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Detection of Heavy Metal Tolerance among different MLSB Resistance Phenotypes of Methicillin-Resistant *S. aureus* (MRSA)

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are widespread globally. Besides their virulence factors, the co-occurrence of antimicrobial and metal resistance has been reported. This study was designed to evaluate the antibiotic resistance and resistance phenotypes, investigate the occurrence of virulence factors, and detect heavy metal tolerance among MRSA strains. Antibiogram profiling was done as recommended by CLSI instructions. Resistance phenotypes were detected by D test, followed by characterization of enzymatic activities and biofilm formation assay. Antibacterial activity of different heavy metals was tested, and predictable synergistic assay was performed. Among MRSA strains collected, high resistance to ampicillin and amoxicillin/clavulanate (100%) and high susceptibility to clindamycin (70%) were obtained. Resistance phenotypes were detected as S, constitutive MLSB, inducible MLSB, and MS by percentages of 10%, 30%, 30% and 30% respectively. Virulence factors like lipolytic (50%) and hemolytic (70%) activity, and biofilm formation ability (100%) were detected. High resistance towards potassium and magnesium was observed. MTC of 500 ppm was detected for all isolates in case of cobalt and iron. In case of zinc and copper, MTC was detected as 500 ppm except for one isolate which was highly resistant, and 500 ppm for all isolates except for two isolates which were highly sensitive respectively. Magnesium in different concentrations (500 and 2000 ppm) showed synergistic activity with erythromycin and clindamycin. Results reveal high heavy metal tolerance among antibiotic resistant MRSA strains, in addition to the presence of virulence factors. Upcoming studies must be focused on the combination of sub-inhibitory concentration of different heavy metals with the available antibiotics.

Keywords: MRSA, biofilm, MLSB, heavy metals, *S. aureus*

INTRODUCTION

Staphylococcus aureus is a bacterial pathogen, which is considered as one of the most common agents in a variety of human infections including skin, soft tissue as well as eye[1]. Those bacterial species shows some virulence factors which are tightly regulated specially during bacterial growth and pathogenesis process [2].

Formation of biofilms is one of the important virulence factors that provide hard bacterial growth mode making them resist the unfavorable conditions more than those without biofilms[3]. A central role in the regulation of such virulence is controlled by bacterial quorum-sensing system[4]. Bacterial biofilm formation ability always allow more advantages for bacterial cell survival compared to planktonic growth, including protection from shear stress, disinfectants, and antibiotics by its special structure[5-7]. This mode of bacterial growth induces cellular dormancy associated with multidrug tolerance, and non-heritable phenotype that differs from classic mechanisms of antibiotic resistance[8,9].

Resistance to antimicrobial agents has become a difficult problem all over the world[10,11]. Nowadays, the emergence of methicillin-resistant *S. aureus* (MRSA) became a great concern as it causes life-threatening nosocomial and community-acquired infections[12]. Due to the limited number of effective antibiotics, treatment of such infections is sometimes considered very difficult[13]. *S. aureus* isolates qualify to have a resistance to erythromycin which is commonly associated with resistance to other macrolides. Three genes were found in *S. aureus* responsible for this resistance viz. *ermA*, *ermB* and *ermC*, and encode methylase enzymes which have a role in modifying the ribosomal target site leading to *S. aureus* MLSB phenotype[14].

Treatment of patients infected with *S. aureus* with clindamycin harboring inducible clindamycin resistance (iMLSb phenotype) might lead to the evolution of resistant strains (cMLSB) and subsequently, causing more therapeutic failure[15]. Erythromycin and clindamycin are different classes of antibiotics which bind to the 50S ribosomal subunit of bacteria and inhibit protein synthesis. *S. aureus* resistance to these antibiotics is created by methylation of the target site on the ribosome mostly related to methylase gene erm (rRNA)[16].

The widespread metal contamination of human surrounding is considered a recalcitrant selection pressure in bacteria with clinical and environmental importance that donates some antibiotic resistance factors[17]. Indeed, bacteria are continually changing their genetic compositions, which makes them able to adapt and alter their living conditions. Therefore, the heavy metal-induced bacterial resistance plays an important role in the development of bacteria[18] and may play an important role in disseminating bacterial antibiotic resistance, but the selective effects of heavy metals on bacterial antibiotic resistance are largely unclear. To investigate this, the effects of heavy metals on antibiotic resistance were studied in a genome-sequenced bacterium, LSJC7. The results showed that the presence of arsenate, copper, and zinc were implicated in fortifying the resistance of LSJC7 towards tetracycline. The concentrations of heavy metals required to induce antibiotic resistance, i.e., the minimum heavy metal concentrations (MHCs. Much like antibiotics, heavy metals often have toxic effects on bacteria. Therefore, due to their antimicrobial properties, they are mostly incorporated into products[19].

