THE INFLUENCE OF HEAVY METALS AND ANTIMICROBIAL ON STAPHYLOCOCCUS AUREUS AND PSEUDOMONAS AEROGINOSA ISOLATES

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Keywords; *Staphylococcus aureus* , gentamycin, chloramphenicol

ABSTRACT

Two isolates of *Staphylococcus aureus* and Pseudomonas aeroginosa of bacteria were exposed to different concentrations (5 , 10 or 25 mg /l)of heavy metals ions (Silver Ag2+ , Cobalt Co2+ or Lead Pb2+) for different exposure periods (0 , 1, 2, 24 and 48 hrs.) .The results showed that the inhibitory effect of these metals on the growth of the two isolates were in the following sequences :- Pb < Co < Ag

Some of these metals caused an elongation of lag phase in liquid medium for these isolates . Whene antibiotic discs (chloramphenicol 30 mcg , clindamycin 2 mcg , erythromycin 15 mcg , gentamycin 10 mcg ,tetracyclin 30 mcg and vancomycin 30 mcg) were added to solid medium which was previously supplemented with the above heavy metals ions , a synergestics effect was observed between antibiotics and heavy metals ions (specially silver ions at 25 mg/ l)to increase the inhibition zone significantly ($P < 0.01$) for both isolates .

INTRODUCTION

The high frequency of antibiotic resistant staphylococci , even among those not known to have been treated with antibiotics would obscure subtle changes in the microbial flora (1). Multidruges resistance strains of Pseudomonas aerogenosa are the major cause of mortality in various clinical conditions , such as cystic fibrosis (2 , 3 , 4 , and 5) and burn-wound infections (6 , 7 and 8) especially in nosocomial situations . This condition has led to the development of new or modified antimicrobial agentes

for the therapy of such infections . Heavy metals , particulary silver and mercury , have a variety of applications in controlling microbial populations (9) .

Microorganisims require some metals like Cu 2+ , Zn 2+ ,Co2+ and Ni2+ at low concentrations as essential micronutrients for vital cofactors for metalloprotiens and certein enzymes , however , at high concentration it has been reported that these metals interact with nucleic acids and enzyme active sites. Toxic effect include ion displacement and / or substituation of essential ions from cellular sites and blocking of functional groups of important molecules , e.g. , enzymes , polypeptides , and essential nutrients transport system (10).

Silver salts alone or in combination with other druges appeare to have a significant potential as antimicrobial agents (11 , 12 , and 13) . Similarly , mercury in the form of less toxic organic compoundes is being used as skin disinfection (14) .

Some novel transition metal (Cu 2+ , Zn 2+ ,Co2+ and Ni2+) complexes of substituted pyriden schiff- bases have been prepared in order to evaluate the effect of metals ions upon chelation , the schiff- bases and their complexes as antibacterial against Escherichia coli , Staphylococcus aureus and Pseudomonas aeroginosa , the complexed Schiff- bases have showen to be more antibacterial against one more bacterial species as compared to uncomplexed schiff- bases (15).

Ahmed et al (16) synthesized complexes of Co2+,Ni2+ , Cu2+ , Mn2+ , Zn2+ , Fe2+ ,Fe3+ and La3+with methylenedisalicylic acid (MDSA) and revealed a high antimicrobial activity for S . aureus and P. aeroginosa . Very little information is available in the literature regarding the action of heavy metals against clinical isolates (17).

This study carried out to assess the effects of heavy metals ions on S. aureus and P. aeroginosa , and to assess the effect of compensation between heavy metals and antibiotics on the above microorganisims.

MATERIALS AND METHODS

Tow isolates Staphylococcus aureus and Pseudomonas aeroginosa were obtained from bacteriological lab. at College of Science, University of Basrah . Neutrient Agar (NA) was used for culturing and activation of these isolates for 24 hrs. ,then bacterial cell suspension was prepared in the concentration 1×106 cell / ml by using Petroff-Hausser counting chamber(18) . One ml of this cell suspension was added to each

tube which containing 20 ml of neutrient broth (NB) medium supplemented with one concentration (5 , 10 or 25 mg/ l)of heavy metals ions : Ag as AgNO3 ,pb as PbNO3 or Co as Co(NO3)2 .6H2O) , triplicates were done for each treatment in addition to control . These cultures were incubated at 37 ◦C . The growth rate (OD at 600 nm) was measured after different exposure periods (0 , 1 , 2 , 24 or 48 hrs.) . From each treatment 0.2 ml was streaked (after 24 hrs. exposured period) on Muller – Hinton Agar (MHA) medium free of metals ions , left for 15 min. to dry . Commercial antibiotics discs(4 discs / plate) were used (Table 1) .

The diameter of inhibition zones were recorded after overnight incubation at 37◦ C.

Another experiment for antibiotic sensitivity test was done on a defined medium (1.5 % w/ v MHA) using plates containing solidified medium (15 ml / plate) supplemented with heavy metals ions . A sample for a late - exponential phase culture(0.3 ml) containing about 1×106 cell / ml was streaking onto the plates and allowed to dry . Commercial antibiotics discs were used at the concentrations that mentioned in table 1. The diameter of the inhibition zones were recorded after overnight incubation at 37 ◦C .

Data were analyzed statistically by using the analysis of variance (ANOVA test) and revised least significant differences test (RLSD) to compare the means .

Table (1) : Interpretation of inhibition zone diameter (Bioanalyse sensitivity discs Ankara / Turkey) .

