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Resistance to Lead, Copper, And Mercury in *P. aeruginosa* Isolated From Hospitals In Iraq.

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ABSTRACT

The aim of this study was to investigate the correlation between biofilm production and heavy metals resistance (HM) in *P. aeruginosa* isolated from clinical samples. Forty-three *P. aeruginosa* isolates were tested for their susceptibility to lead, copper, and mercury using agar pouring method. Biofilm production of *P. aeruginosa* isolates were also investigated. Results revealed that 37/43 of the isolates were resistant to lead nitrate (400µg/ml). Most of the isolates were resistant to these HM in some of concentrations. Results showed that 20/43 (47%) of isolates had biofilm. The results of bacterial curing showed survived resistance to all HM used, which may be due to that HM resistance trait was carried on chromosome rather than plasmid. Our findings support the fact that the heavy metal resistance of *P. aeruginosa* is not correlated with production of the biofilm except for mercury chloride.

Keywords: *Ps. aeruginosa*, Resistance, Lead, Copper, Mercury, Biofilm, Hospital.

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INTRODUCTION

Heavy metals, particularly silver and mercury, have a variety of applications in controlling microbial population [1]. Mercury in the form of less toxic organic compounds is being used as skin disinfectant [2]. Copper is considered as a safe agent to humans, as demonstrated by the widespread and prolonged use by women of copper intrauterine devices [3].

Pseudomonas aeruginosa is frequently multi drug resistant, which contributes to the high morbidity and mortality of patients in burn units, surgery wards, and intensive care units (ICUs). A major reason for its prominence as a pathogen is its high intrinsic resistance to antibiotics and heavy metals [4]. *Ps. aeruginosa* is a prevalent hospital pathogen that is well known for its ability to form biofilms that are recalcitrant to many different antimicrobial treatments [5].

It was found that biofilms were from 2 to 600 times more resistant to heavy metals stress than free-swimming cells. They also showed that biofilms are more resistant to heavy metals than either stationary-phase or logarithmically growing plankotonic cells [6].

The aim of this study was to investigate the correlation between biofilm production and heavy metals resistance in *P. aeruginosa* isolated from clinical samples that resistant to lead, copper, and mercury**.** detect the prevalence of *Ps. aeruginosa* and studying the correlation between biofilm production and HM resistance**.**

MATERIALS AND METHODS

Study design and Bacterial Isolates

This study was designed to assess the prevalence of *Ps. aeruginosa* isolates resistant to lead, copper, and mercury that recovered from clinical samples and studying the correlation between biofilm production and HM resistance.

At the beginning of this study, Out of 296 different clinical samples, 43 (14.5%) isolates belonged to *P. aeruginosa*. These bacterial isolates were identified based on their morphology, Gram-staining, and conventional biochemical tests [7] and as suggested previously [8]. Clinical samples were collected from the main three hospitals in Al-Hilla city/Iraq (Hilla Teaching hospital, Margan Teaching hospital, and Childhood and gynecology hospital), in addition to some private clinics.

Heavy metal susceptibility testing

All isolates were subjected to susceptibility testing by screening test using agar medium supplemented with ($PbNO_3$ 400µg/ml). The isolates were also tested for their susceptibility to three heavy metals (HM) represented by; Lead nitrate, Copper Sulfate, and Mercury chloride. The minimum inhibitory concentration (MIC) of these three HM was detected by agar pouring method, based on standard method [9].

The following concentrations of heavy metals were prepared: (100, 200, 400, 800, 1600, 2400, and 3200 µg/ml) for lead; (100, 200, 400, 800, 1600, 1750, and 3200 µg/ml) for Copper; and (2.7, 5.4, 10.8, 21.6, 43.2, 54.3, and 86.4 µg/ml) for Mercury.

Biofilm formation

Biofilm formation was determined using tissue culture-treated, 96-well polystyrene plates, based on standard methods [10]. Bacteria were grown in individual wells of 96-well plates at 37 ºC in Brain Heart Infusion (BHI) medium supplemented with 1% glucose. After 24 h growth, the plates were washed vigorously. This involved three rounds of plunging the plates into a large volume of distilled water and decanting to remove unattached bacteria. The plates were subsequently dried for 1 h at 60º C prior to staining with a 0.4% crystal violet solution. The A492 of the adhered, stained biofilms was measured using a microtitre plate reader. Biofilm formation by each strain was measured. A biofilm-positive phenotype was defined as having a value of ≥ 0.17 at absorbance of 492 nm.