Through our investigation, we evaluated the antibiotics resistance of ten clinical *S. aureus* isolates, besides detection of their MLBS phenotypes. The heavy metal tolerance among antibiotic-resistant MRSA strains was investigated. In addition, the occurrence of *S. aureus* virulence factors such as biofilm formation ability as well as hemolytic and lipolytic activities were assessed.

MATERIALS AND METHODS

Bacterial strains

Ten *S. aureus* isolates used in the current study were collected from El-Kasr el Ainy hospital, Cairo, Egypt. Initially, the strains of *S. aureus* were identified using traditional biochemical tests including Gram staining, catalase, coagulase (both slide and tube), and DNase tests, and confirmed by growing in selective Mannitol Salt Agar (MSA) medium[20].

Antibiogram profiling

Antibiotic sensitivity was performed by using the standard disk diffusion method on Muller–

Hinton (MH; Laboratories Conda SA, Madrid, Spain) agar plate according to recommended CLSI guidelines[21]. Standard antimicrobial disks (Oxoid, Basingstoke, UK) representing seven different drug classes such as amikacin (30 μg), ciprofloxacin (5 μg), amoxicillin/clavulanic (30 μg), cefoxitin (FOX), sulphamethoxazole-trimethoprim (1.25/23.75 μg), ampicillin (10 μg), erythromycin (15 μg), and clindamycin (2 μg) were used.

Detection of Macrolide-Lincosamide-Streptogramin B (MLSB) resistance phenotypes

MLSB resistance pattern was evaluated for all *S. aureus* isolates using the D-test[13]. At a distance of 15mm (edge to edge), erythromycin (15 μg) and clindamycin (2 μg) discs were placed on MH agar plate which was previously inoculated with 0.5 MacFarland bacterial suspensions. Following overnight incubation at 37°C, D shaped zone formed around clindamycin in the area between the two discs indicated positive result. The results were interpreted as erythromycin-sensitive, clindamycin-sensitive (S); erythromycin-resistant, clindamycin-resistant (constitutive MLSB); erythromycin-resistant, clindamycin-sensitive, D-test positive (inducible MLSB); erythromycin-resistant, clindamycin-sensitive, D-test negative (MS)[22].

Characterization of the *S. aureus* enzymatic activity

Lipolytic activity was carried out using agar plates containing tween 20 (1% v/v) as described previously [23]. The plates were inoculated with a single colony of a culture on nutrient agar and incubated for (1-5) days at 37°C. The turbid zone around colonies, with the change to blue color after the addition of a saturated solution of $CuSO_4 .5H_2O$ reagent, indicates a positive result.

Hemolysis test on blood agar with defibrinated sheep blood 5% (v/v) was used. Isolates were inoculated and incubated for a day at 37 °C. After incubation, the formation of zones around the bacterial growth was detected and characterized as α (partial hemolysis), β (complete hemolysis), and γ (no hemolysis) depending on the activities of each bacterial strain[24].

Microtiter plate biofilm assay

In order to perform the biofilm assay, wells of the 96 polystyrene plate were first filled with 100μl of the bacteria inoculated in Brain Heart Infusion broth, at which uninoculated broth was considered as negative control. After 24 hours incubation at 37°C, the contents of wells were gently removed. The wells were washed as recommended, dried, and the adherent bacteria biofilms formed were then stained with 1% aqueous solution of crystal violet (CV). After 10 minutes, excess stain was washed gently, and the plate was kept for drying. To solubilize the stained adherent biofilm, absolute ethanol was used[25].

By using an auto-reader (Stat Fax-2100; GMI, Inc. USA), the optical density was measured at a wavelength of 570 nm. Each experiment was performed three times. Optical density cut-off value (O.Dc) was calculated as mentioned[7].

