Study on mechanism of cobalt resistance in Enterobacter species

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Abstract
The aim of this study was to understand the mechanism of resistance in *Enterobacter* spp. towards cobalt toxicity. The MIC (minimum inhibitory concentration) and cobalt tolerance determination was done. The cellular biochemistry of this microorganism was checked with the help of estimations of macromolecules, dehydrogenases activity, and determination of cobalt through cell fractionation. Cobalt inhibited the growth and viability of sensitive organisms. Addition of cobalt affected the growth curve phases, which was ultimately an impact of concentration of cobalt. The minimum inhibitory concentration (MIC) determined was 250 ppm in sensitive *Enterobacter* spp. that completely inhibited the growth, synthesis of protein, DNA, and activity of dehydrogenases of the TCA cycle. The resistant *Enterobacter* spp. tolerated even 500 ppm concentration of cobalt. The cobalt incorporated in to the cell wall, membrane and cytoplasm was 66%, 11% and 23% and 9%, 58% and 33% in resistant *Enterobacter* spp. and sensitive *Enterobacter* spp. respectively when 250 ppm cobalt was added in the culture flasks. The mechanism of resistance was accumulation of cobalt in the cell wall and presence of plasmid.

Keywords: Cobalt, Resistant enterobacter, Sensitive enterobacter, Mechanism, Inhibition.

Introduction
A variety of mechanisms have been acquired by microorganisms for adaptation to the presence of toxic heavy metals. These adaptation mechanisms include 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.[1-4] The mechanism of resistance towards the heavy metals by the microbial cells has been studied throughout the world in regards to their mode of action. In various pathogens the phenomenon of resistance has been found to be related with permeability of the cell membrane, genetic alterations and the presence of plasmid or production of enzymes such as β lactamase etc.[5,6] The divalent cations of cobalt, zinc, and nickel are essential nutrients for bacteria, required as trace elements at Nanomolar concentrations. However, at micro- or mili molar concentrations, Co2+, Zn2+, and Ni2+ (and "bad ions" without nutritional roles such as Cd2+) are toxic. These cations are transported into the cell by constitutively expressed divalent cation uptake systems of broad specificity, i.e., basically Mg2+ transport systems. Therefore, in case of a heavy metal stress, uptake of the toxic ions cannot be reduced by a simple down-regulation of the transport activity. As a response to the resulting metal toxicity, metal resistance determinants got evolved which are mostly plasmid-encoded in bacteria.[3]

The best known mechanisms of nickel resistance was mediated by efflux pumps such as cnr CBA (cobalt- nickel resistance) from *Cupriavidus metallidurans* CH34 (formerly *Ralstonia metallidurans* CH34), NccCBA (Nickel-cobalt-cadmium) and NreB (nickel resistance) from *Achromobacter xylosoxidans* 31A, CznABC (Cadmium-zinc-nickel) from *Helicobacter pylori*.[7] Overall, the structural and functional characteristics of antibiotic resistance share common themes with those of metal resistance.[8] Mercury resistance was co-transferred with antibiotic resistances in a subset of mating between Enterobacteriaceae and recipients.[9]

Ghosh[10] reported that bacteria *Acidocella* showed high resistance to cadmium and zinc and from organism having more than one plasmids were isolated and hence resistance was located on plasmid. Similar observations were made by Lankeshwar and Bagde[11] wherein chromium resistance was assigned to plasmid in *E coli* and zinc resistance was also mediated by plasmid in *Shigella dysenteriae*,[12] and lead and nickel resistance was mediated by plasmid in *Enterobacter*.[13] By helper-assisted and unassisted conjugation the plasmids of strain 31A were shown to carry nickel and cobalt resistance determinants.[14]

It is reported that the isolation and primary characterization of the yohM gene of *Escherichia coli*. yohM encodes a membrane-bound polypeptide conferring increased nickel and cobalt resistance in *E. coli*. yohM was specifically induced by nickel or cobalt but not by cadmium, zinc, or copper. Mutation of yohM increased the accumulation of nickel inside the cell, whereas cells harbouring yohM in multicopy displayed reduced intracellular nickel content.[15]

The availability of reliable data through this type of experiment could be a useful tool in purpose of modification in drugs and its proper use at the edge of update information, current status of efficacy of the specific antimicrobials towards the specific microorganisms. The phenomenon of resistance has been already revealed by the former researches in all aspects. In this study we have tried to ostensibly appraise the mechanism of resistance of *Enterobacter* species towards heavy metal cobalt.