Antibiotic	**Symbol**	**Concentration**	**Diameter of inhibition zone**	
			Resistant	**Sensitive**
Chloramphenicol	**C**	**30 mcg**	**≤ 12**	**≥ 18**
Clindamycin	**DA**	**2 mcg**	**≤ 14**	**≥ 17**
Erythromycin	**E**	**15 mcg**	**≤ 13**	**≥ 18**
Gentamycin	**CN**	**10 mcg**	**≤ 12**	**≥ 15**
Tetracyclin	**TE**	**30 mcg**	**≤ 14**	**≥ 19**
Vancomycin	**VA**	**30 mcg**	**≤ 9**	**≥ 12**

RESULTS AND DISCUSSION

The effect of heavy metals ions on the growth of P. aeroginosa in the liquid medium.

1- The effect of silver ions (Ag1+):-

The exposure of bacteria to silver ions at 5 or 10 mg/l concentrations lead to increase the lag phase period comparable with the control treatment , while the concentration 25 mg / l lead to significant decreasing ($p < 0.01$) in the growth of bacteria without increasing in the lag phase period(Fig. 1)

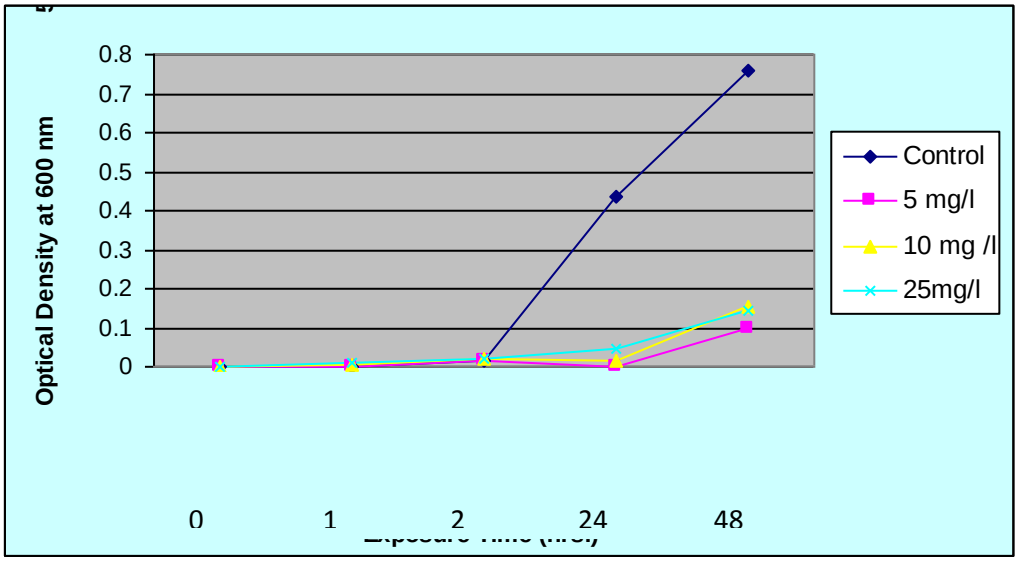

Fig (1) : Effect of silver ions on the growth of Pseudomonas aeroginosa in the liquid medium at different exposure periods.

2- The effect of cobalt ions (Co 2+) :-

The effect of cobalt ions at 5 mg /l concentration was not differ significantly comparable with the control treatment . On the other hand ,10 mg /l concentration lead to relative decreases in the growth comparable with control treatment , while 25mg /l of cobalt ions lead to elongation the lag phase more than 24 hrs., with relative decreases in the growth rate (Fig. 2).

3- The effect of lead ions (Pb2+) :-

The growth of bacteria was not differ significantly comparable with the control treatment at all concentrations of lead ions , and at all the period of the experiment (Fig. 3).

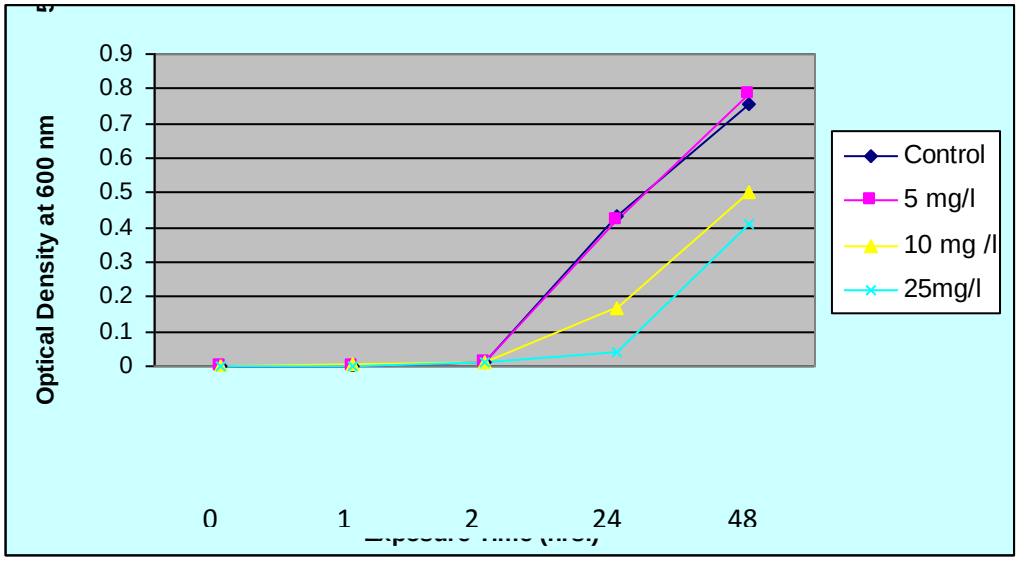

Fig (2) : Effect of cobalt ions on the growth of Pseudomonas aeroginosa in the liquid medium at different exposure periods.

