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A study on Heavy Metals and Antibiotic Resistance of *Staphylococcus aureus* Isolated from Clinical Specimens

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Abstract

Background The trace heavy metals such as Cobalt, Zinc, Copper and Nickel play important roles in bacteria; they regulate a wide array of metabolic function as coenzyme or cofactors. However, some metals like arsenic, mercury and cadmium, are not essential for growth and extremely toxic. Understanding of metal resistance in Staphylococci in association with antibiotics resistance has progressed rapidly in the last years with well-established cadmium, mercury, antimony and arsenic resistance system encoded by plasmids.

Objective To evaluate antibiotic and heavy metal resistance in *Staphylococcus aureus* isolates.

Methods Thirty *S. aureus* isolates were collected from different clinical specimens. The minimum inhibitory concentration of thirty *S. aureus* isolates was determined for four types of antibiotics, which were tetracycline, gentamicin, cefotaxime and penicillin-G. Resistance of *S. aureus* isolates to heavy metals ions (Cobalt, Zinc, Mercury and Cadmium) were tested. Ethidium bromide was used as a curing agent with freshly growing *S. aureus* to study resistance features link with antibiotic and heavy metal resistance.

Results The minimum inhibitory concentration of thirty *S. aureus* 83.3% of the isolates were resisting tetracycline 80% of the isolates were resisting gentamicin 93.3% of the isolates were resisting cefotaxime, and 80% of the isolates were resisting penicillin-G. While, 93.3% of isolates found to be resistant for Cobalt ions, 86.6% resisted Zinc ions, 86.6% resisted Mercury ions. While, 83.3% of isolates resisted Cadmium ions. Using Ethidium bromide as a curing agent showed two groups of cured colonies.

Conclusions There is strong relationship between multiple antibiotic resistances and multiple heavy metal resistance In addition; there may be two to three types of plasmids depending on results obtained from curing experiment.

Key wards Heavy metals, *S aureus*, antibiotics, resistance.

Introduction

Staphylococcus aureus was responsible for a wide range of infections, from mild skin infections to wound infections and bacteraemia. Although the introduction of antibiotics over the last 50 yr has lowered the mortality rate from *S. aureus* infections, the bacteria have developed resistance mechanisms to all antimicrobial agents that have been produced [(1).]

Some strains of *S. aureus* express many potential virulence factors that are lack in other strains. *Staphylococcus aureus* infections can be treated with commonly used antibiotics [(2)]. In recent years some strains of *S. aureus* have become resistant to some antibiotics

which means that it is not killed by antibiotics that's take great attention [3].

Heavy metals consist of a group of about 40 elements. Many are essential for growth of both prokaryotic and eukaryotic organisms, and therefore are required at low concentration. However, some metals like arsenic, mercury and cadmium, are not essential for growth and extremely toxic even at low concentration [4]. The trace heavy metals such as Cobalt, Zinc, Copper and Nickel play important roles in bacteria; they regulate a wide array of metabolic function as coenzyme or cofactors, as catalysts or acid in the enzymes and as structural stabilizer of enzymes and DNA binding protein [5].

Understanding of metal resistance in Staphylococci has progressed rapidly in the last years with well-established cadmium, mercury, antimony and arsenic resistance system encoded by plasmids [6]. Little is known about transport of the resistance to zinc and cobalt (chromosomal encoded) ions in *S. aureus* [7].

The aim of this study was to evaluate antibiotic resistance and tolerance to some heavy metals linked with antibiotic resistance in *S. aureus* and making a curing experiment to demonstrate the relationship of antibiotic resistance and heavy metal tolerance with any cured plasmid could harboring such traits.

Methods

Collection of Isolates: One hundred thirty isolates of *s. aureus* were obtained from different clinical specimens were collected from Al-Yarmouk and Al-Kadhimiya hospitals from 74 female and 56 male. Of these, 30 isolates were identified as *S. aureus* (17 isolates from female and 13 isolates from male). On the basis of their colony morphology, Gram's stain and positive results in coagulase, DNase, catalase, mannitol fermentation, and for the further confirmation the isolates were identified by API staph system.

Antibiotic and heavy metals solution: Tetracycline stock solution prepared at concentration 25000 µg/ml, cefotaxime stock solution prepared at concentration 10000 µg/ml, gentamicin stock solution prepared at concentration 8000 µg/ml and penicillin-G stock solution prepared at concentration 80000µg/ml, then the stock solutions were sterilized by filtration and kept at 4°C, until used [9]. Heavy metals used were Zn $(CH_3COO)_2.2H_2O$, Co $(CH_3COO)_2.4H_2O$, $CdCl_2$ and $HgCl_2$ prepared as stock solution and sterilized by filtration and kept at 4°C until used.