Mechanism of Microbial Resistance towards the Heavy Metals

In studies on mechanisms of resistance to heavy metals, the chemical form of a metal determines its solubility, mobility, and toxicity towards an organism. Therefore, it also affects the MIC value. Inorganic heavy metals that occur as water-soluble salts exert greater toxicities, than water-insoluble forms of the same metals.[16] Silver reported plasmid mediated reduced accumulation of heavy metals. Resistance of heavy metals due to reduced accumulation has also been reported by Cooksey.[17] Metal resistance is inherited by plasmids in many bacteria.[18,19] Mitsuhashi et al.[20] observed reduced MICs up to 10 fold and complete loss of resistance in some strains of *E. coli*, after treatment with the curing agent acriflavine. Similar observations have been reported by Ghosh et al.[21] When organisms are exposed to metal salts, it is first taken up by the cell and then the metal is localized in different parts of the cell where it exerts its toxic effect on those parts of the cells. Three main fractions of the cells from which the localization of heavy metals could be estimated are: Cell wall, cell membrane, and cytoplasm. Unlike antibiotic resistance, there are no universally acceptable metal ion concentrations which are used to designate microbial metal tolerance.[22] Resistance to heavy metals, pollutants, UV light and other antimicrobial substances such as antibiotics was due to extrachromosomal DNA.[23] Bagde and Varma[24] reported that chromium and lead inhibited the synthesis of protein, DNA and RNA almost equally in *E. coli* and *A. aerogenes*. Bagde and Salvi[25] reported that cobalt and nickel inhibited protein, DNA and RNA synthesis of *S. paratyphi* B and *Shigella flexineri.*

***Enterobacter spp* and its Applications in Medical Microbiology**

Enterobacter causes nosocomial infections. It is ubiquitous and can survive on skin and dry surfaces and replicate in contaminated fluids. Numerous outbreaks have been described, including infections due to contaminated enteral feedings,[26] humidifiers and respiratory therapy equipment[27] and hydrotherapy water in a burn unit.[28] *Enterobacter* may also spread from patient to patient due to inadequate attention to infection control measures, especially hand-washing. *Enterobacter* species causes nosocomial infections, including lungs, urinary tract, intra-abdominal cavity and intravascular devices. *E. sakazakii* causes neonatal sepsis with meningitis.[29,30] The nosocomial infections due to *Enterobacter* are rising and broad resistance to third generation cephalosporins, penicillins and quinolones is a rising problem. A number of agents remain effective for treatment. Aminoglycosides retain good activity but usually require combination with another agent. Quinolones are highly active against most strains, but emerging resistance is a major concern. TMP-SMX is under-utilized as therapy of *Enterobacter* infections. Among the beta-lactams, the fourth generation cephalosporins and carbapenems are the most attractive options.[31]

Materials and Methods

The strains of resistant and sensitive *Enterobacter* spp. were obtained from BAC TEST Laboratory Nashik, Maharashtra and stocked in this laboratory. These organisms were grown on a nutrient agar at 37^0C and maintained at 5^0C. The inoculum was prepared in nutrient broth after subculture and 48 hours incubation. Experiments were carried out in 100 ml Erlenmeyer flask 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 cobalt was used in form of cobalt chloride in the experiment (Hi media, Mumbai). The final concentrations of cobalt taken were 150, 200,230 and 250 ppm for the sensitive strain and 200, 300, 400, 500 ppm for the resistant strain.

Estimation of DNA was done by Diphenyl amine method, and for protein estimation Follin-Lawry method was used. All these estimations were made after 12 hours interval.[32] The dehydrogenase assay was performed to determine the dehydrogenase activity according to the procedure of Guha and Mookerjee.[33] The cell fractionation was carried out and cell wall, cell membrane and cytoplasm fractions were obtained by the procedure given by Mitra et al.[34] Cobalt was estimated by 2Nitroso-1-Napthol method.[35]