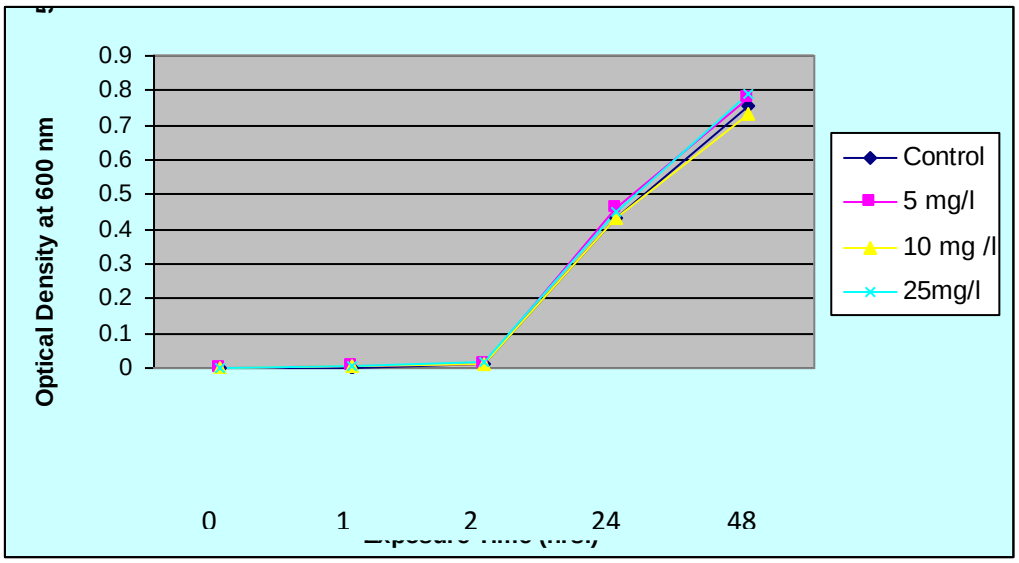

Fig (3) : Effect of lead ions on the growth of Pseudomonas aeroginosa in the liquid medium at different exposure periods.

The effect of heavy metals ions on the growth of S. aureus in the liquid medium .

1- The effect of silver ions (Ag1+):-

Silver ions at 5 mg /l was elongated the lag phase period more than 24 hrs. , and caused decreasing the growth after 48 hrs. While 10 mg /l revealed significant decreasing in the growth of bacteria without increasing in the lag phase periods , but 25 mg /l caused decreasing in the growth rate after 48 hrs. with increasing the lag phase for more than 24 hrs. (Fig. 4).

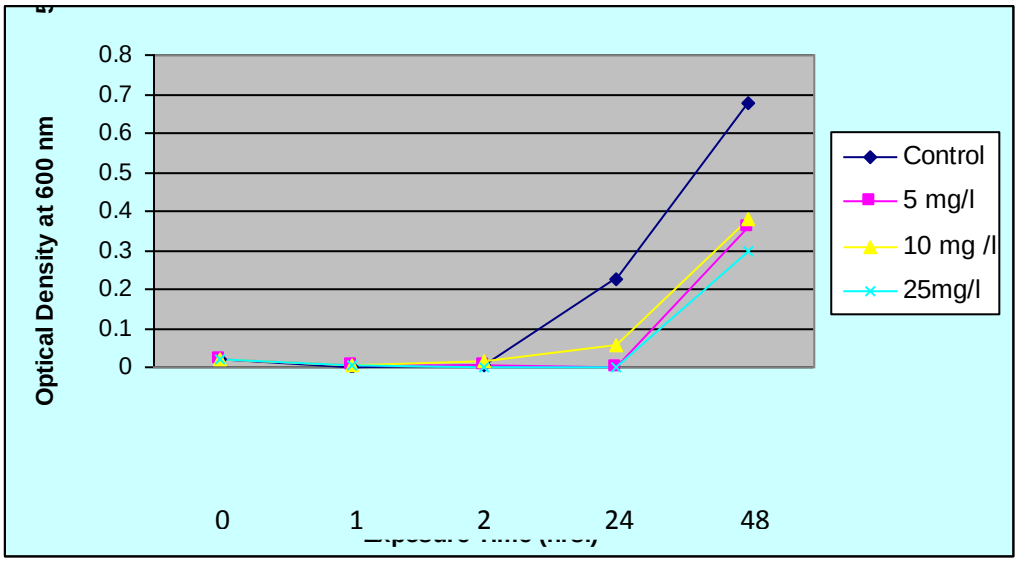

Fig (4) : Effect of silver ions on the growth of Staphylococcus aureus in the liquid medium at different exposure periods.

2- The effect of cobalt ions (Co 2+) :-

The growth rate of bacteria was decreased at 5mg /l concentration without an effect on the lag phase period . Ten mg /l concentration was elongated the lag phase period and decreased the growth rate , while 25 mg /l concentration was decreased the growth after 1 and 2 hrs. of exposure period ., and no growth was appeared after 24 or 48 hrs. of the exposure period (Fig. 5).

