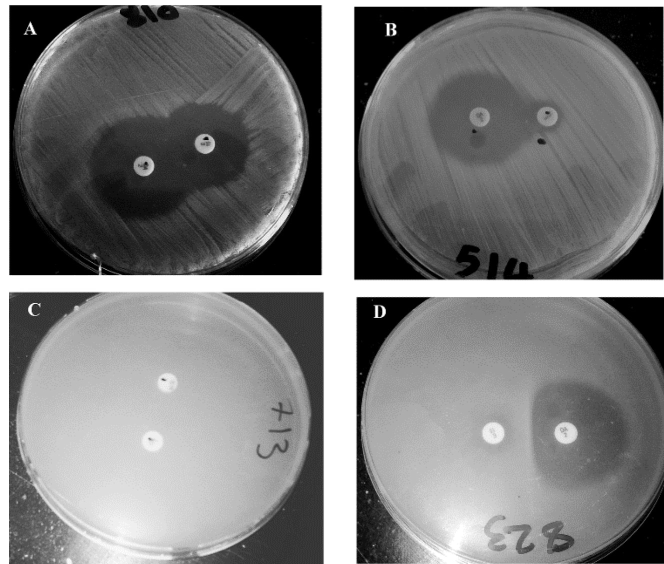
scientific committees of the Faculty of Medicine and Faculty of Graduate studies, Mu'tah University, Jordan.

S. aureus isolates, grown on mannitol salt agar plates (Oxoid, Basingstoke, UK) at 37°C, were identified morphologically and biochemically using standard procedures [6,7] and confirmed by detection of the occurrence of *S. aureus* species-specific (*sau*) gene by polymerase chain reaction (PCR) [8]. MRSA isolates were identified using oxacillin (1 µg) and cefoxitin (30 µg) disks (Oxoid, Basingstoke, UK), and their susceptibility profile was determined using a variety of antibiotics (Oxoid, Basingstoke, UK): gentamycin (10 µg), chloramphenicol (30 µg), tetracycline (30 µg), kanamycin (30 µg), clindamycin (2 µg), erythromycin (15 µg), vancomycin (30 µg), and ampicillin (10 µg), following the Clinical and Laboratory Standards Institute (CLSI) guidelines [9].

Different MLS$_B$ phenotypes were perceived for erythromycin-resistant MRSA isolates using the double diffusion test (D-test) based on CLSI guidelines [9]. Meanwhile, genotypic detection of *erm* genes was carried out for iMLS$_B$ phenotypes. Genomic DNA from iMLS$_B$-MRSA was isolated following a standard protocol [10], adopting the rapid lysis method recommended by Al-Talib *et al.* [11]. DNA amplification was carried out by PCR (XP Thermal cycler, Bioer Technology, Binjiang, China) using specific primer pairs for the *ermB* [4], *ermA*, *ermC*, *sau*, and methicillin-specific resistance (*mecA*) genes as described previously [8]. Single PCR reactions were employed using 2x master mix (i-MAX II, iNtRON Biotechnology, Gyeonggi-do, Korea), while multiplex PCR reactions were employed using 2x master mix (Master/MultiMAX, iNtRON Biotechnology, Gyeonggi-do, Korea) (Table 1). Genomic DNA from MRSA (*S. aureus* ATCC 43300) was used as a control.

The amplified PCR fragments were resolved by electrophoresis through a 1.5% agarose gel containing 0.5 µg/mL ethidium bromide and visualized under UV light (gel documentation, Transilluminator UVP, Upland, USA).

Figure 1. Mueller-Hinton agar plates demonstrating different MLS$_B$ phenotypes.



(A) Er-S, CL-S phenotype; (B) Er-R, CL-S phenotype (MS, D$^-$); (C) Er-R, CL-R phenotype (cMLS$_B$); (D) Er-R, CL-S phenotype (iMLS$_B$, D$^+$). CL: clindamycin; cMLS$_B$: constitutive macrolide-lincosamide-streptogramin B resistant phenotype; D$^-$: D-test negative; D$^+$: D-test positive; Er: erythromycin; iMLS$_B$: inducible macrolide-lincosamide-streptogramin B resistant phenotype; MLS$_B$: macrolide-lincosamide-streptogramin B; MS$_B$: macrolide-streptogramin B resistant phenotype; R: resistant; S: susceptible.

Statistical analysis

Data were analyzed using the Chi-squared test (χ^2) and Fisher's exact test. P values ≤ 0.05 were considered statistically significant.

Results

A total of 71 isolates from the collected samples were identified as *S. aureus* (56%), of which 55 (77.5%) isolates were MRSA and 16 (22.5%) were methicillin-susceptible *S. aureus* (MSSA). Moreover, resistance to

Table 1. Polymerase chain reaction (PCR) components and their conditions used in detection of *erm* and methicillin resistance genes.