Plasmid Isolation was conducted for separation and analysis of nucleic acid by alkaline lysis method.[36] Agarose gel electrophoresis of plasmid DNA was done according to the method of Sambrook et al.[36] Elimination of resistance due to plasmid was carried out by using the sodium dodecyl sulphate (SDS) treatment described by Tomoeda et al.[37] Detection of β lactamase was carried out by idometric method of Livermore and Brown.[38]

Results

Effect of cobalt on growth of *Enterobacter*

Resistant isolate of *Enterobacter* showed growth even up to 500 ppm concentration of cobalt, whereas the sensitive isolate of *Enterobacter* was completely inhibited at 250 ppm concentration of cobalt. The growth of sensitive *Enterobacter* was 95.45% and 68.18% in 150 and 230 ppm concentration of cobalt with respect to their controls (Fig. 1). The growth percentage observed in resistant *Enterobacter* was 86.76% and 44.11% in 200 and 500 ppm concentration of cobalt respectively as compared to the control sets (Fig. 2).

The final results of Estimations of Protein, DNA, and Growth curve were obtained after 72 hours that are given in Table 1. All these results obtained significantly contributed to the identification of the basic effect and mechanism of resistance. Similar to resistant isolate of *Enterobacter* showing growth even up to 500 ppm concentration of cobalt and the sensitive isolate of *Enterobacter* was completely inhibited at 250 ppm concentration of cobalt, macromolecules Protein, (Fig. 3 and 4). DNA, (Fig. 5 and 6) and Dehydrogenase enzymes (Table 1 and 2) depicted exactly same pattern of synthesis.

Table 1: Effect of cobalt (250 ppm) on the activity of dehydrogenases enzymes in resistant *Enterobacter* (250 ppm)

S. No.	Enzymes	OD of Control	OD of Test	% of Activity	% of Inhibition
1	Glutamate Dehydrogenase	0.54	0.37	68.51	31.48
2	Iso citrate Dehydrogenase	0.48	0.39	81.25	18.75
3	Keto glutarate Dehydrogenase	0.5	0.31	62.00	38.00
4	Succinate Dehydrogenase	0.51	0.4	78.43	21.56

Table 2: Effect of cobalt on the activity of dehydrogenases enzymes in sensitive *Enterobacter* at MIC (250 ppm)

S. No.	Enzymes	OD of Control	OD of Test	% of Activity	% of Inhibition
1	Glutamate Dehydrogenase	0.48	0.11	22.91	77.08
2	Iso citrate Dehydrogenase	0.45	0.13	28.88	71.11
3	Keto glutarate Dehydrogenase	0.51	0.1	19.60	80.39
4	Succinate Dehydrogenase	0.46	0.125	27.17	72.82

Table 3: Distribution of cobalt in subcellular fractions of *Enterobacter* after treatment of 48 hours

S. No	Fraction	Resistant *Enterobacter* At 250 ppm	Sensitive *Enterobacter* 250 (MIC)
1	Cell wall	66%	9%
2	Cell Membrane	11%	58%
3	Cytoplasm	23%	33%

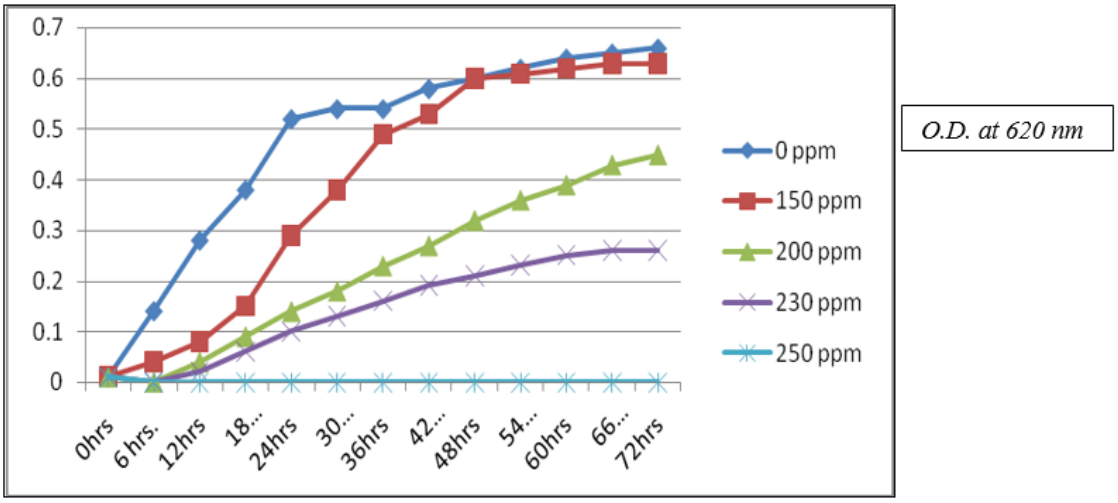

Fig. 1: Growth of sensitive *Enterobacter at* 0 to 250 ppm *cobalt* concentration