Ethidium Bromide solution 10 mg/ml (Curing solution) was prepared by dissolving 0.2gm of ethidium bromide in 20 ml distilled water and stirred on magnetic stirrer for few hours to ensure that the ethidium bromide has dissolved then it was sterilized by filtration, and stored in a dark bottle at 4°C [8].

Minimum Inhibitory Concentration (MIC) tests. Inocula of selected isolates were grown in 5ml nutrient broth, then 0.1ml of each culture were inoculated in series of 5ml fresh nutrient broth containing various concentrations of antibiotics or heavy metals solutions (8, 16, 32, 64, 128, 256, 512 and 1024 µg/ml for antibiotics) and (5, 10, 20, 40, 80, 160, 320, 640 and 1280 µg/ml for heavy metals) for each isolates of *S. aureus,* then all tubes were incubated at 37°C for 24 hours. 100 µl from each tube were spread on brain heart infusion agar plates and all plates were incubated at 37°C for 24 hours. The lowest concentration of the antibiotics or heavy metals solutions that inhibited the growth of bacterial isolates was considered as the minimum inhibitory concentration (MIC) [9].

Plasmid DNA curing. Cells of the selected isolate were grown in 5ml of nutrient broth. 0.1ml samples of each culture were inoculated in series of 5ml fresh nutrient broth tubes containing various concentrations of ethidium bromide (50, 100, 200, 400, 600, 800 and 1000 µg/ml). All tubes were incubated at 37°C for 24-48 hours. The growth density of the deferent tubes was measured visually and compared with the control to determine the

effect of each concentration of curing agent on bacterial growth [10]. The lowest concentration of the curing agent that inhibits the growth of bacterial isolate was considered as the minimum inhibitory concentration (MIC).

Selection of Cured Cells. After treatment of bacterial isolate with standard curing agent and the isolation of survivors on nutrient agar, survivors were analyzed for the presence or absence of drug resistance as a result of elimination of the plasmid by selecting 100 colonies of bacterial isolates from each treatment. These colonies were replica plated (using toothpick) on nutrient agar plate (master plates) and on nutrient agar plates containing an antibiotics and other nutrient agar plate containing a heavy metals to which the original isolate is resistant [10]. If a colony was able to grow on the master plate but not on the selective agar containing the appropriate antibiotic or heavy metal, it means that, the cells of this colony are cured cells that lost plasmid responsible for resistance to the antibiotic or heavy metal. The percentage of the cured cells was determined.

Results

Antibiotic sensitivity test of *S. aureus* isolates.Antibiotic sensitivity test performed with twelve types of antibiotics. The percentage of resistance were 93.3%, 83.3%, 83.3%, 80%, 50%, 33.3%, 30%, 30%, 20%, 20% and 3.3% to the following antibiotics cefotaxime, carbenicillin, tetracycline, gentamicin, cephalixin, fusidic acid, chloramphinicol, bacitracin, vancomycin, streptomycin and imipenem. No resistance was found to amikacin.

The minimum inhibitory concentration (MIC) of antibiotics of *S.aureus* isolates *Staphylococcus aureus* isolates showed high percentage of resistance for the four types of antibiotics that were tested against, 93.3% of *S. aureus* isolates showed resistance for cefotaxime, of these 46.6% showed high resistance level at 64 µg/ml, while 13.3% of isolates showed a lower resistance level at 128 µg/ml. Eighty percent of *S. aureus* isolates showed resistance for penicillin-G. Of these, 50% showed high resistance level at 128 µg/ml, While 6.6% of isolates showed a lower resistance level at 512 µg/. *S. aureus* isolates which represented 83.3 % showed resistant for tetracycline. Of these 56.6% showed high resistance level at 128 µg/ml. While 6.6 of isolates showed a lower resistance level at 256 µg/ml. Eighty percent of *S. aureus* isolates showed resistance for gentamicin. Of these, 56.6% of were resisting 32 µg/ml in high resistance level. While 10% of isolates showed a lower resistance level at 16 µg/ml (Table 1).

Table 1. Resistance percentage of *S.aureus* isolates for different concentration of antibiotics.

