

## Bio-Monitoring of Heavy Metal Resistance in *Pseudomonas* and *Pseudomonas* Related Genus

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### ABSTRACT

The aim of present study was to determine the level of heavy metal resistance patterns and to determine if there is a relationship between heavy metal resistance and plasmid presence. From 28 identified strains, 39.3% corresponded to *Stenotrophomonas maltophilia*, 17.9% to *Chryseomonas luteola*, 14.3% to *Pseudomonas fluorescens*, 10.7% to *Pseudomonas putida*, 7.1% to *Sphingomonas paucimobilis*, 7.1% to *Methylobacterium mesophilicum* and 3.6% *Pseudomonas stutzeri*. The resistance of these Gram-negative bacteria to 8 heavy metals, was investigated by agar dilution method. Most isolates showed tolerance to different concentrations of heavy metals, and minimal inhibition concentrations ranged from 0,005 mmol<sup>-1</sup>-20 mmol<sup>-1</sup>. All strains displayed high resistance to zinc and lead (100% and 96,4% respectively) and high susceptibility to silver, cobalt and mercury (92,9%, 92,9% and 96,4% respectively). *M. mesophilicum* strains were determined as the most resistant strains to studied heavy metals. Isolated bacteria were tested for the presence of plasmids using the modified hot alkaline lysis method. The study also demonstrated that about 17.8% of isolated bacteria carried 0.89-21.59 kb sized plasmids and metal resistance profiles of bacteria carrying the same plasmids were similar. This study reveals the heavy metal resistance profiles of non-aeruginosa *Pseudomonas* species and other related species and the association between the occurrence of plasmids and the resistance to heavy metals.

**Key Words:** Metal resistance, Plasmid profili, *Pseudomonas* spp., *Stenotrophomonas*, *Chryseomonas*, *Sphingomonas*, *Methylobacterium*

## *Pseudomonas* ve *Pseudomonas* İlişkili Cinslerin Ağır Metal Dirençliliklerinin Biyolojik İzlenmesi

### ÖZET

Bu çalışmanın amacı ağır metal dirençliliğinin düzeyinin belirlenmesi ve eğer varsa ağır metal dirençliliği ile plazmid varlığı arasındaki ilişkinin ortaya çıkarılmasıdır. İdentifikasyonu yapılmış 28 suşun %39.3'ünün *Stenotrophomonas maltophilia*, %17.9'unun *Chryseomonas luteola*, %14.3'ünün *Pseudomonas fluorescens*, %10.7'sinin *Pseudomonas putida*, %7.1'inin *Sphingomonas paucimobilis*, % 7.1'inin *Methylobacterium mesophilicum* ve %3.6'sının *Pseudomonas stutzeri* olduğu bulunmuştur. Bu Gram-negatif bakterilerin 8 ağır metale karşı dirençlilikleri agar dilüsyon metodu kullanılarak ortaya çıkarılmıştır. Çoğu izolat ağır metallere karşı farklı konsantrasyonlarda tolerans göstermiş ve minimum inhibisyon konsantrasyonları 0,005 mmol-1-20 mmol-1 aralığında tespit edilmiştir. Bütün suşlar çinko(%100) ve kurşun(%96.4)'a yüksek oranda direnç gösterirken, gümüş(%92.9), kobalt(%92.9) ve civa(%96.4)'ya karşı yüksek oranda duyarlı bulunmuştur. *Methylobacterium mesophilicum* suşları çalışmada kullanılan ağır metallere karşı en dirençli suşlar olarak tespit edilmiştir. Bakterilerde plazmid varlığı modifiye edilmiş hot alkalizasyon metodu kullanılarak ortaya çıkarılmıştır. Çalışmada bakterilerin %17.8'inin 0.89-21.59 kb. aralığında plazmidler taşıdığı ve aynı plazmidleri taşıyan bakterilerin benzer metal dirençliliği profillerine sahip oldukları görülmüştür. Bu çalışma non-aeruginosa *Pseudomonas* türlerinin ve diğer *Pseudomonas* ilişkili türlerin ağır metal dirençlilik profillerini ve bu ağır metal dirençliliği ile plazmid varlığı arasındaki ilişkiyi ortaya koymaktadır.