PCR reactions components	Conditions of PCR reactions
Single PCR: A total volume of 20 µL containing 10 µL of master mix, 100 ng of template DNA, 1.5 µL of each primer (10 pmol/µL, Midland Company/Midland, USA) and nucleic acid free water.	Initial denaturation step of 3 min at 95°C, followed by 35 cycles at 95°C for 15 s of denaturation, 15 s of annealing at 54°C, 30 s of elongation at 72°C, and a final extension step of 5 min at 72°C. An annealing temperature of 47°C was applied in case of *ermB* amplification.
Multiplex PCR: A total volume of 20 µL containing 10 µL of master mix, 100 ng of template DNA, 6 µL of primer mixture (10 pmol/µL, Midland Company, Midland, USA), and nucleic acid free water. Primer mixture included 1 µL from each *ermC* primer, 1.5 µL from each of *sau* and *mecA* primers, and 2 µL from each *ermA* primer.	Initial denaturation step of 5 min at 95°C, followed by 35 cycles at 95°C for 30 s of denaturation, 1 min of annealing at 54°C, 1 min of elongation at 72°C, and a final extension step of 5 min at 72°C.

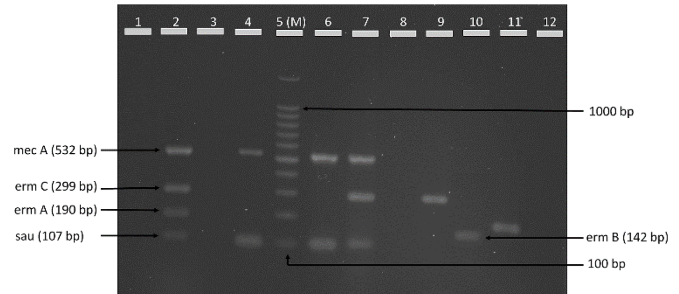
erm: erythromycin ribosomal methylase encoding gene; *mec*: methicillin resistance coding gene; *sau*: *Staphylococcus aureus* specific gene.

methicillin was confirmed by detecting a 532 bp PCR product of the *mecA* gene. MRSA were highly resistant to erythromycin (78%), kanamycin (80%), and tetracycline (63.6%). Meanwhile, 82% and 100% of MRSA samples were susceptible to clindamycin and to vancomycin, respectively. MRSA prevailed in 67.3% of hospital-acquired (HA) infections and 32.7% of community-acquired (CA) diseases. A high rate of resistance to methicillin was detected in 24.6% and 32.7% of nasal and wound isolates, respectively. Furthermore, all isolates of CA-MRSA were susceptibility to clindamycin versus 70% of HA-MRSA.

A total of 43 MRSA isolates were resistant to erythromycin, among which 33 (76.7%) exhibited iMLS$_B$ phenotypes, 2 (4.7%) were MS$_B$, and 8 (18.6%) demonstrated cMLS$_B$ phenotypes (Figure 1). All of CA-MRSA and 61.5% of HA-MRSA showed iMLS$_B$ phenotypes. Statistically, the incidence of inducing clindamycin resistance was 1.6 times greater in CA-MRSA than in HA-MRSA ($p = 0.003$).

Occurrence of merely one *erm* gene or association of more than one *erm* gene, *mecA*, and *sau* genes in iMLS$_B$ is shown in Figure 2. Presence of only the *ermC* gene was frequently demonstrated in both CA-MRSA and HA-MRSA (5/15, 29.4% and 2/16, 12.5%) and significantly in combination with *ermA* in HA-MRSA (5/16, 31.3%, $p = 0.018$) or with *ermB* in CA-MRSA (6/17, 35.3%, $p = 0.05$). A single *ermA* or *ermB* gene was detected in only one isolate. Occurrence of the three *erm* genes in the same isolate was more prevalent in HA-MRSA (6/16, 37.5%) than in CA-MRSA (3/17, 17.6%) (Table 2).

Figure 2. Representative gel electrophoresis revealing the amplicons of *ermA*, *ermB*, *ermC* (199 bp, 142 bp, 299 bp), *sau* (107 bp), and *mecA* (532 bp).



Multiplex polymerase chain reaction (PCR) of gene combinations: lane 2: sample 61; lane 7: sample 712; lanes 4 and 6: methicillin-resistant *Staphylococcus aureus* (ATCC 43300, control); Lanes 9-11: single polymerase chain reaction (PCR) for *erm* genes in sample 61; lane 5: molecular size DNA ladder 100 bp. bp: base pair; *erm*: erythromycin ribosomal methylase encoding gene; *mec*: methicillin resistance coding gene; *sau*: *Staphylococcus aureus* specific gene.