Antibiotic	% Resistance of *S.aureus* for the following concentrations (µg/ml)								% Sensitive isolates
	8	16	32	64	128	256	512	1024	
TE	-	-	10	10	56.6	6.6	-	-	16.6
CN	-	10	56.6	13.3	-	-	-	-	20
CTX	-	-	-	46.6	13.3	33.3	-	-	6.6
P-G	-	-	-	6.6	50	16.6	6.6	-	20

CN: Gentamicin, P-G: Penicillin-G, CTX: Cefotaxime, TE: Tetracycline

Heavy metal resistance of *S.aureus* isolates:

There were 86.6% of isolates resist Zinc ions. About 40 % of the tested *S.aureus* isolates showed high resistance level (most of bacterial isolates resist it) at concentration 0.64 mg/ml, while 6.6 % of isolates showed lower resistance

level at 2 mg/ml concentration. There were 93.3% of the isolates resist Cobalt ions, 30 % of the tested *S.aureus* isolates showed high resistance level (most of bacterial isolates resist it) at concentration 0.16 mg/ml. While 6.6 % of isolates showed lower resisting level at concentration 0.02 mg/ml.There were 83.3% of isolates resisting Cadmium ions. About 33.3% of the tested *S. aureus* isolates showed high resistance level (most of bacterial isolates resist it) at 0.02 mg/ml. while, 3.3 % of isolates showed lower resistance level at 0.01 mg/ml. There were 86.6 % of isolates resist Mercury ions, 56.6 % of the tested *S.aureus* isolates showed high resistance level at 0.02 mg/ml, while 6.6 % of isolates showed low resistance level at 0.04 mg/ml concentration (Table 2).

Table 2. Resistance percentage of locally isolated *S.aureus* for different concentrations of heavy metals.

H.M	% Resistance of *S.aureus* isolates for the following Concentrations (mg/ml)										% Sensitive isolates
	0.005	0.01	0.02	0.04	0.08	0.16	0.32	0.64	1.28	2.0	
Zn	-	-	-	-	-	13.3	16.6	40	10	6.6	13.3
Co	-	-	6.6	20	-	30	16.6	10	10	-	6.6
Cd	-	3.3	33.3	16.6	10	20	-	-	-	-	16.6
Hg	23.3	-	56.6	6.6	-	-	-	-	-	-	13.3

H.M= heavy metals, Zn = Zinc, Co = Cobalt, Cd = Cadmuim, Hg = Mercury

Relationship between heavy metals resistance and antibiotics resistance *S. aureus* isolates which represented 94.4 % (17 from 18-quadruple heavy metal resistance *S. aureus* isolates) were resistant to tetracycline at concentrations ranged between (32 -256 µg/ml). In addition, 94.4 % (17 from 18-quadruple heavy metal resistance *S. aureus* isolates) were resistant to cefotaxime at concentrations ranged between (64-256µg/ml). *S. aureus* isolates which represented 88.8 % (16 from 18-quadruple heavy metal resistance *S. aureus* isolates) was resistant to gentamicin at concentrations ranged between (16-64µg/ml). Also, 88.8% (16 from 18-quadruple heavy metal resistance *S. aureus* isolates) resisted penicillin-G at concentrations ranged between (64-256µg/ml).

Curing of plasmid DNA of *S.aureus* isolate R2 with Ethedium bromide

One isolate had been chosen designated as isolate R2 resistance because it has multi drug and metal resistance and it shows effective growth among the 30 *S.aureus* isolates. Table 3 showed that 100 µg/ml of Ethedium bromide was the less concentration which had noticeable inhibitory effect on bacterial growth for the isolate R2 compared with control growth.

Depending on curing experiment which indicated that may be there were two types of cured colonies; colonies lost resistance for Zinc, Cobalt, Cadmium, Penicillin-G and Tetracycline, colonies lost resistance for Zinc, Cobalt, Cadmium, Penicillin-G, Tetracycline and Cefotaxime this indicated loosing for more than one type of plasmid in the last type of colonies of *S. aureus* isolates. While there were no loss of Genamicin and Mercury resistance, which indicated that these markers are not, located on plasmid DNA (located on chromosome or on mega plasmid). That means that may be there were two to three types of plasmids depending on results obtained from curing experiment as shown in table 3.

Table 3: Number of cured bacterial colonies that lost resistance to antibiotics and heavy metals after treatment with Ethedium bromide.