**Anahtar Kelimeler:** Metal Dirençliliği, Plazmid profili, *Pseudomonas* spp., *Stenotrophomonas*, *Chryseomonas*, *Sphingomonas*, *Methylobacterium*

### INTRODUCTION

Pollution due to heavy metal toxicity is an ever-increasing problem in the developing nations. Heavy metals are major pollutants in marine, ground, industrial and even treated wastewater (Valdman et al. 2001). Presence of high concentration of toxic heavy metals in wastewater directly leads to both contamination of receiving water bodies and deleterious impact on aquatic life (Moten and Rehman 1998). Use of such polluted water for

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consumption and other purposes can bring severe problems to human health. At higher concentration, heavy metals form toxic complex compounds in the cell that are too dangerous for any biological functions (Rajbanshi 2008).

Despite the fact that heavy metals are acutely toxic to microbes, there are metal-resistant bacteria. The toxic effects of heavy metals immediately upon introduction to environmental samples have been documented for a broad array of microbial processes. Long term exposure to metals imposes a selection pressure that favors the proliferation of microbes that are tolerant/resistant to this stress (Ünalı et al. 2003).

Microbes apply various types of resistance mechanisms in response to heavy metals (Bruins et al. 2000, Nies 2003). These mechanisms may be encoded by chromosomal genes, however, most resistance systems appear to be associated with plasmids (Cervantes and Gutierrez-Corona 1994, Wuertz and Mergeay 1997). The incidence of plasmid-bearing strains is higher in polluted sites than in the unpolluted zone (Hada and Sizemore 1981).

Resistance to heavy metals is observed in a wide variety of bacteria, especially in gram negative bacteria (Poole 2002), such as *Pseudomonas*, *Alcaligenes*, *Ralstonia* and *Burkholderia* (Wuertz and Mergeay 1997, Malik and Jaiswal 2000, Kozdro'j and Van Elsas 2001, Ellis et al. 2003, Ünalı et al. 2003, Akinbowale et al. 2007, Zolgharnein et al. 2007, Matyar et al. 2008, Singh et al. 2010). Bacteria of the genus *Pseudomonas* are well-studied and are of great interest not only because of their high resistance to heavy metals and other toxic substances, but also for their simple nutritional requirements and rapid growth on standard laboratory media (Pardo et al. 2003).

In the last decade, numerous studies have reported on the heavy metal resistance of *Pseudomonas aeruginosa* obtained from different heavy metal polluted environments (Filali et al. 2000, Malik and Jaiswal 2000, Kozdro'j and Van Elsas 2001, Raja et al. 2006, Akinbowale et al. 2007, El-Sayed et al. 2008, Raja and Selvam 2009). Therefore, it has been considered as a water quality indicator organism (Kozdro'j and Van Elsas 2001) and as a good candidate for heavy metal removal from polluted sites (Malik and Jaiswal 2000). Surprisingly, little information is available regarding the pattern of heavy metal resistance of the non-aeruginosa *Pseudomonas* species and *Pseudomonas* related genera such as *Chryseomonas*, *Stenothrophomonas*, *Sphingomonas* and *Methylobacterium* species. On the other side, there are very few studies showing the relationship between these species heavy metal resistance patterns and the presence of plasmid.

In the present study, we evidenced the tolerance of the strains that belongs to non-aeruginosa *Pseudomonas* species and *Chryseomonas*, *Stenothrophomonas*, *Sphingomonas* and *Methylobacterium* species to various toxic metal as nickel, zinc, lead, cobalt, copper, chrome, mercury and silver. This study also revealed the relationship between the presence of metal resistance and plasmids.

## MATERIALS AND METHODS

### *Bacterial strains and growth conditions*

In this study, 40 strains which are thought to belong to *Pseudomonas* and *Pseudomonas* related genera were taken from Mugla University culture collection (MU) (Laboratory of Microbiology, Faculty of Arts and Sciences, University of Mugla, Turkey). All strains were cultured in nutrient broth (NB) (Difco) at  $30.0 \pm 0.1^\circ\text{C}$ .

