Heavy Metals Resistance of *Pseudomonas aeruginosa* Isolated from Clinical and Environmental Sources in Hilla City

Raafet M. Al-Enzi Alaa H. Al-Charrakh
Dept. of Microbiology, College of Medicine, University of Babylon, Hilla-Iraq.

Abstract

The study included 300 samples collected from 150 clinical and 150 hospital environmental sources. Only 43 (14.3%) isolates belonged to *Ps. aeruginosa*. The isolates tested for detection of their susceptibility for 7 types of heavy metals (HM) included (Copper Sulfate, Silver Sulfate, Mercury chloride, Lead nitrate, Zinc sulfate, Cadmium sulfate, and Nickel sulfate). The screening tests for all isolates were detected using Lead nitrate in concentration of (400µg/ml). Results revealed that 37:43 were resistant to lead nitrate (400µg/ml); this results may due to the Pseudomonads have P-type ATPase that can resist pb^{2+}. The minimum inhibitory concentration (MIC) of 7 HM was detected by agar dilution method and pouring method. Results revealed that number of isolates were resistant to 7 HM in some of concentrations due to complex enzymes, active efflux, and operon that reduced toxic. The plasmid content investigated for all 37 isolates of *Ps. aeruginosa* (34 clinical and 3 environmental); Results revealed that most isolates 32:37 harbored large (mega) plasmid. The biofilm production of *Ps. aeruginosa* isolates was investigated. Results showed that 20:43 (47%) of isolates had biofilm. This study concluded that the increase of HM resistance was correlated with biofilm production for some HM used. The bacterial curing is proceeding for one isolate *Ps. aeruginosa* (*Ps.*3). The results showed survived resistance to all HM used, may be due to HM resistance trait was carried out on bacterial chromosome rather than plasmid.

الخلاصة

تضمنت هذه الدراسة جمع ٣٠٠ عينة، ١٥٠ سريرية و ١٥٠ أخرى من بيئة المستشفى لغرض عزل بكتريا الزوائف الزنجارية. تم عزل ٤٣ (١٤.٣%) عزلة من الزوائف الزنجارية وتم الكشف عن حساسيتها لعدد من المعادن الثقيلة (كبريتات الفضة، كبريتات الزنك، كبريتات الكادميوم، كبريتات النيكل، كبريتات النحاس، كلوريدات الرئبق، ونترات الرصاص) من خلال تحديد التركيز المثبط الأدنى لهذه المعادن وبطريقتي التخفيف بالأكار وطريقة صب الأطباق. تم الكشف عن المحتوى البلازميدي للعزلات المقاومة للمعادن الثقيلة وأظهرت النتائج أن معظم العزلات ٣٧:٣٢ أحتوت على بلازميد عملاق. تم التحري عن انتاج الغشاء الحيوي للعزلات ووجد ان (٤٧%) من العزلات منتجة له. وأظهرت النتائج ان زيادة المقاومة للمعادن الثقيلة كان مرتبطا مع قابلية بعض العزلات على انتاج الغشاء الحيوي. أظهرت نتائج تحييد البلازميد البكتيري للعزلات بقاء المقاومة للمعادن الثقيلة بعد التحييد مما يشير الى ان صفة مقاومة المعادن الثقيلة محمولة على الكروموسوم وليست على البلازميد البكتيري.

Introduction

Heavy metals, particularly silver and mercury, have a variety of applications in controlling microbial population [1]. Silver salts alone or in combination with other drugs appear to have a significant potential as topical antimicrobial agent [2, 3]. Mercury in the form of less toxic organic compounds is being used as skin disinfectant [4]. Copper is considered as a safe agent to humans, as demonstrated by the widespread and prolonged use by women of copper intrauterine devices [5, 6].