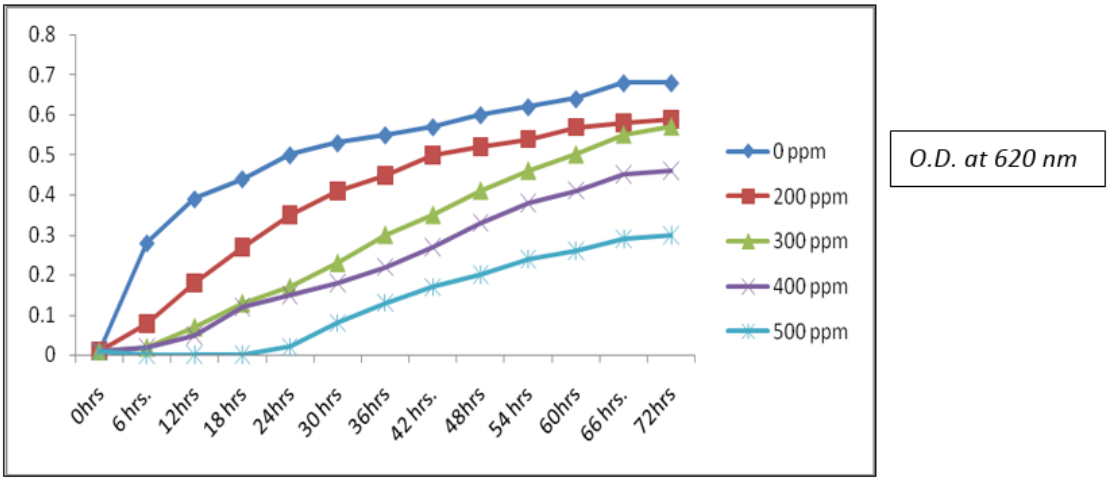

Fig. 2: Growth of resistant *Enterobacter at* 0 to 500 ppm *cobalt* concentration

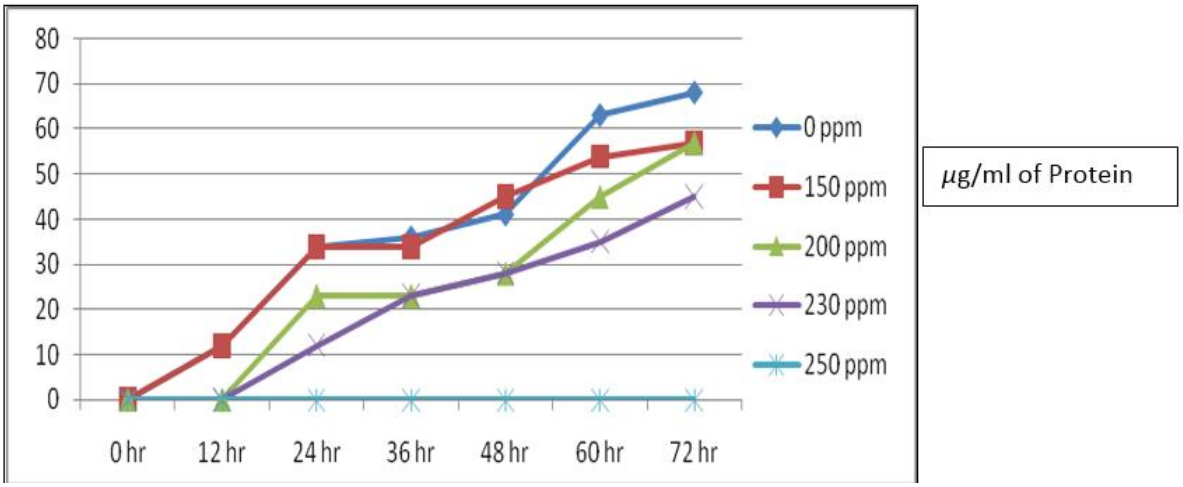

Fig. 3: Effect of cobalt concentration of 0 to 250 ppm on protein synthesis in sensitive *Enterobacter*