### *Phenotypic characterization*

All strains were biochemically identified by conventional tests (Collins et al. 1995) followed by use of API 20 NE system (BioMerieux, Marcy l'Etoile, France). The results were obtained in duplicate and analysed employing the Apilab Plus Software (BioMe'rieux). Test for pigment production were performed on King B medium (Merck) (King et al. 1954). Growth on selective CFC medium (Oxoid) was checked for all strains. Oxidase and catalase tests were performed on Nutrient agar (NA) (Difco). Methyl Green DNase Agar (Difco) was used for DNase test. Finally, enzymatic tests were performed on enzymatic strips (API ZYM, BioMerieux).

### **Heavy metal resistance**

Heavy metal resistance of all isolates were determined by agar dilution method (Washington and Sutter 1980). Plates containing 20 mL of one-half strength NA and different concentrations of metal were poured on day of experiments. Concentrations for metals with tested  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{Pb}(\text{CH}_3\text{COO})_2 \cdot 3\text{H}_2\text{O}$ ,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ,  $\text{K}_2\text{Cr}_2\text{O}_7$ ,  $\text{AgNO}_3$ , and  $\text{HgCl}_2$  were as follows (in  $\text{mmol l}^{-1}$ ): 0.005; 0.01; 0.05; 0.1; 0.5; 1; 2.5; 5; 10; 20 and 40, respectively. Plates were dried at 37 °C for 30 min and inoculated with 0.1 ml from exponentially grown cultures. Plates were incubated at 37 °C for 2 days. Plates containing media with no added metal were inoculated in the same manner to serve as controls. MICs were determined as lowest concentrations of metal ion preventing growth. To define metal resistance, strains not inhibited by 1  $\text{mmol l}^{-1}$   $\text{NiCl}_2$ ,  $\text{ZnSO}_4$ ,  $\text{Pb}(\text{CH}_3\text{COO})_2$ ,  $\text{CoCl}_2$ ,  $\text{CuSO}_4$ ,  $\text{K}_2\text{Cr}_2\text{O}_7$ ,  $\text{AgNO}_3$  and 0.1  $\text{mmol l}^{-1}$   $\text{HgCl}_2$  were regarded as resistant.

On the other hand, it is well known that there are no currently acceptable concentrations of metal ions that can be used to distinguish metal-resistant from metal-sensitive bacteria. However, concentrations used in this study were employed in similar studies reported on heterotrophic bacteria in which testing media utilized nutrient agar (Thompson and Wattling 1983, Sadhukhan et al. 1997). Control strains used for all assays included *P.aeruginosa* ATCC 27853 and *P.aeruginosa* ATCC 29212. All tests were carried out in duplicate.

### **Plasmid profiling**

Plasmid DNA was prepared according to a previously described modification of hot alkaline lysis procedure (Kieser 1984, Foght et al. 1996). Agarose gel 0.7% (wt/vol) was prepared and 12  $\mu\text{l}$  of DNA preparation was loaded into each well. Electrophoresis was conducted for 4 h at 90 V and gel was stained with 0.5  $\mu\text{g/mL}$  ethidium bromide. A plasmid DNA band was observed with 3 UV transilluminator (model LMS-203, UVP, Inc., Upland, CA, USA) and photographed with a Polaroid MP4 camera equipped with red filter and type 667 Polaroid film. Approximate sizes of plasmids were calculated from logarithmic plots against reference plasmids of DNA loader supercoiled (SIGMA D-5292). Plasmid profiles that differed by at least one plasmid band were regarded as different plasmid profiles.

## **RESULTS**

In this study, 40 isolates which are taken from Mugla University culture collection, were studied. All the isolates were characterized by phenotypical characteristics, namely gram staining, colony typing, oxidase and catalase reaction, DNase and pigment production and growth on selective CFC medium. According to results of these tests, 28 isolates determined to belong to non-fermenting Gram negative bacilli and work was carried on with these isolates. After preliminary identification of the isolates were subjected by means of the API 20NE system. From 28 identified strains, 39.3% corresponded to *Stenotrophomonas maltophilia*, 17.9% to *Chryseomonas luteola*, 14.3% to *Pseudomonas fluorescens*, 10.7% to *Pseudomonas putida*, 7.1% to *Sphingomonas paucimobilis*, 7.1% to *Methylobacterium mesophilicum* and 3.6% *Pseudomonas stutzeri* (Table 1). API ZYM enzymatic strips were carried out in order to support the identifications (Table 2).

