

## Study of lead and nickel resistance mechanism in *Enterobacter* species

Nirbhavane H M<sup>1</sup>, Bagde U S<sup>2,\*</sup>

<sup>1</sup>Ph. D Scholar, <sup>2</sup>Ex Professor & HOD, Microbiology Laboratory, Department of Life Sciences, University of Mumbai, Vidyanagari, Mumbai, Maharashtra, India

**\*Corresponding Author:**

Email: bagdeu@yahoo.com

### Abstract

The aim of this study was to understand the mechanism of resistance towards the heavy metal Lead and Nickel using resistant and sensitive *Enterobacter* spp. The cellular biochemistry of this microorganism was checked with the help of estimations of dehydrogenases activity, and at the molecular biology level determination of Lead and Nickel resistant plasmid through Agarose gel Electrophoresis. The Minimum inhibitory concentration (MIC) determined was 100 ppm (Lead) and 50 ppm (Nickel) in sensitive *Enterobacter* spp. that completely inhibited the growth, and activity of dehydrogenases of the TCA (Tricarboxylic Acid) cycle. The tolerance level of Lead and Nickel in the resistant *Enterobacter* spp., were found to be 300 ppm and 200 ppm respectively. Mechanism of resistance was found to be the presence of the resistance plasmid.

**Keywords:** Lead, Nickel, Resistant, Sensitive, Enterobacter, Mechanism.

### Introduction

Microorganisms have acquired a variety of mechanisms for adaptation to the presence of toxic heavy metals. Among the various adaptation mechanisms, metal sorption, mineralization, uptake and accumulation, extracellular precipitation and enzymatic oxidation or reduction to a less toxic form, and efflux of heavy metals from the cell has been reported.<sup>1-4</sup> The mechanism of resistance towards the heavy metals by the microbial cells has been studied throughout the world in regards with their mode of action. In various pathogens the phenomenon of resistance has been found to be related with some essential activities of cell metabolism, such as Permeability of the cell membrane, the synthesis of macromolecules like protein, Deoxyribonucleic acid, Ribonucleic acid, the electron transport and enzyme activity, genetic alterations and the whole cell growth task.<sup>5-8</sup>

Overall, the structural and functional characteristics of antibiotic resistance share common themes with those of metal resistance.<sup>9-10</sup>

In this study we have tried to ostensibly appraise the mechanism of resistance of heavy metals Nickel and Lead with the sensitive and resistant *Enterobacter* species, which is previously tested with the antimicrobials Ceftazidime, Moxifloxacin and Nalidixic acid.<sup>11</sup>

### Materials and Methods

#### Bacterial species and its cultivation

The strains of resistant and sensitive *Enterobacter* spp. were obtained from BAC TEST Laboratory Nashik, Maharashtra and stocked in this laboratory. This particular pathogenic microorganism was selected for the study after testing various pathogens for their susceptibility previously with the antimicrobials and then used towards the heavy metals Lead and Nickel.<sup>11</sup> These organisms were grown on a nutrient agar at 37°C

and maintained at 5°C. The inoculum was prepared in nutrient broth after subculture and 48 hours incubation. The cells of Resistant and sensitive species of *Enterobacter* were cultured on nutrient agar (NA) (HiMedia India) slants containing Beef extract 0.5g; Peptone 2.5g; Sodium chloride 2.5; Agar 15 in a liter of distilled water. pH was maintained at 7.4, slants were incubated at 37°C for 24 hrs.

#### Detection of antimicrobial resistance and sensitivity in Bacteria

**Broth dilution Method:** The broth dilution method was used to determine the minimum Inhibitory Concentrations (MICs) of antibacterial agents according to the guidelines given by CLSI protocol.<sup>12</sup> The MH broth and the Heavy metals were purchased from HiMedia India.