Pseudomonas aeruginosa is pathogen that causes a substantial portion of hospital infections. It is frequently multi drug resistant, which contributes to the high morbidity and mortality of patients in intensive care units (ICUs), burn units and surgery wards. A major reason for its prominence as a pathogen is its high intrinsic resistance to antibiotics and heavy metals [7]. This a ubiquitous, environmentally important microbe that may employ many resistance mechanisms against different heavy metals, such as the *mer* operon that reduces toxic Hg^{2+} to volatile Hg^{0}, which then diffuses out of the cell [8].

Pseudomonas aeruginosa is a prevalent hospital pathogen that is well known for its ability to form biofilms that are recalcitrant to many different antimicrobial treatments [9].

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 [10].

The aim of this study was to detect the prevalence of heavy metals resistant (HMR) *Ps. aeruginosa* isolates recovered from clinical and environmental samples and studying the correlation between biofilm production and HM resistance**.**

Materials and Methods

This study included 300 samples (150 Clinical and 150 Environmental samples. The clinical specimens were collected from patients suffering from burns, wounds, and otitis media, who attending Hilla teaching hospital; whereas hospital environmental samples included catheters, beds, bath rooms, fomites, wall of wards, and some type of disinfectants at the same hospital.

All samples were inoculated on MacConkey agar, Nutrient agar, and selective medium (cetrimide agar), then incubated at 37ºC for 24-48 hrs. *Ps. aeruginosa* were identified by routine diagnostic tests including cellular, cultural and biochemical characteristics.

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 7 heavy metals (HM) represented by; Copper Sulfate, Silver Sulfate, Mercury chloride, Lead nitrate, Zinc sulfate, Cadmium sulfate, and Nickel sulfate. The minimum inhibitory concentration (MIC) of 7 HM was detected by agar dilution method (silver, zinc, cadmium, nickel) and pouring method (lead, copper, mercury), based on standard methods [11, 12].

The following Concentrations of heavy metals were prepared:

1- 0.0001M, 0,001M, 0.01M for Cadmium and 0.0001M, 0.001M, 0.01M, 0.1M for Nickel, Zinc, and Silver.

2- (100, 200, 400, 800, 1600, 2400, 3200 µg/ml) for lead; (100, 200, 400, 800, 1600, 1750, 3200 µg/ml) for Copper; and (2.7, 5.4, 10.8, 21.6, 43.2, 54.3, 86.4 µg/ml) for Mercury.

Biofilm production, Plasmid profile, and Plasmid curing were studied as follows:

1- biofilm formation were determined using tissue culture-treated, 96-well polystyrene plates, based on the methods of [13,14].

2- Plasmid DNA extraction of gram negative bacteria was performed by using Geneaid kit, the steps of the method was according to the manufacturing company (Geneaid kit, USA) and plasmid profile was carried out by electrophoresis [15].

3- Plasmid curing was carried out using Elevated Temperature method according to [16].

Results and Discussion

Out of the 300 samples, only 43 (14.3%) isolates belonged to *Ps. aeruginosa*. 40(26.6%) of these isolates belonged to clinical samples and 3 (2%) isolates belonged to the *Ps. aeruginosa*.

Heavy metals resistance and MIC:

All 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 (Table 1).

Bacterial resistance to heavy metals, (Table 2, 3) shows the MIC of *Pseudomonas aeruginosa* to all studied heavy metals. In case of copper sulfate ($CuSO_4$). Results indicated that all isolates were resistant $CuSO_4$ in a concentration of 100, 200 µg/ml. The MIC of all isolates were 3200 µg/ml.

In case of silver sulfate ($AgSO_4$). The results showed that the 34:37 isolates were resistant $CuSO_4$ in concentration 0.0001M. The MIC of all isolates were 0.01M.

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

In case of lead nitrate ($PbNO_3$). The results revealed that the 30:37 isolates were resistant $PbNO_3$ in concentration 2400 µg/ml. The MIC of all isolates were 3200 µg/ml.

In case of zinc sulfate ($ZnSO_4$). Four of isolates were sensitive to $ZnSO_4$ in low concentration 0.0001M. The MIC of all isolates were 0.1M.