**Table 1.** Number of species of Gram negative bacteria.

Genus	Number of isolates	%
<i>Stenotrophomonas maltophilia</i>	11	39.3
<i>Chryseomonas luteola</i>	5	17.9
<i>Pseudomonas fluorescens</i>	4	14.3
<i>Pseudomonas putida</i>	3	10.7
<i>Sphingomonas paucimobilis</i>	2	7.1
<i>Methylobacterium mesophilicum</i>	2	7.1
<i>Pseudomonas stutzeri</i>	1	3.6
Total	28	100

**Table 2.** Identifications and characteristics that differentiate groups of Gram negative strains.

Characteristics	Reaction <sup>a</sup> of the following Gram negative strains						
	<i>S.maltophilia</i> (11)	<i>C. luteola</i> (5)	<i>P. fluorescens</i> (4)	<i>P.putida</i> (3)	<i>P.stutzeri</i> (1)	<i>S.paucimobilis</i> (2)	<i>M.mesophilicum</i> (2)
<b>Classical tests</b>							
Colony type	S	S	S	S	R	S	S
Oxidase reaction	-	-	+	+	+	+	+
Catalase reaction	+	+	+	+	+	+	+
DNase production	+	+	+	+	-	-	-
Production of pigment	-	-	-	-	-	+	+
<b>API 20 NE</b>							
Indol production on tryptophan	-	-	-	-	-	-	-
Glucose acidification	-	+	-	-	-	-	-
Arginine dihydrolase	-	-	+	+	-	-	-
Urease	-	-	-	-	-	-	+
Esculin hydrolysis	+	+	-	-	-	+	-
Gelatin hydrolysis	+	-	d	-	-	-	-
β-Galactosidase	+	+	-	-	-	+	-
D-Glucose	+	+	+	+	+	+	-
L-Arabinose	-	+	+	+	-	d	d
D-Mannose	+	+	+	-	-	d	-
D-Mannitol	-	+	+	-	+	-	-
N-Acetyl-D-glucosamine	+	+	+	-	+	d	-
Maltose	+	+	d	-	+	+	-
Gluconate	-	+	+	+	+	-	-
Caprate	D	-	+	+	+	-	-
Adipate	-	-	d	-	-	-	-
L-Malate	+	+	+	+	+	-	+
Citrate	+	+	+	+	+	-	-
Phenylacetate	-	+	d	+	-	-	-
<b>API ZYM</b>							
Alkaline phosphatase	+	+	-	-	+	+	d
Esterase(C4)	+	+	+	+	+	+	+
Esterase Lipase(C8)	+	-	+	+	-	+	+
Lipase (C14)	-	-	-	-	-	-	-
Leucine arylamidase	+	+	+	+	+	+	+
Valine arylamidase	+	+	d	-	-	+	-
Cystine arylamidase	-	+	-	-	-	-	-
Trypsin	+	+	+	+	+	d	+
α-chmotrypsin	-	-	d	+	-	d	-
Acid phosphotase	+	+	+	+	-	+	d
Naphtol-AS-phosphohydrolase	+	+	+	+	-	+	+
α-galactosidase	D	-	-	-	-	-	-
β-galactosidase	+	+	-	-	-	+	-
β-glucuronidase	-	-	-	-	-	-	-
α-glucosidase	+	+	-	-	-	+	-
β-glucosidase	+	-	-	-	-	-	-
N-acetyl-β glucosidase	-	d	-	-	-	d	-
α-mannosidase	-	-	-	-	-	-	-
α-fucosidase	-	-	-	-	-	-	-

<sup>a</sup> +, 65% or more the total, -, 35% or less the total; d, 36 to 64% the total.