3- The effect of lead ions (Pb2+) :-

The growth rate was decreased whene lead was used at 5 , 10 or 25 mg /l concentrations , without an effect on the lag phase period . The growth rate was significantly decreased ($p< 0.01$) in comparable with the control treatment (Fig. 6)

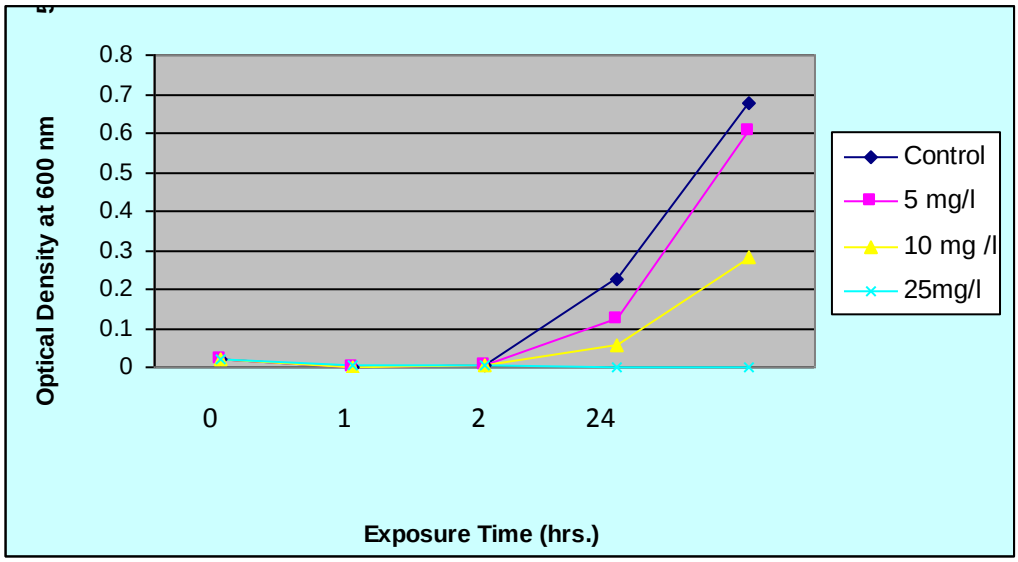

Fig (5) : Effect of cobalt ions on the growth of Staphylococcus aureus in the liquid medium at different exposure periods.

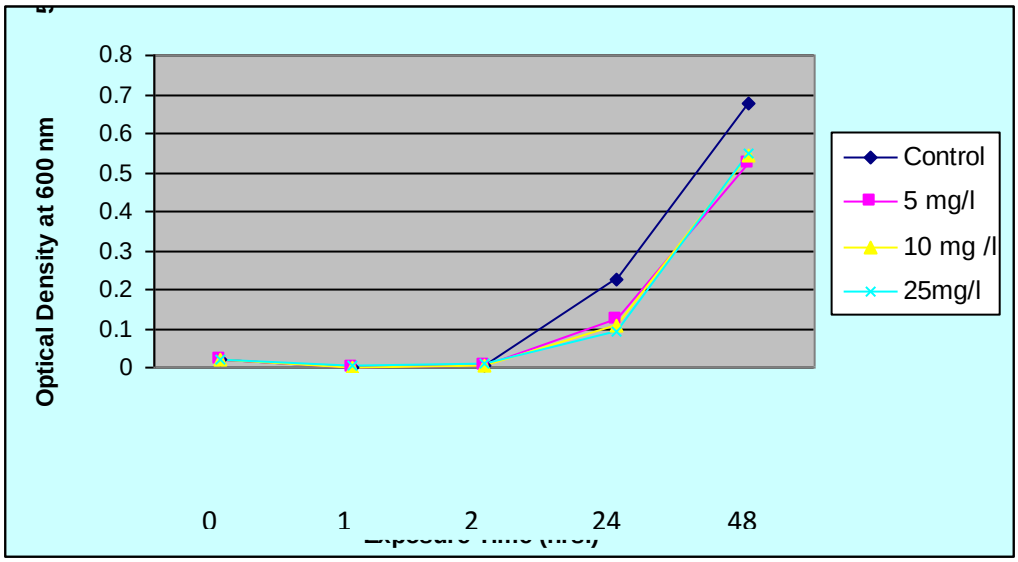

Fig (6) : Effect of lead ions on the growth of Staphylococcus aureus in the liquid medium at different exposure periods.

The different effect probably depended on the strain and on the experimental conditions such as: strain source , time of exposure (19), and type of element , the mechanism of efflux system that differ between S. aureus and P. aeroginosa (10).

Also the difference depended on the differences in the wall binding sites to interact with the metal ions , and differ in metal ions affinity to interact with the wall binding sites (20).

Pseudomonas aeroginosa exhibited increasing lag phase in growth after exposure to high concentrations of the heavy metals .This observation appeare to be due to the selection for metal resistant phenotype (10).

The synergistic effect of heavy metals ions and antibiotics on the growth of P. aeroginosa in the solid medium

To investigate the co-effect of heavy metals with antibiotics on the tested bacteria . The results of experiment was revealed significant increasing ($P< 0.01$) in the inhibition zone when used chloramphenicol antibiotic with silver or cobalt ions in the solid medium plates (1,2) , and no growth appeared at chloramphenicol antibiotic with 25 mg/l silver ions . On the other hand , the inhibition zone do not differ significantly ($P> 0.05$) in treatments which contaning chloramphenicol with lead ions, plate(3) comparable with the control treatment which was free from any addition of metals ions, table (2).