Resistance phenotype	*Staphylococcus aureus*	
	Wild type	Cured cells
Zn, Co, Cd, p-G, TE, CTX	100 % resistance	3 % sensitive
Zn, Co, Cd, P-G, TE	100 % resistance	97 % sensitive
Hg	100 % resistance	100 % resistance
CN	100 % resistance	100 % resistance

Zn = zinc, Co = cobalt, Cd = cadmium, Hg = mercury, P-G = Penicillin-G, TE = Tetracycline

Discussion

The high percentage of *S.aureus* might be due its role as the main cause of nosocomial infections. It is also one of the most important infectious agents, which can cause an opportunistic infection because it is a part of body normal flora [(11)]. *Staphylococcas aureus* isolates showed high percentage of resistance for the four types of antibiotics tested against .These results are in agreement with that of Booth *et al* (2001) which found that 90% of isolates were resistant to β-lactam antibiotics. Production of β-lactamase is the main cause of high resistance of *S.aureus* to β-lactam antibiotics since the β- lactam ring is the main constituent of β-lactam antibiotics molecules [(12)]. The frequent use of tetracycline to treat wound infections locally may elevate resistance percentage of *S.aureus* for this antibiotic. The mechanism of resistance for tetracycline performed by ribosomal protection, active efflux and decrease uptake [(1)]. Kuroda *et al.* reported that, about 45% of the total isolates of *S.aureus* carried a 35.5 kb plasmid and these isolates always showed resistance to gentamicin, tobramycin, kanamycin, amikacin, astromicin, and arbekacin. The plasmid carried resistance here may be transferred easily and that explain the elevated percentage of resistance to gentamicin [(13)].

The highest zinc resistance among bacterial isolates was also reported by Xiong and Jayaswal (1998) [(7)]. The molecular mechanism of resistance involves a number of proteins. These protein molecules either export the metal ions out of the cell or detoxify or sequester them so that the cells can grow in an environment containing high level of toxic metals [(7)]. The highest Cobalt resistance among bacterial isolates was also reported by Xiong and Jayaswal [(7)]. There were 83.3% of isolates resisting Cadmium ions. While there were 86.6 % of isolates resist Mercury ions. Novick and Roth (1968) reported that certain isolates of *S.aureus* carried resistance factors to some inorganic ions including Mercuric, Cadmium, Arsenate and Lead; also they reported that penicillinase plasmids In *S.aureus* carried resistance factors to some inorganic ions including Arsenate, Lead, Mercuric and Cadmium [(14)].

The results of current study revealed that there is strong relation between resistance to antibiotics and heavy metals.

Genes encoding for metal and antibiotic resistance may be located on the same plasmids and/or transposons, conferring co-resistance [(15)]. Curing and transfer experiments revealed that the 26-kb plasmid encoded resistance to Cadmium, Mercuric chloride, Propamidine isothionate and ethidium bromide [(16)]. However, since bacteria are very likely to be confronted with toxic Mercury concentrations, Mercury resistance determinants are very widespread [(6)]. The mechanism of resistance of *S. aureus* to Mercury considered was referred to the category that the detoxication of noxious substances introduced into bacterial cells by some intracellular mechanisms, which

somehow change them into non-noxious form by reduce Mercury ions to a less toxic oxidation state by the bacterial cell [17]. Plasmids might be capable of encoding resistance to antibiotics specifically related to heavy metals (Silver, Mercury, and Copper) resistance [18].

Depending on curing experiment which indicated that there may be two types of cured colonies; colonies lost resistance for Zinc, Cobalt, Cadmium, penicillin-G and tetracycline, and colonies lost resistance for Zinc, Cobalt, Cadmium, penicillin-G, tetracycline and cefotaxime. This indicated loosing for more than one type of plasmid in the last type of colonies of *S. aureus* isolates. While there were no loss of genamicin and Mercury resistance, which indicated that these markers are not, located on plasmid DNA (located on chromosome or on mega plasmid). Such results indicate the presence of two to three types of plasmids depending on results obtained from curing experiment. The effect of ethedium bromide as interchalating dyes with preferential inhibition of plasmid replication. If the resistance is plasmid mediated, those bacteria with clustered resistance genes are more likely to simultaneously pass on those genes to other bacteria, and those bacteria would then have a better chance at survival. In such a situation, one may suggest an association with antibiotic resistance and metal tolerance [19]. If both or all enes clustered are useful to the organism, it is beneficial to the survival of that organism and its species because those genes are more likely to be transferred together in the event of conjugation. Thus, in an environment with multiple stresses, for example antibiotics and heavy metals, it would be more ecologically favorable, in terms of survival, for a bacterium to acquire resistance to both stresses.

Conclusions

The relationship between multiple antibiotic resistances and multiple heavy metal resistance indicates an environmental biohazard of heavy metal pollution in Iraq. In addition, there may be two to three types of plasmids depending on results obtained from curing experiment. The genes responsible for resistance for some heavy metals and antibiotics may be located on the same plasmid DNA.

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