The status of resistance against eight different heavy metals i.e. NiCl<sub>2</sub>, ZnSO<sub>4</sub>, Pb(CH<sub>3</sub>COO)<sub>2</sub>, CoCl<sub>2</sub>, CuSO<sub>4</sub>, K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>, HgCl<sub>2</sub> and AgNO<sub>3</sub> was investigated. Heavy metal resistance rates against all the bacteria in the study are as follows; ZnSO<sub>4</sub>, 100%; Pb(CH<sub>3</sub>COO)<sub>2</sub>, 96.4%; Cu SO<sub>4</sub>, 60.7%; NiCl<sub>2</sub>, 32.1%; CoCl<sub>2</sub>, K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> and AgNO<sub>3</sub>, 7.1%. All strains showed uniform tolerance of ZnSO<sub>4</sub> and Pb(CH<sub>3</sub>COO)<sub>2</sub>. CoCl<sub>2</sub> and AgNO<sub>3</sub> resistance was only detected in *M.mesophilicum* (Table 3).

Among the tested bacterial strains, *S. maltophilia* (formerly *Pseudomonas maltophilia*) was the most frequently detected microorganism. The present study indicated that frequencies of resistance in *S. maltophilia* strains to each metal ion tested were as follows: ZnSO<sub>4</sub>, Pb(CH<sub>3</sub>COO)<sub>2</sub> and CuSO<sub>4</sub>, 100%, NiCl<sub>2</sub>, 72.7%. All *S.maltophilia* strains were sensitive to CoCl<sub>2</sub>, K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>, AgNO<sub>3</sub> and HgCl<sub>2</sub> (Table 3).

**Table 3.** Metal resistance patterns and plasmid profiles of isolated strains .

Strains	Resistance patterns	No of plasmids	Molecular weight
<i>P.fluorescens</i>			
MU 66	Zn,Pb	0	-
MU 75	Zn,Pb	0	-
MU 87	Zn,Pb	2	21.59,12.68
MU 97	Zn,Pb	0	-
<i>P. putida</i>			
MU 73	Zn,Pb,Cu	0	-
MU 83	Zn,Pb,Cu	0	-
MU 139	Zn,Pb	0	-
<i>P.stutzeri</i>			
MU 70	Zn,Pb,Cr	0	-
<i>C. luteola</i>			
MU 18	Zn,Pb,Cu	0	-
MU 56	Zn,Pb	0	-
MU 58	Zn,Pb	0	-
MU 65	Zn,Pb	0	-
MU 96	Zn,Pb,Cu	0	-
<i>S.maltophilia</i>			
MU 23	Zn,Pb,Cu	0	-
MU 25	Ni,Zn,Pb,Cu	0	-
MU 52	Ni,Zn,Pb,Cu	5	13.86,6.24,5.71,2.35,0.89
MU 53	Ni,Zn,Pb,Cu	0	-
MU 63	Ni,Zn,Pb,Cu	2	6.24,2.57
MU 64	Ni,Zn,Pb,Cu	0	-
MU 69	Ni,Zn,Pb,Cu	1	13.86
MU 94	Zn,Pb,Cu	0	-
MU 99	Zn,Pb,Cu	0	-
MU 136	Ni,Zn,Pb,Cu,Cr	0	-
MU 137	Ni,Zn,Pb,Cu	0	-
<i>S.paucimobilis</i>			
MU 67	Zn	0	-
MU 145	Zn,Pb	0	-
<i>M. mesophilicum</i>			
MU 140	Ni,Zn,Pb,Co,Cu,Hg,Ag	0	-
MU 141	Zn,Pb,Co,Cu,Cr,Ag	2	21.59,11.61

In this study, *C. luteola* was determined as a second prevalent species. While all *C.luteola* strains were resistant to ZnSO<sub>4</sub> and Pb(CH<sub>3</sub>COO)<sub>2</sub>, two of them were resistant to CuSO<sub>4</sub> additionally.

*Pseudomonas* strains were resistant to ZnSO<sub>4</sub> and Pb(CH<sub>3</sub>COO)<sub>2</sub>. In addition, two *P.putida* (MU 73 and MU 83) were resistant to CuSO<sub>4</sub> and *P.stutzeri* MU 70 was resistant to K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>. Trends in heavy metal toxicity was in order of Hg=Ag=Co=Ni>Cr>Cu>Pb=Zn for non-aeruginosa *Pseudomonas* spp. (Table 4).