Table (2) : Inhibition zone diameter (mm) of antibiotic alon and with heavy metals ions on P. aeroginosa at the solid medium

Antibiotic	Alone	With heavy metals ions (mean± SD)								
		Ag			Co			Pb		
		5	10	25	5	10	25	5	10	25
C	26.5±0.5	28.5 ±1.5	28 ±2	N.G.	29±1	27.5 ±0.5	28.25± 0.25	27 ±3	27.5±0.5	27.5 ±0.5
CN	21.5 ±0.5	20.5 ±0.5	17 ±3	N.G.	20.25±1.75	22 ±0	20.5 ±0.5	22±0	18 ±2	19 ±0
TE	22.5 ±0.5	19.5 ±1.5	23.5 ±0.5	N.G.	23.25 ±1.25	22±1	22.25 ±0.25	20 ±0	23.5 ±0.5	23 ±0
E	R	17 ±1	13.5 ±0.5	N.G.	13 ±3	13.5 ±0.5	12.5 ±0.5	R	12.5 ±2.5	13.75 ±0.75

SD: Standard Deviation NG: No Growth R:Resistant

The addition of gentamycin to the medium that supplemented with heavy metals ions showed a significant increasing (P<0.01) in the inhibition zone in the treatment which supplemented with 25 mg / l of silver ions, and no growth was appeared at this treatment , plate (1) .When the addition of tetracycline also a significant increasing (P< 0.01) in the inhibition zone and no growth appeared at treatments which contaning 25 mg /l of silver ions . On the other hand , the least inhibition zone was found at the treatment which supplemented with 5 mg /l of silver ions , plate (1) and at the treatment with 5 mg /l of lead ions plate (3). When erythromycin used with the supplemented medium with the previous concentrations of heavy metals ions , a significant increasing (P < 0.01)was revealed in the inhibition zone at the treatment contain 25 mg /l of silver ions (no growth was appeared) followed by treatment with 5 mg /l of the same metals ions . The inhibition zones were not differ at the other treatment as compared with the control treatment which was free from an additions of heavy metals ions .

In general the inhibition zone in the presence of chloramphenicol was higher significantly ,while leas inhibition zone was appeared with erythromycin when added to the medium supplemented with different heavy metals ions .

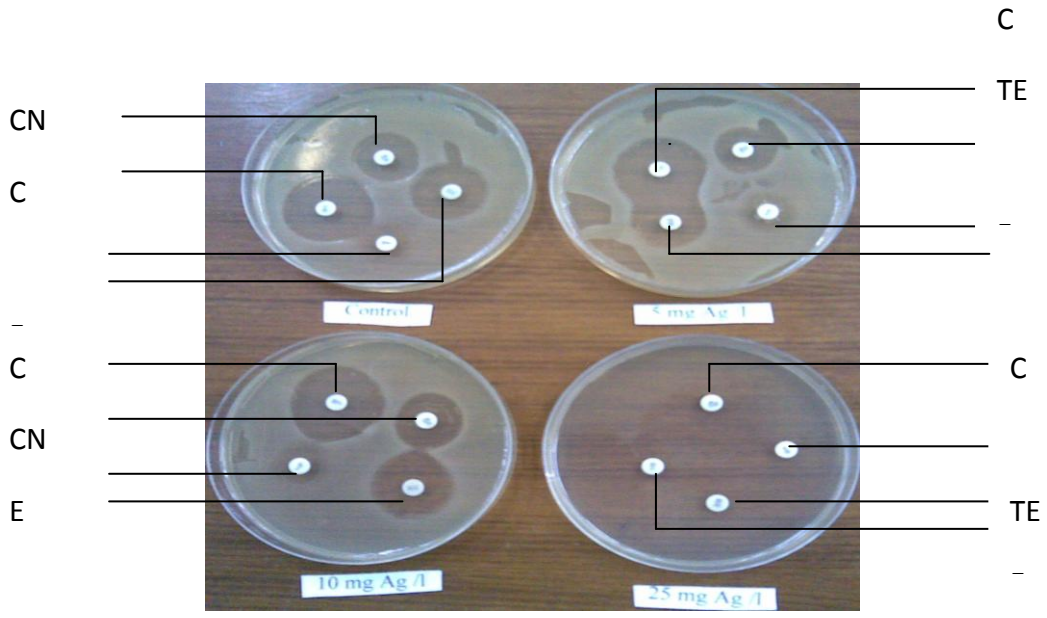

Plate (1) : Effect of silver ions with antibiotics on the growth of P. aeroginosa on solid medium.