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DNA extraction and Plasmid curing

Plasmid DNA extraction of gram negative bacteria was performed using Geneaid kit according to the manufacturing company (Geneaid, USA) and plasmid profile was carried out by gel electrophoresis. *E. coli* standard strain MM294 was used as negative control.

Plasmid curing was carried out using Elevated temperature method [11]. After that, the isolates were cultured on Mueller Hinton agar supplemented with heavy metals at different concentration. Results were recorded by loss of ability of the tested bacteria to survive on this medium. However, if there was growth detected, this means that the gene responsible for heavy metal resistance is carried on chromosome.

RESULTS

Heavy metals resistance and MIC of isolates

All *P. aeruginosa* isolates were subjected to susceptibility testing by screening test using agar medium supplemented with $PbNO_3$ 400µg/ml. Results revealed that 37 isolates (85%) were resistant to lead nitrate, these isolates were distributed into 34 clinical and 3 environmental samples. Bacterial resistance to heavy metals (Table 1) shows the MIC of *P. aeruginosa* to all studied heavy metals.

Table (1): MIC values of *Pseudomonas aeruginosa* isolates to copper sulfate, mercury chloride, and lead nitrate in (µg/ml) concentrations.

Isolates	MIC of Copper sulfate	MIC of Mercury chloride	MIC of Lead nitrate
*Ps.*1	1600	2.7	3200
*Ps.*2	1750	54.3	3200
*Ps.*3	1750	86.4	3200
*Ps.*4	1600	86.4	3200
*Ps.*5	1750	43.2	3200
*Ps.*6	1750	54.3	3200
*Ps.*8	1750	86.4	3200
*Ps.*9	1750	86.4	3200
*Ps.*12	1600	54.3	3200
*Ps.*13	1750	43.2	3200
*Ps.*14	1750	86.4	800
*Ps.*15	1600	86.4	3200
*Ps.*16	1600	86.4	3200
*Ps.*17	1600	54.3	3200
*Ps.*19	1600	86.4	3200
*Ps.*20	3200	86.4	3200
*Ps.*21	1600	86.4	800
*Ps.*22	1600	2.7	2400
*Ps.*24	400	54.3	3200
*Ps.*25	1600	43.2	3200
*Ps.*26	1600	86.4	3200
*Ps.*27	1600	21.6	2400
*Ps.*28	800	43.2	2400
*Ps.*29	400	21.6	3200
*Ps.*30	1600	21.6	3200
*Ps.*31	1600	21.6	1600
*Ps.*32	1600	21.6	3200
*Ps.*33	1600	21.6	3200
*Ps.*34	1600	21.6	3200
*Ps.*36	1600	86.4	3200
*Ps.*37	800	21.6	3200
*Ps.*38	3200	21.6	3200
*Ps.*39	1600	43.2	1600
*Ps.*40	1600	86.4	3200
*Ps.*41	1600	54.3	3200
*Ps.*42	1600	54.3	3200
*Ps.*43	1600	43.2	3200

In case of copper sulfate ($CuSO_4$), results indicated that all isolates were resistant in concentrations of 100, and 200 µg/ml and the MIC of most of the isolates was 1600 µg/ml.

In case of mercury chloride ($HgCl_2$), two isolates were sensitive to $HgCl_2$ in low concentration 2.7 µg/ml. The MIC of all isolates was 86.4µg/ml.

According to lead nitrate ($PbNO_3$), results showed that all isolates were resistant and the MIC values ranged from 800-3200 µg/ml (Table 1). Results also showed that most of the isolates (30:37) were tolerant to lead nitrate at concentration 2400 µg/ml; however six of isolates were sensitive to lead nitrate at concentration of 400 µg/ml. According to environmental isolates (*Ps.*39, *Ps.*40, *Ps.*41), results revealed that they were also resistant and the MIC ranged from 1600-3200 µg/ml.