Antibacterial effect of antibiotics against bacterial isolates

Antibacterial activity assay of antibiotics was carried out by microtiter broth dilution method[26]. As a diluent, MH broth was used, where about 10^6 CFU/mL cells (0.5 MacFarland bacterial cultures) were inoculated, at which the final volume in each microtiter plate well was 100 μl and antibiotics concentration ranged from 0.25-16 μl/ml, 0.06-4 μl/ml, and 0.125-8 μl/ml for gentamycin, ciprofloxacin, and erythromycin respectively. After 24 hours incubation at 37°C, the minimum inhibitory concentration (MIC) values, defined as the lowest antibiotic concentration which inhibited visual bacterial growth, were measured.

Detection of heavy metals/essential elements maximum tolerance concentration (MTC)

The MTC values of heavy metals/essential elements for the bacterial isolates were determined by agar dilution method, using nutrient agar supplemented with different heavy metals like: copper (Cu^{2+}), cobalt (Co^{2+}), zinc (Zn^{2+}) and iron (Fe^{2+}), and different essential elements like: potassium (K^+) and magnesium (Mg^{2+}). The concentrations of the utilized metals included: 500, 1500 and 2000 ppm. After 18 hours incubation at 37°C, the MTC, defined as the highest metal/element concentration that allow the bacterial growth, were measured[27].

Effect of biofilm formation

The antibiofilm activity of different antibiotics at sub-inhibitory concentrations was done as described with minor modifications[28]. In

case of no MIC detected, the ½ MIC represents the highest antibiotic concentration. 100 µl of bacterial suspension (0.5 McFarland) were dispensed into each well of 96-well polystyrene microtiter plates, and different antibiotic concentrations (100 µl) were added, and plates were incubated at 37°C for 18 hours. Antibiotic free wells (biofilm growth) act as positive controls. After incubation, the medium and non-adherent cells were removed and wells were washed three times with sterile PBS. The plates were well dried and then the dye was re-solubilized with absolute ethanol. The OD of each well was measured at 570 nm using ELISA reader. Each assay was performed in duplicates.

The antibiotic/ potassium (K+) and magnesium (Mg2+) synergistic assay

Synergistic activity was assessed by using the agar disk diffusion method using antibiotic disks of erythromycin (15 µg), and clindamycin (2 µg). MH agar supplemented with different elements; K^+ and Mg^{2+} concentrations was used. The bacterial inoculum of 0.5 McFarland concentrations was applied on the surface of the agar plate and antibiotic disks were placed onto the inoculated agar surface. After incubation for 18 hours at 37°C, the zones of growth inhibition around each of the antibiotic disks were measured. MH agar plates without K^+ and Mg^{2+} supplementation were considered as negative control[6].

Statistical analysis

Chi-square test (χ^2) was conducted to analyze the significance of the resistance level to various antibiotics. A value of $p < 0.05$ was considered to be statistically significant. The calculated mean values are presented with standard deviation (SD) bars using the Duncan test using SPSS software (Statistical Package for the Social Sciences, ver. 12.0, New York)

RESULTS
Antibiogram profiling

In this study, ten methicillin-resistant *S. aureus* bacterial isolates were used. The sensitivity test was performed using the Kirby-Bauer antibiogram method using antibiotic discs representing seven different classes of antibiotics and results were interpreted according to CLSI. Results presented in Table (1) showed that high resistance to ampicillin and amoxicillin/clavulanic by percentages of 100% was obtained. On the other hand, clindamycin was found to be the most effective antibiotic against the isolates with 70% of strains exhibiting sensitivity to this drug (Table 1).

Detection of MLSB resistance phenotypes

Resistant phenotypes among *S. aureus* strains were detected using D test. Different resistance phenotypes were detected as
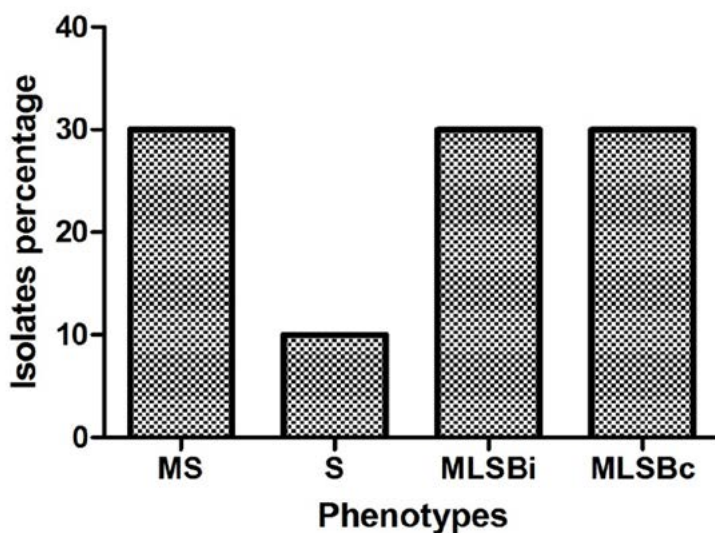