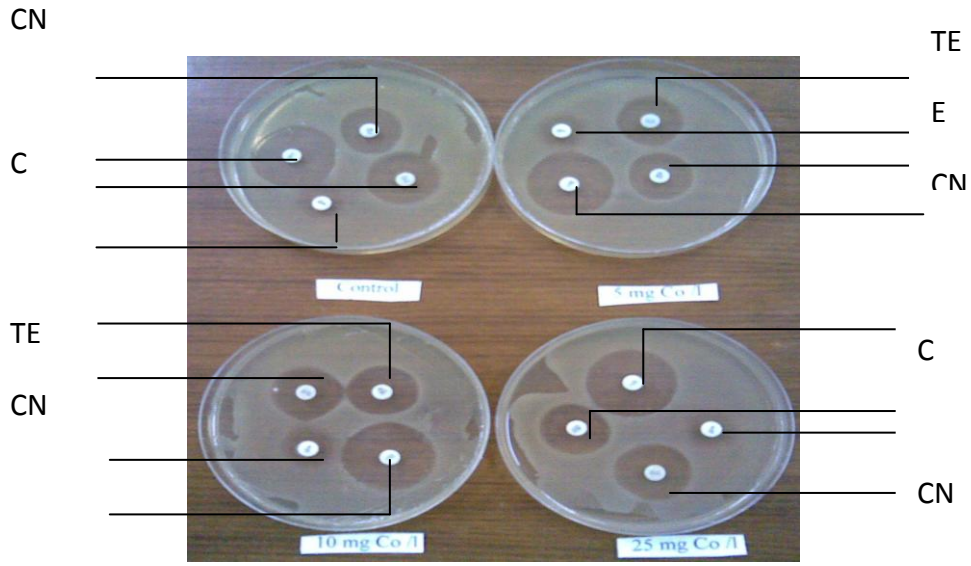

Plate (2): Effect of cobalt ions with antibiotic on the growth of P. aeroginosa on solid medium.

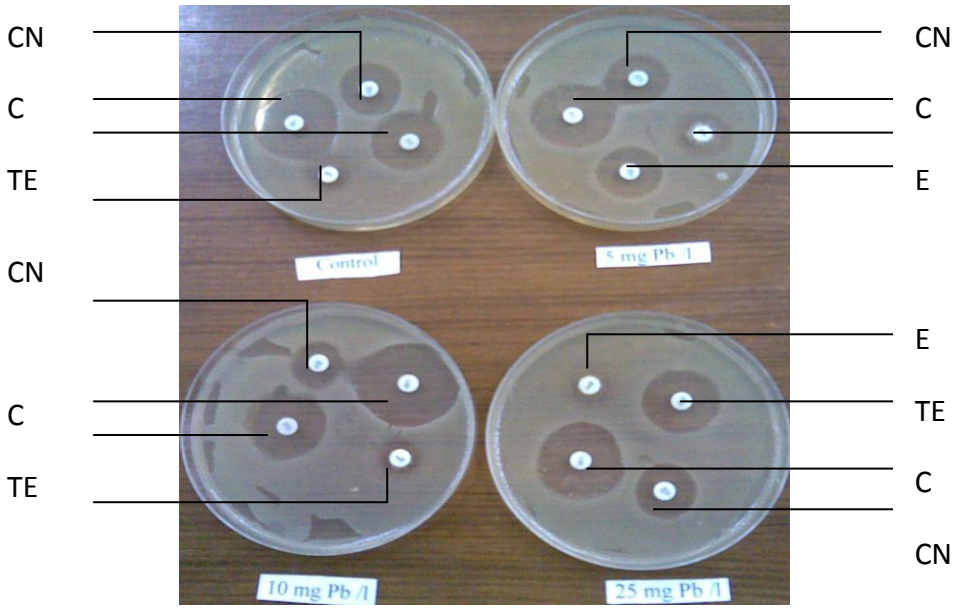

Plate (3): Effect of lead ions with antibiotic on the growth of P. aeroginosa on solid medium.

The synergistic effect of heavy metals ions and antibiotics on the growth of S. aureus in the solid medium

The addition of chloramphenicol antibiotic to the medium which supplemented with 5 or 25 mg/l of silver ions, plate (4) , or cobalt at 25 mg/l , plate (5) or lead at 5 mg/l , plate (6)caused a significant increasing($p<0.01$) in the inhibition zone comparable with the control treatment (free from any addition of heavy metals ions) , table (3).The addition of gentamycin antibiotic to the medium which supplemented with 25 mg/l of silver ions , plate (4) caused a significant increasing($p<0.01$) in the inhibition zone comparable with the control treatment .Also, the addition of vancomycin to the medium which supplemented with different concentration of heavy metals ions showed a significant increasing ($p< 0.01$)in the inhibition zone at the treatment which has 25 mg/l of silver ions , or treatments which have 10 mg/l of cobalt ions ,while the inhibition zone was decreasing largly at the treatment that has 5 mg / l of silver ions, plate (4) .

Table (3):- The inhibition zone diameter (mm) of antibiotic alon and with heavy metals ions on S. aureus in the solid medium

Antibiotic	**Alone**	**With heavy metals ions (mean± SD)**								
		Ag			**Co**			**Pb**		
		5	**10**	**25**	**5**	**10**	**25**	**5**	**10**	**25**
C	**27.5±0.5**	**29 ±1**	**25±1**	**31±3**	**23±1**	**28±4**	**30±2**	**33±5**	**27.5±2.5**	**26±2**
CN	**27± 2**	**27±3**	**28±4**	**37±3**	**23±3**	**25±1**	**27.5±0.5**	**24±0**	**25±1**	**22±4**
VA	**R**	**R**	**R**	**24±4**	**R**	**19±1**	**R**	**R**	**R**	**R**
DA	**R**	**29 ± 1**	**R**	**40±0**	**R**	**30.5±1.5**	**R**	**R**	**R**	**R**