In case of cadmium sulfate ($CdSO_4$). Five of isolates were sensitive to $CdSO_4$ in low concentration 0.0001M. The MIC value of all isolates were 0.1M.

In case of nickel sulfate ($NiSO_4$), the results indicated the all of isolates were resistant to $CdSO_4$ in concentration 0.0001M and 0.001M. The MIC of all isolates were 0.01M. The interpretation of these results may be due to the fact that *Ps. aeruginosa* has many mechanisms for heavy metals resistance; firstly, the accumulation of specific ions can be diminished, not by interference with uptake but by active extrusion of the heavy metals ion from the cells. This mechanism is specific only for *Pseudomonas* spp. Secondly, cations can be segregated in to complex compound by thiol-containing molecules and then ejected from cell. Thirdly, some metal ions may be reduced to a less toxic oxidative state by the complex enzymes and special oxidation mechanisms in the cells and finally, for many metals resistance and homoeostasis is a combination of two or three of the mentioned basic mechanisms that is the case which *Ps. aeruginosa* success [17].

Biofilm formation:

The biofilm formation by *Ps. aeruginosa* isolates was investigated as shown in (Table 4). The results show that 20:43 (47%) of isolates had biofilm. Also the results showed that all environmental isolates are biofilm producers (3 isolates) whereas only 17:40 (42.5%) of clinical isolates are biofilm production. The relationship between biofilm production and heavy metal resistance (HMR) is studied. It is found that the HMR of *Ps. aeruginosa* isolates is not correlated with production of the biofilm. From these results, this study concluded that the increase of to HM resistance was correlated with biofilm production for some (but not all) HM used.

***Plasmid profile**:*

The plasmid content was investigated for all 37 isolates of *Ps. aeruginosa* (34 clinical and 3 environmental) (Figure 1). Results revealed that most isolates 32:37 harbored large (mega) plasmid with large (huge) molecular weight that cannot be detected using 3000 bp size marker (ladder) used in the present study.

***Curing of bacterial plasmids**:*

The bacterial curing was concluded for one isolate *Ps. aeruginosa* (*Ps.*3) (Figure 2). The results showed survived resistance to all HM. This result indicates that the HM resistance trait was carried on chromosome rather than plasmid.

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Table 1 Numbers and percentage of clinical and environmental *Pseudomonas aeruginosa* detected by screening test:

Susceptibility to $PbNO_3$ (400µg/ml)	**No. of isolates (%)**		**Total**	**(%)**
	Clinical	**Environmental**		
Resistant	34(85%)	3 (100%)	37	85%
Sensitive	(15%)	0 6	6	15%
Total	40(92.8%)	3(6.9%)	43	100%

Table 2 MIC values of *Pseudomonas aeruginosa* isolates to Silver sulfate, Zinc sulfate, Cadmium sulfate, Nickel sulfate in Molar concentrations.