**Table 4.** MIC values against the metals of isolated strains.

Strains	Metal Ions							
	Ni	Zn	Pb	Co	Cu	Cr	Hg	Ag
Minimum Inhibition Concentrations (mmol <sup>-1</sup> )								
<u><i>P.fluorescens</i></u>								
MU 66	0.5	1	10	0.05	0.1	0.5	0.005	0.005
MU 75	0.1	1	5	0.05	0.1	0.1	0.005	0.005
MU 87	0.5	5	2.5	0.1	0.5	0.5	0.05	0.05
MU 97	0.5	2.5	2.5	0.1	0.5	0.5	0.05	0.01
<u><i>P. putida</i></u>								
MU 73	0.5	20	2.5	0.25	1	0.5	0.01	0.01
MU 83	0.5	20	2.5	0.25	1	0.5	0.01	0.05
MU 139	0.5	5	2.5	0.25	0.25	0.25	0.01	0.1
<u><i>P.stutzeri</i></u>								
MU 70	0.5	1	5	0.1	0.5	1	0.005	0.005
<u><i>C. luteola</i></u>								
MU 18	0.5	10	2.5	0.25	1	0.25	0.01	0.01
MU 56	0.5	20	5	0.1	0.5	0.5	0.01	0.005
MU 58	0.5	20	5	0.1	0.5	0.5	0.01	0.005
MU 65	0.1	10	5	0.1	0.5	0.5	0.005	0.005
MU 96	0.5	20	2.5	0.5	1	0.5	0.05	0.01
<u><i>S.maltophilia</i></u>								
MU 23	0.5	10	5	0.1	1	0.5	0.005	0.005
MU 25	1	20	10	0.1	1	0.5	0.01	0.005
MU 52	1	20	5	0.1	1	0.5	0.01	0.05
MU 53	1	20	5	0.1	1	0.5	0.01	0.005
MU 63	1	20	5	0.1	1	0.5	0.01	0.005
MU 64	1	20	10	0.1	1	0.5	0.005	0.005
MU 69	1	20	5	0.1	1	0.5	0.01	0.005
MU 94	0.5	20	2.5	0.25	1	0.5	0.05	0.05
MU 99	0.5	10	2.5	0.5	2.5	0.5	0.05	0.1
MU 136	1	20	5	0.1	1	1	0.01	0.005
MU 137	1	20	5	0.1	1	0.5	0.01	0.005
<u><i>S.paucimobilis</i></u>								
MU 67	0.5	0.5	0.5	0.1	0.5	0.5	0.005	0.005
MU 145	0.5	10	5	0.1	0.5	0.1	0.005	0.005
<u><i>M. mesophilicum</i></u>								
MU 140	2.5	20	10	2.5	1	0.25	0.1	1
MU 141	0.5	20	5	2.5	2.5	2.5	0.05	2.5

While one of the *S.paucimobilis* strains was resistant to ZnSO<sub>4</sub> and Pb(CH<sub>3</sub>COO)<sub>2</sub>, the other one was resistant only ZnSO<sub>4</sub>. In this study, *S. paucimobilis* strains were sensitive to the other six heavy metals. *M.mesophilicum* strains were the most resistant bacteria against heavy metals in this study. While *M.mesophilicum* MU 141 was sensitive to NiCl<sub>2</sub> and HgCl<sub>2</sub>, *M.mesophilicum* MU 140 was sensitive to only K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> (Table 4).

During the evaluation of plasmid profile, it was recorded that 5 strains harbored plasmids, illustrating different plasmid profiles; while the remaining 23 strains carried no detectable plasmids. The majority of strains

that contained plasmid were *S.maltophilia* strains and three strains of eleven *S.maltophilia* contained plasmids with different molecular weights. Among these strains, MU 52 contained five plasmids with molecular weights of 0.89, 2.35, 5.71, 6.24 and 13.86 kb, respectively. MU 63 contained two plasmids with molecular weights of 2.57 and 6.24 kb, respectively, while MU 69 contained one plasmid with molecular weight of 13.86 kb. These three strains of *S.maltophilia* show different plasmid profiles; results regarding heavy metal susceptibility and plasmid profile are shown in Table 3.