**Disc diffusion method:** Antimicrobial susceptibility of resistant and sensitive *Enterobacter* Species was determined with the help of disc diffusion method of Kirby et al.<sup>13</sup> with modification by the CLSI.<sup>14,12</sup> The sterile discs impregnated in to the solution of heavy metals were used according to its MIC value. The Muller Hinton (MH) Agar was procured from HiMedia India.

**Detection of  $\beta$  lactamase activity:** Detection of  $\beta$  lactamase was carried out by Tube method and paper strip method of Idometric Test and Acidometric Test.<sup>15</sup> and  $\beta$  lactamase activity testing was done by Qualitative plate test.<sup>16</sup> Nitrocefin test was not done due to unavailability of nitrocefin.

**Plasmid Isolation:** Plasmid Isolation was conducted for separation and analysis of nucleic acid by alkaline lysis method.<sup>17</sup>

**Spectrophotometric method:** Qualitative determination of DNA was carried out with help of the spectrophotometric method given by Maniatis.<sup>18</sup>

**Agarose gel Electrophoresis:** Agarose gel Electrophoresis of plasmid DNA was done according to the method of Sambrook et al.<sup>17</sup> Plasmid DNA from *Enterobacter* resistant species was electrophoresed with DNA ladder in 1.0 % agarose gel slab at 50 V in Tris Acetate buffer (TAE) running buffer (0.5M Tris base, 0.5M EDTA, 1 M Glacial acetic acid). Stained with Ethidium bromide and photographed under UV illumination. The plasmid was estimated by comparing with standard Supermix DNA ladder obtained from Bangalore Genei, (Bangalore, India). It consist of DNA fragments ranges from 500 to 33500-24500 bp generated from number of propriotory plasmids and lambda DNA.

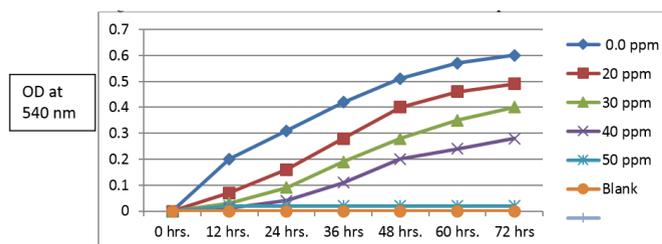
**Elimination of resistance due to plasmid:** Elimination of resistance due to plasmid was carried out by using the sodium dodecyl sulphate (SDS) treatment described by Tomoeda et al.<sup>19</sup> An overnight culture of resistant (R) cells in Nutrient broth containing various concentrations of Heavy metals (ppm) and sensitive species cells were diluted up to  $10^3$  cells/ml in broth, and added to the tubes containing 10% (w/v) SDS and shaken at 37°C. After appropriate dilutions in saline cells were spread on nutrient agar and the sensitivity discs were placed on it.

**Growth curve with various concentrations of Heavy metals:** The experiments were carried out in 100 ml Ehrlenmeyer flasks with side arm. These flasks were inoculated with 1.0 ml inoculums prepared as above. The final volume in the flask was 50 ml. The Extra pure analytical grade heavy metal Lead was used in form of Lead acetate (Qualigenes fine chemical Ltd. India) Nickel was used in form of Nickel chloride in the experiment (Hi media Mumbai). The final concentrations of Nickel taken were 20,30,40,40, and 50 ppm for the Sensitive species and 50, 100, 150, and 200 ppm for the resistant species. The concentrations of Lead taken for resistant species was in the range of 150, 200,230,250 and 300 ppm and for the sensitive species the range was 50, 70, 90, and 100 ppm.