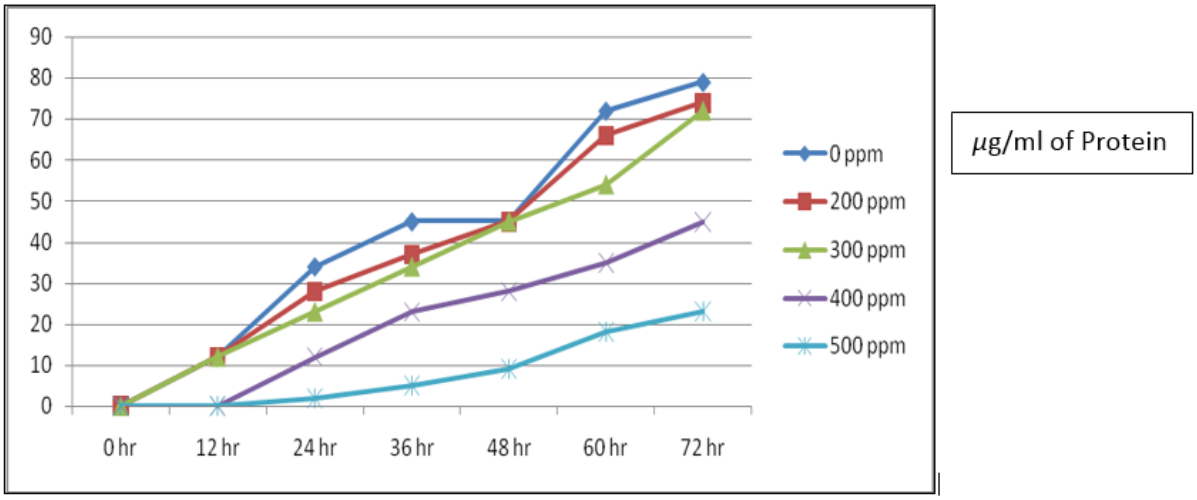

Fig. 4: Effect of cobalt concentration of 0 to 500 ppm on protein synthesis in resistant *Enterobacter*

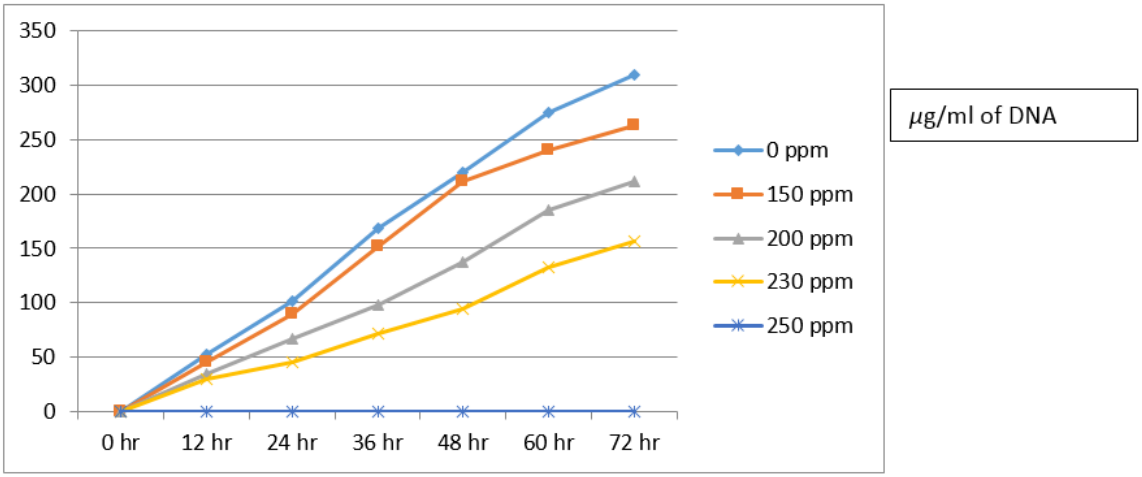

Fig. 5: Effect of cobalt concentration of 0 to 250 ppm on DNA synthesis in sensitive *Enterobacter*