On the other hand, *P.fluorescens* MU 87 contained two plasmids with molecular weights of 12.68 and 21.59 kb, while *M.mesophilicum* MU 141 contained two plasmids with molecular weights of 11.61 and 21.59 kb, respectively (Table 3).

## DISCUSSION

This study was carried out *S. maltophilia*, *C. luteola*, *S. paucimobilis*, *M. mesophilicum* and members of the *Pseudomonas* spp. (*P. fluorescens*, *P. putida* and *P. stutzeri*) that are cause significant clinical and environmental problems. And also revealed correlating heavy metal resistance and plasmid presence.

*S.maltophilia* has recently emerged as an important nosocomial pathogen (Al-Jasser 2006). In severely ill patients, *S.maltophilia* causes a wide range of infections such as bacteremia, pulmonary infections, urinary tract infections, wound infections, meningitidis and endocarditis (Denton and Kerr 1998, Micozzi et al. 2000, Koseoglu et al. 2004). Treatment of invasive infections caused by this organism is difficult as the bacterium is frequently resistant to a wide range of commonly used antimicrobials (Al-Jasser 2006). *S. maltophilia* exhibits high-level intrinsic resistance to a variety of structurally unrelated antibiotics, including  $\beta$ -lactams, quinolones, aminoglycosides, tetracycline, disinfectants, and heavy metals (Alonso and Martinez 1997, Zhang et al. 2000). In the present study, resistance to three heavy metals (Zn, Pb, Cu) was investigated for all *S.maltophilia* strains. Holmes et al. (2009) reported the *S.maltophilia* O2 appeared to be much more resistant to Hg(II), Cd(II), Zn(II), Cu(II), Au(III), and Cr(VI) than the other study strain (*Enterobacter* sp. YSU). While, In this work the *S.maltophilia* O2 MIC for Zn and Cu was 5 mM, our MIC results were 10-20 mM and 1-2.5 mM, respectively. Our strains appeared to be much sensitive to Cr, Hg and Ag than their *S.maltophilia* O2. Pages et al. (2008) in their study found that *S.maltophilia* Sm777 tolerated up to 0.05 mM Hg(II), 5 mM Cu(II), 0.02 mM Ag(I), and 5 mM Pb(II). These concentrations are higher than our results except as Pb(II).

*C.luteola* has only rarely been reported as a human bacterial pathogen. It has been shown that this organism in particular affects patients with health and indwelling disorders. Most reported cases showed septicemia, meningitidis, endocarditis, or peritonitis (Chihab et al. 2004). In our study, all *C.luteola* strains showed uniform resistance to Zn and Pb. In another study, *C.luteola* is reported to be the most resistant to Pb and Cd in Egypt. In contrast to our results, Özdemir and Baysal 2004 reported that their *C.luteola* strains had been Cr resistant. Two strains of *C.luteola* strains were observed lead and zinc resistance as well as copper resistance.

In this study, all *Pseudomonas* spp. were resistant to Zn and Pb. In addition 2 *P.putida* strains were resistant to Cu and *P.stutzeri* was resistant to Cr. These strains were found to be sensitive to Ni, Co, Hg and Ag metals. The incidence of multiple resistance either to metal or antibiotics was observed in the *Pseudomonas* spp. strains. Malik and Jaiswal (2000) reported the frequency of metal resistance in *Pseudomonas* strains isolated from soil to be 80% for Cu, 73.3% for Cd, 71.1% for Cr and Zn, 48.8% for Hg. Similar observation were made by earlier researchers (Bopp et al. 1983, Horitsu et al. 1986, Chaudhary and Kumar 1996). Bopp et al. (1983) isolated a chromate-resistant strain of *P.fluorescens* LB 300 from chromium-contaminated sediment and Horitsu et al. (1986) isolated *P.putida* strain from soil, which exhibited resistance to cadmium at a concentration of 1280 $\mu$ g/ml. Bhagat and Srivastava (1991) isolated some zinc-resistant strains of *P.stutzeri* from industrially polluted area of Delhi (India) which were simultaneously resistant to other heavy metals including Cu, Ni, Cd, Co, Mn and Pb. In our study, *P.stutzeri* has only shown resistance to Zn, Pb and Cr.