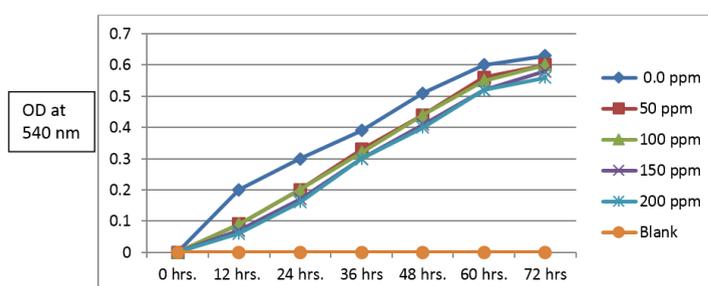
**Dehydrogenase assay:** The dehydrogenase assay was performed to determine the dehydrogenase activity according to the procedure of Guha and Mookerjee.<sup>6</sup>

## Results

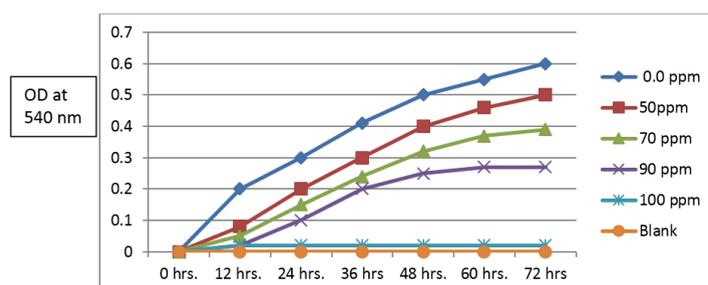
Results of Growth curve study carried out at different concentrations of Nickel and Lead with the appropriate ranges for MIC and tolerance level for sensitive and resistant species of *Enterobacter* are shown in Fig. 1 to 4.



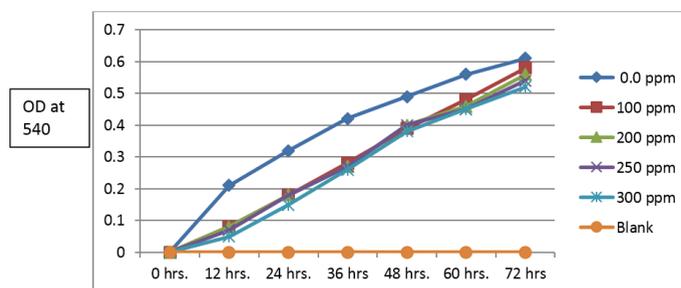
**Fig. 1: Growth of Sensitive Enterobacter species up to 50 ppm concentration of Nickel**



**Fig. 2: Growth of Resistant Enterobacter species up to 200 ppm concentration of Nickel**



**Fig. 3: Growth of Sensitive Enterobacter species up to 100 ppm concentration of Lead**



**Fig. 4: Growth of Resistant Enterobacter species up to 300 ppm concentration Lead**

### Growth Inhibition

Whereas the sensitive strain of *Enterobacter* was completely inhibited at 50 ppm of Nickel (Fig. 1), resistant strain of *Enterobacter* tolerated up to 200 ppm concentration of Nickel (Fig. 2). When the sensitive strain of *Enterobacter* was completely inhibited at 100 ppm of lead (Fig. 3), resistant strain of *Enterobacter* tolerated up to 300 ppm concentration of lead (Fig. 4).

**Table 1: Effect of Heavy metals on sensitive and resistant Enterobacter species**

S. No.	Pathogen	Heavy Metal	Size of Zone of Inhibition	MIC value/tolerance value
1	Sensitive Enterobacter	Nickel	25mm	50 ppm
2	Sensitive Enterobacter	Lead	20 mm	100 ppm
3	Resistant Enterobacter (Before SDS Treatment)	Lead	0 mm	300 ppm
4	Resistant Enterobacter (After SDS Treatment)	Lead	21 mm	
5	Resistant Enterobacter (Before SDS Treatment)	Nickel	0 mm	200 ppm
6	Resistant Enterobacter (After SDS Treatment)	Nickel	24 mm	

The effect of heavy metals when tested in sensitive and resistant strains in terms of size of zone of inhibition in mm, it was as shown in table 1. In case of sensitive strain zone of inhibition was 25 mm for nickel and 20mm for lead. In resistant strain zone of inhibition was 0 and 24 mm for nickel and 0 mm and 21 mm for lead before and after SDS treatment respectively.