Fig. 1. Resistant phenotypes among *S. aureus* strains. Erythromycin-sensitive, Clindamycin-sensitive (S); Erythromycin-resistant, Clindamycin-resistant (constitutive MLSB); Erythromycin-resistant, Clindamycin-sensitive, D-test positive (inducible MLSB); Erythromycin-resistant, Clindamycin-sensitive, D-test negative (MS).

constitutive MLSB, inducible MLSB, S, and MS by percentages of 10%, 30%, 30% and 30% respectively (Fig. 1).

Characterization of the enzymatic activity and biofilm formation ability

The virulence factors of MRSA including lipolytic and hemolytic activity were tested among the ten strains (Table 2), the results showed that 50% of isolates had lipolytic activity, whereas 70% of them had hemolytic activity. Regarding the biofilm formation of MRSA, it was observed that all tested isolates were capable of producing biofilm and most of the isolates (70%) forms strong biofilms while 30% were categorized as moderate biofilm formers (Table 2).

Minimum inhibitory concentrations of different antibiotics

The susceptibility of three antibiotics (gentamycin, ciprofloxacin and erythromycin) was confirmed using micro-broth dilution method (Table 3). High resistance rates among tested MRSA strains were observed.

Detection of heavy metals maximum tolerance concentration (MTC)

Detection of heavy metals MTC was performed using MH supplemented with three

Table 1. antibiotic susceptibility pattern among MRSA isolates

| Antibiotic class | Antibiotics | Antibiogram profile | | | | p-value* |
| | | Resistant | | Non resistant | | |
		No.	%	No.	%	
Aminoglycosides	Amikacin	5	50	5	50	0.740
β-lactam	Cefoxitin	10	100	0	0	-
	Amoxicillin/clavulanic	10	100	0	0	-
Fluoroquinolones	Ciprofloxacin	8	80	2	20	0.678
Sulphonamides	Sulphamethoxazole-trimethoprim	3	30	7	70	0.717
Aminopenicillins	Ampicillin	10	100	0	0	-
Macrolide	Erythromycin	9	90	1	10	0.580
Lincosamide	Clindamycin	3	30	7	70	0.717

*A value of $p < 0.05$ was considered to be statistically significant.

Table 2. Virulence factors and resistance phenotypes assessment among *S. aureus* stains.

| Strains | MLSB resistance phenotypes | Virulence factors | | | |
| | | Enzymatic activity | | **Biofilm formation | |
		*Lipolytic activity	Hemolytic activity	Average O.D. ± SD	Biofilm ability categorization
S1	MS	+	α hemolysis	3.469 ± 0.057	+++
S2	MLSBi	-	γ hemolysis	3.131 ± 0.053	+++
S3	MLSBi	+	γ hemolysis	3.661 ± 0.008	+++
S4	MS	-	α hemolysis	2.797 ± 0.192	++
S5	MLSBi	+	β hemolysis	3.382 ± 0.171	+++
S6	MS	+	β hemolysis	3.569 ± 0.020	+++
S7	S	+	α hemolysis	3.312 ± 0.074	+++
S8	MLSBc	-	γ hemolysis	3.300 ± 0.050	+++
S9	MLSBc	-	β hemolysis	2.123 ± 0.404	++
S10	MLSBc	-	β hemolysis	1.993 ± 0.506	++

MLSBi: inducible MLSB; MLSBc: constitutive MLSB.
*Lipolytic activity was interpreted as negative (-) or positive (+).
**Biofilm production was interpreted as: negative (-), weak (+), moderate (++) strong (+++).