Isolates	MIC of Silver sulfate	MIC of Zinc sulfate	MIC of Cadmium sulfate	MIC of Nickel sulfate
*Ps.*1	0.01	0.1	0.001	0.01
*Ps.*2	0.01	0.1	0.001	0.01
*Ps.*3	0.01	0.1	0.01	0.01
*Ps.*4	0.01	0.1	0.001	0.01
*Ps.*5	0.01	0.1	0.001	0.01
*Ps.*6	0.01	0.1	0.01	0.01
*Ps.*8	0.01	0.01	0.01	0.01
*Ps.*9	0.01	0.1	0.01	0.01
*Ps.*12	0.01	0.1	0.001	0.01
*Ps.*13	0.01	0.1	0.001	0.01
*Ps.*14	0.01	0.1	0.001	0.01
*Ps.*15	0.01	0.1	0.01	0.01
*Ps.*16	0.001	0.01	0.001	0.01
*Ps.*17	0.001	0.1	0.0001	0.01
*Ps.*19	0.0001	0.0001	0.001	0.01
*Ps.*20	0.001	0.01	0.001	0.01
*Ps.*21	0.001	0.01	0.001	0.01
*Ps.*22	0.01	0.01	0.001	0.01
*Ps.*24	0.001	0.01	0.001	0.01
*Ps.*25	0.01	0.01	0.001	0.01
*Ps.*26	0.001	0.01	0.001	0.01
*Ps.*27	0.01	0.01	0.01	0.01
*Ps.*28	0.001	0.1	0.01	0.01
*Ps.*29	0.001	0.01	0.001	0.01
*Ps.*30	0.001	0.01	0.001	0.01
*Ps.*31	0.0001	0.0001	0.01	0.01
*Ps.*32	0.001	0.1	0.01	0.01
*Ps.*33	0.001	0.01	0.01	0.01
*Ps.*34	0.001	0.01	0.001	0.01
*Ps.*36	0.0001	0.0001	0.01	0.01
*Ps.*37	0.001	0.01	0.0001	0.01
*Ps.*38	0.001	0.01	0.01	0.01
*Ps.*39	0.001	0.01	0.001	0.01
*Ps.*40	0.01	0.01	0.0001	0.01
*Ps.*41	0.001	0.01	0.01	0.01
*Ps.*42	0.001	0.1	0.0001	0.01
*Ps.*43	0.001	0.0001	0.0001	0.01

Table 3 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	١٦٠٠
*Ps.*40	1600	86.4	3200
*Ps.*41	1600	54.3	3200
*Ps.*42	1600	54.3	3200
*Ps.*43	1600	43.2	3200

Table 4 Biofilm production by *Pseudomonas aeruginosa* isolates recovered by Clinical and Environment samples:

No. of isolates	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 number between brackets indicates the standard value of Biofilm production by ELIZA technique.