The growth percentages of sensitive *Enterobacter* ( $10^3$  CFU/ml) at 40 ppm and 90 ppm of Nickel and

Lead were found to be 46.66 % and 45 % respectively after 72 hrs. (Table 2). At MIC concentrations of Nickel (50ppm) and Lead (100ppm) there was no growth at all. The growth percentages of resistant *Enterobacter* at  $10^3$  CFU/ml in 300 ppm and 200 ppm of Nickel and Lead were found to be 88.88% and 85.24% respectively after 72 hrs. (Table 3).

**Table 2: Growth Percentages of sensitive Enterobacter after 72 hrs at different concentrations of Heavy metals**

Sr. No.	Heavy Metal	Concentration (ppm)	OD at 540 nm	% of Growth
1	Nickel	0	0.6	100
2	Nickel	20	0.49	81.66
3	Nickel	30	0.4	66.66
4	Nickel	40	0.28	46.66
5	Nickel	50	0.01	0
6	Lead	0	0.6	100
7	Lead	50	0.5	83.33
8	Lead	70	0.39	65
9	Lead	90	0.27	45
10	Lead	100	0.02	0

**Table 3: Growth Percentages of Resistant Enterobacter after 72 hrs at different concentrations of Heavy metals**

Sr. No.	Heavy Metal	Concentration (ppm)	OD at 540 nm	% of Growth
1	Nickel	0	0.63	100
2	Nickel	50	0.6	95.23
3	Nickel	100	0.6	95.23
4	Nickel	150	0.58	92.06

5	Nickel	200	0.56	88.88
6	Lead	0	0.61	100
7	Lead	100	0.58	95.08
8	Lead	200	0.56	91.80
9	Lead	250	0.54	88.52
10	Lead	300	0.52	85.24

### $\beta$ Lactamase detection

It was observed that there was no decolorization occurred in 5 minutes in Idometric method within the tube or on the strip. Violet to Yellow color change was not observed within 5 minutes in Acidimetric method. No yellow color was noticed around the colony within 60 minutes in Qualitative plate test. Thus  $\beta$  Lactamase was not detected.

### Dehydrogenase Assay

The percentages of activity of various dehydrogenase enzymes found in the resistant *Enterobacter* were tested in citric acid cycle substrate

Glutamate, Isocitrate, Ketoglutarate and Succinate at MIC concentration of Heavy metal Nickel and Lead at 200 ppm and 300 ppm respectively. The activity of Glutamate dehydrogenase, Isocitrate dehydrogenase, Ketoglutarate dehydrogenase and Succinate dehydrogenase was measured as 68.82%, 80.85%, 64.0 % and 82.35 % respectively in resistant *Enterobacter* treated with Nickel. (Table 4) The resistant *Enterobacter* showed the dehydrogenase activity Glutamate dehydrogenase 73.68 %, Isocitrate dehydrogenase 83.01%, Ketoglutarate dehydrogenase 68.62, %, and Succinate dehydrogenase 81.25 % when treated with Lead (Table 5).

**Table 4: Effect of Nickel (200 ppm) on the activity of dehydrogenases enzymes in resistant *Enterobacter***

S. No.	Enzymes	OD of Control	OD of Test	% of Activity	% of Inhibition
1	Glutamate Dehydrogenase	0.51	0.35	68.62	31.18
2	Iso citrate Dehydrogenase	0.47	0.38	80.85	19.15
3	Ketoglutarate Dehydrogenase	0.5	0.32	64	36
4	Succinate Dehydrogenase	0.51	0.42	82.35	17.65

**Table 5: Effect of Lead (300 ppm) on the activity of dehydrogenases enzymes in resistant *Enterobacter***

S. No.	Enzymes	OD of Control	OD of Test	% of Activity	% of Inhibition
1	Glutamate Dehydrogenase	0.57	0.42	73.68	26.32
2	Iso citrate Dehydrogenase	0.53	0.44	83.01	16.99
3	Keto glutarate Dehydrogenase	0.51	0.35	68.62	31.38
4	Succinate Dehydrogenase	0.48	0.39	81.25	18.75