Statistically, it was noticed that *ermA* was significantly detected 2.55 more times in HA-MRSA ($p = 0.01$) than in CA-MRSA, with insignificant differences in the prevalence of *ermB* or *ermC* between the two MRSA groups. However, within the same group, *ermC* was significantly detected in CA-MRSA

Table 2. Prevalence of *erm* genes in erythromycin-resistant iMLS$_B$-MRSA.

Genotype	CA-MRSA n= 17	HA-MRSA n= 16	Pvalue	Relative risk	CI (95%)
ermA	1 (6%)	0	0.52	0.94	0.84–1.1
ermB	0	1 (6.3%)	0.51	0.94	0.84–1.1
ermC	5 (29.4%)	2 (12.5%)	0.22	2.35	0.53–10.45
ermA+ermB	2 (11.7%)	1 (6.3%)	0.52	1.88	0.19–18.80
ermA+ermC	0	5 (31.3%)*	0.018	1.45	1.05–2.20
ermB+ermC	6 (35.3%)*	1(6.3%)	0.05	5.65	0.76–41.89
ermA+ermB+ermC	3 (17.6%)	6 (37.5%)	0.19	0.47	0.14-1.57

*Significant ($p \leq 0.05$). CA-MRSA: community-acquired methicillin-resistant *Staphylococcus aureus*; CI: confidence intervals; *erm*: erythromycin ribosomal methylase encoding gene; HA-MRSA: hospital-acquired methicillin-resistant *Staphylococcus aureus*; iMLS$_B$: induciblemacrolide-lincosamide-streptogramin B resistant phenotype; MRSA: methicillin resistant *Staphylococcus aureus.*

Table 3. Frequency and overall prevalence of *erm* genes detection in erythromycin-resistant iMLS$_B$-MRSA.

Genotype	CA-MRSA n= 17	HA-MRSA n= 16	Pvalue	Relative risk	CI (95%)
ermA	5 (29.4%)	12 (75%)*	0. 01	2.55	1.16–5.61
ermB	10 (58.8%)	9 (56.3%)	0.58	1.05	0.58–1.88
ermC	14 (82.4%)**	14 (87.5%)	0.53	0.94	0.70–1.25
Overall prevalence (pvalue)	0.007	0.13			

*Significant ($p \leq 0.05$) among groups; **Significant ($p \leq 0.05$) within same group. CA-MRSA: community-acquired methicillin-resistant *Staphylococcus aureus*; CI: confidence intervals; *erm*: erythromycin ribosomal methylase encoding gene; HA-MRSA: hospital-acquired methicillin-resistant *Staphylococcus aureus*; iMLS$_B$: induciblemacrolide-lincosamide-streptogramin B resistant phenotype; MRSA: methicillin-resistant *Staphylococcus aureus.*

(p = 0.007) (Table 3). Interestingly, *ermB* was detected at relatively high frequency in both HA-MRSA and CA-MRSA (56.3% and 58.8%, respectively).

Discussion

The emergence of MRSA led to difficulties in treating *S. aureus* infections, especially in developing countries [12]. Therefore, clindamycin has been used to treat soft tissue and pediatric infections and is used in patients allergic to β-lactams due to its pharmacokinetics and low cost when compared to other newer agents used to treat MRSA infections [13]. However, harboring *erm* genes may lead to therapeutic failure due to inducible resistance to clindamycin in those patients.

Coinciding with our results, a high prevalence rate (57%–70%) of MRSA was documented among Jordanian hospitalized adults [14]; the MRSA samples and were recovered mainly from upper respiratory tract and wound swabs [15]. Moreover, herein we reported a higher incidence of inducible clindamycin resistance in MRSA (76.7%) with a higher rate of iMLS$_B$ phenotypes in CA-MRSA than in HA-MRSA. Conversely, in studies conducted in Europe, Turkey, Japan, and India, the incidence of iMLS$_B$ was 24%–39% in MRSA, and inducible phenotypes were detected more frequently in HA-MRSA [16-18]. This could be attributed to differences in drug usage recommendations in each country and inconsistent use of erythromycin in different infectious cases.

High prevalence of *ermA* was documented previously in HA-MRSA [19]. In addition, presence of *ermA* on the transposon Tn*554* within SCC*mec* (staphylococcal cassette chromosome mec) I, II, and III, and absence on SCC*mec* IV explains the low prevalence of this gene in CA-MRSA [20]. The occurrence of the *ermB* gene, originally identified from *Streptococcus* species isolated from animals [21], in high frequency in this study may reflect the high capacity of this gene to be horizontally transferred from *Streptococcus* species to *S. aureus.*

Conclusions

The demonstrated high incidence of iMLS$_B$ in clinical practice and community supports limiting the use of erythromycin for prophylaxis and treatment of MRSA. Performing the D-test on isolates conferring erythromycin-resistant and clindamycin-susceptible phenotypes is prudent to exclude inducible clindamycin resistance.

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