Table 3. Minimum inhibitory concentration values of different antibiotics

Isolates	Minimum inhibitory concentration (mg/l)		
	Ciprofloxacin	Gentamycin	Erythromycin
S1	1	≥ 16	≥ 8
S2	≥ 4	≥ 16	≥ 8
S3	≥ 4	≥ 16	≥ 8
S4	≥ 4	≥ 16	≥ 8
S5	≥ 4	≥ 16	≥ 8
S6	2	4	≥ 8
S7	≥ 4	8	2
S8	≥ 4	≥ 16	≥ 8
S9	≥ 4	≥ 16	≥ 8
S10	≥ 4	16	≥ 8

concentrations (500, 1500 and 2000 ppm) of different heavy metals used. The recorded results in Table 4 demonstrated that the bacteria can grow at high concentrations of K^+ and Mg^{2+}. On the other hand, the MTC of heavy metals was evaluated as 500 ppm for all isolates in case of cobalt and iron. For zinc, the MTC was 500 ppm in all isolates except for isolate number 7 which was highly resistant, while in case of copper the MTC was 500 ppm for all isolates except for isolates 2 and 5 which were highly sensitive even in the lowest concentration.

Antibiofilm activity of commercial antibiotics

The effect of three antibiotics was studied to inhibit the MRSA strains biofilm formation ability. Different inhibition percentages were showed (Fig. 2). Generally, low biofilm inhibition percentages
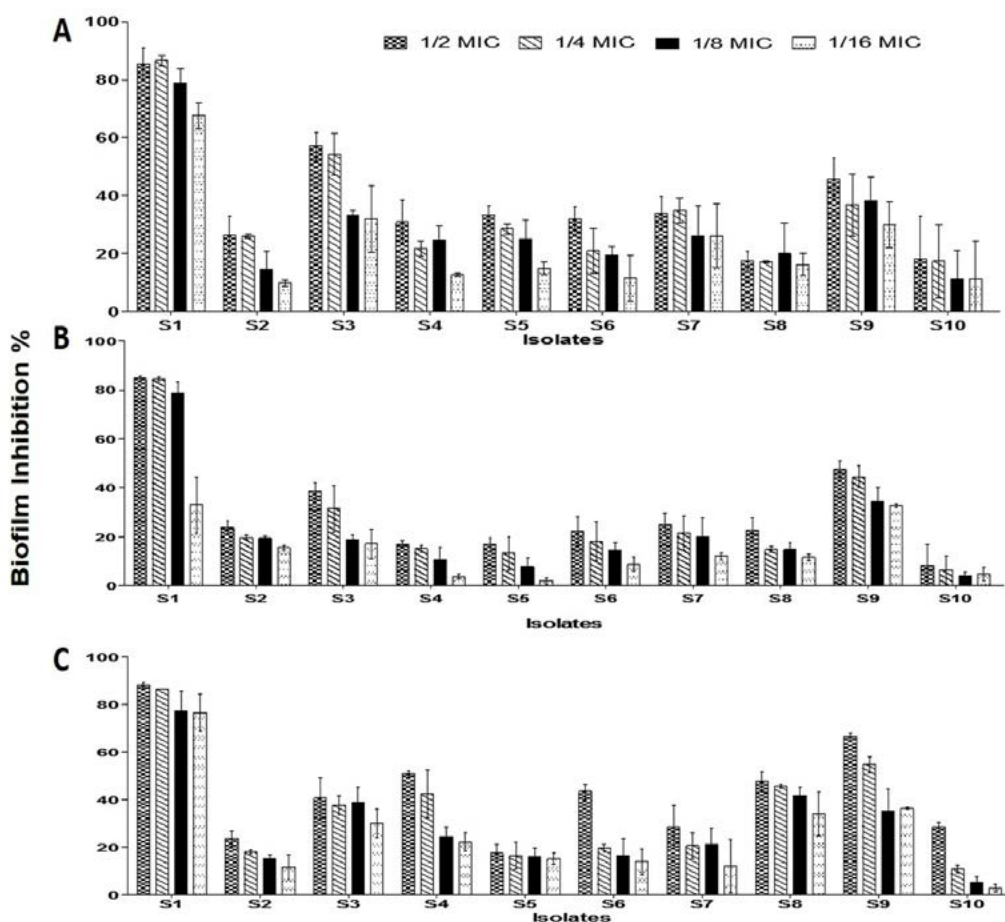

Fig. 2. Effect of different antibiotics concentrations on MRSA strains biofilm formation. (a) Gentamycin (b) Ciprofloxacin and (c) Erythromycin. The bars on the graph represent mean ± SD as a percentage of biofilm inhibition/eradication of triplicate independent experiments (n = 2).