But the activity of all these enzymes in sensitive *Enterobacter* showed activity such as Glutamate dehydrogenase 25% and 26.53 %, Isocitrate dehydrogenase 31.11% & 26.66 %, Ketoglutarate dehydrogenase 22% & 27.08 %, and Succinate dehydrogenase about 27.27% & 28.46 % in Nickel and Lead respectively. (Table 6 and 7)

**Table 6: Effect of Nickel on the activity of dehydrogenases enzymes in sensitive *Enterobacter* at MIC (50 ppm)**

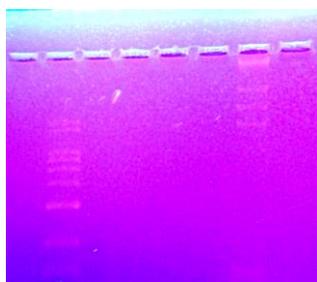
S. No.	Enzymes	OD of Control	OD of Test	% of Activity	% of Inhibition
1	Glutamate Dehydrogenase	0.48	0.12	25.0	75
2	Iso citrate Dehydrogenase	0.45	0.14	31.11	69.81
3	Keto glutarate Dehydrogenase	0.50	0.11	22	78
4	Succinate Dehydrogenase	0.44	0.12	27.27	62.73

**Table 7: Effect of Lead on the activity of dehydrogenases enzymes in sensitive *Enterobacter* strain at MIC(100 ppm)**

S. No.	Enzymes	OD of Control	OD of Test	% of Activity	% of Inhibition
1	Glutamate Dehydrogenase	0.49	0.13	26.53	73.47
2	Iso citrate Dehydrogenase	0.45	0.12	26.66	73.44
3	Keto glutarate Dehydrogenase	0.48	0.13	27.08	72.92
4	Succinate Dehydrogenase	0.46	0.13	28.46	71.54

### Plasmid Isolation and Agarose Gel Electrophoresis

The purity of isolated plasmid DNA was noted as 1.685 during the qualitative determination after plasmid isolation. Agarose gel Electrophoresis of plasmid DNA isolated from *Enterobacter* species resistant to Heavy metal Nickel and Lead Showed 3 different bands in lane 7 (Fig. 5) and the Supermix DNA ladder showed bands of different molecular weight in lane 2, while the sensitive species of *Enterobacter* did not show any band in lane 4. The plasmid DNA has been run along with the DNA ladder for the purpose of conformation only. There are more than 1 plasmid DNA found nearby the bands of ladder having molecular weight of 15000 bp (at lower side), and molecular weight more than 33500bp (both at upper side).



**Fig. 5:** Agarose gel Electrophoresis of plasmid DNA isolated from *Enterobacter* species resistant to Nickel and Lead

### Plasmid Curing

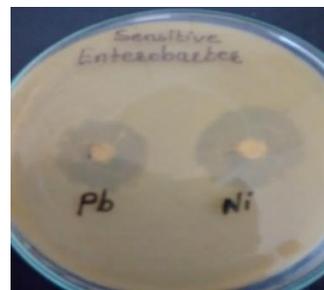
Elimination of resistance in resistance was observed in case of resistant *Enterobacter* spp. after SDS treatment (Fig. 6, and 7). Plasmid was not detected or isolated in sensitive *Enterobacter* spp (Fig. 8).



**Fig. 6:** Resistant *Enterobacter* with Heavy metal Nickel and Lead before SDS Treatment



**Fig.7:** Resistant *Enterobacter* showing sensitivity towards the Heavy metal Nickel and Lead after SDS Treatment



**Fig. 8:** Sensitive *Enterobacter* showing sensitivity towards the Heavy metal Nickel and Lead

### Discussion

Many bacterial heavy metal resistances have been studied for many years especially in contaminated regions. Resistance to toxic heavy metals has been found in bacteria from clinical and environmental origins. The genetic determinants of resistance are frequently located on plasmids or transposons.<sup>20</sup>

The objectives of this study were to screen *Enterobacter* spp. resistance to heavy metals Nickel and Lead, and to determine presence of the plasmid mediated heavy metal resistance.