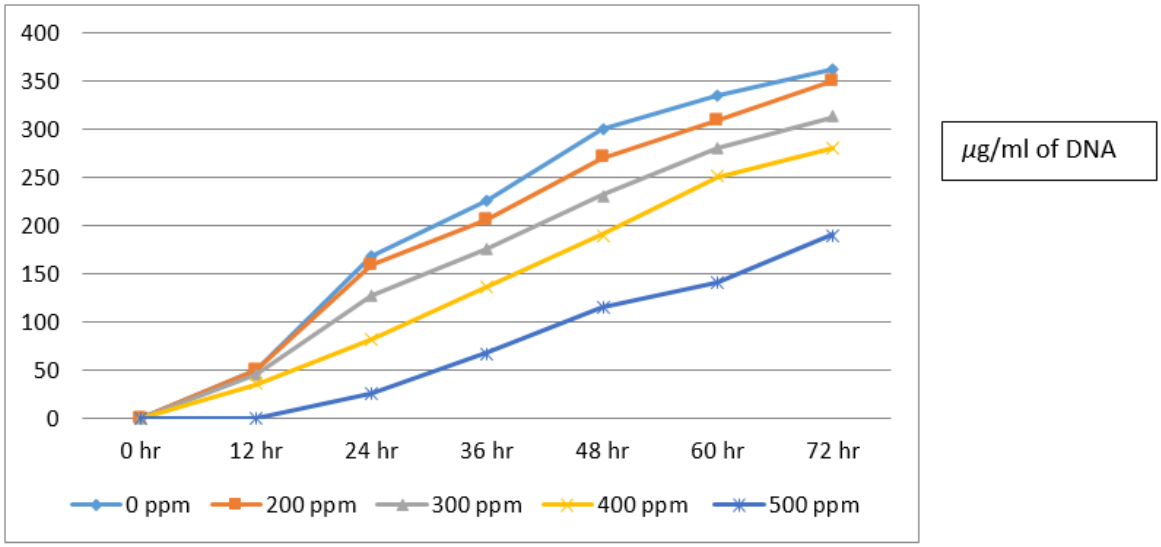

Fig. 6: Effect of cobalt concentration of 0 to 500 ppm on DNA synthesis in resistant *Enterobacter*

Effect of Cobalt on Protein and DNA Synthesis in *Enterobacter*

Protein estimation in sensitive *Enterobacter* showed inhibition of 16.18% in 150 ppm and 33.83% in 230 ppm of cobalt. In resistant *Enterobacter* the inhibition was 6.63% and 29.11% in 200 ppm and 500 ppm cobalt respectively. The protein percentage found in sensitive *Enterobacter* was 83.82% and 33.83% at 150 ppm and 250 ppm respectively. The resistant *Enterobacter* showed 93.67% and 70.89% of protein at 200ppm and 500 ppm cobalt respectively. (Fig. 3-4). After 72 hrs the concentration of protein was found to be 68 µg/ml, 57 µg/ml, 57 µg/ml, 45 µg/ml and 0 µg/ml at 0 ppm, 150 ppm, 200 ppm, 230 ppm, 250 ppm concentration of cobalt in sensitive *Enterobacter* respectively. In resistant *Enterobacter* at the concentration of 0 ppm, 200 ppm, 300 ppm, 400 ppm, and 500 ppm of cobalt, the concentration of protein found was 79 µg/ml, 72 µg/ml, 45 µg/ml, 23 µg/ml and 0 µg/ml respectively (Fig. 3 and 4).

The amount of DNA present in sensitive *Enterobacter* was found to be 84.83% at 150 ppm concentration of cobalt and 49.36% in 230 ppm. In resistant *Enterobacter* the concentration of DNA estimated was about 96.68% and 47.52% in 200 and 500 ppm of cobalt respectively. Therefore the inhibition of DNA calculated was 15.17% and 50.64% at 150 ppm and 230 ppm concentration of cobalt in sensitive *Enterobacter*, *whereas* 3.32% and 42.48% of DNA was inhibited at 200 ppm and 500 ppm by resistant species of this organism respectively (Fig. 5-6). After 72 hrs In resistant *Enterobacter* at the concentration of 0 ppm, 200 ppm, 400 ppm, 400 ppm, and 500 ppm of cobalt, the concentration of DNA was 362 µg/ml, 312 µg/ml, 280 µg/ml, 190 µg/ml and 0 µg/ml respectively. The concentration of DNA was found to be 310 µg/ml, 263 µg/ml, 212 µg/ml, 157 µg/ml and 0 µg/ml at 0 ppm, 150 ppm, 200 ppm, 230 ppm, 250 ppm concentration of cobalt in sensitive *Enterobacter* respectively after 72 hrs (Fig. 5 and 6).