Biofilm formation

The biofilm formation by *P. aeruginosa* isolates was investigated. The results showed that 20**/**43 (47%) of isolates had biofilm (Table 2). The relationship between biofilm production and heavy metal resistance (HMR) was studied. It was found that the HMR of *P. aeruginosa* isolates is not correlated with production of the biofilm.

Table (2): Biofilm production by *Pseudomonas aeruginosa* isolates recovered from clinical and environment samples

Isolate No.	A 492 (≥ 0.17)*	Biofilm production
Ps. 1	0.11	-
Ps. 2	0.20	+
Ps. 3	0.23	+
Ps. 4	0.17	+
Ps. 5	0.09	-
Ps. 6	0.11	-
Ps. 7	0.22	+
Ps.8	0.12	-
Ps. 9	0.22	+
Ps. 10	0.21	+
Ps.11	0.14	-
Ps. 12	0.11	-
Ps. 13	0.14	-
Ps. 14	0.19	+
Ps. 15	0.10	-
Ps. 16	0.15	-
Ps. 17	0.24	+
Ps. 18	0.14	-
Ps.19	0.11	-
Ps.20	0.20	+
Ps.21	0.16	-

No. of isolates	A 492 (≥ 0.17)*	Biofilm production
Ps.22	0.09	-
Ps.23	0.09	-
Ps.24	0.17	+
Ps.25	0.20	+
Ps.26	0.26	+
Ps. 27	0.11	-
Ps.28	0.30	+
Ps.29	0.11	-
Ps.30	0.11	-
Ps. 31	0.16	-
Ps. 32	0.11	-
Ps. 33	0.12	-
Ps.34	0.20	+
Ps.35	0.14	-
Ps. 36	0.16	-
Ps. 37	0.29	+
Ps.38	0.24	+
Ps. 39	0.20	+
Ps.40	0.25	+
Ps.41	0.24	+
Ps.42	0.12	-
Ps.43	0.83	+

* The No. between brackets indicates the standard value of biofilm production by ELIZA technique.

Results shown in Table-1 revealed that the isolate *Ps.* 22 (planktonic) and the isolate *Ps*.43 (biofilm) were similar in their resistance to copper sulfate (1600 µg/ml), which indicate that the biofilm production had no role in increasing of resistance to HM compared to the resistance of free-swimming (planktonic) organisms.

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This result was also detected for copper and lead in which there was no relationship between biofilm production and HM resistance (Table 2). However there was a clear correlation between biofilm production and mercury resistance. It was found that the resistance to mercury was increased in biofilm isolate *Ps*.43 (43.2 µg/ml) compared to planktonic isolate *Ps.* 22 (2.7 µg/ml) (Table 1).

Curing of bacterial plasmids

The bacterial curing was concluded for one isolate *P. aeruginosa* (*Ps.*3) (Figure 1). The results showed survived resistance to all HM. This result indicates that the HM resistance trait was carried on chromosome rather than plasmid.

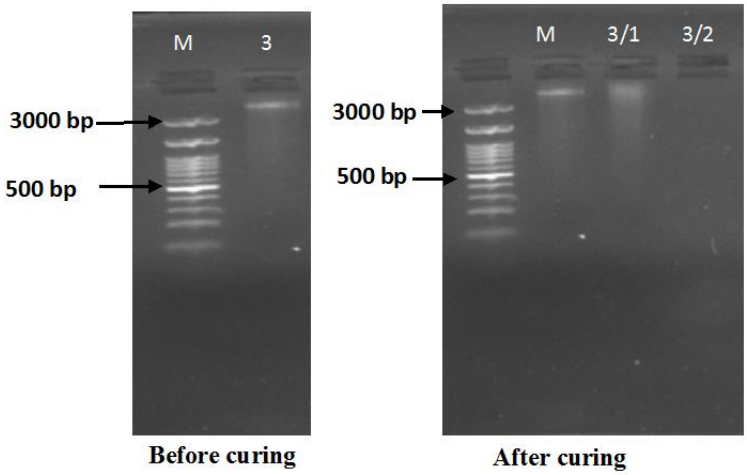

Figure (1): Gel electrophoresis of plasmid DNA content of *Ps. aeruginosa* isolate before and after curing after (1:30) hr. at(60) voltage.