SD: Standard Deviation R: Resistant

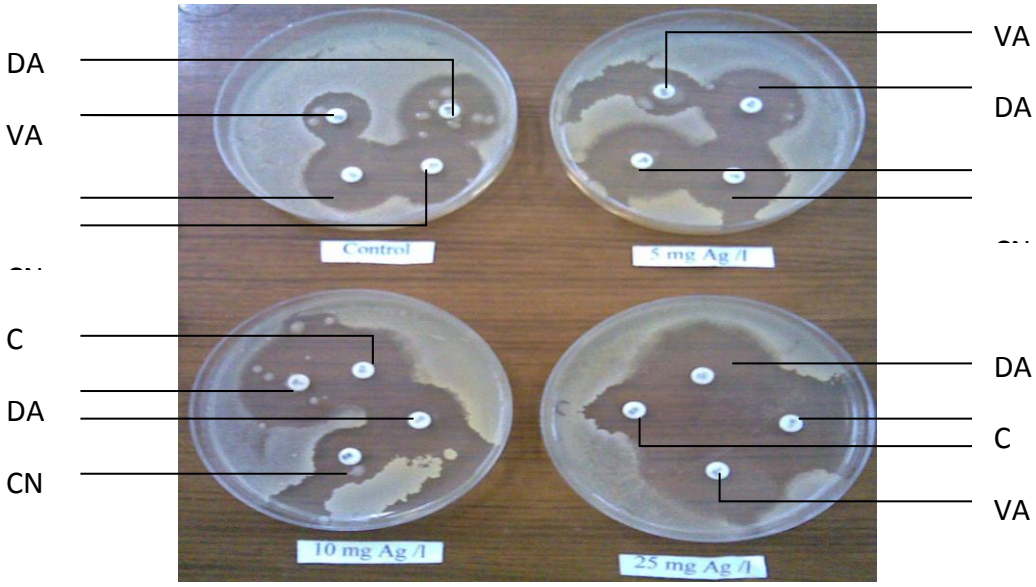

Plate (4) : Effect of silver ions with antibiotic on the growth of S. aureus on solid medium .

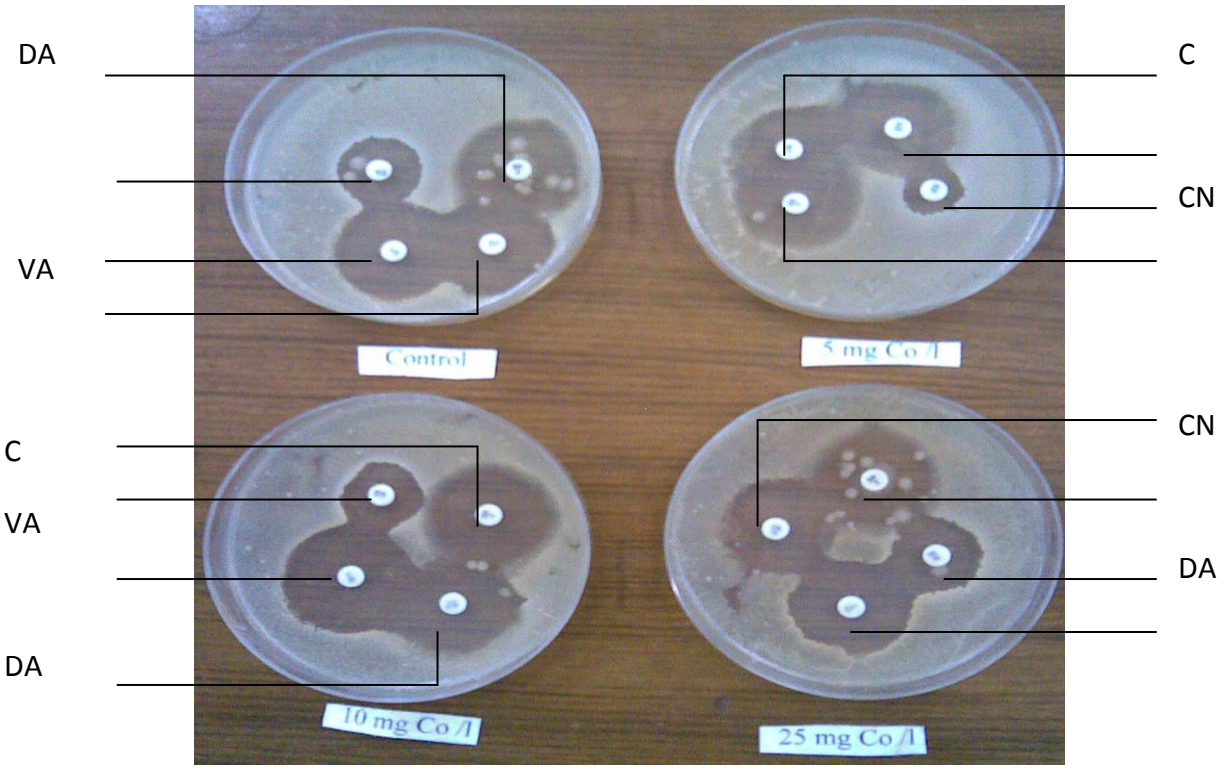

Plate (5) : Effect of cobalt ions with antibiotic on the growth of S. aureus on solid medium .