*Enterobacter aerogenes* has an ability to completely degrade 0.6 mM Lead concentration in 60 hours while the MIC of Lead for the strain was observed to be 3.6 mM. Also it had an optimum pH and Temperature of 7.5 and 37°C, exhibited multiple metal tolerances and showed an improved reduction rate of Pb in presence of Glucose in the medium.<sup>21</sup> In the present study the resistance to the heavy metal nickel and lead were found to be 200 ppm and 300 ppm by the resistant *Enterobacter*. Lee et al. (22) isolated a Nickel-resistant bacterium from soil in order to identify a novel nickel resistance determinant. Using 16S rRNA gene sequencing, an isolate was identified as *Enterobacter* sp. Ni15. This species showed a medium-level (resistant to up to 10 mM) nickel resistance in nutrient-rich media.

The MIC of Lead and Nickel were recorded as 100 ppm and 50 ppm in this study by the sensitive *Enterobacter* spp. It is in agreement with the previous studies. The MIC of Pb towards the strains *Enterobacter cloacae*, *Enterobacter ludwigii* was noted to be about 4 mM and other 2 strains of *Enterobacter ludwigii* showed very high degree of resistance towards Pb. All the different isolates including *Enterobacter* spp. were tested for the presence of plasmids, showed three distinct bands of open circular, linear and supercoiled plasmid on 1% agarose gel.<sup>23</sup> In an investigation reported by Banergee et al.<sup>24</sup> the isolation and characterization of a potent heavy metal accumulating bacterial strain *Enterobacter cloacae* B1 from polluted soil at Ghaziabad, India was carried out. The minimum inhibitory concentration of the selected bacterial strain was recorded to be 1100 ppm for lead, 900 ppm for cadmium, and 700 ppm for nickel.

During the study of growth at various concentrations of Nickel and Lead, sensitive species of *Enterobacter* showed decreased growth with increasing concentration of these heavy metals due to the increase in toxicity. In a study it is reported that the isolated strain of *Enterobacter* was found to give low tolerance with CdCl<sub>2</sub> and was found to be highly tolerant to Pb(NO<sub>3</sub>)<sub>2</sub>. The MICs of Cd<sup>2+</sup>, Cu<sup>2+</sup>, Co<sup>2+</sup>, Zn<sup>2+</sup> and Pb<sup>2+</sup> were 0.4, 1.0, 1.0, 2.0 and 3.0 mM/L respectively. This varying response of tested bacteria might be due to variation in resistance mechanisms.<sup>25</sup>

The result obtained in present study is in accordance with the above statement, as the sensitive *Enterobacter* showed no growth in 50 ppm of Nickel and 100 ppm of Lead. In 1971 Komura and Lazaki<sup>26</sup> reported that the MIC of two different metals could be same for any particular microorganism.

It is reported that, the *Enterobacter* BN4 was able to grow at high concentrations of Cd<sup>2+</sup>, Zn<sup>2+</sup> and Pb<sup>2+</sup> in liquid medium, which might be important for the capacity of this bacterium to survive in different sources of pollution with elevated heavy metal levels. It was found that the toxicity of Cd<sup>2+</sup> was higher than that of Zn<sup>2+</sup> and Pb<sup>2+</sup> that was consistent with the results in solid media in the present study and many other reports.<sup>27</sup>

The growth, and dehydrogenase activity was not inhibited at 300 ppm concentration of Lead and even 200 ppm concentration of Nickel by the resistant species of *Enterobacter*. The final results of Estimation of Dehydrogenase activity and Growth curve obtained after 72 hours are given in Table – 4, 5, 6, and 7. All these results obtained significantly contributed to the identification of the basic effect and mechanism of resistance. There are remarkable differences seen in the activity of various dehydrogenases between the sensitive and resistant *Enterobacter* species at various concentrations of heavy metal in the present work. These results are found to be very useful to illustrate one of the well-known mechanisms of resistance that is related to the effect on enzymes in relation with Production of ATP and TCA cycle. There are remarkable differences seen in the activity of various dehydrogenases between the sensitive and resistant *Enterobacter* species at various concentrations of heavy metal in the present work.