Lane (M): DNA molecular size marker (3000-bp ladder).
Lane ($P_{3/1)}$: shows clinical isolate (first dilution).
Lanes ($P_{3/2}$): shows clinical isolate (second dilution).
Lanes ($P_{3/3}$): shows clinical isolate (third and last dilution).

DISCUSSION

Heavy metals have a variety of applications in controlling microbial population. Lead nitrate was used as a screening test for detection of heavy metals resistance in *P. aeruginosa*. The use of this HM as a screening test was also reported by Vaca Pacheco *et al*. [12] who found all their isolates were resistant to lead nitrate ($PbNO_3$) at a concentration of 400µg/ml.

The interpretation of bacterial resistance to heavy metals be due to the fact that *P. aeruginosa* has many mechanisms for heavy metals resistance like the accumulation of specific ions can be diminished by active extrusion of the heavy metals ion from the cells; or may be reduced to a less toxic oxidative state by the complex enzymes and special oxidation mechanisms in the cells [13].

Prasad *et al.* [14] found that all isolates were sensitive to heavy metals Hg^{2+}, and Pb^{2+} at a concentration of 0.1M, and most of them were resistant to these heavy metals at a concentration of 0.0001M.

Regarding to mercury chloride, Karbasizaed *et al* [15] revealed that coliforms were tolerant to mercury chloride at 54.3 µg/ml, while Prasad *et al* [14] found that all isolates of *P. aeruginosa* were sensitive to mercury chloride in concentration 0.0001M, 0.001M, 0.01M, and 0.1M. *Pseudomonas aeruginosa* were able to resist mercury because it has *mer* operon that reduced toxic Hg^{2+} to volatile Hg^0, which then diffuses out of the cell.

According to lead nitrate, the results showed that all isolates were resistant to lead nitrate (Table 1). These results are similar to that obtained by Karbasized *et al* [15] who revealed the coliforms were tolerant to

lead nitrate was in a MIC of 3200 µg/ml. Prasad *et al*. [14] found that all isolates of *P. aeruginosa* were sensitive to lead nitrate at concentrations of 0.001M, 0.01M and 0.1M.

Biofilms are slimy aggregates of microbes that are likely responsible for many chronic infections as well as for contamination of clinical and industrial environments.

Results of this study found that the heavy metal resistance (HMR) of *P. aeruginosa* isolates is not correlated with production of the biofilm except for mercury chloride ($HgCl_2$) where there was a clear correlation between biofilm production and mercury resistance (Table 2).

A hallmark trait of biofilms is increased resistance to antimicrobial agent compared to the resistance of free-swimming organism [6]. Teizel and Parsek [6] reported that biofilms are more resistant to HM than either stationary-phase or logarithmically growing plankotonic cells. The exterior of the biofilm was preferentially killed after exposure to elevated concentrations of copper. A potential explanation for this is that EPS that encase a biofilm may be responsible for protecting cells from heavy metals stress by binding the heavy metals and retarding their diffusion within the biofilm.

Many researchers worldwide reported that HMR in *P. aeruginosa* is carried on large (mega) plasmids. Raja and Selvam [16] revealed *P. aeruginosa* exhibited resistance to heavy metals such as lead, cadmium, and nickel, due to the presence of plasmid DNA, which was designated as pBC15. The size of this plasmid DNA was approximately 23 kb, and they suggested that nickel and ampicillin resistance gene was conferred by plasmid DNA. The survived resistance of the cured isolates (*P. aeruginosa Ps.*3) to all HM (Figure 1) indicates that the HM resistance trait was carried on chromosome rather than plasmid. This could be due to that the plasmid was not cured out because it is really difficult to cure large mega plasmids. Many isolates of *P. aeruginosa* have no plasmid content and still show heavy metals resistance that lead to think that gene responsible for these resistances found on the chromosome [16].

CONCLUSIONS

Heavy metals, particularly silver and mercury, have a variety of applications in controlling microbial population. *P. aeruginosa* is a prevalent hospital pathogen because of its high intrinsic resistance to antibiotics and heavy metals. In this study, *P. aeruginosa* isolated from different clinical were tested for their susceptibility to seven three heavy metals. It was found that most of the isolates were resistant to these heavy metals in some of concentrations. Our findings support the fact that the heavy metal resistance of *P. aeruginosa* is not correlated with production of the biofilm except for mercury chloride.

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