Dehydrogenase Assay Results

The percentages of activity of various dehydrogenase found in the resistant *Enterobacter* were tested in citric acid cycle substrate Glutamate, Isocitrate, Ketoglutarate and Succinate at MIC concentration of Heavy metal cobalt (250 ppm). The activity of Glutamate dehydrogenase, Isocitrate dehydrogenase, Ketoglutarate dehydrogenase and Succinate dehydrogenase measured was 68.51%, 81.25%, 62.0% and 78.43% respectively in resistant *Enterobacter*. But the activity of all these enzymes in sensitive *Enterobacter* was lessened due to the effect of treatment of MIC concentration of cobalt. In sensitive *Enterobacter* the enzymes Glutamate dehydrogenase, Isocitrate dehydrogenase, Ketoglutarate dehydrogenase and Succinate dehydrogenase showed activity of about 22.9%, 28.28%, 19.60%, and 27.0% respectively (Table 1 and 2).

Thus the inhibition of enzyme activity calculated were 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(Table 1 and 2).

Cobalt Estimation in Cell Fractions

In this experiment the results obtained were in terms of concentration of cobalt in µg /ml by using the standard graph. These concentrations were then converted in to percentage of cobalt consumed by the cells. The cobalt incorporated in the cell wall, membrane and cytoplasm were 66%, 11% and 23% and 9%, 58% and 33% in resistant *Enterobacter* spp. and sensitive *Enterobacter* spp. respectively when 250 ppm cobalt was added in the culture flasks (Table 3). Thus in resistant *Enterobacter* accumulation of cobalt was highest in cell wall, while in sensitive *Enterobacter* accumulation of cobalt was found highest in membrane.

Plasmid was isolated and successful curing of plasmid was carried out with 10% Sodium dodecyl sulfate (SDS) in

resistant *Enterobacter* while no plasmid was isolated from sensitive *Enterobacter* as has been reported in earlier communication.[13, 39]

Discussion

Heavy-metal resistance mechanisms in bacteria have been shown to exist in various species.[40] During the study of growth at various concentrations of cobalt, sensitive strain showed decreased growth with increasing concentration of cobalt due to increase in toxicity.[40] Rajbhansi in 2008[41] reported the cobalt tolerance up to 250 ppm and 200 ppm respectively in *Methylobacterium* spp., and *Flavobacterium*spp. The isolated strain of *Enterobacter* was found to give low tolerance with CdCl2 and was found to be highly tolerant to Pb $(NO3)_2$. The MICs of Cd^{2+}, Cu^{2+} Co^{2+}, Zn^{2+} and Pb^{2+}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.[42] The concentrations of cobalt chloride ranging from 100 µM up to 400 µM were found suitable for optimal *E. coli* growth and expression of cobalt-substituted iron-type nitrile hydratase.[43] In an investigation by Banergee et al.[44] 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. Bioaccumulation of lead by this bacterial strain was extremely high (95.25%), followed by cadmium (64.17%) and nickel (36.77%). The findings in present study were in accordance with the above statement, as the sensitive strain showed no growth in 250ppm while resistant *Enterobacter* tolerated 500 ppm of cobalt. In 1971 Komura and Lazaki reported that the MIC of two different metals could be same for anyone particular microorganism.[45]

Several authors have reported high capability of heavy metals bioaccumulation by Gram negative bacteria.[46,47] The results of cell fractionation experiment yielded during cobalt estimation from cell fractions are in this line. The mechanism of resistant can be understood with this in terms of decreased permeability of cell membrane as the accumulation of cobalt found was 11% and 58% in cell membrane of resistant *Enterobacter* and sensitive *Enterobacter* respectively at 250 ppm. It is related with the mechanism of resistant to the cobalt. The transport of cobalt from cell wall to cell membrane got ceased at 250 ppm in the resistant *Enterobacter*. In a study Mitra et al.[34] reported maximum percentage of cadmium present in cell wall instead of cell membrane of resistant cell of *E. coli.*