The inhibition of enzyme activity was calculated as 77.08 % and 31.48 % for Glutamate dehydrogenase, 71.11 % and 18.75 % for Isocitrate dehydrogenase, 80.39 % and 38 % for Ketoglutarate dehydrogenase and 72.82 % and 21.56 % for Succinate dehydrogenase in case of resistant and Sensitive *Enterobacter* respectively. It is in agreement with earlier report<sup>28</sup> where it is reported that the inhibition of dehydrogenases activity in *S. epidermidis* as Glutamic 93.4%, Succinic 84.6%,  $\alpha$ -ketoglutaric 85.7% and Isocitric dehydrogenases 91.7% and in *K. pneumoniae* it was 93%, 91%, 92% and 89% respectively. The four

dehydrogenases involved in the TCA cycle, glutamic, succinic,  $\alpha$ - ketoglutaric and isocitric dehydrogenases were inhibited to a greater extent, when bacterial cells were exposed to arsenic

The activity of all the dehydrogenases was checked after 48 hours of inoculation. As shown in table the activity of enzymes was noted in form of optical density on colorimeter. In case of the resistant *Enterobacter* the dehydrogenase activity was seen much greater than the dehydrogenase activity found in sensitive *Enterobacter*. This can surely help to understand the mechanism of resistance of the organism in this study (Table - 4, 5, 6, 7). This is an evidence for the inhibition in generation of ATP molecules. The inhibition of all the four type of dehydrogenases in due course concern with the production of Energy rich compounds and inhibits the growth and multiplication of the cell. It is in agreement with the earlier studies.<sup>29,28,8</sup>

Bacterial plasmids have genes that confer highly specific resistances to As, Bi, Cd, Cu, Cr, Hg, Zn, and other toxic heavy metals. For each toxic cation and anion, generally a different resistance system exists, and these systems may be linked together on multiple resistance plasmids.<sup>30</sup>

Plasmid was isolated and cured successfully which is a big evidence to reveal the resistance mechanism in the present work. In a study<sup>(31)</sup> the plasmid DNA was isolated from *K. pneumoniae* with approximate size of 4.9 kb. Curing of plasmid was carried out with SDS. Plasmid curing was achieved by growing the strain treated with SDS. A plasmid isolated from *Klebsiella* spp. was treated with 10 % SDS that leads to loss of a plasmid. A Strain of *Enterobacter* (Ent- 5) tolerated high concentrations of copper (23 mM), nickel (16 mM), chromium (8 mM) and cadmium (14 mM). Cured Ent-5 was not able to grow on Ni and Cu. The sensitivities of the plasmid cured Ent-5 to nickel and copper indicated that copper and nickel resistance is correlated with plasmids.<sup>32</sup>

The absence of B lactamase enzyme in resistant *Enterobacter* was reported previously.<sup>10</sup> The availability of reliable data through this type of experiment could be a useful tool in purpose of determination of heavy metal uptake and its metabolism in bacterial cell or antimicrobial drug preparations with the help of heavy metals at the edge of update information. The current status of Nickel and Lead action towards *Enterobacter* spp., can be understood with help of the obtained results in this study.

## Conclusion

This particular study has highlighted the mechanism of resistance in *Enterobacter* towards heavy metals Lead and Nickel that was due to presence of resistance plasmid in resistant *Enterobacter*. Plasmid was not present in *Enterobacter* sensitive strain.

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