Table 4. Maximum tolerance concentration of different heavy metals/elements by agar dilution method

Strains	Used concentrations (ppm)																	
	Essential elements									Heavy metals								
	K			Mg			Cu			Co			Zn			Fe		
	500	1500	2000	500	1500	2000	500	1500	2000	500	1500	2000	500	1500	2000	500	1500	2000
S1	+	+	+	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-
S2	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	+	-	-
S3	+	+	+	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-
S4	+	+	+	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-
S5	+	+	+	+	+	+	-	-	-	+	-	-	+	-	-	+	-	-
S6	+	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+	-	-
S7	+	+	+	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-
S8	+	+	+	+	+	+	+	-	-	+	-	-	+	-	-	+	-	-
S9	+	+	+	+	+	+	+	+	-	+	-	-	+	-	-	+	-	-
S10	+	+	+	+	+	+	+	+	-	+	-	-	+	-	-	+	-	-

K: potassium; Mg: magnesium; Cu: copper; Co: cobalt; Zn: zinc; Fe: iron; (+): Bacterial growth; (-): No bacterial growth
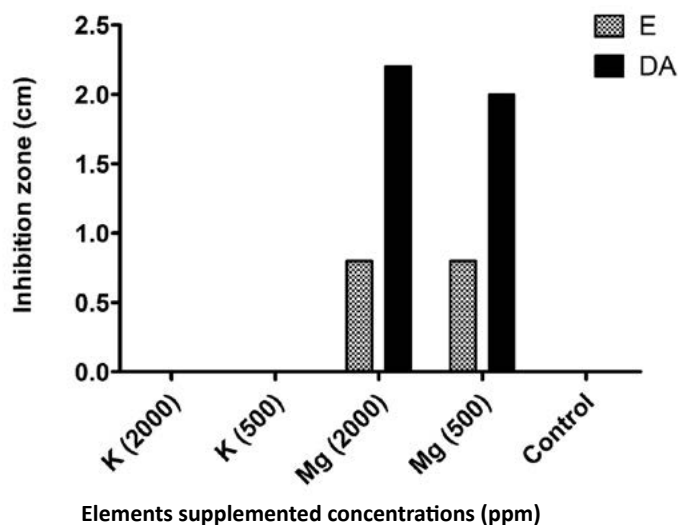
Fig. 3. Synergistic effect of potassium (K) and magnesium (Mg) supplementation with antibiotic discs of erythromycin (E) and clindamycin (DA) against MRSA strain (S8).

were observed for the three tested antibiotics, at which the best inhibition percentages observed by using ½ MIC antibiotics concentration, followed by ¼, 1/8 and 1/16 MICs. The most sensitive isolates which showed reduction in biofilm formation ability were S1, S3 and S9.

Antibiotic/heavy metals synergistic assay

Synergistic activity of K^+ and Mg^{2+} at concentrations of 500 and 2000 ppm (Fig. 3), showed that enhanced inhibition zones were detected in case of magnesium supplementation, while on the other hand no zones were detected in case of potassium supplementation.

DISCUSSION

Recently, *Staphylococcus aureus* has drawn much attention among researchers due to its adaptation ability to a range of antibiotics causing resistance[29], and these bacterial characters can complicate infection treatment and control, specially MRSA strains[30]. Over the past few years, reports about MRSA prevalence were obtained showing different prevalence over countries, at which MRSA prevalence of 53.4% in East Africa [29], 29% in United Arab Emirates (UAE)[31], 34.2% in India [32].

In the present investigation, the antibiotic of *S. aureus* reported a high resistance to ampicillin and amoxicillin/clavulanic by percentages of 100%, followed by resistance to erythromycin (90%) and ciprofloxacin (60%). The resulted ciprofloxacin resistance percentage is in agreement with many reported studies, However, noticeable elevated rate of erythromycin resistance was recorded compared to other reports[12,33,34]. On the other hand, the bacteria showed high sensitivity to clindamycin, which is in line with the study reporting 73.2% sensitivity percentage[33], but considered higher than the percentage reported in Palestine (34.8%)[34].