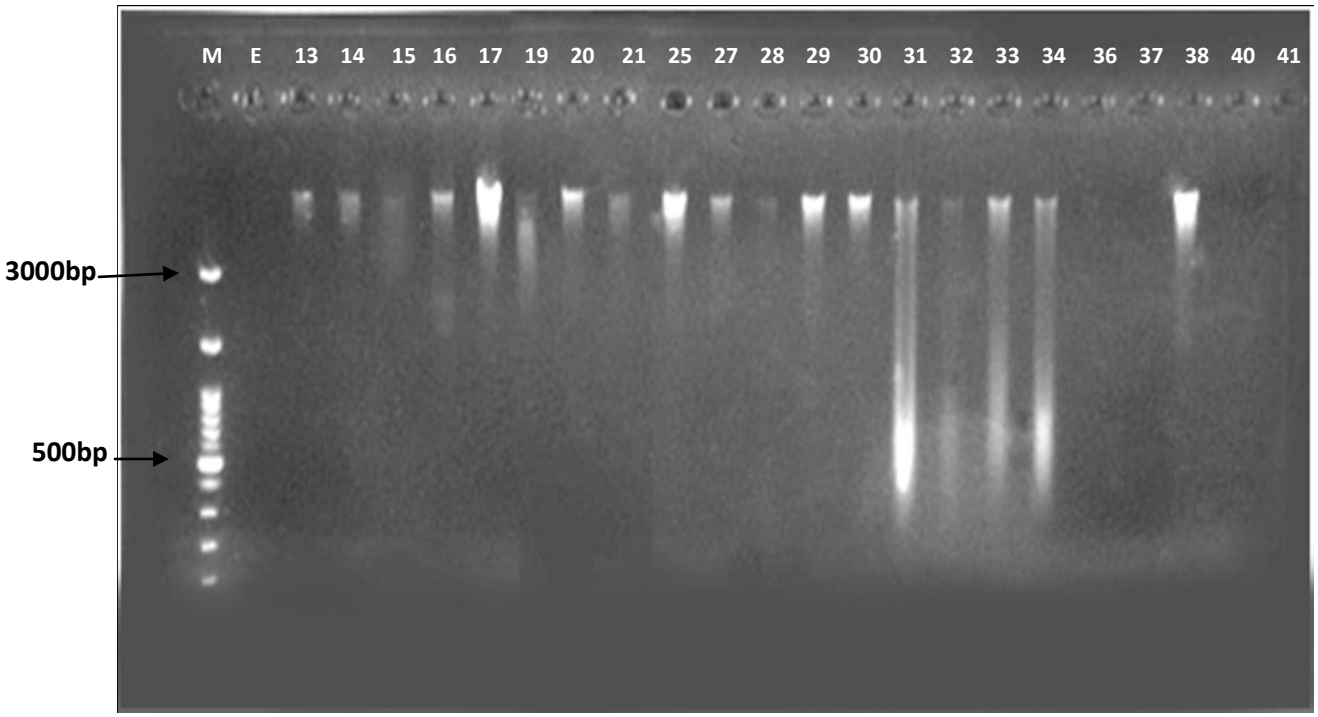

Figure 1 Gel electrophoresis of plasmid DNA content of *Ps. aeruginosa* isolates after (1:30) hr. at(60) voltage.
Lane (M): DNA molecular size marker (3000-bp ladder).
Lane (E): Show negative control (*E. coli standard strain MM294).*
Lanes: (P13), (P14), (P15), (P16), (P17), (P19), (P20), (P21), (P25), (P27), (P28), (P29), (P30), (P31), (P32), (P33), (P34), (P36), (P37), and (P38) shows clinical isolates.
Lanes :(P40), (P41) Show environmental isolates.

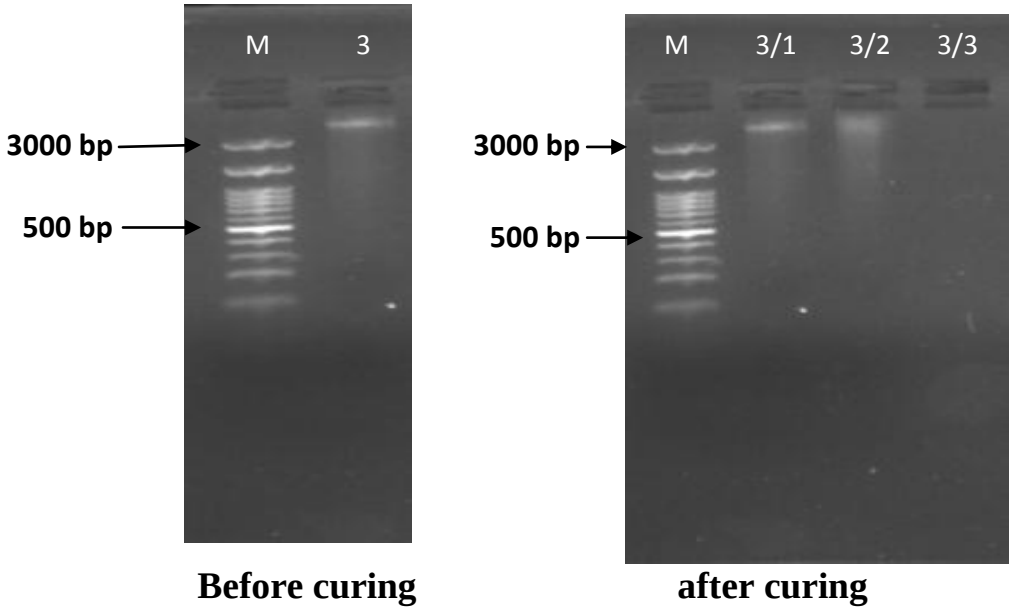

Figure 2 Gel electrophoresis of plasmid DNA content of *Ps. aeruginosa* isolate before and after curing after (1:30) hr. at(60) voltage.
Lane (M): DNA molecular size marker (3000-bp ladder).
Lane ($P_{3/1)}$: show Clinical isolates (first dilution).
Lanes ($P_{3/2}$): show Clinical isolates (second dilution).
Lanes ($P_{3/3}$): show Clinical isolates (third and last dilution).