It can be considered that this phenomenon also influenced growth and the macromolecule synthesis in the cell at various concentrations of cobalt. The Differences in the inhibition of protein, DNA synthesis was clearly indicated by the action of cobalt in both resistant and sensitive *Enterobacter at various* concentrations. High intracellular concentrations of cobalt as well as any other transition metal are toxic; however, molecular basis of cobalt toxicity has not been well documented until recently. Several studies have explored the deleterious effects of cobalt on bacterial metabolism revealing the cobalt competition with iron at several metabolic pathways and adaptive changes that occur in response to elevated cobalt concentration in the growth medium.[48-51] The decrease in the synthesis of protein and DNA, was associated with the inhibition of growth in sensitive organism which indicates direct influence of cobalt on the process of synthesis, as has also been reported earlier.[33, 34]

There were remarkable differences seen in the amount of cobalt present in the various cell organs of both the sensitive and resistant *Enterobacter* species when treated with 250 ppm cobalt concentrations in the present work. These results are found to be very useful to illustrate one of the well-known mechanisms of antimicrobial resistance that is related with the permeability of cell membrane and cell wall (Table 3). In resistant organism maximum percentage of cobalt got accumulated in cell wall unlike in sensitive strain maximum percentage of metal was in membrane, a site of action. Hence there was no inhibition of growth, and macromolecules Protein and DNA synthesis in resistant *Enterobacter*.

Raudrigue et al. in 2005[15] obtained data supporting the hypothesis that YohM is the first described efflux system for nickel and cobalt in *E. coli*. We propose rcnA (resistance to cobalt and nickel) as the new denomination of yohM.

In addition to antibiotic resistance R plasmids are known to provide resistance to heavy metals.[52] Similarly the resistant *Enterobacter spp.* studied here showed resistance to cobalt has been reportedly also showed resistance towards the antimicrobials Ceftazidime, Moxifloxacin, and Nalidixic acid. There was complete inhibition of growth of sensitive *Enterobacter spp*. at 8 µg/ml Ceftazidime, 0.1258 µg/ml of Moxifloxacin and 16 µg/ml of Nalidixic acid, however resistant Enterobacter spp. even tolerated 256, 32 and 1536µg/ml Ceftazidime, Moxifloxacin and Nalidixic acid respectively. Mechanism of resistance was found to be presence of plasmid.[13, 39]

As shown in table 1and 2, the activity of enzymes was noted in form of optical density on colorimeter, the activity of all the dehydrogenases when checked after 48 hours of inoculation. 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 1 and 2). This is an evidence for the inhibition of 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 not uncommon for organisms to manifest resistance by using combination of the mechanisms.[53] Macrolide resistance in *Streptococcus* was due to a combination of increased efflux and ribosomal modification and in *P. aeruginosa* carbapenem resistance was due to combination of β lactamase production, increase in efflux pumps and

changes in bacterial cell wall.[54] Tran and Jacoby[55] reported a multi resistance plasmid that encodes transferable resistance to quinolones.

Several different mechanisms of resistance to cadmium have been reported in the strains of *Staphylococcus aureus*, the presence of penicillinase plasmids altered the permeability properties of cell and conferred resistance to low concentrations of cadmium and number of other metals.[56]

While Plasmid mediated reduced accumulation of heavy metals has been reported by some workers,[57,58] resistance of heavy metals due to reduced accumulation has also been reported.[59, 60]

There may be correlation between the emergence of resistance to antibiotics and heavy metals.[61] There was reported a high correlation between certain types of antibiotic patterns and resistance to heavy metals.[5,11-13,39]

Conclusion

Present investigation rests on mechanism of resistance in *Enterobacter* towards heavy metal cobalt. Cobalt inhibited growth, protein**,** DNA synthesis in sensitive *Enterobacter*. But resistant *Enterobacter* tolerated high concentration of cobalt which was due to accumulation of cobalt in cell wall and due to presence of plasmid. Plasmid was absent in sensitive *Enterobacter*. Hence, the mechanism of resistance was accumulation of cobalt in the cell wall and presence of plasmid.

Conflict of Interest: None.

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