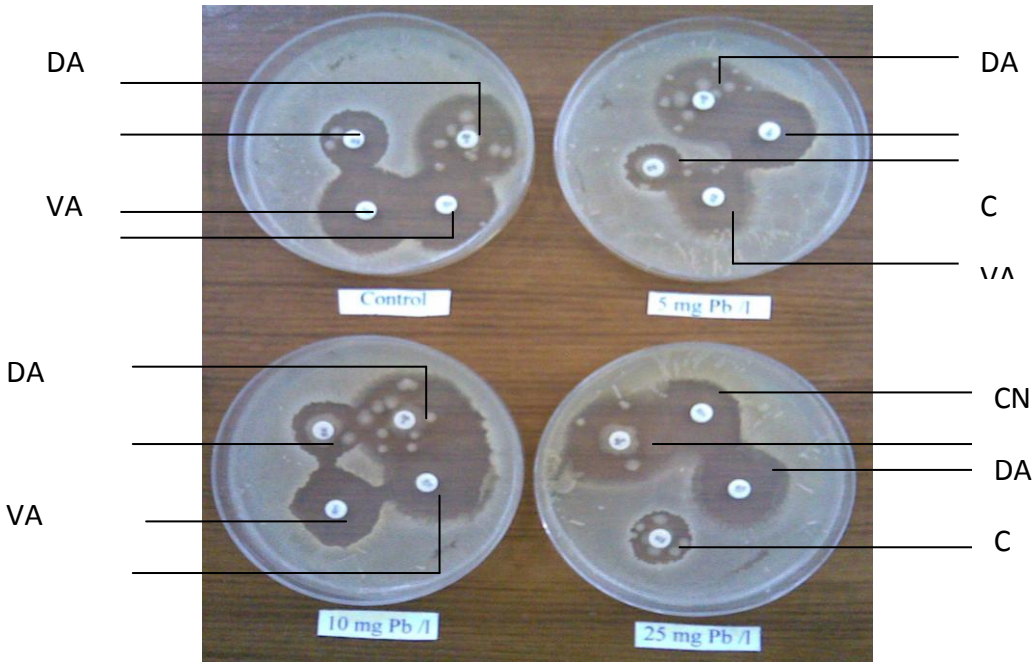

Plate (6) : Effect of lead ions with antibiotics on the growth of S. aureus on solid medium.

The addition of clindamycin antibiotic to the medium which supplemented with different heavy metals ions , a significant increasing ($p< 0.01$) in the inhibition zone was found at the treatment with 5 or 25 mg/ l of silver ions , plate (4) or 10 mg/l of cobalt ions , plate (5) . On the other hand the isolate exhibite a resistant to the antibiotic in the treatment which containe 10 mg /l of silver ions , and 5 or 25 mg/l of cobalt ions , plate (5) ,and all treatments of lead ions , plate (6).

In general , the inhibition zone of chloramphenicol was the highest , while the vancomycin antibiotic has the the lowest effect as compared with the other antibiotics on the growth of bacteria in the presence of heavy metals ions .

Some S. aureus strains resistance to mostly antibiotics (21). The cause of this resistance beyond to production of some enzymes in bacteria and mis used of antibiotic which lead to multidrugs resistance (22). Antibiotic resistance of bacteria is increasing worldwide may result public health problems (23). Multidrugs resistance strains of P.aeruginosa are the major cause of mortality in various clinical conditions , such as cystic fibrosis (5) and burn-wound infections (6), especially in nosocomial situations . This situation has lead to the development of new or modified antimicrobial agents for the therapy of such infection . Heavy metals , particularly silver and mercury , have a variety of applications in controlling microbial

populations (9) . Silver salts alon or in compensations with other drugs appear to have a significant potential as topical antimicrobial agents (13) .

The present study revealed increased susccebtibility of tested isolates toward certin antibiotics when supplemented with heavy metals ions . This results was inline with the results of Higham et al (24) who found that cadmium adapted cells of Pseudomonas putida showed greatly increased sensitivity against some antibiotics , including : aminoglycosides , cyclic polypeptides, and doxycyclinen . It is suggested that this is related to changes in outer membrane structure . Also , the resuls inagreement with results obtained by Ahmed et al (16)

In conclusion heavy metals ions like silver and cobalt alon or in combination with other drugs appear to have a significant potential antimicrobial agents against the major causes of skin infection of human and animals , and burn – wound infections . In addition the heavy metals ions (silver and cobalt) lead to increase the inhibition zones of some antibiotics under study .

تاثير المعادن الثقيلة والمضادات الحياتية على العزلتين الجرثوميتين *Pseudomonas aeroginosa* و *Staphylococcus aureus*

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الخلاصة

تم تعريض العزلتين الجرثوميتين *Pseudomonas aeroginosa* و *Staphylococcus aureus* الى تراكيز مختلفة (5 ، 10 او 25) ملغم/ لتر من ايونات المعادن الثقيلة (الفضة Ag^{2+} ، الكوبلت Co^{2+} او الرصاص Pb^{2+})لفترات تعريض مختلفة (0 ، 1 ،2 ، 24 ، او 48 ساعة).

اوضحت نتائج الدراسة ان التاثير التثبيطي لتلك الايونات على نمو العزلتين كان بالتسلسل التالي

$Pb < Co < Ag$

ادت اضافة بعض تلك المعادن الى زيادة الطور التمهيدي lag phase لتلك العزلتين في الوسط الزرعي السائل . و عند اضافة اقراص المضادات الحياتية (Chloramphenicol 30 mcg , Clindamycin2 Vancomycin mcg , Erythromycin 15 mcg , Gentamycin 10 mcg ,Tetracyclin 30 mcg 30, mcg) الى الاوساط الزرعية الصلبة المدعمة بايونات المعادن بالتراكيز السابقة، اظهرت النتائج حصول تاثير تعاوني بين المضادات الحياتية والمعادن الثقيلة (وبشكل خاص ايونات الفضة بتركيز 25 ملغم / لتر) في زيادة منطقة التثبيط معنويا ($P < 0.01$) لتلك العزلتين .

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