*Sphingomonas* strains were found to be highly susceptible to heavy metals. While one of the *S.paucimobilis* was resistant to zinc, the other one was resistant to zinc and lead. In a similar study in Japan, *S.paucimobilis* KPS01 was reported as highly susceptible to heavy metals (Tada and Inoue 2000). A year later, Tada et al.

(2001) suggested that *S. paucimobilis* KPS01 can be useful for routine monitoring of heavy metals as environmental contaminants, particularly in water sources. Xie et al. (2010) investigated and showed that strain of *S. paucimobilis* DT-T3-03 had high tolerance to heavy metal Zn, and had high resistant ability to many heavy metals such as copper (1.5mM), lead (1.0 mM) and nickel (1.0mM). Similar results for Zn and Pb were found in our study.

In this study, the highest tolerance to heavy metals were also found on *Methylobacterium* strains. These strains were found to highly resistance to heavy metals as Zn, Pb, Co, Cu and Ag. In this study only those strains showed resistance to silver and mercury. One of the strains were resistant to nickel and the other was resistant to chromium. Rajbanshi (2008) reported that most isolates have shown multiple tolerances to both heavy metals and antibiotics. In addition, Rajbansi (2008) has detected a cobalt resistant *Methylobacterium* spp. was only resistant to chloramphenicol and sensitive to tetracycline, ciprofloxacin, cotrimoxazol, gentamicin and ampicillin. So-Yeon and Kyung-Suk (2007) reported that *Methylobacterium* sp. SY-NiR1 showed resistance against multiple heavy metals such as cadmium, chromium, copper, lead, nickel and zinc. In addition study, one *M. mesophilicum* and two *Methylobacterium* sp. strains were reported resistant to Cu, Pb, Cd and Zn. Similar observations were reported for Zn, Ni, Co, Cr, Cd and Cu resistance in *M. mesophilicum* and *M. extorquens* strains (Piotrowska-Seget et al. 2005, Idris et al. 2006). This observation corroborates our results.

Out of the 28 bacterial tested for the presence of plasmids, 5(17.9%) isolates showed plasmid DNA bands on the agarose gel. Among the 5 plasmid containing bacterial strains, 3 strains belonged to *S. maltophilia*, one strain belonged to *P. fluorescens* and one strain belonged to *M. mesophilicum*. *S. maltophilia* MU 52 with highest numbers of plasmids was found resistant to NiCl<sub>2</sub>, ZnSO<sub>4</sub>, Pb(CH<sub>3</sub>COO)<sub>2</sub>, and CuSO<sub>4</sub> metals. It was also shown that plasmid carrying *S. maltophilia* have same size plasmids as 13.86 and 6.24 kb. However, high-metal multi-resistance was observed in *S. maltophilia* strains carrying slightly same size plasmids. The other two strains that contained plasmid were *P. fluorescens* MU 87 and *M. mesophilicum* MU 141. Among these strains, *P. fluorescens* MU 87 contained two plasmids with molecular weights of 21.59 and 12.68 kb, respectively, while *M. mesophilicum* MU 141 contained two plasmids with molecular weights of 21.59 and 11.61 kb, respectively. These two strains contained the plasmid 21.59 kb in size. Double resistance to Zn and Pb was detected in these strains contained 21.59 kb size plasmid. These results demonstrated that some of these bacteria obtained their heavy metal multi resistance trait through their plasmids. These isolated bacteria demonstrate that resistance to heavy metals and antibiotics by genes present on their plasmids suggests the exertion of selective pressure on such bacteria through contamination with antibiotics and heavy metals in their environment. Antibiotic-heavy metal multi resistance in some similar studies have shown the relationship between the presence of the plasmid (Piotrowska-Seget et al. 2005, Zolgharnein et al. 2007).

As a result, heavy metal resistance in *S. maltophilia*, *S. paucimobilis*, *C. luteola*, *M. mesophilicum* and non-aeruginosa *Pseudomonas* spp. (*P. fluorescens*, *P. putida* and *P. stutzeri*) were determined and plasmid profiles were obtained, relationship between the plasmid existence and heavy metal resistance was displayed.

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