Resistance to macrolides, lincosamides, and group B streptogramins is commonly referred to as "MLSB resistance". The *erm* gene encodes methylation of the 23S rRNA−binding site that is shared by these three drug classes[10,12]. Erythromycin and clindamycin are antimicrobials which belong to the MLSB group widely recommended to treat staphylococcal infections [35]. In this study, different MLSB resistance phenotypes percentages for *S. aureus* including S, cMLSB, iMLSB and MS respectively were recorded. In literature survey of many countries, it was observed that in India, 64.8% had cMLSB, 25% had iMLSB and 8% had the MS phenotype among the tested MRSA isolates[15]. In South Ethiopia, inducible clindamycin resistance was detected

in 19/22 of the erythromycin-resistant strains[36]. The prevalence of iMLSB and cMLSB phenotypes was 11.48% and 29.25% respectively, where both phenotypes predominated in MRSA strains were reported[37]. This reported resistance to macrolides is suggested to be due to modification of the bacterial intracellular drug-binding site on the ribosome[10].

Indeed, several virulence factors involved in the pathogenesis character of *S. aureus* strains have been described in the literature[38]. Because of the bacterial ability to grow in biofilms, this bacterial pathogens exhibited drug tolerance to a broad-spectrum of antibiotics, which is attributed to the biofilm itself. Pathogens are able to produce biofilms, which can be explained by many factors including the decreased permeability of the drug inside the bacterial cells, the appearance of persisting bacteria, and intracellular bacterial survival[39]. In literature, different reports for biofilm occurrence were found, Piechota *et al.,* (2018) reported that strong biofilm was formed by 39.7% of MRSA[40]. A percentage of 50% was demonstrated in another study as weak biofilm producers followed by moderate and strong biofilm ones by 27% and 23% respectively[41]. Furthermore, higher biofilm formation was also reported at different percentages of 69.8%, 68.3%, and 53.5%[1,5,42].

Low inhibition percentages were observed by using different antibiotics concentrations (Fig. 3). Recently, sub-inhibitory concentrations of different commercially used antibiotics were tested on the *Staphylococcus aureus* biofilms, and as a result different reductions in those biofilms were observed[26,43,44]. Antibiotics at sub-MIC levels were found to affect some biofilm virulence expression, suggesting that it is antibiotic dose dependent[28,45].

Potentially toxic metal contaminants may interact with ecosystem native inhabitant; therefore those bacteria can develop some mechanisms of tolerance allowing them to survive[46]. This co-occurrence of such antibiotic/metal resistance has been reported [47]. In this study, high MTC values were recorded, 500 ppm for all isolates, in case of cobalt, iron and zinc (except for isolate 7) in addition to copper (except for isolates 2 and 5). On the other hand, the bacteria were grown normally at elevated concentrations of K^+ and Mg^{2+}.

The first-row d-block metal ions, which are manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), and zinc (Zn), are essential micronutrients. Cu and Zn are now recognized to be involved in the phagosomal killing of bacteria engulfed by macrophages, defined as an important defense mechanism[48]. The rapid killing of bacteria is thought to be due to cellular damage caused by very high concentrations of Cu dissolving from the surface, which causes membrane disturbance and rupture, coupled with internal oxidative burst due to the generation of ROS leading to further cellular destruction, including degradation of plasmid and chromosomal DNA[49].

According to our results, no effect for both K^+ and Mg^{2+} was detected even in the highest concentration. On the other hand, by using predictable synergistic disc diffusion method, Mg^{2+} supplementation at concentrations of 500 and 2000 ppm showed enhancement activity for both antibiotics used (Fig. 2). K^+ is responsible for many cellular processes, including regulation of intracellular pH, maintenance of turgor, and activation of enzymes[50]. Whereas, Mg^{2+} is well known to be important in the normal homeostatic mechanisms of the cells[51,52].

CONCLUSION

Our results reveal high heavy metal tolerance among antibiotic-resistant MRSA strains, in addition to the presence of virulence factors such as biofilm formation and enzymatic activity (hemolytic and lipolytic). The supplementation of Mg at different concentrations (500 and 2000 ppm) showed synergistic activity with the antibiotics; erythromycin and clindamycin. To enhance the antibiotic activities, upcoming studies must be focused on the combination of a sub-inhibitory concentration of different heavy metals with the available antibiotics.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial,

direct and intellectual contribution to the work, and approved it for publication.

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ETHICS STATEMENT
Not applicable.

DATA AVAILABILITY
All datasets generated or analyzed during this study are included in the manuscript.

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